

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

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Abstract

The *agr* quorum-sensing system links *Staphylococcus aureus* metabolism to virulence, in part by increasing bacterial survival during exposure to lethal concentrations of H₂O₂, 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 aerobic 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 H₂O₂ doses. Increased survival of wild-type *agr* cells during H₂O₂ exposure required *sodA*, which detoxifies superoxide. Additionally, pretreatment of *S. aureus* with respiration-reducing menadione protected Δ *agr* cells from killing by H₂O₂. 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.

eLife assessment

This **important** study helps to elucidate the mechanism by which the *agr* quorum-sensing system in *Staphylococcus aureus* contributes to virulence. The results are supported by **solid** evidence using state-of-the-art methods. The linkage of metabolism and virulence in this pathogen will be of interest to the areas of microbiology, infectious diseases and immunology.

Introduction (main text 4,779 words)

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

Knowledge of factors that govern the biology of ROS has advanced considerably in recent years. For example, studies have centered on how specific metabolic features, such as aerobic respiration, affect killing by ROS (3, 4), and small-molecule enhancers of ROS-mediated lethality are emerging (5, 6). Less well characterized is how defense against ROS and metabolism changes integrate with the virulence regulatory network that promotes *S. aureus* pathogenesis.

The *agr* quorum-sensing system provides a way to study this dynamic: *agr* is a major virulence regulator that responds to oxidative stress (H₂O₂). The response occurs through a redox sensor in AgrA that attenuates *agr* activity, thereby increasing expression of glutathione peroxidase (BsaA), an enzyme that detoxifies ROS (7). Whether protection from ROS also occurs from positive *agr* action is unknown and likely to be an important issue in development of Agr-targeted therapies (8).

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

The *agr* locus consists of two divergent transcription units driven by promoters P2 and P3 (11). The P2 operon encodes the quorum-signaling module, which contains four genes, *agrB*, *agrD*, *agrC*, and *agrA*. AgrC is a receptor histidine kinase, and AgrA is a DNA-binding response regulator. AgrD is an autoinducing, secreted peptide derived from a pro-peptide processed by AgrB. The autoinducing peptide binds to and causes autophosphorylation of the AgrC histidine kinase, which

phosphorylates and activates the DNA-binding AgrA response regulator. AgrA then stimulates transcription from the P2 (RNAII) and P3 (RNAIII) promoters. RNAIII is a regulatory RNA that additionally contains the gene for delta-hemolysin (*hld*). The DNA-binding domain of AgrA contains an intramolecular disulfide switch (7 [↗](#)). Oxidation leads to dissociation of AgrA from DNA, thereby preventing an AgrA-mediated down-regulation of the BsaA peroxidase.

When we used antimicrobials to study bacterial responses to lethal stress involving the accumulation of ROS, we found that inactivation (deletion) of *agr* reduces lethality arising from treatment with antimicrobials, such as fluoroquinolones, in a largely *bsaA*-dependent manner (12 [↗](#)). Thus, oxidation sensing appears to be an intrinsic checkpoint that ameliorates the endogenous oxidative burden generated by certain antimicrobials. Surprisingly, deletion of *agr* increases the lethal effects of exogenous H₂O₂ (12 [↗](#)), in contrast to the expected expression of the protective *bsaA* system (7 [↗](#)). Thus, *agr* must help protect *S. aureus* from exogenous ROS, a principal host defense, through mechanisms other than *bsaA*.

In the present work we found that protection by wild-type *agr* against lethal concentrations of H₂O₂ was unexpectedly long-lived and 1) associated with decreased expression of respiration genes, and 2) potentially aided by defense systems that suppress the oxidative surge triggered by subsequent, high-level H₂O₂ exposure. The redox switch in AgrA, plus these additional protective properties, indicate that *agr* increases resilience to oxidative stress in *S. aureus* both when it is present and when it is absent. Thus, *agr* integrates protection from host defense into the regulation of staphylococcal virulence.

Results

agr protects *S. aureus* from lethal concentrations of H₂O₂ throughout the growth cycle

Because *agr* is a quorum-sensing regulon, maximal *agr* activity occurs during exponential growth (Figure 1, figure supplement 1 [↗](#)) and is followed by a sharp drop during stationary phase (12 [↗](#), 13 [↗](#)). Surprisingly, protection from H₂O₂ toxicity by wild-type *agr*, assessed by comparison with an *agr* deletion mutant, was observed throughout the growth cycle (Figure 1A [↗](#)). Indeed, maximal protection occurred shortly after overnight growth, long after induction and expression of *agr* transcripts. Comparison of survival rates of Δ *agr* mutant and wild-type cells, following dilution of overnight cultures and regrowth for 1 h prior to challenge with 20 mM H₂O₂, revealed an initial rate of killing that was ~1,000-fold faster for the Δ *agr* mutant (Figure 1B [↗](#)). Peroxide concentration dependence was observed up to 10 mM during a 60-min treatment; at that point, mutant survival was about 100-fold lower (Figure 1C [↗](#)). Complementation tests confirmed that the *agr* deletion elevated killing by H₂O₂ (Figure 1D [↗](#)).

We also monitored the time required for the wild-type *agr* survival advantage against H₂O₂ to manifest itself (Figure 1, figure supplement 2 [↗](#)). Overnight cultures were not readily killed by H₂O₂, as expected from previous results with other lethal stressors (14 [↗](#)). Following dilution to fresh medium, wild-type survival dropped gradually, while mutant survival, although lower, was constant for 20 min. By 40 min, mutant survival exhibited a precipitous 10-fold drop not seen with wild-type cells (Figure 1, figure supplement 2 [↗](#)). This drop in mutant survival correlated temporally with changes in cell density (Figure 1, figure supplement 2 [↗](#)); i.e., the first cell division following dilution to fresh medium. Overall, the *agr*-mediated survival advantage during H₂O₂ exposure was absent in stationary-phase cells and small during lag phase (before exponential growth resumes), but it increased markedly during early growth.

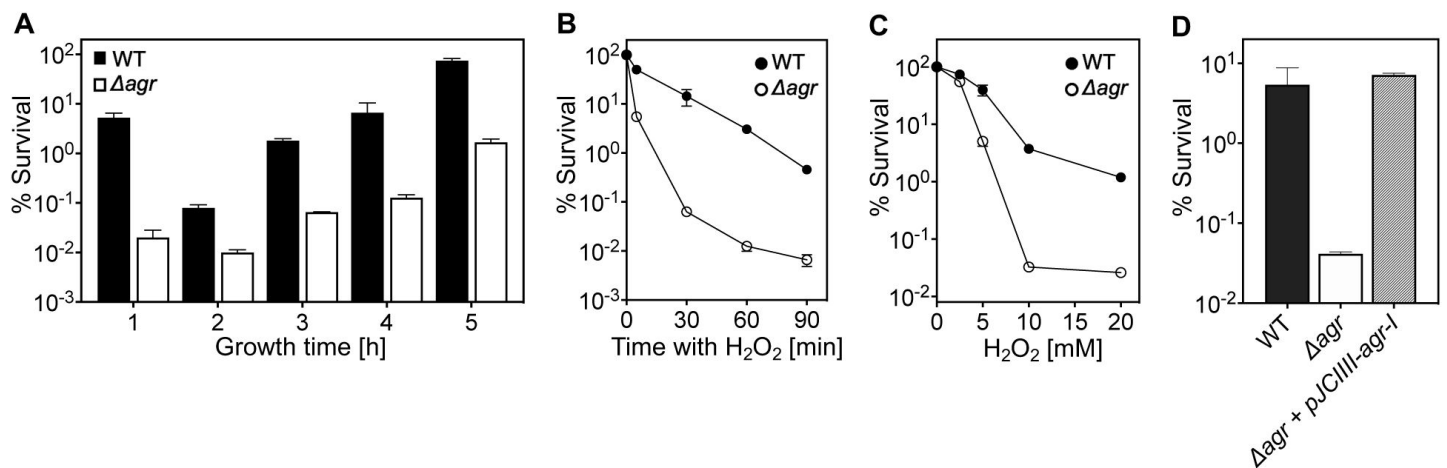


Figure 1.

***agr* deficiency protects from killing by H_2O_2 throughout the growth cycle.**

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

Lag-time differences between strains were more obvious in experiments using nutrient-limited, chemically defined medium (CDM) with highly diluted starting cultures and automated growth analysis (**Figure 1. figure supplement 3** [↗](#)). In CDM, wild-type cells divided within ~150 min, while the lag times with the *Δagr* mutant were more than 205 min (in Tryptic Soy Broth the lag time is 30 min for both). These observations suggest a novel *agr*-mediated decrease in time to enter exponential growth following dilution of stationary phase cultures. The poor killing of *agr* mutant cells by H₂O₂ early in lag phase is consistent with other work in which cells experiencing long lag times are less readily killed ([15](#) [↗](#)), presumably due to remaining longer in a dormant, protected state. To focus on effects during growth, subsequent experiments were performed after incubation of overnight cultures for 1 h in fresh tryptic soy broth unless otherwise specified.

The elevating effect of *agr* inactivation on H₂O₂-mediated lethality was observed across a variety of *S. aureus* strains, although differences in wild-type survival were observed (**Figure 1. figure supplement 4** [↗](#)). Thus, *agr*-mediated protection from H₂O₂ appears to be common among *S. aureus* lineages.

Expression of RNAIII and repression of Rot is required for protection from H₂O₂-mediated lethality

ΔrnaIII and *Δagr* mutants showed identical loss of protection from H₂O₂-mediated killing (**Figure 2A** [↗](#)), indicating that protection is RNAIII-dependent. Since RNAIII represses translation of the downstream regulator Rot ([16](#) [↗](#)), a transcription factor having a key role in *agr* regulation of staphylococcal virulence, we also examined the effects of *rot* on the protective action of *agr* against H₂O₂. When the wild-type strain, a *Δagr* mutant, a *Δrot* mutant, and a *Δagr Δrot* double mutant were compared for survival following treatment with 20 mM H₂O₂, survival of the *Δagr Δrot* double mutant phenocopied that of the wild-type strain (**Figure 2B** [↗](#)): the *rot* deletion reversed the effect of an *agr* deficiency. These data are consistent with *agr* activity allowing induction of *rot*-repressed genes important for protection from peroxide (RNAIII repression of the Rot repressor).

When a low-copy-number plasmid expressing *rot* was introduced into a wild-type *agr* strain, the transformant was more readily killed by H₂O₂, indicating that expression of *rot* is sufficient for increased lethality (**Figs. 2C-D** [↗](#)). These data suggest that wild-type Rot down-regulates expression of protective genes. The observed epistatic effect of *agr* and *rot* did not apply to other downstream, potentially epistatic regulators, such as *saeRS*, *mgrA*, and *sigB* (**Figure 2—figure supplement 1** [↗](#)) ([17](#) [↗](#)). Thus, the epistatic relationship between *agr* and protection from H₂O₂ appears to be *rot*-specific.

agr-mediated protection from H₂O₂ stress

is kinetically uncoupled from *agr* activation

Since *agr*-mediated protection from H₂O₂ occurs throughout the growth cycle, it was possible that protection arises from constitutive, low-level *agr* expression rather than from autoinduction and thereby quorum sensing. To test for a requirement of quorum in the *agr*-mediated oxidative-stress phenotypes, we characterized the role of *agr* activation using a mixed culture strategy in which one strain, an in-frame deletion mutant of *agrBD*, is activated *in trans* by AIP produced by a second, *ΔrnaIII* mutant strain (**Figure 3A** [↗](#)). The AIP-responsive *ΔagrBD* strain carried an intact RNAIII, while the *ΔrnaIII* mutant was wild-type for *agrBD*. As shown in **Figure 3B** [↗](#), hemolytic activity (a marker for RNAIII) of the *ΔagrBD* mutant was restored by mixing it with the *ΔrnaIII* mutant strain that secreted AIP into the surrounding medium. This result confirmed that *agrCA*-directed *trans*-activation of RNAIII by AIP remained intact in the *ΔagrBD* mutant.

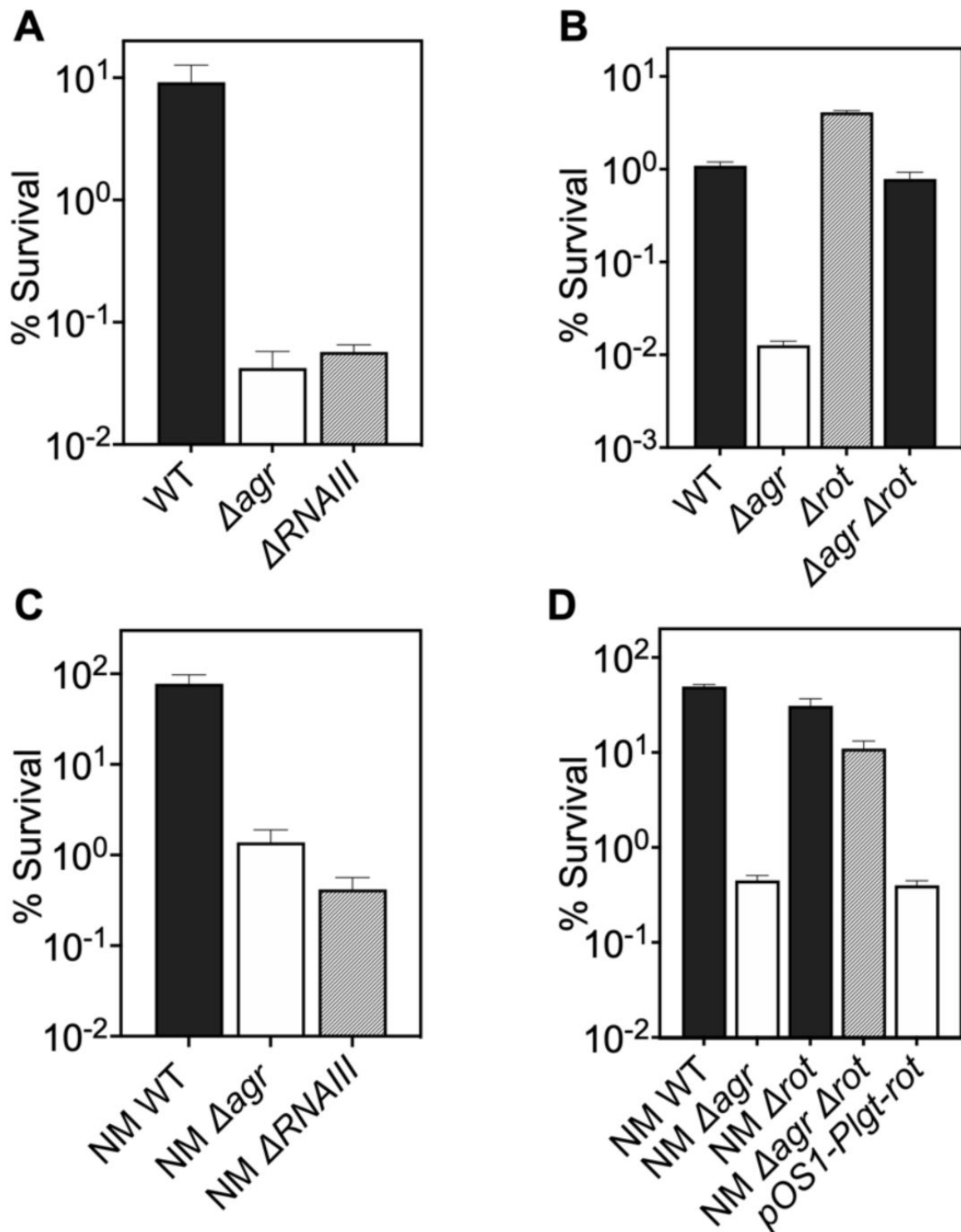


Figure 2.

Involvement of *RNAIII* and *rot*-dependent pathways in *agr*-mediated protection from H_2O_2 -mediated killing.

Cultures were grown for 1 h following dilution from overnight cultures to early log phase ($OD_{600} \sim 0.15$) and then treated with 20 mM H_2O_2 for 60 min before determination of percent survival by plating and enumeration of colonies. (A) Wild-type (WT) LAC (BS819), Δagr mutant (BS1348), $\Delta rnaIII$ mutant (GAW183), and Δrot (BS1301). (B) Δrot and $\Delta agr \Delta rot$ double mutant (BS1302). (C) Wild-type (WT) strain Newman (NM, BS12), Δagr mutant (BS13), and $\Delta RNAIII$ mutant (BS669). (D) Overexpression of *rot*. *rot* was expressed from a plasmid-borne wild-type *rot* (pOS1-*Plgt-rot*, strain VJT14.28). Data represent the means $\pm$ S.D. from biological replicates ($n = 3$).

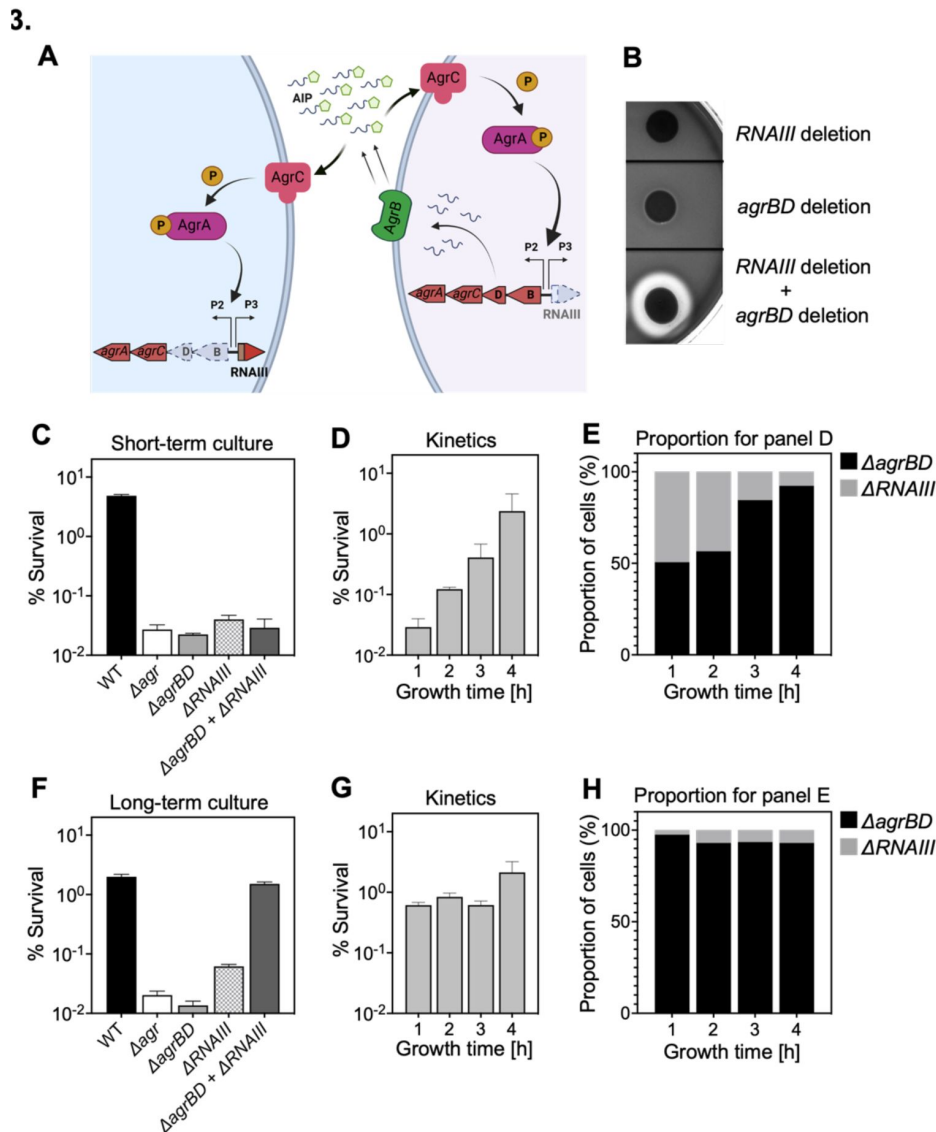


Figure 3.

agr-mediated protection from H₂O₂ stress is uncoupled from *agr* activation kinetics.

(A) Assay design. An *ΔagrBD* deletion mutant (GAW130) was complemented *in trans* by the autoinducing product (AIP) of *AgrBD* in a *ΔrnaIII* (GAW183) mutant that produces AIP endogenously; *AgrC* activation in the *ΔagrBD* 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 *ΔrnaIII* 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 H₂O₂. (B) Trans-activation demonstrated by hemolysis activity on sheep blood agar plates. Bottom of figure shows zone of clearing (hemolysis activity) after mixing 10⁸ *ΔagrBD* CFU with an equal number of *ΔrnaIII*. Zone of clearance is a consequence of *AgrC* receptor activation *in trans* by AIP produced by the *ΔrnaIII* mutant. (C) Absence of trans-activation with short-term culture. The *agr* wild-type strain RN6734 (WT, BS435), *ΔrnaIII* (GAW183), *ΔagrBD* (GAW130), and *ΔrnaIII* and *ΔagrBD* mutants were mixed 1:1 immediately before growth from overnight culture. Overnight cultures were diluted (OD₆₀₀~0.05) into fresh TSB medium, mixed, and grown to early log phase (OD₆₀₀~0.15) when they were treated with 20 mM H₂O₂ for 60 min and assayed for percent survival by plating. (D) Kinetics of killing by H₂O₂, performed as in panel C, but grown from early exponential (1 h, OD₆₀₀~0.15) through late log (5 h, OD₆₀₀~4) phase in TSB. Cultures were treated with H₂O₂ (20 mM for 1 h) at the indicated time points. (E) Proportion of mixed population for panel D represented by each mutant after incubation. The *ΔagrBD* mutant contained an erythromycin-resistance marker to distinguish the strains following plating of serial dilutions on TS agar with or without erythromycin (5 μg/ml). Data represent the means ± S.D. from biological replicates (*n* = 3). (F) Trans-activation during long-term culture. The *agr* wild-type strain RN6734 (WT, BS435), *ΔrnaIII* (strain GAW183), *ΔagrBD* (strain GAW130), and *ΔrnaIII* and *ΔagrBD* mutants mixed 1:1 prior to overnight culture. Survival assays employing *ΔrnaIII* and *ΔagrBD* mixtures, performed as in panel C. (G) Kinetics of killing by H₂O₂. Survival assays employing *ΔrnaIII* and *ΔagrBD* mixtures, performed as in panel D. Cultures were treated with H₂O₂ (20 mM for 1 h) at the indicated time points. (H) Proportion of mixed population for panel G represented by each mutant after incubation, performed as in panel E. Data represent the means ± S.D. from biological replicates (*n* = 3).

Mixed culture tests using these mutants, scored by differential plating for the presence of an erythromycin resistance marker in the $\Delta agrBD$ mutant, showed no protection from lethality of H_2O_2 when the two strains were mixed 1:1 immediately prior to growth from stationary phase (**Figure 3C**). Autoinducer accumulated during subsequent growth, activating *agr* expression and commencing protection from exogenous H_2O_2 (**Figs. 3D-E**). During H_2O_2 treatment, the percentage of the $\Delta agrBD$ mutant ($rnaIII^+$) increased while the percentage of the $\Delta rnaIII$ mutant decreased; this cis-acting result is consistent with the idea that pathways downstream from RNAIII, such as those regulated by *rot*, are the primary drivers of *agr*-mediated protection from H_2O_2 . These results confirm an intimate link between *agr*-mediated protection and the quorum-controlled *agr* gene expression program of late exponential phase. However, after an overnight co-culture of the $\Delta rnaIII$ and $\Delta agrBD$ mutant strains, the $\Delta agrBD$ mutant demonstrated the same degree of protection expected for *agr* wild-type cells during exposure to H_2O_2 (**Figure 3F-H**). Thus, protection by *agr* after overnight co-culture extends to growth resumption from stationary phase, prior to reaching quorum, and therefore protection is uncoupled from the constraint of strict cell-density dependence. These results indicate that protection lasts long after maximal transcription of *agr*, when *agr* expression has largely halted (12, 13). This phenomenon is a critical feature of the *agr* system not appreciated in previous analyses of *agr* activation kinetics.

agr deficiency increases transcription of genes involved in respiration and overflow metabolism in the absence of stress

To explore mechanisms underlying protection from H_2O_2 , we performed RNA-seq with the Δagr and wild-type strains after growth to late exponential growth phase, a point when *agr* expression is maximal. As expected, *agr* up-regulated the transcription of many known virulence genes (Supplementary file 1). The Δagr strain showed elevated expression of genes involved in respiration (*cydA*, *qoxA-D*) and fermentation (18, 19), including *nrdGD*, alcohol dehydrogenases (*adhE* and *adh1*) and lactate dehydrogenases (*ldh*, *ddh*) (**Figure 4A** and Supplementary file 1). Increased respiration and fermentation are expected to increase energy generation. However, metabolic modeling of transcriptomic data showed a ~30% reduction in tricarboxylic acid (TCA) cycle and lactate flux per unit of glucose taken up by the Δagr mutant (**Figure 4B**, Supplementary file 2). Additionally, intracellular ATP levels were ~50% lower in the Δagr mutant compared to the wild-type control, suggesting reduced metabolic efficiency during exponential growth (**Figure 5A**). Moreover, although the *agr* deletion has little effect on growth in the rich medium in which RNA-seq was performed (20), analysis in nutrient-constrained medium (CDM) revealed decreased growth rate and yield of the Δagr mutant relative to wild-type *S. aureus* (**Figure 1—figure supplement 3**). Collectively, these data suggest that Δagr increases respiration and aerobic fermentation to compensate for low metabolic efficiency. Consistent with this idea, *agr* deficiency also increases ATP-yielding carbon “overflow” pathways, as evidenced by increased acetate production (**Figure 5B**) (21, 22). The increase in accumulated acetate in the culture medium during exponential growth was largely consumed after 20 h of growth (**Figure 5B**). Thus, Δagr mutants exhibit TCA cycle proficiency (20) and, despite some expense of efficiency, an increased catabolism of acetate.

Differential transcription of selected genes was confirmed by RT-PCR measurements (**Figure 4—figure supplement 1**). We also confirmed that respiration levels were lower (15%) in *agr* wild-type compared to Δagr (**Figure 5C-D**). Although the stimulatory effect of the *agr* deletion on production of the fermentation product lactate was not observed in optimally aerated broth cultures after growth to late exponential growth phase, it was confirmed for organisms grown in broth under more metabolically demanding suboptimal aeration conditions (**Figure 5E**). Overall, these results are consistent with transcription-level up-regulation of respiratory and fermentative pathways in *agr*-deficient strains.

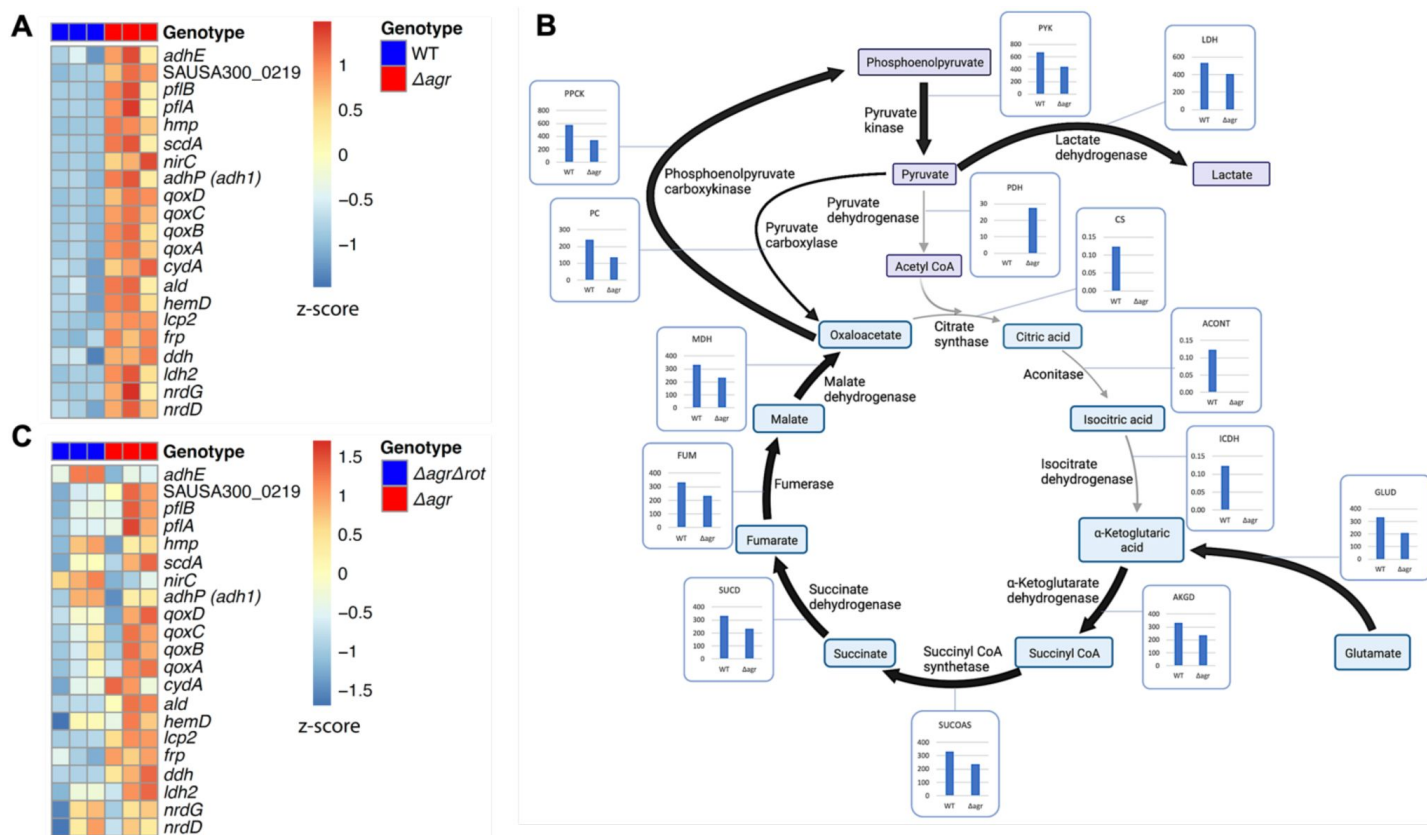


Figure 4.

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 *agr* wild-type (BS819) and Δagr mutant (BS1348) grown to late exponential phase (OD_{600} ~4.0). Shown are significantly up-regulated genes in the Δagr 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 is denoted by arrowhead thickness. Boxed charts indicate relative flux activity levels in *agr* wild-type versus Δagr 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 $\Delta agr \Delta rot$ double mutant (BS1302) with its parental Δagr strain (BS1348). Heatmap colors indicate expression z-scores. Sample preparation and figure labelling as for **Figure 4A**. See Supplementary file 2 for supporting information.

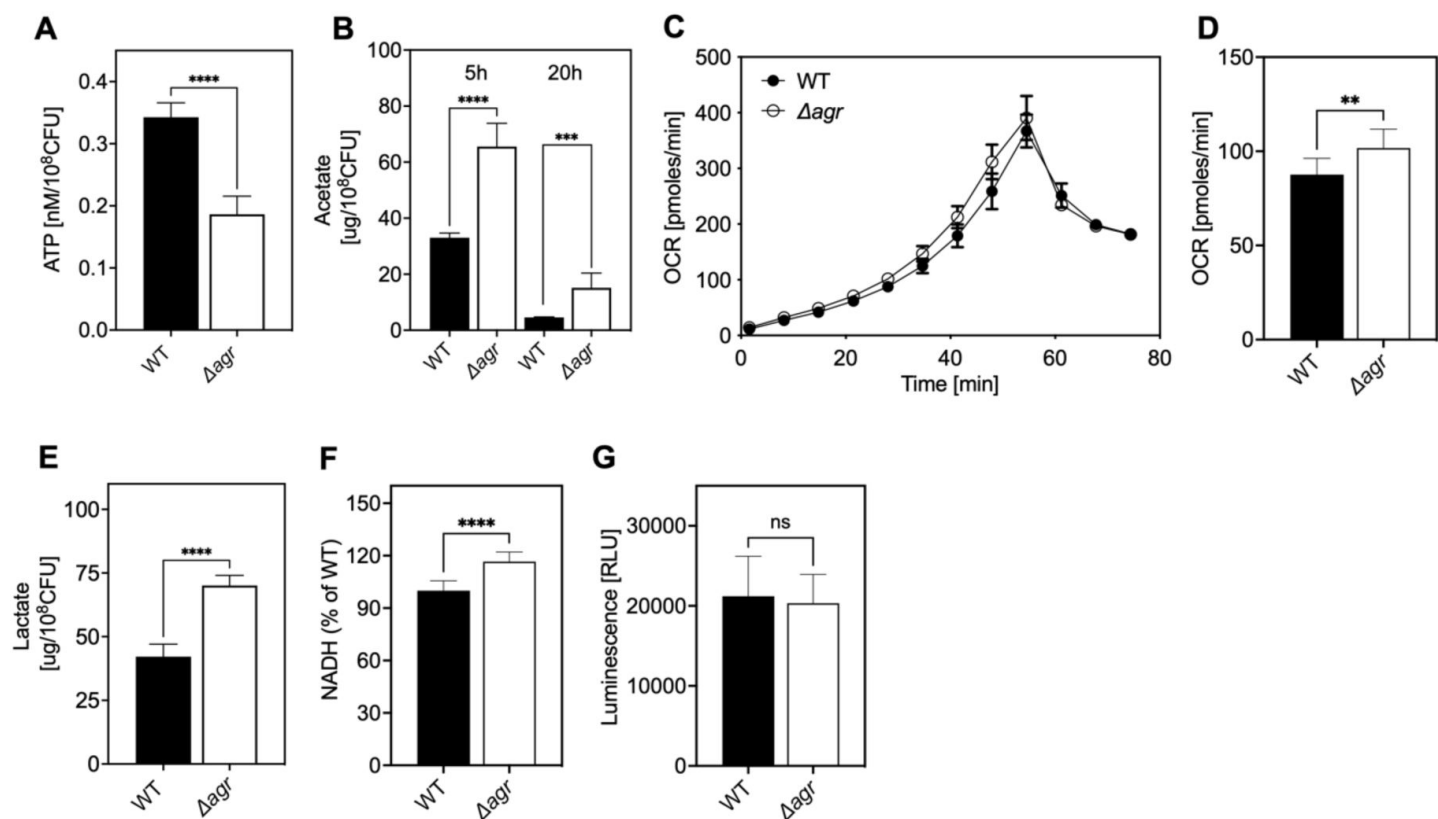


Figure 5.

Association of *agr* deficiency with a metabolic flux shift toward fermentive metabolism during aerobic growth.

(A) Intracellular ATP levels. Comparison of *S. aureus* LAC *agr* wild-type (BS819) and *Δagr* mutant (BS1348) strains for ATP expressed as nM/10⁸ cells after growth of cultures in TSB medium to late-exponential phase (OD₆₀₀~4.0). (B) Intracellular acetate levels. Samples were taken after 5 h and 20 h of growth in TSB medium; strains were *agr* wild-type (BS819) and *Δagr* mutant (BS1348). (C-D) Oxygen consumption. Strains *agr* wild-type (BS819) and *Δagr* mutant (BS1348) were compared using Seahorse XFp analyzer (C), and the rate of oxygen consumption (D) was determined from the linear portion of the consumption curve. Representative experiments from at least 3 independent assays are shown. (E) Lactate production during low oxygen culture. *agr* wild-type (BS819) and *Δagr* mutant (BS1348) were grown in TSB medium with suboptimal aeration to late-exponential phase (OD₆₀₀~4.0). (F-G) *agr* deletion induces NADH reductive stress. (F) NADH levels. Luminescence assay of NADH for *agr* wild-type (BS819) and *Δagr* mutant (BS1348) grown to late-exponential phase (OD₆₀₀~4.0) determined by an alcohol dehydrogenase-based redox cycling assay (shown as % of WT). (G) NAD⁺/NADH ratio. For all panels, data points are the mean value ± SD (n = 3). *P < 0.05; ****P < 0.0001, by Student's two-tailed *t* test. Seahorse statistical significances are compared to TSB medium.

The tendency of Δagr cells to forgo the additional ATP yield from acetate production in favor of NAD^+ -generating lactate (23 [↗](#), 24 [↗](#)) underscores the importance of redox balance in Δagr cells. Since respiration and fermentation generally lower $NAD^+/NADH$ ratios and since these activities are increased in Δagr strains (Figure 5F-G [↗](#)), we expected a lower $NAD^+/NADH$ ratio relative to wild-type agr cells. However, we observed an increase in both NAD^+ and $NADH$ compared to wild-type: the $NAD^+/NADH$ ratio remained tightly bounded. Collectively, these observations suggest that a surge in $NADH$ consumption and reductive stress in the Δagr strain induces a burst in respiration and fermentation.

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

We reasoned that the transcriptional changes due to the Δagr mutation likely persist, as does this strain's susceptibility to killing by H_2O_2 , after growth from overnight culture. With this in mind, and to determine whether agr -mediated changes act through *rot*, we performed RNA-seq experiments after 1-hr growth from overnight cultures of a $\Delta agr \Delta rot$ double mutant that phenocopies wild-type agr with respect to H_2O_2 -mediated death and with respect to its parental Δagr strain (Supplementary file 3). Fold-changes and number of genes differentially expressed were lower in the Δagr mutant relative to the wild-type culture, potentially because a significant portion of the population, even after an hour of growth (early exponential phase), still consisted of cells experiencing stationary phase at the time of sampling. Nevertheless, we did observe a shift to expression of fermentation-associated genes (*ilvA*, *pflAB*, *aldh1*, *ddh*, *lctp2*) in the Δagr strain (Figure 4C [↗](#) and Supplementary file 3). Thus, up-regulation of metabolic genes in the Δagr mutant extends beyond post-exponential growth to the exit from stationary phase and into subsequent cell proliferation, as does the long-lived protection from H_2O_2 -mediated killing seen with the wild-type strain.

To examine induction of genes by lethal levels of H_2O_2 , our gene expression analysis included a comparison between untreated and H_2O_2 -treated cells after growth from overnight culture (Supplementary file 3). The $\Delta agr \Delta rot$ double mutant that phenocopies wild-type agr had elevated expression of many genes involved in lowering oxidative stress compared to the Δagr mutant. Those genes are involved in the regulation of misfolded proteins (*mcsA*, *mcsB*, *clpC*, *clpB*), Fe-S cluster repair (*iscS*), DNA protection and repair (*dps*), and genes regulated by the protein-damage repair gene *bshA* (*fhuB/G*, *queC-E*) (25 [↗](#)) (Figure 7 [↗](#), Figure 7—figure supplement 1 [↗](#), and Supplementary file 3). Elevated expression of protective genes indicates that the double mutant survives damage from H_2O_2 better because protective genes are rendered inducible (loss of *Rot*-mediated repression). Overall, the data show that agr wild-type cells assume a long-lived stage after activation at high cell density in which they are primed to express genes (e.g., *clpB/C*, *dps*) that protect against high levels of exogenous oxidative stress.

Endogenous ROS is involved in *agr*-mediated protection from lethal, exogenous H_2O_2 stress

We next monitored the effect of reducing respiration and ATP levels by adding subinhibitory doses of the redox cycling agent menadione (26 [↗](#)) to cultures of Δagr and wild-type cells prior to lethal levels of H_2O_2 . Addition of menadione for 30 min, which induces a burst of ROS that inactivates

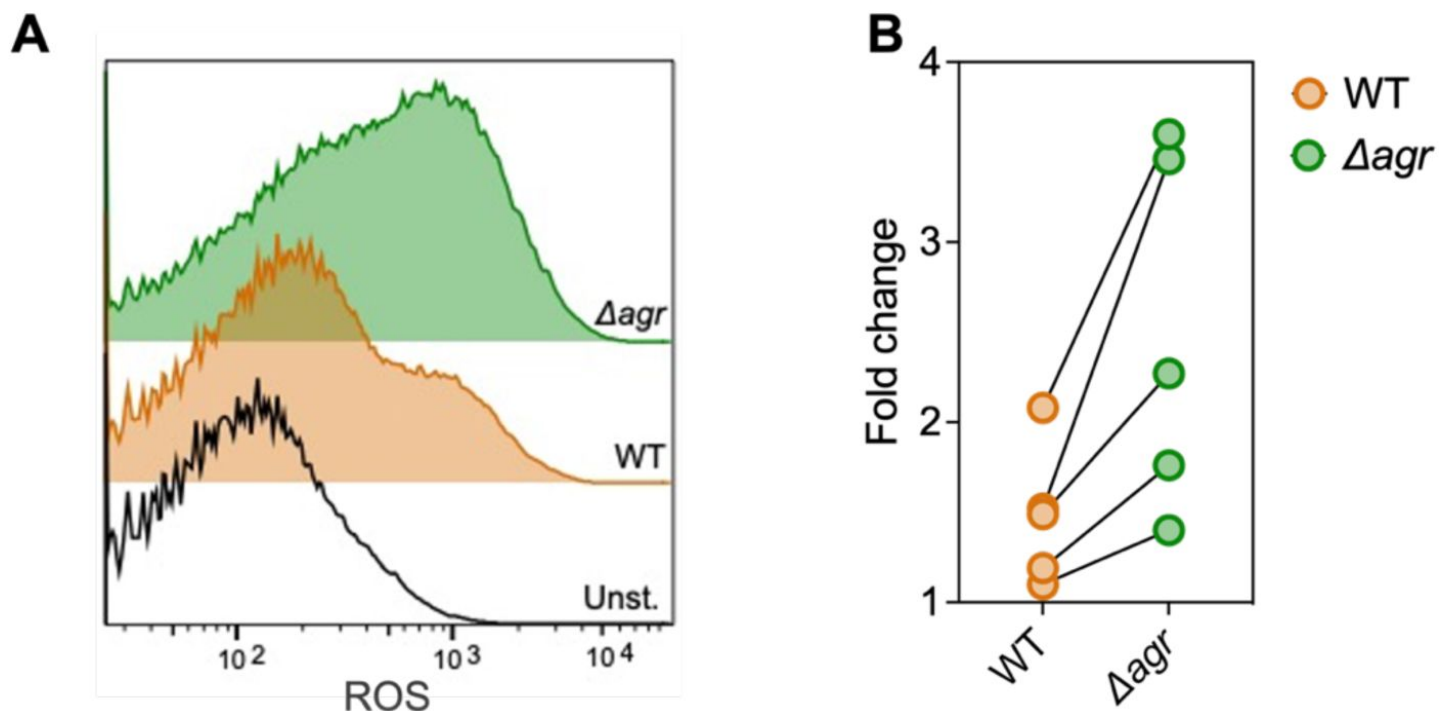


Figure 6.

Increase in ROS levels associated with Δagr deficiency.

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

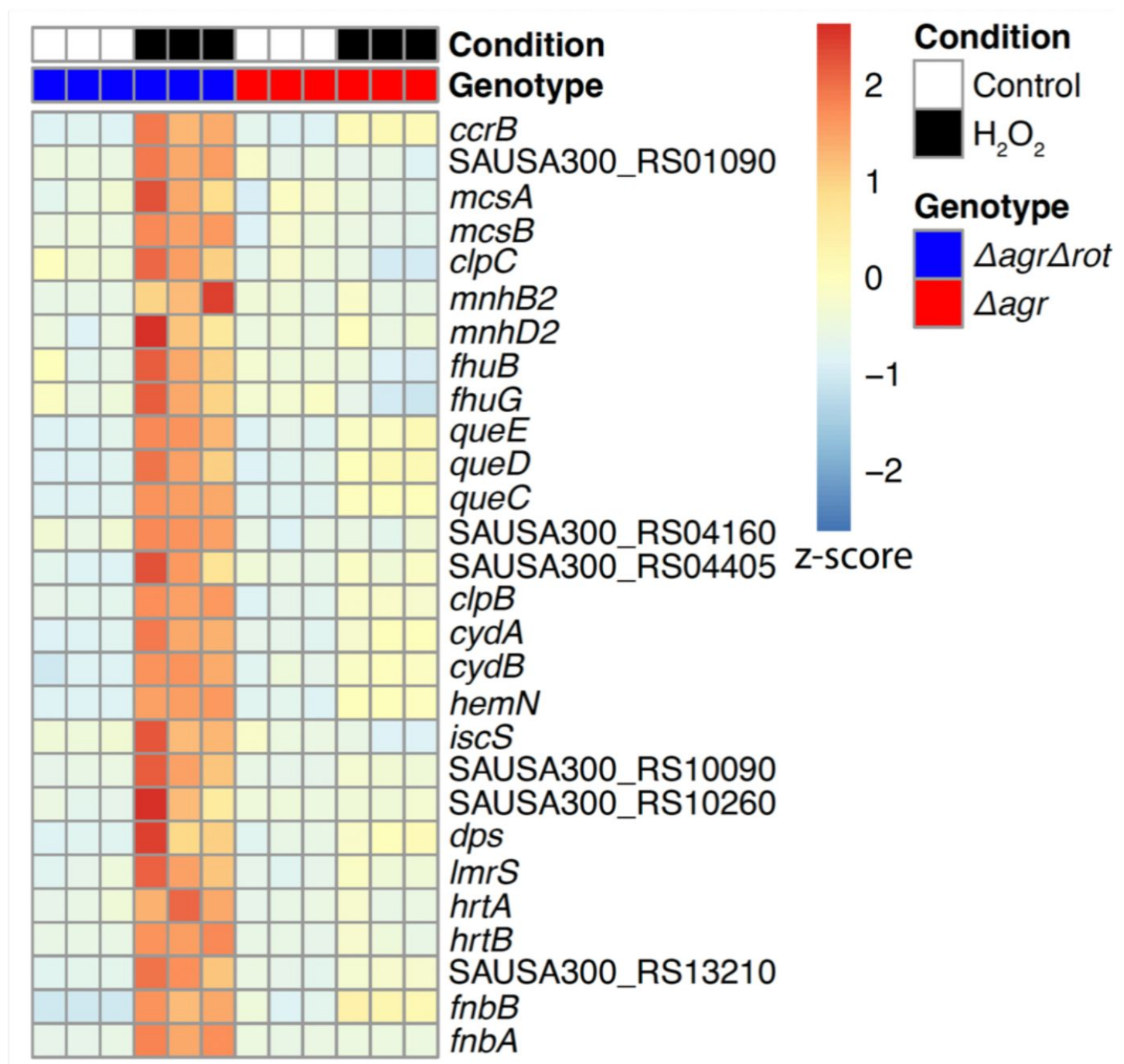


Figure 7.

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 $\Delta agr \Delta rot$ 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 $\Delta agr \Delta rot$ (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.

the TCA cycle and thereby respiration (26 [↗](#)), protected the Δagr mutant but had little effect on the wild-type *agr* strain (**Figure 8A** [↗](#)). Menadione's effect on respiration and ATP can be reversed by N-acetyl cysteine (26 [↗](#)). Addition of N-acetyl cysteine in the presence of menadione restored H_2O_2 susceptibility to the *agr* mutant (**Figure 8A** [↗](#)). Thus, blocking endogenous ROS production/accumulation reverses the lethal effect of an *agr* deficiency with respect to a subsequent exogenous challenge with H_2O_2 .

S. aureus has multiple enzymes that control the endogenous production and detoxification of ROS. SodA and SodM dismutate superoxide ($O_2^{\bullet-}$) to H_2O_2 , and catalase and AhpC then convert H_2O_2 to water, limiting the formation of toxic hydroxyl radical ($OH^{\bullet}$). Accordingly, we asked whether mutations in these pathways affect *agr*-dependent phenotypes with respect to lethal H_2O_2 exposure. A deficiency in the *sodA* superoxide dismutase (27 [↗](#)) resulted in lower survival of the wild-type *agr* strain, similar to that observed with the Δagr mutant (**Figure 8B-C** [↗](#)). The effect was reversed by complementation with *sodA* on a low-copy-number plasmid (**Figure 8D** [↗](#)). The $\Delta sodA$ mutation had no effect on killing with the Δagr strain. Moreover, *sodA* expression (Supplementary file 1) and activity levels (**Figure 8E** [↗](#)) were similar for wild-type *agr* and the Δagr mutant. Together, these observations suggest that the contribution of *sodA* toward protective priming by wild-type *agr* involves dismutation of low levels of endogenous superoxide generated by respiration. In contrast, endogenous levels of ROS are saturating for *sodA* in Δagr cells. Inactivation of *sodM*, which is thought to be primarily induced by exogenous oxidative stress (28 [↗](#)), had no noticeable effect on the H_2O_2 susceptibility of the wild-type or the Δagr mutant. We conclude that scavenging enzymes, such as SodA, are better able to control the threat posed by endogenous ROS in *agr* wild-type than in Δagr cells. They render the former better able to survive a subsequent lethal dose of H_2O_2 , a compound that freely enters cells (29 [↗](#)) and would add to endogenous ROS levels.

Other oxidative-stress-response mutations in genes encoding catalase, thiol-dependent peroxidases, and bacillithiol showed little effect on the relative lethality of H_2O_2 between wild-type *agr* and Δagr mutant strains (**Figure 8—figure supplement 1** [↗](#)). Thus, protection against H_2O_2 lethality by these genes is not *agr*-specific. Paradoxically, a deficiency of *ahpC* (*ahpC::bursa*), which encodes a peroxidase (30 [↗](#)), almost completely reversed the elevated killing associated with the Δagr mutation (**Figure 8F** [↗](#)). An *ahpC* deficiency had no effect on the response of the otherwise wild-type *agr* strain. A deficiency in other downstream genes in the *ahpC* operon (*ahpF*, SAUSA300_0377-0378) showed no effect, indicating that the protective behavior of mutant *ahpC* was not caused by polar effects (**Figure 8—figure supplement 2** [↗](#)).

Results with *ahpC* deficiencies were initially surprising, because reduced ROS detoxification should increase rather than decrease killing. Compensatory expression of other protective genes, such as *katA* in the $\Delta ahpC$ Δagr double mutant (30 [↗](#)), might enable cells to better survive damage from subsequent stress-stimulated ROS increases. Indeed, *katA* expression increased > 10-fold in the $\Delta ahpC$ and $\Delta ahpC$ Δagr double mutants (**Figure 8G** [↗](#)). Thus, $\Delta katA$ overcomes $\Delta ahpC$ -mediated protection, consistent with the idea expressed previously that *katA* is more protective than *ahpC* against high levels of exogenous oxidative stress (30 [↗](#), 31 [↗](#)). We conclude that the protective action of an *ahpC*-deficient mutant is due to a pre-induced, compensatory increase in expression of another protective catalase.

Importance of the long-lived “memory” of *agr*-mediated protection in a murine intraperitoneal infection model

To determine whether long-lived *agr*-mediated protection is important for *S. aureus* pathogenesis, we used the mixed infection strategy (outlined in **Figure 3** [↗](#)) in which a $\Delta agrBD$ mutant is “primed” in response to AIP produced by a $\Delta rnaIII$ mutant after overnight co-culture containing an equal ratio of the two mutant strains (**Figure 9A** [↗](#) and **Figure 9—figure supplement 1** [↗](#)). Then mice were infected via intraperitoneal inoculation; 2 h later, we lavaged the peritoneal

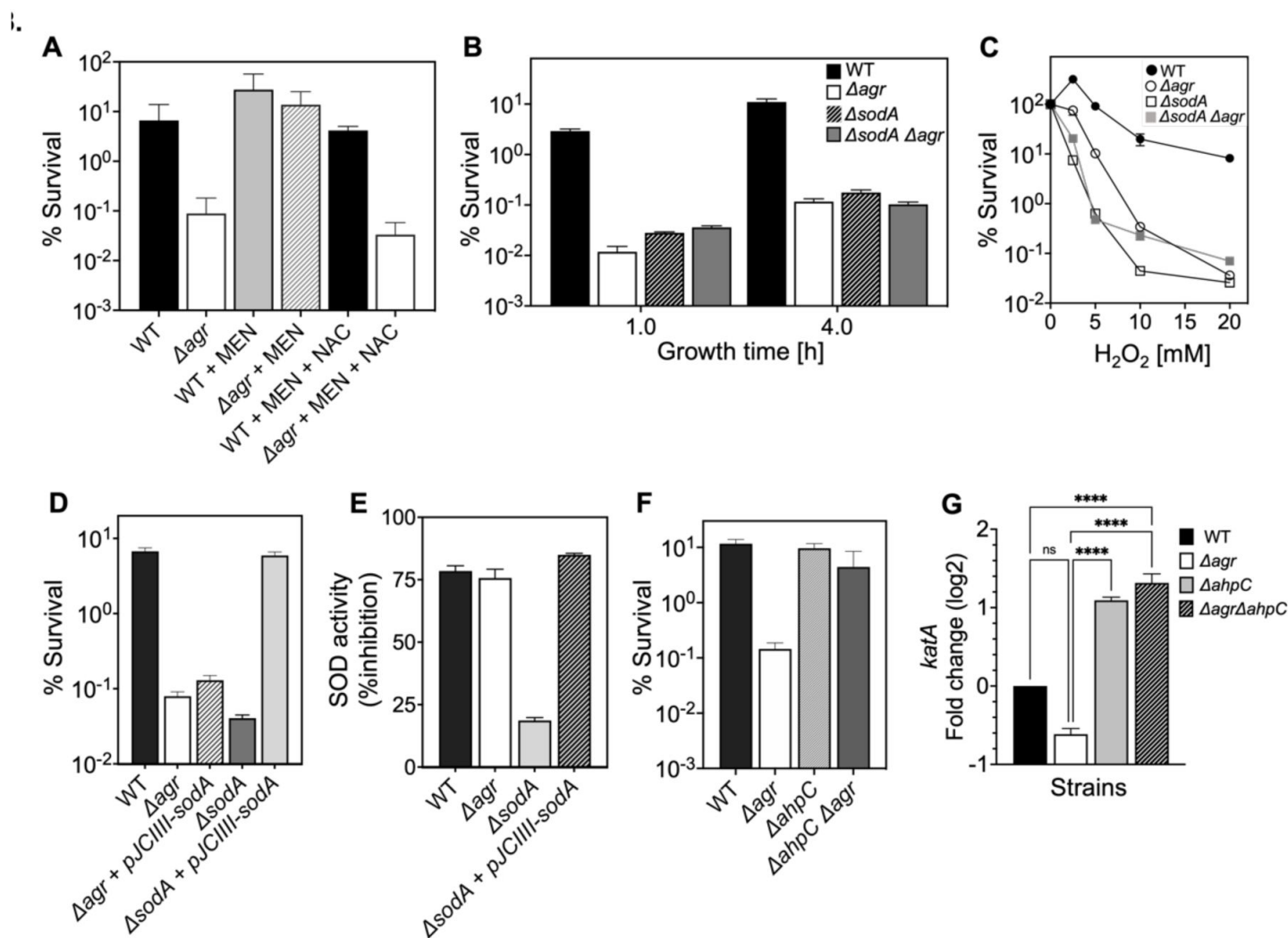


Figure 8.

Involvement of endogenous ROS in *agr*-mediated protection from lethal H₂O₂ stress.

(A) Protective effect of menadione on survival. *S. aureus* LAC wild type (BS819) and Δagr mutant (BS1348) cultures were grown to late exponential phase (4 h after dilution of overnight cultures), exposed to 80 μ M menadione (MD) with or without 4 mM N-acetyl cysteine (NAC) for 30 min prior to treatment with H₂O₂ (20 mM for 1 h) and measurement of survival. (B) Effect of *sodA* deletion on survival. Cultures of *agr* wild-type (BS819), Δagr (BS1348), a *sodA::tetM* (BS1422), and *sodA::tetM-agr* double mutant (BS1423) were grown to early (1 h after dilution, OD₆₀₀~0.15) or late log (4 h after dilution, OD₆₀₀~4.0) prior to treatment with 20 mM H₂O₂ for 60 min. (C) Effect of H₂O₂ concentration on survival. Late log (4 h, OD₆₀₀~4.0) cultures of the *agr* wild-type and Δagr mutant strains were treated with indicated concentrations of H₂O₂ 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, OD₆₀₀~4.0) cells treated with 20 mM H₂O₂ for 60 min. (E) SodA activity. *agr* wild-type or the indicated mutants were grown to late-exponential phase (OD₆₀₀~4.0); 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 $\Delta ahpC::bursa-agr$ double-mutant (BS1487) cells were treated with 20 mM H₂O₂ for 60 min. (G) Effect of *ahpC* deletion on expression of *katA* in the indicated mutants. Total cellular RNA was extracted from late exponential-phase cultures (OD₆₀₀~4.0), followed by reverse transcription and PCR amplification of the indicated genes, using *rpoB* as an internal standard. mRNA levels were normalized to those of each gene to wild-type control. Data represent the means $\pm$ S.D. from (*n* = 3) biological replicates. One-way ANOVA was used to determine statistical differences between samples (*****P* < 0.0001).

cavity and harvested organs for determination of colony forming units (CFU). By 2 h after bacterial administration, the number of *S. aureus* cells injected as inoculum had declined by 1000-fold (**Figure 9B** and **Figure 9—figure supplement 1**). Mutant proportions, identified by differential plating, demonstrated that $\Delta agrBD$ cells were enriched by ~30% in both peritoneum and organs compared to the $\Delta rnaIII$ mutant. The fraction of $\Delta agrBD$ ($rnaIII^+$) mutants in sites of bacterial dissemination (heart, kidney, liver, lungs, and spleen) was similar to their elevated fraction in the peritoneum, thereby suggesting that *agr* enhances intraperitoneal infection and access to, rather than entry into extraperitoneal organs. In a control infection in which $\Delta agrBD$ was “unprimed” by mixing $\Delta agrBD$ and $\Delta rnaIII$ mutants immediately before growth from stationary phase, the proportion of $\Delta agrBD$ bacterial burden was lower at all tissue sites (**Figure 9A** and **Figure 9—figure supplement 1**). This drop represented a decline in long-lived *agr* induction of virulence.

To study *agr*-ROS effects during infection, we repeated *in vivo* studies using $Nox2^{-/-}$ (also known as $Cybb^{-/-}$) mice deficient in enzymes associated with host phagocyte production of ROS (the gp91 [phox] component of the phagocyte NADPH oxidase)(32). We found that *agr*-mediated priming (mixing $\Delta agrBD$ and $\Delta rnaIII$ before overnight co-culture) failed to increase hematogenous dissemination to lung and spleen tissues following infection of $Nox2^{-/-}$ mice (**Figure 9C**). Thus, when the host makes little ROS, long-lived *agr*-mediated protection has little effect in these tissues. The data also indicate that *agr*-mediated protection against ROS enhances fitness in lung and spleen, but it is dispensable for full virulence in other organs. Collectively, the murine experiments indicate that the long-lived “memory” of *agr* induction enhances overall pathogenicity of *S. aureus* during sepsis. They also support data previously published indicating that clearance of disseminating bloodstream pathogens (33) and protection from ROS buildup (34) are tuned to particular sites in the host organism.

Discussion

We report that *agr*, a quorum-sensing regulator of virulence in *S. aureus*, provides surprisingly long-lived protection from the lethal action of exogenous H_2O_2 . The protection, which is uncoupled from *agr* activation kinetics, arises in part from limiting the accumulation of endogenous ROS. This apparent tolerance to lethal stress derives from an RNAIII-*rot* regulatory connection that couples virulence-factor production to metabolism and thereby to levels of ROS. Collectively, the results suggest that *agr* anticipates and protects the bacterium from increases in ROS expected from the host during *S. aureus* infection.

Details of *agr*-mediated protection are sketched in **Figure 10**. At low levels of ROS, *agr* is activated by a redox sensor in AgrA, RNAIII is expressed and represses the Rot repressor, thereby rendering protective genes (e.g., *clpB/C*, *dps*) inducible via an unknown mechanism.

Superoxide dismutase and scavenging catalases/peroxidases detoxify superoxide and peroxide, respectively (scavenging deficiencies reduce the protective effect of wild-type *agr*). Deletion of *agr* eliminates expression of RNAIII and repression of Rot, resulting in a metabolic instability associated with a 100-fold increase in H_2O_2 -mediated death.

The *agr* system directly reduces H_2O_2 -mediated killing by reducing levels of endogenous ROS, much like intrinsic tolerance to lethal antimicrobial stress (35). However, the protective system we describe is distinct in that it primes cells for induction of genes (e.g., *clpB/C*, *dps*) that mitigate damage upon subsequent exposure to high levels of ROS. Still unidentified protective genes exist; thus, *agr*-mediated protection may be further shaped by both known (*ahpC*) and unidentified pathways and factors that modify the redox state. Another distinctive feature of *agr*-mediated

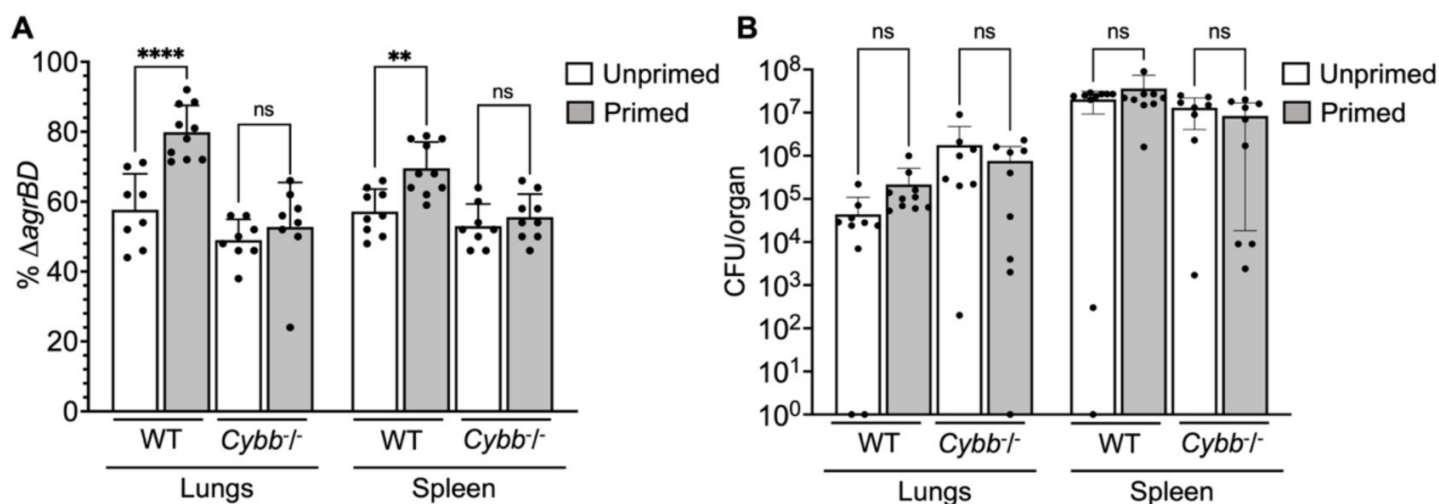


Figure 9.

Survival advantage of *agr* priming of *S. aureus* absent in phagocyte NADPH-deficient murine infection.

(A) percentage of $\Delta agrBD$ (AIP-responsive in-frame deletion mutant carrying an intact RNAIII) cells and (B) bacterial burden in lung or spleen after 2 h of intraperitoneal infection of wild-type (WT) C57BL/6 mice or phagocyte NADPH oxidase-deficient (*Cybb*^{-/-}) mice (see [Figure 9](#)—figure supplement 1 for data with other organs). $\Delta agrBD$ and $\Delta rnaIII$ mutant cultures were grown separately and mixed at a 1:1 ratio either before (primed) or after (unprimed) overnight growth, as for [Figure 3](#). Both primed and unprimed mixtures were diluted after overnight growth, grown to early log phase (OD₆₀₀~0.15), and used as inocula (1×10^8 CFU) for intraperitoneal infection (n = 2 groups of 10 mice each). After 2 h, lungs and spleen were harvested and homogenized; aliquots were 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 (panel 9A) or Student's two-tailed *t* test (panel 9B) were used to determine the statistical significance of the difference between primed and unprimed cultures. Error bars indicate standard deviation (***P* < 0.01; *****P* < 0.0001).

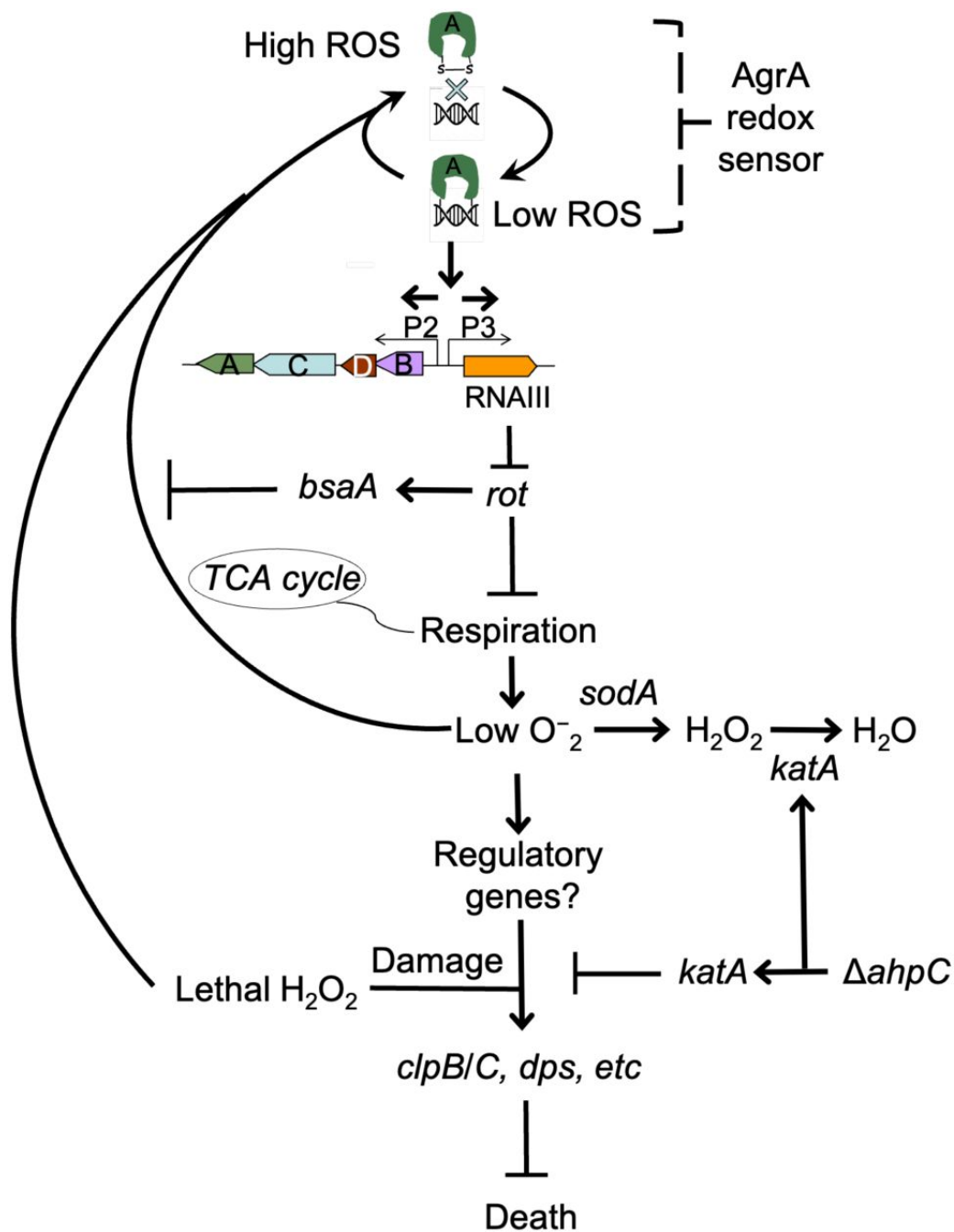


Figure 10.

Schematic representation of *agr*-mediated protection from ROS.

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

protection is its manifestation even in early log-phase cultures, long after the maximal transcription of *agr* at high cell density, i.e., quorum. In a sense, *S. aureus* has a “memory” of the *agr*-activated state.

Transcriptional profiling during growth from diluted, overnight cultures revealed that the Δ *agr* mutation elevated expression of several respiration and fermentation genes. Acceleration of cellular respiration is likely a source of ROS, as it appears to be for bactericidal antibiotics (3). Our work supports this idea by showing that increased respiration caused by deletion of *agr* is associated with increased ROS-mediated lethality. Given that Δ *agr* mutants are unable to downregulate surface proteins during stationary phase (36, 37), it is possible that deletion of *agr* perturbs the cytoplasmic membrane or the machinery that sorts proteins across the cell wall. In support of this notion, jamming SecY translocation machinery of *E. coli* results in downstream events shared with antibiotic lethality, including accelerated respiration and accumulation of ROS (38). In this scenario, the formation of a futile macromolecular cycle may accelerate cellular respiration to meet the metabolic demand of unresolvable problems caused by elevated surface sorting.

As noted above, *agr* is inactivated by oxidation, which elevates levels of the antioxidant BsaA during exposure to H₂O₂ (7). That would make our finding that H₂O₂-mediated killing is increased in the Δ *agr* mutant paradoxical. This apparent inconsistency can be explained by a focus of prior work on growth-related phenotypes (7) rather than on lethality (the underlying mechanisms are distinct (2)). Additionally, we note that *bsaA* was not upregulated in either our RNA-seq experiments (Supplementary file 1) or in previous transcriptional profiling data (39). Thus, an alternative, but not mutually exclusive, hypothesis is that the growth-related effect of *bsaA* on *agr*-mediated responses to stress is strain dependent. Another complexity involves test conditions, as indicated by consideration of previous work in which *agr* wild-type cells exhibited greater oxidative stress than the *agr*-deficient mutant due to *agrA*-mediated production of ROS-inducing phenol-soluble modulins (39). The present experiments were performed in highly diluted cultures in which levels of these modulins are likely low (40, 41). The complex relationship between *agr*, ROS-mediated lethality, and physiological state illustrates the importance of understanding *agr* biology before applying therapies that inactivate *agr* (8).

We also note that although the absence of *agr* increases killing by high levels of H₂O₂, it has the opposite effect for lethal concentrations of ciprofloxacin (12). In the latter case, the absence of *agr* upregulates expression of *bsaA* in the strain examined; *bsaA* counters endogenous ROS induced by ciprofloxacin (12). The present work shows that excess endogenous ROS is generated during *agr* deficiency. Thus, protection from endogenous lethal stress via *agr* inactivation may not be only through the redox-dependent *bsaA* but also by a second pathway involving increased respiratory metabolism. Together these two scenarios may help explain why *agr* in certain types of infection is selected for loss of function, especially during antimicrobial treatment (42–45). The present work also supports the idea that exposing bacteria to exogenous H₂O₂ does not fully recapitulate the intracellular environment created by antibiotics and other stresses that act via ROS-mediated cell death (38), emphasizing that inactivation of *agr* can be either destructive or protective, depending on the type of lethal stress. Similar results have been reported with other bacteria: *mazF*, *lepA*, and *cpx* are destructive or protective based on the level of lethal stress (46, 47).

The protective activity of *agr* activity carried over to *in vivo* studies using mice, as it was largely absent if the mice were deficient in host phagocyte production of ROS (*Nox2*^{-/-} mice with a null allele of NADPH oxidase). The benefits of *agr* to *S. aureus* fitness seen with NADPH oxidase-proficient mice were observed largely in lungs, a key host defense niche for neutrophil-mediated clearance of disseminating pathogens (33). The redox switch in AgrA, plus the protective properties associated with *agr* activation, lead to a clinical model in which *agr* links virulence-factor expression to an intrinsic protection against a lethal, H₂O₂-mediated immune response

during infection (**Figure 11** [↗](#)). In this model, *agr* quorum-sensing renders cells better prepared to respond to lethal, exogenous oxidative stress. We note, however, that *agr*-mediated fitness benefits were present in certain tissues even in NADPH oxidase-deficient mice, indicating the existence of long-lived factors other than those that suppress oxidative stress. Thus, such a pre-emptive defense system may apply to many challenges experienced by *S. aureus* during infection, especially during bloodstream dissemination and conditions within inflamed tissues ([48](#) [↗](#), [49](#) [↗](#)).

In conclusion, uncoupling of *agr*-mediated tolerance from bacterial population density anticipates increases in exogenous ROS expected during *S. aureus*-host interactions, thereby contributing to virulence. The ubiquity of quorum sensing suggests that it protects many bacterial species from oxidative damage. A next step is to find RNA, protein, and/or epigenetic markers underlying the *agr*-mediated “memory” that improves protection against subsequent H₂O₂ exposure, since that will provide insights into the role of *agr* in cellular survival and adaptation during infection. Discovering ways to manipulate the lethal stress response, as seen with supplementation of antimicrobials with N-acetyl-cysteine during treatment of *Mycobacterium tuberculosis* ([50](#) [↗](#)) and development of inhibitors of enzymes that produce protective H₂S ([5](#) [↗](#)), could reveal novel approaches for enhancing antimicrobial therapy and host defense systems ([51](#) [↗](#)–[53](#) [↗](#)).

Materials and Methods

Bacterial strains, plasmids, primers and growth conditions

S. aureus strains, plasmids, and primers used in the study are described in **Tables 1** [↗](#) and **2** [↗](#). Bacterial cells were grown in tryptic soy broth (TSB) at 37°C with rotary shaking at 180 rpm. For suboptimal aeration, broth cultures were grown in a closed-capped 15 mL conical tube with 10 mL of TSB. Colony formation was on tryptic soy agar (TSA) with or without defibrinated sheep blood, incubated at 37°C or 30°C. Phages 80α and Φ11-mediated transduction was used for strain construction ([54](#) [↗](#)); transductants were selected on TSA plates containing the appropriate antimicrobial.

For analysis of *in vitro* growth curves, overnight cultures grown in TSB were diluted 1:1000 in chemically defined medium (CDM) ([55](#) [↗](#)), and growth was monitored at 37°C in 96-well plates (100 μL/well) using an Agilent LogPhase 600 Microbiology Reader (Santa Clara, CA) with 1 mm orbital shaking, measuring OD₆₀₀ at 40-min intervals. The curves represent averaged values from three biological replicates.

Measurement of bioluminescence

Overnight cultures were diluted to OD₆₀₀ ~0.05 and grown in TSB at 37°C with rotary shaking at 180 rpm. Aliquots (100 μL) were inoculated into flat bottom 96-well microtiter plates (Corning, Corning, NY), and bioluminescence was detected using a BioTek Synergy Neo2 plate reader (Agilent, Santa Clara, CA).

Antimicrobials and chemicals

Antimicrobials, chemicals, and reagents were obtained from MilliporeSigma (Burlington, MA) or Thermo Fisher Scientific (Waltham, MA).

Construction of mutants

Transposon mutants were generated by transducing *Bursa aurealis* insertions, obtained from the University of Nebraska transposon mutant (ΦNE) library ([56](#) [↗](#)), into LAC or LAC *agr::tetM* using phages 80α and Φ11.

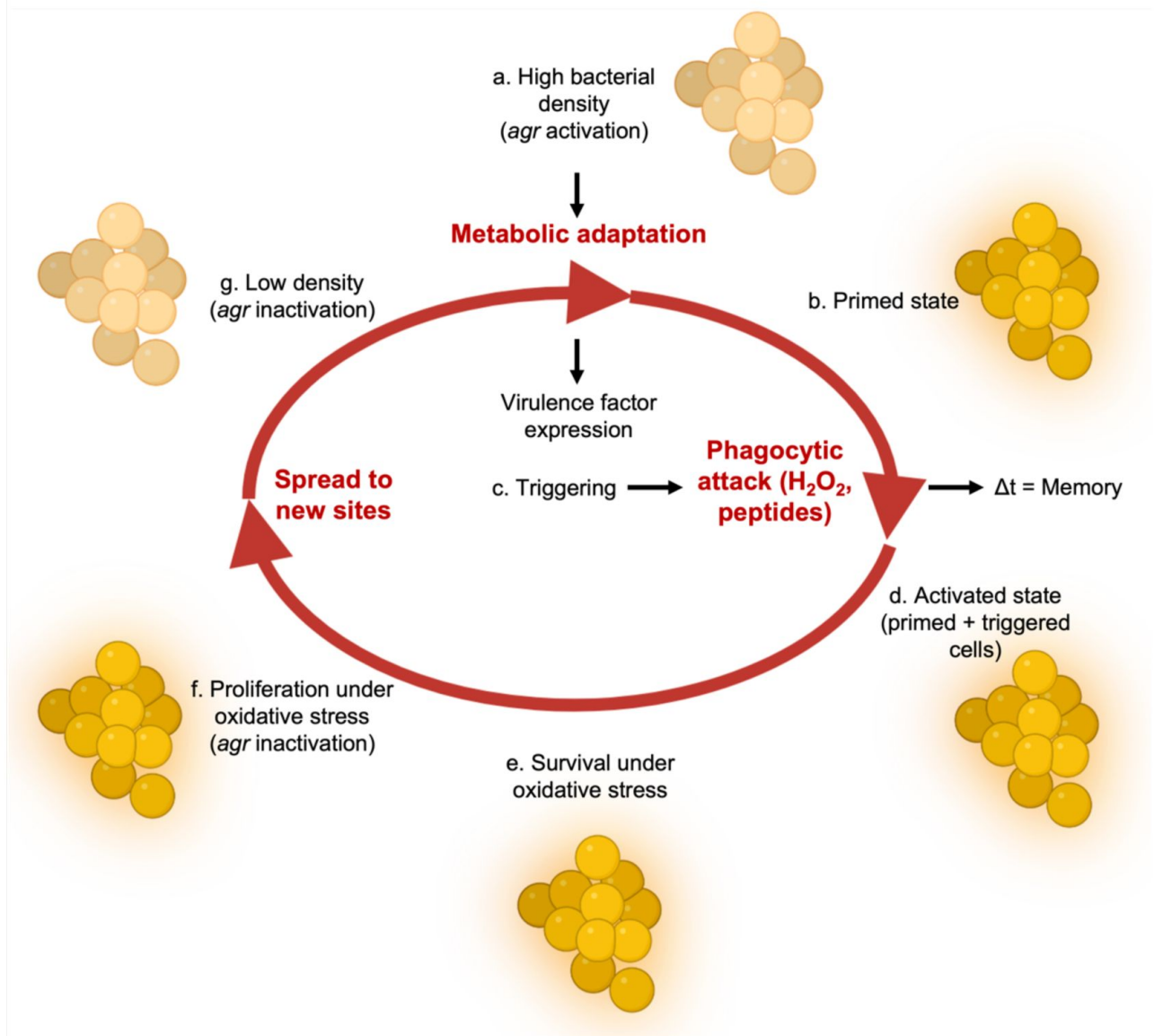


Figure 11.

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) (68). (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 (69), 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.

Strain	Background	Relevant Genotype	Reference or Source
BS819	LAC	<i>agr</i> group I wild-type (CC8), Erm ^S	(70)
BS1348	BS819	<i>agr::tetM</i>	(12)
BS820	BS819	<i>agr::ermC</i>	(12)
BS821	BS819	<i>rnall::cadA</i>	(71)
BS12	Newman	<i>agr</i> group I wild-type (CC8)	(72)
BS13	BS12	<i>agr::tet</i>	(13)
BS669	BS12	<i>rnall::cadA</i>	(12)
BS39	BS39 clinical strain	<i>agr</i> (+) clinical isolate (CC45)	(73)
BS40	BS40 clinical strain	<i>agr</i> (-) clinical isolate (CC45)	(73)
BS867	JE2	<i>agr</i> group I wild-type (CC8)	(56)
BS1010	BS867	<i>agr::cadA</i> in <i>S. aureus</i> JE2	This study
BS1280	BS12	<i>saeQRS::spec</i>	(74)
BS1282	BS12	<i>agr::tetM</i> , <i>saeQRS::spec</i>	(74)
BS653	<i>E. coli</i>	Top10 with pJC1111 (amp ^R in <i>E. coli</i> ; cd ^R in <i>S. aureus</i>)	(57)
BS656	RN4220	RN4220 with pRN7023 [shuttle vector (amp ^R in <i>E. coli</i> ; Cm ^R in <i>S. aureus</i>) containing SaPI1 <i>intI</i>]	(57)
BS435	RN6734	<i>agr</i> group-I prototype strain, derivative of NCTC 8325	(75)
BS688	BS435	<i>agr::cadA</i>	This study
GAW130	BS435	<i>agr::cadA</i> , SaPI1 <i>attC::pGAW98</i> (<i>agr</i> -I Δ <i>agrBD</i>)	This study
GAW183	BS435	<i>rnall::cad</i>	This study
BS450	MW2	<i>agr</i> group I wild-type (CC1)	(76)
BS451	MW2	<i>agr::tetM</i>	This study
BS988	126a	<i>agr</i> (+) clinical isolate (CC5)	(73)
BS989	127b	<i>agr</i> (-) clinical isolate (CC5)	(73)
BS842	BS819	BS820 with SaPI- <i>attC::agr</i> -IpJC1111 (<i>agr</i> -I, 8325-4)	(12)
BS1301	BS819	<i>rot::Tn917</i>	This study
BS1302	BS819	<i>agr::tetM</i> , <i>rot::Tn917</i>	This study
BS1279	BS12	<i>rot::Tn917</i>	(74)
BS1281	BS12	<i>agr::tetM</i> , <i>rot::Tn917</i>	(74)
VJT14.28	BS12	<i>pOS1-Plgt-sodARBS-rot</i>	(74)

Table 1.

Bacterial strains^a

BS1486	BS819	<i>ahpC::bursa</i> (NE911)	This study (56)
BS1487	BS819	<i>agr::tetM, ahpC::bursa</i>	This study (56)
BS1488	BS819	<i>katA::bursa</i> (NE1366)	This study (56)
BS1489	BS819	<i>agr::tetM, katA::bursa</i>	This study (56)
BS1399	BS12	<i>sodA::tetM, sodM::ermC</i>	(77)
BS1422	BS819	<i>sodA::tetM</i>	This study
BS1423	BS819	<i>agr::ermC, sodA::tetM</i>	This study
BS1435	BS819	<i>sigB</i> clean deletion	(78)
BS1436	BS819	<i>agr::tet, sigB</i> clean deletion	(78)
BS1246	BS12	<i>mgrA::cat</i>	(79)
BS999	BS819	BS819 with SaPI-1 <i>attC::pGYlux</i> (vector containing promoterless <i>lux</i>)	(80)
BS1222	BS819	BS819 with SaPI-1 <i>attC::Pagrp3-lux</i>	(81)
BS1518	BS12	<i>agr::tet, mgrA::cat</i>	This study
BS1527	BS867	<i>bshC::bursa</i> (NE230)	(56)
BS1528	BS867	<i>agr::tetM, bshC::bursa</i>	This study
BS1522	BS867	<i>gpxA2::bursa</i> (NE563),	(56)
BS1523	BS867	<i>agr::tetM, gpxA2::bursa</i>	This study
BS1490	BS819	<i>bsaA::bursa</i> (NE1730)	This study
BS1491	BS819	<i>bsaA::bursa, agr::tetM</i>	This study
BS1707	BS819	BS1422 with SaPI- <i>attC::P_{sarA}-sodRBS-sodA</i>	This study
BS1708	BS819	BS1348 with SaPI- <i>attC::P_{sarA}-sodRBS-sodA</i>	This study
BS1494	BS867	<i>ahpF::bursa</i> (NE1571)	(56)
BS1504	BS867	<i>agr::tetM, ahpF::bursa</i>	This study
BS1495	BS867	SAUSA300_0377::bursa (NE725)	(56)
BS1501	BS867	<i>agr::tetM, SAUSA300_0377::bursa,</i>	This study
BS1496	BS867	SAUSA300_0378::bursa (NE537)	(56)
BS1502	BS867	<i>agr::tetM, SAUSA300_0378::bursa</i>	This study

^aAll bacterial strains are *S. aureus*, unless otherwise indicated. Abbreviations: CC, clonal complex; NEx, strain designation in the Nebraska Transposon Mutant Library (56).

Table 1. (continued)

#	Name	Gene/Target	Sequence 5' → 3'	Source
1	pflBRT.1a	<i>pflB</i>	AAAAATGGAAGATGGAACAGACAC	(82)
2	pflBRT.1b		TCGATAACTGCATTACTTGTTC	
3	pflART.1a	<i>pflA</i>	TGACAAACATATTAGATTGACAGGAAAGC	(83)
4	pflART.1b		ATCATCAGAATAACCAGGCACAAGG	
5	ldh2RT.1a	<i>ldh2</i>	GGATCTGTAGGATCAAGCTATGCC	(48)
6	ldh2RT.2b		TGGTGAAGGACTGTGGACTGTACC	
7	nrdGRT.1a	<i>nrdG</i>	CAGTGTTTATGTATCAGGATGTCC	(82)
8	nrdGRT.1b		GTTCGCCACCTAATAGACTTAGCC	
9	qoxB-RT.3A	<i>qoxB</i>	GTTGTA CTGGCATGTTCGCC	(84)
10	qoxB-RT.3B		GGCATTATGGTGCATCTTACC	
11	cydA-RT.1A	<i>cydA</i>	CATTTGATACATCTTCCCATGCC	(84)
12	cydA-RT.1B		ATCTGCTAAGAACTCAATAGTCC	
13	hmp-RT.1A	<i>hmp</i>	TGACTTTAGTGAATTTACACCAGG	(84)
14	hmp-RT.1B		CGTTTAACGCCAAAAGTTAAATGG	
15	spaRT1	<i>spA</i>	CAAACCTGGTCAAGAACTTGTTGTTG	(85)
16	spaRT2		GCTAATGATAATCCACCAAATACAGTTG	
17	clfB RT1	<i>clfA</i>	GGATAGGCAATCATCAAGCACAAG	(85)
18	clfB RT2		GCTATCTACATTGCGACTGTTTGTG	
19	ahp RT For	<i>ahpC</i>	CGTAAAAACCTGGCGAAGTAT	(86)
20	ahp RT Rev		TGCAATGTTTTAGCGCCTTCT	
21	kat RT For	<i>katA</i>	TGGTGTTTTTGGGCATCCA	(6)
22	kat RT Rev		CCCTAGGCCCTGCTGTCATA	
23	rpoB F	<i>rpoB</i>	GAACATGCAACGTCAAGCAG	(87)
24	rpoB R		AATAGCCGCACCAGAATCAC	

Table 2.

Oligonucleotides

25	MPsodA#1	<i>sodA</i>	AGGCGCGCCTTTATTTTGTTCATTATATAAT TCGTCAACTTTTTCCCAG	This study
26	MPsodA#2		GGATGATTATTTATGGCTTTTGAATTACCAAA ATTACCATACGC	This study
27	MPsodA#3	<i>PsarA</i>	TTCAAAAGCCATAAATAATCATCCTCCTAAGG TACCCGG	This study
28	MPsodA#4		GCGGCCGCTCTGATATTTTGGACTAAACCAA ATGCTAACCCAG	This study
29	MPsodA#5	pJC1111	AAAATATCAGAGCGGCCGCCAG	This study
30	MPsodA#6		ACAAAATAAAGGCGCGCCTATTCTAAATG	This study
31	pJC1111 FOR	pJC1111- <i>PsarA</i> - <i>sodRBS-sodA</i>	TGGCCTTTTGCTCACATGTTCTTTCCTGCGTT ATCCCCTGATTC	This study
32	pJC1111 REV		TGATATCAAAATTATACATGTCAACG	This study
33	GWO#27	<i>agrBD</i>	CAATTTTACACCACTCTCCTCACTGTCATTAT ACGATTTAG	This study
34	GWO#28		TAATTTAAATAGAGAGTGTGATAGTAGGTGG AATTATTAAATAG	This study
35	JCO#339	<i>agr</i> flanking regions	GGTACCTGAAGCGGGCGAGCGAG	This study
36	JCO#340		GGATCCGATAATAAAGTCAGTTAACGACGTA TTCAATTGTAAATCTTGTTGG	This study
37	JCO#342		CTCGAGAAGAAGGGATGAGTTAATCATCATT ATGAGAC	This study
38	JCO#343		GCATGCGATCTATCAAGGATGTGATGTTATG AAAGTCCAAATTTATCAATTACCG	This study

Table 2. (continued)

Construction of the $\Delta agrBD$ mutant: *S. aureus* $\Delta agrBD$ mutant GAW128 was generated by a chromosomal integration strategy outlined in Chen *et al.* (57) in an *agr*-null background strain, BS687. Plasmid pJC1111, a suicide plasmid containing a cadmium resistance (Cd^R) cassette and the *SaPII attS* site that enables single-copy insertion into the corresponding chromosomal *SaPII attC* site, was used as the backbone vector for the *S. aureus agrBD* construct. pJC1000 contains the RN6734 *agr* locus cloned into pUC18. Inverse PCR of pJC1000 was performed using *agrBD* primers GWO#27 and GWO#28, re-ligated following treatment with polynucleotide kinase, and designated pGAW98. The *SphI-EcoRI* fragment of pGAW98 was ligated into the *SaPII* integration vector pJC1111 and designated pGAW119. Strain RN9011 (RN4220 with pRN7023 [vector (Cm^R) containing *SaPII* integrase]) was electroporated with plasmid pGAW119 and plated on GL agar containing 0.1 mM $CdCl_2$. Phage 80a lysates of Cd^R colonies were used to transduce BS687 (RN6734 $\Delta agr::ermC$, *Erm*), generating GAW128 ($\Delta agrBD$).

To construct *agr* mutant BS687, *agr* flanking regions were amplified with primer pairs JCO#339, JCO#340, and JCO#342, JCO#343 and cloned into the *HincII* site of pUC18 to generate pJC1527 and pJC1528, respectively. pJC1530 was generated by four-way ligation of the *KpnI-BamHI* fragment of pJC1527 (*agr* left flank), *XhoI-SphI* fragment of pJC1528 (*agr* right flank), and *BamHI-XhoI* fragment from pJC1073 (*Erm* cassette) to *KpnI-SphI* digested pJC1202 (replacement vector). Plasmid pJC1530 was electroporated into strain RN4220 with selection on GL agar containing 10 $\mu g/mL$ of chloramphenicol at 30°C. Phage 80a lysates of Cm^R colonies were used to transduce strain JCSA18 (*rpsL** mutant of RN6734 that results in streptomycin resistance) and then allelic exchange of the $Em^R Sm^S Cm^R$ colonies was performed as previously described. Phage 80a lysates of $Em^R Sm^R Cm^S$ colonies were then used to transduce RN6734 with selection for Em^R , generating BS687.

sodA complementation: Plasmid *PsarA-sodA*-pJC1111, expressing *sodA* under the control of the constitutive promoter *PsarA*, was integrated into the *S. aureus* chromosome at the *SaPII attC* site of strain LAC (58), LAC *sodA::tetM*, and LAC *agr::ermC*. Complementation plasmid *PsarA-sodRBS-sodA* was generated by Gibson assembly and inserted into the *SaPII* integration vector pJC1111. Wild-type *sodA* and the *sarA* promoter were amplified from *S. aureus* gDNA using primers MPsodA#1-2 (*sodA* gene and RBS) and MPsodA#3-4 (*PsarA*). Primers MPsodA#5-6 were used to linearize pJC1111. Primers introduced relevant oligonucleotide overlaps that enabled Gibson assembly (6), generating *PsarA-sodRBS-sodA*. *PsarA-sodRBS-sodA* was transformed into *E. coli* DH5a for amplification, purification, and sequence validation via primers pJC1111 FOR and pJC1111 REV. Purified *PsarA-sodRBS-sodA* was electroporated into RN9011 and positive chromosomal integrants at the *SaPII* chromosomal attachment (*attC*) site were selected with 0.1 mM $CdCl_2$. Phage 80a lysates of positive integrants were used to transduce BS1422 (LAC *sod::tetM*) and BS1348 (LAC *agr::tetM*), generating BS1707 and BS1708, respectively.

Survival measurements

To measure lethal action, overnight cultures were diluted ($OD_{600} \sim 0.05$) in fresh medium and grown with shaking to early exponential ($OD_{600} \sim 0.15$) or late log ($OD_{600} \sim 4$) phase, conditions when *agr* expression is largely absent (12) or maximally activated, respectively. Early (undiluted) and late exponential phase cultures (diluted into fresh TSB medium to $OD_{600} \sim 0.15$) were incubated with H_2O_2 under aerobic conditions either at a fixed concentration for one or more time points or at various concentrations for a fixed time. At the end of treatment, aliquots were removed, concentrated by centrifugation and serially diluted in phosphate-buffered saline to remove H_2O_2 , and plated for determination of viable counts at 24 h. Percentage of survival was calculated relative to a sample taken at the time of H_2O_2 addition. When menadione and N-acetylcysteine were used to inhibit or potentiate killing by H_2O_2 , they were added prior to lethal treatments as described previously (14). For experiments involving menadione pretreatment, cultures were grown for 3.5 h, and menadione (40 mM solution in 96% EtOH, final concentration 80 μM) was added for the last 0.5 h of culture, preceding the H_2O_2 treatment at 4h. N-acetylcysteine was used to counter the action of menadione; it was added simultaneously with

menadione, at a final concentration of 30 mM (640 mM stock in sterile ddH₂O was used). All experiments were repeated at least three times; similar results were obtained from the biological replicates.

Measurement of excreted metabolites

Excreted metabolites were assayed in the supernatant fluids of bacterial cultures following centrifugation at 12,000 x g for 10 min for late exponential (OD₆₀₀~4) or overnight culture (20 h cultures), as indicated in figures. Aliquoted supernatants were stored at -80°C and thawed on ice prior to analysis. Cells were plated for determination of viable counts; L(+)-lactate and acetate concentrations were measured using commercially available colorimetric and fluorometric kits (cat. no. MAK065, MAK086), according to manufacturer's recommendations (MilliporeSigma, Burlington, MA).

Measurement of oxygen consumption

Overnight cultures were diluted into fresh TSB (OD₆₀₀~0.05), grown for 5 hours at 37°C with shaking at 180 rpm (OD₆₀₀~4), diluted (OD₆₀₀~0.025) in fresh TSB, and added to a microtiter plate (200 µL/well). Oxygen consumption rate (OCR) was measured using a Seahorse XF HS Mini Analyzer (Agilent, Santa Clara, CA) according to the manufacturer's instructions. The Seahorse XF sensor cartridge was hydrated in a non-CO₂ 37°C incubator with sterile water (overnight) and pre-warmed XF calibrant for 1 hour prior to measurement. OCR measurements were recorded in 15 measurement cycles with 3 minutes of measurement and 3 minutes of mixing per cycle. CFU were enumerated to confirm equal concentrations of *agr*-deficient mutant and wild-type cells

Measurement of ATP, NADH, NAD/NADH levels

Overnight cultures were diluted into fresh TSB (OD₆₀₀~0.05), grown for 5 hours at 37°C with shaking at 180 rpm (OD₆₀₀~4), diluted (OD₆₀₀~1.0) in fresh TSB, and incubated at room temperature with reagent for determination of ATP, NADH, and NAD/NADH using BacTiter-Glo Microbial Cell Viability Assay (Promega, Madison, WI), NAD/NADH-Glo Assay (Promega), and NAD(P)-Glo Detection (Promega) kits (cat. no G8231, G9071, G9061), respectively, according to the manufacturer's instructions. The amount of NADH in the *agr*-deficient mutant was calculated normalized to wild-type. Luminescence was detected using a SpectraMax i3x luminometer (Molecular Devices, San Jose, CA). The amount of ATP was calculated and normalized to cell numbers.

Measurement of baseline ROS levels

Overnight cultures were diluted into fresh TSB (OD₆₀₀~0.05), and grown with shaking to early exponential phase (OD₆₀₀~0.2). 200 µL of culture was removed and cell density was normalized before staining with carboxy-H2DCFDA fluorescent dye (final concentration 10 µM) (Invitrogen, Waltham, MA). Samples were incubated at room temperature for 5 minutes, then 800 µL of PBS + EDTA buffer (100 mM) was added to each sample, and ROS levels were measured by fluorescence-based flow cytometry (BD Fortessa, BD Biosciences, San Jose, CA). All tubes with cultures were wrapped with aluminum foil to avoid light. A sample containing LAC *agr* wild-type cells lacking carboxy-H2DCFDA was included as a control for auto-fluorescence. Forward and side scatter parameters were acquired with logarithmic amplification. ROS was detected using the 488 laser and a 530/30nm bandpass filter. Data were analyzed using FlowJo software version 10.8.1 (BD Biosciences, San Jose, CA).

Measurement of superoxide dismutase (SOD) activity

Overnight cultures were diluted (OD₆₀₀~0.05) into fresh TSB, grown to late exponential phase (OD₆₀₀~4), diluted to OD₆₀₀=1, centrifuged at 12,000 x g for 5 min, and the cell pellet was homogenized in 300 µL of ice-cold lysis buffer (100 mM Tris-HCl pH 7.4 + 0.5% Triton + 5 mM 2-

mercaptoethanol + 0.2 mM PMSF). SOD activity was measured using a commercially available kit (cat. no CS0009-1KT), according to manufacturers' instructions (MilliporeSigma, Burlington, MA). The experiment was repeated three times with similar results.

RNA sequencing and data analysis

Overnight cultures were diluted ($OD_{600} \sim 0.05$) into fresh TSB medium and grown at 37 °C to early exponential phase ($OD_{600} \sim 0.5$) (Δagr single mutant and $\Delta agr \Delta rot$ double mutant) or late exponential phase ($OD_{600} \sim 4$) (agr wild-type and Δagr strains).

Samples of Δagr and $\Delta agr \Delta rot$ were divided into two 3 mL aliquots, and the aliquots were incubated at 37 °C for another 30 min, with or without treatment with H_2O_2 . Peroxide concentrations for Δagr and $\Delta agr \Delta rot$ were normalized to expected killing at the time of harvest (Figure 7—figure supplement 1 [↗](#)).

Three independent cultures for each sample were used for determination of transcriptional profiles. Briefly, cultures were concentrated by centrifugation (12,000 x g for 5 min), and resuspended cells were disrupted using Lysing Matrix B tubes in a FastPrep-24 homogenizer (MP Biomedicals, Irvine, CA) at 6 M/s, for 30s, 3 times (samples were resting on ice between homogenizer runs), and RNAs were extracted from the collected bacterial cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA). RNA was isolated using RNeasy (Qiagen, Germantown, MD) mini spin columns. Sequence libraries were generated using the TruSeq Stranded Total RNA Library Prep kit (Illumina, San Diego, CA) following the manufacturer's recommendations. The rRNAs were removed by the Ribo-zero Kit (Illumina) to enrich mRNA, using 13 cycles of PCR amplification of the final library. Amplified libraries were purified using AMPure beads (Beckman Coulter, Brea, CA), quantified by Qubit (Thermo Fisher Scientific, Waltham, MA) and qPCR, and visualized in an Agilent Bioanalyzer (Santa Clara, CA). Pooled libraries were sequenced as paired-end 50-bp reads using an Illumina NovaSeq instrument.

Reads were initially trimmed using Trimmomatic version 0.39 ([59 ↗](#)) to remove adaptors as well as leading or trailing bases with a quality score less than 3, filtering reads with minimum length 36. Reads were mapped to reference strain USA300 FPR3757 (RefSeq identifier GCF_000013465.1) using Bowtie2 version 2.2.5 ([60 ↗](#)). Using gene annotations from the same assembly, reads mapped to each gene were counted with featureCounts version 2.0.1 ([61 ↗](#)), producing a counts matrix. Additional analysis was performed in R (R Core Team 2021) using the package DESeq2 version 1.32 ([62 ↗](#)).

Normalization to account for inter-sample library size variation was performed using the built-in normalization function of DESeq2. All RNA-seq heatmaps were colored according to row (gene) z-scores of DESeq2 normalized counts. For differential expression testing, the Wald test was used with a log-2 fold-change threshold of 0.5 and an FDR of 0.1. For simple pairwise comparisons (e.g., the effect of strain under control conditions), datasets were split so that analysis was performed independently for strains used in the comparison. To determine the interaction between strain and condition variables, all samples were included with the experimental design (formula “expression ~ condition + strain + condition:strain”, where condition:strain is the interaction between variables).

Metabolic flux prediction

The SPOT (Simplified Pearson cOrrelation with Transcriptomic data) computational method ([63 ↗](#)) was used to analyze the difference in intracellular metabolic fluxes between wild-type LAC and $agr::tetM$ mutant grown in TSB to late exponential phase ($OD_{600} \sim 4$). SPOT is similar to the E-Flux2 method described previously ([64 ↗](#)), but a recent validation study ([65 ↗](#)) shows that SPOT generally outperforms E-Flux2. SPOT infers metabolic flux distribution by integrating

transcriptomic data in a genome-scale metabolic model of *S. aureus* (66 [DOI](#)) that was adapted for use with strain LAC. For a list of the metabolic reactions ranked by unit of flux per 100 units of glucose uptake flux, see Supplementary file 2.

Real-time qRT-PCR assays

Briefly, RNA was purified as described above from late exponential (OD_{600} ~4.0) cells, cDNAs were synthesized using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA), and real-time reverse transcription quantitative PCR (qRT-PCR) was performed using QuantiNova™ SYBR Green PCR Kit (Qiagen, Hilden, Germany). Primers were synthesized by IDT Inc. (Coralville, IA). Three independent biological samples were run in triplicate and *rpoB* was used to normalize gene expression. $2^{-\Delta\Delta Ct}$ method was used to calculate the relative fold gene expression (67 [DOI](#)).

Peritoneal infection of mice

C57BL/6 mice and C57BL/6 *Cybb*^{-/-} (also known as gp91phox/nox2) were purchased from the Jackson Laboratory and bred onsite to generate animals for experimentation. Age and gender-matched, 8–10-week-old mice were used. *S. aureus* strains harboring *RNAIII* or *agrBD* deletion in the NCTC 8325 background were grown overnight in TSB (37°C, 180 rpm) separately or mixed at a 1:1 ratio. Overnight cultures were diluted (OD_{600} ~0.05) into fresh TSB medium (subcultured separately for the cultures mixed overnight (‘primed’) or mixed 1:1 for *RNAIII* or *agrBD* mutant single cultures (‘unprimed’) and grown at 37°C to early exponential phase (OD_{600} ~0.5)). Bacteria were washed one time by centrifugation with PBS and adjusted to 10⁹ CFU/mL. Twenty C57BL/6 WT mice and 17 *Cybb*^{-/-} mice were injected intraperitoneally with 100 μ L of either ‘primed’ or ‘unprimed’ inoculum. After 2 h, internal organs, peritoneal lavage and blood were collected. The organs were homogenized in sterile PBS and serial dilutions were plated for viable counts on TS agar. Collected blood was lysed with saponin and plated for viable counts on TSA plates. Peritoneal lavage fluid was serially diluted and plated for viable counts. All animal studies were performed as per an NYU Grossman School of Medicine Institutional Animal Care and Use Committee (IACUC) approved protocol to the Shopsin Lab.

Statistical analysis

Prism software (GraphPad, Inc.) was used to perform statistical analyses. Statistical significance was determined using the Student *t* test, Mann–Whitney *U* test, one-way analysis of variance (ANOVA), or the Kruskal–Wallis test, depending on the data type. Statistical significance was considered to be represented by *P* values of <0.05.

Data availability

The RNA-seq data are available through the NCBI GEO repository using the accession number GSE207045.

Author Contributions

M.P., Conceptualization, Data curation, Formal analysis, Investigation, Visualization,

Methodology, Writing - original draft and review and editing

A.I.P, Investigation, Writing - review and editing

G.P, Data curation, Formal analysis, Visualization, Methodology, Writing - review and editing

A.P., Data curation, Formal analysis, Methodology, Writing - review and editing

J.K., Investigation, Visualization, Writing - review and editing

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R.J.U., Investigation, Writing - review and editing

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J.N.W., Resources, Writing - review and editing

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D.S.L., Formal analysis, Visualization, Writing – review and editing

D.P., Supervision, Writing – review and editing

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X.Z., Conceptualization, Writing – review and editing

K.D., Conceptualization, Writing – original draft and review and editing

I.Y., Supervision, Funding acquisition

V.J.T., Resources, Supervision, Funding acquisition

B.S., Conceptualization, Resources, Supervision, Funding acquisition, Project Administration, Writing – original draft and review and editing

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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.

Figures and Figure supplements

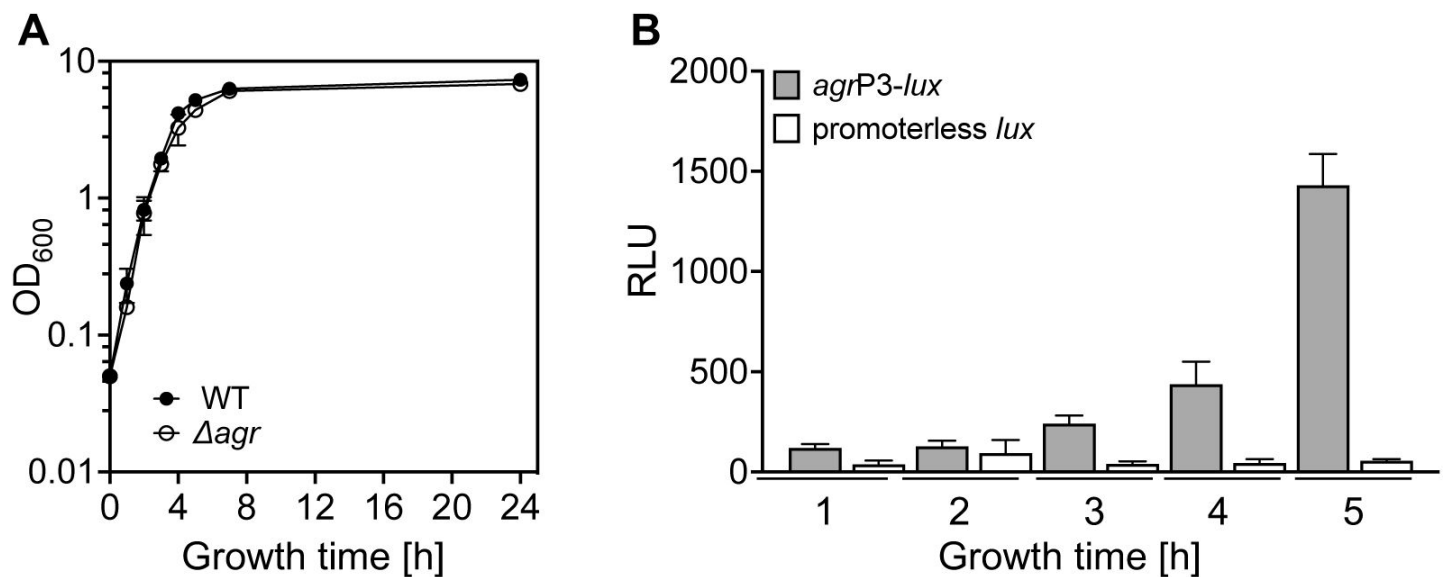


Figure 1—figure supplement 1.

Correlation of growth phase and *agr* expression.

(A) Growth curves. Overnight cultures of *S. aureus* LAC *agr* wild-type (WT, BS819) or Δagr mutant (BS1348) were diluted ($OD_{600} \sim 0.05$) in fresh TSB medium and growth was monitored by measuring the optical density at 600 nm (OD_{600}). (B) Tests of *agrP3* promoter activity. *S. aureus* LAC *agr* wild-type (WT, BS819) containing *agrP3-lux* (SaPI1 *attC::agrP3-lux*; strain BS1222) or control containing a promoterless *lux* gene within the *attC* site (SaPI1 *attC::pGyLux*, strain BS999) grown as in (A) for the indicated times. *agrP3* activity (relative luminescence units [RLU]) was assayed at the indicated times (see Materials and Methods). Data represent the means $\pm$ S.D. from biological replicates ($n = 3$).

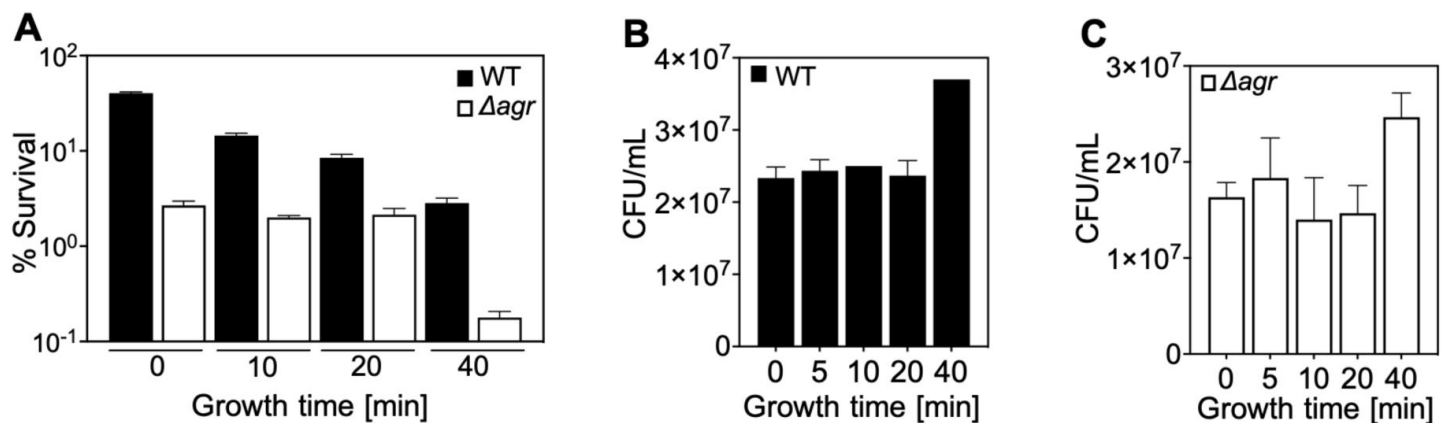


Figure 1—figure supplement 2.

Correlation of lag-time and *agr*-mediated protection from H₂O₂-mediated killing.

Overnight cultures of *S. aureus* LAC *agr* wild-type (WT, BS819) and Δagr mutant (BS1348), grown for the indicated times following dilution to fresh medium, were treated with H₂O₂ (20 mM for 60 min) (Fig. S1A). Data represent the means $\pm$ S.D. from biological replicates ($n = 3$). Survival of Δagr mutant cells was unchanged up to the 40 min time point, and then it dropped sharply. The sharp drop coincided with the time to first division (i.e., the lag time), as evidenced by an increase in colony-forming units (CFUs) at the 40 min time point in the absence of treatment (Fig. S1B and C). In contrast to results with the Δagr strain, survival of the *agr* wild-type strain gradually decreased throughout the experiment (Fig. S1A). Increased lag-time is associated with tolerance to lethal stress owing to a delay in growth when switched to a new environment (15). Thus, our observations suggest that a subpopulation of Δagr mutant cells remains longer in a dormant state, decreasing the lethality of H₂O₂. The differential effect of the lag time on the *agr* wild-type and Δagr mutant cultures was absent during exponential growth (40 min). These results suggest that *agr* contributes to at least two forms of protection from H₂O₂-mediated killing: tolerance by a transient lag state and tolerance during growth phase. To focus on the latter form, assays involving cultures after overnight growth were grown for ~65 min (OD₆₀₀~0.15).

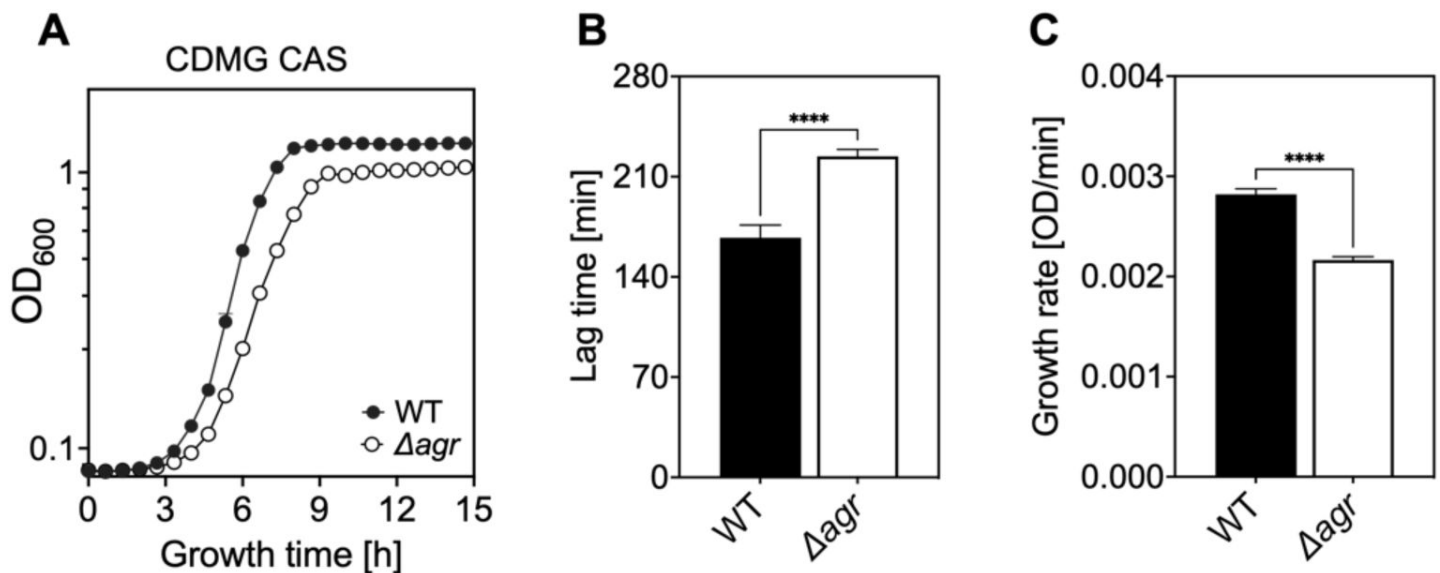


Figure 1—figure supplement 3.

Extended lag phase and decreased growth rate and yield of an Δagr mutant.

(A) Growth curves. *S. aureus* LAC *agr* wild-type (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 OD₆₀₀ 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. Corresponding lag times and growth rates are displayed in panels (B) and (C), respectively. Data represent the means $\pm$ S.D. from biological replicates ($n = 3$). Statistical significance was calculated with a Student's two-tailed t test (****, $P \leq 0.0001$).

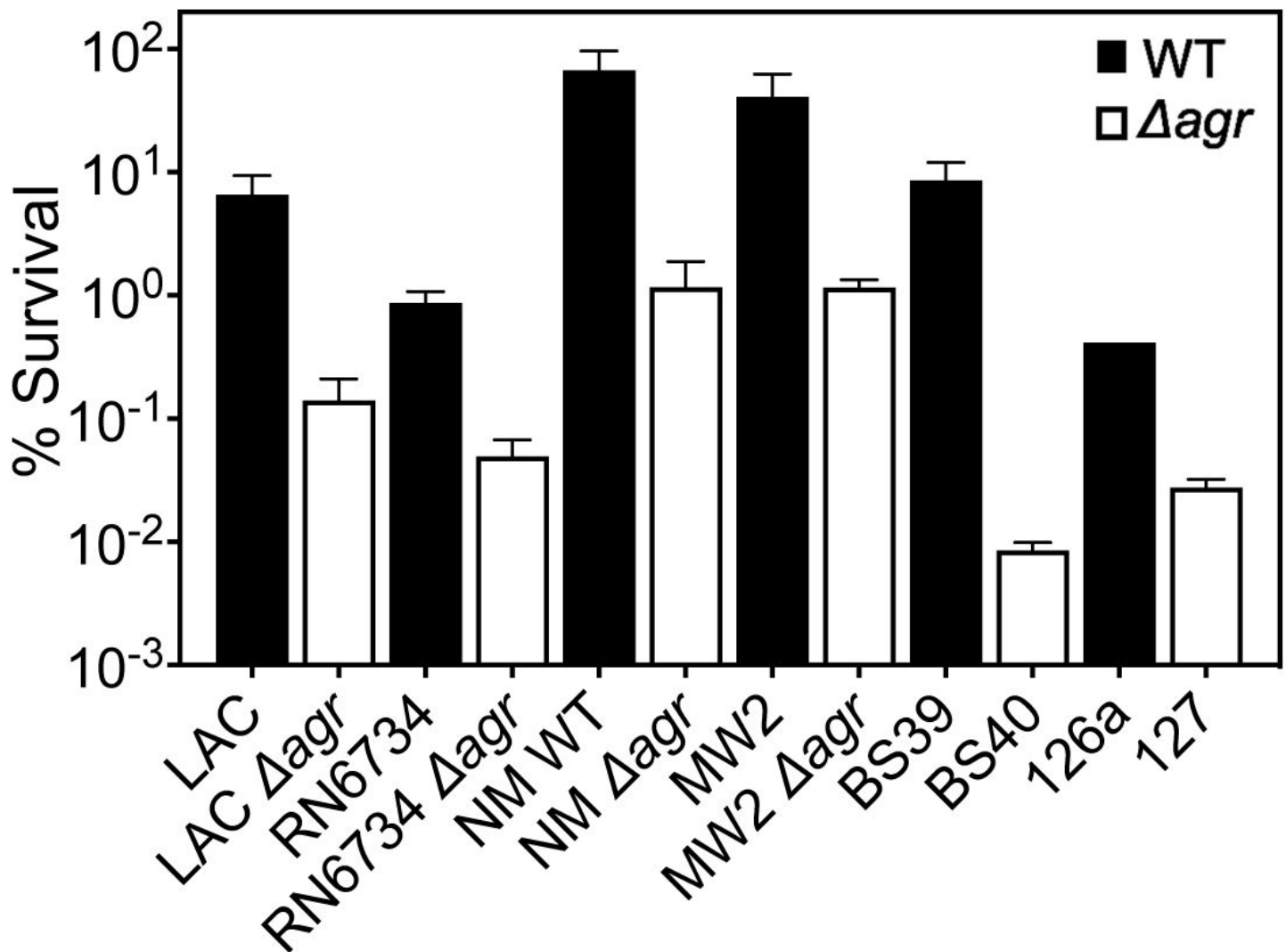


Figure 1—figure supplement 4.

Agr-mediated protection from H₂O₂-mediated killing among diverse *S. aureus* strains.

Laboratory strains LAC, RN6734, Newman (NM, BS12), MW2 (BS450), and clinical isolates BS39 and 126a with *agr* deficient mutant derivatives were compared for survival following treatment with 20 mM of H₂O₂ for 60 min. In this experiment overnight cultures were diluted in TSB and grown to early log phase (OD₆₀₀~0.15). Percent survival was determined relative to samples taken at the time of peroxide treatment. Some mutants were created by transduction of marker-disrupted alleles (LAC, RN6734, Newman, MW2) while others were naturally occurring (BS40, 127) (see Table 1). Data represent the means ± S.D. from biological replicates (*n* = 3).

The data show that peroxide lethality varies among strains, but in each case deletion of *agr* increases killing.

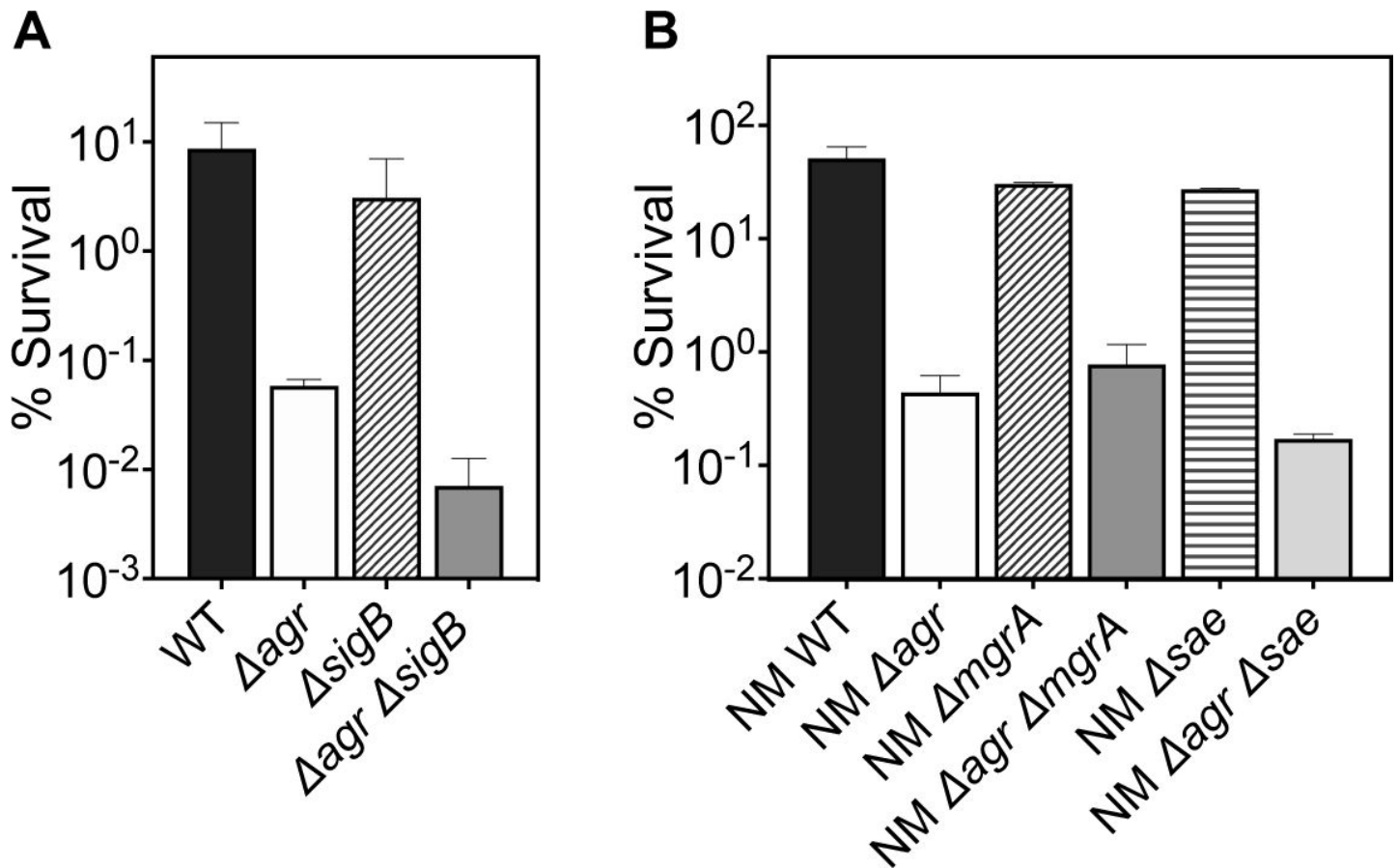


Figure 2—figure supplement 1.

Deficiency of downstream global regulators does not differentially affect *agr*-mediated protection from H₂O₂-mediated cell death.

The effect of (A) *sigB*, (B) *mgrA* and *sae* on survival in the presence or absence of *agr* during treatment with H₂O₂ was measured. Cells were grown to early log phase (OD₆₀₀~0.15) and treated with 20 mM of H₂O₂ for 60 min. Data represent the means ± S.D. from biological replicates (*n* = 3). Bacterial strains were BS819 (LAC) and BS12 (Newman, NM) for WT and BS1348 (LAC) and BS13 (NM) for the *agr*, and BS BS1435-36, BS1280, BS1282 and BS1246, BS1518 for *sigB*, *sae* and *mgrA* mutants, respectively.

The genes tested were either part of known two-component systems or SarA protein-family regulatory circuits involved in virulence gene expression. They are all downstream/epistatic to *agr*-RNAIII (reviewed in (17)). Mutations in *sigB*, *mgrA*, and *sae* showed little or no effect with respect to the protective *agr*-mediated phenotype. These results support the idea that *rot* is the primary regulator pathway that protects the *agr* wild-type from H₂O₂-mediated killing.

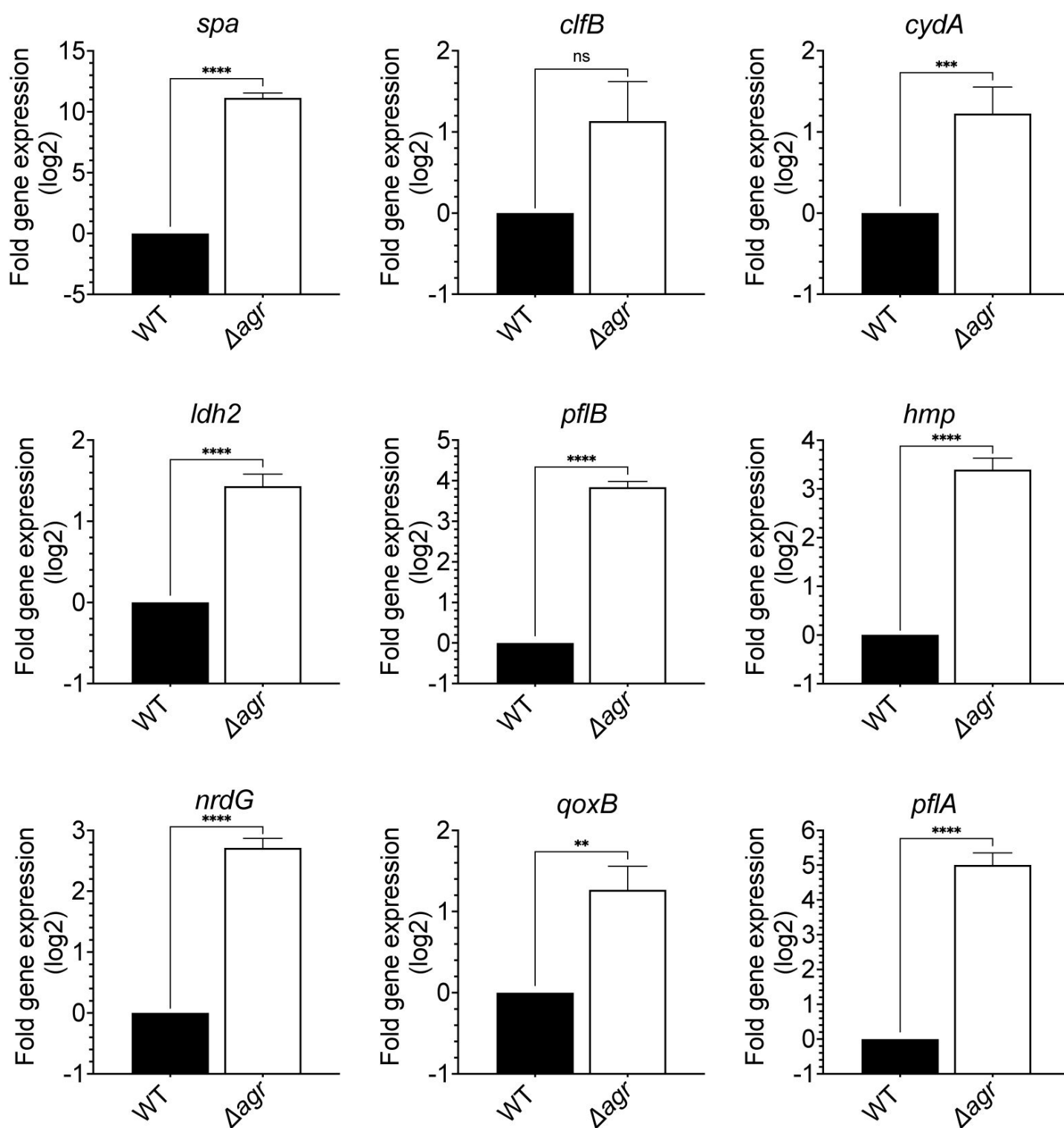


Figure 4—figure supplement 1.

Induction of expression of selected fermentive/anaerobic genes stimulated by deletion of *agr*.

Total cellular RNA was extracted from late exponential phase cultures ($OD_{600} \sim 4.0$) of *agr* wild-type (BS819) or Δagr mutant (BS1348), followed by reverse transcription and PCR amplification of the indicated genes from Fig. 5A, using *rpoB* as an internal standard. mRNA levels were normalized to those of each gene with an untreated wild-type control. Data represent the mean $\pm$ SEM of three independent experiments. Student's t test was used to determine statistical differences between samples (** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$). In each case the *agr* deletion increased expression, indicating elevated metabolism.

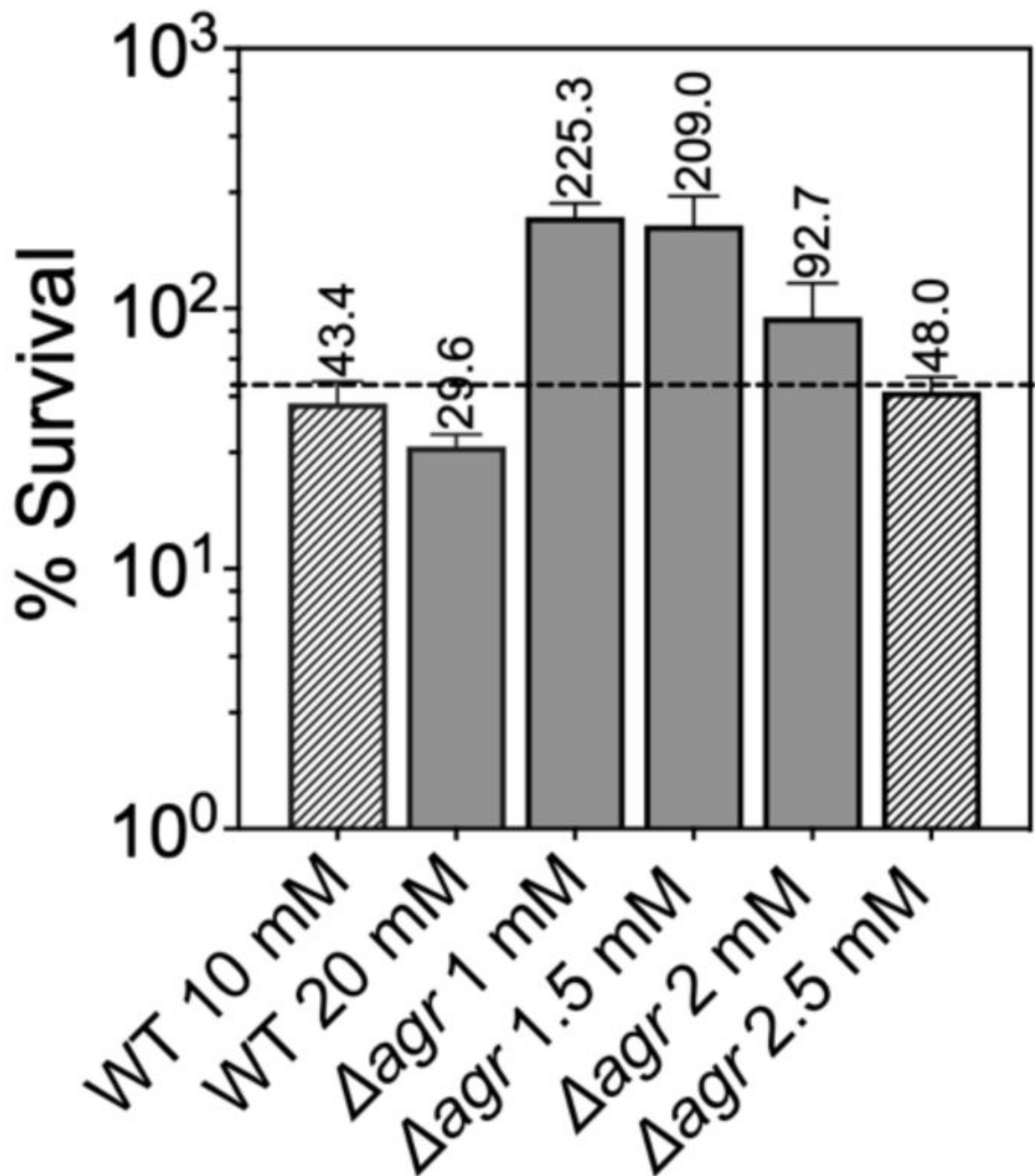


Figure 7—figure supplement 1.

Normalization of the lethal concentration of H₂O₂ with *agr* wild-type and *Δagr* 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 H₂O₂ 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 H₂O₂ concentrations and thereby lethality to achieve ~50% (dotted line) cell survival, normalized to *agr* wild-type and *Δagr* mutant strains. Survival of the *Δagr* mutant with H₂O₂ for 30 min at a concentration of 2.5 mM closely approximated 50% survival of the *agr* wild-type with 10 mM H₂O₂, providing a basis for choice of concentrations and treatment time for RNA-seq analysis.

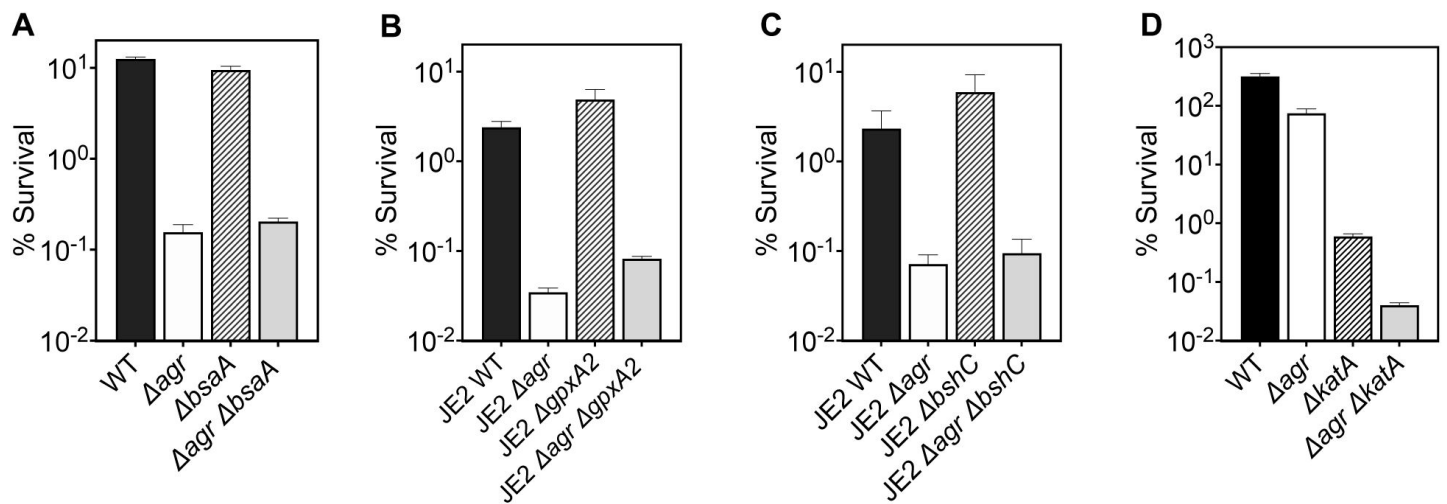


Figure 8—figure supplement 1.

Deficiency in ROS detoxification genes (*katA*, *bsaA1/gpxA1*, *bsaA2/gpxA2*, and bacilliothiol (BSH)) have no effect on *agr*-mediated protection from H₂O₂-mediated cell death. Effect of (A) *bsaA* (B) *gpxA2*, (C) *bshC* and (D) *katA* on survival during treatment with H₂O₂. Cells were grown to early log phase (OD₆₀₀~0.15) and treated with 20 mM of H₂O₂ for 60 min for A-C or with 2 mM of H₂O₂ 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 H₂O₂ stress. Mutations in additional genes involved in H₂O₂ detoxification that included catalase, (*katA*), two thiol-dependent peroxidases (*gpxA1* and *gpxA2*), and the low-molecular-weight thiol bacilliothiol (*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 H₂O₂-mediated growth inhibition (7). The *ΔkatA* mutation was hyperlethal with the wild-type and *Δagr* mutant, even when otherwise sub-inhibitory concentrations of H₂O₂ were used. Collectively, the data support the idea that *agr*-mediated phenotypes are detoxification pathway-specific.

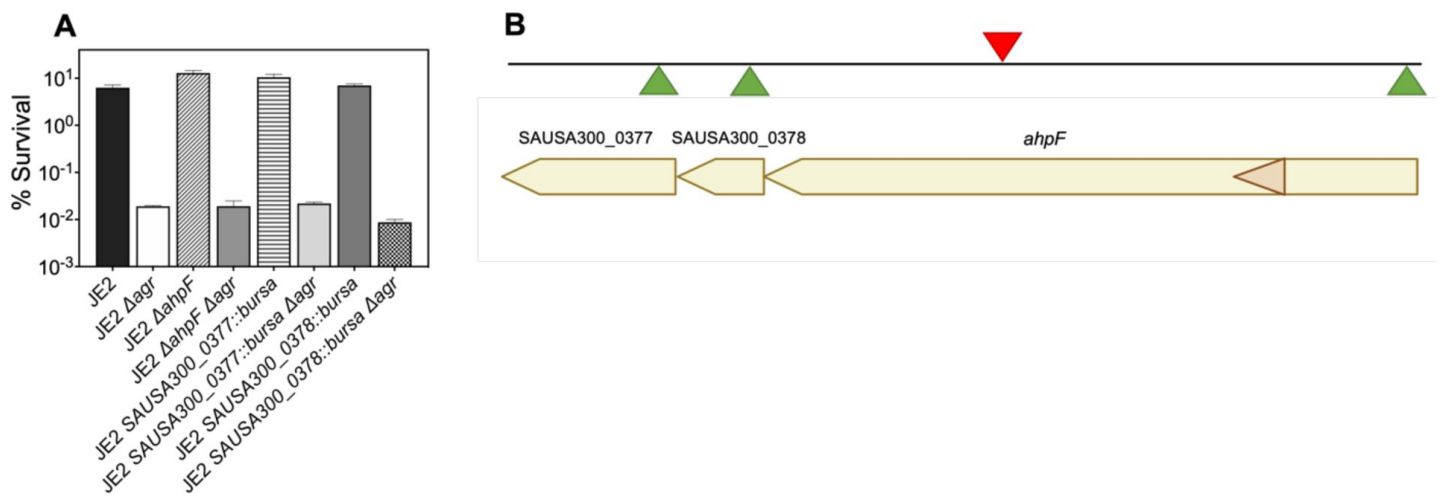


Figure 8—figure supplement 2.

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) (56) used in this study are indicated by triangles. Green triangles, plus-strand insertion; red triangle, minus-strand insertion. Data represent the means $\pm$ 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 *ahpC*-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.

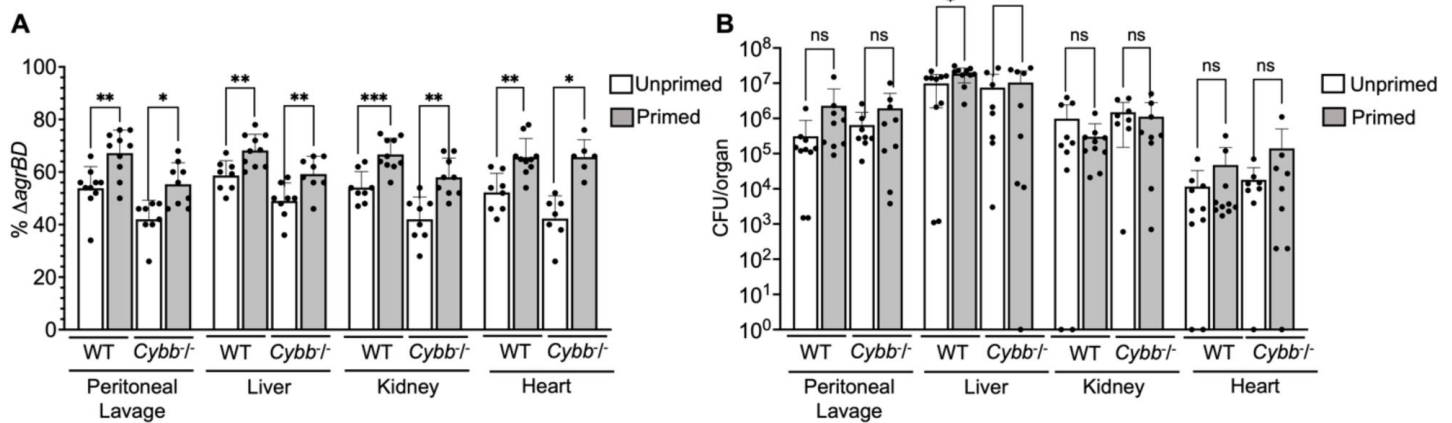


Figure 9—figure supplement 1.

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 $\Delta agrBD$ or (B) CFU of *S. aureus* RN6734 $\Delta rnaIII$ (GAW183) and $\Delta agrBD$ 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 *agr* 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 (OD₆₀₀~0.15), and used as inoculum for intraperitoneal infection with 1×10^8 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.

References

1. Spaan AN, Surewaard BG, Nijland R, van Strijp JA (2013) **Neutrophils versus Staphylococcus aureus: a biological tug of war** *Annu Rev Microbiol* **67**:629–650
2. Drlica K, Zhao X (2021) **Bacterial death from treatment with fluoroquinolones and other lethal stressors** *Expert review of anti-infective therapy* **19**:601–618
3. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ (2007) **A common mechanism of cellular death induced by bactericidal antibiotics** *Cell* **130**:797–810
4. Lobritz MA, Belenky P, Porter CB, Gutierrez A, Yang JH, Schwarz EG, Dwyer DJ, Khalil AS, Collins JJ (2015) **Antibiotic efficacy is linked to bacterial cellular respiration** *Proc Natl Acad Sci U S A* **112**:8173–8180
5. Shatalin K, Nuthanakanti A, Kaushik A, Shishov D, Peselis A, Shamovsky I, Pani B, Lechpammer M, Vasilyev N, Shatalina E, Rebatchouk D, Mironov A, Fedichev P, Serganov A, Nudler E (2021) **Inhibitors of bacterial H(2)S biogenesis targeting antibiotic resistance and tolerance** *Science* **372**:1169–1175
6. Shee S, Singh S, Tripathi A, Thakur C, Kumar TA, Das M, Yadav V, Kohli S, Rajmani RS, Chandra N, Chakrapani H, Drlica K, Singh A (2022) **Moxifloxacin-mediated killing of Mycobacterium tuberculosis involves respiratory downshift, reductive stress, and accumulation of reactive oxygen species** *Antimicrob Agents Chemother* **66**
7. Sun F, Liang H, Kong X, Xie S, Cho H, Deng X, Ji Q, Zhang H, Alvarez S, Hicks LM, Bae T, Luo C, Jiang H, He C (2012) **Quorum-sensing agr mediates bacterial oxidation response via an intramolecular disulfide redox switch in the response regulator AgrA** *Proc Natl Acad Sci U S A* **109**:9095–9100
8. Khan BA, Yeh AJ, Cheung GY, Otto M (2015) **Investigational therapies targeting quorum-sensing for the treatment of Staphylococcus aureus infections** *Expert Opin Investig Drugs* **24**:689–704
9. Novick RP (2003) **Autoinduction and signal transduction in the regulation of staphylococcal virulence** *Mol Microbiol* **48**:1429–1449
10. Novick RP, Geisinger E (2008) **Quorum sensing in staphylococci** *Annu Rev Genet* **42**:541–564
11. Novick RP, Projan SJ, Kornblum J, Ross HF, Ji G, Kreiswirth B, Vandenesh F, Moghazeh S (1995) **The agr P2 operon: an autocatalytic sensory transduction system in Staphylococcus aureus** *Mol Gen Genet* **248**:446–458
12. Kumar K, Chen J, Drlica K, Shopsis B (2017) **Tuning of the lethal response to multiple stressors with a single-site mutation during clinical infection by Staphylococcus aureus** *mBio* **8**
13. Geisinger E, Chen J, Novick RP (2012) **Allele-dependent differences in quorum-sensing dynamics result in variant expression of virulence genes in Staphylococcus aureus** *J Bacteriol* **194**:2854–2864

14. Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, Zalis EA, Clair G, Adkins JN, Cheung AL, Lewis K (2016) **Persister formation in *Staphylococcus aureus* is associated with ATP depletion** *Nat Microbiol* **1**
15. Fridman O, Goldberg A, Ronin I, Shores N, Balaban NQ (2014) **Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations** *Nature* **513**:418–421
16. Geisinger E, Adhikari RP, Jin R, Ross HF, Novick RP (2006) **Inhibition of rot translation by RNAIII, a key feature of agr function** *Mol Microbiol* **61**:1038–1048
17. Bronesky D, Wu Z, Marzi S, Walter P, Geissmann T, Moreau K, Vandenesch F, Caldelari I, Romby P (2016) ***Staphylococcus aureus* RNAIII and its regulon link quorum sensing, stress responses, metabolic adaptation, and regulation of virulence gene expression** *Annu Rev Microbiol* **70**:299–316
18. Fuchs S, Pane-Farre J, Kohler C, Hecker M, Engelmann S (2007) **Anaerobic gene expression in *Staphylococcus aureus*** *J Bacteriol* **189**:4275–4289
19. Pagels M, Fuchs S, Pane-Farre J, Kohler C, Menschner L, Hecker M, McNamarra PJ, Bauer MC, von Wachenfeldt C, Liebeke M, Lalk M, Sander G, von Eiff C, Proctor RA, Engelmann S. (2010) **Redox sensing by a Rex-family repressor is involved in the regulation of anaerobic gene expression in *Staphylococcus aureus*** *Mol Microbiol* **76**:1142–1161
20. Somerville GA, Beres SB, Fitzgerald JR, DeLeo FR, Cole RL, Hoff JS, Musser JM (2002) **In vitro serial passage of *Staphylococcus aureus*: changes in physiology, virulence factor production, and agr nucleotide sequence** *J Bacteriol* **184**:1430–1437
21. Sadykov MR, Thomas VC, Marshall DD, Wenstrom CJ, Moormeier DE, Widhelm TJ, Nuxoll AS, Powers R, Bayles KW (2013) **Inactivation of the Pta-AckA pathway causes cell death in *Staphylococcus aureus*** *J Bacteriol* **195**:3035–3044
22. Somerville GA, Chaussee MS, Morgan CI, Fitzgerald JR, Dorward DW, Reitzer LJ, Musser JM (2002) ***Staphylococcus aureus* aconitase inactivation unexpectedly inhibits post-exponential-phase growth and enhances stationary-phase survival** *Infect Immun* **70**:6373–6382
23. Hochgrafe F, Wolf C, Fuchs S, Liebeke M, Lalk M, Engelmann S, Hecker M (2008) **Nitric oxide stress induces different responses but mediates comparable protein thiol protection in *Bacillus subtilis* and *Staphylococcus aureus*** *J Bacteriol* **190**:4997–5008
24. Stevanin TM, Ioannidis N, Mills CE, Kim SO, Hughes MN, Poole RK (2000) **Flavohemoglobin Hmp affords inducible protection for *Escherichia coli* respiration, catalyzed by cytochromes bo' or bd, from nitric oxide** *J Biol Chem* **275**:35868–35875
25. Posada AC, Kolar SL, Dusi RG, Francois P, Roberts AA, Hamilton CJ, Liu GY, Cheung A (2014) **Importance of bacillithiol in the oxidative stress response of *Staphylococcus aureus*** *Infect Immun* **82**:316–332
26. Rowe SE, Wagner NJ, Li L, Beam JE, Wilkinson AD, Radlinski LC, Zhang Q, Miao EA, Conlon BP (2020) **Reactive oxygen species induce antibiotic tolerance during systemic *Staphylococcus aureus* infection** *Nat Microbiol* **5**:282–290

27. Clements MO, Watson SP, Foster SJ (1999) **Characterization of the major superoxide dismutase of *Staphylococcus aureus* and its role in starvation survival, stress resistance, and pathogenicity** *J Bacteriol* **181**:3898–3903
28. Gaupp R, Ledala N, Somerville GA (2012) **Staphylococcal response to oxidative stress** *Frontiers in cellular and infection microbiology* **2**
29. Imlay JA (2008) **Cellular defenses against superoxide and hydrogen peroxide** *Annu Rev Biochem* **77**:755–776
30. Cosgrove K, Coutts G, Jonsson IM, Tarkowski A, Kokai-Kun JF, Mond JJ, Foster SJ (2007) **Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*** *J Bacteriol* **189**:1025–1035
31. Seaver LC, Imlay JA (2001) **Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in *Escherichia coli*** *J Bacteriol* **183**:7173–7181
32. Pollock JD, Williams DA, Gifford MA, Li LL, Du X, Fisherman J, Orkin SH, Doerschuk CM, Dinuer MC. (1995) **Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production** *Nature genetics* **9**:202–209
33. Yipp BG, Kim JH, Lima R, Zbytnuik LD, Petri B, Swanlund N, Ho M, Szeto VG, Tak T, Koenderman L, Pickkers P, Tool ATJ, Kuijpers TW, van den Berg TK, Looney MR, Krummel MF, Kubes P (2017) **The lung is a host defense niche for immediate neutrophil-mediated vascular protection** *Sci Immunol* **2**
34. Beavers WN, DuMont AL, Monteith AJ, Maloney KN, Tallman KA, Weiss A, Christian AH, Toste FD, Chang CJ, Porter NA, Torres VJ, Skaar EP (2021) ***Staphylococcus aureus* peptide methionine sulfoxide reductases protect from human whole blood killing** *Infect Immun* **89**
35. Zeng J, Hong Y, Zhao N, Liu Q, Zhu W, Xiao L, Wang W, Chen M, Hong S, Wu L, Xue Y, Wang D, Niu J, Drlica K, Zhao X (2022) **A broadly applicable, stress-mediated bacterial death pathway regulated by the phosphotransferase system (PTS) and the cAMP-Crp cascade** *Proc Natl Acad Sci U S A* **119**
36. Morfeldt E, Taylor D, von Gabain A, Arvidson S. (1995) **Activation of alpha-toxin translation in *Staphylococcus aureus* by the trans-encoded antisense RNA, RNAIII** *EMBO J* **14**:4569–4577
37. Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S (1993) **Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule** *EMBO J* **12**:3967–3975
38. Takahashi N, Gruber CC, Yang JH, Liu X, Braff D, Yashaswini CN, Bhuhhanil S, Furuta Y, Andreescu S, Collins JJ, Walker GC (2017) **Lethality of MalE-LacZ hybrid protein shares mechanistic attributes with oxidative component of antibiotic lethality** *Proc Natl Acad Sci U S A* **114**:9164–9169
39. George SE, Hrubesch J, Breuing I, Vetter N, Korn N, Hennemann K, Bleul L, Willmann M, Ebner P, Gotz F, Wolz C (2019) **Oxidative stress drives the selection of quorum sensing mutants in the *Staphylococcus aureus* population** *Proc Natl Acad Sci U S A* **116**:19145–19154

40. Queck SY, Jameson-Lee M, Villaruz AE, Bach TH, Khan BA, Sturdevant DE, Ricklefs SM, Li M, Otto M (2008) **RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*** *Mol Cell* **32**:150–158
41. Wang R, Braughton KR, Kretschmer D, Bach T-HL, Queck SY, Li M, Kennedy AD, Dorward DW, Klebanoff SJ, Peschel A, DeLeo FR, Otto M (2007) **Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA** *Nat Med* **13**:1510–1514
42. Altman DR, Sullivan MJ, Chacko KI, Balasubramanian D, Pak TR, Sause WE, Kumar K, Sebra R, Deikus G, Attie O, Rose H, Lewis M, Fulmer Y, Bashir A, Kasarskis A, Schadt EE, Richardson AR, Torres VJ, Shopsin B, van Bakel H. (2018) **Genome plasticity of agr-defective *Staphylococcus aureus* during clinical infection** *Infect Immun*
43. Raghuram V, Alexander AM, Loo HQ, Petit RA, Goldberg JB, Read TD (2022) **Species-wide phylogenomics of the *Staphylococcus aureus* Agr operon revealed convergent evolution of frameshift mutations** *Microbiol Spectr* **10**
44. Shopsin B, Copin R., Drlica K (2018) **Adaptation of *Staphylococcus aureus* during infection** *Antimicrobial Resistance in the 21st Century* :431–459
45. Young BC, Wu CH, Gordon NC, Cole K, Price JR, Liu E, Sheppard AE, Perera S, Charlesworth J, Golubchik T, Iqbal Z, Bowden R, Massey RC, Paul J, Crook DW, Peto TE, Walker AS, Llewelyn MJ, Wyllie DH, Wilson DJ (2017) **Severe infections emerge from commensal bacteria by adaptive evolution** *Elife* **6**
46. Dorsey-Oresto A, Lu T, Mosel M, Wang X, Salz T, Drlica K, Zhao X (2013) **YihE kinase is a central regulator of programmed cell death in bacteria** *Cell reports* **3**:528–537
47. Wu X, Wang X, Drlica K, Zhao X (2011) **A toxin-antitoxin module in *Bacillus subtilis* can both mitigate and amplify effects of lethal stress** *PLoS One* **6**
48. Richardson AR, Libby SJ, Fang FC (2008) **A nitric oxide-inducible lactate dehydrogenase enables *Staphylococcus aureus* to resist innate immunity** *Science* **319**:1672–1676
49. Vitko NP, Spahich NA, Richardson AR (2015) **Glycolytic dependency of high-level nitric oxide resistance and virulence in *Staphylococcus aureus*** *mBio* **6**
50. Vilcheze C, Jacobs WR (2021) **The promises and limitations of N-acetylcysteine as a potentiator of first-line and second-line tuberculosis drugs** *Antimicrob Agents Chemother*
51. Cao S, Huseby DL, Brandis G, Hughes D (2017) **Alternative evolutionary pathways for drug-resistant small colony variant mutants in *Staphylococcus aureus*** *mBio* **8**
52. Gusarov I, Shatalin K, Starodubtseva M, Nudler E (2009) **Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics** *Science* **325**:1380–1384
53. Shatalin K, Shatalina E, Mironov A, Nudler E (2011) **H2S: a universal defense against antibiotics in bacteria** *Science* **334**:986–990
54. Novick RP (1991) **Genetic systems in staphylococci** *Methods Enzymol* **204**:587–636
55. Hussain M, Hastings JG, White PJ (1991) **A chemically defined medium for slime production by coagulase-negative staphylococci** *J Med Microbiol* **34**:143–147

56. Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, Bose JL, Bayles KW (2013) **A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes** *mBio* **4**:e00537–512
57. Chen J, Yoong P, Ram G, Torres VJ, Novick RP (2014) **Single-copy vectors for integration at the SaPI1 attachment site for *Staphylococcus aureus*** *Plasmid* **76**:1–7
58. Geisinger E, George EA, Muir TW, Novick RP (2008) **Identification of ligand specificity determinants in AgrC, the *Staphylococcus aureus* quorum-sensing receptor** *J Biol Chem* **283**:8930–8938
59. Bolger AM, Lohse M, Usadel B (2014) **Trimmomatic: a flexible trimmer for Illumina sequence data** *Bioinformatics* **30**:2114–2120
60. Langmead B, Salzberg SL (2012) **Fast gapped-read alignment with Bowtie 2** *Nat Methods* **9**:357–359
61. Liao Y, Smyth GK, Shi W (2014) **featureCounts: an efficient general purpose program for assigning sequence reads to genomic features** *Bioinformatics* **30**:923–930
62. Love MI, Huber W, Anders S (2014) **Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2** *Genome Biol* **15**
63. Kim MK, Lane A, Kelley JJ, Lun DS (2016) **E-Flux2 and SPOT: Validated methods for inferring intracellular metabolic flux distributions from transcriptomic data** *PLoS One* **11**
64. Balasubramanian D, Ohneck EA, Chapman J, Weiss A, Kim MK, Reyes-Robles T, Zhong J, Shaw LN, Lun DS, Ueberheide B, Shopsin B, Torres VJ (2016) ***Staphylococcus aureus* coordinates leukocidin expression and pathogenesis by sensing metabolic fluxes via RpiRc** *mBio* **7**
65. Bhadra-Lobo S, Kim MK, Lun DS (2020) **Assessment of transcriptomic constraint-based methods for central carbon flux inference** *PLoS One* **15**
66. Becker SA, Palsson BO (2005) **Genome-scale reconstruction of the metabolic network in *Staphylococcus aureus* N315: an initial draft to the two-dimensional annotation** *BMC microbiology* **5**
67. Livak KJ, Schmittgen TD (2001) **Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT Method** *Methods* **25**:402–408
68. Wright JS, Jin R, Novick RP (2005) **Transient interference with staphylococcal quorum sensing blocks abscess formation** *Proc Natl Acad Sci U S A* **102**:1691–1696
69. Andrade-Linares DR, Lehmann A, Rillig MC (2016) **Microbial stress priming: a meta-analysis** *Environ Microbiol* **18**:1277–1288
70. Boles BR, Thoendel M, Roth AJ, Horswill AR (2010) **Identification of genes involved in polysaccharide-independent *Staphylococcus aureus* biofilm formation** *PLoS One* **5**
71. Wilde AD, Snyder DJ, Putnam NE, Valentino MD, Hammer ND, Lonergan ZR, Hinger SA, Aysanoa EE, Blanchard C, Dunman PM, Wasserman GA, Chen J, Shopsin B, Gilmore MS, Skaar EP, Cassat JE (2015) **Bacterial hypoxic responses revealed as critical determinants of the host-pathogen outcome by TnSeq analysis of *Staphylococcus aureus* Invasive Infection** *PLoS Pathog* **11**

72. Duthie ES, Lorenz LL (1952) **Staphylococcal coagulase; mode of action and antigenicity** *J Gen Microbiol* **6**:95–107
73. Benson MA, Lilo S, Wasserman GA, Thoendel M, Smith A, Horswill AR, Fraser J, Novick RP, Shopsin B, Torres VJ (2011) **Staphylococcus aureus regulates the expression and production of the staphylococcal superantigen-like secreted proteins in a Rot-dependent manner** *Mol Microbiol* **81**:659–675
74. Benson MA, Lilo S, Nygaard T, Voyich JM, Torres VJ (2012) **Rot and SaeRS cooperate to activate expression of the staphylococcal superantigen-like exoproteins** *J Bacteriol* **194**:4355–4365
75. Ji G, Beavis R, Novick RP (1997) **Bacterial interference caused by autoinducing peptide variants** *Science* **276**:2027–2030
76. Baba T, Takeuchi F, Kuroda M, Yuzawa H, Aoki K, Oguchi A, Nagai Y, Iwama N, Asano K, Naimi T, Kuroda H, Cui L, Yamamoto K, Hiramatsu K (2002) **Genome and virulence determinants of high virulence community-acquired MRSA** *Lancet* **359**:1819–1827
77. Kehl-Fie TE, Chitayat S, Hood MI, Damo S, Restrepo N, Garcia C, Munro KA, Chazin WJ, Skaar EP (2011) **Nutrient metal sequestration by calprotectin inhibits bacterial superoxide defense, enhancing neutrophil killing of Staphylococcus aureus** *Cell host & microbe* **10**:158–164
78. Lauderdale KJ, Boles BR, Cheung AL, Horswill AR (2009) **Interconnections between Sigma B, agr, and proteolytic activity in Staphylococcus aureus biofilm maturation** *Infect Immun* **77**:1623–1635
79. Luong TT, Dunman PM, Murphy E, Projan SJ, Lee CY (2006) **Transcription profiling of the mgrA regulon in Staphylococcus aureus** *J Bacteriol* **188**:1899–1910
80. Mesak LR, Yim G, Davies J (2009) **Improved lux reporters for use in Staphylococcus aureus** *Plasmid* **61**:182–187
81. Figueroa M, Jarmusch AK, Raja HA, El-Elmat T, Kavanaugh JS, Horswill AR, Cooks RG, Cech NB, Oberlies NH. (2014) **Polyhydroxyanthraquinones as quorum sensing inhibitors from the guttates of Penicillium restrictum and their analysis by desorption electrospray ionization mass spectrometry** *J Nat Prod* **77**:1351–1358
82. Kinkel TL, Roux CM, Dunman PM, Fang FC (2013) **The Staphylococcus aureus SrrAB two-component system promotes resistance to nitrosative stress and hypoxia** *mBio* **4**:e00696–613
83. Chen PR, Nishida S, Poor CB, Cheng A, Bae T, Kuechenmeister L, Dunman PM, Missiakas D, He C (2009) **A new oxidative sensing and regulation pathway mediated by the MgrA homologue SarZ in Staphylococcus aureus** *Mol Microbiol* **71**:198–211
84. Dmitriev A, Chen X, Paluscio E, Stephens AC, Banerjee SK, Vitko NP, Richardson AR (2021) **The intersection of the Staphylococcus aureus Rex and SrrAB regulons: an example of metabolic evolution that maximizes resistance to immune radicals** *mBio* **12**
85. Brignoli T, Manetti AGO, Rosini R, Haag AF, Scarlato V, Bagnoli F, Delany I (2019) **Absence of protein A expression is associated with higher capsule production in staphylococcal isolates** *Front Microbiol* **10**

86. Mashruwala AA, Boyd JM (2017) **The *Staphylococcus aureus* SrrAB regulatory system modulates hydrogen peroxide resistance factors, which imparts protection to aconitase during aerobic growth** *PLoS One* 12
87. Dyzenhaus S, Sullivan MJ, Albuquerque B, Boff D, van de Guchte A, Chung M, Fulmer Y, Copin R, Ilmain JK, O’Keefe A, Altman DR, Stubbe FX, Podkowik M, Dupper AC, Shopsin B, van Bakel H, Torres VJ. (2022) **MRSA lineage USA300 isolated from bloodstream infections exhibit altered virulence regulation** *Cell host & microbe*

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Reviewer #1 (Public Review):

As a pathogen, *S. aureus* has evolved strategies to evade the host's immune system. It effectively remains 'under the radar' in the host until it reaches high population densities, at which point it triggers virulence mechanisms, enabling it to spread within the host. The agr quorum sensing system is central to this process, as it coordinates the pathogen's virulence network in response to its cell density. When a threshold cell density (quorum) is reached, individual cells in the population detect the quorum peptide (AgrD). This activates the Agr two-component system comprising of a histidine kinase (AgrC) and response regulator (AgrA), leading to the expression of exoenzymes and toxins that facilitate the pathogenicity of *S. aureus*.

However, previous research has indicated that oxidative stress can possibly bypass agr quorum signaling and inhibit agr-dependent gene expression. Specifically, when exposed to high concentrations of hydrogen peroxide (H₂O₂), the redox-sensitive cysteines in AgrA can form an intramolecular disulfide bond, preventing AgrA from binding to DNA and initiating

gene expression. Moreover, another study has shown that cells which have responded to the quorum peptide are vulnerable to oxidative damage. This damage is mediated by PSM toxins that are produced by quorum peptide responders. Consequently, this oxidative stress leads to the selection of *agr* mutants that are better adapted to growth in oxygen-rich environments and can exploit the benefits of the products released by the quorum-responding cells.

Given this sensitivity of the *agr* system to oxidative modification and the fitness cost arising from PSM expression, it raises a pertinent question: how do cells with a functional *agr* quorum sensing system persist within the population under conditions of oxidative stress without being overtaken by *agr* mutants? The current study by Podkowik et al. offers a plausible explanation. It suggests that cells that respond to the quorum peptide may be primed against oxidative stress by activating intrinsic mechanisms that reduce not only the endogenous production of harmful ROS but also mitigate their adverse effects on the cell, thus providing a unique benefit to cells that maintain an active *agr* system. Interestingly, these protective mechanisms are long-lived, and safeguard the cells against external oxidative stressors such as H₂O₂, even after the *agr* system has been deactivated in the population.

In their study, the authors present compelling evidence that supports the role of *agr* in shielding *S. aureus* from lethal H₂O₂ stress, and they establish that this protection is connected to the activation of *agr*-dependent RNAIII and the subsequent block of Rot translation. Importantly, the protective mechanisms that are activated persist throughout growth and provide *S. aureus* with defense against the host's ROS in a murine intraperitoneal infection model.

However, the study falls short in elucidating the specific intrinsic mechanisms responsible for this long-lasting protection against external ROS. While the authors infer that *agr* mutants, which are more vulnerable to external H₂O₂, display an increased respiratory activity and gene expression profile associated with aerobic fermentation, it remains ambiguous whether controlling these mechanisms alone can confer extended protection from external H₂O₂ in an aerobic environment. Further research is needed to confirm this hypothesis.

In summary, this study reveals that the *agr* quorum sensing system's role in relation to ROS is multifaceted and more complex than previously thought, as it orchestrates a balance between mechanisms that both induce and mitigate endogenous ROS, ultimately contributing to the pathogenesis of *S. aureus*.

Reviewer #2 (Public Review):

In their study, Podkowik et al. elucidate the protective role of the accessory gene regulator (*agr*) system in *Staphylococcus aureus* against hydrogen peroxide (H₂O₂) stress. Their findings demonstrate that *agr* safeguards the bacterium by controlling the accumulation of reactive oxygen species (ROS), independent of *agr* activation kinetics. This protection is facilitated through a regulatory interaction between RNAIII and Rot, impacting virulence factor production and metabolism, thereby influencing ROS levels. Notably, the study highlights the remarkable adaptive capabilities of *S. aureus* conferred by *agr*. The protective effects of *agr* extend beyond the peak of *agr* transcription at high cell density, persisting even during the early log-phase. This indicates the significance of *agr*-mediated protection throughout the infection process. The absence of *agr* has profound consequences, as observed by the upregulation of respiration and fermentation genes, leading to increased ROS generation and subsequent cellular demise. Interestingly, the study also reveals divergent effects of *agr* deficiency on susceptibility to hydrogen peroxide compared to ciprofloxacin. While *agr* deficiency heightens vulnerability to H₂O₂, it also upregulates the expression of *bsaA*, countering the endogenous ROS induced by ciprofloxacin. These findings underscore the complex and context-dependent nature of *agr*-mediated protection.

Furthermore, *in vivo* investigations using murine models provide valuable insights into the importance of agr in promoting *S. aureus* fitness, particularly in the context of neutrophil-mediated clearance, with notable emphasis on the pulmonary milieu. Overall, this study significantly advances our understanding of agr-mediated protection in *S. aureus* and sheds light on the sophisticated adaptive mechanisms employed by the bacterium to fortify itself against oxidative stress encountered during infection.

The conclusions of this paper are mostly well supported by the data; however, certain aspects regarding the impact of agr loss on bacterial metabolic status require additional experimental clarification.

1. The RNA-seq analysis revealed that the Δ agr strain exhibited increased expression of genes involved in respiration and fermentation, suggesting enhanced energy generation. However, metabolic modeling based on transcriptomic data indicated a decrease in tricarboxylic acid (TCA) cycle and lactate flux per unit of glucose uptake in the Δ agr mutant. Additionally, intracellular ATP levels were significantly lower in the Δ agr mutant compared to the wild-type strain, despite the carbon being directed into an acetate-generating, ATP-yielding carbon "overflow" pathway. Furthermore, growth analysis in nutrient-constrained medium demonstrated a decrease in the growth rate and yield of the Δ agr mutant. Given that *S. aureus* actively utilizes the electron transport chain (ETC) to replenish NAD pools during aerobic growth on glucose, supporting glycolytic flux and pyruvate dehydrogenase complex (PDHC) activity while restricting TCA cycle activity through carbon catabolite repression (CCR), it is suggested that the authors analyze glucose consumption rates in conjunction with the determination of intracellular levels of pyruvate, AcCoA, and TCA cycle intermediates such as citrate and fumarate. These additional experiments will provide valuable insights into the metabolic fate of glucose and pyruvate and their subsequent impact on cellular respiration and fermentation in the Δ agr mutant.
2. The authors highlighted the importance of redox balance in Δ agr cells by emphasizing the tendency of these cells to prioritize NAD⁺-generating lactate production over generating additional ATP from acetate. However, the results regarding acetate and lactate production in Δ agr cells during aerobic growth suggest that carbon is directed towards acetate generation rather than lactate.
3. The authors mentioned that respiration and fermentation typically reduce the NAD⁺/NADH ratios, and since these activities are elevated in Δ agr strains (Figure 5F-G), they initially anticipated a lower NAD⁺/NADH ratio compared to wild-type agr cells. However, the increase in respiration and activation of fermentative pathways leads to a decrease in NADH levels, therefore resulting in an increase in the NAD⁺/NADH ratio.