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Host-Microbial Interactions | Full-Length Text

Staphylococcus aureus senses human neutrophils via PerR to coordinate the expression of the toxin LukAB

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ABSTRACT *Staphylococcus aureus* is a gram-positive pathogen that poses a major health concern, in part due to its large array of virulence factors that allow infection and evasion of the immune system. One of these virulence factors is the bicomponent pore-forming leukocidin LukAB. The regulation of *lukAB* expression is not completely understood, especially in the presence of immune cells such as human polymorphonuclear neutrophils (hPMNs). Here, we screened for transcriptional regulators of *lukAB* during the infection of primary hPMNs. We uncovered that PerR, a peroxide sensor, is vital for hPMN-mediated induction of *lukAB* and that PerR upregulates cytotoxicity during the infection of hPMNs. Exposure of *S. aureus* to hydrogen peroxide (H_2O_2) alone also results in increased *lukAB* promoter activity, a phenotype dependent on PerR. Collectively, our data suggest that *S. aureus* uses PerR to sense the H_2O_2 produced by hPMNs to stimulate the expression of *lukAB*, allowing the bacteria to withstand these critical innate immune cells.

IMPORTANCE *Staphylococcus aureus* utilizes a diverse set of virulence factors, such as leukocidins, to subvert human neutrophils, but how these toxins are regulated is incompletely defined. Here, we identified the peroxide-sensitive repressor, PerR, as a required protein involved in the induction of *lukAB* in the presence of primary human neutrophils, a phenotype directly linked to the ability of PerR to sense H_2O_2. Thus, we show that *S. aureus* coordinates sensing and resistance to oxidative stress with toxin production to promote pathogen survival.

KEYWORDS MRSA, cytotoxins, PerR, neutrophils, LukAB, pore-forming toxins, *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) can cause multiple illnesses including skin infections, pneumonia, and bacteremia (1). With the rise of antibiotic resistance, treating infections such as methicillin-resistant *S. aureus* (MRSA) has become increasingly difficult. *S. aureus* success as a pathogen is in part because it possesses a large collection of virulence factors (2, 3). These include the bicomponent pore-forming leukocidins, which target and rupture the membranes of immune cells that the host requires for protection from invasive pathogens (4). *S. aureus* strains associated with human infections, including community-associated MRSA strains from the USA300 lineage, produce up to five leukocidins that target human cells: leukocidin AB (LukAB, also known as LukGH), Panton–Valentine leukocidin (PVL, also known as LukSF-PV), leukocidin ED (LukED), gamma hemolysin HlgAB, and gamma hemolysin HlgCB (5). These toxins preferentially target leukocytes, using specific proteinaceous receptors to do so (6–9). *S. aureus* differentially activates the expression of leukocidin loci (10, 11), which is hypothesized to be important to combat the host-mediated attack during infection and promote pathogen survival. Most of the leukocidins share 60%–80% sequence similarity, aside

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from LukAB, which only shares 30%–40% sequence similarity to the other toxins (12, 13). Interestingly, among the leukocidins, the promoter activity of *lukAB* has been shown to be the most active during tissue culture infection of human polymorphonuclear neutrophils (hPMNs) (10). Additionally, LukAB is the predominant toxin that lyses hPMNs in these tissue culture models (9, 10, 12–14), both through intracellular and extracellular mechanisms, which aids in *S. aureus* escape of the immune system (10). These findings together with the observation that the *lukAB* operon is found in over 99% of *S. aureus* isolates have positioned LukAB as an attractive vaccine candidate (15, 16).

The network involved in the regulation of toxins in *S. aureus* includes two-component systems that activate expression, such as the *S. aureus* exoprotein (Sae) system and the accessory gene regulator (Agr) system. Other transcription factors such as the repressor of toxins (Rot) are also involved in the regulation of leukocidins (17, 18). The SaeRS system is a major activator of all the toxins (5, 19–21), but it is still unknown why *lukAB* specifically is more active in the presence of hPMNs compared to the other leukocidins. We hypothesized that these and/or other uncharacterized regulators may be involved in the upregulation of *lukAB* during infection of hPMNs.

This study aimed to identify transcriptional regulators involved in the hPMN-mediated activation of *lukAB*. We performed a high-throughput screen that utilized a luminescent transcriptional reporter to measure *lukAB* promoter activity in transposon mutants. The work revealed that the inactivation of *perR* reduces *lukAB* promoter activity and *S. aureus*-mediated cytotoxicity in neutrophils. PerR is a peroxide sensor that represses many genes involved in iron storage and oxidative stress response (22, 23). Our data demonstrate that H_2O_2, which is released by hPMNs (24, 25), induces *lukAB* through a PerR-mediated mechanism. Collectively, these data suggest that *S. aureus* uses PerR to upregulate the *lukAB* promoter when the bacterium encounters hPMNs, thus dually coordinating the protection against oxidative damage and a counterattack to kill hPMNs.

RESULTS

Identification of transcriptional regulators that alter promoter activity of *lukAB* during infection of hPMNs

We hypothesize that the *lukAB* promoter (P*lukAB*) may be regulated by various transcriptional regulators in different environmental conditions. To uncover transcription factors involved in the regulation of P*lukAB*, we utilized a sublibrary of the Nebraska Transposon Mutant Library (26) described by Balasubramanian et al. (11), which consists of strains with mutations in genes likely to be involved in transcription and translation. This mutant library was constructed in JE2, a *S. aureus* strain in the USA300 background (26). USA300 strains are associated with the current epidemic of community-associated MRSA infections (27). The regulatory sublibrary was transduced with a plasmid containing the *lukAB* promoter driving expression of the click beetle red luciferase (*CBR-luc*) (28). The reporter sublibrary was then subcultured for 3 hours, followed by a 3-hour infection of hPMNs. D-Luciferin was added, and the promoter activity was measured (Fig. 1A). We included two internal controls, *rot::bursa* (increased promoter activity) (29) and *saeR::bursa* (no to low promoter activity) (19). Indeed, we observed increased *lukAB* promoter activity in the absence of *rot* and decreased *lukAB* promoter activity in the absence of *saeR*, validating the screen. Altogether, the screen uncovered 72 mutants that had at least 1.75-fold less P*lukAB* activity compared to wild-type JE2, and these gene products were categorized as potential activators of P*lukAB* (Fig. 1B; see Table S1). Conversely, we identified 51 mutants with at least 1.75-fold more P*lukAB* activity than wild-type JE2, suggesting that they contained mutations in gene products that could act as repressors of P*lukAB* (Fig. 1C).

Activators differentially regulate *lukAB* in the presence of hPMNs

As we were interested in discovering transcriptional regulators that played a role in the upregulation of P*lukAB* in the presence of hPMNs, we analyzed the potential activators of

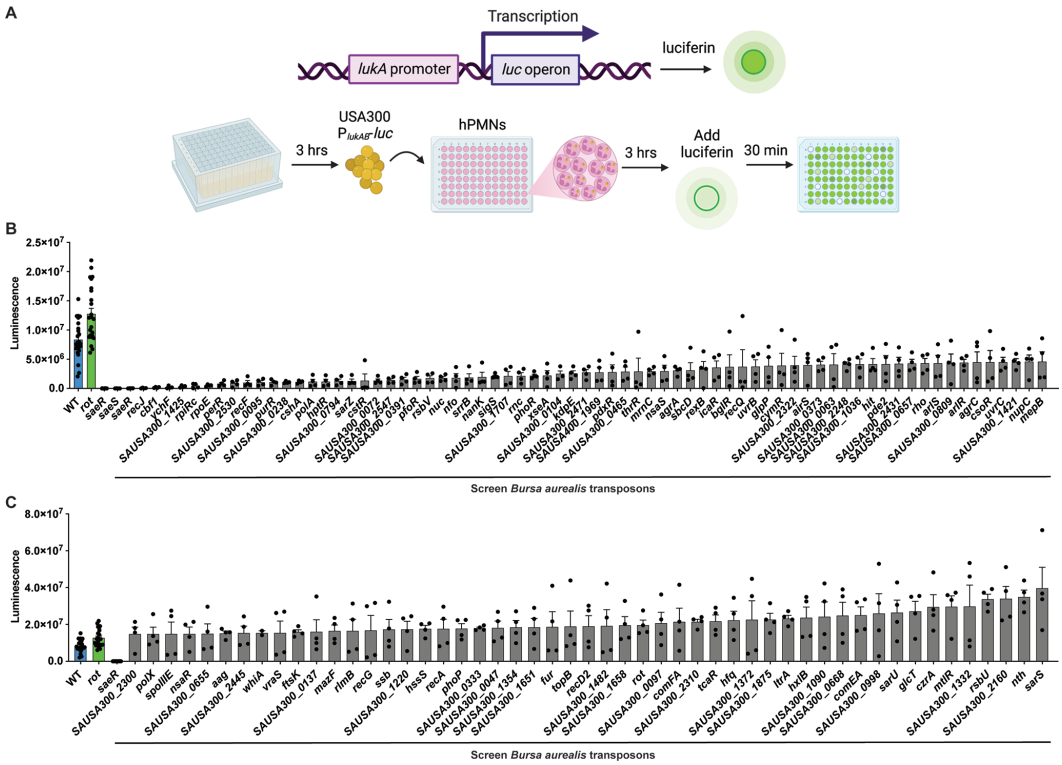

FIG 1 Mutations in non-essential genes affect the regulation of the *lukAB* promoter. (A) A luminescence screen was conducted on a JE2 transposon library containing a reporter plasmid where the *lukAB* promoter (P*lukAB*) was fused to the luciferase gene. The library is composed of 250 mutants with mutations in non-essential genes that may have regulatory roles. The promoter activity was measured by the luminescence of the JE2 transposon library in RPMI + HEPES + 5% normal human serum (NHS) with hPMNs after 3 hours of infection. Created with BioRender.com. (B) Results show potential activators of P*lukAB*, which have an average luminescence less than wild-type JE2 (n = 4 donors; six independent colonies for controls) at a multiplicity of infection (MOI) of 8. Mutant strains shown have a minimum of a 1.75-fold difference compared to wild-type JE2. Error bars indicate the standard error of the mean (SEM). (C) Results show potential repressors of P*lukAB*, which have an average luminescence greater than wild-type JE2 (n = 4 donors; six independent colonies for controls, MOI = 8). Mutant strains shown have a minimum of a 1.75-fold difference compared to wild-type JE2. Error bars indicate SEM.

P*lukAB* further. We selected regulators that were significantly different from wild-type JE2 and possessed features indicative of the direct regulation of downstream genes, such as genes that encoded for proteins that contained a helix-turn-helix motif or were part of two-component systems. These selection criteria narrowed our screen to 15 potential activators (Fig. 2). Utilizing the experimental design described in Fig. 1, we tested the activation of P*lukAB* luminescence by the potential activators in the presence and absence of hPMNs. In the secondary screen, only some of the mutants continued to show attenuated P*lukAB* activity, suggesting that the luciferase reporter is best suited to detect strains with exceedingly impacted gene regulation (Fig. 2A). In contrast, when the assay was repeated in media alone, the luminescence of most of these mutants was greater than wild-type JE2 (Fig. 2B). This suggests that the attenuation of P*lukAB* activity is dependent on the presence of hPMNs for these strains. We infer that the general luminescence is greater for all strains in media alone because *S. aureus* is phagocytosed during infection, which decreases the ability for D-luciferin to diffuse into the bacteria. Therefore, the difference in $\log_2$ fold change of the luminescence for the two conditions was compared (Fig. 2C). Mutants with a negative $\log_2$ fold change had a decreased activation of P*lukAB* compared to wild-type JE2, and mutants with a positive $\log_2$ fold change had an increased P*lukAB* activity. All the mutants tested had a shift in their regulation of P*lukAB* between the two conditions. Specifically, some mutants showed increased P*lukAB* activation in media alone and decreased activation in the presence of hPMNs (Fig. 2C). Therefore, these regulators seem to act as repressors in the absence of

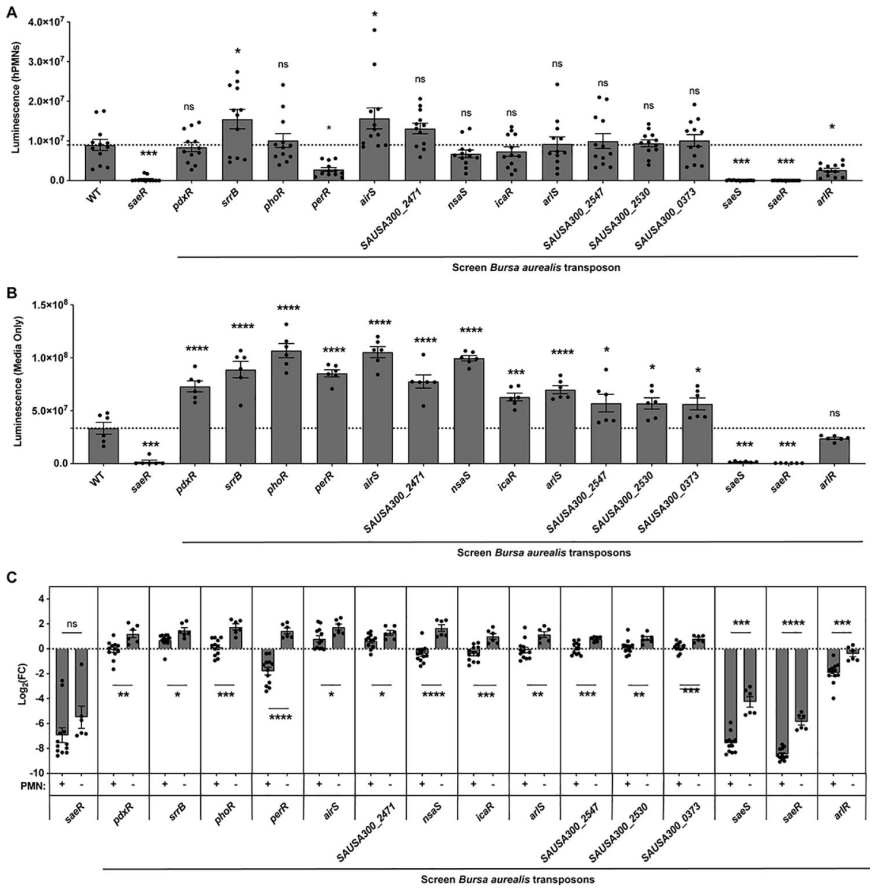

FIG 2 Regulation of *lukAB* promoter activity in the presence or absence of hPMNs. (A) P*lukAB* luminescence values of selected P*lukAB* activators in the presence of hPMNs in media containing RPMI + HEPES + 5% NHS. The results shown are from two independent experiments each performed with three colonies of each strain repeated in four blood donors (n = 12, MOI = 8). The dotted line represents wild-type JE2. Statistical analysis was performed using one-way ANOVA with multiple comparisons to determine the statistical significance of mutants compared to wild-type JE2. Error bars indicate SEM. (B) Luminescence values of selected P*lukAB* activators grown as in panel (A) but in the absence of hPMNs. The results shown are from two independent experiments each performed with three colonies of each strain (n = 6). The dotted line represents wild-type JE2. Statistical analysis was performed using one-way ANOVA with multiple comparisons to determine the statistical significance of mutants compared to wild-type JE2. Error bars indicate SEM. (C) Log_2 fold change of luminescence of mutants compared to wild-type JE2 in the presence or absence of hPMNs (n = 6–12). Statistical analysis was performed using unpaired t-tests with Welch's correction to compare the log_2 fold change of luminescence in the two conditions for each mutant strain. Log_2 fold change of luminescence was used to compare the two conditions to account for the reduction in raw luminescence values because of phagocytosed bacteria, which reduces the efficacy of D-luciferin to cross the bacterial membrane. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$. ns, not significant.

hPMNs and as activators in the presence of hPMNs. This suggests a switch in the regulation of *lukAB* that is dependent on the presence of hPMNs.

PerR regulates *lukAB* promoter activity and cytolytic activity in the presence of hPMNs

LukAB is responsible for the lysis of hPMNs in tissue culture models of *S. aureus* infection (10, 13). Thus, in addition to promoter activity, we analyzed the ability of the 15 potential activators to enhance the lysis of hPMNs. We observed that all the selected mutants were deficient in hPMN killing (Fig. 3A).

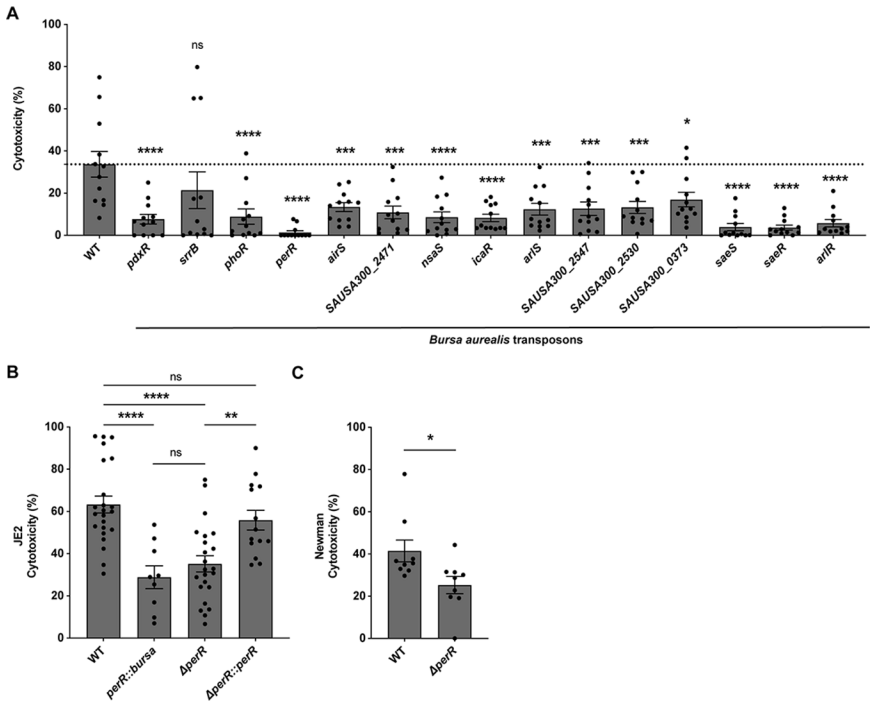

FIG 3 Decreased hPMN cytotoxicity of potential *lukAB* activator mutants. (A) Cytotoxicity values of selected P*lukAB* activators. Cytotoxicity was measured as percent lactate dehydrogenase (LDH) release from lysed hPMNs. The results shown are from two independent experiments each performed with three colonies of each strain repeated in four blood donors (n = 12, MOI = 8). The dotted line represents wild-type JE2. Statistical analysis was performed using one-way ANOVA with multiple comparisons to determine the statistical significance of mutants compared to wild-type JE2. Error bars indicate SEM. (B) Cytotoxicity of strains wild-type JE2, *perR::bursa*, Δ*perR*, and the complement strain (Δ*perR::perR*). The values are averages of eight independent experiments with two colonies of each strain repeated in 23 blood donors (n = 18–46, MOI = 18). The increase in MOI was used to induce increased cytotoxicity. Statistical analysis was performed using one-way ANOVA with multiple comparisons to determine the statistical significance of mutants compared to wild-type JE2. Error bars indicate SEM. (C) Cytotoxicity of Newman wild-type and Δ*perR*. The results shown are averages from three independent experiments with two colonies of each strain repeated in nine blood donors (n = 18, MOI = 18). Statistical analysis was performed using an unpaired t-test with Welch's correction to determine the statistical significance of mutants compared to wild-type JE2. Error bars indicate SEM. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

We then analyzed the transposon mutants for their ability to lyse hPMNs and their P*lukAB* activity in the presence of hPMNs. We identified eight mutants that had attenuated cytotoxicity and an average negative $\log_2$ fold change of P*lukAB* activity in the presence of hPMNs (Table 1). Among these, we observed that *saeR::bursa* and *saeS::bursa*, the response regulator and sensor histidine kinase of the SaeRS two-component system, were both hits as activators in our analysis, further validating the findings of our screen. The two other strains that displayed a pronounced attenuation of cytotoxicity and decreased luminescence in the presence of hPMNs were *perR::bursa* and *arlR::bursa* (Table 1).

Previous studies have established the importance of the ArlRS two-component system for *S. aureus* virulence (30–35), which further validated our study.

PerR is the main peroxide sensor in gram-positive bacteria such as *Bacillus subtilis* and *S. aureus* (36, 37) and is necessary for *S. aureus* pathogenesis in various animal models including a murine skin abscess, *Caenorhabditis elegans*, fruit fly, and zebrafish (22, 23, 38–40). PerR is an oxidation-sensing transcriptional regulator, mainly functioning in iron storage and oxidative stress resistance pathways (22, 36, 41–50). The PerR regulon includes *katA* (catalase), *ahpCF* (alkyl hydroperoxide reductase), *bcp* (bacterioferritin

TABLE 1 List of mutants with low *lukAB* promoter activity and cytotoxicity

Locus tag	NE number	Gene description	Gene name	Luciferase with hPMNs ± SEM	*P*-value hPMNs vs media only	LDH ± SEM	*P*-value LDH	DNA-binding domain
SAUSA300_0691	NE1622	DNA-binding response regulator SaeR	*saeR*	25,790.9 ± 4,072.3	<0.0001	3.7 ± 1.2	<0.0001	Helix-turn-helix
SAUSA300_1842	NE665	Transcriptional regulator, Fur family (repressor)	*perR*	2,790,645.6 ± 509,781.5	<0.0001	1.4 ± 0.8	<0.0001	Helix-turn-helix
SAUSA300_2558	NE1116	Nisin susceptibility-associated sensor histidine kinase	*nsaS*	6,761,540.4 ± 947,821.9	<0.0001	8.6 ± 2.6	<0.0001	
SAUSA300_0690	NE1296	Sensor histidine kinase SaeS	*saeS*	62,617.4 ± 16,194.2	0.0001	3.9 ± 1.7	<0.0001	
SAUSA300_1308	NE1684	DNA-binding response regulator ArlR	*arlR*	2,639,434.3 ± 456,560.1	0.0004	5.8 ± 1.8	<0.0001	Helix-turn-helix
SAUSA300_2599	NE1132	Intercellular adhesion transcription regulator (biofilm operon *icaADBC* repressor IcaR)	*icaR*	7,348,020.1 ± 1,201,711.2	0.0005	8.3 ± 1.7	<0.0001	Helix-turn-helix
SAUSA300_0503	NE354	Transcriptional regulator, gntR family protein (PLP-dependent aminotransferase family protein)	*pdxR*	8,444,604.6 ± 1,142,922.4	0.0052	7.7 ± 2.3	<0.0001	Helix-turn-helix
SAUSA300_1307	NE1183	Sensor histidine kinase protein ArlS	*arlS*	9,235,594.9 ± 1,771,937.7	0.0018	12.4 ± 2.8	0.0004	Helix-turn-helix

comigratory protein), *trxB* (thioredoxin-disulfide reductase), *fur* (ferric uptake regulator), *ftn* (ferritin), and *mrgA* (ferritin-like Dps homolog) (22). PerR is a metal-dependent regulator that binds to Zn^{2+} in combination with either Fe^{2+} or Mn^{2+}, and its function is altered depending on the metal to which it is bound (22, 23, 43, 51). PerR senses low levels of H_2O_2 when bound to Fe^{2+} in *S. aureus* resulting in the derepression of its regulon (22, 23, 52).

To further validate the role of PerR in the regulation of *S. aureus* toxins, we tested the cytotoxicity of isogenic *perR* deletion and complementation strains. The Δ*perR* strain was constructed by phage transducing a *perR::ermC* mutation into JE2. The complement Δ*perR::perR* strain was made using pIMAY* (53) to replace the *erm* cassette with a wild-type *perR*. We observed that both the Δ*perR* and *perR::bursa* mutants exhibit attenuated cytotoxicity toward hPMNs (Fig. 3B). Of note, the phenotype was restored to wild-type levels in the complementation strain. We next tested if the deletion of *perR* resulting in altered cytotoxicity was a USA300-specific phenotype. We tested cytotoxicity in the strain Newman, a methicillin-sensitive *S. aureus* strain (54), and observed that the Δ*perR* strain also exhibits decreased cytotoxicity of hPMNs as compared to the wild-type strain (Fig. 3C). Together, these data demonstrate that PerR is required for the full lytic activity of *S. aureus* when the bacteria are exposed to hPMNs.

Hydrogen peroxide induces *lukAB* promoter activity

One of the major roles of PerR is sensing H_2O_2 in the environment (23, 51, 55, 56). hPMNs generate H_2O_2 by first synthesizing superoxide via NADPH oxidase (NOX2), which then undergoes dismutation to form H_2O_2 (24, 25) to attack pathogens (46, 57). We next tested if H_2O_2 alone could induce *lukAB* promoter activity. Using the luciferase reporter strains described above, we observed that H_2O_2 indeed induces P*lukAB* in wild-type JE2 but not in *perR::bursa*. We also tested the role of SaeR in this H_2O_2-mediated induction of P*lukAB* and observed that *saeR::bursa*, like *perR::bursa*, exhibited very little induction of *lukAB* regardless of H_2O_2 (Fig. 4A). These data suggest that H_2O_2-mediated P*lukAB* activity is dependent on both PerR and SaeRS. Of note, the impact of 0.1 mM H_2O_2 exposure on P*lukAB* induction was independent of H_2O_2 antimicrobial activity as we detected no significant difference in colony-forming units between any of the strains and treatments (Fig. 4B). We also observed that H_2O_2 induces P*lukAB* in a dose-dependent manner (Fig. 4C). Collectively, these data suggest that PerR plays a role in regulating cytotoxicity through the upregulation of *lukAB* in response to H_2O_2 produced by hPMNs.

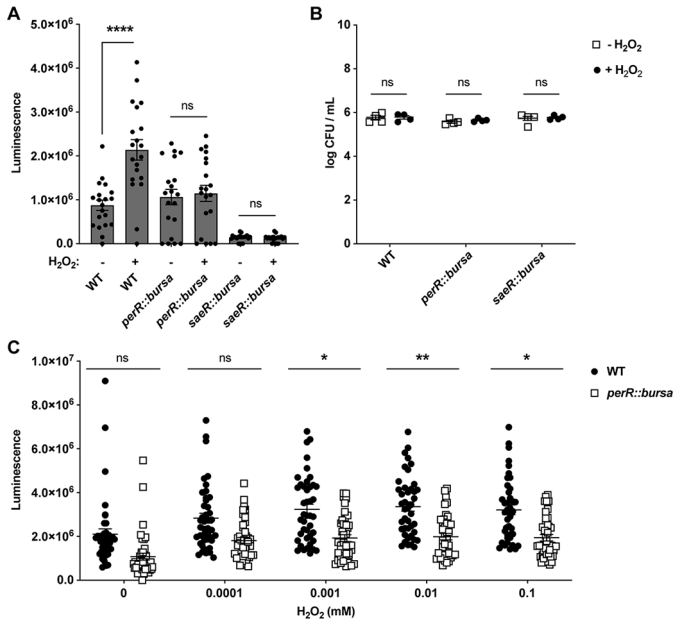

FIG 4 H_2O_2 treatment increases the promoter activity of *lukAB*. (A) P*lukAB* luminescence values after a 1-hour treatment with 0 or 0.1 mM of H_2O_2 in wild-type JE2, *perR::bursa,* and *saeR::bursa*. The results shown are from two independent experiments each performed with 10 colonies of each strain (n = 20). Statistical analysis was performed using unpaired *t*-tests with Welch's correction to determine the statistical significance of 0.1 mM H_2O_2 treatment. Error bars indicate SEM. (B) CFUs per milliliter were calculated by plating the bacteria after exposure to H_2O_2 for 1 hour. The results shown are from two independent experiments each performed with two colonies for each strain (n = 4). Statistical analysis was performed using unpaired *t*-tests with Welch's correction to determine the statistical significance. Error bars indicate SEM. (C) P*lukAB* luminescence values after treating bacteria with various concentrations of H_2O_2 for 1 hour. The results shown are from eight independent experiments each performed with six colonies of each strain (n = 48). Statistical analysis was performed on average luminescence per experiment using unpaired *t*-tests with Welch's correction to determine the statistical significance of mutants compared to wild-type JE2 at each concentration. Error bars indicate SEM. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

PerR binding sites are found throughout the *S. aureus* chromosome

To gain insight into how PerR controls the activation of the P*lukAB*, we performed a bioinformatic analysis where the sequences of the PerR binding site (22, 45), whose consensus is ATTATAATTATTATAAT, were used to query the chromosome of the USA300 strain LAC. Of note, the *S. aureus* PerR consensus sequence was initially identified in strain 8325-4 (22, 45). For this purpose, the sequence motif scanning software FIMO (58) was used. The hits were mapped to the reference annotation of the LAC genome, and the distance of the putative PerR binding site sequence, with respect to the genes in which it occurred, was calculated. Genes were considered to have a putative PerR binding site if an alignment occurred at most 100 bp upstream of the start codon of the gene or within the coding sequence. Figure 5 summarizes our findings. Figure 5A depicts the sequence motif of the PerR binding sites identified in strain 8325-4, while Fig. 5B shows the alignment positions and scores for the PerR binding site motif. Among the loci containing putative PerR binding sites, we observe that binding sites for *ahpC*, *katA*, *ftnA*, *dps*, *fur*, and *perR* were identified, as expected. Some of these genes possessed more than one predicted PerR binding site. In addition, some interesting hits included *SAUSA300_1202* and *SAUSA300_1203*, which are conserved hypothetical proteins that were not tested in our screen, and *SAUSA300_0084* and *SAUSA300_1200*, which are transcriptional regulators. In our screen, *SAUSA300_0084* behaved like an activator of *lukAB*. *SAUSA300_1200* was not tested in our screen. Altogether, these data suggest

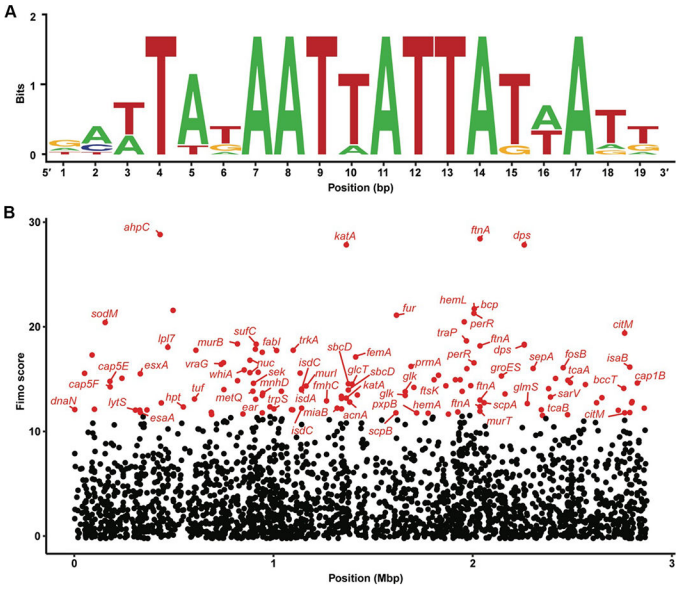

FIG 5 PerR binding site predicted in many potential genes. (A) PerR binding site sequence motif based on binding site sequences from strain *S. aureus* 8325-4. (B) Genes predicted to have a PerR binding site. Each dot represents a predicted binding site that lies within a gene or at most 100 bp upstream of it. The horizontal axis represents the coordinates in the assembly at which the binding site occurs, while the vertical axis represents the alignment score; that is how closely the predicted binding site resembles the sequence motif in (A). Genes in red have a FIMO alignment score in the upper 5% and are labeled with their symbol, unless undefined in the reference assembly.

that PerR may directly regulate many loci in *S. aureus* and that PerR may impact gene expression by acting directly on regulated operators or indirectly by controlling the expression of other master regulators.

DISCUSSION

Neutrophils, a crucial part of the innate immune response, are one of the first immune cells to respond to an infection (59). To better understand *S. aureus* pathogenesis, we were interested in the regulation of *lukAB* during infection of hPMNs. LukAB plays a vital role during tissue culture infection of hPMNs (10, 12, 13), and it is a promising vaccine candidate (16). In this study, we conducted a screen of a transposon mutant library to identify potential new regulators of *lukAB* during infection of hPMNs. We identified PerR, a peroxide regulator, as a key protein for hPMN-mediated induction of P*lukAB*. Our data illustrate that PerR influences *lukAB* regulation to increase *S. aureus* cytotoxicity (Fig. 2 and 3). We observed that *lukAB* promoter activity is induced by H_2O_2 (Fig. 4), suggesting that H_2O_2 released by hPMNs may play a role in the observed regulation (Fig. 6). Collectively, our data show that *S. aureus* can sense H_2O_2 via PerR to increase toxin production in response to hPMNs in an attempt to evade these critical innate immune leukocytes.

Although we found genes that may play a regulatory role in toxin production, we do not know whether this regulation occurs directly or indirectly. Our PerR *in silico* binding site mapping experiment suggests that the impact on P*lukAB* might be indirect as no binding site was identified in the promoter or *lukAB* coding sequence. PerR may instead be regulating one or more regulators that directly bind to the promoter of *lukAB*. Such indirect regulation is seen in *S. aureus* by several different regulators, including RNAIII and Rot. RNAIII, the effector molecule of *agr*, regulates a number of different virulence factors through the activation and repression of downstream regulators such as Rot and SarT (17, 30, 60–67). Rot also indirectly regulates toxins through the repression of the SaeRS system (17, 21, 30). Our data suggest that PerR does not directly regulate *lukAB*

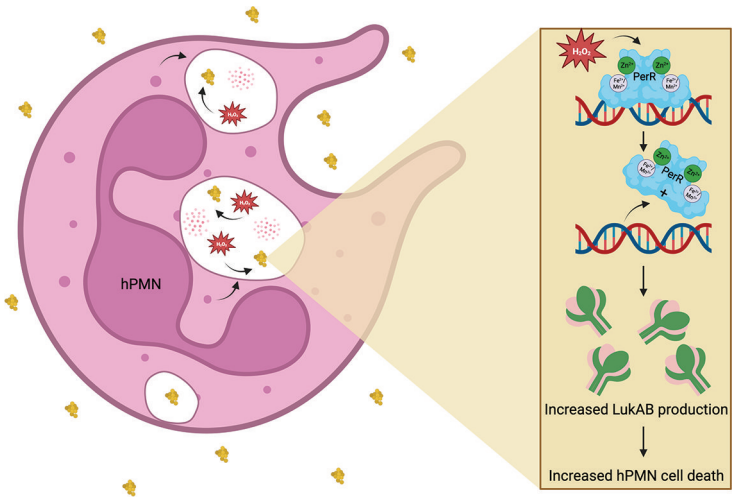

FIG 6 Model of the role of PerR in LukAB-mediated *S. aureus* virulence. hPMNs release H_2O_2 in the presence of *S. aureus* in the phagolysosome. In response, PerR, a dimeric peroxide sensor, stimulates the production of LukAB, which increases hPMN cell death. Figure made with BioRender.

nor global regulators such as *sae, agr,* and *rot*. Instead, PerR binding sites were found on the promoters of *fur, sarV*, a *lysR-like* (SAUSA300_0093), and upstream of the histidine kinases *lytS* and *airS*. Future studies are needed to elucidate the molecular mechanism of PerR-mediated regulation of P*lukAB* and the contribution of these genes.

The data presented herein establish that PerR functions not only in oxidative stress resistance and iron storage pathways but also in *lukAB* regulation. This highlights a trend seen in *S. aureus*, where metabolic or stress response regulators are additionally involved in the regulation of virulence factors. RpiRc, PurR, and CodY are all metabolic regulators that have also been shown to control toxin expression (11, 68–70). RpiRc is involved in the catabolism of sugar and has been established to repress the *rnaIII* promoter, resulting in increased Rot levels and, therefore, decreased toxin expression (11). PurR functions as a repressor of purine biosynthesis and also participates in directly regulating virulence factors and master regulators of virulence (68). The canonical role of CodY enables *S. aureus* to adapt to environments with nutrient limitations and metabolic stress (71). In addition, CodY also functions as a repressor of virulence factors and regulators such as α-toxin and RNAIII (69). We posit that it is advantageous for the bacteria to have regulators that perform dual-functional roles, especially in the case of PerR; having a regulator that is able to sense and respond to H_2O_2 and upregulate a virulence factor that can kill PMNs that are producing H_2O_2 may promote bacterial survival and proliferation during infection.

Altogether, the findings presented here highlight the ability of *S. aureus* to sense host environments and respond in coordinated ways to both protect the bacterium and suppress the host immune system. Additional studies are needed to better understand how PerR is regulating the leukocidins and if other stress response regulators have dual functions. This knowledge will give us a better understanding of the complexities of virulence regulation during infection and may highlight key targets for future therapeutics.

MATERIALS AND METHODS

Purification of human neutrophils

Human PMNs were isolated by a Ficoll–Paque method as described before (72).

Bacterial cultures and growth conditions

All *S. aureus* strains were grown on tryptic soy agar (TSA) or TSA supplemented with appropriate antibiotics (chloramphenicol 10 µg/mL and erythromycin 2.5 µg/mL) at 37°C. Liquid cultures of *S. aureus* were grown in tryptic soy broth (TSB) and supplemented with antibiotics if needed. Liquid cultures were incubated at 37°C while shaking at 180 rpm. They were grown in 96-deep-well plates (Corning, 14-222-353) with 1 mL of growth medium. *E. coli* was grown in a flask containing 20 mL of Luria–Bertani broth. For subculturing *S. aureus,* a dilution of 1:100 was used from the overnight cultures into fresh media.

Construction of mutant strains

For all the strains, plasmids, and oligonucleotides used in this study, see Tables S2 and S3 in the supplemental materials. The P*lukAB_luc* strain was constructed as previously described in Anderson et al. (73). Briefly, the backbone of the P*lukAB_luc* plasmid originated from the plasmid pHC123 (kindly provided by Alex Horswill) (28) and was cut at the SalI and KpnI restriction sites before being ligated with the *lukAB* intergenic region and being transformed into DH5α and electroporated into AH-LAC. The primers pHC123_*lukAB*_F and pHC123_*lukAB*_R were used. The JE2 promoter–reporter library was generated by phage transduction using phage 80α lysate of the AH-LAC P*lukAB_luc* strain. The regulatory library was grown overnight in 400 µL of TSB in a round-bottomed deep-well plate. In the morning, 390 µL of fresh TSB was inoculated with 10 µL of the overnight culture and grown at 120 rpm until an optical density (OD_{600}) of 1. Next, 5 µL of 1 M $CaCl_2$ and 100 µL of phage lysate were added to each well, and this was left at room temperature for 20 minutes. We added 40 µL of 1 M Na citrate, and 10 µL of the mix was spot-platted onto TSA + Cm10 and grew overnight at 30°C. Colonies were picked from this plate and grown overnight, and then, 50 µL of the overnight culture was added to 50 µL of 20% glycerol and frozen down for further use.

The JE2 Δ*perR* strain was generated by phage transduction using phage φ11 lysate from Newman Δ*perR::ermC* (kindly provided by Anthony Richardson). Complementation of *perR* was performed with plasmid pIMAY* (kindly provided by Angelika Gründling via Addgene), which is used to stably integrate DNA into the natural site, resulting in a single-copy chromosomal insertion (53). JE2 Δ*perR::perR* was made by cloning the *perR* allele into the pIMAY* plasmid. pIMAY* was cut with XhoI and XmaI before ligation with the *perR* coding region. Primers PerR_PIMAY_F and PerR_PIMAY_R were used to amplify upstream and downstream regions of *perR* with base pair homology to pIMAY* for ligation. pIMAY*-*perR* plasmid was transformed into IM08B and electroporated in JE2Δ*perR*.

Ex vivo infection assay

Human PMNs were seeded on 96-well flat-bottom white tissue culture-treated plates (Corning, 3917) at a concentration of 2×10^5 cells/well at a final volume of 80 µL of phenol red-free Roswell Park Memorial Institute media (RPMI—Gibco, 11-835-055) supplemented with 10 mM HEPES (Corning, 25-060-CI) and 5% NHS (SeraCare, 1830-0003). One hundred and fifty microliters of the *S. aureus* subcultures was transferred to clear round-bottom plates (Corning, 3788) to measure OD_{600}. The OD_{600} of the subcultures was obtained before infection using the PerkinElmer EnVision 2103 Multilabel Reader. For the primary and secondary screens, the cultures were grown overnight in 96-well plates and then subcultured for 3 hours. For the *perR* deletion and transposon cytotoxicity assay, cultures were grown overnight in 96-well plates and then subcultured for 3.5 hours. hPMNs were infected at an MOI of 8 (10 µL) or 18 (20 µL) and incubated at 37°C in 5% CO_2 for 3 hours. MOI was confirmed by serial dilution and plating for CFU. Ten microliters of TSB was added to the media for a final volume of 100 µL in each well when experiments were conducted at an MOI of 8.

Luminescence reporter assay

The *ex vivo* infection assay described above was conducted on the Nebraska Transposon Mutant Library strains containing the pHC123 plasmid with the promoter of *lukAB* driving expression of the luciferase operon (26, 28). These strains were grown in 1 mL of TSB with 10 µg/mL of chloramphenicol (to retain reporter plasmid) in 96-deep-well plates overnight and subcultured for 3 hours the following day. After the 3-hour infection of hPMNs, background luminescence was measured by PerkinElmer EnVision 2103 Multilabel Reader before adding 15 mg/mL (10 µL/well) of D-luciferin (Thermo Fisher Scientific, 88293) resuspended in water to each well. Plates were stored in the dark for 30 minutes before luminescence was measured again. OD_{600} was accounted for in the analysis. The resulting OD_{600} was ~0.15 in the 96-well plate. The resulting raw luminescence values after infection were divided by the OD_{600} for each strain for the luminescence reporter assay.

Cytotoxicity assay

The *ex vivo* infection assay described above was performed before measuring the cytotoxicity of the strains. Subcultures were grown for 3 hours for the secondary screen and 3.5 hours for the *perR* deletion and transposon experiments to obtain a higher OD_{600}. The resulting OD_{600} was ~0.2 in the 96-well plate. TSB was added to normalize the cultures to an OD_{600} of 0.19. After the 3-hour infection of hPMNs, plates were centrifuged at 1,500 rpm for 5 minutes at 4°C. Twenty-five microliters of supernatants was transferred to clear-bottom black 96-well plates (Corning, 3904). Cytotoxicity was measured by LDH release from hPMNs. Twenty-five microliters of the CytoTox - One Homogeneous Membrane Integrity Assay (Promega, G7892) was also added to the black 96-well plates. LDH release was quantified per the manufacturer's instructions using an EnVision 2103 Multilabel Reader.

Hydrogen peroxide treatment assay

Subcultures were grown for 3.5 hours. H_2O_2 30% (Sigma-Aldrich, 7722-84-1) was freshly diluted with H_2O to varying concentrations (0.0001, 0.001, 0.01, 0.1, 1, and 5 mM). The final concentration of H_2O_2 added to the wells was 0.0001, 0.001, 0.01, and 0.1 mM. Fifty microliters of the subculture and 50 µL of H_2O_2 were added to 96-well flat-bottom white tissue culture-treated plates. Bacterial strains used in the experiment contained the pHC123_*lukAB* plasmid for luminescence readings. After a 1-hour H_2O_2 treatment, background luminescence was measured by the PerkinElmer EnVision 2103 Multilabel Reader before adding 15 mg/mL (10 µL/well) of D-luciferin. Plates were incubated in the dark for 30 minutes at room temperature before luminescence was measured again. To calculate CFU per milliliter, bacteria were plated after 1-hour H_2O_2 treatment.

Analysis of PerR binding sequence and regulon

PerR binding site sequences for *S. aureus* were obtained from the CollecTF database (http://www.collectf.org/browse/home/) and verified to be identical to those reported in Horsburgh et al. (22). A sequence logo for the aligned sequences was created using WEBLOGO (https://weblogo.berkeley.edu/logo.cgi). A *S. aureus* LAC reference assembly (NCBI ID: GCF_015475575.1) was scanned for PerR binding sites using FIMO v. 5.5.3 (https://meme-suite.org/meme/tools/fimo) (58) with the PerR binding site sequences and default parameters. Only FIMO hits falling within the first third of a coding sequence or up to 100 bp upstream of a coding sequence were retained; to this end, we filtered the FIMO hits using Python v. 3.9.2, the BioPython package v. 1.78, and GenBank LAC annotation files from NCBI (GCF_015475575.1). Locus tags corresponding to coding sequences in the annotation files of the LAC assembly (GCF_015475575.1) were translated to those of a USA300 FPR3757 assembly (GCF_000013465.1) via sequence similarity search with blastp v. 2.11.0, only retaining hits at 95% identity with 95% coverage. FIMO hits were plotted in R (74) v. 4.3.0 using ggplot2 v. 3.4.2 (75). Only hits

with a FIMO score in the upper 5% were colored red, additionally labeling those with a gene symbol, if available.

Statistics

Statistical significance was determined using Prism 9.0 (GraphPad Software). One-way ANOVA with multiple comparisons was used to compare data sets with more than two strains. Unpaired *t*-tests with Welch's correction were used for comparing data sets that only had two mutants.

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BioRender was used to create some images.

E.E.A. and V.J.T. designed the study. A.S., E.E.A., S.D., and M.P. generated strains, and A.S. performed the experiments. E.E.A. and S.D. trained A.S. A.P. performed the bioinformatic analyses, and B.S. and V.J.T. managed the project. A.S. wrote the first draft of the manuscript, and E.E.A., S.D., and V.J.T. worked on the final version of the manuscript. All authors commented on the manuscript.

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DIRECT CONTRIBUTION

This article is a direct contribution from Victor J. Torres, a member of the *Infection and Immunity* Editorial Board, who arranged for and secured reviews by Lindsey Shaw, University of South Florida, and Anthony Richardson, University of Pittsburgh.

ETHICS APPROVAL

Buffy coats were obtained from anonymous healthy donors from the New York Blood Center. All experiments were performed according to NIH guidelines and U.S. federal law.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Tables S2 and S3 (IAI00526-23-s0001.docx). Strains and plasmids, as well as oligonucleotides, used in this study.
Table S1 (IAI00526-23-s0002.xlsx). Primary screen measuring *lukAB* promoter activity in *S. aureus* JE2 regulatory transposon library containing pHC123 reporter plasmid.
Table S4 (IAI00526-23-s0003.xlsx). PerR binding sites.

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