

Staphylococcus aureus SaeR/S-regulated factors reduce human neutrophil reactive oxygen species production

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ABSTRACT

Neutrophils are the first line of defense after a pathogen has breached the epithelial barriers, and unimpaired neutrophil functions are essential to clear infections. *Staphylococcus aureus* is a prevalent human pathogen that is able to withstand neutrophil killing, yet the mechanisms used by *S. aureus* to inhibit neutrophil clearance remain incompletely defined. The production of reactive oxygen species (ROS) is a vital neutrophil antimicrobial mechanism. Herein, we test the hypothesis that *S. aureus* uses the SaeR/S two-component gene regulatory system to produce virulence factors that reduce neutrophil ROS production. With the use of ROS probes, the temporal and overall production of neutrophil ROS was assessed during exposure to the clinically relevant *S. aureus* USA300 (strain LAC) and its isogenic mutant LACΔ*saeR/S*. Our results demonstrated that SaeR/S-regulated factors do not inhibit neutrophil superoxide (O_2^-) production. However, subsequent neutrophil ROS production was significantly reduced during exposure to LAC compared with LACΔ*saeR/S*. In addition, neutrophil H_2O_2 production was reduced significantly by SaeR/S-regulated factors by a mechanism independent of catalase. Consequently, the reduction in neutrophil H_2O_2 resulted in decreased production of the highly antimicrobial agent hypochlorous acid/hypochlorite anion ($HOCl/^-OCl$). These findings suggest a new evasion strategy used by *S. aureus* to diminish a vital neutrophil antimicrobial mechanism. *J. Leukoc. Biol.* 100: 1005–1010; 2016.

Introduction

PMNs (leukocytes or neutrophils) are the most abundant WBC in the human body and the first line of defense during bacterial

infection [1]. Following migration to the site of infection and phagocytosis, neutrophils expose pathogens to an abundance of microbicidal components, including cationic peptides, proteases, and potent ROS [2, 3]. Assembly and activation of the NADPH oxidase system result in the production of O_2^- from molecular O_2 , followed by dismutation to H_2O_2 and to the formation of the highly bactericidal agent $HOCl/^-OCl$, catalyzed by the enzyme MPO [4–6]. The production of neutrophil ROS is highly effective at killing many pathogens, including the Gram-positive pathogen *S. aureus* [7, 8]. Microbicidal capacity of ROS against *S. aureus* is exemplified further by the observed increase in susceptibility to infections in individuals with genetic defects in any of the 5 structural components of the NADPH oxidase complex, resulting in chronic granulomatous disease [9, 10]. Collectively, neutrophil microbicidal systems are very efficient at killing ingested bacteria and limiting inflammation.

Despite the neutrophil's capacity to contain most bacterial pathogens, its antimicrobial mechanisms are not fully effective in killing *S. aureus*, and bacterial survival following neutrophil phagocytosis has been proposed as a virulence strategy used by this bacterium [11, 12]. This is best exemplified by the epidemic of CA-MRSA that began in the late 1990s [13]. CA-MRSA can cause uncomplicated skin and soft-tissue infections, as well as invasive, life-threatening illnesses in otherwise healthy individuals [13–15], and its emergence has fostered research investigating the ability of *S. aureus* to survive after neutrophil phagocytosis.

Survival of *S. aureus* following neutrophil phagocytosis is dependent on the concerted effort of multiple virulence factors [11, 12, 16–18]. The *S. aureus* SaeR/S two-component system regulates virulence genes essential for evasion of neutrophil killing [12, 19, 20]. In brief, the *sae* locus consists of 4 open-reading frames (*saePQRS*). *SaeS* and *saeR* code for the two-component module of the SaeR/S system, where *SaeS* is the histidine kinase, and *SaeR* is the cognate response regulator. SaeR/S-regulated genes are up-regulated in response to

Abbreviations: CA-MRSA = community-associated methicillin-resistant *Staphylococcus aureus*, DPBS = Dulbecco's PBS, $HOCl/^-OCl$ = hypochlorous acid/hypochlorite anion, LAC HK = heat-killed LAC, MPO = myeloperoxidase, O_2^- = superoxide, PMN = polymorphonuclear leukocyte, ROS = reactive oxygen species, SOD = superoxide dismutase, WT = wild-type

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neutrophil-derived components, including human α -defensin-1 and H_2O_2 [19, 21, 22]. Studies using isogenic *saeR/S* deletion mutants have shown that neutrophil lysis is reduced, and *S. aureus* has significantly reduced survival following neutrophil phagocytosis in the absence of SaeR/S [11, 12]. SaeR also controls the expression of secreted virulence factors, including those that contribute to neutrophil lysis, such as leukocidin G/H (*lukG/H* also known as *lukA/B*), γ hemolysins (*hlgA, B, C*), and Pantone-Valentine leukocidin (*lukF/S-PV*) [12, 23]. Additionally, SaeR-regulated factors influence neutrophil cell fate contributing to pathogen survival [22].

In this study, we investigate further the role of *S. aureus* SaeR/S in modulating neutrophil function by examining its influence on ROS production. Our results demonstrate that SaeR/S-regulated factors decrease neutrophil-derived H_2O_2 and HOCl production by a mechanism independent of catalase activity.

MATERIALS AND METHODS

Bacterial strains and culture

WT *S. aureus* pulsed-field gel electrophoresis-type USA300 (strain LAC) [24] and its previously generated isogenic *saeR/S* mutant strain (*LAC Δ saeR/S*) [23] were grown in tryptic soy broth containing 0.5% glucose and harvested at midexponential growth, as described previously [11, 22, 23].

Neutrophil and ROS assays

Human neutrophils were isolated from heparinized venous blood of healthy volunteer donors in accordance with a protocol approved by the Institutional Review Board for Human Subjects at Montana State University. Human neutrophils were isolated as described previously [11, 12]. For all ROS assays, 1×10^6 neutrophils (loaded with various probes, described below) were exposed to 1×10^7 bacteria (10:1 bacteria:PMN ratio) in 96-well serum-coated plates in duplicate. Neutrophils were also exposed to LAC HK (5 min at 99°C), 500 ng/ml PMA, or RPMI, and phagocytosis was synchronized [11]. All measurements were done using a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

Luminol. Neutrophil-derived ROS detection by the oxidation of cell-permeable luminol, resulting in chemiluminescence, was measured as described previously [25]. Neutrophils were stained with 100 μ M luminol for 15 min in the dark at 4°C. Chemiluminescence was measured in 1 min intervals. The final concentrations of SOD and catalase were 50 and 2000 U/ml, respectively.

Isoluminol. Neutrophil-derived O_2^- production by the oxidation of cell-impermeable isoluminol, resulting in chemiluminescence, was measured as described previously [25]. Neutrophils were stained with 100 μ M isoluminol for 15 min in the dark at 4°C. Chemiluminescence was measured in 1 min intervals.

Amplex Red. Neutrophil-derived extracellular H_2O_2 production was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's instructions. Amplex Red oxidation was measured in 1 min intervals with 535/595 nm excitation/emission wavelengths.

Secreted catalase. The Amplex Red Catalase Assay Kit (Thermo Fisher Scientific) was used following the manufacturer's suggested protocol. Supernatants from PMN and/or bacterial incubations were collected following 90 min exposure at 37°C and sterile filtered with 0.22 μ m syringe filters.

R19-S. Neutrophil-derived HOCl production was measured using R19-S (FutureChem, Seoul, South Korea) [26, 27]. Oxidation of R19-S was measured in 1 min intervals with 485/535 nm excitation/emission wavelengths. Alternatively, neutrophil intracellular HOCl production at 90 min was measured by flow cytometry with a BD FACSCalibur (BD Biosciences, San Jose, CA, USA) using 488/530 nm emission/excitation wavelengths.

Neutrophil phagocytosis assay

Phagocytosis of *S. aureus* by human neutrophils was determined with fluorescence microscopy, as described previously [11]. FITC-labeled bacteria were added (10:1 bacteria:neutrophil ratio), and phagocytosis was synchronized as above. To counterstain uningested bacteria, samples were stained with anti-FITC conjugated to Alexa Fluor 594 (Thermo Fisher Scientific), and mounted coverslips were evaluated using fluorescence microscopy. The number of *S. aureus* bound and/or ingested was evaluated in 25 or 50 neutrophils per experiment from separate fields of view, and percent phagocytosis was calculated as (number of ingested bacteria per cell/total number of PMN-associated bacteria per cell, bound or ingested) \times 100.

HOCl killing

A mixture of HOCl/ $^-$ OCl was generated by mixing 10 ml commercially available Clorox, 5 ml DPBS, and 160 μ l 36.5–38.0% hydrochloric acid. After overnight incubation in the dark, the concentration of HOCl/ $^-$ OCl was calculated using Beer's Law [28]. The average pH of the solution was 7.51 ± 0.09 , similar to the acid dissociation constant for HOCl/ $^-$ OCl ($pK_a = 7.44$) [28, 29]. Bacteria (1×10^7), resuspended in DPBS, were mixed with HOCl/ $^-$ OCl, diluted in DPBS to desired working concentrations and incubated at 37°C for 30 min. Bacteria were enumerated following overnight incubation at 37°C. Bacterial survival was calculated relative to bacterial concentration following exposure to DPBS only.

Statistical procedures

Statistical analyses were performed using GraphPad Prism version 6.0a (GraphPad Software, La Jolla, CA, USA) with *t* tests and ANOVA as indicated, and error bars represent the SEM.

RESULTS AND DISCUSSION

SaeR/S-regulated factors decrease human neutrophil ROS production

Previous studies have shown that human neutrophils fail to kill *S. aureus* completely after phagocytosis, and SaeR/S-regulated factors are at least partially responsible for reduced bactericidal activity [11, 12]. However, the mechanisms affected by SaeR/S-regulated factors leading to reduced neutrophil staphylococcal killing have yet to be elucidated fully. To this end, neutrophil ROS production in response to WT *S. aureus* strain LAC and its isogenic Δ *saeR/S* mutant strain (*LAC Δ saeR/S*) was analyzed using probes to measure different ROS. First, intracellular neutrophil ROS production was measured with luminol. Whereas there were no significant differences in the time to maximum neutrophil ROS production, total neutrophil ROS production was reduced significantly in response to the *S. aureus* LAC compared with *LAC Δ saeR/S* (Fig. 1A and B). To determine if extracellular ROS contributed to abundance of intracellular ROS, neutrophils were exposed to LAC or *LAC Δ saeR/S*, including SOD or catalase. As expected and in congruence with published findings, the addition of exogenous SOD reduced total neutrophil ROS production in response to LAC and *LAC Δ saeR/S* (Fig. 1C) [30, 31]. In the presence of SOD, significant differences remained in total neutrophil intracellular ROS production during exposure to LAC and *LAC Δ saeR/S* (Fig. 1D). The addition of exogenous catalase also reduced total neutrophil intracellular ROS production in response to LAC and *LAC Δ saeR/S* compared with untreated neutrophils exposed to bacteria (Fig. 1E). However, neutrophils produced similar amounts of intracellular ROS in

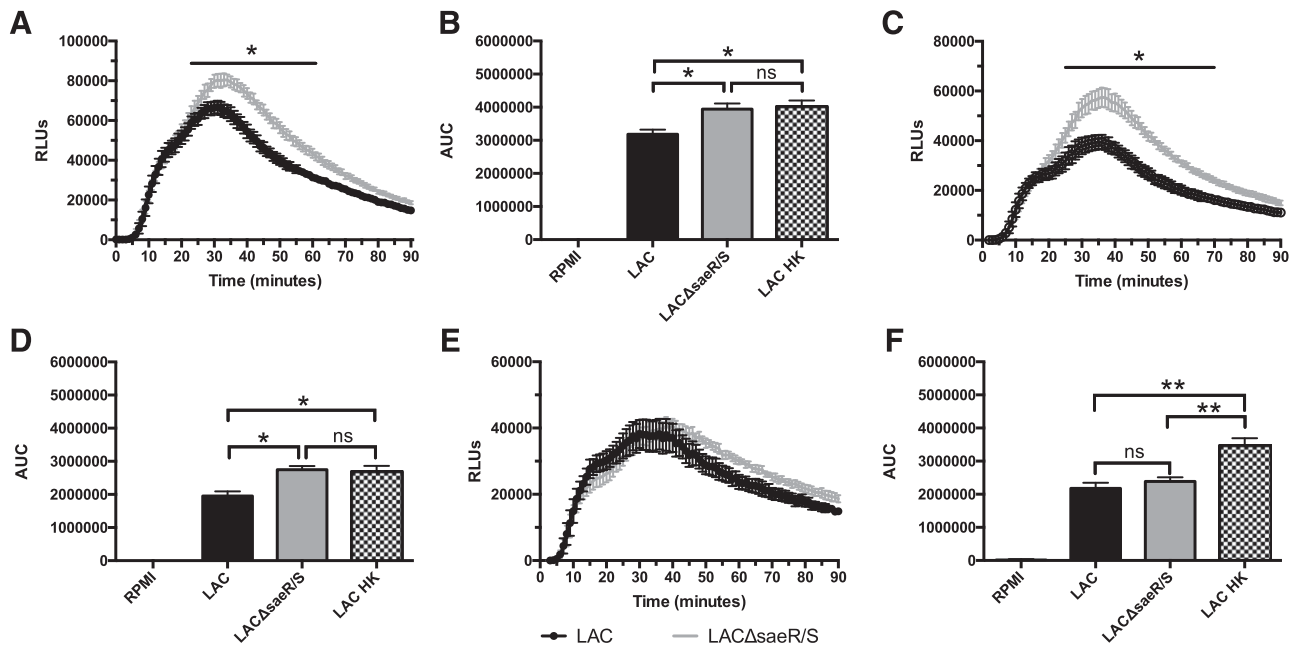


Figure 1. SaeR/S-regulated factors decrease intracellular ROS. Human PMNs were preloaded with luminol, as described in Materials and Methods, and exposed to LAC, LAC Δ saeR/S, or RPMI, and chemiluminescence was measured. (A) Time-dependent neutrophil intracellular ROS production following exposure to *S. aureus* LAC or LAC Δ saeR/S. (B) Total relative neutrophil ROS production determined by calculating the area under the curve (AUC) from A. (C) Time-dependent neutrophil intracellular ROS production following exposure to *S. aureus* LAC or LAC Δ saeR/S in the presence of exogenous SOD. (D) Total relative neutrophil ROS production determined by calculating the area under the curve from C. (E) Time-dependent neutrophil intracellular ROS production following exposure to *S. aureus* LAC or LAC Δ saeR/S in the presence of exogenous catalase. (F) Total relative neutrophil ROS production determined by calculating the area under the curve from E. Data represent 5 separate experiments, using 5 different neutrophil donors; * $P \leq 0.05$, ** $P \leq 0.01$, as determined by two-way ANOVA (A, C, and E) and one-way ANOVA (B, D, and F). RLUs, Relative luminescence units.

response to LAC and LAC Δ saeR/S in the presence of exogenous catalase (Fig. 1F). Collectively, these results suggest that *S. aureus* SaeR/S-regulated factors reduce intracellular ROS production. Additionally, neutrophils exposed to LAC HK produced similar amounts of intracellular ROS as those exposed to LAC Δ saeR/S (Fig. 1B and D), confirming that reduction of neutrophil ROS by *S. aureus* is an active process that requires a viable organism and SaeR/S. Importantly, the neutralization of extracellular H₂O₂ with exogenous catalase eliminated differences in intracellular neutrophil ROS production in response to LAC and LAC Δ saeR/S. These results suggest that *S. aureus* SaeR/S-regulated factors reduce the

production of neutrophil-derived H₂O₂, leading to a reduction in overall ROS production. Of note, neutrophil propidium iodide uptake was not significantly different between neutrophils exposed to LAC or LAC Δ saeR/S after 90 min, confirming that differences in ROS production were not a result of differences in neutrophil membrane damage (data not shown). As differences in uptake of LAC and LAC Δ saeR/S could influence ROS abundance, we assessed neutrophil phagocytosis using fluorescence microscopy. There were no differences in neutrophil ingestion of WT LAC compared with the LAC Δ saeR/S mutant (Supplemental Fig. 1), which is in agreement with previous findings [12].

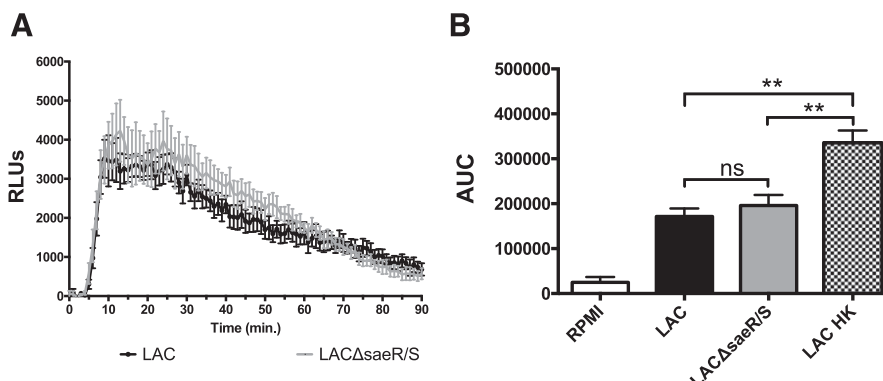


Figure 2. Human neutrophil extracellular O₂⁻ production following exposure to WT *S. aureus* LAC and LAC Δ saeR/S using isoluminol. (A) Time-dependent neutrophil extracellular O₂⁻ production following exposure to *S. aureus* LAC or LAC Δ saeR/S. (B) Total relative neutrophil O₂⁻ production determined by calculating the area under the curve from A. Data represent 4 separate experiments, using 4 different neutrophil donors; ** $P \leq 0.01$, as determined by two-way ANOVA (A) and one-way ANOVA (B).

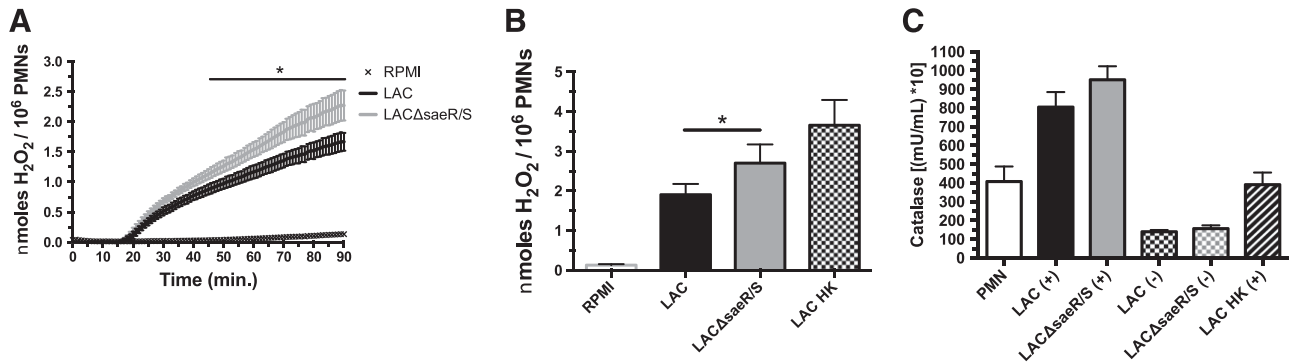


Figure 3. Human neutrophil extracellular H_2O_2 production is significantly reduced by SaeR/S-regulated *S. aureus* factors. (A) Time-dependent neutrophil extracellular H_2O_2 production was measured using Amplex Red, following exposure *S. aureus* LAC or LAC Δ saeR/S or RPMI as a control. (B) Neutrophil-derived extracellular H_2O_2 production calculated from an H_2O_2 standard curve. (C) Secreted catalase following *S. aureus* exposure to human neutrophils. Bacteria exposed (+) or not exposed (-) to neutrophils. Data represent 4 separate experiments (A and C) and 5 separate experiments (B), with $*P \leq 0.05$, as determined by two-way ANOVA (A) and paired *t* test (B).

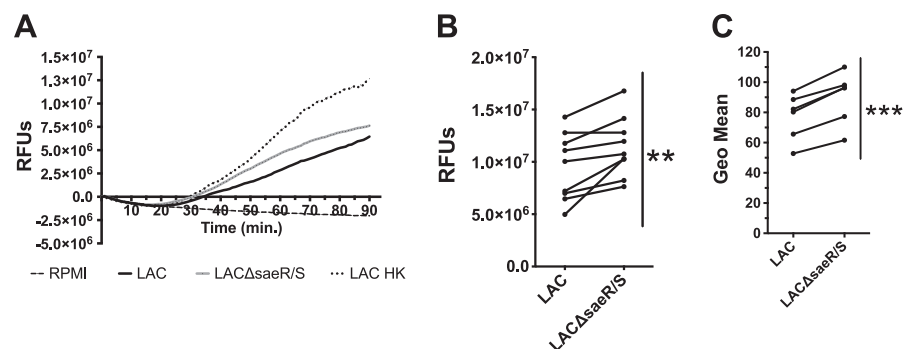
SaeR/S-regulated factors reduce the production of neutrophil-derived H_2O_2

Luminol measures overall intracellular ROS production. As our results showed that extracellular ROS played a significant role in enhancing intracellular neutrophil ROS production (Fig. 1A–F) and suggested that SaeR/S-regulated factors reduce the production of neutrophil-derived H_2O_2 , we used isoluminol and Amplex Red probes to measure specifically O_2^- and H_2O_2 , respectively. Cell-impermeable isoluminol was used to measure neutrophil extracellular O_2^- production in response to LAC and LAC Δ saeR/S. We hypothesized that neutrophil O_2^- production would not be reduced by SaeR/S-regulated factors, as significant differences remained in overall neutrophil intracellular ROS production during exposure to LAC and LAC Δ saeR/S in the presence of exogenous SOD (Fig. 1C and D). The neutrophil O_2^- burst occurred within 10 min after recording luminescence (Fig. 2A). Consistent with results shown in Fig. 1C and D, there were no significant differences in neutrophil O_2^- production during exposure to LAC or LAC Δ saeR/S (Fig. 2B). This suggests that activation and assembly of the NADPH oxidase complex are not affected by SaeR/S-regulated factors. Exposure to LAC HK did result in significantly increased O_2^- production compared with LAC and LAC Δ saeR/S. This is expected, as *S. aureus*

produces SOD to neutralize O_2^- via *sodA* and *sodM*, but these genes are not regulated by SaeR/S [12, 23, 32, 33].

As the measurement of neutrophil intracellular ROS with luminol in the presence of catalase suggested that SaeR/S-regulated factors reduce production of extracellular H_2O_2 (Fig. 1C and D), we used the H_2O_2 -specific probe Amplex Red to measure neutrophil extracellular H_2O_2 production in response to *S. aureus* LAC and LAC Δ saeR/S. Detectable neutrophil-derived H_2O_2 was observed within 20 min of exposure to LAC and LAC Δ saeR/S (Fig. 3A). Significant differences in extracellular H_2O_2 production between neutrophils exposed to LAC and LAC Δ saeR/S were observed, starting at 43 min. In addition, there were significant increases in molar amounts of H_2O_2 produced by neutrophils in response to LAC Δ saeR/S versus LAC at the end of the 90 min assay (Fig. 3B). Differences in extracellular H_2O_2 production by neutrophils confirmed results showing overall reduction in ROS—and no significant differences between neutrophils exposed to LAC and LAC Δ saeR/S—when the cell-impermeable catalase was present (Fig. 1E and F). Importantly, there were no differences in the secreted catalase that could degrade extracellular H_2O_2 (Fig. 3C), confirming that SaeR/S does not regulate catalase following neutrophil phagocytosis.

Figure 4. Human neutrophil intracellular hypochlorite production is reduced significantly by SaeR/S-regulated *S. aureus* factors. HOCl production was measured in human neutrophils using R19-S, following exposure to WT *S. aureus* LAC or LAC Δ saeR/S. (A) Representative plot of time-dependent neutrophil intracellular HOCl production following exposure to *S. aureus* LAC or LAC Δ saeR/S or LAC HK or RPMI as a control. (B) Relative neutrophil-derived HOCl production at the end of the 90 min assay from A. (C) Relative neutrophil-derived intracellular HOCl measured by flow cytometry. Data represent 9 separate experiments (B) and 6 separate experiments (C); $**P \leq 0.01$, $***P \leq 0.001$, as determined by paired *t* test (B and C). RFUs, Relative fluorescence units.



SaeR/S-regulated factors reduce the production of neutrophil-derived HOCl

HOCl is present in neutrophil phagosomes and is highly bactericidal. H_2O_2 is a precursor to the production of MPO-catalyzed HOCl. Therefore, we measured intracellular HOCl production using the cell-permeable R19-S probe to assess how SaeR/S-regulated factors affect HOCl production (Fig. 4A). Consistent with our results showing that SaeR/S reduced H_2O_2 production, SaeR/S-regulated factors significantly decreased neutrophil HOCl production (Fig. 4B and C). Production of neutrophil H_2O_2 and HOCl was detected at ~ 20 min, with the maximum H_2O_2 burst occurring earlier than the HOCl burst (Supplemental Fig. 2).

The formation of H_2O_2 from O_2^- can occur spontaneously or is catalyzed by SOD or MPO [34, 35]. Based on our observations, we propose that unidentified SaeR/S-regulated factor(s) interfere with the enzymatic reactions catalyzed by MPO to produce H_2O_2 and HOCl. Inasmuch as there are no differences in secreted catalase between neutrophils exposed to *S. aureus* LAC and $\text{LAC}\Delta\text{saeR/S}$ (Fig. 3C), we propose that the significant decrease in H_2O_2 produced by neutrophils exposed to LAC compared with $\text{LAC}\Delta\text{saeR/S}$ is a result of SaeR/S-regulated factors inhibiting the SOD activity of MPO to produce H_2O_2 . In addition, SaeR/S-regulated factors may interfere with the chlorination activity of MPO, resulting in decreased production of HOCl. Ongoing studies are determining if decreased HOCl production is a direct result of lower H_2O_2 production or if SaeR/S-regulated factors directly interfere with both the dismutase and chlorination activity of MPO.

WT *S. aureus* LAC and $\text{LAC}\Delta\text{saeR/S}$ are equally susceptible to reagent HOCl

S. aureus SaeR/S-regulated factors significantly decreased neutrophil-derived HOCl production in response to WT LAC versus $\text{LAC}\Delta\text{saeR/S}$ (Fig. 4). To determine if the WT and mutant were differentially susceptible to HOCl, we exposed LAC and $\text{LAC}\Delta\text{saeR/S}$ to varied concentrations of HOCl. As shown in Fig. 5, LAC and $\text{LAC}\Delta\text{saeR/S}$ were equally susceptible to HOCl

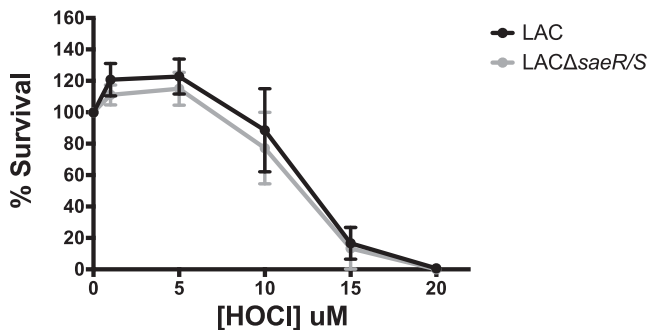


Figure 5. *S. aureus* LAC and $\text{LAC}\Delta\text{saeR/S}$ are equally susceptible to killing by HOCl. Bacteria (1×10^7) were exposed to different concentrations of HOCl for 30 min at 37°C . Subsequently, bacteria were serially diluted and plated on trypticase soy agar plates, and CFUs were enumerated following overnight incubation at 37°C . Data represent 5 separate experiments.

exposure. HOCl concentrations below $5 \mu\text{M}$ did not kill *S. aureus*. However, there was a precipitous decrease in *S. aureus* survival following exposure between 5 and $15 \mu\text{M}$ HOCl. These data are in agreement with previously published findings [36]. Future studies will investigate the physiologically relevant question as to whether reduction in ROS makes WT *S. aureus* more resistant to neutrophil killing reflective of clinical syndromes, demonstrating the importance of ROS in controlling *S. aureus* [9, 10]. It is possible that a SaeR/S-mediated reduction in ROS makes the ROS amount insufficient to kill *S. aureus*, as in vitro studies have demonstrated a fine line between ROS amounts that are effective versus amounts that the pathogen can tolerate (Fig. 5 and ref. [21]).

The importance of detoxifying ROS is demonstrated by the many mechanisms used by *S. aureus* that include scavenging and neutralizing ROS, such as O_2^- and H_2O_2 , with SODs (SodA and SodM) and catalase (KatA), respectively [37, 38]. In addition, the iron-regulated surface-determinant proteins IsdA and IsdB have been implicated in increasing *S. aureus* resistance to killing by H_2O_2 [21], as well as methionine sulfoxide reductases to reduce oxidized methionine residues following oxidative stress that increase *S. aureus* resistance to ROS [39]. Unlike previously described mechanisms to neutralize ROS, our data suggest that *S. aureus* can directly inhibit H_2O_2 and HOCl production, resulting in increased *S. aureus* survival. Future studies will focus on identifying specific SaeR/S-regulated factors that inhibit neutrophil ROS production.

AUTHORSHIP

F.E.G. contributed to project design and experimental procedures, analyzed data, provided the figure presentation, and wrote the manuscript. C.B.A., J.A., N.W.M.d.J., K.B.P., and J.v.S. contributed to project design and experimental procedures. J.M.V. provided oversight and contributed to project design, data analysis, figure presentation, and manuscript writing.

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DISCLOSURES

The authors have declared that there are no conflicts of interest.

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