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HIGHLIGHTED ARTICLE

Oxidation of bacillithiol during killing of *Staphylococcus aureus* USA300 inside neutrophil phagosomes

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Abstract

Targeting immune evasion tactics of pathogenic bacteria may hold the key to treating recalcitrant bacterial infections. *Staphylococcus aureus* produces bacillithiol (BSH), its major low-molecular-weight thiol, which is thought to protect this opportunistic human pathogen against the bombardment of oxidants inside neutrophil phagosomes. Here, we show that BSH was oxidized when human neutrophils phagocytosed *S. aureus*, but provided limited protection to the bacteria. We used mass spectrometry to measure the oxidation of BSH upon exposure of *S. aureus* USA300 to either a bolus of hypochlorous acid (HOCl) or a flux generated by the neutrophil enzyme myeloperoxidase. Oxidation of BSH and loss of bacterial viability were strongly correlated ($r = 0.99$, $p < 0.001$). BSH was fully oxidized after exposure of *S. aureus* to lethal doses of HOCl. However, there was no relationship between the initial BSH levels and the dose of HOCl required for bacterial killing. In contrast to the HOCl systems, only 50% of total BSH was oxidized when neutrophils killed the majority of phagocytosed bacteria. Oxidation of BSH was decreased upon inhibition of myeloperoxidase, implicating HOCl in phagosomal BSH oxidation. A BSH-deficient *S. aureus* USA300 mutant was slightly more susceptible to treatment with either HOCl or ammonia chloramine, or to killing within neutrophil phagosomes. Collectively, our data show that myeloperoxidase-derived oxidants react with *S. aureus* inside neutrophil phagosomes, leading to partial BSH oxidation, and contribute to bacterial killing. However, BSH offers only limited protection against the neutrophil's multifaceted killing mechanisms.

KEYWORDS
hypochlorous acid, low-molecular-weight thiol, methicillin-resistant *Staphylococcus aureus*, myeloperoxidase

Abbreviations: BSH, reduced bacillithiol; BSO_2H, bacillithiol sulfinic acid; BSO_2N, bacillithiol sulfonamide; BSO_3H, bacillithiol sulfonic acid; BSSB, bacillithiol disulfide; BSSP, bacillithiol mixed disulfide with protein thiols; BSSX, bacillithiol mixed disulfide with another LMW thiol; CoA, coenzyme A; DPI, diphenylene iodonium chloride; GO, glucose oxidase; HBSS, Hank's balanced salt solution; HOCl, hypochlorous acid; LB, Lysogeny broth; LMW, low-molecular-weight thiol; MOI, multiplicity of infection; MPO, myeloperoxidase; NEM, *N*-ethylmaleimide; PBS, Phosphate buffered saline; RPMI, Roswell Park Memorial Institute 1640 medium; TSB, tryptic soy broth; WT, wild type.

1 | INTRODUCTION

Staphylococcus aureus is a commensal bacterium, but also a major human pathogen. Approximately 30% of humans carry this Gram-positive bacterium asymptomatically on their nasal mucosa and skin.[1] If this natural barrier is breached, invasive disease can occur, which ranges from localized wound infections and abscesses to life-threatening systemic infections including septicemia and pneumonia.[2] *S. aureus* has evolved various immune evasion mechanisms, which along with the emergence of antibiotic resistance, make this pathogen a significant risk to human health.[3] A better appreciation of how the immune system tries to kill this microbe and how *S. aureus* endeavors to resist these bactericidal mechanisms may reveal new strategies for combating *S. aureus* infections.

Neutrophils are central to human defense against *S. aureus*, as illustrated by the severe infections that patients suffer when their neutrophils are defective.[4] Neutrophils ingest staphylococci into phagosomes where they are exposed to a variety of toxins including antimicrobial peptides, cell wall hydrolases, proteases, and reactive oxidants. Oxidant production is initiated by the assembly of the NADPH oxidase on the phagosomal membrane, which uses electrons from cytosolic NADPH to reduce molecular oxygen to superoxide ($O_2^{\cdot -}$). Following fusion of azurophilic granules, the heme enzyme myeloperoxidase (MPO) is released into the phagosome along with antimicrobial proteins and peptides required for nonoxidative killing. MPO converts superoxide to hydrogen peroxide, which it uses in turn to generate the potent bactericidal oxidant hypochlorous acid (HOCl).[5,6]

Oxidative mechanisms contribute to the killing of *S. aureus* by neutrophils. Furthermore, the use of MPO inhibitors and neutrophils from MPO-deficient individuals has demonstrated that oxidative killing is predominantly an MPO-dependent process.[7] HOCl was proposed to be the most likely product to mediate MPO-dependent killing because it is a potent bactericidal oxidant that effectively kills a variety of bacteria in vitro at low doses.[8–12] However, whether or not *S. aureus* is exposed to lethal doses of this oxidant inside neutrophil phagosomes has not been conclusively demonstrated.

Some *S. aureus* cells can survive within neutrophil phagosomes, known as persisters or small colony variants, which are tolerant to antibiotics and various stresses.[13] The bacteria produce several virulence factors and antioxidant defense systems that provide protection under host-pathogen interactions and disarm MPO.[14] These include catalase to degrade hydrogen peroxide,[15] a protein called SPIN that directly inhibits MPO,[16] an HOCl-induced disulfide reductase MerA that is controlled by the HypR repressor,[17] and the major low-molecular-weight (LMW) thiol bacillithiol (BSH) possibly involved in HOCl detoxification.[18,19] The relative effectiveness of these bacterial antioxidant defenses compared with the neutrophil's oxidative attack may determine the fate of bacteria within phagosomes.

BSH has several physiological functions in *S. aureus* and other Gram-positive bacteria, including processing of intracellular metals and detoxification of oxidants and electrophiles,[18,20–24] which in eukaryotes and many Gram-negative bacteria are carried out by glutathione. Lack of BSH makes *S. aureus* more susceptible to hydrogen peroxide and HOCl, suggesting a role for this LMW thiol in resisting oxidants produced by the innate immune system.[25–28] In support of this proposal, BSH deficiency lowers survival of *S. aureus* in whole blood,[26] human airway cells, and murine macrophages.[27] In addition to direct scavenging of oxidant, the protective role of BSH can be ascribed to the formation of BSH mixed disulfides with metabolic proteins (e.g., GAPDH and MetE) and the redox regulation of thiol-based transcription factors (e.g., OhrR), as revealed in *B. subtilis* and *S. aureus*.[29,30] Consequently, if significant amounts of HOCl react with *S. aureus* in the neutrophil phagosome, then BSH may protect the bacteria.

We recently monitored glutathione and mycothiol oxidation in *Pseudomonas aeruginosa* and *Mycobacterium smegmatis*, respectively, and demonstrated that HOCl produced in the phagosome can account for death of *P. aeruginosa* but not *M. smegmatis*.[31,32] In this work, we have focused our attention on BSH oxidation during phagocytosis of *S. aureus* because it has the potential to demonstrate whether lethal doses of HOCl react with *S. aureus* within phagosomes, and to show whether the major LMW thiol protects *S. aureus* against the oxidative attack of neutrophils.

2 | MATERIAL AND METHODS

2.1 | Reagents

BSH was purchased from Carbosynth Ltd (Compton, UK). HOCl was a commercial chlorine bleach product sold by Pental (Melbourne, Australia).Hank's balanced salt solution (HBSS) and phosphate buffered saline (PBS for cell culture, glucose oxidase (GO) from *Aspergillus niger* (≥100,000 U/g), *N*-ethylmaleimide (NEM), 1,4-dithiothreitol (DTT), taurine, 3,3′,5,5′-tetramethylbenzidine (TMB), diphenylene iodonium chloride (DPI), sodium azide, bovine liver catalase, lysostaphin from *Staphylococcus staphylolyticus*, chloramphenicol and D-(+)-xylose were purchased from Sigma (Merck, Darmstadt, Germany). Roswell Park Memorial Institute 1640 medium (RPMI,Gibco), Lysogeny broth (LB; Miller's) and tryptic soy broth (TSB) powder were from Thermo Fisher (Waltham, MA, USA), and saponin was from Fluka (Buchs, Switzerland). MPO, from human neutrophils, was supplied by PLANTA (Vienna, Austria) and had a purity index (A_{430}/A_{280}) of at least 0.82. Dextran from *Leuconostoc mesenteroides* (average molecular weight: 150,000 Da; Sigma) and Ficoll-Paque (GE Healthcare, Uppsala, Sweden, and Freiburg, Germany) were used for neutrophil isolation. The specific MPO inhibitors TX1 and AZM198, 2-thioxanthine molecules,[33] were provided by AstraZeneca (Mölndal, Sweden).

2.2 | Cultivation of *S. aureus*

We used the *S. aureus* USA300 and USA300JE2 WT strains, their isogenic Δ*bshA* mutants and the *S. aureus* USA300JE2 pRB473-*bshA* complemented strain. Construction of the *S. aureus* USA300 Δ*bshA* mutant was described previously.[26] The *S. aureus* USA300JE2 *bshA* mutant and

its *bshA* complemented strain were constructed using the pMAD-delta-*bshA* and pRB473-*bshA* plasmids, respectively, as described previously for the corresponding *S. aureus* COL strains.[34] Bacteria were stored and grown under standard conditions and maintained on Columbia sheep blood agar plates. To generate stationary phase bacteria, 10 ml LB or TSB was inoculated with a single colony of *S. aureus* and grown overnight in a shaking incubator at 37°C at 200 rpm. The complemented USA300JE2 strain required the addition of 1% xylose and 10 μg/ml chloramphenicol in the inoculation medium. For experiments using log phase bacteria, 1 ml of an overnight culture was diluted the next morning into 9 ml of fresh culture medium and grown until mid-log phase. Bacteria were pelleted by centrifugation at 12,000×*g* for 5 min and washed twice with PBS, then resuspended in HBSS. Bacterial aggregates were removed by a slow spin at 100×*g* for 5 min and the concentration of bacteria in the supernatant was estimated by its OD_{550}. This estimate was based on a standard curve for USA300 WT of CFU versus the OD_{550}, which gave an OD_{550} of 0.12 for 1×10^8 bacteria/ml. The bacterial concentration was determined empirically using the standard practice of spreading bacteria on to Colombia sheep blood agar plates and counting CFUs, which were repeated in four technical replicates.

2.3 | Measurement of BSH, CoA, and cysteine by LC–MS

Acetonitrile and NEM were added to washed bacteria to final concentrations of 50% and 20 mM, respectively, and incubated at 60°C for 15 min. Precipitated protein and cell debris was removed by centrifugation at 12,000×*g* for 5 min. Supernatants were diluted 1:5 either with 0.25% aqueous formic acid for measurement of the NEM adducts of BSH and cysteine or with water for that of coenzyme A (CoA). Diluted supernatants were injected for multiple reaction monitoring (MRM)-based LC–MS analyses as described before.[19,31]

2.4 | Treatment of *S. aureus* with HOCl, ammonia chloramine, or the GO/MPO system

Solutions of HOCl (50–400 μM) were prepared in HBSS by diluting a fresh stock that was prepared daily. The stock's concentration was determined spectrophotometrically using $\varepsilon_{292} = 350\ M^{-1}\ cm^{-1}$ at pH 12.[35] Ammonia chloramine (NH_2Cl, 1 mM) was prepared by mixing equal volumes of HOCl (2 mM) with ammonium chloride (20 mM), then diluted in HBSS to 50–160 μM as required and used within minutes. Preparations of HOCl and NH_2Cl at twice the final concentration, were added while vortexing to an equal volume of *S. aureus* USA300 in HBSS (2×10^9 CFU/ml, $OD_{550} = 2.4$) and incubated for 10, 30, or 60 min at 37°C with end-over-end rotation (Figure S1(A)). To expose bacteria to a flux of HOCl, *S. aureus* (1×10^9 CFU/ml) were incubated with GO (7.2 μg/ml) and MPO (50 nM) in HBSS at 37°C with end-over-end rotation. After 10 and 30 min, catalase (10 μg/ml) and the MPO inhibitor TX1 (20 μM) were added to stop the reaction.[33] The GO/MPO system generated 70 μM HOCl over 10 min as determined by incubating the enzyme system in the presence of 1 mM taurine to convert HOCl to the stable product taurine chloramine, which was subsequently measured colorimetrically using TMB and iodide as described before.[36]

After treating bacteria with oxidant, an aliquot was immediately serially diluted in pH 11 water to minimize bacterial clumping before spreading on plates for CFU of surviving bacteria. Separate aliquots were treated with acetonitrile (50%) and 20 mM NEM, then incubated at 60°C for 15 min. Protein and cell debris was removed by centrifugation at 12,000×*g* for 5 min and the supernatant was diluted 1:5 with 0.25% formic acid in water before LC–MS analysis to determine BSH–NEM as described before.[19]

2.5 | Isolation of human neutrophils from peripheral blood

Blood was collected from healthy human volunteers with informed consent, and ethical approval from the Southern Health & Disability Ethics Committee, New Zealand. Our studies abide by the Declaration of Helsinki principles. Human neutrophils were isolated under sterile conditions from freshly drawn heparinized blood. Dextran sedimentation was followed by Ficoll-Paque centrifugation, and erythrocyte lysis in hypotonic buffer.[37] Purified neutrophils were resuspended in RPMI supplemented with 2% fetal calf serum, kept at room temperature, and used as soon as possible. Neutrophils were ≥96% pure as determined by flow cytometry using their characteristic forward/side scatter, which is known to accurately report the neutrophil population.[38]

2.6 | Measurement of BSH species in *S. aureus* phagocytosed by human neutrophils

S. aureus cells were opsonized just prior to use by incubating them for 20 min at 37° C with end-over-end rotation (6 rpm) in HBSS containing 10% serum. The serum was either freshly collected from the neutrophil donor, or pooled human serum from at least six healthy donors that was stored frozen and thawed prior to use. Bacteria were then incubated at 5×10^7/ml with or without prewarmed neutrophils (5×10^6/ml) in HBSS containing 10% serum with end-over-end rotation. In some cases, neutrophils were preincubated with inhibitors at 37°C for 5 min prior to the addition of bacteria (final concentrations: 0.5 mM methionine, 100 μM sodium azide, 10 μM DPI, 10 μM AZM198). Untreated control bacteria were handled the same way and in parallel with the neutrophil and bacteria mixtures. Bacteria and neutrophils were mixed together and samples taken immediately before (time 0) or after incubation for 15 and 30 min. After addition of 50% acetonitrile and 20 mM NEM, the samples were incubated at 60°C for 15 min. Precipitated proteins and cell debris were removed by centrifugation at 12,000×*g* for 5 min, and the supernatant was diluted 1:5 with 0.25% formic acid in water. These samples were analyzed by LC–MS, as described previously,[19] to determine BSH–NEM, bacillithiol disulfide (BSSB), bacillithiol sulfonamide (BSO_2N), bacillithiol

sulfinic (BSO_2H), and bacillithiol sulfonic acid (BSO_3H). To quantify mixed disulfides with other LMW thiols (BSSX), an aliquot of the supernatant was reduced with 50 mM DTT and alkylated with 100 mM NEM for measurement of BSH–NEM. The concentration of BSSX was calculated by subtracting the concentrations of BSH-NEM and 2×BSSB, determined before reduction with DTT, from the BSH–NEM concentration measured after reduction. To quantify mixed disulfides with protein thiols (BSSP), the protein pellet obtained after precipitation with acetonitrile was washed and reduced with DTT, alkylated with NEM and BSH–NEM was quantified a measure of BSSP.

2.7 | Analysis of *S. aureus* viability during phagocytosis

Neutrophil bacterial killing assays were carried out as described previously.[39] Briefly, *S. aureus* cells were serum-opsonized and incubated with or without prewarmed neutrophils in HBSS containing 10% serum as described above. Time point samples were diluted into ice-cold PBS to stop bacterial growth and phagocytosis, and all subsequent handling was done on ice or at 4°C. For neutrophil-containing samples, a differential spin step of 100×*g* for 5 min was used to pellet the neutrophils leaving the nonphagocytosed bacteria in the supernatant. Each neutrophil pellet was gently resuspended in PBS for two further washes via centrifugation, and supernatants were pooled for quantification of nonphagocytosed bacteria. The phagocytosed bacteria were harvested by lysing the washed neutrophils in ice-cold PBS containing 0.05% saponin using ten strokes of a small tube-and-grinder glass homogenizer. Quantification of viable bacteria in the separate fractions was by serial dilution in pH 11 water, followed by plating on blood agar, and colony counting after overnight incubation at 37°C. For the killing assay, the number of bacteria phagocytosed by neutrophils was calculated from the difference in colony counts of a bacteria-only control and the pooled nonphagocytosed fraction. When comparing bacterial survival with residual BSH levels, the colony counts from the ingested and nonphagocytosed fractions were added together to give the number of bacteria present when the entire neutrophil and bacteria incubations were extracted with acetonitrile for the LC–MS analysis of BSH.

To directly quantify phagocytosis, incubations of neutrophils with bacteria were prepared as above, collecting samples at 5 and 10 min for analysis. After the initial quenching dilution and centrifugation step, the neutrophil pellets were resuspended and immediately spun onto microscope slides at approximately 5×10^5 neutrophils per slide (Shandon Cytospin 2, Warrington, England). The cells were fixed by methanol for Romanowsky-type staining using Diff-Quik Solutions I and II (RAL Diagnostics, Martillac, France) before mounting for light microscopy (Olympus CKX53 microscope with camera, Tokyo, Japan). Multiple images were captured (six per slide, 40× objective) and assessed independently by three people, two of whom were blinded to the identity of the samples. Means of the three counts were used to measure the number of phagocytosed bacteria/neutrophil, and data were normalized against the bacterial concentration of the initial inoculum as determined by colony counting.

For the neutrophil experiments measuring bacterial survival until 3 h post phagocytosis, the protocol was largely as outlined above but included some modifications. These changes in the method were to optimize a synchronous uptake of bacteria into phagosomes, provide nutritious media for the neutrophils over the longer timeframe, and to ensure there was no contamination of the harvested phagosomal intracellular bacteria by residual extracellular bacteria. The points of difference were as follows: neutrophils and opsonized bacteria were incubated together at 1×10^7 and $1–2 \times 10^8$/ml respectively, in RPMI containing 10% serum. This phagocytosis incubation (at 37°C with end-over-end rotation) was for 10 min only, then the entire mixture was spun at 100×*g*, 4°C, for 5 min, followed by one wash of the pellet with ice-cold PBS. The washed neutrophils were incubated at 5×10^6/ml in PBS containing 50 μg/ml lysostaphin for 5 min at 37°C to eliminate any *S. aureus* outside the neutrophils.[39] After centrifugation and a further wash with PBS, the neutrophils were resuspended at 1×10^7/ml in fresh RPMI and 10% serum. An aliquot was immediately removed as the time point zero sample, and the remainder was returned for a second incubation at 37° C with end-over-end rotation for 3 h. Time point aliquots were taken into ice-cold pH 11 water containing 0.05% saponin, and the neutrophils were immediately lysed by syringing five times through a 25-gauge needle. The CFUs of bacterial survivors were reported as a percentage of those phagocytosed in the first 10 min incubation period. In the modified protocol, a bacteria-only control was incubated for the first 10 min period only.

2.8 | Statistics

Data analysis was carried out using GraphPad Prism Software (La Jolla, USA). Statistical significance was determined using appropriate tests as described in the figure legends. A p value of less than 0.05 was considered significant.

3 | RESULTS

3.1 | BSH oxidation correlates with the loss of viability in HOCl-treated *S. aureus*

To establish the optimal growth conditions that promote high BSH levels in bacteria, we first measured the effect of different growth conditions on the BSH content in *S. aureus* USA300. BSH levels were measured using LC–MS and varied by up to 100-fold in the USA300 strain depending on the growth phase and media (Table 1). Known oxidation products of BSH, such as BSSB, BSO_2H, BSO_3H, and BSO_2N,[19] were not detected in *S. aureus* under any of the normal growth conditions. LB-grown bacteria yielded the highest BSH content. BSH levels measured in exponentially growing cells in TSB (1.9 ± 0.5 μmol/g dry weight) were similar to those reported in *S. aureus* USA300 grown under comparable conditions (0.42–1.63 μmol/g dry weight) using monobromobimane labeling and HPLC-fluorescence measurements.[26] *S. aureus* Newman was also previously shown to have higher BSH levels during the exponential growth in TSB than in stationary phase.[27] Hence, for

TABLE 1 Levels of LMW thiols in *S. aureus* USA300 WT in different growth phases and media, and LD_{50} for killing by HOCl

Medium	Growth phase	BSH (pmol/10^8 CFU)	BSH (μmol/g)[a]	CoA (μmol/g)[a]	Cysteine (μmol/g)[a]	LD_{50} HOCl (nmol/10^8 CFU)
LB	Exponential	173.3 ± 25.9	5.8 ± 0.9	0.8 ± 0.3	0.23 ± 0.03	6.9 (6.3–7.6)
LB	Stationary	216.6 ± 40.3	7.2 ± 1.3	1.3 ± 0.4	0.21 ± 0.12	5.9 (5.5–6.3)
TSB	Exponential	55.0 ± 14.8*	1.9 ± 0.5*	0.9 ± 0.1	0.21 ± 0.04	9.3 (8.0–10.4)
TSB	Stationary	1.9 ± 0.8*	0.1 ± 0.03*	0.7 ± 0.3	0.10 ± 0.02	7.7 (7.3–8.1)

Bacteria were grown in either LB or TSB overnight to stationary phase, then either washed straight away or sub-cultured to reach mid-exponential growth phase. Bacteria were washed with PBS, then acetonitrile (50%) and 20 mM NEM were added and incubated at 60°C for 15 min. After precipitated protein was removed by centrifugation, all LMW thiols with NEM adducts were measured in the supernatant by MRM-based LC–MS. Dose–response killing curves for hypochlorous acid (HOCl) were generated as described in Figure 1 and the LD_{50} was derived from a best curve-fit (variable slope, 4 parameters, constrained at top = 100 and bottom = 0).

Data for LMW thiols are presented as mean ± SD and for LD_{50} as mean (95% CI), $n = 3–5$.

[a]Based on a residual dry weight of 0.03 mg per 1×10^8 CFU.

*Denotes a significant difference $p < 0.05$ compared with the respective LMW thiol level in bacteria grown to exponential phase in LB as determined by one-way-ANOVA with Dunnett's test.

experimental studies with HOCl treatments, it is prudent to measure the content of BSH in bacteria under the defined growth conditions, because it varies considerably.

To correlate oxidation of BSH with bacterial survival upon exposure to HOCl, *S. aureus* cells were grown in LB to stationary phase or in TSB to log phase, then suspended in HBSS and treated with oxidant. In both experiments, there was a dose-dependent BSH depletion, which was mirrored by a loss of bacterial viability (Figures 1(A) and 1(B)). Loss of viability was strongly related to the remaining BSH after treatment with increasing HOCl doses (Pearson $r = 0.99$, $p < 0.001$; Figures 1(C) and 1(D)). We have previously shown that at the doses of HOCl used here, the loss of BSH in *S. aureus* can be accounted for by its oxidation to the BSSB, BSO_2H, BSO_3H and BSO_2N as well as disulfides with proteins (BSSP).[19]

3.2 | Intracellular BSH levels of *S. aureus* grown in LB and TSB to different growth phases do not dictate sensitivity to HOCl

The above results suggest that BSH could itself be a critical target when HOCl reacts with bacteria, or help protect the bacteria by shielding a critical target. As described above, however, BSH levels varied considerably depending on culture conditions, while there was little difference in the sensitivity of the bacteria to HOCl, as indicated by the dose required to kill half of the bacteria (LD_{50}) (Table 1). A two-way plot of this data confirmed there was no correlation between initial BSH levels and the LD_{50} for killing of *S. aureus* by HOCl (Figure 1(E)). The tight relationship observed between survival and oxidation of BSH (Figures 1(C) and 1(D)) is therefore independent of the intracellular BSH content. Collectively, these results show that BSH oxidation can indicate the extent of HOCl-mediated lethal damage to *S. aureus*, but variations in the synthesis of intracellular BSH levels do not affect sensitivity to the oxidant.

Next, we tested whether complete lack of BSH in *S. aureus* would increase their sensitivity towards oxidants. *S. aureus* BSH-null mutants in different strain backgrounds have been reported to be more susceptible to HOCl treatments in media.[25,27] However, HOCl reacts with media constituents such as ammonia salts and proteins, generating alternate toxic species such as chloramines, and altering the doses required for killing.[40] Comparative survival assays were performed for the USA300 WT and the isogenic Δ*bshA* mutant, diluted in HBSS to the same OD and treated with increasing concentrations of either HOCl or ammonia chloramine (NH_2Cl) for up to 60 min (Figures 2 and Figure S1(A)). There was no significant difference in viability or growth between the WT and Δ*bshA* strains in HBSS in the absence of oxidants over the course of the experiment (Figure S1(B)). With the oxidant dose normalized in the customary way against the CFU of the untreated control, there was no survival advantage evident for WT over the Δ*bshA* mutant (Figure S1(C) and S1(D)). However, a small but significant difference in survival was revealed when the same dosing experiments were normalized against the starting OD (Figures 2(A) and 2(B)). We have confirmed that treating these strains at the same OD effectively presents the same biomass (bacterial dry weight) to the oxidant (Table S1). The masking of the small difference in oxidant sensitivity by normalizing against CFU can be explained by the fact that at the same OD, the Δ*bshA* mutant has a lower (64%) colony-forming capacity compared with WT (Table S1). Therefore, the lower survival after treating equivalently dense cellular suspensions (Figures 2(A) and 2(B)) revealed a raised sensitivity of the Δ*bshA* mutant relative to WT for both HOCl and NH_2Cl.

We checked whether BSH deletion in USA300 had caused a change in the synthesis of other LMW thiols in *S. aureus*, that is, CoA and cysteine,[41] which might compensate for the lack of BSH. In WT bacteria, coenzyme A and cysteine accounted for less than 20% of the total LMW thiol pool and their levels were not significantly different in the Δ*bshA* deletion mutant (Table S2).

Due to its multiple resistance, we were unable to complement the USA300 strain with plasmid-encoded *bshA* to assess whether the small increase in sensitivity to reactive chlorine species can be restored with ectopic *bshA*. We therefore used the USA300JE2 srtain of *S. aureus* for this purpose. The JE2 strain is deficient for two plasmids

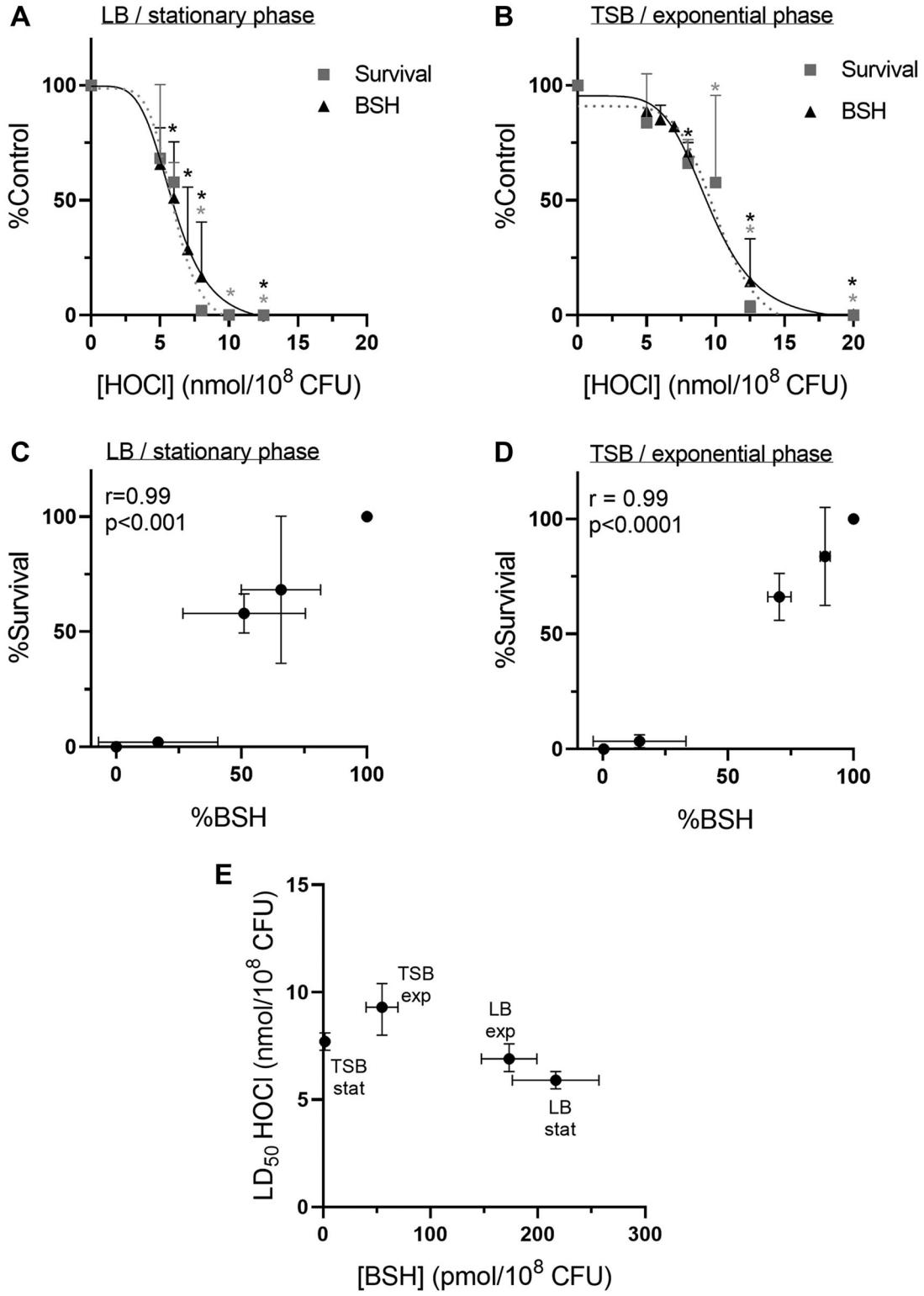

FIGURE 1 Loss of BSH correlates with death of *S. aureus* USA300 treated with HOCl. *S. aureus* USA300 WT cells were grown to stationary phase (stat) in LB (A and C), or exponential growth phase (exp, OD = 0.4) in TSB (B&D), washed with PBS, then 1×10^9 CFU/ml in HBSS were treated with HOCl. Samples were serially diluted onto agar plates for colony counting after overnight incubation at 37°C. BSH levels were measured as described in Table 1 and expressed relative to the untreated control. (A and B) Data are means + SD for at least three independent experiments. Significant differences (*$p < 0.05$) compared with the untreated control were identified using one-way ANOVA with Dunnett's multiple comparison test. (C and D) The association between % survival and % BSH was determined using Pearson's correlation. (E) Bacteria were grown in either LB or TSB to stationary phase (stat), exponential growth phase (exp, OD = 0.4). Bacterial BSH levels and the LD_{50} values for killing by HOCl were determined as described in Table 1. Data for BSH are plotted as mean ± SD, and for LD_{50} as mean ± 95% CI.

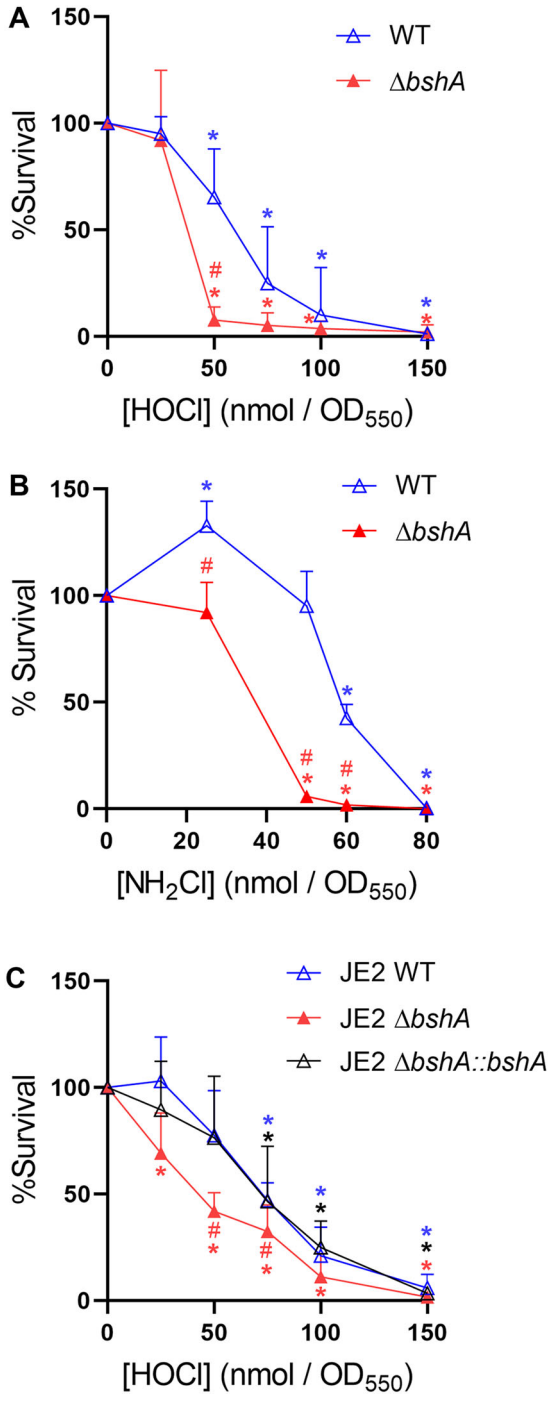

FIGURE 2 BSH-deficiency is associated with an increase in the susceptibility of *S. aureus* USA300 to HOCl and NH_2Cl. (A and B) *S. aureus* USA300 WT and BSH-deficient USA300 Δ*bshA* strains were grown to stationary phase in LB, washed with PBS, adjusted to an OD of 1.2, then treated with increasing concentrations of HOCl or NH_2Cl in HBSS for 60 or 10 min, respectively. Survival was determined as described in Figure 1 and expressed relative to the untreated, time-matched control. Data are interexperiment means + SD from multiple independent experiments for the WT strain treated with HOCl ($n = 12$) or NH_2Cl ($n = 3$), or the Δ*bshA* strain treated with HOCl ($n = 3$) or NH_2Cl ($n = 3$). (C) Stationary phase cells of LB-grown USA300JE2 WT, Δ*bshA* mutant and *bshA* complemented strains were washed and treated with increasing doses of HOCl in HBSS for 10 min as described in (A). Data are interexperiment means + SD from five independent experiments. Significant differences (*$p < 0.05$) from the untreated control (0 μM HOCl) for each strain were identified by one-way ANOVA with Dunnett's multiple comparison test. Significant differences (#$p < 0.05$) of the mutant strains compared with WT at each dose were identified by two-way ANOVA with Sidak's multiple comparison test

carrying multiple antibiotic resistance genes, and we have constructed an isogenic Δ*bshA* mutant, and a *bshA* complemented strain expressing *bshA* under control of a xylose-inducible promoter in the USA300JE2 Δ*bshA* mutant background[34] (Table S2). Survival assays confirmed that the USA300JE2 *bshA* mutant was more sensitive towards HOCl stress compared with the isogenic WT strain (Figure 2(C)). Restoring BSH synthesis increased survival under HOCl stress to the level observed in HOCl-treated WT cells (Figure 2(C)). Thus, BSH confers resistance to HOCl stress in both USA300 and USA300JE2 strains.

3.3 | Oxidation of BSH during phagocytosis by human neutrophils is MPO dependent

To address whether BSH is a target when *S. aureus* is under immune attack within phagosomes, we used purified human neutrophils in an in vitro phagocytosis assay in HBSS with 10% serum (Figure 3). *S. aureus* USA300 WT cells were grown in LB to stationary phase to yield the highest BSH level and thus the best signal-to-noise ratio for measuring changes in reduced and oxidized BSH species. The amount of BSH increased in *S. aureus* incubated without neutrophils, consistent with bacterial growth observed during the experiment. In contrast, when bacteria were incubated with neutrophils, the BSH levels dropped significantly with ongoing phagocytosis (Figure 3(A)). After 30 min of phagocytosis (when 84 ± 9% SD of bacteria were ingested), approximately 30% of the initial BSH was depleted, or 50% if compared with the BSH of untreated *S. aureus* control cells (Figure 3(A)). The concentration of oxidized BSH species, such as BSSB and mixed disulfides were also measured at each time point. While no BSSB could be detected in untreated bacteria, a significant increase of BSSB occurred during neutrophil phagocytosis (Figure 3(B)). There was little change in mixed disulfides with other LMW thiols (BSSX) or proteins (BSSP) in control bacteria without neutrophils (Figures 3(C) and 3(D)). During neutrophil phagocytosis, BSSX levels were elevated at the 15 min time point, but declined to near baseline after 30 min. However, the observed fluctuations in the levels of oxidized species upon phagocytosis, except for BSSB, were not significantly different from control samples. Higher oxidation states of BSH previously detected in HOCl-treated *S. aureus*, that is, sulfonamide, sulfinic, and sulfonic acids,[19] were below the limit of quantification. In summary, during phagocytosis of *S. aureus* by neutrophils, levels of BSH dropped by up to 30 nM, which at earlier time points could be accounted for by the formation of 20 nM BSSX, 2 nM BSSP, and 0.6 nM BSSB. At later time points, the mixed disulfides declined, possibly due to degradation.

The significant decline in BSH after 30 min of phagocytosis was inhibited when the neutrophils were preincubated with MPO

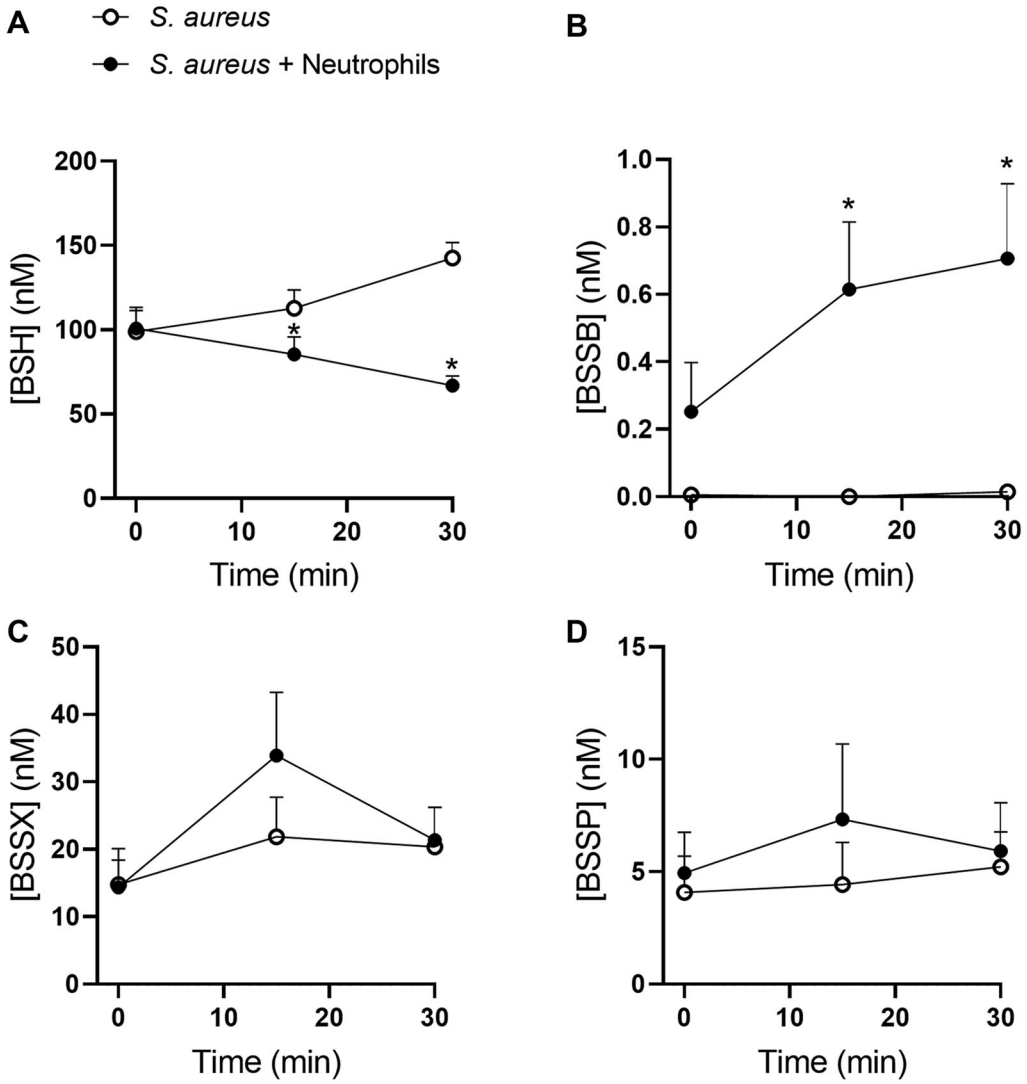

FIGURE 3 Oxidation of BSH in *S. aureus* occurs after phagocytosis by neutrophils. LB-grown stationary phase *S. aureus* USA300 WT cells were opsonized, then incubated at 5×10^7/ml with or without neutrophils (5×10^6 CFU/ml) in HBSS containing 10% serum at 37°C. At the indicated times, samples were analyzed by LC-MS for the bacillithiol species in total cell lysates including (A) BSH as the NEM adduct, (B) BSSB, and (C) BSSX, and (D) BSSP. Data are presented as mean + SEM for at least four independent experiments using different blood donors. A statistical difference (*$p < 0.05$) between samples containing bacteria only and bacteria with neutrophils was determined using a repeated measures two-way ANOVA with Sidak's multiple comparison test.

inhibitors AZM198 and azide, which are known to inhibit phagosomal HOCl production (Figure 4).[31] Methionine, added to scavenge extracellular HOCl, did not affect the fall in BSH levels. This result indicates that BSH oxidation must occur inside neutrophils. Together, our results demonstrate that BSH is oxidized during phagocytosis by an MPO-derived oxidant.

3.4 | Loss of viability exceeds BSH oxidation during phagocytosis of *S. aureus* by neutrophils

Next, we investigated whether the decrease in reduced BSH that occurred during phagocytosis of *S. aureus* aligned with the loss of bacterial viability. Since a tight correlation was observed in the HOCl experiments (Figures 1(A)–1(D)), we hypothesized that comparing the decline of BSH to that of bacterial survival during phagocytosis would indicate whether HOCl contributes to killing inside neutrophil phagosomes. Bacteria were fed to neutrophils and the whole mixture was analyzed for BSH levels to compare with bacterial viability assessed under equivalent conditions. This single-step analysis included both intracellular and extracellular bacteria to minimize loss of BSH due to handling. During incubation with neutrophils, both bacterial survival and BSH levels declined (Figure 5(A)). The decline in bacterial survival at 30 min with neutrophils was 83 ± 6% (mean ± SD), significantly greater ($p < 0.05$) than the drop in BSH which was 50 ± 15%. *S. aureus* cells were also treated with reagent HOCl leading to a loss of BSH similar to that obtained with neutrophils (Figure 5(B)). The exposure of *S. aureus* to HOCl resulted in a sudden drop in viability but it did not exceed the loss in BSH (Figure 5(B)). It is unlikely that any BSH out-

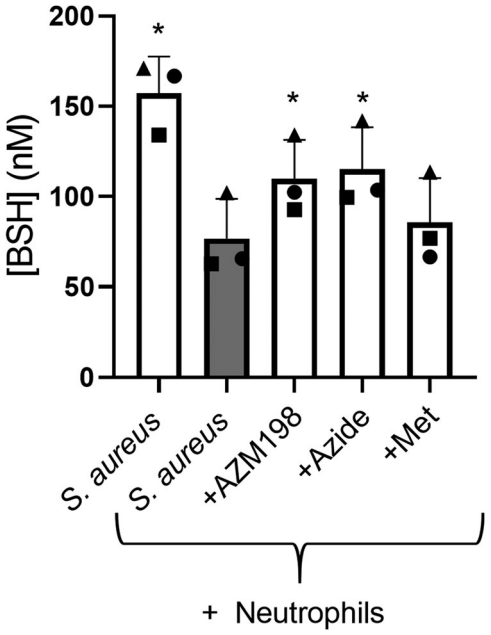

FIGURE 4 BSH oxidation during neutrophil phagocytosis is dependent on MPO activity. Neutrophils (5×10^6/ml in HBSS with 10% serum) were pretreated at 37°C for 5 min with the MPO inhibitors AZM198 (10 μM) and azide (100 μM), and methionine (0.5 mM) before mixing with serum-opsonized *S. aureus* USA300 WT (LB-stat, 5×10^7 CFU/ml). BSH levels were determined by LC–MS in total cell lysates after a 30 min incubation. Each data point is from one of three independent experiments using different blood donors as indicated by symbol shape. The error bar represents the mean + SD. Significant differences ($^*p < 0.05$) compared with untreated neutrophils and bacteria (gray bar) were identified by repeated measures ANOVA with Dunnett's multiple comparison test. When compared with bacteria alone, only the sample containing untreated neutrophils and bacteria, that is, the gray bar, revealed statistically significant differences in BSH levels.

side phagosomal compartments accounted for the lack of association between BSH oxidation and killing by neutrophils because the extracellular proportion of BSH was only 9 ± 6% ($n = 2$) of the total BSH level (data not shown). To more closely resemble the oxidative burden within phagosomes, bacteria were subjected to a flux of HOCl generated by purified MPO from hydrogen peroxide and chloride. Under these conditions, *S. aureus* survival and BSH levels declined gradually within 30 min and in tandem to levels observed with reagent HOCl (Figure 5(C)). The fact that viability loss exceeded the drop in BSH levels during phagocytosis, but not with the HOCl treatments, suggests that within phagosomes *S. aureus* cells are exposed to HOCl along with other factors that must also contribute to microbial death.

In order to investigate whether BSH protects *S. aureus* inside phagosomes, we first looked at manipulating bacterial BSH levels by varying growth conditions as done previously for assessing sensitivity to HOCl. Neutrophils were freshly isolated from healthy donors and incubated with serum-opsonized bacteria for 30 min before the neutrophils were pelleted and washed, then lysed to quantify the ingested bacteria. There was no difference in the phagosomal survival of USA300 WT from different growth conditions (Table 2). Next, we compared the survival of *S. aureus* USA300 WT and Δ*bshA* mutant cells ingested by neutrophils (Figure 6). The USA300 Δ*bshA* mutant was slightly more susceptible to killing compared with the WT (Figures 6(A) and 6(B)). For both strains, survival was significantly enhanced in the presence of DPI (Figure 6(A)), which indicates that oxidants derived from the NADPH oxidase contributed to killing.

To ensure that differences in bacterial survival were not being concealed by altered rates of phagocytosis, we did a quantitative analysis of phagocytosis on USA300WT and Δ*bshA* strains (Figure 6(C)). While the phagocytosed fraction of bacteria was accounted for in the killing assay (Figure 6(A)), this was via an arithmetic measure of the difference between the concentration of the bacteria-only sample and those recovered extracellularly. Therefore, differences in phagocytosis not captured by this indirect method may have masked bona fide differences in bacterial survival of the two strains. Phagocytosis was directly quantified using light microscopy, where the intracellular bacteria were counted after incubation with neutrophils for 5 or 10 min (Figures 6(C) and Figure S3). It was necessary to assess phagocytosis at these early time points before degradation of the ingested bacteria interfered with their detection. Slides of fixed neutrophils and their engulfed bacteria were counted, and on average 119 neutrophils were counted per time point. The uptake of bacteria by neutrophils was consistent between experiments and neutrophil donors, and there was no difference between the bacterial strains. We conclude that the slightly raised survival of USA300 WT over Δ*bshA* after 30 min incubation (Figure 6(A)), occurred under conditions of equal phagocytosis.

TABLE 2 Neutrophil phagocytosis and killing of *S. aureus* USA300 WT after growing bacteria in different media

Medium	Growth phase	Phagocytosed at 30 min (% of starting)	Survival at 30 min (% of phagocytosed)
LB	Stationary ($n = 3$)	81.3 ± 9.7	19.8 ± 11.5
TSB	Exponential ($n = 9$)	86.3 ± 7.9	18.4 ± 7.3

Neutrophils were isolated from peripheral blood and suspended in HBSS containing 10% pooled human serum. LB-grown bacteria were in stationary phase after overnight culture, and TSB-grown bacteria were diluted 10-fold from an overnight culture into fresh TSB to enter exponential growth phase before the killing experiment. Bacteria were harvested by centrifugation, washed in PBS, then serum-opsonized before incubating at approximately 10:1 with neutrophils (5×10^6/ml) at 37°C with gentle mixing. After 30 min, the nonphagocytosed bacteria were separated from neutrophils by centrifugation, and the neutrophils were lysed to release the phagocytosed bacteria. Bacterial concentration for all starting, nonphagocytosed, and phagocytosed samples, was determined by colony counting. See *Material and Methods* section for details
Data are presented as mean ± SD.

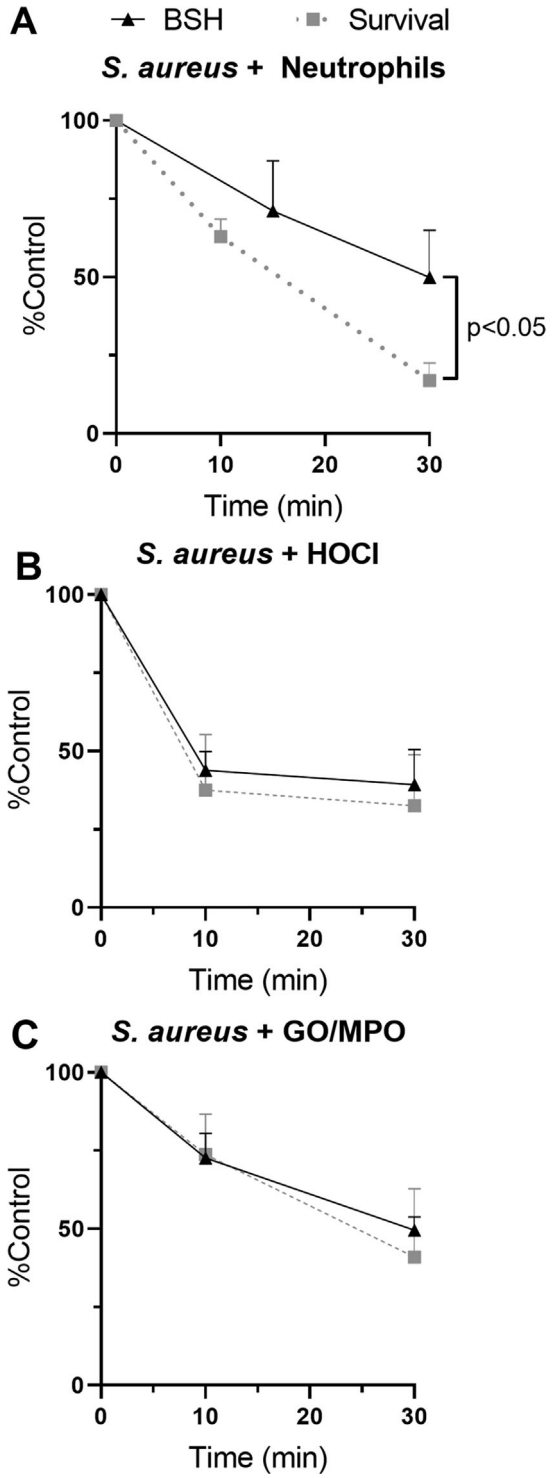

FIGURE 5 The bactericidal activity of neutrophils against *S. aureus* exceeds the degree of BSH oxidation. Changes in viability and levels of reduced BSH were measured in LB-grown stationary phase *S. aureus* USA300 WT (1×10^9 CFU/ml) after exposure to; (A) neutrophils at 5×10^7/ml, (B) a bolus of 70 μM reagent HOCl, or (C) 70 μM HOCl generated by glucose oxidase (GO; 7.2 μg/ml) and MPO (50 nM) over 10 min. Survival (by colony counting), and the concentration of BSH (by LC–MS on total lysates) at each respective time point were in each case expressed as % of untreated bacteria control (see Figure S2 for details). Data are shown from at least three independent experiments, with mean + SD. A significant difference (*$p < 0.05$, Student's *t*-test) between %Survival and %BSH at the 30 min time point was identified in the neutrophil system only.

To see whether regeneration of BSH synthesis would affect survival inside neutrophils, we used the *S. aureus* USA300JE2, for which a *bshA* complemented strain is available. However, in neutrophil killing assays with the USA300JE2 strains, there was no discernable difference in bacterial survival between WT, the Δ*bshA* mutant and the *bshA* complemented strain (Figure 6(D)).

The neutrophil assay above studies the period when the majority of bacterial killing occurs. Elimination of the small number of phagosomal survivors may be just as important in clearing infections in vivo. We used a modified protocol to test whether BSH contributes to long-term bacterial survival of the USA300 strain in neutrophil phagosomes (Figure 7). In this system, initial phagocytosis of bacteria by neutrophils was limited to 10 min, so that uptake into phagosomes was synchronized. This was followed by incubation with lysostaphin to remove any adherent extracellular bacteria. After additional washing to remove lysostaphin, the neutrophils were incubated for a further 3 h to follow ongoing killing of the ingested bacteria. Viability of the USA300 WT and the Δ*bshA* mutant declined to a few percent over the second incubation period. No advantage for survival within the phagosome was evident for the BSH-producing USA300 WT strain compared with the BSH-null mutant. This indicates that BSH does not provide protection against phagosomal killing by neutrophils during long-term infections.

4 | DISCUSSION

By monitoring the oxidation of BSH in *S. aureus* USA300, we have assessed whether oxidants contribute to the killing of these bacteria within neutrophil phagosomes. We have demonstrated that MPO-derived oxidants react with BSH in *S. aureus* during phagocytosis. The extent of BSH oxidation is consistent with MPO-derived oxidants contributing to phagosomal killing. Although BSH was oxidized during phagocytosis, it did not offer substantial protection against either the neutrophil's oxidative or nonoxidative killing mechanisms. Consequently, it is unlikely that BSH alone acts as a defense mechanism to promote the survival of *S. aureus* inside phagosomes.

The precise contribution that oxidants make to bacterial killing within phagosomes has been difficult to untangle from other bactericidal mechanisms because manipulating events within these vacuoles may give misleading outcomes. For example, it is often concluded that oxidants are unnecessary for killing *E. coli* because neutrophils still kill these bacteria effectively when the NADPH oxidase or MPO are absent or inhibited.[42] Yet, MPO activity in normal neutrophil phagosomes is sufficient to account for killing.[43,44] In MPO's absence, however, alternative cytotoxic mechanisms compensate for a lack of this enzyme and the oxidants it generates.

To evaluate the contribution of oxidants to phagosomal killing of *S. aureus*, we have investigated modification of their proteins[8] and staphyloxanthin,[45] and now oxidation of the LMW thiol BSH. Previously, we found that tyrosine residues in bacterial proteins are chlorinated during phagocytosis.[8] From these studies we concluded that HOCl and chloramines are formed inside phagosomes and react with phagocytosed bacteria, but it was debatable as to whether the dose of oxidant was sufficient to kill. This conclusion was reinforced by

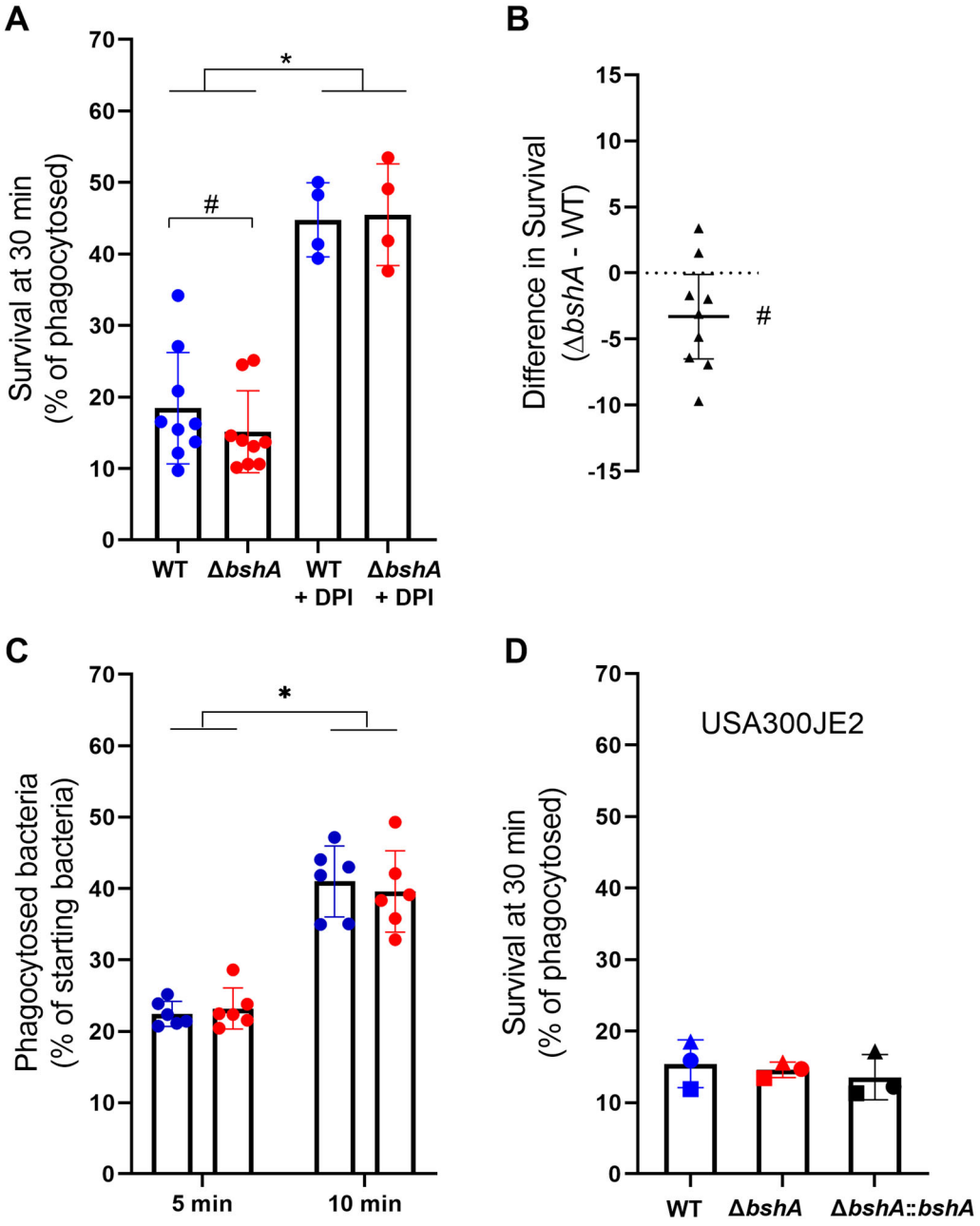

FIGURE 6 BSH provides limited protection of *S. aureus* inside neutrophil phagosomes. (A) The survival of ingested *S. aureus* USA300 WT and USA300 Δ*bshA* was compared after phagocytosis by purified neutrophils. Bacteria grown to exponential phase in TSB were serum-opsonized and incubated with neutrophils (5×10^6/ml) in HBSS with 10% serum, in the absence or presence of DPI (10 μM). Colony counts of the initial inoculum showed the mean multiplicity of infection (MOI) across the nine separate experiments was 7.4 ± 1.3 (SD). After 30 min, the nonphagocytosed bacteria were removed and the neutrophils were washed and lysed to release the phagosomal bacteria. Surviving ingested bacteria were determined by colony counting and expressed relative to the amount phagocytosed at 30 min as determined by subtracting the number of nonphagocytosed bacteria from the initial inoculum. Data are from nine independent experiments using five different donors, mean ± SD. Four experiments included DPI, both strains' survival was significantly higher in the presence of DPI ($^*p = 0.6 \times 10^{-9}$, two-way ANOVA). The difference between the survival of WT and Δ*bshA*, without DPI, is illustrated in (B). Using paired data, the mean difference in survival (% of phagocytosed bacteria) of Δ*bsh* compared with WT was −3.30 (with 95% CI of −0.10 to −6.50). Significance ($^\#p = 0.045$) was determined by a one-sample *t*-test on the differences. (C) The phagocytosis of USA300 WT (blue symbols) and USA300 Δ*bshA* (red symbols) was compared in six experiments (mean MOI 7.7 ± 1.4 SD) using three different neutrophil donors. Incubations were set up as for (A) and after 5 or 10 min, aliquots of neutrophils were removed and washed once by centrifugation in ice-cold PBS before preparing cytospins for staining and counting. See *Material and Methods* section and Figure S3 for details. Data are means of three independent counts of the bacteria internalized by neutrophils, and are reported relative to the initial inoculum. The number of phagocytosed bacteria was significantly higher at 10 min compared with 5 min ($^*p < 0.0001$, two-way ANOVA). (D) Survival in neutrophils was compared for *S. aureus* USA300JE2 WT, Δ*bshA* and complemented Δ*bshA*::*bshA* strains. Bacteria were grown to stationary phase in LB, with the additions of 1% xylose and 10 μg/ml chloramphenicol for the Δ*bshA*::*bshA* construct. Otherwise, the experiment was carried out as for (A). The different symbols represent three separate experiments using different neutrophil donors. The mean ± SD is shown for each strain, the overall mean survival across all strains was 14.5 ± 2.5%.

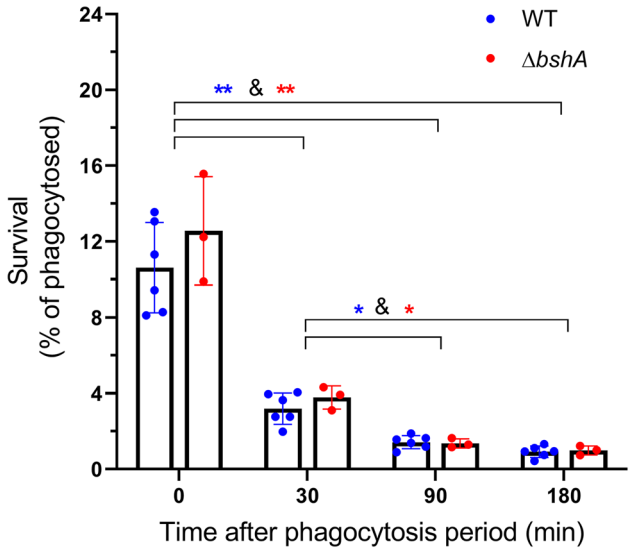

FIGURE 7 Longer term phagosomal survival of *S. aureus* USA300 is unaffected by BSH. *S. aureus* USA300 WT and USA300 Δ*bshA* grown to stationary phase in LB were serum-opsonized and added at ≈10–20:1 ratio to neutrophils in RPMI containing 10% serum. After 10 min at 37°C with end-over-end rotation, the nonphagocytosed bacteria were removed by centrifugation, and the neutrophils treated with lysostaphin, washed and resuspended in fresh RPMI with 10% serum before an extended 3 h incubation at 37°C. At the indicated times, neutrophils were lysed to monitor bacterial survival, which is reported relative to the amount phagocytosed in the initial 10 min period. See *Material and Methods* section for details. The means of separate experiments are plotted ± SD, for WT ($n = 6$) and Δ*bshA* ($n = 3$). Analysis using ANOVA with the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli, identified significant loss in survival over time for both strains (**$p < 0.0001$, for 0 min vs. 30, 90, 180 min, and *$p < 0.05$, for 30 min vs. 90, 180 min). There was no difference at any time point between WT and Δ*bshA*.

our finding that staphyloxanthin, the golden carotenoid pigment of *S. aureus*,[46] was not oxidized when the bacteria were phagocytosed.[45] However, chlorination of tyrosine residues and staphyloxanthin by reactive chlorine species is slow and may underestimate oxidant exposure.

In contrast to other modifications, HOCl reacts rapidly with BSH ($k = 6 \times 10^7$ M^{-1} s^{-1}),[19] making it a good indicator of bacterial exposure to phagosomal oxidants. BSH oxidation cannot be used to quantify total HOCl production in the phagosome because the majority will react with neutrophil proteins.[8] The oxidation of BSH in different *S. aureus* strains following exposure to reagent HOCl has been observed previously.[19,20,25] We have now established that BSH is oxidized at doses of HOCl that are toxic to *S. aureus* USA300. The decline in BSH during phagocytosis via an MPO-dependent process is consistent with its oxidation by HOCl, or chloramines, formed inside the phagosomes. To confirm that BSH was oxidized by reactive chlorine species during phagocytosis, we analyzed bacterial samples for known oxidation products of BSH.[19] While the HOCl-specific sulfonamide of BSH, BSO_2N, was undetectable, there was transient formation of other oxidation products including BSSB, BSSP, and BSSX. Previously, BSO_2N was detected in HOCl-treated bacteria when the majority of BSH was oxidized.[19] In the neutrophil system however, less than half of the BSH was oxidized after 30 min. It is possible that in neutrophil phagosomes, bacterial BSH is not exposed to high enough doses of HOCl to form BSO_2N. Although we could not conclusively identify the oxidant responsible for depleting BSH, other supporting evidence for reactive chlorine species within phagosomes comes from chlorination of protein tyrosine residues[8] and chemical probes,[47,48] as well as bleaching of bacterial green fluorescent protein during phagocytosis[49,50] and oxidation of bacterial glutathione.[31]

BSH afforded only relatively small protection against reagent HOCl suggesting that BSH is not a critical target in *S. aureus*. More abundant protein thiols and methionine residues are expected on kinetic grounds to be the prime targets of HOCl. Indeed, 40% of methionine residues in *S. aureus* 502A are oxidized to methionine sulfoxide during phagocytosis.[51] Proteomic analysis of *S. aureus* COL after HOCl treatment showed significant increases in thiol oxidation of 58 different proteins and enzymes, indicating the multiplicity of oxidative targets and ongoing consequences.[29] It has been shown that if HOCl damages certain targets located on bacterial membranes, such as transport and energy-transducing systems, microbial death can occur even in the absence of oxidation of cytosolic components.[11,12] Although not crucial for killing, decreases in BSH can be considered a barometer of oxidative toxicity experienced by *S. aureus*.

The degree of oxidation of BSH during phagocytosis of *S. aureus* offers considerable insight into how the bacteria were killed. Firstly, given the extent of BSH loss during phagocytosis, it is apparent that oxidants can account for killing half of the ingested bacteria. The remainder of the killing can be attributed to nonoxidative killing mechanisms. Accordingly, since substantial phagosomal killing still occurred in the presence of DPI, it is likely that oxidative and nonoxidative killing mechanisms occurred in tandem and were nonredundant.

Our results from investigating killing of the Gram-positive *S. aureus* USA300 by neutrophils contrast with previous findings using the Gram-negative *E. coli* and *P. aeruginosa*, which were killed equally well by neutrophils regardless of whether MPO-derived oxidants were prevented from reacting with the bacteria.[31,43] The current results also differ from those with mycobacteria where insufficient HOCl formed in phagosomes to contribute to their demise.[32] These differences could be related to cell envelope structures. Gram-negative bacteria have an outer LPS-containing membrane, whereas Gram-positive *S. aureus* have a thick peptidoglycan cell wall. The cell envelope of mycobacteria consists of peptidoglycan, arabinogalactan and a mycolic acid-containing outer membrane.[52] Thus, depending on the specific bacteria phagocytosed by neutrophils, different combinations of bactericidal mechanisms may be involved in killing. These could range from solely oxidative to solely nonoxidative, or various combinations of both. Another twist on the permutations of bactericidal mechanisms employed by neutrophils is that heterogeneity has been observed in the oxidant production within different phagosomes.[47] In some phagosomes, oxidant production starts as soon as bacteria were ingested, whereas in others, oxidants were not detected until bacteria would have been killed. Heterogeneity in phagosomal killing mechanisms may

be important in limiting the ability of bacteria to further evolve their immune escape mechanisms.

Our results suggest that BSH is not a defense mechanism for *S. aureus* within phagosomes. Susceptibility to reagent HOCl was largely independent of their initial BSH level, and *S. aureus* USA300 devoid of BSH was only marginally more susceptible to HOCl or ammonia chloramine. BSH-null mutants of closely related USA300 and USA300JE2 strains were only slightly, or not, more vulnerable within neutrophil phagosomes, respectively. The marginal difference in USA300 was lost when phagocytosis was synchronized and extended over a long time period. The strength of our neutrophil killing assay is that it specifically measures viability of phagocytosed bacteria, and is independent of the rate of phagocytosis or bacterial growth throughout the experiment. It is possible, however, that the assay cannot detect subtle differences in survival between bacterial strains because it does not monitor killing in real time. Also, it provides only an average rate of killing by all the neutrophils, regardless of the number of bacteria they have ingested. Likewise, the oxidation of BSH was measured as an average across all bacteria, and can therefore not be related to the amount of HOCl produced in individual phagosomes. Differences may become apparent by monitoring killing and oxidation in individual phagosomes.

In addition to the present work with the *S. aureus* USA300 strains, the *S. aureus* COL Δ*bshA* mutant and the *S. aureus* SH1000 strain devoid of BSH due to a truncated *bshC* gene, were both previously shown to be more sensitive to HOCl treatments.[25,27] However, the earlier killing assays were performed in media containing high concentrations of proteins, peptides and ammonium salts, which scavenge HOCl and prevent it from reacting directly with bacteria.[40] Therefore, other reactive species, including chloramines, could have been responsible for killing the bacteria. For example, ammonia chloramine (NH_2Cl) is formed in the reaction of ammonia (NH_4^+) with HOCl or is released from organic chloramines.[53] It is cell-permeable and bactericidal. Importantly, while ammonia chloramine and HOCl kill *S. aureus* at similar doses as shown here and elsewhere,[53] the reactivity of the two oxidants with biomolecules and the mechanisms underlying their bactericidal effects differ.[54,55] Thus, the resistance conferred by BSH observed in previous studies is unlikely to reflect direct reaction with HOCl. It is also important to note that BSH depletion may impact expression or functions of other oxidant detoxification systems. For example, Posada et al.[26] showed down-regulation of a putative thioredoxin reductase in the *S. aureus* COL Δ*bshA* mutant.

Other studies have shown that the BSH-deficient *S. aureus* strains SH1000 and COL Δ*bshA* are eradicated more readily in whole blood killing assays compared with their BSH-producing counterparts.[26] These results are consistent with BSH increasing bacterial resistance to oxidative stress, however, a variety of genes involved in virulence are differentially expressed in COL Δ*bshA*, which may independently affect bacterial survival in whole blood.[26] Our neutrophil killing assay, which is independent of the rate of phagocytosis and inherent bacterial fitness in the complex blood environment, revealed that BSH provided only limited protection to *S. aureus* USA300. The BSSB reductase YpdA, an integral part of the recycling system for *S*-bacillithiolated proteins[20] was further shown to protect the BSH-repaired *S. aureus* SH1000 strain (SH1000 *bshC*) in both whole blood and neutrophil killing assays.[56] This finding supports a role for the BSH redox system in combating oxidative stress experienced by the SH1000 *bshC* strain during phagocytosis by neutrophils. Different survival phenotypes observed in various genetic backgrounds of *S. aureus* isolates might indicate that BSH is more important in the less virulent strains (e.g., COL, SH1000 *bshC*) than in highly virulent strains (e.g., USA300), which have other mechanisms, such as specific toxins and surface factors to evade neutrophil killing.[57,58]

Of particular interest in our studies, is the small percentage of bacteria that survived inside neutrophils 3 h after phagocytosis. Others have also found that some *S. aureus* cells survive in neutrophil phagosomes.[13] What enables their continued survival warrants deeper investigation because these bacteria may seed further infections or carry mutations that enable them to foil neutrophils' antimicrobial arsenal. The virulence factors employed by these bacteria and/or the composition of the phagosomes they were contained in, may give clues to how *S. aureus* defenses can be disarmed so that cytotoxic mechanisms of neutrophils prevail. Future studies focused on bacterial killing within individual phagosomes are likely to furnish key insights into how neutrophils deploy their killing mechanisms and how bacteria thwart them.

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AUTHORSHIP

L. V. A. and N. D. performed the majority of the experimental work, analyzed and interpreted the data, and wrote most of the manuscript. N. D. conceptualized the study and wrote the original manuscript draft. R. S. and V. V. L. performed some experiments. V. V. L. and H. A. provided the bacterial strains and intellectual input for the discussion. M. B. H. and A. J. K. provided funding and expertise, helped design and interpret experiments, and edited the manuscript.

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DISCLOSURE

A. J. K has a patent for novel MPO inhibitors. The other authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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