Methionine Sulfoxide Reductases Protect against Oxidative Stress in *Staphylococcus aureus* Encountering Exogenous Oxidants and Human Neutrophils

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Key Words

Methionine sulfoxide reductase · *Staphylococcus aureus* · Neutrophils · Oxidative stress

Abstract

To establish infection successfully, *Staphylococcus aureus* must evade clearance by polymorphonuclear neutrophils (PMN). We studied the expression and regulation of the methionine sulfoxide reductases (Msr) that are involved in the repair of oxidized staphylococcal proteins and investigated their influence on the fate of *S. aureus* exposed to oxidants or PMN. We evaluated a mutant deficient in *msrA1* and *msrB* for susceptibility to hydrogen peroxide, hypochlorous acid and PMN. The expression of *msrA1* in wild-type bacteria ingested by human PMN was assessed by real-time PCR. The regulation of *msr* was studied by screening a library of two-component regulatory system (TCS) mutants for altered *msr* responses. Relative to the wild-type bacteria, bacteria deficient in Msr were more susceptible to oxidants and PMN. Upregulation of staphylococcal *msrA1* occurred within the phagosomes of normal PMN and PMN deficient in NADPH oxidase activity. Furthermore, PMN granule-rich extract stimulated the upregulation of *msrA1*. Modulation of *msrA1* within PMN was shown to be partly dependent on the *VraSR* TCS. Msr contributes to

staphylococcal responses to oxidative attack and PMN. Our study highlights a novel interaction between the oxidative protein repair pathway and the *VraSR* TCS that is involved in cell wall homeostasis.

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Introduction

Staphylococcus aureus is an important human pathogen that is responsible for a broad spectrum of infections, ranging from mild cellulitis to life-threatening endovascular disease. The rising prevalence of antibiotic resistance among staphylococci exacerbates the clinical challenges imposed by staphylococcal disease.

Optimal host defense relies on the capacity of polymorphonuclear neutrophils (PMN) to phagocytose and kill *S. aureus* with antimicrobial agents delivered to or generated in phagosomes. Especially potent among phagosomal oxidants is hypochlorous acid (HOCl), which is generated by the myeloperoxidase-hydrogen peroxide (MPO-H₂O₂)-chloride system [1]. PMN-derived oxidants readily damage bacterial proteins, lipids and DNA. Methionine is

Y.Y.P. and J.S. contributed equally to this article.

Table 1. *S. aureus* strains and plasmids used in this study

Strain or plasmid ID	Background strain	Plasmid or deletion	Details	Reference
Wild-type	USA300-0114 MRSA ΔpUSA03		erm ^s	[7]
Wild-type pCM28	USA300-0114 MRSA ΔpUSA03	pCM28	erm ^s Cm ^r	this study
Wild-type pEPSA5	USA300-0114 MRSA ΔpUSA03	pEPSA5	erm ^s Cm ^r	this study
Δ <i>msrA1B</i>	USA300-0114 MRSA ΔpUSA03		erm ^s	this study
Δ <i>msrA1B</i> pCM28	USA300-0114 MRSA ΔpUSA03	pCM28	erm ^s	this study
Δ <i>msrA1B</i> pCM28- <i>msrA1B</i>	USA300-0114 MRSA ΔpUSA03	pCM28- <i>msrA1B</i>	erm ^s Cm ^r	this study
Δ <i>vraSR</i> pEPSA5	USA300-0114 MRSA ΔpUSA03	pEPSA5	erm ^s Cm ^r	this study
Δ <i>vraSR</i> pEPSA5- <i>vraSR</i>	USA300-0114 MRSA ΔpUSA03	pEPSA5- <i>vraSR</i>	erm ^s Cm ^r	this study
Δ <i>nre</i>	USA300-0114 MRSA ΔpUSA03	SAUSA300_2337-38Δ	erm ^s	this study
Δ 0217-8	USA300-0114 MRSA ΔpUSA03	SAUSA300_0217-18Δ	erm ^s	this study
Δ 1219-20	USA300-0114 MRSA ΔpUSA03	SAUSA300_1219-20Δ	erm ^s	this study
Δ <i>kdp</i>	USA300-0114 MRSA ΔpUSA03	SAUSA300_2035-36Δ	erm ^s	this study
Δ 2558-9	USA300-0114 MRSA ΔpUSA03	SAUSA300_2558-59Δ	erm ^s	this study
Δ 1798-9	USA300-0114 MRSA ΔpUSA03	SAUSA300_1798-99Δ	erm ^s	this study
Δ <i>lyt</i>	USA300-0114 MRSA ΔpUSA03	SAUSA300_0254-55Δ	erm ^s	this study
Δ <i>pho</i>	USA300-0114 MRSA ΔpUSA03	SAUSA300_1638-39Δ	erm ^s	this study
Δ <i>hss</i>	USA300-0114 MRSA ΔpUSA03	SAUSA300_2308-09Δ	erm ^s	this study
Δ <i>vra</i>	USA300-0114 MRSA ΔpUSA03	SAUSA300_1865-66Δ	erm ^s	this study
Δ <i>arl</i>	USA300-0114 MRSA ΔpUSA03	SAUSA300_1307-08Δ	erm ^s	this study
Δ 1441-2	USA300-0114 MRSA ΔpUSA03	SAUSA300_1441-42Δ	erm ^s	this study
Δ <i>agr</i>	USA300-0114 MRSA ΔpUSA03	Δ <i>agr</i> ::tetM	tet ^r	[8]
<i>sae</i> ::spec	USA300-0114 MRSA ΔpUSA03	<i>sae</i> ::spec	spec ^r	[9]
<i>graS</i> ::Tn-mariner	USA300-0114 MRSA ΔpUSA03	<i>graS</i> ::Tn-mariner	erm ^r	
pCM28		empty	amp ^r Cm ^r	[11]
pEPSA5		empty	amp ^r Cm ^r	[12]
pCM28- <i>msrA1B</i>		pCM28- <i>msrA1B</i>	amp ^r Cm ^r	this study
pEPSA5- <i>vraSR</i>		pEPSA5- <i>vraSR</i>	amp ^r Cm ^r	this study
pJB38			amp ^r Cm ^r	[10]
pJMB176		pJB38 Δ <i>msrA1B</i>	amp ^r Cm ^r	this study
pJB204		pJB38 Δ <i>vraSR</i>	amp ^r Cm ^r	this study

For antibiotic sensitivity to ampicillin, erythromycin, spectinomycin and chloramphenicol: ^s = sensitive; ^r = resistant.

highly susceptible to oxidation, and oxidation in proteins can disrupt their normal function [2]. Methionine can be oxidized to either of two enantiomers, methionine-S-sulfoxide and methionine-R-sulfoxide, and each can be selectively repaired by a specific class of methionine sulfoxide reductase (Msr); MsrA and MsrB repair the S and R stereoisomers, respectively. The *S. aureus* genome harbors three *msrA* genes (*msrA1*, *msrA2* and *msrA3*) and a single *msrB* [3]. The adjacent *msrA1* and *msrB* genes are cotranscribed and encode enzymes that provide the majority of the Msr activity in *S. aureus* [4, 5].

To persist and subsequently establish infection, invading bacteria need to evade, resist or tolerate insults inflicted by PMN. Given the role of Msr in the defense against physiological oxidative stress [6], we speculated that *S. aureus* might employ these enzymes to repair and thus limit damage caused by PMN-derived oxidants, thereby providing a mechanism to survive within phagosomes.

In this study, we investigated the contribution of Msr to the fate of *S. aureus* exposed to oxidants and to human PMN. We also explored the regulation of *msr* in *S. aureus* ingested by PMN and the role of staphylococcal two-component regulatory systems (TCS) in regulating the *msrA1* response.

Methods

Bacterial Strains and Culture Conditions

The *S. aureus* strains used in this study were constructed in the community-associated *S. aureus* USA300 LAC strain that is cured of the native plasmid pUSA03 which confers erythromycin resistance [7] unless otherwise indicated. *S. aureus* was cultured in tryptic soy broth (TSB; BD Biosciences) at 37°C with shaking at 200 rpm. For strains harboring empty vector or complementation plasmids, the media was supplemented with 10 µg/ml of chloramphenicol (Sigma-Aldrich; table 1). To obtain stationary phase organisms, bacteria were inoculated from glycerol stocks into TSB

Table 2. Oligonucleotides used in this study

Oligonucleotide	Sequence 5' to 3'	Application
<i>gapdh</i> primer1	TACACAAGACGCACCTCACAGA	real-time PCR
<i>gapdh</i> primer 2	ACCTGTTGAGTTAGGGATGATGTT T	real-time PCR
<i>msrA1</i> primer 1	TCCAGGCATCAAGTCAGTCG	real-time PCR
<i>msrA1</i> primer 2	TTGTCAACATGACCGCCACTA	real-time PCR
<i>vraS</i> primer 1	CGGCAAGTATGATGCTATCTGCTA	real-time PCR
<i>vraS</i> primer 2	TAACTGCGAATCTTGAACCATTTT C	real-time PCR
<i>msrA1_5EcoRI</i>	GGGGAATTCGGGCA-ATTGAATCAATTAATTAAGGTGGCAG	cloning
<i>msrA1_up3 NheI</i>	ACGCGTGGTACCGCTAGCGCTAGCATGAATTACCTCCTCTATCTATCTAATTAT	cloning
<i>msrB_dwn5mluI</i>	GCTAGCGGTACCACGCGTACGCGTGTGGAAAGTATGTTTAAAAAATTATTCGGAAGG	cloning
<i>msrB_dwn3BamHI</i>	CCCGGATCCCACATCAATCACTTTTGTTCACCTTTAATCACAGC	cloning
<i>msrA_veri5</i>	GAGAAAAACATTAATTTATTGTAG	verification
<i>msrB_veri3</i>	CATTGTCACATCAATCAC	verification
<i>msrA1B_comp5EcoRI</i>	GGGGAATTCGGCAATTGAATCAATTAATTAAGGTGGCAG	cloning
<i>msrA1B_comp3BamHI</i>	GGGGGATCCCTACTTATCAAAATGTGATATTAAATCGCC	cloning
<i>1865up5EcoRI</i>	GGGGAATTCGGGCGAAACATGACACACAAATATATATCAACGC	cloning
<i>1865up3fuse</i>	ACGCGTGGTACCGCTAGCGCAGCTAGCATGCTATATACTAAGATGAGCATTG	cloning
<i>1865dwn5fuse</i>	GCTAGCGGTACCACGCGTCGCCAATCACAATATAACATCAAATAGACACC	cloning
<i>1865dwn3SalI 1865veri5</i>	CCCGTCGACGCCGTAATGGAATCGTATTAATACTACTAAGTGGAGAGCAACAGTAAGAAATAATA TCAAGCACTACC	cloning
<i>1865veri3</i>	GCACCCGCTGAAACATCTACTCTAATTAAG	verification
<i>1865 comp5BamHI</i>	CCCGGATCCTTAGGAGGATGATTATTTATGAACCACTACATTAGAACAATTGGTTCAATGCTCATC	cloning
<i>1865 comp3SalI</i>	CCCGTCGACGGTGTCTATTTGATGTTATATTGTGATTGGC	cloning

and grown for 16–18 h. For mid-log phase bacteria, the stationary bacteria were subcultured for approximately 2.5 h from a starting optical density at 550 nm (OD_{550}) of 0.05. In experiments involving PMN, 0.01% of human serum albumin (Talecris Biotherapeutics) was added to the subculture media. For experiments measuring *msrA1* expression in the presence of both oxidants and vancomycin, an overnight starter culture of *S. aureus* grown in TSB was washed once with HEPES-buffered Hank's balanced salt solution (HBSS) before being used to inoculate minimal media (7.6 mM NH_4SO_4 , 33 mM KH_2PO_4 , 60 mM K_2HPO_4 , 11 mM NaCl, 3 mM KCl, 0.2% glucose, 1 mM $MgSO_4$, 0.5 μ g/ml nicotin, 0.5 μ g/ml thiamine, 0.5 μ g/ml panthothenate, 3 ng/ml biotin, and 25 μ g/ml of each of the following amino acids: Gly, Val, Leu, Thr, Phe, Tyr, Cys, Met, Pro, Arg, and His). The bacteria were then cultured between 4 and 6 h before being treated.

Construction of Δ *msrA1B*, Δ *vraSR* and TCS Mutants

S. aureus strains with mutations in the *graRS* [7], *agr* [8] and *saeRS* [9]. TCS were available as part of laboratory collections (table 1). Other TCS chromosomal mutations, Δ *msrA1B* and Δ *vraSR* strains were created by homologous recombination using pJB38 [10]. To create the complementing plasmid and strains, the *msrA1* and *msrB* or *vraSR* genes were amplified by PCR and cloned into the multicopy plasmids pCM28 [11] or pEPSA5 [12]. All clones were passaged through RN4220 [13] and subsequently transduced into the appropriate strains using bacteriophage 80 α [14]. For oligonucleotide (Integrated DNA Technologies) sequences, refer to table 2. See online supplementary information for further details (for all online suppl. material, see www.karger.com/doi/10.1159/000355915).

Neutrophil Isolation

Informed consent was obtained from each individual following a protocol approved by the institutional review board for human subjects at the University of Iowa. Peripheral blood was drawn from normal healthy volunteers or individuals with X-linked chronic granulomatous disease (CGD), and PMN were purified as previously described [15].

Feeding PMN to *S. aureus* and PMN Killing Assay

Mid-log phase bacteria were pelleted for 5 min and resuspended in 20 mM HEPES-buffered HBSS containing Ca^{2+} and Mg^{2+} . The OD_{550} of the suspension was measured and this was converted to colony-forming units (CFU)/milliliter: an OD_{550} reading of 1.0 was equivalent to 3×10^8 CFU/ml. Bacteria were used immediately or held on ice. Bacteria were opsonized by incubation at 37°C for 20 min in the presence of 10% pooled human serum. Opsonized bacteria were fed to PMN or PMN pretreated with 10 μ M diphenylene iodonium (DPI) at the desired multiplicity of infection (MOI) and tumbled end over end at 37°C to allow phagocytosis to proceed. The PMN were then pelleted at 380 g for 5 min, and the extracellular bacteria were aspirated away. The cell pellets were resuspended to the original volume with 20 mM HEPES-buffered HBSS. These samples were processed to quantitate CFU to assess PMN-mediated killing or prepared for gene expression experiments. For PMN killing experiments, a 50- μ l sample of cell suspension was removed at the 0 min (time 0). The remaining samples were tumbled at 37°C, and 50- μ l aliquots were removed at specified time points. For viability testing, the 50- μ l samples were diluted and incubated with 2.5 ml of pH 11 water for 5 min to lyse the PMN. These samples were vortexed and serially diluted

into saline. 10 μ l of each diluted sample was spotted onto tryptic soy agar plates in at least triplicate. After 16 h of growth at 37°C, the CFU were enumerated. From these, the CFU in the total PMN suspension for each sample were calculated. To assess the viability at each time point, the total calculated CFU for each strain were expressed as a percentage of the starting CFU for that strain. The starting CFU for each strain were taken as the CFU recovered from bacteria fed to DPI-treated PMN at time 0, in which there was minimal bacterial killing (data not shown). For gene expression experiments, the samples were tumbled at 37°C and at the desired time point, and the PMN that had ingested bacteria were pelleted, lysed and processed for real-time PCR analysis.

Real-Time PCR

RNA was extracted from bacteria or PMN-ingested bacteria as described previously [11]. cDNA was synthesized with AMV Reverse Transcriptase (Roche) and used as a template for real-time PCR. Reactions were conducted in PerfeCTa SYBR Green fast mix ROX (Quanta Biosciences) with each primer at 200 nM. Expression of *gapdh* was used to normalize all the gene expression data as described previously [11]. The *gapdh* primers amplify *S. aureus gapdh* but do not amplify human *gapdh* (data not shown). After denaturation at 95°C for 30 s, the samples were cycled at 95°C for 15 s, then at 60°C for 30 s, for 40 cycles using the ABI 7000 system. For primer sequences, refer to table 2.

Bacterial Exposure to Oxidants

In experiments used to determine the susceptibility of bacteria to oxidants, *S. aureus* strains in the mid-log phase were resuspended in PBS to an OD₅₅₀ of 0.250. Bacteria (0.5 ml volume) were either left untreated or treated with reagent grade H₂O₂ or HOCl (Sigma-Aldrich) at predetermined concentrations for 1 h at 37°C. Residual H₂O₂ was quenched by diluting samples 1:100 in PBS supplemented with catalase (1,300 U/ml; Sigma). Where H₂O₂-MPO was employed, 75 nmol H₂O₂ was added to each 0.75-ml sample with 0, 1.5, 3.8, 7.5 or 15 pmol of recombinant MPO. In parallel, samples without *S. aureus* were assayed for HOCl generation (n = 3), as described in Dypbukt et al. [16]. The average amount of HOCl generated from 3 experiments was used to plot the data. After the 1-hour incubation, samples were diluted and spot-plated onto tryptic soy agar (BD Biosciences). CFU for each strain were enumerated after overnight growth at 37°C and then calculated as a percentage of non-treated bacteria for the same strain at time 0. For gene expression studies, *S. aureus* was resuspended to an OD₅₅₀ of 0.25 in minimal media before being challenged with H₂O₂ or HOCl at the specified concentrations in the presence or absence of 7 μ g/ml of vancomycin for 1 h at 37°C.

Bacterial Exposure to PMN Granule-Rich Extract

PMN were resuspended in cold relaxation buffer (RB; 10 mM PIPES, pH 7.3, 100 mM KCl, 3 mM NaCl₂, 3.5 mM MgCl₂, 1.25 mM EGTA, 1 mM ATP) and sonicated on ice. Unbroken cells and nuclei were pelleted (200 g at 4°C for 5 min) and the supernatant was centrifuged at 10,000 g at 4°C for 20 min. The granule-enriched pellet was washed with ice-cold RB, spun at 10,000 g, resuspended in RB and lysed by freeze thawing 3 times in a methanol-dry ice bath. Granule proteins (10⁷ cell equivalents) and 1.5 $\times$ 10⁸ bacteria were incubated together in HBSS with calcium and magnesium for 30 min at 37°C.

Treatment with Cell Wall-Active Antibiotics

In experiments involving TCS mutants, *S. aureus* were diluted to an OD₅₅₀ of 0.3 in TSB, in the presence or absence of 7 μ g/ml of vancomycin or 25 μ g/ml of D-cycloserine (Sigma-Aldrich), and shaken at 200 rpm for 1 h at 37°C.

Msr Activity Assay

Bacteria were washed with assay buffer (50 mM TRIS-HCl, pH 7.5, 145 mM NaCl), pelleted and resuspended into 200 μ l of assay buffer. Bacteria were lysed with 25 μ g of lysostaphin (Sigma-Aldrich) at 37°C for 10 min. Lysates were sonicated on ice and then incubated with 2 mM dabsyl-methionine sulfoxide (a mixture of the S and R enantiomers; Anaspec) and 20 mM DTT in assay buffer. After 1 h at 37°C, reactions were quenched with acetonitrile. Samples were analyzed on an LC-Dabs HPLC column (Supelco) to separate dabsyl-methionine sulfoxide from dabsyl-methionine. For further details, refer to the online supplementary material.

Statistical Analysis

Statistical analyses were performed using Graphpad Prism 5 software. Data were analyzed using paired t tests or one-way ANOVA with post-tests as indicated in the figure legends. p values <0.05 were considered to be statistically significant.

Results

Validation of the *msrA1 msrB* Deletion Mutant

To study the role of Msr in *S. aureus*, we created a mutant in the wild-type background in which both the *msrA1* and *msrB* genes were deleted (Δ *msrA1B*). The growth of the Δ *msrA1B* double-mutant strain was indistinguishable from that of the parent strain (online suppl. fig. 1).

To assess the impact of *msrA1B* deletion on Msr activity in *S. aureus*, we studied bacteria in the absence or presence of vancomycin, since Msr expression increases upon exposure to cell wall antibiotics such as vancomycin [17]. In the absence of vancomycin, Msr activity in the wild-type lysates was low, and in Δ *msrA1B*, it was below the level of detection (fig. 1). Msr activity was restored when both *msrA1* and *msrB* were reintroduced into the Δ *msrA1B* strain via a multicopy plasmid (pCM28). Upon treatment with vancomycin, Msr activity increased in the wild-type and the complemented deletion mutant (Δ *msrA1B* with pCM28-*msrA1B*), but not in Δ *msrA1B*. Thus, the data confirm that under these conditions, MsrA1 and MsrB support the majority of Msr activity in *S. aureus*, which was eliminated in our *msrA1B* deletion mutant.

Susceptibility of Wild-Type and *msr* Mutants to Oxidants and PMN

Reasoning that the capacity to repair oxidant-mediated damage by Msr would provide a survival advantage, we

Fig. 1. Msr activity in wild-type and *msr* mutants. Conversion of dabsyl-methionine sulfoxide to dabsyl-methionine by lysates from wild-type, $\Delta msrA1B$ and complemented $\Delta msrA1B$ mutants were measured. Bacteria were treated with buffer (non-treated) or with vancomycin for 1 h. Data represent the mean $\pm$ SEM (n = 3 experiments).

compared the abilities of the wild-type, $\Delta msrA1B$ and complemented mutant strains to grow after exposure to H_2O_2 for 1 h in PBS. The number of culturable bacteria present after treatment with H_2O_2 was expressed as a percentage of the initial number of bacteria present prior to treatment.

At the lowest concentration of H_2O_2 tested (25 mM), there was no significant difference in the growth of the wild-type or $\Delta msrA1B$ mutant after exposure to the oxidant. At higher concentrations of H_2O_2 (50, 75 and 100 mM), the viability of the wild-type mutant was greater than that of $\Delta msrA1B$ mutant ($p < 0.05$; fig. 2a). Viability of the complemented mutant treated with 25–75 mM of H_2O_2 was likewise better than that of the $\Delta msrA1B$ strain, suggesting that *msrA1B* contributed to better survival of bacteria in the presence of H_2O_2 at these concentrations. At 100 mM H_2O_2 , $\Delta msrA1B$ and its complement were equally susceptible to killing by H_2O_2 , suggesting that expression of *msrA1B* driven from the complementation plasmid where gene dosage is not precisely controlled may be limited.

Although H_2O_2 contributes to oxidative stress imposed on bacteria within PMN phagosomes, most of the oxygen consumed by activated PMN is converted into HOCl [18–20], a more potent antimicrobial agent [21]. To determine whether Msr-deficient *S. aureus* was more vulnerable to damage by the physiologically relevant oxidant HOCl, we subjected *S. aureus* to sublethal amounts of HOCl or a HOCl-generating system (MPO- H_2O_2 -chloride). The latter system generates toxic oxidants continuously and better mirrors conditions that exist within PMN phagosomes.

The number of bacteria recovered after treatment with HOCl was expressed as a percentage of the initial number of bacteria prior to treatment (fig. 2b). Wild-type bacteria were more resistant to killing by 100 μ M HOCl than was $\Delta msrA1B$ ($p < 0.01$; fig. 2b). In the presence of the HOCl-generating system, the wild-type and $\Delta msrA1B$ strains also exhibited a significant difference in survival at the high end of concentrations of HOCl produced ($p < 0.05$; fig. 2c).

Given that Msr can influence the susceptibility of wild-type *S. aureus* to the antimicrobial effects of H_2O_2 and HOCl, oxidants that are generated by activated PMN, we reasoned that Msr-deficient bacteria would be more vulnerable to PMN killing. We fed opsonized wild-type, $\Delta msrA1B$ and complemented mutants to PMN for 10 min to allow maximum ingestion. Subsequently, extracellular bacteria were removed and the fate of ingested *S. aureus* assessed immediately (time 0) and after 30 and 60 min (fig. 2d). Because PMN begin to kill bacteria soon after the ingestion of bacteria and during the 10 min that are allowed for phagocytosis to occur (data not shown), we measured in parallel the viability of bacteria fed to DPI-treated PMN. The pharmacological inhibition of the NADPH oxidase by DPI [22] ensured that minimal killing of bacteria was achieved by time 0, thus providing us with the total number of ingested bacteria at the start of the time course. To quantitate PMN killing of ingested *S. aureus* over time, we enumerated viable bacteria recovered from normal PMN at each time point relative to the number of bacteria that was recovered at time 0 from the same strain fed to DPI-treated PMN. There was a reduction in the viability of all strains over time, but viable bacteria persisted even 1 h after ingestion. The $\Delta msrA1B$ mutant was more susceptible to PMN killing than was the wild-type mutant at 30 and 60 min ($p < 0.05$).

Together, these data indicate that *msrA1B* contributed to the recovery of bacteria, not only after exposure to oxidants in isolation but also in the more complicated context of the PMN phagosome, where oxidants and granule proteins synergize to damage ingested targets.

msrA1 Induction in *S. aureus* Exposed to PMN

To examine the expression of *msr* in PMN-ingested bacteria, we recovered *S. aureus* from PMN that had been fed bacteria. After 10 min of phagocytosis by PMN, extracellular bacteria were aspirated away and the time course initiated. At time 0, the level of *msrA1* in the ingested bacteria was higher in organisms grown to the stationary phase relative to those grown to the mid-log phase (fig. 3a, b), with levels in both increasing further at 15 min and

Fig. 2. Susceptibility of wild-type and *msr* mutants to oxidants and PMN. Wild-type, $\Delta msrA1B$ and complemented $\Delta msrA1B$ strains were grown to the mid-log phase, resuspended in PBS and treated with H₂O₂ (**a**), HOCl (**b**) or HOCl generated by the MPO-H₂O₂ system (**c**) at the stated concentrations for 1 h. After 1 h at 37°C, the bacteria were plated and CFU enumerated after overnight growth. Data represent CFU/ml as a percentage of non-treated control for each strain at time 0. Data are the mean $\pm$ SEM (n = 6 experiments for **a** and **b**, and n = 3 experiments for **c**). At each concentration of the oxidant, a paired t test was used to compare

the wild-type and $\Delta msrA1B$ strains (* p < 0.05, ** p < 0.01). **d** Each bacterial strain was opsonized in pooled human serum and then fed to normal or DPI-treated PMN (MOI of 5:1). Ingested bacteria were recovered at the indicated time points and CFU were enumerated. At each time point, the viability of a strain is expressed as bacteria recovered from normal PMN as a percent of the same strain recovered from DPI-treated PMN at time 0. A paired t test was used to compare the wild-type and $\Delta msrA1B$ strains at each time point (* p < 0.05). Data represent the mean $\pm$ SEM (n = 6 experiments).

remaining high at 30 and 60 min. Incubation in the absence of PMN did not promote the expression of *msrA1*.

Given that Msr participates in cellular oxidant defense, we sought to determine whether oxidants generated within PMN phagosomes could drive *msrA1* expression in ingested *S. aureus*. To that end, we compared *msrA1* expression in *S. aureus* fed to normal PMN, PMN pretreated with DPI and PMN isolated from individuals with CGD, thereby providing PMN with pharmacologic or genetic absence of a functional phagocyte NADPH oxidase as a source of reactive oxygen species [23]. We detected high levels of

msrA1 expression in *S. aureus* ingested by DPI-treated and CGD PMN, despite the absence of PMN-generated oxidants in those PMN. The magnitude of the induction was highest in bacteria ingested by DPI-treated PMN, followed by bacteria ingested by CGD PMN at 60 min (fig. 3c).

The Role of Oxidants in the Expression of *msrA1*

Given that the induction of *msrA1* was more marked in PMN that were unable to produce oxidants, we studied whether oxidants may have a role in dampening the *msrA1* response. We treated bacteria grown in minimal

Fig. 3. *msrA1* expression in wild-type mutant ingested by normal and oxidase-deficient PMN. Serum-opsonized wild-type *S. aureus* in the mid-log (a) and stationary phase (b) were incubated alone or fed to PMN at an MOI of 1:1. At 0, 15, 30 and 60 min after phagocytosis, both ingested bacteria had recovered from PMN and bacteria incubated alone were prepared for real-time PCR. The expression of *msrA1* was normalized to the expression of *gapdh*, and fold change in gene expression was relative to that in opsonized *S. aureus* prior to exposure to PMN. Data represent

the mean $\pm$ SEM (n = 3 experiments). At each time point, a paired t test was used to analyze the difference between the ingested and non-ingested bacteria (* p < 0.05, ** p < 0.01). c Opsonized mid-log phase wild-type *S. aureus* was incubated alone or fed at an MOI of 1:1 to suspended normal PMN, DPI-treated PMN or PMN derived from 1 of 4 unrelated individuals with X-linked CGD. Data represent the mean $\pm$ SEM (n = 4 experiments). Data after 60 min were analyzed by 1-way ANOVA followed by a Tukey post-test (* p < 0.05).

media with vancomycin to induce *msrA1* expression in the presence or absence of H₂O₂ or HOCl. Since HOCl is such a potent antimicrobial agent [1], much lower concentrations of HOCl (10 and 30 μ M) were used relative to H₂O₂ (100 mM). At the tested concentrations, neither oxidant induced *msrA1* expression when used alone, but vancomycin alone induced *msrA1* more than 15-fold (fig. 4). In the presence of 10 or 30 μ M of HOCl, the level of *msrA1* induced by vancomycin was similar to that observed with vancomycin treatment alone. However, in the presence of 100 mM H₂O₂, the effect of vancomycin on *msrA1* induction was markedly reduced (fig. 4). These data suggest that although oxidants did not directly induce *msrA1*, exposure to H₂O₂ but not to HOCl may limit the *msrA1* response to stimuli arising from disruption of the bacterial cell wall. The observed decrease in vancomycin-induced *msrA1* response in the presence of H₂O₂ did not reflect a global inhibition of transcription by oxidant treatment, as the expression of the H₂O₂-responsive gene alkyl hydroperoxide reductase was increased as expected [24] (online suppl. fig. 2).

Modulation of *msrA1* by *Staphylococcal* TCS

We sought to determine which pathways in *S. aureus* participate in the detection and relay of signals that modulate the induction of *msrA1*. To sense and respond to the

Fig. 4. *msrA1* expression in *S. aureus* treated with oxidants and vancomycin (vanc). Wild-type *S. aureus* cultured in minimal media was treated with H₂O₂ or HOCl in the presence or absence of vancomycin (7 μ g/ml) or with vancomycin alone for 1 h at 37°C. Bacteria were then assessed for *msrA1* expression by real-time PCR and the data were normalized to *gapdh* expression. The data represent the fold change in *msrA1* expression relative to the non-treated control (mean $\pm$ SEM, n = 6). A paired t test was used to compare sample conditions (* p < 0.05, ** p < 0.01).

Fig. 5. Regulation of *msrA1* by TCS. **a** Wild-type and mutant strains of *S. aureus* that were each deficient in one TCS were grown to the mid-log phase and treated with vancomycin for 1 h (table 1). TCS mutants are labeled in the graph with the gene locus or TCS name where known. The expression of *msrA1* measured by real-time PCR was normalized to the expression of *gapdh*. Fold change in *msrA1* is expressed relative to the untreated wild-type strain. The data represent the mean of two independent experiments. **b** Wild-type and Δ *vraSR* grown to the stationary phase were diluted to an OD₅₅₀ of 0.3 and then treated with buffer or with D-cycloserine. Msr activity in bacterial lysates was measured. Data shown represent the mean $\pm$ SEM (n = 4). A paired t test was performed between the cycloserine-treated wild-type and mutant samples (* p < 0.05). **c** Serum-opsonized wild-type, Δ *vraSR* and complemented Δ *vraSR* grown to the mid-log phase were fed to PMN (MOI of 1:1), and *msrA1* expression in the ingested bacteria was measured and normalized to *gapdh* expression. Fold change is expressed relative to opsonized wild-type bacteria at time 0. Data represent the mean $\pm$ SEM (n = 9) and are analyzed by 1-way ANOVA with a Tukey post-test (* p < 0.05, *** p < 0.001).

environment, prokaryotes utilize TCS [25], which typically consist of a cell surface histidine kinase as sensor and a cytoplasmic regulator to coordinate a transcriptional response. Only a few of the 16 TCS encoded in the *S. aureus* genome have been thoroughly characterized.

In a screen of a library of 15 nonessential TCS mutants for impaired *msrA1* induction in response to vancomycin, deletion of *vraSR* resulted in loss of *msrA1* induction under our experimental conditions (fig. 5a). To confirm the observed relationship between *VraSR* and

Msr, we quantitated Msr activity in the *vraSR* deletion mutant (Δ *vraSR*) and its isogenic wild-type mutant after treatment with a cell wall-active antibiotic structurally unrelated to vancomycin. We chose D-cycloserine for its ability to induce *msrA1* and, more importantly, because its antistaphylococcal activity, in contrast to that of vancomycin, is unchanged in the absence of a functional *VraSR* system [26, 27]. At a concentration of D-cycloserine that modulated Msr activity with the least impact on viability (data not shown), D-cycloserine-treated wild-type mutants showed approximately 2-fold more Msr activity than did the non-treated controls. In contrast, treatment of Δ *vraSR* resulted in less Msr activity relative to the treated wild-type strain ($p < 0.05$; fig. 5b). These data suggested that *VraSR* contributed to the regulation of *msrA1* in response to cell wall-active antibiotics.

To determine whether *VraSR* participated in the regulation of *msrA1* in PMN-ingested bacteria, we measured *msrA1* expression in wild-type, Δ *vraSR* and Δ *vraSR* mutants complemented with a multicopy plasmid (Δ *vraSR* with pEPSA5-*vraSR*) after phagocytosis by PMN. Phagocytosis of *S. aureus* by PMN was the same for all the strains (data not shown). Both wild-type and complemented mutants significantly upregulated *msrA1* in response to ingestion by approximately 11- and 15-fold, respectively. In contrast, Δ *vraSR* exhibited a relatively muted response, with only an approximately 7-fold induction in *msrA1* ($p < 0.05$; fig. 5c). Similar to the trend observed in *msrA1*, *vraS* expression in *S. aureus* ingested by DPI-treated PMN and CGD PMN was higher than that in normal PMN (online suppl. fig. 3). Taken together, these data provide support for a link between *VraSR*, a TCS that can be activated by perturbations to the cell wall, and Msr, one of the oxidant repair systems. However, they also highlight the complexity of *msrA1* regulation in a biological setting composed of multiple interacting antimicrobial toxins, as occurs in PMN phagosomes, and implicate factors other than *VraSR*.

PMN Granules Induce the Expression of *msrA1*

Optimal PMN antimicrobial action relies not only on oxidants from the NADPH oxidase but also on granule proteins released into phagosomes during degranulation [28]. Granule proteases (e.g., elastase, cathepsin G) [29–31] and cationic antimicrobial proteins (e.g., defensins and cathelicidins) [32] can compromise the stability of bacterial membranes that in turn may disrupt the cell wall [33–35]. Because our data demonstrate that up-regulation of *msrA1* in ingested *S. aureus* could be medi-

Fig. 6. *msrA1* expression in *S. aureus* treated with PMN granule proteins. Wild-type, Δ *vraSR* and complemented Δ *vraSR* strains were treated with PMN granule extracts (Gex), PBS (negative control) or RB (vehicle control). The levels of *msrA1* in the samples at 0 and 30 min were assessed by real-time PCR. The expression levels were normalized to the expression of *gapdh*. Data represent the mean $\pm$ SEM ($n = 3$ experiments). To compare the RB sample with the Gex sample at 30 min, a paired t test was used for each strain (* $p < 0.05$).

ated by factors other than PMN-derived oxidants (fig. 3c) and could be induced by cell wall stress, we hypothesized that granule proteins could trigger a response that was relayed through *VraSR*. To test this hypothesis, we measured *msrA1* in bacteria exposed to PMN granule-rich extracts in the absence of exogenous reactive oxygen species. The level of *msrA1* expression was lowest at the start of the experiment prior to any treatment in all the strains. After a 30-min incubation period, *msrA1* expression was slightly higher in bacteria that were not treated or in the RB control. This increase in *msrA1* expression may reflect changes in the growth status of the bacteria, since *msrA1* expression varies during different growth phases [3]. In bacteria treated with granule extract, *msrA1* expression was significantly higher in the wild-type when compared to the RB-treated bacteria at 30 min ($p < 0.05$; fig. 6). In contrast, the level of *msrA1* in granule extract-treated samples in Δ *vraSR* was not significantly elevated compared to its RB control. These data suggest that granule proteins alone promoted increased *msrA1* expression and that *VraSR* contributed to the modulation of *msrA1* in response to granule proteins.

Discussion

Optimal antimicrobial action in PMN relies heavily on phagocyte-generated oxidants. In such an inhospitable environment, ingested *S. aureus* rapidly initiates a program of transcriptional responses that promote its survival and dissemination [11, 36], making it likely that the net balance between sustained damage and repair dictates the fate of ingested bacteria. Defensive strategies employed by bacteria in response to oxidants involve both genes encoding proteins that target oxidants, such as catalase, superoxide dismutase, thioredoxin, and alkyl hydroperoxide reductase [36], as well as those that repair oxidant-mediated damage and restore normal function. Msr are key enzymes in the defense against damage mediated by reactive oxygen species that are produced as a by-product of aerobic respiration, and their evolutionary conservation across nearly all species underscores their importance [37, 38]. HOCl oxidizes protein methionines in *Escherichia coli* in a dose-dependent fashion, and methionine oxidation closely correlates with loss of bacterial viability [39]. Conversely, the capacity of Msr to repair damaged methionines parallels the increased resistance of *E. coli* to killing by HOCl [39] and likewise contributes to the defenses of *Mycobacterium tuberculosis* [40] and *Helicobacter pylori* against HOCl [41]. Thus, we reasoned that oxidation of key proteins in *S. aureus* may also result in reduced viability when the Msr-mediated repair system is inadequate to meet demands.

We demonstrate that the absence of Msr activity increased the susceptibility of *S. aureus* to the oxidants H₂O₂ and HOCl. We found that the ability of the Msr system to temper the effects of oxidant damage was less marked when *S. aureus* was treated with low doses of H₂O₂ or HOCl, which may indicate that the role of Msr in oxidant defense becomes more important when *S. aureus* is subjected to higher levels of oxidant stress and greater levels of protein damage (fig. 2b). In addition, we observed that even in the absence of any oxidant treatment, the percentage of recovered Δ *msrA1B* was sometimes slightly lower than that of the wild-type and complemented strain (fig. 2a–c). Although this difference did not reach statistical significance, it suggested that Δ *msrA1B* may be slightly more sensitive to the aerobic environment during the 1-hour incubation in PBS than the other strains. This behavior contrasts with our observation that all the strains showed similar growth in TSB. Since TSB is a rich source of substrates that could serve as oxidant sinks, it is likely that the enriched media both promotes microbial growth directly and neutralizes some

of the environmental oxidants, thus limiting their deleterious effects on the bacteria.

The absence of Msr activity increased the susceptibility of *S. aureus* not only to reagent H₂O₂ and HOCl but also to the antimicrobial effects of PMN. In contrast to the immediate exposure to high concentrations of oxidants imposed by adding H₂O₂ or HOCl to suspensions of *S. aureus*, oxidant generation within phagosomes occurs gradually, following the kinetics of NADPH oxidase assembly and activation. As a result, oxidants within PMN may be generated sufficiently slowly to allow reparative responses such as the induction of *msr* to be engaged. Given the role of Msr in oxidant repair, we speculated that PMN-derived oxidants may drive the upregulation of *msrA1* that we observed in *S. aureus* ingested by PMN. Our data show that bacteria ingested by normal PMN upregulated *msrA1* rapidly after phagocytosis. *S. aureus* ingested by PMN that have impaired NADPH oxidase activity and were not exposed to phagosomal oxidants also upregulated *msrA1* after phagocytosis and to higher levels. These data suggest that the *msrA1* response to ingested *S. aureus* may be muted by the presence of oxidants in the phagosome, either directly by oxidant attack on bacteria or indirectly by oxidative modifications of the host-derived phagosomal contents. In the absence of PMN, *S. aureus* exposed simultaneously to H₂O₂ and a cell wall-active antibiotic (vancomycin) had a blunted *msrA1* transcriptional response relative to that seen in vancomycin alone. Unexpectedly, HOCl, a much more powerful oxidant than H₂O₂, did not influence vancomycin-induced *msrA1*. The mechanistic basis for the differential responses to the two oxidants is unknown and merits further study. These findings illustrate how oxidants and granule proteins present in phagosomes can act synergistically on target bacteria.

The *S. aureus* genome encodes three other *msr* genes besides *msrA1*, including *msrB*, *msrA2* and *msrA3*. Since *msrB* is cotranscribed and regulated by the same promoter, the pattern of expression follows that of *msrA1* and it is responsive to the same stimuli. The other two *msr* genes are located at different genomic loci and under the regulation of promoters that are responsive to differing stresses than that of *msrA1* and *msrB*. Not only do *msrA2* and *msrA3* show a different pattern of expression to *msrA1* and *msrB* during varied phases of growth, they also do not respond to the same stress signals [3]. For these reasons and also because MsrA1 and MsrB account for almost all the Msr activity in *S. aureus* (fig. 1), the expression of *msrA2* and *msrA3* was not evaluated in this study.

The regulation of *msrA1* in *S. aureus* is not yet fully elucidated, although *msr* is one of a set of genes known as the cell wall stress stimulon, which is regulated by stresses inflicted by cell wall-active antibiotics such as β -lactams, vancomycin and D-cycloserine [42]. Genes in the cell wall stress stimulon overlap with those regulated by *VraSR*, a TCS that influences susceptibility of *S. aureus* to vancomycin by modulating cell wall biosynthesis [26]. The *VraSR* system senses disruptions of cell wall biosynthesis in target bacteria, which may explain why both cell wall-active antibiotics and antimicrobial peptides induce the *VraSR* system [43]. In our study, PMN granule proteins alone stimulated *msr* expression in a *VraSR*-dependent fashion. The mechanism by which *VraSR* is activated and how it subsequently modulates *msrA1* remain to be elucidated, including whether *VraR* directly or indirectly regulates *msr*. We noted that the introduction of the complementation plasmid into Δ *vraSR* increased *msrA1* expression to higher levels than that observed in Δ *vraSR* both in the presence and absence of granule extracts but did not restore it to wild-type levels. The levels of *msrA1* were also similar in the RB and granule extract-treated samples for this strain. Complementation of a mutation can be challenging and, given that the restoration of *vraSR* expression was driven from a multicopy plasmid and from an inducible promoter rather than from its native promoter, the gene dosage and regulation of *vraSR* may not accurately emulate the wild-type strain under these conditions. Nonetheless, the levels of *vraSR* in Δ *vraSR* with pEPSA5-*vraSR* were higher than in Δ *vraSR* and allowed for greater *msrA1* expression.

The transcriptional changes that occur in phagocytosed bacteria reflect a coordinated response to the multitude of stresses experienced within the host environment. Given the complexity of this environment, multiple signaling pathways underpin the net global response,

and their cross-talk can contribute to the modulation of stress response genes such as *msr*. Indeed, our data show that there are factors other than *VraSR* that can modulate the expression of *msrA1* in *S. aureus* within the PMN environment. The identification and characterization of signaling systems in addition to *VraSR* in the regulation of *msrA1* modulation remain an area of interest.

Our study highlights *Msr* as one of the mechanisms by which *S. aureus* may resist the consequences of the multifaceted antimicrobial attack that occurs in PMN phagosomes and reemphasizes that the relationships between stress and repair pathways are not necessarily straightforward. We speculate that *Msr* and functionally related proteins in the antioxidant pathway serve important roles in cell wall biosynthesis and homeostasis. Further understanding of the relationship between oxidant stress, both exogenous and endogenous, and cell wall damage in the context of elucidating how *S. aureus* survives within PMN may provide insights into the pathogenesis of staphylococcal disease and into the development of novel therapeutic approaches.

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Disclosure Statement

None of the authors have conflicts of interests.

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