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The Extracytoplasmic Function Sigma Factor σ^S Protects against both Intracellular and Extracytoplasmic Stresses in *Staphylococcus aureus*

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Previously we identified a novel component of the *Staphylococcus aureus* regulatory network, an extracytoplasmic function σ-factor, σ^S, involved in stress response and disease causation. Here we present additional characterization of σ^S, demonstrating a role for it in protection against DNA damage, cell wall disruption, and interaction with components of the innate immune system. Promoter mapping reveals the existence of three unique *sigS* start sites, one of which appears to be subject to autoregulation. Transcriptional profiling revealed that *sigS* expression remains low in a number of *S. aureus* wild types but is upregulated in the highly mutated strain RN4220. Further analysis demonstrates that *sigS* expression is inducible upon exposure to a variety of chemical stressors that elicit DNA damage, including methyl methanesulfonate and ciprofloxacin, as well as those that disrupt cell wall stability, such as ampicillin and oxacillin. Significantly, expression of *sigS* is highly induced during growth in serum and upon phagocytosis by RAW 264.7 murine macrophage-like cells. Phenotypically, σ^S mutants display sensitivity to a broad range of DNA-damaging agents and cell wall-targeting antibiotics. Furthermore, the survivability of σ^S mutants is strongly impacted during challenge by components of the innate immune system. Collectively, our data suggest that σ^S likely serves dual functions within the *S. aureus* cell, protecting against both cytoplasmic and extracytoplasmic stresses. This further argues for its important, and perhaps novel, role in the *S. aureus* stress and virulence responses.

Staphylococcus aureus is an exceedingly virulent and successful pathogen, capable of causing a wide range of infections, from relatively benign skin lesions to life-threatening septicemia. With an overwhelming ability to adapt to its environment, *S. aureus* has become the most common cause of both hospital- and community-acquired infections and is believed to be the leading cause of death by a single infectious agent in the United States (20, 34). The threat posed by this organism to human health is further heightened by the rapid and continued emergence of multidrug-resistant isolates (1, 20, 34, 43).

Many components govern the adaptive nature of *S. aureus*, including complex regulatory networks, which allow it to respond to constantly changing environments via rapid shifts in gene expression. There are a number of different elements that mediate this fine-tuning, including DNA-binding proteins, two-component systems, regulatory RNAs, and alternative σ factors (10, 11, 18, 21, 22, 32, 44, 50, 51). The last class acts by binding to core RNA polymerase and redirecting promoter recognition to coordinate gene expression, bringing about expedient and wide-reaching alterations within the cell.

From a classification perspective, σ factors are divided into five discrete subfamilies, with the essential housekeeping factors (σ^A or σ^{70}), which are responsible for the majority of transcription, constituting group 1. The remaining families (groups 2 to 5) contain alternative σ factors, which are important for niche-specific transcriptional regulation in response to environmental change (24, 27, 40, 41). These elements provide the ability to readily adapt to an ever-changing environment by discrete alterations in transcription profiles. As such, bacteria typically encode a number of alternative σ factors within their genome that fulfill a wide range of functions. Of the alternative families, group 4, comprising the ECF (extracytoplasmic function) σ factors, contains by far the most numerous of all such elements (27); for example, *Streptomyces coelicolor* contains approximately 65 σ factors, around 50 of which are of the ECF subtype (27). Interestingly, *S. aureus* relies on only 4 σ factors to oversee the execution of its gene expression. In addition to a primary σ factor, σ^A (16, 17), *S. aureus*, as with the majority of firmicutes, possesses a σ^B alternative σ factor, which controls the general stress response (18, 28, 37, 55, 64). A third σ factor, σ^H, has recently been reported, demonstrating homology to σ^H from *Bacillus subtilis*, and has been shown to regulate competence genes and the integration and excision of prophages (46, 68). Finally, a recent discovery in our laboratory demonstrated the existence of a fourth σ factor, σ^S, belonging to the ECF family (65). Unlike many other organisms, which commonly possess multiple ECF σ factors, σ^S is the only such element discovered in this organism thus far (27, 65).

Previous work by our group revealed a role for σ^S in the stress and virulence response of *S. aureus* (65). Specifically, we showed that σ^S is important in extended survival during starvation and by lysis with Triton X-100. Competitive growth analysis revealed a decreased ability of a *sigS* mutant to compete against its parental strain both under standard conditions and in the presence of stress. Interestingly, transcriptional analysis of *sigS* in the laboratory strain SH1000 revealed only baseline expression during growth in rich media over a 72-h period. Finally, using a murine model of septic arthritis, we demonstrated a role for σ^S in systemic infections, as mice infected with a *sigS* mutant displayed significantly decreased weight loss, mortality, severity of infection, systemic dissemination, and mounted immune response by the host.

In this study, we have further explored the role and regulation of σ^S, in an effort to understand the conditions under which *S.*

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TABLE 1 Strains, plasmids, and primers used in this study[a]

Strain, plasmid, or primer	Genotype or description	Reference or source
E. coli strains		
DH5α	ϕ80 *lacZ*ΔM15 Δ(*argF-lacZYA*)*U169 endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1*	60a
S. aureus strains		
RN4220	Restriction-deficient transformation recipient	Lab stocks
8325-4	Wild-type laboratory strain, *rsbU* mutant	Lab stocks
SH1000	Wild-type laboratory strain, *rsbU* functional	28
Newman	Wild-type laboratory strain, human clinical isolate	Lab stocks
USA300	USA300-LAC MRSA isolate cured of pUSA300-LAC-MRSA	Paul Fey, UNMC
LES57	SH1000 pAZ106::*sigS-lacZ sigS*$^+$	65
HKM01	RN4220 pAZ106::*sigS-lacZ sigS*$^+$	This study
HKM02	8325-4 pAZ106::*sigS-lacZ sigS*$^+$	This study
HKM03	Newman pAZ106::*sigS-lacZ sigS*$^+$	This study
HKM04	USA300 pAZ106::*sigS-lacZ sigS*$^+$	This study
HKM05	RN4220 *sigS*::*tet* (lacking *sigS*)	This study
HKM06	RN4220 *sigS*::*tet* pMK4::*sigS*$^+$	This study
HKM07	USA300 *sigS*::*tet* (lacking *sigS*)	This study
HKM08	USA300 *sigS*::*tet* pMK4::*sigS*$^+$	This study
Plasmids		
pMK4	Shuttle vector	67a
pHKM1	pMK4 containing a 2.1-kb *sigS* fragment	This study
Primers		
OL281	ACT GGA TCC CAG TTG CAG ATG CAT CTC TCC	
OL1715	ATG CTG CAG CAA GTC TAT CTG GCG TAC	
OL1036	CCG CGC ACA TTT CCC CGA AA	
OL1275	ACC TTG AAG GAT ACA AGC AA	
OL1276	GGC ATT TAC GCT TAA CGG AC	
OL1528	GTG GTG TTT GTT GTA TAC GTC	

[a] Primer restriction sites are underlined. Abbreviations: MRSA, methicillin-resistant *S. aureus*; UNMC, University of Nebraska Medical Center.

aureus utilizes this transcriptional regulator. We show that not only is *sigS* transcription seemingly subject to genetic control in *S. aureus* cells, but it is also highly inducible in response to a variety of stresses, including those that elicit DNA damage and cell wall perturbations. Additionally, we reveal that *sigS* is strongly upregulated upon exposure to serum and following phagocytosis by macrophage-like cells. Finally, we present a role for σ^S in the response to DNA damage and cell wall stress, as well as a role in protection against components of the innate immune system.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. aureus* and *Escherichia coli* strains and plasmids are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB) medium at 37°C. *S. aureus* was grown in 100 ml tryptic soy broth (TSB) (1:2.5 flask/volume ratio) at 37°C with shaking at 250 rpm, unless otherwise indicated. Synchronized cultures were obtained as described previously (65). When required, antibiotics were added at the following concentrations: ampicillin, 100 mg liter^{-1} (*E. coli*); tetracycline, 5 mg liter^{-1} (*S. aureus*); erythromycin, 5 mg liter^{-1} (*S. aureus*); lincomycin, 25 mg liter^{-1} (*S. aureus*); and chloramphenicol, 5 mg liter^{-1} (*S. aureus*). Where specified, chemically defined minimal media (CDM) and metal ion-limiting media (CL) were prepared as described previously (29, 74). Porcine serum agar was created by adding filter-sterilized porcine serum (Sigma) to preautoclaved and cooled 2% agar in distilled water.

Construction of the *sigS* mutant and *sigS-lacZ* fusion strains. All strains used in this study, other than those described below, were created via ϕ11-mediated transduction from strains previously described (Table 1).

Construction of *sigS* complement strains. The complement construct generated contains approximately 1 kb of upstream and 710 bp of downstream DNA, relative to the *sigS* coding region. This was PCR amplified using primer pair OL281 and OL1715 (Table 1) and cloned into the Gram-positive shuttle vector pMK4, creating pHKM1. *S. aureus* RN4220 was transformed with this construct, with clones confirmed by PCR analysis, using a combination of gene- and vector-specific primers (OL281/OL1036). A representative clone was selected to transduce the RN4220 and USA300 *sigS* mutants. Clones were again confirmed by PCR analysis, creating strains HKM06 and HKM08.

β-Galactosidase assays. Levels of β-galactosidase activity were measured as described previously (35). The results presented here are representative of three independent replicates, which showed less than 10% variability.

Real-time PCR. Quantitative real-time PCR analysis was conducted as described previously (35) using primers listed in Table 1 specific for *sigS* (OL1275/OL1276). Control primers were for the 16S rRNA gene, as described previously (36). Values were calculated from three independent replicates, and the data analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

Primer extension analysis. Primer extension analysis was carried out as described previously (63) using the AMV reverse transcriptase (RT) primer extension system (Promega) according to the manufacturer's guidelines. RNA for primer extension reactions was extracted using an RNeasy kit (Qiagen) as described previously (35). For primer extension analysis, 32 μg RNA was used with primer OL1528.

Plate-based stress assays. Plate-based assays to determine alterations in transcription resulting from external stress were performed using *sigS-lacZ* fusion strains as described previously (65), with the following stress

chemicals: 6 M HCl, 85% phosphoric acid, 100% trichloroacetic acid (TCA), 88% formic acid, 0.2 M acetic acid, 6 M sulfuric acid, 6 M nitric acid, 6 M sodium hydroxide, 2 M NaCl, 1 M glucose, 95% ethanol, 100% methanol, 100% isopropanol, 10% SDS, 10% Triton X-100, 10% Tween 20, 1 M *N*-lauroyl sarcosine, 30% hydrogen peroxide, 1 M methyl viologen, 1% menadione, 2 mg ml^{-1} pyrogallol, 1 M sodium nitroprusside, 1 M ethyl methane sulfonate, 1 M methyl methanesulfonate (MMS), 5 mg ml^{-1} penicillin G, 5 mg ml^{-1} ciprofloxacin, 5 mg ml^{-1} nalidixic acid, 5 mg ml^{-1} cefotaxime, 5 mg ml^{-1} vancomycin, 2 mg ml^{-1} phosphomycin, 5 mg ml^{-1} spectinomycin, 100 mg ml^{-1} ampicillin, 100 mg ml^{-1} oxacillin, 5 mg ml^{-1} gramicidin, 5 mg ml^{-1} tetracycline, 50 mg ml^{-1} kanamycin, 50 mg ml^{-1} neomycin, 10 mg ml^{-1} chloramphenicol, 20 mg ml^{-1} puromycin, 2 mg ml^{-1} bacitracin, 2 mg ml^{-1} mupirocin, 500 mM diamide, 12.8 mg ml^{-1} berberine chloride, 4.21 M peracetic acid, 0.1 M EDTA, 1 M dithiothreitol (DTT). Plates were incubated for 24 h at 37°C and screened for blue halos, indicating expression.

Transcriptional analysis during growth in porcine serum. Synchronous cultures of the *sigS-lacZ* fusion strains were standardized to an optical density at 600 nm (OD_{600}) of 0.5, pelleted, and washed twice in phosphate-buffered saline (PBS) before being resuspended in 1 ml of filter-sterilized porcine serum (Sigma). The suspension was then incubated at 37°C in a rotator device for a period of 1, 5, or 24 h. At the appropriate time point 1-ml samples were pelleted and stored at −20°C for future analysis. Concomitantly, the CFU per ml for each sample was determined via serial dilution and plating on tryptic soy agar (TSA). Harvested bacterial cells were assayed for β-galactosidase production as described previously (35), with the following alterations. Arbitrary expression units were calculated as a measure of substrate cleavage (4-MUG [4-methylumbelliferyl-β-glucuronide]) by β-galactosidase into 4-MU (methylumbelliferyl), which was evaluated by measuring the fluorescence of each sample at 355/460 nm, 0.1 s, divided by the CFU ml^{-1}. Samples collected from the initial inocula were analyzed for β-galactosidase activity and used as a measure of baseline expression to identify changes in transcription, as described previously (52, 73). The data presented were generated from 3 independent replicates and analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

Macrophage cell culture and *S. aureus* intracellular transcriptional analysis. Assays were carried out using the RAW 264.7 murine leukemic monocyte macrophage cell line (ATCC TIB-71) as described previously (73). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (Invitrogen) and a 1% penicillin/streptomycin solution (Sigma) until infection, at which time antibiotics were used as described below. RAW 264.7 cells were seeded into 6-well plates and allowed to grow to a density of 2.5×10^6 cells per well. These were then infected with *S. aureus* strains resuspended in cell culture medium at 2.5×10^8 CFU per well to give a multiplicity of infection (MOI) of 100. To synchronize infections and facilitate contact between bacteria and RAW 264.7 cells, plates were centrifuged at 1,000 rpm for 10 min. Cells were subsequently incubated for 1 h at 37°C in a humidified atmosphere containing 5% CO_2 to allow phagocytosis. After this time, wells were washed twice with PBS, and any remaining nonphagocytosed bacteria were killed by the addition of medium containing 30 μg ml^{-1} gentamicin for 1 h. This was then replaced with fresh DMEM containing 5 μg ml^{-1} gentamicin and incubated for 24 h. Following this, RAW 264.7 cells were washed twice with PBS and lysed using 500 μl PBS containing 0.5% Triton X-100. Samples were withdrawn to determine bacterial numbers, and the remaining bacteria were pelleted by centrifugation. Harvested bacterial cells were assayed for β-galactosidase production as described previously (35), with the modifications described above for pig serum studies. The data presented were generated from 6 independent replicates and analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

DNA damage sensitivity assays. Exponentially growing cultures were washed and resuspended in PBS before the addition of DNA-damaging agents: 150 mM H_2O_2, 20 mM MMS, or 2 mg ml^{-1} ethidium bromide (EtBr). These were placed at 37°C with shaking, and aliquots removed at the time intervals specified. Samples were then serially diluted, and CFU ml^{-1} determined alongside control samples that were removed prior to exposure. Percent survival was calculated by comparing initial CFU ml^{-1} to final CFU ml^{-1} from three independent assays, and the data were analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance. Data are presented as fold change of percent survival relative to that of the wild-type strain.

UV radiation survival assay. The UV radiation survival assay was performed as previously described (9). Briefly, strains were synchronized to an OD_{600} of 0.05 and allowed to grow for 4 h. Cultures were then serially diluted, and 10^{-2} through 10^{-6} dilutions plated on TSA. Dilutions 10^{-2} and 10^{-3} were subjected to UV irradiation at 4,000 μJ/cm^2 using a CL-1000 UV Cross-linker (UVP). Dilutions 10^{-4} through 10^{-6} served as unexposed controls. All plates were incubated in the dark at 37°C overnight. Survival rates were calculated from three independent experiments, and the data analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

MICs of cell wall-targeting antibiotics. MIC determinations were performed using a variety of cell wall-targeting antibiotics and a broth microdilution assay, described previously (5).

Whole-blood survival assay. The USA300 wild-type and its isogenic *sigS* mutant strains were subjected to analysis using a whole-human-blood model of survival as described previously (35). Pooled and deidentified whole human blood was purchased from Bioreclamation. Survival rates were calculated from three independent replicates, and the data analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

Macrophage cell culture and *S. aureus* intracellular survival assay. For macrophage cell culture and *S. aureus* intracellular survival assays, infections were carried out as described above for transcription studies, with the following alterations. RAW 264.7 cells were infected with *S. aureus* strains resuspended in cell culture medium at 2.5×10^6 CFU per well to give an MOI of 1. Samples were withdrawn 24 h postphagocytosis, and CFU ml^{-1} determined via serial dilution and plating on TSA. The data presented were generated from 3 independent replicates and analyzed using a Student *t* test with a 5% confidence limit to determine statistical significance.

RESULTS

σ^S is differentially expressed in *S. aureus* wild-type strains. An unusual finding from our previous study on σ^S was that no expression of the *sigS* gene was detected in SH1000 under standard conditions (65). To assess whether this is a conserved phenomenon, transcriptional analysis using a *sigS-lacZ* fusion was performed in a variety of laboratory strains, including RN4220, 8325-4, SH1000, and Newman, as well as the clinical isolate USA300. Expression of *sigS* again exhibited only baseline activity over a 24-h period in complex liquid media in every strain, apart from RN4220 (Fig. 1A). Surprisingly, in the last strain we observed an approximate 5-fold increase in *sigS* expression compared to other *S. aureus* isolates. To ensure our findings were not an artifact of the fusion construct, we performed quantitative real-time PCR (qRT-PCR) on these wild-type strains during a window of maximal *sigS* expression (3 h). Using this approach, we again observed robust expression of *sigS* in RN4220, with minimal transcription detected in the other isolates (Fig. 1B). Curiously, while low expression was observed for the other backgrounds, transcriptional activity in Newman was almost entirely negligible. To determine if nucleotide alterations in the promoter region contributed to this variable expression, we sequenced a 945-bp region immediately 5′ of the annotated *sigS* start codon for each of these strains. Interestingly, all sequences were identical to each other and, where

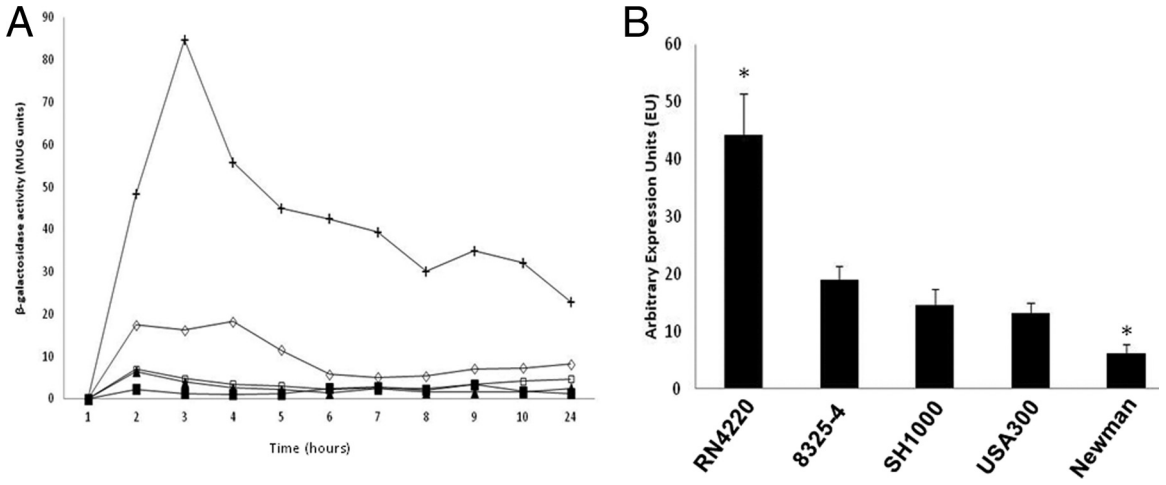

FIG 1 Transcription profiling of *sigS* in a variety of *S. aureus* wild-type strains. (A) *sigS-lacZ* fusion strains in RN4220 (+), 8325-4 (◇), SH1000 (▲), USA300 (■), and Newman (□) were grown in TSB at 37°C and sampled every hour for 10 h and again at 24 h. β-Galactosidase activity was measured to determine levels of expression. Assays were performed on duplicate samples and the values averaged. The results presented here were representative of three independent experiments that showed less than 10% variability. (B) Quantitative real-time PCR was performed on *S. aureus* wild-type strains grown for 3 h under the same conditions as described for panel A, with primers specific to *sigS*. The data presented are from at least 3 independent experiments. Error bars indicate ± standard error of the mean; *, $P < 0.05$ by the Student *t* test.

available, matched publicly available genome data for the requisite strains. As such, the differential expression of *sigS* is seemingly not mediated by SNPs within the promoter or regulatory regions of the *sigS* gene.

Mapping the *sigS* transcriptional start site. We previously demonstrated that σ^S acts to upregulate itself from its own promoter region; therefore, in order to elucidate this promoter and any others, we set out to map the *sigS* promoters using RN4220 as a model. To this end, we performed primer extension analysis on RNA extracted from strain RN4220 from exponentially growing cultures (3 h) using a primer located 12 nucleotides (nt) downstream of the *sigS* initiation codon. We identified 3 unique transcription start sites (Fig. 2), the longest of which bears an adenine plus 1 residue, located 150 bp upstream of the translation start site. This is 7 nt away from a putative σ^A promoter, denoted P1, with a sequence of aTtACA, followed by a 17-bp spacer, and then TATtta (where lowercase typeface indicates nucleotides that differ from consensus). Promoter P2 is located 126 nt upstream of the trans-

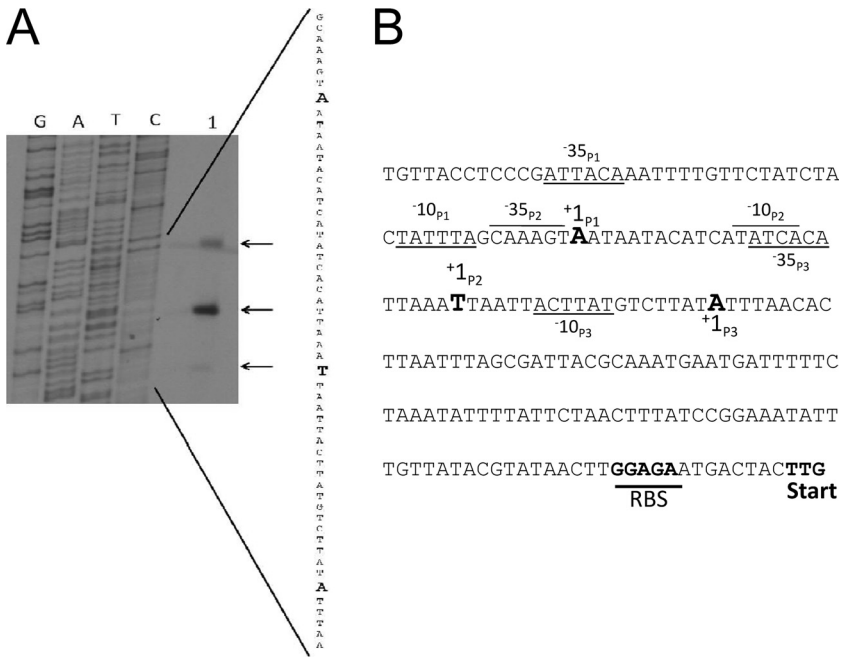

FIG 2 Primer extension analysis reveals three *sigS* promoters. (A) Mapping of the 5′ ends of the *sigS* transcripts by primer extension. RNA was extracted from RN4220 grown to exponential phase (3 h) and used in reactions (lane 1). (B) Transcriptional start sites (+1) for promoters P1, P2, and P3 are denoted with corresponding −35 and −10 regions.

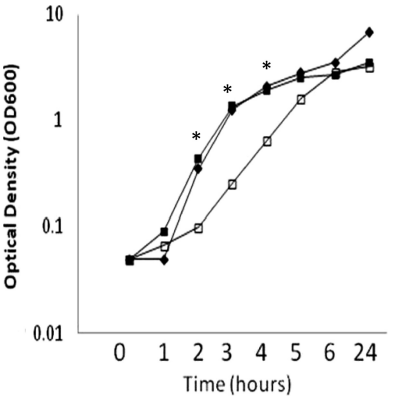

FIG 3 RN4220 *sigS* mutants have a growth defect upon exit from stationary phase. Optical density readings (OD_{600}) of the RN4220 wild-type (■), *sigS* mutant (□), and *sigS* complement (◆) strains were taken every hour for 6 h and again at 24 h during growth at 37°C with shaking in TSB. Growth curves are representative of at least three independent experiments that showed less than 10% variability. *, $P < 0.05$ by the Student *t* test, indicating significant difference in growth between the *sigS* mutant and its parental and complemented strains.

lational start site, beginning with a thymine residue, and appears to have no notable σ^A or σ^B promoter sequences; however, a possible σ^S consensus was identified as CAAAGT 12 bp upstream of TATCA, the putative −10 site. The third transcription start site, positioned 107 nt upstream of the coding region, contains an adenine plus 1 residue and is 7 nucleotides away from a putative σ^A promoter, denoted as P3, with a sequence of aTcACA, followed by an 11-bp spacer, and then acTtAT.

***sigS* deletion results in a growth defect in strain RN4220.** We previously reported that the *sigS* mutant in the SH1000 background displayed no notable growth defect under standard conditions (65). Given the data presented above regarding differential expression of *sigS* in *S. aureus* wild-type strains, we next performed growth analysis of the *sigS* mutant in the RN4220, 8325-4, SH1000, and USA300 LAC (Los Angeles County clone) backgrounds. Interestingly, while mutation of σ^S led to no notable growth defects in the last 3 strains, we observed a significant growth defect in strain RN4220 (Fig. 3). Specifically, *sigS* deletion in this strain resulted in a significant defect upon exit from stationary phase, which continued through exponential growth. At hour 2, we noted a 4.4-fold decrease in optical density of the σ^S mutant compared to the wild type, which peaks at hour 3 with a fold change of 5.3. This trend continues through hour 4 with a fold decrease of 3. By hour 5, growth of the σ^S mutant is comparable to that of the parent strain. This observation is particularly interesting, as *sigS* expression peaks at hour 3 in RN4220, which corresponds to the time point at which we observe the highest fold decrease in growth for the mutant strain.

***sigS* expression is inducible in response to external stimuli.** Given the differential nature of *sigS* expression among *S. aureus* strains, we next set out to explore whether transcription could be induced from this locus via external stress, as with other ECF sigma factors (27, 56, 67). This was performed using a disk diffusion assay previously described by us (35, 65), which builds on a pilot screening conducted with SH1000 (65). As such, *sigS-lacZ* fusion strains in 8325-4, Newman, SH1000, and USA300 were grown in the presence of a plethora of stress conditions (see Materials and Methods). While we were unable to detect upregulation of *sigS* in most strains, we did observe significant inducibility in 8325-4. Specifically, we noted *sigS* expression in the presence of a variety of chemicals (Table 2), including a number of agents that induce cell wall stress, as well as compounds known to elicit DNA damage. We also noted *sigS* upregulation in amino acid-limiting media, in metal ion-limiting media, and during growth on pig serum. These minimal medium studies were of particular interest, as they correlate with our previous studies demonstrating a starvation survival defect for *sigS* mutant strains (65). Again to rule out artifacts of the screen, we sought to verify these findings during continuous growth in liquid media. This was performed with the 8325-4 *sigS-lacZ* fusion strain grown in TSB containing sublethal concentrations of select chemicals (MMS, H_2O_2, and NaOH) shown in Table 2 and revealed increased expression of *sigS* in each instance (Fig. 4A to C). Specifically, in the presence of MMS, expression peaked at 5 h, with a 48.7-fold increase compared to standard conditions. Maximal expression with both NaOH and H_2O_2 occurred at 10 h, with fold increases of 10 and 4.4, respectively, compared to unsupplemented media. An additional qRT-PCR analysis was performed (Fig. 4D) to verify these

TABLE 2 Compounds found to induce expression of a *sigS-lacZ* reporter fusion in strain 8325-4

Agent/conditions	Stress/mode of action	Overall effect
NaOH	Alkali stress	DNA damage
H_2O_2	Oxidative stress	DNA damage
MMS	Alkylates DNA	DNA damage
EMS[a]	Alkylates DNA	DNA damage
Ciprofloxacin	Inhibits DNA gyrase	DNA damage
Nalidixic acid	Inhibits DNA gyrase	DNA damage
Chloramphenicol	Inhibits protein synthesis	Miscellaneous
Pig serum	Components of the humoral immune system	Miscellaneous
Amino acid-limiting media	Minimal media	Miscellaneous
Metal-limiting media	Minimal media	Miscellaneous
Cefotaxime	Inhibits transpeptidation	Cell wall weakening/disruption
Ampicillin	Inhibits transpeptidation	Cell wall weakening/disruption
Oxacillin	Inhibits transpeptidation	Cell wall weakening/disruption
SDS	Disrupts cell walls	Cell wall weakening/disruption
Phosphomycin	Inhibits UDP-*N*-acetylglucosamine-3-enolpyruvyltransferase	Cell wall weakening/disruption

[a] EMS, ethyl methanesulfonate.

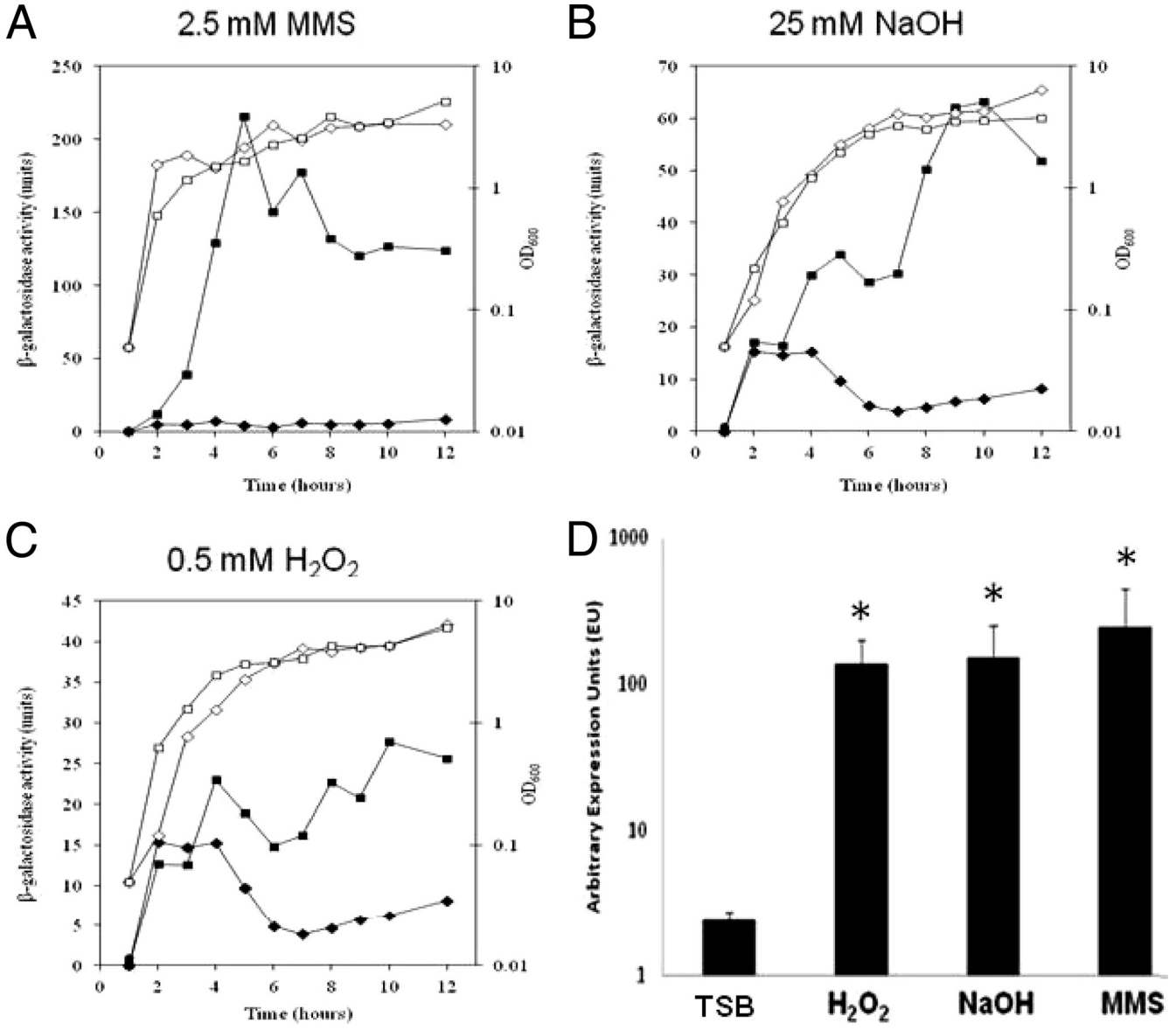

FIG 4 *sigS* transcription is inducible in response to external stress. (A to C) The 8325-4 *sigS-lacZ* strain was grown in either TSB (◆) or TSB supplemented with sublethal concentrations of the indicated stress chemicals (■). Cultures were sampled every hour for 10 h, and again at 24 h, to determine β-galactosidase activity. Additionally, growth was monitored via OD_{600} at the times indicated for both standard (◇) and supplemented (□) growth conditions. Assays were performed on duplicate samples and the values averaged. The results presented here were representative of three independent replicates that showed less than 10% variability. (D) Quantitative real-time PCR analysis was performed with strain 8325-4 grown for 5 h under conditions identical to those described for panels A to C, using primers specific to *sigS*. The data presented are from at least 3 independent experiments. Error bars indicate ± standard error of the mean; *, $P < 0.05$ by the Student *t* test.

data and again confirmed that the greatest fold increase in *sigS* expression was induced by exposure to MMS, resulting in a 102.6-fold increase in transcription. Transcription levels of *sigS* upon exposure to NaOH and H_2O_2 were 63.3- and 57.2-fold higher, respectively.

To explore if this upregulation was conserved for other *S. aureus* strains, but perhaps below the limit of detection for our plate-based assay, we performed experiments with MMS, NaOH, and H_2O_2 using SH1000 and USA300 *sigS-lacZ* fusion strains. Interestingly, despite a lack of blue coloration in the plate-based assay, we again detected upregulation of *sigS* during growth in liquid media with sublethal concentrations of MMS and H_2O_2 (Fig. 5A and B). Specifically, expression with MMS in both SH1000 and USA300 was highest at 2 h, with fold increases of 3.6 and 8.1, respectively, compared to standard growth conditions. In the presence of H_2O_2, *sigS* expression in SH1000 increased 2.6-fold (2 h) and 2.3-fold in USA300 (7 h). Conversely, we observed no increase in expression in the presence of NaOH when grown under these conditions, suggesting that greater, and more lethal, concentrations of this agent may be required to induce expression. We again confirmed these data by qRT-PCR in SH1000 and USA300 grown in the presence of MMS and H_2O_2 (Fig. 5C). We determined that SH1000 displays a 16.9-fold increase in *sigS* expression when cultured with MMS and a 13.5-fold increase when

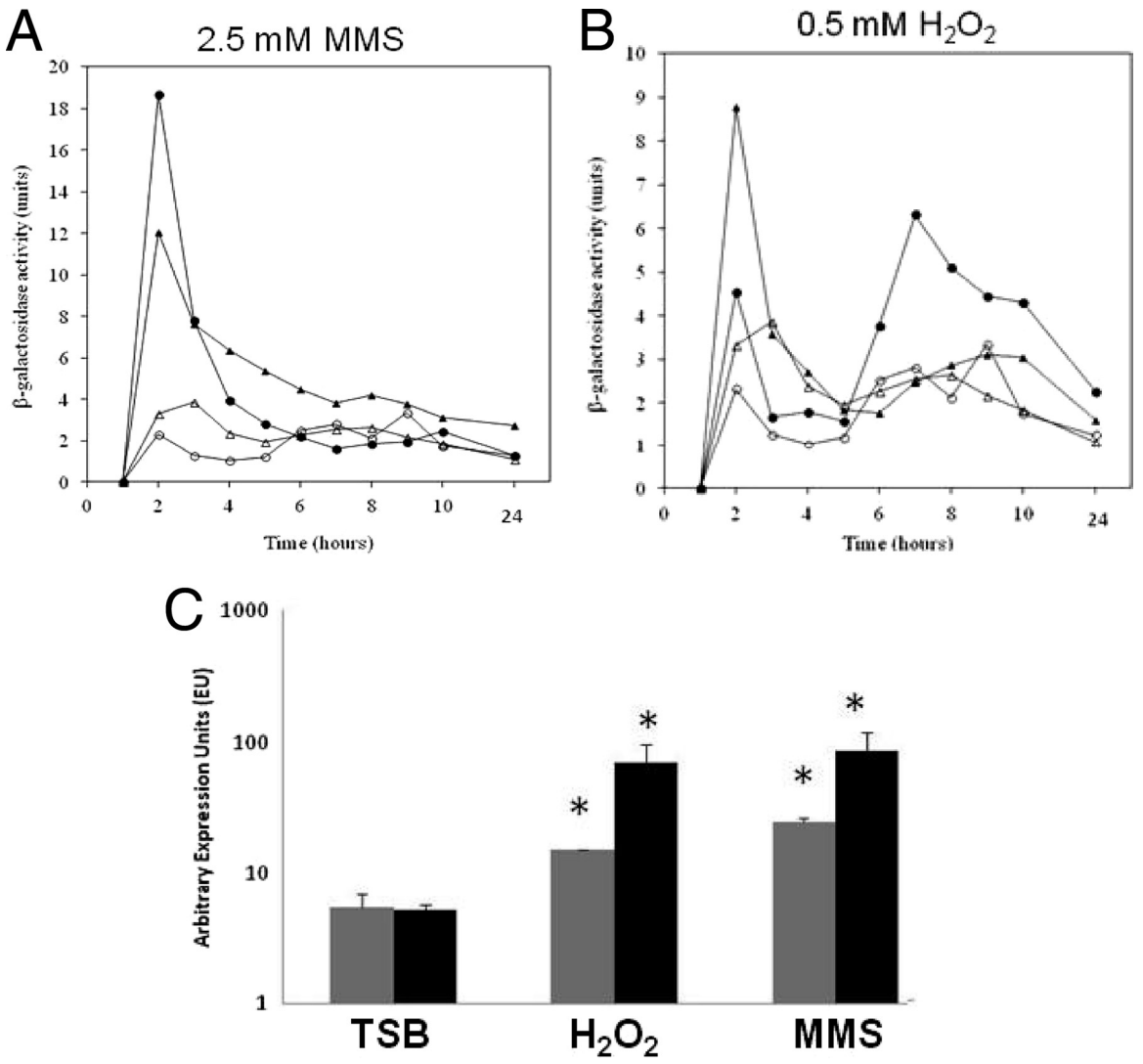

FIG 5 The inducibility of *sigS* expression is conserved across *S. aureus* strains. (A and B) The SH1000 (Δ) and USA300 (○) *sigS-lacZ* fusion strains were grown in either TSB (open symbols) or TSB supplemented with sublethal concentrations of the indicated stress chemicals (closed symbols). Cultures were sampled every hour for 10 h, and again at 24 h, to determine β-galactosidase activity. Assays were performed on duplicate samples and the values averaged. The results presented here were representative of three independent replicates that showed less than 10% variability. (C) Quantitative real-time PCR analysis was performed with strains USA300 (gray) and SH1000 (black) grown for 2 h under conditions identical to those for panels A and B, using primers specific to *sigS*. The data presented are from at least 3 independent experiments. Error bars indicate ± standard error of the mean; *, $P < 0.05$ by a Student *t* test, indicating significant variation from standard conditions (TSB).

cultured with H_2O_2. Expression of *sigS* in USA300 grown with MMS or H_2O_2 displayed 4.6- and 2.8-fold increases in transcription, respectively. Collectively, these findings demonstrate a significant inducibility of *sigS* in response to external stimuli, which is conserved across *S. aureus* strains.

***sigS* is strongly upregulated during challenge by components of the innate immune system.** Despite limited expression under standard laboratory growth conditions, we have previously demonstrated a role for σ^S in the virulence of *S. aureus* (65). Working on the hypothesis that *sigS* transcription would be increased during infection, we performed expression profiling of *sigS-lacZ* fusion strains upon *ex vivo* challenge by components of the innate immune system. Indeed, our plate-based analysis already suggests that *sigS* expression is increased by exposure to pig serum (Table 2). In order to confirm these findings and quantify this increase across different strains, we performed transcriptional analysis using *sigS-lacZ* fusion strains grown in TSB and then subcultured into pig serum. We determined that after just 1 h of growth in serum, *sigS* expression increased 3.4-fold in strain 8325-4 compared to TSB (Fig. 6A). Expression continued to rise over time, with fold increases of 21.2 and 20.5 observed at hours 5 and 24, respectively. Additionally we observed a similar effect in strains SH1000 and USA300. Specifically, over the course of growth, we noted fold increases of 13.8, 36.2, and 44.0 in SH1000 for hours 1, 5, and 24, respectively. Finally, in USA300, *sigS* expression increased 6.8-, 15.6-, and 26.3-fold at hours 1, 5, and 24, respectively. We continued this line of investigation by assessing *sigS* expression upon phagocytosis by RAW 264.7 macrophage-like cells. Accordingly, macrophages were infected with strains 8325-4, SH1000, and USA300 bearing a *sigS-lacZ* fusion for a period of 24

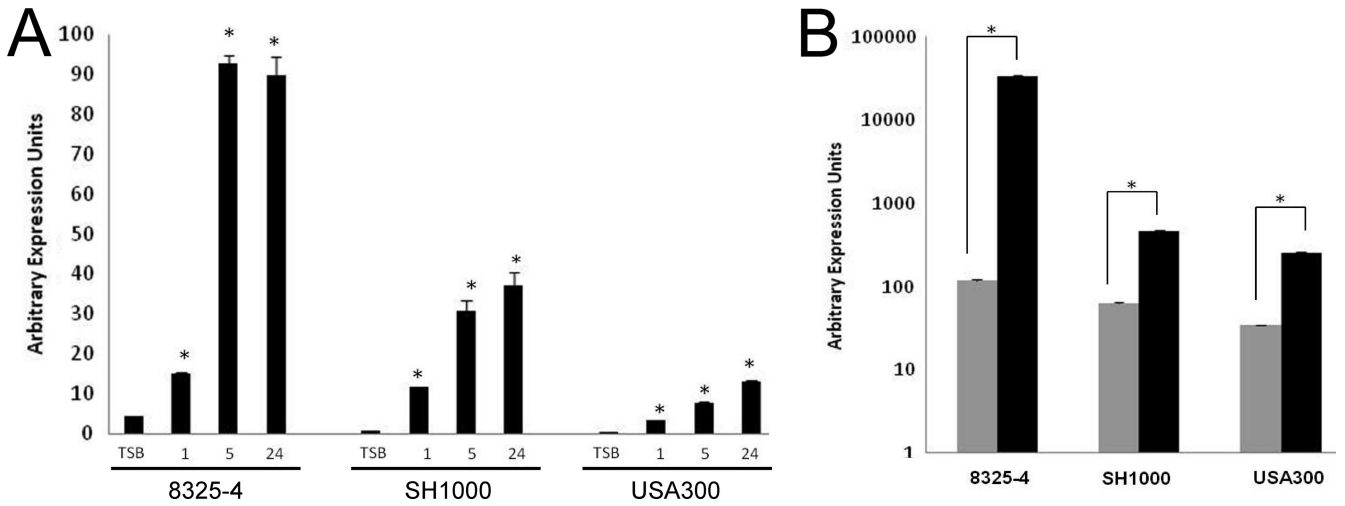

FIG 6 Profiling of *sigS* expression during challenge by components of the innate immune system. Fusion strains were assayed for β-galactosidase activity prior to (TSB), and during (1 h, 5 h, and 24 h), growth in pig serum (A); and prior to phagocytosis (gray bars) and 24 h postphagocytosis (black bars) by RAW 264.7 murine macrophage-like cells (B). Cells were infected at an MOI of 1:100 and incubations carried out at 37°C in a humidified atmosphere of 5% CO_2. The data presented are from at least 3 independent experiments. Error bars indicate ± standard error of the mean; *, $P < 0.001$ by a Student *t* test.

h, before β-galactosidase activity was measured. Expression of *sigS* was significantly increased after phagocytosis in all strains tested (Fig. 6B), with the highest levels observed in 8325-4. In this strain we found a 286.6-fold increase in *sigS* expression compared to background levels. Expression in SH1000 and USA300 increased 7.5- and 7.4-fold, respectively, compared to background levels. As such, these findings support our hypothesis and suggest that σ^S is required during the interaction of *S. aureus* with its host.

σ^S mutants are sensitive to DNA damage stress and cell wall-targeting antibiotics. Thus far, we have demonstrated that chemicals known to induce DNA damage and cell wall stress strongly impact *sigS* transcription. Accordingly, we next sought to perform death-curve kill studies to examine the viability of *sigS* mutants during exposure to lethal concentrations of these agents in strains 8325-4, SH1000, and USA300. We found that when exponentially growing cultures were exposed to 5× the MIC of MMS for 30 min, a consistent decline in mutant cell viability was seen across all strains tested (the data for USA300, which are representative, are presented in Fig. 7A). Specifically, we recovered 3.33-fold fewer *sigS* mutant cells than wild-type cells when cultures were exposed to this agent. Complementation of the σ^S mutation reduced the observed growth impairment significantly, although not completely to wild-type levels. This lack of full complementation is likely attributed to plasmid instability in the presence of DNA-damaging conditions, as suggested by others previously (3, 26, 49, 60, 72, 75). Further to this, in order to determine whether the role of σ^S was limited to protection against DNA alkylation (as induced by MMS), we next examined the ability of the USA300 σ^S mutant to survive exposure to agents that induce other types of DNA damage. As such, analysis was carried out during exposure to oxidative stress resulting from the addition of H_2O_2. Following a 5-min exposure to this agent, we observed a 3.9-fold decrease in *sigS* mutant viability compared to the parent strain, which was fully complementable (Fig. 7B). We next used the DNA-intercalating agent ethidium bromide and found that the *sigS* mutant displayed a 2.1-fold decrease in viability compared to the wild-type strain after 15 min of exposure (Fig. 7C). We again saw that complementation was able to abrogate these effects, but not completely to the levels of the wild-type strain. This is likely attributed to the ability of EtBr to cure plasmids upon exposure, as observed by others previously (3). Finally, in order to determine if σ^S mediates protection against UV-induced lesions and double-strand breaks, we compared the survivability of the wild-type strain and its *sigS* mutant. Exponentially growing cultures were serially diluted on TSA and subjected to UV at a dosage of 4,000 μJ cm^{-2}. Exposure at this level resulted in a 2.1-fold decrease in viability for the mutant (Fig. 7D). Complementation in this assay is not possible because of plasmid instability, as we observed >83% loss upon exposure (data not shown).

Following this, we next assessed the sensitivity of *sigS* mutants to a variety of cell wall-targeting antibiotics (Fig. 8). Analysis using bacitracin in the USA300 background revealed a 4.5-fold decrease in MIC for the mutant strain (30 μg ml^{-1}) compared to the parent (135 μg ml^{-1}). We observed a similar degree of sensitivity with ampicillin, resulting in a 4-fold decrease in MIC for the mutant (25 μg ml^{-1}) compared to wild-type USA300 (100 μg ml^{-1}). Finally, analysis performed using penicillin G yielded a striking 60-fold decrease in MIC for the *sigS* mutant (5 μg ml^{-1}) compared to the parent (300 μg ml^{-1}).

σ^S aids in protection of the *S. aureus* cell during interaction with components of the innate immune system. Previously we have shown a role for σ^S in virulence, using a murine model of septic arthritis. We have also demonstrated here that *sigS* expression increases not only upon exposure to serum but also during phagocytosis by macrophage-like cells. Therefore, we sought to determine the importance of σ^S during challenge by components of the innate immune system. This was first performed using whole human blood and the USA300 wild type, its *sigS* mutant, and its complemented strains. Exponentially growing cells were inoculated into whole human blood and incubated for 4 h. After this time, we recovered 2.1-fold-fewer viable cells of the *sigS* mutant than of the wild-type strain (Fig. 9A). Complementation analysis restored viability to levels similar to that of the wild type. Following this, we also conducted macrophage survival assays,

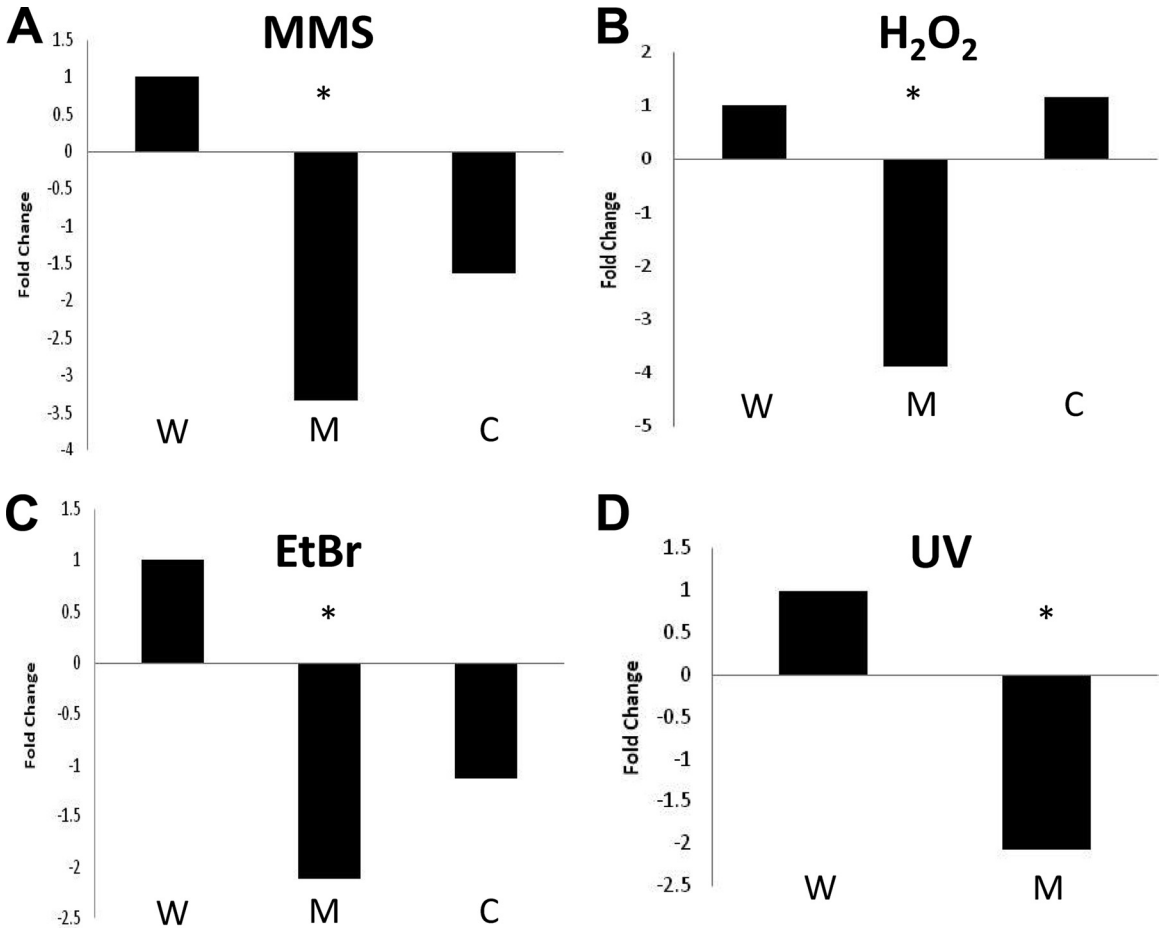

FIG 7 *sigS* mutants are sensitive to a variety of DNA damage-inducing stresses. The USA300 wild-type (W) and *sigS* mutant (M) strains, along with a *sigS* complement strain (C), were analyzed for viability in the presence of DNA damage-inducing stressors. CFU counts were determined both pre- and postexposure, and the survivability was determined. The data are presented as fold change relative to the wild-type strain and are representative of at least three independent experiments that showed less than 10% variability. Shown are exposures of 30 min to 25 mM MMS (A), of 5 min to 150 mM H_2O_2 (B), of 15 min to 5 mM EtBr (C), and to UV at 4,000 μJ per cm^2 (D). *, $P < 0.05$ by a Student *t* test.

again in the USA300 background, to assess the ability of the *sigS* mutant to persist upon phagocytosis. At 24 h postphagocytosis by RAW 264.7 cells, we observed a 4.5-fold decrease in survivability of the *sigS* mutant compared to the parent (Fig. 9B). Complementation of this finding increases survivability of the mutant cells, but not completely to levels of the wild-type strain. This is likely explained by the significant instability of the plasmid during phagocytosis, which we routinely observed when performing this

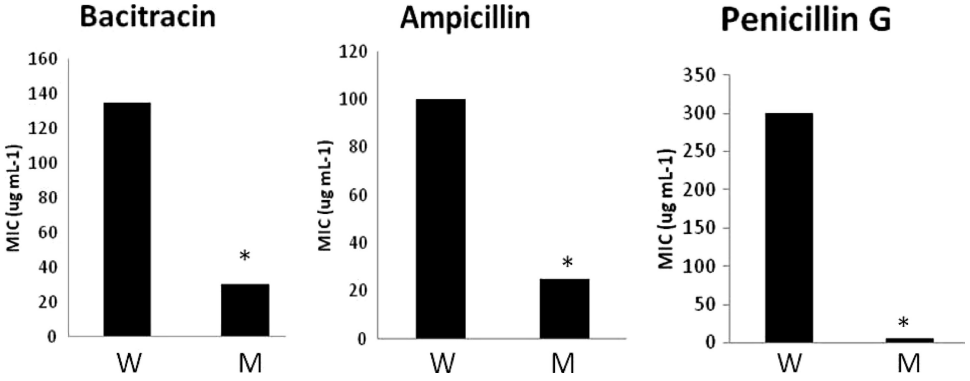

FIG 8 *sigS* mutants are sensitive to a number of cell wall-targeting antibiotics. The USA300 wild-type (W) and *sigS* mutant (M) strains were grown in TSB containing increasing concentrations of the cell wall-targeting antibiotics bacitracin, ampicillin, and penicillin G in a 96-well plate format. The cultures were allowed to grow overnight at 37°C and subsequently analyzed for growth, and the MICs were determined. The data are representative of at least three independent experiments that showed less than 10% variability. *, $P < 0.05$ by a Student *t* test.

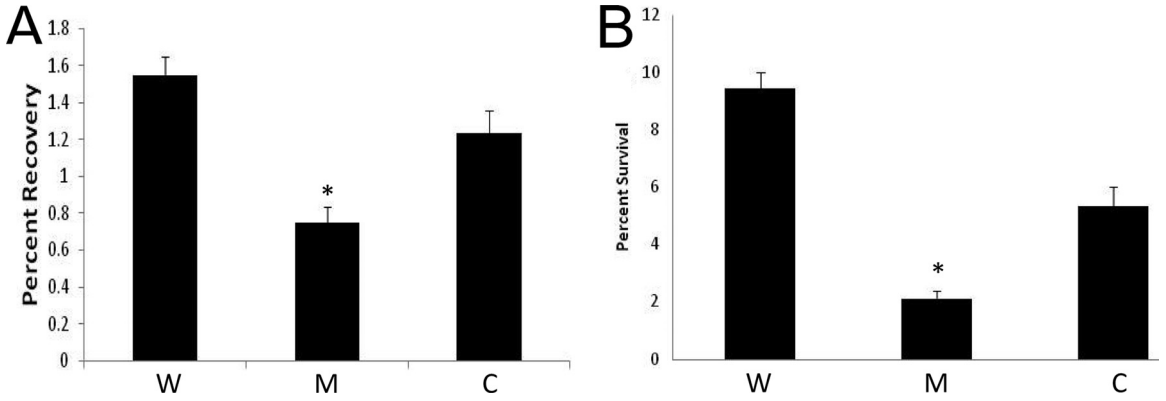

FIG 9 σ^S aids in protection of the *S. aureus* cell during interaction with components of the innate immune system. The USA300 wild type (W) and *sigS* mutant (M), along with a *sigS* complement strain (C), were analyzed for viability 4 h after exposure to whole human blood (A) and 24 h postphagocytosis by 264.7 RAW murine macrophage-like cells (B). CFU counts were determined both pre- and postexposure, and the percent survival was determined. Error bars indicate ± standard error of the mean; *, $P < 0.05$ by a Student *t* test.

assay. Collectively, these findings support our earlier work, which indicates an important role for σ^S in the virulence of *S. aureus* and confirms our expression analysis, demonstrating *sigS* upregulation during interaction with components of the innate immune system.

DISCUSSION

In this study, we provide new evidence for the role of σ^S, a novel ECF σ factor in *S. aureus*. In our previous works we have shown that σ^S is a functioning sigma factor that controls its own expression (65). Additionally, we have demonstrated a role for it in the stress and virulence responses of this organism. From a gene expression standpoint, we have previously shown that *sigS* expression is minimal during growth under standard laboratory conditions in SH1000 (65). In this study, we reveal that this phenomenon is conserved across a variety of *S. aureus* strains, including laboratory (8325-4, SH1000, and Newman) and clinical (USA300) isolates. In each case, we observed low levels of expression of *sigS* during growth in rich media. These results may not be entirely surprising, as the majority of ECF σ factors are employed to protect the cell during times of stress and are often transcribed only when required (27, 56, 67).

Interestingly, we did observe robust *sigS* expression in the highly mutated laboratory strain RN4220. Among the many mutations present in this strain are those that render the activity of other global regulators nonfunctional, including *agr* and *sigB* (15, 70). As such, the regulatory circuits in place in this strain are likely to be highly disordered, potentially explaining *sigS* dysregulation and therefore upregulation. Interestingly, these effects do not appear to be mediated directly through either Agr or σ^B, as mutations in the genes encoding these proteins alone do not affect *sigS* expression in either SH1000 or USA300 (data not shown). Recent sequencing of the RN4220 genome has revealed a number of single-nucleotide polymorphisms (SNPs) and deletions relative to the parental strain NCTC 8325 (2, 48). Of interest, a number of these are in genes involved in DNA metabolism, replication, recombination, and repair. Most notably, RN4220 carries an SNP in UvrC, a component of the UvrABC exonuclease, which in *Escherichia coli* repairs DNA damage induced by a number of mechanisms, including UV light (61). Interestingly, our analysis here demonstrates σ^S mutants are less able to survive exposure to UV stress. Additionally, SNPs in RN4220 are located in a putative helicase, SAOUHSC_02790, as well as a truncated resolvase, SAOUHSC_02392. Collectively, these observations suggest that RN4220 is perhaps more prone to DNA damage than other wild-type strains, as a result of mutated and nonfunctional repair pathways. This would then perhaps explain why this strain exhibits stronger *sigS* expression than other wild types, as we implicate σ^S in influencing the response of *S. aureus* to DNA damage in this study. Indeed, we have observed that the increasing levels of *sigS* transcription in different wild-type strains directly correlate with their sensitivity to DNA-damaging agents, such as MMS (data not shown).

Promoter mapping of the *sigS* locus reveals three discrete transcriptional start sites. Promoters P1 and P2 both appear to be under the control of the housekeeping σ factor σ^A; however, both are severely corrupted from consensus sequences and/or spacing. Due to the relative weakness of these promoters, it is likely that other regulatory elements must act to activate transcription from these sequences. This again likely explains the low levels of *sigS* expression observed in the majority of *S. aureus* strains and argues for a genetic regulatory network that controls expression of this regulator. Previously we demonstrated that *sigS*, as with other ECF sigma factors (27, 45, 65), controls its own expression via autoregulation. During promoter mapping in the present study, we reveal a likely σ^S-controlled transcript, P2 (CAAAGT-12 bp-TATCA). Typically, ECF sigma factor consensus sequences display a conserved AAC motif in the −35 region (27, 45); however, exceptions exist. Specifically, the ECF sigma factor of *Neisseria gonorrhoeae* does not recognize an AAC motif (25), while σ^R of *Streptomyces coelicolor* recognizes an AAT motif (54). More importantly, σ^X, an ECF sigma factor in several *Pseudomonas* spp., specifically recognizes an AAG motif, as seen here for σ^S (4, 39). ECF σ factors typically have significant divergence and decreased homology within their region 2.4 (41, 45), which specifically recognizes −10 promoter elements. Accordingly, such sites are often difficult to ascertain; however, the identified putative −10 element is strikingly similar to the TCTGA recognized sequence of σ^E in *E. coli* (13).

In addition to examining expression in wild-type strains, we have also assessed the level to which *sigS* is upregulated in response

to external stress. We show that a variety of stressors can induce expression of *sigS*, ranging from those that elicit alkali stress to those that affect protein synthesis. Interestingly, this is most pronounced in strain 8325-4, which, like RN4220, lacks natural σ^B activity. We are able to demonstrate that the conditions of *sigS* inducibility in 8325-4 hold true for the σ^B functional strains SH1000 and USA300, although not always at the same levels. Of note, when we inactivate *sigB* in SH1000, we do not observe the same robust increases in *sigS* inducibility seen in 8325-4 (data not shown), despite these strains being very closely related. This observation is perhaps explained by the fact that 8325-4 is an *rsbU* mutant, which is an activator of σ^B activity, rather than a true *sigB*-null strain. Given recent findings showing a role for RsbU outside its influence of σ^B activity (71), it is possible that these differences are mediated by RsbU, rather than σ^B, mechanisms. It has also recently been shown that 8325-4 and SH1000 have genetic differences beyond the 11-bp deletion in *rsbU* (53), perhaps suggesting that SNPs and other genetic variations between these 2 strains influence *sigS* expression.

With regard to environmental influence on *sigS* expression, those chemicals that induce DNA damage, such as methyl methanesulfonate, appear to have the most profound effects. These findings correlate well with our phenotypic studies, showing that *sigS* mutants have increased sensitivity to a broad range of DNA damage-inducing stresses. These include alkylating and intercalating agents, reactive oxygen species, and UV-induced damage, each of which leads to the activation of specific and distinct repair pathways. Interestingly, when we analyzed the transcription of a number of DNA repair pathway genes (*ogt*, *uvrB*, and *mutM*) in both *sigS* mutants and *S. aureus* wild-type strains, we observed no alterations in expression (data not shown). As such, our findings suggest that σ^S is involved in mediating a comprehensive response to DNA damage by an as-yet-unknown mechanism. These findings are somewhat novel, as the majority of ECF σ factors typically respond to perturbations in the cell wall. However, reports on ECF σ factors from other organisms reveal several examples of these factors that function in sensing and responding to cytoplasmic stress. Specifically, both RpoE of *Rhodobacter sphaeroides* and Ecf of *Neisseria gonorrhoeae* respond to oxidative stress, which can in turn lead to DNA damage (6, 19, 25).

Interestingly, a number of agents that were identified as inducing *sigS* expression are not typically thought of as inducing DNA damage but can also induce this kind of stress. For example, H_2O_2 resulted in *sigS* upregulation and can react with intracellular iron to form hydroxyl radicals, which cause damage to DNA (8, 30, 31, 57). Additionally, SOS and DNA damage repair genes have previously been shown in *Escherichia coli* to be upregulated during alkali stress caused by excess NaOH, which also upregulates *sigS* expression (23, 62). Finally, the protein synthesis-inhibiting antibiotic chloramphenicol upregulated *sigS* and has been shown to lead to the degradation of double-stranded DNA and the inhibition of DNA synthesis (47).

A consideration with these DNA damage agent studies is that these agents may not be directly upregulating *sigS* expression but might cause mutations within the *S. aureus* genome, leading to SNPs. In such a scenario, this could lead to dysregulation of regulatory circuits, leading to *sigS* upregulation in a manner akin to that proposed for RN4220 and 8325-4. To examine this, we analyzed 8325-4 *sigS-lacZ* fusion strains exposed to DNA-damaging agents for a 24-h period. Upon removal of the stressor, strains were grown on agar plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). We found no detectable blue coloration on such plates (data not shown), indicating that DNA damage-induced upregulation of *sigS* does not appear to be mediated via heritable SNPs but results directly from exposure to these agents. As such, the increase in expression of *sigS* is due solely to exposure to agents such as MMS and suggests that σ^S is utilized by the cell to adapt during times of DNA damage.

We also observed substantial increases in expression of *sigS* following exposure to a number of cell wall-targeting chemicals, suggesting a role for σ^S in protection against this type of stress. This correlates well with other works presented in this study, which demonstrate *sigS* mutants have increased sensitivity to the cell wall-targeting antibiotics. These findings also corroborate our previous work, which demonstrates that *sigS* mutants are sensitive to growth in the presence of a number of cell wall-disrupting agents, including Triton X-100 and SDS (65). This suggests a role for σ^S in the *S. aureus* cell wall stress response, which is typical of ECF σ factors. For example, RpoE in *Escherichia coli* serves to upregulate genes involved in the heat shock response and is triggered by misfolded proteins accumulating in the periplasm and outer membrane (13). Furthermore, σ^W and σ^M of *Bacillus subtilis* both respond to cell wall biosynthesis-inhibiting antibiotics, with σ^M proving vital for survival during exposure to phosphomycin (7, 69). This information, alongside the observation that σ^S is the lone ECF σ factor in *S. aureus*, suggests it likely serves dual functions within the cell, protecting against both cytoplasmic and extracytoplasmic stresses.

We have also demonstrated here that *sigS* transcription is increased considerably when *S. aureus* is challenged by complement during growth in pig serum. We also present evidence for *sigS* upregulation during *ex vivo* infection, revealing high levels of expression upon phagocytosis by murine macrophage-like cells. Phenotypically, we show that σ^S is important for survival during growth in whole human blood and following phagocytosis. Collectively, this supports our previous work, which reveals a major requirement for σ^S during virulence (65). As part of the microbicidal mechanism employed by macrophages, reactive oxygen species and reactive nitrogen intermediates (RNI) are excreted at very high levels, leading to DNA damage in invading organisms during infection (33, 38, 42, 59, 66). Moreover, it has been observed that pathogenic organisms such as *Burkholderia* spp., *Brucella abortus*, and *Vibrio cholerae* defective in DNA damage repair mechanisms are attenuated in virulence, underscoring their importance during infection (12, 14, 58, 76). Together these findings suggest that, upon entry into the host, bacterial pathogens are faced with an array of DNA-damaging conditions. Given that these conditions lead to activation of σ^S in *S. aureus*, this likely goes some way toward explaining the avirulent phenotype of *sigS* mutants.

In summary, we present extended characterization of the lone, and novel, ECF σ factor σ^S in *S. aureus*. We reveal that, under standard conditions, its transcription remains low in a range of wild-type strains but can be upregulated in response to external stimuli. Specifically, chemicals leading to DNA damage and cell wall disruption strongly induce expression of *sigS*. This upregulation is seemingly of importance, as functional characterization reveals that *sigS* mutants are sensitive to both of these types of stress. Additionally, we reveal strong upregulation of this gene during growth in pig serum as well as upon phagocytosis by murine macrophage-like cells, which is seemingly protective to the

cell. Collectively, our data suggest that σ^S likely serves dual functions within the cell, protecting against both cytoplasmic and extracytoplasmic stresses. This further argues for its important, and perhaps novel, role in the *S. aureus* stress and virulence responses.

ACKNOWLEDGMENT

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