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Site-Specific Mutation of the Sensor Kinase GraS in *Staphylococcus aureus* Alters the Adaptive Response to Distinct Cationic Antimicrobial Peptides

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The *Staphylococcus aureus* two-component regulatory system, GraRS, is involved in resistance to killing by distinct host defense cationic antimicrobial peptides (HD-CAPs). It is believed to regulate downstream target genes such as *mprF* and *dltABCD* to modify the *S. aureus* surface charge. However, the detailed mechanism(s) by which the histidine kinase, GraS, senses specific HD-CAPs is not well defined. Here, we studied a well-characterized clinical methicillin-resistant *S. aureus* (MRSA) strain (MW2), its isogenic *graS* deletion mutant (Δ*graS* strain), a nonameric extracellular loop mutant (ΔEL strain), and four residue-specific ΔEL mutants (D37A, P39A, P39S, and D35G D37G D41G strains). The Δ*graS* and ΔEL strains were unable to induce *mprF* and *dltA* expression and, in turn, demonstrated significantly increased susceptibilities to daptomycin, polymyxin B, and two prototypical HD-CAPs (hNP-1 and RP-1). Further, P39A, P39S, and D35G-D37G-D41G ΔEL mutations correlated with moderate increases in HD-CAP susceptibility. Reductions of *mprF* and *dltA* induction by PMB were also found in the ΔEL mutants, suggesting these residues are pivotal to appropriate activation of the GraS sensor kinase. Importantly, a synthetic exogenous soluble EL mimic of GraS protected the parental MW2 strain against hNP-1- and RP-1-mediated killing, suggesting a direct interaction of the EL with HD-CAPs in GraS activation. *In vivo*, the Δ*graS* and ΔEL strains displayed dramatic reductions in achieved target tissue MRSA counts in an endocarditis model. Taken together, our results provide new insights into potential roles of GraS in *S. aureus* sensing of HD-CAPs to induce adaptive survival responses to these molecules.

Host defense against *Staphylococcus aureus* infections involves rapid immune responses, which include neutrophils (PMNs), effector proteins such as complement, and cationic antimicrobial peptides (CAPs) that are part of the innate immune system (1–3). Different host defense CAPs (HD-CAPs) are expressed in distinct human tissues and are integral to mitigating bacterial infections, including those caused by *S. aureus* (3–5).

Endovascular infections caused by *S. aureus* are among the most common staphylococcal clinical syndromes and are associated with high morbidity and mortality rates (6–8). The persistence and progression of endovascular *S. aureus* infections, such as infective endocarditis (IE), necessitate the pathogen to resist the microbicidal actions of HD-CAPs (6, 8), including those of PMNs (e.g., neutrophil defensins, such as hNP-1) and platelets (e.g., the family of thrombin-induced platelet microbicidal proteins [tPMPs]), as well as clinically utilized CAPs (e.g., calcium-daptomycin). Of importance, *S. aureus* appears to deploy a number of mechanisms to subvert inhibition or killing by HD-CAPs, including changes in cytoplasmic membrane (CM) biophysics or energetics, perturbations in surface charge, and modifications of the phospholipid repertoire (9–12).

Two-component regulatory systems (TCRS) have come to the forefront of the staphylococcal "virulon" as signal transduction systems commonly employed by *S. aureus* to sense and respond to potentially noxious external stimuli (13). In this pathogen, the GraRS TCRS (also termed antibiotic peptide sensor [APS]) is involved in promoting resistance to distinct HD-CAPs by upregulating target genes such as *mprF* and *dltABCD*. In turn, the proteins encoded by the genes modify the net positive surface charge of the *S. aureus* envelope (14–17). The protein MprF is a lysylphosphatidylglycerol (L-PG) synthase, which attaches positively charged lysine molecules onto negatively charged phosphatidylglycerol (PG) within the *S. aureus* CM. In addition, MprF also functions as an outer CM flippase for L-PG, translocating the lysinylated PG to the outer CM leaflet (9, 18, 19). The *dltABCD* operon also contributes to the net surface positive charge by covalently incorporating D-alanine to cell wall teichoic acids (16, 20).

The expression of GraRS-mediated effector genes appears to be preferentially induced by certain HD-CAPs, such as tPMP-1 (platelets) and polymyxin B, *in vitro*. This observation raised the notion that this TCRS selectively senses and responds to HD-CAPs having specific structural or mechanistic determinants (14, 15, 17). Mutagenesis studies have revealed that a *graS* mutation abolished the induction of *mprF* and *dltABCD* genes, further supporting the notion of GraS as a sensor kinase for selective CAPs in *S. aureus* (14, 15, 17). A comparative alignment of the GraS protein sequences of *S. aureus* versus *Staphylococcus epidermidis* revealed a common motif consisting of two transmembrane seg-

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ments flanking a 9-amino-acid extracellular loop (14). Of note, Li et al. demonstrated that differences in induction by CAPs between *S. aureus* and *S. epidermidis* were due to structural changes within the respective loop motifs of the GraS histidine kinase (14). These findings suggested that the extracellular loop is an important determinant for the ability of the GraS protein to detect and transduce *S. aureus* adaptive responses to certain HD-CAPs.

In the present study, we utilized isogenic *graS* deletion (Δ*graS*) and nonameric extracellular loop (ΔEL) mutants of a well-characterized clinical *S. aureus* strain, MW2, to investigate potential mechanism(s) by which GraS senses specific HD-CAPs. In addition, key residues in the EL of GraS predicted to mediate detection of HD-CAPs were mutated to evaluate residue-specific functions of GraS. These strains were then used to examine the contribution of the entire *graS* locus, as well as the EL and specific residues thereof in relation to (i) the induction of *mprF* and *dlt* expression by sublethal concentrations of a range of HD-CAPs *in vitro*, (ii) the modulation of net cell surface charge in *S. aureus*, (iii) *in vitro* susceptibility to HD-CAPs of distinct structure and tissue origin, and (iv) potential *in vivo* correlates of virulence during the induction and maintenance of a prototypical endovascular infection (infective endocarditis [IE]).

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains used in this study are listed in Table 1. All *S. aureus* strains were grown in either tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) or Mueller-Hinton broth (MHB; Difco Laboratories) as indicated, depending on the individual experiments. *Escherichia coli* DH5α was grown in Luria-Bertani medium (Fisher Scientific). Liquid cultures were grown in Erlenmeyer flasks at 37°C with shaking (250 rpm) in a volume that was no greater than 10% of the flask volume. All antibiotics were purchased from either Sigma Chemical Co. or Fisher Scientific and were used at the following concentrations: ampicillin, 100 μg/ml; erythromycin, 3 μg/ml; chloramphenicol, 5 μg/ml.

CAPs. Human neutrophil peptide-1 (hNP-1), a prototypical α-defensin, was purchased from Peptide International (Louisville, KY). RP-1 (an 18-amino-acid congener modeled in part upon α-helical microbicidal domains of the platelet factor 4 family of platelet kinocidins) was synthesized and authenticated as detailed before (21, 22). The antistaphylococcal mechanisms of RP-1 recapitulate those of native thrombin-induced platelet microbicidal protein 1 (tPMP-1) (21). Polymyxin B (PMB) was purchased from Sigma Chemicals Co. (St. Louis, MO). Peptides hNP-1 and RP-1 were used for both *in vitro* killing assays and gene induction studies for the study strains, as described before (17). PMB was employed in selected gene induction experiments. The above-described cationic antimicrobial peptides (CAPs) differ in source, primary structures, mechanisms of action, and net positive charge (range, +4 to + 6 at pH 7.0) (21, 23).

Preparation of soluble GraS. The EL sensor domain of GraS features a 9-amino-acid motif (DYDFPIDSL) that is hypothesized to detect certain HD-CAPs and initiate transduction of response regulator functions of GraSR (14, 17). To test this concept, we synthesized a soluble peptide representing this sensor domain and assessed its ability to modify *S. aureus* responses to various peptides. To enhance biological functionality, the soluble trimeric GraS EL mimetic was synthesized to contain three sensor motifs interposed by diglycine hinges. Thus, the full-length synthetic trimeric sensor peptide sequence was NH_2-DYDFPIDSLGGDYDF PIDSLGGDYDFPIDSL-COOH.

A nonsense (scrambled) peptide of the same composition, but with randomized sequence, was also synthesized as a control. Both of these polypeptides were generated by solid-phase 9-fluorenyl-methyloxycarbonyl (Fmoc) chain assembly and purified using reverse-phase high-performance liquid chromatography (HPLC), and the purified product

TABLE 1 Strains used in this study

Strain or plasmid	Description	Reference or source
S. aureus		
MW2	Community-acquired MRSA, wild-type strain, human clinical isolate	47
Δ*graS* strain	*graS* in-frame deletion mutant of MW2	Present study
ΔEL strain	*graS* extracellular loop deletion mutant (deletion of 9 amino acids within GraS)	Present study
Δ*graS*$_{comp}$ strain	Δ*graS* complemented with pEPSA5-expressing *graRS* genes from MW2	Present study
ΔEL$_{comp}$ strain	ΔEL strain complemented with pEPSA5-expressing *graRS* genes from MW2	Present study
D37A strain	Extracellular loop mutant generated by site-directed mutagenesis (D→A at position 37)	Present study
P39A strain	Extracellular loop mutant generated by site-directed mutagenesis (P→A at position 39)	Present study
P39S strain	Extracellular loop mutant generated by site-directed mutagenesis (P→S at position 39)	Present study
D35G D37G D41G strain	Extracellular loop mutant generated by site-directed mutagenesis (D→G at positions 35, 37, 41)	Present study
E. coli DH5α	Host strain for construction of recombinant plasmids	48
Plasmids		
pEPSA5	Shuttle vector for ectopic gene expression in *S. aureus*	49
pMAD	Allelic replacement vector to generate *S. aureus* mutant strain	30

(>95%) was authenticated by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectroscopy, as we have detailed previously (24).

Antibiotics/CAP susceptibility testing. The MICs to PMB were determined by the standardized microdilution assay in MHB medium, following CLSI protocols, using $\sim 1 \times 10^5$ CFU/ml in 96-well microtiter plates with MIC assessments performed at 48 h (25). The MICs to daptomycin (DAP), vancomycin (VAN), and oxacillin (OX) were determined by standard micro-Etest according to the manufacturer's recommended protocols. A minimum of three independent experimental runs were performed to determine MICs to each antibiotic.

Standard MIC testing in nutrient broth may underestimate CAP activities (23, 26). Accordingly, *in vitro* bactericidal assays were carried out with hNP-1 and RP-1 as described previously by using a 2-h microdilution method in Eagle's minimal essential medium (9, 23). These assays were performed with hNP-1 (10 μg/ml) and RP-1 (1 μg/ml) using an initial inoculum of 5×10^3 CFU *S. aureus* cells (9, 23). These CAP concentrations were selected based on extensive pilot studies showing their inability to completely eradicate the starting inoculum of the parental MW2 strain over the 2-h exposure period. Data were calculated and expressed as the relative percentage of surviving CFU (±standard deviation [SD]) of CAP-exposed versus CAP-unexposed cells. A minimum of three independent studies were performed for each CAP.

DNA manipulations. Genomic DNA was isolated from *S. aureus* as described previously (27). Plasmid DNA purification was performed using a Wizard Plus kit from Promega, Inc. (Madison, WI). The restriction enzymes and T4 DNA ligase used in this study were purchased from New

England BioLabs (Beverly, MA). Preparation and transformation of *Escherichia coli* DH5α were accomplished using the method described by Inoue et al. (28). Electroporation of recombinant plasmids into *S. aureus* was carried out using the procedures of Shenk and Laddaga (29).

Construction of *S. aureus* mutants. All mutant strains were generated in an *S. aureus* MW2 background with in-frame deletion of target genes by allelic replacement, using the temperature-sensitive plasmid pMAD as described previously (15, 30). *S. aureus* MW2 (USA400), a prototypical clinical methicillin-resistant *S. aureus* (MRSA) isolate, has been well characterized genotypically (e.g., available genome sequence information) and is virulent *in vivo* in animal models (31–33). Briefly, PCR was used to amplify an ~2-kb fragment comprising a 1-kb fragment upstream and another 1-kb fragment downstream of *graS* using genomic DNA as the template. The PCR fragment was cloned into pMAD, resulting in pMAD-*graS*. The recombinant shuttle vector was transformed first into *E. coli*, then into RN4220 as an intermediary, and finally into *S. aureus* strain MW2 (34). Specific mutations of individual residues within the EL loop of *graS* were introduced by PCR using primers with altered nucleotides, cloned into pMAD, and transformed into *S. aureus* MW2. Allelic exchanges were performed as described previously (30). Selected mutants were subsequently complemented by reintroducing pMAD-*graS* into the chromosome by homologous recombination, as described previously (30). All the mutant strains created were confirmed with PCR and sequencing.

Preparation of RNA. To assess CAP induction of *mprF* and *dlt* by CAPs, RNA samples were isolated from cultures of the strains exposed to hNP-1, RP-1, or PMB. Briefly, overnight cultures of the strain sets were used to inoculate 10 ml of MHB to an optical density at 600 nm (OD_{600}) of 0.1 and allowed to grow for 2.5 h (~1×10^8 to 5×10^8 CFU/ml) before the addition of peptide. For transcription induction, CAPs were used at the following exposure concentrations in artificial conditions *in vitro*: hNP-1, 30 μg/ml; PMB, 30 μg/ml (~0.25× MIC). The cultures were incubated for an additional 30 min before the RNA was harvested. The sublethality of these CAP concentrations over the 30-min exposure period (≥90% survival) was confirmed by quantitative culture as described before (17). Total cellular RNA was isolated from the *S. aureus* cell pellets by using the RNeasy kit (Qiagen, Valencia, CA) and the FASTPREP FP120 instrument (BIO 101, Vista, CA), according to the manufacturer's recommended protocols.

Quantification of transcript levels by qRT-PCR. Quantitative real-time PCR (qRT-PCR) analyses were performed as described previously (35). Briefly, 2 μg of DNase-treated RNA was reverse transcribed using the SuperScript III first-strand synthesis kit (Invitrogen) according to the manufacturer's protocols. Quantification of cDNA levels was performed by following the instructions of the Power SYBR green master mix kit (Applied Biosystems) on an ABI PRISM 7000 Sequence detection system (Applied Biosystems). The primers used to amplify *mprF* were qRT-mprF-F and qRT-mprF-R (17). The *dltA* and *gyrB* genes were similarly detected using respective specific primers as described before (35).

Determination of net cell surface charge. To estimate relative positive cell surface charge that may contribute to HD-CAP resistance (9, 12, 16, 19, 36), cytochrome *c* binding assays were performed as described before by spectrophotometric assay (OD_{530}) (15, 37). The binding of cytochrome *c* (pI = 10; Sigma) is approximated from the amount of the polycation remaining within reaction mixture supernatants following exposure to a study strain for 15 min. Larger amounts of residual cytochrome *c* in the supernatants correlate with a more relatively positive surface charge (9, 12, 15, 38). A minimum of three independent experiments was performed for each strain-CAP combination.

CM fluidity analyses. It has been shown that CM biophysical characteristics affect the interaction of *S. aureus* with HD-CAPs (11, 39). Principal among these parameters is the CM order, a composite measure of the fluidity versus rigidity properties of PL bilayers. The comparative CM orders of study strains were determined by fluorescence polarization spectroscopy using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) as described before (11, 40). Data were quantified from the polarization index (±SD) (9, 23), in which there is an inverse relationship between the polarization index and CM fluidity/rigidity (i.e., higher index indicates greater CM rigidity) (9, 11, 38, 41). These assays were performed at least six times for each strain on separate days.

Experimental rabbit IE model. Animals were maintained in accordance with the American Association for Accreditation of Laboratory Animal Care criteria. The Animal Research Committee (IACUC) of the Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center approved these animal studies.

A well-characterized catheter-induced rabbit model of aortic IE was used as described previously (16, 42, 43) to assess the role of GraS EL sensor on relative *S. aureus* virulence *in vivo*. Briefly, female New Zealand White rabbits (2.0 to 2.5 kg body weight; Harlan Laboratories, Indianapolis, IN) underwent indwelling transcarotid-transaortic valve catheterization with a polyethylene catheter (to induce sterile aortic valve vegetations). Twenty-four hours after catheter placement, animals were infected intravenously (i.v.) with ~1×10^6 CFU/animal, a 95% infective dose (ID_{95}) for the parental MW2 strain as established in pilot studies.

For virulence comparisons, five strains were studied: the parental MW2, its *graS* knockout (Δ*graS* strain), the EL knockout (ΔEL strain), a D→G substitution EL mutant (in which 3 aspartic acid [D] residues are substituted with glycines [G] in the EL sensor), and the respective *graRS*-complemented strains. At the time of sacrifice, cardiac vegetations, kidneys, and spleens were aseptically excised and quantitatively cultured as detailed before (44). Virulence assessments were then based on comparisons of the *S. aureus* CFU densities of the infecting strains in three target tissues. The mean $\log_{10}$ CFU/g of tissue (±SD) was calculated for each target tissue in each group for statistical comparisons. The lower limit of microbiologic detection in the target tissues is ≤1 $\log_{10}$ CFU/g of tissue.

For verification of plasmid (pEPSA5) maintenance within the Δ*graS* complemented strain during *in vivo* studies, all tissue samples from animals infected with this construct were quantitatively cultured in the presence and absence of chloramphenicol (10 μg/ml; plasmid selection marker). These studies confirmed stability of the plasmid during animal passage (data not shown).

Statistical analysis. *S. aureus* tissue CFU densities among the various groups were compared using the Kruskal-Wallis analysis of variance (ANOVA) test with the Tukey *post hoc* correction for multiple comparisons. Significance was determined at P values of <0.05.

RESULTS

***In vitro* CAP susceptibility profiles.** To determine the role of GraS sensor kinase and to identify critical residues of extracellular loop (EL) in resistance to structurally distinct host defense CAPs, we compared the *in vitro* susceptibility profiles of the Δ*graS* and ΔEL mutants and the four EL point-mutated constructs (D37A, P39A, P39S, and D35G D37G D41G strains) to the parental MW2 against DAP, PMB, RP-1, or hNP-1. Site-specific D→A, D→G, P→A, or P→S amino acid substitutions were designed to alter charge (D→A, D→G) propensity for secondary structure stability (P→A) or conservation of hydrogen bond potential (P→S).

As shown in Table 2, deletion of the entire *graS* open reading frame (ORF) in the MW2 parental strain resulted in significantly increased susceptibilities to calcium-DAP and PMB compared to those of the parental MW2 strain ($P < 0.01$). Notably, the EL sensor mutant (ΔEL mutant) also exhibited significantly increased susceptibilities to DAP and PMB, suggesting that the 9-amino-acid extracellular sensing loop of the GraS histidine kinase is important for CAP resistance in *S. aureus*. Similarly, susceptibilities of both the Δ*graS* and ΔEL strains to hNP-1 and RP-1 were significantly higher than those of the parental MW2 ($P <$ 0.001; Fig. 1A and B). Complementation of the Δ*graS* and ΔEL

TABLE 2 MICs of the study strains

Strain	MIC (μg/ml) DAP	VAN	OX	PMB
MW2	0.5	2	48	125
Δ*graS* strain	0.094	1	48	7.8
ΔEL strain	0.125	1	48	7.8
Δ$graS_{comp}$ strain	0.5	2	48	125
$ΔEL_{comp}$ strain	0.5	2	48	125
D37A strain	0.5	2	48	125
P39A strain	0.25	2	48	62.5
P39S strain	0.25	2	48	62.5
D35G D37G D41G strain	0.25	2	48	62.5

strains with a *graRS*-expressing plasmid restored parental-level susceptibilities to all four CAPs tested in most assays.

Next, we used the mutant strains that have either single mutations (D37A, P39A, and P39S) or triple mutations (D35G D37G D41G) in the EL of GraS. In contrast to the two deletion mutants described above (Δ*graS* and ΔEL strains), most of the point-mutated EL constructs demonstrated only a moderate reduction in MICs to DAP and PMB (Table 2). Of note, the exception to this pattern was D37A, which had no apparent effect on MICs of DAP or PMB. However, the P39A, P39S, and D35G D37G D41G mutant constructs showed significantly increased susceptibilities to hNP-1 and RP-1 relative to those of the parental MW2 strain (Fig. 1). Interestingly, the D37A mutant resulted in enhancement of resistance to hNP-1 and RP-1-induced killing *in vitro* ($P < 0.01$ and $P < 0.05$ compared to MW2, respectively).

Effect of GraS EL mutations on induction of *mprF* and *dlt-ABCD* expression. To assess the role of the GraS EL and specific amino acids therein on the transcriptional response, mutant strains were exposed to subinhibitory concentrations of hNP-1 or PMB *in vitro*, and transcription levels of *mprF* and *dltA* genes were measured (Fig. 2 and 3). Consistent with previous experiments (17), the expression of *mprF* and *dltA* was significantly upregu-

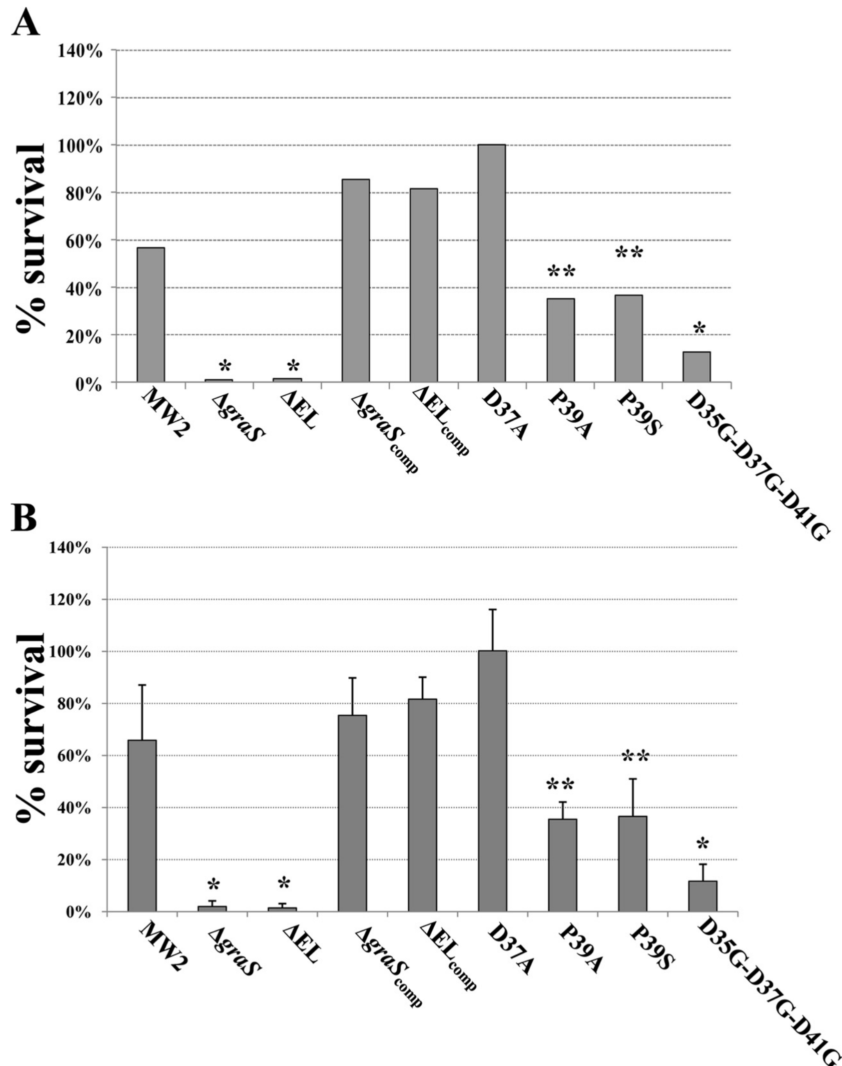

FIG 1 *In vitro* susceptibilities of the *S. aureus* strain set to hNP-1 (A) and RP-1(B). *In vitro* bacterial survival assays were carried out with hNP-1 (10 μg/ml) and RP-1 (1 μg/ml) as described previously using a 2-h microdilution method (9, 23). These data represent the means (±SD) from three independent experiments. *, $P < 0.01$; **, $P < 0.05$ compared to the MW2 parental strain.

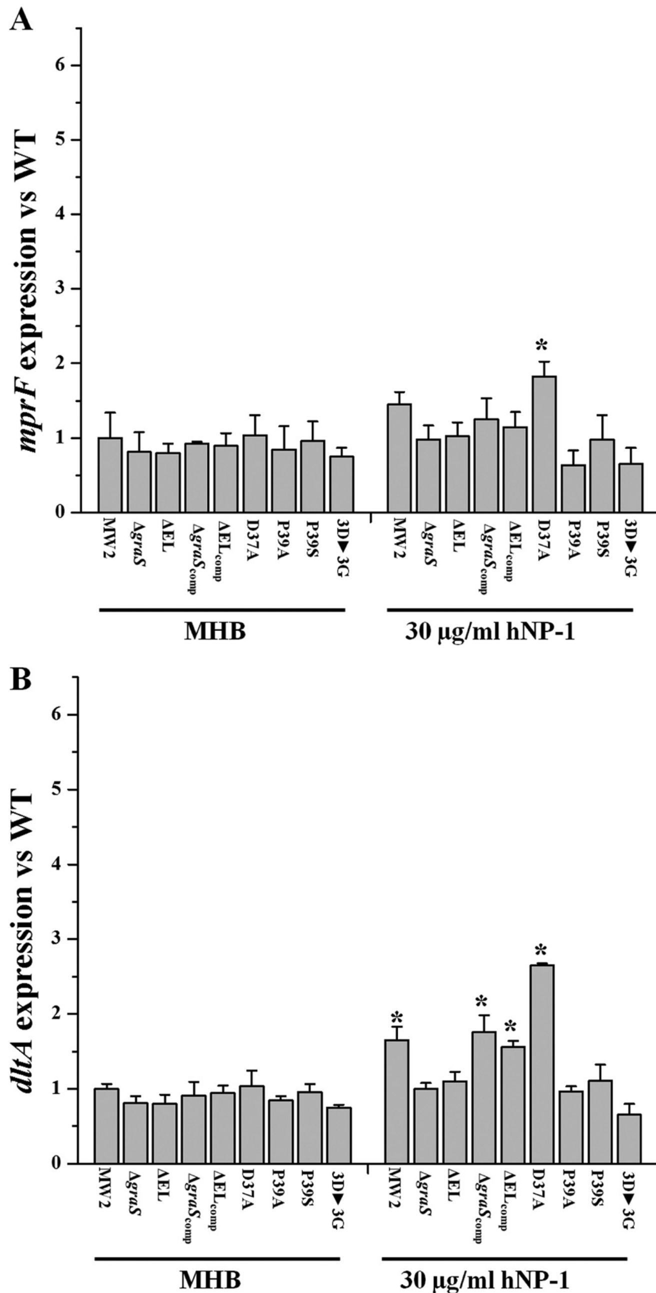

FIG 2 Induction of *mprF* (A) and *dltA* (B) transcription by hNP-1. Gene expression analyses were performed on RNA samples from cultures of the study strains exposed to hNP-1 (30 μg/ml) for 30 min during exponential growth. MHA, CAP-free medium alone; 3D▶3G, D35G D37G D41G strain. *, $P < 0.01$ compared to condition without hNP-1 treatment.

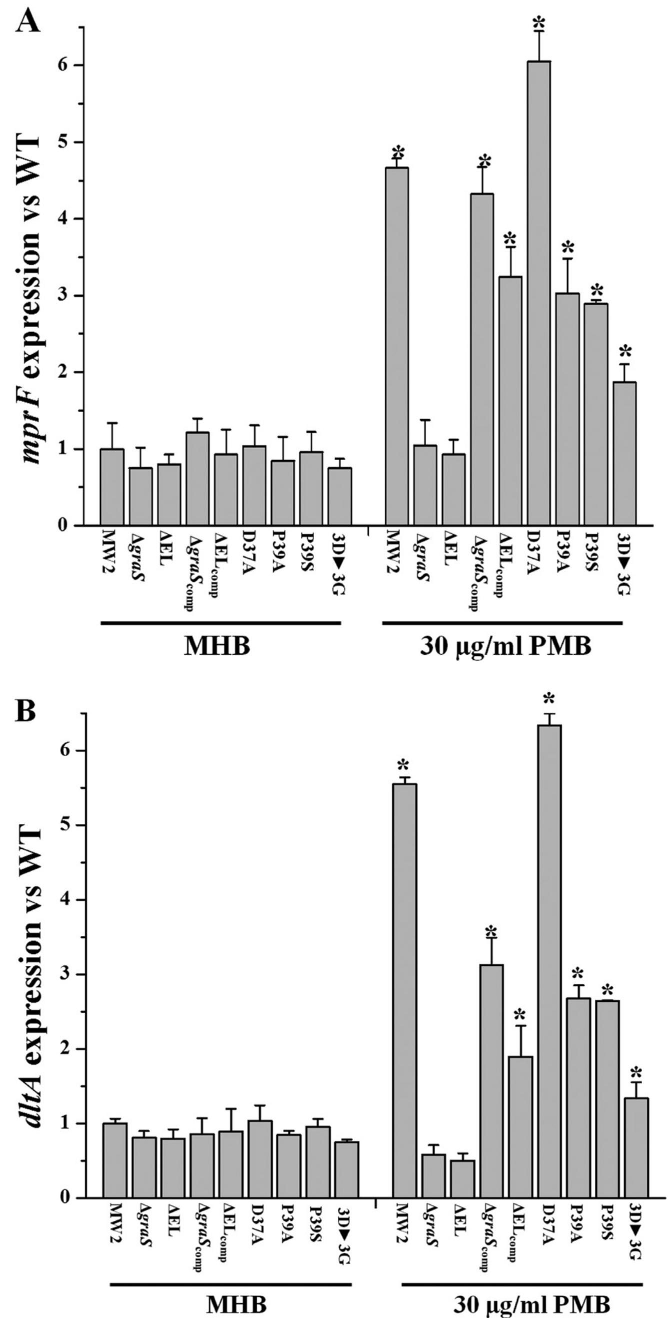

FIG 3 Induction of *mprF* (A) and *dltA* (B) transcription by polymyxin B. Gene expression analyses were performed on RNA samples from cultures of the study strains exposed to polymyxin B (30 μg/ml) for 30 min during exponential growth. MHB, CAP-free medium alone; 3D▶3G, D35G D37G D41G strain. *, $P < 0.01$ compared to the condition without hNP-1 treatment.

lated by PMB (~4.7- and 5.5-fold higher than in the absence of PMB, respectively; Fig. 3A and B) but not substantially by hNP-1 (Fig. 2A and B) in the parental MW2 strain. As expected, the Δ*graS* and ΔEL strains showed no induction of *mprF* and *dltA* transcription by PMB (Fig. 3A and B), confirming the essential role of the EL of GraS in inducing downstream target gene expression. Complementation of the Δ*graS* and ΔEL strains with a *graRS*-expressing plasmid restored the inducibility of *mprF* expression by PMB (Fig. 3A). In contrast to *mprF* expression, induction of the *dltA* transcription by PMB in these two complemented strains was restored only partially (Fig. 3B).

Consistent with a previous publication (17), vancomycin (2 μg/ml) and oxacillin (50 μg/ml) did not induce expression of *mprF* and *dltA* in any of the tested strains (data not shown).

Next, we studied critical residues within the EL of GraS that may be involved in the induction of *graRS*-regulated genes upon exposure to HD-CAPs. To do so, qRT-PCR analyses were per-

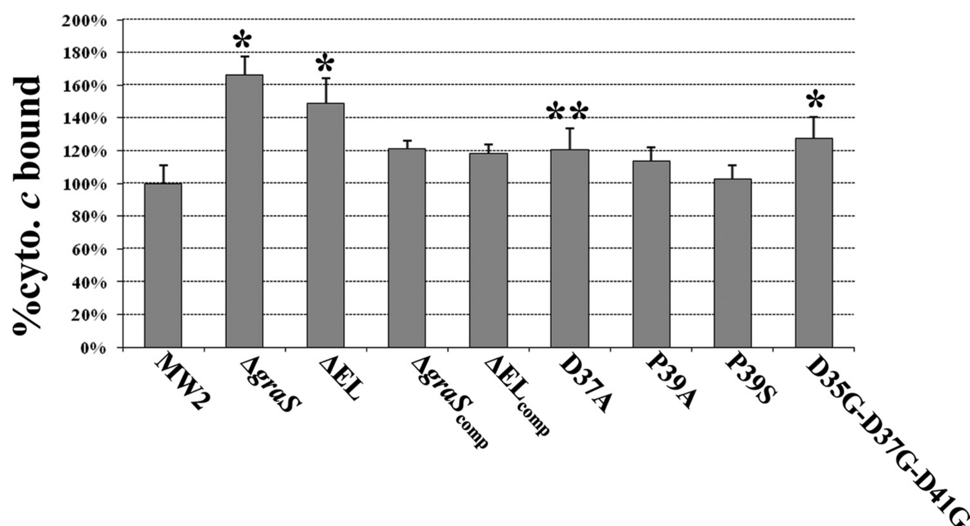

FIG 4 Binding of positively charged cytochrome *c* to whole *S. aureus* cells. The graph shows the percentage of cytochrome *c* unbound after 15 min of incubation with *S. aureus* at room temperature. Data represent the means (±SD) from three independent experiments. The MW2 parental strain was normalized to 100%. *, $P < 0.01$; **, $P < 0.05$ compared to the MW2 parental strain.

formed on RNA samples from EL mutant strains, in the presence and absence of hNP-1 or PMB. Surprisingly, enhanced induction of both *mprF* and *dltA* expression by hNP-1 was observed in the D37A strain (~2- and 3-fold induction versus medium-only control, respectively), while all the other EL point-mutated strains (P39A, P39S, and D35G D37G D41G constructs) showed no induction at all. Similar to hNP-1 induction, the D37A mutant strain exhibited a significantly enhanced level of induction of both *mprF* and *dltA* transcription in response to CAPs compared to those of the parental MW2 strain ($P < 0.01$). These observations suggested that the aspartic acid (D) at position 37 may be important for CAP induction of the GraRS TCRS. Although expression of *mprF* and *dltA* in the P39A, P39S, and D35G D37G D41G variants was induced by PMB, levels of induction were significantly reduced compared with those of MW2. Replacement of three aspartic acids within the EL with glycine residues (D35G D37G D41G) resulted in the most significant impact on induction of *mprF* and *dltA* transcription by PMB. Similar induction profiles of *mprF* and *dltA* expression were observed when the cultures were incubated with a lower concentration of PMB (~0.1× MIC; data not shown). Collectively, these data indicated that induction of *mprF* and *dltA* gene expression by specific CAPs is dependent on the sensor kinase, GraS, and, more specifically, likely involves anionic residues (i.e., aspartic acid) within the EL of GraS.

Net cell surface charge. GraRS positively regulates expression of *mprF* and *dltABCD*, both of which are critical for maintaining overall positive surface charge in *S. aureus* (14, 16, 17, 36, 45, 46). Therefore, we sought to determine the relative surface charge of the isogenic strain set in the present study. As shown in Fig. 4, deletion of *graS* or the EL of *graS* in MW2 corresponded to increased net cytochrome *c* binding compared to the isogenic parental MW2 or respective complemented strains (i.e., less positive surface charge; $P < 0.001$). Reductions in the net positive surface charge were also observed in D37A ($P < 0.05$), P39A, and D35G D37G D41G ($P < 0.01$) strains, with the highest reduction seen for the latter variant strain.

CM fluidity/rigidity. CM fluidity analyses revealed no substantial differences among the 9 study strains (data not shown).

Protection of *S. aureus* from CAP killing by a soluble exogenous GraS EL mimetic. To assess whether the exogenous soluble EL of GraS contributes to *S. aureus* subversion of the antistaphylococcal effects of HD-CAPs, susceptibility assays to hNP-1 and RP-1 were performed in the presence of the soluble EL mimetic peptide. As shown in Fig. 5A and B, the exogenous EL (10 μg/ml), but not the nonsense peptide, enhanced survival of all the strains tested against both hNP-1 (10 μg/ml) and RP-1 (1 μg/ml). To ensure that this protection of cells from HD-CAP killing by the exogenous soluble EL mimetic was not related to a direct impact of this synthetic peptide on induction of *mprF* and *dltABCD*, we performed qRT-PCR analyses on the MW2 and Δ*graS* strains either in the presence of the soluble EL or nonsense peptide control. Resulting data confirmed that transcription of *mprF* and *dltA* was not independently affected by either synthetic peptide (not shown).

Comparative *in vivo* virulence. In the comparative virulence assessments, the Δ*graS* and ΔEL strains exhibited dramatically lower CFU densities in all three target tissues than those in animals infected by the parental MW2 strain (reductions ranging from ~5 to 6 $\log_{10}$ CFU/g versus MW2). Moreover, for all tested *S. aureus* strains, CFU densities in cardiac vegetations were significantly higher than those in kidney and spleens at 24 h postinfection ($P < 0.01$) (Table 3). The Δ*graS* complementation strain resulted in return to near-parental bacterial densities in all three target tissues. Paralleling our *in vitro* gene expression profiles and HD-CAP susceptibility data, the D35G D37G D41G mutant strain showed only a modest reduction in target tissue counts and failed to reach statistical significance.

DISCUSSION

HD-CAPs have evolved as integral components of the innate immune system. However, successful pathogens such as *S. aureus* have developed a variety of adaptive resistance strategies to circumvent the antimicrobial effects of a wide range of HD-CAPs. Recently, we and others have shown that the TCRS GraRS plays a pivotal role in resistance of *S. aureus* to distinct CAPs by functioning as a CAP sensor. Transcription of *graRS*-regulated genes, such

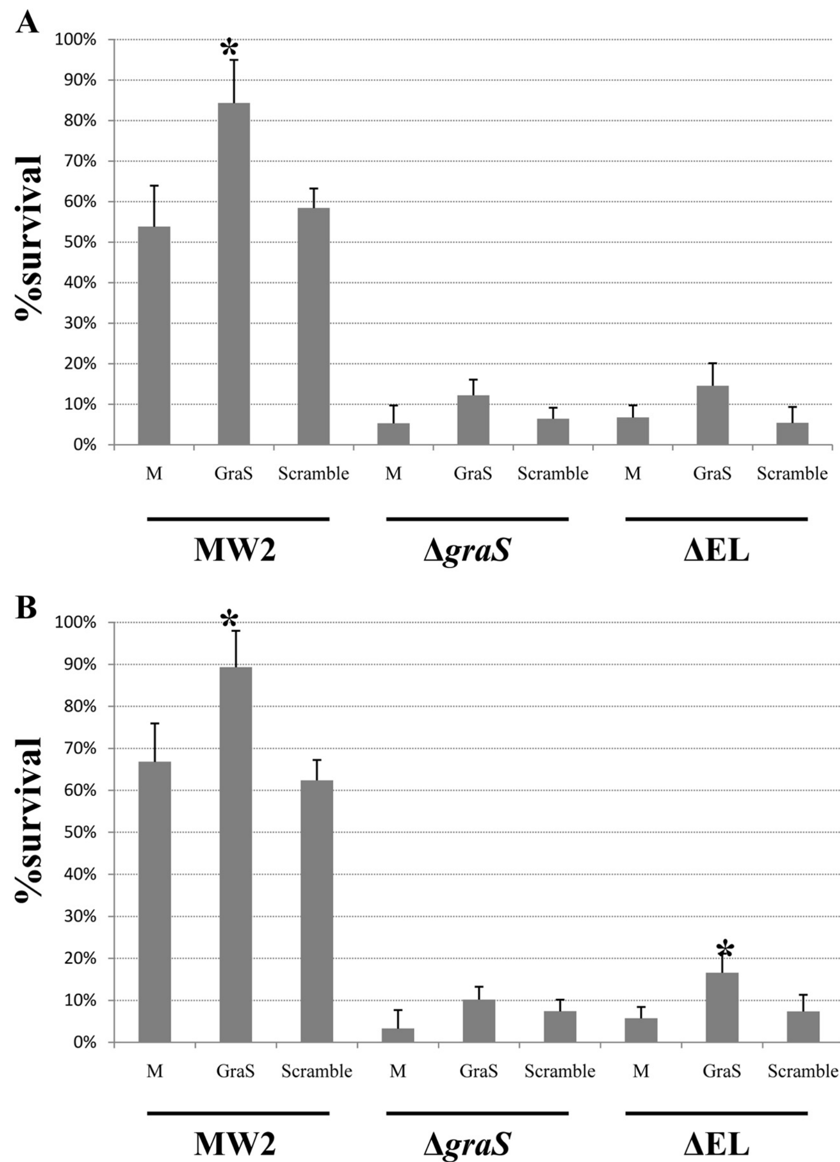

FIG 5 Effect of soluble exogenous GraS EL mimetic on survival of *S. aureus* cells upon exposure to hNP-1 (A) or RP-1 (B). *In vitro* bacterial survival assays were carried out with hNP-1 (10 μg/ml) and RP-1 (1 μg/ml) using the 2-h microdilution method (9, 23). The data were calculated and expressed as the relative percentage of surviving CFU of CAP-exposed versus CAP-unexposed cells. Data represent the means (±SD) from three independent experiments. M, medium-only control; GraS, synthetic trimeric GraS EL; Scramble, random nonsense peptides. *, $P < 0.01$ compared to the medium-only control.

as *mprF* and *dltABCD*, has been shown to be dependent on specific HDP/CAPs (14, 17), indicating additional mechanisms of target gene regulation via distinct CAP stimuli. For example, our studies (17) and those of Li et al. (14) demonstrated *graRS*-mediated regulation of the downstream *mprF* and *dltABCD* genes, the products of which are important for surface charge maintenance in *S. aureus*. In these prior investigations, it was demonstrated that a relatively selective range of CAPs appear to activate the *graRS* system in *S. aureus* (e.g., LL-37, RP-1, or PMB but not hBD-3 or hNP-1) (14, 17). Furthermore, we previously found that *vraFG* (a putative ABC efflux pump downstream of *graRS*) (15) could also alter the expression of *mprF* and *dltABCD* via an autocrine system involving *graRS*, indicating interdependency of *graRS-vraFG* expression (17).

TABLE 3 Comparative *in vivo* virulence of study strains

Strain (no. of animals)	Mean $\log_{10}$ CFU/g of tissue ± SD		
	Vegetation	Kidney	Spleen
MW2 (6)	7.42 ± 0.89	6.02 ± 0.91	5.92 ± 0.36
Δ*graS* strain (7)	1.54 ± 0.78[a]	1.07 ± 0.87[a]	1.09 ± 0.80[a]
ΔEL strain (7)	1.61 ± 0.71[a]	1.05 ± 0.90[a]	1.15 ± 0.89[a]
Δ$graS_{comp}$ strain (6)	7.33 ± 0.50	5.35 ± 1.21	5.64 ± 0.45
D35G D37G D41G strain (7)	6.50 ± 1.05	4.96 ± 1.21	5.43 ± 0.46

[a] $P < 0.001$ compared to MW2.

GraRS homologs exist in *Listeria* spp. and *S. epidermidis*. For example, the GraS homolog in *S. epidermidis* is also involved in CAP sensing and activation of downstream target genes. Although there is a significant structural homology (70% at the amino acid level) between GraS sensor kinases of *S. epidermidis* and *S. aureus*,

sequence divergence exists in the 9-amino-acid EL flanked by two transmembrane segments at the N terminus. Moreover, when the GraS loop of *S. aureus* is replaced with that of *S. epidermidis* in a parental *S. aureus* strain, GraS was activated rather "promiscuously" (instead of selectively) upon exposure to a large cadre of HD-CAPs, mirroring the loop donor *S. epidermidis* isolate. Thus, these domain swapping studies indicated that the nonameric GraS EL of *S. aureus* is important in the CAP-selective induction of GraRS-regulated genes. However, the amino acid residue-specific contributions within the nonameric EL to CAP-selective activation of GraRS TCRS are unknown.

In the present investigation, we used a well-characterized MRSA strain, MW2, and its isogenic *graS* deletion (Δ*graS*) mutant as well as a *graS* mutant specifically devoid of the 9 amino acid EL (ΔEL strain). In addition, mutagenesis of individual residues within the EL (D37A, P39A, P39S, and D35G D37G D41G) was introduced in the MW2 parental strain. These strategic substitutions were designed to alter physicochemical properties believed to be integral to CAP-*S. aureus* interactions (e.g., charge, secondary structure, hydrogen bonding propensity), while preserving net biochemical context of the target EL sequence.

A number of interesting findings emerged from these investigations. First, the Δ*graS* strain as well as the ΔEL strain displayed significantly increased susceptibilities to calcium-DAP (a positively charged lipopeptide antibiotic) and PMB (a highly cationic peptide of bacterial origin) compared to those of the parental MW2 and respective complemented strains. In addition, the two deletion mutants (Δ*graS* and ΔEL strains) also displayed dramatically enhanced *in vitro* susceptibilities to the two prototypical HD-CAPs (hNP-1 and RP-1), implying a requisite role for the 9-amino-acid EL of GraS in sense and response mechanisms correlating with resistance to selective CAPs. Furthermore, *in vitro* CAP susceptibility assays of the P39A, P39S, and D35G D37G D41G EL point mutant strains demonstrated moderate reduction in susceptibilities to all tested CAPs. These specific data further suggest that the proline (at position 39 in GraS) and the two aspartic acid residues (at positions 37 and 41 in GraS) are important for *graRS*-mediated CAP sensing. It is important to emphasize that these specific residues are absent in the *S. epidermidis* EL counterpart of the GraS protein. These findings imply that the GraS sensor function involves both charge and three-dimensional conformation in its capacity to detect and transduce activation of GraR-mediated adaptive responses to HD-CAPs. Further studies on the EL residue-specific impacts on GraS CAP sensing characteristics are in progress.

Second, since previous studies have shown regulation of *mprF* and *dltABCD* by the *graRS-vraFG* regulatory network, we examined the ability of the study strains to induce *mprF* and *dltA* expression upon exposure to hNP-1 or PMB by qRT-PCR. Previously, we have demonstrated that the expression of the *graRS*-mediated effector genes, *mprF* and *dltABCD*, was induced upon exposure to PMB but not by hNP-1. As anticipated, all tested strains showed either no or only minimal induction of *mprF* and *dltA* expression upon exposure to hNP-1, except for the D37A mutant. The significant induction of *mprF* and *dltA* expression by hNP-1 in the D37A mutant (Fig. 2A and B) suggests that the aspartic acid residues in EL affect CAP specificity of GraS. Furthermore, the D37A mutant displayed enhanced induction of *mprF* and *dltA* expression by PMB compared to that of the parental MW2 strain. This outcome suggests that the D37 residue contributes to the magnitude of activation of GraS sensor for target gene expression, and the D37A mutation may represent a "gain-in-function" substitution. The enhanced transcription of *mprF* and *dltA* in D37A correlated with the enhanced survival of the mutant strain in hNP-1 and RP-1 killing assays (Fig. 1A and B). However, the present analyses failed to reveal the expected impacts on surface charge, despite enhanced expression of *mprF* and *dltABCD* in the D37A mutant. In contrast, reduction of *mprF* and *dltA* induction by PMB was found in the P39A, P39S, and D35G D37G D41G mutants, indicating these latter residues are fundamental to the appropriate activation of the GraS sensor kinase.

Third, our *in vitro* data led us to evaluate the *in vivo* virulence impacts of specific mutations in the *graRS* operon in the rabbit IE model. Achievable target organ bacterial densities were found to be dramatically reduced in animals infected with either the Δ*graS* or ΔEL mutants. Both hNP-1 (prototypic neutrophil HD-CAP) and secreted mammalian platelet microbicidal proteins (represented by the synthetic congener, RP-1) play crucial roles in bacterial clearance in endovascular infections. These functions are believed to include (i) platelet-mediated clearance of bacteria from vegetations by way of secreted kinocidins and (ii) neutrophil-mediated clearance of bacteria from within hematogenous abscesses in target organs, such as kidneys and spleens, as a result of intracellular HD-CAPs (e.g., hNP-1), in combination with traditional neutrophil-based oxidative antimicrobial mechanisms. Thus, the inability to induce *mprF* and *dlt* expression in the various *graS* mutants, and in turn their increased susceptibilities to HD-CAPs, may explain their highly significant reductions in bacterial fitness in the IE model. In contrast, but consistent with our *in vitro* data on CAP susceptibility and induction of *mprF and dltA* expression, the virulence impacts of the D35G D37G D41G EL mutant were rather modest. It should be mentioned that in previous work from our laboratories (16), individual knockouts of *dltA* and *mprF* in a different background *S. aureus* strain yielded significant albeit rather modest impacts on virulence in the same experimental IE model (16). These latter data speak to the key role of *graS* in virulence networks beyond *dltA* and *mprF*.

Lastly, recently published studies by Li et al. (45) demonstrated that antisera raised against the EL of GraS rendered *S. epidermidis* unable to induce downstream target genes in response to human β-defensin 3. Consistent with our present data, these findings suggest the importance of the EL sensor in the interaction with HD-CAPs. Moreover, our findings that the exogenous soluble EL mimetic of GraS protects *S. aureus* against hNP-1 or RP-1 suggest that direct interactions of the EL with the HD-CAP(s) are required for triggering of the *graRS*-mediated sense-response system. These data suggest that the *graRS*-mediated protection against anti-staphylococcal effects of CAPs necessitates the interaction of EL of GraS with HD peptides of the innate immune system.

The current study offers important new insights into the potential roles of the GraRS TCRS in sense-and-response adaptations for *S. aureus* survival to specific HD-CAPs or cationic anti-infectives. This investigation also advances our understanding of the role of specific residues within the GraS sensing loop in the outcome of infections based on the specific host contexts that *S. aureus* may encounter *in vivo*. We recognize the limitations of these studies, including (i) only one genetic background was investigated, (ii) a limited cadre of HD and other CAPs were evaluated, (iii) an incomplete set of EL point mutants was assessed, and (iv) assessment of the impact on *in vivo* virulence was restricted to

a single animal model (IE). These limitations are currently being addressed in our laboratories.

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