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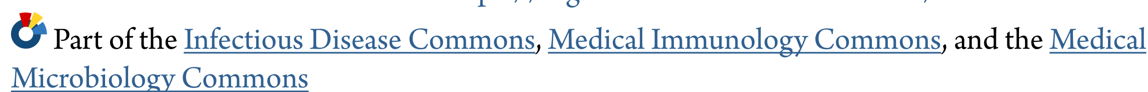
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Chaili, Siyang; Cheung, Ambrose L. L.; Bayer, Arnold S.; Xiong, Yan Q.; Waring, Alan; Memmi, Guido; and Donegan, Niles, "The GraS Sensor in Staphylococcus Aureus Mediates Resistance to Host Defense Peptides Differing in Mechanisms of Action" (2016). *Open Dartmouth: Faculty Open Access Articles*. 914.
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AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity

The GraS Sensor in *Staphylococcus aureus* Mediates Resistance to Host Defense Peptides Differing in Mechanisms of Action

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Staphylococcus aureus **uses the two-component regulatory system GraRS to sense and respond to host defense peptides (HDPs). However, the mechanistic impact of GraS or its extracellular sensing loop (EL) on HDP resistance is essentially unexplored. Strains with null mutations in the GraS holoprotein (Δ*graS*) or its EL (ΔEL) were compared for mechanisms of resistance to HDPs of relevant immune sources: neutrophil α-defensin (human neutrophil peptide 1 [hNP-1]), cutaneous β-defensin (human β-defensin 2 [hBD-2]), or the platelet kinocidin congener RP-1. Actions studied by flow cytometry included energetics (ENR); membrane permeabilization (PRM); annexin V binding (ANX), and cell death protease activation (CDP). Assay conditions simulated bloodstream (pH 7.5) or phagolysosomal (pH 5.5) pH contexts. *S. aureus* strains were more susceptible to HDPs at pH 7.5 than at pH 5.5, and each HDP exerted a distinct effect signature. The impacts of Δ*graS* and ΔEL on HDP resistance were peptide and pH dependent. Both mutants exhibited defects in ANX response to hNP-1 or hBD-2 at pH 7.5, but only hNP-1 did so at pH 5.5. Both mutants exhibited hyper-PRM, -ANX, and -CDP responses to RP-1 at both pHs and hypo-ENR at pH 5.5. The actions correlated with Δ*graS* or ΔEL hypersusceptibility to hNP-1 or RP-1 (but not hBD-2) at pH 7.5 and to all study HDPs at pH 5.5. An exogenous EL mimic protected mutant strains from hNP-1 and hBD-2 but not RP-1, indicating that GraS and its EL play nonredundant roles in *S. aureus* survival responses to specific HDPs. These findings suggest that GraS mediates specific resistance countermeasures to HDPs in immune contexts that are highly relevant to *S. aureus* pathogenesis in humans.**

Host defense peptides (HDPs) represent a critical first line of immune protection against *Staphylococcus aureus* infections (1–3). Distinct HDPs are deployed via constitutive or inducible processes by neutrophils, keratinocytes, platelets, or other human tissues that this organism commonly encounters (3, 4). Moreover, distinct HDPs appear to have evolved for optimal host defense in specific immunologic and anatomic compartments (4, 5). In turn, pathogenic *S. aureus* strains have coevolved specific and rapidly adaptive systems to sense and respond to host cues to achieve immune avoidance or subversion.

Recently, we showed that the two-component regulatory system (TCRS) GraSR plays a key role in *S. aureus* survival in the face of HDPs (6–8). Also known to be a component of the antibiotic peptide sensor (APS) (9, 10), GraSR upregulates adaptive resistance genes such as *mprF* and *dltA*, which encode proteins that modulate net surface charge and influence the composition of the *S. aureus* envelope (11, 12). In a previous report (7), we demonstrated that *S. aureus* strains with deletions in *graS* (Δ*graS*) or its extracellular sensor loop (ΔEL) become hypersusceptible to several cationic peptides, including calcium-complexed daptomycin (DAP) (a cationic lipopeptide complex), polymyxin B (PMB) (a prokaryotic cationic cyclopeptide), and human neutrophil peptide 1 (hNP-1). Importantly, we observed a direct correlation between induction of *mprF* and *dltABCD* and survival in the face of PMB, but not hNP-1. Thus, certain HDPs may trigger more efficient sense/countermeasure response functions mediated by GraS.

These prior findings provided a logical basis for our hypothesis that GraS or its extracellular loop (EL) mediates critical adaptive countermeasures to specific mechanisms of HDP action in distinct anatomic contexts. Thus, the present study was designed to explore the role of the GraS holoprotein versus its EL in mediating resistance responses to relevant HDPs under pH conditions reflecting bloodstream (pH 7.5) or phagolysosomal (pH 5.5) settings.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Table 1 summarizes the methicillin-resistant *Staphylococcus aureus* (MRSA) strain panel used in this study, generated from the community-acquired MRSA strain MW2. MW2 (USA400) is a prototypic and well-characterized clinical isolate with a known genome that has been demonstrated to be virulent in multiple animal models. *graS* mutant strains were generated by in-frame deletion of target genes by allelic replacement using the temperature-sensitive plasmid pMAD as previously detailed (7). Organisms were cultured in brain heart infusion broth (BHI) (Becton Dickinson) at 37°C with shaking overnight (~16 h), subcultured under identical conditions for 3 h to

Received 7 August 2015 **Returned for modification** 20 September 2015 **Accepted** 16 November 2015

Accepted manuscript posted online 23 November 2015

Citation Chaili S, Cheung AL, Bayer AS, Xiong YQ, Waring AJ, Memmi G, Donegan N, Yang S-J, Yeaman MR. 2016. The GraS sensor in *Staphylococcus aureus* mediates resistance to host defense peptides differing in mechanisms of action. Infect Immun 84:459–466. doi:10.1128/IAI.01030-15.

Editor: A. Camilli

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.01030-15.

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TABLE 1 *Staphylococcus aureus* strains used in the current investigation

Strain	Description
MW2	Wild type, community-acquired MRSA, human clinical isolate
Δ*graS*	In-frame *graS* deletion mutant of MW2
Δ$graS_{comp}$	Δ*graS* mutant complemented with pEPSA5-expressing *graRS* from MW2
ΔEL	*graS* extracellular loop deletion mutant (9 amino acids encoded in *graS*)
ΔEL_{comp}	ΔEL complemented with pEPSA5-expressing *graRS* from MW2

logarithmic phase, harvested by centrifugation, washed, and resuspended to the appropriate CFU (culture confirmed) by spectrophotometry for each specific assay.

HDPs. Peptides representing relevant immunologic contexts were studied. Human neutrophil peptide 1 (hNP-1) (Peptides International, Louisville, KY) is a prototypic α-defensin found in human neutrophil-specific granules and is important in phagolysosomal killing of *S. aureus*. Human β-defensin 2 (hβD-2; Peptides International) is a predominant host defense peptide (HDP) elaborated by epithelial tissues throughout the body. The mimetic peptide RP-1 is a synthetic 18-amino-acid congener engineered in part from the microbicidal α-helix domains of the platelet factor 4 family of kinocidins (8). RP-1 was synthesized, purified, and authenticated as previously detailed (9). Each study peptide has demonstrated *in vitro* antimicrobial activity against *S. aureus* (3, 7, 8, 10).

sEL sensor domain. A soluble *graS* extracellular loop (sEL) sensor domain mimetic has been previously shown to alter the survival of *S. aureus* in response to certain HDPs (7). This molecule was designed to contain three sensor motifs interposed by diglycine hinges (DYDFPIDSL-GG-DYDFPIDSL-GG-DYDFPIDSL). A "nonsense" peptide of the same composition but having a randomized (scrambled) sequence was also generated and was included in assays as a control. The sEL and nonsense peptides were synthesized, purified, and authenticated as previously described (8, 9).

Susceptibility of *graS* mutants to host defense peptides. The susceptibility of *graS* mutants to distinct HDPs was assessed using an established radial diffusion method (10). In brief, logarithmic-phase cells adjusted to 10^6 CFU/ml were seeded into 10 ml of buffered 1% agarose. Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) or 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer was used to adjust assay conditions to pH 7.5 or pH 5.5. These pH conditions were chosen to represent relevant anatomical contexts, namely, bloodstream or acidic phagolysosome, respectively. Based on pilot studies, 10 μg of each HDP, alone or in combination with an equivalent mass (10 μg) of *graS* EL mimetic or nonsense peptide, was added to wells in the seeded underlay matrix and incubated at 37°C for 3 h. The sEL peptide, nonsense peptide, and vehicle (phosphate-buffered saline [PBS]) alone were included in each assay as internal controls. After 3 h of incubation, plates were overlaid with nutrient medium (Trypticase soy) and incubated for 24 h at 37°C. Zones of inhibition (ZOI) were measured to the nearest millimeter in diameter. A minimum of two independent experiments were conducted on separate days for statistical analysis.

Mechanisms of HDP action. Six-parameter multicolor flow cytometry was used to analyze four specific mechanisms of HDP action and two global effects associated with these mechanisms in the parental, Δ*graS*, or ΔEL *S. aureus* strains: (i) perturbation of cell membrane (CM) energetics (ENR) (e.g., transmembrane potential), (ii) CM permeabilization (PRM), (iii) annexin V binding (ANX) (negatively charged phospholipids and CM turnover (13, 14), (iv) caspase-like/metacaspase-like cell death protease induction (CDP) (11, 14, 15), (v) osmodisruption (forward scatter [FSC]), and (vi) membrane invagination/chromosomal condensation/cytoplasmic refractivity (side scatter [SSC] granularity). The following fluorophores were used with a FACSCalibur instrument (Becton Dickinson): 3,3-dipentyloxacarbocyanine (DiOC5) (excitation, 484 nm; emission, 660 nm) (Invitrogen, Carlsbad, CA) for ENR, propidium iodide (PI) (excitation, 535 nm; emission, 620 nm) (Sigma, St. Louis, MO) for PRM, annexin V-allophycocyanin conjugate (ANX-V) (excitation, 650 nm; emission, 660 nm) (Invitrogen, Carlsbad, CA) for ANX, and CellEvent caspase-3/7 green (C-3/7) (excitation, 502 nm; emission, 530 nm) (Invitrogen, Carlsbad, CA) for CDP. FSC and SSC were measured in parallel. Logarithmic-phase organisms were adjusted to 10^6 CFU/ml in PIPES (pH 7.5) or MES (pH 5.5) and exposed to 20 μg of each HDP of interest for 1 h at 37°C. Based on extensive pilot data, this peptide concentration was used to achieve approximately 50% survival given the high inoculum of bacteria exposed. A triple-stain cocktail containing $DiOC_5$ (0.5 μM), PI (5.0 μg/ml), and ANX-V (2.5 μl/ml) in 50 mM potassium-containing minimal essential medium (K^+ MEM) (without phenol red; Sigma) was added to each sample following incubation. Samples were stained at room temperature for 15 min before flow cytometry. Parallel samples were incubated with 30 μl of C-3/7 reagent for 30 min at 37°C following peptide exposure. After incubation, 400 ml of PBS was added to remove any background signal. Sodium dodecyl sulfate (SDS) (10%, wt/vol; Ambion) (a nonspecific perturbant of ENR and PRM) or buffers alone (K^+ MEM or PBS) were included as controls in each experiment. Fluorescence of a minimum of 10,000 cells was acquired from each sample, and results from a minimum of two independent studies conducted on different days were used for statistical analysis.

Statistical analysis. The Mann-Whitney U test was used as appropriate to determine significant differences in susceptibility phenotypes and mechanisms of HDP action in *S. aureus* strains.

RESULTS

Impact of pH on HDP susceptibility. The susceptibility of *S. aureus* strains to prototypic HDPs under different pH conditions is summarized in Fig. 1 and 2. The wild-type strain was susceptible to hNP-1 and RP-1 at both pH 7.5 and 5.5. However, this strain was susceptible to hBD-2 only at pH 7.5. The susceptibility was greatest for RP-1, substantially less for hNP-1, and least for hBD-2. Notably, the wild-type strain was more susceptible to the HDPs at pH 7.5 than at pH 5.5 (Fig. 1A and C). These susceptibility rankings were consistent for HDPs under both pH conditions, even though absolute susceptibilities were reduced at pH 5.5.

Impact of *graS* or EL mutation on HDP susceptibility. At pH 7.5, the Δ*graS* and ΔEL mutants were significantly more susceptible to hNP-1 or RP-1 than the parental strain (Fig. 1 and 2) ($P < 0.05$). The Δ*graS* mutant trended toward greater hBD-2 susceptibility than the parental strain, but this did not reach significance. At pH 5.5, the mutants were significantly more susceptible to all HDPs than the respective controls at pH 7.5. Notably, only the Δ*graS* mutation conferred significantly greater susceptibility to hBD-2 at pH 5.5, while the ΔEL mutation did not achieve significance. Complementation of mutants largely restored susceptibility to wild-type-equivalent levels (Fig. 1 and 2).

Effect of soluble EL on HDP susceptibility. The exogenous soluble EL (sEL) did not exert intrinsic anti-*S. aureus* efficacy alone (Fig. 2). At pH 7.5, it significantly protected the Δ*graS* and ΔEL mutants against hNP-1 ($P < 0.05$) but not hBD-2 or RP-1. At pH 5.5, the sEL protected against hNP-1 and hBD-2 (Fig. 2). Interestingly, the sEL did not protect any strain against RP-1 at either pH.

Impact of *graS* or EL mutations on mechanisms of HDP action. The comparative impact of HDP mechanisms on the panel of *S. aureus* strains is shown in Fig. 3 and 4 and in Fig. S1 and S2 in the supplemental material. The specific mechanisms of action of

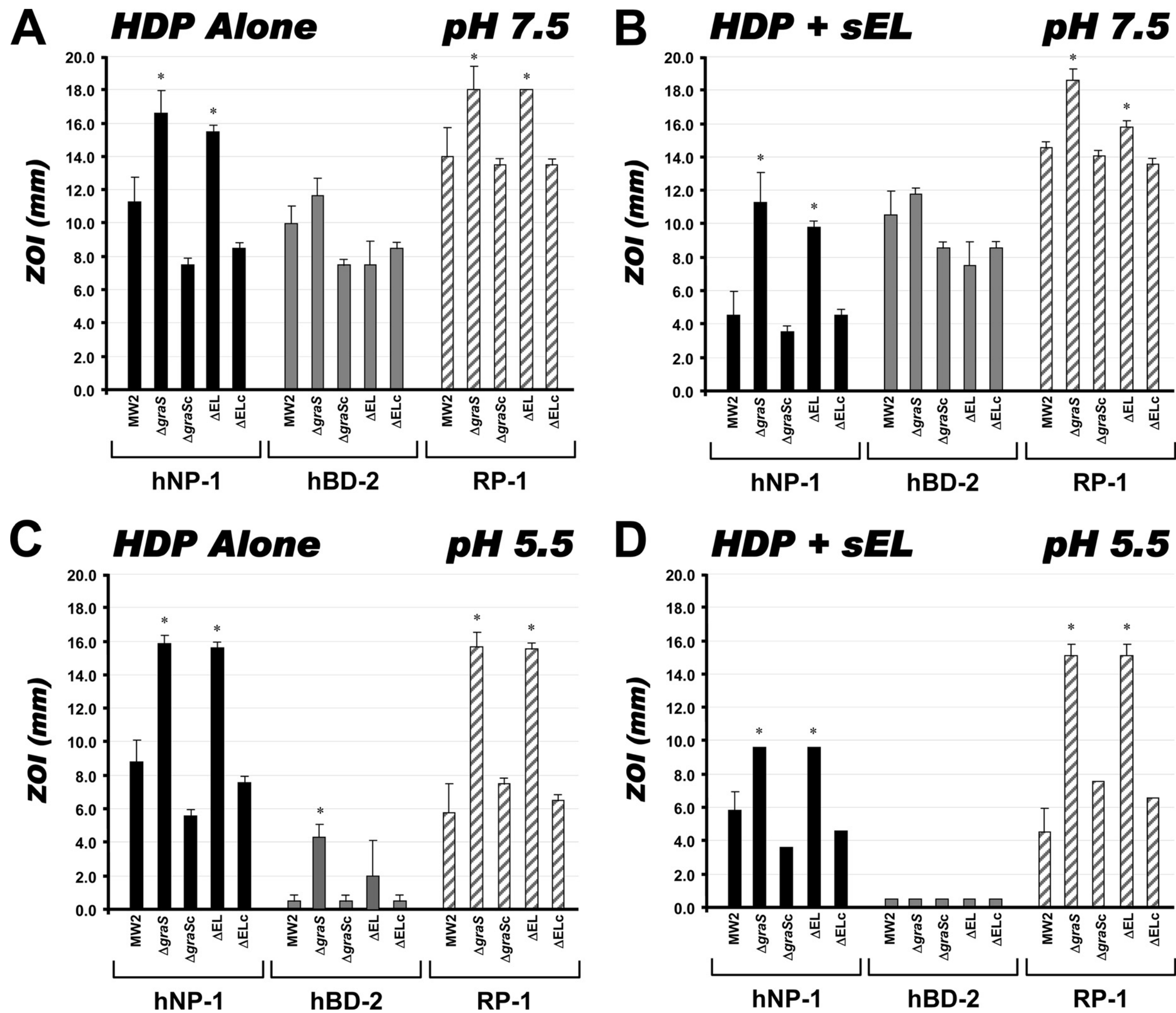

FIG 1 Comparative efficacy of HDPs alone or in combination with the sEL against the wild type (MW2), Δ*graS*, and ΔEL *S. aureus* study strains at pH 7.5 versus pH 5.5 *in vitro*. Quantitative analysis of the impact of Δ*graS* or ΔEL deletions on the susceptibility of *Staphylococcus aureus* to HDPs at pH 7.5 or 5.5 in the presence or absence of the soluble extracellular loop (sEL) mimetic is shown. *, $P < 0.05$ versus wild-type control.

the study HDPs versus SDS relative to the Δ*graS* or ΔEL mutants are detailed below.

(i) hNP-1. In the wild-type strain, hNP-1 caused significant increases in side scatter (SSC), energetics (ENR), and annexin V binding (ANX) compared to those for untreated controls ($P < 0.05$) (Fig. 3 and 4). At pH 7.5, both the Δ*graS* and ΔEL mutants exhibited decreased SSC and ANX compared to the wild type (Fig. 3). These effects reached significance in the Δ*graS* and ΔEL strains for SSC or ANX, respectively. These results corresponded to biological relevance as evidenced by significantly increased susceptibility of the mutants to hNP-1 (Fig. 1 and 2). Interestingly, at pH 5.5, only the Δ*graS* mutant exhibited reduced SSC and ANX versus the parent strain (Fig. 3). Complementation largely reverted hNP-1 mechanisms to wild-type equivalence. No detectable impact of hNP-1 in Δ*graS* or ΔEL mutants was observed regarding forward scatter (FSC), ENR, cell membrane permeabilization (PRM), or cell death protease activation (CDP) at either pH (Fig. 3 and 4).

(ii) hBD-2. Exposure to hBD-2 at pH 7.5 caused significant increases in SSC, ENR, and ANX in wild-type *S. aureus* (Fig. 3 and 4). Relative to that for the wild type, Δ*graS* and ΔEL mutations led to further increases in ANX (Fig. 3) ($P < 0.05$). Only the Δ*graS* mutant displayed significantly reduced ENR versus the parental strain. In contrast to the case for hNP-1, increased ANX was not associated with significantly greater susceptibility to hBD-2 in the Δ*graS* mutant at pH 7.5 (Fig. 1 and 2). The ΔEL mutant also exhibited greater ANX than the wild type, but this effect did not translate to greater hBD-2 susceptibility. At pH 5.5, hBD-2 caused significantly increased ENR in the ΔEL mutant (Fig. 3 and 4). Complementation reverted hBD-2-induced mechanisms to wild-type equivalence in both mutant strains and at pH 5.5 appeared to hypercompensate with respect to ANX in both revertants and with respect to SSC and ENR in the ΔEL complemented strain (Fig. 3). No detectable impact of hBD-2 was observed regarding FSC, PRM, or CDP at either pH for either the Δ*graS* or ΔEL mutants (Fig. 3 and 4).

(iii) RP-1. At pH 7.5, RP-1 exerted multiple actions in the wild-type strain, including significant increases in SSC, PRM, ANX, and CDP (Fig. 3 and 4). Oppositely, RP-1 caused a significant decrease in ENR in the wild-type strain at pH 7.5. Impor-

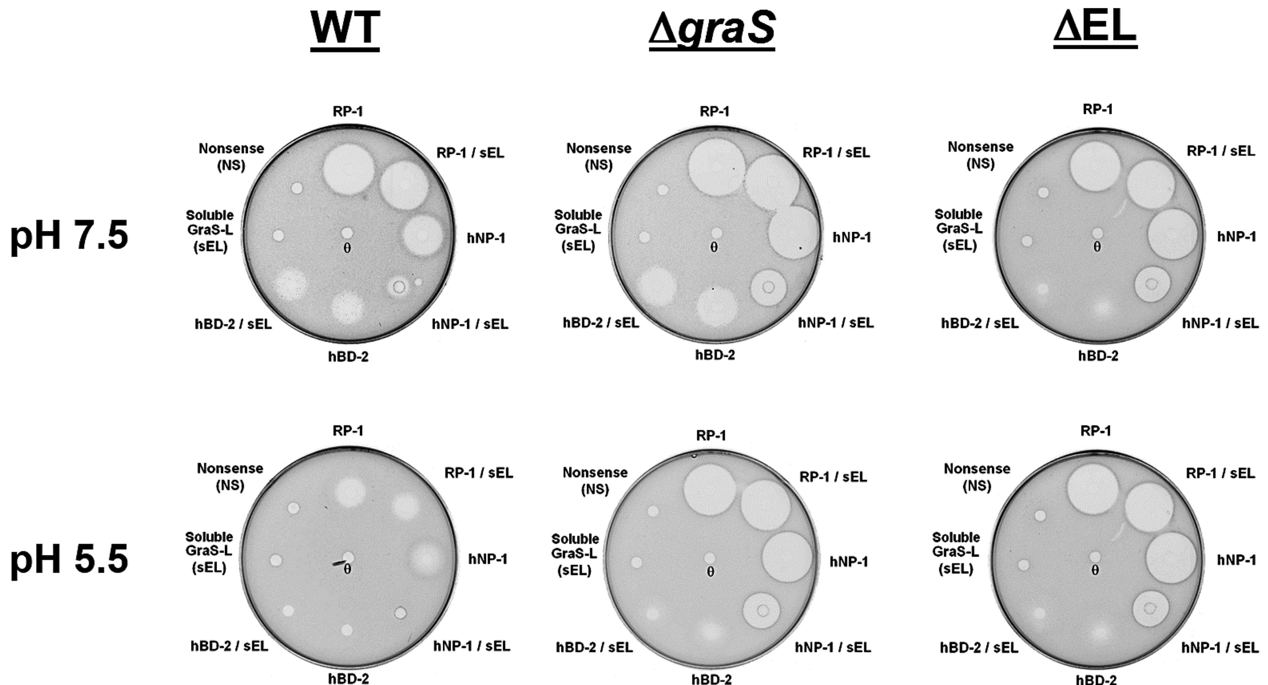

FIG 2 Susceptibility of *S. aureus* study strains to HDPs at pH 7.5 versus 5.5 in the presence or absence of the sEL or nonsense peptide (NS; scrambled sEL). A negative control (double-distilled water [ddH$_2$O]) is shown as the center well. Note the differential consequences of Δ*graS* versus ΔEL mutations under distinct pH conditions, with overall lower absolute efficacy of HDPs against the wild-type (WT) strain at pH 5.5 and of hBD-2 against the WT and Δ*graS* (but not ΔEL) strains at pH 5.5.

tantly, both the Δ*graS* and ΔEL (albeit to a lesser extent) mutants exhibited significant increases in PRM and CDP, with concurrent significant decreases in SSC and ENR compared to those for the wild-type control (Fig. 3 and 4) ($P < 0.05$). These effects translated to increased RP-1 susceptibility compared to the control (Fig. 1 and 2). At pH 5.5, overall the effects of RP-1 were less extensive that those at pH 7.5 in the wild-type strain. However, both the Δ*graS* and ΔEL mutants exhibited significant reductions in SSC and ENR and increases in PRM and CDP compared to the parental strain at pH 5.5 (Fig. 3) ($P < 0.05$). Only the Δ*graS* mutant exhibited a significant decrease in ANX at pH 5.5. Complementation largely failed to restore other RP-1-induced mechanisms to wild-type levels, suggesting that RP-1 functions through mechanisms that are less amenable to GraS-mediated countermeasures that those of hNP-1 or hBD-2.

(iv) SDS. As anticipated, exposure of strains to the nonspecific detergent sodium dodecyl sulfate (SDS) caused a significant reduction in ENR and a significant increase in PRM at pH 7.5 or 5.5. These effects are clearly seen in the mechanistic signature map (Fig. 4). By comparison, each HDP exhibited effects distinct from that of SDS, which were pH dependent (Fig. 4; see Fig. S1 and S2 in the supplemental material).

DISCUSSION

The concept of adaptive bacterial systems for sensing and responding to host cues to achieve immune avoidance or subversion is well established (16). Among these mechanisms, the GraSR multicomponent system appears to be integral to *S. aureus* defense against innate immune effector HDPs (6, 7). The current investigation builds upon our previous work by exploring the mechanisms of resistance mediated through GraS and/or its EL in response to HDPs of relevant immune context and under cognate physiologic conditions (3, 4, 17). Several key findings which shed new light on the role of GraSR in the pathogenic relationship of *S. aureus* with the human host emerged from these studies.

Overall, the present data established that wild-type and mutant *S. aureus* strains were more susceptible to HDPs at pH 7.5 than at pH 5.5. This finding is consistent with our prior reports, demonstrating that *S. aureus* susceptibility to α-defensins and certain other HDPs is greater at pH 7.5 than at pH 5.5 (5, 13). Importantly, the current data revealed that distinct HDPs, even structurally related defensins of different immune compartments, exert unambiguously distinct actions against *S. aureus*. Furthermore, these effects appear to be pH dependent. For example, in the wild-type strain, hNP-1-mediated killing at either pH 7.5 or 5.5 correlated significantly with increases in ANX and SSC. These findings are consistent with our prior report showing that the HDP thrombin-induced platelet microbial protein 1 (tPMP-1) induces cytoplasmic membrane invagination and nucleic acid condensation correlating with chromosomal condensation, cytoplasmic refractivity, and inhibition of macromolecular synthesis (18, 19). hBD-2 exerted anti-*S. aureus* efficacy predominantly at pH 7.5. This efficacy was associated with significant hyperpolarization (ENR) and ANX in the wild-type strain. The staphylocidal effects of these mechanisms appear to be pH dependent, given that similar events observed at pH 5.5 were not associated with hBD-2 efficacy. In contrast, the platelet kinocidin mimetic RP-1 exerted multiple actions at both pH 7.5 and 5.5. The key mechanistic signature of RP-1 efficacy in wild-type *S. aureus* included simultaneous increases in SSC, PRM, ANX, and CDP, with a significant decrease in ENR. These mechanisms were amplified at pH 7.5 versus 5.5.,

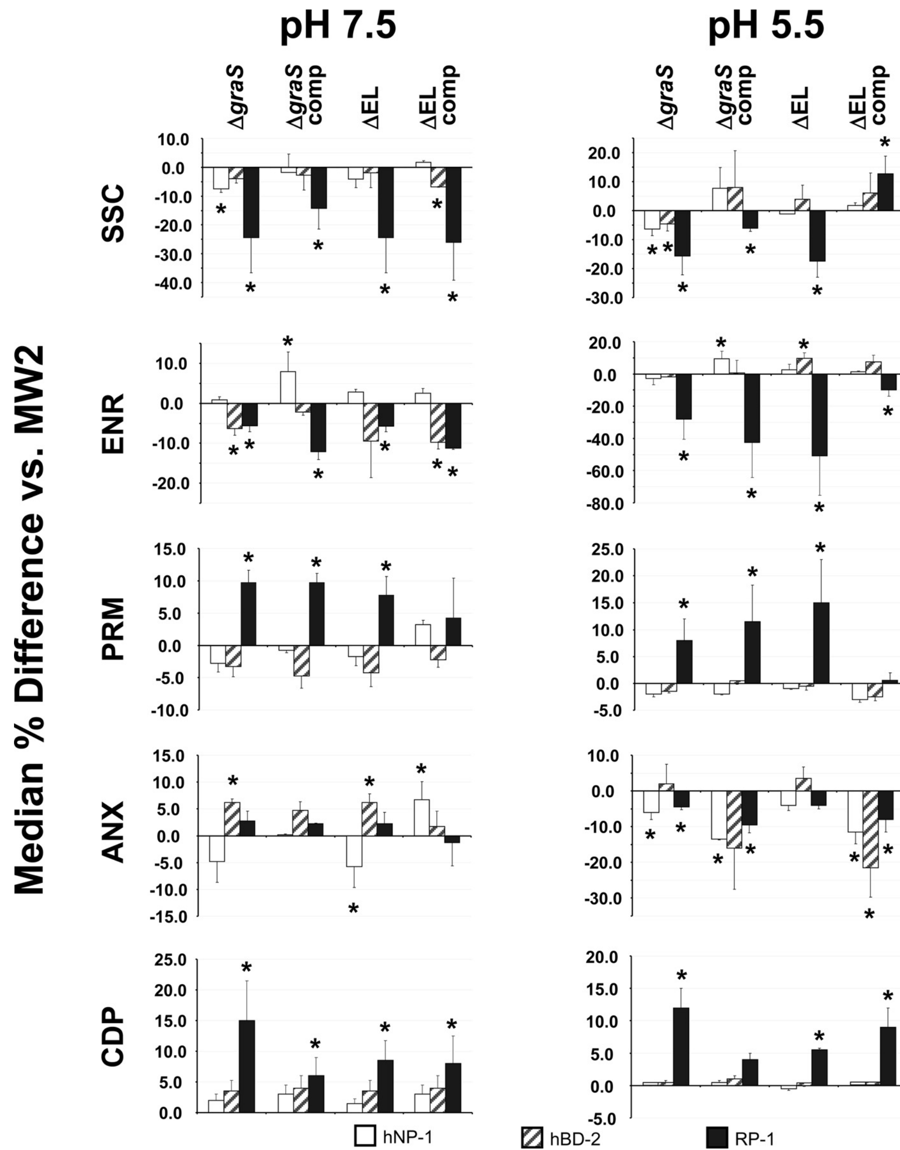

FIG 3 Quantitative mechanisms of HDPs against *Staphylococcus* study strains at pH 7.5 versus 5.5 *in vitro*. Data represent median percent differences versus wild-type MW2 normalized to buffer alone. *, significance defined as a change of ≥5% which also achieved a P value of <0.05 versus the WT control (Mann-Whitney U test of 10,000 event sample population). The following mechanisms of action (y axis) were determined using multicolor flow cytometry: side scatter (SSC) (intracellular refraction/cytoplasm condensation), cellular energetics (ENR) (transmembrane potential), cell membrane permeabilization (PMR) (propidium iodide uptake), negatively charged phospholipid accessibility (ANX) (cytoplasmic membrane turnover [e.g., extracellular exposure of intracellular leaflet bilayer]), and caspase-like/metacaspase-like cell death protease activation (CDP). In contrast to HDPs, the nonspecific detergent SDS caused strong cellular de-energization and increased membrane permeabilization in all strains independent of pH (see Fig. 4). In contrast, study HDPs exerted one or more specific mechanisms of action that were influenced by pH.

consistent with greater RP-1 efficacy at pH 7.5. Importantly, the mechanistic signature of each HDP mentioned above was clearly distinct from that of the nonspecific detergent SDS. This observation affords cogent evidence that HDPs exert specific, targeted, and context-dependent effects that confront *S. aureus* and its GraSR system.

The Δ*graS* and ΔEL mutants exhibited significant and equivalent increases in susceptibility to hNP-1 under pH conditions designed to reflect bloodstream (pH 7.5) or phagolysosomal (pH 5.5) immune contexts. Mechanistically, increases in hNP-1-induced killing of these mutants at pH 7.5 corresponded to decreased SSC and ANX compared to those for the wild-type strain. These results suggest that the GraS holoprotein confers adaptive responses to *S. aureus* that yield intracellular countermeasures manifesting as increased CM turnover, including changes in phospholipid location and/or composition. At pH 7.5, the finding that these events were equivalent in the EL and *graS* mutants strongly suggests that, at least for hNP-1, the EL is sufficient for the sensing function of the GraSR system. At pH 5.5, hNP-1 exhibited less efficacy than at pH 7.5 for all strains tested. However, the Δ*graS* mutant exhibited greater susceptibility to hNP-1 at pH 5.5 than the wild type, and in inverse relation to ANX. Further, the ANX response was dependent on the GraS system, as both the Δ*graS* and ΔEL mutants were deficient in mounting a protective ANX

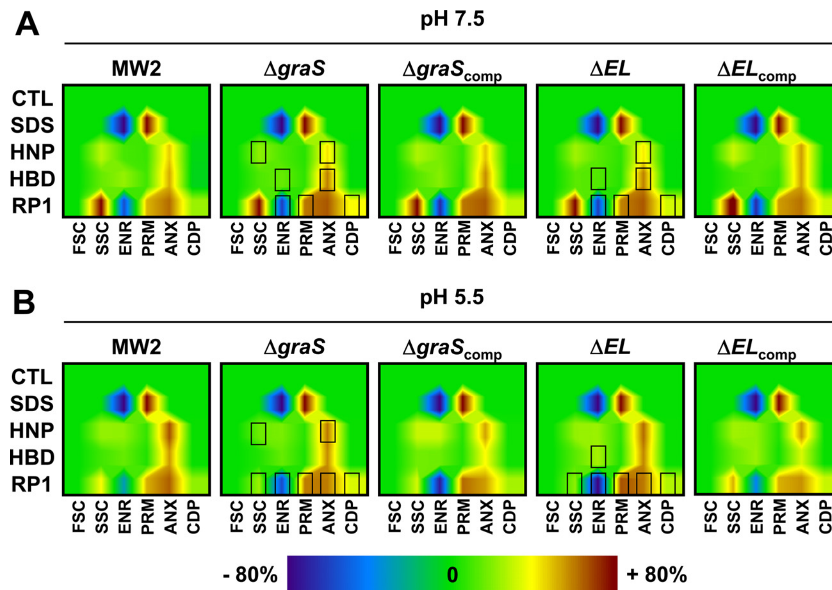

FIG 4 Mechanistic signature mapping of HDPs against *Staphylococcus* study strains at pH 7.5 versus 5.5 *in vitro*. Exposure conditions (*y* axis) included control (CTL) (buffer alone), the indiscriminant membrane detergent sodium dodecyl sulfate (SDS), hNP-1 (HNP), hBD-2 (HBD), and RP-1 (RP1). Mechanisms of action (*x* axis) were determined using multicolor flow cytometry: forward scatter (FSC) (cell size/shape), side scatter (SSC) (intracellular refraction indicative of cytoplasm condensation [see Materials and Methods]), cellular energetics (ENR) (transmembrane potential), cell membrane permeabilization (PMR) (propidium iodide uptake), negatively charged phospholipid accessibility (ANX) (cytoplasmic membrane turnover [e.g., extracellular exposure of intracellular leaflet bilayer]), and cell death protease activation (CDP). Percent increases (yellow-orange-red) or decreases (blue-indigo-violet) in mechanisms of action recorded 1 h postexposure are integrated in each signature map. Significant changes compared to the untreated control are indicated by black squares. Mechanistic signatures are shown at pH 7.5 (A) and pH 5.5 (B). As anticipated, de-energization and increased membrane permeabilization were strongly induced by the SDS control at either pH. In comparison, the study HDPs exerted one or more specific mechanisms of action that were unambiguously distinct from that of SDS and that in many cases differed at pH 7.5 versus 5.5.

response observed in the wild-type parent. This theme was amplified at pH 5.5, a condition likely encountered within the neutrophil phagolysosome.

Several new insights also emerged regarding the interaction of hBD-2 with the GraS system in *S. aureus*. At pH 7.5, neither the Δ*graS* nor the ΔEL mutation rendered *S. aureus* more susceptible to hBD-2. However, at pH 5.5, the Δ*graS* mutation led to an increase in susceptibility to this HDP; a lesser effect was also observed with the ΔEL mutant. This pattern of results suggests novel intersections of GraS function and hBD-2 activity under distinct pH conditions. First, efficacy at pH 7.5 corresponded to impairment of ANX in the Δ*graS* mutant, indicating that GraS mediates important CM turnover required for survival in response to hBD-2. Second, given that neither the charge nor the conformation of hBD-2 is significantly different at pH 7.5 versus 5.5 (13, 17), the GraSR system rather than the peptide hBD-2 may function more differently under distinct pH conditions. Third, the observation that the full Δ*graS* deletion, and not the ΔEL mutation alone, conferred distinct vulnerability to hBD-2 indicates that the GraS holoprotein is more influenced by pH than is its EL. Several mechanisms underlying this relationship may exist, including conformational dynamics, the impact of a ΔpH gradient (electron motive force), or the influence of charge or osmotic conditions targeting the holoprotein rather than the EL sensor motif. Thus, it appears that at neutral pH (e.g., in the bloodstream), the holoprotein and the EL domain of the sensor are functionally equivalent. However, at acidic pH (e.g., in phagolysosomes), EL sensing of an HDP may not be as effectively transduced by the GraS holoprotein and thus may fail to convey an adaptive survival response(s) to the organism. This finding is supported by the observation that the sEL protected the Δ*graS* mutant against hBD-2 at pH 5.5. Therefore, the GraS holoprotein and its EL motif appear to play complementary, nonredundant roles in sensing specific hBD-2 and conveying adaptive responses to *S. aureus*.

Compared to defensin hNP-1 or hBD-2, the platelet kinocidin mimetic peptide RP-1 exerted a much more complex mechanistic signature that correlated with overall greater susceptibility of *S. aureus* under pH conditions reflecting the bloodstream (pH 7.5) or phagolysosome (pH 5.5). The Δ*graS* mutant, and to a lesser extent the ΔEL mutant, exhibited significant increases in key mechanisms of RP-1 action compared to controls. These mechanisms included increases in CM functions (increased PRM and decreased ENR) and the rapid induction of cell death-like pathway(s) (e.g., increased ANX and CDP) compared to the case for the wild type. Consistent with these findings, the sEL failed to protect any *S. aureus* strain from killing by RP-1 at either pH. Collectively, these findings indicate that RP-1 exerts multiple effects against which the GraS holoprotein or its EL afford protective responses. As RP-1 is not a defensin and differs markedly from hNP-1 or hBD-2 in physicochemical parameters, it is likely that HDPs of distinct structure exert differential actions against which the GraSR system may or may not convey effective resistance. Thus, the considerably greater efficacy compared to other study HDPs suggests that multiple *S. aureus* countermeasures are required for survival in response to RP-1 in the bloodstream or upon interactions of platelets with neutrophils in the acidic phagolysosome (3).

The current findings are consistent with prior data which suggested that GraSR is involved in stress responses and potential mechanisms of action of other peptide anti-infective agents, including daptomycin, LL-37, and the experimental agent brilacidin (20). Other studies have suggested that GraX as well as distinct two-component regulatory systems, such as VraSR and NsaSR, are also involved in *S. aureus* responses to antibiotics (21, 22). Likewise, Stk1/Stp1 signaling appears to cross talk with GraSR in modulating *S. aureus* cell wall charge (23). GraSR may have special relevance to sensing peptide-based agents, including vancomycin (glycopeptide), daptomycin (lipopeptide), and structurally or functionally similar host defense peptides.

In a broader view, the present results also support hypotheses regarding novel mechanisms by which peptide anti-infectives may target *S. aureus* via the GraSR or other systems (30–33). The current data suggest that certain HDPs induce cell death pathways in *S. aureus* such as exist in many other pathogenic bacteria (14, 15, 24–28). For example, when exposed to certain HDPs, *S. aureus* exhibits a sharp increase in annexin V binding (ANX) consistent with access to hydroxylated or otherwise negatively charged lipids (29). *S. aureus* is not known to make phosphatidylserine, the classic binding ligand of annexin V; whether a novel phosphatidylserine intermediate or like lipid emerges as a result of HDP exposure remains to be determined. Likewise, the present data suggest that certain peptides lead to activation of proteases associated with cell death (CDP) which cleave amino acid motifs of caspase- or metacaspase-like substrates. These findings align with responses to HPD actions reflecting pathways (14, 15) that are initiated by altered membrane integrity (e.g., permeability [PRM] and potential [ENR]), leading to chromosomal condensation (e.g., SSC [18; this study) and perturbed macromolecular synthesis (19) and yielding rapid and irreversible cell death (see Fig. S2 in the supplemental material). GraS-mediated countermeasures to these mechanisms appear to involve cell membrane potential (ENR) equilibration and membrane lipid turnover (ANX) to limit further HDP-mediated injury and minimize HDP access to intracellular targets. Thus, deficient GraS holoprotein or EL-mediated functions render *S. aureus* less able to adaptively respond to HDPs and more vulnerable to killing by certain HDPs. While HDP-mediated cell death in *S. aureus* has been understudied to date, the current findings suggest coordinated programs that have the potential to be targeted by novel anti-infective peptides or other agents.

Limitations of the current studies should also be understood. Most importantly, studying the effects of HDPs *in vitro* cannot fully recapitulate the complex conditions under which they function *in vivo*. In addition, only selected mechanistic parameters were studied in the current investigations. Although our novel approach to define mechanistic signatures led to new insights into HDP interactions with *S. aureus*, there are likely other mechanisms of HDP action that are counteracted by GraS/EL which contribute to differences in susceptibility under distinct conditions. Studies using increasingly specific GraS or EL mutations, broader GraSR TCS disruptions, and more specific mechanistic probes should afford a more complete understanding. Investigation of potentially unique structure-mechanism relationships of HDPs with the GraSR system is in progress in our laboratory. Nonetheless, the present results offer new insights into the host-pathogen relationships between HDPs and *S. aureus* adaptive resistance responses, which are likely more complex than has been understood previously.

ACKNOWLEDGMENTS

This study was supported in part by grants from the U.S. National Institutes of Health (1 R21/R33-AI-111661-01 [M.R.Y.], 2 R01-AI091801-06 [A.L.C.], and 5 RO1-AI-039108-17 [A.S.B.]).

FUNDING INFORMATION

U.S. National Institutes of Health provided funding to Michael R Yeaman under grant number 1 R21/R33-AI-111661-01. U.S. National Institutes of Health provided funding to Ambrose Cheung under grant number 2 R01-AI091801-06. U.S. National Institutes of Health provided funding to Arnold Bayer under grant number 5 RO1-AI-039108-17.

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