IAI
Journals.ASM.org

The *Staphylococcus aureus* Two-Component Regulatory System, GraRS, Senses and Confers Resistance to Selected Cationic Antimicrobial Peptides

Soo-Jin Yang,[a,b] Arnold S. Bayer,[a,b] Nagendra N. Mishra,[a] Michael Meehl,[c] Nagender Ledala,[c] Michael R. Yeaman,[a,b] Yan Q. Xiong,[a,b] and Ambrose L. Cheung[c]

Division of Infectious Diseases, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, California, USA[a]; The David Geffen School of Medicine at UCLA, Los Angeles, California, USA[b]; and Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire, USA[c]

The two-component regulatory system, GraRS, appears to be involved in staphylococcal responses to cationic antimicrobial peptides (CAPs). However, the mechanism(s) by which GraRS is induced, regulated, and modulated remain undefined. In this study, we used two well-characterized MRSA strains (Mu50 and COL) and their respective mutants of *graR* and *vraG* (encoding the ABC transporter-dependent efflux pump immediately downstream of *graRS*), and show that (i) the expression of two key determinants of net positive surface charge (*mprF* and *dlt*) is dependent on the cotranscription of both *graR* and *vraG*, (ii) reduced expression of *mprF* and *dlt* in *graR* mutants was phenotypically associated with reduced surface-positive charge, (iii) this net reduction in surface-positive charge in *graR* and *vraG* mutants, in turn, correlated with enhanced killing by a range of CAPs of diverse structure and origin, including those from mammalian platelets (tPMPs) and neutrophils (hNP-1) and from bacteria (polymyxin B), and (iv) the synthesis and translocation of membrane lysyl-phosphatidylglycerol (an *mprF*-dependent function) was substantially lower in *graR* and *vraG* mutants than in parental strains. Importantly, the inducibility of *mprF* and *dlt* transcription via the *graRS-vraFG* pathway was selective, with induction by sublethal exposure to the CAPs, RP-1 (platelets), and polymyxin B, but not by other cationic molecules (hNP-1, vancomycin, gentamicin, or calcium-daptomycin). Although *graR* regulates expression of *vraG*, the expression of *graR* was codependent on an intact downstream *vraG* locus. Collectively, these data support an important role of the *graRS* and *vraFG* loci in the sensing of and response to specific CAPs involved in innate host defenses.

Two-component regulatory systems (TCRS) are prototypical signal transduction mechanisms utilized by most bacteria to monitor and respond to environmental stimuli. These systems typically use a membrane protein sensor and a response regulator activated via a phosphorelay to control target gene transcription (48). It has been recently shown that GraRS, a TCRS in *Staphylococcus aureus*, plays a functional role in expression of the heterotypic versus homotypic vancomycin-intermediate resistance phenotypes (VISA) (9, 18, 26). In addition, mutations in *graRS* or its adjacent ABC transporter genes *vraFG* (encoding an ATPase and a permease) render strains hypersusceptible to vancomycin, as well as to polymyxin B (PMB; a cyclic cationic bacterium-derived peptide) (26). Extending this observation, Li et al. demonstrated that *graRS* (also called *aps*, for antibiotic peptide sensor) and *vraFG* are coinvolved in promoting resistance to distinct cationic antimicrobial peptides (CAPs) in *S. aureus* (23). In several strain backgrounds, GraRS has been shown to regulate expression of the immediate downstream locus, *vraFG*, as well as *mprF*, and *dltABCD* (17, 23, 26). MprF is a lysyl-phosphatidylglycerol (L-PG) synthase which adds positively charged lysine molecules to phosphatidylglycerol within the *S. aureus* cell membrane and also functions as an outer membrane translocase for L-PG (33, 44, 46). Besides MprF, the *dltABCD* operon also contributes to the net positive surface charge by covalently attaching D-alanine to cell wall teichoic acids(46). Since both the *mprF* and *dlt* operons participate in maintaining overall staphylococcal surface positive charge (33, 36, 44), we proposed that mutations in *graRS* could impact susceptibility to CAPs, potentially via a surface charge-dependent mechanism. Indeed, downregulation of these *graRS*-regulated genes in *graRS* mutants has been linked to increased susceptibility to selected CAPs (23, 26). However, the exact molecular mechanisms by which GraRS regulates expression of *mprF*, *dlt*, and *vraFG* genes in mediating CAP resistance are not well understood.

In the present study, we utilized isogenic *graR* and *vraG* parent-mutant strain pairs in two distinct methicillin-resistant *S. aureus* (MRSA) genetic backgrounds, Mu50 and COL, to characterize the contribution of these two linked loci to (i) the induction of *mprF* and *dlt* expression by sublethal concentrations of a range of CAPs, (ii) the modulation of cell surface charge, and (iii) *in vitro* resistance to a cadre of CAPs of distinct structures and origins.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in the present study are listed in Table 1. *S. aureus* Mu50, a prototypical clinical VISA isolate, has been well characterized phenotypically (e.g., homotypic VISA) and is virulent *in vivo* in animal models (7, 8, 22, 45). Similarly, *S. aureus* COL, a prototypical MRSA laboratory strain with a known genome, has been studied extensively *in vitro* and is virulent in a number of animal models (10, 15). All mutant strains were generated by allelic re-

Received 14 July 2011 Returned for modification 26 August 2011
Accepted 4 October 2011
Published ahead of print 10 October 2011
Editor: J. N. Weiser
Address correspondence to Arnold S. Bayer, abayer@labiomed.org.

doi:10.1128/IAI.05669-11

TABLE 1 Strains and plasmid used in this study

S. aureus strain or plasmid	Description	Reference
S. aureus strains		
Mu50	MRSA, VISA, wild-type strain	22
Mu50 Δ*graR*	*graR* in-frame deletion mutant of Mu50	26
Mu50 Δ*vraG*	*vraG* in-frame deletion mutant of Mu50	26
COL	MRSA, wild-type strain	15
COL Δ*graR*	*graR* in-frame deletion mutant of COL	26
COL Δ*vraG*	*vraG* in-frame deletion mutant of COL	26
Plasmid		
pEPSA5::*vraFG*	pEPSA5 expressing *vraFG* genes from Mu50	26

placement using the plasmid pMAD, resulting in deletion of the coding sequence, as described previously (2, 26). For selected studies, we utilized the MU50 parental strain, its *vraG* deletion mutant, and a complemented *vraG* mutant containing a plasmid expressing *vraFG* in *trans* (Table 1).

All *S. aureus* strains were grown in either tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) or Mueller-Hinton broth (MH; Difco Laboratories, Detroit, MI) for individual experiments. 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 strains were maintained at −70°C until thawed before each experimental run.

CAPs. PMB was purchased from Sigma Chemicals Co. (St. Louis, MO). Human neutrophil peptide-1 (hNP-1), a prototypical α-defensin, was purchased from Peptide International (Louisville, KY). RP-1 (a synthetic 18-amino-acid congener modeled in part upon α-helical microbicidal domains of platelet factor-4 family PMPs), was prepared and authenticated as detailed before (49, 56). Of note, the antistaphylococcal mechanisms of RP-1 recapitulate that of native PMP-1 (49). Because of the large amounts of peptide required for both susceptibility and gene induction studies, the RP-1 peptide was used instead of thrombin-induced platelet microbicidal proteins isolated and purified from fresh mammalian platelets (49, 56). Peptides hNP-1 and RP-1 were used for both *in vitro* killing assays (see below) and gene induction studies for the study strains; PMB was used in selected gene induction experiments, The CAPs described above differ in primary and secondary structures, mechanisms of action, and cationicity (ranging from +4 to +6 at neutral pH) (49, 50). For gene induction experiments, these three CAPs were utilized at the following exposure concentrations: hNP-1, 50 μg/ml; RP-1, 50 μg/ml; and PMB, 60 μg/ml. These concentrations did not exert substantial killing of the bacterial inoculum over a 30-min exposure period (data not shown).

In addition to the cationic peptides above, we utilized the following cationic molecules to examine for target gene inducibility: vancomycin, calcium-daptomycin, and gentamicin. Each agent was purchased from their respective pharmaceutical sources, reconstituted according to manufacturer's instructions and utilized at the following concentrations for gene induction studies: vancomycin, 8 μg/ml; calcium-daptomycin, 2 μg/ml; and gentamicin, 512 μg/ml. As for the CAPs described above, these peptide concentrations did not exert substantial killing of the bacterial inoculum over the 30-min gene induction period (data not shown).

CAP susceptibility testing. Standard MIC testing in nutrient broth may underestimate most CAP activities (50, 57). Accordingly, *in vitro* bactericidal assays were carried out with RP-1 and hNP-1 as described previously using a 2-h microdilution method in Eagle minimal essential medium (20, 50). We used an inoculum of 10^3 CFU of exponential-growth-phase cells and CAP concentrations of RP-1 at 3 μg/ml and hNP-1 at 20 μg/ml. These CAP concentrations were selected based on extensive pilot studies showing their inability to substantially reduce starting inocula of either parental strain over the 2-h exposure period. The data were calculated and expressed as the relative percent surviving CFU (± the standard deviation [SD]) of CAP-exposed versus CAP-unexposed cells, with the survival of each parental strain set at 100%. A minimum of three independent runs was performed for each CAP.

Isolation of RNA. For RNA isolation, fresh overnight cultures of *S. aureus* strains were used to inoculate TSB to an optical density at 600 nm (OD_{600}) of 0.1. Cells were harvested during the exponential growth phase (at 2 h), the early stationary phase (at 6 h), and the late stationary phase (at 12 h). Total RNA was isolated from the cell pellets using an RNeasy kit (Qiagen, Valencia, CA) and a FastPrep FP120 instrument (Bio 101, Vista, CA), according to the manufacturer's recommended protocols.

Transcription analyses. Quantitative real-time PCR (qRT-PCR) analyses were performed as described previously (5). Briefly, 1 μg of DNase-treated RNA was reverse transcribed using a SuperScript III first-strand synthesis kit (Invitrogen) according to the manufacturer's protocols. Quantification of the cDNA levels was performed according to 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 (5′-TTGTAGGTTTCGGTGGCTTT-3′) and qRT-mprF-R (5′-GATGCATCGAAAACATGGAA-3′). The *dltA* and *gyrB* genes were similarly detected using respective specific primers as described before (5).

RT-PCR was performed as described previously (52). Briefly, *mprF*, *dltA*, and *vraF* cDNAs were generated using Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, MA) and gene-specific reverse primers (26). The RT-PCR primers used for the detection of the *gyrA* transcripts have been described previously (39). Amplification was performed with initial denaturation at 95°C for 1 min, followed by 18 to 20 cycles of denaturation at 95°C for 30s, annealing at 52°C for 30s, and extension at 72°C for 30s, followed in turn by a final extension at 72°C for 5 min. For all quantitation studies, gene expression of the parental strain was normalized to "1", and those of all mutants were quantified relative to the parental strain accordingly.

To assess the induction of *mprF* and *dlt* by CAPs and other cationic molecules, RT-PCR analyses were performed on RNA samples from cultures of the strain sets exposed to hNP-1, RP-1, PMB, vancomycin, calcium-daptomycin, or gentamicin. Briefly, overnight cultures of the strain sets were used to inoculate 20 ml of TSB to an OD_{600} of 0.1 and allowed to grow for 2 h (~10^8 CFU/ml) before the addition of hNP-1 (50 μg/ml), RP-1 (50 μg/ml), PMB (60 μg/ml), vancomycin (8 μg/ml), calcium-daptomycin (2 μg/ml), or gentamicin (512 μg/ml). The cultures were incubated for an additional 30 min before the RNA was harvested. The sublethality of these CAP concentrations over 30 min was confirmed by quantitative culture (data not shown).

Net cell surface charge. To quantify relative cell surface charge in our parental and mutant constructs, we used one of two assays with polycations: (i) poly-L-lysine (PLL) binding or (ii) cytochrome *c* binding (Sigma). The binding of fluorescein isothiocyanate (FITC)-labeled PLL to the *S. aureus* isolates was performed with a well-described flow cytometric assay (20, 30). In this analysis, the extent of FITC-labeled PLL inversely reflects the relative surface positive charge. The data are expressed as mean relative fluorescence units (± the SD). In selected assays requiring large amounts of reagent, (e.g., CAP induction studies), we also determined binding to cytochrome *c* as a complementary surface charge assay (26, 37). Previous studies have documented a close correlation between these two surface charge assays in *S. aureus* (20, 46, 51, 53). The binding of cytochrome *c* was measured with a spectrophotometric assay which quantifies the amount of the polycation remaining within reaction mixture supernatants following exposure to the study strains, with higher amounts of residual cytochrome *c* in the supernatants correlating with a more positive surface charge (20, 26, 30, 51). The data are calculated and expressed as the

TABLE 2 Effect of *graR* and *vraG* mutations on CAP susceptibilities and PL profiles

Strain	Mean ± SD[a]			
	% Survival after 2-h exposure to:		% L-PG among overall PL content	
	3 μg of RP-1/ml	20 μg of hNP-1/ml	Total L-PG (inner + outer)	Outer L-PG only
S. aureus Mu50 (parental)	100†	100	12.89 ± 0.16	4.42 ± 1.13
Mu50 Δ*vraG*	65.23 ± 2.03*	79.47 ± 7.63*	9.46 ± 2.81*	1.57 ± 0.4*
Mu50 Δ*graR*	33.91 ± 0.81*	59.78 ± 4.01*	9.69 ± 0.49*	1.65 ± 0.24*
S. aureus COL (parental)	100†	100	15.63 ± 0.33	5.28 ± 0.05
COL Δ*vraG*	49.17 ± 12.96*	32.19 ± 2.77*	12.65 ± 1.09*	4.29 ± 1.83
COL Δ*graR*	53.33 ± 18.86*	53.23 ± 10.09*	12.04 ± 1.12*	4.31 ± 0.86

[a] *, $P < 0.05$ versus the parental strains. †, Parental strains were normalized to 100%.

percentage of cytochrome *c* bound to the cell. The data shown for both surface charge assays are the means (± the SD) of three independent experiments.

Membrane PL contents. MprF is an enzyme involved in the synthesis and outer membrane translocation of lysyl-phosphatidylglycerol (L-PG), one of the three major *S. aureus* membrane phospholipids (PLs; L-PG, PG, and cardiolipin [CL]) (12, 33, 36). To quantify the relative proportions of these three PLs in our strain sets, membrane PLs were extracted from *S. aureus* cell pellets as described previously (30). The target PLs were separated by two-dimensional thin-layer chromatography, removed from the plates, and then quantified spectrophotometrically by a previously described chemical assay (20, 30). The proportion of synthesized L-PG that was translocated to the outer cell membrane leaflet was quantified spectrophotometrically, as detailed before using the L-PG outer-membrane-impermeable UV probe, fluorescamine (29, 30). The data were expressed as the proportionalities (± the SD) of the three PLs. At least three independent experiments were performed to analyze the PL contents.

Cell membrane fluidity. The relative membrane order characteristics (i.e., fluidity/rigidity) of *S. aureus* can independently modify interactions with CAPs (29). To assure that any differences seen among the study strains in CAP susceptibility profiles were not attributable to membrane order perturbations, membrane fluidity was determined by fluorescence polarization spectroscopy using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) as described previously (4, 29). The data were quantified by determination of the polarization index (± the SD) (20, 50). These assays were performed at least six times for each strain on separate days.

Statistics. Data were analyzed by using Kruskal-Wallis analysis of variance, with a *P* value of <0.05 considered significant.

RESULTS

CAP susceptibility. To assess the role of *graRS* and *vraFG* in resistance to structurally distinct host defense CAPs, we examined the *in vitro* susceptibility profiles of *graR* and *vraG* knockout mutants of Mu50 and COL against two prototypical antistaphylococcal CAPs: RP-1 (a synthetic congener of platelet factor-4 family of microbicidal molecules) (55, 56) and hNP-1 (a human neutrophil CAP). As shown in Table 2, deletion of *graR* or *vraG* resulted in significantly increased susceptibilities to both RP-1 and hNP-1 killing of the mutants compared to the respective parental strains ($P < 0.05$). Meehl et al. have also shown that both knockout mutants above were significantly more susceptible to vancomycin and polymyxin B (PMB) in these two strain backgrounds (26), but exhibited only slighly enhanced susceptibility to, calcium-daptomycin and gentamicin, both cationic antimicrobials (26).

Regulation of *mprF* and *dltABCD* expression by GraRS and VraFG. To determine the effect of *graR* and *vraG* mutations on expression of *mprF* and *dlt* genes, qRT-PCR analyses were performed on RNA samples isolated from cultures of Mu50 strain set. As shown in Fig. 1A and B, in agreement with previous findings with MRSA strain MW2 (23), transcription of the *mprF* and *dlt* genes in VISA strain Mu50 was significantly decreased in the isogenic *graR* mutant ~4-fold and ~8-fold, respectively, during the early exponential growth phase (2 h of growth; $P < 0.01$). Similar to the *graR* mutant, the *vraG* mutant strain also displayed significantly decreased early-exponential-phase expression of *mprF* and *dlt* compared to the parental Mu50 strain (~2.5- and ~5-fold, respectively). Similar, statistically relevant expression outcomes were noted at the late exponential phase (6 h growth) for both *mprF* and *dlt* expression. By the stationary phase (12 h growth), the expression differences between parental and *graR* or *vraG* mutants were minimal. These data demonstrate that *graRS* regulates expression of *mprF* and *dlt*, at least in part, via a *vraFG*-dependent mechanism(s), in a growth-phase-dependent manner.

Furthermore, the effect of *vraG* on the expression of *graRS* was also assessed by qRT-PCR. As shown in Fig. 1C, the *vraG* mutant exhibited ~9-fold-lower *graS* expression than the parent during exponential growth, whereas the complemented *vraG* mutant (containing the plasmid pEPSA5 expressing *vraFG*) exhibited restored parental-level *graS* expression. These data demonstrated the interdependency of *graRS* and *vraFG* genes and suggest the presence of a positive-feedback loop between *graRS* and *vraFG*.

Effect of the *graRS* and *vraFG* mutations on cell surface charge. Since GraRS and VraFG positively coregulate expression of *mprF* and *dltABCD*, each of which, in turn, is critical for maintaining overall positive surface charge, we assessed the relative surface charges of our strain sets. As shown in Fig. 2, the net positive surface charges of both the *graR* and *vraG* mutants were significantly lower than that of the parental strain Mu50, as determined by enhanced fluorescent-PLL binding ($P < 0.01$). Similar reductions in the net positive surface charge were also observed in the *graR* and *vraG* mutant strains compared to the parental COL strain ($P < 0.01$; data not shown).

Effect of the *graR* and *vraG* mutations on synthesis and translocation of L-PG. The synthesis and translocation of the positively charged L-PG molecule (which confers increased net positive charge to the cell surface) is dependent on expression of *mprF* (12, 20, 31, 44, 46, 54). Since *graRS* regulates the expression of *mprF* and *graRS* expression appears to be dependent on an intact *vraFG*, the impact of *graR* and *vraG* on the synthesis and "flipping" of L-PG to the outer surface of the cell membrane was investigated. As shown in Table 2, the total proportion of L-PG synthesized within the overall PL content was significantly re-

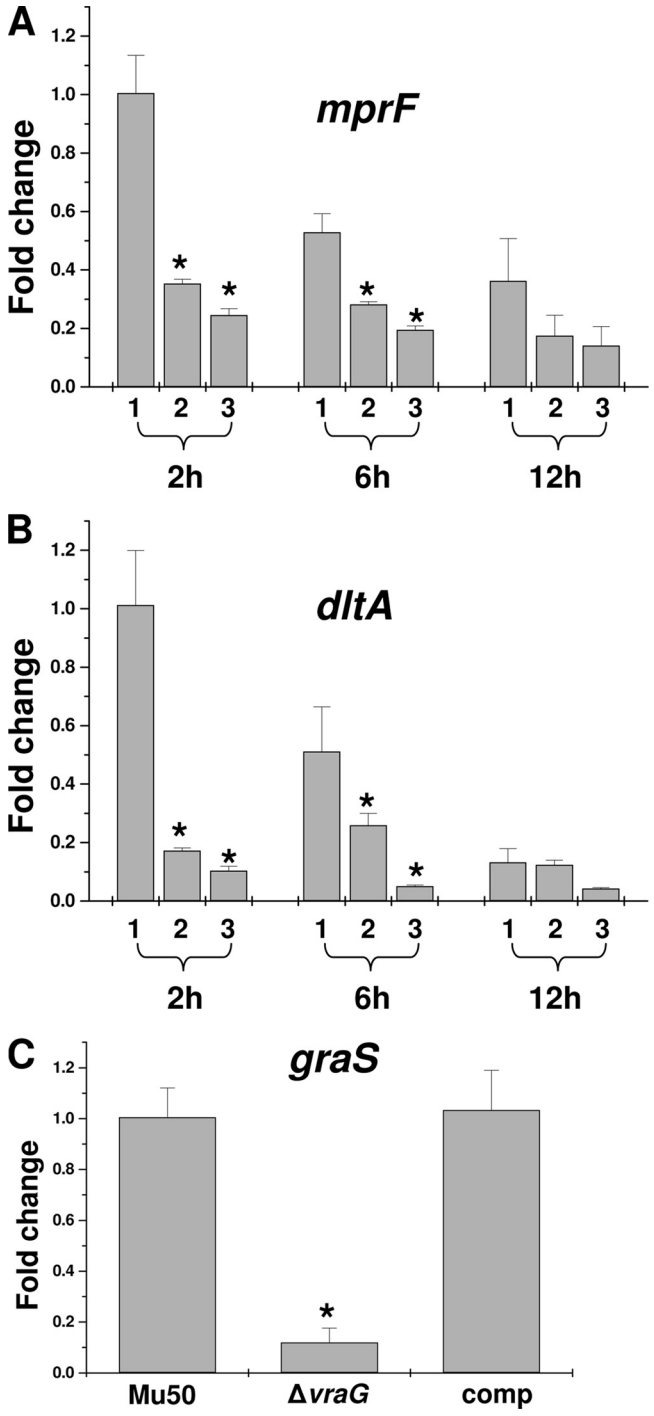

FIG 1 Transcriptional regulation of *mprF* and *dlt* expression by GraRS and VraFG. (A) Relative transcription level of *mprF* in parental Mu50 (1), its Δ*vraG* mutant (2), and its Δ*graR* mutant (3) strains. (B) Relative transcription of the *dlt* in Mu50 (bar 1), Δ*vraG* (bar 2), and Δ*graR* (bar 3) strains. (C) Effect of *vraG* mutation on *graRS* transcription. RNA samples from parental Mu50, its Δ*vraG* mutant, and its complemented Δ*vraG* mutant containing pEPSA5::*vraFG* were isolated at 2 h postinoculation (exponential growth phase) and subjected to a qRT-PCR analysis. *, $P < 0.01$.

duced in the *graR* and *vraG* mutant strains compared to both respective parental strains ($P < 0.05$). The amount of L-PG translocated to the outer cell membrane was also reduced, especially for the Mu50 strain set. The impact of *vraG* and *graR* mutations on translocation of L-PG failed to reach statistical significance in the COL strain set, indicating that the net impact of the GraRS-VraFG regulatory system on global MprF protein function (i.e., L-PG synthesis and translocation) may be strain specific. Of note, these phenotypic data on L-PG production and flipping roughly parallel the impact of the *graR* and *vraG* mutations on relative positive surface charge noted above.

Membrane fluidity. Cell membrane fluidity analyses revealed no substantive differences among the parental, *graR*, and *vraG* mutant isolates in either strain background (data not shown).

Induction of *mprF* and *dlt* expression by specific CAPs via GraRS-VraFG network. Previous studies have shown that certain CAPs (e.g., hBD-3; indolicidin) can induce expression of *graRS* and its downstream regulated genes, including *mprF* and *dlt* (23). To assess whether expression of *mprF* is inducible by our study CAPs (hNP-1, RP-1, or PMB), RT-PCR analyses were performed in parental strain Mu50. As shown in Fig. 3, both RP-1 (50 μg/ml) and PMB (60 μg/ml), but not hNP-1 (50 μg/ml), were associated with increased transcription of *mprF*. Similarly, expression of *dlt* was induced by RP-1 and PMB, but not by hNP-1. The expression of both *mprF* and *dlt* in MRSA strain COL was also inducible with PMB at the 30- and 60-μg/ml concentrations (data not shown).

Next, using PMB as a prototypical inducer of *mprF* and *dlt* expression in Mu50, we hypothesized that this induction event is dependent on an intact *graRS-vraFG* network. Thus, RT-PCR analyses were also performed on RNA samples from the parental Mu50 strain and its isogenic *graR* and *vraG* mutant strains, in the presence or absence of PMB. As predicted, induction of *mprF* and *dlt* expression by PMB was dependent on an intact *graRS* locus (Fig. 4). Similar to the *graR* mutant, the *vraG* mutant strain also failed to induce expression of *mprF* and *dltA* in the presence of PMB. In addition, the *vraG* mutant strain exhibited a more pronounced effect on *dlt* expression than on *mprF* expression. Collectively, these data indicate that induction of *mprF* and *dlt* gene expression is dependent on the TCRS GraRS, as well as the ABC transporter system, VraFG.

Induction of positive cell surface charge by PMB. To determine whether the inducibility of *dlt* and *mprF* by PMB documented above translated into a detectable phenotype in terms of enhanced surface positive charge, we utilized the cytochrome *c* binding assay. As seen in Fig. 5, the relative positive surface charge of the parental strain Mu50 increased significantly with PMB induction compared to the uninduced control (i.e., reduced cytochrome *c* binding; $P < 0.05$). In contrast, there was no substantial change in surface charge in the presence of PMB for the *vraG* and *graR* mutant strains. Of interest, the association of PMB-induced *dlt* and *mprF* gene expression with enhancement of the positive surface charge also correlated with increased synthesis and outer membrane translocation of L-PG (1.52- and 1.62-fold increases in total L-PG and outer L-PG, respectively, in the presence of 30 μg of PMB/ml versus the uninduced control; $P < 0.05$). Thus, PMB-triggered induction of *dlt* and *mprF* gene expression translated well into the predicted phenotypic outcomes.

DISCUSSION

Cationic antimicrobial peptides (CAPs) are crucial components of the innate immune system. Their production is evolutionarily conserved in virtually all groups of organisms, including vertebrates, invertebrates, and plants (3, 16, 34, 47, 55). Such molecules are typically amphipathic, with a net positive charge at physiolog-

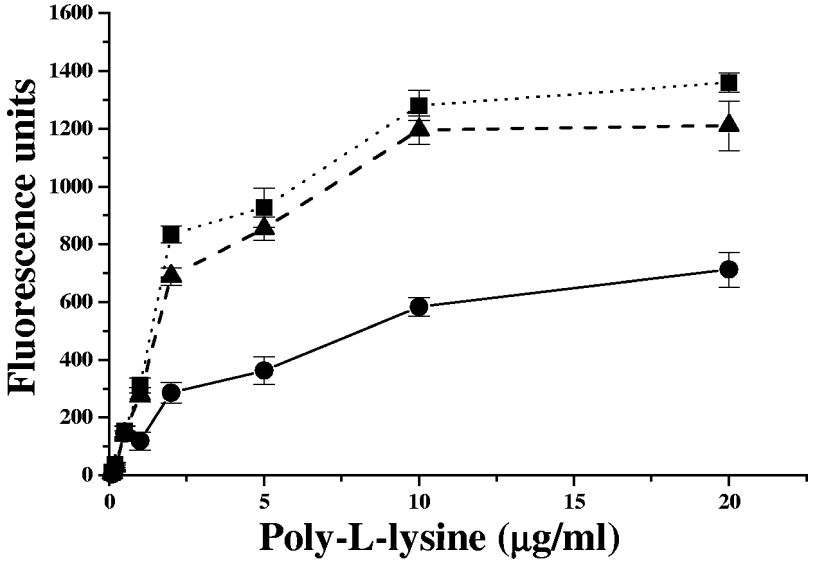

FIG 2 Binding of FITC-labeled PLL to whole *S. aureus* cells. The graph shows the relative fluorescence units (± the SD) of FITC-labeled PLL bound to Mu50 (●), Δ*vraG* (■), and Δ*graR* (▲) whole cells: the lower the number of fluorescence units, the greater the PLL repulsion and the more positively charged the *S. aureus* cell envelope (30).

ical pH, features believed to be important in targeting these molecules to the relatively negatively charged bacterial cell membranes (3, 55, 57). In addition to damaging target bacterial membranes by a variety of mechanisms, CAPs may also affect vital intracellular processes including biosynthesis of nucleic acids, proteins, and cell wall components (6, 35, 50). Accordingly, *S. aureus* has developed a variety of resistance strategies to prevent peptide-induced lethality for a wide range of CAPs from epithelial cells (e.g., LL-37 and hBD-3), phagocytic cells (e.g., hNP-1), and platelets (e.g., tPMPs) (3, 23, 38, 46, 55, 57). These putative "CAP-evasive" mechanisms in *S. aureus* may include proteolytic degradation of CAPs (42), trapping of CAPs within the cell wall to inhibit access to target cell membranes (19) and increase the net surface positive charge to reduce interactions with CAPs at the bacterial surface (46), efflux of the CAPs by transport pumps (26), and enhanced production of carotenoid pigments to alter membrane fluidity properties (28).

Recently, it has been shown that the TCRS, GraRS, may well play a pivotal role in resistance of *S. aureus* to CAPs by acting as a CAP "sensor" (23, 26) (Fig. 6). In these studies, it was apparent that there was a relatively selective range of CAPs that could activate the *graRS* system in *S. aureus* (e.g., the human cathelicidin, LL-37, or indolicidin, but not hBD-3 or histadin). Li et al. also showed that GraRS appeared to regulate expression of *dltABCD* and *mprF* (two important positive surface charge maintenance genes in *S. aureus*). Furthermore, deletion of the *graRS* locus led to significantly increased *in vitro* susceptibility to the positively charged molecule, vancomycin, and the bacterial-derived CAP, PMB as we previously reported (26). In addition to the *graR* and *graS*, the first gene of *graRS* (*apsRS*) operon encodes GraX (ApsX), a protein of unknown function that may also play a role in CAP resistance (23, 24, 26). Our studies, as well as that of Li et al., demonstrated *graRS*-mediated regulation of the downstream *vraFG*, encoding an ABC-transporter-dependent efflux pump (23, 26). This pattern of downstream regulation of TCRS following CAP sensing has also been described in other Gram-positive bacteria. For example, upon exposure to bacitracin, the BceRS TCRS in *B. subtilis* induces expression of the bacitracin transporter, BceAB, by upregulating *bceRS* transcription and subsequent expression of the *bceAB* operon (32).

Despite these findings, the specific contribution of GraRS to *mprF* and *dltABCD* expression and how this regulation system might, in turn, impact susceptibility to specific host defense CAPs

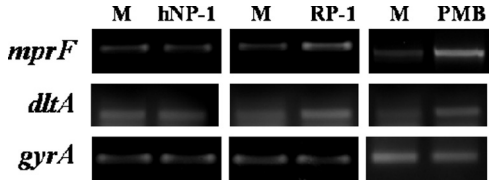

FIG 3 Induction of *mprF* and *dlt* transcription by specific CAPs. RT-PCR analyses were performed on RNA samples from cultures of Mu50 strain exposed to hNP-1 (50 μg/ml), RP-1 (50 μg/ml), or PMB (60 μg/ml) for 30 min during exponential growth. M, CAP-free medium alone.

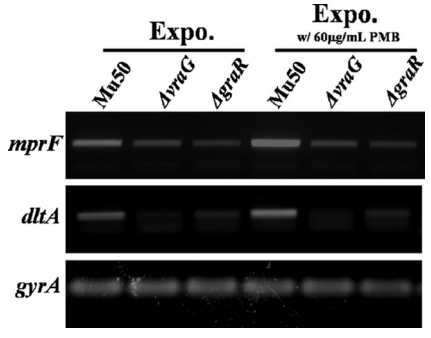

FIG 4 Effect of *vraFG* and *graRS* on PMB-induced expression of *mprF* and *dlt* genes. RNA samples were isolated from exponential-phase cultures of the strain set in the presence or absence of PMB (60 μg/ml) and subjected to RT-PCR to detect the transcription of *mprF*, *dltA*, and *gyrA*.

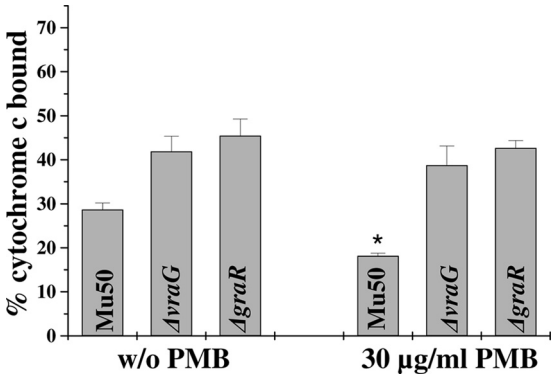

FIG 5 Effect of PMB induction of the *graRS* network on relative positive surface charge. The graph shows the percentage of cytochrome *c* bound after 10 min of incubation in parental strain Mu50 compared to its Δ*vraG* and Δ*graR* deletion mutants. The data represent the means and SDs from three independent experiments. *, $P < 0.05$. Mu50 parent, PMB-induced versus uninduced.

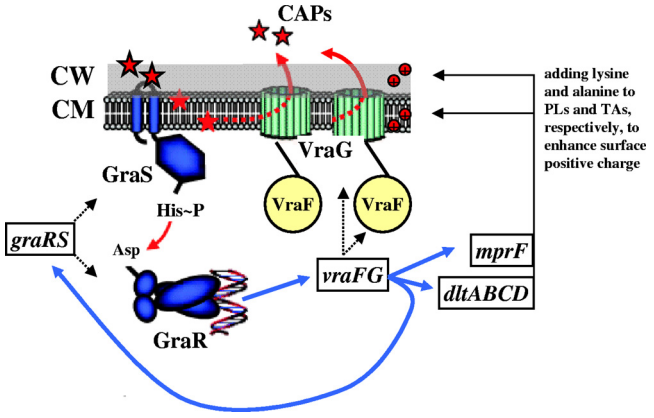

FIG 6 Putative model of the GraRS-VraFG network. In this model, GraS senses CAPs selectively, resulting in its autophosphorylation. GraS then phosphorylates GraR, driving *mprF* and *dlt* expression in a *vraFG*-dependent manner. VraFG can also affect expression of *mprF* and *dlt* via a positive feedback mechanism. MprF and DltABCD encode proteins which add positively charged lysine and D-alanine to membrane phospholipids and cell wall teichoic acids, respectively, to increase the surface positive charge (44, 46, 53, 54). VraFG may function to efflux CAPs (26). PLs, phospholipids; TAs, teichoic acids. A blue arrow indicates transcriptional upregulation.

remained undefined. In the present study, we used two well-known and well-characterized *S. aureus* strains (VISA Mu50 and MRSA COL) and their isogenic *graR* and *vraG* mutants to explore these issues. A number of interesting findings emerged from these investigations. First, both *vraG* and *graR* mutant strains displayed significantly decreased intrinsic transcription of *mprF* and *dltA* compared to their respective parental strains. Of interest, *graRS* has been known to influence *mprF* and/or *dlt* expression in some, but not all, previously studied strains (17, 23), Importantly, the present data show that *vraFG* could also alter the expression of *mprF* and *dlt* via *graRS*, a phenomenon not well appreciated previously (i.e., *graRS* expression was downregulated in *vraG* mutant strains).

Recently, it has been shown that the *agr* operon (via *agrC-agrA*), a quorum-sensing system, is involved in the regulation of *graRS* expression (13, 25). In addition, the *graRS* operon also appears to interact with the WalKR (YycFG) TCRS, which is involved in cell wall lipid metabolism (11). Thus, it is possible that the mutation in *vraG* may have affected regulation of overlapping TCRS, such as Agr, resulting in indirect downregulation of *graRS* expression. Taken together, these data provide strong support for our notion of a *graRS-vraFG* regulatory "loop" with a positive-feedback limb (Fig. 6).

Second, although previous studies have shown regulation of *mprF* and/or *dlt* by *graRS* (23, 40), these studies did not assess the phenotypic consequence of these expression pathways in detail. In the current study, we documented changes in L-PG synthesis and outer membrane translocation that paralleled perturbations in the *mprF* expression profiles in *graR* or *vraG* mutant strains. It should be emphasized that, although the absolute quantitative changes in L-PG synthesis and flipping between parental and mutant strains was relatively small, such modest alterations in membrane lipids can have profound physiologic impacts (1, 41). In addition, lowered expression of both *mprF* and *dlt* in the above mutants correlated well with both their reduced positive surface charge phenotype and their increased susceptibility to killing by two mammalian host defense CAPs of platelet and polymorphonuclear leukocyte origins. Recent data from our laboratory and others suggest an alternative to a strict surface-mediated charge-repulsion mechanism in circumstances of enhanced *mprF*-mediated L-PG synthesis and/or translocation. Kilelee et al. (21) demonstrated the likelihood of formation of "lipid domains" consisting of CAPs plus anionic membrane PLs (e.g., PG or cardiolipin). Thus, in the presence of excess L-PG (e.g., during *graRS* induction), the relative proportion of membrane PG and/or cardiolipin is reduced, providing fewer "docking sites" for initial membrane localization of CAPs. Whether the *vraFG* ABC transporter system can also impact CAP susceptibilities in *S. aureus* via a putative CAP-efflux mechanism remains to be defined.

Third, as noted above, *graRS* in *S. aureus* induces expression of *mprF*, *dltABCD*, and *vraFG* in a relatively CAP-selective manner (23). In the present study, we found that the expression of the *graRS*-mediated effector genes, *mprF* and *dlt*, was induced upon exposure to the platelet CAP congener, RP-1, and the bacterial CAP, PMB, but not by the neutrophil CAP, hNP-1. In addition, three other positively charged compounds—vancomycin, gentamicin, and calcium-daptomycin—also failed to induce transcription of *mprF* (data not shown). Collectively, these findings are in line with those of Li et al. (23) and suggest a relative selectivity in the induction of the sensor GraS by specific CAPs, thus suggesting that GraS may be a putative CAP membrane sensor protein in *S. aureus*. Ongoing investigations are focused on defining specific structural features of individual CAPs that may trigger GraRS sense and response functions.

Fourth, it is noteworthy that there was a relative disconnect between the ability to induce the *graRS* TCRS and phenotypic resistance to the same cadre of potential inducer molecules. Thus, for example, both RP-1 and PMB each induced the *graRS* network, and strains with an intact *graRS* pathway were substantially more resistant to killing *in vitro* by these same peptides. In contrast, neither hNP-1 nor vancomycin could induce the *graRS* network, and yet strains with an intact *graRS* pathway were substantially more resistant to killing *in vitro* by these same peptides versus corresponding *graRS* mutants (26). Furthermore, certain cationic antibiotics (gentamicin and daptomycin) were not sensed by the *graRS* system, and *in vitro* susceptibility profiles to these

compounds were not impacted by genetic perturbations within this network. Collectively, these observations (i) confirm relatively selective cationic peptide sensing by the *graRS* system and (ii) phenotypic changes induced by sense response to one peptide may provide *in vitro* cross-resistance to killing by other structurally unrelated cationic molecules (e.g., induction by RP-1 with resultant enhanced resistance to the noninducer, hNP-1). This suggests that there may be common mechanisms of resistance among such diverse CAPs.

We recognize that there are several limitations to the CAP susceptibility and induction studies above: (i) these investigations were performed in relatively austere artificial media, not closely reflective of the host milieu (e.g., absence of plasma proteins); (ii) moreover, invading bacteria are likely exposed to a cadre of CAPs at sites of infection; and (iii) physiologic CAP concentrations are undoubtedly orders of magnitude above those used in our *in vitro* analyses (14, 27, 43). Future studies will be designed to adjudicate these limitations.

In contrast to *S. aureus*, the GraRS homolog in *S. epidermidis* induced *mprF* and *dltABCD* expression upon exposure to CAPs nonselectively (24). Although there is a significant amino acid sequence homology (70%) between the GraS (sensor kinase) of *S. aureus* and *S. epidermidis*, sequence divergence occurs in the 9-amino-acid extracellular loop (33%) flanked by two transmembrane domains at the N terminus of the *S. epidermidis* GraS protein (23, 26). Of interest, when the GraS extracellular loop of *S. aureus* is replaced with that from *S. epidermidis*, expression of *dlt* was induced promiscuously upon exposure to a number of CAPs (e.g., hBD3), similar to what has been found with the parental *S. epidermidis* strain (23). In addition, we have recently shown that deletion of this 9-amino-acid extracellular loop in *S. aureus* could render the resultant mutant strain incapable of responding to CAPs that normally induce *mprF* and *dltABCD* expression in the parent (e.g., RP-1 and PMB [unpublished data]). At present, we are attempting to define the critical residues within the GraS extracellular loop of *S. aureus* that are involved in induction of GraRS-regulated genes upon exposure to host defense CAPs.

ACKNOWLEDGMENTS

This study was supported by grants from the National Institutes of Health (AI-39108 to A.S.B., AI-39001 to M.R.Y., and AI-56114 to A.L.C.) and American Heart Association grants SDG0630219N and AID09GRNT2180065 to Y.Q.X.

We thank Andres Taboada for excellent technical assistance with the CAP susceptibility testing.

REFERENCES

1. **Aricha B, et al.** 2004. Differences in membrane fluidity and fatty acid composition between phenotypic variants of *Streptococcus pneumoniae.* J. Bacteriol. **186**:4638–4644.
2. **Arnaud M, Chastanet A, Debarbouille M.** 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. Appl. Environ. Microbiol. **70**:6887–6891.
3. **Bader MW, et al.** 2005. Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell **122**:461–472.
4. **Bayer AS, et al.** 2000. *In vitro* resistance of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein is associated with alterations in cytoplasmic membrane fluidity. Infect. Immun. **68**:3548–3553.
5. **Bertsche U, et al.** 2011. Correlation of daptomycin resistance in a clinical *Staphylococcus aureus* strain with increased cell wall teichoic acid production and D-alanylation. Antimicrob. Agents Chemother. **55**:3922–3928.
6. **Brogden KA.** 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. **3**:238–250.
7. **Climo MW, Patron RL, Archer GL.** 1999. Combinations of vancomycin and β-lactams are synergistic against staphylococci with reduced susceptibilities to vancomycin. Antimicrob. Agents Chemother. **43**:1747–1753.
8. **Cui L, Murakami H, Kuwahara-Arai K, Hanaki H, Hiramatsu K.** 2000. Contribution of a thickened cell wall and its glutamine nonamidated component to the vancomycin resistance expressed by *Staphylococcus aureus* Mu50. Antimicrob. Agents Chemother. **44**:2276–2285.
9. **Cui L, Tominaga E, Neoh H-M, Hiramatsu K.** 2006. Correlation between reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus.* Antimicrob. Agents Chemother. **50**:1079–1082.
10. **De Lencastre H, et al.** 1999. Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in *Staphylococcus aureus* strain COL that impact on the expression of resistance to methicillin. Microb. Drug Resist. **5**:163–175.
11. **Dubrac S, Bisicchia P, Devine KM, Msadek T.** 2008. A matter of life and death: cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. Mol. Microbiol. **70**:1307–1322.
12. **Ernst C, et al.** 2009. The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. PLoS Pathog. **5**:e1000660.
13. **Falord M, Mader U, Hiron A, Debarbouille M, Msadek T.** 2011. Investigation of the *Staphylococcus aureus* GraSR regulon reveals novel links to virulence, stress response and cell wall signal transduction pathways. PLoS One **6**:e21323.
14. **Ganz T, Selsted ME, Lehrer RI.** 1990. Defensins. Eur. J. Haematol. **44**:1–8.
15. **Gill SR, et al.** 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. J. Bacteriol. **187**:2426–2438.
16. **Hancock REW, Diamond G.** 2000. The role of cationic antimicrobial peptides in innate host defenses. Trends Microbiol. **8**:402–410.
17. **Herbert S, et al.** 2007. Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. PLoS Pathog. **3**:e102.
18. **Howden BP, et al.** 2008. Genomic analysis reveals a point mutation in the two-component sensor gene *graS* that leads to intermediate vancomycin resistance in clinical *Staphylococcus aureus.* Antimicrob. Agents Chemother. **52**:3755–3762.
19. **Jin T, et al.** 2004. *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. J. Immunol. **172**:1169–1176.
20. **Jones T, et al.** 2008. Failures in clinical treatment of *Staphylococcus aureus* infection with daptomycin are associated with alterations in surface charge, membrane phospholipid asymmetry, and drug binding. Antimicrob. Agents Chemother. **52**:269–278.
21. **Kilelee E, Pokorny A, Yeaman MR, Bayer AS.** 2010. Lysylphosphatidylglycerol attenuates membrane perturbation rather than surface association of the cationic antimicrobial peptide 6W-RP-1 in a model membrane system: implications for daptomycin resistance. Antimicrob. Agents Chemother. **54**:4476–4479.
22. **Kuroda M, et al.** 2001. Whole genome sequencing of meticillin-resistant *Staphylococcus aureus.* Lancet **357**:1225–1240.
23. **Li M, et al.** 2007. The antimicrobial peptide-sensing system *aps* of *Staphylococcus aureus.* Mol. Microbiol. **66**:1136–1147.
24. **Li M, et al.** 2007. Gram-positive three-component antimicrobial peptide-sensing system. Proc. Natl. Acad. Sci. U. S. A. **104**:9469–9474.
25. **Matsuo M, Oogai Y, Kato F, Sugai M, Komatsuzawa H.** 2011. Growth-phase dependence of susceptibility to antimicrobial peptides in *Staphylococcus aureus.* Microbiology **157**:1786–1797.
26. **Meehl M, Herbert S, Gotz F, Cheung A.** 2007. Interaction of the GraRS two-component system with the VraFG ABC transporter to support vancomycin-intermediate resistance in *Staphylococcus aureus.* Antimicrob. Agents Chemother. **51**:2679–2689.
27. **Mezzano S, et al.** 1992. Glomerular localization of platelet factor 4 in streptococcal nephritis. Nephron **61**:58–63.
28. **Mishra NN, et al.** 2011. Carotenoid-related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. Antimicrob. Agents Chemother. **55**:526–531.
29. **Mishra NN, et al.** 2009. Analysis of cell membrane characteristics of *in vitro*-selected daptomycin-resistant strains of methicillin-resistant *Staphylococcus aureus* (MRSA). Antimicrob. Agents Chemother. **53**:2312–2318.

30. **Mukhopadhyay K, et al.** 2007. *In vitro* susceptibility of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein-1 (tPMP-1) is influenced by cell membrane phospholipid composition and asymmetry. Microbiology **153**:1187–1197.
31. **Nishi H, Komatsuzawa H, Fujiwara T, McCallum N, Sugai M.** 2004. Reduced content of lysyl-phosphatidylglycerol in the cytoplasmic membrane affects susceptibility to moenomycin, as well as vancomycin, gentamicin, and antimicrobial peptides, in *Staphylococcus aureus.* Antimicrob. Agents Chemother. **48**:4800–4807.
32. **Ohki R, et al.** 2003. The BceRS two-component regulatory system induces expression of the bacitracin transporter, BceAB, in *Bacillus subtilis*. Mol. Microbiol. **49**:1135–1144.
33. **Oku Y, Kurokawa K, Ichihashi N, Sekimizu K.** 2004. Characterization of the *Staphylococcus aureus mprF* gene, involved in lysinylation of phosphatidylglycerol. Microbiology **150**:45–51.
34. **Pelegrini P, et al.** 2009. A novel antimicrobial peptide from *Crotalaria pallida* seeds with activity against human and phytopathogens. Curr. Microbiol. **59**:400–404.
35. **Peschel A.** 2002. How do bacteria resist human antimicrobial peptides? Trends Microbiol. **10**:179–186.
36. **Peschel A, et al.** 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. J. Exp. Med. **193**:1067–1076.
37. **Peschel A, et al.** 1999. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J. Biol. Chem. **274**:8405–8410.
38. **Peschel A, Sahl H-G.** 2006. The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat. Rev. Microbiol. **4**:529–536.
39. **Rice KC, et al.** 2003. The *Staphylococcus aureus cidAB* operon: evaluation of its role in regulation of murein hydrolase activity and penicillin tolerance. J. Bacteriol. **185**:2635–2643.
40. **Sass P, Bierbaum G.** 2009. Native *graS* mutation supports the susceptibility of *Staphylococcus aureus* strain SG511 to antimicrobial peptides. Int. J. Med. Microbiol. **299**:313–322.
41. **Shinitzky M, Barenholz Y.** 1978. Fluidity parameters of lipid regions determined by fluorescence polarization. Biochim. Biophys. Acta **515**: 367–394.
42. **Sieprawska-Lupa M, et al.** 2004. Degradation of human antimicrobial peptide LL-37 by *Staphylococcus aureus*-derived proteinases. Antimicrob. Agents Chemother. **48**:4673–4679.
43. **Soong LB, Ganz T, Ellison A, Caughey GH.** 1997. Purification and characterization of defensins from cystic fibrosis sputum. Inflamm. Res. **46**:98–102.
44. **Staubitz P, Neumann H, Schneider T, Wiedemann I, Peschel A.** 2004. MprF-mediated biosynthesis of lysylphosphatidylglycerol, an important determinant in staphylococcal defensin resistance. FEMS Microbiol. Lett. **231**:67.
45. **Utaida S, Pfeltz RF, Jayaswal RK, Wilkinson BJ.** 2006. Autolytic properties of glycopeptide-intermediate *Staphylococcus aureus* Mu50. Antimicrob. Agents Chemother. **50**:1541–1545.
46. **Weidenmaier C, et al.** 2005. DltABCD- and MprF-mediated cell envelope modifications of *Staphylococcus aureus* confer resistance to platelet microbicidal proteins and contribute to virulence in a rabbit endocarditis model. Infect. Immun. **73**:8033–8038.
47. **Welling MM, et al.** 1998. Antibacterial activity of human neutrophil defensins in experimental infections in mice is accompanied by increased leukocyte accumulation. J. Clin. Invest. **102**:1583–1590.
48. **West AH, Stock AM.** 2001. Histidine kinases and response regulator proteins in two-component signaling systems. Trends Biochem. Sci. **26**: 369.
49. **Xiong YQ, Bayer AS, Elazegui L, Yeaman MR.** 2006. A synthetic congener modeled on a microbicidal domain of thrombin-induced platelet microbicidal protein-1 recapitulates staphylocidal mechanisms of the native molecule. Antimicrob. Agents Chemother. **50**:3786–3792.
50. **Xiong YQ, Mukhopadhyay K, Yeaman MR, Adler-Moore J, Bayer AS.** 2005. Functional interrelationships between cell membrane and cell wall in antimicrobial peptide-mediated killing of *Staphylococcus aureus.* Antimicrob. Agents Chemother. **49**:3114–3121.
51. **Yang S-J, et al.** 2010. Cell wall thickening is not a universal accompaniment of the daptomycin nonsusceptibility phenotype in *Staphylococcus aureus*: evidence for multiple resistance mechanisms. Antimicrob. Agents Chemother. **54**:3079–3085.
52. **Yang S-J, et al.** 2005. A LysR-type regulator, CidR, is required for induction of the *Staphylococcus aureus cidABC* operon. J. Bacteriol. **187**: 5893–5900.
53. **Yang SJ, et al.** 2009. Enhanced expression of *dltABCD* is associated with development of daptomycin nonsusceptibility in a clinical endocarditis isolate of *Staphylococcus aureus.* J. Infect. Dis. **200**:1916–1920.
54. **Yang SJ, et al.** 2009. Regulation of *mprF* in daptomycin-nonsusceptible *Staphylococcus aureus.* Antimicrob. Agents Chemother. **53**:2636–2637.
55. **Yeaman MR, Bayer AS, Koo S-P, Foss W, Sullam PM.** 1998. Platelet microbicidal proteins and neutrophil defensin disrupt the *Staphylococcus aureus* cytoplasmic membrane by distinct mechanisms of action. J. Clin. Invest. **101**:178–187.
56. **Yeaman MR, Gank KD, Bayer AS, Brass EP.** 2002. Synthetic peptides that exert antimicrobial activities in whole blood and blood-derived matrices. Antimicrob. Agents Chemother. **46**:3883–3891.
57. **Yeaman MR, Puentes SM, Norman DC, Bayer AS.** 1992. Partial characterization and staphylocidal activity of thrombin-induced platelet microbicidal protein. Infect. Immun. **60**:1202–1209.