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RESEARCH ARTICLE

Novel Functions and Signaling Specificity for the GraS Sensor Kinase of *Staphylococcus aureus* in Response to Acidic pH

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ABSTRACT Although the GraS sensor kinase of *Staphylococcus aureus* is known for the sensing of and resistance to cationic antimicrobial peptides (CAMPs), we recently established that it also signals in response to acidic pH, which is encountered on human skin concurrently with CAMPs, antimicrobial unsaturated free fatty acids (uFFA), and calcium. We therefore evaluated how these environmental signals would affect GraS function and resistance to antimicrobial uFFA. Growth at pH 5.5 promoted increased resistance of *S. aureus* USA300 to linoleic and arachidonic acids but not palmitoleic or sapienic acid. However, enhanced resistance to these $C_{16:1}$ uFFA was achieved by supplementing acidic medium with 0.5 mM calcium or subinhibitory CAMPs. Enhanced resistance to uFFA at acidic pH was dependent on GraS and GraS-dependent expression of the lysyl-phosphatidylglycerol synthase enzyme MprF, through a mechanism that did not require the lysyl-transferase function of MprF. In addition to enhanced resistance to antimicrobial uFFA, acidic pH also promoted increased production of secreted proteases in a GraS-dependent manner. During growth at pH 5.5, downstream phenotypes of signaling through GraS, including resistance to uFFA, MprF-dependent addition of positive charge to the cell surface, and increased production of secreted proteases, all occurred independently of acidic amino acids in the extracytoplasmic sensor loop of GraS that were previously found to be required for sensing of CAMPs. Cumulatively, our data indicate that signaling through GraS at acidic pH occurs through a mechanism that is distinct from that described for CAMPs, leading to increased resistance to antimicrobial uFFA and production of secreted proteases.

IMPORTANCE *Staphylococcus aureus* asymptomatically colonizes 30% of humans but is also a leading cause of infectious morbidity and mortality. Since infections are typically initiated by the same strain associated with asymptomatic colonization of the nose or skin, it is important to understand how the microbe can endure exposure to harsh conditions that successfully restrict the growth of other bacteria, including a combination of acidic pH, antimicrobial peptides, and antimicrobial fatty acids. The significance of our research is in showing that acidic pH combined with antimicrobial peptide or environmental calcium can signal through a single membrane sensor protein to promote traits that may aid in survival, including modification of cell surface properties, increased resistance to antimicrobial fatty acids, and enhanced production of secreted proteases.

KEYWORDS *Staphylococcus aureus*, cell signaling, sensor kinase, mechanisms of resistance, acidic pH, antimicrobial fatty acids, antimicrobial peptides, proteases

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Staphylococcus aureus is a Gram-positive opportunistic pathogen that asymptomatically colonizes up to 30% of humans (1) but is also a leading cause of infectious morbidity and mortality, such that death attributed to *S. aureus* in the United States has now exceeded that caused by AIDS, tuberculosis, and viral hepatitis combined (2). Its preferred site of colonization in asymptomatic carriage is the anterior nares, and among those who exhibit nasal carriage, the bacteria are also frequently found on exposed skin, including the hands, perineum, and axillae. This asymptomatic colonization plays a key role in the epidemiology of *S. aureus* disease, since infections are nearly always caused by the endogenous nasal carriage strain (3). Congruently, the ability of *S. aureus* to resist local innate immunity at sites of colonization is critical to its success as a pathogen, and our recent work has focused on how *S. aureus* is able to sense and respond to these signals of innate immunity (4–7).

The anterior nares of the nose are exposed to secretions of the upper respiratory tract, including antimicrobial unsaturated free fatty acids (uFFA), of which, linoleic acid ($C_{18:2}$) is the most abundant in human nasal secretions (8), while the major antimicrobial uFFA on skin is sapienic acid ($C_{16:1}$), an isomer of palmitoleic acid that is uniquely produced by human sebaceous glands (9, 10). We found that these antimicrobial uFFA induce the expression of secreted proteases and also an RND family efflux pump that contributes to resistance (4, 5, 11). Other environmental signals relevant to innate immunity on skin and the anterior nares include acidic pH (12–14) and antimicrobial peptides (15–18). Extracellular calcium also has an important role in maintaining the dermal barrier function of the skin (19). Nevertheless, although *S. aureus* is concurrently exposed to multiple environmental signals and mediators of innate immunity at sites of colonization, its ability to sense and respond to these signals is typically studied in a singular manner (20–22). In this context, our recent work has alluded to the possibility that *S. aureus* could effectively multitask in response to sensing disparate environmental signals through the GraS sensor kinase (7, 23).

GraS and its cognate response regulator GraR are part of a five-component signaling system composed of the cotranscribed *graXRS* genes and coassociated *vraFG*, where GraX is a cytoplasmic accessory protein and VraFG comprise a two-component ABC transporter (24–26). GraS is known for its role in sensing cationic antimicrobial peptides (CAMPs), attributed to a short extracellular sensor loop, leading to autophosphorylation and phosphorelay to the response regulator GraR, which in turn promotes expression of genes required for resistance, including *mprF* and *dltABCD* (27–29). MprF promotes synthesis of lysyl-phosphatidylglycerol (lysyl-PG), while the *dlt* genes promote D-alanylation of teichoic acids (27, 30), and these two activities confer a positive charge to the cell envelope, which repels CAMPs. The role of the accessory GraX and VraFG proteins is less well understood, although GraX likely functions as a scaffold to promote protein interactions with GraS, GraR, and VraFG to fine tune the signaling mechanism (25, 26). Adding to the complexity, GraS is considered to belong to the intramembrane sensor kinase family of proteins, which signal in response to membrane perturbation and are characterized by having a minimal extracytoplasmic sensor segment and coassociating with two-component ABC transporters or other accessory signaling proteins (31–33).

Recent work by ourselves and others has expanded the sensory capabilities of GraS to include acidic pH, including its requirement for growth at the pH extreme of 4.5, activation of GraS-dependent expression of MprF at pH 5.5, and a requirement for growth in acidified macrophage phagosomes (7, 34). In the current model of GraS function, acidic amino acids in its short nonapeptide extracellular sensor loop promote recognition of and signaling in response to CAMPs (29). However, exposure to acidic pH should reduce the charge on these acidic amino acids and also affect the properties of membrane phospholipid, which would include a reduction in repulsive forces between polar lipid head groups and tighter lateral packing (35). As such, signaling through GraS at acidic pH may not be critically dependent on these acidic amino acids that contribute to recognition of CAMPs.

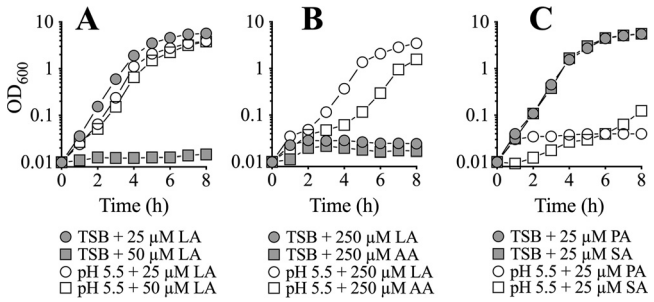

FIG 1 Influence of acidic pH on growth of *S. aureus* USA300 in the presence of linoleic acid (LA; $C_{18:2}$), arachidonic acid (AA; $C_{20:4}$), palmitoleic acid (PA; $C_{16:1}$), or sapienic acid (SA; $C_{16:1}$). Triplicate flasks of TSB or TSB (pH 5.5) were supplemented with indicated concentrations of LA (A), LA or AA (B), and PA or SA (C) and inoculated to an initial optical density measured at 600 nm (OD_{600}) of 0.01. Growth (OD_{600}) was assessed at hourly intervals, and each data point represents the mean ± SE from triplicate flasks.

In view of these considerations, the purpose of our present study was 2-fold. First, since concurrent exposure to antimicrobial uFFA, acidic pH, calcium, and antimicrobial peptides should be key environmental features of *S. aureus* persistence on skin and the anterior nares, we queried how the interplay of these environmental signals would influence its resistance to antimicrobial uFFA. Second, we investigated the role of the GraS sensor kinase and signaling mechanism in response to these combined environmental signals. Our experiments were conducted with the hypervirulent and pandemic USA300 strain of community acquired methicillin-resistant *S. aureus* (CA-MRSA), which is known for its efficient community transmission (36). Here, we report that acidic pH, antimicrobial peptides, and environmental calcium all promote increased resistance of *S. aureus* USA300 to antimicrobial uFFA and that during growth at acidic pH, this was dependent on signaling through GraS independently of acidic amino acids in its extracellular sensor loop. We further reveal a role for GraS in promoting the production of *S. aureus* secreted proteases in response to acidic pH. Cumulatively, these findings are consistent with the function of GraS as an intramembrane sensor kinase.

RESULTS

***S. aureus* sensitivity to antimicrobial uFFA is differentially influenced by carbon chain length and acidic pH.** Linoleic acid ($C_{18:2}$) is the major unsaturated free fatty acid (uFFA) in tissue abscesses and nasal secretions, while sapienic acid ($C_{16:1}$) is predominant in sebaceous secretions. Its isomer palmitoleic acid is the major antimicrobial uFFA in skin of mice and other mammals and is also abundant in adipose triglyceride and membrane phospholipid (8, 10, 37). Since the skin and nasal mucosa are maintained at acidic pH (12, 13), we evaluated how this affects *S. aureus* resistance to antimicrobial uFFA by conducting growth assays in unmodified tryptic soy broth (TSB; initial pH 7.3) or TSB buffered at pH 5.5. Consistent with our previous work (5), *S. aureus* USA300 grew well in TSB plus 25 μM linoleic acid but exhibited an extended lag phase with 50 μM linoleic acid (Fig. 1A). At pH 5.5, USA300 grew equally well in 25 or 50 μM linoleic acid (Fig. 1A) and also grew with 250 μM linoleic or arachidonic acid ($C_{20:4}$) (Fig. 1B). Surprisingly, an opposite effect was observed with $C_{16:1}$, where USA300 grew well in TSB plus 25 μM palmitoleic or sapienic acid but not with this same concentration at pH 5.5 (Fig. 1C). However, if the incubation time was extended, acidic pH permitted outgrowth after 48 h at palmitoleic acid concentrations ranging from 200 to 500 μM, and this was not evident in unbuffered TSB (see Fig. S1 in the supplemental material). Therefore, we conclude that acidic pH favors increased resistance to $C_{18:2}$ and $C_{20:4}$ antimicrobial uFFA but appears to have a bimodal effect with $C_{16:1}$, initially potentiating the inhibitory activity but also permitting outgrowth after extended incubation.

Cationic antimicrobial peptides and extracellular calcium also stimulate increased resistance to antimicrobial uFFA. Although acidic pH promoted increased sensitivity to $C_{16:1}$ antimicrobial uFFA, we considered that *S. aureus* is concurrently exposed to multiple environmental signals on human skin, including antimicrobial

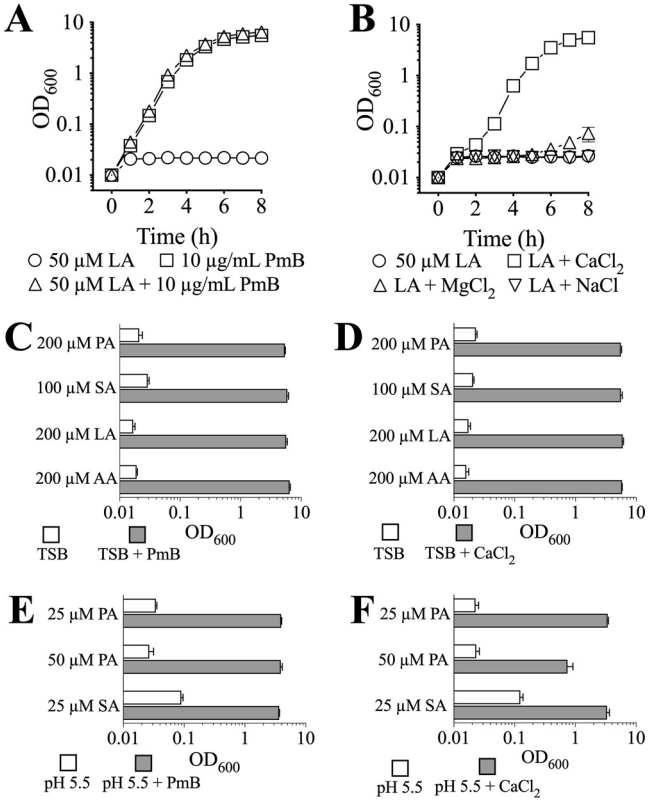

FIG 2 Influence of polymyxin B (PmB) and cation supplements on growth of *S. aureus* USA300 in the presence of antimicrobial uFFA. (A) Growth in TSB supplemented with 50 μM LA, 10 μg/ml PmB, or 50 μM LA plus 10 μg/ml PmB as indicated. (B) Growth in TSB supplemented with 50 μM LA or 50 μM LA containing 0.5 mM $CaCl_2$, $MgCl_2$, or NaCl. (C) Growth in TSB plus 10 μg/ml PmB and containing indicated concentrations of PA, SA, LA, or AA. (D) Growth in TSB plus 0.5 mM $CaCl_2$ and containing indicated concentrations of PA, SA, LA, or AA; (E) growth in TSB (pH 5.5) or TSB (pH 5.5) plus 10 μg/ml PmB, with indicated concentrations of PA or SA. (F) Growth in TSB (pH 5.5) or TSB (pH 5.5) plus 0.5 mM $CaCl_2$, with indicated concentrations of PA or SA. All data points represent the means $\pm$ SEs from triplicate cultures. For panels A and B, OD_{600} was monitored at hourly intervals, while for panels C to F, growth was monitored after 12 h. For panels C to F, statistical significance between medium alone and medium supplemented with PmB or $CaCl_2$ was measured using an unpaired one-tailed *t* test. In all cases, $P < 0.0001$, with the exception of panel F, 50 μM PA and 25 μM SA, where $P = 0.066$ and $P = 0.0003$, respectively.

peptides and extracellular calcium, which has a key role in promoting the structural integrity of the dermal barrier and has been measured in human sweat at 16 μg/ml, equivalent to 0.4 mM (19, 38). We therefore tested whether these additional signals alone or in combination with acidic pH would influence *S. aureus* resistance to antimicrobial uFFA. Using polymyxin B (PmB) as a model cationic antimicrobial peptide, we observed that subinhibitory PmB eliminated the lag phase that normally occurs in TSB plus 50 μM linoleic acid (Fig. 2A), and the same effect was achieved with 0.5 mM calcium but not sodium or magnesium (Fig. 2B).

Supplemental PmB or calcium also promoted growth at higher concentrations of uFFA, including 200 μM linoleic, arachidonic, or palmitoleic acid and 100 μM sapienic acid (Fig. 2C and D). Moreover, although acidic pH impaired *S. aureus* growth in 25 μM palmitoleic or sapienic acid (Fig. 1C), this effect was eliminated in TSB (pH 5.5) supplemented with either PmB or calcium, which permitted growth in 50 μM palmitoleic acid and 25 μM sapienic acid (Fig. 2E and F). To determine the full extent of resistance, we conducted MIC determinations with different modifications to basal TSB (Table 1). In unbuffered TSB, the MICs of palmitoleic and linoleic acid were 100 and 400 μM, respectively. At pH 5.5, the MIC of palmitoleic acid decreased to 75 μM, while that of linoleic acid increased to 1,200 μM. At pH 5.5, addition of supplemental calcium

TABLE 1 Influence of acidic pH, subinhibitory PmB, and supplemental calcium on MICs of linoleic and palmitoleic acids for *S. aureus* USA300

	MIC (μM) of:	
Medium	**Linoleic acid**	**Palmitoleic acid**
TSB	400	100
TSB plus 10 μg/ml PmB	1,200	300
TSB plus 0.5 mM $CaCl_2$	1,800	800
TSB (pH 5.5)	1,200	75
TSB (pH 5.5) plus 20 μg/ml PmB	1,600	700
TSB (pH 5.5) plus 0.5 mM $CaCl_2$	>2,500	300

or subinhibitory Pmb increased the MICs for palmitoleic acid to 300 μM and 700 μM, respectively, compared to 1,600 and >2,500 μM for linoleic acid under the same conditions.

Although subinhibitory PmB promoted enhanced resistance to antimicrobial uFFA, we considered that this could be due to it being a lipopeptide, which might exhibit lipid-mediated interactions with free fatty acids to render them less effective. However, this was considered unlikely, since 10 μg/ml PmB is equivalent to 7.7 μM, which promoted *S. aureus* growth in concentrations of linoleic and palmitoleic acid that were far in excess of that of PmB. To provide additional evidence that subinhibitory CAMPs confer increased resistance of *S. aureus* to antimicrobial uFFA, we conducted experiments with cathelicidin-2 (CATH-2). Although CATH-2 is of chicken origin, it belongs to the cathelicidin family of CAMPs and has high activity toward *S. aureus*; compared to that of LL-37, its antimicrobial activity is less sensitive to variations in acidity and salt (39–41). Growth assays were conducted in microtiter plates, with preliminary experiments establishing a higher threshold of resistance to uFFA than in flask cultures (see Fig. S2A and B) and that CATH-2 was inhibitory at >6.0 μM (Fig. S2C). Based on these trials, we compared the ability of 10 μg/ml PmB or 1.5 μM CATH-2 to influence growth in TSB containing 500 μM linoleic acid or 300 μM palmitoleic acid. Although we could not do a direct physiologic comparison for CATH-2, dermcidin is an antimicrobial peptide that has been measured at 2.1 μM in eccrine gland secretions (42), comparable to our use of 1.5 μM CATH-2. As expected, PmB eliminated the lag phase with both uFFA (Fig. 3), while CATH-2 reduced the lag phase from 20 h to 6 h in 500 μM linoleic acid and eliminated the lag phase in 300 μM palmitoleic acid (Fig. 3).

As an additional test of specificity, we queried whether enhanced resistance could be achieved with vancomycin, a cationic antimicrobial glycopeptide that does not stimulate signaling through GraS (43). In unbuffered TSB, vancomycin was inhibitory at >0.5 μg/ml (Fig. S3A), and in contrast to PmB, subinhibitory vancomycin did not confer enhanced resistance to either linoleic or palmitoleic acid (Fig. S3B and C). Subinhibitory vancomycin also did not stimulate growth in 100 μM palmitoleic acid at pH 5.5,

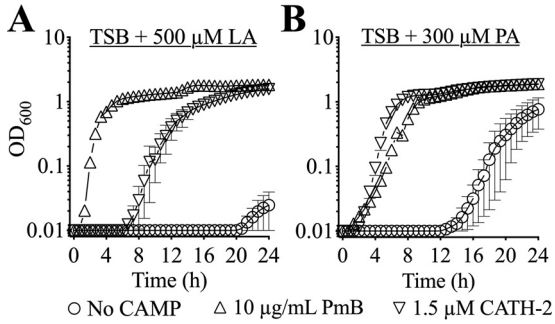

FIG 3 Influence of cationic antimicrobial peptides PmB and CATH-2 on growth of *S. aureus* USA300 under inhibitory concentrations of antimicrobial uFFA. Growth of USA300 in TSB with 500 μM LA (A) or 300 μM PA (B) supplemented with subinhibitory concentrations of 10 μg/ml PmB or 1.5 μM CATH-2. Each data point represents mean ± SE from triplicate cultures.

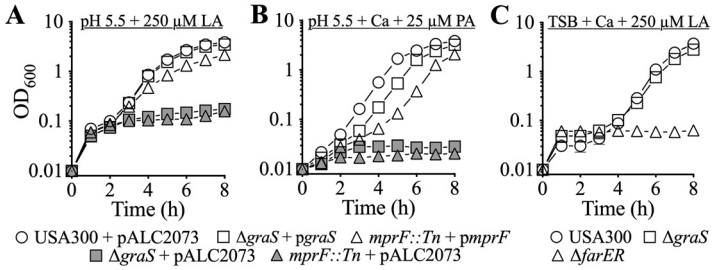

FIG 4 Genetic requirements for enhanced resistance of *S. aureus* USA300 to linoleic acid (A and C) or palmitoleic acid (B) in response to calcium and acidic pH. Growth of USA300, isogenic Δ*graS* or *mprF*::Tn mutants, and respective p*graS* or p*mprF* complemented strains in TSB (pH 5.5) plus 250 μM LA (A) or TSB (pH 5.5) plus 0.5 mM $CaCl_2$ and 25 μM PA (B). (C) Growth of USA300 and isogenic Δ*graS* or Δ*farER* mutants in TSB plus 0.5 mM $CaCl_2$ and 250 μM. Each data point represents the mean ± SE from triplicate cultures.

whereas PmB and CATH-2 both stimulated growth to similar extents (Fig. S3D). Cumulatively, these findings establish that selected CAMPs and supplemental calcium both promote increased resistance of *S. aureus* to antimicrobial uFFA, including amelioration of $C_{16:1}$ toxicity at acidic pH.

GraS and the GraS-regulated gene *mprF* are required for resistance to antimicrobial uFFA at acidic pH. We recently established that efflux pump FarE is induced by and required for resistance to antimicrobial uFFA through a mechanism that is dependent on the fatty acid kinase encoded by *fakA* (5, 11), which is also required for metabolic incorporation of uFFA into phospholipids (7, 44), while GraS responds to acidic pH in macrophage phagosomes (7). We therefore queried the role of these genes in enhanced resistance to antimicrobial uFFA that is manifested at acidic pH. We first assessed viability by plating exponential-phase cultures of USA300 and isogenic variants on TSB agar (TSA), TSA plus 200 μM linoleic acid (LA), TSA (pH 5.5), or TSA (pH 5.5) plus 500 μM LA (see Fig. S4). As expected, USA300 Δ*farER* exhibited a loss of viability on TSA plus 200 μM LA. However, on TSA (pH 5.5) plus 500 μM LA, there was no loss of viability for either USA300 Δ*farER* or USA300 Δ*fakA*, whereas USA300 Δ*graS* exhibited a severe loss of viability (Fig. S4). The requirements for *graS* and the GraS-regulated gene *mprF* were then evaluated through growth analyses in TSB (pH 5.5) plus 250 μM LA, under which condition both mutants failed to grow, and growth was restored with the respective p*graS* and p*mprF* complementation vectors (Fig. 4A). Both genes were also required for resistance to 25 μM palmitoleic acid in TSB (pH 5.5) plus 0.5 mM calcium (Fig. 4B). Under nonacidic growth conditions, the *farER* genes were once again required for resistance to 250 μM LA in TSB plus 0.5 mM calcium, whereas USA300 Δ*graS* exhibited unrestricted growth (Fig. 4C). From these data, it is evident that FarE-mediated efflux contributes to enhanced resistance that is manifested in response to supplemental calcium under nonacidic growth conditions, whereas GraS and MprF are both essential for enhanced resistance to antimicrobial uFFA at acidic pH.

GraS exhibits unique requirements for signaling at acidic pH. Since signaling through GraS in response to cationic antimicrobial peptides depends on three aspartate residues in a short nonapeptide extracytoplasmic loop (29), we queried whether this requirement is maintained at acidic pH, using polymyxin B (PmB) as a model CAMP. As expected, USA300 Δ*graS* failed to grow in TSB plus 10 μg/ml PmB, and although there was a significant difference in the stationary-phase cell densities of USA300 and USA300 Δ*graS* plus p*graS*, there was successful complementation of growth. However, no complementation was evident in USA300 Δ*graS* plus p$graS^{3D>G}$, encoding a variant GraS where three aspartate residues in the extracytoplasmic sensor loop are replaced with glycines (Fig. 5A and S5A). Although this supports a role for these acidic amino acids in recognition of and signaling in response to CAMPs (29), when growth was assessed in TSB (pH 5.5) plus 20 μg/ml PmB, both p*graS* and p$graS^{3D>G}$ were equally effective in restoring growth of USA300 Δ*graS* (Fig. 5A and S5B). Both complementation vectors also restored growth of USA300 Δ*graS* in TSB (pH 5.5) plus 250 μM LA (Fig. 5B).

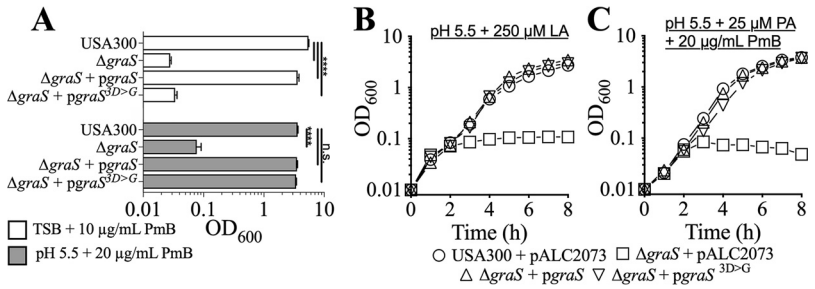

FIG 5 Requirement for acidic amino acids in extracellular sensor loop of GraS is dependent on growth conditions. (A) OD_{600} after 12 h of growth in TSB plus 10 μg/ml PmB or TSB (pH 5.5) plus 20 μg/ml PmB. (B) Growth in TSB (pH 5.5) containing 250 μM LA. (C) Growth in TSB (pH 5.5) supplemented with 20 μg/ml PmB and 25 μM palmitoleic acid. Each data point represents the mean ± SE from triplicate cultures. Statistical significance for panel A was measured using one-way ANOVA. ****, $P < 0.0001$; n.s., not significant.

Similar results were obtained in TSB (pH 5.5) supplemented with 20 μg/ml PmB and 25 μM palmitoleic acid, where USA300 Δ*graS* failed to grow, but growth was restored with both p*graS* and p*graS*$^{3D>G}$ vectors (Fig. 5C).

Since signaling through GraS promotes expression of MprF, which confers resistance to CAMPs through lysine modification of membrane phospholipids (45), we conducted assays of cytochrome *c* binding to monitor MprF-dependent modification of cell surface charge. Consistent with our previous work where acidic pH promoted a GraS-dependent increase in transcription of an *mprF*::*lux* reporter (7), growth at pH 5.5 also promoted a GraS-dependent increase in cell surface positive charge, as evident in reduced binding of cytochrome *c* (Fig. 6). Once again, p*graS* and p*graS*$^{3D>G}$ were equally effective in restoring cell surface charge to USA300 Δ*graS* grown at acidic pH (Fig. 6). Cumulatively, these data support the established paradigm for signaling through GraS during growth in unbuffered TSB, where acidic amino acids in the extracellular sensor loop contribute to the sensing of antimicrobial peptides (29). However, our experiments now reveal a novel specificity at acidic pH, where these same amino acids are dispensable for resistance to PmB and antimicrobial uFFA.

The lysyl-transferase function of MprF is not required for resistance to antimicrobial uFFA during growth at acidic pH. MprF confers resistance to CAMPs through its ability to promote lysine modification of membrane phospholipids. This occurs through a two-step mechanism whereby the lysyl-phosphatidylglycerol synthase domain transfers lysine to phosphatidylglycerol on the inner surface of the cytoplasmic membrane, after which a flippase domain translocates the nascent lysyl-PG to the outer leaflet of the membrane (30). Since MprF was required for enhanced

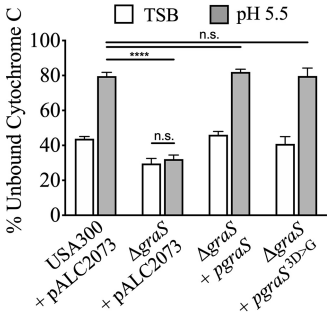

FIG 6 Restoration of cell surface positive charge is independent of acidic amino acids in the extracellular sensor loop of GraS during growth at acidic pH. Cultures were grown to an OD_{600} of 0.5 in TSB or TSB (pH 5.5) and then processed for assay of cytochrome *c* binding. Each data point represents the mean ± SE from three replicate determinations from each of three cultures. Statistical significance was measured using two-way ANOVA. ****, $P < 0.0001$; n.s., not significant.

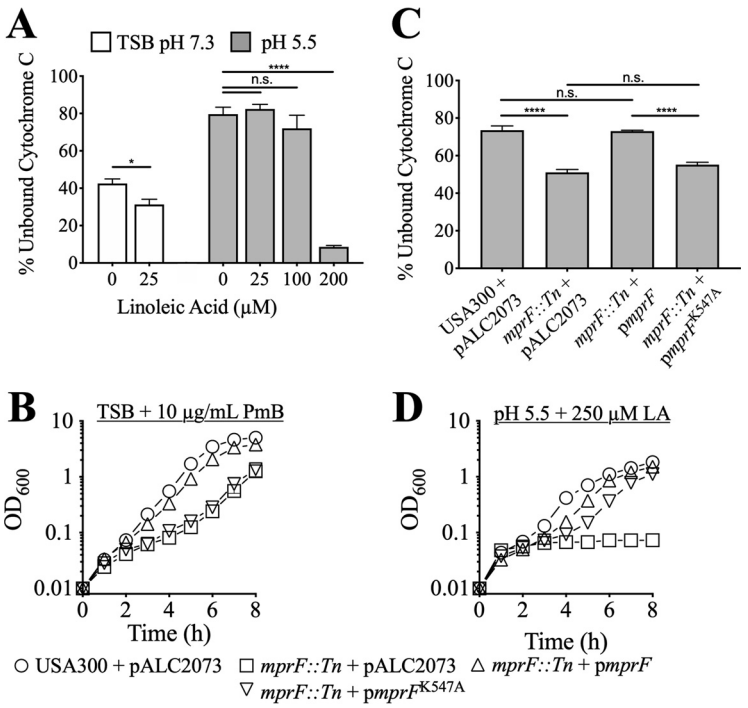

FIG 7 The lysyl-PG synthase function of MprF is not required for *S. aureus* USA300 resistance to 250 μM linoleic acid during growth at acidic pH. (A) Cytochrome *c* binding as a measure of MprF function in cultures of USA300 grown in TSB or TSB (pH 5.5) containing the indicated concentration of linoleic acid (LA). (B) Growth in TSB plus 10 μg/ml PmB of USA300 and isogenic *mprF*::Tn mutant complemented with pALC2073, p*mprF*, or p*mprF*^{K547A}. (C) Cytochrome *c* binding after growth in TSB (pH 5.5) of USA300 and isogenic *mprF*::Tn mutant complemented with pALC2073, p*mprF*, or p*mprF*^{K547}. Cultures were supplemented with 120 ng/ml anhydrotetracycline (aTc) to induce expression from the $P_{xyl/tetO}$ promoter. (D) Growth assay as described for panel B, except that cultures were grown in TSB (pH 5.5) plus 250 μM LA. Statistical significance was measured using two-way ANOVA in panel A and one-way ANOVA in panel C. *, $P < 0.05$; ****, $P < 0.0001$; n.s., not significant.

resistance to antimicrobial uFFA during growth at acidic pH, we queried whether this was dependent on its lysyl-transferase function. We observed that during growth at acidic pH, cell surface positive charge was maintained up to 100 μM linoleic acid, followed by a sharp decrease at 200 μM (Fig. 7A), consistent with a reduction in cell surface lysyl-PG. From this we surmised that the lysyl-PG-synthase function of MprF should be dispensable for *S. aureus* resistance to 250 μM linoleic acid during growth at pH 5.5, and to assess this hypothesis, we constructed a K547A substitution in MprF, which eliminates lysyl-transferase activity without affecting the stability of the protein (30).

To confirm the MprF^{K547A} phenotype, we first evaluated growth of USA300 *mprF*::Tn complemented with p*mprF* or p*mprF*^{K547A} in TSB plus 10 μg/ml PmB. As expected, USA300 *mprF*::Tn exhibited impaired growth, which was restored with pALC*mprF* but not pALC*mprF*^{K547A}, consistent with abrogation of lysyl-PG synthase activity (Fig. 7B). This was confirmed through cytochrome *c* binding, where p*mprF* restored cell surface charge to USA300 *mprF*::Tn but p*mprF*^{K547A} did not (Fig. 7C). These data are consistent with abrogation of lysyl-PG synthase activity in MprF^{K547A} as previously reported (30). Nevertheless, p*mprF*^{K547A} was able to rescue growth of USA300 *mprF*::Tn in TSB (pH 5.5) plus 250 μM linoleic acid (Fig. 7D). Therefore, MprF is needed to support *S. aureus* resistance to antimicrobial uFFA during growth at acidic pH, through a mechanism that is independent of its lysyl-transferase function.

Signaling through GraS also contributes to production of secreted proteases during growth at acidic pH. Having established that signaling through GraS is required for *S. aureus* resistance to antimicrobial uFFA at acidic pH, we considered whether other phenotypic traits might also exhibit a GraS-dependent phenotype at

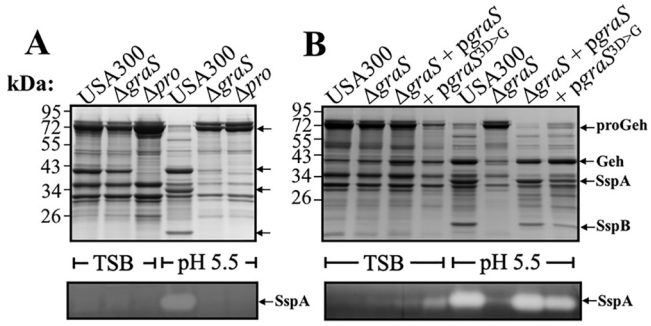

FIG 8 Signaling through GraS contributes to *S. aureus* USA300 production of secreted proteases during growth at acidic pH. (A) SDS-PAGE profile of secreted proteins (top) and zymogram for detection of SspA serine protease (bottom) in culture supernatants of USA300 and isogenic Δ*graS* or protease-deficient Δ*pro* mutants after growth for 20 h in TSB or TSB (pH 5.5). Arrows indicate the positions of proteins that represent the signature protein profile of the staphylococcal proteolytic cascade, including the precursor of glycerol ester hydrolase proGeh, mature Geh lipase, mature SspA serine protease, and SspB cysteine protease. (B) Same as described for panel A, with strains USA300 and Δ*graS* complemented with p*graS* or p*graS*3$^{D>G}$. Expression from the $P_{xyl/tetO}$ promoter of pACL2073 was induced using 120 ng/ml aTc. For SDS-PAGE profiles of secreted proteins, TCA-precipitated protein equivalent to 3.0 OD_{600} units of culture supernatant were applied to each lane, while for zymogram analyses, a volume of culture supernatant equivalent to 0.075 OD_{600} unit was applied to each lane.

acidic pH. Foremost, our previous work revealed that antimicrobial uFFA induce expression of secreted proteases (4), producing a characteristic change in the profile of secreted proteins attributed to the staphylococcal proteolytic cascade. Notably, the 72-kDa precursor of glycerol ester hydrolase proGeh is converted to a mature 40-kDa form by the metalloprotease aureolysin, which is also required for maturation of the SspA serine protease, and SspA then activates the proSspB cysteine protease precursor producing a 20-kDa mature SspB (4, 6, 46, 47). Knowing that *S. aureus* is concurrently exposed to antimicrobial uFFA and acidic pH on human skin, we queried whether acidic pH could also stimulate protease expression. Accordingly, compared to growth in unbuffered TSB, culture supernatant from USA300 grown in TSB (pH 5.5) exhibited the signature protein profile of the staphylococcal proteolytic cascade, including a reduction in proGeh, and appearance of new proteins consistent with production of SspA and SspB. These changes were not evident in USA300 Δ*graS* or in USA300 Δ*pro*, where *aur* (aureolysin) and the *sspABC* serine protease operon are inactivated (Fig. 8), but were restored in USA300 Δ*graS* with both p*graS* and p*graS*$^{3D>G}$ (Fig. 8B). These observations were mirrored in an accompanying casein hydrolysis zymogram for detection of the SspA serine protease (Fig. 8). We further note that during growth in nonbuffered TSB, induction of *graS* expression in USA300 Δ*graS* plus p*graS* was not sufficient to induce protease production, indicating that acidic pH is a prerequisite for signaling through GraS to induce production of secreted proteases.

DISCUSSION

In this study, we assessed the genetic requirements for *S. aureus* resistance to antimicrobial uFFA when exposed to signals that would be encountered at sites of colonization and infection, including acidic pH, antimicrobial peptides, and environmental calcium. It is now evident that these commonly encountered environmental signals confer enhanced resistance to antimicrobial uFFA, with different genetic requirements depending on acidic or nonacidic growth conditions and exposure to calcium or subinhibitory antimicrobial peptides. We previously found that the RND family efflux pump FarE was required for *S. aureus* resistance to antimicrobial uFFA (5, 11), and in our present work, this requirement was maintained under nonacidic growth conditions for enhanced resistance to linoleic acid in unbuffered TSB supplemented with calcium. However, at acidic pH, enhanced resistance to both $C_{18:2}$ and $C_{16:1}$ uFFA was dependent on GraS and GraS-dependent expression of MprF. Moreover, this occurred independently of acidic amino acids in the extracellular sensor segment of

GraS and the lysyl-transferase function of MprF that are essential for resistance to antimicrobial peptides (29, 30). Growth at acidic pH also promoted GraS-dependent production of secreted proteases through a mechanism that was again independent of acidic amino acids in the extracellular sensor segment. These novel findings broaden our understanding not only of *S. aureus* mechanisms for resistance to antimicrobial uFFA but also of mechanisms through which *S. aureus* senses and responds to combined stresses that would be encountered at sites of colonization and infection.

Central to our work is the novel signaling capacity and expanded function of GraS. Previous work on GraS signaling in response to CAMPs focused on acidic amino acids in the extracytoplasmic nonapeptide segment DYDFPIDSL, finding that substitution of the three aspartate residues with glycine led to loss of *graS*-dependent expression of *mprF* and *dltA* in response to CAMPs concomitant with increased sensitivity (29). Our experiments with antimicrobial peptides under nonacidic growth conditions confirmed this requirement, as also reported for the CovS and PhoQ sensor kinases, where acidic amino acids in their large extracytoplasmic sensor domain interact with cations on the cytoplasmic membrane to maintain homeostasis, and signaling is initiated when this interaction is disrupted by CAMPs (48, 49). Nevertheless, these same amino acids were dispensable to GraS function at acidic pH, irrespective of whether the phenotypic readout was cell surface positive charge, resistance to antimicrobial peptide, resistance to antimicrobial uFFA, or production of secreted proteases. However, a key difference in comparison to CovS and PhoQ is that GraS has a minimal sensor domain, as does the SaeS global regulator of virulence in *S. aureus* (32, 50). Importantly, GraS and SaeS both have a minimal N-terminal sensor domain composed of two transmembrane helices separated by an eight- or nine-amino-acid extracellular linker segment, and this organization conforms to a family of intramembrane sensor kinases that sense membrane perturbation (31).

For GraS, this may be especially relevant to signal transduction at acidic pH, which reduces the charge on polar lipid head groups, leading to reduced repulsive forces and tighter lateral packing of phospholipids (35). Acidic pH should also reduce the charge on acidic amino acids in the extracytoplasmic linker segment of GraS, which would render them less effective in sensing cationic antimicrobial peptides. It is therefore logical that GraS should have the capacity to signal in response to altered membrane properties at acidic pH as opposed to a strict dependence on acidic amino acids in the extracellular linker segment. In this respect, it is salient to note that antimicrobial peptides also cause changes in membrane structure (51), which could potentially allow GraS to respond to membrane damage caused by these peptides. For SaeS, it was proposed that the entire N-terminal sensor domain functions as a trip wire, such that any stimulus that elicits conformational changes in the N-terminal domain would trigger kinase activity, while amino acids in the extracellular linker segment serve to fine-tune the response to different stimulants (32, 50). Indeed, deletion of this linker segment in GraS rendered *S. aureus* more sensitive to antimicrobial peptides (28). Therefore, while our present data indicate that acidic amino acids in the extracellular linker segment are dispensable to signaling at acidic pH, it is feasible that the entire N-terminal segment of GraS functions as a molecular tripwire to sense membrane perturbation as described for SaeS (32, 50). As such, changes in phospholipid composition and physical properties in response to acidic pH or antimicrobial peptide would be sufficient to trigger GraS kinase activity independently of the need to recognize a physical antimicrobial peptide ligand. The DesK sensor kinase of *Bacillus subtilis* operates on such a principle, whereby assembly of a thicker cytoplasmic membrane at low temperature stimulates signaling through DesK independently of the need for an extracellular sensor segment (52).

While previous and present data confirm that signaling through GraS is critical for expression of MprF, which confers resistance to CAMPs through lysine modification of phospholipids (7, 45), we were surprised to find that during growth at acidic pH, MprF was also required for enhanced resistance to both $C_{16:1}$ and $C_{18:2}$ antimicrobial uFFA through a mechanism that was independent of its lysyl-transferase function. However,

similar observations were noted on the role of MprF in promoting reduced susceptibility to the cationic lipopeptide antibiotic daptomycin (53, 54). It is not completely understood how daptomycin kills *S. aureus*, but current evidence supports a model whereby it targets fluid microdomains in the membrane followed by oligomerization and translocation to the inner leaflet, where it then blocks the interaction between essential membrane proteins and fluid membrane microdomains (55). Although clinical isolates with reduced susceptibility to daptomycin often have nonsynonymous polymorphisms in MprF, there is no general consensus that this is due to increased production of lysyl-PG, and one of the most commonly occurring polymorphisms causes a T345A/I/K substitution at the junction of the flippase and lysyl-PG synthase domains (53).

Notably, a T345A substitution at this juncture is alone sufficient to promote reduced susceptibility to daptomycin, and it was proposed that this may allow the flippase domain to accommodate daptomycin and translocate the antibiotic out of the fluid inner membrane microdomains (53, 54). Since our data indicate that abrogation of the lysyl-PG synthase activity of MprF does not interfere with its ability to complement growth of *S. aureus mprF*::Tn at pH 5.5 in the presence of antimicrobial uFFA, this would be consistent with a mechanism whereby under these conditions, the flippase domain promotes translocation of phosphatidylglycerol containing an unsaturated fatty acid instead of its physiologic lysyl-PG substrate. In support of this analysis, we note that growth at pH 5.5 promotes a GraS-dependent increase in cell surface positive charge (Fig. 6), which is maintained at 25 μM and 100 μM linoleic acid but then drops sharply at 200 μM linoleic acid (Fig. 7A), under which condition growth remained dependent on MprF (Fig. 7D). This could be accounted for if an excess of linoleoyl-PG competed with lysyl-PG for translocation by MprF under these conditions or, alternatively, if membrane properties under these conditions were not conducive to synthesis of lysyl-PG.

Another novel feature of signaling through GraS and downstream phenotypic traits revealed through our work is its requirement for the production of secreted proteases during growth at acidic pH. Although the focus of research on GraS has been its role in signaling through GraR to promote expression of genes that modify cell surface properties as required for resistance to antimicrobial peptides (28, 29, 56), one study alluded to a broader role, including promotion of growth at high temperatures and resistance to oxidative stress (57). In the latter respect, it is noteworthy that polyunsaturated arachidonic acid is reported to have bactericidal activity toward *S. aureus* through a lipid peroxidation mechanism (58). Expression profiling also revealed that several major virulence factors exhibited increased GraS-dependent expression in response to antimicrobial peptide, including the accessory gene regulator *agr* as well as secreted hemolysins and cell surface proteins (57). Nevertheless, the gene encoding SspA serine protease was not among those that were reported as being regulated through GraS (57). Another recent study aimed at mapping the global network of extracellular protease regulation in *S. aureus* identified seven major regulators and seven secondary regulators, but GraS and GraR were not among these (59). Consequently, it is likely that the role of GraS in promoting production of secreted protease is limited to signaling at acidic pH, which is encountered by *S. aureus* on human skin and macrophage phagosomes but also on nasal mucosa (12) and in chronic abscesses (60).

As with acidic pH and CAMPs, our findings revealed that extracellular calcium also promoted increased resistance of *S. aureus* to antimicrobial uFFA, representing a convergence of signals that could promote persistence of *S. aureus* on human skin. Although acidic pH initially promoted increased sensitivity of *S. aureus* to palmitoleic and sapienic acids, this effect was ameliorated with 0.5 mM calcium. While the mechanistic basis of this finding has yet to be elucidated, recent studies have highlighted the role of environmental calcium in promoting microbial persistence strategies, including enhanced biofilm formation of *Vibrio fischeri* through a mechanism that was dependent on the SypS sensor kinase and calcium-dependent activation of the LadS histidine

kinase in *Pseudomonas aeruginosa* to induce an acute-to-chronic transition in virulence (61, 62). Therefore, future work will focus on understanding how environmental calcium may influence *S. aureus* persistence strategies and on elucidating how changes in membrane properties and composition during growth at acidic pH trigger signaling through GraS.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacteria and plasmids that were used or constructed in this study are listed in Table S1 in the supplemental material. *S. aureus* cultures were maintained as frozen stocks (−80°C) in 20% glycerol and streaked on TSB agar (TSA) when required. Tryptic soy broth (TSB; Difco) used for this study contained 2.5 g/liter glucose (~14 mM). Metabolism of glucose can acidify the culture medium as glucose is consumed, reaching a minimum pH of ~5.9 in early stationary phase and then increasing again as the acetate by-product is consumed (63). In consideration of this, we conducted pH measurements during growth in TSB and confirmed that pH was not affected during early exponential growth when bacteria are highly susceptible to antimicrobial uFFA. TSB or TSA was supplemented, when needed, with 10 μg/ml erythromycin or chloramphenicol and 2 μg/ml tetracycline for propagation of strains bearing resistance markers. Where indicated, TSB or TSA was supplemented by addition of 0.1 M morpholineethanesulfonic acid (MES) buffer (Bio Can Scientific) and adjusted to pH 5.5 with HCl prior to autoclaving. To supplement media with fatty acids, a 10 mM stock concentration was first prepared in TSB containing 0.1% dimethyl sulfoxide (DMSO) and then diluted into TSB or warm TSA plus 0.1% DMSO to achieve the desired concentration of fatty acids. Linoleic acid (*cis*,*cis*-9,12-octadecadienoic acid; 18:2) was purchased from Sigma, arachidonic acid (*cis*,*cis*,*cis*,*cis*-5,8,11,14-eicosatetraenoic acid; 20:4) and palmitoleic acid (*cis*-9-hexadecenoic acid; 16:1) were purchased from Cayman Chemicals, and sapienic acid (*cis*-6-hexadecenoic acid; 16:1) was purchased from Abcam. As required, media were also supplemented with polymyxin B (Sigma) or CATH-2 (64). *Escherichia coli* strains were grown on LB agar or LB broth supplemented with 100 μg/ml ampicillin when needed. Unless otherwise stated, all cultures were grown at 37°C, and liquid cultures were incubated on an orbital shaking platform at 220 rpm.

Strain and plasmid construction. Genetic manipulation of *S. aureus* was conducted according to established guidelines and as described in previous work (4, 11, 65). All recombinant plasmids were initially constructed in *E. coli* DH5α. The integrity of plasmids was confirmed through nucleotide sequencing of cloned DNA segments prior to electroporation into USA300 or isogenic derivatives, using *S. aureus* RN4220 as an intermediate host. Primer sequences used for PCR amplification of gene segments for plasmid construction or site-directed mutagenesis of cloned genes are defined in Tables S1 and S2 in the supplemental material and are based on the reference genome sequence of USA300 FPR3757 (66). Plasmid pALC2073, which provides a basal level of gene expression from the $P_{xyl/tetO}$ promoter and a stronger inducible level of expression with anhydrotetracycline (67, 68), was used for ectopic expression *graS* and *mprF*. Site-directed mutagenesis was conducted on p*graS* using primers *graS*-SDM-F and *graS*-SDM-R with Phusion DNA polymerase to produce p$graS^{3D>G}$, where codons for Asp35, Asp37, and Asp41 were changed to those for glycine, using guidelines described in the Stratagene QuikChange manual. Similarly, p*mprF* was used as the template with mutagenic primers *mprF*-SDM-F and *mprF*-SDM-R to produce p$mprF^{K547A}$, where the codon at Lys547 was altered to encode alanine.

Growth, viability, and MIC assays. For growth analyses, cultures of *S. aureus* were prepared by inoculating 3 ml of TSB in a 13-ml polypropylene tube containing antibiotic as required and grown overnight for 16 h. After determining the optical density at 600 nm (OD_{600}), aliquots were subcultured into 125-ml-capacity flasks containing 25 ml of TSB or TSB modified by addition of buffer, fatty acid, antimicrobial peptide, or cation supplements to achieve an initial OD_{600} of 0.01. Growth (OD_{600}) was monitored at hourly intervals. Alternately, bacteria were subcultured into 200 μl of medium in wells of 96-well flat-bottom assay plates (Fisher) to an OD_{600} of 0.01, and growth was monitored at 37°C using a Synergy H4 temperature-controlled microplate reader (BioTek Instruments) with measurement of OD_{600} every 20 min for 18 to 24 h. For viability assays, *S. aureus* inoculum cultures in polypropylene tubes as described above were grown for 4 h and diluted to an OD_{600} of 0.01 in fresh TSB, followed by preparation of serial 10^{-1}, 10^{-2}, and 10^{-3} dilutions. Triplicate aliquots of 10 μl from each dilution were then plated on different formulations of TSA, and viable bacteria were enumerated after 24 h of growth.

For MIC assays, inoculum cultures were grown to mid-exponential phase in flasks as for growth assays and then subcultured at an OD_{600} of 0.01 into triplicate 20- by 150-mm glass culture tubes containing 3 ml of medium supplemented with 0.1% DMSO and designated concentrations of linoleic or palmitoleic acid. Cultures were incubated at 37°C with vigorous shaking, and OD_{600} values were determined after 24 h.

Cytochrome *c* binding assay. Cell surface charge was measured as a function of cytochrome *c* binding as previously described (69). Briefly, bacterial cultures were grown to an OD_{600} of 0.5 before being washed twice in morpholinepropanesulfonic acid (MOPS) buffer (20 mM, pH 7.0). Cells were resuspended to an OD_{600} of 7.0 in MOPS buffer, and 360-μl aliquots were mixed with 40 μl of bovine cytochrome *c* (Sigma) to a final concentration of 0.5 mg/ml. Samples were incubated for 15 min at 37°C, followed by centrifugation at 6,000 × *g* for 8 min, and the remaining unbound cytochrome *c* was quantified by measuring absorbance at 530 nm (A_{530}) relative to a MOPS buffer blank containing 0.5 mg/ml cytochrome *c*.

SDS-PAGE and zymography. For SDS-PAGE analysis of secreted protein profiles, *S. aureus* cultures were grown for 20 h, and proteins in cell-free culture supernatant were precipitated by mixing with equal

volumes of ice-cold 20% trichloroacetic acid (TCA), washed in ice-cold 70% ethanol, and then air dried prior to dissolving in SDS-PAGE reducing buffer as described previously (46). Protein equivalent to 3.0 OD_{600} units of culture supernatant was then loaded for protein separation on a 10% acrylamide gel using the Laemmli buffer system (70), and after electrophoresis, proteins were stained using Coomassie blue. For detection of protease activity by zymogram assay, the resolving gel was copolymerized with 1 mg/ml casein and protein equivalent to 0.075 OD_{600} unit was applied to each lane. Details on sample processing, electrophoresis, and zymogram development are as described previously (71).

Data analysis. Prism 8 version 8.4.0 was used to create all graphs and perform statistical analyses in this study. In all experiments, triplicate cultures were used, and means ± standard errors (SEs) are used to represent the data in graphs. Unpaired one-tailed *t* tests, one-way analysis of variance (ANOVA) with multiple comparisons, or two-way ANOVA with multiple comparisons was used to test statistical significance depending on the nature of the experiment. Significance was defined as stated in the figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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REFERENCES

1. Sakr A, Brégeon F, Mège JL, Rolain JM, Blin O. 2018. *Staphylococcus aureus* nasal colonization: an update on mechanisms, epidemiology, risk factors, and subsequent infections. Front Microbiol 9:2419. https://doi.org/10.3389/fmicb.2018.02419.
2. van Hal SJ, Jensen SO, Vaska VL, Espedido BA, Paterson DL, Gosbell IB. 2012. Predictors of mortality in *Staphylococcus aureus* bacteremia. Clin Microbiol Rev 25:362–386. https://doi.org/10.1128/CMR.05022-11.
3. Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, Nouwen JL. 2005. The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect Dis 5:751–762. https://doi.org/10.1016/S1473-3099(05)70295-4.
4. Arsic B, Zhu Y, Heinrichs DE, McGavin MJ. 2012. Induction of the staphylococcal proteolytic cascade by antimicrobial fatty acids in community acquired methicillin resistant *Staphylococcus aureus*. PLoS One 7:e45952. https://doi.org/10.1371/journal.pone.0045952.
5. Alnaseri H, Arsic B, Schneider JET, Kaiser JC, Scinocca ZC, Heinrichs DE, McGavin MJ. 2015. Inducible expression of a resistance-nodulation-division-type efflux pump in *Staphylococcus aureus* provides resistance to linoleic and arachidonic acids. J Bacteriol 197:1893–1905. https://doi.org/10.1128/JB.02607-14.
6. Cadieux B, Vijayakumaran V, Bernards MA, McGavin MJ, Heinrichs DE. 2014. Role of lipase from community-associated methicillin-resistant *Staphylococcus aureus* strain USA300 in hydrolyzing triglycerides into growth-inhibitory free fatty Acids. J Bacteriol 196:4044–4056. https://doi.org/10.1128/JB.02044-14.
7. Flannagan RS, Kuiack RC, McGavin MJ, Heinrichs DE. 2018. *Staphylococcus aureus* uses the GraXRS regulatory system to sense and adapt to the acidified phagolysosome in macrophages. mBio 9:e01143-18. https://doi.org/10.1128/mBio.01143-18.
8. Do TQ, Moshkani S, Castillo P, Anunta S, Pogosyan A, Cheung A, Marbois B, Faull KF, Ernst W, Chiang SM, Fujii G, Clarke CF, Foster K, Porter E. 2008. Lipids including cholesteryl linoleate and cholesteryl arachidonate contribute to the inherent antibacterial activity of human nasal fluid. J Immunol 181:4177–4187. https://doi.org/10.4049/jimmunol.181.6.4177.
9. Wille JJ, Kydonieus A. 2003. Palmitoleic acid isomer ($C_{16:1}$delta6) in human skin sebum is effective against Gram-positive bacteria. Skin Pharmacol Appl Skin Physiol 16:176–187. https://doi.org/10.1159/000069757.
10. Takigawa H, Nakagawa H, Kuzukawa M, Mori H, Imokawa G. 2005. Deficient production of hexadecenoic acid in the skin is associated in part with the vulnerability of atopic dermatitis patients to colonization by *Staphylococcus aureus*. Dermatology 211:240–248. https://doi.org/10.1159/000087018.
11. Alnaseri H, Kuiack RC, Ferguson KA, Schneider JET, Heinrichs DE, McGavin MJ. 2018. DNA binding and sensor specificity of FarR, a novel TetR family regulator required for induction of the fatty acid efflux pump FarE in *Staphylococcus aureus*. J Bacteriol 201:e00602-18. https://doi.org/10.1128/JB.00602-18.
12. England RJA, Homer JJ, Knight LC, Ell SR. 1999. Nasal pH measurement: a reliable and repeatable parameter. Clin Otolaryngol 24:67–68. https://doi.org/10.1046/j.1365-2273.1999.00223.x.
13. Elias PM. 2007. The skin barrier as an innate immune element. Semin Immunopathol 29:3–14. https://doi.org/10.1007/s00281-007-0060-9.
14. Baraniuk JN, Merck SJ. 2009. New concepts of neural regulation in human nasal mucosa. Acta Clin Croat 48:65–73.
15. Cottrill EE, Chen B, Adappa ND, Palmer JN, Kennedy DW, Lee RJ, Cohen NA. 2017. Expression of dermcidin in human sinonasal secretions. Int Forum Allergy Rhinol 7:154–159. https://doi.org/10.1002/alr.21851.
16. Schittek B, Hipfel R, Sauer B, Bauer J, Kalbacher H, Stevanovic S, Schirle M, Schroeder K, Blin N, Meier F, Rassner G, Garbe C. 2001. Dermcidin: a novel human antibiotic peptide secreted by sweat glands. Nat Immunol 2:1133–1137. https://doi.org/10.1038/ni732.
17. Kisich KO, Howell MD, Boguniewicz M, Heizer HR, Watson NU, Leung DYM. 2007. The constitutive capacity of human keratinocytes to kill *Staphylococcus aureus* is dependent on β-defensin 3. J Invest Dermatol 127:2368–2380. https://doi.org/10.1038/sj.jid.5700861.
18. Zanger P, Nurjadi D, Vath B, Kremsner PG. 2011. Persistent nasal carriage of *Staphylococcus aureus* is associated with deficient induction of human-defensin 3 after sterile wounding of healthy skin *in vivo*. Infect Immun 79:2658–2662. https://doi.org/10.1128/IAI.00101-11.
19. Lee SE, Lee SH. 2018. Skin barrier and calcium. Ann Dermatol 30:265–275. https://doi.org/10.5021/ad.2018.30.3.265.
20. Weinrick B, Dunman PM, McAleese F, Murphy E, Projan SJ, Fang Y, Novick RP. 2004. Effect of mild acid on gene expression in *Staphylococcus aureus*. J Bacteriol 186:8407–8423. https://doi.org/10.1128/JB.186.24.8407-8423.2004.
21. Kenny JG, Ward D, Josefsson E, Jonsson IM, Hinds J, Rees HH, Lindsay JA, Tarkowski A, Horsburgh MJ. 2009. The *Staphylococcus aureus* response to unsaturated long chain free fatty acids: survival mechanisms and virulence implications. PLoS One 4:e4344. https://doi.org/10.1371/journal.pone.0004344.
22. Li M, Cha DJ, Lai Y, Villaruz AE, Sturdevant DE, Otto M. 2007. The antimicrobial peptide-sensing system *aps* of *Staphylococcus aureus*. Mol Microbiol 66:1136–1147. https://doi.org/10.1111/j.1365-2958.2007.05986.x.
23. El-Halfawy OM, Czarny TL, Flannagan RS, Day J, Bozelli JC, Kuiack RC, Salim A, Eckert P, Epand RM, McGavin MJ, Organ MG, Heinrichs DE, Brown ED. 2020. Discovery of an antivirulence compound that reverses β-lactam resistance in MRSA. Nat Chem Biol 16:143–149. https://doi.org/10.1038/s41589-019-0401-8.
24. Li M, Lai Y, Villaruz AE, Cha DJ, Sturdevant DE, Otto M. 2007. Gram-positive three-component antimicrobial peptide-sensing system.

Proc Natl Acad Sci U S A 104:9469–9474. https://doi.org/10.1073/pnas.0702159104.
25. Falord M, Karimova G, Hiron A, Msadek T. 2012. GraXSR proteins interact with the VraFG ABC transporter to form a five-component system required for cationic antimicrobial peptide sensing and resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother 56:1047–1058. https://doi.org/10.1128/AAC.05054-11.
26. Muzamal U, Gomez D, Kapadia F, Golemi-Kotra D, Muzamal U, Gomez D, Kapadia F, Golemi-Kotra D. 2014. Diversity of two-component systems: insights into the signal transduction mechanism by the *Staphylococcus aureus* two-component system GraSR. F1000Res 3:252. https://doi.org/10.12688/f1000research.5512.1.
27. Weidenmaier C, Peschel A, Kempf VA, Lucindo N, Yeaman MR, Bayer AS. 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. https://doi.org/10.1128/IAI.73.12.8033-8038.2005.
28. 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. https://doi.org/10.1128/IAI.01030-15.
29. Cheung AL, Bayer AS, Yeaman MR, Xiong YQ, Waring AJ, Memmi G, Donegan N, Chaili S, Yang S-J. 2014. Site-specific mutation of the sensor kinase GraS in *Staphylococcus aureus* alters the adaptive response to distinct cationic antimicrobial peptides. Infect Immun 82:5336–5345. https://doi.org/10.1128/IAI.02480-14.
30. Ernst CM, Staubitz P, Mishra NN, Yang S-J, Hornig G, Kalbacher H, Bayer AS, Kraus D, Peschel A. 2009. The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. PLoS Pathog 5:e1000660. https://doi.org/10.1371/journal.ppat.1000660.
31. Mascher T. 2006. Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in *Firmicutes* bacteria. FEMS Microbiol Lett 264:133–144. https://doi.org/10.1111/j.1574-6968.2006.00444.x.
32. Liu Q, Yeo W, Bae T. 2016. The SaeRS two-component system of *Staphylococcus aureus*. Genes 7:81. https://doi.org/10.3390/genes7100081.
33. Jeong D-W, Cho H, Jones MB, Shatzkes K, Sun F, Ji Q, Liu Q, Peterson SN, He C, Bae T. 2012. The auxiliary protein complex SaePQ activates the phosphatase activity of sensor kinase SaeS in the SaeRS two-component system of *Staphylococcus aureus*. Mol Microbiol 86:331–348. https://doi.org/10.1111/j.1365-2958.2012.08198.x.
34. Villanueva M, García B, Valle J, Rapún B, Ruiz De Los Mozos I, Solano C, Martí M, Penadés JR, Toledo-Arana A, Lasa I. 2018. Sensory deprivation in *Staphylococcus aureus*. Nat Commun 9:523. https://doi.org/10.1038/s41467-018-02949-y.
35. Lähdesmäki K, Ollila OHS, Koivuniemi A, Kovanen PT, Hyvönen MT. 2010. Membrane simulations mimicking acidic pH reveal increased thickness and negative curvature in a bilayer consisting of lysophosphatidylcholines and free fatty acids. Biochim Biophys Acta 1798:938–946. https://doi.org/10.1016/j.bbamem.2010.01.020.
36. Miller LG, Eells SJ, Taylor AR, David MZ, Ortiz N, Zychowski D, Kumar N, Cruz D, Boyle-Vavra S, Daum RS. 2012. *Staphylococcus aureus* colonization among household contacts of patients with skin infections: risk factors, strain discordance, and complex ecology. Clin Infect Dis 54:1523–1535. https://doi.org/10.1093/cid/cis213.
37. Shryock TR, Kapral FA. 1992. The production of bactericidal fatty acids from glycerides in staphylococcal abscesses. J Med Microbiol 36:288–292. https://doi.org/10.1099/00222615-36-4-288.
38. Montain SJ, Cheuvront SN, Lukaski HC. 2007. Sweat mineral-element responses during 7 h of exercise-heat stress. Int J Sport Nutr Exerc Metab 17:574–582. https://doi.org/10.1123/ijsnem.17.6.574.
39. Coorens M, Scheenstra MR, Veldhuizen EJA, Haagsman HP. 2017. Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis. Sci Rep 7:40874. https://doi.org/10.1038/srep40874.
40. Schneider VAF, Coorens M, Bokhoven JLMT, Posthuma G, van Dijk A, Veldhuizen EJA, Haagsman HP. 2017. Imaging the antistaphylococcal activity of CATH-2: mechanism of attack and regulation of inflammatory response. mSphere 2:e00370-17. https://doi.org/10.1128/mSphere.00370-17.
41. Banaschewski BJH, Baer B, Arsenault C, Jazey T, Veldhuizen EJA, Delport J, Gooyers T, Lewis JF, Haagsman HP, Veldhuizen RAW, Yamashita C. 2017. The antibacterial and anti-inflammatory activity of chicken cathelicidin-2 combined with exogenous surfactant for the treatment of cystic fibrosis-associated pathogens. Sci Rep 7:15545. https://doi.org/10.1038/s41598-017-15558-4.
42. Flad T, Bogumil R, Tolson J, Schittek B, Garbe C, Deeg M, Mueller CA, Kalbacher H. 2002. Detection of dermcidin-derived peptides in sweat by ProteinChip technology. J Immunol Methods 270:53–62. https://doi.org/10.1016/S0022-1759(02)00229-6.
43. Yang S-J, Bayer AS, Mishra NN, Meehl M, Ledala N, Yeaman MR, Xiong YQ, Cheung AL. 2012. The *Staphylococcus aureus* two-component regulatory system, GraRS, senses and confers resistance to selected cationic antimicrobial peptides. Infect Immun 80:74–81. https://doi.org/10.1128/IAI.05669-11.
44. Parsons JB, Broussard TC, Bose JL, Rosch JW, Jackson P, Subramanian C, Rock CO. 2014. Identification of a two-component fatty acid kinase responsible for host fatty acid incorporation by *Staphylococcus aureus*. Proc Natl Acad Sci U S A 111:10532–10537. https://doi.org/10.1073/pnas.1408797111.
45. 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 231:67–71. https://doi.org/10.1016/S0378-1097(03)00921-2.
46. Nickerson NN, Prasad L, Jacob L, Delbaere LT, McGavin MJ. 2007. Activation of the SspA serine protease zymogen of *Staphylococcus aureus* proceeds through unique variations of a trypsinogen-like mechanism and is dependent on both autocatalytic and metalloprotease-specific processing. J Biol Chem 282:34129–34138. https://doi.org/10.1074/jbc.M705672200.
47. Massimi I, Park E, Rice K, Muller-Esterl W, Sauder D, McGavin MJ. 2002. Identification of a novel maturation mechanism and restricted substrate specificity for the SspB cysteine protease of *Staphylococcus aureus*. J Biol Chem 277:41770–41777. https://doi.org/10.1074/jbc.M207162200.
48. Tran-Winkler HJ, Love JF, Gryllos I, Wessels MR. 2011. Signal transduction through CsrRS confers an invasive phenotype in group A *Streptococcus*. PLoS Pathog 7:e1002361. https://doi.org/10.1371/journal.ppat.1002361.
49. Cho US, Bader MW, Amaya MF, Daley ME, Klevit RE, Miller SI, Xu W. 2006. Metal bridges between the PhoQ sensor domain and the membrane regulate transmembrane signaling. J Mol Biol 356:1193–1206. https://doi.org/10.1016/j.jmb.2005.12.032.
50. Liu Q, Cho H, Yeo W-S, Bae T. 2015. The extracytoplasmic linker peptide of the sensor protein SaeS tunes the kinase activity required for staphylococcal virulence in response to host signals. PLoS Pathog 11:e1004799. https://doi.org/10.1371/journal.ppat.1004799.
51. Lee T-H, Hall K, Aguilar M-I. 2016. Antimicrobial peptide structure and mechanism of action: a focus on the role of membrane structure. Curr Top Med Chem 16:25–39. https://doi.org/10.2174/1568026615666150703121700.
52. Inda ME, Vandenbranden M, Fernández A, de Mendoza D, Ruysschaert J-M, Cybulski LE. 2014. A lipid-mediated conformational switch modulates the thermosensing activity of DesK. Proc Natl Acad Sci U S A 111:3579–3584. https://doi.org/10.1073/pnas.1317147111.
53. Ernst CM, Slavetinsky CJ, Kuhn S, Hauser JN, Nega M, Mishra NN, Gekeler C, Bayer AS, Peschel A. 2018. Gain-of-function mutations in the phospholipid flippase MprF confer specific daptomycin resistance. mBio 9:e01659-18. https://doi.org/10.1128/mBio.01659-18.
54. Ernst CM, Peschel A. 2019. MprF-mediated daptomycin resistance. Int J Med Microbiol 309:359–363. https://doi.org/10.1016/j.ijmm.2019.05.010.
55. Müller A, Wenzel M, Strahl H, Grein F, Saaki TV, Kohl B, Siersma T, Bandow JE, Sahl H-G, Schneider T, Hamoen LW. 2016. Daptomycin inhibits cell envelope synthesis by interfering with fluid membrane microdomains. Proc Natl Acad Sci U S A 113:E7077–E7086. https://doi.org/10.1073/pnas.1611173113.
56. Howden BP, Stinear TP, Allen DL, Johnson PD, Ward PB, Davies JK. 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. https://doi.org/10.1128/AAC.01613-07.
57. Falord M, Mäder U, Hiron A, Débarbouillé 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. https://doi.org/10.1371/journal.pone.0021323.
58. Beavers WN, Monteith AJ, Amarnath V, Mernaugh RL, Roberts LJ, Chazin WJ, Davies SS, Skaar EP. 2019. Arachidonic acid kills *Staphylococcus aureus* through a lipid peroxidation mechanism. mBio 10:e01333-19. https://doi.org/10.1128/mBio.01333-19.
59. Gimza BD, Larias MI, Budny BG, Shaw LN. 2019. Mapping the global

network of extracellular protease regulation in *Staphylococcus aureus*. mSphere 4:e00676-19. https://doi.org/10.1128/mSphere.00676-19.
60. Zhou C, Bhinderwala F, Lehman MK, Thomas VC, Chaudhari SS, Yamada KJ, Foster KW, Powers R, Kielian T, Fey PD. 2019. Urease is an essential component of the acid response network of *Staphylococcus aureus* and is required for a persistent murine kidney infection. PLoS Pathog 15: e1007538. https://doi.org/10.1371/journal.ppat.1007538.
61. Broder UN, Jaeger T, Jenal U. 2016. LadS is a calcium-responsive kinase that induces acute-to-chronic virulence switch in *Pseudomonas aeruginosa*. Nat Microbiol 2:16184. https://doi.org/10.1038/nmicrobiol.2016.184.
62. Tischler AH, Lie L, Thompson CM, Visick KL. 2018. Discovery of calcium as a biofilm-promoting signal for *Vibrio fischeri* reveals new phenotypes and underlying regulatory complexity. J Bacteriol 200:e00016-18. https://doi.org/10.1128/JB.00016-18.
63. Rice KC, Nelson JB, Patton TG, Yang SJ, Bayles KW. 2005. Acetic acid induces expression of the *Staphylococcus aureus cidABC* and *lrgAB* murein hydrolase regulator operons. J Bacteriol 187:813–821. https://doi.org/10.1128/JB.187.3.813-821.2005.
64. Banaschewski BJH, Veldhuizen EJA, Keating E, Haagsman HP, Zuo YY, Yamashita CM, Veldhuizen RAW. 2015. Antimicrobial and biophysical properties of surfactant supplemented with an antimicrobial peptide for treatment of bacterial pneumonia. Antimicrob Agents Chemother 59: 3075–3083. https://doi.org/10.1128/AAC.04937-14.
65. Novick RP. 1991. Genetic systems in staphylococci. Methods Enzymol 204:587–636. https://doi.org/10.1016/0076-6879(91)04029-n.
66. Diep BA, Gill SR, Chang RF, Phan TH, Chen JH, Davidson MG, Lin F, Lin J, Carleton HA, Mongodin EF, Sensabaugh GF, Perdreau-Remington F. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. Lancet 367:731–739. https://doi.org/10.1016/S0140-6736(06)68231-7.
67. Corrigan RM, Foster TJ. 2009. An improved tetracycline-inducible expression vector for *Staphylococcus aureus*. Plasmid 61:126–129. https://doi.org/10.1016/j.plasmid.2008.10.001.
68. Bateman BT, Donegan NP, Jarry TM, Palma M, Cheung AL. 2001. Evaluation of a tetracycline-inducible promoter in *Staphylococcus aureus in vitro* and *in vivo* and its application in demonstrating the role of *sigB* in microcolony formation. Infect Immun 69:7851–7857. https://doi.org/10.1128/IAI.69.12.7851-7857.2001.
69. Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, Götz F. 1999. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem 274:8405–8410. https://doi.org/10.1074/jbc.274.13.8405.
70. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685. https://doi.org/10.1038/227680a0.
71. Rice K, Peralta R, Bast D, de Azavedo J, McGavin MJ. 2001. Description of staphylococcus serine protease (*ssp*) operon in *Staphylococcus aureus* and nonpolar inactivation of *sspA*-encoded serine protease. Infect Immun 69:159–169. https://doi.org/10.1128/IAI.69.1.159-169.2001.