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Heterogeneity of *mprF* Sequences in Methicillin-Resistant *Staphylococcus aureus* Clinical Isolates: Role in Cross-Resistance between Daptomycin and Host Defense Antimicrobial Peptides

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Over the past several years, single-nucleotide polymorphisms (SNPs) within the *mprF* open reading frame (ORF) have been proposed to be associated with a gain-of-function phenotype in terms of daptomycin (DAP) nonsusceptibility (referred to as daptomycin resistance [DAP-R] herein for ease of presentation) in *Staphylococcus aureus*. We investigated the frequencies of SNPs within the *mprF* ORF and the relationships of such SNPs to cross-resistance between DAP and cationic host defense peptides (HDPs). Thirty-five well-characterized, unique DAP-susceptible (DAP-S) and DAP-R methicillin-resistant *S. aureus* (MRSA) isolates of the clonal complex 5 genotype were used. In addition to *mprF* SNPs and DAP-HDP cross-resistance, several other key genotypic and phenotypic metrics often associated with DAP-R were delineated, as follows: (i) *mprF* expression, (ii) membrane phospholipid content, (iii) positive surface charge, (iv) DAP binding, and (v) cell wall thickness profiles. A number of DAP-S strains (MICs of ≤1 μg/ml) exhibited *mprF* SNPs, occasionally with high-level *mprF* sequence variation from the genotype reference strain. However, none of these SNPs were localized to well-chronicled *mprF* hot spot locations associated with DAP-R in *S. aureus*. In contrast, all 8 DAP-R isolates demonstrated SNPs within such known *mprF* hot spots. Moreover, only the DAP-R strains showed MprF gain-of-function phenotypes, enhanced *mprF* expression, higher survival against two prototypical HDPs, and reduced DAP binding. Although a heterogenous array of *mprF* SNPs were often found in DAP-S strains, only selected hot spot SNPs, combined with concurrent *mprF* dysregulation, were associated with the DAP-R phenotype.

There have been many recent reports of clinical *Staphylococcus aureus* strains that have evolved *in vitro* daptomycin (DAP) resistance (DAP-R) (note that although the official term is daptomycin nonsusceptibility, we will utilize daptomycin resistance in this paper for ease of presentation) in the context of failing DAP treatment regimens, especially in endovascular infections (1–5). One of the key features of many DAP-R strains is the acquisition of one or more single-nucleotide polymorphisms (SNPs) in a relatively restricted cadre of genes, especially in *mprF* (multiple peptide resistance factor) (3, 6–8). The *mprF* gene is responsible for the synthesis and translocation (flipping) of the positively charged phospholipid (PL) lysyl-phosphatidylglycerol (L-PG) within its cell membrane (CM). This process contributes substantially to the net positive surface charge in *S. aureus*, particularly in adaptive responses to cationic antimicrobial agents (3, 7, 9). Although DAP is inherently an anionic molecule, its bactericidal activity is absolutely dependent on it undergoing extensive complexing with calcium, rendering the functional DAP complex positively charged. Therefore, genes such as *mprF* that affect surface charge are hypothesized to be integral to DAP-R, potentially via charge repulsive electrostatic mechanisms (10). In addition, recent data from our laboratories have shown that both clinical and *in vitro*-derived DAP-R *S. aureus* strains frequently exhibit cross-resistance with cationic host defense peptides (HDPs), such as those derived from polymorphonuclear leukocytes (PMNs) and platelets (8, 11, 12).

The cataloguing of *mprF* SNPs associated with DAP-R in *S. aureus* has principally emerged from studies of individual isogenic DAP-susceptible (DAP-S) and DAP-R strain pairs. However, such data have not been systematically evaluated in a population-based survey of *mprF* sequences from well-defined groups of *S. aureus* strains with differing DAP MICs. Thus, in the current study, we investigated the frequencies of *mprF* gain-of-function SNPs and their relationships to both DAP-R and cross-resistance to prototypical HDPs. We also compared these outcomes with several genotypic and phenotypic determinants previously linked to *mprF*-mediated DAP-R in *S. aureus*, including positive surface charge (3, 13), CM phospholipid content and asymmetry (9, 14), *mprF* transcription profiles (7, 15), DAP surface binding (16), and cell wall thickening (8).

MATERIALS AND METHODS

Bacterial strains. The 27 methicillin-resistant *S. aureus* (MRSA) bloodstream study isolates were selected from a recently described overall strain collection of 47 MRSA strains (11, 17). Since these 47 strains were from distinct genotypic backgrounds, we focused only on isolates genotyped as clonal complex 5 (CC5; the most common clonotype in this collection) to

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maintain relative strain homogeneity. Sixteen of the 27 CC5 strains had DAP MICs of 0.25 to 0.5 μg/ml, while the remaining 11 strains had DAP MICs of 1 μg/ml; both groups are DAP-S by presumptive breakpoints (18). In addition, 8 DAP-R isolates (MICs of >2 μg/ml) previously genotyped as CC5 were randomly selected from our DAP-R strain collection. All of the DAP-S MRSA isolates were from bacteremic patients who had never received DAP and have been characterized previously (11, 17). The DAP-R MRSA isolates (MICs of >2 μg/ml) were from patients who had been treated with DAP and failed therapy. The latter strains have not been reported before.

All strains were grown in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI), Mueller-Hinton broth (Difco Laboratories, Detroit, MI), or Luria broth (LB; Difco Laboratories) as indicated below, depending on the individual experiment. 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. Preliminary studies showed that all MRSA isolates in this investigation had similar *in vitro* growth kinetics and growth yields (data not shown).

The MICs of the strains to DAP were determined by standard Etest (AB Biodisk, Dalvagen, Sweden) on Mueller-Hinton agar plates, following the manufacturer's protocol (including calcium supplementations) (Difco Laboratories, Detroit, MI). DAP-R was defined as an Etest MIC of ≥2 μg/ml (19).

DNA isolation and *mprF* sequencing. Genomic DNA was isolated from *S. aureus* using the method of Dyer and Iandolo (20). PCR amplification of the *mprF* ORFs was performed as previously described, using the primer pair *mprF*-F-bam and *mprF*-R-sph (15). DNA sequencing of the *mprF* ORFs was kindly performed at City of Hope, Duarte, CA. The *mprF* sequences from *S. aureus* MU50 (CC5) (GenBank accession number BA000017.4) were used as the consensus reference sequences to identify *mprF* SNPs among the study strains.

Host defense peptide susceptibility assays. We studied prototypical HDPs from human PMNs and rabbit platelets. Human neutrophil peptide-1 (hNP-1), an archetypal α-defensin, was purchased from Peptide International (Louisville, KY). Thrombin-induced platelet microbicidal proteins (tPMPs) from fresh rabbit platelets were obtained as previously detailed; this preparation contains a mixture of tPMPs but is predominantly tPMP-1 (20–22). The bioactivity of these preparations (μg/ml equivalents) was determined by the standard *Bacillus subtilis* killing assay as outlined before (21, 22).

Standard MIC testing in nutrient broth may underestimate HDP activities (22, 23). Accordingly, *in vitro* bactericidal assays were carried out with hNP-1 and tPMPs using a well-characterized 2-h microdilution method in Eagle's minimal essential medium (3, 23). These assays were performed with either tPMPs (1 and 2 μg/ml bioactivity equivalents) or hNP-1 (10 and 20 μg/ml), using an initial inoculum of 5×10^3 CFU *S. aureus* cells (3, 23). These HDP concentrations were selected based on extensive pilot studies showing their inability to completely eradicate the starting inocula of the strain collections over the 2-h exposure period. Data were calculated and expressed as the relative percentage of surviving CFU (± standard deviation [SD]) of HDP-exposed versus HDP-unexposed cells, with the survival of each parental strain set at 100%. A minimum of three independent experiments was performed for each of the HDP assays.

Surface charge assays. To quantify the relative positive cell surface charges in the strain sets, cytochrome *c* binding assays were performed as described before (24, 25). The binding of the highly cationic cytochrome *c* (pI = 10) (Sigma) was measured spectrophotometrically (optical density at 530 nm [OD_{530}]), which quantifies the amount of this polycation remaining within the reaction mixture supernatants following exposure to the study strains for 30 min; the higher the amounts of residual cytochrome *c* in the supernates, the more relative positive surface charge exists (3, 8, 24, 26). A minimum of three independent experiments was performed for each evaluation.

RNA isolation and quantitative real-time PCR (qRT-PCR). Four isolates from each of the three DAP MIC groups were randomly selected for characterizing *mrpF* expression profiles. To quantify the expression of the *mprF* gene, total RNA was isolated from the *S. aureus* cell pellets using the RNeasy kit (Qiagen, Valencia, CA) and the FastPrep FP120 instrument (BIO 101, Vista, CA) according to the manufacturer's recommended protocols. For RNA isolation, fresh overnight cultures of *S. aureus* strains were used to inoculate TSB to an OD_{600} of 0.1. Cell pellets were then obtained at either exponential (2 h) or late stationary (12 h) growth phases using previously described methods (27).

For qRT-PCR analyses, 2 μg of DNase-treated RNA was reverse transcribed using the SuperScript III first-strand synthesis kit (Invitrogen) according to the manufacturer's protocols. Quantification of cDNA levels was performed following the instructions of the Power SYBR green master mix kit (Applied Biosystems) on an ABI Prism 7000 sequence detection system (Applied Biosystems). The *mprF* and *gyrB* genes were detected using their respective specific primers as described before (27, 28). Fold changes in the expression levels of *mprF* were quantified in relation to the levels of *gyrB*.

CM phospholipid contents. The three major *S. aureus* CM phospholipids (PLs) are L-PG, PG, and cardiolipin (CL) (9, 10, 29). To quantify the relative proportions of these three PLs in our strain sets, CM PLs were extracted from the selected *S. aureus* strains as described previously (26). The target PLs were separated and identified via two-dimensional thin-layer chromatography by their electrophoretic mobilities and ninhydrin staining properties (3, 26) and then removed from the plates and chemically quantified by spectrophotometer as described previously (3, 26). In addition, the proportion of synthesized L-PG which was translocated to the outer CM leaflet was quantified spectrophotometrically as detailed before, using the L-PG-targeting outer-CM-impermeable UV probe fluorescamine (14, 26). The latter fluorophore only binds to outer CM amine-containing PLs, such as L-PG. The composite data were expressed as the relative proportions (±SD) of the three PLs and the percentages of L-PG translocated to the outer CM, respectively. At least three independent experiments were performed to analyze the overall PL contents and L-PG translocations.

DAP binding assays. To determine the relative profiles of whole-cell DAP binding to the selected *S. aureus* strains, bacteria were grown to an OD_{600} of 0.4 to 0.6 in LB supplemented with calcium (50 μg/ml) and then incubated with Bodipy-fluorescein-labeled DAP (Bodipy-DAP, 8 μg/ml; supplied courtesy of Cubist Pharmaceuticals, Lexington, MA) for 20 min at 37°C with shaking. Excess unincorporated label was removed by washing the cells three times in LB. In the final wash step, the cells were counterstained with DAPI (4′,6-diamidino-2-phenylindole; 2 μg/ml) DNA stain to visualize the cells (16) and then imaged using a DeltaVision deconvolution microscope (Applied Precision, Inc., Issaquah, WA) (16). The DAP-binding phenotype of the MRSA cells was quantified by the relative number of DAP-binding fluorescent spots/cell (more than 100 cells were queried for each strain).

Cell wall thickness. The cell wall thicknesses of all study strains were compared by transmission electron microscopy (TEM) (8). The mean thickness (nm ± SD) of 100 cells was determined for the set of strains at a constant magnification of ×190,000 (model number 100CX; JEOL, Tokyo, Japan) using digital image capture and morphometric measurement (Advanced Microscopy Techniques version 54, Danvers, MA).

Statistical analysis. The Kruskal-Wallis analysis of variance (ANOVA) test with the Tukey *post hoc* correction for multiple comparisons was utilized where indicated below. Significance was determined at a *P* value of <0.05.

RESULTS

SNPs within *mprF* genes. A total of 35 *S. aureus* strains were subjected to *mprF* open reading frame (ORF) sequencing. Sequencing analyses of *mprF* genes revealed that 11/16 isolates with DAP MICs of ≤0.5 μg/ml and 6/11 isolates with DAP MICs of 1

TABLE 1 Host defense peptide susceptibility among the study strain sets

HDP (concn used)[a]	% Survival (mean ± SD) after 2-h exposure to HDP in group with DAP MIC of:			*P* value		
	≤0.5 μg/ml	1 μg/ml	≥2 μg/ml	0.5 vs 1 μg/ml	1 vs 2 μg/ml	0.5 vs 2 μg/ml
tPMPs (2 μg/ml)	10.1 ± 12.9	35.3 ± 24.9	43.1 ± 30.2	<0.01	NS[b]	<0.001
tPMPs (1 μg/ml)	19.1 ± 15.7	47.8 ± 26.2	59.0 ± 27.8	<0.01	NS	<0.001
hNP-1 (20 μg/ml)	17.2 ± 14.3	19.5 ± 15.9	29.1 ± 10.3	NS	<0.05	<0.001
hNP-1 (10 μg/ml)	31.7 ± 21.9	39.8 ± 22.3	65.4 ± 20.3	NS	<0.05	<0.001

[a] tPMPs, thrombin-induced platelet microbicidal proteins; hNP-1, human neutrophil defensin-1.
[b] NS, not significant.

μg/ml had *mprF* sequences identical to that of the *S. aureus* MU50 reference strain. In contrast, 5/16 isolates with DAP MICs of ≤0.5 μg/ml exhibited an amino acid substitution changing isoleucine at position 498 to asparagine (I498N) versus the MU50 sequence. In addition, analyses of 3/11 isolates with DAP MICs of 1 μg/ml had a distinct point mutation (Q692E; glutamine to glutamic acid). Interestingly, two strains in the latter group (MICs of 1 μg/ml) had 30 other amino acid substitutions within their *mprF* ORFs, suggesting high levels of heterogeneity within *mprF* ORFs in some *S. aureus* strains. All isolates with DAP MICs of ≥2 μg/ml had only one amino acid substitution, either at position 341 (L341S; leucine to serine) or 826 (L826F; leucine to phenylalanine), within their *mprF* ORFs. These latter SNPs have been well defined in the literature as high-frequency *mprF* mutational loci associated with DAP-R via gain-of-function phenotypes (9, 12, 13, 30, 31).

***In vitro* susceptibilities to HDPs.** In general, strain groups with higher DAP MICs exhibited significantly higher survival profiles when exposed *in vitro* to the two prototypical HDPs in this study (Table 1). Specifically, strains with DAP MICs of 1 μg/ml exhibited lower susceptibility to tPMP killing than strains with DAP MICs of ≤0.5 μg/ml ($P < 0.01$). In contrast, strains with DAP MICs of 1 μg/ml failed to show significantly reduced susceptibility to hNP-1 compared to those with DAP MICs of ≤0.5 μg/ml. Importantly, DAP-R isolates (DAP MICs of ≥2 μg/ml) exhibited significantly reduced killing by hNP-1 compared to strains in the other two MIC groups ($P < 0.001$).

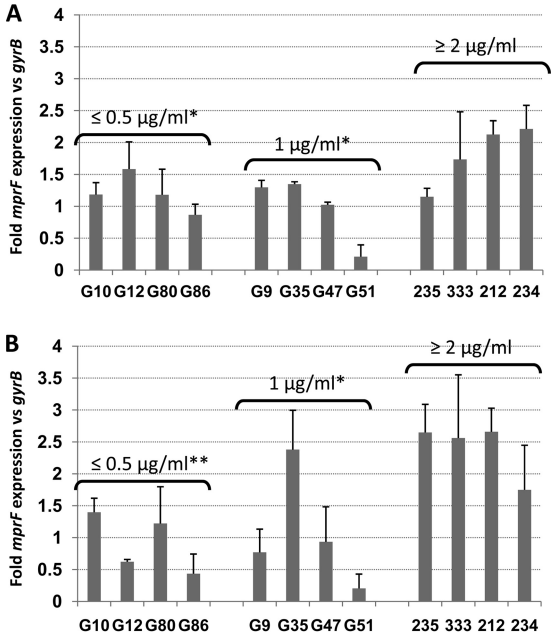

FIG 1 Transcription of *mprF* genes among the study strains during exponential growth phase (A) and stationary phase (B). Total cellular RNA samples from the strains grown in Trypticase soy broth were isolated at 2 h (exponential growth phase) or 12 h (stationary phase) postinoculation and subjected to qRT-PCR analyses. Means ± SDs are shown. *, $P < 0.05$ versus results for group with DAP MICs of ≥2 μg/ml; **, $P < 0.01$ versus results for group with DAP MICs of ≥2 μg/ml.

Expression of *mprF*. As demonstrated by the results in Fig. 1A and B, isolates with DAP MICs of ≥2 μg/ml exhibited significantly higher mean fold *mprF* expression levels than the two DAP-S groups of isolates during both exponential and stationary growth phases. However, there were no significant differences in the mean fold levels of *mprF* expression between strains with DAP MICs of ≤0.5 and those with DAP MICs of 1 μg/ml, irrespective of the growth phase queried.

PL content and translocation of L-PG. The proportion of total L-PG within the overall PL content was significantly increased (~2-fold) in isolates with DAP MICs of ≥2 μg/ml compared to its proportions in strains in the other two MIC groups (Table 2). This profile was mainly observed relative to increases in net L-PG synthesis rather than enhanced translocation (flipping) profiles. As expected, in addition to the enhanced L-PG synthesis phenotype, the DAP-R group displayed a substantial compensatory reduction in the amount of CM PG compared to the amounts in the other two DAP-S isolate groups.

Bodipy-DAP whole-cell binding. As shown by the results in Fig. 2A, B, and C, isolates in the two DAP-S MIC groups bound significantly more Bodipy-DAP than DAP-R strains ($P < 0.05$). In agreement with a previous publication (16), the DAP binding in DAP-S cells occurred focally and preferentially on the staphylococcal surface, especially near the septal division plane. When quantified by the mean number of fluorescent DAP spots/cell, this

TABLE 2 Phospholipid profiles among the three DAP MIC groups of MRSA isolates[a]

DAP MIC strain group	Proportion (mean % ± SD) among overall PL content				
	Inner L-PG	Outer L-PG	L-PG	PG	CL
≤0.5 μg/ml	11.2 ± 1.0†	2.0 ± 1.3	13.2 ± 1.3†	81.5 ± 2.2†	5.3 ± 1.3*
1 μg/ml	13.6 ± 4.1†	2.3 ± 1.0	15.9 ± 4.8†	75.5 ± 8.3†	8.7 ± 5.2
≥2 μg/ml	22.0 ± 5.3	2.5 ± 0.4	24.5 ± 5.6	68.0 ± 11.5	7.5 ± 6.0

[a] L-PG, lysyl-phosphatidylglycerol; PG, phosphatidylglycerol; CL, cardiolipin; *, $P < 0.05$ versus results for group with DAP MICs of ≥2 μg/ml; †, $P < 0.01$ versus results for group with DAP MICs of ≥2 μg/ml.

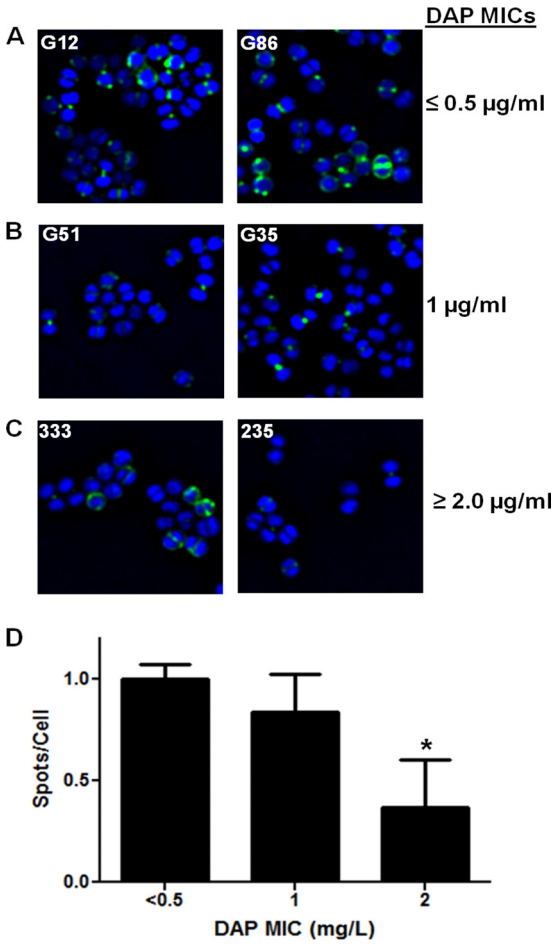

FIG 2 Representative binding of fluorescent DAP (Bodipy-DAP) to *S. aureus* bacteria of different DAP MIC groups. *S. aureus* strains from groups with DAP MICs of ≤0.5 μg/ml (A), 1 μg/ml (B), and ≥0.5 μg/ml (C) were stained with Bodipy-labeled DAP. (D) Intensity of fluorescence from cells stained with Bodipy-DAP. Mean results ± SD are shown. *, $P < 0.05$ versus results for DAP-S groups.

confirmed the significantly decreased surface binding observed among the DAP-R isolates (Fig. 2D).

Net surface charge. Cytochrome *c* binding analyses of all 35 strains revealed that the DAP-R isolates had significantly enhanced mean positive surface charge compared to both groups of DAP-S isolates ($P = 0.0029$ versus strains with DAP MICs of 1 μg/ml and $P = 0.0034$ versus strains with DAP MICs of ≤0.5 μg/ml) (see Table S1 in the supplemental material). There were no significant differences in relative surface charge between isolates in the two DAP-S MIC groups ($P = 0.57$).

Cell wall thickness. The cell wall thicknesses determined by TEM did not differ significantly among the three MIC groups of study strains (see Table S1 in the supplemental material). The average cell wall thicknesses for the groups with DAP MICs of ≤0.5, 1, and ≥2 μg/ml were 25.0 ± 2.3, 25.7 ± 2.4, and 24.8 ± 3.7 nm, respectively.

DISCUSSION

In the present investigation, we studied a total of 35 well-characterized, unique DAP-S and DAP-R MRSA strains to evaluate potential interrelationships among the frequencies of SNPs within *mprF*, DAP-R phenotype, and HDP cross-resistance phenotypes. A number of interesting findings emerged from this investigation.

First, *mprF* sequencing revealed that there are distinct SNPs within the *mprF* ORFs in a substantial proportion of DAP-S strains of the CC5 genotype (i.e., groups with DAP MICs of ≤0.5 or 1 μg/ml). Thus, there are *mprF* sequence variations not only in the DAP-R *S. aureus* strains, as previously published (3, 7, 8, 13), but also in selected DAP-S strains of this genotypic complex. However, the two SNPs found in the DAP-S strains, I498N and Q692E, have never been described before in association with enhanced L-PG synthesis/translocation or the DAP-R phenotype. The five DAP-S strains with the I498N SNP (DAP MICs of ≤0.5 μg/ml) and the three DAP-S strains with the Q692E SNP (DAP MICs of 1 μg/ml group) did not show any significant difference in CM PL contents, surface positive charge, or *in vitro* HDP susceptibilities compared to these characteristics in the DAP-S strains without such SNPs. These data imply that these mutations are not in essential locations within *mprF* that dictate changes in MprF structure or function or translate to modifications of CM PL profiles. Surprisingly, in addition to these latter SNPs, two strains in the group with DAP MICs of 1 μg/ml had 30 different amino acid substitutions within their MprF proteins, connoting a high frequency of *mprF* sequence heterogeneity in some DAP-S *S. aureus* strains. This hypermutational *mprF* heterogeneity, however, did not yield enhanced MprF functionality (i.e., in L-PG synthesis or translocation). In contrast, unlike the DAP-S strains, all isolates with DAP MICs of ≥2 μg/ml had *mprF* SNPs which have been well characterized as being associated with the DAP-R phenotype (8, 9, 11, 28, 29). In line with previous studies (12–14), the DAP-R strain group exhibited excess CM synthesis of L-PG, enhanced surface positive charge, and a reduced DAP-binding phenotype. These sequencing data, in combination with the PL profiling data described above, indicate that whereas *mprF* SNPs and, even, frequent *mprF* heterogeneities were found in some DAP-S isolates, SNPs within hot spot loci of the *mprF* ORF are required for the MprF gain-of-function phenotypes.

Second, we have previously shown that some DAP-R *S. aureus* isolates express more *mprF* transcripts over a standard *in vitro* growth cycle than their DAP-S parental strains (7, 8). As shown by the results in Fig. 1, DAP-R isolates (DAP MICs of ≥2 μg/ml) showed higher levels of *mprF* transcription than isolates from the two DAP-S groups during both exponential and stationary phases, indicating that the DAP-R isolates used in this study express more net *mprF* transcripts in their mutated form (L341S or L826F).

Third, recently published studies by our group and others have suggested that DAP-R *S. aureus* strains commonly exhibit cross-resistance to certain cationic HDPs, especially those associated with defense against endovascular infections, i.e., tPMPs from platelets and hNP-1 from neutrophils (4, 9, 10, 29). In agreement with these prior investigations (12), the present strain groups having higher DAP MICs showed significantly reduced susceptibilities to these two prototypical HDPs (Table 1). In particular, DAP-R isolates exhibited the highest levels of resistance to these two HDPs. These data indicate that only hot spot SNPs (L341S and L826F) within *mprF* are correlated with the DAP-R and HDP cross-resistance phenotypes.

Fourth, in previous publications (10, 12), the latter two SNPs have been associated with excess production of L-PG. In agree-

ment with these reports (12–14), all the DAP-R isolates in this study with either the L341S or the L826F SNP showed excess synthesis of L-PG. Excess synthesis and translocation of L-PG are believed to contribute to the relative positive surface charge in *S. aureus*; thus, as expected, the increased L-PG synthesis profiles of our DAP-R isolates versus those of the DAP-S isolates paralleled such surface charge analyses. The observation that only inner L-PG synthesis and not outer leaflet translocation was considerably increased in the DAP-R strains should mainly be viewed in the context of the concomitant reduction in PG content. Since PG serves as a putative initial docking site for DAP within the staphylococcal CM, reduced levels of PG would be associated with greater DAP-R (28, 32). In addition, DAP molecules which cross the outer CM could also theoretically be charge repulsed at the level of the inner CM in DAP-R strains. The observation that such DAP-R isolates bound significantly less Bodipy-DAP than DAP-S strains is likely due to a composite of reduced CM DAP docking sites and inner-CM charge repulsion mechanisms.

Last, in addition to the above-described concepts of hot spot mutations in *mprF*, dysregulation of *mprF* expression, and DAP/HDP cross-resistance, we investigated cell wall thickness phenotypes in our strain sets. Prior investigations from our laboratory and others have documented a common (albeit not universal) association between a thickened cell wall phenotype and DAP/HDP resistance in *S. aureus* (8, 12, 13). The mechanistic relationship between DAP-R and a thickened cell wall phenotype is hypothesized to rest in either a mechanical barrier to DAP penetration and/or affinity trapping of calcium-DAP within the cell wall structure. However, in the present study, the cell wall thicknesses of DAP-R isolates were not significantly different from those of isolates in the two DAP-S groups. This observation further underscores the importance of mutant *mprF*-associated electrostatic perturbations as one mechanism of DAP-R phenotypes.

It is important to recognize the limitations of the present studies. Our data are somewhat restricted in that only a single clonal complex (CC5) genotype was interrogated. It will be important to repeat such analyses with other relevant genotype strains commonly causing clinical syndromes, such as CC8, CC22, and CC30 (33–35). Moreover, we did not query other gene perturbations that may influence *mprF* which also have been linked to the DAP-R phenotype, including *yycFG*, *dltA*, and *rpoB* (36). Furthermore, we studied a relatively small MRSA strain sample size. Nonetheless, the results of the current studies reveal novel insights regarding the multiple functions of *mprF* heterogeneity in antibiotic and host defense avoidance by MRSA.

In conclusion, our data suggest the following: (i) there are individual point mutations (SNPs), as well as high levels of heterogeneity within *mprF* ORFs in some DAP-S *S. aureus* strains; (ii) however, only selected hot spot point mutations resulting in *mprF* dysregulation appear to yield the MprF gain-of-function phenotype (e.g., enhanced L-PG synthesis); (iii) in turn, the latter phenotype appears to be linked to surface positive charge modifications; and (iv) these genotypic and phenotypic abnormalities are observed predominantly in DAP-R isolates and are associated with HDP cross-resistance.

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