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Impact of Multiple Single-Nucleotide Polymorphisms Within *mprF* on Daptomycin Resistance in *Staphylococcus aureus*

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A number of single nucleotide polymorphisms (SNPs) within the *mprF* open reading frame (ORF) have been associated with daptomycin-resistance (DAP-R) in *Staphylococcus aureus*. Such SNPs have been found throughout the *mprF* ORF, although there are clearly preferred ''hot spots'' within this gene frequently linked to DAP-R phenotype. These *mprF* SNPs are often correlated with a gain-in-function phenotype, either in terms of increased production (synthase activity) and/or enhanced translocation (translocase activity) of lysyl-phosphatidylglycerol (L-PG) within its cell membrane. However, it is unclear if multiple hot spot *mprF* SNPs can accumulate within *mprF* ORFs and cause additive elevations of DAP minimum inhibitory concentrations (MICs). In this study, we used a previously well-characterized plasmid complementation system in *S. aureus* Newman Δ*mprF* mutant to express: (1) single point–mutated forms of *mprF* ORFs cloned from two DAP-R *S. aureus* strains ($mprF_{S295L}$ or $mprF_{T345A}$) and (2) dual point–mutated forms of *mprF* ORFs simultaneously harboring SNPs in the central bifunctional domain and synthase domain in MprF, respectively ($mprF_{S295L+L826F}$ or $mprF_{T345A+L826F}$). The current study revealed that, although individual hot spot point mutations within *mprF* ORF can recapitulate signature DAP-R–associated phenotypes (*i.e.*, increased DAP MICs, enhanced surface positive charge, and increased L-PG synthesis), accumulation of such hot spot point mutations paradoxically caused reduction in these latter three metrics.

Keywords: daptomycin, *Staphylococcus aureus, mprF,* antimicrobial peptides

Introduction

One rather consistent feature of daptomycin-resistant (DAP-R) *Staphylococcus aureus* strains is acquisition of one or more point mutations in a relatively limited set of genes, especially in *mprF*.[1–4] MprF is responsible for lysinylation of phosphatidylglycerol (PG) to generate the unique positively charged phospholipid (PL) species, lysyl-phosphatidylglycerol (L-PG), within the *S. aureus* cell membrane (CM),[5] as well as its translocation (flipping) to the outer CM leaflet.[6,7] Over the past several years, studies from our laboratory and others showed that most DAP-R *S. aureus* strains have acquired single nucleotide polymorphisms (SNPs) within either the C-terminal L-PG synthase domain, the N-terminal L-PG flippase domain, or most commonly within the central bifunctional domain.[1–3,8–10] Such SNPs in *mprF* of DAP-R strains have often been associated with gain-in-function phenotypes in terms of enhanced L-PG synthesis and/or flipping, as well as positive surface charge modifications.[1,2,9–13] In addition, *mprF* open reading frame (ORF) sequencing of 44 MRSA strains in our laboratory (22 DAP-susceptible [DAP-S]/DAP-R pairs) revealed that the majority of the 22 DAP-R MRSA strains had single point mutations mainly clustered between the 9th and 10th transmembrane

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Note: In this article, we will use the terminology ''DAP-R'' interchangeably with ''DAP nonsusceptibility'' for a more facile presentation.

domains or in the C-terminal domain (''hot spot'' loci).[1,2] However, there have been no reports to-date of clinically derived DAP-R strains containing multiple hot spot point mutations within the *mprF* ORF associated with DAP-R by minimum inhibitory concentration (MIC) testing.

In the current study, we utilized an isogenic *mprF* deletion (Δ*mprF*) mutant generated in the well-studied methicillin-susceptible *S. aureus* strain, Newman, and several *in trans* complemented Δ*mprF* variant strains. For complementation of Δ*mprF*, the previously described plasmid system was used to express either a single point–mutated form of *mprF* ORFs derived from previously characterized DAP-R strains[1,9] or double point–mutated forms of the *mprF* ORFs that have hot spot SNPs in the central bifunctional domain, as well as in the synthase domain, respectively. We investigated the impact of such single or dual point mutations on *in vitro* susceptibilities to DAP and two neutrophil-derived cationic antimicrobial peptides (CAMPs), hNP-1 and LL-37. Moreover, PL profiles, surface positive charges, and DAP binding to the staphylococcal surface were analyzed to measure if the dual hot spot point mutations in *mprF* yielded further perturbations in those signature phenotypes typically associated with DAP-R among clinically- or *in vitro*-derived DAP-R *S. aureus*.[1,2,10,13–16]

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are shown in Table 1. We used the wild-type *S. aureus* Newman strain,[17] its isogenic *mprF* knockout mutant strain (Δ*mprF*[9]), and several *in trans* complemented Δ*mprF* strains, employing the previously described pRB474[18] plasmid expressing: (1) a single point–mutated form of *mprF* cloned from two previously well-characterized DAP-R strains (MSSA701 and REF2145 harboring $mprF_{S295L}$ and $mprF_{T345A}$, respectively) and (2) a double point–mutated form of *mprF* ($mprF_{S295L+L826F}$ or $mprF_{T345A+L826F}$) as described before.[4,9,10,13,16,19] Combinations of the two SNPs in the double point–mutated form of *mprF*s represent SNPs (S295L or T345A) from the central transmembrane domain between the synthase- and translocase domain (bifunctional domain) combined with a SNP (L826F) from the C-terminal synthase domain.[1,2] The L826F substitution was the most common *mprF* SNP observed in our previous studies and has been associated with enhanced L-PG synthesis and DAP-R phenotype by MICs.[1,2,10,11,20] A plasmid-based complementation construct of the L826F single mutation in the Newman Δ*mprF* strain was also utilized in selected assays. Wild-type Newman and Newman Δ*mprF* strains harboring the empty plasmid were included as controls.

All *S. aureus* strains were cultured in either Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, MI) or Mueller-Hinton Broth (MHB; Difco Laboratories) depending on each assay. Liquid cultures were grown in Erlenmeyer flasks at 37°C with shaking (250 rpm) for aeration in a volume that was less than 10% of the flask volume.

Table 1. Bacterial Strains and Plasmids Used in This Study

Strain	*Description*	*References*
Staphylococcus aureus		
Newman	MSSA; WT strain isolated from a human infection in 1952	17
Newman Δ*mprF*	Newman *mprF*::Em; Em^r	26
C_{S295L}	Newman Δ*mprF* strain expressing $mprF_{S295L}$	9
$C_{S295L+L826F}$	Newman Δ*mprF* strain expressing $mprF_{S295L+L826F}$	This study
C_{T345A}	Newman Δ*mprF* strain expressing $mprF_{T345A}$	9
$C_{T345A+L826F}$	Newman Δ*mprF* strain expressing $mprF_{T345A+L826F}$	This study
C_{L826F}	Newman Δ*mprF* strain expressing $mprF_{L826F}$	This study
Escherichia coli DH5α	Host strain for construction of recombinant plasmids	36
Plasmid pRB474	Shuttle vector carrying *Bacillus subtiltis vegII* promoter; Cm^r	18
pCR2.1	*E. coli* plasmid; Amp^r	Invitrogen

Amp^r, ampicillin resistance; CL, cardiolipin; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance.

DNA sample preparations and mprF *complementation*

Genomic DNA samples were isolated from *S. aureus* cells as described by Dyer and Iandolo.[21] Extraction and purification of plasmid DNA samples were performed using Wizard Plus kits (Promega, Inc., Madison, WI.). Preparation of competent *Escherichia coli* DH5α and transformation of plasmid constructs were accomplished as described previously.[22]

Electroporation of plasmids into electrocompetent *S. aureus* cells was performed as described by Schenk and Laddaga.[23] Briefly, overnight *S. aureus* cultures grown in B2 broth (1.0% casein hydrolysate, 2.5% yeast extract, 0.1% K_2HPO_4 [pH 7.5], 0.5% glucose, 2.5% NaCl) at 37°C with shaking (250 rpm) were diluted into 25 ml of fresh B2 broth at OD_{600} of 0.25. The cells were grown until they reached mid-log phase of growth ($\sim OD_{600}$ of 0.5), pelleted by centrifugation, washed thrice with deionized water, and then resuspended in 5 ml of 10% glycerol solution. The cells were then incubated for 15 min at RT, pelleted again, and resuspended in 0.5 ml of 10% glycerol solution and used for electroporation. The cells and plasmid DNA were electroporated at 100 ohms resistance, 25 μF capacitance, and 2.3 kV in a Gene Pulser™ apparatus (Bio-Rad Laboratories).

Complementation with double point–mutated forms of *mprF* genes in Newman Δ*mprF* was accomplished by PCR amplification of the *mprF* ORFs with primers mprF-F-bamHI[9] and mprF-R-L826F (5′-GCACTTGGATTTTAA TTATTTGTGACGTATTACACG CATTACTTTAGAAA GTGATTCCCAAA**A**CG-3′) using purified DNA samples from DAP-R *S. aureus* strains, 701 and REF2145 strains. The amplified PCR products, each containing S295L+L826F or T345A+L826F in *mprF*, were cloned into the polylinker of pCR®2.1 vector (TA Cloning™ Kit; Thermo fisher), then treated with *Bam*HI and *Eco*RI to liberate the PCR inserts, and cloned into the *Bam*HI and *Eco*RI sites of the pRB474.[18]

DNA sequences of the *mprF* ORFs cloned into the pRB474 were confirmed by sequencing services at City of Hope, Duarte, CA.

Antibiotics and MIC determination

Ampicillin and chloramphenicol were used at 100 and 5 μg/ml, respectively, during the cloning experiments. DAP was kindly provided by Cubist Pharmaceuticals (Lexington, MA). The MICs to DAP and oxacillin (OX) were determined by standard E-test. Three independent experimental runs were performed to determine the DAP and OX MICs.

Population analysis

To determine DAP-R profiles of the study strains, DAP population analyses were performed as described before.[16,24] Briefly, initial inoculum of $\sim 2\times 10^8$ CFU/ml *S. aureus* cells was exposed to 0–8 μg/ml of DAP concentrations representing sublethal-to-lethal levels. All DAP population analyses were done in the presence of 50 μg/ml calcium (Ca^{2+}). The area-under-the curve was calculated as before.[10] Three independent experimental runs were performed.

hNP-1 and LL-37 susceptibility assays

Since standard MIC testing in MHB or TSB may inhibit CAMP activities, *in vitro* survival assays were performed with hNP-1 and LL-37 using a 2-hr microdilution method in Eagle's minimal Essential medium.[19,25] The two prototypical antimicrobial peptides, hNP-1 (an α-defensin in neutrophils) and LL-37 (a human cathelicidin found in neutrophils and skin), were purchased from Peptide International (Louisville, KY). A final inoculum of 5×10^3 CFUs of overnight grown *S. aureus* cells was exposed to hNP-1 (10 μg/ml) and LL-37 (1 μg/ml).[9] These concentrations of hNP-1 and LL-37 represented a sublethal level against the wild-type Newman *S. aureus* strain, as determined in pilot experiments. Three independent runs were performed on separate days.

CM PL contents

To quantify in our study strains the relative proportions of L-PG among the three major staphylococcal PLs (*i.e.*, L-PG, PG, and cardiolipin [CL]),[5,6,26] CM PLs were extracted, separated, and identified using two-dimensional thin-layer chromatography (2D-TLC) as described previously.[19,27] Individual TLC PL spots were scraped from the TLC plates and then quantified by a well-defined spectrophotometric analysis.[19,27] Data are expressed as the proportionalities (±standard deviation [SD]) of the L-PG, PG, and CL for each staphylococcal strain. Four independent experiments were performed on separate days to analyze the PL profiles.

Determination of surface positive charge

To assess relative net charges on the study strains, cytochrome *c* (Sigma, St. Louis, MO) binding to cell surface was measured by quantifying the amount of the unbound cytochrome *c* in reaction mixtures.[10,19,27,28] For the assay, *S. aureus* cells were grown overnight for ~17 hr in TSB, washed thrice with 20 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.0), and resuspended in the MOPS buffer at OD_{600} of 1.0 ($\sim 10^9$ CFUs). The bacterial cells were then incubated with cytochrome *c* (50 μg/ml) for 15 min and pelleted; the amount of cytochrome *c* unbound to cell surfaces in the supernatant was determined by measuring OD_{530}. The more unbound cytochrome *c* in the supernatant indicates the more positively charged the cell surface. Three independent cytochrome *c* binding assays were performed on separate days.

DAP binding analysis

Whole cell DAP binding assays were performed as described before.[9,19] Briefly, $\sim 10^8$ CFUs of each strain were exposed to 5 μg/ml of DAP for 10 min, centrifuged, and then supernatants were analyzed for residual unbound DAP by a radial diffusion assay and standard curve technique as described previously.[19,29] The amount of bound DAP (±SD) was then calculated by subtracting unbound DAP amounts from the initial 5 μg/ml of DAP. These assays were repeated at least four times for each *S. aureus* strain on separate days.

Statistical analyses

Data were analyzed by the Kruskal–Wallis analysis of variance test with the Tukey *post hoc* correction for multiple comparisons. Differences less than *p*-value of 0.05 were considered statistically significant.

Results

DAP and CAMP susceptibility profiles

In agreement with our prior observations,[9] Newman Δ*mprF* strains with plasmids expressing single point–mutated forms of *mprF* ($C_{S295L,}$ C_{T345A}, or C_{L826F}) resulted in increased DAP MICs versus the parental Newman strain. However, the two Newman Δ*mprF* strains expressing dual point–mutated *mprF* genes ($C_{S295L+L826F}$ and $C_{T345+L826F}$) each showed decreased DAP MICs versus the single C_{S295L}, C_{T345A}, or C_{L826F} strains, respectively (Table 2). All the strains, including the C_{L826F}, displayed identical OX MICs (0.38 μg/ml), indicating that there was no impact of *mprF* single- or dual-point mutations on OX susceptibilities (*i.e.*, no ''seesaw'' effect[10,30]). The qRT-PCR analyses confirmed that all the cloned *mprF* ORFs in pRB474 expressed at similar levels during exponential growth phase (data not shown).

As shown in our previous publication,[9] disruption of *mprF* in the Newman parental strain resulted in significantly enhanced susceptibility to the innate host defense CAMPs, hNP-1 and LL-37. Complementation of the Newman Δ*mprF* strain with the single point–mutated *mprF* genes *in trans* restored susceptibilities to hNP-1 and LL-37 toward parental level. In contrast, complementation of the Newman Δ*mprF* strain with plasmids expressing dual point–mutated form of *mprF* genes ($C_{S295L+L826F}$ and $C_{T345+L826F}$) yielded increased susceptibilities to both hNP-1 and LL-37 compared to the single point–mutated C_{S295L} or C_{T345A} strains ($p < 0.05$ and $p < 0.01$, respectively).

Population analyses

The Newman Δ*mprF* strains complemented with either of the dual point–mutated *mprF* genes ($C_{S295L+L826F}$ and $C_{T345+L826F}$) displayed substantial shift to the left in the

Table 2. *In Vitro* Susceptibility Assays, Lysyl-Phosphatidylglycerol Synthesis, and Daptomycin Binding Assays of the Study Strains

Strain	mprF *SNP*	*DAP MICs (μg/ml)*	*% survival (mean ± SD) after 2 h exposure to:*		*Amount (μg) of bound DAP (5 μg)*
			hNP-1 (10 μg/ml)	*LL-37 (1 μg/ml)*	
Newman	—	0.5	34.8 ± 7.7	36.4 ± 4.7	0.72 ± 0.12
Newman Δ*mprF*	—	0.125	5.0 ± 2.9[a]	2.1 ± 1.6[a]	1.32 ± 0.11[a]
C_{S295L}	S295 L	1	28.2 ± 5.4	32.8 ± 6.4	0.62 ± 0.12
$C_{S295L+L826F}$	S295 L+L826F	0.38	19.7 ± 6.2[b]	10.8 ± 4.6[c]	0.98 ± 0.20[b]
C_{T345A}	T345A	2	28.5 ± 7.7	26.7 ± 3.6	0.59 ± 0.18
$C_{T345A+L826F}$	T345A+L826F	0.38	17.8 ± 6.5[b]	6.8 ± 2.4[c]	0.84 ± 0.23[b]
C_{L826F}	L826F	0.75	—	—	—

[a] $p < 0.01$ versus Newman wild-type (WT) strain.
[b] $p < 0.05$ versus C_{S295L} and C_{T345A} strains, respectively.
[c] $p < 0.01$ versus C_{S295L} and C_{T345A} strains, respectively.
DAP, daptomycin; MICs, minimum inhibitory concentrations; SD, standard deviation; SNPs, single nucleotide polymorphisms.

DAP population curves compared to strains complemented with the single point–mutated *mprF* genes, C_{S295L} or C_{T345A} (Fig. 1A, B). Area under the curve (AUC) values were approximately twofold and approximately sevenfold less for the $C_{S295L+L826F}$ and $C_{T345+L826F}$ strains versus their single point–mutated variants, C_{S295L} or C_{T345A}, respectively (mean ± SD, 5.18 ± 0.52 for C_{S295L} versus 2.25 ± 0.24 for $C_{S295L+L826F}$ and 14.36 ± 0.48 for C_{T345A} vs. 2.17 ± 0.22 for $C_{T345+L826F}$; $p < 0.01$ for both comparisons). AUC value for the C_{L826F} was 3.18 ± 0.32.

Synthesis of L-PG

L-PG content of both SNP constructs was at near-parental levels. In contrast, the L-PG content was not detectable in the two strains expressing dual point–mutated *mprF* genes (Table 3). Interestingly, the two strains expressing dual point–mutated *mprF* genes showed significantly increased PG amounts, as a compensatory adaptation to the inability to produce L-PG.

Net surface positive charge

As anticipated, the $C_{S295L+L826F}$ and $C_{T345+L826F}$ strains had a significantly decreased surface positive charge compared to the single point–mutated C_{S295L} and C_{T345A} strains, respectively (Fig. 2). These reductions of surface positive charge for the $C_{S295L+L826F}$ and $C_{T345+L826F}$ strains were well correlated with the nondetectable level of L-PG in the two dual SNP variant constructs (Table 3).

DAP whole cell binding

Mirroring our prior investigation,[9] the Newman Δ*mprF* strain bound significantly more DAP than the wild-type Newman strain (Table 2). The $C_{S295L+L826F}$ and $C_{T345+L826F}$ dual point–mutated strains exhibited significantly enhanced DAP binding versus the two strains expressing single point–mutated *mprF* genes ($p < 0.05$).

Discussion

Although several adaptive changes have been proposed to explain the DAP-R phenotype in *S. aureus* and other Gram-positive pathogens,[31] the mechanisms of DAP-R still remain incompletely understood. There have been a number of recent reports, including ours, indicating the presence of SNPs within the *mprF*. ORF is associated with a gain-in-function phenotype in terms of DAP-R in *S. aureus*.[1,2,4,9,10,12,19,32] These SNPs have mainly been observed in previously defined ''hot spots'' within the *mprF* ORF, usually within the

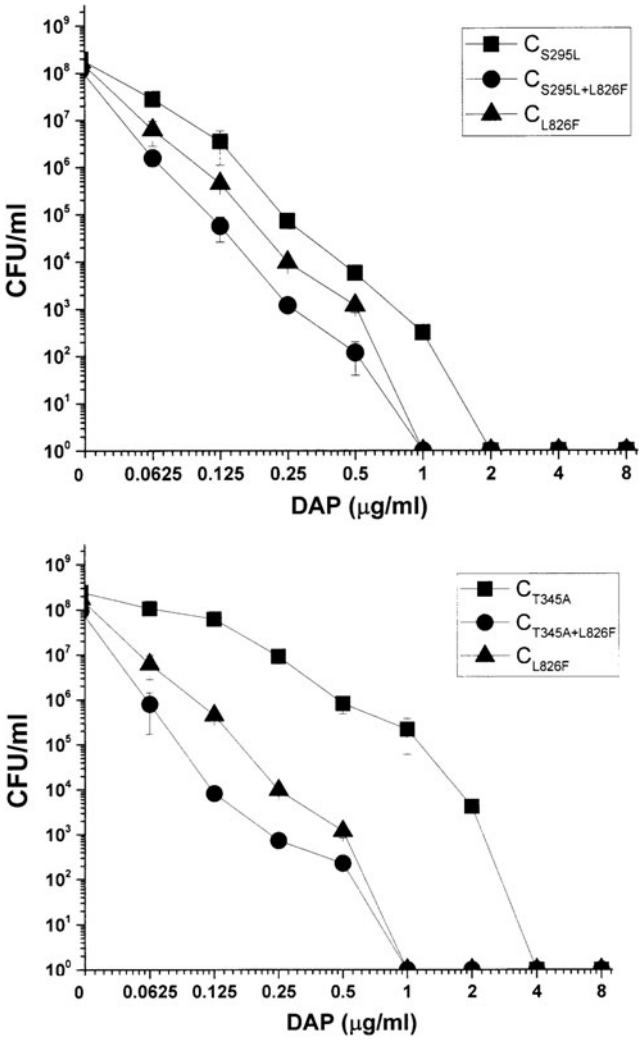

FIG. 1. DAP population analyses of study strains. Data represent the mean ± SD for three independent experiments. DAP, daptomycin; SD, standard deviation.

TABLE 3. PHOSPHOLIPID COMPOSITION OF THE STUDY STRAINS

	% of total PL content (mean ± SD)		
Strains	*L-PG*	*PG*	*CL*
Newman	17 ± 3	72 ± 5	11 ± 4
Δ*mprF*	ND	86 ± 5[a]	14 ± 5
C_{T345A}	18 ± 7	72 ± 10	10 ± 4
$C_{T345A+L826F}$	ND	91 ± 2[a]	9 ± 2
C_{S295L}	12 ± 3	82 ± 5	7 ± 2
$C_{S295L+L826F}$	ND	91 ± 4[a]	9 ± 4

[a] $p < 0.05$ versus Newman wild-type (WT) strain, C_{S295L}, and C_{T345A} strains, respectively.

L-PG, lysyl-phosphatidylglycerol; ND, not detectable; PG, phosphatidylglycerol; PL, phospholipid.

central bifunctional domain or the C-terminal synthase domain of this protein.[1,2] These SNPs have generally resulted in enhanced L-PG synthesis and/or translocation, with resultant modifications in surface positive charge modifications.[1,2,9,12,19] Among the hot spot point mutations within the *mprF* ORF, the S295L, T345A, and L826F SNPs (S295L and T345A in the bifunctional domain; L826F in the C-terminal domain) were most frequently observed among DAP-R *S. aureus* strains in association with excess production of L-PG.[1,2,9,10,12,19] However, no clinically derived or laboratory-derived DAP-R *S. aureus* strains have been reported to concomitantly contain two or more such hot spot SNPs in correlation with increased DAP MICs in the DAP-R range (≥2 μg/ml).

In this current investigation, we used a previously well-characterized *in trans* plasmid complementation strategy to express dual point–mutated forms of *mprF* ORFs (one in the bifunctional domain [S295L or T345A] and the other in the synthase domain [L826F]) within the Newman Δ*mprF* strain.[9] We investigated the impact of such dual SNPs on *in vitro* susceptibilities to two prototypical host defense CAMPs, hNP-1 and LL-37, and DAP.[26] A number of interesting findings emanated from these investigations that might explain why there have been no reports of clinical *S. aureus* strains that accumulated multiple hot spot point mutations in *mprF* ORF associated with high-level DAP-R phenotype by MIC.

The incorporation of dual point–mutated form of *mprF*s ($C_{S295L+L826F}$ and $C_{T345+L826F}$) into the plasmid system yielded significantly decreased DAP MICs below the Newman parental level (0.5 μg/ml). In contrast, and in agreement with a previous publication,[9] single point–mutated form of *mprF*s (C_{S295L}, C_{T345}, C_{L826F}) caused an increase in DAP MICs (Table 1). Correlating with the MIC results, there were significant leftward shifts in DAP-R population analyses in the two dual point–mutated constructs compared to the each single point–mutated form of *mprF*s (Fig. 1). These data suggest that, although individual *mprF* hot spot point mutations can cause the DAP-R phenotype, accumulation of these SNPs within *mprF* in a single strain does not confer further elevation of DAP-R, but in fact paradoxically renders such isolates more DAP-S.

Next, PL profile analyses revealed that the proportional amount of L-PG synthesized was decreased to essentially undetectable levels in the dual point–mutated $C_{S295L+L826F}$ and $C_{T345+L826F}$ complemented strains. We speculate that the accumulation of multiple hot spot point mutations within *mprF* ORFs may have caused loss of synthase activity of MprF. Furthermore, the inability to synthesize L-PG in the dual SNP complemented strains well correlated with decreased net positive surface charge (Fig. 2) and enhanced DAP binding in those strains (Table 1). These data support the notion that a ''charge repulsion'' mechanism for the DAP-R phenotype in the single point–mutated *mprF* is likely in-play.[9,13,16,19,33]

Recent publications from our group and others have indicated that DAP-R MRSA or MSSA strains frequently display cross-resistance to prototypical host defense CAMPs.[1,2,15,19] Correlating with the L-PG synthesis and net surface positive charge data, the dual SNP-complemented strains displayed enhanced susceptibilities to the host defense CAMPs, hNP-1 and LL-37, versus the SNP-complemented strains, respectively. These data indicate that, at least in part, reduced net surface positive charge, as a result of loss in L-PG synthesis, is responsible for the reduced hNP-1 and LL-37 susceptibilities in the dual SNP-complemented strains.

We recognize that there are limitations in the current studies, including: (1) the limited number of combinations of *mprF* SNPs tested; (2) evaluating only one single MSSA parental strain, and no MRSA background isolates; (3) use of a plasmid-based complementation approach rather than chromosomal *mprF* point mutation constructs; and (4) since the plasmid used in our studies only expresses during exponential growth, this may well underlie the relatively blunted PL and surface charge responses seen in our single *mprF* mutant constructs compared to the parental strain. This latter phenomenon has been previously noted by our group.[9] Collectively, these limitations are currently being addressed in our laboratory.

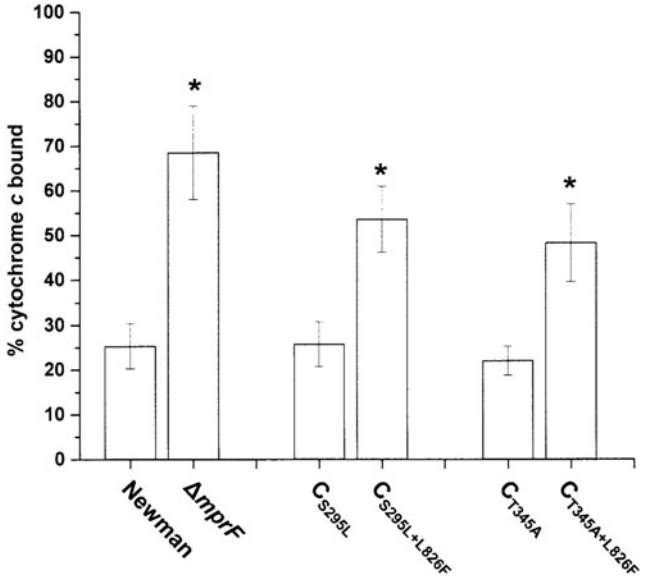

FIG. 2. Cytochrome *c* binding analyses for determination of staphylococcal net positive surface charge. Percent of cytochrome *c* bound after 15 min of incubation with *Staphylococcus aureus* strains at room temperature was shown. Data are depicted as the mean ± SD from three independent assays. *$p < 0.01$.

In combination with previously published results,[9] our data suggest that: (1) individual hot spot point mutations within *mprF* gene can recapitulate the DAP-R phenotype observed in clinically-derived DAP-R donor strains through gain-in-function mechanisms (*i.e*., increased L-PG synthesis and net surface positive charge); and (2) accumulation of multiple hot spot point mutations results in decrease of L-PG synthase activity, which likely reduces DAP MICs caused by mitigation of surface charge repulsion of calcium-DAP. Our current findings are in line with those of Friedman *et al*.[3] exhibiting that the selective pressure of serial *in vitro* growth in sublethal concentrations of DAP results in single, but not multiple, *mprF* SNPs. Our data may also explain, at least in part, why DAP MICs among DAP-R *S. aureus* clinical isolates are never very high (*i.e*., >4 μg/ml). If such cumulative SNPs would have been additive, high-level DAP-R isolates would have likely been observed.

Our data leave open the possibility that clinical *S. aureus* strains exposed to DAP *in vivo* may well accumulate multiple *mprF* point mutations, while remaining ''DAP-S'' by MIC testing. Such strains would not be identified easily in the microbiology laboratory, and being DAP-S, such isolates would likely be readily cleared from infection sites by DAP. It would now be interesting to reexamine clinical strains from patient receiving DAP therapy for emergence of multiple hot spot SNPs in *mprF*.

The mechanism(s) by which multiple *mprF* SNPs lead to reduced DAP MICs and other phenotypic perturbations remain to be defined. This scenario could represent a ''fitness or metabolic burden'' on the organism. In contrast, it is conceivable that compensatory mutations in other genes previously associated with DAP-R (*e.g*., *yycFG*; *rpoBC*; or *vraSR*[32,34,35]) could also be playing a role.

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Disclosure Statement

No competing financial interests exist.

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