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In Vitro Cross-Resistance to Daptomycin and Host Defense Cationic Antimicrobial Peptides in Clinical Methicillin-Resistant *Staphylococcus aureus* Isolates[▽]

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We investigated the hypothesis that methicillin-resistant *Staphylococcus aureus* (MRSA) isolates developing reduced susceptibilities to daptomycin (DAP; a calcium-dependent molecule acting as a cationic antimicrobial peptide [CAP]) may also coevolve reduced *in vitro* susceptibilities to host defense cationic antimicrobial peptides (HDPs). Ten isogenic pairs of clinical MRSA DAP-susceptible/DAP-resistant (DAP^s/DAP^r) strains were tested against two distinct HDPs differing in structure, mechanism of action, and origin (thrombin-induced platelet microbicidal proteins [tPMPs] and human neutrophil peptide-1 [hNP-1]) and one bacterium-derived CAP, polymyxin B (PMB). Seven of 10 DAP^r strains had point mutations in the *mprF* locus (with or without *yyc* operon mutations), while three DAP^r strains had neither mutation. Several phenotypic parameters previously associated with DAP^r were also examined: cell membrane order (fluidity), surface charge, and cell wall thickness profiles. Compared to the 10 DAP^s parental strains, their respective DAP^r strains exhibited (i) significantly reduced susceptibility to killing by all three peptides ($P < 0.05$), (ii) increased cell membrane fluidity, and (iii) significantly thicker cell walls ($P < 0.0001$). There was no consistent pattern of surface charge profiles distinguishing DAP^s and DAP^r strain pairs. Reduced *in vitro* susceptibility to two HDPs and one bacterium-derived CAP tracked closely with DAP^r in these 10 recent MRSA clinical isolates. These results suggest that adaptive mechanisms involved in the evolution of DAP^r also provide MRSA with enhanced survivability against HDPs. Such adaptations appear to correlate with MRSA variations in cell membrane order and cell wall structure. DAP^r strains with or without mutations in the *mprF* locus demonstrated significant cross-resistance profiles to these unrelated CAPs.

Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are often a challenge for clinicians due to limited treatment options and resistance to multiple antibiotics (15, 34). In this regard, daptomycin (DAP; a calcium-dependent antimicrobial lipopeptide) has become a relevant therapeutic option (28, 29).

Cationic antimicrobial host defense peptides (HDPs), including those of hematogenous origin, such as platelets and neutrophils, kill many important blood-borne pathogens, especially *S. aureus* (35). Although most HDPs initially interact with the bacterial cell membrane, their overall mechanism(s) of action can be quite distinct and multifactorial, involving the cell membrane, the cell wall, and intracellular targets (37). Since both DAP and HDPs target the bacterial cell membrane to initiate their lethal mechanism(s), we hypothesized that common resistance pathways between these molecules might exist (21, 22). Of interest, previous studies from our laboratories indicated that selected DAP-resistant (DAP^r) *S. aureus* strains isolated from clinical infections, as well as following *in vitro* passage in DAP, exhibited *in vitro* cross-resistance to several HDPs (13, 21). (Although the official terminology is "daptomycin nonsusceptible," the term "daptomycin resistant" was employed in this study for a more facile presentation.) However, the overall frequency of the co-occurrence of such reduced *in vitro* susceptibilities to DAP and HDPs, especially among clinically derived MRSA strains, remains undefined.

The most prevalent and well-known genetic perturbations associated with DAP^r in *S. aureus* are single point mutations in various regions of the *mprF* open reading frame (ORF), with or without concomitant point mutations in the *yyc* operon (12, 14, 27, 42). The *mprF* locus is principally involved in the maintenance of a relative positive surface charge in *S. aureus* via lysinylation of cell membrane phosphatidylglycerol (PG) (10). Phenotypic mechanisms which have been linked to DAP^r in *S. aureus* include increases in net positive surface charge ostensibly imparting a charge-repulsive milieu against cationic peptides (related to gain-in-function mutations in *mprF* or *dlt* [39, 42]), thickened cell walls (5), and/or altered cell membrane order (2, 13, 21, 22). Of interest, most of the same phenotypic perturbations have also been linked to reduced *in vitro* susceptibilities to cationic HDPs (13, 41). The purposes of the current investigation were to (i) determine the frequency and extent of *in vitro* concurrence of reduced susceptibility to DAP and selected HDPs in MRSA clinical isolates; (ii)

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TABLE 1. Genotypes of study strain pairs[a]

Strain	*spa* type	*spa* motif	Clonal complex	SCC*mec* type	*agr* type
CB1483	2	TJMBMDMGMK	5	II	II
CB185	2	TJMBMDMGMK	5	II	II
CB5079	1	YHGFMBQBLO	8	IV	I
CB5080		YHGFMBQBLO	8	IV	I
CB5083	1	TMDMGMK	5	II	II
CB5082	1	TMDMGMK	5	II	II
CB5088	47	YHGFMBQBLO	8	II	I
CB5089	47	YHGFMBQBLO	8	II	I
CB1631	1	TJMBMDMGMK	5	II	II
CB1634	1	TJMBMDMGMK	5	II	II
CB1663	2	TJMBMDMGMK	5	II	II
CB1664	2	TJMBMDMGMK	5	II	II
CB5057	1	YHGFMBQBLO	8	IV	I
CB5059	1	YHGFMBQBLO	8	IV	I
CB5062	16	WGKAKAOMQQQ	30	II	III
CB5063	16	WGKAKAOMQQQ	30	II	III
CB5015	2	TJMBMDMGMK	5	II	II
CB5016	2	TJMBMDMGMK	5	II	II
CB5021	2	TJMBMDMGMK	5	II	II
CB5020	2	TJMBMDMGMK	5	II	II

[a] Pairs of isolates are represented by alternative shading and no shading, with the first strain in each pair being the DAP^s parental strain and the second one in each pair being the DAP^r strain.

TABLE 2. MICs, PFGE types, and *mprF*/*yycG* SNPs among 10 study strain pairs[a]

Strain	MIC (μg/ml)[b]				USA group	SNP	
	DAP	VAN	OX	GM		*mprF*	*yycG*
CB1483	0.25	1	>8	2	USA100		
CB185	4	2	>8	2		L826F[c]	None
CB5079	0.5	1	>8	2	USA300		
CB5080	2	2	>8	2		L826F[c]	None
CB5083	0.25	1	>8	2	USA100		
CB5082	4	2	>8	2		L341S[d]	None
CB5088	0.5	1	8	2	USA300		
CB5089	2–4	2	>8	2		S295L[d]	None
CB1631	0.5	2	>8	2	USA100		
CB1634	4	4	>8	2		L826F[c]	Frameshift
CB1663	0.5	1	>8	>256	ND		
CB1664	4	4	4	>256		L826F[c]	R86H
CB5057	0.5	1	>8	2	USA300		
CB5059	4	4	0.5	2		I420N[c]	T474I
CB5062	0.5	1	>8	2	ND[e]		
CB5063	8	2	>8	2		None	None
CB5015	1	4	>8	2	ND		
CB5016	4	4	>8	2		None	None
CB5021	0.25	1	>8	2	ND		
CB5020	1	4	>8	2		None	None

[a] Pairs of isolates are represented by alternative shading and no shading, with the first strain in each pair being the DAP^s parental strain and the second one in each pair being the DAP^r strain.
[b] VAN, vancomycin; OX, oxacillin; GM, gentamicin.
[c] Mutation in putative *mprF* synthase domain.
[d] Mutation in putative *mprF* translocase domain.
[e] ND, not determined.

compare the antistaphylococcal efficacies of HDPs that are involved in endovascular defense but that differ in structure, charge, mechanism(s), and origin; (iii) correlate potential cross-resistance with cell surface charge, cell membrane fluidity, and cell wall thickness; and (iv) define the relationship of such concurrently reduced *in vitro* susceptibilities to the presence of *mprF* mutations.

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MATERIALS AND METHODS

Bacterial strains. The 10 DAP^s/DAP^r MRSA study pairs employed in this investigation were clinical bloodstream isolates selected from the Cubist Pharmaceuticals isolate collection by one of the investigators (A.R.), including an initial pre-DAP therapy strain and a matched strain which developed *in vitro* DAP^r during DAP treatment. The selection of this strain set was prioritized to encompass DAP^r strains with and without *mprF* and/or *yyc* operon mutations. Isolates studied were identical on pulse-field gel electrophoresis (PFGE). To confirm the putative isogenicity of each strain pair, these isolates were evaluated by the following genotypic assays: *agr* typing (20), *spa* typing and clonal complexing (30), and staphylococcal cassette chromosome *mec* (SCC*mec*) typing (6). These studies confirmed the genetic relatedness of each of the 10 strain pairs (Table 1). The *in vitro* growth rates of all strain pairs were virtually identical over a 24-h period (data not shown).

The MICs of the strain set to DAP, vancomycin, oxacillin, and gentamicin were determined by standard Etest (AB Biodisc, Dalvagen, Sweden) on Mueller-Hinton agar (MHA) plates (Difco Laboratories, Detroit, MI). To assess the DAP Etest, plates were calcium supplemented according to the manufacturer's recommendations (50 μg/ml $CaCl_2$). DAP MICs are shown in Table 2; the breakpoint distinguishing the DAP^s and DAP^r phenotypes was considered ≥2 μg/ml. Vancomycin MICs were 1 μg/ml for 8/10 parental strains and 2 and 4 μg/ml for one strain each. Vancomycin MICs commonly increased 2- to 4-fold among DAP^r isolates (9/10 isolates), with 4 isolates exhibiting a vancomycin-intermediate susceptible *S. aureus* (VISA) phenotype (MIC = 4 μg/ml). For gentamicin, 9/10 DAP^s/DAP^r pairs exhibited MICs of 2 μg/ml (gentamicin susceptible); one parental strain was intrinsically gentamicin resistant. As expected, oxacillin MICs were >8 μg/ml for all DAP^s parental strains, with no change in oxacillin MICs observed in 8/10 DAP^r strains. Of interest, a significant reduction of oxacillin MIC was seen in one DAP^r strain (the so-called seesaw effect [31, 32, 40]). Seven of the 10 DAP^r strains exhibited single nucleotide polymorphisms (SNPs) within the *mprF* gene locus, with or without concomitant SNPs within the *yyc* operon in *yycG*. DAP^r strains isolated either clinically or following serial *in vitro* passage in DAP have often been shown to temporally accumulate mutations in these loci (12). Table 2 lists the specific SNPs identified in these 7 DAP^r strains. It should be noted that SNPs in *mprF* occurred within both its putative lysyl-PG (L-PG) synthase and translocase domains (10) and represented the most common SNP hot spots for mutations in DAP^r strains, as previously reported (4, 12, 41).

HDP and CAP susceptibilities. HDP and cationic antimicrobial peptide (CAP) bactericidal assays were performed in minimal liquid nutrient medium (Eagle's minimal essential medium [MEM]) in appropriate buffers (37, 43) by a 2-h timed-kill method as previously detailed (22). The following peptides were studied: (i) two prototypical mammal-derived HDPs, thrombin-induced platelet microbicidal proteins (tPMPs) from rabbits and human neutrophil-derived defensin-1 (hNP-1) (both of these HDPs have previously been shown to play a role in innate host defenses against endovascular infections [1, 9, 18, 38, 45]), and (ii) a bacterium-derived cyclic CAP, polymyxin B (PMB). The concentrations of peptides used in the killing assays were 0.25 to 0.50 μg/ml for tPMPs, 5 to 10 μg/ml for hNP-1, and 20 to 40 μg/ml for PMB. These sublethal concentrations were selected on the basis of (i) their ability to reduce survival of the parental DAP^s strains by >50% in preliminary studies and (ii) peptide concentrations used in prior investigations of HDP-*S. aureus* interactions (23, 37). The hNP-1 was purchased from Peptide International (Louisville, KY); PMB was purchased from Sigma-Aldrich (St. Louis, MO). DAP was obtained from Cubist Pharmaceuticals (Lexington, MA). The tPMP was prepared from thrombin-stimulated rabbit platelets as previously described (44). The bioactive tPMP concentration equivalency was determined as detailed elsewhere (22). Stationary-phase cells (overnight cultures) were utilized in all assays.

All HDPs described above were reconstituted in appropriate diluents as described elsewhere (37, 44). *S. aureus* cells were diluted into the peptide solutions to achieve the desired final inoculum (10^3 CFU/ml) (38, 39) and peptide concentrations and were then incubated at 37°C. After 2 h exposure, samples were obtained and processed for quantitative culture to evaluate the extent of killing by each CAP. Final data were expressed as mean percent surviving CFU/ml ± standard deviation (SD). Since there is no bona fide resistance breakpoint for HDPs, we compared only the mean percent survivability ± SD in the DAP^s

TABLE 3. *In vitro* susceptibilities to three distinct CAPs, cell membrane fluidity, and cell wall thickness of 10 study strain pairs[a]

Strain	% survival (mean ± SD) after 2 h of exposure to:						Cell membrane fluidity (PI value)	Cell wall thickness (nm)
	tPMPs		hNP-1		PMB			
	0.5 μg/ml	0.25 μg/ml	10 μg/ml	5 μg/ml	40 μg/ml	20 μg/ml		
CB1483	18 ± 12	32 ± 18	45 ± 11	59 ± 11	7 ± 8	31 ± 18	0.333 ± 0.012	35.9 ± 3.8
CB185	67 ± 22*	95 ± 10*	56 ± 8	69 ± 14	9 ± 11	38 ± 16	0.331 ± 0.004	38.8 ± 3.4**
CB5079	3 ± 4	22 ± 8	3 ± 6	21 ± 7	0 ± 0	40 ± 18	0.453 ± 0.042	30.7 ± 3.2
CB5080	48 ± 7*	74 ± 11*	40 ± 5*	60 ± 13*	37 ± 7*	65 ± 19	0.369 ± 0.047	39.3 ± 4.2***
CB5083	14 ± 7	32 ± 15	0 ± 0	0 ± 0	0 ± 0	33 ± 18	0.394 ± 0.064	30.6 ± 3.1
CB5082	78 ± 10*	83 ± 12*	33 ± 13*	48 ± 8*	52 ± 7*	77 ± 14*	0.325 ± 0.046	37.0 ± 4.1***
CB5088	3 ± 2	17 ± 8	9 ± 9	20 ± 13	5 ± 8	52 ± 10	0.388 ± 0.033	33.7 ± 3.3
CB5089	41 ± 3*	76 ± 17*	19 ± 9	40 ± 9	26 ± 7*	59 ± 3	0.343 ± 0.008	34.6 ± 2.6*
CB1631	22 ± 12	31 ± 24	15 ± 14	30 ± 24	6 ± 7	46 ± 25	0.375 ± 0.021	39.7 ± 3.5
CB1634	87 ± 8*	100 ± 12*	72 ± 16*	79 ± 10*	12 ± 9	56 ± 28	0.280 ± 0.045*	39.8 ± 3.5
CB1663	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	20 ± 5	0.422 ± 0.081	29.4 ± 3.0
CB1664	84 ± 36*	82 ± 39*	67 ± 17*	64 ± 14*	57 ± 21*	69 ± 5*	0.334 ± 0.075	44.7 ± 5.3*
CB5057	80 ± 10	80 ± 6	24 ± 6	34 ± 5	40 ± 24	85 ± 1	0.321 ± 0.022	34.2 ± 4.4
CB5059	94 ± 13	95 ± 19	70 ± 21*	76 ± 20*	69 ± 20	83 ± 13	0.265 ± 0.054	36.0 ± 3.6*
CB5062	69 ± 27	74 ± 35	23 ± 9	44 ± 8	0 ± 0	7 ± 12	0.332 ± 0.098	32.3 ± 3.0
CB5063	85 ± 2	96 ± 7	64 ± 33	69 ± 28	20 ± 21	56 ± 23*	0.331 ± 0.014	36.2 ± 4.0***
CB5015	80 ± 36	82 ± 28	65 ± 7	66 ± 3	44 ± 12	58 ± 1	0.375 ± 0.051	41.4 ± 6.7
CB5016	85 ± 32	92 ± 19	87 ± 8*	82 ± 3*	50 ± 10	48 ± 8	0.342 ± 0.035	43.1 ± 6.1[b]
CB5021	24 ± 11	57 ± 14	16 ± 12	28 ± 24	22 ± 13	66 ± 13	0.295 ± 0.020	30.4 ± 3.8
CB5020	101 ± 16*	111 ± 5*	83 ± 5*	73 ± 6*	82 ± 30*	95 ± 41	0.083 ± 0.04*	47.7 ± 7.5***

[a] Pairs of isolates are represented by alternative shading and no shading, with the first strain in each pair being the DAP^s parental strain and the second one in each pair being the DAP^r strain. *, $P < 0.05$ versus parental strain; **, $P = 0.05$ versus parental strain; ***, $P < 0.0001$ versus parental strain.
[b] $P = 0.057$ versus parental strain value.

versus DAP^r groups for statistical assessments. A minimum of three experimental runs were performed on separate days.

Cell membrane fluidity. Relative cell membrane order was determined by polarization spectrofluorometry as described previously by Mishra et al. (22) using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH). An inverse relationship exists between polarization indices and the degree of cell membrane order (i.e., a lower polarization index [PI] value equates to a greater cell membrane fluidity). These assays were performed a minimum of three times for each strain set on separate days.

Cell wall thickness. All strain pairs were prepared for assessment of cell wall thickness profiles by transmission electron microscopy as described previously (41). The mean thickness ± SD of 100 cells was determined for our strain set at a magnification of ×190,000 (model 100CX; Jeol, Tokyo, Japan) using digital image capture and morphometric measurement (version 54; Advanced Microscopy Techniques, Danvers, MA). Cells were prepared for microscopy by previously published techniques (17). The electron microscopy measurements were performed by one of the authors (C.C.N.), who was blinded to the identity of the strains as DAP^s or DAP^r.

Surface charge. To measure relative net surface charge, a fluorescein isothiocyanate (FITC)-labeled poly-L-lysine (PLL) binding assay was performed using flow cytometry (FACS Calibur apparatus; Beckman Instruments, Alameda, CA) as described previously (21, 23). Data were expressed as mean fluorescent units ± SDs. The lower that the residual cell-associated label was, the more positively charged that the *S. aureus* cell envelope is (21, 23). At least three independent runs were performed on separate days.

Statistical analysis. Means and SDs were calculated for all variables. Differences between strains for killing and cell membrane/cell wall profile assays were analyzed with the Wilcoxon rank-sum test or two-tailed Student *t* test, as appropriate. For analysis of the relationships between CAP susceptibilities, DAP MIC, or cell membrane/cell wall profiles, comparisons for individual variables were performed using simple linear regression and multiple linear regression, and these techniques were then used to assess the joint relationship of the predictors with the outcome. All variables with P values of <0.2 were included in a stepwise regression analysis, with the criterion for remaining in the model being significance at α equal to 0.05. P values of ≤ 0.05 were considered significant.

RESULTS

HDP and CAP susceptibilities. In general, among the individual isogenic pairs, DAP^r strains exhibited higher survival profiles than their respective parental DAP^s strains when they were exposed *in vitro* to the cadre of test CAPs (Table 3). For example, 7/10 DAP^r strains were significantly more resistant to both peptide exposure concentrations of tPMPs and hNP-1 than their respective DAP^s parental strain. In five of these seven comparisons, reduced killing profiles for these two HDPs tracked together for individual strain pairs. For PMB, the concomitant cross-resistance phenomenon was less common and did not consistently track with the two HDPs.

When they were analyzed as collective groups of DAP^s and DAP^r strains, DAP^r strains demonstrated significant reductions in killing by all three CAPs assessed at all peptide exposure concentrations tested; this was especially striking for sublethal concentrations of tPMPs and hNP-1 *in vitro* (Table 4).

TABLE 4. Group comparison of CAP susceptibility, cell membrane fluidity, and cell wall thickness of all DAP^s and DAP^r strains[a]

Parameter	DAP^s strains ($n = 10$)	DAP^r strains ($n = 10$)	P value
% survival after 2 h of exposure to CAP (concn[b])			
tPMP (0.50)	31 ± 32	77 ± 19	0.0012
tPMP (0.25)	43 ± 29	90 ± 11	<0.001
hNP-1 (10)	20 ± 21	59 ± 22	<0.001
hNP-1 (5)	30 ± 22	66 ± 13	<0.001
PMB (40)	12 ± 17	41 ± 24	0.0067
PMB (20)	44 ± 23	65 ± 16	0.0328
Cell membrane fluidity (PI value)	0.369 ± 0.05	0.300 ± 0.08	0.0358
Cell wall thickness (nm)	33.84 ± 4.1	39.70 ± 4.10	0.0051

[a] Values are means ± SDs.
[b] Concentrations are in micrograms per milliliter.

TABLE 5. Comparative analysis of CAP susceptibility, cell membrane fluidity, and cell wall thickness of the DAP^s parental strains and the 7 DAP^r strains with mutations in *mprF* (with or without *yycG* mutations)[a]

Parameter	DAP^s strains (n = 10)	DAP^r strains (n = 7)	P value
% survival after 2 h of exposure to CAP (concn[b])			
tPMP (0.50)	31 ± 32	71 ± 20	0.0112
tPMP (0.25)	43 ± 29	86 ± 10	0.016
hNP-1 (10)	20 ± 21	51 ± 21	0.0085
hNP-1 (5)	30 ± 22	62 ± 14	0.0041
PMB (40)	12 ± 17	37 ± 23	0.0207
PMB (20)	44 ± 23	64 ± 15	0.0599
Cell membrane fluidity (PI value)	0.369 ± 0.05	0.321 ± 0.04	0.0441
Cell wall thickness (nm)	33.84 ± 4.1	38.60 ± 3.3	0.0218

[a] Values are means ± SDs.
[b] Concentrations are in micrograms per milliliter.

We next examined the potential correlation of HDP-resistant or CAP-resistant profiles with acquisition of an *mprF* mutation(s), with or without concomitant *yyc* operon mutations, in DAP^r strains (Table 5). Collectively, acquisition of *mprF* mutations in DAP^r strains was associated with significantly reduced killing by both HDPs (tPMPs and hNP-1). A similar but less robust statistical trend was noted for PMB. To further dissect the specific impact of *mprF* mutations alone (n = 4 strains) or in combination with *yycG* mutations (n = 3 strains), we analyzed CAP susceptibility phenotypes in relation to one or more mutations. As seen in Table 6, there was a notable trend of increasing CAP-resistant phenotypes with accumulation of both the *mprF* and *yycG* mutations combined compared to *mprF* mutations alone. However, the small sample sizes precluded definitive statistical evaluation. It should be underscored that the three DAP^r strains lacking SNPs in either locus still demonstrated substantially reduced killing by all study peptides compared to their parental DAP^s strains.

Cell membrane fluidity. When they were compared individually, each DAP^r strain exhibited a clear trend toward more fluid cell membranes than the respective DAP^s parental strain (Table 3). This comparison reached statistical significance for two individual strain pair comparisons. When they were compared as collective DAP^s versus DAP^r isolate groups, the cell membranes of the DAP^r strains were significantly more fluid than those of the isolates in the DAP^s group (Table 4).

Cell wall thickness. All DAP^r strains had significantly thicker cell walls than their respective DAP^s parental strains (Table 3). Similarly, when they were analyzed as collective groups of DAP^s and DAP^r strains, DAP^r strains exhibited significantly thicker cell walls than the DAP^s isolates (Table 4). This same relationship held when strains with *mprF* mutations were compared to the DAP^s strains (Table 5).

To refine the phenotypic associations between cell wall/cell membrane profiles with CAP-DAP susceptibility profiles, we performed simple linear regression analysis comparing cell wall thickness and cell membrane fluidity with the outcome variables of CAP and DAP efficacies (reductions in numbers of CFU/ml versus MICs, respectively). Cell wall thickness was directly and significantly associated with reduced killing by hNP-1 ($P < 0.001$), tPMPs ($P < 0.05$), and PMB ($P < 0.01$) among the DAP^r strains. Cell wall thickness was related to higher DAP MICs, although this did not reach statistical significance ($P = 0.12$). Increased cell membrane fluidity correlated well with reduced killing of DAP^r strains by all peptides (hNP-1, $P < 0.01$; tPMPs, $P < 0.01$; and PMB, $P < 0.01$). Similarly, enhanced cell membrane fluidity tracked somewhat with higher DAP MICs in the DAP^r strains, although not significantly ($P = 0.4$). Although multiple linear regression analyses with both predictor variables of cell wall thickness and cell membrane fluidity were attempted, they proved to not be feasible due to limited sample sizes.

Genotypic and phenotypic associations. To further explore phenotypic-genotypic correlates, DAP^r strains were subcategorized as to the presence of mutations in *mprF* alone, mutations in *mprF* plus *yycG*, or no mutations in either locus. Of interest, DAP^r strains with mutations in *mprF* alone or in combination with *yycG* mutations exhibited obvious trends in thicker cell walls than DAP^s strains and increased cell membrane fluidity compared to DAP^s strains

TABLE 6. Comparative analysis of CAP susceptibility, cell membrane fluidity, cell wall thickness, and MIC profiles of all DAP^s strains and DAP^r strains with or without specific point mutation profiles[a]

Parameter	DAP^s (n = 10)	DAP^r mutations in *mprF* alone (n = 4)	DAP^r mutations in *mprF* plus *yycG* (n = 3)	DAP^r neither mutation (n = 3)
% survival after 2 h of exposure to CAP (concn[b])				
tPMP (0.50)	31 ± 32	59 ± 17	88 ± 5	90 ± 9
tPMP (0.25)	43 ± 29	82 ± 9	92 ± 9	100 ± 10
hNP-1 (10)	20 ± 21	37 ± 15	70 ± 3	78 ± 12
hNP-1 (5)	30 ± 22	54 ± 13	73 ± 8	75 ± 7
PMB (40)	12 ± 17	31 ± 68	46 ± 30	51 ± 31
PMB (20)	44 ± 23	60 ± 16	69 ± 13	66 ± 25
Cell membrane fluidity (PI value)	0.369 ± 0.05	0.343 ± 0.19	0.293 ± 0.04	0.252 ± 0.15
Cell wall thickness (nm)	33.84 ± 4.1	37.43 ± 2.1	40.16 ± 4.4	42.13 ± 5.5
MIC (μg/ml)	0.475 ± 0.22	3.25 ± 0.96	4.00 ± 0.00	4.33 ± 3.5

[a] Values are means ± SDs. See text for comparative assessments of these data sets.
[b] Concentrations are in micrograms per milliliter.

(Table 6). However, small sample sizes precluded adequate statistical comparisons.

Surface charge. No consistent pattern of surface charge differences was observed between the DAP^r and DAR^s strain pairs, either individually or as overall groups (data not shown).

DISCUSSION

There have been a number of recent reports concerning development of DAP^r among clinical strains of *S. aureus* during the therapy of invasive infections with this agent (13, 33). Recent studies of a limited number of DAP^s/DAP^r MRSA and methicillin-susceptible *S. aureus* (MSSA) strain pairs from our laboratories have shown a trend toward coevolution of relative resistance to several HDPs and DAP^r (13, 22, 39). In the present investigation, we used a well-characterized set of DAP^s and DAP^r MRSA strains to examine (i) the frequency and extent of this phenomenon and (ii) potential genotypic and phenotypic associations that may serve as biomarkers of such a co-occurrence in reduced *in vitro* susceptibilities (relative cross-resistance) of these agents.

A number of interesting findings emerged from this study. First, we employed two HDPs which have been well chronicled to defend against endovascular infections: tPMPs from platelets and hNP-1 from neutrophils (44, 46). DAP^r MRSA strains in this study demonstrated a clear trend of reduced *in vitro* susceptibility to these HDPs, whether they were assessed in individual (head-to-head) or group strain comparisons. In addition, this *in vitro* cross-resistance phenotype extended to PMB, a cyclic bacterium-derived CAP with no overt structural or mechanistic similarities to either DAP or the two HDPs tested (22). In contrast, no such cross-resistance was found for the cationic ribosome-targeting molecule gentamicin. Since the principle mechanistic feature shared by DAP and the three peptides above is cell membrane targeting (22), this suggested that a general adaptive paradigm for such cross-resistance was operative. This hypothesis prompted our comparison of several prototypical cell membrane and cell surface parameters between the DAP^s and DAP^r strain pairs. Of interest, 9/10 DAP^r isolates exhibited 2- to 4-fold increases in MICs to vancomycin, a minimally cationic cell wall-targeting antibiotic. Previous studies have documented a temporal linkage between vancomycin usage, increased vancomycin MICs, and subsequent resistance *in vitro* to DAP (8, 14, 24).

Second, recently published studies by our group and others (10, 13, 21, 22, 41) have suggested an important role in DAP^r for at least two genes involved in maintenance of staphylococcal positive surface charge, *mprF* and *dlt* (13, 21, 39). For *mprF*, several investigations have identified a series of gain-in-function point mutations within its ORF. These mutations have been associated with either excess production or increased outer cell membrane translocation of the positively charged phospholipid species L-PG. The net result of these effects is believed to be enhancement of relative positive surface charge (10, 13). Our current data were somewhat in line with these prior observations, showing that DAP^r strains commonly exhibited SNPs within the *mprF* ORF (in either its putative synthase or translocase domain) (10). Surprisingly, the presence of such SNPs in *mprF* among DAP^r strains was not consistently associated with significant changes in the relative surface charge profiles compared with the profiles of their respective DAP^s parental strains. This suggested several possible explanations: (i) these SNPs may not have altered the *mprF* gene expression profile in these strains. This phenomenon was recently seen in a DAP^r MSSA strain in which a well-defined SNP was identified within the *mprF* ORF without enhancement of *mprF* gene expression (39). In that instance, an increased positive surface charge was identified in association with enhanced expression of the *dlt* operon. (ii) *mprF* expression was enhanced in our DAP^r strains of interest, but compensatory adaptations in other genes involved in surface charge maintenance occurred (e.g., *dlt* or genes regulating cell wall amidation [7, 25, 26, 36]). (iii) *mprF* expression and L-PG synthesis/translocation were indeed enhanced, but DAP^r in these strains was unrelated to a charge-mediated effect on peptide interactions. In this regard, recent investigations from our laboratory and others provide evidence of an increased association of certain CAPs with the cell membrane and potentially with PG-cardiolipin-enriched regions. In this case, we hypothesize that any effect of charge repulsion would be a secondary mechanism of DAP^r (16, 19). Gene expression and phospholipid compositional profiling are currently in progress to examine these possible scenarios. It should be underscored that although acquisition of *mprF* SNPs (with or without concomitant *yycG* SNPs) was frequently found in our DAP^r strains, isolates without either mutation demonstrated similar frequencies and extents of DAP-CAP cross-resistance. Thus, other genotypic mechanisms of DAP^r are likely at play for such strains.

As pointed out above, SNPs within the *yyc* operon have previously been noted in both *in vitro*-derived (by serial DAP passage) and clinically derived DAP^r strains of *S. aureus* (13, 21). Among *in vitro*-generated DAP^r strains, accumulation of *yyc* operon SNPs appears to temporally follow mutations in the *mprF* operon (12). The precise mechanism(s) by which SNPs within the *yyc* operon (e.g., *yycG*, as in three of our strains) cause the DAP^r phenotype is not known. This multifunctional *S. aureus* regulatory operon has been described to be a factor potentially important in (i) virulence (through impacts on *ssaA* and *lytM* expression), (ii) influence upon cell wall biosynthesis through regulation of the *tag* operon expression (involved in cell wall teichoic acid synthesis), (iii) cell membrane fatty acid homeostasis, and (iv) biofilm formation (11).

Third, in addition to the potential issue of surface charge impacts on DAP-CAP cross-resistance, we investigated two other phenotypic characteristics of the DAP^s/DAP^r strain pairs that might influence peptide-*S. aureus* interactions: (i) cell membrane order and (ii) cell wall thickness. Our laboratories have reported a number of instances in which *S. aureus* strains with highly disordered cell membranes demonstrated significantly reduced abilities to be killed *in vitro* by prototypical HDPs, including tPMPs and hNP-1 (2, 13, 21, 37). The mechanism(s) by which increased cell membrane fluidity leads to reduced HDP-induced killing of *S. aureus* is not clear but may include perturbations of the HDP-cell membrane association, a reduced capacity for HDP insertion into the target cell membrane, and/or CAP partitioning within such disordered cell membranes (13, 37). Similarly, *S. aureus* strains which have excessively ordered (rigid) cell membranes by virtue of robust pigment production also show DAP-CAP cross-resistance *in*

vitro (22). In the present study, there was a notable trend among all 10 DAP^r strains to have substantially more fluid cell membranes than their respective DAP^s parental strains. Studies are in progress to determine whether enhanced cell membrane fluidity is causal in DAP^r or whether it is a secondary consequence and surrogate biomarker of this phenotype.

Lastly, the cell walls of these DAP^r strains were substantially thicker by electron microscopy than those of their respective DAP^s parental isolates. The association of thickened cell walls with DAP^r has been previously documented as a common, but not universal, accompaniment of this phenotype in *S. aureus*, similar to VISA strains (5, 8, 21, 41). It has been postulated that, as with VISA strains, thickened cell walls may represent a physical barrier or an affinity trap to DAP and other HDPs or CAPs, preventing their accessibility to their principle cell membrane target(s) (8, 21). As noted before, 9/10 DAP^r strains studied showed 2- to 4-fold increases in vancomycin MICs correlating with such thickened cell walls. The metabolic pathways leading to the thickened cell wall phenotype and associated DAP-CAP relative cross-resistance in *S. aureus* are likely to be multifactorial and complex (21). Recent data from our laboratories implicate excess synthesis of cell wall teichoic acids as an important contributor to the thickened cell wall and DAP^r phenotypes in selected strains (3).

It should be emphasized that the current investigation had several important limitations: (i) the small sample size of DAP^r strains with and without mutations within the *mprF* locus precluded adequate statistical analysis of the impacts of this operon on DAP-CAP cross-resistance phenotypes; (ii) only a limited breadth of cell membrane and cell wall profiling was queried in comparing the strain pairs; (iii) a relatively narrow range of host defense CAPs was investigated, leaving open the question of how specific or nonspecific the DAP-CAP cross-resistance phenotype really is; and (iv) CAPs were assessed for their inhibitory activities against study strains individually, at low-inoculum challenges, using peptide concentrations that are likely well below their physiological concentrations and in assays conducted within austere artificial media *in vitro*. Such conditions are unlikely to effectively represent those under which *S. aureus* strains encounter DAP and HDPs within the endovascular compartment *in vivo*. These limitations are being addressed in current investigations in our laboratories. Finally, the precise sequence of events by which *in vitro* DAP^r coevolves with CAP^r in *S. aureus* is not clear. Thus, do organisms first develop DAP^r upon prolonged exposure to this agent *in vivo*, with the co-occurrence of relative resistance to HDPs being a secondary phenomenon, or are bloodstream organisms first selected *in vivo* for reduced susceptibility to HDPs by exposure to endovascular host defenses (i.e., those from platelets and neutrophils), setting the stage for DAP^r? These and other potential paradigms, while not mutually exclusive, are also under active investigation in our laboratories.

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