Heterogeneity of Genetic Pathways toward Daptomycin Nonsusceptibility in *Staphylococcus aureus* Determined by Adjunctive Antibiotics

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Daptomycin is increasingly used in combination with other antibiotics to enhance antimicrobial efficacy and/or to mitigate the emergence of daptomycin nonsusceptibility (DNS). This study used a clinical methicillin-resistant *Staphylococcus aureus* (MRSA) strain in which DNS emerged upon therapy to examine the influence of antibiotic combinations on the development of mutations in specific genes (*mprF*, *rpoBC*, *dltA*, *cls2*, and *yycFG*) previously associated with DNS. Whole genomes of bacteria obtained following 28 days of *in vitro* exposure to daptomycin with or without adjunctive clarithromycin, linezolid, oxacillin, or trimethoprim-sulfamethoxazole were sequenced, and the sequences were compared to that of the progenitor isolate. The addition of oxacillin to medium containing daptomycin prevented the emergence of *mprF* mutation but did not prevent *rpoBC* mutation (*P* < 0.01). These isolates maintained susceptibility to daptomycin during the combined exposure (median MIC, 1 mg/liter). Daptomycin plus clarithromycin or linezolid resulted in low-level (1.5 to 8 mg/liter) and high-level (12 to 96 mg/liter) DNS, respectively, and did not prevent *mprF* mutation. However, these same combinations prevented *rpoBC* mutation. Daptomycin alone or combined with linezolid or trimethoprim-sulfamethoxazole resulted in high-level DNS and mutations in *mprF* plus *rpoBC*, *cls2*, and *yycFG*. Combining daptomycin with different antimicrobials alters the mutational space available for DNS development, thereby favoring the development of predictable collateral susceptibilities.

Daptomycin (DAP) is frequently used in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infection. However, DAP-nonsusceptible (DNS) strains are observed clinically and often associated with treatment failure (1). The development of DNS subpopulations is of particular concern in three clinical scenarios: (i) prolonged DAP therapy, particularly with underdosing, (ii) sequestered foci of infection where DAP penetration is reduced, such as osteomyelitis or infective endocarditis (2, 3), and (iii) refractory or recurrent infection where there has been previous exposure to host antimicrobial peptide defenses and/or glycopeptide antimicrobials (4, 5).

DNS MRSA emerges via the accumulation of single or multiple mutations, each resulting in a slight increase in the DAP MIC (6, 7). Several DNS MRSA strains have been characterized, with causal mutations identified in *mprF*, *yycFG* (*walKR*), *dltA*, *rpoBC*, and/or *cls2* (8). These elements share a role in maintaining cell envelope homeostasis, either by directly modifying the cell membrane or as a regulatory response to cell surface perturbations. The identification of a direct mechanism for DNS development with any given mutation is frequently confounded by simultaneous changes in the expression and/or activity of the MprF, Cls2, and DltA enzymes (9). While there is significant strain-to-strain variability in the pathways affected, a frequent consequence of the development of DAP resistance is a reduced anionic cell membrane potential, resulting in reduced charge-charge attraction between Gram-positive membranes and either DAP or host cationic defense peptides (10). Although no single mutation is uniquely responsible for the DNS phenotype (11) and clinical DNS strains have emerged that have the wild-type *mprF* sequence (4), the first

and most clinically relevant mutation to emerge typically occurs as a gain-of-function mutation in *mprF* (12). Thus, preventing or delaying the emergence of *mprF* mutation may result in a longer effective therapeutic window for DAP.

DAP has been increasingly used with additional antimicrobials either for empirical coverage of complex infection or targeted toward a specific infection type or organism to improve clinical efficacy (13, 14). It is unknown whether these adjunctive antibiotics exert a treatment-dependent influence on the pattern and/or frequency of mutation in genes associated with DNS development. Previous work in our laboratory demonstrated that combinations of DAP plus oxacillin or DAP plus clarithromycin suppressed the emergence of DNS (15). In this prior study, a DAP-susceptible clinical MRSA isolate from which DNS emerged upon therapy was exposed *in vitro* to escalating concentrations of DAP with or without the presence of additional anti-

biotics for a period of 28 consecutive days. This current study expands significantly on these initial results by comparing the frequencies of mutation in genes previously associated with DAP nonsusceptibility between strains and the efficiencies of different antibiotic combinations with DAP in preventing these mutations.

(Portions of this work were presented at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 9 September 2014.)

MATERIALS AND METHODS

Bacterial strains. MRSA strain J01 and the generation of DNS strains from it *in vitro* have been described in previous work (15). Briefly, DAP-susceptible clinical isolate J01 was collected from a patient presenting with left-sided endocarditis and multiple septic-embolic complications. A second clinical isolate, J03, was collected after 24 days of antimicrobial therapy and had become DNS. In order to explore mechanisms involved in the transition to a DNS phenotype, five independent replicates of J01 were exposed in liquid culture to escalating concentrations of DAP stepwise over a period of 28 days in the presence or absence of a static concentration of adjunctive antimicrobial at one-half the MIC. This current work assesses the five replicate strains obtained from each of the following exposures: (i) DAP monotherapy (D1 to D5), (ii) DAP plus clarithromycin (CLR; 100 mg/liter) (DC1 to DC5), (iii) DAP plus linezolid (LZD; 0.5 mg/liter) (DL1 to DL5), (iv) DAP plus oxacillin (OXA; 16 mg/liter) (DO1 to DO5), and (v) DAP plus trimethoprim-sulfamethoxazole (SXT; 6 mg/liter [sulfamethoxazole component]) (DS1 to DS5).

Antimicrobials and media. CLR, erythromycin (ERY), OXA, and SXT were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). DAP was obtained from Cubist (Cubist Pharmaceuticals, Lexington, MA, USA). LZD was obtained from Pfizer (Pfizer, Inc., New York, NY, USA). Mueller-Hinton broth II (BD, Sparks, MD, USA) supplemented with 50 µg/ml calcium (as $CaCl_2$) and 12.5 µg/ml magnesium (as $MgCl_2$) was used to grow *S. aureus* in liquid culture. Isolates that exhibited poor growth in liquid culture due to aromatic compound auxotrophy were cultivated in medium supplemented with 1 mM shikimic acid.

Susceptibility testing. The MICs of the study bacteria to DAP, OXA, and vancomycin (VAN) were determined by Etest as suggested by the manufacturer (bioMérieux, Marcy l'Etoile, France). The MICs to other antibiotics were determined by broth microdilution according to the Clinical and Laboratory Standards Institute guidelines (17). All samples were incubated at 35°C for 24 h. Reference strain ATCC 29213 was included in all MIC determination testing as an internal control. All values obtained were within the acceptable range for this organism.

Whole-genome sequencing. Genomic DNA was extracted, and multiplex short-read DNA libraries were created with the Nextera XT DNA preparation kit (Illumina). Whole-genome sequencing was performed on a MiSeq instrument (Illumina) using 2× 300-bp chemistry; all steps were performed according to the manufacturer's recommendations.

A read-mapping approach was used to align the short-read sequences for all isolates to the assembled genome of *S. aureus* FRP3757-USA300 (GenBank accession number NC_007793.1) using Bowtie2 (18). Single nucleotide polymorphisms, insertions/deletions up to 10 bp in length, and the predicted amino acid consequences were identified using Nesoni version 0.128 with a stringency threshold of 80% (http://bioinformatics.net.au). The effects of mutations in loci that have not previously been associated with the DNS phenotype will be explored in future work. This study focused exclusively on mutations identified in the following loci that have previously been associated with the DNS phenotype: SAUSA300_0020 (*yycF*), SAUSA300_0021 (*yycG*), SAUSA300_0527 (*rpoB*), SAUSA300_0528 (*rpoC*), SAUSA300_0835 (*dltA*), SAUSA300_1255 (*mprF*), and SAUSA300_2044 (*cls2*) (7, 12, 19). The full list of predicted changes to the products of genes of interest (*mprF*, *rpoBC*, *dltA*, *yycFG*, and *cls2*) is provided in Table 1.

Cytochrome *c* binding. The cytochrome *c* binding assay was adapted from previously published methods (20, 21). Briefly, overnight cultures of

S. aureus grown in Trypticase soy broth (TSB) were diluted in fresh medium and allowed to regrow to logarithmic phase. Cells were harvested, washed twice with MOPS (morpholinepropanesulfonic acid) buffer (20 mM, pH 7.0), adjusted to an optical density at 600 nm (OD_{600}) of 1.0, and collected from 1-ml aliquots via centrifugation. Cell pellets were resuspended in 200 µl MOPS buffer and combined with 50 µl of cytochrome *c* solution (equine heart, 2.5 mg/ml in MOPS buffer; Sigma). Samples were incubated for 10 min at room temperature and separated by centrifugation at top speed. Supernatants were recovered, and the OD_{530} was measured spectrophotometrically. The amount of cell-bound cytochrome *c* was determined using an extemporaneously prepared standard curve. The data presented are the average results of at least 3 independent replicates.

Statistical analysis. Comparisons of the MIC values of different populations were conducted using the Mann-Whitney test. Comparisons of the development of specific mutations between populations exposed to different antibiotics were conducted using Fisher's exact test.

Nucleotide sequence accession number. Sequence reads have been submitted to the European Nucleotide Archive under project accession number ERP009667.

RESULTS

In our data set of 175 loci previously associated with the DNS phenotype (seven target genes times 25 genomes analyzed), 49 (28%) had altered sequences compared to the genome sequence of *S. aureus* USA300-FPR3757, resulting from 26 unique mutations (Table 1). Of these, two involved insertion/deletion events (indels), while 24 involved single nucleotide polymorphisms (SNPs). Only one of the SNPs, in *cls2*, is predicted to result in the introduction of a premature stop codon; the remaining 25 SNPs and indels are predicted to result in single-amino-acid changes (substitutions, additions, or removals) in the protein product. None of the mutations are predicted to result in frameshift or synonymous changes. The end-of-study DAP MICs among the 25 strains analyzed were significantly increased in isolates with a genetic mutation in *mprF* and further increased when additional mutations existed within *rpoBC*, *yycFG*, or *cls2* (Fig. 1).

Polymorphisms in *mprF* were the most prevalent mutation observed, occurring in 20/25 strains. Mutations were not localized to one specific region of MprF but did cluster within known DNS hot spots (22) in the bifunctional central transmembrane domain (S295L [an S-to-L change at position 295], A302V, P314L, S337L, M347R, H376Y, and W424C) or occurred to one specific residue near the C terminus of the cytosolic synthase domain (L826I and L826F) (7, 23). One strain contains a 3-nucleotide insertion and is predicted to incorporate an additional asparagine residue early in the MprF peptide sequence (D40DN). The combination of DAP and OXA prevented the development of an *mprF* mutation, whereas isolates from single-antibiotic exposure to DAP and from every other DAP combination displayed *mprF* mutations after 28 days ($P < 0.01$). A previous study had observed the establishment of a stable G-to-A transitional mutation in the noncoding region upstream from the AMP-dependent synthetase *acsA* in *S. aureus* MW2 (locus tag MW2528) prior to the development of an *mprF* mutation (12). *S. aureus* FPR3757 does not carry this transitional mutation, and none of the 25 strains analyzed in this study had any detectable sequence variation in the region upstream from *acsA* (locus tag SAUSA300_2542). Therefore, an intergenic transition upstream from *acsA* is not a necessary prerequisite for an *mprF* mutation.

Polymorphisms in *rpoC* were frequent in the study population, occurring in 14/25 strains. Mutations in *rpoB* were less common (2/25) and did not appear to localize to a single region of the gene

TABLE 1 Bacterial study strains, antibiotic susceptibilities, and genetic mutations in select loci

Strain,[a] drug exposure	MIC of[b]:				Mutation(s)[c] in:						
	DAP	VAN	OXA	CLR	*mprF*	*rpoB*	*rpoC*	*dltA*	*cls2*	*yycF*	*yycG*
Clinical isolates											
J01	0.5	2	32	256	—	—	—	—	—	—	—
J03	3	2	16	256	T345X	S464P	—	—	—	—	—
In vitro strains											
DAP											
D1	32	2	0.01	16	L826F	—	N735K	—	R320L	—	—
D2	32	2	0.01	32	L826I	—	N735K	—	R320S	—	369delQ
D3	12	2	0.01	32	L826I	—	N735K	—	R320S	—	369delQ
D4	8	2	0.01	8	L826F	—	N735K	—	—	—	—
D5	24	1.5	0.01	32	P314L	—	—	—	F85X	—	—
DAP+CLR											
DC1	3	2	0.01	64	H376Y, W424C	—	—	—	—	—	—
DC2	1.5	1.5	0.01	64	A302V	—	—	—	—	—	—
DC3	8	3	0.01	32	P314L	—	—	—	—	—	—
DC4	3	1.5	0.02	32	M347R	—	—	—	—	—	—
DC5	3	2	0.01	32	S337L	—	—	—	—	—	—
DAP+LZD											
DL1	32	2	0.01	32	S337L	—	—	—	F85X	—	—
DL2	64	4	0.01	0.03	41insN	—	—	—	—	—	—
DL3	192	1.5	0.01	0.01	L826F	—	—	—	L77F	—	G199E
DL4	64	3	0.03	1	S295L	—	—	—	—	—	—
DL5	192	2	0.01	0.01	L826F	—	—	—	L77F	—	G199E
DAP+OXA											
DO1	0.02	1.5	256	2	—	—	N735K	A426E	—	—	—
DO2	1	3	64	4	—	—	N735K	—	—	—	—
DO3	3	3	128	4	—	—	N735K	—	—	—	—
DO4	0.05	2	128	4	—	—	N735K	—	—	—	—
DO5	3	3	128	0.06	—	S366F	—	—	—	—	—
DAP+SXT											
DS1	24	2	4	32	L826I	—	N735K	—	—	—	—
DS2	96	2	4	32	L826I	—	N735K	—	—	—	—
DS3	32	3	0.5	16	L826I	—	N735K	—	R320L	—	—
DS4	12	3	32	8	S295L	—	N735K	—	—	K151N	—
DS5	64	2	16	8	L826I	V1130K, V1162I	—	—	A56G, T58N	—	—

[a] All *in vitro* strains were derived from J01 following 28 days of antibiotic exposure.
[b] MICs to antibiotics are provided in mg/liter. Susceptibility breakpoints for *S. aureus* are as follows: DAP, ≤1 mg/liter; VAN, ≤2 mg/liter; OXA, ≤2 mg/liter; CLR, ≤2 mg/liter (17).
[c] —, the strain has the wild-type sequence.

or to a single domain of the encoded protein (S746F in replicate DO5, and S464P, V1130K [nucleotide change, GT3388AA], and V1162I in replicate DS5). However, each mutation in *rpoB* did map to one of the three regions shown to alter the response of RNA polymerase to transcriptional pausing and termination signals in *Escherichia coli* (nucleotides 500 to 575, 740 to 840, and 1225 to 1342 in the *E. coli* RpoB sequence) (24). Changes in *S. aureus* RpoB have already been described elsewhere as contributing to the DNS phenotype (12), potentially via increased DltA activity (9); however, in contrast to the mutations identified in this study, only one (A1086V) of the three RpoB changes associated with the DNS phenotype in previous work (A621E, I953S, and A1086V) localizes to any of the regions associated with modified termination signal recognition. Unlike the mutations in MprF and RpoB, all mutation events in *rpoC* resulted in the same single-amino-acid change in domain 4 (N735K). This region constitutes the funnel domain and is a site involved in binding of transcription factors (25). While changes in *S. aureus* RpoC have already been described elsewhere as contributing to the DNS phenotype

(12), the F632S and Q961K modifications identified in that work occur outside domain 4. No strains exposed to either CLR or LZD during DAP selection developed one of the otherwise nearly ubiquitous *rpoBC* mutations ($P < 0.001$).

Our study population occasionally contained polymorphisms in cardiolipin synthase (*cls2*), which occurred in 9/25 strains. Mutation events localized to either the linker region between the transmembrane domain and the first phospholipase D domain (A56G, T58N, L77F, or F85X) or to one residue in the *N*-terminal region of the second phospholipase D domain (R320L or R320S) (26). No strains exposed to either CLR or OXA during DAP selection developed any *cls2* mutation ($P = 0.028$). Furthermore, strains that had developed a mutation resulting in a change to amino acid residue 826 of MprF (i.e., L826I or L826F) were strongly associated with the development of an additional mutation in *cls2* (Fisher's exact test, $P = 0.009$). Mutations in *yycFG* or *dltA* were infrequent, occurring in only five and one strain, respectively, and did not correlate with exposure to any particular antibiotic. We do note that none of the strains exposed to either CLR
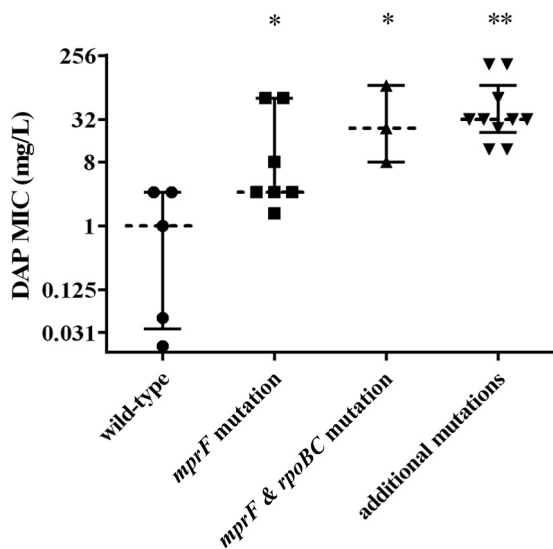
FIG 1 End-of-study DAP MICs grouped by patterns of mutation in genes that contribute to the DNS phenotype. Whiskers are the interquartile ranges, and the dashed line is the median for each group. Groups, left to right, comprise MICs for strains with the wild-type *mprF* sequence (*n* = 5), an *mprF* mutation only (*n* = 7), *mprF* and *rpoBC* mutations only (*n* = 3), or an *mprF* mutation and at least one additional mutation in either *cls2* or *yycFG* (*n* = 10). ∗, *P* < 0.05 versus the results for the wild type; ∗∗, *P* < 0.001 versus the results for the wild type (Mann Whitney test).

or OXA during DAP selection developed any *yycFG* mutation, but the trend was not statistically significant (*P* = 0.061).

Treatment-dependent changes in the MICs of DAP, VAN, OXA, and CLR are reported in Table 1. The addition of either OXA or CLR during the 28 days of DAP selection resulted in a significantly lower end-of-study DAP MIC than selection with DAP monotherapy (*P* < 0.05). Furthermore, these combinations delayed the development of the DNS phenotype compared to its development during selection with DAP monotherapy or, in some replicates, prevented the emergence of the DNS phenotype altogether. In contrast, the addition of either LZD or SXT did not result in any difference in the end-of-study DAP MIC or any delay in DNS development compared to the results for selection with DAP monotherapy (*P* > 0.05), as reported previously (15). VAN MICs remained stable during selection, with no treatment-dependent variability and no MIC changes greater than one doubling dilution. While changes to VAN MICs were modest overall, the progenitor strain is at the borderline of VAN susceptibility (MIC, 2 mg/liter) and one end-of-study strain developed intermediate VAN susceptibility (MIC, 4 mg/liter). In contrast, end-of-study strains developed markedly (>3 $\log_{10}$) increased susceptibilities to OXA, from an initial MIC value of 32 mg/liter to an end-of-study average of approximately 0.01 mg/liter. Exceptions to this trend occurred when OXA or SXT was added during DAP selection, resulting in end-of-study median OXA MICs of 128 mg/liter and 4 mg/liter, respectively (*P* < 0.05). An increase in end-of-study OXA MICs in strains exposed to OXA during DAP selection can be attributed to the selective pressure exerted by continuous OXA exposure. The mechanism by which exposure to SXT could result in a partial reversal of the OXA MIC decreases observed during DNS selection remains unclear. Of note, a wild-type *mecA* sequence was confirmed in 23/25 strains, indicating that OXA

susceptibility is not dependent on loss of the staphylococcal cassette chromosome *mec* (SCC*mec*) element, as has been discussed elsewhere (27). In contrast, the *mecA* sequence was not detectable in DC4 and DL3, suggesting that loss of the cassette chromosome may contribute to OXA susceptibility in these isolates.

Analogous to the overall decreases observed for OXA MICs, all end-of-study strains developed increased sensitivity to CLR (0.01 to 64 mg/liter), although the absolute change in CLR MICs was less pronounced. The addition of LZD, OXA, or SXT during DAP selection did not affect the magnitude or the direction of changes in CLR MICs, although there were higher end-of-study CLR MICs in strains exposed to DAP and CLR than in other combination treatment arms, again attributable to selective pressure exerted by continuous CLR exposure (*P* < 0.01).

The initial characterization of the *yycFG* two-component regulator system in *S. aureus* identified a marked reduction in the MICs of macrolides upon *yycF* disruption (28). Since perturbations in the *yycFG* system have also been associated with the development of the DNS phenotype (4) and DNS study strains frequently show reduced CLR MICs (Table 1), one consequence of DNS development may be altered *yycFG* signaling, resulting in increased macrolide sensitivity. However, in the population of MRSA strains containing *yycFG* disruption, there was no significant difference in CLR or ERY MICs compared to those of DNS strains that maintain a wild-type *yycFG* system (*P* > 0.05 for both comparisons). Therefore, while the MICs to macrolide antibiotics were frequently reduced in DNS study strains, that reduction was not dependent on *yycFG* mutation.

One well-characterized consequence of DAP exposure is the evolution of a less anionic bacterial envelope (10). Mutations associated with DNS MRSA may contribute to this phenotype via direct membrane modification (MprF) (29), modified proton gradient homeostasis (Cls2) (30), altered membrane biosynthesis regulation (YycFG) (31), or increased lipoteichoic acid modification (RpoBC) (9). Consistent with this model, the study strains overall demonstrated a reduced anionic membrane potential, as shown by the results in Fig. 2. The binding of cationic cytochrome *c* to bacterial membranes was reduced for all study strains relative to its binding to the progenitor strain (*P* < 0.05), with the exception of strains exposed to DAP and OXA, where the binding was overall similar to that of the parent strain (*P* = 0.54) and consistent with the low daptomycin susceptibility compared to the results for other combinations.

DISCUSSION

Due to the complexities and treatment failures associated with serious *S. aureus* infection, clinicians are increasingly interested in alternatives to standardized therapy. Many practitioners prescribe DAP with an adjunctive antibiotic for complex or prolonged infection, to enhance antimicrobial efficacy that results in improved therapeutic outcomes (32). It is not known whether these additional antimicrobials alter the metabolic cost associated with establishing and maintaining various mutations that result in DNS development. Our group and others have previously demonstrated that the addition of antistaphylococcal β-lactams during DAP exposure prevents the phenotypic emergence of DNS subpopulations (15, 21). This combination has gained acceptance among practitioners and has been used in patients, resulting in clinical successes (33–35). This has been attributed partially to β-lactam-mediated enhancement of DAP binding to the bacterial
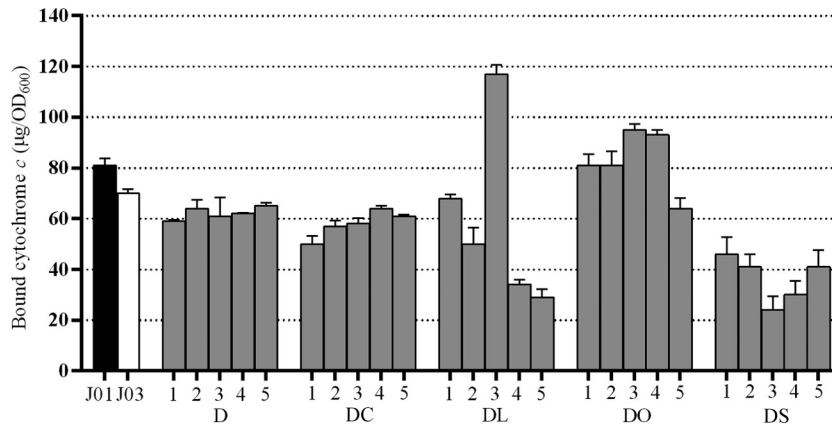
FIG 2 Cytochrome *c* binding affinities of study strains. Isolates J01 and J03 were derived *in vivo* during patient treatment. The *in vitro*-exposure strains (gray bars) were derived over 28 days of exposure to daptomycin plus an adjunctive antibiotic. Higher values correspond to a more anionic membrane. Data represent the mean values ± standard deviations from three independent replicates for each of the five replicate strains obtained for each drug exposure. DC, DAP+CLR; DL, DAP+LZD; DO, DAP+OXA; DS, DAP+SXT.

surface, but increased binding is not essential for improved activity, and other mechanisms are being explored (36).

In this study, we analyzed the DNA sequences of known determinants of DAP susceptibility in a collection of *in vitro*-derived MRSA strains to determine whether adjunctive treatment can alter patterns of DNS mutation. We have summarized these findings in Fig. 3, which provides a conceptual map delineating the

complex contributions of genetic mutations and phenotypic adaptations associated with DNS development. Our primary findings were that (i) strains exposed to OXA during 28 days of DAP selective pressure uniquely maintained wild-type *mprF* sequences and remained DAP susceptible and (ii) strains exposed to CLR or LZD during 28 days of DAP selective pressure uniquely maintained wild-type *rpoBC* sequences but did not develop DNS. Be-
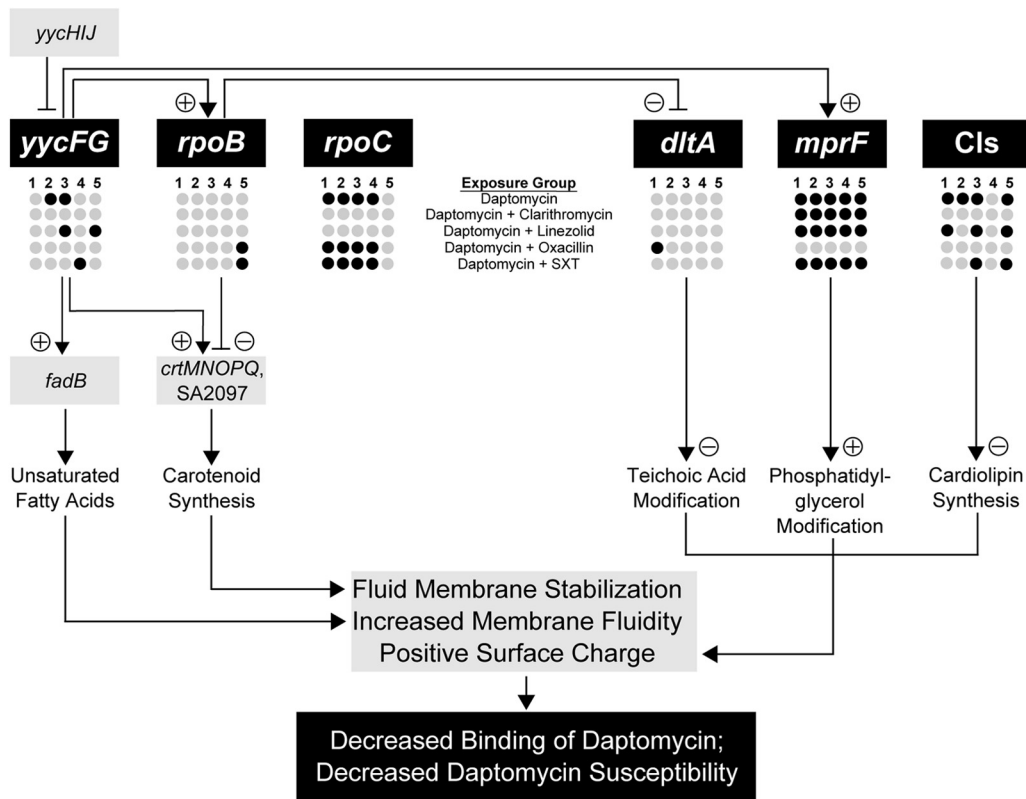

FIG 3 Biological model of the contributions of genetic mutations and phenotypic adaptations to the development of daptomycin-nonsusceptible *Staphylococcus aureus*. Genes in which mutations emerged following treatment-dependent exposure conditions in the current study are represented by dark circles, and light-gray circles indicate wild-type sequences. Implications of mutations for the downstream elements (regulated gene or phenotype) are indicated by a plus or minus sign in the pathway. SXT, trimethoprim-sulfamethoxazole.

cause strains exposed to OXA maintained DAP susceptibility even after *rpoBC* mutation developed, we conclude that the primary mediator of the DNS phenotype in the larger collection of strains is MprF derangement.

MprF was first identified in 2001 as FmtC, a membrane protein that confers OXA susceptibility when inactivated (37), and later was characterized as a factor that confers resistance to host cationic peptides via membrane lysinylation (38). Soon after FDA approval and introduction of DAP into clinical practice, mutations in *mprF* were found to be associated with reduced susceptibility to DAP (12) and later shown to be causal of the phenotype (39). Recent work shows that depletion of MprF using antisense RNA technology not only restores the DAP-susceptible phenotype in DNS MRSA but also decreases the susceptibility to OXA (40). Thus, MprF activity has consistently been associated with both OXA and DAP susceptibility, usually with an inverse relationship.

Study strains exposed over 28 days to both DAP and OXA showed heterogeneous cytochrome *c* binding profiles, but overall, the binding was similar to that of the parent strain. Two replicates (DO1 and DO2) displayed progenitor strain-level cytochrome binding ($P > 0.85$). Two replicates (DO3 and DO4) displayed enhanced cytochrome *c* binding ($P < 0.01$), consistent with previous findings where the addition of β-lactam antibiotics during DAP exposure resulted in more anionic membranes and enhanced the binding of the cationic peptide (and presumably DAP) (21). These two strains (DO3 and DO4) share a unique frameshift mutation that results in truncation of a hypothetical membrane protein (nt334delA; SAUSA300_0694, accession number CP000255.1). The full-length hypothetical membrane protein is predicted to be highly basic, with an isoelectric point of 9.63 and a net charge at pH 7 of +6. The loss of such a protein would potentially result in a more anionic membrane and enhanced cytochrome binding. One strain (DO5) displayed reduced cytochrome binding, analogous to the cytochrome affinity of the other DAP exposure strains ($P < 0.01$, J01 versus DO5). This heterogeneity in binding profiles in strains exposed to both DAP and OXA, rather than the enhanced binding reported previously, is perhaps a consequence of the more prolonged exposure of study strains to both agents.

Based upon these results, it is tempting to speculate on why exposure to OXA prevents the development of DNS. The establishment of multiple mutations resulting in the DNS phenotype may be facilitated by an initial derangement in MprF function. Therefore, prevention of *mprF* mutation with OXA may prevent the first committed step during the development of DNS. Alternatively, the DNS phenotype itself may reduce the ability of the cell to withstand β-lactam-mediated stresses regardless of the causative genetic mechanism. In this scenario, MprF does not play a gatekeeper role but, rather, is one of many mutations that experiences negative selection in the presence of OXA. With therapeutic concentrations in the patient setting, β-lactams increase DAP killing and effectiveness (33, 41); therefore, this may leave fewer bacterial cells available to develop *mprF* mutation and DAP resistance. Further work is needed to understand the clinical effects of prolonged exposure to this antibiotic combination.

While this work has focused on the use of adjunctive therapy to reduce the emergence of DNS subpopulations, clinicians often consider combining antimicrobials to take advantage of synergistic activity. Alternatively referred to as the seesaw effect or collat-

eral susceptibility, it has been reported that improved activity of OXA improves as the MRSA strain becomes DNS. This appears to be a β-lactam class effect, although the phenomenon appears to be more pronounced for β-lactams that preferentially target PBP1 (41). This study suggests that adjunctive therapy with OXA is reasonable for both synergy and prevention of the development of resistance, possibly by maintaining wild-type *mprF*. This effect may extend to additional antistaphylococcal β-lactams and perhaps to ceftaroline, but so far it has only been demonstrated experimentally with OXA.

To our knowledge, this is the first study to identify the potential utility of therapy using DAP plus an antistaphylococcal β-lactam both for increased antibiotic activity and for the prevention of specific genetic changes associated with DNS development during treatment. While the adjunctive antibiotic exposures were at one-half the MIC and not therapeutic exposures, this approach provides a useful direct comparison among antibiotics to identify different phenotypic and genotypic pathways for the suppression or emergence of daptomycin resistance with combination exposures. Further studies are warranted to identify the clinical significance and risks of long-term combination therapy using DAP plus antistaphylococcal β-lactams to treat prolonged infection. This study is limited by the analysis of only previously known mediators of the DNS phenotype. Future work will examine the influence of novel mutations in other loci on the development of DNS. The results of this study indicate that clinicians can affect the mutational space permitted for DNS development by using adjunctive antibiotic therapy.

ACKNOWLEDGMENTS

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