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MECHANISMS OF RESISTANCE

Prolonged Exposure to β-Lactam Antibiotics Reestablishes Susceptibility of Daptomycin-Nonsusceptible *Staphylococcus aureus* to Daptomycin

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ABSTRACT Daptomycin-nonsusceptible (DAP-NS) *Staphylococcus aureus* often exhibits gain-in-function mutations in the *mprF* gene (involved in positive surface charge maintenance). Standard β-lactams, although relatively inactive against methicillin-resistant *S. aureus* (MRSA), may prevent the emergence of *mprF* mutations and DAP-NS. We determined if β-lactams might also impact DAP-NS isolates already possessing an *mprF* mutation to revert them to DAP-susceptible (DAP-S) phenotypes and, if so, whether this is associated with specific penicillin-binding protein (PBP) targeting. This study included 25 DAP-S/DAP-NS isogenic, clinically derived MRSA bloodstream isolates. MICs were performed for DAP, nafcillin (NAF; PBP-promiscuous), cloxacillin (LOX; PBP-1), ceftriaxone (CRO; PBP-2), and cefoxitin (FOX; PBP-4). Three DAP-NS isolates were selected for a 28-day serial passage in subinhibitory β-lactams. DAP MICs and time-kill assays, host defense peptide (LL-37) susceptibilities, and whole-genome sequencing were performed to associate genetic changes with key phenotypic profiles. Pronounced decreases in baseline MICs were observed for NAF and LOX (but not for CRO or FOX) among DAP-NS versus DAP-S isolates ("seesaw" effect). Prolonged (28-d) β-lactam passage of three DAP-NS isolates significantly reduced DAP MICs. LOX was most impactful (~16-fold decrease in DAP MIC; 2 to 0.125 mg/liter). In these DAP-NS isolates with preexisting *mprF* polymorphisms, accumulation of additional *mprF* mutations occurred with prolonged LOX exposures. This was associated with enhanced LL-37 killing activity and reduced surface charge (both *mprF*-dependent phenotypes). β-lactams that either promiscuously or specifically target PBP-1 have significant DAP "resensitizing" effects against DAP-NS *S. aureus* strains. This may relate to the acquisition of multiple *mprF* single nucleotide polymorphism (SNPs), which, in turn, affect cell envelope function and metabolism.

KEYWORDS cationic peptide, methicillin-resistant *Staphylococcus aureus*, *mprF*, penicillin-binding protein

Staphylococcus aureus has developed resistance to virtually every class of antimicrobials (1, 2). Although daptomycin (DAP) has been effective in treating methicillin-resistant *S. aureus* (MRSA) infections, including those refractory to vancomycin (VAN), a number of published clinical reports document the *in vivo* development of DAP nonsusceptibility (DAP-NS) during treatment (3, 4). Currently, the Infectious Diseases Society of America (IDSA) recommends DAP plus β-lactam therapy as a principal option for the treatment of such persistent MRSA infections (1). This recommendation reflects

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previous findings suggesting oxacillin (or nafcillin) can enhance the antimicrobial effects of DAP and prevent or delay the emergence of the DAP-NS (5, 6).

The characterization of a number of DAP-NS MRSA isolates demonstrates that mutations within cell wall-associated and global regulatory genes contribute to the DAP-NS phenotype (5). However, the most clinically relevant and frequent mutations cluster within recognized DAP-NS "hot spots" of the multipeptide resistance factor (*mprF*) gene (5, 6). The *S. aureus mprF* locus is responsible for increasing the synthesis of cationic lysyl-phosphatidylglycerol (L-PG) and its translocation into the outer cytoplasmic membrane (CM) leaflet; this presumably results in an increase in net positive surface charge and repulsion of the calcium-DAP complex, preventing insertion of the cationic oligomer (6, 7). Interestingly, DAP-NS isolates with these hot spot *mprF* mutations can become "resensitized" *in vitro* to DAP when additional *mprF* point mutations are gained. Isolates with such genotypes are associated with a loss of *mprF* functionality in terms of reduced lysinylation of PG and/or its outer CM translocation (8, 9).

A common finding among DAP-NS isolates is a paradoxical increase in β-lactam susceptibility, a phenomenon termed the DAP-β-lactam "seesaw effect" (10, 11). Although the seesaw mechanism has not been fully elucidated, it appears to involve at least two genes which impact either the cell wall stimulon (*vraSR*) and/or PBP2a chaperoning, folding, and CM localization (*prsA*) (11, 12). The synergy of DAP and β-lactams exploits this effect through a combination of enhanced DAP binding to the cell wall divisome, as well as blockade of specific penicillin-binding proteins (PBPs) (4, 13, 14). The latter effect has been shown to be most potent with β-lactams that inhibit PBP-1. This monofunctional transpeptidase is responsible for cell division and separation, and it locates at the divisome of *S. aureus* (15). The cell divisome is also where DAP binding focally occurs to exert its most potent CM depolarization, as well as essential cell division protein mislocalization (16). Given these elegantly linked mechanisms of DAP-β-lactam combined activity, we hypothesized that exposure to β-lactams might potentially resensitize DAP-NS *S. aureus* to DAP. In this study, DAP-NS isolates were passaged in β-lactams possessing specific versus promiscuous PBP-binding profiles and then evaluated for their resulting phenotypic and genotypic modifications.

This study was presented in part at the 18th International Symposium on Staphylococci and Staphylococcal Infection in Copenhagen, Denmark, August 2018.

RESULTS

Antibiotic susceptibilities. The susceptibilities to the tested antibiotics in the 25 DAP-susceptible (DAP-S) and DAP-nonsusceptible (DAP-NS) isolate pairs and their previously identified *mprF* mutations are provided in Table 1 (14, 17–19). Overall, baseline DAP MICs ranged from 0.19 to 0.75 mg/liter in the DAP-S isolates and 2 to 4 mg/liter for DAP-NS isolates derived *in vivo* following DAP treatment. The isolate JKD6005 displayed a DAP MIC of 1 mg/liter by broth dilution and 2 mg/liter by Etest, which is similar to the reported value (20). However, this isolate was derived *in vivo* from a patient receiving VAN treatment only, and it lacks an *mprF* mutation, so this discordance is not unexpected. Overall, the seesaw effect between elevated DAP MICs and lower β-lactam MICs was apparent among many β-lactams tested (Fig. 1). A $\geq$4-fold decrease in the MICs for at least one β-lactam in the DAP-NS versus DAP-S isolates occurred in 48% of pairs, most notably with the PBP-1 specific cloxacillin (LOX) (44% of pairs). Analysis of the correlation between DAP-NS and β-lactam susceptibilities revealed a significant negative association between DAP and LOX MICs ($P = 0.032$), while nafcillin (NAF), meropenem (MEM), and ceftriaxone (CRO) susceptibilities demonstrated similar trends toward negative association but lacked statistical significance ($P \geq 0.118$) (Fig. 1).

Serial passage. DAP-NS isolates J03, D712, C25, and JKD6005 were passaged in triplicate daily for 28 d in exposure arms of no antibiotic (media alone), NAF, LOX, CRO, or cefoxitin (FOX). The selection of these isolates for passage was based on their containing common but distinct hot spot mutations within the bifunctional domain of

TABLE 1 Isolate characteristics[a]

Isolate	*mprF* SNP	MIC (mg/liter) of:					
		DAP	NAF	LOX	MEM	CRO	FOX
J01		0.5	32	16	8	128	64
J03	T345I	2	16	2	4	128	64
D592		0.5	256	512	512	256	256
D712	L341S	2	128	512	512	256	256
JKD6004		0.5	256	512	512	512	512
JKD6005		2	128	512	512	512	512
C1		0.19[b]	16	32	32	4	32
C2	L826F	2	16	2	16	16	32
C3		0.5	32	64	32	128	32
C4	P314L	4	32	2	16	16	128
C5		0.25	32	2	32	128	32
C6	T345A	3[b]	32	0.125	16	32	32
C7		0.5	64	16	32	64	64
C8		3[b]	64	2	32	8	32
C9		0.5	32	1	16	32	32
C10	L826F	3[b]	16	0.063	8	4	32
C13		0.75[b]	4	4	16	16	64
C14	T472K	4	0.25	0.125	2	4	32
C15		0.75[b]	64	256	32	32	64
C16	M347R	4	32	32	16	16	128
C17		0.5	64	128	32	64	32
C18	L341S	4	64	64	32	64	64
C19		0.38[b]	128	64	32	64	64
C21	L826F	4	32	0.5	8	16	32
C22		0.5	8	0.125	4	8	64
C23		4	64	64	32	128	128
C24		0.5	4	4	8	64	32
C25	S295L	3	0.25	0.25	4	16	32
C26		0.38[b]	128	256	128	512	64
C27	T345K	2	128	512	128	512	128
C30		0.25	32	8	8	16	32
C31	L826F	2	32	2	4	4	32
C32		0.5	4	2	4	32	32
C33	S337L	2	4	4	4	16	16
C34		0.38[b]	64	32	8	32	64
C35		4	64	2	8	32	32
C36		0.5	128	512	128	128	64
C37	V351E	3[b]	1	0.25	8	16	32
C38		0.75[b]	32	16	8	16	64
C39	L826F	3[b]	32	16	8	8	64
C40		0.25	16	0.5	8	4	32
C41	M347R	3[b]	2	0.25	16	16	32
C42		0.75[b]	16	4	8	64	64
C43	S337L	3[b]	8	2	8	16	64
C46		0.38[b]	32	8	16	32	16
C47	L826F	3[b]	128	8	32	32	64
C48		0.5	16	0.25	8	4	32
C49	T345I	2	16	0.25	32	16	64
C50		0.5	64	512	128	256	128
C51	T345I	2	64	128	128	128	64

[a]These include previously identified *mprF* SNPs (149) and DAP and β-lactam susceptibilities in DAP-S and DAP-NS isolates. Results include both broth dilution and Etest method confirmation.
[b]Etest result is displayed when discriminate differences are found between broth dilutions (e.g., 2 to 4 mg/liter).

MprF for J03 (T345I), D712 (L341S), and C25 (S295L) (6). The isolate JKD6005 was selected as a "negative" control to interrogate the importance of preexisting *mprF* mutations, as it contained a wild-type *mprF* sequence. Figure 2 displays the DAP MIC fold change over the 28-d exposures with or without different β-lactams. Of note, in isolates with preexisting *mprF* mutations, passage in β-lactams was often able to resensitize the isolate to DAP. This occurred as early as day 7 of passage and continued throughout the 28-d exposure. Overall, LOX was most effective at resensitizing isolates to DAP, followed by NAF. In isolate C25, CRO was also highly effective in DAP

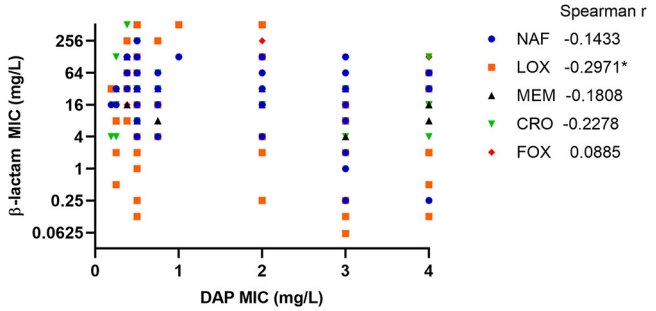

FIG 1 DAP-β-lactam seesaw effect demonstrated by correlation of DAP and β-lactam MICs in all 50 isolates. *, $P = 0.032$.

resensitization, but a limited resensitizing effect of this agent was noted in other isolates. Enhanced DAP susceptibility with β-lactam passage did not occur in JKD6005 lacking a preexisting *mprF* polymorphism. This strain does contain a mutation in WalR (YycF/VicR), an essential response regulator implicated in both DAP-NS and the seesaw effect, so this may play a role in preventing resensitization (20). The DAP-β-lactam seesaw effect was present in two of these three DAP-NS strains used for long-term β-lactam passage (J03 and C25). In these two latter strains, DAP resensitization post-passage was accompanied by at least a 2-fold MIC increase in the respective β-lactam used for passage (Table 2).

Whole-genome sequencing. The isolates selected for serial passage were sequenced at day zero prior to β-lactam passage and then at the end of treatment (day 28). Passage isolates maintained the preexisting *mprF* mutations identified in the J03, D712, and C25 backgrounds and gained additional mutations in *mprF*, a cell division gene (*div1b*), the beta and beta′ subunits of the RNA polymerase (*rpoBC*), and several genes associated with metabolic function (Table 2; Table S1 in the supplemental material). Of particular interest was the accumulation of additional *mprF* mutations. This was observed in all three isolate backgrounds with LOX passage; the passage isolates with these genotypes also demonstrated increased sensitivity to DAP, with up to

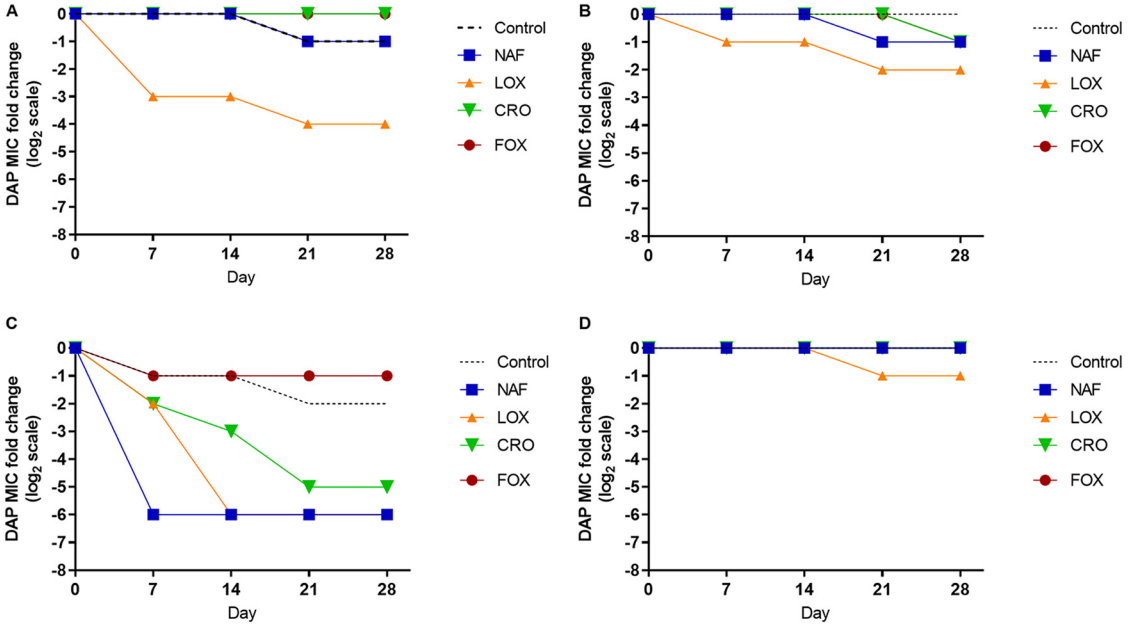

FIG 2 Serial passage in isolates J03 (A), D712 (B), C25 (C), and JKD6005 (D) with no antibiotic or β-lactams. Data represent median DAP MIC changes over 28 days with different exposures.

TABLE 2 Daptomycin susceptibility and *mprF* polymorphisms with β-lactam passage after 28 days[a]

Isolate	Passage	Replicate	DAP MIC (mg/liter)	β-lactam MIC (mg/liter)[b]	*mprF* SNP	MprF domain	*div1b* mutation	*rpoB*/C mutation
J01	None		0.5					
J03	None		2		$T_{345}I$	Bifunctional		*rpoB* $S_{464}P$
	Media	i	2		None			
	Media[a]	ii	1		$Y_{325}H$	Bifunctional		
	Media[a]	iii	1		$R_{437}P$	Synthase		
	CRO[c]	ii	0.75	512	$V_{152}G$	Translocase		
	LOX[c]	ii	0.125	32	$R_{788}L$	Synthase	Q_{425}[d]	
	LOX[c]	iii	0.125	32	$R_{788}L$	Synthase	Q_{415}[d]	
D592	None		0.5					
D712	None		2		$L_{341}S$	Bifunctional		
	Media	i	2		None			
	Media	ii	1		None			
	Media	iii	2		None			
	NAF[e]	iii	1	256	$S_{337}P$	Bifunctional		
	CRO[e]	i	2	2,048	$M_{609}T$	Synthase		*rpoC* $A_{567}V$
	CRO[e]	iii	0.5	2,048	$G_{389}A$	Synthase		*rpoB* $G_{767}C$
	FOX[e]	ii	0.5	512	$F_{657}L$	Synthase		
	LOX[e]	i	0.5	1,024	$S_{136}L$	Translocase		
	LOX[e]	ii	0.5	1,024	$S_{136}L$	Translocase		
C24	None		0.5		-	-		
C25	None		3–4		$S_{295}L$	Bifunctional		
	Media[f]	i	1		S_{825}[d]	Synthase		
	Media[f]	ii	1		S_{825}[d]	Synthase		
	Media	iii	2		None	-		
	FOX[f]	Iii	1	64	$A_{315}S$	Bifunctional		
	LOX[f]	i	0.125	8	L_{84}[d]	Translocase	$A_{420}E$	
	LOX[f]	ii	0.125	8	L_{84}[d]	Translocase		
	LOX[f]	iii	0.125	8	L_{84}[d]	Translocase	E_{416}[d]	

[a]For the β-lactam-exposed strains, only the passages with additional mprF mutations (versus DAP-NS parent strain) are presented.
[b]β-lactam MIC represents the MIC in the respective β-lactam after passage.
[c]Passage isolates maintained MprF $T_{345}I$ and RpoB $S_{464}P$.
[d]Nonsense stop-gain mutation resulting in premature end of translation.
[e]Passage isolates maintained MprF $L_{341}S$.
[f]Passage isolates maintained MprF $S_{295}L$.

32-fold difference in susceptibility (e.g., MIC changes from 3 to 4 mg/liter to 0.125 mg/liter). The largest shifts in DAP susceptibility were also associated with concomitant gains of *div1b* mutations. Mutations in *mprF* were not identified in the JKD6005 background with any β-lactam passage, indicating that a preexisting *mprF* mutation may be necessary for β-lactams to induce this latter effect.

To determine the temporal relationship between these identified mutations and phenotype, interim-passaged strains at days 7, 14, and 21 were also whole-genome sequenced. This identified, first, that no new mutations occurring in any passaged strains during this 7- to 21-day period compared to those identified at day 28 of passage (Table 2 and Table S1). Second, this also determined the approximate time of appearance and sustainability of these mutations throughout continued passage. With *mprF*, *div1b*, and rpoB/C mutations, this interim sequencing identified that *mprF* single nucleotide polymorphisms (SNPs) were present at day 7, while *div1b* was first detected at day 21 and *rpoB*/C only at day 28. Table 3 represents the *mprF* SNP frequencies in strains J03 and D712 at days 7, 21, and 28 passaged in LOX. In both strains, replicates with high *mprF* allele frequency correlated with a DAP resensitization, while replicates with no *mprF* alternative allele frequency maintained DAP MICs >1 mg/liter. In J03 passaged in LOX, mutations in *div1b* occurred along with an additional *mprF* mutation. In SNP frequency analysis, this mutation was detected initially at day 21 (0.71 to 0.72 alternative allele frequency) and then at day 28 (0.99 to 1.00 alternative allele frequency). This *div1b* appearance aligned with the greatest DAP resensitization (MIC = 0.125 mg/liter) occurring at days 21 to 28.

TABLE 3 *mprF* SNP frequencies in strains J03 and D712 at days 7, 14, 21, and 28 of LOX passage[a]

Isolate	Passage	Replicate	Additional *mprF* SNP frequency at day:			
			7	14	21	28
J03[b]	LOX	i	0.58	0.74	0.11	0.00[c]
J03[b]	LOX	ii	0.11	0.08	0.78	0.98
J03[b]	LOX	iii	0.51	0.61	0.79	0.99
D712[d]	LOX	i	0.91	0.67	0.86	1.00
D712[d]	LOX	ii	1.00	1.00	1.00	0.99
D712[d]	LOX	iii	0.00	0.00	0.00	0.00

[a]Frequency of mapped reads containing the alternative allele is represented.
[b]Passage isolates maintained MprF T_{345}I.
[c]Value of 0.00 indicates that all mapped reads contained the reference allele.
[d]Passage isolates maintained MprF L_{341}S.

Time-kill assays. To determine if the MIC reductions with β-lactam passage translated to enhanced DAP killing, isolates J01/J03 and J03 passaged in β-lactam for 28 days were evaluated in time-kill curves with DAP at 3.9 mg/liter. As displayed in Fig. 3, no significant growth defect occurred after the 28-day passage. Upon exposure to DAP, the DAP-S isolate J01 was rapidly killed, while the DAP-NS J03 isolate regrew in the presence of DAP. However, the β-lactam-passaged isolates were all significantly killed with DAP over the first 8 h of exposure, paralleling the substantive decline in DAP MICs on passage. The most rapid, extensive, and sustained DAP killing occurred with the J03 LOX passage isolate ($P < 0.01$ versus J03) and was similar in DAP killing to the susceptible wild-type isolate J01. Moreover, only the LOX-passaged isolate did not exhibit substantial regrowth between 24 and 48 h exposure to DAP. To assess the contribution of *mprF* along with other mutations developed during passage on DAP killing, J03-LOX (additional *mprF* plus *div1b* mutation), D712-LOX (additional *mprF* plus *rpoB* mutation), and JKD6005-LOX (no *mprF* mutation) were assessed in kill curves. As displayed in Fig. 4, DAP killing was enhanced with additional *mprF* mutations and most pronounced when with the *div1b* mutation. These results also indicate β-lactam passage alone does not substantially enhance DAP activity without additional *mprF* mutations.

LL-37 susceptibility. The evolution of DAP-NS is often accompanied by increased resistance to killing by human host defense cationic peptides, such as LL-37 (18, 19). The J01/J03 pair and J03-passaged isolates were assessed for LL-37 susceptibility. Like previous studies, DAP-NS (J03) was associated with reduced LL-37 killing compared to DAP-S (J01) (18). Among the β-lactam-passaged isolates, the LOX-passaged strains exhibited the lowest survival profiles when exposed *in vitro* to LL-37 concentrations of either 2 mg/liter or 5 mg/liter (Table 4) ($P < 0.01$); the LL-37 susceptibility pattern was virtually identical to the parental DAP-S J01 isolate. The other β-lactam-passaged isolates exhibited similar LL-37 survival profiles to J03, except for CRO- and FOX-passaged isolates, which exhibited enhanced survival at the higher concentration of LL-37 ($P < 0.05$). Similar to time-kill curves with DAP, LL-37 killing was impacted by additional *mprF* plus other mutations. LL-37 killing was most pronounced in J03-LOX

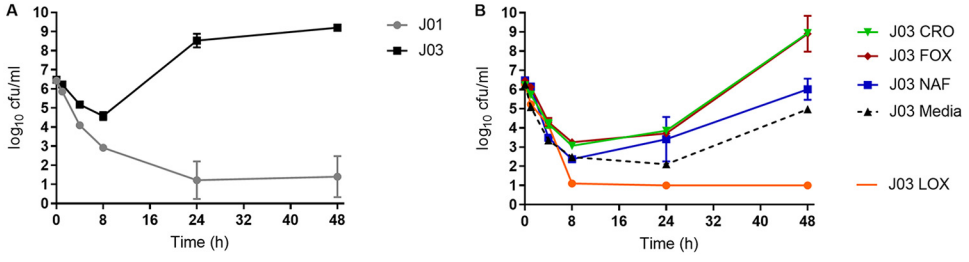

FIG 3 Time-kill curves. (A) Kill curves J01 and J03 versus DAP of 3.9 mg/liter. (B) Kill curves of J03-passaged isolates versus DAP of 3.9 mg/liter.

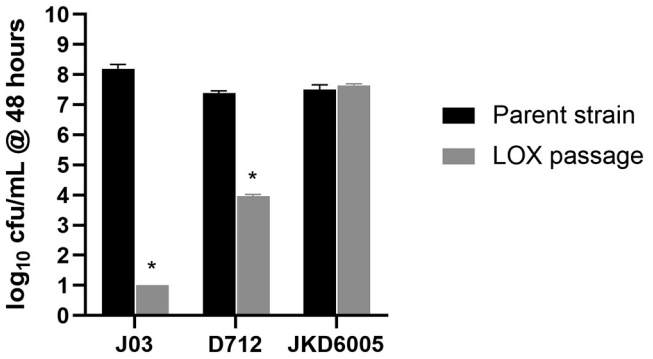

FIG 4 Bacteria quantification (CFU/ml) at 48 h in time time-kill curves with DAP 3.9 mg/liter against individual strains passaged in LOX. *, $P < 0.001$ versus parent strains.

with additional *mprF* plus *div1b* mutations (Table 4). However, minimal killing with LL-37 of 2 to 5 mg/liter was observed against D712-LOX with additional *mprF* plus *rpoB* mutations (93% to 98% survival) and JKD6005-LOX with no additional mutations (89% to 98% survival).

DISCUSSION

Several studies have described the synergistic relationship between β-lactam antibiotics and both DAP and cationic host defense peptides (HDPs) (4, 21). This synergy with HDPs is thought to be advantageous in enhancing β-lactam treatment, and it may contribute to the unambiguously superior clinical outcomes with β-lactams over VAN in methicillin-susceptible *S. aureus* (MSSA) bacteremic syndromes (22, 23). Although prior studies have shown that β-lactams can suppress the evolution of DAP or VAN resistance *in vitro* (24–26), few studies have determined the impact of β-lactams to prevent emergence of resistance to these agents *in vivo*. In the case of DAP-NS, this impact has been linked to the ability of β-lactams to prevent emergence of *mprF* mutations (24, 25). However, to our knowledge, no report has conclusively documented the ability of β-lactams to resensitize clinically derived DAP-NS isolates to DAP. The results of our current investigation indicate that long-term β-lactam passage can, indeed, resensitize DAP-NS isolates to DAP; this event appears to be mediated, at least in part, through the accumulations of additional point mutations in *mprF*.

Based on our data and others, the PBP-binding profile of those β-lactams that seem to be associated with DAP synergy in MRSA are likely selective, not global (11, 12). We previously found that β-lactams that target PBP-1 either as part of promiscuous PBP binding (PBPs 1 to 4 and 2a) or via PBP-1 specifically result in highly synergistic interactions with DAP versus DAP-NS MRSA. This synergy does not appear to occur exclusively through enhanced DAP binding to the CM, but rather through a dual mechanistic effect at distinct β-lactam and DAP cell wall divisome targets (13).

TABLE 4 *In vitro* susceptibility to DAP and host defense peptide (HDP) LL-37 in J01/J03 and J03 passage isolates

Isolate	Passage	DAP MIC (mg/liter)	% survival (mean + SD) after 2 h of exposure to LL-37 concn of:	
			2 mg/liter	5 mg/liter
J01		0.5	82 ± 9	8 ± 1
J03		3 to 4	92 ± 21	46 ± 8
J03	Media	1	95 ± 2	61 ± 7[a]
	NAF	1	84 ± 8	51 ± 9
	LOX	0.125	75 ± 13[a]	7 ± 10[a]
	CRO	2	97 ± 1	63 ± 4[a]
	FOX	2	98 ± 1	68 ± 6[a]

[a]$P < 0.05$ versus DAP-NS J03.

In the current study, using a large collection of well-characterized DAP-S/DAP-NS isolate pairs derived from patients, we evaluated the ability of β-lactams with a range of PBP-binding specificity profiles to resensitize DAP-NS strains. The most profound synergistic effects with DAP occurred with LOX, a PBP-1-specific antibiotic, although similar trends were observed with NAF, MER, and CRO (Table 1; Fig. 1). We were somewhat surprised to find a substantial seesaw effect with CRO, a PBP-2-specific antibiotic (8/25 pairs [32%]). Of note, the seesaw effect among specific DAP-S/DAP-NS isolate-pairs was generally harmonious for LOX versus CRO. CRO MICs were the highest among all the β-lactams tested in the 25 DAP-S isolates ($MIC_{50} = 64$), perhaps allowing for a greater "window" for disclosing a seesaw relationship. In contrast, The DAP-S isolates were more susceptible to LOX and MEM ($MIC_{50} = 16$). This work emphasizes that the seesaw effect is variable among distinct β-lactams and strain specific and that inhibition of PBP-1 or PBP-2 particularly can foster a synergistic interaction with DAP versus DAP-NS isolates (27).

This work has potentially important clinical translational implications. As previously described, DAP-NS isolates derived from patients treated with DAP who failed DAP therapy quite frequently exhibited point mutations in *mprF* (20/25 isolates [80%] in this investigation) (5, 8, 28). Although DAP-NS can evolve without DAP treatment, this has been shown to occur primarily in VAN treatment in which cross-resistance to both VAN and DAP develops, most likely via a combination of a cell wall-thickening phenotype as well as distinct metabolic adaptations (29–32). In this study, we identified that prolonged β-lactam passage can reverse the elevated DAP MICs of DAP-NS isolates, resulting in some passaged isolates becoming up to 16-fold more DAP susceptible versus their respective DAP-NS isolates and 4-fold lower than the original DAP-S parental isolate. This effect was noted in all LOX-passaged isolates from backgrounds with preexisting *mprF* polymorphisms, while no significant resensitization occurred with prolonged β-lactam passage in JKD6005 possessing the wild-type *mprF* sequence. This analysis also delineated that these additional *mprF* mutations can occur as early as day 7 of passage and were directly related to this resensitization event as one of the first mutations identified. These data indicate that existing *mprF* perturbations may be essential for the β-lactam-induced DAP resensitization event to occur. Although the β-lactam passage was done without DAP in culture, there may be benefits to adding β-lactams to DAP treatment during prolonged courses of therapy to either prevent DAP-NS development or revert emerging DAP-NS subpopulations toward a more DAP-S phenotype (17). Of interest, we demonstrated the latter phenomenon occurring in a patient in which the DAP-NS phenotype reverted to DAP-S when ceftaroline was added to DAP treatment (33).

In a recent study by Yang and colleagues (8), introduction of dual-point mutations in *mprF* via genetic complementation substantially lowered DAP MICs compared to the DAP-NS host isolate, as well as the DAP-S parental isolate. These investigators incorporated combinations of two common hot spot *mprF* mutations in DAP-NS isolates, S295L plus L826F and T345A plus L826F, resulting in (i) enhanced DAP susceptibility, and (ii) evidence of reduced MprF functionality (i.e., significant reductions in outer CM L-PG flipping). In our current passage experiments, these same hot spot mutations were present in J03 (T345X) and C25 (S295L), while another common hot spot *mprF* mutation was present in isolate D712 (L341S). We were able to recapitulate the novel findings of the Yang group (8) "pharmacologically" by using distinct β-lactams to generate additional *mprF* mutations in these isolates. Notably, the PBP-1-specific LOX generated such additional *mprF* mutations in all three isolates. Isolates exposed to LOX with the same preexisting *mprF* mutations as studied by Yang et al. resulted in exquisite susceptibility to DAP (MIC = 0.125 mg/liter) in these dual-point *mprF* mutation, postpassage isolates. These additional point mutations occurred in either the synthase or translocase domain, while none were mapped to the bifunctional domain (Table 2). However, the present study presents an interesting association but does not indicate causality of these dual-point *mprF* SNPs. The changes associated with CM phospholipid composi-

tion and CM order with these dual-point *mprF* mutations and their causality for DAP-NS reversal are a focus of future studies.

One well-described mechanism of DAP action is via calcium-activated DAP oligomerization and subsequent CM depolarization. This mechanism recently was found to be linked to DAP binding at the divisome of the CM (16, 34). We previously identified that DAP-NS cells have increased divisome formation and increased PBP-1 transcription, indicating that this may be an adaptive response to DAP-induced CM damage (13).

In this present study, several mutations besides *mprF* occurred in β-lactam-passaged isolates (Table 2). Many of these mutations are associated with fundamental cellular metabolism, such as purine biosynthesis, sensor histidine kinases, asparaginyl-tRNA synthetase, and phosphoglycerate dehydrogenase (Table S1 in the supplemental material); in turn, these perturbations may well be expected during prolonged exposure to subinhibitory antibiotic levels (35, 36). Furthermore, mutations were identified post-passage in *div1b*, encoding a putative cell division protein. This occurred exclusively with isolates passaged in LOX and, when present, resulted in highly DAP-S variants (MIC of 0.125 mg/liter). We noted that β-lactam-passaged isolates were more susceptible to DAP killing; it should be emphasized that LOX-passaged isolates with a *div1b* mutation were killed to the detection limit within 8 h, faster than either the DAP-NS progenitor or DAP-S parental isolate (Fig. 3). We hypothesize that mutations within divisome proteins may alter the compensatory response mechanism to DAP, thus rendering isolates more DAP-S. Current work is in progress to identify the functional role of alterations in cell divisome proteins on DAP activity, including introduction of these novel *div1b* mutations via allelic exchange into a naive background strain(s).

There are certain limitations to our work to note. First, although a large and well-characterized MRSA strain collection was used for susceptibility screening, only four backgrounds were used in passage experiments. These four strains were phylogenetically diverse with USA300/ST8, USA100/ST5, and ST239 represented (17, 20, 37). Important trends emerged with these latter isolates, but further variability may be documented when additional isolates are evaluated. Our investigation did not evaluate a PBP-3-selective antibiotic such as cefaclor, although we have previously noted limited effects of this agent in combination with DAP (14). Moreover, the exact mechanistic basis for the CM changes observed in our resensitization studies require further clarification. Most interestingly, the mechanism(s) by which selected β-lactams induce DAP-NS isolates with preexisting *mprF* SNPs to accumulate additional *mprF* mutations remains to be elucidated. Finally, the mechanisms by which multiple *mprF* mutations render such strains significantly more DAP-S remain to be delineated. It does not appear that a general "growth" defect is in-play, as the LOX-postpassage DAP-S variant had growth kinetics similar to its DAP-NS progenitor. It should be noted that the growth and killing assays used quantitative CFU assays, which have some limitations to determine bacterial viability due to cell aggregation compared to turbidity measurements.

In summary, this study provides novel insights on the activity of β-lactam antibiotics in DAP-NS MRSA. It points toward an important role of PBP-1-targeting antibiotics to induce mutations that may potentially reverse or prevent the DAP-NS phenotype. These findings support the previous notion of β-lactam prevention of DAP-NS through inhibiting *mprF* mutation development (17). However, it also introduces the exciting notion that β-lactams can reverse DAP-NS by inducing additional mutations in signature genes related to DAP-NS, such as *mprF*.

MATERIALS AND METHODS

Bacterial isolates. This study used a well-characterized collection of 50 clinical MRSA isolates that represent DAP-S and DAP-NS (MICs ≥ 2 mg/liter) pairs derived from bacteremic patients (Table 1). Previous publications with targeted or whole-genome sequence data have described these isolates in detail (5, 17, 20). This collection includes 22 DAP-S/DAP-NS pairs of clinical bloodstream isolates from the Cubist Pharmaceuticals Isolate Collection, two DAP-S/DAP-NS MRSA pairs from patients successfully treated with DAP plus nafcillin following DAP-NS emergence, and one DAP-S/DAP-NS MRSA pair isolated after vancomycin (VAN) treatment (these latter three isolates were kindly supplied by George Sakoulas and Benjamin Howden).

Antimicrobials and media. The antibiotics used in this study and their PBP-binding profiles include nafcillin (NAF), PBP-nonselective; cloxacillin (LOX), PBP-1 selective; meropenem (MEM), PBP-1 selective; ceftriaxone (CRO), PBP-2 selective; and cefoxitin (FOX), PBP-4 selective. The β-lactams were purchased from Sigma-Aldrich (St. Louis, MO, USA). DAP was commercially purchased and its activity confirmed by quality control susceptibility testing against ATCC 29231 per Clinical and Laboratory Standards Institute (CLSI) guidelines, version M100 ED28:2018 (38). Mueller-Hinton broth II (MHB) (BD, Sparks, MD, USA) supplemented with 25 mg/liter calcium (as $CaCl_2$), 12.5 mg/liter magnesium (as $MgCl_2$), and 2% sodium chloride were used to grow *S. aureus* in liquid culture with β-lactams. All DAP assays used MHB with 50 mg/liter calcium as recommended (38).

Antibiotic susceptibility testing. The MICs of all isolates ($n = 25$ pairs, 50 isolates) to DAP and β-lactams were determined in triplicate by broth microdilution according to the CLSI guidelines (38). DAP MICs were also confirmed by Etest. The passaged isolates were evaluated for DAP MIC in triplicate following 7, 14, 21, and 28 days of passage in each β-lactam. Visual inspection for MIC determination occurred following 18 to 24 h of incubation at 35°C. Isolate pairs with a positive seesaw effect were defined by a $\geq$ 4-fold decrease in β-lactam MIC in the DAP-NS isolate compared to its respective parental DAP-S strain.

Serial passage. Four DAP-NS isolates were passaged in triplicate daily for 28 days in exposure arms of no antibiotic, NAF, LOX, CRO, or FOX. The free average concentration in human serum (fC_{avg}) after standard dosing was used for β-lactam daily passage (2.6 mg/liter NAF, 1.4 mg/liter LOX, 24 mg/liter MEM, and 19 mg/liter CRO. If these concentrations were above the β-lactam MIC, then 0.5$\times$ MICs were used to allow for bacterial growth as previously described (14). On day zero, bacterial colonies from overnight growth on Mueller-Hinton agar were suspended in normal saline and turbidity adjusted to the equivalent of 0.5 McFarland standard. Each culture was diluted 1:100 into three replicates with fresh MHB25 plus 2% salt to a total volume of 1 ml and containing each β-lactam. Each sample was grown overnight at 37°C with shaking at 160 rpm. Following incubation, 10 μl of each culture was transferred into fresh media and placed back on the shaker to grow as previously described (17). This process continued for 28 consecutive days with subsequent DAP susceptibility testing on samples obtained on days 7, 14, 21, and 28 of passage.

Whole-genome sequencing of serial passage isolates. Genomic changes in all isolates and replicates pre- and postpassage were analyzed by whole-genome sequencing. Genomic DNA was extracted with the Janus automated workstation, using the Chemagic DNA/RNA kit (Perkin Elmer). Unique dual-index libraries were prepared using the Nextera XT DNA preparation kit (Illumina), and libraries were sequenced on a NextSeq (Illumina) with 2 $\times$ 150-bp chemistry as per the manufacturer's instructions. The short-read sequence data for all isolates were mapped to reference *S. aureus* J01 (RefSeq accession no. NZ_CP040619.1), D712 (RefSeq accession no. NZ_CP040665.1), or JKD6004 (RefSeq accession no. NZ_CP040622.1), using Snippy v4.4.3 (https://github.com/tseemann/snippy). Variants were called using a minimum mapped read depth of five and base call stringency of 90%. Allelic frequencies were calculated from the mapped alignment without application of the listed thresholds, displayed as the proportion of mapped reads containing the alternative allele compared to those containing the reference allele. Predicted protein consequences of variants were identified using snpEff v4.4 (39), with custom databases constructed from the above-listed RefSeq genomes and configured to use the bacterial and plastid codon table.

Time-kill assays. Time-kill studies with DAP against the three DAP-NS isolates were conducted both prior to β-lactam passage and on the resulting isolates of each β-lactam passage. Time-kill studies were performed at 37°C in MHB50 containing DAP at the active unbound (free average concentration [fC_{avg}]) of 3.9 mg/liter from a standard human dose of 6 mg/kg (40). This was determined with the formula $fC_{avg} = (fC_{max} + fC_{min})/2$ (14). The rationale for using this test concentration was to approximate a clinically relevant concentration that would be achieved regardless of variation in volume of distribution or patient-dependent clearance.

LL-37 susceptibility. DAP-NS MRSA frequently exhibits cross-resistance to multiple host defense peptides (HDPs), especially those from either mammalian PMNs or platelets (18). We studied the relative HDP susceptibility profiles of one prototype isogenic DAP-S/DAP-NS isolate-set (J01-J03), including its β-lactam-postpassage variants using LL-37, a linear cathelicidin HDP found in mammalian PMNs and epithelium (41).

The LL-37 bactericidal assay was carried out in minimal liquid nutrient medium, phosphate-buffered saline (PBS; pH 7.4), and 1% brain heart infusion (BD, Sparks, MD, USA) by a 2-h time-kill method as previously described (18, 42). We used 2 to 5 μg/ml of the LL-37 peptide concentrations for the time-kill assay. These sublethal concentrations were employed on the basis of (i) their ability to decrease survival of the parental DAP-S isolates by $<$50% in preliminary studies, and (ii) peptide concentrations used in previous investigations of HDP-*S. aureus* interactions (18, 42). LL-37 was obtained from Peptide International, Louisville, KY. A final inoculum (10^3 CFU/ml) of *S. aureus* cells was used to assess LL-37, and *S. aureus* cells were incubated at 37°C. Post-2 h exposure, samples were collected and further processed for quantitative culture to evaluate the degree of killing. Final data were represented as mean percent surviving CFU/ml ($\pm$ standard deviation [SD]). Since there is no bona fide resistance breakpoint for LL-37, we compared only the mean percent survivability $\pm$ SD of study isolates for statistical comparison. A minimum of three experiments were performed on distinct days.

Statistical analysis. DAP MIC results were evaluated using the Wilcoxon rank sum test. A two-tailed Student's *t* test was used for statistical analysis of all other quantitative data. Spearman's *r* was used to determine antibiotic susceptibility correlations. *P* values of $\leq$0.05 defined significance.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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REFERENCES

1. Liu C, Bayer A, Cosgrove SE, Daum RS, Fridkin SK, Gorwitz RJ, Kaplan SL, Karchmer AW, Levine DP, Murray BE, Rybak MJ, Talan DA, Chambers HF, Infectious Diseases Society of America. 2011. Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children. Clin Infect Dis 52:e18–e55. https://doi.org/10.1093/cid/ciq146.
2. Rodvold KA, McConeghy KW. 2014. Methicillin-resistant *Staphylococcus aureus* therapy: past, present, and future. Clin Infect Dis 58(Suppl 1): S20–S27. https://doi.org/10.1093/cid/cit614.
3. Capone A, Cafiso V, Campanile F, Parisi G, Mariani B, Petrosillo N, Stefani S. 2016. *In vivo* development of daptomycin resistance in vancomycin-susceptible methicillin-resistant *Staphylococcus aureus* severe infections previously treated with glycopeptides. Eur J Clin Microbiol Infect Dis 35:625–631. https://doi.org/10.1007/s10096-016-2581-4.
4. Dhand A, Bayer AS, Pogliano J, Yang SJ, Bolaris M, Nizet V, Wang G, Sakoulas G. 2011. Use of antistaphylococcal beta-lactams to increase daptomycin activity in eradicating persistent bacteremia due to methicillin-resistant *Staphylococcus aureus*: role of enhanced daptomycin binding. Clin Infect Dis 53:158–163. https://doi.org/10.1093/cid/cir340.
5. Bayer AS, Mishra NN, Cheung AL, Rubio A, Yang SJ. 2016. Dysregulation of *mprF* and *dltABCD* expression among daptomycin-non-susceptible MRSA clinical isolates. J Antimicrob Chemother 71:2100–2104. https://doi.org/10.1093/jac/dkw142.
6. Bayer AS, Mishra NN, Chen L, Kreiswirth BN, Rubio A, Yang SJ. 2015. Frequency and distribution of single-nucleotide polymorphisms within *mprF* in methicillin-resistant *Staphylococcus aureus* clinical isolates and their role in cross-resistance to daptomycin and host defense antimicrobial peptides. Antimicrob Agents Chemother 59:4930–4937. https://doi.org/10.1128/AAC.00970-15.
7. Bayer AS, Schneider T, Sahl HG. 2013. Mechanisms of daptomycin resistance in *Staphylococcus aureus*: role of the cell membrane and cell wall. Ann N Y Acad Sci 1277:139–158. https://doi.org/10.1111/j.1749-6632.2012.06819.x.
8. Yang SJ, Mishra NN, Kang KM, Lee GY, Park JH, Bayer AS. 2018. Impact of multiple single-nucleotide polymorphisms within *mprF* on daptomycin resistance in *Staphylococcus aureus*. Microb Drug Resist 24:1075–1081. https://doi.org/10.1089/mdr.2017.0156.
9. Oku Y, Kurokawa K, Ichihashi N, Sekimizu K. 2004. Characterization of the *Staphylococcus aureus mprF* gene, involved in lysinylation of phosphatidylglycerol. Microbiology 150:45–51. https://doi.org/10.1099/mic.0.26706-0.
10. Vignaroli C, Rinaldi C, Varaldo PE. 2011. Striking "seesaw effect" between daptomycin nonsusceptibility and beta-lactam susceptibility in *Staphylococcus haemolyticus*. Antimicrob Agents Chemother 55:2495–2496. https://doi.org/10.1128/AAC.00224-11.
11. Renzoni A, Kelley WL, Rosato RR, Martinez MP, Roch M, Fatouraei M, Haeusser DP, Margolin W, Fenn S, Turner RD, Foster SJ, Rosato AE. 2017. Molecular bases determining daptomycin resistance-mediated resensitization to beta-lactams (seesaw effect) in methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 61:e01634-16. https://doi.org/10.1128/AAC.01634-16.
12. Jousselin A, Manzano C, Biette A, Reed P, Pinho MG, Rosato AE, Kelley WL, Renzoni A. 2015. The *Staphylococcus aureus* chaperone *prsA* is a new auxiliary factor of oxacillin resistance affecting penicillin-binding protein 2A. Antimicrob Agents Chemother 60:1656–1666. https://doi.org/10.1128/AAC.02333-15.
13. Berti AD, Theisen E, Sauer JD, Nonejuie P, Olson J, Pogliano J, Sakoulas G, Nizet V, Proctor RA, Rose WE. 2016. Penicillin binding protein 1 is important in the compensatory response of *Staphylococcus aureus* to daptomycin-induced membrane damage and is a potential target for beta-lactam-daptomycin synergy. Antimicrob Agents Chemother 60: 451–458. https://doi.org/10.1128/AAC.02071-15.
14. Berti AD, Sakoulas G, Nizet V, Tewhey R, Rose WE. 2013. Beta-lactam antibiotics targeting PBP1 selectively enhance daptomycin activity against methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 57:5005–5012. https://doi.org/10.1128/AAC.00594-13.
15. Pereira SF, Henriques AO, Pinho MG, de Lencastre H, Tomasz A. 2009. Evidence for a dual role of PBP1 in the cell division and cell separation of *Staphylococcus aureus*. Mol Microbiol 72:895–904. https://doi.org/10.1111/j.1365-2958.2009.06687.x.
16. Pogliano J, Pogliano N, Silverman JA. 2012. Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins. J Bacteriol 194:4494–4504. https://doi.org/10.1128/JB.00011-12.
17. Berti AD, Baines SL, Howden BP, Sakoulas G, Nizet V, Proctor RA, Rose WE. 2015. Heterogeneity of genetic pathways toward daptomycin nonsusceptibility in *Staphylococcus aureus* determined by adjunctive antibiotics. Antimicrob Agents Chemother 59:2799–2806. https://doi.org/10.1128/AAC.04990-14.
18. Mishra NN, McKinnell J, Yeaman MR, Rubio A, Nast CC, Chen L, Kreiswirth BN, Bayer AS. 2011. *In vitro* cross-resistance to daptomycin and host defense cationic antimicrobial peptides in clinical methicillin-resistant *Staphylococcus aureus* isolates. Antimicrob Agents Chemother 55: 4012–4018. https://doi.org/10.1128/AAC.00223-11.
19. Mishra NN, Bayer AS, Moise PA, Yeaman MR, Sakoulas G. 2012. Reduced susceptibility to host-defense cationic peptides and daptomycin coemerge in methicillin-resistant *Staphylococcus aureus* from daptomycin-naive bacteremic patients. J Infect Dis 206:1160–1167. https://doi.org/10.1093/infdis/jis482.
20. Howden BP, McEvoy CR, Allen DL, Chua K, Gao W, Harrison PF, Bell J, Coombs G, Bennett-Wood V, Porter JL, Robins-Browne R, Davies JK, Seemann T, Stinear TP. 2011. Evolution of multidrug resistance during *Staphylococcus aureus* infection involves mutation of the essential two component regulator *walKR*. PLoS Pathog 7:e1002359. https://doi.org/10.1371/journal.ppat.1002359.
21. Sakoulas G, Okumura CY, Thienphrapa W, Olson J, Nonejuie P, Dam Q, Dhand A, Pogliano J, Yeaman MR, Hensler ME, Bayer AS, Nizet V. 2014. Nafcillin enhances innate immune-mediated killing of methicillin-resistant *Staphylococcus aureus*. J Mol Med (Berl) 92:139–149. https://doi.org/10.1007/s00109-013-1100-7.
22. Kim SH, Kim KH, Kim HB, Kim NJ, Kim EC, Oh MD, Choe KW. 2008. Outcome of vancomycin treatment in patients with methicillin-susceptible *Staphylococcus aureus* bacteremia. AAC 52:192–197. https://doi.org/10.1128/AAC.00700-07.
23. Fowler VG, Jr, Boucher HW, Corey GR, Abrutyn E, Karchmer AW, Rupp ME, Levine DP, Chambers HF, Tally FP, Vigliani GA, Cabell CH, Link AS, DeMeyer I, Filler SG, Zervos M, Cook P, Parsonnet J, Bernstein JM, Price CS, Forrest GN, Fatkenheuer G, Gareca M, Rehm SJ, Brodt HR, Tice A, Cosgrove SE, *S. aureus* Endocarditis and Bacteremia Study Group. 2006. Daptomycin versus standard therapy for bacteremia and endocarditis caused by *Staphylococcus aureus*. N Engl J Med 355:653–665. https://doi.org/10.1056/NEJMoa053783.
24. Zheng X, Berti AD, McCrone S, Roch M, Rosato AE, Rose WE, Chen B. 2017. Combination antibiotic exposure selectively alters the development of vancomycin intermediate resistance in *Staphylococcus aureus*.

Antimicrob Agents Chemother 62:e02100-17. https://doi.org/10.1128/AAC.02100-17.
25. Berti AD, Wergin JE, Girdaukas GG, Hetzel SJ, Sakoulas G, Rose WE. 2012. Altering the proclivity towards daptomycin resistance in methicillin-resistant *Staphylococcus aureus* using combinations with other antibiotics. Antimicrob Agents Chemother 56:5046–5053. https://doi.org/10.1128/AAC.00502-12.
26. Mehta S, Singh C, Plata KB, Chanda PK, Paul A, Riosa S, Rosato RR, Rosato AE. 2012. Beta-lactams increase the antibacterial activity of daptomycin against clinical methicillin-resistant *Staphylococcus aureus* strains and prevent selection of daptomycin-resistant derivatives. Antimicrob Agents Chemother 56:6192–6200. https://doi.org/10.1128/AAC.01525-12.
27. Kosowska-Shick K, McGhee PL, Appelbaum PC. 2010. Affinity of ceftaroline and other beta-lactams for penicillin-binding proteins from *Staphylococcus aureus* and *Streptococcus pneumoniae*. Antimicrob Agents Chemother 54:1670–1677. https://doi.org/10.1128/AAC.00019-10.
28. Bayer AS, Mishra NN, Sakoulas G, Nonejuie P, Nast CC, Pogliano J, Chen KT, Ellison SN, Yeaman MR, Yang SJ. 2014. Heterogeneity of *mprf* sequences in methicillin-resistant *Staphylococcus aureus* clinical isolates: role in cross-resistance between daptomycin and host defense antimicrobial peptides. Antimicrob Agents Chemother 58:7462–7467. https://doi.org/10.1128/AAC.03422-14.
29. Howden BP, Johnson PD, Ward PB, Stinear TP, Davies JK. 2006. Isolates with low-level vancomycin resistance associated with persistent methicillin-resistant *Staphylococcus aureus* bacteremia. Antimicrob Agents Chemother 50:3039–3047. https://doi.org/10.1128/AAC.00422-06.
30. Cui L, Ma X, Sato K, Okuma K, Tenover FC, Mamizuka EM, Gemmell CG, Kim MN, Ploy MC, El-Solh N, Ferraz V, Hiramatsu K. 2003. Cell wall thickening is a common feature of vancomycin resistance in *Staphylococcus aureus*. J Clin Microbiol 41:5–14. https://doi.org/10.1128/JCM.41.1.5-14.2003.
31. Cui L, Neoh HM, Shoji M, Hiramatsu K. 2009. Contribution of *vraSR* and *graSR* point mutations to vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. Antimicrob Agents Chemother 53:1231–1234. https://doi.org/10.1128/AAC.01173-08.
32. Cui L, Tominaga E, Neoh HM, Hiramatsu K. 2006. Correlation between reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. Antimicrob Agents Chemother 50:1079–1082. https://doi.org/10.1128/AAC.50.3.1079-1082.2006.
33. Rose WE, Schulz LT, Andes D, Striker R, Berti AD, Hutson PR, Shukla SK. 2012. Addition of ceftaroline to daptomycin after emergence of daptomycin-nonsusceptible *Staphylococcus aureus* during therapy improves antibacterial activity. Antimicrob Agents Chemother 56:5296–5302. https://doi.org/10.1128/AAC.00797-12.
34. Muller A, Wenzel M, Strahl H, Grein F, Saaki TNV, Kohl B, Siersma T, Bandow JE, Sahl HG, Schneider T, Hamoen LW. 2016. Daptomycin inhibits cell envelope synthesis by interfering with fluid membrane microdomains. Proc Natl Acad Sci U S A 113:E7077–E7086. https://doi.org/10.1073/pnas.1611173113.
35. Berti AD, Shukla N, Rottier AD, McCrone JS, Turner HM, Monk IR, Baines SL, Howden BP, Proctor RA, Rose WE. 2018. Daptomycin selects for genetic and phenotypic adaptations leading to antibiotic tolerance in MRSA. J Antimicrob Chemother 73:2030–2033. https://doi.org/10.1093/jac/dky148.
36. Dey S, Gudipati S, Giuliano C, Zervos MJ, Monk JM, Szubin R, Jorgensen SCJ, Sakoulas G, Berti AD. 2019. Reduced production of bacterial membrane vesicles predicts mortality in ST45/USA600 methicillin-resistant *staphylococcus aureus* bacteremia. Antibiotics (Basel) 9:2. https://doi.org/10.3390/antibiotics9010002.
37. Clinical and Laboratory Standards Institute. 2019. Performance standards for antimicrobial susceptibility testing. CLSI M100-ED29. Clinical and Laboratory Standards Institute, Wayne, PA.
38. Cingolani P, Platts A, Wang Le L, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM. 2012. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. Fly (Austin) 6:80–92. https://doi.org/10.4161/fly.19695.
39. Dvorchik B, Arbeit RD, Chung J, Liu S, Knebel W, Kastrissios H. 2004. Population pharmacokinetics of daptomycin. Antimicrob Agents Chemother 48:2799–2807. https://doi.org/10.1128/AAC.48.8.2799-2807.2004.
40. Kahlenberg JM, Kaplan MJ. 2013. Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease. J Immunol 191:4895–4901. https://doi.org/10.4049/jimmunol.1302005.
41. Mishra NN, Liu GY, Yeaman MR, Nast CC, Proctor RA, McKinnell J, Bayer AS. 2011. Carotenoid-related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. Antimicrob Agents Chemother 55:526–531. https://doi.org/10.1128/AAC.00680-10.
42. Kang KM, Mishra NN, Park KT, Lee GY, Park YH, Bayer AS, Yang SJ. 2017. Phenotypic and genotypic correlates of daptomycin-resistant methicillin-susceptible *Staphylococcus aureus* clinical isolates. J Microbiol 55:153–159. https://doi.org/10.1007/s12275-017-6509-1.