

# 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

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**The activity of daptomycin (DAP) against methicillin-resistant *Staphylococcus aureus* (MRSA) is enhanced in the presence of  $\beta$ -lactam antibiotics. This effect is more pronounced with  $\beta$ -lactam antibiotics that exhibit avid binding to penicillin binding protein 1 (PBP1). Here, we present evidence that PBP1 has a significant role in responding to DAP-induced stress on the cell. Expression of the *pbpA* transcript, encoding PBP1, was specifically induced by DAP exposure whereas expression of *pbpB*, *pbpC*, and *pbpD*, encoding PBP2, PBP3, and PBP4, respectively, remained unchanged. Using a MRSA COL strain with *pbpA* under an inducible promoter, increased *pbpA* transcription was accompanied by reduced susceptibility to, and killing by, DAP *in vitro*. Exposure to  $\beta$ -lactams that preferentially inactivate PBP1 was not associated with increased DAP binding, suggesting that synergy in the setting of anti-PBP1 pharmacotherapy results from increased DAP potency on a per-molecule basis. Combination exposure in an *in vitro* pharmacokinetic/pharmacodynamic model system with  $\beta$ -lactams that preferentially inactivate PBP1 (DAP-meropenem [MEM] or DAP-imipenem [IPM]) resulted in more-rapid killing than did combination exposure with DAP-nafcillin (NAF) (nonselective), DAP-ceftriaxone (CRO) or DAP-cefotaxime (CTX) (PBP2 selective), DAP-cefclor (CEC) (PBP3 selective), or DAP-cefoxitin (FOX) (PBP4 selective). Compared to  $\beta$ -lactams with poor PBP1 binding specificity, exposure of *S. aureus* to DAP plus PBP1-selective  $\beta$ -lactams resulted in an increased frequency of septation and cell wall abnormalities. These data suggest that PBP1 activity may contribute to survival during DAP-induced metabolic stress. Therefore, targeted inactivation of PBP1 may enhance the antimicrobial efficiency of DAP, supporting the use of DAP– $\beta$ -lactam combination therapy for serious MRSA infections, particularly when the  $\beta$ -lactam undermines the PBP1-mediated compensatory response.**

The presence of subinhibitory concentrations of  $\beta$ -lactam antibiotics increases daptomycin (DAP) activity against both DAP-susceptible and -nonsusceptible (DNS) methicillin-resistant *Staphylococcus aureus* (MRSA) (1, 2). The mechanisms for this are not fully understood, but some  $\beta$ -lactams have been observed to increase binding of DAP to bacterial cell membranes (3, 4) or to target binding to membrane regions where DAP is most effective (5). This is consistent with reports of a DAP– $\beta$ -lactam “seesaw effect,” whereby *S. aureus* frequently gains susceptibility to  $\beta$ -lactams upon acquisition of the DAP-nonsusceptible (DNS) phenotype (6). Collectively, these *in vitro* observations have been translated to antimicrobial therapy combinations to successfully manage difficult-to-treat MRSA infections (1, 3, 6, 7).

The targets of  $\beta$ -lactam antibiotics are the penicillin binding proteins (PBPs) that assemble and cross-link the bacterial cell wall through transglycosylation and transpeptidation. *S. aureus* produces four PBPs, all of which retain transpeptidase activity but only one of which (PBP2) also demonstrates transglycosylase activity (8). The *mecA* element found in methicillin-resistant *S. aureus* (MRSA) encodes a fifth PBP (PBP2a), a transpeptidase that is resistant to  $\beta$ -lactam inactivation (9, 10). The relative affinity of different  $\beta$ -lactams for PBPs varies: some  $\beta$ -lactams bind to and inactivate one PBP preferentially, while other  $\beta$ -lactams demonstrate relatively nonselective binding to multiple PBPs (11, 12). Previous work has suggested that the degree to which different

$\beta$ -lactams potentiate the anti-MRSA activity of DAP may be associated with the relative affinity for PBP1 (13). This specific differential effect echoes other recent studies that observed increased toxin expression (12) and induction of DNA repair systems (14) upon inhibition of PBP1 but not upon inhibition of other PBPs (12, 15–17).

We hypothesize that the components of the cellular divisome, including PBP1, may form a critical adaptive response to DAP-mediated surface injury and that  $\beta$ -lactams which compromise PBP1 activity may enhance the efficiency of DAP killing without necessarily increasing DAP binding.

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Importantly, PBP1 of *S. aureus* is homologous to PBP2x of *Streptococcus pneumoniae* and PBP3 of *Escherichia coli*, each comprised of a C-terminal transpeptidase domain and an N-terminal structural domain recently identified as a critical component in the bacterial divisome complex responsible for mediating cell division (18, 19). In fact, these homologous PBPs, including PBP1 in *S. aureus*, are located in division and cell wall synthesis clusters on their respective bacterial chromosomes. Furthermore, mediation of cell division appears to supersede peptidoglycan synthesis in the hierarchy of PBP1 function, given that depletion of PBP1 has been shown to induce abnormal cell morphology and incomplete septation but does not appear to alter peptidoglycan cross-linking (18), whereas depletion of PBP2 or PBP4 results in significantly altered peptidoglycan (10, 20).

This study provides evidence that PBP1 contributes to survival in the presence of DAP and that modulation of PBP1 activity can alter the *in vitro* efficacy of DAP killing.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *S. aureus* strains D592 and D712 are paired isogenic MRSA isolates from a clinical series using DAP plus antistaphylococcal  $\beta$ -lactam in refractory bacteremia (3). Strain D592 is the DAP-susceptible index isolate, whereas D712 is a subsequent isolate that had become DNS as well as vancomycin intermediate following multiple unsuccessful antimicrobial regimens, as previously characterized (13). The genetic relatedness of the two patient isolates was confirmed by whole-genome sequencing (21). *S. aureus* strain COL is a prototypical MRSA strain that is DAP susceptible (18, 22), and constructs placing *pbpA* under an isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)-inducible promoter have been previously described (18). All antibacterials were purchased as commercial agents. MICs were determined by broth microdilution per the Clinical and Laboratory Standards Institute guidelines and with *S. aureus* ATCC 29213 for quality control (23). *S. aureus* was propagated in calcium-supplemented (50-mg/liter) Mueller-Hinton broth. For each antibiotic, the  $fC_{avg}$  is here defined as the average free concentration of drug achieved in serum during a typical dosing interval, selected with the formula  $fC_{avg} = (fC_{max} + fC_{min})/2$ , where  $fC_{max}$  and  $fC_{min}$  are maximum and minimum free concentrations of drug in serum, respectively.

**Cathelicidin LL37 killing assays.** Cationic peptides have an antibiotic mechanism similar to that of DAP, and yet distinct differences in potency and membrane activity have been identified (24). Therefore, the primary antimicrobial peptide LL37 was evaluated in combination with  $\beta$ -lactams. *S. aureus* D712 was grown in antibiotic-free LB broth or broth containing 5 mg/liter of the test  $\beta$ -lactam antibiotic overnight (15 to 18 h), resuspended in phosphate-buffered saline (PBS), and subjected to killing assays in 128  $\mu$ M LL37, as previously described (25).

**DAP binding assays.** Enhanced DAP binding in the presence of  $\beta$ -lactams is one proposed mechanism for synergy, but only select  $\beta$ -lactams have been previously evaluated. This study provides a more comprehensive evaluation of  $\beta$ -lactams with distinct PBP inhibition profiles. *S. aureus* D712 was grown in antibiotic-free LB broth or broth containing 5 mg/liter of the test  $\beta$ -lactam antibiotic to an optical density at 600 nm ( $OD_{600}$ ) of 0.4 to 0.6, stained for 30 min with dipyrromethene boron difluoride (BODIPY)-DAP (16 mg/liter) and  $Ca^{2+}$  (50 mg/liter), and visualized microscopically as previously described (25). DAP binding was evaluated visually and measured by binding intensity and spots per cell to determine the quality and focus of binding. A  $\beta$ -lactam concentration of 5 mg/liter was used for the binding and LL37 killing assays in order to study this effect at physiologically relevant concentrations and provide relative comparisons among the  $\beta$ -lactams.

**Autolysis assays.** Resistance to cationic peptides has been linked to reduced autolysis (26), so it is of interest to evaluate autolysis during  $\beta$ -lactam exposure to understand how  $\beta$ -lactams might alter DAP activ-

ity. *S. aureus* D712 was grown in antibiotic-free LB broth or broth containing 5 mg/liter of the test  $\beta$ -lactam antibiotic overnight (15 to 18 h), resuspended in 0.1% Triton X-100 in PBS to an  $OD_{600}$  of 0.8, and measured spectrophotometrically over time. Results were expressed graphically as percent OD remaining versus time zero.

**FITC-labeled PLL binding.** DAP activity has been previously associated with alterations in cell surface charge (26), although such changes are not observed in all DAP-nonsusceptible *S. aureus* isolates (24). Fluorescein isothiocyanate (FITC)-labeled poly-L-lysine (PLL) binding studies were performed to evaluate surface charge in the presence of  $\beta$ -lactams. Assays were performed using a flow cytometric method as previously described (27). PLL is a polycationic molecule used to study the interactions of cationic peptides with charged bacterial envelopes. In this analysis, the extent of bacterium-bound FITC-labeled PLL inversely reflects the relative surface positive charge. A total of 10,000 events were counted and analyzed using a BD FACSCalibur system (Becton Dickinson Labware, San Jose, CA). At least two independent experiments of triplicate samples were performed.

**qPCR.** Reverse transcription-PCR (RT-PCR) was performed to identify the effect of DAP exposure on PBP regulation in *S. aureus*. Samples were prepared and analyzed as previously described (12). Briefly, overnight cultures were diluted 1:100 in fresh medium and propagated at 37°C (250 rpm) for 6 h in the presence or absence of 0.125 mg/liter DAP. Total RNA from pelleted cultures was isolated using phenol-chloroform and treated with DNase (Life Technologies, Madison, WI, USA) per manufacturer recommendations. Purified RNA (500 ng) was reverse transcribed (iScript; Bio-Rad, Hercules, CA, USA), and the resulting cDNA was used as the template for real-time PCR (StepOne Plus; Applied Biosystems, Foster City, CA, USA) with Kapa Sybr green universal quantitative qPCR (qPCR) mix (Kapa Biosystems, Wilmington, MA, USA) using primers as described by Dumitrescu et al. (12). Relative expression levels were normalized to *gyrB* (StepOne Plus software; Applied Biosystems).

**Effects of PBP1 expression modulation on DAP activity.** A strain of MRSA COL with *pbpA* under the control of a *spac1* promoter with the corresponding relative PBP1 expression previously described (18) was grown overnight (14 to 18 h) in LB containing erythromycin at 10 mg/liter and IPTG at 35, 100, or 1,000  $\mu$ M and then diluted 20 $\times$  into fresh LB containing the same IPTG concentration plus 50 mg/liter  $Ca^{2+}$  and either 16 mg/liter DAP or no antibiotic. CFU were enumerated at time zero and 90 min by plating 10- $\mu$ l aliquots in triplicate. The experiment was performed 3 times, and all results were pooled prior to analysis.

***In vitro* PK/PD model.** A previously described *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) model was used for simulating one-compartment antibiotic exposures of DAP and/or study  $\beta$ -lactams (28, 29). All model experiments were performed in duplicate. Overnight cultures of DNS MRSA strain D712 were adjusted to obtain a starting model inoculum of  $\sim 10^6$  CFU/ml. The following antibiotic regimens were evaluated: (i) DAP at 6 mg/kg of body weight every 24 h (targeted maximum free drug concentration [ $fC_{max}$ ], 7.2 mg/liter; half-life, 8 h), (ii) imipenem (IPM) at 1,000 mg every 8 h ( $fC_{max}$ , 50 mg/liter; half-life, 1 h), (iii) nafcillin (NAF) at 2,000 mg every 4 h ( $fC_{max}$ , 5 mg/liter; half-life, 1 h), (iv) cefotaxime (CTX) at 2,000 mg every 6 h ( $fC_{max}$ , 128 mg/liter; half-life, 1.2 h), (v) cefaclor (CEC) at 500 mg every 8 h ( $fC_{max}$ , 13 mg/liter; half-life, 1 h), (vi) cefoxitin (FOX) at 2,000 mg every 6 h ( $fC_{max}$ , 43 mg/liter; half-life, 1 h), (vii) meropenem (MEM) at 1,000 mg every 8 h ( $fC_{max}$ , 110 mg/liter; half-life, 1 h), and (viii) ceftriaxone (CRO) at 1,000 mg every 24 h ( $fC_{max}$ , 20 mg/liter; half-life, 8 h). Modeling of antibiotics in combination with two different elimination rates was performed according to the methods described by Blaser (30). Areas under the growth curve were calculated using the trapezoidal method.

**Electron microscopy.** Transmission electron microscopy (TEM) images were obtained as described previously (31). Samples were collected following overnight growth in  $\beta$ -lactam antibiotic at the average unbound serum concentration obtained from the regimens modeled in the *in vitro* pharmacokinetic/pharmacodynamic experiment. Cell wall thickness

TABLE 1 MICs of single agents and of DAP in the presence of  $\beta$ -lactams

Strain	MIC (mg/liter) of <sup>a</sup> :								DAP in presence of $\beta$ -lactam ( $fC_{avg}$ ):						
	Single agent:								IPM (11)	MEM (55)	ERT (4)	NAF (2.7)	CTX (65)	CEC (6.5)	FOX (16)
	DAP	IPM	MEM	ERT	NAF	CTX	CEC	FOX							
D592	0.5	64	128	64	256	256	128	256	0.13	0.03	0.13	0.13	0.13	0.13	0.13
D712	2	32	32	32	256	256	64	256	0.5	NG	0.5	0.5	0.5	1	1
COL	0.5	16	16	64	64	256	128	256							

<sup>a</sup> Abbreviations: ERT, ertapenem; NG, no growth.

measurements were determined using ImageJ 1.39t software on a minimum of 25 cells per treatment using four separate quadrants of each cell. Septation frequency was determined by examination of a minimum of 25 cells per treatment.

**Statistical analysis.** Areas under the growth curve for *in vitro* model cultures were assessed via one-way analysis of variance (ANOVA) at pre-defined time points (4 h, 12 h, 24 h, and 48 h), and combination exposures were compared using Tukey’s honestly significant difference (HSD) *post hoc* test. Groupings of combination exposures comparing BBP1-selective to non-BBP1-selective  $\beta$ -lactams were assessed using Student’s *t* test. All statistical analyses were performed using GraphPad Prism version 6.05 (GraphPad Software, Inc., La Jolla CA).

**RESULTS**

**Antibiotic susceptibilities.** Results of susceptibility testing with study antibiotics are reported in Table 1. All study strains were resistant to  $\beta$ -lactam antibiotics; however, the presence of  $\beta$ -lactam antibiotics reduced the amount of DAP required to inhibit organism growth regardless of the PBP binding profile of the  $\beta$ -lactam or the strain background.

**Effects of DAP on PBP expression in *S. aureus* D712.** To identify potential effects of DAP exposure on PBP transcription, qPCR was performed on *S. aureus* D712 exposed to subinhibitory DAP. Results are presented in Fig. 1. We observed that DAP exposure differentially affected expression of PBPs. Expression of *pbpA* (encoding BBP1) in cultures exposed to DAP increased 5.3-fold compared to a no-antibiotic control ( $P < 0.01$ ). In contrast, exposure to DAP did not result in significant changes to expression of transcripts encoding other PBPs (*pbpB* [BBP2], 1.1-fold; *pbpC* [BBP3], 0.8-fold; *pbpD* [BBP4], 1.9-fold;  $P > 0.05$  for all comparisons).

**Effects of inducible BBP1 production on DAP susceptibility.** To assess if BBP1 expression contributes to DAP nonsusceptibility, the MIC of DAP was determined in *S. aureus* strain

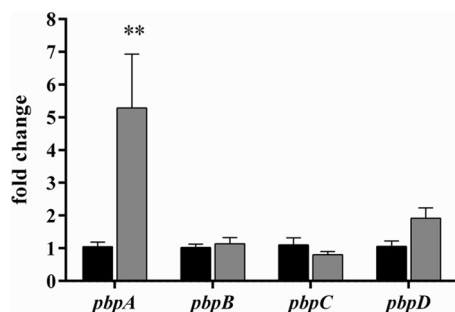


FIG 1 PBP expression profile following exposure to subinhibitory daptomycin. Black bars, no-antibiotic control; gray bars, 1/4 $\times$  daptomycin MIC. Values marked with an asterisk denote statistically significant differences between daptomycin exposure and the no-antibiotic control (\*\*,  $P < 0.01$ ).

COL $spac1$  in the presence of increasing IPTG concentrations. COL $spac1$  is a derivative of COL in which expression of *pbpA* (encoding BBP1) is controlled by the IPTG-inducible *Pspac* promoter (18). Results are presented in Table 2. DAP MICs increased in an IPTG dose-dependent manner, resulting in DAP nonsusceptibility with IPTG concentrations in excess of 100  $\mu$ M. DAP bacterial killing is presented in Fig. 2. Recoverable CFU after 90 min of DAP exposure increased significantly in an IPTG dose-dependent manner, suggesting that DAP is less effective under conditions of high BBP1 production.

**Effect of adjunctive  $\beta$ -lactams on DAP activity in an *in vitro* PK/PD model.**  $\beta$ -Lactams with different specificities for *S. aureus* PBPs were modeled with DAP in an *in vitro* PK/PD simulation to investigate if inactivation of BBP1 chemically would result in enhanced antimicrobial activity. Results are presented in Fig. 3 and Table 3. Addition of any  $\beta$ -lactam enhanced the efficacy of DAP, and this activity was particularly relevant during the initial synergy phase of exposure in terms of both area under the concentration-time curve from 0 to 4 h ( $AUC_{0-4}$ ) and time to 3-log reduction in recoverable CFU per milliliter. This was anticipated, as the initial  $\beta$ -lactam concentration following a dose (the  $fC_{max}$ ) was predicted to exceed the 50% inhibitory concentration ( $IC_{50}$ ) for all four PBPs. However, the duration of PBP blockade during the dosing interval will depend on the specificity of the individual  $\beta$ -lactam for different PBPs. Comparing DAP simulations containing BBP1-specific  $\beta$ -lactams (DAP-MEM and DAP-IPM) and simulations containing non-BBP1-specific  $\beta$ -lactams (DAP-NAF, DAP-CRO, DAP-CTX, DAP-CEC, and DAP-FOX), simulations containing BBP1-specific  $\beta$ -lactams were consistently superior in terms of lower AUCs throughout the simulation and times to 3-log reductions in recoverable CFU per milliliter ( $P < 0.05$  at all time points assessed).

**Effect of adjunctive  $\beta$ -lactams on DAP binding, autolysis, and killing by cathelicidin LL37.** *S. aureus* D712 was grown in medium containing 5 mg/liter of antibiotic selected from a diverse panel of  $\beta$ -lactams with differential specificities in PBP binding, and the effects of  $\beta$ -lactam exposure on DAP binding, autolysis,

TABLE 2 Increase in daptomycin MICs in *S. aureus* COL upon *pbp1* induction

IPTG concn added ( $\mu$ M)	DAP MIC (mg/liter) in strain:	
	<i>S. aureus</i> COL	<i>S. aureus</i> COL $_{PspacBBP1}$
1	0.5	0.5
10	0.5	1
100	0.5	4
1,000	0.5	4

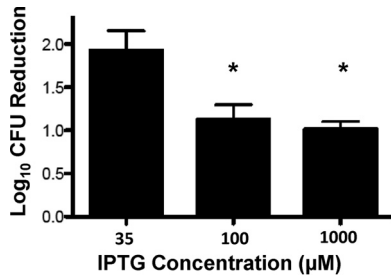


FIG 2 Bacterial recovery after daptomycin exposure with varied amounts of IPTG inducer. Data represent the differences in the number of recoverable CFU evaluated at 90 min relative to the number of recoverable CFU at baseline. Values marked with an asterisk denote statistically significant differences between the respective treatment arm and the 35 µM IPTG control required for basal-level expression of *pbpA* (\*,  $P = 0.03$ ).

PLL binding, and susceptibility to LL37 killing were measured. As shown in Fig. 4, exposure to most β-lactams increased both the number of BODIPY-DAP spots per cell and the intensity of binding at those spots. The exception to this trend was IPM, a β-lactam with high specificity for PBP1. Treatment with IPM resulted in no significant increase in the number of binding foci on the surface of the bacteria, even though it was one of the most effective synergistic agents in combination with DAP (Fig. 4B). This finding paralleled the results of the other experiments whereby IPM was unique among the β-lactams tested in that exposure did not significantly alter surface charge (see Fig. S1 in the supplemental material), autolysis in Triton X-100 (Fig. 5A), or sensitization to killing by LL37 (Fig. 5B).

**Effect of adjunctive β-lactams on cell morphology.** Electron microscopy images of *S. aureus* D712 exposed to β-lactam were examined to investigate if specific chemical inactivation of differ-

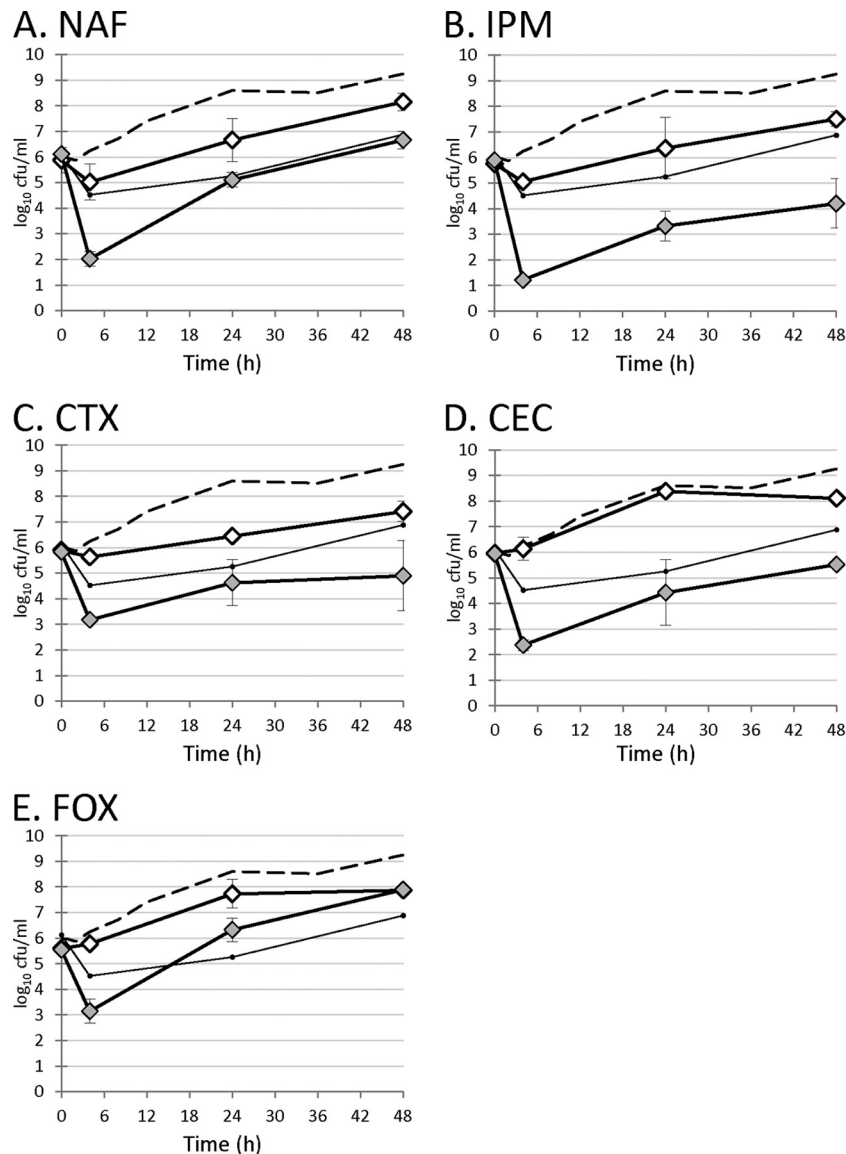


FIG 3 Activity of daptomycin and/or β-lactam in a 48-hour *in vitro* pharmacokinetic/pharmacodynamic model. Dashed line, growth control; small black circles, daptomycin; white symbols, β-lactam monotherapy; gray symbols, daptomycin-β-lactam.



TABLE 3 Effectiveness of DAP with or without  $\beta$ -lactam in *in vitro* pharmacokinetic/pharmacodynamic model<sup>a</sup>

Antibacterial(s)	AUC (log <sub>10</sub> CFU · h/ml)				Time to achieve 3-log <sub>10</sub> reduction (h)
	0–4 h	0–12 h	0–24 h	0–48 h	
None (growth)	24.0 ± 0.7	78.2 ± 5.7	174.3 ± 14.0	384.8 ± 19.8	
DAP	19.2 ± 0.6	42.2 ± 1.4	84.4 ± 5.4	191.4 ± 31.9	6.93 ± 0.30
DAP + IPM	10.1 ± 0.4	25.2 ± 4.3	58.5 ± 10.3	147.9 ± 33.4	1.10 ± 0.02
DAP + MEM	10.7 ± 0.1	28.3 ± 0.0	63.6 ± 11.3	140.1 ± 19.8	1.08 ± 0.18
DAP + NAF	13.7 ± 0.1	35.8 ± 3.9	84.7 ± 6.6	212.8 ± 21.4	1.76 ± 0.08
DAP + CTX	13.6 ± 0.2	39.0 ± 5.4	85.6 ± 18.9	192.6 ± 46.9	1.71 ± 0.04
DAP + CRO	15.5 ± 0.2	39.4 ± 1.6	93.0 ± 8.6	208.8 ± 22.9	1.74 ± 0.03
DAP + CEC	14.1 ± 1.3	29.5 ± 4.7	68.2 ± 14.0	171.6 ± 28.9	2.68 ± 0.56
DAP + FOX	12.9 ± 0.1	35.2 ± 2.0	95.1 ± 1.9	248.9 ± 16.4	1.83 ± 0.12

<sup>a</sup> Values represent the average ± standard deviation from two independent experiments.

ent PBPs would result in distinct alterations in cell morphology. Results are presented in Fig. 6 and Table 4. Addition of  $\beta$ -lactam with affinity for PBP1 significantly increased the frequency of cells undergoing septation events relative to exposure to DAP alone ( $P < 0.001$ ). Addition of  $\beta$ -lactam with nonspecific PBP binding also significantly increased the frequency of septation ( $P < 0.05$ ). In contrast, addition of  $\beta$ -lactam with affinity for PBP2, PBP3, or PBP4 resulted in no difference in the septation frequency ( $P > 0.05$ ). *S. aureus* cell walls demonstrated significant thickening upon exposure to  $\beta$ -lactam regardless of PBP specificity, with the exception of the PBP3-specific drug CEC, which resulted in significantly thinner cell walls ( $P < 0.01$  for all comparisons). Exposure to  $\beta$ -lactams with affinity for either PBP1 or PBP2 resulted in larger cell diameters ( $P < 0.01$ ), whereas exposure to PBP3- or PBP4-specific agents had no effect on cell size ( $P > 0.05$ ).

## DISCUSSION

Several published studies have noted synergy between DAP and  $\beta$ -lactam antibiotics, both *in vitro* (2, 6, 32) and *in vivo* (1, 3, 7). This improved activity has largely been attributed to enhanced DAP binding to *S. aureus* in the presence of  $\beta$ -lactam antibiotics (3). However, alterations to the cell membrane surface charge

thought to result in enhanced binding are not observed in all strains that show synergy (33). The demonstrated effectiveness of this combination therapy despite heterogeneity in the specific  $\beta$ -lactam employed reflects the possibility that DAP- $\beta$ -lactam synergy may be a  $\beta$ -lactam class effect. However, a recent study observed significant diversity among different  $\beta$ -lactams in their relative efficacies in combination with DAP and reported that synergy is dependent on the relative affinity of a  $\beta$ -lactam for PBP1 (13).

Here, we have established several additional lines of evidence that increased production of PBP1 is a crucial compensatory response to DAP injury and that undermining this response is a viable strategy in enhancing DAP activity pharmacodynamically using currently available  $\beta$ -lactams in combination therapy: (i) exposure to subinhibitory DAP resulted in increased transcription of *pbpA*, the gene encoding PBP1, but not of genes encoding other *S. aureus* PBPs; (ii) using a previously characterized bacterial construct placing *pbpA* under control inducible by IPTG in MRSA COL, increased *pbpA* transcription was accompanied by reduced susceptibility to, and killing by, DAP *in vitro*; (iii) exposure to  $\beta$ -lactams exhibiting high selectivity for PBP1 resulted in more-rapid bactericidal activity and lower areas under the inhibitory

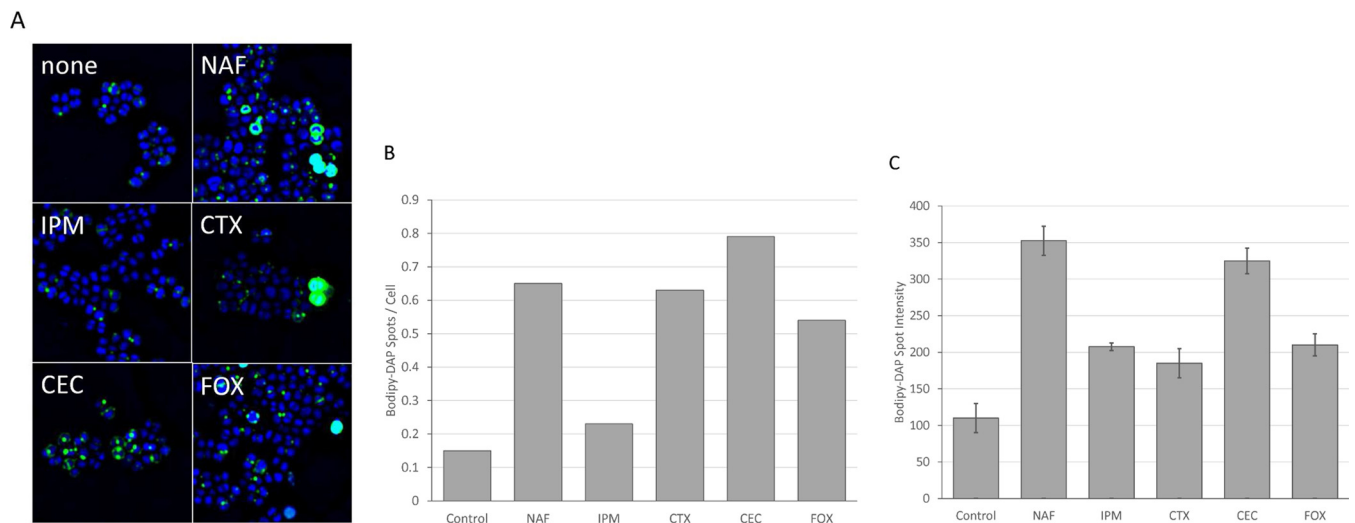
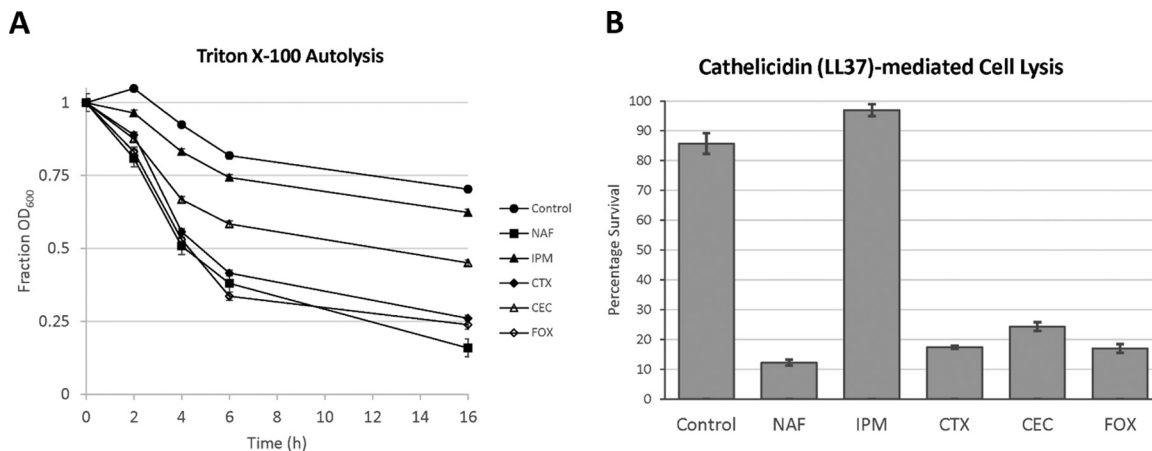


FIG 4 Binding of BODIPY-daptomycin to *S. aureus* D712 following overnight  $\beta$ -lactam exposure. (A) Fluorescence microscopy of *S. aureus* pretreated for 24 h in the presence of 5 mg/liter of the  $\beta$ -lactam indicated and subsequently exposed to 16 mg/liter BODIPY-daptomycin for 20 min. (B) Number of BODIPY-daptomycin spots per cell. (C) Signal intensity of BODIPY-daptomycin bound to *S. aureus* membranes.



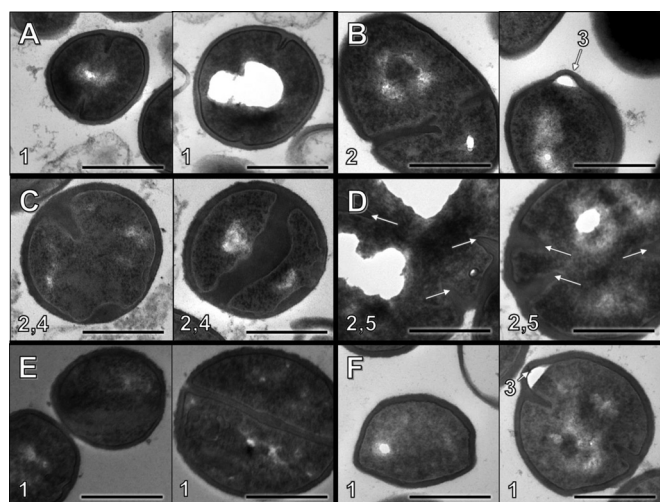
**FIG 5** Effects of pretreatment with subinhibitory  $\beta$ -lactam on *S. aureus* autolysis. (A) Triton X-100 autolysis. Bacteria were pretreated overnight with  $\beta$ -lactam. Antibiotic was removed at time zero, and bacteria were resuspended in 0.1% Triton X-100. Data represent the percentages of the initial OD<sub>600</sub> reading evaluated at preset time points following Triton X-100 exposure. (B) Cathelicidin (LL37)-mediated lysis. Bacteria were pretreated overnight with  $\beta$ -lactam. Antibiotic was removed at time zero, and bacteria were resuspended in PBS containing 128  $\mu$ M LL37. Data represent the percentages of recoverable CFU remaining after 2 h of incubation with LL37.

curve (AUCs) relative to  $\beta$ -lactams with low selectivity for PBP1 in pharmacokinetic/pharmacodynamic modeling of DAP; (iv) the PBP1-selective  $\beta$ -lactams enhanced killing without enhancing DAP binding, suggesting a mechanism of enhancing the efficiency of DAP killing per molecule of DAP membrane insertion, rather than increasing the number of DAP molecules binding to the membrane; (v) supporting the latter, PBP1-selective  $\beta$ -lactams, unlike  $\beta$ -lactams binding selectively to other PBPs, did not enhance PLL binding (i.e., reduce net surface charge) or increase autolysis. These findings are supportive of prior work demonstrating that mediation of cell division appears to be a primary role for PBP1 in *S. aureus* that supersedes its C-terminal transpeptidase function (18). One response of bacteria to DAP exposure appears

to be an increased rate of septation and cell division (5). Interference with this aspect of PBP1 function, therefore, appears to dominate the mechanism of synergy with DAP in MRSA, given that DAP binding is not enhanced by PBP1-selective agents such as the carbapenems. To this point, there was an overall lack of cell surface charge alteration with  $\beta$ -lactams, which is in contrast to previous studies with nafcillin and cephalosporins (3, 34). This effect may play a role in moderately enhancing DAP activity, but the PBP1 target is essential for synergistic effect.

In addition, these data demonstrate that PBP1-specific  $\beta$ -lactams appear to discriminate between enhancement of DAP and of cationic antimicrobial peptides such as cathelicidin (LL37). We have previously shown that penicillins and cephalosporins sensitize MRSA to killing by LL37 and other host defense peptides, but we did not include carbapenems in that analysis (25). Interestingly, in this study we found that growth of MRSA in the PBP1-specific carbapenems did not enhance LL37 killing, despite potentiation of DAP activity. This paralleled their inability to induce autolysis or alter surface charge. Enhanced autolysis has been consistently linked to increased susceptibility to host defense peptide killing, with bacterial strains that show a relative resistance to host defense peptide killing having a reduced autolysis phenotype (26).

It appears, therefore, that while killing by the DAP-calcium



**FIG 6** Cell morphology observations following overnight  $\beta$ -lactam exposure at  $fC_{avg}$  concentration. Each panel consists of two representative images of *S. aureus* D712 exposed to the following conditions: antibiotic-free control (A), NAF (B), MEM (C), CTX (D), CEC (E), and FOX (F). Notable features: 1, well-defined borders and/or septa; 2, abnormal septation/separation; 3, membrane invagination; 4, inconsistent cell wall thickness; 5, atypical cell shape/size. Bar, 500 nm.

**TABLE 4** Differential alteration of cell morphology characteristics by  $\beta$ -lactam inhibition of discrete PBPs<sup>a</sup>

Exposure	<i>S. aureus</i> PBP specificity (12, 13)	% of cells containing septa	Cell wall thickness (nm)	Cell diam (nm)
Medium-only control		12.8	26.7 $\pm$ 5.36	662 $\pm$ 61
NAF	Nonspecific	38.9*	35.4 $\pm$ 5.65**	914 $\pm$ 155**
MEM	PBP1	73.9**	33.7 $\pm$ 6.07**	1,002 $\pm$ 135**
CTX	PBP2	37.5**	47.1 $\pm$ 11.86**	1,307 $\pm$ 215**
CEC	PBP3	24.0	21.7 $\pm$ 4.65**	729 $\pm$ 99
FOX	PBP4	25.4	34.1 $\pm$ 4.70**	748 $\pm$ 92*

<sup>a</sup> Values marked with asterisks denote statistically significant differences between  $\beta$ -lactam exposure and medium-only control (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

complex parallels that of host defense peptides, there are critical differences that are distinguished by carbapenems, presumably due to the effects on PBP1. The activity of DAP is dependent not only on the quantity of binding but also on the quality of binding, as some  $\beta$ -lactams induce marked increases in DAP surface binding but do not increase DAP potency. This feature has been recently highlighted in the mechanism of DAP resistance in *Enterococcus faecalis*, whereby DAP is bound and diverted to membrane sites away from the septum and, therefore, bound drug is sequestered to a site where it is functionally inactive in killing the bacterium (35).

NAF, which is considered to be the optimal agent against serious methicillin-susceptible *S. aureus* infections, appears to exhibit a “best of both worlds” phenotype of potentiating the activity of both DAP and LL37. Carbapenems are effective in potentiating DAP activity but offer no enhancement of killing by host defense peptides of the innate immune system, whereas cephalosporins do potentiate innate host defense peptides. While DAP binding is enhanced by NAF, it may be that its effects on PBP1 through its broader PBP binding profile may actually be responsible for its potentiation of DAP activity, rather than the increased DAP binding. These findings highlight that the potency of antimicrobial therapy should be gauged not simply through *in vitro* killing but rather through a concerted effect of direct killing and the enhancement of defense peptides that are already being produced in the host.

Differences in morphology identified following exposure to  $\beta$ -lactams with different PBP specificities may illuminate why  $\beta$ -lactams that inactivate PBP1 appear to have the strongest synergy with DAP. Recent work in *Bacillus subtilis* suggests that DAP preferentially binds to regions of extreme membrane strain, such as those found around nascent septa (5). Upon DAP damage of the cell membrane in *B. subtilis*, relocalization of proteins involved in cell division and cell wall synthesis occurs and results in defects in cell wall and cell membrane formation. We hypothesize that DAP exposure in *S. aureus* results in a similar disruption in essential proteins involved in cell division, and selective or nonselective  $\beta$ -lactam inhibition of PBP1, which is the PBP in *S. aureus* responsible for cell division, leads to enhanced lethality by disrupting this important response mechanism. In our study, strains exposed to  $\beta$ -lactams that inactivate PBP1 show a significantly increased prevalence of cells in the process of nascent septation. Additionally, *S. aureus* exposed to  $\beta$ -lactams that inactivate PBP1 may experience further membrane strain due to the significant inconsistencies in cell wall thickness within a single cell. While exposure to most  $\beta$ -lactams tested resulted in thickened cell walls, the significance of this finding is unclear, as previous studies have demonstrated that cell wall thickness has no significant direct effect on DAP activity (36). However, for isolates exposed to  $\beta$ -lactams specific for PBP1, the cell wall thickening was not uniform within a single cell, resulting in a wavy cell wall-membrane interface. These extremes of cell wall thickening and thinning were not observed following exposure to  $\beta$ -lactams with alternative binding profiles (both specific and nonspecific) and may result in strained membranes providing targeted sites for more-effective DAP binding rather than less-effective diffuse binding.

In summary, *S. aureus* PBPs differ in their role with respect to cell wall maintenance and response to cell injury. Their common property of binding  $\beta$ -lactam antibiotics has led some to presume that they are variations of the same theme and represent bacterial

redundancy. On the contrary, we have found that PBP1 of *S. aureus* stands out as a protein whose production is enhanced by DAP-induced cell injury and that increasing PBP1 renders MRSA less susceptible to DAP killing and growth inhibition. Carbapenems that preferentially bind PBP1 allow for enhanced DAP activity by not necessarily increasing DAP binding, presumably because of efficiency in killing. In contrast, cephalosporins appear to increase DAP binding but not necessarily activity. NAF stands out as a  $\beta$ -lactam that enhances DAP binding and host defense peptide killing, perhaps laying the foundation of its excellent clinical performance among the other  $\beta$ -lactams in serious *S. aureus* infections. In order to further explore this PBP1 target and mechanism, we are pursuing additional studies on how individual regulation and selective inactivation of the PBPs in *S. aureus* impact DAP activity. The effect of DAP exposure on the localization of cell wall and membrane proteins, including PBP1, is also being investigated. Further studies are necessary to reveal the maximal benefits of  $\beta$ -lactam antibiotics as adjunctive therapies against MRSA.

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