The Journal of Infectious Diseases

MAJOR ARTICLE

Mechanistic Insights Into the Differential Efficacy of Daptomycin Plus β-Lactam Combinations Against Daptomycin-Resistant *Enterococcus faecium*

Razieh Kebriaei,[1] Kyle C. Stamper,[1] Kavindra V. Singh,[2,3] Ayesha Khan,[2,3,4] Seth A. Rice,[1] An Q. Dinh,[2,3] Truc T. Tran,[2,3] Barbara E. Murray,[2,3,4] Cesar A. Arias[2,3,4,5,6] and Michael J. Rybak[1,7]

[1]Anti-Infective Research Laboratory, Eugene Applebaum College of Pharmacy & Health Sciences, Detroit, Michigan, USA, [2]Division of Infectious Diseases, [3]Center for Antimicrobial Resistance and Microbial Genomics, [4]Department of Microbiology and Molecular Genetics, UTHealth McGovern Medical School, Houston, Texas, USA, [5]Center for Infectious Diseases, UTHealth School of Public Health, Houston, Texas, USA, [6]Molecular Genetics and Antimicrobial Resistance Unit, International Center for Microbial Genomics; Universidad El Bosque, Bogotá, Colombia, and [7]School of Medicine, Wayne State University, Detroit, Michigan, USA

Background. The combination of daptomycin (DAP) plus ampicillin (AMP), ertapenem (ERT), or ceftaroline has been demonstrated to be efficacious against a DAP-tolerant *Enterococcus faecium* strain (HOU503). However, the mechanism for the efficacy of these combinations against DAP-resistant (DAP-R) *E. faecium* strains is unknown.

Methods. We investigated the efficacy of DAP in combination with AMP, ERT, ceftaroline, ceftriaxone, or amoxicillin against DAP-R *E. faecium* R497 using established in vitro and in vivo models. We evaluated *pbp* expression, levels of penicillin-binding protein (PBP) 5 (PBP5) and β-lactam binding affinity in HOU503 versus R497.

Results. DAP plus AMP was the only efficacious regimen against DAP-R R497 and prevented emergence of resistance. DAP at 8, 6, and 4 mg/kg in combination with AMP was efficacious but showed delayed killing compared with 10 mg/kg. PBP5 of HOU503 exhibited amino acid substitutions in the penicillin-binding domain relative to R497. No difference in *pbp* mRNA or PBP5 levels was detected between HOU503 and R497. labeling of PBPs with Bocillin FL, a fluorescent penicillin derivative, showed increased β-lactam binding affinity of PBP5 of HOU503 compared with that of R497.

Conclusions. Only DAP (10 mg/kg) plus AMP or amoxicillin was efficacious against a DAP-R *E. faecium* strain, and *pbp5* alleles may be important contributors to efficacy of DAP plus β-lactam therapy.

Keywords. Vancomycin-resistant *E. faecium*; *E. faecium*; Daptomycin; β-lactam; PBP.

Enterococci are major nosocomial pathogens with an important ability to acquire resistance determinants [1–4]. Infections caused by multidrug-resistant *Enterococcus faecium* are a challenge owing to the paucity of efficacious antibiotics [5]. Daptomycin (DAP) is a cell membrane acting lipopeptide antibiotic that has become a key drug in the treatment of multidrug-resistant *E. faecium* infections. DAP displays concentration-dependent bactericidal activity against most *E. faecium*, and the area under the concentration-time curve (AUC) from 0 to 24 hours (AUC_{0-24h}) divided by the minimum inhibitory concentration (MIC) is the pharmacokinetic-pharmacodynamic (PK/PD) parameter that best predicts the in vivo efficacy of the antibiotic against these organisms [6].

Emergence of in vivo of DAP resistance in *E. faecium* seems to occur commonly, mainly mediated by changes in the *liaFSR* system [2, 7–9], a 3-component regulatory system that controls cell membrane adaptation. Resistance development can be prevented by combining DAP with β-lactams resulting in higher relative exposures (AUC_{0-24h}/MIC) because the DAP MICs are lower with the combination [10–13]. We previously demonstrated [14] that DAP monotherapy (doses from 6 to 10 mg/kg) against a DAP-tolerant strain of *E. faecium* (HOU503; DAP MIC of 2 μg/mL, designated as DAP-susceptible (DAP-S) dose dependent by the current break points) was marginally effective [15, 16]) This strain harbors *liaFSR* substitutions, resulting in the emergence of resistance with all DAP monotherapy regimens. In contrast, addition of ampicillin (AMP), ceftaroline (CPT) or ertapenem (ERT) led to increased killing and abolished emergence of resistance over the 14-day in vitro model experiments [14].

Although these combinations seem promising, there are few data to suggest which β-lactam works best with DAP and whether these combinations would be effective against DAP-resistant (DAP-R) strains (including those with mutations in the *liaFSR* system). In the current study, we evaluated the effect

Received 18 February 2020; editorial decision 28 May 2020; accepted 2 June 2020; published online June 8, 2020.

Correspondence: Michael J. Rybak, Anti-Infective Research Laboratory Pharmacy Practice–4148, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, 259 Mack Ave, Detroit, MI 48201 (m.rybak@wayne.edu).

The Journal of Infectious Diseases® 2020;222:1531–39

DOI: 10.1093/infdis/jiaa319

of DAP monotherapy (10 mg/kg) and DAP in combination with AMP, CPT, or ERT (and others) using the simulated endocardial vegetation (SEV) PK/PD model, validating the results in a rat model of infective endocarditis [14]. We demonstrated that the combination of DAP plus AMP was the only regimen that achieved therapeutic efficacy and prevented development of resistance against DAP-R R497.

To gain insights into the mechanistic basis of the so-called see-saw effect (resensitization to β-lactams in DAP-R strains), we characterized the possible role of penicillin-binding proteins (PBPs) in the phenotype. We found that the synergistic activity of the DAP plus AMP combination was correlated with the *pbp5* allele sequence and the binding affinity of Bocillin-FL (BOC-FL), a fluorescent penicillin derivative, to PBP 5 (PBP5). No alterations in transcript levels of the *pbp* genes or protein levels of PBP5 were observed. Our results suggest that β-lactam interactions with PBP5 isotypes may be important mediators of the see-saw phenomenon in enterococci.

MATERIALS AND METHODS

Bacterial Strains

E. faecium R497 is a DAP-R (DAP MIC, 16 μg/mL) clinical isolate [17–19] that harbors the T120S and W73C substitutions in LiaS and LiaR, respectively, and was used in all SEV and in vivo experiments. The other strains investigated in this research include HOU503, a vancomycin-resistant *E. faecium* and DAP-tolerant clinical isolate (MIC, 2 μg/mL; DAP-S dose dependent), S447 a vancomycin-resistant *E. faecium* DAP-S (dose dependent) strain, lacking substitutions in the LiaFSR system, and R496, a DAP-R with LiaFSR substitutions (Supplementary Table 1).

Antimicrobial Agents and Media

DAP and ERT were obtained from Merck; AMP, ceftriaxone (CRO), and amoxicillin (AMX) powder were purchased from Sigma-Aldrich; and CPT was obtained from Allergan Pharmaceuticals. Mueller-Hinton broth II (MHB; Difco) with 50 μg/mL of calcium and 12.5 μg/mL magnesium was used for susceptibility testing. Because of the dependency of DAP on calcium for antimicrobial activity and calcium loss from the media due to calcium binding to albumin, MHB supplemental to a concentration of 75 μg/mL was used in the in vitro SEV model experiments, as described elsewhere [20]. Colony counts were determined using brain-heart infusion (BHI) agar supplemented with 50 μg/mL of calcium.

Susceptibility Testing

All MICs were determined in duplicate using microbroth dilution method [21] at approximately 5×10^5 colony-forming units (CFUs)/mL, following the Clinical and Laboratory Standards Institute guidelines [15]. Combination MIC values for DAP in the presence of β-lactams were determined by supplementing the broth with concentrations of β-lactam at their respective free peak concentration biological (C_{max}) and included AMP (70 μg/mL), CPT (17 μg/mL), and ERT (15.5 μg/mL). The combination MICs were performed at one-half, one-quarter, and one-eighth times the peaks, respectively, to investigate the impact of varying concentrations. The DAP MIC fold reduction from baseline was calculated by dividing DAP MICs by the MIC obtained in the presence of listed β-lactams.

Time-kill experiments were performed in the broth containing approximately 3 to 3.5 g/dL of albumin and equivalent of 50 μg/mL calcium in 24 hours to reproduce the protein binding characteristic of the aforementioned antibiotics. Time-kill plots were generated by plotting mean colony counts versus time to compare 24-hour time killing effects of the different antibiotic combinations. Bactericidal activity was defined as a decrease of ≥3 $\log_{10}$ CFUs/mL, and synergy between 2 agents was defined as ≥2 $\log_{10}$ CFUs/mL reduction at the end of 24 hours in comparison with the most potent single agent alone [15]. Antibiotic carryover was addressed by serial dilutions of the samples, and the lower limit of detection was 100 CFUs/g.

In Vitro PK/PD Model

A SEV PK/PD model [22] was used for all antibiotic experiments and has been described elsewhere [23, 24]. DAP was administered once daily via an injection port. The simulated DAP regimens with a targeted half-life ($t_{½}$) of 8 hours were 6, 8, 10, and 14 mg/kg/d, with peaks of 93.9, 123.3 141.1, and 197.54 μg/mL, respectively [14]. The DAP regimens were tested alone and in combination with AMP (2 g) continuous infusion (70 μg/mL) and AMX (2 g) continuous infusion (16.1 μg/mL), using AMP and AMX supplied daily, to ensure drug stability, CRO (2 g) ($C_{max,}$ 257 μg/mL; $t_{½}$, 8 hours) every 24 hours, ERT (1 g) ($C_{max,}$ 155 μg/mL; $t_{½}$, 4 hours) every 24 hours, or CPT (600 mg) (C_{max}, 20.448 μg/mL; $t_{½}$, 2.66 hours) every 12 hours.

SEV samples were removed in duplicate from each model over the sampling period of 0–336 hours. All samples were plated for CFU counts (CFUs per gram), and emergence of resistance was tested by plating on DAP-containing agar at 3 times the baseline DAP MIC. Any colonies detected on drug plates were tested for changes in MIC, using microbroth dilution MIC testing according to Clinical and Laboratory Standards Institute guidelines [15].

Humanized Model of Rat Infective Endocarditis

Aortic valve endocarditis induction, infective dose ($ID_{90–100}$) determination, bacterial inoculation in anesthetized male Sprague-Dawley, jugular vein catheterized rats, and the use of a programmable pump to infuse the test drugs intravenously via jugular vein were carried out, following methods published elsewhere [14, 25]. Animals were inoculated with ≥10^7 CFUs per rat, inoculum representing ≥10 times the ID_{90} and antibiotic infusion therapy starting 24 hours after bacterial inoculation. At

the time of therapy initiation, 2–3 animals per each experiment were euthanized as baseline untreated controls to determine the CFU counts of bacteria in vegetations, as described elsewhere [14, 25, 26].

We used a humanized DAP dose of 6 mg/kg/d (45.3 mg/kg/d for rats) for 3 days, as published elsewhere [14, 25]. Experimental efficacious doses used in published rat endocarditis or murine models were used for CPT (40 mg/kg; intravenous infusion via jugular vein for 30 minutes, every 8 hours for 3 days) [26] AMP (333.33 mg/kg; intravenous infusion for 30 minutes, every 8 hours for 3 days) [27] and ERT (20 mg/kg; infusion for 30 minutes, every 8 hours for 3 days) [28]. Animals were euthanized ≥16 hours after the last antibiotic dose and CFU counts of vegetation (CFUs per gram) were determined and compared with each other and with controls. The unpaired *t* test was used for statistical analysis with Prism 4 software for Windows (GraphPad). The minimum detection limit was 10^1 CFUs/g of tissue. The ID_{90} values were determined using the method outlined by Reed and Muench [29, 30].

PBP5 Sequence Alignment, Protein Quantification, and Gene Expression Analyses

Whole-genome sequences for S447, HOU503, and R497 are available on the National Center for Biotechnology Information Web site and were used to retrieve the *pbp5* sequence. DNA and amino acid sequence multiple alignments were performed using the MUSCLE tool from the European Bioinformatics Institute Web site (http://www.ebi.ac.uk/Tools/msa/muscle/). The results of the bioinformatics analysis were confirmed by means of polymerase chain reaction (PCR) amplification and Sanger sequencing. For expression analyses, all strains were grown in BHI broth at 37°C to the exponential phase (optical density at 600 nm, approximately 0.8), and RNA was extracted using a PureLink RNA Mini Kit (Ambion) in 3 technical and 3 biological replicates.

Subsequently, treatment with Turbo DNAse kit (Ambion) was performed to remove genomic DNA. The complementary DNA was generated from approximately 1 µg of purified RNA, using SuperScript II Reverse Transcriptase (Invitrogen). Gene expression was evaluated with 5 ng of complementary DNA using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Relative expression ratios were calculated by normalizing to the housekeeping genes *gyrB* and *ddl*.

Because the efficiency was different with the primers investigated, fold changes were calculated using an efficiency-corrected calculation model described by Pfaffl [31]. Primer efficiency was determined by the LinRegPCR software, version 11.0, program in each reaction. Differences in gene expression between pairs of strains were calculated using the normalized expression for each gene and considered significant at $P < .05$ (based on 2-tailed unpaired Student *t* test). Results are an average of 3 independent experiments with 3 biological replicates each. S447 gene expression was considered baseline for relative comparison with HOU503 and R497 levels.

BOC-FL Labeling of PBPs

Cells grown overnight were inoculated at a 1:50 dilution into fresh 50 mL of BHI and grown until the midexponential phase (optical density at 600 nm, approximately 0.8–1). Cells were spun down and washed in 1× phosphate-buffered saline before being disrupted with glass beads in the FastPrep24 instrument. Cell debris were spun down, and supernatants centrifuged at 100 000*g* for 1 hour. Membrane pellets were resuspended in 150 µL of phosphate-buffered saline with protease inhibitor cocktails (Roche) and mechanically disrupted for optimal homogenization without chemical agents.

Whole-protein content was quantified using a Pierce bicinchoninic assay (Thermo Fisher Scientific). Next, 100 µg of whole-membrane extracts was incubated for 30 minutes at 37°C with 100 µmol/L of BOC-FL in the dark. The reaction was stopped with 0.1% Triton-X 100 for 20 minutes at 4°C before spinning down at 15 000 rpm for 10 minutes and collection of the supernatants for Western blotting. Then 25 µL of the samples were loaded and run on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and visualized with the Bio-Rad ChemiDoc Alpha Imager under the Alexa 488 filter. Individual PBP proteins were identified by bands corresponding to their known molecular weights, based on previous findings [32, 33].

RESULTS

DAP MIC Reduction in Presence of β-Lactams and Synergy with DAP Plus AMP Against R497

The presence of β-lactams (AMP, CPT, ERT, AMX, piperacillin, or CRO) reduced the DAP MIC between 16- and 32-fold (Supplementary Table 2). The MICs against *E. faecium* R497 were as follows: DAP, 16 µg/mL; CPT, >64 µg/mL; AMP, >64 µg/mL; ERT, >64 µg/mL; DAP plus CPT, 4 µg/mL; DAP plus AMP, 2 µg/mL; and DAP plus ERT, 4 µg/mL. The concentration of the β-lactam antibiotic in the medium did not seem to affect the DAP MIC change, because concentrations below the C_{max} (one-half, one-quarter, and one-eighth times the C_{max}) produced similar reductions in the DAP MIC, compared with the C_{max}. Nonetheless, despite the important effect of all the β-lactam antibiotics on the DAP MIC, only the combination of AMP plus DAP was synergistic in time-kill experiments (Figure 1), although the effect was not bactericidal.

β-Lactam-Dependence of DAP Combinations Against *E. faecium* R497

DAP monotherapy (at 10 mg/kg/d) and DAP in combination with CPT, ERT, or CRO showed no activity against R497 in the SEV PK/PD models, with selection of resistant isolates with elevated MICs as high as >64 µg/mL (Figure 2 and Supplementary Table 3).

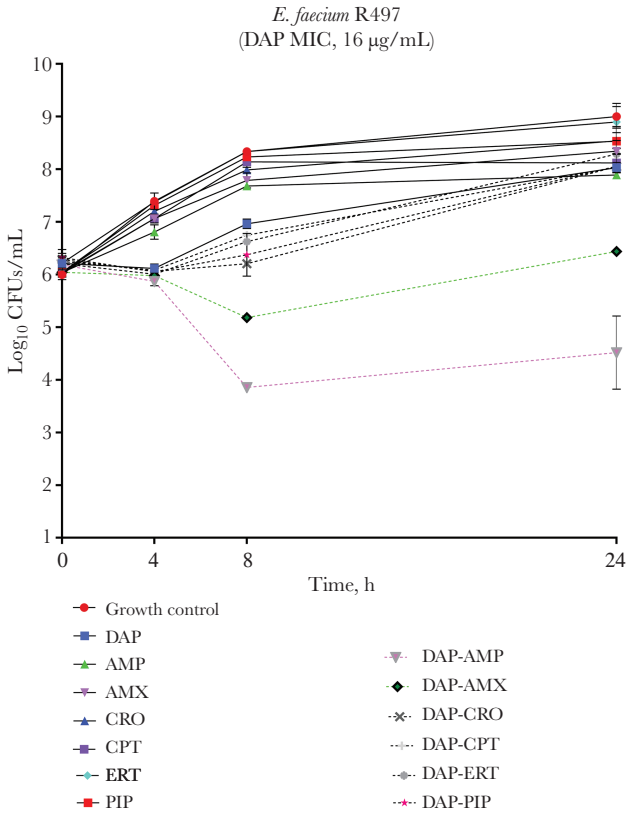

Figure 1. Results of 24-hour time-kill experiments against *Enterococcus faecium* R497 using various daptomycin (DAP) and β-lactam combinations. Abbreviations: AMP, ampicillin; AMX, amoxicillin; CPT, ceftaroline; CRO, ceftriaxone; ERT, ertapenem; PIP, piperacillin.

Even regimens of DAP at 14 mg/kg with ERT or CPT exhibited no efficacy. In contrast, the combination of DAP (10 mg/kg/d) plus AMP demonstrated enhanced killing activity compared with DAP alone. This regimen reached CFU counts below detection limits at 24 hours and maintained that level for the duration of the in vitro model. Of note, DAP doses <10 mg/kg combined with AMP had similar killing activity and, compared with 10 mg/kg/d, only a delay in reaching CFU counts below the detection limit was observed (Figure 2B). DAP in combination with AMX caused a reduction in CFU count, to at or slightly above the level of detection during the 14-day experiments (Figure 2C). Emergence of DAP resistance was prevented in all experiments using DAP in combination with AMP or AMX. The achieved pharmacokinetics parameters for DAP, CPT, AMP, AMX, CRO and ERT are shown in Supplementary Table 4 and coefficients of variation between all standards for each assay are listed in Supplementary Table 5.

Agreement Between Humanized Rat Endocarditis Model and SEV PK/PD Model Results

To validate the in vitro findings in the SEV PK/PD model, we tested the in vivo efficacy of DAP combinations with AMP, ERT, or CPT in the rat infective endocarditis model. DAP was used at a humanized dose of 6 mg/kg/d for 3 days. Results for controls (infected; no treatment for 24 hours) and all infected animals plus treatment bacterial counts ($\log_{10}$ CFUs per gram) after 3 days of monotherapy or combination therapy are shown in Figure 3. DAP plus AMP was the most efficacious combination, showing sterile vegetation in 50% (4 of 8 rats) and a statistically significant ($P < .001$) reduction in geometric mean (standard deviation) CFU count (1 [1] $\log_{10}$ CFUs) compared with controls (5.4 [0.7] $\log_{10}$ CFUs) and compared with all the other regimens (Table 1).

Effect of PBP Sequence on Affinity of β-Lactams for PBP5

We determined the PBP5 sequence to evaluate the mechanistic basis for the varying efficacies of DAP and β-lactam combinations in killing and preventing the emergence of DAP resistance in HOU503 versus R497. We hypothesized that R497 responds only to the DAP plus AMP combination owing to decreased PBP5 β-lactam binding affinity relative to that of HOU503. BOC-FL labeling of PBPs embedded in the membrane revealed that the low-molecular-weight PBP5 of HOU503 had increased β-lactam binding affinity relative to PBP5 from a DAP-S strain S447, or the DAP-R, R497 (Figure 4A). Relative gene expression levels of all *pbp* genes were evaluated and compared between S447, HOU503, and R497, by means of quantitative reverse-transcription PCR with normalization to the housekeeping genes, *gyrB* and *ddl*. There were no significant changes in *pbp* transcript levels in the strains (Figure 4B). Immunoblotting also indicated no significant changes in PBP5 levels between the strains (Figure 4C).

These results suggested that *pbp5* allele sequence variation is likely contributing to alterations in β-lactam binding affinity. Thus, we compared the *pbp5* sequence of the strains. All 3 clinical strains (S447, HOU503, and R497) had classic mutations widely associated with high-level AMP-resistance (M485A and 466'S) [17, 34–36]. Interestingly, the *pbp5*-predicted amino acid sequences of the DAP-S S447 were identical to those of the DAP-R R497. In contrast, PBP5 of HOU503 had substitutions in the transpeptidase domain (H408Q, A462V, T546N, T558A, S582G, and V586L) and the penicillin-binding domain (Q632K and L642P), compared with that of HOU503 (Supplementary Figure 1). Thus, HOU503 harbors, to our knowledge, a previously unreported *pbp5* allele that includes both classic AMP-susceptible and AMP resistance–related mutations in the *E. faecium* population. We speculate that these substitutions produce a hybrid *pbp5* allele and contribute to increased β-lactam binding affinity to PBP5. Thus, *pbp5* alleles are likely to correlate with the activity of specific β-lactams in the see-saw effect associated with nonsusceptibility to DAP.

DISCUSSION

Treatment of infections caused multidrug-resistant *E. faecium* is challenging, and previous in vitro and limited clinical data suggest that the combination of DAP plus β-lactams is a promising

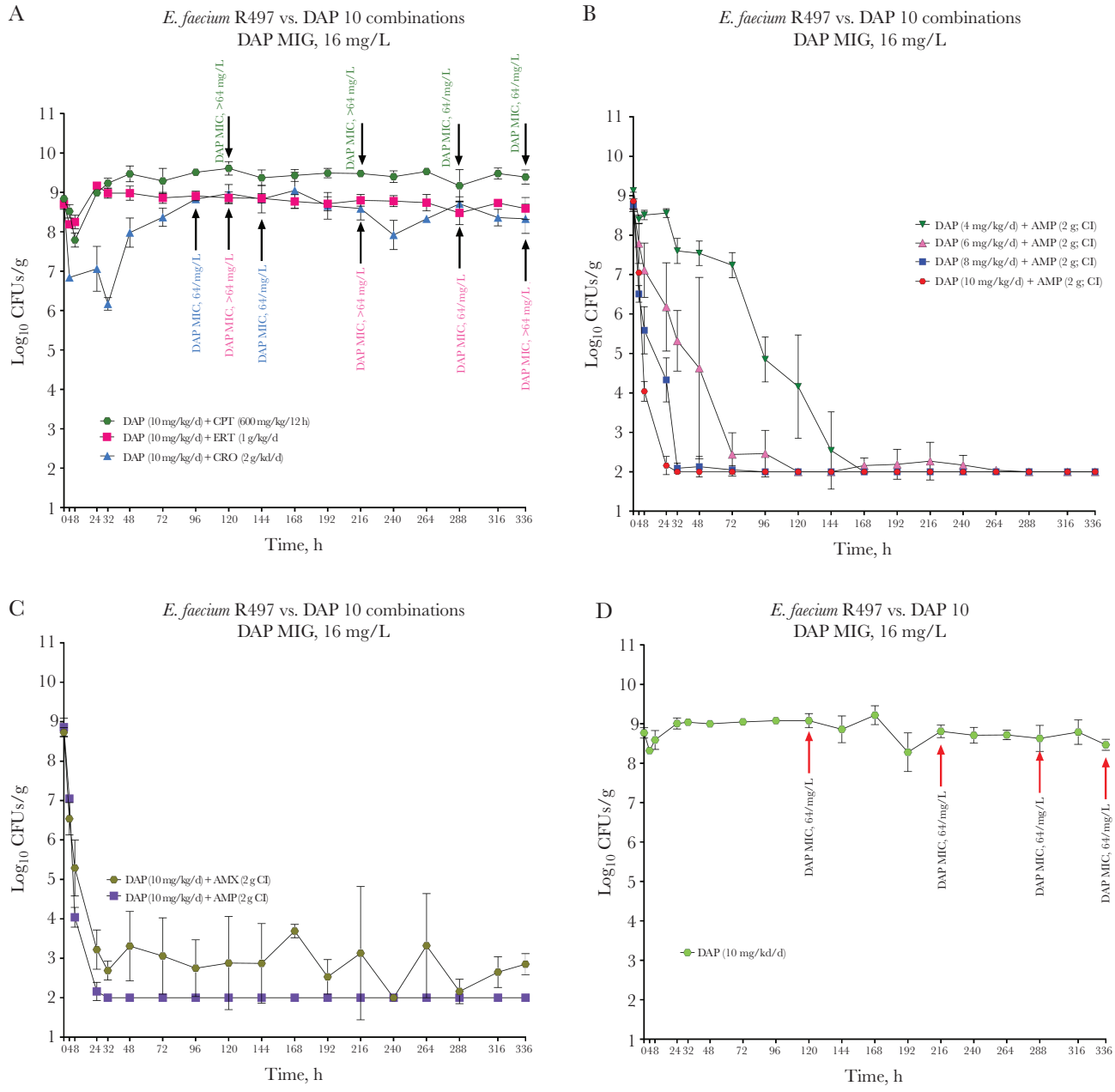

Figure 2. Results of simulated endocardial vegetation pharmacokinetic-pharmacodynamic model *A,* Comparison of daptomycin (DAP) activity against *Enterococcus faecium* R497 with ceftaroline (CPT), ceftriaxone (CRO), or ertapenem (ERT); arrows show development of resistance at various time points. *B,* DAP–ampicillin (AMP) combinations using dose deescalation of DAP. *C,* Comparison between combinations of DAP-AMP and DAP–amoxicillin (AMX) *D,* DAP monotherapy (10 mg/kg/d); arrows show development of resistance at various time points. Abbreviation: CI, continuous infusion.

strategy. We have previously shown that DAP combined with AMP, CPT, or ERT not only was efficacious in vitro and in vivo against a DAP-tolerant strain of *E. faecium* (HOU503) harboring known substitutions in *liaFSR* but also prevented emergence of resistance during therapy [16]. Moreover, the therapeutic efficacy of DAP was similar even at doses as low as 6 mg/kg when any of the β-lactams tested (AMP, CPT, or ERT) was added to the regimen. Although the mechanistic basis of the phenomenon is unknown, these encouraging results with a DAP-tolerant strain (exhibiting MICs within the susceptible range) raised the possibility of using the same strategy for fully DAP-R *E. faecium* strains exhibiting high MICs.

In the current study, using *E. faecium* (R497) (a DAP-R strain with MIC of 16 μg/mL) and harboring *liaFSR* changes (among others previously associated with DAP-R), we show that the only efficacious regimen against this strain, both in vitro and in vivo, was the combination of DAP plus AMP. DAP plus AMX (a derivative of AMP) had therapeutic efficacy similar to that of

Table 1. Animal Model Results

Treatment[a]	Vegetation, Mean (SD), Log_{10} CFUs/g	Comparison	Difference Between Means (CFU/g)[b]	P Value[c]
DAP + AMP	1 (1)	Versus baseline untreated controls (5.4 [0.7])	−4.4 (0.5)	<.001
DAP + CPT	3.3 (1)		−2.1 (0.8)	.03
DAP	4.6 (1)		−0.7 (0.7)	.31
CPT	6.4 (0.1)		+0.9 (0.3)	.01
DAP + AMP	1 (1)	Versus DAP + CPT	−2.3 (0.9)	.03
		Versus DAP	−3.6 (0.8)	<.001
		Versus CPT	−5.3 (0.5)	<.001
		Versus AMP	−4.2 (0.7)	<.001
		Versus DAP + ERT[d]	−4.7 (0.9)	<.001
DAP	4.6 (1)	Versus CPT	−1.7 (0.7)	.04
AMP	5.2 (1)	Versus CPT	−1.1 (0.7)	.13

Abbreviation: AMP, ampicillin; CFUs, colony-forming units; CPT, ceftaroline; DAP, daptomycin; ERT, ertapenem.

[a]All antibiotics were administered daily day for 3 days against *Enterococcus faecium* strain R497. The dosages were as follows: DAP, 45.3 mg/kg (7-hour intravenous infusion every 24 hours); AMP, 333 mg/kg (30-minute infusion every 8 hours); and CPT, 40 mg/kg (30-minute infusion every 8 hours).

[b]Negative values in this column indicate that CFU counts were lower than in the comparison group (control or other treatment group); positive values, that CFU counts were higher than in controls.

[c]Data were log-transformed, and unpaired *t* tests were performed to obtain *P* values.

[d]ERT dosage: 20 mg/kg (infusion for 30 minutes, every 8 hours for 3 days).

NOTE: The first 4 rows are compared with controls.

DAP plus AMP, albeit with a less prominent reduction in bacterial counts. Furthermore, the success of this combination was dependent on DAP exposure; the time required for killing to the limits of detection was reliant on the dose (AUC/MIC) exposure to DAP. Interestingly, CPT and ERT, which were previously shown to improve DAP therapeutic efficacy (*E. faecium* HOU503), failed against *E. faecium* R497. Thus, our current results implicate the importance of β-lactam specific structure differences and DAP concentration exposure dependence in the final efficacy of DAP–β-lactam combination.

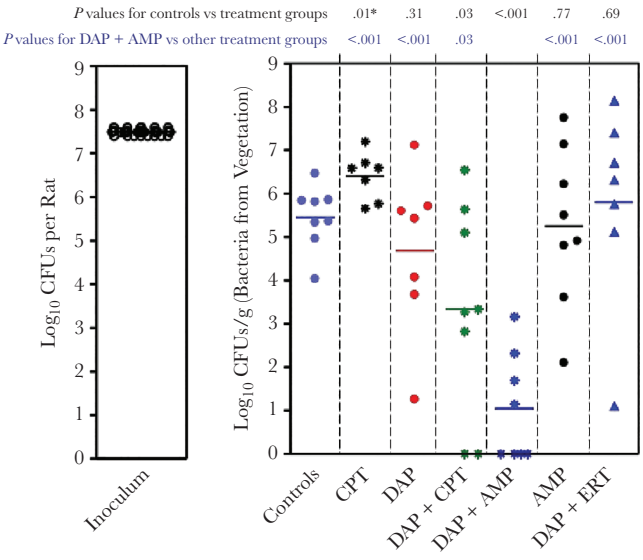

Figure 3. The results of in vivo efficacy of daptomycin (DAP) in combination with ampicillin (AMP), ertapenem (ERT), or ceftaroline (CPT) in the rat infective endocarditis model. CPT (40 mg/kg) and DAP (45.3 mg/kg; both by 30-minute intravenous infusion via the jugular vein, every 8 hours for 3 days) were used against *Enterococcus faecium* strain R497. Horizontal lines represents geometric mean colony-forming unit (CFU) counts. Previously published experimental efficacious doses of AMP (333.33 mg/kg/8 h), ERT (20 mg/kg/8 h), and CPT (40 mg/kg/8 h) for 3 days were used. Controls represent no antibiotic treatment.

These results were highly reproducible in both our SEV PK/PD model and a humanized model of rat endocarditis. Moreover, in vitro MIC determinations did not predict the SEV PK/PD model results, because MIC reduction was shown in all DAP–β β-lactam combinations. Of interest, cation-adjusted MHB with 50 mg/L of calcium ion was used for MIC experiments and albumin broth in time-kill experiments and SEV models. Thus, the discrepancy between the results may be due to additional protein binding of β-lactams in the medium containing albumin. In time-kill experiments, only the DAP plus AMP combination displayed synergy, while the DAP plus AMX combination stayed at the initial inoculum level at the end of 24-hour exposure. These results confirm one of the major limitations of DAP MIC determination and the lack of translatability of this test to in vivo situations. Furthermore, the bacterial density used in our models is significantly higher than that for MIC determination, and both DAP and β-lactams are affected by the inoculum [16].

The discrepancy in the effect of the DAP plus β-lactam combinations between *E. faecium* HOU503 and R497 prompted us to provide insights into the actual mechanism of the DAP plus β-lactam synergism. Both HOU503 and R497 harbor the classic substitutions in the response regulator LiaR (W73C) and the LiaS sensor histidine kinase (T120S) previously associated with activation of the LiaFSR system [35].

In *E. faecalis*, we recently showed [9] that the major effector of the LiaFSR response is through LiaX, a novel sentinel protein that

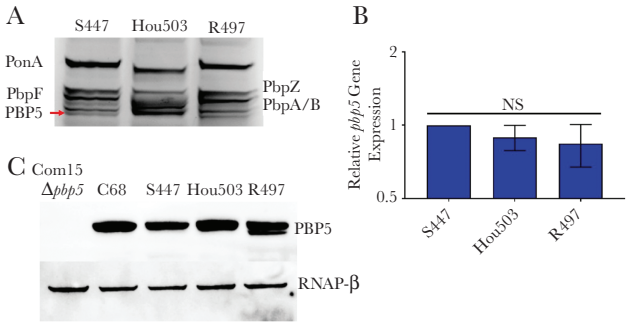

Figure 4. Penicillin-binding protein (PBP) 5 (PBP5) of HOU503 has increased β-lactam binding affinity, independent of gene expression and protein levels. *A,* PBP β-lactam affinity determined by Bocillin FL labeling of PBPs embedded in enterococcal membrane fractions, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualization of fluorescence intensities. PBP5 (*red arrow*) has increased band intensity in HOU503 relative to S447 and R497. *B,* There are no significant differences in *pbp5* relative gene expression, as determined with quantitative reverse-transcription. Statistics were calculated with data from 3 biological replicates and 3 internal technical replicates. Data represent means with standard deviations (*error bars*). *C,* PBP5 expression levels determined by Western blotting cell lysates. Com15, a clade B strain lacking *pbp5,* was used as a negative control. C68, a hospital-associated clade A1 strain used to purify PBP5 for antibody production, served as a positive control. An antibody against the RNA polymerase β subunit (RNAP-β) was used as the loading control.

detects the presence of DAP and antimicrobial peptides in the extracellular milieu leading to activation of cell membrane adaptation and redistribution of anionic phospholipid microdomains. LiaX-mediated resistance to DAP and innate immune antimicrobial peptides also leads to increased virulence in vivo [9]. Interestingly, LiaX has a homolog in *E. faecium* (designated PBP5-binding protein, and also regulated by LiaR) [35, 36], which seems to be involved in β-lactam resistance, connecting the LiaFSR system not only to membrane homeostasis but also to peptidoglycan synthesis. Thus, LiaX-mediated changes in the cell membrane can hypothetically affect the functions or affinity of PBPs and, ultimately, lead to the see-saw effect or β-lactam resensitization. Indeed, the differential affinity of β-lactams to PBPs has been shown before in *Staphylococcus aureus* [11, 37].

Using the available sequences of PBP5 of R497 and HOU503, we show that there are 7 changes in the PBP5 sequence of R497 compared with HOU503 (Supplementary Figure 1). All the amino acid substitutions are located in the transpeptidase and penicillin-binding domains. The changes did not seem to affect the expression or amount of PBP5 available. However, using BOC-FL labeling of PBPs in the membrane, we showed that PBP5 of HOU503 had increased β-lactam binding affinity compared with R497 and a DAP-S strain (S447). Of note, R497 and S447 have identical *pbp5* alleles. Thus, our results suggest the possibility that the PBP5 allele sequence is a major determinant of the see-saw effect in terms of β-lactam selectivity. Indeed, because AMP seems to have the highest affinity for PBP5 [38, 39], it seems reasonable to select this compound as the best partner for DAP when treating deep-seated infections due to *E. faecium*. Moreover, our data suggest that a sequence-based approach (*pbp5* sequence) could be used to determine the ability of β-lactams to synergize with DAP. Our findings also support the notion that the initial activation of *liaFSR* is the critical genetic event for the evolution of resistance both in vivo and in vitro, and, after such occurrence, multiple pathways are plausible.

In summary, we present evidence that the success of DAP–β-lactam combination therapy against multidrug-resistant *E. faecium* with LiaFSR substitutions is likely dependent on β-lactam interactions with PBPs. The combination of DAP (even at 4 mg/kg) with AMP, is effective in infections of high bacterial density, preventing developing of resistance. Our findings suggest a possible new approach to severe enterococcal infections using a combination of DAP plus AMP antibiotics initially, as well as the potential to use lower DAP doses.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Financial support. This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (grant R01 AI121400 to M. J. R. and grants K24-AI121296, R01AI134637, and R21AI143229 to C. A. A.)

Potential conflicts of interest. B. E. M. and C. A. A. have received grant support from Merck, MeMed Diagnostics, and Entasis Pharmaceuticals. MJR has received support from, consulted for, or provided lectures on behalf of Allergan and Merck. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Mishra NN, Bayer AS, Tran TT, et al. Daptomycin resistance in enterococci is associated with distinct alterations of cell membrane phospholipid content. PLoS One **2012**; 7:e43958.
2. Diaz L, Tran TT, Munita JM, et al. Whole genome analyses of *Enterococcus faecium* with diverse daptomycin minimal inhibitory concentrations. Antimicrob Agents Chemother **2014**; 58:4527–34.
3. Humphries RM, Kelesidis T, Tewhey R, et al. Genotypic and phenotypic evaluation of the evolution of high-level daptomycin nonsusceptibility in vancomycin-resistant *Enterococcus faecium*. Antimicrob Agents Chemother **2012**; 56:6051–3.

4. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis **2004**; 39:309–17.
5. Arias CA, Contreras GA, Murray BE. Management of multidrug-resistant enterococcal infections. Clin Microbiol Infect **2010**; 16:555–62.
6. Safdar N, Andes D, Craig WA. In vivo pharmacodynamic activity of daptomycin. Antimicrob Agents Chemother **2004**; 48:63–8.
7. Arias CA, Panesso D, McGrath DM, et al. Genetic basis for in vivo daptomycin resistance in enterococci. N Engl J Med **2011**; 365:892–900.
8. Prater AG, Mehta HH, Kosgei AJ, et al. Environment shapes the accessible daptomycin resistance mechanisms in *Enterococcus faecium*. Antimicrob Agents Chemother **2019**; 63: e00790-19.
9. Khan A, Davlieva M, Panesso D, et al. Antimicrobial sensing coupled with cell membrane remodeling mediates antibiotic resistance and virulence in *Enterococcus faecalis*. Proc Natl Acad Sci USA **2019**; 116:26925–32.
10. Smith JR, Barber KE, Raut A, Aboutaleb M, Sakoulas G, Rybak MJ. β-Lactam combinations with daptomycin provide synergy against vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium*. J Antimicrob Chemother **2015**; 70:1738–43.
11. Cotroneo N, Harris R, Perlmutter N, Beveridge T, Silverman JA. Daptomycin exerts bactericidal activity without lysis of *Staphylococcus aureus*. Antimicrob Agents Chemother **2008**; 52:2223–5.
12. Berti AD, Sakoulas G, Nizet V, Tewhey R, Rose WE. β-Lactam antibiotics targeting PBP1 selectively enhance daptomycin activity against methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **2013**; 57:5005–12.
13. Barber KE, Werth BJ, Rybak MJ. The combination of ceftaroline plus daptomycin allows for therapeutic de-escalation and daptomycin sparing against MRSA. J Antimicrob Chemother **2015**; 70:505–9.
14. Kebriaei R, Rice SA, Singh KV, et al. Influence of inoculum effect on the efficacy of daptomycin monotherapy and in combination with β-lactams against daptomycin-susceptible *Enterococcus faecium* harboring LiaSR substitutions. Antimicrob Agents Chemother **2018**; 62:e00315-18.
15. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 26th ed. M100 2016. Clinical Laboratory Standards Institute, **2018**.
16. Humphries RM. The new, new daptomycin breakpoint for *Enterococcus* spp. J Clin Microbiol **2019**; 57:e00600-19.
17. Panesso D, Reyes J, Gaston EP, et al. Deletion of *liaR* reverses daptomycin resistance in *Enterococcus faecium* independent of the genetic background. Antimicrob Agents Chemother **2015**; 59:7327–34.
18. Reyes J, Panesso D, Tran TT, et al. A *liaR* deletion restores susceptibility to daptomycin and antimicrobial peptides in multidrug-resistant *Enterococcus faecalis*. J Infect Dis **2015**; 211:1317–25.
19. Munita JM, Tran TT, Diaz L, et al. A *liaF* codon deletion abolishes daptomycin bactericidal activity against vancomycin-resistant *Enterococcus faecalis*. Antimicrob Agents Chemother **2013**; 57:2831–3.
20. Steed ME, Vidaillac C, Rybak MJ. Novel daptomycin combinations against daptomycin-nonsusceptible methicillin-resistant *Staphylococcus aureus* in an in vitro model of simulated endocardial vegetations. Antimicrob Agents Chemother **2010**; 54:5187–92.
21. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc **2008**; 3:163–75.
22. Marron A, Carratalà J, Alcaide F, Fernández-Sevilla A, Gudiol F. High rates of resistance to cephalosporins among viridans-group streptococci causing bacteraemia in neutropenic cancer patients. J Antimicrob Chemother **2001**; 47:87–91.
23. Hall AD, Steed ME, Arias CA, Murray BE, Rybak MJ. Evaluation of standard- and high-dose daptomycin versus linezolid against vancomycin-resistant *Enterococcus isolates* in an in vitro pharmacokinetic/pharmacodynamic model with simulated endocardial vegetations. Antimicrob Agents Chemother **2012**; 56:3174–80.
24. Hershberger E, Coyle EA, Kaatz GW, Zervos MJ, Rybak MJ. Comparison of a rabbit model of bacterial endocarditis and an in vitro infection model with simulated endocardial vegetations. Antimicrob Agents Chemother **2000**; 44:1921–4.
25. Smith D, Singh K, Tran T, Arias C, Murray B. Daptomycin plus ceftaroline against daptomycin-resistant *Enterococcus faecium* in a rat model of experimental endocarditis. Open Forum Infect Dis **2016**; 3. doi:10.1093/ofid/ofw172.1528
26. Singh KV, Tran TT, Nannini EC, Tam VH, Arias CA, Murray BE. Efficacy of ceftaroline against methicillin-susceptible *Staphylococcus aureus* exhibiting the cefazolin high-inoculum effect in a rat model of endocarditis. Antimicrob Agents Chemother **2017**; 61:e00324-17.
27. Pefanis A, Thauvin-Eliopoulos C, Eliopoulos GM, Moellering RC Jr. Activity of ampicillin-sulbactam and oxacillin in experimental endocarditis caused by beta-lactamase-hyperproducing *Staphylococcus aureus*. Antimicrob Agents Chemother **1993**; 37:507–11.
28. Catena F, Ansaloni L, Gazzotti F, et al. Effect of early antibiotic prophylaxis with ertapenem and meropenem in

experimental acute pancreatitis in rats. J Hepatobiliary Pancreat Surg **2009**; 16:328–32.
29. Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. World J Virol **2016**; 5:85–6.
30. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Epidemiol **1938**; 27:493–7.
31. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **2001**; 29:e45.
32. Henry X, Amoroso A, Coyette J, Joris B. Interaction of ceftobiprole with the low-affinity PBP 5 of *Enterococcus faecium*. Antimicrob Agents Chemother **2010**; 54:953–5.
33. Arbeloa A, Segal H, Hugonnet JE, et al. Role of class A penicillin-binding proteins in PBP5-mediated beta-lactam resistance in *Enterococcus faecalis*. J Bacteriol **2004**; 186:1221–8.
34. Pietta E, Montealegre MC, Roh JH, Cocconcelli PS, Murray BE. *Enterococcus faecium* PBP5-S/R, the missing link between PBP5-S and PBP5-R. Antimicrob Agents Chemother **2014**; 58:6978–81.
35. Montealegre MC, Roh JH, Rae M, et al. Differential penicillin-binding protein 5 (PBP5) levels in the *Enterococcus faecium* clades with different levels of ampicillin resistance. Antimicrob Agents Chemother **2017**; 61:e02034-16.
36. Rice LB, Bellais S, Carias LL, et al. Impact of specific *pbp5* mutations on expression of beta-lactam resistance in *Enterococcus faecium*. Antimicrob Agents Chemother **2004**; 48:3028–32.
37. Cha R, Rybak MJ. Daptomycin against multiple drug-resistant *Staphylococcus* and *Enterococcus* isolates in an in vitro pharmacodynamic model with simulated endocardial vegetations. Diagn Microbiol Infect Dis **2003**; 47:539–46.
38. Sifaoui F, Arthur M, Rice L, Gutmann L. Role of penicillin-binding protein 5 in expression of ampicillin resistance and peptidoglycan structure in *Enterococcus faecium*. Antimicrob Agents Chemother **2001**; 45:2594–7.
39. Rice LB, Carias LL, Hutton-Thomas R, Sifaoui F, Gutmann L, Rudin SD. Penicillin-binding protein 5 and expression of ampicillin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother **2001**; 45:1480–6.