Differential Effects of Penicillin Binding Protein Deletion on the Susceptibility of Enterococcus faecium to Cationic Peptide Antibiotics

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Beta-lactam antibiotics sensitize Enterococcus faecium to killing by endogenous antimicrobial peptides (AMPs) of the innate immune system and daptomycin through mechanisms yet to be elucidated. It has been speculated that beta-lactam inactivation of select E. faecium penicillin binding proteins (PBPs) may play a pivotal role in this sensitization process. To characterize the specific PBP inactivation that may be responsible for these phenotypes, we utilized a previously characterized set of E. faecium PBP knockout mutants to determine the effects of such mutations on the activity of daptomycin and the AMP human cathelicidin (LL-37). Enhanced susceptibility to daptomycin was dependent more on a cumulative effect of multiple PBP deletions than on inactivation of any single specific PBP. Selective knockout of PBPZ rendered E. faecium more vulnerable to killing by both recombinant LL-37 and human neutrophils, which produce the antimicrobial peptide in high quantities. Pharmacotherapy targeting multiple PBPs may be used as adjunctive therapy with daptomycin to treat difficult E. faecium infections.

In the United States, Enterococcus has emerged as a leading cause of nosocomial infections accounting for nearly 200,000 infections and at least 1,300 deaths annually. Management of systemic infections caused by Enterococcus faecium can be extremely challenging due to intrinsic antibiotic resistance, lack of potent bactericidal therapies, and frequent comorbidities of the patients in which they occur (1–3). Standard treatment is further hampered by the emerging and rising incidence of resistance to daptomycin, the only available antibiotic which may demonstrate bactericidal activity as monotherapy against E. faecium (4).

Our recent studies have revealed that several beta-lactam antibiotics render E. faecium more vulnerable to killing by the cationic peptide antibiotic, daptomycin, and to endogenous host antimicrobial peptides (AMPs) of the innate immune system such as human cathelicidin LL-37 (5–7). These observed phenomena have been utilized through daptomycin plus ampicillin in the successful treatment of refractory bacteremia due to vancomycin-resistant Enterococcus (VRE). However, the underlying mechanism of this drug synergy remains poorly understood (5).

Additional in vitro work has shown that the advanced-generation cephalosporin antibiotic cefaroline has very potent synergy with daptomycin (6). Other investigators have demonstrated that cefaroline binds to all of the high molecular-weight penicillin binding proteins (PBPs) of E. faecium, including PBP5 (8). It is unclear whether inactivation of a specific PBP is responsible for beta-lactam synergy with daptomycin. In the current investigation, we utilized the wild type (WT) and previously constructed class A PBP knockout strains in E. faecium (9) to determine the effects of loss of individual PBP functions on susceptibility to daptomycin and LL-37 in E. faecium.

MATERIALS AND METHODS

Bacterial strains. E. faecium strains used to examine effects of PBP mutations are listed in Table 1 and were obtained from prior studies (9–11). A vancomycin-resistant E. faecium strain was examined for daptomycin plus cefaroline synergy using simulated dosing of the two drugs in combination (6).

Antimicrobial susceptibility (MIC) testing: daptomycin susceptibility assays. Daptomycin susceptibility testing was performed three times using Etest (bioMérieux, Inc., Durham, NC) on brain heart infusion (BHI) agar plates on three different days. Daptomycin kill curves were performed in cation-adjusted Mueller–Hinton broth (MHB) supplemented with CaCl2 at 50 mg/liter using a starting inoculum of 107 CFU/ml. Samples were obtained at 0 and 24 h and serially diluted 1:10 to 1:107, and then 10 μl was plated in duplicate on Todd–Hewitt agar (THA) plates. Assays were performed in duplicate for each experiment and completed twice on separate days. Colonies were enumerated after 24 h, and the log CFU/ml was calculated for graphical presentation. The limit of detection was 1,000 CFU/ml (log10 = 3).

Daptomycin population analyses were performed on BHI agar plates supplemented with CaCl2 at 50 mg/liter. These conditions were chosen in order to allow for more subtle differences in susceptibility between the different strains to be determined compared to standard conditions. We have previously determined that the relative ranking of differences in daptomycin heteroresistance is the same under these conditions, but differences between the strains are amplified and therefore more easily analyzed (12).

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TABLE 1 *Enterococcus faecium* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
<th>Genotype</th>
<th>MICa (mg/liter)</th>
<th>COL (mg/liter)</th>
<th>PB (mg/liter)</th>
<th>DAP (mg/liter)</th>
<th>LL-37 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D344R</td>
<td>9</td>
<td>Wild-type <em>E. faecium</em></td>
<td>32</td>
<td>128</td>
<td>128</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>D344S</td>
<td>10</td>
<td>ΔpbpF ΔpbpZ (ΔPBPS) derivative of D344R</td>
<td>32</td>
<td>&gt;256</td>
<td>192</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>CV536</td>
<td>11</td>
<td>ΔpbpF (ΔPBPS) derivative of D344R</td>
<td>32</td>
<td>&gt;256</td>
<td>256</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>CV537</td>
<td>11</td>
<td>ΔpbpZ (ΔPBPS2) derivative of D344R</td>
<td>8</td>
<td>192</td>
<td>192</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>CV535</td>
<td>11</td>
<td>ΔpbpF ΔpapA derivative of D344R</td>
<td>32</td>
<td>192</td>
<td>128</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>CV538</td>
<td>11</td>
<td>ΔpbpF ΔpapZ ΔpapA derivative of D344R</td>
<td>32</td>
<td>256</td>
<td>192</td>
<td>1.5</td>
<td>32</td>
</tr>
<tr>
<td>CV598</td>
<td>11</td>
<td>ΔpapA (ΔPBPF) cv588 complemented</td>
<td>16</td>
<td>48</td>
<td>48</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>CV571/pCWR1686</td>
<td>11</td>
<td>with pbbP plasmid; Km</td>
<td>16</td>
<td>192</td>
<td>192</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>8019</td>
<td>6</td>
<td>Contemporary vancomycin-resistant <em>E. faecium</em></td>
<td>NP</td>
<td>NP</td>
<td>NP</td>
<td>0.38</td>
<td>2</td>
</tr>
</tbody>
</table>

a CPT, ceftaroline (broth microdilution); COL, colistin (Etest); PB, polymyxin B (Etest); DAP, daptomycin (Etest); LL-37 (broth microdilution); NP, not performed. Km, kanamycin resistance. For D344R and all its mutants, the MICs were as follows: vancomycin, 2 mg/liter (Etest); telavancin, <0.5 mg/liter (broth microdilution); linezolid, 0.75 to 1.0 mg/liter (Etest); and minocycline, 16 mg/liter (Etest).

Susceptibility testing to vancomycin, linezolid, colistin, polymyxin B, and minocycline (bioMérieux) were performed by Etest on BHI agar. BHI was utilized as described previously (9). Susceptibility testing to telavancin (Astellas Pharmaceuticals, Northbrook, IL) and ceftaroline (Forest Pharmaceuticals, New York, NY) was performed in BHI broth according to Clinical and Laboratory Standards Institute methods.

Polymyxin B (Sigma-Aldrich, St. Louis, MO) population analyses were performed in BHI agar according to daptomycin population analyses without calcium supplementation.

**In vitro PK/PD model and PD analysis.** An *in vitro*, one-compartment, pharmacokinetic/pharmacodynamic (PK/PD) model with a 250-ml capacity and input and output ports was used. The apparatus was prefilled with cation-adjusted MHB supplemented with calcium at 50 mg/ml, and antimicrobials were administered as boluses over a 48-h time period. Prior to each experiment, bacterial lawns from an overnight growth on BHI agar were suspended and added to each model to obtain a starting inoculum of ~10⁶ CFU/ml. Fresh medium was continuously supplied and removed from the compartment, along with the drug via a peristaltic pump (Masterflex; Cole-Parmer Instrument Company, Chicago, IL) at an appropriate rate to compensate for the organisms’ abilities to survive in the model. Models were performed in duplicate to ensure reproducibility. Supplemental daptomycin was added at an appropriate rate to CPT combination models to compensate for the higher flow rate required to simulate CPT clearance.

Samples from each model were collected at 0, 4, 8, 24, 32, and 48 h in duplicate and diluted in cold 0.9% saline. Colony counts were determined by spiral plating of appropriate dilutions by using an automatic spiral plater (WASP, DW Scientific, West Yorkshire, England) to enumerate CFU/ml and avoid antibiotic carryover. Colonies were counted using a laser colony counter (ProtoCOL, Synoptics, Ltd., Frederick, MD). If the anticipated dilution was near the MIC, then vacuum filtration was used to avoid antibiotic carryover. When vacuum filtration was used, samples were washed through a 0.45-µm-pore-size filter with normal saline to remove the antimicrobial agent. For both methods, plates were incubated at 35°C for 24 h before colonies were counted. These methods have a lower limit of reliable detection of 1 log₁₀ CFU/ml. The total reduction in log₁₀ CFU/ml over 48 h was determined by plotting time-kill curves based on the number of surviving organisms. Bactericidal activity (99.9% kill) was defined as a ≥3-log₁₀ CFU/ml decrease in colony count from the initial inoculum. Bacteriostatic activity was defined as a <3-log₁₀ CFU/ml reduction in colony count from the initial inoculum, and inactivity was defined as no observed reductions in the initial inoculum. The time to achieve a 99.9% bacterial load reduction was determined by linear regression or by visual inspection (if r² ≤ 0.95). Therapeutic enhancement of combinations was defined as ≥2-log₁₀ CFU/ml reduction over the most active single agent.

**Autolysis assays.** Bacteria were grown overnight (14 to 16 h) with shaking (200 rpm, 37°C) in 5 ml ofuria-Bertani (LB) broth supplemented with CaCl₂ at 50 mg/ml, washed once in phosphate-buffered saline (PBS), resuspended to optical density at 600 nm (OD₆₀₀) of 1.0 in 0.2% Triton X-100–PBS, and placed at 37°C with gentle shaking. The OD₆₀₀ was measured at specified intervals and expressed graphically. The experiment was performed three times on different days in duplicate, and the results of one representative experiment are shown (see Fig. 5). To test the effects of daptomycin on autolysis, similar methods were used, except that 5 μl of daptomycin at 16 mg/ml was added to the 5-ml overnight culture (final daptomycin concentration, 16 mg/ml), the sample was returned to the 37°C shaker for 1 h, and the autolysis assay was then performed as described above. Stationary-phase bacteria were used in order to reduce the effects on cell viability in the daptomycin pulsing experiment.

**Daptomycin binding.** Daptomycin binding assays were performed as previously described (5, 6). For these studies, bacteria were grown to an OD₆₀₀ of 0.5 in LB broth, to which was added BODIPY-daptomycin (Cubist Pharmaceuticals) at 0.5, 1, 2, or 4 mg/liter plus CaCl₂ at 50 mg/ml for 15 to 20 min. The antimicrobial activity of DAPT-daptomycin is =-2× the MIC of unlabeled daptomycin. Bacteria were then washed three times with fresh LB medium, with the final wash containing DAPI (4′,6′-diamidino-2-phenylindole) nucleic acid stain (Molecular Probes/Invitrogen) at 2 μg/ml, resuspended in 5% of the original volume, and transferred onto a 1.2% agarose pad containing 20% LB medium for microscopy. Imaging analysis was performed using ImageJ software v1.48f and CellProfiler 2.0. Using deconvolved images, BODIPY spot outlines were detected using the 2× the background signal cut off to get the outline of the spot. Spot outlines were overlaid onto corresponding nondeconvolved images for intensity analysis. For individual BODIPY spot intensity, the average signal in the spot and finally subtracted from its own image background signal to get the actual intensity. Two representative fields of each condition were analyzed. In every condition except for the wild-type parent (~150 cells) and the ΔPBPS mutant (~250 cells), more than >350 cells were included into analysis. To determine the number of spots per cell using the cell outline, we were able to count the BODIPY.
The document contains scientific research on the use of daptomycin and ceftaroline in the treatment of E. faecium infections. It discusses the susceptibility testing and killing assays performed in RPMI medium. There is a section on neutrophil killing assays, where neutrophils are isolated from healthy donors and incubated with bacteria to determine killing efficacy. The results show that the triple PBP knockout strain is more susceptible to daptomycin compared to the wild-type strain. The study also investigates the synergy between daptomycin and ceftaroline, demonstrating that the combination is more effective against VRE than each drug alone.

The results are visualized in various graphs and charts, which illustrate the killing efficacy over time and the MIC values for different strains. The document concludes with a discussion on the potential clinical implications of these findings, highlighting the importance of understanding the interaction between different antibiotics and the bacterial strains.
increased binding compared to the parent strain, both in terms of number of the foci/cell and the intensity of the bound label (Fig. 4). The assay was applied to Δphp5, ΔphpF, and ΔphpZ mutants compared to the parent using 2- and 4-mg/liter BODIPY-daptomycin concentrations in order to determine whether the differences seen in the daptomycin killing assays could tease out increased Δphp5 mutant binding compared to the rest of the mutants. Increased binding intensity was seen for Δphp5 and ΔphpZ mutants, compared to the ΔphpF knockout and the parent strain. The results of the - mg/liter BODIPY-daptomycin assays are shown in Fig. S2 in the supplemental material. The cytochrome c binding studies revealed that the enhanced daptomycin binding of Δphp5 and ΔphpZ strains was not dependent upon a global change in surface charge compared to the wild-type parent E. faecium strain (data not shown).

Autolysis studies showed that all of the PBP knockouts had increased autolysis compared to the parental WT strain (Fig. 5A). This difference was further accentuated after the strains were pulsed with daptomycin at 16 mg/liter for 1 h, where autolysis was reduced in the WT strain by daptomycin exposure but not in the various PBP knockout strains (Fig. 5B).

Given the interesting effects selective PBP knockouts had on the activity of daptomycin described above, we sought to determine whether these changes conferred differences in susceptibility to killing by an endogenous cationic AMP produced by the human innate immune system, cathelicidin LL-37. LL-37 MICs for the different strains are shown in Table 1. The WT parental E. faecium showed an LL-37 MIC of 32 μM, with reductions to 8 μM in the ΔphpZ, ΔponA, and ΔphpF ΔphpZ knockouts. LL-37 killing assays were performed at 0.5× the MIC of the parental E. faecium WT strain (16 μM) (Fig. 6). Paralleling the results of the daptomycin studies, the ΔphpZ and ΔphpZ ΔphpF knockouts demonstrated significant increases in LL-37 killing within 2 h.

**FIG 3** Daptomycin killing (reduction in log_{10} CFU/ml) at 24 h of E. faecium and Δphp5, ΔphpF, ΔphpZ, and ΔponA knockout derivatives. Using daptomycin at 1 and 2 mg/liter, the Δphp5 strain showed increased killing compared to the other strains (*, P < 0.05 compared to wild-type parental strain [Mann-Whitney U test]).

**FIG 4** BODIPY-labeled daptomycin binding of E. faecium and single PBP deletion mutants alone or in various combination. Binding was assessed both in terms of number of binding foci/cells (left panel) and the intensity of the foci (right panel). All of the deletion mutants showed increased binding by both parameters compared to the control wild-type strain (P < 0.0001 [Student t test]), except ΔponA foci/cell (*, P = 0.0036 [Student t test]).
Human neutrophil killing assays were also performed on the \( \Delta pbp5 \), \( \Delta pbpZ \), \( \Delta pbpF \), and WT strains to further assess LL-37 susceptibility differences and by utilizing two-way analysis of variance. The results in Fig. 7 demonstrate that only the \( \Delta pbpZ \) knockout strain showed a modest but statistically significant drop in viable bacteria from 15 to 45 min after neutrophil exposure, reinforcing that inhibition of \( pbpZ \) renders \( E. faecium \) more susceptible to innate immune system killing. An observed trend for \( \Delta pbp5 \) to confer increased neutrophil killing did not achieve statistical significance.

**FIG 5** Autolysis of \( E. faecium \) and PBP knockouts in 0.2% Triton X-100 in PBS after overnight growth to stationary phase (A) or after overnight growth and then pulsing with daptomycin at 16 mg/liter for 1 h.

**FIG 6** LL-37 (16 \( \mu \)M) killing assay results for \( E. faecium \) and PBP knockout mutants as specified. The percent survival results at 2 h are shown (*, \( P < 0.05 \) [Mann-Whitney U test]).
that genetic inactivation of PBPs through knockouts recapitulates pharmacological inactivation through beta-lactam antibiotics, which is likely but not proven. Nevertheless, the findings are in line with previously published data examining the pharmacodynamics interaction between peptide and beta-lactam antibiotics. For example, prior work showed that ampicillin reduced the daptomycin MIC by ~3-fold in VRE treated successfully with daptomycin plus ampicillin (5). The knockout mutants in the present study showed up to a 4-fold reduction in daptomycin Etest MICs in the triple ΔpbpF ΔponA ΔpbpZ knockout. Although small in magnitude, this 4-fold reduction in MIC could nevertheless have important clinical implications, given that daptomycin monotherapy has shown reduced efficacy in treating VRE bacteremia as the daptomycin MIC increases from 1 to 4 mg/liter (15). Interestingly, in that same study, the addition of concomitant beta-lactams increased the daptomycin treatment efficacy for VRE bacteremia in cases where the daptomycin MIC was 4 mg/liter up to the treatment efficacy rate seen for daptomycin monotherapy of VRE bacteremia with a daptomycin MIC of ≥2 mg/liter (15). Some experts have suggested that the daptomycin susceptibility breakpoint be reduced from 4 to 2 mg/liter for Enterococcus spp., with perhaps 4 mg/liter as an “intermediate” requiring combination therapy for bacteremia (16). Although the optimal dose of daptomycin for treating Enterococcus bacteremia has not been established, doses higher than the 6 mg/kg/day approved for S. aureus bacteremia have been shown to be bacteriostatic against isolates harboring mutations in LiaFSR (16).

We also evaluated the activity of the endogenous cationic AMP, cathelicidin LL-37, against our WT and knockout strains. LL-37 is mainly expressed by neutrophils and epithelial cells, and LL-37 concentrations of 15 to 25 μg/ml have been documented at sites of inflammation (17, 18). Evaluation of the different E. faecium mutants for LL-37 susceptibility revealed that the ΔpbpZ knockout was extremely sensitive to the AMP, showing a 4-fold drop in MIC from 32 to 8 μM (a physiologically achievable concentration) and a decrease in bacterial survival in LL-37 killing assays performed at one-half the WT strain LL-37 MIC. In neutrophil killing, the ΔpbpZ strain showed a modest yet statistically significant drop in viable bacteria surviving from 15 to 45 min, suggesting that inhibition of PBZ renders E. faecium more susceptible to the first responders of the innate immune system and secretors of LL-37 (i.e., neutrophils).

We used various concentrations of BODIPY-daptomycin in binding studies to determine whether the differences in daptomycin susceptibility could be demonstrated by differences in binding. We found that all of the PBP deletion mutants showed increased intensity and foci of BODIPY-daptomycin compared to the parent strain. However, using various concentrations BODIPY-daptomycin, we were not able to detect differences between these strains that correlated with daptomycin killing differences at low concentrations or with daptomycin MICS. This lack of correlation of the daptomycin binding studies to activity of daptomycin between the PBP deletion mutants raises the possibility that PBP deletion may result in compensatory bacterial membrane compositions that affect qualitative and quantitative daptomycin binding. Specifically, recent data suggest cardiolipin-mediated diversion away from the bacterial division septum may play a critical role in reducing the antibacterial activity of daptomycin (19). Preliminary data in our laboratories suggest that this may be the case and is under investigation. Thus, the bacterial cell surface com-
prised of the cell wall and cell membrane may represent a tightly regulated network, with changes in one inducing compensatory effects on the other. The logical next step in pharmacotherapy would therefore to apply antibiotics that disrupt both simultaneously for maximal bactericidal effect.

Taken together, these results present an extension of our understanding of how the inhibition of specific PBPs influences the activity of daptomycin and LL-37 (and therefore other cationic AMPs) against E. faecium. Inactivation of PBPs appears to make daptomycin more active at sub-MICs of daptomycin. However, daptomycin susceptibility is enhanced upon inactivation of multiple PBP targets and therefore is likely to be maximized by beta-lactams with the broadest PBP-binding profiles. Furthermore, while there is some overlap, the activity of cathelicidin appears to center specifically around PBPZ inactivation.

Inhibition of PBPs increased bacterial autolysis compared to the WT E. faecium parent strain. Of greater interest, however, is that daptomycin exposure of the WT E. faecium parent triggered a reduction in autolysis that was not seen in the PBP mutants (Fig. 5). It therefore appears that the wild-type E. faecium can compensate by strengthening its surface to resist daptomycin-mediated cellular injury upon daptomycin exposure and that this compensatory response is optimal when all PBPs are functional. In S. aureus, reduced autolysis has been associated with diminished susceptibility to vancomycin, daptomycin, and cationic AMPs such as platelet microbicidal proteins (20).

While the present study lays the foundation for further potentially clinically relevant studies, the analysis described here has some important limitations. First, the clinical strain 8019 used in the PK/PD model, although isolated from a patient who had developed a daptomycin-nonsusceptible VRE infection, was more susceptible to daptomycin than most clinical VRE strains, so its behavior in this model may not be representative of all VRE strains. Second, our investigation of PBP knockout mutants utilized only one strain background of E. faecium that was not from a contemporary clinical strain and that was vancomycin susceptible. Future studies with contemporary VRE strains must be performed to confirm the broad applicability of our findings. Third, the present study was not entirely complete in its PBP analysis. For example, we did not examine the effects of the other class B PBPs of E. faecium, i.e., PBP A and PBP B, since they were not included in the prior studies from where these strains were derived. In addition, the immunologic studies here focused on the single-knockout mutants in more detail rather than the double and triple knockouts. Finally, as pointed out previously, we assumed that PBP deletion could serve as a surrogate of pharmacological PBP inhibition by beta-lactam antibiotics, but the changes in the membrane characteristic of these processes may not be identical. Nevertheless, the findings demonstrate from yet another perspective the potential benefits that PBP-inactivating compounds may have on the treatment of infections caused by multiderug-resistant bacteria, even though these compounds do not compromise bacterial viability on their own.

Although these findings are interesting and anecdotal provide some good clinical results in difficult cases, we caution clinicians when extrapolating in vitro data studying the potential benefits of combination antibiotic therapy such as daptomycin plus a beta-lactam to the treatment of patients with VRE infections without a greater published clinical experience such as a clinical trial.

In summary, using a series of PBP deletion mutants, we determined that the synergistic activity between daptomycin plus beta-lactams may not be mediated by inactivation of a particular single PBP but rather a collective simultaneous inhibition of multiple PBPs. Inactivation of multiple class A and class B high-molecular-weight PBPs, as seen with ceftaroline, appears to exert the maximal daptomycin plus beta-lactam effect. Clinical studies will be crucial to guide the incorporation of this combination therapy treatment paradigm in increasingly common serious E. faecium infections.

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Figure S1. Polymyxin B population analysis in BHI agar of wild-type (WT) *E. faecium* compared to various PBP knockout mutants as shown in the legend.
Figure S2. Bodipy-labeled daptomycin binding of *E. faecium* and Δpbp5, ΔpbpF, ΔpbpZ knockouts. Representative microscopic imaging are shown above and quantitation of binding shown below. Δpbp5, ΔpbpZ mutants showed increased binding compared to the parental strain (p<0.05, t-test).