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Ampicillin Enhances Daptomycin- and Cationic Host Defense Peptide-Mediated Killing of Ampicillin- and Vancomycin-Resistant *Enterococcus faecium*

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We studied an ampicillin- and vancomycin-resistant *Enterococcus faecium* (VRE) isolate from a patient with endocarditis and bacteremia refractory to treatment with daptomycin (6 mg/kg of body weight) plus linezolid. Blood cultures cleared within 24 h of changing therapy to daptomycin (12 mg/kg) plus ampicillin. We examined the effects of ampicillin on daptomycin-induced growth inhibition and killing, surface charge, and susceptibility to several prototypical host defense cationic antimicrobial peptides. MICs and time-kill curves with daptomycin were assessed in the presence and absence of ampicillin. The impact of ampicillin on surface charge was assessed by flow cytometry and a poly-L-lysine binding assay. The effects of ampicillin preexposures upon VRE killing by five distinct cationic peptides of different structure, charge, origin, and mechanism of action were analyzed using the epidermal cathelicidin LL-37, thrombin-induced platelet microbicidal proteins (tPMPs), and a synthetic congener modeled after tPMP microbicidal domains (RP-1), human neutrophil peptide-1 (hNP-1), and polymyxin B (bacteria derived). Fluoroscein-Bodipy-labeled daptomycin was used to evaluate daptomycin binding to VRE membranes in the presence or absence of ampicillin. In media containing ampicillin (25 to 100 mg/liter), daptomycin MICs decreased from 1.0 to 0.38 mg/liter. Based on time-kill analysis and an *in vitro* pharmacodynamic model, ampicillin enhanced daptomycin activity against the study VRE from a bacteriostatic to a bactericidal profile. VRE grown in ampicillin (25 to 150 mg/liter) demonstrated an incremental reduction in its relative net positive surface charge. When grown in the presence (versus absence) of ampicillin (25 and 100 mg/liter), the VRE strain (i) was more susceptible to killing by LL-37, tPMPs, hNP-1, and RP-1 but not to polymyxin B and (ii) exhibited greater binding to Bodipy-labeled daptomycin. We conclude that ampicillin induces reductions in net positive bacterial surface charge of VRE, correlating with enhanced bactericidal effects of cationic calcium-daptomycin and a diverse range of other cationic peptides *in vitro*. While the mechanism(s) of such β-lactam-mediated shifts in surface charge remains to be defined, these finding suggest a potential for β-lactam-mediated enhancement of activity of both daptomycin and innate host defense peptides against antibiotic-resistant bacteria.

Daptomycin, a cyclic lipopeptide antibiotic, associates with calcium to form a cationic complex that targets the bacterial cytoplasmic membrane, causing rapid membrane depolarization and subsequent lethality against susceptible Gram-positive organisms (21). There have been a number of recent reports of isolation of daptomycin-resistant *Staphylococcus aureus* (17, 20–23, 27–29, 31, 39, 41) and enterococcus strains (24, 26) emerging during daptomycin treatment in patients with recalcitrant infections. Although the accepted term is "daptomycin nonsusceptibility," we will utilize the term "daptomycin resistant" for ease of data presentation and discussion in this paper. The mechanisms leading to daptomycin resistance in *S. aureus* are complex, although some studies have implicated mutations and changes in expression of genes involved in the modulation of bacterial surface charge, such as *dlt* and *mprF* (17). Of interest, development of daptomycin resistance in *S. aureus* has commonly been associated with coevolution of reduced susceptibility to killing by a variety of host defense molecules, such as cationic antimicrobial peptides (22, 34). The mechanisms of enterococcal resistance to daptomycin remain largely undefined, but in short appear to parallel the phenotypic changes of daptomycin resistance in *S. aureus* with different genotypic changes (2, 3, 32, 36).

Previously, we identified a reciprocal relationship in the *in vitro* susceptibility of enterococci to conventional cationic antibiotics (e.g., gentamicin, streptomycin) or cationic host defense peptides (e.g., platelet microbicidal proteins [PMPs]) versus noncationic antibiotics of different classes (e.g., cell wall-active agents, DNA gyrase inhibitors, protein synthesis inhibitors) (5). Similarly, subsequent studies demonstrated that comparative *in vitro* enterococcal susceptibility profiles for vancomycin versus cationic host defense peptides exhibited reciprocal phenotypes among clinical isolates of *Enterococcus faecium* (6, 7). These data emphasized two important themes regarding enterococci: (i) reduced *in vitro* susceptibility to cationic host defense peptides tracked with reduced susceptibility to conventional cationic antibiotics; (ii) reduced *in*

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vitro susceptibility to such cationic agents correlated inversely with susceptibility to noncationic antibiotics, particularly ampicillin and vancomycin. Finally, we demonstrated additive interactions *in vitro* between cationic host defense peptide congeners and noncationic antibiotics against selected enterococcal strains (47).

In the present study, we analyzed an ampicillin- and vancomycin-resistant *E. faecium* (VRE) strain from a case of aortic valve endocarditis in a hemodialysis patient with bacteremia refractory to 7 days of therapy with daptomycin (6 mg/kg of body weight every 48 h) plus linezolid (600 mg intravenously [i.v.] every 12 h). Based on prior studies showing *in vitro* synergy between daptomycin and ampicillin against *Enterococcus* spp. (3, 8, 13, 30, 37), a combined daptomycin-ampicillin therapeutic regimen was employed for this patient. Of interest, the patient's persistent bacteremia was rapidly cleared within 24 h of beginning a combination regimen of high-dose daptomycin (12 mg/kg every 48 h) plus ampicillin (1 g every 6 h).

In light of this dramatic microbiological response in our patient, combined with the prior *in vitro* reports cited above demonstrating potential antienterococcal "synergy" with daptomycin plus ampicillin, we investigated selected effects of ampicillin on our ampicillin-resistant VRE and demonstrated that ampicillin exposures induced a notable reduction in net positive surface charge that was associated with increased surface binding of daptomycin.

MATERIALS AND METHODS

Antimicrobial susceptibility testing. For the initial (pretherapy) bloodstream VRE strain, MICs of vancomycin, ampicillin, linezolid, and daptomycin were determined by standard Etest (AB Biodisk, Solna, Sweden) using CLSI methods (9). MICs were also determined for daptomycin and polymyxin B by using the standard Etest on Mueller-Hinton agar (MHA) containing 0, 5, 10, 25, 50, or 100 mg/liter of ampicillin. For all experiments, a minimum of two assays were performed on different days. The complete antibiotic susceptibility profile report from the Clinical Microbiology Laboratory for the final bloodstream isolate on day 7 was identical to that of the original isolate. However, only the original bloodstream VRE was stored and available for further study.

Time-kill assays were performed in duplicate with an initial bacterial inoculum of 10^6 CFU/ml (to reflect a high-inoculum infection, such as endocarditis) in Mueller-Hinton broth (MHB) supplemented to a calcium concentration of 50 mg/liter containing no antibiotic (growth control), ampicillin (20 mg/liter) alone, daptomycin (4 mg/liter; 4× the MIC]) with or without ampicillin (20 mg/liter), or daptomycin (10 mg/liter; 10× the MIC]) with or without ampicillin (20 mg/liter). These antibiotic concentrations were chosen to encompass readily achievable free serum concentrations of each agent during clinical treatment regimens (11, 14, 18). Quantitative bacterial counts were determined by sampling, serially diluting 1:10^0 to 1:10^7 in fresh MHB, and plating 20 μl in duplicate after 0, 6, and 24 h of incubation at 37°C. In order to eliminate antibiotic carryover, the undiluted sample (1 ml) was microcentrifuged in an Eppendorf tube at 13,000 rpm for 5 min, and the pellet was resuspended in 100 μl fresh MHB, allowing for a limit of detection of 0.7 $\log_{10}$ CFU/ml.

***In vitro* pharmacodynamic model.** To simulate daptomycin plus ampicillin combination regimens, an *in vitro* pharmacodynamic model was used, consisting of a one-compartment 500-ml glass chamber (working model volume, 250 ml of broth) with multiple ports for the removal of brain heart infusion (BHI) broth, delivery of antibiotic, and collection of bacterial and antimicrobial samples (19). Briefly, overnight cultures of the VRE isolate were diluted in fresh BHI broth and adjusted to a 1.0 McFarland turbidity. The suspension was added to BHI broth in the chamber model, yielding a final volume of 270 ml and a starting inoculum of ~10^8 CFU/ml. Serial samples were taken at 0 (predose), 1, 2, 4, 6, 8, 24, 28, 32, and 48 h to quantify viable counts. The following regimens for daptomycin were simulated using a mean 8-h terminal half-life: 4 mg/kg every 24 h (q24h; free-drug area under the concentration-time curve at 24 h [$f\mathrm{AUC}_{24}$] of 154 mg · h/liter), 6 mg/kg q24h (251 mg · h/liter), 8 mg/kg (329 mg · h/liter), and 10 mg/kg (377 mg · h/liter) alone and in combination with ampicillin at 2 g q4h (maximum concentration of drug in serum [$C_{\max}$], 70 mg/liter), based on a 1-h mean terminal half-life (11, 14, 18). For combination regimen experiments, the elimination rate was set for the drug with the shortest half-life, and the drug with the longer half-life was supplemented (4, 40).

FITC-labeled PLL binding. Assays were performed using a flow cytometric method as previously described (22, 33). Poly-L-lysine (PLL) is a polycationic molecule used to study the interactions of cationic peptides with charged bacterial envelopes. In this analysis, the extent of bacteria-bound fluorescein isothiocyanate (FITC)-labeled PLL inversely reflected 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). Data are expressed as means relative fluorescent units (± the standard deviation [SD]). At least two independent experiments of triplicate samples were performed.

Cationic antimicrobial peptides. We studied a panel of cationic host defense antimicrobial peptides differing in anatomic and host source, molecular mass, net charge at pH 7.5, and proposed mechanism(s) of action. Human LL-37 (net charge, +6 at pH 7.5), a cationic cathelicidin antimicrobial peptide prevalent in skin and neutrophils, was purchased from AnaSpec, Inc. (Fremont, CA); the human neutrophil α-defensin hNP-1 (net charge, +3 at pH 7.5) was purchased from Peptide International (Louisville, KY); tPMPs were prepared from freshly collected rabbit platelets, and their bioactive equivalencies were determined as previously described (42, 43). RP-1 (a synthetic 18-amino-acid congener modeled after the α-helical microbicidal domain of the tPMP family of platelet peptides; net charge, +8 at pH 7.5) was synthesized and authenticated as previously detailed (45). As a negative-control peptide, a bacteria-derived and membrane-targeting cyclic cationic molecule, polymyxin B, was used (Sigma, St. Louis, MO); the latter peptide has limited activity against enterococci due to its poor capacity to penetrate the Gram-positive cell wall.

Antimicrobial peptide microbicidal assays. The VRE strain was grown to stationary phase (16 to 20 h) in LB in either the absence of ampicillin or presence of various ampicillin concentrations, pelleted, and then washed in assay buffer (RPMI plus 5% LB for LL-37; phosphate-buffered saline for polymyxin B, RP-1, and tPMP; 10 mM KH_2PO_4, pH 7.4, for hNP-1 assays). Initial bacterial inocula of 10^3 CFU/ml were used in tPMP, polymyxin B, RP-1, and hNP-1 assays as previously described (42–44). For LL-37 assays, a starting inoculum of 10^5 CFU/ml was used, as it was more discriminatory of dose-dependent differences in killing of the study isolate by this peptide in extensive preliminary investigations. Pilot studies were used to determine cationic peptide concentrations that caused a <50% reduction in enterococcal counts in the 2-h survival assay. Thus, we utilized the following peptide concentrations: LL-37, 36 mg/liter; RP-1, 5 mg/liter; hNP-1, 2 mg/liter; tPMPs, 2.5 mg/liter equivalent; polymyxin B, 640 mg/liter. The percentage of surviving bacteria (± SD) after 2 h of incubation at 37°C was calculated by plating on blood agar plates. Results represent three separate experiments performed in duplicate.

Daptomycin binding assays. To determine if ampicillin was able to impact the ability of daptomycin to bind to the VRE membrane, the organism was grown in LB broth overnight (14 to 16 h) at 37°C in the presence or absence of ampicillin (50 to 100 mg/liter) and then incubated for 10 min with Bodipy-fluorescein-labeled daptomycin (16 mg/liter; courtesy of Cubist Pharmaceuticals, Lexington, MA). This concentration of labeled daptomycin was established by pilot studies as the optimal concentration for fluorescence microscopy. Excess unincorporated label was removed by washing the cells three times in LB broth. The cells were counterstained with FM 4-64 to visualize the membrane and 4′,6-

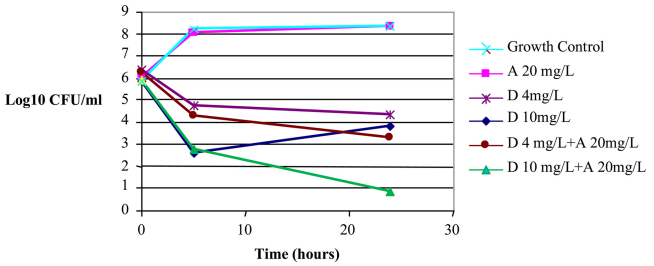

FIG 1 Time-kill curves against vancomycin-resistant *Enterococcus faecium* for daptomycin (D) and ampicillin (A) at the specified concentrations in Mueller-Hinton broth. The results of one representative experiment are shown.

diamidino-2-phenylindole (DAPI) to visualize the nucleoid and then imaged using a Delta Vision deconvolution microscope (Applied Precision, Inc. Issaquah, WA) as previously described (36).

Statistics. Statistical evaluations of the differences in survival in the presence of various cationic peptides and differences in PLL binding were performed with the Mann-Whitney U test (Prism 5.0; GraphPad Software, Inc., San Diego, CA). *P* values of <0.05 were considered statistically significant.

RESULTS

***In vitro* susceptibility to antibiotics: MICs.** By Etest, the VRE strain was resistant to both vancomycin and ampicillin (MICs of >128 mg/liter for both drugs) but susceptible to linezolid (MIC, 2 mg/liter) and daptomycin (MIC, 1 mg/liter). The polymyxin MIC was 160 mg/liter. The daptomycin MIC (1 mg/liter) did not change when tested in MHA containing ampicillin concentrations of 5, 10, or 25 mg/liter, but the MIC was reduced approximately 3-fold (0.38 mg/liter) in MHA containing 50 or 100 mg/liter ampicillin. No changes were noted for the MIC of polymyxin B in MHA containing the same range of ampicillin concentrations.

Microbicidal assays. Figure 1 demonstrates the 24-h time-kill assay results for the VRE isolate in different combinations of ampicillin and daptomycin. The most relevant finding was that exposure of the VRE isolate to either daptomycin at 10 mg/liter (10× the MIC) alone or daptomycin at 10 mg/liter plus ampicillin at 20 mg/liter yielded the same degree of killing (>3 $\log_{10}$ CFU/ml) after 6 h of incubation but significantly more killing than lower-dose daptomycin (4 mg/liter) with or without ampicilllin. However, at 24 h, VRE exposed to daptomycin at 10 mg/liter alone showed regrowth to a net 2 $\log_{10}$ CFU/ml reduction compared to time zero; in contrast, the combination of daptomycin at 10 mg/liter plus ampicillin at 20 mg/liter demonstrated continued bacterial killing, with reductions of VRE counts to below the limit of detection (>5 $\log_{10}$ CFU/ml versus the time zero baseline value). As expected, growth curves in ampicillin alone (20 mg/liter) paralleled those seen in antibiotic-free medium.

An *in vitro* pharmacodynamic model was employed to further investigate the relative potency of killing of various simulated human-like doses of daptomycin alone or with standard, high-dose ampicillin therapy (2 g i.v. q4h). The results in Fig. 2 demonstrate that daptomycin monotherapy at 4 to 10 mg/kg/day provided only a bacteriostatic effect against this VRE isolate. However, the addition of ampicillin significantly increased the activity of all daptomycin doses tested ($P < 0.05$ for the 24- to 28-h time points), exceeding bactericidal thresholds at 24 h. Notably, daptomycin at 4 mg/kg/day with ampicillin provided significantly greater killing than daptomycin monotherapy at 10 mg/kg/day.

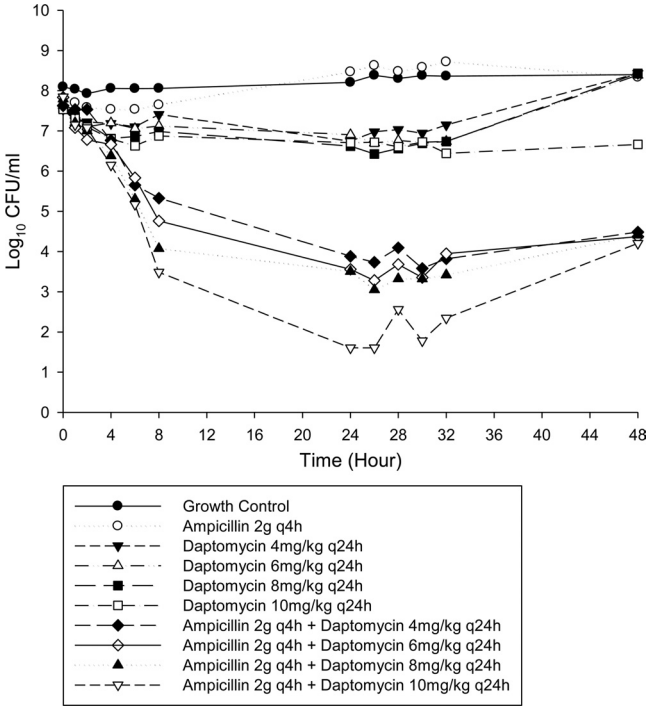

FIG 2 *In vitro* pharmacodynamic model simulating ampicillin at 2 g i.v. q4h and daptomycin at 4 to 10 mg/kg alone or in combination. Daptomycin up to 10 mg/kg provided only bacteriostatic activity against this VRE isolate. Ampicillin markedly potentiated the killing activity of daptomycin to bactericidal levels, such that the combination regimen provided more killing than any concentration of daptomycin monotherapy up to 10 mg/kg.

Effect of ampicillin on VRE net surface charge. Because of the rapid clearance of this patient's bacteremia and the marked potentiation of activity *in vitro* with the daptomycin plus ampicillin combination, we hypothesized that ampicillin (to which the VRE was highly resistant *in vitro*) was somehow modifying the enterococcal envelope, resulting in enhancement of daptomycin-mediated killing. Given the potential of the surface charge to impact the interaction of cationic calcium-daptomycin (17) with the enterococcal membrane, the capacity of ampicillin to alter this parameter was examined. Of interest, there was an increased affinity to PLL when the isolate was grown in ampicillin at 50 mg/liter compared to growth in antibiotic-free medium ($P < 0.05$) (Fig. 3), indicating a net decrease in the relative positive surface charge with growth in ampicillin.

Effect of ampicillin preexposures on microbicidal efficacies of cationic peptides. We had previously observed that *E. faecium* was relatively susceptible to killing by the cationic cathelicidin LL-37 compared to other Gram-positive bacteria, such as *S. aureus* and *Streptococcus pyogenes* (G. Sakoulas, unpublished observations). The effect of ampicillin on LL-37 susceptibility was therefore examined. Pilot susceptibility studies performed in ampicillin-free medium and medium containing a high dose of ampicillin (100 mg/liter) showed a 2-fold reduction in the LL-37 MIC (72 to 36 mg/liter) in the presence of ampicillin (concomitant exposures; not when pregrown in ampicillin). In contrast, susceptibility testing of VRE that was pregrown in ampicillin (100 mg/liter) showed a further reduction in the LL-37 MIC to 18 mg/liter.

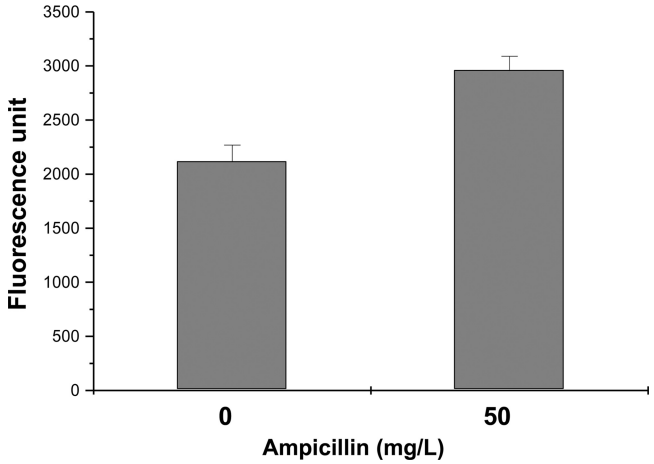

FIG 3 FITC-labeled PLL binding assay using flow cytometry results. The degree of FITC-labeled PLL inversely reflects the relative surface positive charge. The VRE isolate demonstrated an increased affinity to PLL when it was grown in ampicillin at 50 mg/liter compared to growth in antibiotic-free medium ($P < 0.05$), indicating a net decrease in relative positive surface charge with growth in ampicillin. Data are expressed as mean relative fluorescent units (± SD). Two independent experiments with triplicate samples were performed.

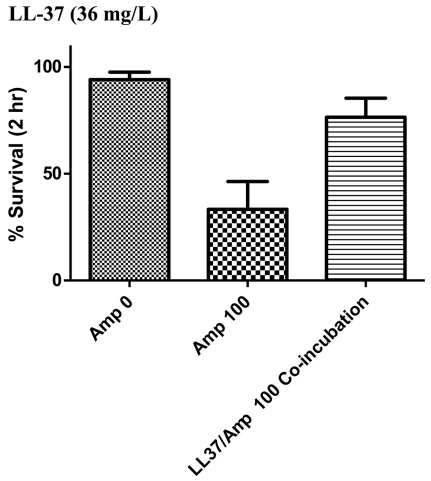

FIG 4 Killing activity of cathelicidin LL-37 at 36 mg/liter (8 μM), expressed as the percentage of surviving CFU at 2 h compared to time zero against VRE grown to stationary phase in antibiotic-free LB broth (far left) and LB containing ampicillin at 100 mg/liter (middle) ($P = 0.027$). The column on the far right demonstrates the killing activity of LL-37 at 36 mg/liter coincubated in the presence of ampicillin at 100 mg/liter against VRE grown in antibiotic-free LB.

The bactericidal activity of LL-37 (36 mg/liter) in the concomitant presence or the absence of ampicillin (100 mg/liter) was assessed, and we found a slight increase in killing (Fig. 4). However, when the VRE was pregrown overnight in LB containing ampicillin (100 mg/liter), a marked increase in LL-37 killing was observed ($P = 0.027$) (Fig. 4).

The microbicidal effects of 4 additional cationic peptides were determined against the VRE strain after pregrowth in either antibiotic-free broth or in broth containing ampicillin at various concentrations. Results were as follows: tPMP, 2.5 mg/liter equivalent (Fig. 5A); hNP-1, 2 mg/liter (Fig. 5B); RP-1, 5 mg/liter (Fig. 5C); polymyxin B, 640 mg/liter (Fig. 5D). Paralleling the data above but employing daptomycin plus ampicillin versus the LL-37 cationic peptide, pregrowth in ampicillin (100 mg/liter) prior to peptide exposures was associated with significantly greater enterococcal killing by 3 of the 4 cationic molecules ($P < 0.05$). As expected for Gram-positive bacteria, the VRE showed relative resistance to polymyxin B killing at 4× the MIC, without any significant enhancement in killing induced by pregrowth in ampicillin. Among the cationic peptides that did demonstrate increased killing of the VRE isolate when grown in ampicillin, only RP-1 exhibited enhanced killing following pregrowth in ampicillin at both 25 mg/liter and 100 mg/liter compared to growth in ampicillin-free medium.

Effect of ampicillin on daptomycin membrane binding. As a complement to the microbicidal assays described above, daptomycin membrane binding studies were performed using Bodipy-fluorescein-labeled daptomycin with VRE pregrown with or without ampicillin (50 mg/liter). VRE pregrown in ampicillin demonstrated increased binding to labeled daptomycin compared to VRE pregrown in antibiotic-free medium when viewed by fluorescence microscopy and measured by pixelgrams (Fig. 6).

DISCUSSION

In the current study, we identified interesting relationships between ampicillin and daptomycin on both *in vitro* and *in vivo* bases by utilizing a daptomycin-susceptible but ampicillin- and vancomycin-resistant *E. faecium* clinical isolate. Our studies translated observations from "bedside to bench," following the rapid clearance of this strain from the bloodstream after administration of high-dose daptomycin plus ampicillin in a patient with refractory VRE bacteremia. Relevant to this striking clinical outcome, we confirmed *in vitro* that ampicillin at clinically relevant but sub-MIC levels enhanced the activity of daptomycin from a bacteriostatic to a bactericidal potency, even though ampicillin alone had no measureable effect on bacterial growth. These findings were further validated in an *in vitro* pharmacodynamic model that demonstrated that simulated coexposures of low-dose daptomycin (4 mg/kg/day) plus ampicillin (2 g i.v. q4h) yielded much greater killing potency than simulated high-dose daptomycin alone (10 mg/kg/day). Given the apparent increase in the number of cases of daptomycin-resistant enterococcal infections (24, 26) and the relatively high cost of the drug, the latter finding connotes not only clinical efficacy impacts but also potentially significant pharmaco-economic implications. Thus, daptomycin administered at standard approved doses (4 to 6 mg/kg/day) plus ampicillin would be significantly less costly than high-dose daptomycin (8 to 12 mg/kg/day).

Of interest, in terms of the salutary influence of ampicillin upon daptomycin-induced killing, ampicillin appeared to substantially reduce the net positive surface charge on our VRE strain, in a concentration-dependent manner. Consistent with this effect, we found that ampicillin preexposure led to increased susceptibility to killing by a number of other cationic peptides, including those of diverse structures, charges, sources (i.e., skin, polymorphonuclear leukocytes, and platelets), and mechanism(s) of action (e.g., pore formers and non-pore formers). Furthermore, VRE grown in the presence of ampicillin showed significantly increased binding to labeled daptomycin compared to VRE grown in medium without ampicillin. These observations strongly suggest a charge-based mechanism for the impact of ampicillin on

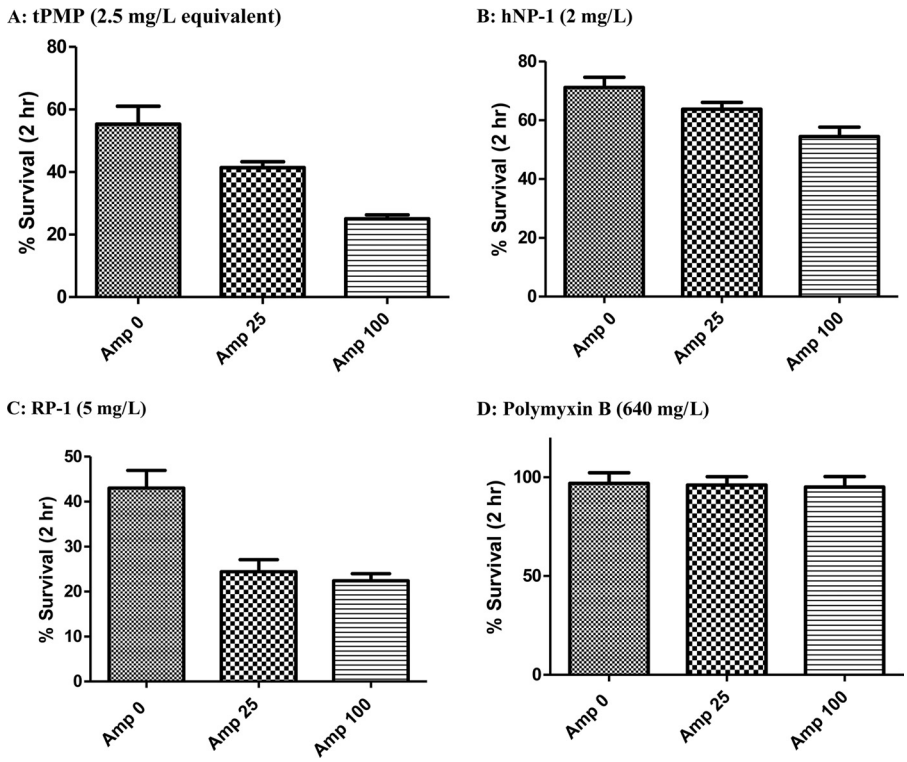

FIG 5 Killing activities, expressed as percentage of surviving CFU at 2 h compared to time zero, against VRE grown in antibiotic-free BHI broth and broth containing 25 or 100 mg/liter ampicillin by tPMP at 2.5 mg/liter equivalent ($P < 0.01$) (A), hNP-1 at 2 mg/liter ($P < 0.02$) (B), RP-1 at 5 mg/liter ($P < 0.01$) (C), and polymyxin B at 640 mg/liter ($P < 0.9$) (D). Mean results of 3 experiments are shown.

daptomycin-mediated killing of this study's VRE strain. It should also be emphasized that disclosure of this effect required pregrowth in ampicillin (to which the organism was resistant) at least 16 to 18 h prior to exposures to the above cationic peptides. These observations point to a probable "ampicillin-sensitizing" metabolic effect underlying this phenomenon.

Growth in ampicillin failed to induce significant enhancement in killing by polymyxin B at 4× the MIC (640 mg/liter). In addition, measurement of the polymyxin B MIC by Etest in agar containing various concentrations of ampicillin failed to induce any changes in the MICs. These finding suggest that changes in VRE surface charge induced by ampicillin are not sufficient to overcome the physical cell wall barrier, which mediates relative resistance of Gram-positive bacteria to polymyxin B.

The present findings support our previous documentation of definable interactions between cationic peptides and selected cell wall-active antibiotics in other enterococcal strains. For example, we identified an inverse relationship between susceptibility to cationic antimicrobial peptides and ampicillin or vancomycin, suggesting that mechanisms of resistance to conventional antibiotics may render enterococci more vulnerable to certain host cationic peptides (5–7). Moreover, the present findings are consistent with our prior observations of favorable interactions between β-lactam or other cell wall-active antibiotics and host defense peptides versus other Gram-positive organisms. For example, we previously reported that PMPs enhance oxacillin- or vancomycin-induced killing of *S. aureus* (46). Similarly, our prior studies demonstrated that PMPs in combination with antistaphylococcal antibiotics reduce *S. aureus* adherence to platelets, a mechanism used by this organism as a potential immune subversion strategy (48).

The precise mechanisms responsible for this enhanced daptomycin and cationic host defense peptide killing following putative ampicillin "sensitization" of VRE are under current investigation. In this regard, recent data have demonstrated that ampicillin can suppress the emergence of daptomycin resistance in enterococci *in vitro*, also suggesting a favorable interaction between these two antibiotics (15). Those authors speculated that ampicillin may prompt the release of lipoteichoic acid (LTA) from the cell envelope which, in turn, could either increase cell wall autolysin activity or reduce substrate availability for *dlt*-mediated LTA D-alanylation (1). A lack of D-alanylated LTA may, in turn, result in an enhancement of the relative net negative envelope charge and, thus, increased susceptibility to killing by daptomycin and other cationic peptides (16). It is also conceivable that ampicillin may affect biofilm formation *in vivo*, which may also have played a role in the clearance of bacteremia, given the importance of this phenotype in endocarditis and persistent bacteremia (38). Additional studies are needed, not only to evaluate the effects of ampicillin on penicillin binding protein expression and LTA release in enterococci but also on biofilm formation and other virulence determinants.

Although hypothetical at this point, it is also feasible that the substitution of D-alanine by D-lactate in VRE within the peptidoglycan pentapeptide precursor concomitantly results in an increased anionic surface charge of the organism (10) and thereby contributes to greater surface binding of cationic peptides, such as daptomycin. Such an effect is supported by our earlier demonstration of additive *in vitro* interactions between β-lactam or glycopeptide antibiotics with cationic host defense peptides against a number of *Enterococcus* strains (47, 49). Interestingly, our

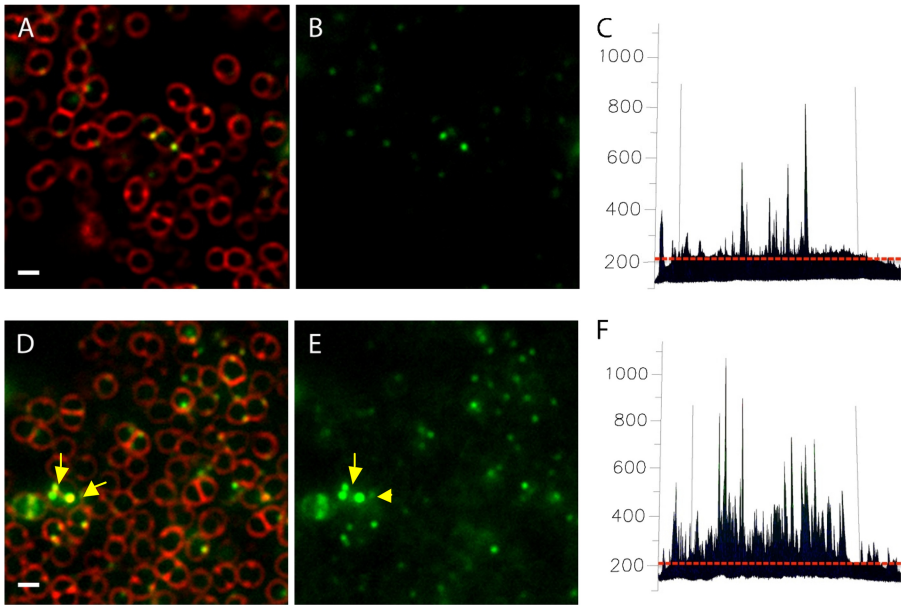

FIG 6 Incorporation of fluorescently labeled daptomycin by VRE grown in the presence or absence of ampicillin. The strain was labeled with 16 mg/liter Bodipy-labeled daptomycin (green) (A, B, D, and E) for 10 min at 37°C after growth to log phase in either antibiotic-free LB broth (A to C) or in LB broth containing ampicillin at 50 mg/ml (D to F). Cells were stained with FM 4-64 (red) (A, B, D, and E) or DAPI (blue) (B and E). Bar, 1 μm. (C and F) Comparison of daptomycin-Bodipy incorporation, demonstrating higher pixel intensities for daptomycin-Bodipy fluorescence for cells grown with (F) or without (C) 50 mg/ml ampicillin. The yellow arrows highlight Bodipy-daptomycin staining (D and E).

groups have recently confirmed the enhancement of daptomycin activity by antistaphylococcal β-lactams against *mecA*-positive methicillin-resistant *S. aureus* (MRSA) strains *in vitro*, as well as in animal models of endocarditis (44) and in several cases of refractory MRSA bacteremia (12). In addition, we previously demonstrated the potentiation of killing of *S. aureus* strains by combinations of cell wall-active antibiotics (oxacillin) plus cationic tPMPs (42, 46). Collectively, these findings underscore both similarities as well as probable differences in the mechanism(s) between staphylococci and enterococci, vis á vis β-lactam interactions with daptomycin. For example, although potentiation of killing and reductions in the relative positive surface charge by β-lactams were observed in both *S. aureus* and VRE, the enhancement of daptomycin staphylocidal activity was seen with either preexposure or concomitant exposure strategies. In contrast, the β-lactam-associated "sensitization" of VRE to subsequent killing by cationic antimicrobial peptides required long-duration pregrowth in ampicillin and was not observed with concomitant exposures.

Although limited to a single isolate, the findings from this study may have much broader clinical implications. First, these results provide a potential therapeutic option for patients with daptomycin-refractory VRE bacteremia. Second, these data suggest that β-lactam antimicrobials may be beneficial beyond their direct antimicrobial properties, in terms of enhancing bacterial clearance by the innate host defense system, in particular as related to cationic host defense peptides. These two issues justify an in-depth examination of the frequencies and mechanisms of such favorable interactions of various β-lactams with a range of cationic antimicrobial molecules in additional VRE strains. Lastly, it will be important to examine the potential salutary interactions of ampicillin-daptomycin combinations against other daptomycin-resistant VRE strains.

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