Characterizing Vancomycin-Resistant Enterococcus Strains with Various Mechanisms of Daptomycin Resistance Developed in an In Vitro Pharmacokinetic/Pharmacodynamic Model

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Two daptomycin (DAP) regimens were evaluated in a pharmacokinetic/pharmacodynamic (PK/PD) model, and the mutants recovered were examined for changes in phenotypic characteristics. Three Enterococcus faecium strains (vancomycin-resistant Enterococcus [VRE] ATCC 51559, VRE 12311, and VRE SF 12047) were utilized in a 7-day, 1-compartment in vitro PK/PD model. The simulated dosing regimens were DAP at 6 mg/kg/day (free \( C_{max} = 7.9 \, \mu g/ml \), half-life \( t_{1/2} = 8 \, h \)) and DAP at 10 mg/kg/day (\( C_{max} = 13.17 \, \mu g/ml \), \( t_{1/2} = 8 \, h \)). Samples were plated daily on Mueller-Hinton agar containing DAP at 16 \( \mu g/ml \) and 50 \( \mu g/liter \) Ca\(^{2+} \) to assess the emergence of DAP resistance. For each strain, the mutant with the highest DAP MIC was then evaluated for changes in relative surface charge, cell wall thickness, and cytoplasmic membrane depolarization induced by DAP. The initial DAP MICs were 4 \( \mu g/ml \) for all 3 strains. A dose-dependent response and regrowth were observed for DAP 6 mg/kg/day and DAP 10 mg/kg/day against all 3 strains. Mutants of VRE ATCC 51559 (MIC = 128 and 64 \( \mu g/ml \)) and VRE 12311 (MIC = 256 and 32 \( \mu g/ml \)) were recovered from the DAP 6 mg and DAP 10 mg regimen, respectively. For VRE SF 12047, a mutant (MIC = 64 \( \mu g/ml \)) was recovered from the DAP 6 mg model. All mutants displayed an increase in relative surface charge compared to those of their respective parent strains. The DAP-resistant mutants displayed a 43 to 58% increase in cell wall thickness \((P < 0.0001)\), while DAP membrane depolarization decreased by 53 to 65% compared to that of the susceptible strains. VRE with DAP resistance displayed increased surface charge, increased cell wall thickness, and decreased depolarization induced by DAP, consistent with previous observations in Staphylococcus aureus with reduced DAP susceptibility. Further characterization of DAP-resistant VRE is warranted.

Daptomycin is a cyclic lipopeptide antibiotic that has bactericidal activity against a wide variety of Gram-positive bacteria, including methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci (VRE) (4). Although clinical strains of S. aureus and enterococci displaying nonsusceptibility and resistance to daptomycin have been reported, the mechanism of this phenotype has not been clearly elucidated (2, 4, 16, 17, 22, 29, 37, 38). The majority of work in this area has concentrated on S. aureus. Mutations and/or changes in the expression of several genes (mprF, yycG, rpoB, rpoC, and dlt-ABC-D) and alterations within the cell membrane and cell wall (fluidity, membrane potential, membrane proteins, and the amount and distribution of phospholipids) have been discovered but are not universally found in nonsusceptible strains (17, 22, 29, 37, 38).

Recent studies investigating S. aureus strains developing daptomycin nonsusceptibility during daptomycin therapy have revealed a decrease of cell membrane depolarization secondary to daptomycin exposure (20, 22). In addition, daptomycin nonsusceptibility has been associated with vancomycin-intermediate S. aureus (VISA) and an increase in cell wall thickness (6, 11). The mechanism of reduced daptomycin susceptibility is hypothesized to be secondary to an affinity trapping of antibiotic molecules in the thickened cell wall (11). Daptomycin-nonsusceptible S. aureus strains have been shown to have diminished daptomycin and cationic peptide binding to the cytoplasmic membrane secondary to an increase in positive cell surface charge (20, 22, 37).

Mutations in the mprF gene have been commonly found in postexposure clinical isolates that have developed nonsusceptibility to daptomycin (37, 38). The MprF protein is responsible for modifying net surface charge by lysinylation of the membrane phosphatidylglycerol (PG) to generate lysyl-PG (LPG) and translocation of the positively charged phospholipids to the outer leaflet of the cytoplasmic membrane (14, 29). Recently, Yang et al. have identified increased expression of mprF and dltABCD in daptomycin-nonsusceptible strains of S. aureus (29, 37, 38). The operon dltABCD contributes to the net positive surface charge by the \( \delta \)-alanylation of wall teichoic acids (37). A shift in the cytoplasmic membrane surface charge toward a more positive state is thought to impede binding via repulsion of the daptomycin-Ca\(^{2+} \) complex, the active form that simulates a cationic antimicrobial peptide (20, 37).

In stark contrast, only three studies have examined genetic
changes in enterococci resistant to daptomycin (1, 5, 30). Two gene clusters (EF2694 to EF2701 and EF1751 to EF1753) associated with cell membrane proteins and the phage shock protein C were found to be upregulated in daptomycin-resistant Enterococcus faecalis (5). Examination of a clinical isologenic pair of E. faecalis isolates found alterations in genes associated with phospholipid metabolism and a cell envelope stress response system (1). It has previously been demonstrated that deletion of dltA in Enterococcus faecium leads to increased susceptibility to cationic peptides; therefore, it seems logical that an increase in the expression of the dltABCD operon would lead to decreased daptomycin susceptibility (15). E. faecalis possesses the ability to increase its cell wall thickness as a mechanism to develop resistance to monolaurin, a fatty acid ester with antibacterial activity via action on the membrane (13). Therefore, verification of the changes in the cytochrome (SpectraMax M5; Molecular Devices, Sunnyvale, CA), and the concentration of bance of the supernatant was measured at 530 nm using a spectrophotometer

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chance of colony count determination is 2.0 log10 CFU/ml. For all samples, antimicrobial

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fluorescence measured with daptomycin, Fe is the fluorescence measured with the buffer, and Fn is the fluorescence measured with nisin. The results presented are the means of 2 independent experiments.

Statistical analysis. Paired continuous data were evaluated with a paired t test or sign test (if distribution was not normal). A P value of ≤0.05 was considered significant.

RESULTS

Susceptibility testing. Vancomycin and daptomycin MICs for study isolates are displayed in Table 1.

One-compartment in vitro PK/PD model. The results of the 7-day, 1-compartment models are displayed in Table 2. Daptomycin simulated regimens of 6 mg/kg/day and 10 mg/kg/day produced dose-dependent responses in VRE SF 12047 and VRE 12311. Daptomycin simulated regimens of 6 mg/kg/day produced mutants with elevated daptomycin MIC values for all strains, while 10 mg/kg/day produced mutants for VRE 12311 and VRE ATCC 51559. Regrowth occurred with both dosing regimens in all strains.

Emergence of derivative mutants with increased daptomycin MICs. For VRE SF 12047, only one mutant (MIC 16 to 32 μg/ml) was recovered at 168 h during exposure to the daptomycin-simulated regimen of 6 mg/kg/day. For both VRE 12311 and VRE ATCC 51559, mutants were recovered from both simulated regimens at the time of first regrowth and all time points tested thereafter. The mutants recovered from the DAP 6 mg/kg/day 168 h sample were chosen as the isogenic pair to be tested secondary to having the highest MIC values.

PFGE. Pulsed-field gel electrophoresis (PFGE) confirmed the strain relatedness of the three strains utilized for the in vitro PK/PD model and the respective mutants recovered from the 168 h PD sample for the daptomycin 6 mg/kg simulation.

Cytochrome c binding. The relative cell surface charges as determined by cytochrome c binding experiments are displayed in Fig. 1. For the isogenic pairs, all E. faecium mutants with elevated daptomycin MIC values had a decrease in cytochrome c binding, suggesting an increase in cell surface positivity compared to that of the daptomycin-susceptible parent strain.

TEM. The TEM results for cell wall thickness are displayed in Table 1 and Fig. 2. For the isogenic pairs, the E. faecium mutants with elevated daptomycin MIC values displayed significant increases in mean cell wall thickness, increasing from 30.3 nm to 43.5 nm (43% increase), from 22.2 nm to 32.4 nm (46% increase), and from 24.9 nm to 39.5 nm (58% increase) for VRE SF 12047, VRE 12311, and VRE ATCC 51559, respectively (P < 0.0001 for all comparisons).

Determination of cytoplasmic membrane depolarization. The effect of daptomycin on the cytoplasmic membrane in E. faecium isolates was evaluated using the membrane potential-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Daptomycin dose</th>
<th>Maximum kill (log10 CFU/ml)</th>
<th>Time of 1st regrowth (h)</th>
<th>Inoculum at 168 h (log10 CFU/ml)</th>
<th>MIC (μg/ml)*</th>
<th>Regrowth (Mut)</th>
<th>96 h</th>
<th>168 h</th>
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<tbody>
<tr>
<td></td>
<td>Pop Mut</td>
<td>Pop Mut</td>
<td></td>
<td></td>
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<tr>
<td>VRE SF 12047</td>
<td>6 mg/kg</td>
<td>−3.73</td>
<td>72</td>
<td>8.65</td>
<td>NA</td>
<td>16</td>
<td>NA</td>
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<tr>
<td></td>
<td>10 mg/kg</td>
<td>−6.96</td>
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<td>4.32</td>
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<tr>
<td>VRE ATCC 51559</td>
<td>6 mg/kg</td>
<td>−3.22</td>
<td>24</td>
<td>7.86</td>
<td>16</td>
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<td>64–128</td>
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<td>10 mg/kg</td>
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<td>16–32</td>
<td>32</td>
<td>16</td>
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<td>VRE 12311</td>
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<td>7.94</td>
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<td>16–32</td>
<td>64</td>
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<tr>
<td></td>
<td>10 mg/kg</td>
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<td>7.44</td>
<td>32</td>
<td>8</td>
<td>32</td>
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</table>

* Mut, recovered mutant; Pop, overall bacterial population; NA, not applicable.

TABLE 1. Susceptibility testing and transmission electron microscopy results for Enterococcus faecium strains and their mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (μg/ml)</th>
<th>Cell wall thickness ± SD (nm)</th>
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<tr>
<td></td>
<td>Vancomycin</td>
<td>Daptomycin</td>
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<tr>
<td>VRE SF 12047</td>
<td>&gt;256</td>
<td>4</td>
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<tr>
<td>VRESF 12047 M</td>
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<tr>
<td>VRE ATCC 51559</td>
<td>&gt;256</td>
<td>256</td>
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<tr>
<td>VRE ATCC 51559 M</td>
<td>&gt;256</td>
<td>2–4</td>
</tr>
<tr>
<td></td>
<td>&gt;256</td>
<td>128</td>
</tr>
</tbody>
</table>

* M, mutant recovered from pharmacodynamic sample at 168 h in model simulating daptomycin at 6 mg/kg every 24 h.
sensitive fluorescent dye DiSC3. Dye was added into the medium containing bacteria and allowed to penetrate and accumulate inside the cells, and the fluorescence measured during the assay represents the amount of dye present in the medium. If the bacterial membrane is depolarized in the presence of drug, dye will escape from inside the cells out to the medium, causing an increase in measurable fluorescence.

All daptomycin-resistant mutants demonstrated a decrease in depolarization due to daptomycin compared to that of the daptomycin-susceptible parent strains (Fig. 3). For VRE 12311 and the VRE 12311 mutant, daptomycin-induced depolarization decreased significantly, from 93.03% ± 9.86% (mean ± standard deviation) to 27.65% ± 4.95% (P = 0.017). VRE ATCC 51559 and its mutant showed a similar decrease (75.95% ± 2.64% versus 22.29% ± 11.45%, P = 0.06). The daptomycin depolarization was 98.52% ± 5.29% for VRE SF 12047 and 45.89% ± 4.42% for the VRE SF 12047 mutant (P = 0.008), illustrating a >50% loss in daptomycin’s depolarization ability for this VRE strain.

DISCUSSION

To our knowledge, this is the first published paper to discuss phenotypic changes and potential mechanisms associated with daptomycin-resistant E. faecium strains, the predominant species responsible for nosocomial infections caused by VRE. It is also the first study to utilize multiple isogenic pairs developed in vitro. The three strains utilized in the in vitro PK/PD models, VRE SF 12047, VRE 12311, and VRE ATCC 51559, varied in their susceptibility to daptomycin activity and in their propensity to develop daptomycin-resistant mutants during therapy, therefore allowing the recovery of mutants with MICs ranging from 32 to 256 μg/ml. Our study examined the activity of daptomycin and the development of resistance in a high-inoc-
Daptomycin displayed the most potent and sustained activity against VRE SF 12047. The propensity of this strain to develop resistance (only one mutant recovered at 168 h under daptomycin pressure of 6 mg/kg/day) was strikingly less than that of the other two strains tested. VRE ATCC 51559 and VRE 12311 developed resistance to daptomycin at 24/24 h and 24/48 h under exposure to daptomycin at 6 and 10 mg/kg/day, respectively. The variability among strains highlights the need for further research to determine the optimal daptomycin dose for enterococcal infections and to prevent the emergence of resistance.

Comparison of the isogenic in vitro-derived pairs found a small increase in cell surface positivity (decrease in cytochrome c binding) in the daptomycin-resistant mutants. An increase in the positive cell surface charge of E. faecium strains relative to that of the daptomycin-susceptible parent strains may be asso-

FIG. 3. Cytoplasmic membrane depolarization of VRE ATCC 51559 (A) and the VRE ATCC 51559 mutant (B), VRE 12311 (C) and the VRE 12311 mutant (D), and VRE SF 12047 (E) and the VRE SF 12047 mutant (F). Black lines show results with nisin, dark-gray lines show results with daptomycin, and light-gray lines show results for control.
cated with daptomycin resistance. Preliminary data from one study examining a clinical Enterococcus faecalis isogenic pair also found a decrease in cytochrome c binding in the daptomycin-resistant strain (MIC = 16 μg/ml) compared to that of the daptomycin-susceptible strain (MIC = 1 μg/ml) parent (27). As stated previously, daptomycin is bound to calcium in its active state, forming a positively charged complex that simulates a cationic peptide antibiotic. It is hypothesized that, in S. aureus, a change in the cytoplasmic membrane surface charge from negative to more positive contributes to the increase in daptomycin MIC values by charge repulsion of the positively charged daptomycin-Ca²⁺ complex (20, 37). This increase in membrane surface charge in S. aureus with increased daptomycin MIC values has been demonstrated in clinical, in vitro, and genetically manipulated isolates via decreased binding of cytochrome c or other cationic peptide antibiotics (14, 20, 24, 29, 37). It would appear that enterococci also possess the ability to alter cytoplasmic membrane surface charge and this contributes to daptomycin resistance. Further analysis, including the use of more sensitive assays, is required to confirm these findings in clinical Enterococcus faecium strains and explore the mechanisms by which enterococci are able to alter cytoplasmic membrane surface charge.

With regard to cell wall thickness, comparison of the in vitro-derived isogenic pairs revealed a significant increase for all the daptomycin-resistant Enterococcus faecium mutants compared to the cell wall thickness of the parent strain. This finding suggests that thickening of the cell wall is one mechanism by which Enterococcus faecium may increase its resistance to daptomycin under prolonged exposure. Previous studies have shown that enterococci possess the ability to thicken cell walls in an effort to survive prolonged exposure. Previous studies have shown that enterococci possess the ability to thicken cell walls in an effort to survive prolonged exposure. 

Daptomycin acts like a cationic antimicrobial peptide in its active calcium-bound form and exerts bactericidal activity in S. aureus through the insertion of a lipophilic acyl tail into the cytoplasmic membrane, leading to potassium efflux, destruction of the ion concentration gradient, membrane depolarization, and probably, more generalized disruption of the cell membrane (9, 19). While the mechanism of action of daptomycin has not been specifically studied in Enterococcus spp., it is logical that it would act via similar mechanisms in these two Gram-positive organisms. Clinical S. aureus strains with decreased susceptibility to daptomycin have been found to have decreases in membrane depolarization from daptomycin compared with their respective isogenic susceptible strains (20, 22). The in vitro-derived Enterococcus faecium mutants in this study displayed similar characteristics, with decreased depolarization due to daptomycin compared to that in their susceptible isogenic parent strains. In addition, preliminary data examining the clinical Enterococcus faecalis isogenic pair also found decreased membrane depolarization by daptomycin in the resistant strain (MIC = 16 μg/ml) compared to that in the susceptible parent (MIC = 1 μg/ml) (27).

This is the first study to examine phenotypic characteristics associated with daptomycin-resistant Enterococcus faecium strains and is strengthened by the number of in vitro-derived strains examined. Even with its novelty, positive findings, and larger number of strains, this study does have limitations. Since this study utilized an in vitro model to simulate clinical daptomycin exposure, it is logical that the mechanisms by which the examined Enterococcus faecium strains developed resistance to daptomycin will more closely mimic in vivo conditions than other laboratory methods, such as serial passage. It is still possible, however, that daptomycin-resistant Enterococcus faecium strains developed in vivo may differ from the in vitro-developed strains here, because Enterococcus faecium isolates in the human body are also exposed to cationic peptides that are part of the immune system. The lack of white cells in the model compared to the immune system may also alter the rate of resistance selection in vitro compared to the rate in vivo. We also did not examine any genetic changes associated with the emergence of daptomycin-resistant strains.

Clearly, more research is needed to continue to explore the mechanisms by which Enterococcus faecium strains change their cell wall and cytoplasmic membrane, leading to the development of daptomycin resistance. In S. aureus, changes in surface charge are secondary to alterations in phospholipid asymmetry (translocation of the positively charged phospholipids to the outer leaflet of the cytoplasmic membrane) and increased alanylation of cell wall teichoic acid (14, 20, 29, 37). Research into the phosphatidylglycerol content of the cytoplasmic membrane of various species of bacteria has found differences in amino acid substrate specificity (32). Enterococcus faecium TX1330 possesses a multispecific amino acid aminocacylphosphatidylglycerol synthase, allowing it to synthesize greater amounts of phosphatidylglycerols and perform elaborate remodeling of these lipids within the cell membrane in response to environmental stress (32). Therefore, while changes in phospholipids may also be responsible for the daptomycin resistance in enterococci, the exact mechanisms may vary between the species. Additional work examining the mechanisms, including the dose exposure, leading to the development of daptomycin resistance in enterococci is needed.

ACKNOWLEDGMENT

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