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Defining Daptomycin Resistance Prevention Exposures in Vancomycin-Resistant *Enterococcus faecium* and *E. faecalis*

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Daptomycin is used off-label for enterococcal infections; however, dosing targets for resistance prevention remain undefined. Doses of 4 to 6 mg/kg of body weight/day approved for staphylococci are likely inadequate against enterococci due to reduced susceptibility. We modeled daptomycin regimens *in vitro* to determine the minimum exposure to prevent daptomycin resistance (Dap^r) in enterococci. Daptomycin simulations of 4 to 12 mg/kg/day (maximum concentration of drug in serum [C_{max}] of 57.8, 93.9, 123.3, 141.1, and 183.7 mg/liter; half-life [$t_{1/2}$] of 8 h) were tested against one *Enterococcus faecium* strain (S447) and one *Enterococcus faecalis* strain (S613) in a simulated endocardial vegetation pharmacokinetic/pharmacodynamic model over 14 days. Samples were plated on media containing 3× the MIC of daptomycin to detect Dap^r. Mutations in genes encoding proteins associated with cell envelope homeostasis (*yycFG* and *liaFSR*) and phospholipid metabolism (cardiolipin synthase [*cls*] and cyclopropane fatty acid synthetase [*cfa*]) were investigated in Dap^r derivatives. Dap^r derivatives were assessed for changes in susceptibility, surface charge, membrane depolarization, cell wall thickness (CWT), and growth rate. Strains S447 and S613 developed Dap^r after simulations of 4 to 8 mg/kg/day but not 10 to 12 mg/kg/day. MICs for Dap^r strains ranged from 8 to 256 mg/liter. Some S613 derivatives developed mutations in *liaF* or *cls*. S447 derivatives lacked mutations in these genes. Dap^r derivatives from both strains exhibited lowered growth rates, up to a 72% reduction in daptomycin-induced depolarization and up to 6-nm increases in CWT ($P < 0.01$). Peak/MIC and AUC_{0-24}/MIC ratios (AUC_{0-24} is the area under the concentration-time curve from 0 to 24 h) associated with Dap^r prevention were 72.1 and 780 for S447 and 144 and 1561 for S613, respectively. Daptomycin doses of 10 mg/kg/day may be required to prevent Dap^r in serious enterococcal infections.

Enterococcus spp. are among the leading causes of nosocomial infections (1). Vancomycin-resistant *Enterococcus faecium* has been recognized as an important multidrug-resistant pathogen for which new or improved therapies are urgently needed (2, 3). Daptomycin is a lipopeptide antibiotic with potent *in vitro* bactericidal activity against Gram-positive bacteria, including vancomycin-resistant enterococci (VRE). Although daptomycin lacks an approved indication from the FDA, it is frequently utilized as an alternative agent for the management of VRE infections. However, the optimal pharmacokinetic/pharmacodynamic (PK/PD) targets for resistance prevention and therapeutic success with daptomycin have not been defined (4). In fact, the susceptibility breakpoint designating resistance in enterococci also remains questionable; however, for ease of presentation, we will use the term resistance to refer to nonsusceptible enterococci with daptomycin MICs of >4 mg/liter. Most experts agree that the daptomycin doses of 4 to 6 mg/kg of body weight/day used for staphylococcal infections are likely inadequate for VRE infections due to reduced susceptibility observed in enterococci (3–5). Daptomycin MIC_{50} and MIC_{90} are 2 and 4 mg/liter for *E. faecium*, and 0.5 and 1 mg/liter for *Enterococcus faecalis* versus 0.25 and 0.5 mg/liter for staphylococci (6). Reports of daptomycin resistance are now becoming more common, emphasizing the need for improved daptomycin dosing paradigms to help preserve the utility of this drug against these difficult to treat pathogens (7–10). Due to the substantial morbidity and mortality associated with enterococcal infections, it is critical to identify characteristics that correlate with treatment failure and therefore improve antimicrobial optimization and patient outcomes.

In order to derive daptomycin exposure targets for resistance prevention, we evaluated simulated daptomycin regimens from 4 to 12 mg/kg/day in a 14-day PK/PD model of simulated endocardial vegetations (SEVs) against two daptomycin-susceptible clinical VRE strains (*E. faecium* S447 and *E. faecalis* S613). Resistant strains derived from these models were then subjected to a variety of phenotypic and genotypic analyses to evaluate characteristics associated with development of daptomycin resistance.

MATERIALS AND METHODS

Bacterial strains. Two daptomycin-susceptible and vancomycin-resistant enterococcal strains, *E. faecalis* S613 and *E. faecium* S447 recovered from the bloodstream and urine, respectively, of patients before daptomycin therapy, were evaluated (11, 12). Both isolates developed daptomycin-resistant derivatives *in vivo* during daptomycin therapy (11, 12) and

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have been previously characterized. Additionally, the draft sequences of both genomes are available (11, 12).

Antimicrobial agents and media. Daptomycin was commercially purchased (Cubist Pharmaceuticals, Inc., Lexington, MA). Mueller-Hinton broth II (MHB) (Difco, Detroit, MI) with 50 mg/liter of calcium and 12.5 mg/liter magnesium was used for susceptibility testing. Due to the dependency of daptomycin on calcium for antimicrobial activity and protein binding of calcium, MHB supplemented to 75 mg/liter of calcium was used for *in vitro* SEV model experiments (13). Colony counts were determined using brain heart infusion agar (BHIA) (Difco, Detroit, MI). Emergence of resistance was assessed with BHIA supplemented with 50 mg/liter of calcium and 3× MIC of daptomycin of each original strain, *E. faecalis* S613 and *E. faecium* S447.

Susceptibility testing. Daptomycin MICs were determined in duplicate by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (14). Any colonies growing on resistance screening plates were evaluated for changes in susceptibility by broth microdilution.

***In vitro* PK/PD model.** A well-characterized *in vitro* PK/PD model of simulated endocardial vegetations (SEVs) previously validated against an *in vivo* endocarditis model with *Staphylococcus* and *Enterococcus* spp. and developed by our laboratory was utilized (15). The SEVs were prepared as previously described using human cryoprecipitate as the source of fibrin, a platelet suspension, bovine thrombin in siliconized microcentrifuge tubes, and an organism suspension to reach a final bacterial density of 10^9 CFU/g. The resultant SEVs were removed from the tubes and suspended in the model via a sterile monofilament line. The model apparatus was prefilled with growth media containing human albumin at a concentration of 3.5 g/dl. Fresh medium was continuously added and removed from the compartment along with the drug via a peristaltic pump set to simulate the half-life of daptomycin. Daptomycin was administered once daily via an injection port. The following daptomycin exposures with a targeted half-life ($t_{1/2}$) of 8 h were evaluated: 4 mg/kg every 24 h (q24h) (peak 57.8 mg/liter) (16), 6 mg/kg q24h (peak 93.9 mg/liter) (17), 8 mg/kg q24h (peak 123.3 mg/liter) (17), 10 mg/kg q24h (peak 141.1 mg/liter) (17), 12 mg/kg q24h (peak 183.7 mg/liter) (17), and a drug-free growth control with medium clearance set to mimic an 8-h $t_{1/2}$ similar to drug experiments. All models were performed in duplicate.

Pharmacokinetic analysis. Pharmacokinetic samples were obtained and stored as previously described (18). Concentrations of daptomycin were determined using a validated high-performance liquid chromatography assay that conforms to the guidelines set forth by the College of American Pathologists (17). This assay has demonstrated an interday coefficient of variation between 0.6 and 7.3% for all standards. The daptomycin peak concentrations, $t_{1/2}$, and the area under the concentration-time curve from 0 to 24 h (AUC_{0-24}) were determined using PK Analyst software (version 1.10; MicroMath Scientific Software, Salt Lake City, UT). The trapezoidal method was utilized to calculate AUC_{0-24}.

Pharmacodynamic analysis. Two SEVs were removed from each model at 0, 4, 8, 24, 32, 48, 56, 72, 96, 120, 144, 168, 192, 216, 240, 264, 288, 312, and 336 h. All models were run in duplicate. The SEVs were homogenized and diluted in cold saline to be plated on BHIA plates. For all samples, antimicrobial carryover was accounted for by serial dilution of the plated samples. If the anticipated dilution was near the MIC, samples were processed via vacuum filtration and washed through a 0.45-μm filter (Pall Corporation, Ann Arbor, MI) with normal saline to remove any daptomycin. The limit of detection for determination of colony counts was 1 $\log_{10}$ CFU/g. The plates were incubated at 35°C for 24 h, and the viable CFU were enumerated. Changes in $\log_{10}$ CFU/g were plotted against time to construct curves to describe the antibacterial activity of the simulated regimens. Bactericidal activity (99.9% kill) was defined as a $\geq$3-$\log_{10}$ CFU/g reduction in colony count from the initial inoculum. Bacteriostatic activity was defined as a $<$3-$\log_{10}$ CFU/g reduction in colony count from the initial inoculum, while inactivity was defined as no observed reductions in bacterial counts from the initial inoculum. Changes in CFU/g over time were reported graphically as the mean and standard deviation of the duplicate models for each exposure.

Resistance. Emergence of resistance was evaluated at multiple time points throughout the simulation by plating 100-μl samples of homogenized clots at each time point on BHIA plates supplemented with 50 mg/liter of calcium and daptomycin at a concentration 3× the MIC for the specific test organism. The plates were examined for growth after 48 h of incubation at 35°C. Colonies growing on resistance screening plates were evaluated for changes in MIC by broth microdilution. Isolates with a daptomycin MIC of $>$4 mg/liter were saved in glycerol tubes at −80°C for further phenotypic and genetic workup.

Characterization of daptomycin-resistant derivatives. (i) Cationic peptide and daptomycin binding determination. When daptomycin is bound with elemental calcium, the daptomycin-calcium complex is positively charged, and its degree of binding with the cell membrane has been postulated to be dependent on the attractive forces between the drug and cell surface (19). Cytochrome *c* is a cationic peptide that has been used as a surrogate marker for changes in surface charge. A cytochrome *c* binding study was performed by the method of Kraus et al. (20) Resistant derivatives from each model were evaluated for changes in cytochrome *c* binding and compared to the unexposed parent strain.

(ii) Binding of fluorescent daptomycin. Bacteria were grown to an optical density at 600 nm (OD_{600}) of 0.6 and then incubated with 16 μg/ml daptomycin–boron-dipyrromethene (bodipy) for 10 min, washed three times in medium to remove unincorporated label, stained with 2 μg/ml DAPI (4,6-diamidino-2-phenylindole), and placed on a 1.2% agarose pad for imaging in an Applied Precision deconvolution fluorescence microscope as described previously (21). For quantitation of daptomycin-bodipy fluorescence, images from each sample were collected using identical camera exposures. Cell Profiler was used to measure the average fluorescence intensity of individual pixels for $>$200 cells for the parent and resistant strains. The average fluorescence intensity of individual pixels for the background was also measured and subtracted from the cells to generate an accurate measurement of daptomycin-bodipy binding. Daptomycin binding to the resistant strains was compared to the binding observed with the parent strains.

(iii) Binding of fluorescent cathelicidin LL37. Bacteria were grown in LB broth to an OD_{600} of 0.5 to 0.6, diluted 1:1 in phenol red-free RPMI medium (Invitrogen) in a final volume of 200 μl with 6-carboxytetramethylrhodamine (TAMRA)-labeled LL37 (American Peptide, Sunnyvale, CA) (TAMRA-LL37) to a final concentration of 1 μM for *E. faecium* and 32 μM for *E. faecalis*, and incubated for 45 min at 37°C with shaking. These concentrations were chosen based on previous LL37 susceptibility data in order to maintain bacterial integrity for microscopy. Bacteria were pelleted, washed three times with RPMI medium, counterstained with DAPI (2 μg/ml) in the final wash, and visualized using a Delta Vision Deconvolution microscope (Applied Precision, Inc., Issaquah, WA).

(iv) Determination of cytoplasmic membrane depolarization. The ability of daptomycin to depolarize the cytoplasmic membrane of parent and resistant strains was carried out as previously described using a kinetic spectrofluorometric assay (22). Upon membrane potential dissipation from daptomycin or another cationic antimicrobial peptide, 3,3-dipropylthiadicarbocyanine iodide ($DiSC_3$) is released from the cell membrane into the surrounding medium, causing an increase in the fluorescence intensity at a wavelength of 670 nm. Daptomycin concentrations of 11.3 mg/liter (correlating with an average free peak of a 10-mg/kg/day dose) were utilized for each experiment. The membrane depolarization profiles over 1 h of the resistant strains and the parent strains were determined and compared. Nisin was used as a positive control to demonstrate that the assay was functioning properly.

(v) Cell wall thickness. Cell wall thickness was determined by transmission electron microscopy (TEM) as described previously (23). Ultrathin sections were evaluated at a magnification of ×48,000 with a JEOL 100CX electron microscope, and images were captured with a MegaView III side-mounted digital camera. The resulting images were analyzed us-

TABLE 1 Oligonucleotide primers used in this study

Bacterial species and gene(s)	Primer[a]	Sequence	Product size (bp)
E. faecalis			
liaFSR	liaFS-F	TGCAACACCTGGTGGCGACATG	1,570
	liaFS-R	CGCGCGCATTTCCGATTGGG	
	liaSR-F	TGGCGCGGGAGTTGCATGA	1,387
	liaSR-R	TGCTGCCGCCAAAACGTCCT	
cls	cls-F	ACATCAAAAAGACCCCCTCA	1,806
	cls-R	AAAAGATGTCCCTTTTTCACA	
E. faecium			
liaFSR	liaFS-F	CATCTAAAGTCCTATAGCCAACAGT	1,358
	liaFS-R	TGCTTGCTCCGTCAATGCCGA	
	liaSR-F	GATTAGCCCGTGAGCTGCATGA	1,505
	liaSR-R	GCCACAACAGCATGAGTGGCT	
yycF	yycF-F	GGGGATGCGAAAGAAGAAGCA	1,539
	yycF-R	AAGAGCCCCGATAACGGCAT	
yycG	yycG-F	AGAAGAGGCAGCACCTGCGGA	2,533
	yycG-R	AAGGCGCATTATAGGCCAACTCAAA	
cls	cls-F	TATTCACCTTGGTAGGCTTC	1,554
	cls-R	TTTTTGTCACAGTCCCTTTAATCTT	
cfa	cfa-F	GCCAACTCAAGTGTTTGCGG	2,400
	cfa-R	AAGAGCGCTGTTCGCTACTG	

[a] The forward and reverse primers are indicated by the letter F and R, respectively, at the end of the primer name.

ing ImageJ 1.39t software. Cell wall thickness was determined for at least 15 cells per sample using four separate quadrants of each cell for a total of ≥100 measurements per sample.

(vi) Growth kinetics. Bacterial growth kinetics of daptomycin-resistant strains derived from the final time points of each model were evaluated and compared to the parent strains. Briefly, using a 96-well, flat-bottom plate, 200 μl of MHB was inoculated with 10^6 CFU/ml of each test organism in triplicate. The plate was incubated in a spectrophotometer at 37°C for 8 h, and the optical density at 600 nm was automatically mixed and measured every 15 min. Turbidity measurements were plotted over time to construct growth curves. Daptomycin-resistant strains were compared to unexposed parent strains.

(vii) Mutation screening by PCR. Mutations in genes previously associated with daptomycin resistance were evaluated using Sanger sequencing of PCR products obtained from the entire open reading frame. The following genes were investigated: (i) *yycFG* encoding a predicted two-component regulatory system involved in cell wall homeostasis (*yycF* and *yycG* encode the putative response regulator and histidine kinase of the system, respectively) (12); (ii) *liaFSR*, encoding a putative three-component regulatory system that is part of the cell envelope response to antibiotics and antimicrobial peptides (11, 24); and (iii) *cls* and *cfa*, encoding two enzymes (cardiolipin synthase and cyclopropane fatty acid synthase, respectively) that are likely to be involved in cell membrane phospholipid metabolism and have been previously associated with daptomycin resistance in *E. faecalis* (11) and *E. faecium* (11, 12, 25). A nonsynonymous mutation was defined as a nucleotide change that resulted in an amino acid substitution not present in daptomycin-susceptible parental enterococci (S447 and S613), whose genomes are sequenced and publicly available. *E. faecalis* V583 (26) and *E. faecium* DO (27) (closed genomes) were used as the template for sequence assembly. Primers utilized to amplify the genes above are listed in Table 1.

(viii) Statistical analysis. Changes in CFU/g, cytochrome *c* binding, daptomycin-bodipy binding, TAMRA-LL37 binding, total daptomycin-induced depolarization, and growth curve AUCs were compared by one-way analysis of variance with Tukey's posthoc test or by *t* test as appropriate. A *P* value of ≤0.05 was considered significant. All statistical analyses were performed using SPSS statistical software (release 21.0; SPSS, Inc., Chicago, IL).

RESULTS

Susceptibility testing. Daptomycin MICs for *E. faecium* S447 and *E. faecalis* S613 were 2 and 1 mg/liter, respectively. MICs of daptomycin-resistant derivatives recovered from the *in vitro* models ranged from 8 to 256 mg/liter.

***In vitro* PK/PD model.** Observed PK parameters and achieved PK/PD ratios are summarized in Table 2. Quantitative changes in $\log_{10}$ CFU/g for each regimen are illustrated in Fig. 1 and 2 for each

TABLE 2 PK/PD relationships achieved in the model

Dose in mg/kg/day	Achieved PK parameter[a]			*E. faecium* S447 with a DAP MIC of 2 mg/liter[b]		*E. faecalis* S613 with a DAP MIC of 1 mg/liter	
	C_{max}	$t_{1/2}$	AUC_{0-24}	AUC_{0-24}/MIC	Peak/MIC	AUC/MIC	Peak/MIC
12	184.1 ± 2.5	7.75 ± 0.35	2,036.3 ± 145.1	1,018.15	92.05	2,036.3	184.1
10	144.3 ± 1.8	8.0 ± 0.14	1,561.7 ± 37.2	780.85	72.15	1,561.7	144.3
8	122.4 ± 0.1	8.15 ± 0.07	1,342.9 ± 7.6	671.45	61.2	1,342.9	122.4
6	94.3 ± 1.9	7.25 ± 0.21	959.1 ± 35.7	479.55	47.15	959.1	94.3
4	57.6 ± 1.7	8.05 ± 0.07	627.3 ± 22.9	313.65	28.8	627.3	57.6

[a] C_{max}, maximum concentration of drug in serum; $t_{1/2}$, half-life; AUC_{0-24}, area under the concentration-time curve from 0 to 24 h.
[b] DAP, daptomycin.

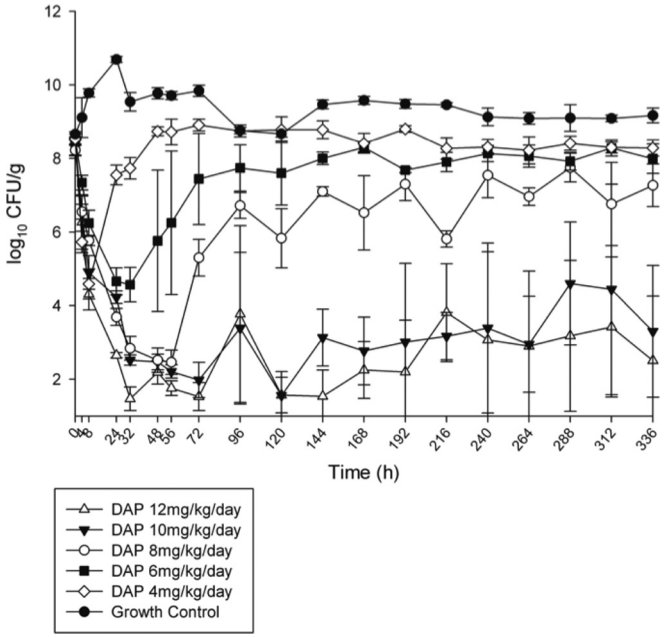

FIG 1 Pharmacodynamic response of *Enterococcus faecium* S447 in the SEV model to different dose exposures of daptomycin (DAP) over 14 days. Data are presented as mean CFU/g with error bars indicating standard deviations of the means of results from duplicate experiments.

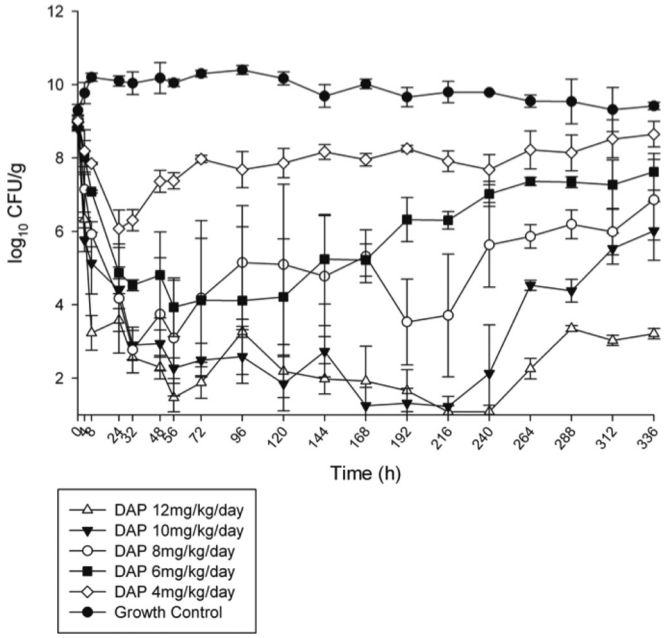

FIG 2 Pharmacodynamic response of *Enterococcus faecalis* S613 in the SEV model to different dose exposures of daptomycin over 14 days. Data are presented as mean CFU/g with error bars indicating standard deviations of the means of results from duplicate experiments.

organism. Against *E. faecium* S447, all regimens were bactericidal within the first 24 h, but only 10- and 12-mg/kg/day exposures remained bactericidal for the duration of the models. These exposures produced similar reductions in bacterial densities ($P = 0.55$) by 336 h but offered significantly greater CFU/g reductions compared to 4- to 8-mg/kg/day regimens ($P = 0.0005$). Against *E. faecalis* S613, 6- to 12-mg/kg/day regimens were bactericidal within the first 24 h, but only 12-mg/kg/day exposures remained bactericidal for the duration of the model. This regimen maintained statistically superior CFU/g reductions compared to 4- to 10-mg/kg/day regimens by 336 h ($P = 0.0005$). Daptomycin resistance, defined as recovery of isolates with an MIC of >4 mg/liter, emerged in both strains from models simulating 4-, 6-, and 8-mg/kg/day regimens but not during simulated exposures of 10 and 12 mg/kg/day. Resistant isolates were observed as early as 120 h and 144 h from the *E. faecalis* S613 strain and the *E. faecium* S447 strain, respectively. The peak/MIC and AUC_{0-24}/MIC ratios observed with the 10-mg/kg/day simulations were 72 and 781 for strain S447 and 144 and 1,562 for strain S613, respectively.

Characterization of daptomycin-resistant derivatives. (i) Cationic peptide and daptomycin binding determination. Cytochrome *c* binding studies revealed a significant reduction in the bound fraction of cytochrome *c* in daptomycin-resistant isolates derived from our models. On average, *E. faecalis* S613-derived resistant strains bound 42% (median) less cytochrome *c* than the parent strain, with a range from 2% to 94% ($P = 0.01$). *E. faecium* S447-derived resistant strains bound 28% (median) less cytochrome *c* than the parent strain with a range of 13% to 40%. This difference was significant for some of the S447-derived resistant strains tested, but not all.

(ii) Binding of fluorescent daptomycin. Two representative daptomycin-resistant derivatives were chosen from the SEV model for microscopy experiments: (i) a derivative of *E. faecalis* S613 that was obtained on day 10 at a simulated dose of 4 mg/kg (*E. faecalis* $S613_{D4}$ harboring an insertion of isoleucine in position 177 of the putative LiaF protein with an MIC of 16 μg/ml), and (ii) a derivative of *E. faecium* S447, obtained on day 14 at a simulated dose of 4 mg/kg (*E. faecium* $S447_{D4}$ with no mutations in target genes with a MIC of 128 μg/ml). Results of daptomycin-bodipy binding for selected parent and derivative strains are depicted in Fig. 3. The mean fluorescent intensity of bound daptomycin-bodipy at 16 μg/ml was 2.3× higher for *E. faecalis* S613 than for *E. faecalis* $S613_{D4}$ (671.29 ± 11.80 versus 282.16 ± 19.40, respectively [mean ± standard error shown]; $P < 0.0001$). Similarly, the mean fluorescent intensity of bound daptomycin-bodipy for *E. faecium* S447 cells was 334.83 ± 5.65 but was below the level of detection for *E. faecium* $S447_{D4}$.

(iii) Binding of fluorescent LL37. Changes in fluorescent labeled LL37 binding between *E. faecalis* S613 and *E. faecium* S447 versus their daptomycin-resistant derivatives (*E. faecalis* $S613_{D4}$ and *E. faecium* $S447_{D4}$, respectively) are illustrated in Fig. 4. Similar to daptomycin binding, TAMRA-labeled LL37 (TAMRA-LL37) was found to bind less to their daptomycin-resistant derivatives obtained from the SEV model. The mean fluorescence intensity of bound TAMRA-LL37 at 1 μM was 3.4× higher in *E. faecium* S447 than in *E. faecium* $S447_{D4}$ (601.99 ± 37.99 versus 177.30 ± 17.08, respectively [mean ± standard error shown]; $P < 0.0001$). Similarly, the mean fluorescence intensity of bound TAMRA-LL37 at 32 μM in *E. faecalis* S613 cells was 2.4× higher than those in *E. faecalis* $S613_{D4}$ (154.35 ± 20.83 versus 65.09 ± 17.08, respectively; $P < 0.05$).

(iv) Determination of cytoplasmic membrane depolarization. Daptomycin-induced membrane depolarization of parent strains and selected resistant derivatives is depicted in Fig. 5A and

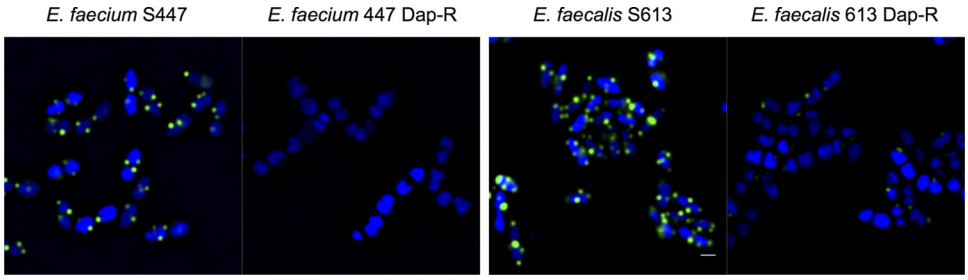

FIG 3 Membrane-bound fluorescently labeled daptomycin (green) in the parent strains (*E. faecium* S447 and *E. faecalis* S613) (left images) and representative daptomycin-resistant (Dap-R) strains (right images). Cells are stained with DAPI (blue).

B. Both the total amount of membrane depolarization and the rate of depolarization were substantially reduced among daptomycin-resistant strains. The total percentage depolarization was 19.7% to 37.2% for *E. faecalis* S613 derivatives and 12.8% to 53.3% in *E. faecium* S447-derived strains compared to 100% for both parent strains ($P < 0.0001$).

(v) Cell wall thickness. Transmission electron microscopy (Fig. 6 and 7) revealed multiple morphological abnormalities in *E. faecium* $S447_{D4}$ compared to its daptomycin-susceptible parental strain. *E. faecium* $S447_{D4}$ was found to have a 4.5-nm increase in cell wall thickness compared to *E. faecium* S447 (31.7 ± 4.9 nm versus 27.2 ± 4.9 nm, respectively; $P < 0.001$), while the cell wall thickness of *E. faecalis* $S613_{D4}$ increased by an average of 6.2 nm compared to S613 (26.2 ± 4.4 nm versus 20.0 ± 4.1 nm, respectively; $P < 0.001$). Qualitatively, the daptomycin-resistant derivatives of both species were found in clusters, demonstrating poor cell separation and abnormal septum formation. Another prominent finding observed in this study was the development of abnormal protrusions from the cell wall. These abnormalities have previously been reported in daptomycin-resistant VRE, but it is unclear whether they are a cause or effect of daptomycin resistance (11, 24).

(vi) Growth kinetics. Growth kinetic analysis revealed that the majority of the resistant derivatives from either strain developed daptomycin resistance at the cost of fitness. Interestingly, the degree of growth rate inhibition appeared to be proportional to the daptomycin exposures in the model from which they were derived. Daptomycin-resistant strains derived from the 4-mg/kg/day simulations had less growth rate inhibition, while resistant derivatives from 6- and 8-mg/kg/day simulations tended to have lower growth rates. The growth rates of *E. faecalis* $S613_{D4}$ (harboring an insertion in *liaF*) and an S613-derived mutant with a three-codon deletion in *cls* were unchanged compared to that of the parent strain ($P = 0.28$ and $P = 0.055$, respectively). The time to mid-exponential growth was 286 ± 3.15 min versus 323 ± 34.5 min for *E. faecalis* S613 and its resistant derivatives, respectively, and 338 ± 0.9 min versus 405 ± 50.2 min for *E. faecium* S447 and its resistant derivatives, respectively. Exponential-phase doubling times for strains S613 and S447 were both 50 min. *E. faecalis* S613-derived strains demonstrated a median doubling time of 75 min with a range of 52 to 91 min. *E. faecium* S447-derived strains demonstrated a median mid-exponential doubling time of 60 min with a range of 57 to 74 min. The overall growth rate, assessed by comparison of the area under the growth-versus-time curves, was significantly lower in 50% of *E. faecalis* S613-derived strains compared to S613 ($P \leq 0.001$) and 83% of *E. faecium* S447-derived strains relative to S447 ($P \leq 0.001$).

(vii) Mutation screening by PCR. Mutations in *liaFSR* and *cls* were assessed in all derivative strains. Additionally, *yycFG* and *cfa* were also evaluated in *E. faecium* derivatives. The mutations identified are summarized in Table 3. Nonsynonymous point mutations in *liaF* and *cls* were identified in six *E. faecalis* S613 mutants derived from the 4-mg/kg/day simulations but were not found in strains derived from other exposures. No mutations in the target genes were identified in *E. faecium* S447-derived strains.

DISCUSSION

Infections with VRE are a major therapeutic challenge due to intrinsic resistance to multiple antibiotic classes, a paucity of bactericidal agents, and the rapid emergence of acquired antibiotic resistance. Antimicrobial dose optimization is necessary for the preservation of the clinical utility of currently available antimicrobials, but there are insufficient data to recommend resistance prevention dosing targets for most drugs. In this study, we have demonstrated that for daptomycin-susceptible strains of vancomycin-resistant *E. faecium* and *E. faecalis*, daptomycin exposures associated with doses of at least 10 mg/kg/day are likely to be

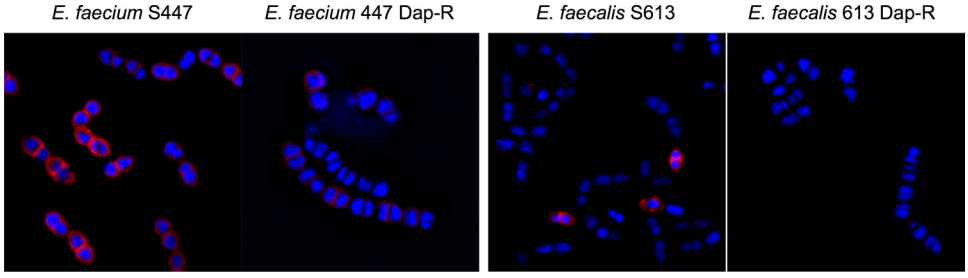

FIG 4 Membrane-bound fluorescently labeled human cathelicidin LL37 (red) in the parent strains (*E. faecium* S447 and *E. faecalis* S613) (left images) and representative daptomycin-resistant (Dap-R) strains (right images). Cells are stained with DAPI (blue).

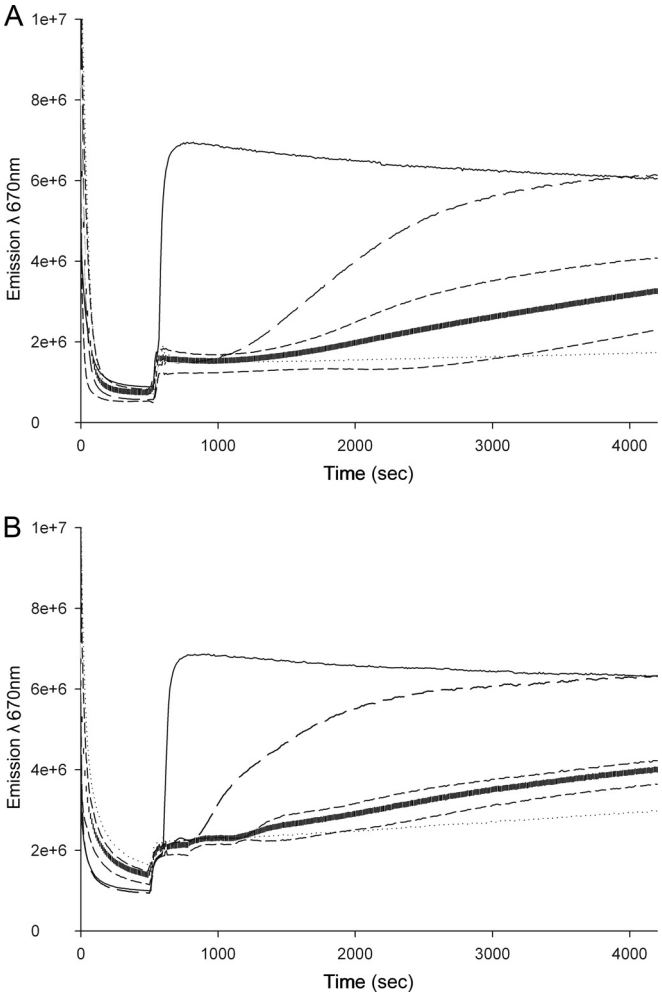

FIG 5 Daptomycin-induced membrane depolarization over 1 h in *E. faecium* S447 and resistant derivatives (A) and *E. faecalis* S613 and resistant derivatives (B). Nisin (25 mg/liter) was used as a positive control for membrane depolarization (thin solid line). The parent strain exposed to daptomycin (11.3 mg/liter) (line of long dashes), average resistant derivative exposed to daptomycin (11.3 mg/liter) (thick solid line) with upper and lower standard deviation (lines of short dashes), and negative control (dotted line) are shown. The emission wavelength is shown on the *y* axes.

required to prevent resistance emergence. These doses were associated with an AUC_{0-24}/MIC ratio of 781 for *E. faecium* S447 and 1,562 for *E. faecalis* S613. These exposures are clinically achievable and have been shown to be safe in multiple studies (17, 28–30). While we did not detect resistant derivatives from the models at ≥10-mg/kg/day simulations, bacterial regrowth began to occur in *E. faecalis* S613 after 240 h in both the 10- and 12-mg/kg/day simulations (Fig. 2). While this regrowth phenomenon may occur in *in vitro* models in the absence of meaningful susceptibility changes, it is possible that if the model was run for longer that resistance may have emerged from even these higher exposures.

It is interesting that there is a 2-fold-higher daptomycin resistance prevention AUC_{0-24}/MIC ratio in *E. faecalis* compared to *E. faecium*; this is associated with a much higher intrinsic resistance to the cationic antimicrobial peptide LL37 in the former species, even though daptomycin MIC_{50}/MIC_{90} values are considerably higher in *E. faecium* (2/4 mg/liter versus 0.5/1 mg/liter) (31). The increased intrinsic resistance to antimicrobial peptides in *E. faecalis* may be related to the fact that it causes 90% of enterococcal endocarditis, compared to 5 to 10% for *E. faecium* (32). Reflective of this difference in LL37 MIC, which approximates 1 to 4 μM in *E. faecium* and 16 to 64 μM in *E. faecalis*, was the concentrations of LL37 used in the LL37 binding assay experiments. These findings highlight the cross-resistance between endogenous antimicrobial peptides produced by innate immune defenses and pharmaceutical antibiotics like daptomycin. It also points to the important role that *in vivo* peptide exposure might play in driving antibiotic resistance in the absence of administered drugs. Peptide-mediated predisposition for daptomycin resistance *in vivo* makes achieving the critical AUC_{0-24}/MIC threshold of utmost importance in the treatment of infective endocarditis, where suboptimal exposure to cationic antimicrobial peptides is likely.

The mechanisms of daptomycin resistance in enterococci have not been fully elucidated. It appears that resistance to daptomycin can emerge through a variety of genetic pathways (33), but a common theme involves mutations in two group of genes: (i) genes involved in cell envelope homeostasis (*yycFG* and *liaFSR* systems) and (ii) genes encoding enzymes involved in phospholipid metabolism (i.e., *cls*, *gdpD*, and *cfa* encoding cardiolipin synthase, glycerophosphodiester phosphodiesterase, and cyclopropane fatty acid synthase, respectively) (11, 12, 24, 34). However, the absence of mutations in some of these target genes in some of our resistant derivatives suggests that alternative genetic pathways remain to be discovered. In *E. faecalis* S613, mutations in *liaF* and *cls* were observed in resistant strains derived from the 4-mg/kg/day exposures. However, these mutations, although present in the same genes, were different from those previously described under daptomycin exposure *in vivo* (11). Moreover, resistant derivatives of *E. faecium* S447 selected in the SEV model harbored none of the mutations in *yycG*, *cls*, or *cfa* previously described in an S447-resistant derivative (designated R446) obtained from the bloodstream of a patient after daptomycin therapy. Our findings suggest that there are multiple alternative genetic pathways leading to daptomycin resistance. Our results also highlight the remarkable ability of enterococci to adapt to the attack of antimicrobial peptides.

Recent insights into the complex mechanism of daptomycin resistance suggest that in certain enterococcal strains, the mechanism of resistance may not involve "repulsion" of cation-decorated daptomycin from the cell surface, as previously shown. Tran et al. recently postulated that diversion of daptomycin from the bacterial septum with subsequent "trapping" of the antibiotic away from septal areas may be an alternative mediator of daptomycin resistance in *E. faecalis* (24). This antibiotic diversion was associated with redistribution of cardiolipin arrays away from the septum, which appeared to be mediated by an Ile177 deletion in the predicted LiaF protein. Interestingly, instead of an Ile177 deletion, our *E. faecalis* S613-derived mutant ($S613_{D4}$) harbored an Ile insertion at the same position. However, our microscopy data with bodipy-labeled daptomycin and TAMRA-labeled LL37 indicate that the mechanism of daptomycin resistance for this mutant involves the classical "repulsion" of the antibiotic from the cell surface, highlighting different strategies under various conditions. Resistant derivatives also developed thicker cell walls with more septum formation and poor cell separation. It is believed that some organisms may use this strategy of forming "false" division septa in order to draw daptomycin molecules away from critical binding sites at the "true" division septum. It is possible that di-

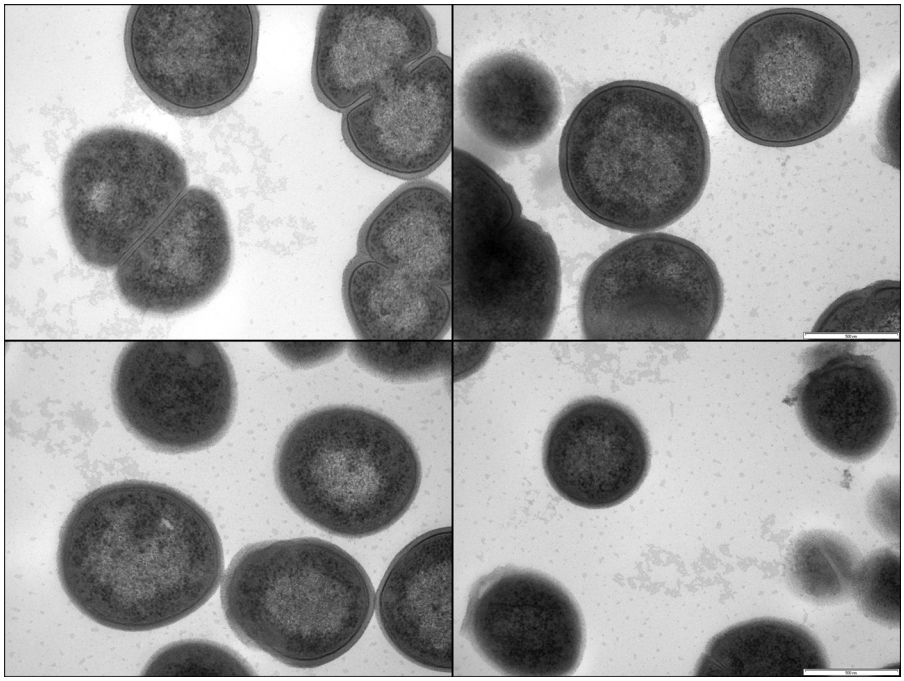

FIG 6 TEM of the parent strain *E. faecium* S447 illustrating baseline cell wall thickness and the absence of abnormal surface projections and division septa. Bars, 500 nm.

version of daptomycin from critical sites is an initial strategy at early stages of the development of daptomycin resistance. While daptomycin is not shown to be diverted in the daptomycin-resistant mutant *E. faecalis* $S613_{D4}$ (as previously shown in other strains), the genotypic characterization of such resistant derivatives suggest that different mutations than those previously described *in vivo* occurred, making comparisons difficult to establish. Additionally, other unknown mutations may have occurred, and their role in manifestation of this phenotype is unclear at this time. Given that it was not feasible to perform fluorescent drug

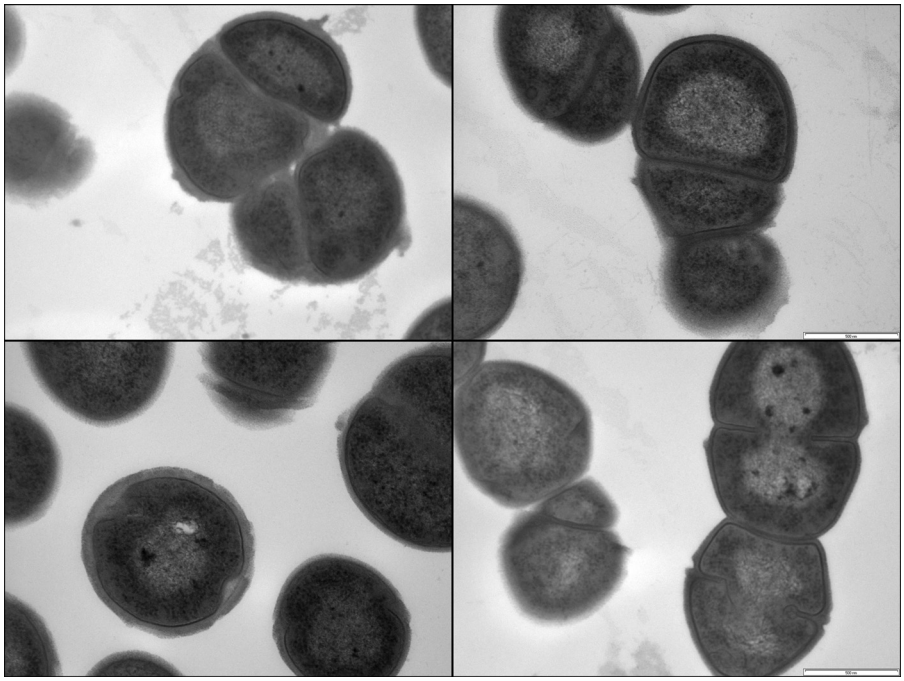

FIG 7 TEM of the parent strain *E. faecium* $S447_{D4}$ illustrating increased cell wall thickness and the presence of abnormal surface projections and division septa. Bars, 500 nm.

TABLE 3 *E. faecalis* S613 mutants derived from 4-mg/kg/day exposures

Time derived (h)	DAP MIC (mg/liter)	Gene	Mutation in gene
120	32	*liaF*	Insertion of Ile at position 177
144	32	*cls*	G653→A (R218Q)
	16	*cls*	G800→A (R267H)
168	8	*cls*	Deletion of NFQ77–79
240	16	*liaF*	Insertion of Ile at position 177
336	16	*cls*	Deletion of NFQ77–79

binding studies or TEM on more than two of the daptomycin-resistant derivatives, it may also be possible that some of the other strains would have behaved differently in these experiments. Further studies are warranted to clarify the genetic changes associated with these phenotypes in *E. faecalis* (24).

To our knowledge, this is the first study that explores the daptomycin resistance prevention breakpoint in enterococci using clinically relevant drug exposures over the substantial time period of 14 days. Based on our observations, it is clear that the dose of daptomycin for the management of VRE infections and prevention of resistance is much higher than the labeled dose of 6 mg/kg/day indicated for staphylococcal bacteremia and is likely around 10 mg/kg/day for average patients with MICs in the susceptible range. However, this study is limited to some extent by the number of strains tested and the potential variability between enterococcal species (i.e., *E. faecium* versus *E. faecalis*). Furthermore, this resistance prevention breakpoint was derived in a model with very high bacterial densities (9 $\log_{10}$ CFU/g), so these data may be more representative of serious high-inoculum infections such as infective endocarditis and less relevant to more-benign infections. Emerging data suggest that certain strains of VRE with genetic predisposition toward daptomycin resistance, specifically those with *liaFSR* mutations, may require clinically unachievable daptomycin exposures to prevent resistance. Further research in this area should focus on characterizing this resistance prevention exposure in other strains and exploring strategies, such as combination therapy, to lower this breakpoint.

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