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Deletion of *liaR* Reverses Daptomycin Resistance in *Enterococcus faecium* Independent of the Genetic Background

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We have shown previously that changes in LiaFSR, a three-component regulatory system predicted to orchestrate the cell membrane stress response, are important mediators of daptomycin (DAP) resistance in enterococci. Indeed, deletion of the gene encoding the response regulator LiaR in a clinical strain of *Enterococcus faecalis* reversed DAP resistance (DAP-R) and produced a strain hypersusceptible to antimicrobial peptides. Since LiaFSR is conserved in *Enterococcus faecium*, we investigated the role of LiaR in a variety of clinical *E. faecium* strains representing the most common DAP-R genetic backgrounds. Deletion of *liaR* in DAP-R *E. faecium* R446F (DAP MIC of 16 μg/ml) and R497F (MIC of 24 μg/ml; harboring changes in LiaRS) strains fully reversed resistance (DAP MICs decreasing to 0.25 and 0.094 μg/ml, respectively). Moreover, DAP at concentrations of 13 μg/ml (achieved with human doses of 12 mg/kg body weight) retained bactericidal activity against the mutants. Furthermore, the *liaR* deletion derivatives of these two DAP-R strains exhibited increased binding of boron-dipyrromethene difluoride (BODIPY)-daptomycin, suggesting that high-level DAP-R mediated by LiaR in *E. faecium* involves repulsion of the calcium-DAP complex from the cell surface. In DAP-tolerant strains HOU503F and HOU515F (DAP MICs within the susceptible range but bacteria not killed by DAP concentrations of 5× the MIC), deletion of *liaR* not only markedly decreased the DAP MICs (0.064 and 0.047 μg/ml, respectively) but also restored the bactericidal activity of DAP at concentrations as low as 4 μg/ml (achieved with human doses of 4 mg/kg). Our results suggest that LiaR plays a relevant role in the enterococcal cell membrane adaptive response to antimicrobial peptides independent of the genetic background and emerges as an attractive target to restore the activity of DAP against multidrug-resistant strains.

Enterococcus faecium has become one of the most recalcitrant nosocomial pathogens due to the emergence of strains that exhibit multidrug resistance. Vancomycin resistance is now almost universal in *E. faecium* isolates recovered from U.S. hospitals, and the Centers for Disease Control and Prevention has deemed this pathogen a serious public health threat (1). This difficult situation has also been recognized by the Infectious Diseases Society of America by the inclusion of *E. faecium* as one of the "No-ESKAPE" pathogens (*E. faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Enterobacter* spp.) (2), against which new therapies are urgently needed. Interestingly, the rise of *E. faecium* as an important nosocomial pathogen has been associated with the dissemination of a hospital-associated (HA) genetic lineage, which differs from that of community-associated subpopulations (3, 4).

The only FDA-approved option for the treatment of vancomycin-resistant *E. faecium* (VRE) is linezolid (quinupristin-dalfopristin [Q/D] FDA approval has been withdrawn). Both linezolid and Q/D have several limitations, such as toxicity, problems due to the administration, bacteriostatic effects, and emergence of resistance (5). Daptomycin (DAP), a lipopeptide antibiotic with bactericidal activity against *E. faecium*, has emerged as a key frontline option for the treatment of severe VRE infections. However, the main challenge when DAP is used against VRE is the development of resistance during the course of treatment, which has been reported extensively (6–8). Using genomic and biochemical analyses of DAP-resistant (DAP-R) strains of *Enterococcus faecalis* and *E. faecium*, we have provided robust evidence that development of DAP resistance mainly results from mutations in two major groups of genes, i.e., those encoding (i) proteins involved in the regulation of cell envelope homeostasis and (ii) enzymes responsible for cell membrane (CM) phospholipid metabolism (9–13). Among the first group of gene products, the most studied is the LiaFSR system, a three-component regulatory system present in all Gram-positive organisms of clinical importance that is predicted to orchestrate the cell envelope stress response to antimicrobial peptides (AMPs). The LiaFSR system is composed of a predicted transmembrane protein (LiaF) that, in *Bacillus subtilis* and *S. aureus*, has been shown to negatively regulate the system, a histidine kinase (LiaS), and a classic helix-turn-helix (HTH)-type response regulator (LiaR) (14–16).

We recently showed that deletion of *liaR* in a clinical strain of vancomycin-resistant *E. faecalis* not only fully reversed DAP resis-

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TABLE 1 *Enterococcus faecium* strains used in this study

E. faecium strain	Relevant characteristic(s)[a]	DAP MIC (μg/ml)	Reference(s) or source
R446	DAP- and VAN-resistant clinical isolate harboring eight changes in predicted proteins compared to its parental strain, including an S333L substitution in YycG, along with other changes in Cls, Cfa, RrmA, SulP, XpaC, and PTS-EIIA and in a protein harboring an HD domain	16	6, 9, 20,21
R446F	Fusidic acid-resistant derivative of R446	16	This study
R446F Δ*liaR*	Derivative of R446F harboring a nonpolar deletion of *liaR*	0.25	This study
R497	DAP-resistant isolate harboring W73C and T120A substitutions in LiaR and LiaS, respectively, and an additional insertion of MPL at position 110 in Cls	24	9, 21
R497F	Fusidic acid-resistant derivative of R497	24	This study
R497F Δ*liaR*	Derivative of R497F harboring a nonpolar deletion of *liaR*	0.094	This study
R497F Δ*liaR*::*liaR*	Complementation of *liaR* in *cis* (native chromosomal location)	24	This study
HOU503	DAP-tolerant and VAN-resistant clinical isolate harboring W73C and T120A substitutions in LiaR and LiaS, respectively	3	21
HOU503F	Fusidic acid-resistant derivative of HOU503	3	This study
HOU503F Δ*liaR*	Derivative of HOU503F harboring a nonpolar deletion of *liaR*	0.064	This study
HOU503 Δ*liaR*::*liaR*	Complementation of *liaR* in *cis* (native chromosomal location)	3	This study
HOU515	DAP-tolerant clinical isolate harboring an A414T substitution in YycG and no LiaFSR substitutions	3	21
HOU515F	Fusidic acid-resistant derivative of HOU515	3	This study
HOU515F Δ*liaR*	Derivative of HOU515F harboring a nonpolar deletion of *liaR*	0.047	This study

[a] VAN, vancomycin; DAP, daptomycin; YycG, putative histidine kinase of the essential two-component regulatory system YycFG; PTS, phosphotransferase system; Cls, cardiolipin synthase.

tance but also yielded a strain hypersusceptible to DAP, with MICs decreasing below the value of the DAP-susceptible (DAP-S) parental strain (13). Similarly, deletion of *liaR* in a DAP-S laboratory strain of *E. faecalis* (OG1RF) decreased the DAP MIC 8-fold, indicating that LiaR mediates the DAP response in these organisms. Interestingly, the reversion of DAP resistance in *E. faecalis* was associated with an increased susceptibility to a cadre of AMPs of different origins and mechanisms of action and with a marked decrease in the MIC of telavancin, another CM-acting antimicrobial used in clinical practice (13).

In *S. aureus*, the mechanism of DAP resistance has been postulated to depend on electrostatic repulsion from the cell surface of the positively charged DAP-calcium complex (17). However, unlike in *S. aureus*, the mechanism of DAP resistance in *E. faecalis* appears to be related to diversion of the antibiotic molecule away from the septum, which is the principal target of DAP (12). This phenomenon is associated with redistribution of CM cardiolipin (CL) microdomains from septal locations to other CM regions. Our previous data in *E. faecalis* (12, 13) support the notion that LiaR controls the redistribution of CL microdomains responsible for decreased susceptibility to DAP, suggesting that LiaR is the "master" regulator of the enterococcal cell response to the antimicrobial peptide attack. Additionally, our recent crystallographic studies on the response regulator LiaR and an adaptive LiaR mutant ($LiaR^{D191N}$) complexed with the target DNA indicate that the structural basis for increased resistance to DAP hinges on a transition of the LiaR dimer to a tetramer that increases the affinity for target promoters. Crystal structures of the LiaR DNA binding domain complexed with DNA suggest that LiaR induces DNA binding that potentially increases recruitment of RNA polymerase to the transcription start site (18, 19).

The LiaFSR system is conserved in *E. faecium*, and the predicted sequences of LiaR exhibit 89% amino acid identity to those of *E. faecalis*. Moreover, our recent evidence suggests that the mechanism of DAP resistance in *E. faecium* is more similar to that described in *S. aureus* (i.e., repulsion of the antibiotic from the cell surface) (20–22). In this work, we aimed to characterize the role of LiaR in DAP resistance in diverse clinical strains of *E. faecium* that exhibit DAP resistance or are tolerant to this antibiotic. Our results show that LiaR is required for DAP resistance in *E. faecium*, independent of the genetic background or the presence of substitutions in LiaFSR, highlighting the important role of LiaR in antimicrobial resistance and CM homeostasis.

MATERIALS AND METHODS

Bacterial strains. Four *E. faecium* strains whose genome sequences have been obtained (21) were included in this study and are described in Table 1 (6, 9, 20, 21). Briefly, two DAP-R clinical strains (R446 and R497) with DAP MICs of 16 μg/ml and 24 μg/ml, respectively, and two DAP-tolerant *E. faecium* strains (HOU503 and HOU515; MICs of 3 μg/ml) (21) were chosen. Tolerance was defined by the inability of DAP to kill the DAP-S strains (HOU503 and HOU515) at concentrations of 5× the MIC in time-kill studies, as previously reported (21). The rationale for choosing these strains was that they represent the most common genetic pathways for DAP resistance based on a previous whole-genome analysis study that included 19 *E. faecium* strains with diverse DAP MICs (21). R497 and HOU503 harbor substitutions in LiaS and LiaR (T120A and W73C, respectively) and are representatives of the LiaFSR pathway, the most common system affected in DAP-R *E. faecium*. R446 and HOU515 lack substitutions in LiaFSR but harbor substitutions in YycG (S333L and A414T, respectively), the putative histidine kinase of the YycFG system, a two-component regulatory system implicated in cell wall homeostasis. Changes in YycFG (or accessory proteins YycHIJ) were the second most frequent changes observed in DAP-R strains of *E. faecium* after LiaFSR (21).

Mutagenesis strategy. We generated in-frame *liaR* deletions in the four *E. faecium* strains mentioned above (Table 1) and complemented R497 and HOU503 by placing their native *liaR* genes in their original chromosomal locations (the predicted LiaR harbors a W73C substitution). We used a *p*-chlorophenylalanine (p-Chl-Phe) sensitivity counterselection system ($PheS^*$) (23) to obtain the mutants and deliver the genes back into the chromosomes (complementation) using plasmid pHOU1,

as described previously (9, 20, 24). Briefly, ~500-bp regions upstream and downstream of *liaR* were amplified by crossover PCR using DNA of the corresponding strain as the target and the primers shown in Table S1 in the supplemental material. Each fragment was cloned into pHOU1 using EcoRI and BamHI. The recombinant plasmids were electroporated into *E. faecalis* CK111 and delivered into fusidic acid-resistant derivatives of the target *E. faecium* strains by conjugation. First recombination integrants were selected on gentamicin (125 μg/ml) and fusidic acid (25 μg/ml) and subsequently plated in medium containing *p*-chlorophenylalanine (9, 20, 24). Colonies obtained from the counterselection medium were tested by replica plating in the presence of different DAP concentrations. Clones that were susceptible (or resistant in the case of complementation) to DAP were further purified, and the deletions (or complementations) were confirmed by PCR and sequencing. All candidate colonies were subjected to pulsed-field gel electrophoresis (PFGE) to confirm their genetic relationship with the parental strains. Growth curves of mutants and parental strains (fusidic acid-resistant derivatives) were performed to determine if the deletions altered the growth kinetics of the mutants. The mutagenesis strategy deleted 633 nucleotides of *liaR* in R446F and HOU515F and 615 nucleotides in R497F and HOU503F.

Susceptibility testing. We determined the MICs of DAP, telavancin, β-lactams (ampicillin, cephalothin, and ceftaroline), tetracyclines (doxycycline, minocycline, tetracycline, and tigecycline), fosfomycin, and colistin for the wild type, mutant derivatives, and complemented strains by Etest (bioMérieux, Marcy l'Etoile, France) on Mueller-Hinton agar according to instructions from the manufacturer with incubation for 24 h. The MICs for each strain were determined in triplicate with readings performed by two independent observers, and the results were interpreted using breakpoints issued by the Clinical and Laboratory Standards Institute (CLSI) (25).

Time-kill assays. Time-kill assays were performed with an initial bacterial inoculum of 10^7 CFU/ml in Mueller-Hinton broth (MHB) supplemented with calcium (50 mg/liter). We selected concentrations of DAP that were similar to those predicted to be the free peak serum DAP concentrations when the antibiotic is given in humans at doses of 4 and 12 mg/kg body weight (4 and 13 μg/ml, respectively). Bacteria were enumerated at 0, 6, and 24 h. Antibiotic carryover was controlled by centrifugation to discard the supernatant, and the pelleted bacteria were suspended in 0.9% saline solution before plating (11, 26, 27). DAP bactericidal activity was defined as a reduction of 3 $\log_{10}$ in CFU/ml at 24 h in comparison to the value in the initial inoculum. The limit of detection, assuming maximum plating efficiency, was 200 CFU/ml.

Binding of BDP-DAP. In order to evaluate the interactions of DAP with the bacterial CM, we used boron-dipyrromethene difluoride (BODIPY)-daptomycin (BDP-DAP), a fluorescent derivative of DAP, as previously described (12). The assays were performed in all wild-type *E. faecium* isolates (fusidic acid-resistant derivatives) (Table 1) and the corresponding *liaR* deletion mutants. BDP-DAP staining was performed according to previously published protocols (12, 28–30). The *E. faecium* isolates were grown in Luria-Bertani (LB) broth at 37°C and exposed to two concentrations of BDP-DAP (4 and 64 μg/ml in LB broth supplemented with Ca^{2+} at 50 mg/liter) for 10 min in the dark. In order to measure fluorescence emission, we used a standard fluorescein isothiocyanate (FITC) filter set (excitation at 490 nm and emission at 528 nm). Three independent experiments were performed for each strain on different days. The fluorescence intensity was quantitated and normalized to the protein concentration of the sample in order to estimate the amount of binding of BDP-DAP, as described previously (12).

NAO staining of *E. faecium* strains. We had previously shown (12, 13) that the fluorescent dye 10-*N*-nonyl acridine orange (NAO) can be used to visualize anionic phospholipids (PL) in the CM. We examined the effect of development of DAP-R on the distribution of PL in *E. faecium* strains as previously described in *E. faecalis* (12, 13). For microscopic examination, *E. faecium* cells were grown in Trypticase soy broth (TSB) to exponential phase (A_{600} of ~0.3). NAO (Molecular Probes) at a concentration of 1 μM was added to the growth medium. This concentration of NAO was found not to inhibit the growth of *E. faecium*. Samples were stained for 3.5 h at 37°C in the dark with gentle agitation. Subsequently, cells were washed three times with 0.9% saline and immobilized on a poly-L-lysine (Sigma-Aldrich)-treated coverslip. Fluorescent images were captured by an Olympus IX71 microscope with a PlanApo N 100× objective according to previously described protocols (12, 13). Emission of green fluorescence from NAO was detected using a standard fluorescein isothiocyanate (FITC) filter (excitation at 490 nm and emission at 528 nm). Image acquisition was performed using the SlideBook, version 5.0, software package. Three independent experiments were performed for each strain on different days. Captured images were processed using Adobe Photoshop CS5.

RESULTS

Deletion of *liaR* reverses high-level DAP resistance in *E. faecium*, independent of the genetic background. Strains R497 and R446 are DAP-R isolates with DAP MICs of 24 μg/ml and 16 μg/ml, respectively. R497 harbors substitutions in LiaS (T120S) and LiaR (W73C) and insertion of MPL residues at position 110 in the putative cardiolipin synthase. R446 is a derivative of the DAP-S strain S447 isolated from a patient (Table 1) (6, 20). R446 harbors eight changes in predicted proteins (Table 1), including an S333L substitution in the histidine kinase YycG, a member of the essential YycFG two-component regulatory system that has been implicated in DAP resistance in staphylococci (31). Of note, R446 lacks changes in LiaFSR. We used these two distinct DAP-R strains with completely different genetic backgrounds and targeted *liaR* under the hypothesis that LiaR plays a pivotal role in CM homeostasis and DAP resistance in all *E. faecium* isolates, regardless of the genetic pathway leading to DAP resistance/tolerance. We were able to obtain nonpolar deletions of both strains and complemented R497F Δ*liaR* with the native gene (which encodes a predicted LiaR protein harboring a W73C substitution). No differences in growth kinetics of the wild-type versus the *liaR*-deletion derivatives were seen in the absence of DAP (see Fig. S1 in the supplemental material). As shown in Table 1, deletion of *liaR* markedly reduced the DAP MIC from 24 μg/ml to 0.094 μg/ml and from 16 μg/ml to 0.25 μg/ml in R497F Δ*liaR* and R446F Δ*liaR*, respectively. Of note, the observed DAP MICs are much lower than any DAP MIC reported for clinical strains of *E. faecium* in our examination of the available comprehensive multinational surveillances (32, 33), suggesting that the deletion not only reverses DAP resistance but also generates hypersusceptibility to the antibiotic. Our results support our main hypothesis that LiaR is crucial for DAP resistance and CM homeostasis in enterococci, independent of the strain background or the presence of substitutions in the LiaFSR system.

Deletion of *liaR* causes hypersusceptibility to DAP in DAP-S *E. faecium*. We had shown previously that *E. faecalis* and *E. faecium* with DAP MICs close to the current CLSI breakpoint (4 μg/ml) and reported as DAP-S harbor mutations in genes associated with DAP resistance (10, 11). Moreover, we have shown that these changes lead to tolerance, as assessed by time-kill curves. Therefore, we chose two such strains (HOU503 and HOU515) with different genetic backgrounds for further analysis. *E. faecium* HOU503 (21) has a DAP MIC of 3 μg/ml and harbors only the T120A and W73C substitutions in LiaS and LiaR but no other substitution previously associated with DAP resistance (Table 1). Strain HOU515 also exhibits an MIC of 3 μg/ml and harbors an A414T substitution in the predicted YycG without changes in

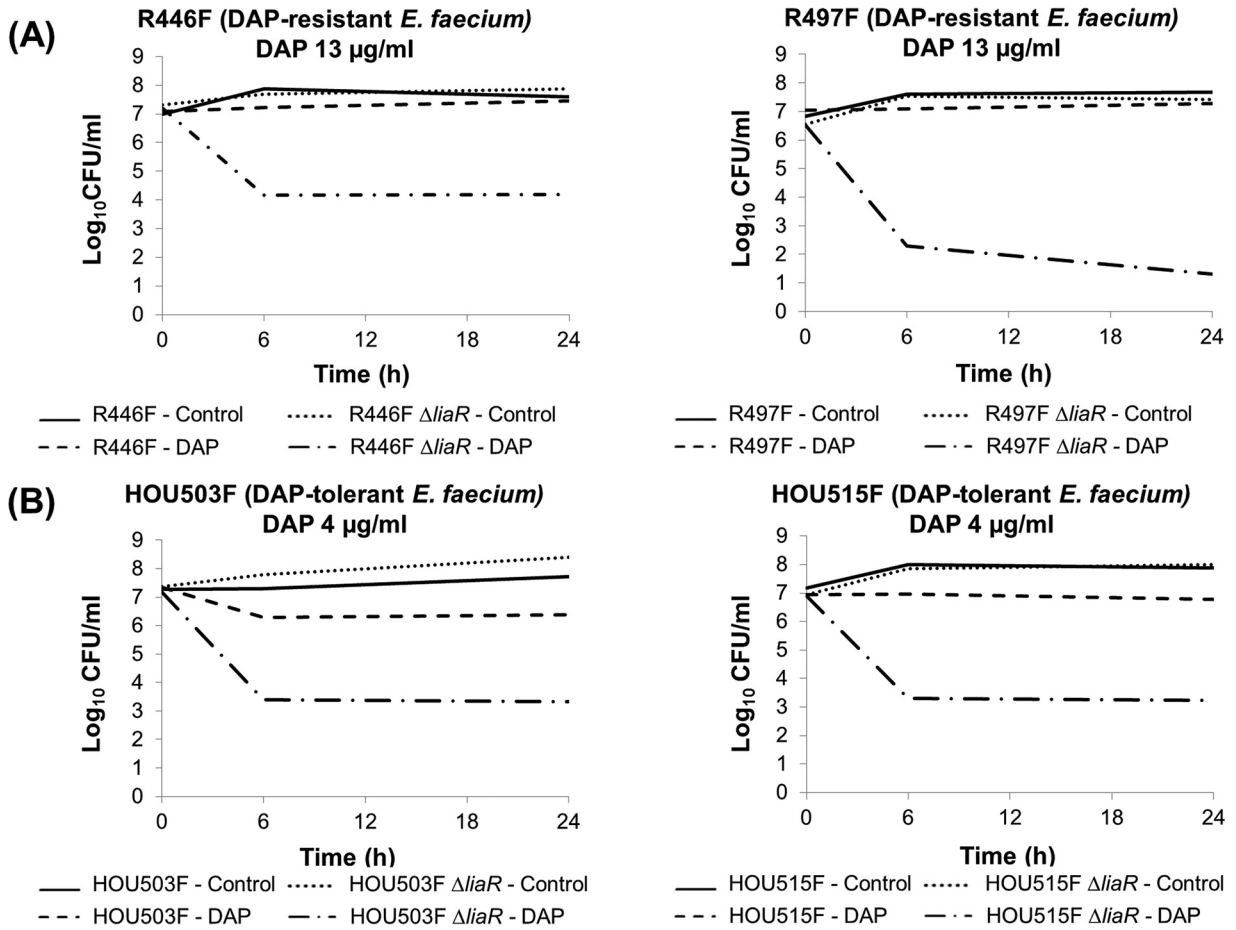

FIG 1 Time-kill assays for DAP-R and tolerant *E. faecium* strains and *liaR* deletion derivatives. (A) DAP-R R446F and R497F strains and *liaR* deletion derivatives R446F Δ*liaR* and R497F Δ*liaR* were grown in MHB supplemented with DAP (13 μg/ml) and calcium (50 μg/ml) and in the absence of DAP. (B) DAP-tolerant HOU503F and HOU515F and *liaR* deletion derivatives (HOU503F Δ*liaR* and HOU515F Δ*liaR*) were grown in MHB supplemented with DAP (4 μg/ml) and calcium (50 mg/liter) and in the absence of DAP. The limit of detection was 200 CFU/ml. Time-kill curves are representative of at least two assays performed on different days.

LiaFSR. Similarly to results in the DAP-R strains, deletion of *liaR* markedly decreased the MIC of DAP from 3 μg/ml to 0.064 and 0.047 μg/ml in HOU503F and HOU515F, respectively. Complementation (placing the gene in its native chromosomal location) of *liaR* in HOU503F Δ*liaR* restored the DAP MIC to wild-type levels (3 μg/ml). Thus, our results confirm that LiaR is likely to play a role in CM homeostasis in *E. faecium* clinical strains.

The *liaR* deletions are specific for DAP but no other antibiotics. In order to determine if the *liaR* deletion effect was specific to DAP, we assessed susceptibility of the strains to other antibiotics. We evaluated the MICs of several groups of antimicrobials, including cell wall-acting antibiotics (β-lactams and fosfomycin), a lipoglycopeptide (telavancin), and protein synthesis inhibitors (tetracycline class of drugs). Table S2 in the supplemental material shows the results of the MIC determinations. We did not observe any changes in the MICs of the tested antibiotics upon deletion of *liaR* in the majority of the strains, suggesting that the deletion was specific for DAP. However, a notable exception was the *liaR* deletion derivative of R446F (representative of the YycFG pathway), which exhibited a 21-fold decrease in the MIC of fosfomycin (6 μg/ml) compared to the MIC for the wild-type R446F (128 μg/ml). The fosfomycin change was associated with a decrease of 16-fold in the ampicillin MIC (see Table S2).

DAP is bactericidal against derivatives of *E. faecium* lacking *liaR* at concentrations achievable in humans. We had shown previously (10, 11, 21) that mutations in *liaFSR* and *yycFG* are associated with abolishment of DAP bactericidal activity at concentrations 5× to 7× the MIC. Here, we aimed to determine if deleting *liaR* would restore the bactericidal activity of DAP. In order to test this hypothesis, we used time-kill assays with DAP concentrations that correlate with human free-drug concentrations at doses of 4 and 12 mg/kg (which have been used in clinical practice). Figure 1 shows the time-kill curves for the four strains. Interestingly, deletion of *liaR* restored the bactericidal activity of DAP against R497F and R446F (DAP MICs of 24 and 16 μg/ml, respectively) but only at free peak DAP concentrations achieved by an equivalent human dose of 12 mg/kg (DAP concentration of 13 μg/ml). In DAP-tolerant strains HOU503F and HOU515F (DAP MIC of 3 μg/ml), deletion of *liaR* restored DAP bactericidal activity (reversed tolerance) at concentrations that correlate with a human dose of 4 mg/kg, the FDA-approved dose for skin and soft tissue infections.

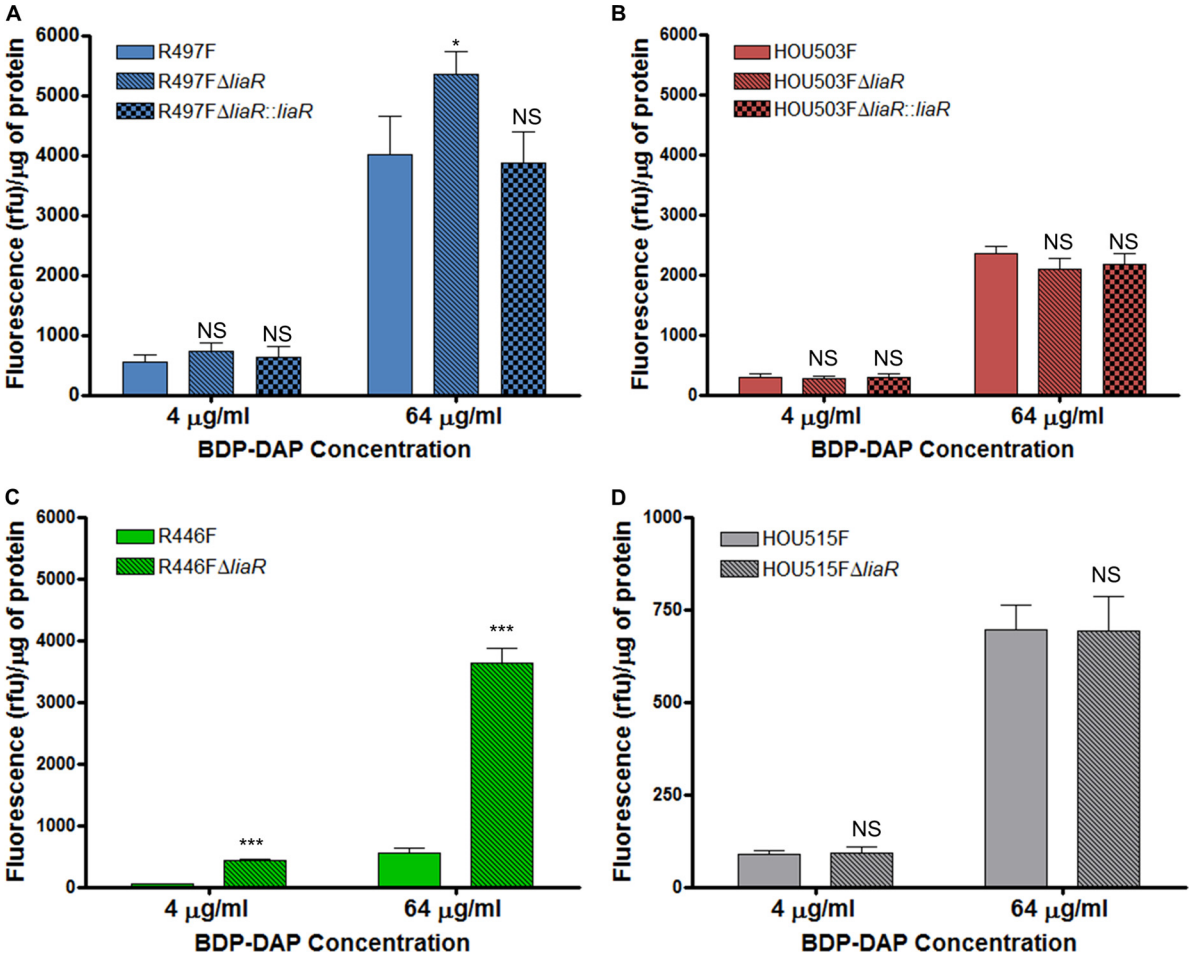

FIG 2 Fluorescence intensities of BODIPY-labeled daptomycin (BDP-DAP) binding to *E. faecium* strains. Cells were treated with BDP-DAP at low (4 μg/ml) and high (64 μg/ml) concentrations. Fluorescence was normalized to the cell protein content for each sample. Intensities were compared to levels of wild-type/parental cells. Strains are grouped by their representative pathway: *liaFSR* (A and B) and *yycFGHIJ* (C and D). rfu, relative fluorescence unit; NS, nonsignificant; *, $P < 0.01$; ***, $P < 0.0001$.

Deletion of *liaR* restores binding of BDP-DAP to the cell surface of *E. faecium*. Using BDP-DAP to study the interactions of the antibiotic to the cell membrane of representative strains of *E. faecium*, we previously demonstrated that antibiotic repulsion is the prominent mechanism of resistance in DAP-R *E. faecium* R497 and R446 (21). In contrast, the patterns of BDP-DAP binding to DAP-tolerant strains, HOU503 and HOU515, were similar to the pattern of a DAP-S control (*E. faecium* DO/TX16) at low concentrations, and only HOU515 displayed significantly lower BDP-DAP binding at high concentrations (64 μg/ml) (21). To determine whether deletion of *liaR* also affected antibiotic interactions with the CM, we also used BDP-DAP to evaluate binding of DAP to *E. faecium* strains and *liaR* deletion mutant derivatives (Table 1). Figure 2 and Fig. S2 in the supplemental material show that deletion of *liaR* significantly increased the binding of the antibiotic molecule to the CM in DAP-R isolates (R497F and R446F with MICs of 24 and 16 μg/ml, respectively), a phenomenon that was most evident at high BDP-DAP concentrations (64 μg/ml). In R497F, *cis*-complementation with *liaR* decreased the BDP-DAP binding to levels similar to the level of the wild-type strain (Fig. 2; see also Fig. S2A in the supplemental material), confirming the involvement of *liaR* in the resistance phenotype. Interestingly, in DAP-tolerant strains (HOU503F and HOU515F, both exhibiting a DAP MIC of 3 μg/ml), we did not find differences in BDP-DAP binding between wild-type and *liaR* deletion mutant derivatives (Fig. 2; see also Fig. S2B and D), similar to what has been previously reported (21). These findings suggest that tolerance in these strains is not mediated by repulsion of the antibiotic molecule from the cell surface.

DAP resistance in *E. faecium* is not associated with redistribution of anionic PL microdomains. We have previously used the hydrophobic fluorescent dye NAO to visualize CL-enriched microdomains in *E. faecalis* (12, 13). NAO was shown previously to associate with CL and produces fluorescence due to the ability of CL molecules to cluster in microdomains in the CM, providing the opportunity for NAO to form arrays between CL domains (34, 35). Recent work has demonstrated that NAO is promiscuous in its binding to anionic phospholipids such as CL and phosphatidylglycerol in *Escherichia coli* (36). In *E. faecalis*, we showed that development of DAP resistance is associated with redistribution of these presumed CL microdomains, which move away from the division septum to other CM areas. As we cannot

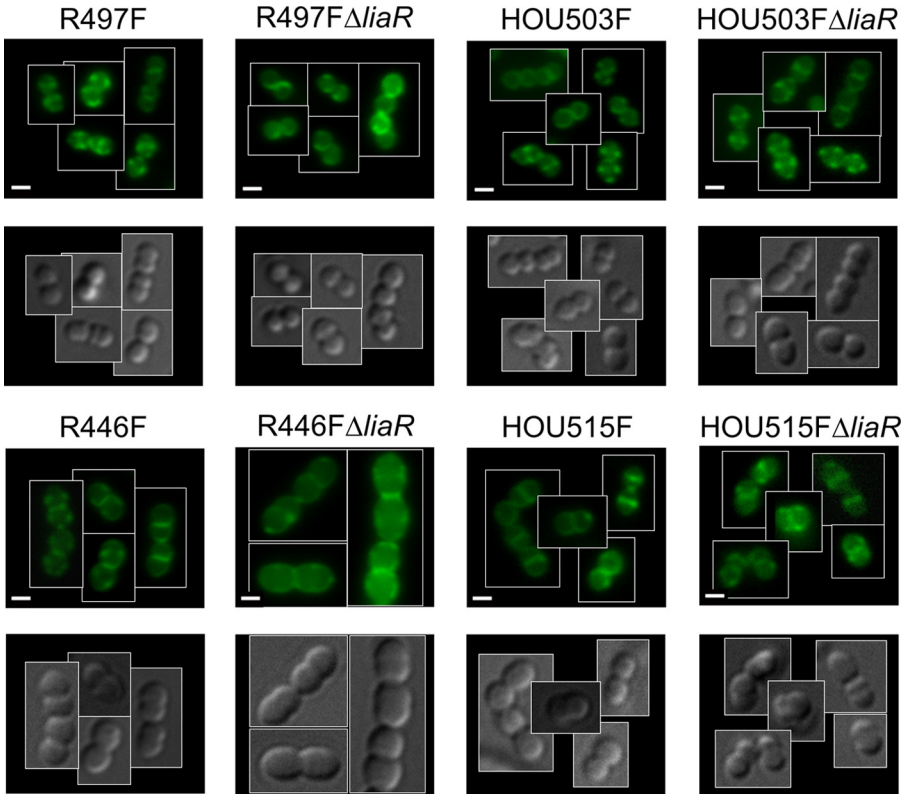

FIG 3 Staining of representative cells of *E. faecium* and derivatives with 10-*N*-nonyl acridine orange (1 μM). Top panels display fluorescence microscopy images of bacterial cells. Phase-contrast images of the same cells are shown in the bottom panels. Scale bar, 1 μm.

differentiate binding of this dye to specific PL species, we postulated that fluorescence seen in this experiment represents the interaction of NAO with anionic PLs. Figure 3 shows that, as described previously in *E. faecalis* and *B. subtilis* (12, 13, 37), anionic PL microdomains concentrate at the septum, including potential future septal areas, and polar regions in all wild-type *E. faecium* strains. However, unlike *E. faecalis* (12, 13), no change in the distribution of such anionic PL microdomains was observed upon deletion of *liaR*, independent of the MIC. Our findings support the notion that high-level resistance to DAP in *E. faecium* is mediated by electrostatic repulsion of the DAP-calcium complexes from the cell surface without apparent redistribution of CM anionic phospholipid microdomains (15, 17).

DISCUSSION

Bacteria have evolved sophisticated mechanisms to protect their CMs from the attack of different stressors, including AMPs. CM integrity is of paramount importance for bacterial physiological processes, and therefore the CM is a vital structure required for cell homeostasis and survival. AMPs are the most common bacterial CM-targeting molecules found in nature, produced by competing bacteria and host innate immune systems. DAP is a lipopeptide antibiotic whose mechanism of action resembles that of AMPs, including the disruption of CM structure and function that eventually leads to bacterial cell death. In the course of our investigations directed toward the elucidation of the molecular basis for DAP resistance in enterococci (9–13, 18–22), we have found that one of the major systems involved in the enterococcal CM response to DAP is the LiaFSR three-component regulatory system. Indeed, we recently showed (13) that a *liaR* deletion generated in a DAP-R strain of *E. faecalis* fully reversed DAP resistance and increased susceptibility to telavancin, another CM-targeting antibiotic used in clinical practice. Most importantly, the absence of *liaR* generated DAP hypersusceptibility in a laboratory strain of *E. faecalis*, suggesting that LiaR is a master regulator of the enterococcal CM stress response to AMPs and a possible target for nontraditional therapeutic approaches to restore the activity of potent bactericidal antibiotics such as DAP.

Using biophysical and structural approaches to study LiaR from *E. faecalis* (19), we recently provided evidence (i) that activation of LiaR hinges on a dimer-to-tetramer transition permitting LiaR to recognize regulatory regions that extend beyond the predicted consensus sequence, (ii) that an adaptive LiaR mutation (D191N), associated with DAP resistance, produces structural changes in LiaR that favor the formation of the tetrameric structure even in the absence of phosphorylation, leading to constitutive activation of the response regulator, and (iii) that LiaR is likely to bend its target DNA as part of its potential recruitment of RNA polymerase. Interestingly, substitutions in LiaR of *E. faecium* were one of the most frequent changes associated with DAP resistance in clinical strains. Therefore, following our experience in *E. faecalis*, we decided to target *liaR* in several *E. faecium* strains in order to determine if the response regulator plays an important role in DAP resistance and if such function depends on the presence of mutations in *liaFSR*.

We deleted *liaR* in four different clinical *E. faecium* strains that we had previously characterized by whole-genome sequencing

(20, 21). The strains are not related and represent isolates with different genetic backgrounds and DAP MICs. We decided to include two strains that exhibit high-level resistance to DAP but harbor different mutations (R497 possesses LiaFSR substitutions, whereas R446 exhibits mutations in other genes without changes in LiaFSR) (Table 1) and two strains previously shown to be tolerant to DAP, with MICs below the current breakpoint (HOU503 also harbors LiaFSR substitutions but HOU515 does not). The strains also represent the most common genetic changes associated with DAP resistance (designated the LiaFSR and YycFG pathways) in *E. faecium* (21).

Our results indicate that, as previously reported for *E. faecalis*, LiaR also mediates DAP-R in *E. faecium*. Most importantly, the role of LiaR in the resistance phenotype was independent of the genetic background, the pathway for DAP resistance, or the presence of mutations in *liaFSR*. Moreover, deletion of *liaR* not only reversed DAP resistance but also decreased the MIC beyond the values obtained for the parental strains, suggesting that LiaR orchestrates the mechanisms leading to preservation of the CM stress response in *E. faecium*, a phenomenon that seems to be conserved in all enterococci. Thus, LiaR emerges as an appealing target to interfere with the CM adaptive response in enterococci, restore the activity of antibiotics that target the CM, and, perhaps, enhance the clearance of infecting bacteria by the innate immune system.

Interestingly, deletion of *liaR* in DAP-R strain R446 (representative of the YycFG pathway, a two-component regulatory system implicated in controlling cell wall homeostasis and cell division in staphylococci) (31) also affected the susceptibilities of fosfomycin and ampicillin, producing 21- and 16-fold decreases in the MICs, respectively. We postulate that this phenomenon might be related to the predominance of the YycFG system and peptidoglycan homeostasis in DAP-R strains. This effect seems to be strain dependent since we did not observe susceptibility changes in other strains. The molecular basis for this effect is the subject of future investigations.

Our time-kill assays suggest that the *liaR* deletion also restores the bactericidal activity of DAP against DAP-tolerant *E. faecium* at concentrations likely obtained with the human dose of DAP of 4 mg/kg, a dose that is now considered suboptimal for serious infections, emphasizing the fact that hypersusceptibility to DAP is the hallmark of the *liaR* deletion. Interestingly, higher concentrations of DAP were required to achieve a bactericidal effect in derivatives of DAP-R *E. faecium* lacking *liaR* (R497F Δ*liaR* and R446F Δ*liaR*) than in HOU503F Δ*liaR* and HOU515F Δ*liaR* (DAP tolerant), albeit still within concentrations achievable by equivalent human doses. This discrepancy in the killing activity of DAP was observed despite the fact that all *liaR* deletion derivatives exhibited similar DAP MICs (≤0.25 μg/ml) (Table 1). This observation could be explained by the presence of additional mutations associated with high-level DAP-R that may lead to reduced susceptibility to DAP, independent of *liaFSR*. For example, overexpression of mutated cardiolipin synthase (Cls) has been associated with DAP-R in *E. faecalis* (38). Both R497F and R446F harbor Cls substitutions, and it is plausible that changes in expression of mutated Cls could potentially reduce the activity of DAP even in the absence of LiaR although such a strategy does not appear to be as successful as when LiaR is present. An alternative explanation is that unidentified LiaR-independent pathways to DAP resistance may be present.

Finally, our BDP-DAP experiments suggest that the mechanism of high-level DAP resistance in *E. faecium* is likely to be more similar to that described in *S. aureus* than that in *E. faecalis*. Indeed, unlike *E. faecalis* (where we have previously provided evidence that diversion of DAP from the septum is the predominant strategy to prevent killing by the antibiotic), our present results suggest that electrostatic repulsion is more likely to play a prominent role in DAP resistance in *E. faecium*. Moreover, our NAO experiments also suggest that DAP-R in *E. faecium* is not associated with redistribution of anionic PL microdomains in the CM, supporting even further the repulsion hypothesis.

In summary, we provide compelling evidence that LiaR is a master response regulator of the enterococcal CM response and of the development of DAP-R in all enterococci. Since the LiaFSR system is present in all Gram-positive organisms of clinical importance (designated VraTSR in *S. aureus*), targeting this system may be a novel approach to restore the activity of important anti-enterococcal antibiotics such as DAP.

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REFERENCES

1. **Centers for Disease Control and Prevention.** 2013. Antibiotic resistance threats in the United States, 2013. U.S. Department of Health and Human Services, CDC, Atlanta, GA. http://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf.
2. **Rice LB.** 2008. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. J Infect Dis **197:**1079–1081. http://dx.doi.org/10.1086/533452.
3. **Galloway-Peña J, Roh JH, Latorre M, Qin X, Murray BE.** 2012. Genomic and SNP analyses demonstrate a distant separation of the hospital and community-associated clades of *Enterococcus faecium*. PLoS One **7:**e30187. http://dx.doi.org/10.1371/journal.pone.0030187.
4. **Lebreton F, van Schaik W, McGuire AM, Godfrey P, Griggs A, Mazumdar V, Corander J, Cheng L, Saif S, Young S, Zeng Q, Wortman J, Birren B, Willems RJ, Earl AM, Gilmore MS.** 2013. Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains. mBio **4**(4):e00534-13. http://dx.doi.org/10.1128/mBio.00534-13.
5. **Arias CA, Contreras GA, Murray BE.** 2010. Management of multidrug-resistant enterococcal infections. Clin Microbiol Infect **16:**555–562. http://dx.doi.org/10.1111/j.1469-0691.2010.03214.x.
6. **Lewis JS, II, Owens A, Cadena J, Sabol K, Patterson JE, Jorgensen JH.** 2005. Emergence of daptomycin resistance in *Enterococcus faecium* during daptomycin therapy. Antimicrob Agents Chemother **49:**1664–1665. http://dx.doi.org/10.1128/AAC.49.4.1664-1665.2005. (Erratum, **49:**2152, http://dx.doi.org/10.1128/AAC.49.5.2152.2005.)
7. **Lesho EP, Wortmann GW, Craft D, Moran KA.** 2006. De novo daptomycin nonsusceptibility in a clinical isolate. J Clin Microbiol **44:**673. http://dx.doi.org/10.1128/JCM.44.2.673.2006.
8. **Munoz-Price LS, Lolans K, Quinn JP.** 2005. Emergence of resistance to daptomycin during treatment of vancomycin-resistant *Enterococcus faecalis* infection. Clin Infect Dis **41:**565–566. http://dx.doi.org/10.1086/432121.
9. **Arias CA, Panesso D, McGrath DM, Qin X, Mojica MF, Miller C, Diaz L, Tran TT, Rincon S, Barbu EM, Reyes J, Roh JH, Lobos E, Sodergren E, Pasqualini R, Arap W, Quinn JP, Shamoo Y, Murray BE, Weinstock GM.** 2011. Genetic basis for *in vivo* daptomycin resistance in enterococci. N Engl J Med **365:**892–900. http://dx.doi.org/10.1056/NEJMoa1011138.
10. **Munita JM, Panesso D, Diaz L, Tran TT, Reyes J, Wanger A, Murray BE, Arias CA.** 2012. Correlation between mutations in *liaFSR* of *Enterococcus faecium* and MIC of daptomycin: revisiting daptomycin break-

points. Antimicrob Agents Chemother **56:**4354–4359. http://dx.doi.org/10.1128/AAC.00509-12.
11. **Munita JM, Tran TT, Diaz L, Panesso D, Reyes J, Murray BE, Arias CA.** 2013. A *liaF* codon deletion abolishes daptomycin bactericidal activity against vancomycin-resistant *Enterococcus faecalis*. Antimicrob Agents Chemother **57:**2831–2833. http://dx.doi.org/10.1128/AAC.00021-13.
12. **Tran TT, Panesso D, Mishra NN, Mileykovskaya E, Guan Z, Munita JM, Reyes J, Diaz L, Weinstock GM, Murray BE, Shamoo Y, Dowhan W, Bayer AS, Arias CA.** 2013. Daptomycin-resistant *Enterococcus faecalis* diverts the antibiotic molecule from the division septum and remodels cell membrane phospholipids. mBio **4**(4):e00281-13. http://dx.doi.org/10.1128/mBio.00281-13.
13. **Reyes J, Panesso D, Tran TT, Mishra NN, Cruz MR, Munita JM, Singh KV, Yeaman MR, Murray BE, Shamoo Y, Garsin D, Bayer AS, Arias CA.** 2015. A *liaR* deletion restores susceptibility to daptomycin and antimicrobial peptides in multidrug-resistant *Enterococcus faecalis*. J Infect Dis **211:**1317–1325. http://dx.doi.org/10.1093/infdis/jiu602.
14. **Wolf D, Kalamorz F, Wecke T, Juszczak A, Mäder U, Homuth G, Jordan S, Kirstein J, Hoppert M, Voigt B, Hecker M, Mascher T.** 2010. In-depth profiling of the LiaR response of *Bacillus subtilis*. J Bacteriol **192:**4680–4693. http://dx.doi.org/10.1128/JB.00543-10.
15. **Mehta S, Cuirolo AX, Plata KB, Riosa S, Silverman JA, Rubio A, Rosato RR, Rosato AE.** 2012. VraSR two-component regulatory system contributes to *mprF*-mediated decreased susceptibility to daptomycin in *in vivo*-selected clinical strains of methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **56:**92–102. http://dx.doi.org/10.1128/AAC.00432-10.
16. **Boyle-Vavra S, Yin S, Jo DS, Montgomery CP, Daum RS.** 2013. VraT/YvqF is required for methicillin resistance and activation of the VraSR regulon in *Staphylococcus aureus*. Antimicrob Agents Chemother **57:**83–95. http://dx.doi.org/10.1128/AAC.01651-12.
17. **Ernst CM, Staubitz P, Mishra NN, Yang SJ, Hornig G, Kalbacher H, Bayer AS, Kraus D, Peschel A.** 2009. The bacterial defensin resistance protein MprF consists of separable domains for lipid lysinylation and antimicrobial peptide repulsion. PLoS Pathog **5:**e1000660. http://dx.doi.org/10.1371/journal.ppat.1000660.
18. **Miller C, Kong J, Tran TT, Arias CA, Saxer G, Shamoo Y.** 2013. Adaptation of *Enterococcus faecalis* to daptomycin reveals an ordered progression to resistance. Antimicrob Agents Chemother **57:**5373–5383. http://dx.doi.org/10.1128/AAC.01473-13. (Erratum, **58:**631, 2014, http://dx.doi.org/10.1128/AAC.02297-13.)
19. **Davlieva M, Shi Y, Leonard PG, Johnson TA, Zianni M, Arias CA, Ladbury JE, Shamoo Y.** 2015. A variable DNA recognition site organization establishes the LiaR mediated cell envelope stress response of enterococci to daptomycin. Nucleic Acids Res **43:**4758–4773. http://dx.doi.org/10.1093/nar/gkv321.
20. **Tran TT, Panesso D, Gao H, Roh JH, Munita JM, Reyes J, Diaz L, Lobos EA, Shamoo Y, Mishra NN, Bayer AS, Murray BE, Weinstock GM, Arias CA.** 2013. Whole-genome analysis of a daptomycin-susceptible *Enterococcus faecium* strain and its daptomycin-resistant variant arising during therapy. Antimicrob Agents Chemother **57:**261–268. http://dx.doi.org/10.1128/AAC.01454-12.
21. **Diaz L, Tran TT, Munita JM, Miller WR, Rincon S, Carvajal LP, Wollam A, Reyes J, Panesso D, Rojas NL, Shamoo Y, Murray BE, Weinstock GM, Arias CA.** 2014. Whole-genome analyses of *Enterococcus faecium* isolates with diverse daptomycin MICs. Antimicrob Agents Chemother **58:**4527–4534. http://dx.doi.org/10.1128/AAC.02686-14.
22. **Munita JM, Mishra NN, Alvarez D, Tran TT, Diaz L, Panesso D, Reyes J, Murray BE, Adachi JA, Bayer AS, Arias CA.** 2014. Failure of high-dose daptomycin for bacteremia caused by daptomycin-susceptible *Enterococcus faecium* harboring LiaSR substitutions. Clin Infect Dis **59:**1277–1280. http://dx.doi.org/10.1093/cid/ciu642.
23. **Kristich CJ, Chandler JR, Dunny GM.** 2007. Development of a host-genotype-independent counterselectable marker and a high-frequency conjugative delivery system and their use in genetic analysis of *Enterococcus faecalis*. Plasmid **57:**131–144. http://dx.doi.org/10.1016/j.plasmid.2006.08.003.
24. **Panesso D, Montealegre MC, Rincón S, Mojica MF, Rice LB, Singh KV, Murray BE, Arias CA.** 2011. The hyl_{Efm} gene in $pHyl_{Efm}$ of *Enterococcus faecium* is not required in pathogenesis of murine peritonitis. BMC Microbiol **11:**20. http://dx.doi.org/10.1186/1471-2180-11-20.
25. **Clinical and Laboratory Standards Institute.** 2014. Performance standards for antimicrobial susceptibility testing; twenty-first informational supplement. CLSI document MS100-S23. Clinical and Laboratory Standards Institute, Wayne, PA.
26. **Berti AD, Sakoulas G, Nizet V, Tewhey R, Rose WE.** 2013. β-Lactam antibiotics targeting PBP1 selectively enhance daptomycin activity against methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother **57:**5005–5012. http://dx.doi.org/10.1128/AAC.00594-13.
27. **Vignaroli C, Rinaldi C, Varaldo PE.** 2011. Striking "seesaw effect" between daptomycin nonsusceptibility and beta-lactam susceptibility in *Staphylococcus haemolyticus*. Antimicrob Agents Chemother **55:**2495–2496. http://dx.doi.org/10.1128/AAC.00224-11.
28. **Pogliano J, Pogliano N, Silverman JA.** 2012. Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins. J Bacteriol **194:**4494–4504. http://dx.doi.org/10.1128/JB.00011-12.
29. **Hachmann AB, Sevim E, Gaballa A, Popham DL, Antelmann H, Helmann JD.** 2011. Reduction in membrane phosphatidylglycerol content leads to daptomycin resistance in *Bacillus subtilis*. Antimicrob Agents Chemother **55:**4326–4337. http://dx.doi.org/10.1128/AAC.01819-10.
30. **Hachmann AB, Angert ER, Helmann JD.** 2009. Genetic analysis of factors affecting susceptibility of *Bacillus subtilis* to daptomycin. Antimicrob Agents Chemother **53:**1598–1609. http://dx.doi.org/10.1128/AAC.01329-08.
31. **Türck M, Bierbaum G.** 2012. Purification and activity testing of the full-length YycFGHI proteins of *Staphylococcus aureus*. PLoS One **7:**e30403. http://dx.doi.org/10.1371/journal.pone.0030403.
32. **Mendes RE, Farrell DJ, Sader HS, Streit JM, Jones RN.** 2015. Update of the telavancin activity *in vitro* tested against a worldwide collection of Gram-positive clinical isolates (2013), when applying the revised susceptibility testing method. Diagn Microbiol Infect Dis **81:**275–279. http://dx.doi.org/10.1016/j.diagmicrobio.2014.12.011.
33. **Sader HS, Farrell DJ, Flamm RK, Jones RN.** 2014. Daptomycin activity tested against 164,457 bacterial isolates from hospitalised patients: summary of 8 years of a Worldwide Surveillance Programme (2005–2012). Int J Antimicrob Agents **43:**465–469. http://dx.doi.org/10.1016/j.ijantimicag.2014.01.018.
34. **Mileykovskaya E, Dowhan W, Birke RL, Zheng D, Lutterodt L, Haines TH.** 2001. Cardiolipin binds nonyl acridine orange by aggregating the dye at exposed hydrophobic domains on bilayer surfaces. FEBS Lett **507:**187–190. http://dx.doi.org/10.1016/S0014-5793(01)02948-9.
35. **Mileykovskaya E, Dowhan W.** 2000. Visualization of phospholipid domains in *Escherichia coli* by using the cardiolipin-specific fluorescent dye 10-N-nonyl acridine orange. J Bacteriol **182:**1172–1175. http://dx.doi.org/10.1128/JB.182.4.1172-1175.2000.
36. **Oliver PM, Crooks JA, Leidl M, Yoon EJ, Saghatelian A, Weibel DB.** 2014. Localization of anionic phospholipids in *Escherichia coli* cells. J Bacteriol **196:**3386–3398. http://dx.doi.org/10.1128/JB.01877-14.
37. **Kawai F, Shoda M, Harashima R, Sadaie Y, Hara H, Matsumoto K.** 2004. Cardiolipin domains in *Bacillus subtilis* Marburg membranes. J Bacteriol **186:**1475–1483. http://dx.doi.org/10.1128/JB.186.5.1475-1483.2004.
38. **Palmer KL, Daniel A, Hardy C, Silverman J, Gilmore MS.** 2011. Genetic basis for daptomycin resistance in enterococci. Antimicrob Agents Chemother **55:**3345–3356. http://dx.doi.org/10.1128/AAC.00207-11.