

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

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Running Head: Deletion of *liaR* in *E. faecium* restores DAP susceptibility

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## ABSTRACT

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 *E. faecalis*, reversed DAP resistance and produced a strain hypersusceptible to antimicrobial peptides. Since LiaFSR is conserved in *E. 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 16 µg/mL) and R497F (MIC 24 µg/mL, harboring changes in LiaRS) 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) retained bactericidal activity against the mutants. Furthermore, the *liaR*-deletion derivatives of these two DAP-R strains exhibited increased binding of 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 HOU503RF and HOU515F (DAP MICs within susceptible range but not killed by DAP concentrations 5X the MIC), deletion of *liaR* not only markedly decreased the DAP MICs (0.064 and 0.047 µg/mL) 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.

## INTRODUCTION

*E. faecium* have 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 US 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 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 front-line 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 *E. faecalis* and *E. faecium*, we have provided robust evidence that development of DAP resistance mainly results from mutations in two major groups of genes 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. The LiaFSR system is composed of a predicted transmembrane protein (LiaF) that, in *B. 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 resistance 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 susceptibility in *E. faecalis* was associated with an increased susceptibility to a cadre of antimicrobial peptides of different origins and mechanisms of action and a marked decrease in the MICs of telavancin, another CM membrane-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 *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<sup>D191N</sup>) complexed with the target DNA indicate that the structural basis for increased resistance to DAP

hinges in a transition of the LiaR dimer to 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-R 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 shown in Table 1 (6, 9, 20, 21). Briefly, two DAP-R clinical strains (R446 and R497) with DAP MIC of 16 µg/mL and 24 µg/mL, respectively, and two DAP-tolerant *E. faecium* (HOU503 and HOU515, MICs 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 5X 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* 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 change 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 location (the predicted LiaR harbors a W73C substitution). We used the p-chloro-phenylalanine (p-Chl-Phe) sensitivity counterselection system (PheS\*) (23) to obtain the mutants and deliver the genes back into the chromosome (complementation) using plasmid pHOU1, as described previously (9, 20, 24). Briefly, ~500 bp regions upstream and downstream of the *liaR* were amplified by crossover PCR using DNA of the corresponding strain as target and primers shown in Table S1. 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 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 parental strains. Growth curves of mutants and parentals (fusidic acid-resistant derivatives) were performed to determine if the deletion 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 MICs of DAP, telavancin, β-lactams (ampicillin, cephalotin, ceftaroline), tetracyclines (doxycycline, minocycline, tetracycline, tigecycline), fosfomycin and colistin of wild-type, mutant derivatives and complemented strains by Etest (bioMérieux, Marcy l'Etoile, France) on Mueller-Hinton agar following instructions from the manufacturer and incubated for 24 hours. The MICs for each strain were determined in triplicate with readings performed by 2 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<sup>7</sup> CFU/mL in Mueller-Hinton broth (MHB) supplemented with calcium (50 mg/L). 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 (4 and, 13 µg/mL, respectively). Bacteria were enumerated at 0, 6 and 24 hours. 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<sub>10</sub> in CFU/mL at 24 h in comparison to the initial inoculum. The limit of detection, assuming maximum plating efficiency, was 200 CFU/mL.

**Binding of bodipy-daptomycin (BDP-DAP).** In order to evaluate the interactions of DAP with the bacterial CM, we used 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 following 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<sup>2+</sup> at 50 mg/L) for 10 min in the dark. In order to measure fluorescence emission, we used a standard fluorescein isothiocyanate 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 protein concentration of the samples in order to estimate the amount of binding of BDP-DAP, as described previously (12).

**10-N-nonyl acridine orange (NAO) staining of *E. faecium* strains.** We had previously shown (12, 13) that the fluorescence dye 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* were grown in trypticase soy broth (TSB) to exponential phase (*A*<sub>600</sub> 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 thrice 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 100X objective following 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 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), LiaR (W73C) and insertion of MPL at position 110 in the putative cardiolipin synthase. R446 is a derivative of a DAP-S strain S447 isolated from a patient (Table 1) (6, 20). R446 harbors 8 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-R/tolerance. We were able to obtain non-polar deletions of both strains and complemented R497FΔ*liaR* with the native gene (which encodes a predicted LiaR protein harboring a W73C substitution). No differences in growth kinetics of wild-type vs the *liaR*-deletion derivatives were seen in the absence of DAP (Fig. S1). 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 R497FΔ*liaR* and R446FΔ*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 only harbors 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 a A414T substitution in the predicted YycG without changes in LiaFSR. Similar to 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 HOU503FΔ*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 to several groups of antimicrobials including cell-wall acting antibiotics (β-lactams, fosfomycin), lipoglycopeptides (telavancin) and protein synthesis inhibitors (tetracycline class of drugs). Table S2 shows the results of the MICs. 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 MICs of fosfomycin (6 µg/ml) compared to the wild type R446F (128 µg/ml). The fosfomycin change was associated with a decrease of 16 fold in the ampicillin MIC (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 5X to 7X 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). Fig. 1 shows the time-kill curves for the 4 strains. Interestingly, deletion of *liaR* restored the bactericidal activity of DAP against R497F and R446F (DAP MIC 24 and 16 µg/mL, respectively) but only at free peak DAP concentrations achieved by an equivalent human dose of 12 mg/kg (DAP 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.

**Deletion of *liaR* restores binding of BDP-DAP to 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 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). Fig. 2 and S2 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 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 wild-type strain (Fig. 2 and Fig.S2, panel a), confirming the involvement of *liaR* in the resistance phenotype. Interestingly, in DAP-tolerant strains (HOU503F and HOU515F both exhibiting 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 and Fig.2S, panel b 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 10-N-nonyl-acridine orange (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 *E. 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 differentiate binding of this dye to specific PL species, we postulated that fluorescence seen in this experiment represent interaction of NAO with anionic PLs. Fig. 3 shows that, as described previously in *E. faecalis* and *B. subtilis*, (12, 13, 37), anionic PL microdomains concentrate at the septum and polar regions in all wild-type *E. faecium* strains including potential future septal areas. 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 CM from the attack of different stressors, including antimicrobial peptides (AMP). CM integrity is of paramount importance for bacterial physiological processes and, therefore, 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 towards the elucidation of the molecular bases 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 antimicrobial peptides and a possible target for non-traditional therapeutic approaches in order 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 that *i*) activation of LiaR hinges on a dimer to tetramer transition permitting LiaR to recognize regulatory regions that extend beyond the predicted consensus sequence, *ii*) 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*) LiaR is likely to bend its target DNA as part of its potential recruitment of RNA polymerase.

363 Interestingly, substitutions in LiaR of *E. faecium* were one of the most frequent changes  
364 associated with DAP-resistance in clinical strains. Therefore, following our experience in *E.*  
365 *faecalis*, we decided to target *liaR* in several *E. faecium* strains in order to determine if the  
366 response regulator plays an important role in DAP-resistance and if such function depends on  
367 the presence of mutations in *liaFSR*.

368  
369 We deleted *liaR* in four different clinical *E. faecium* strains that we had previously  
370 characterized by whole genomic sequencing (20, 21). The strains are not related and represent  
371 isolates with different genetic backgrounds and DAP MICs. We decided to include two strains  
372 that exhibit high-level resistance to DAP but harbor different mutations (R497 possesses  
373 LiaFSR substitutions whereas R446 exhibit mutations in other genes without changes in  
374 LiaFSR, Table 1) and two strains previously shown to be tolerant to DAP with MICs below the  
375 current breakpoint (HOU503 also harbors LiaFSR substitutions but HOU515 does not). The  
376 strains also represent the most common genetic changes associated with DAP-resistance  
377 (designated LiaFSR and YycFG “pathways”) in *E. faecium* (21).

378  
379 Our results indicate that, as previously reported for *E. faecalis*, LiaR also mediates DAP-  
380 R in *E. faecium*. Most importantly, the role of LiaR in the resistance phenotype was independent  
381 of the genetic background, the pathway for DAP resistance, or the presence of mutations in  
382 *liaFSR*. Moreover, deletion of *liaR* not only reversed DAP resistance but also decreased the  
383 MIC beyond the values obtained for the parental strains, suggesting that LiaR orchestrates the  
384 mechanisms leading to preserve CM stress response in *E. faecium*, a phenomenon that seems  
385 to be conserved in all enterococci. Thus, LiaR emerges as an appealing target to interfere with  
386 the CM adaptive response in enterococci and restore the activity of antibiotics that target the  
387 CM and, perhaps, favor 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 the cell wall homeostasis and cell division in staphylococci) (31), also affected the susceptibility of fosfomycin and ampicillin producing a 21 and 16 fold decrease 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. 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 human doses of DAP 4 mg/kg, a dose that is now considered suboptimal for serious infections and emphasizing the fact that hypersusceptibility to DAP is the hallmark of the *liaR* deletion. Interestingly, higher concentrations of DAP were required to achieve bactericidal effect in derivatives of DAP-R *E. faecium* lacking *liaR* (R497F $\Delta$ *liaR* and R446F $\Delta$ *liaR*) compared to HOU503F $\Delta$ *liaR* and HOU515F $\Delta$ *liaR* (DAP-tolerant), albeit, still within concentrations achievable by human dosing. This discrepancy in the killing activity of DAP was observed despite the fact that all *liaR* deletion derivatives exhibited similar DAP MICs ( $\leq$  0.25  $\mu$ g/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 (CIs) has been associated with DAP-R in *E. faecalis* (38). Both R497F and R446F harbor CIs substitutions and it is plausible that changes in expression of mutated CIs could potentially reduce the activity of DAP even in the absence of LiaR, although such 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 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 the 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 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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## FIGURE LEGENDS

**Figure 1.** Time-kill assays for DAP-R and tolerant *E. faecium* strains and *liaR*-deletion derivatives. (A) DAP-R R446F/R497F and *liaR*-deletion derivatives R446F $\Delta$ *liaR*/R497F $\Delta$ *liaR* were grown in Mueller-Hinton broth (MHB) supplemented with DAP (13  $\mu$ g/mL) and calcium (50  $\mu$ g/mL) and in the absence of DAP. (B) DAP-tolerant HOU503F and HOU515F and *liaR* deletion derivatives (HOU503F $\Delta$ *liaR* and HOU515F $\Delta$ *liaR*) were grown in Mueller-Hinton broth (MHB) supplemented with DAP (4  $\mu$ g/mL) and calcium (50 mg/L) and in the absence of DAP. CFU, colony-forming units. The limit of detection was 200 CFU/mL. Time kill curves are representative experiments of at least two assays performed in different days.

**Figure 2.** Fluorescence intensities of BODIPY-labeled daptomycin (BDP-DAP) binding to *E. faecium* strains. Cells were treated with BDP-DAP at low (4  $\mu$ g/mL) and high (64  $\mu$ g/mL) concentrations. Fluorescence was normalized to cell protein content for each sample. Intensities were compared to wild-type/parental cells. Strains are grouped by their representative pathway: *liaFSR* (A and B) and *ycyFGHIJ* (C and D). Rfu – relative fluorescence unit; NS – non-significant; \*  $P < 0.01$ ; and \*\*\*  $P < 0.0001$ .

**Figure 3.** Staining of representative cells of *E. faecium* and derivatives with 10-N-nonyl acridine orange (1  $\mu$ M). Top panels display fluorescence microscopy images of bacterial cells. Phase-contrast images of the same cells are in bottom panels. Bars, 1  $\mu$ m.

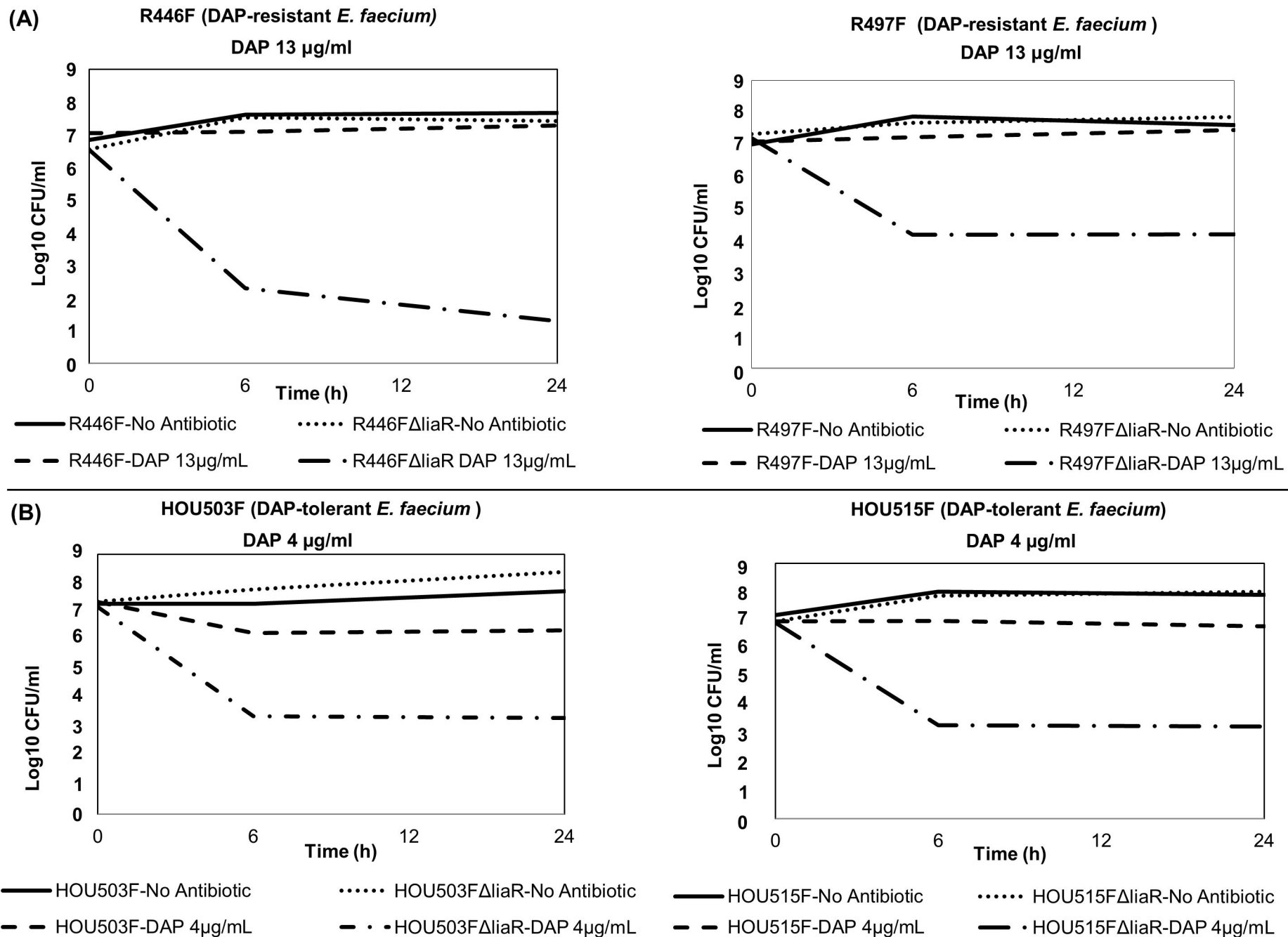


Figure 1



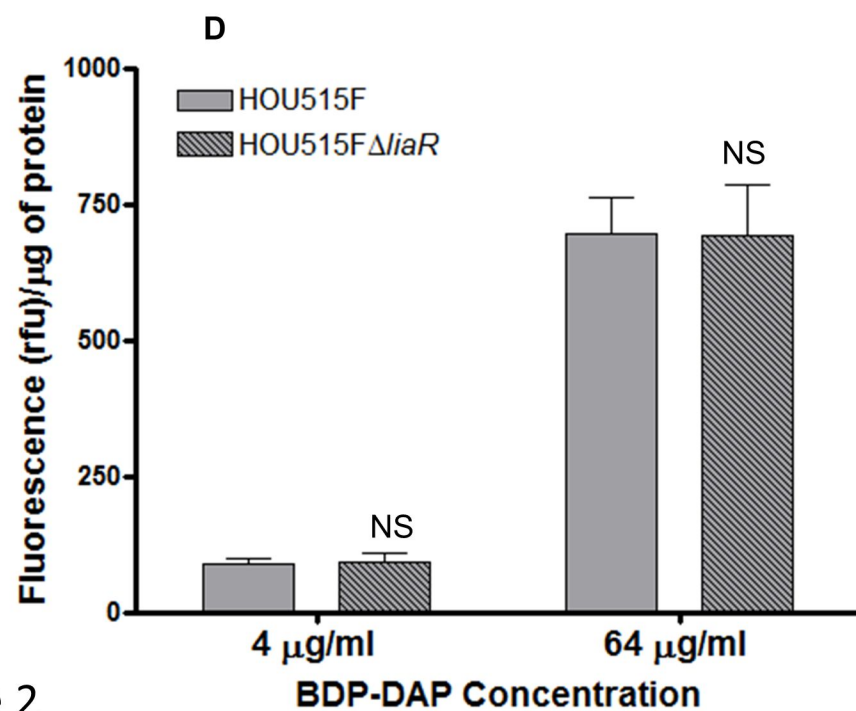
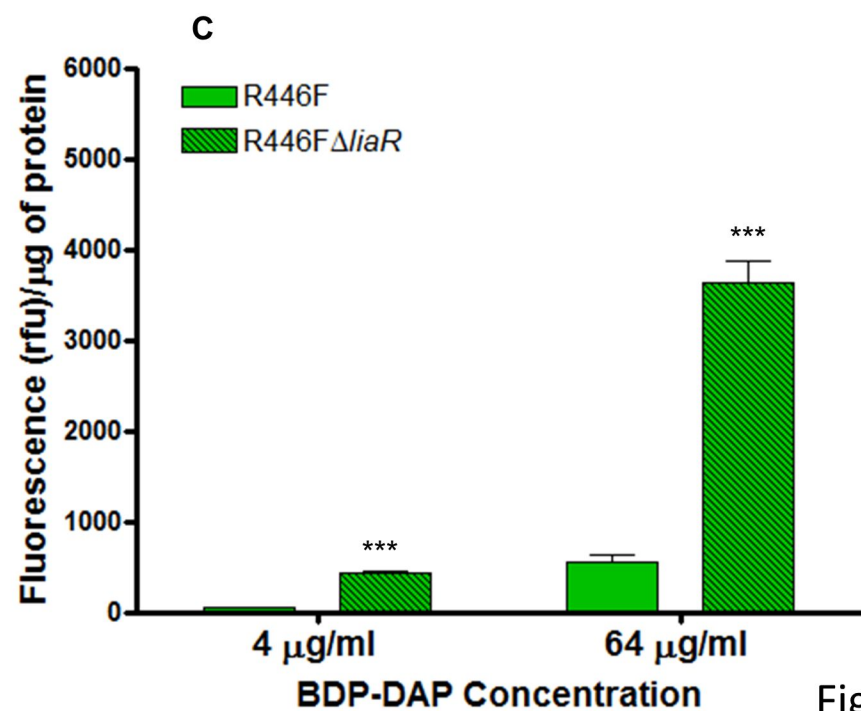
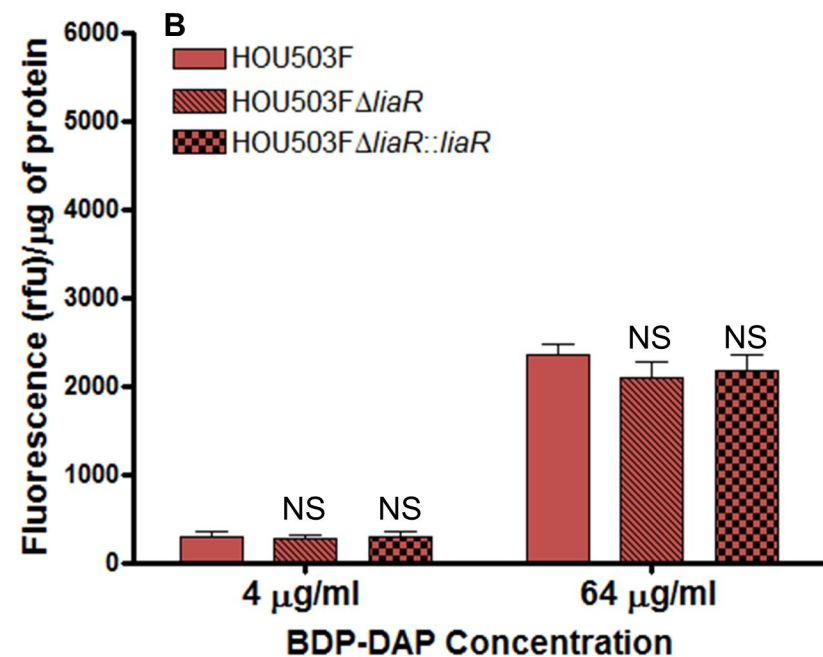
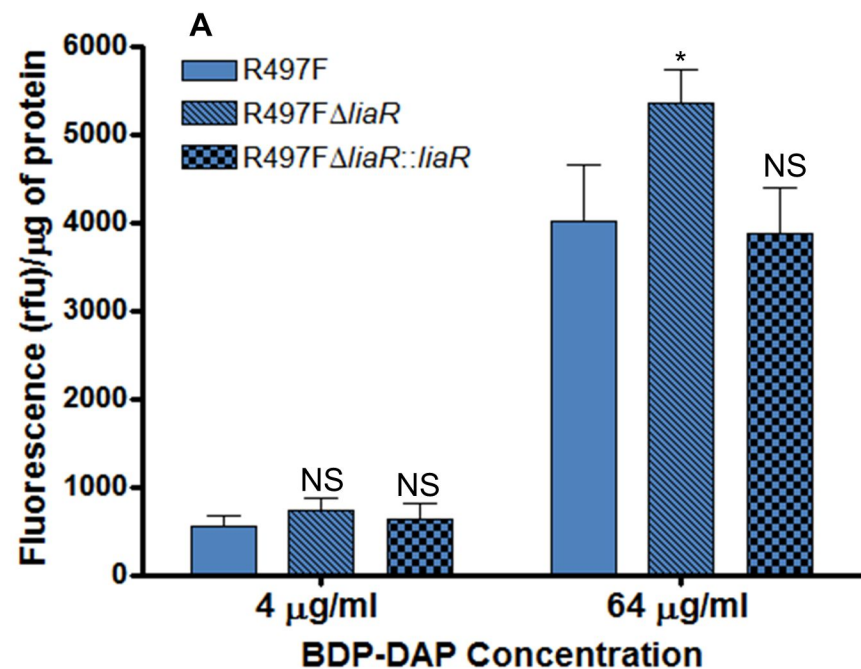


Figure 2

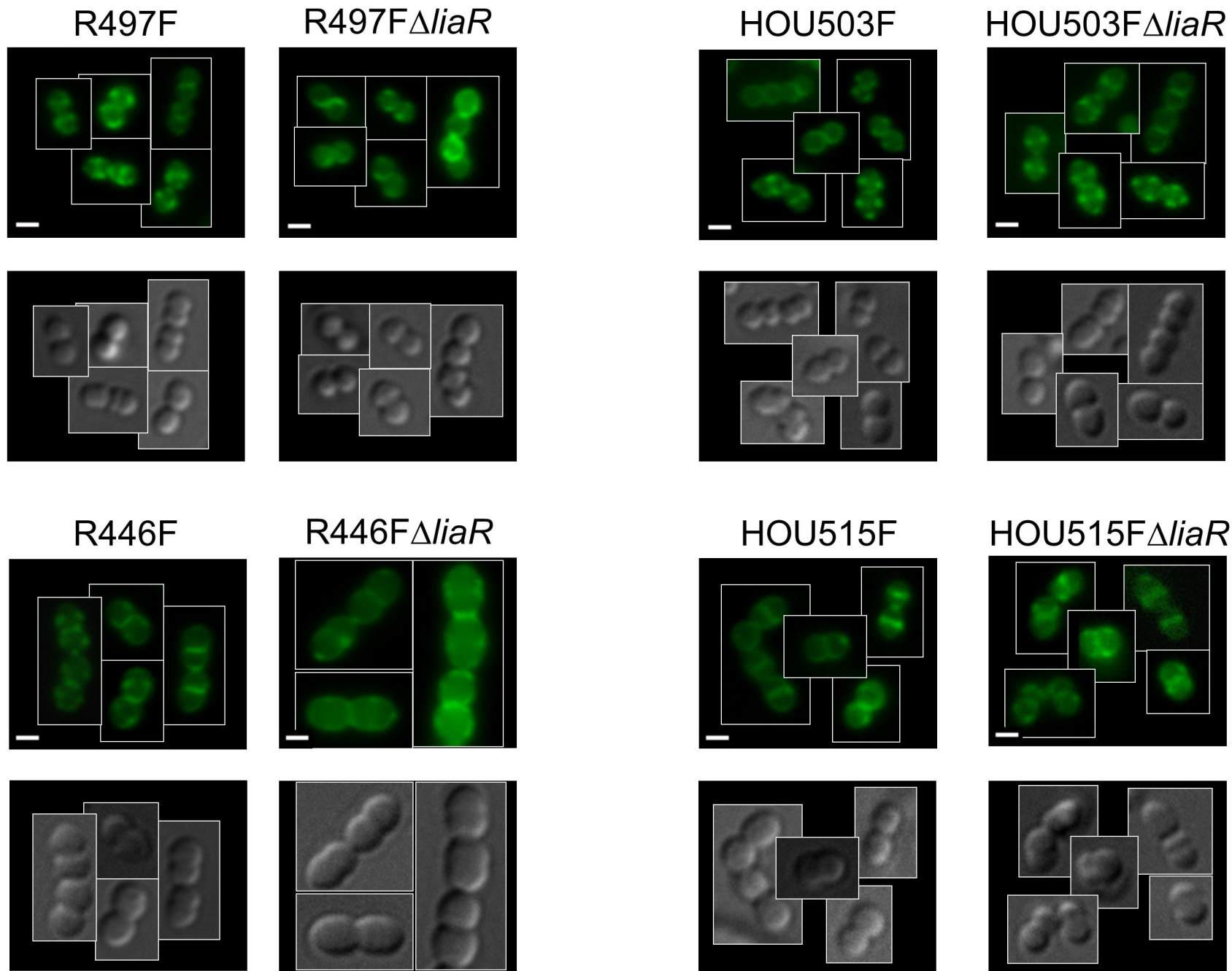


Figure 3

**TABLE 1. *Enterococcus faecium* strains used in this study.**

<i>E. faecium</i> strains	Relevant characteristics	DAP MIC (µg/mL)	Reference
R446	DAP and VAN-resistant clinical isolate harboring 8 changes in predicted proteins compared to its parental strain including a S333L substitution in YycG <sup>a</sup> . The other changes are in Cls, Cfa, RrmA, SulP, XpaC, 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
R446FΔ <i>liaR</i>	Derivative of R446F harboring a non-polar deletion of <i>liaR</i>	0.25	This study
R497	Daptomycin-resistant isolate harboring W73C and T120A substitutions in LiaR and LiaS, respectively. Additionally, the strain harbors an insertion of MPL at position 110 in Cls <sup>b</sup> .	24	9, 21
R497F	Fusidic acid-resistant derivative of R497	24	This study
R497FΔ <i>liaR</i>	Derivative of R497F harboring a	0.094	This study

	non-polar deletion of <i>liaR</i>		
R497F $\Delta$ <i>liaR</i> :: <i>liaR</i>	Complementation of <i>liaR</i> in <i>cis</i> (native chromosomal location)	24	This study
HOU503	Daptomycin-tolerant and vancomycin-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 $\Delta$ <i>liaR</i>	Derivative of HOU503F harboring a nonpolar deletion of <i>liaR</i>	0.064	This study
HOU503 $\Delta$ <i>liaR</i> :: <i>liaR</i>	Complementation of <i>liaR</i> in <i>cis</i> (native chromosomal location)	3	This study
HOU515	Daptomycin-tolerant clinical isolate harboring a A414T substitution in YycG and no LiaFSR substitutions	3	21
HOU515F	Fusidic acid-resistant derivative of HOU515	3	This study
HOU515F $\Delta$ <i>liaR</i>	Derivative of HOU515F harboring a nonpolar deletion of <i>liaR</i>	0.047	This study

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<sup>a</sup>YycG, putative histidine kinase of an essential two-component regulatory system YycFG

<sup>b</sup>CIs, cardiolipin synthase