

# A *liaR* Deletion Restores Susceptibility to Daptomycin and Antimicrobial Peptides in Multidrug-Resistant *Enterococcus faecalis*

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**Daptomycin is a lipopeptide antibiotic that is used clinically against many gram-positive bacterial pathogens and is considered a key frontline bactericidal antibiotic to treat multidrug-resistant enterococci. Emergence of daptomycin resistance during therapy of serious enterococcal infections is a major clinical issue. In this work, we show that deletion of the gene encoding the response regulator, LiaR (a member of the LiaFSR system that controls cell envelope homeostasis), from daptomycin-resistant *Enterococcus faecalis* not only reversed resistance to 2 clinically available cell membrane-targeting antimicrobials (daptomycin and telavancin), but also resulted in hypersusceptibility to these antibiotics and to a variety of antimicrobial peptides of diverse origin and with different mechanisms of action. The changes in susceptibility to these antibiotics and antimicrobial peptides correlated with in vivo attenuation in a *Caenorhabditis elegans* model. Mechanistically, deletion of *liaR* altered the localization of cardiolipin microdomains in the cell membrane. Our findings suggest that LiaR is a master regulator of the enterococcal cell membrane response to diverse antimicrobial agents and peptides; as such, LiaR represents a novel target to restore the activity of clinically useful antimicrobials against these organisms and, potentially, increase susceptibility to endogenous antimicrobial peptides.**

**Keywords.** LiaFSR; daptomycin; *E. faecalis*; antimicrobial peptides.

The World Health Organization has named antimicrobial resistance as one of the greatest public health threats of the 21st century [1, 2]. Multidrug-resistant enterococci are among the most difficult microorganisms to treat in clinical settings because of resistance to all available antimicrobials. Moreover, infections caused by such multidrug-resistant bacteria often occur in patients who are critically ill and/or significantly immunocompromised, further complicating the treatment of these patients.

Daptomycin (DAP), a key front-line bactericidal agent for treating multidrug-resistant enterococci, is a cyclic lipopeptide that targets the bacterial cell membrane (CM) of gram-positive bacteria, becoming a de facto cationic antimicrobial peptide. The antibacterial mechanism of DAP action involves interactions with the CM in a calcium-dependent manner [3–5]. Calcium-complexed DAP seems to insert initially into the outer leaflet of the bacterial CM with preference for septal division planes [6]. Subsequently, DAP appears to oligomerize in a process that has been shown to depend on the presence of phosphatidylglycerol [4]. Oligomerization of DAP leads to disruption of the integrity of the CM and alterations of cell division that eventually lead to cell death [6]. We have previously shown that mutations in genes encoding the 3-component regulatory system LiaFSR (involved in the bacterial response to cell envelope-acting antibiotics and antimicrobial

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peptides [AMPs]), markedly reduced the activity of DAP [7–11]. The LiaFSR system [12–17], which is present in all gram-positive pathogens of clinical importance, including streptococci and *Staphylococcus aureus* [12–17], is composed of a transmembrane protein (LiaF/VraT), a membrane bound histidine-kinase (LiaS/VraS), and a response regulator (LiaR/VraR). In *Bacillus subtilis*, LiaR regulates the expression of a gene cluster (*liaIHGFSR*) that orchestrates the CM stress response against attack by antibiotics and AMPs [12, 18–20], although the mechanisms of this response are unknown. We previously showed that, in *Enterococcus faecalis*, adaptation against DAP-mediated CM disruption involves redistribution of cardiolipin microdomains in the CM, which are relocated away from the septum to other CM areas [10]. We postulated that the phospholipid remodeling diverted DAP from the septum to other cardiolipin-rich areas in the CM, thus preventing the antibiotic from interacting with its main septal target [10] and, perhaps, from reaching the inner leaflet of the CM [3]. The remodeling of cardiolipin microdomains was associated with a deletion of isoleucine in position 177 of LiaF [10]. Moreover, our previous studies using quantitative experimental evolution revealed that development of DAP resistance in a clinical strain of *E. faecalis* follows a clear order and hierarchy of genetic changes [11]. We also showed that substitutions in LiaFSR appear to be the first pivotal event in the evolution of DAP resistance and, possibly, membrane adaptations [11]. In this work, we present data that the response regulator, LiaR, mediates CM adaptation in *E. faecalis* by controlling the remodeling of CM cardiolipin microdomains. Deletion of the *liaR* gene not only reversed resistance to DAP and AMPs but also produced hypersusceptibility to these molecules in vitro and resulted in

attenuation of in vivo virulence. Thus, we postulate that interfering with the LiaFSR response may provide a novel strategy to restore and preserve the activity of antimicrobials (eg, DAP) and potentiate the innate immune clearance of resistant microorganisms. Such an approach may become a viable antimicrobial strategy against multidrug-resistant gram-positive organisms in the future.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

Bacterial strains and their phenotypes are listed in Table 1. *E. faecalis* S613 is a vancomycin-resistant and DAP-susceptible clinical isolate and was recovered from the bloodstream of a patient with bacteremia in 2005 [22]. S613F $\Delta_{liaF177gdpD170cls61}$  is a DAP-resistant derivative of S613 generated in vitro that harbors 3 alleles associated with DAP resistance (*liaF*, *gdpD*, and *cls*) [22]. All enterococci were routinely grown on brain-heart infusion agar or broth at 37°C with gentle agitation. *Escherichia coli* strains used for cloning and genetic manipulations were grown in Luria-Bertani (LB) broth or LB broth supplemented with chloramphenicol (10 µg/mL) when harboring derivatives of pHOU3.

### Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MICs) of DAP and telavancin were determined by the Etest, using the manufacturer's recommendations (Biomérieux), on Mueller-Hinton agar and incubated for 24 hours. Telavancin susceptibilities were assessed because of similarities in the mechanism of action of telavancin, compared with that of DAP (targeting CM). The results were interpreted using breakpoints issued by the Clinical and

**Table 1. Characteristics of *Enterococcus faecalis* Strains Used in This Study**

<i>E. faecalis</i> Strain	Relevant Characteristic(s)	DAP MIC, µg/mL <sup>a</sup>	Reference(s)
S613	Vancomycin-resistant and daptomycin-susceptible bloodstream isolate exhibiting HLR resistance to both gentamicin and streptomycin	0.5	[7, 10]
S613F	Fusidic acid-resistant derivative of S613	0.5	This study
S613F $\Delta_{liaF177gdpD170cls61}$	Daptomycin-resistant derivative of S613F harboring <i>liaF</i> <sub>177</sub> , <sup>b</sup> <i>gdpD</i> <sub>170</sub> , <sup>c</sup> and <i>cls</i> <sub>61</sub> <sup>d</sup> resistance-associated alleles	8	[7, 10]
S613F $\Delta_{liaF177gdpD170cls61}\Delta_{liaR}$	Derivative of S613F $\Delta_{liaF177gdpD170cls61}$ harboring a nonpolar deletion of <i>liaR</i>	0.094	This study
S613F $\Delta_{liaF177gdpD170cls61}\Delta_{liaR}::liaR$	S613F $\Delta_{liaF177gdpD170cls61}\Delta_{liaR}$ complementation of <i>liaR</i> in <i>cis</i> (native chromosomal location)	8	This study
OG1RF	Laboratory strain of <i>E. faecalis</i>	2	[21]
OG1RF $\Delta_{liaR}$	OG1RF harboring a nonpolar deletion of <i>liaR</i>	0.094	This study
OG1RF $\Delta_{liaR}::liaR$	OG1RF $\Delta_{liaR}$ complementation of <i>liaR</i> in <i>cis</i> (native chromosomal location)	2	This study
OG1RF $\Delta_{liaR}::pAT392_{liaR}$	OG1RF $\Delta_{liaR}$ complemented with <i>liaR</i> in <i>trans</i> , using plasmid pAT392 under the control of the constitutive P2 promoter	2	This study

Abbreviation: HLR, high-level resistance.

<sup>a</sup> Daptomycin (DAP) minimum inhibitory concentrations (MICs) were determined by the use of Etest (bioMérieux) on Mueller-Hinton agar.

<sup>b</sup> Mutated allele encoding the transmembrane protein LiaF, resulting in deletion of isoleucine at position 177 of the putative protein.

<sup>c</sup> Mutated allele encoding a glycerophosphoryl diester phosphodiesterase, resulting in deletion of isoleucine at position 170 of the putative enzyme.

<sup>d</sup> Mutated allele encoding a cardiolipin synthase, resulting in deletion of lysine at position 61 of the putative protein.

Laboratory Standards Institute (CLSI) [23, 24], except for telavancin (for which no breakpoint was available).

### Mutant Construction and Genetic Manipulations

To create nonpolar deletions of *liaR* in S613FΔ<sub>liaF177gdpD170cls61</sub> (Table 1), we used the *p*-chloro-phenylalanine sensitivity counterselection (PheS\*) system [25] with plasmid pHOU3 (which carries the chloramphenicol acetyltransferase gene), as described previously [7, 26, 27]. Briefly, fragments upstream and downstream of *liaR* (Supplementary Table 1) were amplified by crossover polymerase chain reaction (PCR), using the genomic DNA of *E. faecalis* S613 as a template. PCR products were cloned into pHOU3, using specific restriction enzymes (Supplementary Table 1). The plasmid constructs were electroporated into *E. faecalis* CK111 and, subsequently, delivered to *E. faecalis* S613FΔ<sub>liaF177gdpD170cls61</sub> and *E. faecalis* OG1RF by conjugation [7, 10, 26, 27]. First-recombination integrants were selected on agar containing chloramphenicol (10 μg/mL) and fusidic acid (25 μg/mL). To obtain the desired replacement, first-event integrants were subsequently grown on *p*-chloro-phenylalanine, and colonies were tested by replica plating in the presence of different DAP concentrations [7, 10, 26, 27]. DAP-susceptible mutants were characterized by pulsed-field gel electrophoresis and the open read frames of *liaFSR*, *gdpD*, and *cls* were sequenced in their entirety in both directions.

For complemented strains, the *liaR* gene was amplified by PCR with primers 1 and 4 (Supplementary Table 1), using S613 DNA as a template and the fragment (2113 bp) cloned into pHOU3. The recombinant plasmids were delivered into the *E. faecalis* S613FΔ<sub>liaF177gdpD170cls61</sub>Δ*liaR* and *E. faecalis* OG1RFΔ*liaR* mutants, using the same procedures specified above. The *liaR* gene was also cloned into plasmid pAT392 [28], using primers 9 and 10 (Supplementary Table 1), for *trans* complementation in *E. faecalis* OG1RF, and recombinant colonies were selected in the presence of gentamicin (125 μg/mL). The complemented strains were tested by replica plating in the presence of different DAP concentrations and characterized by pulsed-field gel electrophoresis.

### Evaluation of Activity of AMPs

The in vitro bactericidal assays with AMPs were carried out as described previously [29–31] using a 2-hours microdilution method in Eagle minimal essential medium. We used an inoculum of 10<sup>3</sup> colony-forming units (CFU) of exponential phase cells at 2 concentrations of AMPs (Table 2). These AMPs concentrations were selected based on extensive pilot studies showing their inability to substantially reduce starting inocula of either parental strain over the 2-hour exposure period. The data were calculated and expressed as the relative percent of surviving colony-forming units of AMP-exposed versus AMP-unexposed cells. The percentage survival at 2 hours of *E. faecalis* S613FΔ<sub>liaF177gdpD170cls61</sub>Δ*liaR* was compared to the survival of

**Table 2. Survival of *Enterococcus faecalis* Strains After 2 Hours of Exposure to Antimicrobial Peptides**

<i>E. faecalis</i> Strain	Survival, %, Mean ± SD													
	LL37, μg/mL	HBD-3, μg/mL	Nisin, μg/mL	GAL, μg/mL	RP-1, μg/mL	MER, μg/mL	FRU, μg/mL	LL37, μg/mL	HBD-3, μg/mL	Nisin, μg/mL	GAL, μg/mL	RP-1, μg/mL	MER, μg/mL	FRU, μg/mL
<i>E. faecalis</i> S613FΔ <sub>liaF177gdpD170cls61</sub>	20	10	83 ± 6 <sup>a</sup>	83 ± 6 <sup>a</sup>	83 ± 6 <sup>a</sup>	83 ± 6 <sup>a</sup>	83 ± 6 <sup>a</sup>	83 ± 6 <sup>a</sup>	83 ± 6 <sup>a</sup>	83 ± 6 <sup>a</sup>	83 ± 6 <sup>a</sup>	83 ± 6 <sup>a</sup>	83 ± 6 <sup>a</sup>	83 ± 6 <sup>a</sup>
<i>E. faecalis</i> S613FΔ <sub>liaF177gdpD170cls61</sub> Δ <i>liaR</i>	20	10	81 ± 6 <sup>a</sup>	81 ± 6 <sup>a</sup>	81 ± 6 <sup>a</sup>	81 ± 6 <sup>a</sup>	81 ± 6 <sup>a</sup>	81 ± 6 <sup>a</sup>	81 ± 6 <sup>a</sup>	81 ± 6 <sup>a</sup>	81 ± 6 <sup>a</sup>	81 ± 6 <sup>a</sup>	81 ± 6 <sup>a</sup>	81 ± 6 <sup>a</sup>
<i>E. faecalis</i> S613FΔ <sub>liaF177gdpD170cls61</sub> Δ <i>liaR</i> Δ <sub>liaR::liaR</sub>	20	10	17 ± 21	46 ± 28	38 ± 21	85 ± 18	85 ± 18	85 ± 18	85 ± 18	85 ± 18	85 ± 18	85 ± 18	85 ± 18	85 ± 18
<i>E. faecalis</i> S613FΔ <sub>liaF177gdpD170cls61</sub> Δ <sub>liaR::liaR</sub> Δ <sub>liaR::liaR</sub>	20	10	84 ± 14 <sup>a</sup>	94 ± 22 <sup>a</sup>	85 ± 22 <sup>a</sup>	95 ± 18	95 ± 18	95 ± 18	95 ± 18	95 ± 18	95 ± 18	95 ± 18	95 ± 18	95 ± 18

Abbreviations: FRU, frutillucin, a cationic lipopeptide; GAL, gallidermin, type A lantibiotic; HBD3, human β-defensin 3; LL-37, a cathelicidin neutrophil-derived antimicrobial peptide; MER, mersacidin, type B lantibiotic; nisin, type A lantibiotic; RP-1, synthetic antimicrobial peptide; SD, standard deviation.

<sup>a</sup> *P* < .05, compared with S613FΔ<sub>liaF177gdpD170cls61</sub>Δ*liaR* survival at 2 h.

its parental strain (*E. faecalis* S613F $\Delta_{\text{liaF177gdpD170cls61}}$ ) and complemented derivative (*E. faecalis* S613F $\Delta_{\text{liaF177gdpD170cls61}}\Delta_{\text{liaR}}::\text{liaR}$ ). A minimum of 3 independent experiments was performed for each experiment.

### ***Caenorhabditis elegans* Infection Model**

The method used for the *C. elegans* infection model has been previously described [32]. Briefly, 60–90 synchronized young adult nematodes were infected with *E. faecalis* S613F $\Delta_{\text{liaF177gdpD170cls61}}$ , S613F $\Delta_{\text{liaF177gdpD170cls61}}\Delta_{\text{liaR}}$ , and S613F $\Delta_{\text{liaF177gdpD170cls61}}\Delta_{\text{liaR}}::\text{liaR}$  on brain-heart infusion agar medium containing gentamicin (10  $\mu\text{g}/\text{mL}$ ). The plates were incubated at 25°C, and worm death was scored daily. Worms were considered dead when they exhibited no response to a platinum wire pick. Kaplan–Meier log rank analysis was used to compare survival curves pairwise. *P* values of .05 were considered statistically significant. The software GraphPad Prism (version 5.0) was used for analyses.

### **10-*N*-nonyl Acridine Orange (NAO) Staining**

Visualization of CM cardiolipin-rich domains was performed as described before [10], using the fluorescent dye *N*-nonyl acridine orange (NAO). NAO has been previously shown to bind preferentially to cardiolipin-arrays in the CM in several bacteria, including enterococci [33], and the association of NAO with cardiolipin molecules results in a green-to-red shift in fluorescence [34]. Fluorescence images were captured by an Olympus IX71 microscope with a PlanApo N 100X objective. At least 3 independent experiments were performed for each strain on different days. Fluorescence was viewed using the PicoQuant Imaging System. Excitation was at 490 nm, and emission was at either 528 or 617 nm at exposure times of 500 ms. Fluorescence areas identified in both wavelengths indicate a green-to-red shift, a property exhibited by NAO when bound to cardiolipin (Figure 3 and Supplementary Figure 2).

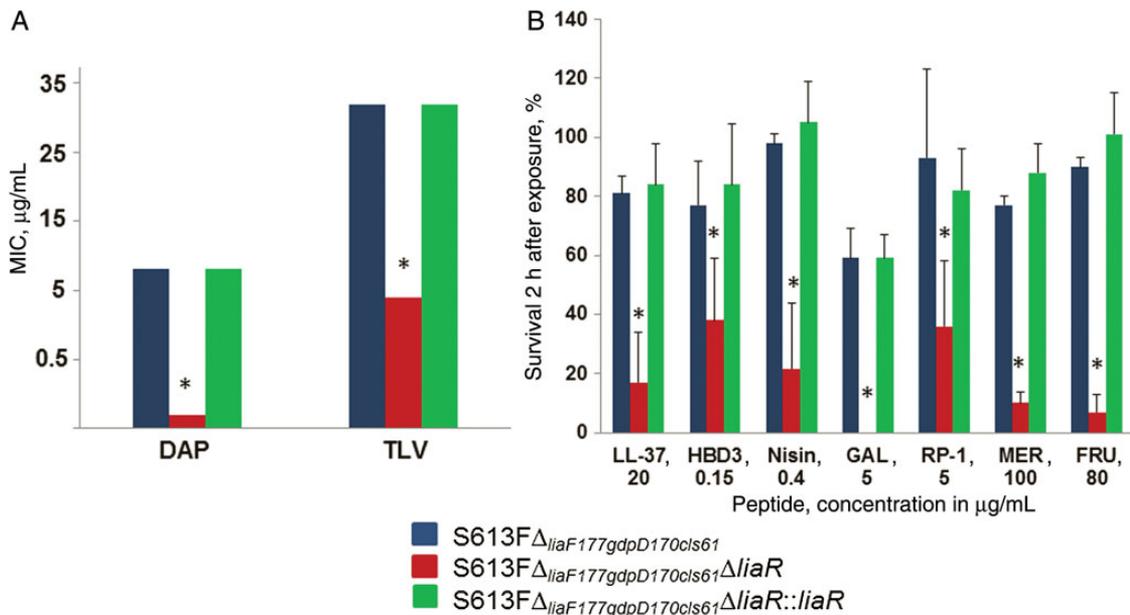
## **RESULTS**

### **Deletion of *liaR* Restores Susceptibility to CM-Targeting Antibiotics and AMPs**

To understand the role of LiaFSR in the enterococcal response to antimicrobials, we targeted the gene encoding the response regulator, LiaR, in *E. faecalis* strain S613F $\Delta_{\text{liaF177gdpD170cls61}}$ , a DAP- and fusidic acid-resistant derivative previously generated from *E. faecalis* S613 [22]. S613F $\Delta_{\text{liaF177gdpD170cls61}}$  contains amino acid changes in 3 putative proteins associated with DAP resistance: (1) LiaF $\Delta_{177}$ , a derivative of LiaF (a member of the LiaFSR system) lacking Ile177; (2) GdpD $\Delta_{170}$ , a glycerophosphodiester phosphodiesterase with a deletion of Ile170 that is involved in CM phospholipid metabolism; and (3) Cls $\Delta_{61}$ , a cardiolipin synthase harboring a deletion of Lys61 [7]. We generated a markerless nonpolar deletion of

*liaR* (nucleotides 15–620) in *E. faecalis* S613F $\Delta_{\text{liaF177gdpD170cls61}}$  (S613F $\Delta_{\text{liaF177gdpD170cls61}}\Delta_{\text{liaR}}$ ), using the PheS\* system, as described before [25]. Of note, deletion of *liaR* did not affect the growth of the mutant (Supplementary Figure 1A). Using the same PheS\* system, we complemented the mutant (S613F $\Delta_{\text{liaF177gdpD170cls61}}\Delta_{\text{liaR}}::\text{liaR}$ ) by delivering the original allele to the same chromosomal location. As shown in Figure 1A, deletion of *liaR* generated hypersusceptibility to DAP, fully reversing resistance to this antibiotic, with the MIC decreasing from 8  $\mu\text{g}/\text{mL}$  to 0.094  $\mu\text{g}/\text{mL}$ , far below the usual MIC of enterococci (breakpoint, 4  $\mu\text{g}/\text{mL}$ ). Complementation of the mutated strain with *liaR* restored the DAP resistance phenotype, indicating that *liaR* is necessary for DAP resistance in this *E. faecalis* strain. To confirm the role of *liaR*, we generated another *liaR* deletion in a laboratory strain of *E. faecalis* (OG1RF; DAP MIC, 2  $\mu\text{g}/\text{mL}$ ). Deletion of *liaR* in OG1RF also produced hypersusceptibility to DAP (reduction, 8 fold; MIC, 0.094), a phenomenon that was reversed by reintroducing *liaR* into its chromosomal location or on a multicopy plasmid (pAT392; Table 1), confirming *liaR*'s role in the level of DAP susceptibility. Additionally, the *liaR* deletion also markedly increased the susceptibility to telavancin, resulting in an 8-fold MIC decrease (Figure 1A). All these MICs changes were reversed by reintroduction of *liaR* into its native chromosomal location (Figure 1A).

AMPs are among the most ancient antibacterial mediators of the mammalian innate immune response [35]. Additionally, several prokaryotic organisms produce similar peptides that target competing bacteria. The main targets of prokaryotic- and eukaryotic-derived AMPs are the CM-associated precursors of peptidoglycan synthesis (lipid I and II), cell-wall teichoic acid and/or lipoteichoic acid precursors (lipid III and IV) [36], and the lipopolysaccharide (LPS) of gram-negative bacteria [35]. To determine whether *liaR* is also an important mediator of the *E. faecalis* response to AMPs (like calcium-complexed DAP), we tested the activity of a variety of AMPs against S613F $\Delta_{\text{liaF177gdpD170cls61}}$ , as well as its *liaR* deletion mutant and complemented derivatives. These peptides are from different origins and exhibit distinct mechanisms of action [35–40]. The peptides tested included (1) LL-37, a cathelicidin peptide produced by mammalian neutrophils and epithelium that has been shown to be active against both gram-positive and gram-negative pathogens (via binding of LPS) [35]; (2) human  $\beta$ -defensin 3, which is produced by epithelial cells and neutrophils and interacts with lipid II and LPS [37]; (3) nisin, a class I prokaryote bacteriocin and type A lantibiotic produced by *Lactococcus lactis* that interacts with lipid II, III, and IV [38]; (4) gallidermin, a nisin-related lantibiotic produced by *Staphylococcus gallinarum* that also targets lipid II, III, and IV [38]; (5) RP-1, a synthetic AMP mimicking the bactericidal domain of the PF-4 family of kinocidins [39, 41]; (6) mersacidin, a type B lantibiotic peptide produced by *Bacillus* species that targets lipid II [38]; and (7) friulimicin B, a naturally occurring

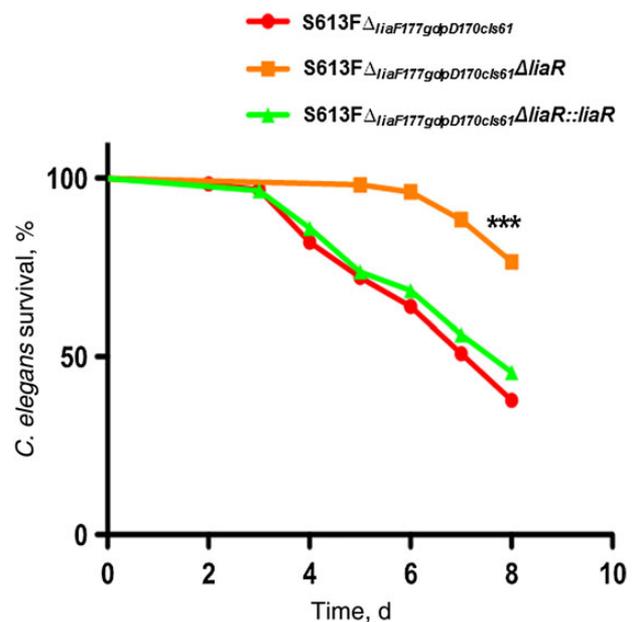


**Figure 1.** Minimum inhibitory concentrations (MICs) and susceptibility to antimicrobial peptides of *Enterococcus faecalis* derivatives. **A**, Change of daptomycin (DAP) and telavancin (TLV) MICs determined by the Etest (Biomérieux) on Mueller-Hinton agar. MIC determination was performed in triplicate for all strains, with the same result in each experiment. Of note, breakpoints for TLV are not available. **B**, Survival after exposure to antimicrobial peptides (AMPs). Results are from 3 independent experiments. \* $P < .001$ , compared with S613FΔ*liaF177gdpD170cls61*. Abbreviations: FRU, friulimicin, naturally occurring lipopeptide; GAL, gallidermin, type A lantibiotic; HBD3, human  $\beta$ -defensin 3; LL-37, human cathelicidin; MER, mersacidin, type B lantibiotic; nisin, type A lantibiotic; RP-1, synthetic antimicrobial peptide.

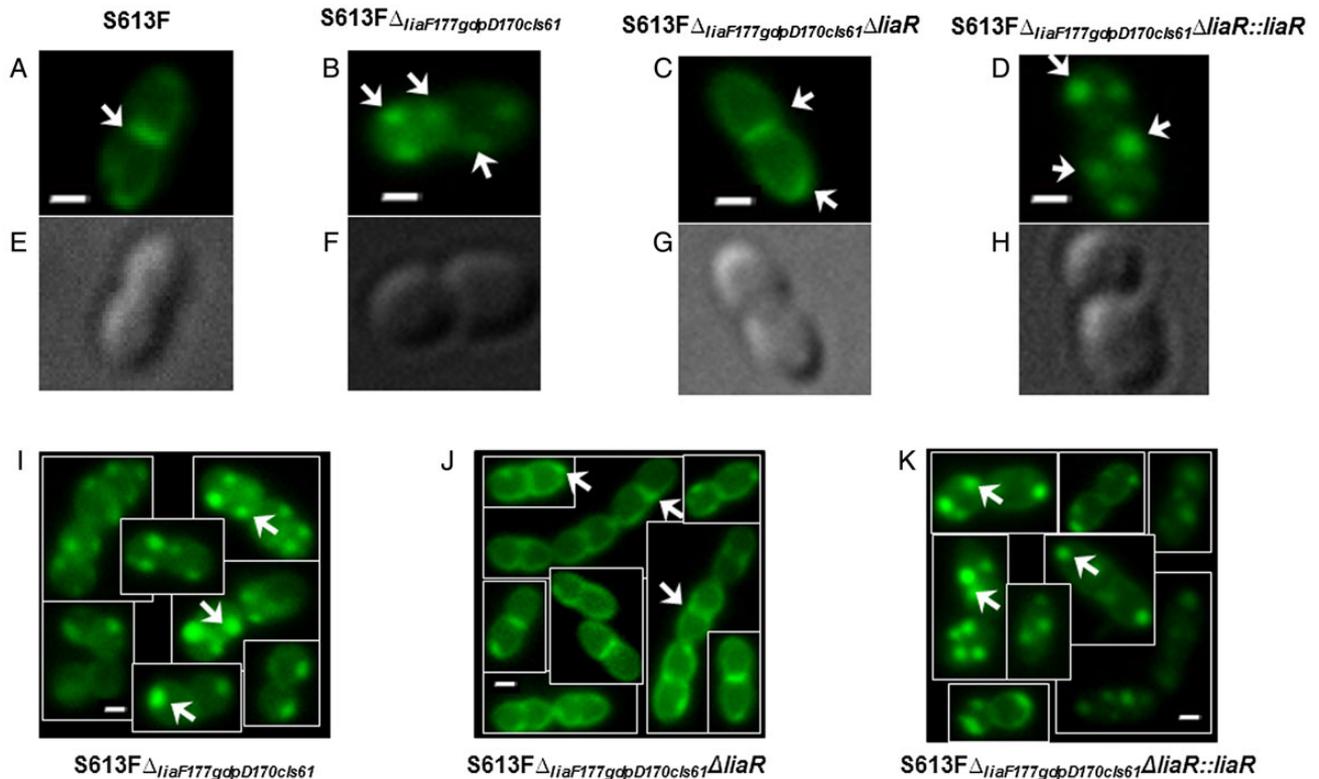
cyclic lipopeptide produced by *Actinoplanes friuliensis* that interferes with peptidoglycan synthesis through the formation of a  $\text{Ca}^{2+}$ -dependent complex with the bactoprenol phosphate carrier C(55)-P [38]. We evaluated survival of S613FΔ*liaF177gdpD170cls61*Δ*liaR*, as well as its parental and complemented derivative, at 2 different concentrations of each AMP (Figure 1B and Table 2). Figure 1B shows that the *liaR* deletion had a profound and uniform impact on susceptibility to all AMPs tested, paralleling the effects seen with DAP and telavancin. Survival in the presence of AMPs was markedly reduced in the *liaR* deletion mutant, compared with its parental strain, and this effect was reversed by *liaR* complementation. Our results suggest that LiaR is a master regulator of the enterococcal CM response to both CM-acting antimicrobials and AMPs.

#### Deletion of *liaR* Also Affects In Vivo Virulence

To test whether the *liaR* deletion also affects the in vivo virulence of multidrug-resistant *E. faecalis*, we used the *C. elegans* model, which has been extensively used to examine the pathogenic properties of *E. faecalis* [32, 42]. AMPs are important mediators of the immune response in *C. elegans*, and this nematode possesses a complex signaling cascade that regulates the production of AMPs such as caenopores, lysozymes, and lectins [43–46] that behave as an important line of defense against bacterial pathogens. *E. faecalis* readily causes infection in the



**Figure 2.** Killing of *Caenorhabditis elegans* by *Enterococcus faecalis* derivatives. Exposure of *C. elegans* to *E. faecalis* S613FΔ*liaF177gdpD170cls61*Δ*liaR* results in significant attenuation, compared with the parental strain (S613FΔ*liaF177gdpD170cls61*). The in vivo phenotype was restored by introducing *liaR* back into its native chromosomal location (S613FΔ*liaF177gdpD170cls61*Δ*liaR::liaR*). The experiment was repeated 3 times with similar results (n=60–90 worms). \*\*\* $P < .0001$ .



**Figure 3.** Staining of representative cells of *Enterococcus faecalis* S613F and derivatives with 10-N-nonyl acridine orange (500 nM). A–D and I–K, Fluorescence microscopy images of bacterial cells. E–H, Phase-contrast images of the same cells in panels A–D. A, S613F displays cardiolipin microdomains in septum and poles of the cell (white arrows). B and I, S613F $\Delta$ *liaF177gdpD170cls61* exhibits cardiolipin microdomains located away from the main septum and distributed throughout the cells. C and J, Deletion of *liaR* redistributes the cardiolipin microarrays back to the septum and poles, as shown in DAP-susceptible *E. faecalis* S613F. D and K, Reintroducing *liaR* in its native chromosomal location restored the remodeling of cardiolipin microdomains in the cell membrane. White arrows indicate the cardiolipin domains in the cell membrane, and bars denote lengths of 1  $\mu$ m.

*C. elegans* model, colonizing the intestinal lumen and resulting in persistent infection leading to eventual death [32]. We tested the effect of the *liaR* deletion on the ability of S613F $\Delta$ *liaF177gdpD170cls61* to kill *C. elegans*. As shown in Figure 2, deletion of *liaR* significantly attenuated the virulence seen with the parental strain S613F $\Delta$ *liaF177gdpD170cls61* ( $P < .05$ ), an effect that was initially evident 3 days after infection and continued over the 8-day observation period. Since the deletion did not affect the in vitro growth (Supplementary Figure 1), we postulate that the attenuation is likely due to increased susceptibility to AMPs.

#### LiaR Controls Localization of CM Cardiolipin Microdomains

We previously showed that emergence of DAP resistance in *E. faecalis* S613 was associated with important redistribution of cardiolipin microdomains in the CM, which relocate away from the principle DAP target, the division septum [10]. This CM phospholipid remodeling was observed after we generated an Ile177 deletion in LiaF, suggesting that this mutation was likely to produce the CM effect via its response regulator,

LiaR. Thus, to gain further insights into the mechanism of CM adaptation and the effect on susceptibility to DAP mediated by LiaR, we used the hydrophobic fluorescent dye NAO to assess localization of cardiolipin-enriched microdomains in the CM, using fluorescence microscopy, as previously described [33, 34]. As shown in Figure 3, NAO staining (500 nm) of DAP-resistant S613F $\Delta$ *liaF177gdpD170cls61* revealed the presence of cardiolipin domains that were localized away from the main division septum as previously reported [10]. Deletion of *liaR* produced a marked change in the cardiolipin-microdomain distribution so that, in the absence of LiaR, the CM cardiolipin-microdomains were redirected back to the septum, a pattern similar to that observed in the parental *E. faecalis* S613 strain (Figure 3 and Supplementary Figure 1). The pattern of cardiolipin-domain distribution in the CM was fully restored after complementation with *liaR*. This finding provided evidence that LiaR mediates CM phospholipid redistribution and that this structural phenomenon is mechanistically associated with resistance to a broad range of antibiotics and AMPs.

## DISCUSSION

Our previous studies [7–10] focused on the mechanisms of DAP resistance in enterococci. These investigations provided insights into the molecular events associated with CM adaptation and the bacterial stress response in multidrug-resistant *E. faecalis* and suggested that interfering with such a process might be a novel antimicrobial strategy [7–10]. Indeed, we have recently shown that, in a clinical strain of *E. faecalis*, specific mutations triggered CM adaptation mechanisms to withstand the attack by AMPs. Using a pair of clinical DAP-susceptible (S613) and DAP-resistant *E. faecalis* (R712) strains obtained from a patient before and after DAP therapy, we demonstrated that development of DAP resistance involved initial diversion of the antibiotic molecule away from the septum. Importantly, this effect was associated with redistribution of cardiolipin microdomains in the CM [10]. We provided compelling evidence that the initial remodeling of cardiolipin microdomains was related to a deletion of Ile from position 177 of LiaF, one of the members of the LiaFSR system that, in *B. subtilis*, controls the bacterial CM stress response (LiaF appears to negatively regulate the histidine kinase LiaS) [18]. Moreover, by using an in vitro quantitative experimental evolution system, we showed that changes in the *E. faecalis* LiaFSR pathway were the initial changes associated with the adaptive response that led to successful selection of DAP-resistant strains [11]. The CM adaptive response in *E. faecalis* also involved alterations in phospholipid composition of the CM, that, among other effects, reduced the content of phosphatidylglycerol, which we presume interferes with the activity of DAP by reducing the ability to oligomerize inside of the CM [10]. The change in phospholipid composition was associated with alterations in enzymes involved in phospholipid metabolism (GdpD and CIs). However, the changes in phospholipid enzymes appeared late in the selection process, after exposure to serially increasing concentrations of DAP, and occurred in the presence of initial changes of the LiaFSR system.

Here, we targeted the LiaR response regulator, the effector of the LiaFSR system, to further dissect the role of this system in CM homeostasis in DAP resistance. Toward this end, we generated a nonpolar deletion of *liaR* from both a DAP-resistant clinical strain and from a laboratory isolate and then complemented the gene deletion. Our findings suggest that LiaR is a master regulator of the *E. faecalis* CM adaptive response to the challenge by a broad range of unrelated CM-targeting cationic antimicrobials of different origins, structures, and mechanisms of action. Indeed, our results further indicate that LiaR plays a major role in the development of resistance to CM-targeting compounds. Interestingly, deletion of *liaR* in DAP-resistant *E. faecalis* S613 $\Delta_{liaF177gdpD170cls61}$  not only reversed resistance but also resulted in hypersusceptibility to the antibiotic, with MICs decreasing far below the values of the original parent strain. Thus, it is tempting to speculate that the LiaR regulon is crucial

in phospholipid remodeling as part of the CM adaptive response and that, in its absence, membrane adaptation is weakened to such extent that CM disruptions from different origins have profound deleterious effects.

Importantly, the LiaR-mediated structural CM adaptations and changes in AMP susceptibilities in vitro were paralleled by changes in the in vivo virulence of this important pathogen. It should be emphasized that, although the nematode model used in our in vivo studies does not contain mammalian-type granulocytes, platelets, or epithelial cells, *C. elegans* possesses functional homologues therein [32]. Therefore, our finding of in vitro pan-hypersusceptibility of the *liaR* mutant to a wide-ranging array of such peptides suggests that the response to AMP homologues in *C. elegans* is likely playing a role in virulence attenuation. Our findings have important implications since they likely indicate that LiaR plays a major role in the overall CM homeostasis and stress response to such antimicrobial molecules and confirms that interfering with LiaR function would be an intriguing approach to not only restore antimicrobial susceptibility to current anti-enterococcal antimicrobials but also to increase the innate immune-mediated clearance of such pathogens. Also, since the LiaFSR system is present in all clinically important gram-positive bacteria [12–17, 47, 48], a potential small-molecule approach to interfere with LiaR function may have broader applicability. Indeed, deletion of *liaR* orthologs in other gram-positive pathogens has been associated with alterations of virulence and response to AMPs [16, 17, 20, 47, 48].

Additionally, in the current study, we show that one mechanism by which LiaR orchestrates the CM response in *E. faecalis* involve cardiolipin microdomain redistribution. Cardiolipin plays a crucial role in the organization of the bacterial CM with the formation of microarrays localized in septal and polar areas [49]. The cardiolipin domains appear to contribute to the function of multiprotein complexes that include CM transport, cell division, and metabolism [49]. Moreover, recent evidence suggests that increased cardiolipin content is associated with inhibition of DAP-mediated membrane permeabilization [3]. Interestingly, using a liposomal model, it was shown that when membranes containing phosphatidylglycerol were saturated with cardiolipin at molar fractions of 10% and 20%, DAP was unable to form pores or translocate to the inner leaflet of the CM despite the fact that oligomerization of the antibiotic molecule was not affected [3]. These findings are consistent with our working hypothesis of the DAP diversion mechanism, in which cardiolipin microarrays may trap DAP and prevent the antibiotic from reaching the critical targets at the septal division plane. Thus, this LiaR-mediated cardiolipin-microdomain redistribution appears to be the first step in preventing cationic AMP-mediated CM damage, a sophisticated evolutionary adaptive response devised by *E. faecalis*.

In conclusion, we provide evidence that LiaR is a master regulator of the multidrug-resistant *E. faecalis* response to antibiotics and AMPs and have identified the CM-associated mechanism for such regulation. LiaR (and its orthologs) emerges as a potential target for developing antiadaptation molecules as antimicrobials and may restore the clinical utility of current antibiotics, a novel strategy to overcome multidrug resistance in recalcitrant bacterial pathogens.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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