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Bacteria have developed several evolutionary strategies to protect their cell membranes (CMs) from the attack of antibiotics and antimicrobial peptides (AMPs) produced by the innate immune system, including remodeling of phospholipid content and localization. Multidrug-resistant Enterococcus faecalis, an opportunistic human pathogen, evolves resistance to the lipopeptide daptomycin and AMPs by diverting the antibiotic away from critical septal targets using CM anionic phospholipid redistribution. The LiaFSR stress response system regulates this CM remodeling via the LiaR response regulator by a previously unknown mechanism. Here, we characterize a LiaR-regulated protein, LiaX, that senses daptomycin or AMPs and triggers protective CM remodeling. LiaX is surface exposed, and in daptomycin-resistant clinical strains, both LiaX and the N-terminal domain alone are released into the extracellular milieu. The N-terminal domain of LiaX binds daptomycin and AMPs (such as human LL-37) and functions as an extracellular sentinel that activates the cell envelope stress response. The C-terminal domain of LiaX plays a role in inhibiting the LiaFSR system, and when this domain is absent, it leads to activation of anionic phospholipid redistribution. Strains that exhibit LiaX-mediated CM remodeling and AMP resistance show enhanced virulence in Caenorhabditis elegans, an animal model that is abolished in animals lacking an innate immune pathway crucial for producing AMPs. In conclusion, we report a mechanism of antibiotic and AMP resistance that couples bacterial stress sensing to major changes in CM architecture, ultimately also affecting host-pathogen interactions.

Significance

Vancomycin-resistant enterococci are hospital-associated pathogens that evolved resistance to most antibiotics used in clinical practice. Daptomycin, a lipopeptide antibiotic used as frontline therapy for severe multidrug-resistant enterococcal infections, targets the bacterial cell membrane (CM). The LiaFSR stress response system orchestrates daptomycin and antimicrobial peptide (AMP) resistance by modulating CM phospholipid content or localization. Here, we identify a single protein (LiaX) that senses antimicrobial molecules and regulates changes in CM phospholipid architecture. We show that LiaX-mediated modulation of antibiotic and AMP resistance affects virulence during infection caused by a recalcitrant hospital pathogen. Targeting this response in multidrug-resistant organisms may be a therapeutic intervention to restore susceptibility to cell envelope-targeting antibiotics and increase the ability of the immune system to clear pathogens.

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Antibiotic resistance has been recognized as a major global health threat of the 21st century (1). Vancomycin-resistant enterococci (VRE) are among the most challenging organisms to treat, as some display resistance to all available antimicrobials used in clinical practice (2). Enterococci evolved resistance under the selective pressures of the modern health care environment due to their genomic plasticity and ability to acquire and disseminate a wide repertoire of antibiotic resistance determinants (3).

Daptomycin (DAP) is a frontline antibiotic against deep-seated VRE infections (4); however, the emergence of daptomycin resistance (DAP-R) in clinical settings emphasizes the need for novel therapies (5). DAP is a cell membrane (CM)-acting, cationic antibiotic that targets anionic phospholipids at the bacterial division septum (6). DAP insertion into the CM (6) leads to mislocalization of membrane proteins involved in maintaining cell envelope homeostasis, leading to cell death (7). Similarly, cationic antibiotic resistance | Enterococcus faecalis | daptomycin | cell membrane adaptation | antimicrobial peptides
antimicrobial peptides (AMPs) produced by the innate immune system exert their antibacterial activity through CM disruption (8, 9).

Emergence of DAP-R in clinical and laboratory settings is described in Enterococcus faecalis and Enterococcus faecium (5). In *E. faecalis*, DAP-R is associated with changes in CM phospholipid composition (10) and redistribution of anionic phospholipids away from the septum, diverting DAP (11, 12). These changes are linked to mutations in genes encoding the LiaFSR system and enzymes involved in phospholipid metabolism (13). LiaFSR (for lipid-H-interacting antibiotics) is a 3-component stress response system conserved among *Firmicutes* that regulates cell envelope integrity (14). A deletion of the gene encoding the LiaR response regulator leads to hypersusceptibility to DAP and AMPs (15). *E. faecalis* lacking LiaR is more susceptible to killing by innate immune cells (16). Quantitative experimental evolution has shown that pathways to DAP-R require initial activation of the LiaFSR response followed by changes in phospholipid enzymes (17). However, the mechanism by which LiaR affects cell envelope integrity on exposure to antibiotics and AMPs is unknown.

Here, we identify the genetic and biochemical basis of the adaptive response to CM active antibiotics in *E. faecalis*. We characterize LiaX, a protein of previously unknown function, as the main mediator of the LiaR-directed CM response to DAP and AMPs. Each domain of LiaX plays a unique function in modulating DAP-R. The C-terminal domain of LiaX inhibits the LiaFSR system in wild-type strains. Thus, the absence of the C-terminal domain releases this inhibition and acts as a signal to trigger the CM stress response. The N-terminal domain functions as a sentinel that can bind DAP and AMPs in the extracellular environment, leading to activation of the LiaFSR system and protection against antimicrobials. DAP-R strains show enhanced virulence in vivo in a *Caenorhabditis elegans* infection model relative to daptomycin-susceptible (DAP-S) strains due to resistance to the innate immunity. Supplementing the *C. elegans* infection assay with the N-terminal domain of the LiaX is sufficient to enhance the virulence of DAP-S strains. Thus, LiaX functions as a modulator of the CM stress response linking membrane adaptation, antibiotic resistance, and pathogenesis.

**Results**

**Determination of the LiaR Regulon.** We hypothesized that the biochemical mechanism of DAP-R is mediated by genes regulated by the LiaFSR system. Thus, we used transcriptomics to identify target genes in the LiaR regulon. Genes mediating DAP-R were identified by comparing 1) a clinical strain pair of DAP-S and DAP-R *E. faecalis* (S613 and R712) recovered from the bloodstream of a patient before and after DAP therapy, respectively (13), and 2) a DAP-R derivative of S613 that harbors the mutated alleles from R712 [S613ΔliaR/p77pgkDiXΔliaR; referred herein as TM (13)] with its derivative that has a deletion of *liaR* and is hypersusceptible to DAP (12). We identified 3 proteins encoded by the *liaXYZ* operon (*SI Appendix, Fig. S1*) as well as genes coding for transmembrane proteins of unknown function and proteins involved in cell envelope stress responses, cell division, and cell wall synthesis among others (*SI Appendix, Fig. S2 and Table S3*). Previous structural studies showed that, upon activation, LiaR oligomerizes, increasing its ability to bind regulatory DNA sequences upstream of LiaFSR and liaXYZ and recruit RNA polymerase (18). Thus, we focused on liaXYZ and used qRT-PCR to verify them as potentially important effectors of LiaR (*SI Appendix, Figs. S1 and S2 and Table S3*).

The first gene of the liaXYZ cluster ([liaX]) encodes a 533-amino acid (AA) protein with 2 distinct predicted domains: an N-terminal domain composed mainly of α-helices and a C-terminal domain formed largely of β-strands (*SI Appendix, Fig. S1*). LiaX lacks predicted classical secretion signals, an LPXTG motif, and transmembrane domains. *liaX* encodes a 107-AA transmembrane protein harboring a PspC domain that is involved in cell envelope stress adaptation in gram-negative bacteria (19). *liaZ* encodes a 115-AA transmembrane protein with homology to bacterial holins involved in the response to phase-mediated membrane damage (20).

**The C-Terminal Domain of LiaX Regulates CM Adaptation.** Experimental evolution of the DAP-S clinical isolate S613 identified that mutations in *liaF*, *liaA*, or a frameshift stop codon at a predicted AA position 289 in LiaX were crucial in the initial adaptive trajectory to DAP-R (17). Position 289 is within a protease susceptible linker region that bridges the N- and C-terminal domains of LiaX, suggesting that a C-terminal domain truncation is important for adaptation to DAP. Thus, to verify the role of LiaX and the function of the C-terminal domain, we generated a nonpolar deletion of *liaX* (OG1RFΔliaX) and truncation of the C-terminal domain (stop codon at AA 289, OG1RF*liax*Δ289) in a DAP-S laboratory strain of *E. faecalis* (OG1RF). Deletions of *liaX* and the C-terminal domain-encoding region made OG1RF resistant to DAP (8-fold increase in minimum inhibitory concentration [MIC]) with anionic phospholipids redistributed away from the division septum as evidenced by the pattern of 10-N-nonyl acridine orange staining (Fig. 1 and *SI Appendix, Fig. S3*). Complementation of *liaX* in the mutants reverted the resistance phenotype and reestablished septal localization of anionic phospholipids (Fig. 1 and *SI Appendix, Fig. S3*). Analysis of membrane composition of major lipids in exponential-phase cells showed an increase in phosphatidylglycerol (PG) and a decrease in cardiolipin in DAP-R laboratory (OG1RFΔliaX, OG1RF*liax*Δ289) and clinical (R712) strains relative to their DAP-S counterparts, with no changes observed in lysis-l-PG (*SI Appendix, Fig. S4*). Complementation of *liaX* (OG1RFΔliaX pAT392ΔliaX) fully reverted this phenotype (*SI Appendix, Fig. S4*). Of note, these lipid composition changes differ from previous studies conducted on stationary-phase cells (10).

The deletion of *liaX* or its C-terminal domain led to the activation of the LiaFSR stress response system as indicated by the up-regulation of the liaFSR and liaXYZ gene clusters relative to the DAP-S, OG1RF (*SI Appendix, Fig. S5*). Thus, the C-terminal domain of LiaX acts as a negative regulator of the LiaFSR system, and in the absence of this domain, constitutive activation of the system leads to CM remodeling that modulates antibiotic resistance.

**LiaX and Its N-Terminal Domain Are Released into the Extracellular Environment in DAP-R Strains.** Since LiaX has 2 distinct domains, we hypothesized that the subcellular localization of LiaX is important for its function. Localization was probed with an enzyme-linked immunosorbent assay (ELISA) and immunoblotting on whole cells, cell wall or CM extracts, and concentrated supernatants using an affinity purified antibody to the N terminus of LiaX. All experiments were performed with pairs of DAP-R and DAP-S isolated from clinical and laboratory strains. The 60-kDa LiaX or the N terminus alone (~30 kDa) was detected on the cell surface and extracellular milieu in significantly higher amounts in all DAP-R strains compared with the DAP-S parental strains (Fig. 2). Notably, the N-terminal domain of LiaX is detected along with full-length LiaX in the supernatants of the DAP-R clinical strains (Fig. 2C).

To identify changes in LiaX localization under antibiotic exposure, DAP-S and DAP-R strains were incubated with increasing DAP concentrations prior to immunoblotting. LiaX was detected in the whole-cell lysates and supernatants of DAP-S strains in a concentration-dependent manner, while DAP-R strains constitutively produced and released high amounts of LiaX (*SI Appendix, Fig. S6*). A band corresponding to the N terminus was also detected in strains that harbored the full-length LiaX in the lysates or supernatants, indicating that LiaX is likely processed and that the N-terminal end is released...
separately. To determine if overproduction of LiaX is sufficient for extracellular secretion, we cloned liaX in plasmid pMSP3535 under a nisin-inducible promoter and delivered the construct to OG1RFΔliaX. Induction with ascending concentrations of nisin led to increased expression and eventual secretion of both full-length and N-terminal LiaX (SI Appendix, Fig. S7). Additionally, we sought to visualize LiaX in the extracellular milieu using immunogold labeling and transmission electron microscopy. More LiaX was detected in the extracellular milieu of DAP-R compared with DAP-S strains (SI Appendix, Fig. S8). Our results
indicate that development of DAP-R involving CM remodeling was associated with increased cell surface exposure and extracellular release of LiaX and/or the N terminus of the protein.

LiaX Binds DAP and the Human Cathelicidin LL-37. To dissect the extracellular role of LiaX, we postulated that LiaX could bind DAP or AMPs to “shield” the cell from the antibiotic attack. The human cathelicidin LL-37 (21) is a peptide secreted by polymorphonuclear leukocytes to disrupt the bacterial CM and is functionally similar to DAP. Enterococci lacking liaR are more susceptible to killing by human neutrophils, presumably by enhancing the ability of LL-37 to kill the invading bacteria (16). DAP-R has been associated with cross-resistance to AMPs, including LL-37 (22). In agreement with those studies, all laboratory and clinical DAP-R strains (including OG1RFΔliaX) were resistant to LL-37 killing compared with their DAP-S counterparts (SI Appendix, Fig. S9). Furthermore, reintroduction of liaX in OG1RFΔliaX reverted the resistance phenotype. We then tested the ability of both LiaX and the N-terminal domain to bind DAP using fluorescence spectroscopy. The dissociation constant (Kd) for the full LiaX protein was 30.4 ± 6.1 nM in the presence of 1 mM Ca2+, while the N-terminal domain had a modestly lower affinity (Kd = 83.8 ± 6.9 nM) (Fig. 3A and Table S4). DAP MICs for enterococci range from ~0.25 to over 20 μg/mL (1.5 to 12 μM), which is more than 10 times our measured in vitro Kd levels. Thus, LiaX binding to DAP occurs within physiologically relevant concentrations. LL-37 binding to LiaX was measured using microscale thermophoresis (MST) by quantifying changes in fluorescence emitted by NT-647-labeled LiaX (Fig. 3B). The Kd for LiaX was 8.26 ± 0.28 μM, and it was 10.6 ± 0.43 μM for the N terminus of LiaX (SI Appendix, Table S4). There is a modest sigmoidal character to the binding isotherms, suggesting cooperativity during binding (Fig. 3B). The binding affinity of the N terminus of LiaX to LL-37 was also verified by MST with a His tag-specific NT-674 dye labeling LiaX, yielding a Kd similar to full-length protein (5.268 ± 1.141 μM) (SI Appendix, Fig. S10). To determine if the binding of the N terminus of LiaX to AMPs is largely charge mediated, we tested an active form of dermcin (DCD-1L), a membrane-anion anionic AMP that is expressed in human eccrine sweat glands. DCD-1L has broad-spectrum antimicrobial activity, including against E. faecalis, with a mechanism of action predicted to be distinct from that of cationic AMPs (23, 24). We confirmed that DCD-1L (50 μg/mL) was active against OG1RF and S613 (SI Appendix, Fig. S11). However, in contrast to LL-37, the N-terminal domain of LiaX exhibited no detectable binding affinity to DCD-1L, even at high concentrations (1,000 μM) (SI Appendix, Fig. S10 and Table S4). To determine if a reduction in the cationic charge of LL-37 (+5.9) would impact binding, we tested neutral (substituting alanine and lysine for serine) and charge reversed (substituting some aspartate or arginine residues for lysine or glutamate, net charge –6.1) mutants of LL-37. The binding affinity of the N-terminal domain of LiaX to neutral LL-37 decreased by ~10-fold, with no binding observed in charge reversed LL-37 (SI Appendix, Fig. S10 and Table S4). These findings confirm that LiaX, specifically the N-terminal domain, binds DAP and cationic AMPs and that charge likely plays an important role in this interaction.

Protection by LiaX against DAP Is Dependent on a Viable LiaFSR System. Since LiaX binds DAP with high affinity, we postulated that releasing LiaX into the environment is a bacterial strategy to prevent DAP or AMPs from reaching the CM. Thus, we performed a modified spent media assay with MIC determinations that explored the ability of supernatants from DAP-R strains (with excess LiaX) to protect DAP-S strains against DAP. The DAP MIC of the DAP-S clinical isolate S613 increased when grown in the presence of supernatants derived from DAP-R R712 (Fig. 4A). Of note, R712 constitutively releases higher amounts of LiaX into the medium than S613 in both the presence and absence of DAP (SI Appendix, Fig. S6). Supernatants collected from R712 at late exponential phase yielded maximum protection to S613 relative to those harvested from earlier growth phases (SI Appendix, Fig. S12). This implies that higher extracellular accumulation of LiaX by late exponential phase correlates with increased protection. No change in the DAP MIC was observed when OG1RF was exposed to supernatants from OG1RFΔliaX (Fig. 4A), confirming that LiaX must be present in the environment for protection. The DAP MIC of DAP-S OG1RF increased in the presence of supernatants from DAP-R OG1RFΔliaX (which secretes the N-terminal domain of LiaX), indicating that the N terminus is sufficient to mediate protection (Fig. 4A). We then investigated if exogenous addition of purified LiaX or the N terminus alone can protect DAP-S strains. Addition of 50 nM N-terminal LiaX was enough to increase the DAP MIC of OG1RF and S613 over 8-fold (Fig. 4B). Full-length LiaX was less potent, with a 2- to 3-fold increase in the DAP MICs. Addition of a range of 1 to 100 nM N-terminal LiaX yielded a high level of protection to OG1RF in a concentration-dependent manner, with the full-length LiaX having a less potent effect even at 200 nM (Fig. 4C). Of note, LiaX and its N terminus at their functional concentrations had no impact on the growth of E. faecalis (SI Appendix, Fig. S13). Furthermore, the N terminus of LiaX (and to a lesser extent, full-length LiaX) protected OG1RF and S613 from LL-37-mediated killing (SI Appendix, Fig. S14) in a concentration-dependent manner (SI Appendix, Fig. S14B).

To assess if the LiaX-mediated protection against DAP was due to direct binding and titration of the antibiotic in the environment or alternatively, through activation of an enterococcal signaling network (i.e., LiaFSR), we determined the DAP MIC of DAP-S Staphylococcus aureus ATCC29213 (DAP MIC of 0.5 μg/mL) in the presence of supernatants from OG1RFΔliaX (Fig. 4D) and with the addition of exogenous LiaX or the N terminus of LiaX (Fig. 4B). The DAP MIC did not change under any condition, suggesting that the LiaX-mediated effect is specific to E. faecalis and that LiaX does not merely sequester DAP to prevent it from binding to the CM. Furthermore, we examined whether the LiaX-protective effect relied on an active LiaFSR system by testing changes on DAP MICs of OG1RF lacking liaR using supernatants from OG1RFΔliaX and after addition of exogenous LiaX or N-terminal LiaX. The supernatant of OG1RFΔliaX (Fig. 4D) or the addition of LiaX or N-terminal LiaX (Fig. 4B) did not protect OG1RFΔliaX against DAP. Moreover, LiaX and the N terminus of LiaX did not protect OG1RFΔliaX from LL-37-mediated killing (SI Appendix, Fig. S14A). Thus, we conclude that the mechanism of

![Fig. 3. LiaX and the N terminus of LiaX can bind antimicrobial molecules with high affinity.](https://www.pnas.org/cgi/doi/10.1073/pnas.1916037116 Khan et al.)
LiaX-mediated protection against antimicrobials requires an intact LiaFSR system.

**LiaX-Mediated Activation of LiaFSR Requires the Presence of DAP.** To determine if the basis of the mechanism of extracellular protection mediated by LiaX or the N terminus of LiaX was a specific response dependent on the presence of cell envelope stress or a stochastic phenomenon due to production of high levels of LiaX in the cellular environment, we assessed the transcriptional activation of liaFSR and liaXYZ in the presence of LiaX or its N-terminal domain by qRT-PCR. Fig. 4D shows that adding 50 nM purified LiaX or the N terminus of LiaX in the media of DAP-S OG1RF had no effect on the levels of transcription of the gene clusters relative to untreated controls. However, when DAP (at subinhibitory concentrations) and purified N terminus of LiaX were added, the liaFSR and liaXYZ operons were highly up-regulated, correlating with maximal activation of the LiaFSR stress response. This is consistent with our earlier studies (Fig. S4 and Table S4) that suggest that the majority of LiaX would be bound to DAP under these conditions. Most notably, LiaFSR activation with addition of the N terminus of LiaX plus DAP was significantly higher and more pronounced than that of treatment with DAP alone or DAP with full-length LiaX (Fig. 4D).

**LiaX Mediates Enhanced Virulence In Vivo Due to Resistance to Host Innate Immunity.** Inactivation of the LiaFSR system increases the ability of human innate immune cells to clear both E. faecalis and E. faecium (16). Our binding studies showed that LiaX binds LL-37 with high affinity (Fig. 4B and SI Appendix, Fig. S10), which led us to postulate that LiaX may play a role in E. faecalis pathogenesis and mediate resistance to other host-derived AMPs. We tested this hypothesis by using the C. elegans infection model (25). Both clinical- (Fig. 5A) and laboratory-derived (Fig. 5B) DAP-R strains displayed increased virulence as measured by nematode killing compared with their DAP-S parental strains. To determine if the enhanced virulence was mediated by increased resistance to AMPs produced by the innate immune system, we tested the ability of E. faecalis to infect nematodes lacking the p38 MAP kinase pathway (Δpmk-1), a key immune pathway that regulates important immune effectors, including CM disrupting AMPs (26). Consistent with our hypothesis, DAP-R strains had no virulence advantage over their DAP-S counterparts when infecting the Δpmk-1 worms (Fig. 5 C and D).

Since the N terminus of LiaX serves as a potent signaling molecule that can interact with AMPs, we hypothesized that supplementing the N terminus of LiaX into the media would modulate virulence of E. faecalis in the C. elegans model. Indeed, OG1RF and S613 were more virulent on addition of the N terminus of LiaX (Fig. 6) in a dose-dependent manner (SI Appendix, Fig. S15A). Addition of exogenous N terminus of LiaX did not, however, alter the virulence of OG1RF ΔliaR and TM ΔliaR (Fig. 6 and SI Appendix, Fig. S15B). Of note, addition of the N terminus alone was not toxic to the worms (SI Appendix, Fig. S15C). These results suggest that the N terminus of LiaX contributes to increased virulence in vivo by mediating E. faecalis protection against AMPs produced by the innate immune system.

**Discussion**

We have previously shown that the LiaFSR system regulates DAP and AMP resistance in enterococci (12, 15) by causing major changes in the cell envelope, including alterations of CM phospholipid content and architecture (11, 27). Here, we characterize a protein LiaX, a previously unknown mediator of the CM adaptive stress response in OG1RF. Data are shown from 3 experiments with 3 biological replicates each. ns, not significant; WT, wild type. **P < 0.001.
response to antibiotics and AMPs, as a main modulator of the LiaFSR system. Each domain of LiaX serves a different function. In a DAP-S strain, LiaX is surface exposed, and the C-terminal domain inhibits the LiaFSR system, potentially through the membrane-embedded regulators, LiaF and LiaS (Fig. 7A). Thus, DAP-R can evolve via 2 pathways. A C-terminal domain truncation of liaX (17) releases the inhibition that this domain has on LiaFSR, leading to CM remodeling and DAP-R. Additionally, mutations in liaFSR that occur in clinical settings (11, 13) can activate the system and lead to overexpression and extracellular release of LiaX. When LiaX is overexpressed, both the full-length and N-terminal domains are detected in the extracellular environment. We hypothesize that these 2 forms of LiaX play distinct roles, where the N terminus of LiaX specifically serves as a sentinel to sense and bind antimicrobials to activate the envelope stress response, potentially signaling through other transmembrane components of the LiaFSR system (Fig. 7B). In the absence of environmental stressors, the full-length LiaX is able to again bind the membrane-associated regulators, down-regulating gene expression. This would turn “OFF” the stress response in the absence of antimicrobials, allowing anionic phospholipids to localize to the division septum. Both pathways (namely, a C-terminal truncation or secretion of LiaX) lead to CM remodeling, with anionic phospholipids diverted from the septum causing DAP to bind away from its septal targets (11). Each of these strategies converges in clinical resistance, with elimination of the bactericidal activity of DAP and AMPs and also, resulting in increased virulence in vivo. Interestingly, despite lacking transmembrane domains, LPXTG motifs, or a classical secretion signal, LiaX is surface exposed and detected in CM fractions. We thus speculate that other genes in the operon (liaYZ) that encode transmembrane proteins are likely to contribute to the signaling cascade and function as interacting partners of LiaX. Further study is needed to elucidate how LiaX is transferred to the extracellular environment.

CM remodeling can have important consequences on enterococcal virulence in vivo (12). We have previously shown that DAP-R enterococci have an increased ability to survive inside human phagocytes (16). Here, we show that LiaX and specifically, the N-terminal domain bind LL-37 with high affinity. LiaX mediates increased virulence in C. elegans, and DAP-R strains have no virulence advantage over DAP-S strains when the innate immune system, responsible for the production of AMPs, is not functional. The N terminus of LiaX alone is able to increase the virulence of E. faecalis (laboratory and clinical isolates) in C. elegans. Thus, when mutations in liaFSR lead to the extracellular release of the N-terminal domain, AMPs produced by the innate immune system are sensed by LiaX, triggering the CM adaptive response (Fig. 7B) and enhancing virulence. Furthermore, LiaFSR mediates bacterial resistance to environmental stressors in natural niches (28). Mutations in liaR were shown to enhance VRE gastrointestinal (GI) tract colonization in a mouse model by promoting resistance to lithocholic acid, a bile acid found exclusively in the GI tract (29). Thus, in clinical settings, changes in LiaX could favor enterococcal colonization, resistance against metabolic stressors, and the host innate immunity. Such an adaptive response could also serve as a major selective pressure for developing resistance to DAP during the colonization/
infection process, jeopardizing the therapeutic efficacy of this antibiotic (30). Moreover, our findings on LiaX mediating resistance to the innate immunity could also explain the fact that some clinical enterococcal strains exhibit resistance to DAP, even in the absence of actual exposure to the antibiotic (5).

In summary, our results highlight the major role of a single protein in orchestrating the cell envelope stress response to antibiotics, influencing membrane adaptation and virulence. This critical function suggests that LiaX could be a potential target for developing antiadaptation molecules that could potentially restore the effectiveness of widely used antimicrobials and enhance innate immunity mechanisms to clear multidrug-resistant infecting bacteria.

Materials and Methods

Microbiology and Molecular Biology Methods. Strains and primers used in this study are listed in SI Appendix, Table S1 and S2, respectively. Standard culture media were used for routine strain and plasmid propagation as described in SI Appendix, SI Materials and Methods. The liaX mutants (full deletion, C-terminal truncation) were constructed as described to create an in-frame, nonpolar deletion using the Phes* counterselection system (31, 32) and complemented with pAT392 (33). Recombinant LiaX and the N-terminal domain with a His tag and SUMO tag approach were expressed in pET-Duet vector in Escherichia coli BL-21 and purified using a nickel column method (18). For immunoblotting, cell wall extracts were prepared from midexponential-phase cultures using the lysosome treatment method (34) with membranes isolated by ultracentrifugation at 100,000 × g for 1 h, and supernatants were isolated from the same cultures by 10% trichloroacetic acid (TCA) precipitation. A spent medium assay was used to determine MICs on DAP-S strains in the presence of their own or DAP-R strains spent medium collected at early, mid, and late exponential phases following clinical and laboratory standards institute guidelines. Broth microdilution was performed to determine MICs in the presence of purified LiaX. MICs were read 24 h postinoculation. AMP killing assays were performed in RPMI 1640 media + 5% Luria–Bertani broth for 2 h with a 10^3 inocula or 10 mM sodium phosphate buffer + 100 mM NaCl for 4 h with a 10^6 inocula for 50 μg/mL LL-37 or dermcidin (DCD-1L), respectively (12, 23). Colony-forming unit counts compared survival with the peptide relative to the untreated control. Statistics were calculated between 3 independent experiments with 3 biological replicates with 1-way ANOVA (P < 0.01).

Transcriptional Profiling and Quantitative Real-Time PCR. Transcriptome shotgun sequencing comparing DAP-R and DAP-S strains was performed on midexponential-phase cultures in 3 biological replicates with standard RNA extraction. RNA was quality controlled by bioanalyzer and Nanodrop prior to subjecting to RNA sequencing analysis. DNA Illumina libraries were then sequenced on the HiSeq2000. Complete genome of R712 was sequenced for reference with PacBio. Reads per kilobase million values, read mapping, and alignment (35) for differential expression analysis (36) were performed using a negative binomial fitted model with a Fischer’s exact test. qRT-PCR analysis conducted in 3 experiments and 3 biological replicates was performed on exponential-phase cultures grown in the presence or absence of DAP in some instances, as indicated, followed by standard RNA extraction and subsequently, by complementary DNA (cDNA) synthesis and evaluation of gene expression with 5 ng cDNA using SYBR Green. The gyrB and gdhA housekeeping genes were used for normalization. Differential gene expression was analyzed with 1-way ANOVA (P < 0.01).

Lipid Extraction and 2-Dimensional Thin-Layer Chromatography (TLC). Strains were grown to midexponential phase in tryptic soy broth, extracted using a modified Bligh and Dyer method prior to spotting on HPTLC Silica Gel 60 Plates (Millipore), and separated by 2-dimensional TLC (37, 38). Spots developed in CuSO4 + 8% phosphoric acid heated at 180 °C were identified based on previous studies and lipid standards (Avanti Polar Lipids). Relative quantification was performed through ImageJ, with results displayed as an average of 4 biological replicates and statistics calculated by a 1-way ANOVA (P < 0.05).

Protein Localization Analysis. Affinity-purified polyclonal anti-rat antibodies raised in house were used for all protein detection assays. Surface exposure and extracellular LiaX were analyzed with an ELISA (39) using an antibody to the N terminus of LiaX performed on exponentially growing whole cells or filter-sterilized and TCA-precipitated supernatants, respectively. Data were compiled from 3 independent experiments with 24 technical replicates for each strain. Two-tailed unpaired t test was used for statistics comparing DAP-R and DAP-S pairs in addition to all strains relative to OGR1RFαliaX as negative control. Cell wall extracts and supernatants were normalized using the bicinchoninic acid assay prior to immunoblotting with the Thermo Fischer BLT2 and IBlDx Flex systems. Antibodies to RNA polymerase β-subunit and EbpA, a pilin subunit embedded in the enterococcal cell wall, were used as a cell wall extract quality control.

Microscopy Methods. Anionic phospholipids were visualized by staining with 10-N-nonyl acridine orange and fluorescence microscopy as described before (11) with Olympus 1 × 71 or Keyence BX-700 using a cumulative of 3 experiments with 3 biological replicates. Fluorescence intensity was quantified by averaging the relative fluorescence units across cell length for 25 to 50 cells per strain on ImageJ. Immunoglobulin labeling and transmission electron microscopy were performed, as described previously (40), with antibody to...
the N terminus of LiaX with anti-rat Immunoglobulin G antibody conjugated to 18-nm gold nanoparticles. Samples with 3 biological replicates were imaged using a JEOL JEM 1400 120-kV microscope.

LiaX and Antimicrobial Molecule Binding Assays. Determination of LiaX binding affinity to DAP, LL-37 variants, and dermicin was performed with fluorescence spectroscopy or MST (41). For DAP, the excitation was at 365 nm with emission at 435 nm. For LL-37, the excitation was at 280 nm using 25% light-emitting diode with LiaX NT-647 labeled (in 0.1 M phosphate buffer, pH 7.4, 0.0027 M potassium chloride, 0.137 M NaCl, and 1 mM Tris [2-carboxyethyl] Phosphine with or without 5% dimethyl sulfoxide) and tested in the presence of increasing LL-37 variant or dermicin concentrations. Data analysis was performed with Nanotemper software v1.5.41.

C. elegans Infection Model. In order to assess virulence of DAP-R strains relative to their susceptible counterparts, we used a C. elegans infection model as previously described (25). Briefly, 60 to 90 synchronized young adult nematodes [wild type or pmk-1(okm25)] (26) were infected with E. faecalis strains on brain heart infusion (BHI) agar containing gentamicin (10 μg/mL). For assays with exogenous LiaX, a modified liquid killing assay was performed (42) by transferring worms to 20% BHI broth, 80% M9W buffer, and the N terminus of LiaX (0.1 to 500 nM). The plates were incubated at 25 °C, and worm death was scored daily. Kaplan–Meier log rank analysis was used to compare survival curves pairwise (P < 0.05) calculated from 3 independent experiments.

Data Availability Statement. All datasets, including genome sequences for S613 and R712, have been made publicly available. Genomes are deposited to the National Center for Biotechnology Information database, https://www.ncbi.nlm.nih.gov/genome, under accession numbers CP036247–CP036249. SI Appendix includes detailed experimental protocols and references for the original protocols that were modified or adapted. All unique materials (e.g., genetically altered bacterial strains, plasmids, antibodies) will be made available from the corresponding author on request.

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