Mechanisms of Daptomycin Resistance and the Seesaw Effect in Multi-Drug Resistant Enterococci
[redacted] *(Microbiology and Infectious Diseases, On-topic Exam)*
Advisor: [redacted].

SPECIFIC AIMS

Enterococci are one of the most problematic hospital-associated pathogens due to resistance to most antibiotics used in clinical practice. The CDC conservatively estimates that vancomycin-resistant enterococci cause 20,000 infections and 1,300 deaths per year in the US. Daptomycin (DAP), a lipopeptide antibiotic, which acts by disrupting bacterial cell membrane integrity, is a key drug of "last resort" for the treatment of multi-drug resistant (MDR) enterococcal infections. However, increasing clinical resistance to DAP is a daunting challenge. The three-component regulatory system, LiaFSR, orchestrates the cell envelope (CE) stress response to antibiotics and mediates DAP resistance (DAP-R) through CM remodeling in *E. faecalis* (*Efs)* and *E. faecium (Efm)*. In *Efs,* LiaR (the response regulator) modulates DAP-R by rearranging anionic phospholipids. Transcriptional profiling identified a gene cluster, *liaXYZ,* encoding three novel proteins, as the main targets of LiaR.

My long-term goal is understanding LiaFSR modulated mechanisms of antibiotic resistance and bacterial adaptation that could be exploited for novel therapeutics. The objective of my project is the characterization of LiaX**,** a novel, surface exposed, liaFSR effector, and study its role in DAP-R and the "seesaw" effect (β-lactam hypersusceptibility observed in parallel with DAP resistance and vice-versa). Our preliminary work indicates that the C-terminal (Ct) of LiaX alters DAP-R by regulating CM remodeling, and the N-terminal (Nt) is involved in the "seesaw" effect. **The central hypothesis is that LiaX protects the CM from antibiotic attack by, *i)* activating the LiaFSR system, *ii)* regulating the transmembrane proteins, LiaYZ, that directly mediate phospholipid remodeling, and *ii)* interacting with key penicillin-binding proteins (PBPs) through the Nt thereby altering their localization and susceptibility to β-lactams.** The hypothesis will be tested with these aims:

Specific Aim 1: Characterize the localization of LiaX in *E. faecalis* as it pertains to the CE stress response to antimicrobial peptides. *I hypothesize that LiaX alters its localization in response to antibiotic stress or upon the development of DAP-R, that activates the LiaFSR mediated stress response.* I will perform cell fractionation of DAP sensitive (DAP-S) and DAP-R strains with or without sub-minimum inhibitory concentration (MIC) DAP exposure; and observe localization and quantify protein levels of LiaX with ELISAs, western blots and immunogold labeling with transmission electron microscopy (TEM). My preliminary data shows that LiaX is highly secreted in DAP-R strains and that spent media of DAP-R strains can protect DAP-S strains from antibiotic stress. I will determine if this protection is mediated by LiaX through activation of *liaR*. I will also study LiaXs role in antimicrobial peptide resistance *in vitro* and *in vivo* with a *Caenorhabditis elegans* infection model.

Specific Aim 2: Dissect the role of LiaX in regulating DAP resistance through protein interactions. *I hypothesize that LiaX interacts with LiaYZ by negatively regulating their function through the Ct in the absence of antibiotic stress.* While a deletion of *liaX* or its Ct leads to DAP-R and CM remodeling, a double deletion of *liaYZ* in these backgrounds restores susceptibility and abolishes phospholipid rearrangement. I hypothesize that a secondary activation signal for CM remodeling is through LiaX mediated protein interactions. I will study the LiaX-LiaYZ interaction with the bacterial two-hybrid system and far western blotting analysis. The LiaX "interactome" in DAP-S versus DAP-R strains will be characterized with proximity-dependent biotin identification using a biotin ligase fused to full length LiaX, the Ct or Nt LiaX fragment that results in DAP-R. Mass spectrometry of streptavidin affinity purified proteins will be used to identify biotinylated proteins.

Specific Aim 3: Elucidate the role of LiaX in mediating the "Seesaw" effect through interactions with PBP5. Previous pull down assays and *in vivo* tandem affinity purifications show that the Nt of LiaX interacts with PBP5 of *Efm* and *Efs,* mediating β-lactam resistance. This interaction is likely critical for proper localization of this key PBP to sites of active septal peptidoglycan (PG) synthesis. *I hypothesize that when the CE stress response is activated by DAP, the LiaX-PBP5 interaction is disrupted, leading to mislocalization of PBP5 and increased access of β-lactams to the enzyme.* To observe colocalization of LiaX and PBP5, I will use thin-section TEM, labeling α-LiaX and α-PBP5 antibodies with different sized gold nanoparticles, and fluorescence microscopy using mCherry tagged PBP5 and GFP tagged LiaX. I will study PBP5 mislocalization and aberrant PG synthesis in DAP-R strains with fluorescence microscopy using mCherry-PBP5, FL-vancomycin and fluorescent D-amino acids. The activity of β-lactams on DAP-R strains will be evaluated by, *i)* determining MICs of a range of β-lactams, cephalosporins and carbapenems, and *ii)* monitoring the degree of β-lactam binding to each PBP with fluorescent bocillin labeling of PBPs and whole cells.

We postulate that results from the above aims will place LiaX at the fulcrum of CM and CW homeostasis regulation in enterococci. Characterization of LiaX will provide insights into novel mechanisms of antibiotic resistance and may expose it as a target for future therapeutic interventions against MDR enterococci.

A. Significance

Antibiotic resistance has been declared as one of the biggest global health threats of the 21st century by the United Nations. Every year nosocomial infections caused by multi-drug resistant (MDR) bacteria cause 23,000 deaths in the US alone and an estimated $25 billion in economic costs[1]. By 2050, this is estimated to rise to 10 million deaths a year and $100 trillion in economic burden[2]. Among MDR organisms, vancomycin-resistant enterococci (VRE) are major recalcitrant, nosocomial pathogens[1]. Genetic plasticity makes *Enterococcus faecalis (Efs)* and *E. faecium (Efm)* intrinsically resistant to many antibiotics [3]. Linezolid is the only FDA-approved drug for MDR *Efm* but its toxicity and bacteriostatic nature limit its use. Daptomycin (DAP), a lipopeptide antibiotic that targets cell membranes, is used off-label for the treatment of severe MDR enterococcal infections[4, 5]. However, **emerging rates of clinical DAP resistance in VRE leave few to no treatment options, with a dire need for novel therapies**[6, 7].

LiaFSR, a three-component system (TCS), regulates DAP and antimicrobial peptide (AMP) resistance in MDR *Efs* and *Efm*[8-12]. Transcriptional profiling identified a gene cluster, *liaXYZ*, regulated by *liaR.* **The focus of this study** is on characterization of one of the novel proteins, **LiaX**, to understand its role in antibiotic resistance. Experimental evolution of a DAP susceptible (DAP-S) clinical isolate showed that all DAP resistant (DAP-R) trajectories had initial mutations in *liaR, liaF* or *liaX*[13]. While mutations in *liaF* and *liaR* relied on alterations in other genes as well, a C-terminal (Ct) truncation of *liaX* (**Fig 1**) alone was sufficient for high level DAP-R. Thus, LiaX is a major modulator of stress adaptation. DAP-R is often observed with β-lactam resensitization, a phenomenon called the "seesaw" effect that is exploited for combination therapies against MDR MRSA, *Efs* and *Efm*[14-17]. The mechanism for the seesaw effect in enterococci, however, is unknown. LiaX-mediated DAP-R is seen in parallel with β-lactam resensitization (**Table 1**). Thus, **I hypothesize** that LiaX is a multifunctional protein, that regulates DAP-R through cell membrane (CM) remodeling and modulates the seesaw effect through interactions with key penicillin binding proteins (PBPs) in *Efs* (**Fig 1**). **My ultimate goal** is to understand the molecular mechanisms by which LiaX modulates the cell envelope (CE) stress response, that can reveal insights on the multifaceted approaches bacteria utilize to evolve and adapt to antibiotic stress. The proposed research will characterize the role of a novel protein, that is at the *fulcrum of CM adaptation and cell wall (CW) alterations*, and expose it as a therapeutic target for the treatment of MDR enterococcal infections.

B. Innovation

Cell membrane remodeling, extracellular protection and cell wall homeostasis regulation by a single protein is an unprecedented antibiotic resistance mechanism; and, anti-adaptation is a novel therapeutic strategy. LiaX is highly secreted in DAP-R strains and plays a protective role for the cell during antibiotic attack. This situates a single protein at the intersection of three distinct antibiotic resistance strategies. Characterizing LiaX will *i)* provide novel insights into CM and CW homeostasis regulation in bacteria, *ii)* identify a previously unknown mechanistic basis for the seesaw effect in enterococci and *iii)* dissect the mechanism by which extracellular stress sensing and signaling leads to CE architectural changes. Disabling the LiaFSR system in MDR pathogens by targeting LiaX can reverse susceptibility to CE targeting antibiotics, and limit the cells ability to adapt, which can augment host immune system-mediated infection clearance[11]. The ability of DAP-R cells to secrete a protein that allows DAP-S cells to be protected from antibiotic attack could also play an integral role in heterogeneous resistance *in vivo* where the resistant phenotype of a small subset of cells can allot protection to the larger population[18-20]. The techniques and mentorship in this project bring together genetics, biochemistry, proteomics, microbial physiology, animal models, and clinical medicine that allows direct bench-to-patient translation of my findings on LiaX to antimicrobial target discovery and development.

C. Approach

C.1 Preliminary Data

LiaX, an effector of the LiaFSR stress response system, mediates DAP resistance. The LiaFSR system consisting of the histidine kinase sensor liaS, negative regulator liaF, and response regulator liaR, is well-conserved across *Firmicutes* and orchestrates the CE stress response to antibiotics [21-23]. It mediates DAP-R in clinical and laboratory MDR enterococci, through CM remodeling[8, 10, 24]. *LiaXYZ*, a three-gene cluster is regulated by LiaR[25]. Mutations in genes encoding these effectors have been implicated in DAP-R clinical isolates[24, 26]. LiaX is a 533 amino acid (AA) protein in *Efs* (59% homology to *Efm*), with α-helices at the N-terminal (Nt) domain and β-sheets at the C-terminal (Ct) domain (**Fig 1**). The *PheS** counterselection system[27] was used to manipulate DAP-S *Efs* strain, OG. The deletion (OGΔ*liaX*) and Ct truncation of *liaX* (OG-*liaX*^{NT}, **Fig 1**), leads to high level DAP-R (**Table 1**). DAP-R is associated with a diversion of anionic phospholipids (APLs) away from the division septum (**Fig 2**). Trans complementations of the strains reversed these phenotypes. Thus, LiaX, and specifically its Ct, mediates

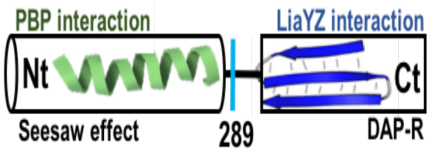

Figure 1: LiaX domains schematic. Nt interacts with PBP5 in the CW and Ct likely interacts with LiaYZ. Stop codon at AA289 leads to Ct truncation.

DAP-R and CM remodeling through negative regulation. The CE stress response is regulated by multiple circuits, and pathways[28] and LiaX could be a connector protein that modulates DAP-R in addition to liaFSR signaling.

LiaX regulates CM remodeling through LiaYZ. APL redistribution protects the division septum from DAP binding, averting cell death[10]. LiaY is a 107AA transmembrane protein with a PspC domain, that maintains CM integrity during phage attack in *E. coli*[29]. LiaZ is a 118 AA transmembrane protein with homology to bacterial holins that alter CW homeostasis[30]. A double deletion of *liaYZ* in OG-*liaX*^{NT} leads to DAP hypersusceptibility and abolishes APL redistribution (**Fig 2**), while single deletions are not sufficient. Thus, the Ct of LiaX likely regulates DAP-R, through negative regulation of direct CM remodeling by LiaYZ. I hypothesize that direct interactions between these proteins are the key to this regulation.

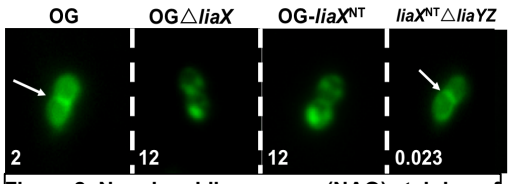

Figure 2: Nonyl acridine orange (NAO) staining of anionic phospholipids. White arrows mark septal localization. DAP MICs of strains on bottom left.

Type	Strain	DAP MIC	Characteristics
Laboratory-S	OG	2	Referred to as OG1RF in literature- Ceftriaxone (CRO) MIC= 32ug/ml
Laboratory-R	OG△*liaX*	12	Non-polar deletion- CRO MIC= 8ug/ml
Laboratory-R	OG-*liaX*^{NT}	12	C-terminal truncation, with LiaX N-terminal only- CRO MIC= 8ug/ml
Laboratory-S	OG△*liaX::liaX*	4	Complementation of liaX in trans- CRO MIC= 32ug/ml
Laboratory-S	OG-*liaX*^{NT}△*liaYZ*	.023	Nonpolar deletion of liaYZ in OG-liaX^{NT}
Clinical-S	S613	1	Bloodstream strain before DAP therapy [8]
Clinical-R	R712	12	Derivative of S613 isolated after DAP therapy (with liaF, cls, gdpD mutations) [8]
Clinical derivative-R	TM	8	S613 derivative with DAP-R allelic replacements [12]
Clinical derivative-S	TM△*liaR*	.094	TM with nonpolar liaR deletion [12]

Table 1: Strains used in this study. S sensitive, R resistant, MICs in ug/ml, DAP breakpoint is 4ug/ml.

C.1.3 LiaX is highly surface exposed and secreted in DAP-R strains. Whole-cell ELISAs[31]and Western Blot (WB) analysis with antibodies (Ab) against the Nt of LiaX revealed that surface exposure of LiaX is greatly increased in DAP-R strains (**Fig 3A**) compared to their DAP-S counterparts (**Table 1,** R and S labeled strains); moreover, LiaX and the Nt alone were detected at high levels in the supernatants of DAP-R strains (**Fig 3B)**. This is corroborated by immunogold-labeled LiaX visualized by TEM[32], where the DAP-R clinical strain shows more LiaX in the extracellular milieu than its DAP-S counterpart (**Fig 3C,D**). No LiaX was detected around OG△*liaX* (not shown). WB analysis indicated the presence of high molecular weight (HMW) species in DAP-R strains harboring the full length *liaX*, indicating that the full-length protein is possibly able to oligomerize. Thus, LiaX localization and conformation seems to be of paramount importance in DAP-R enterocci, regardless of origin.

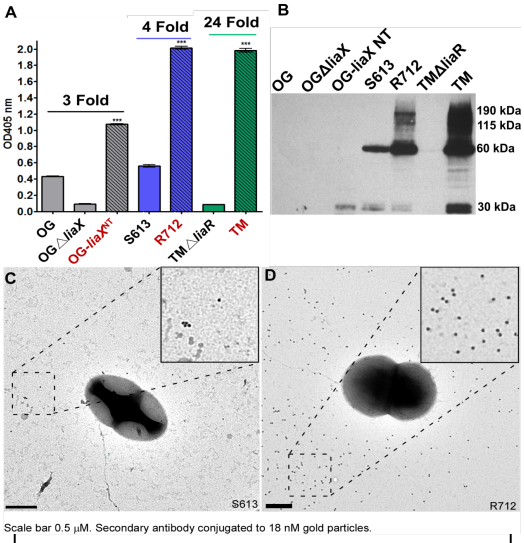

Figure 3: LiaX surface exposure and secretion. A) Whole-cell ELISAs with αNt-LiaX Abs, Y-axis absorbance at 405nm, DAP-R strains in red. B) Supernatant western blot. 60kDa full length LiaX, 30kDa Nt-LiaX detected. C) S613 and D) R712 immunogold labeling and TEM with αNt-LiaX Abs.

C.1.4 Extracellular LiaX protects DAP-S strain from antibiotic attack. Binding assays revealed that the Nt of LiaX binds DAP and the human AMP, LL-37, with K_D=0.05 and 8.3μM, respectively. Thus, extracellular LiaX could play a protective role in DAP-R. I developed a modified spent media assay, used to study quorum sensing molecules[33, 34], with broth macrodilutions[35] to assess if filter sterilized media from a DAP-R strain can protect a DAP-S from antibiotic death. While the DAP MIC of a DAP-S strain with fresh or its own spent media was 3μg/ml, addition of media from a DAP-R clinical isolate, R712, increased the MIC to 14μg/ml, making it highly resistant. Addition of OG-*liaX*^{NT} media to OG yielded the same results, indicating that the Nt of LiaX is likely responsible for the protective effect. The exact mechanism of protection, however, is unknown.

C.1.5 LiaX binds PBP5 and modulates the seesaw effect, altering DAP-R and β-lactam susceptibility. Enterococci are intrinsically resistant to many cephalosporins, mediated by the low-affinity- PBP5[3]. Deletion of *liaX* or the Ct truncation in OG led to DAP-R, and resulted in a 4-fold decrease in ceftriaxone (CRO) susceptibility (**Table 1**). Thus, LiaX likely regulates the seesaw effect in enterococci. Tandem affinity purification of PBP5 in *Efm* found LiaX to be tightly bound to PBP5 in a β-lactam resistant strain[36]. This was tested in *Efs* using a pull-down with column affinity purification that showed LiaX, with its Nt alone, can interact with PBP5 (**Fig 4**). I then verified that PBP5 and LiaX interact with their Nt domains *in vivo* in *E. coli* using the bacterial two hybrid system (BACTH)[37], with positive interaction indicated by β-galactosidase production (**Fig 5C, D**). In DAP-R *S. aureus,* β-lactam susceptibility is associated with mislocalization of PBP2 and aberrant peptidoglycan (PG)

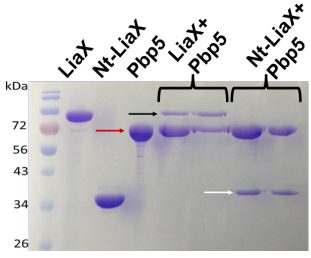

Figure 4: Pull down assay with LiaX or Nt of LiaX with PBP5 from DAP-S *Efs* S613.

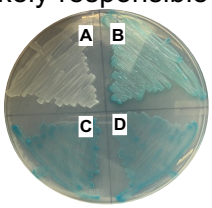

Figure 5: Bacterial two-hybrid testing PBP5-LiaX interaction. A) Negative control B) Positive control, interacting leucine zippers. C, D) Reporter strain showing positive interaction of LiaX-T25 and PBP5-T18 indicated by β-galactosidase activity (blue).

synthesis, facilitated by protein interactions with a chaperone, PsrA[38]. We monitored PG synthesis with fluorescent D-alanine (NADA) that is incorporated into sites of nascent CW synthesis[39]. DAP-S S613, showed normal septal and side wall synthesis while the DAP-R R712 showed increased peripheral PG and no side wall synthesis (**Fig 6**). I hypothesize that PBP5 mislocalization, in the absence of the LiaX-PBP5 interaction, makes PBP5 more accessible to β-lactams which is the possible mechanism underlying the seesaw effect.

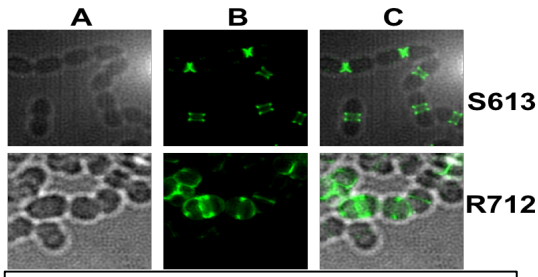

Figure 6: NADA (NBD-amino-D-alanine) staining. A) Phase contrast. B) Fluorescence C) Overlay

C.2 Research Plan

Mechanistic Model. Based on published and preliminary data, we have a proposed model of the LiaFSR mediated response to antibiotics. My project aims to explore LiaX's role in this model. In the absence of antibiotics (**Fig 7A**), the system is kept in the "OFF" state by the negative regulator, LiaF[40, 41], and possibly the Ct of LiaX as well (**Table 1, Fig 2**). The Nt of LiaX is likely bound to PBP5. Under antibiotic stress (**Fig 7B**), the system is activated by LiaR[25, 42]. High surface exposure and/or secretion of LiaX likely: ***i)*** triggers CM remodeling since the Ct no longer inhibits LiaYZ, and ***ii)*** maintains a positive feedback loop as LiaX senses antibiotic stress and keeps the system "ON". Conformational changes due to increased protein levels or mutations in *liaX* likely disrupt the LiaX-PBP5 interaction, leading to PBP5 mislocalization and increased susceptibility to β-lactams. Thus, CM remodeling can be regulated by liaFSR signaling or by LiaX directly. As indicated (**Fig 7**), *Aim 1 studies LiaX localization and function in AMP resistance, Aim 2 dissects the LiaX "interactome", and Aim 3 studies the mechanism behind the seesaw effect.* LiaX emerges as a major modulator of DAP-R, indicating that a complex circuit beyond TCS' regulates the CE stress response. My alternative hypotheses that will be tested if expected results are not obtained are that LiaX inhibits the system through interactions with LiaF or LiaS, modulating the ultimate phosphorylation of LiaR[21, 43]; and that LiaX mislocalizes other PBPs or allosterically regulates PBP5, altering its conformation and susceptibility of the active site to β-lactams as has been proposed for the homolog PBP2a in *S. aureus*[44].

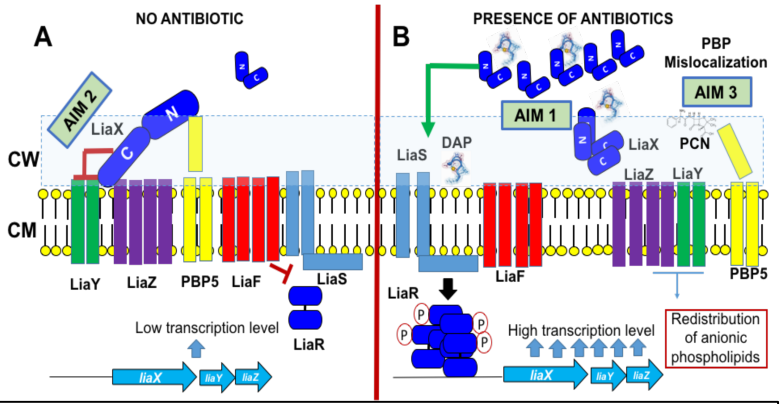

Figure 7: Proposed model of the CE stress response. A) "OFF" state B) "ON" state. Cell Membrane (CM), Cell Wall (CW), Daptomycin (DAP), Penicillin (PCN).

C.2.1. Aim 1: Characterize the localization of LiaX in *Efs* as it pertains to the CE stress response to AMPs.

Sub-Aim 1A. Evaluate LiaX protein levels and localization under DAP stress and in DAP-R strains.

Rationale and Hypothesis: RNA Seq and qRT-PCR analysis showed a 6-fold upregulation of *liaXYZ* in a DAP-R clinical *Efs* strain compared to a DAP-S strain. LiaX is surface exposed and secreted in higher amounts in DAP-R strains, of all origins, compared to their DAP-S counterparts (**Fig 3**). HMW species were detected in the supernatant of DAP-R strains, indicating possible oligomerization of LiaX (**Fig 3B**). Thus, LiaX protein levels and localization are of functional importance in DAP-R strains. *I hypothesize that LiaX undergoes an alteration in localization under DAP stress and upon the development of resistance.* This alteration can be due to liaFSR-mediated activation and increase in *liaX* expression or due to mutations in *liaX* itself.

Experimental Approach

LiaX levels and localization under DAP stress and in DAP-R. DAP-R and DAP-S strain pairs (**Table 1**) will be grown to mid-log phase, normalized and fractionated into supernatant, mutanolysin extracted CW, ultracentrifugation separated CM and cytoplasmic fractions with defined protocols for *Efs*[45]. The fractions and whole-cell lysates will be subject to WB analysis[31] using α-Nt LiaX antibodies (Abs), with α-EbpA[46](pilin subunit) as a CW control and α-RNA Polymerase (β-subunit) as a cytoplasmic control. To observe changes under antibiotic stress, DAP-S strains normalized at mid-log phase will be exposed to DAP (0.5x MIC) for 15, 30 or 60 min before being subject to whole-cell ELISAs[47] or fractionation and WB analysis. Exact protein levels will be quantified on ImageJ by using a standard purified protein curve[48].

Determine LiaX Oligomerization in DAP-R strains. To determine if the HMW species detected in the supernatant fractions of DAP-R strains (**Fig 3B**) are LiaX oligomers, ammonium sulfate precipitated supernatants will be subjected to Blue native (BN) PAGE[49] using 4-16% Bis-Tris gels, visualized by Commassie staining and analyzed by LC/MS/MS[50] at the UTHealth MS core[51] with Swiss-Prot peptide analysis on Mascot software.

Expected results, pitfalls and alternative approaches: If LiaFSR is locked in the "ON" state in R712 and TM, I expect to detect high levels of LiaX in the lysates, CW and supernatant while low, basal levels of LiaX are

detected in their DAP-S counterparts, localizing to the CW only. LiaX is detected in the supernant of OG-*liaX*^{NT} and highly surface displayed (**Fig 3**) compared to OG but total protein levels will likely not differ drastically since there are no mutations in the *liaFSR* regulators. If the LiaFSR system is activated upon DAP exposure in DAP-S strains, higher levels of LiaX will be detected in the CW and supernatants in a time-dependent manner, but will be lower than those detected in DAP-R strains. This can identify the "threshold" of LiaX required for liaFSR-mediated DAP-R. While DAP induction conditions for LiaFSR activation have not been studied in *Efs,* stress studies in homologous systems have shown activation of *liaFSR* and its effectors within 15-30mins of induction; so, I expect *Efs* to behave similarly[5, 22, 52, 53]. If BN PAGE cannot resolve LiaX oligomers, I will use analytical size exclusion chromatography[54] to assess purified LiaX protein oligomeric states at increasing concentrations.

Sub-Aim 1B. Determine LiaXs role in resistance to AMPs *in vitro* and *in vivo*.

Rationale and Hypothesis: AMPs are ancient antibacterials in mammalian innate immunity and play integral roles in clearing infections and augmenting the effect of antibiotics[55]. Deletion of *liaR* in MDR *Efs* restored susceptibility to DAP and a broad spectrum of AMPs[11]. Deletion of *liaX* or its Ct led to DAP-R (**Table 1**) while binding assays showed that the Nt of LiaX binds the human AMP defensin, LL37, with a K_D of 8.3μM. Thus, *I hypothesize that LiaX mutants will be more resistant to AMPs and thus increase the likelihood of host death.*

Experimental Approach

Evaluation of *in vitro* activity of AMPs. The following AMPs with broad-spectrum gram-positive activity that cause CM perturbations will be tested: LL37, RP-1, hNP-1, and gramicidin D[11, 56]. AMPs that target CM-associated CW synthesis precursors (lipid II) will also be tested: human β-defensin 3, nisin, gallidermin, mersacidin, and friulimicin B[57]. 2-hour *in vitro* bactericidal microdilution assays[11, 58, 59] will be conducted in buffers appropriate for AMP solubility and activity to test the parental OG, the mutants OGΔ*liaX,* OG-*liaX*^{NT} and their respective complements. An inoculum of 10^3 colony-forming units (CFU) will be exposed to concentrations of AMPs that reduce the starting inocula of OG by 25, 50 and 75% over a 2h exposure. Relative survival rates will be determined by CFU counts on BHI agar of AMP-exposed versus AMP-unexposed cells.

The role of LiaX *in vivo*. To test if *liaX* mutations affect mortality through enhanced resistance to AMPs, the *Caenorhabditis elegans* model[60, 61] developed by Dr. Danielle Garsin will be used, to compare survival upon infection with OG, the mutants OGΔ*liaX,* OG-*liaX*^{NT} and their complements. 60-90 synchronized young adult nematodes will be infected with each strain on BHI agar with gentamycin, and incubated at 25°C with daily scoring of worm death. Kaplan-Meier log rank analysis ($p< 0.05$) will compare survival curves using uninfected worms feeding on *E.coli* OP50 as a negative control.

Expected results, pitfalls and alternative approaches: If LiaX regulates resistance to CE active agents, the *liaX* mutants will be more resistant to AMPs *in vitro* and more lethal *in vivo*. TM and TMΔ*liaR* from our previous study[11] can validate the *in vitro* and *in vivo* experiments. If AMP concentrations are not optimal for the assays, standard MIC broth microdilution assays[56] will be used. If differences in mortality are not distinct, other aspects of host infection will be assessed such as daily bacterial burden and localization of intact bacteria with TEM[60] or with fluorescence microscopy using a chromosomal *gfp* insertion [62, 63]. My alternative hypothesis is that *liaX* mutants are more resistant to DAP treatment that prolongs survival of nematodes infected with DAP-S *Efs*; which can be tested with a nematode killing assay in liquid media supplemented with DAP.

Sub-Aim 1C: Assess extracellular LiaXs protection of DAP-S strains from DAP stress.

Rationale and Hypothesis: LiaX is secreted in high amounts in DAP-R strains (**Fig 3B,D**), and binds DAP with a K_D of 0.5uM. The addition of spent media from a DAP-R strain increased the MIC of a DAP-S strain 4-fold, making it resistant to DAP killing. High concentration of LiaX in the media could mediate DAP protection. *I postulate that secreted LiaX is a secondary signal for LiaFSR activation, that protects the cell during the presence of extracellular stressors.*

Experimental Approach

Determine if LiaX protects cells by activating the CE stress response. To determine if LiaX in the R712 or OG-*liaX*^{NT} media increased the MIC of DAP-S strains, I will perform spent media assays with filter-sterilized media from OGΔ*liaX*. To assess if this protection is dependent on *liaR*, the spent media of all DAP-R strains will be tested on OGΔ*liaR* and TMΔ*liaR;* strains lacking the response regulator. To determine if LiaX upregulates *liaR* expression, I will use qRT-PCR to quantitate the fold-difference of *liaR* in OG and S613 on exposure to 0.5x MIC DAP in the presence or absence of DAP-R media. OGΔ*liaX* spent media, DAP alone, and DAP-R media alone will be negative controls. Housekeeping genes *gdh* (glucose-6-phosphate dehydrogenase) and *gyrB* will be references for normalization. Differences in transcript levels >2 fold will be considered significant ($p <0.05$). The above assays and qRT-PCR will also be done with the exogenous addition of recombinant protein LiaX, Nt-LiaX or Ct-LiaX purified with a HisTrap affinity column, and gel filtration chromatography[25].

Expected results, pitfalls and alternative approaches: If extracellular LiaX or the Nt specifically is protecting cells from DAP stress, addition of DAP-R media, purified LiaX or Nt of LiaX will increase DAP MICs while addition of OGΔ*liaX* spent media and purified Ct of LiaX will not. If the signaling is via *liaR,* DAP MICs of OGΔ*liaR* and

TMΔ*liaR* will not change. If LiaX or Nt-liaX in the media activates LiaR, qRT-PCR will show a greater upregulation of *liaR* in DAP-S strains with DAP-R spent media, purified LiaX or Nt of LiaX following DAP exposure compared to DAP alone, DAP and purified Ct-LiaX or no DAP. My alternative hypothesis is that LiaX is a connector protein mediating cross talk and altering the transcription of other CE stress response genes that can be tested with comparative RNA-Seq on DAP-S strains exposed to DAP plus DAP-R spent media or purified LiaX.

C.2.2 Aim 2: Dissect the role of LiaX in regulating DAP resistance through protein interactions.

Sub-Aim 2A: Characterize the LiaX interactome in DAP-R versus DAP-S strains.

Rationale and Hypothesis: Protein interactions are the key to regulation of the LiaFSR system in other Firmicutes[42, 64, 65]. Characterizing LiaX interactions will help us understand the mechanism of LiaFSR-mediated stress adaptation in enterococci and other bacteria (**Fig 7**). *I hypothesize that in a DAP-S strain, LiaX interacts with and inhibits components of the LiaFSR system and these interactions are disrupted in a DAP-R strain.*

Experimental Approach

Identify all LiaX interacting partners. Proximity-dependent biotinylation (BioID) screens for proximate proteins to a bait *in vivo* in a native environment and can capture stable or transient protein interactions[66]. Using a published protocol[67] I will make full-length, Nt and Ct fusions of *liaX* to *birA**, a promiscuous biotin ligase, in a constitutive expression vector[68]. The constructs will be transformed into OGΔ*liaX* to prevent interference from chromosomal *liaX*. Functionality will be verified by westerns and MICs, since full and Ct *liaX* should complement the DAP MIC (**Table 1**). Cells will be grown in BHI with biotin for 24 hours, washed, lysed in SDS buffer, and centrifuged. Supernatants will be incubated with streptavidin magnetic beads for 4h and proteins bound to the beads will be reduced, alkylated, and digested with trypsin proteases[69] before analysis with LC/MS/MS.

Expected results, pitfalls and alternative approaches: Potential interacting partners of LiaX can be identified[66] at all localization zones, leaving a web of interactions. The LiaX-BirA* fusion can reveal the WT "interactome" of LiaX while the Nt LiaX-BirA* fusion can reveal the "resistome". If the fusions are not functional, I will complement OGΔ*liaX in cis* for endogenous regulation[27]. My alternative approach is a tandem affinity purification (TAP) pull-down[36] assay to identify proteins in complex with LiaX or Nt of LiaX.

Sub-Aim 2B: Study the LiaX and LiaYZ interaction in the context of DAP-R.

Rational and Hypothesis: Deletion of *liaX* and the Ct alone leads to DAP-R and CM remodeling but this effect is abolished when *liaYZ* are also deleted (**Fig 2**). The localization and function of the homolog of *liaY* in *B. subtilis* is regulated by protein interactions with components of the *liaFSR* system[41, 65]. Thus, *I hypothesize that LiaX interacts with and inhibits LiaYZ through its Ct domain, and changes in LiaX conformation disrupt this interaction.*

Experimental Approach

Bacterial two hybrid and Far Western[70] verification of LiaX-YZ interaction. BACTH is based on the reconstitution of adenylate cyclase activity in *E. coli* by interacting proteins fused to catalytic domains of *cyaA* from *B. pertussis*[37]. I have used the system to verify LiaX-PBP5 interactions (**Fig 5**). Full-length, Ct or Nt of *liaX* will be fused to N or C terminus of T18 while *liaY*, *liaZ* or *liaYZ* will be fused to the N or C terminus of T25. The constructs will be cotransformed into the reporter strain, incubated at 30^{o} for 40h[71], and assayed for β-galactosidase activity. Empty vectors are negative controls and GCN4 leucine zipper motifs are positive controls[72]. For far westerns, I will clone *liaY, liaZ, liaYZ* or *gfp* alone (negative control) into pBAD/HisA, an over-expression vector in *E. coli* LMG194. Cell lysates will be separated by SDS-PAGE on 10-20% Tricine gels. PVDF membranes with proteins will be renatured with gradient reducing guanidine-HCl buffer and blocked before overnight incubation with purified LiaX, Nt LiaX or Ct LiaX in protein-binding buffer[73]. The membranes will be probed with αHis$_6$ Abs to confirm protein expression or with αLiaX Ab to identify interactions.

Determine relative *liaYZ* expression in liaFSR-activated and liaX-activated DAP-R strains. I propose that LiaX regulates LiaYZ through protein interactions, adding another level of regulation that is independent of *liaFSR* mediated transcriptional upregulation. To observe differences in *liaYZ* gene expression, I will perform qRT-PCR comparing relative transcript levels in S613 versus R712 (locked in the "ON" state due to a *liaF* mutation) with OG versus OGΔ*liaX* or OG-*liaX*^{NT}, which are DAP-R through the sole contribution of LiaX.

Expected results, pitfalls and alternative approaches: I will be able to confirm if the Ct of LiaX interacts with LiaY or Z more specifically. If the far westerns are ineffective, I will perform *in vivo* crosslinking of OG and S613 with WB and mass spectrometry analysis of membrane fractions. If LiaX regulates liaYZ through protein interactions only, there will be no significant upregulation of *liaYZ* in OGΔ*liaX* and OG-*liaX*^{NT} compared to OG. If not, my alternative hypothesis is that LiaX inhibits the system through interactions with, LiaF or LiaS, modulating the dephosphorylation or phosphorylation of LiaR[21, 74]; which can be tested with the above protein interaction experiments and *in vitro* phosphorylation [γ-^{32}P]ATP assays using purified proteins[25] and SDS-PAGE with autoradiography[75, 76] to assess if LiaX impacts the autophosphorylation of LiaS, phosphotransfer between LiaS-LiaR, or phosphatase activity of LiaF or LiaS.

C.2.3 Aim 3: Elucidate the role of LiaX in mediating the seesaw effect through its interaction with PBP5.

Sub-Aim 3A. Study PBP5-LiaX colocalization in DAP-S strains and PBP5 mislocalization in DAP-R strains

Rationale and Hypothesis: LiaX plays a vital role in the seesaw effect as a full or Ct deletion of *liaX* leads to DAP-R with increased CRO susceptibility (**Table 1**). LiaX interacts with PBP in *Efm*[36] and *Efs* (**Fig 4, 5**)**.** In *S. aureus*, β-lactam susceptibility in DAP-R strains is associated with PBP2 mislocalization and aberrant CW synthesis[38], and we observed a similar abnormal PG synthesis pattern in a DAP-R *Efs* clinical strain (**Fig 6**). *I postulate that LiaX interacts with PBP5 in DAP-S strains and that disruption of this interaction in DAP-R strains leads to PBP5 mislocalization.*

Experimental Approach

PBP5 and LiaX colocalization. I will use thin-sectioning TEM and immunogold labeling[77] where sections of OG, OGΔ*liaX,* OG-*liaX*^{NT} and their complements will first be labeled by α-LiaX Abs and 18nm nanoparticles followed by subsequent labeling by α-PBP5 Abs with 12nm particles. Colocalization will also be studied with fluorescence miscroscopy using GFP-*liaX* and mCherry-*pbp5* fusions cotransformed into OGΔ*liaX.* GFP-*liaX* will be transformed first to verify complementation of the DAP MIC, making the strain DAP-S (**Table 1**). WB analysis of membranes with α-mCherry Abs and septal localization will confirm functionality of mCherry-PBP5.

PBP5 and PG mislocalization with fluorescence. PBP5 mislocalization will be studied using the mChery-*pbp5* fusion transformed into OG, OGΔ*liaX,* OG-*liaX*^{NT} or their complement strains. Expression will be confirmed by WB analysis of membrane fractions with α-mCherry Abs and cells will be visualized by fluorescence microscopy. To observe delocalized PG insertion[78] associated with DAP-R, the above strains will be stained with Bodipy FL-vancomycin (VAN)[79] or with NADA-green[39] and visualized by fluorescence microscopy.

Expected results, pitfalls and alternative approaches: If LiaX and PBP5 colocalize, TEM should reveal coaggregation of the 18nm and 12 nm goldparticles and fluorescence microscopy should reveal septal overlaying signals from GFP-LiaX and mCherry-PBP5 in DAP-S strains. If PBP5 mislocalizes, mCherry-pbp5 will localize at the septum in DAP-S strains but produce a random, dispersed signal in DAP-R strains. FL-VAN and NADA-green labeling should show septal and side wall staining in DAP-S strains but abnormal staining in DAP-R strains, suggesting aberrant PG synthesis[38]. Since phospholipid alterations can mislocalize proteins[5], my alternative hypothesis is that APL redistribution is mislocalizing PBPs. This can be tested by NAO staining to visualize co-localization of APLs like cardiolipin with the mCherry-PBP5 fusion protein using fluorescence miscroscopy.

Sub-Aim 3B: Assess PBP5 levels and β-lactam binding to PBPs in DAP-R *liaX* mutants.

Rationale and Hypothesis: Cephalosporin resistance in *Efm* and *Efs* has been associated with overproduction of PBP5[80, 81]. In *S. aureus*, β-lactam susceptibility in DAP-R strains was reflected by increased β-lactam binding to HMW PBPs with a decrease in membrane bound PBP2a [38]. *I hypothesize that DAP-R liaX mutants either have decreased levels of membrane bound low-affinity PBP5 and/ or increased β-lactam binding to PBP5.*

Experimental Approach

Determine PBP5 protein levels. Membrane fractions and cell lysates of OG, OGΔ*liaX,* OG-*liaX*^{NT} and their complements will be subject to α-PBP5 WB analysis to assess total protein and membrane bound PBP5 levels.

Evauate β-lactam susceptibility of DAP-R mutants. DAP-R *S. aureus* strains are sensitive to penicillins and carbanpenems or cephalosporins that target PBP1 and PBP2[16]. To determine the extent of the seesaw effect in enterococci, MICs of OG, OGΔ*liaX,* OG-*liaX*^{NT} and their complement strains will be determined to a range of 1st to 5th generation cephalosporins, as well as relevant carbapenems[17, 38].

Assess β-lactam binding to PBPs. Membrane fractions of the above strains will be labeled with (Boc-Fl) for 30 min[81] before SDS-PAGE separation with individual PBPs visualized by a Typhoon 9400 scanner. Relative intensities will be quantified with ImageJ. For whole-cell visualization of β-lactam binding, cell pellets incubated with Boc-Fl for 10 min will be subject to fluorescence imaging[82].

Expected results, pitfalls and alternative approaches: If PBP5 in DAP-R strains has higher β-lactam binding affinity, Boc-FL should stain it and DAP-R cells more intensely. If PBP5 membrane anchoring is altered, levels of membrane-bound PBP5 will be lower in the DAP-R strains. Total PBP5 protein levels should not change since there are no mutations in *pbp5*; and if they do, I will assess transcriptional changes by measuring *pbp5* mRNA levels with RT-PCR. My alternative hypotheses are that β-lactam affinity of other PBPs is altered, and this can be visualized in the Boc-Fl labeled, SDS-PAGE analysis of PBPs; or that LiaX binds PBP5 at an allosteric site, and disruption of the interaction leads to increased active site access by β-lactams, like PBP2a in *S. aureus*[44]. The latter can be tested by deleting *pbp5* in OG, that will decrease the CRO MIC[83, 84]. Site-Directed mutagenesis can be used for random AA insertions in the predicted *pbp5* allosteric site[85] at the interface of lobes 1,2, and 3 to generate *in trans* complements for OGΔ*pbp5*. If LiaX interacts with PBP5 at the allosteric site, mutations in the region will disrupt the interaction and the mutated *pbp5* will not complement the CRO MIC in OGΔ*pbp5.*

Future Directions: This study can identify unknown interacting partners of LiaX, that can be anti-adaptation, antimicrobial targets and open avenues to study novel pathways that bridge CM and CW homeostasis regulation in bacteria. Understanding the role of LiaX in DAP-R and the seesaw effect in *Efs* will then lead to further studies that dissect its role in *Efm* using similar techniques and tools developed and validated in this study.

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