LiaX, a member of the LiaFSR system, is Essential for Cell Envelope Adaptation System in *Enterococcus faecium*

Tran TT[1,2], Panesso D[1,2], Khan A[3-5], Axell-House DB[1,2], Shamoo Y[6], Arias CA[1,2]

[1]Center for Infectious Diseases [2]Division of Infectious Diseases, Department of Medicine, Houston Methodist Hospital, Houston, TX [3]Genomics and Resistant Microbes (GeRM) Group, Facultad de Medicina, Clínica Alemana de Santiago, Santiago, Chile [4]Instituto de Ciencia e Innovación en Medicina, Santiago, Chile [5]Universidad del Desarrollo, Santiago de Chile, Chile [6]Rice University, Houston, TX

Abstract

Background: Daptomycin (DAP) is an important antibiotic for enterococci. Our previous studies identified LiaX as a surface-exposed protein that is a mediator of cell envelope homeostasis in *Enterococcus faecalis* (*Efs*) upon exposure to DAP via activation of the LiaFSR system. LiaX encodes for a soluble protein with an N-terminal of α-helices, and a C-terminus which is β-pleated sheets. Its role in *E. faecium*, a species of clinical relevance, remains unknown. In this work, we aim to elucidate the function of LiaX of *E. faecium* (*Efm*).

Methods: We used the commensal strain of *Efm* TX1330RF (DAP minimum inhibitory concentration [MIC] 3 µg/ml), its Δ*liaR* derivative (TX1330RFΔ*liaR* [DAP MIC 0.125 µg/ml]) and targeted the *liaX* gene for mutagenesis. Using the PheS* counterselection system, we aimed to create a truncation or in-frame deletion of *liaX*. Mutants were characterized by determination of DAP MIC and visualization of anionic phospholipid domains by 10-N-*nonyl*-acridine orange (NAO). The nisin-controlled expression vector, pMSP3535, was used to express LiaX from *Efm* ($LiaX_{TX1330RF}$) in *Efs* host OG1RFΔ*liaX*, which lacks *liaX*. Expression of LiaX of *Efs* ($LiaX_{OG1RF}$) was used as a control.

Results: In the presence of *liaR*, we were unable to create truncation or in-frame deletion of *liaX* after many attempts. In *Efm* TX1330RFΔ*liaR*, we successfully created an in-frame deletion of *liaX*, TX1330RFΔ*liaRliaX* (DAP MIC 0.125 µg/ml). NAO staining of all strains of TX1330RF, TX1330RFΔ*liaR* and its Δ*liaX* mutant showed localization of anionic phospholipid domains at septa of the cells. Interestingly, multiple attempts to complement *liaR* in TX1330RFΔ*liaRliaX* were also futile. NAO staining of *Efs* OG1RFΔ*liaX* demonstrated that anionic phospholipid microdomains redistributed away from septa. Expression of $LiaX_{OG1RF}$ in *Efs* OG1RFΔ*liaX* successfully restored microdomains to septal and polar regions of *Efs* while expression of $LiaX_{TX1330RF}$ did not show any significant difference in the fluorescence staining compared to its parental host (*Efs* OG1RFΔ*liaX*).

Conclusion: Our findings suggest that LiaX has divergent functions as a mediator of cell envelope homeostasis in enterococci and may be essential in the *Efm* response to DAP. Efforts to elucidate its role in DAP resistance in *Efm* are ongoing.

Background

- Enterococci are among the leading causes of hospital-associated infections including urinary tract, bloodstream, intra-abdominal and surgical site infections [1,2].
- Daptomycin (DAP) is a cyclic lipopeptide antibiotic that has *in vitro* bactericidal activity against enterococci, including vancomycin-resistant strains. However, evolving resistance to daptomycin during therapy has been reported in infections caused by *E. faecalis* and *E. faecium* [3-6].
- The LiaFSR stress response system has been shown to orchestrate DAP and antimicrobial peptide resistance in *E. faecalis* by modulating cell membrane phospholipid content and localization.
- LiaX, the effector of the LiaFSR system in *E. faecalis*, senses antimicrobial molecules and regulates changes in cell membrane phospholipid architecture.
- The function of LiaX in *E. faecium*, a species of clinical significance, remains to be elucidated.

Aim

To gain insights into the functional role of *liaX* in *Enterococcus faecium*

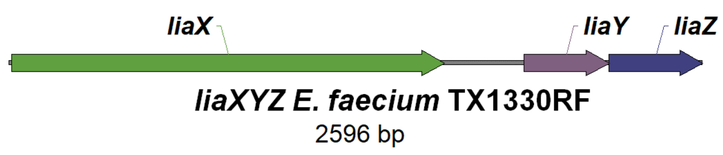
Methods

Bacterial strains –*Enterococcus faecium* TX1330RF and its *liaR (*TX1330RFΔ*liaR)* derivative were used to generate derivatives (**Table 1**). *E. faecalis* mutant of *liaX* (OG1RFΔ*liaX*) was used for complementation with pMSP3535.

Mutagenesis Strategy – In-frame and partial deletions of *liaX* were attempted using the *p*-chlorophenylalanine (p-Chl-Phe) sensitivity counterselection system (PheS*) to obtain the mutants. Briefly, ~500 bp regions upstream and downstream of *liaX* were amplified by crossover PCR using DNA from the corresponding strain as the target. Each fragment is cloned into pHOU1 using EcoRI and BamHI. The recombinant plasmids were electroporated into *E. faecalis* CK111 and delivered into rifampicin-resistant derivatives of the target *E. faecium* strain by conjugation. First recombinant integrants were selected on gentamicin (150 µg/ml) and rifampin (100 µg/ml) and subsequently plated in medium containing *p*-chlorophenylalanine. Colonies obtained from the counterselection medium were tested by replica plating in the presence of varying DAP concentrations. Candidate colonies were subjected to pulsed-field gel-electrophoresis to confirm their genetic relatedness with the parent strain. Regions of *liaX* was amplified and subjected to Sanger sequencing to confirm deletion(s) and DAP MICs by Etest.
Complementation was achieved using the nisin-controlled expression vector, pMSP3535. *E. faecalis* OG1RF *liaX* and *E. faecium* TX1330RF *liaR* and *liaX* were cloned downstream of PnisA. Plasmids pMSP3535::$liaR_{TX1330RF}$, pMSP3535::$liaX_{TX1330RF}$ and pMSP3535::$liaX_{OG1RF}$ were introduced by electroporation into the appropriate hosts. Strains harboring pMSP3535 were cultured with 50-100 ng/ml nisin.

Fluorescence Microscopy – 10-*N*-nonyl acridine orange (NAO) fluorescent dye was obtained from Molecular Probes. In brief, bacterial cells were grown to early exponential phase in BHI broth. Nisin 50 ng/ml and NAO 1 µM were added and bacterial cells were incubated for 3 h at 37°C with rotary shaking. After staining, 8 µl of cells were immobilized in 1% agarose pads on object slides coated with poly-L-lysine. Cells were viewed with a Keyence BZ-X710 fluorescence microscope using FITC (excitation 495 nm, emission 519 nm).

Results

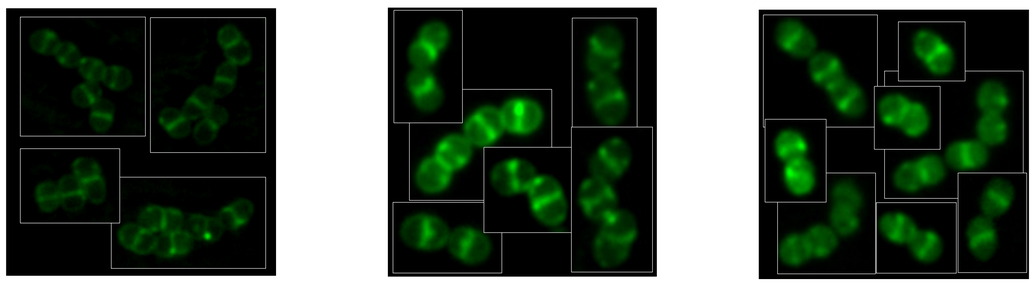


***liaXYZ E. faecium* TX1330RF**
2596 bp

Figure 1. *liaXYZ* of *E. faecium* TX1330RF

Table 1. *Enterococcus faecium* and derivatives

Strain	DAP MIC (µg/ml)
TX1330RF	1
TX1330RFΔ*liaR*	0.125
TX1330RFΔ*liaR* (pMSP3535::*liaR*)	1
TX1330RFΔ*liaR*Δ*liaX*	0.125
TX1330RFΔ*liaRliaX* (pMSP3535::*liaR*)	0.5

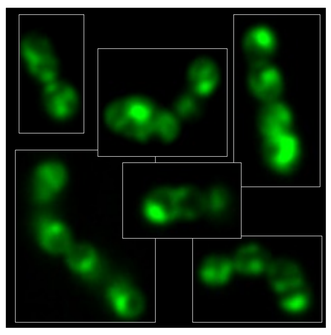


TX1330RF | TX1330RFΔ*liaR* | TX1330RFΔ*liaRliaX*

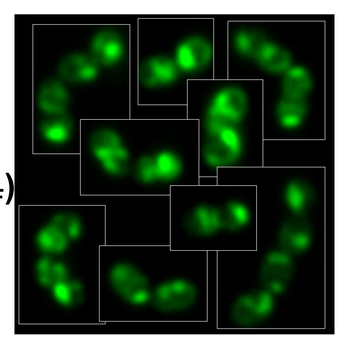
Figure 2. Localization of anionic phospholipids with NAO in *E. faecium* TX1330RF and derivatives

Results

OG1RFΔ*liaX* (pMSP3535)



OG1RFΔ*liaX* (pMSP3535::$liaX_{OG1RF}$)



OG1RFΔ*liaX* (pMSP3535::$liaX_{TX1330RF}$)

Figure 3. Localization of anionic phospholipids by NAO in *E. faecalis* OG1RFΔ*liaX*

Conclusion

- Our findings suggest that LiaX has divergent functions as a mediator of cell membrane homeostasis in enterococci
- LiaX may be essential in *E. faecium* whose LiaFSR is intact
- Efforts to elucidate the role of LiaX in daptomycin resistance in *E. faecium* are ongoing

Acknowledgments

This work is supported by the National Institute of Allergy and Infectious Diseases (NIAID) of the NIH (award numbers K24AI121296, R01AI134637, R01AI48342, and P01AI152999 to C. A. A).

References

1. **Sievert DM, Ricks P, Edwards JR, et al.** 2013. Infect Control Hosp Epidemiol **34**:1-14.
2. **Arias CA, Murray BE.** 2012. Nat Rev Microbiol **10:**266-78.
3. **Arias CA, Panesso D, McGrath DM, et al**. 2011. N Engl J Med **365**:892-900.
4. **Tran TT, Panesso D, Gao H, et al**. 2013. Antimicrob Agents Chemother **57**:261-8 **Tran TT, Panesso D, Mishra NN, et al.** 2013. mBio **4**:e00281-13
5. **Khan A, Davlieva M, Panesso D, et al** 2019. *Proc Natl Acad Sci U S A*. pii: 201916037