Dynamics of *Enterococcus faecalis* cardiolipin synthase gene expression reveal compensatory roles in daptomycin resistance

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Abstract

Background: Daptomycin (DAP) is a lipopeptide antibiotic targeting membrane anionic phospholipids (APLs) at the division septum[1], and resistance (DAP-R) has been linked to mutations in genes encoding ***i***) the LiaFSR stress response system or its effector LiaX, and ***ii***) cardiolipin synthase (Cls) [2,3]. Activation of the *E. faecalis (Efs)* LiaFSR response is associated with DAP-R and redistribution of APL microdomains away from the septum, and cardiolipin is predicted to be a major component of these APL microdomains [1,7]. *Efs* harbors two putative *cls* genes, *cls1* and *cls2*. While changes in Cls1 have been implicated in DAP-R, the exact roles of each enzyme in resistance are unknown. We aim to characterize the contributions of Cls1 and Cls2 in the development of DAP-R.

Methods: *cls1* and *cls2* were deleted individually and in tandem from DAP-S *Efs* OG117[4] and DAP-R *Efs* OG117Δ*liaX* (a DAP-R derivative with an activated LiaFSR response). Mutants were characterized by DAP minimum inhibitory concentration (MIC) using E-test on Mueller-Hinton II agar and localization of APL microdomains with 10-N-nonyl-acridine orange staining[6]. Quantitative PCR (qRT-PCR) was used to study gene expression profiles of *cls1* and *cls2* in *Efs* OG117Δ*liaX* relative to *Efs* OG117 across the cell growth cycle.

Results: qRT-PCR revealed differential expression profiles of *cls1* and *cls2* associated with DAP-R. *cls1* was highly upregulated in stationary phase concurrent with a decrease in *cls2* expression. However, independent deletion of *cls1* or *cls2* in the DAP-R background resulted in no significant changes in DAP MICs or localization of APL microdomains (remaining non-septal). Further studies revealed that *cls2* expression is upregulated upon deletion of *cls1* in both the DAP-S and DAP-R background, suggesting a potential compensatory role for Cls2. Double deletion of both *cls* genes in the DAP-R strain decreased DAP MIC restored the septal localization of APL microdomains.

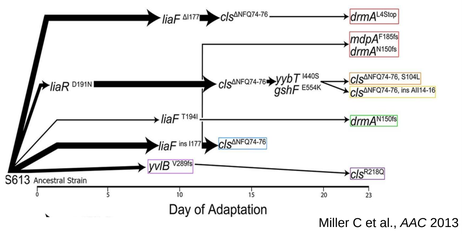
Conclusions: Cls1 is the major and predominant enzyme involved in cell membrane adaptation associated with the development of DAP-R in *E. faecalis*. However, we describe a novel compensatory and overlapping role for cardiolipin synthases to ensure bacterial survival upon attack from antimicrobial peptides and related antibiotics.

Background

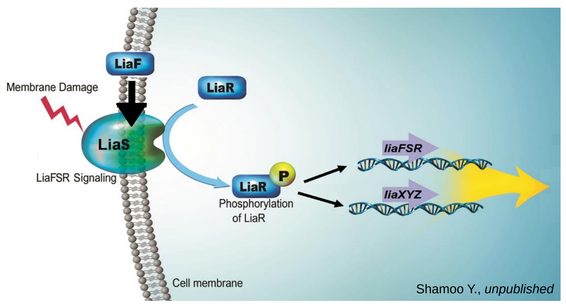
Daptomycin (DAP):
- Lipopeptide antibiotic
- Used in multi-drug resistant enterococcal infections
- Targets anionic phospholipids (APL) in cell membrane at division septum[1]
- Disrupts cell division and lipid biogenesis[5]

DAP-Resistance (DAP-R):
- Mediated by LiaFSR[2,3]
- Causes re-distribution of APL away from septum as visualized with 10-n-nonyl acridine orange (NAO)[1]
- LiaY may be involved in membrane adaptation through unknown downstream partners

Cardiolipin synthase (Cls):
- *E. faecalis: cls1* and *cls2*
- Synthesizes cardiolipin, proposed component of APL microdomains[6,7]
- DAP-R-associated mutations found in *cls1*[2,3]
- Cls may act in downstream of LiaY in mediating membrane adaptation



Miller C et al., *AAC* 2013



Shamoo Y., *unpublished*

	OG1RF	Δ*liaX289*	289Δ*liaYZ*
DAP MIC (ug/mL)	2	12	8
APL microdomain localization	Septal	Redist.	Septal

Aim

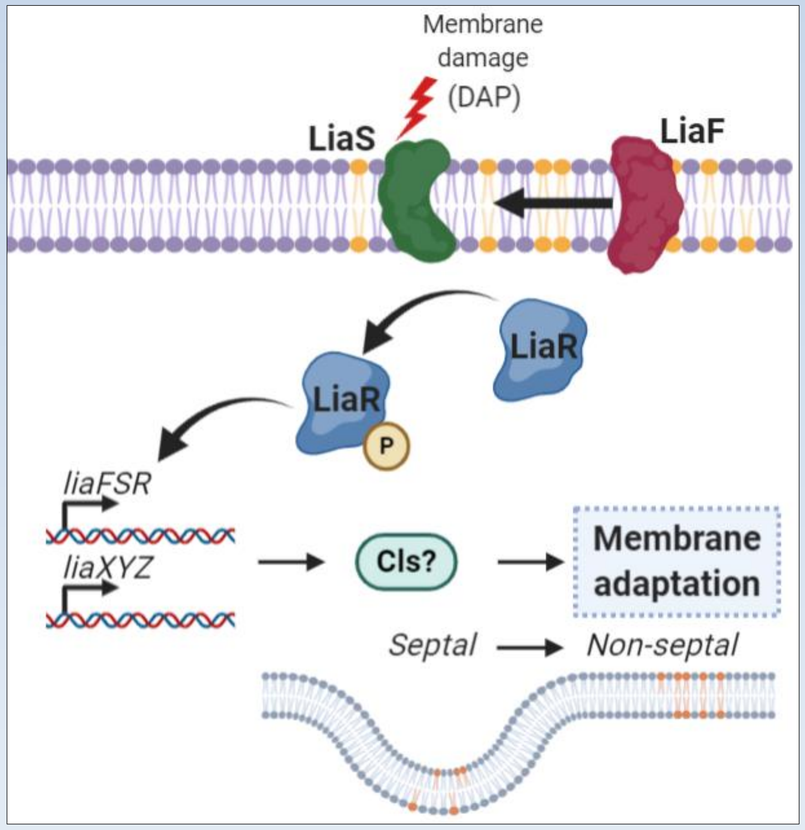
Cardiolipin Synthase?

Activation of LiaFSR/LiaXYZ → **Membrane Adaptation DAP Resistance**

Methods

- **Mutant Generation**: Complete deletion mutants of *cls1*, *cls2*, or both were generated in *E. faecalis* OG117 (DAP-susceptible) and *E. faecalis* OG117Δ*liaX* (DAP-R) using the CRISPR-Cas9 system adapted for use in *E. faecalis*[4].
- **DAP Minimum Inhibitory Concentration**: Strains were diluted to a concentration of approximately $1x10^8$ CFU/mL and plated on Mueller Hinton agar. A DAP E-test (bioMerieux) strip was placed onto the plate and incubated for 24 hours at 37C prior to evaluation.
- **APL Microdomain Localization**: NAO is a hydrophobic fluorescent dye that specifically binds APLs in the membrane[6]. Strains were grown in tryptic soy broth with 1uM of NAO to exponential phase prior to visualization (Keyence BZ-X710).
- ***cls* Gene Expression**: Strains were grown from t=1h to 8h in tryptic soy broth, and RNA was extracted (PureLink RNA Extraction Kit, Invitrogen). All RNA samples were treated with DNase (TurboDNAse, Ambion) prior to cDNA synthesis (SuperScript II, Invitrogen). qRT-PCR was used to evaluate differences in gene expression using the Pfaffl method, relative to 16S rRNA expression.

Mechanistic Model



Conclusions

- DAP-R is associated with increased expression of *cls1,* especially in stationary phase
- However, deletion of *cls1* alone does not prevent re-distribution of anionic phospholipid microdomains
- Cls2 may have role in DAP-R secondary to Cls1, as qRT-PCR shows increased expression of *cls2* when *cls1* is deleted
- Deletion of both genes restores septal APL microdomain localization

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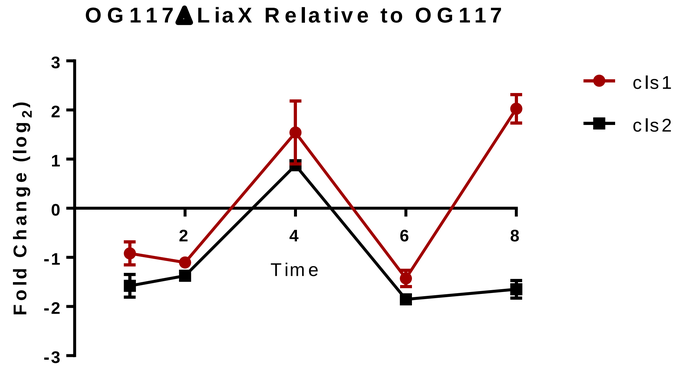
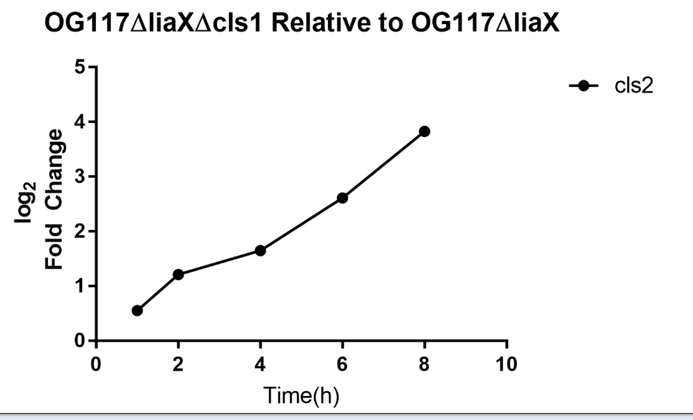
Results

Deletion of *cls* restores septal APL microdomain localization

Efs OG117Δ*liaX*	DAP MIC (μg/mL)	NAO Staining
Wild-type	8	Redist.
Δ*cls1*	6-8	Redist.
Δ*cls2*	8	Redist.
Δ*cls1*Δ*cls2*	4-6	Septal

cls1 and *cls2* are differentially expressed in DAP-R





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