Enterococcus faecalis CL Synthases Play Redundant Roles in Membrane Homeostasis and Daptomycin Resistance

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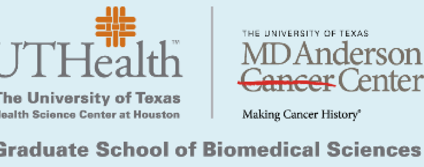


Abstract

Background: Daptomycin (DAP) is a lipopeptide antibiotic targeting anionic phospholipids (APLs) at the division septum, and resistance (DAP-R) has been associated with activation of the E. faecalis (Efs) LiaFSR response and redistribution of APL microdomains (predicted to contain cardiolipin, CL) away from the septum. Efs encodes two CL synthase genes, cls1 and cls2. While changes in Cls1 are associated with DAP-R, the exact roles of each enzyme are unknown. This work aims to establish the roles of both enzymes in Efs and the LiaFSR system.

Methods: *cls1* and/or *cls2* were deleted from Efs OG117 and OG117Δ*liaX* (a DAP-R strain with an activated LiaFSR response). qRT-PCR was used to study gene expression profiles of *cls1* and *cls2* in the cls mutants. Membrane lipid content was analyzed using hydrophilic interaction chromatography-mass spectrometry. Mutants were characterized by DAP minimum inhibitory concentration (MIC) using E-test and localization of APL microdomains with 10-N-nonyl-acridine orange.

Results: qRT-PCR shows upregulation of *cls1* and *cls2* in exponential phase of Efs OG117Δ*liaX* relative to Efs OG117, with *cls1* continuing to be upregulated in stationary phase. Regardless of the genetic background, deletion of either *cls* results in upregulation of the remaining *cls*.

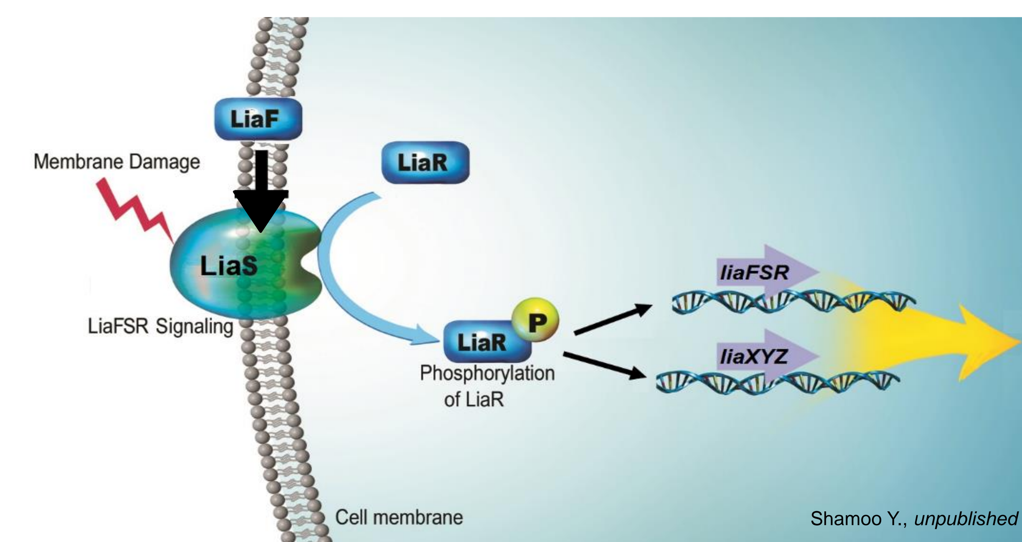
Lipidomics analysis confirms that deletion of both *cls* is required to eliminate all CL content. When comparing CL profiles of Δ*cls1* relative to Δ*cls2* in both DAP-S and DAP-R, both strains produced similar levels and species of CL to each other. However, development of DAP-R causes a change in membrane lipid content, of note, an increase in CL with no significant difference in phosphatidylglycerol compared to DAP-S. Evaluation of CL species in DAP-R shows a shift towards species containing longer fatty acid chains and higher saturation.

Independent deletion of *cls1* or *cls2* in the DAP-R strain shows no significant phenotypic changes from the parent strain. Ultimately, double deletion of both cls genes lowered the DAP MIC relative to the parent strain and restored septal localization of APL microdomains. DAP MIC was able to be restored upon in trans complementation of either cls1 or cls2 into the double deletion background.

Conclusions: While Cls1 is predominantly associated with DAP-R, here we show a functional redundancy between Cls1 and Cls2 in both cell membrane homeostasis and in DAP-R.

Background

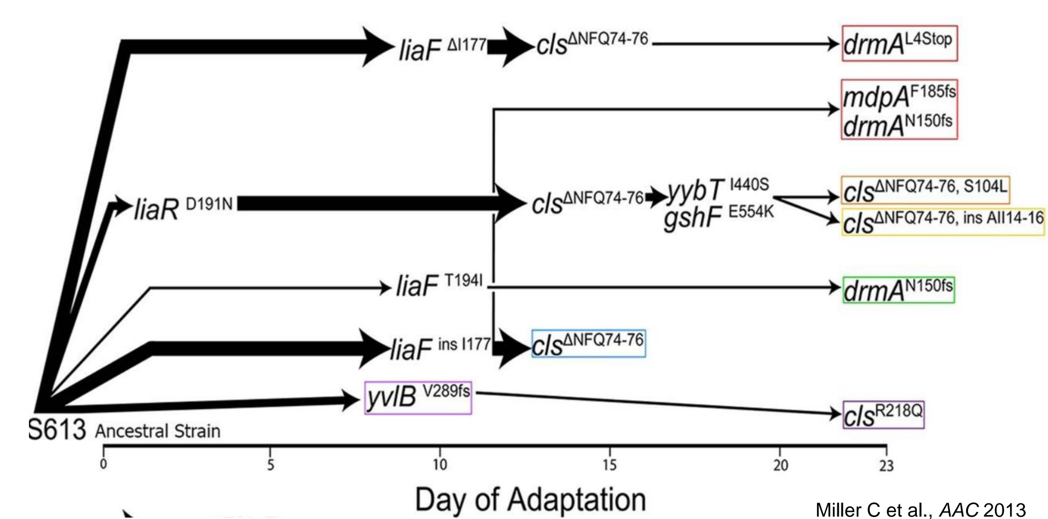
Daptomycin (DAP):
- Lipopeptide antibiotic
- Used in multi-drug resistant enterococcal infections
- Targets anionic phospholipids (APL) in cell membrane at division septum[1]
- Disrupts cell division and lipid biogenesis[5]



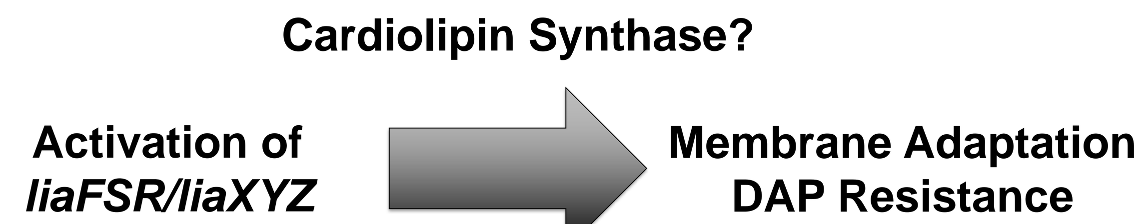
DAP-Resistance (DAP-R):
- Mediated by LiaFSR[2,3]
- Causes re-distribution of APL away from septum as visualized with 10-n-nonyl acridine orange (NAO)[1]
- LiaY may be involved in membrane adaptation through unknown downstream partners

	OG1RF	Δ*liaX289*	289Δ*liaYZ*
DAP MIC (ug/mL)	2	8	4
APL microdomain localization	Septal	Redist.	Septal

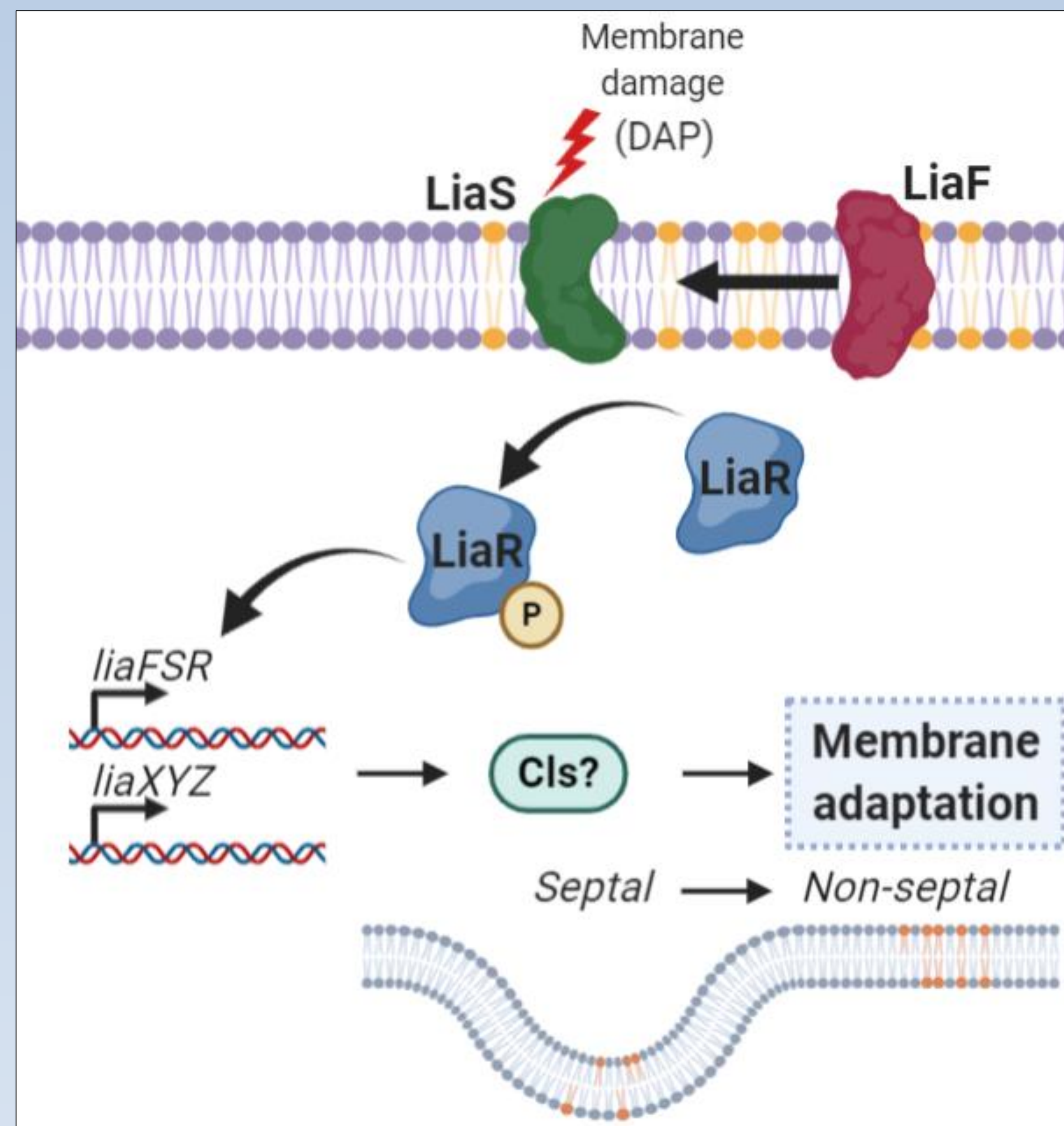
Cardiolipin synthase (Cls):
- *E. faecalis: cls1* and *cls2*
- Synthesizes cardiolipin, proposed component of APL microdomains[6,7]
- DAP-R-associated mutations found in *cls1*[2,3]
- Cls may act in downstream of LiaY in mediating membrane adaptation



Aim



Mechanistic Model



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 brain heart infusion agar containing appropriate selective antibiotics and/or nisin for induction *(trans* complementation with pMSP3535). A DAP E-test (bioMerieux) strip was placed onto the plate and incubated for 24 hours at 37C prior to evaluation.

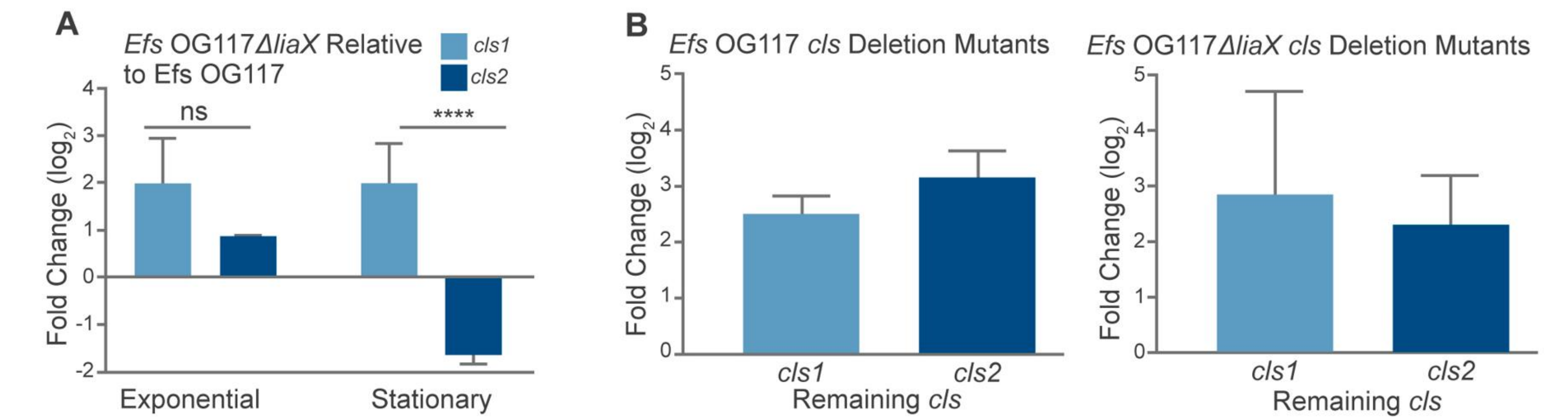
APL Microdomain Localization: NAO is a hydrophobic fluorescent dye that specifically binds APLs in the membrane[6]. Strains were grown in brain heart infusion broth with 1uM of NAO to stationary phase prior to visualization (Keyence BZ-X710).

***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.

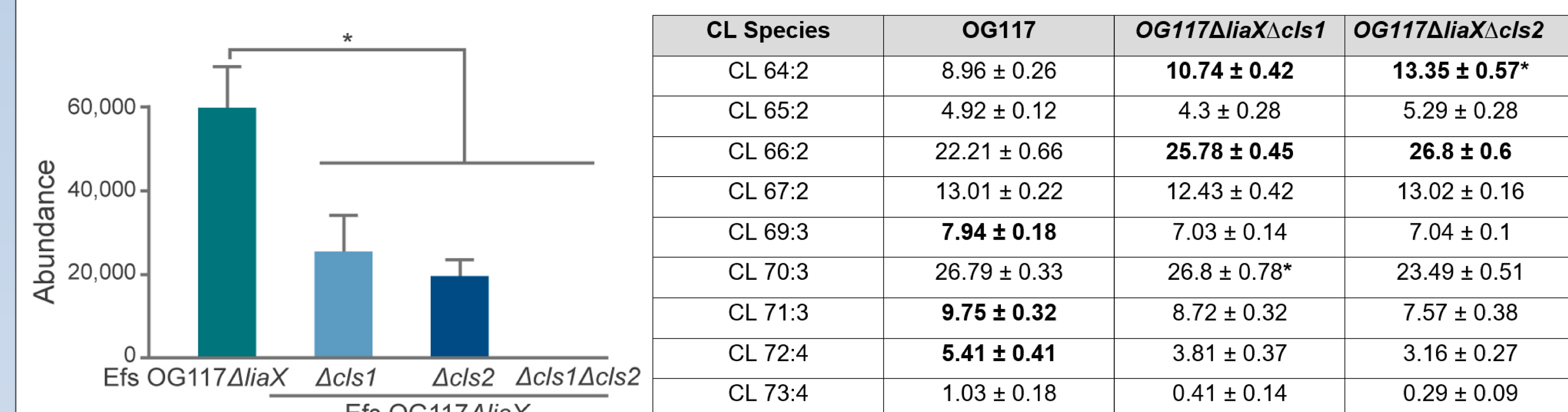
Lipidomics Analysis
Membrane lipid content was analyzed using hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS) on membranes extracted via the Bligh and Dyer method.

Results

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



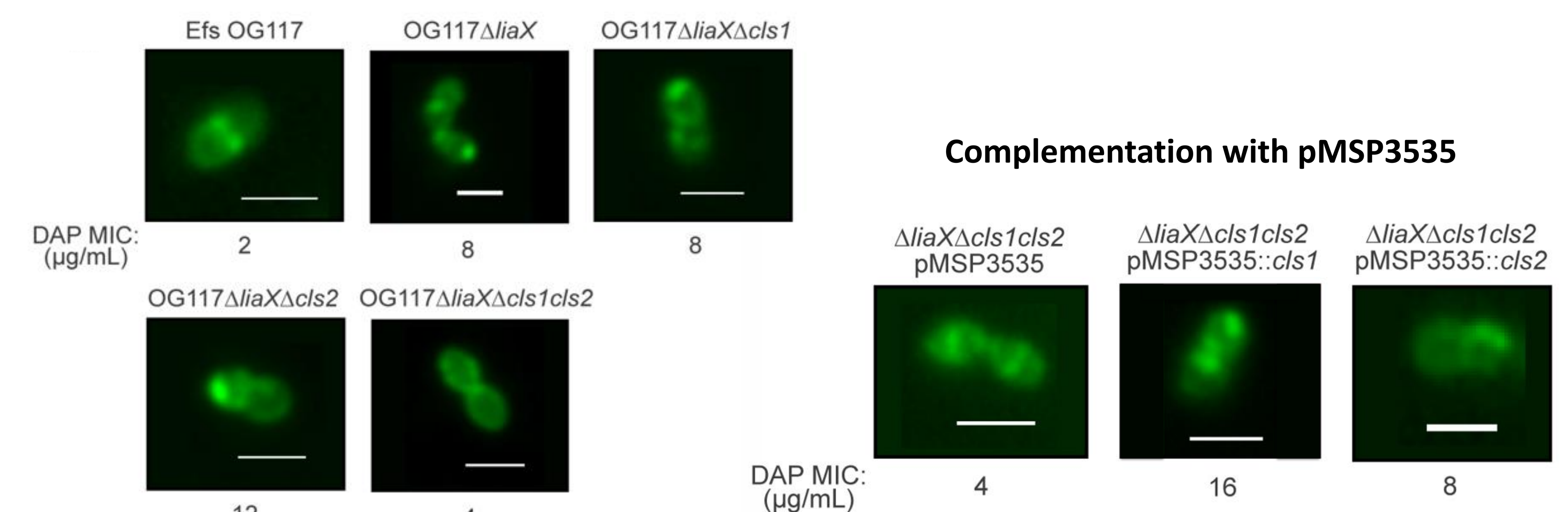
Membrane Cardiolipin Content



CL Species	OG117	*OG117ΔliaXΔcls1*	*OG117ΔliaXΔcls2*
CL 64:2	8.96 ± 0.26	**10.74 ± 0.42**	**13.35 ± 0.57***
CL 65:2	4.92 ± 0.12	4.3 ± 0.28	5.29 ± 0.28
CL 66:2	22.21 ± 0.66	**25.78 ± 0.45**	**26.8 ± 0.6**
CL 67:2	13.01 ± 0.22	12.43 ± 0.42	13.02 ± 0.16
CL 69:3	**7.94 ± 0.18**	7.03 ± 0.14	7.04 ± 0.1
CL 70:3	26.79 ± 0.33	26.8 ± 0.78*	23.49 ± 0.51
CL 71:3	**9.75 ± 0.32**	8.72 ± 0.32	7.57 ± 0.38
CL 72:4	**5.41 ± 0.41**	3.81 ± 0.37	3.16 ± 0.27
CL 73:4	1.03 ± 0.18	0.41 ± 0.14	0.29 ± 0.09

Proportion (by %) of each CL species. **Text in bold** (p<0.01, n=3) represents the strain with statistically significantly higher proportion of each individual CL species between either the parent strain or the *cls* mutants. Asterisk* represents individual *cls* mutants that have significantly different (*p<0.05) levels of that CL species to each other.

Deletion of *cls* restores septal APL microdomain localization



Funding

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Conclusions

- DAP-R is associated with increased expression of *cls1,* especially in stationary phase
- Cls2 may have role in DAP-R secondary to Cls1, as qRT-PCR shows increased expression of *cls2* when *cls1* is deleted
- Both synthases produce similar amounts and species of cardiolipin.
- Deletion of both genes restores septal APL microdomain localization and DAP MIC

References

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