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Lipid A Phosphorylation Modulates *Porphyromonas gingivalis* Outer Membrane Vesicle Function in Biofilm Dispersal

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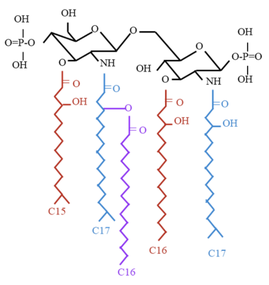
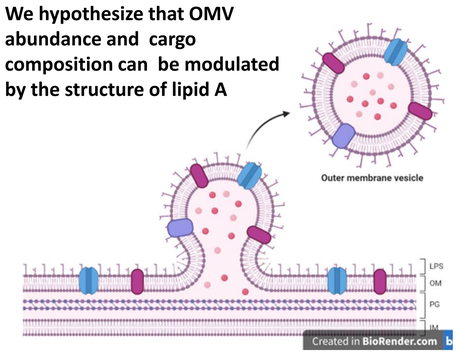
Background



We hypothesize that OMV abundance and cargo composition can be modulated by the structure of lipid A



Modification	by	mutant lipid A
1. Removal of C1-P	lpxE	4-acyl, C1-P
2. Removal of C4'-P	lpxF	5-acyl, C4'-P

Deletion of both lpxF and lpxE (ΔlpxF/lpxE mutant) will retain the precursor form of lipid A (5-acyl bis-P).

Figure 1. *P. gingivalis* possesses lipid A phosphatases that modify LPS structure. The majority of LPS found in the outer membrane of WT *P. gingivalis* has been dephosphorylated by two phosphatases, encoded by the *lpxE* and *lpxF* genes. We predict that removal of lipid A phosphates impacts how LPS fits alongside other membrane lipids, as well as the interactions between LPS and membrane proteins.

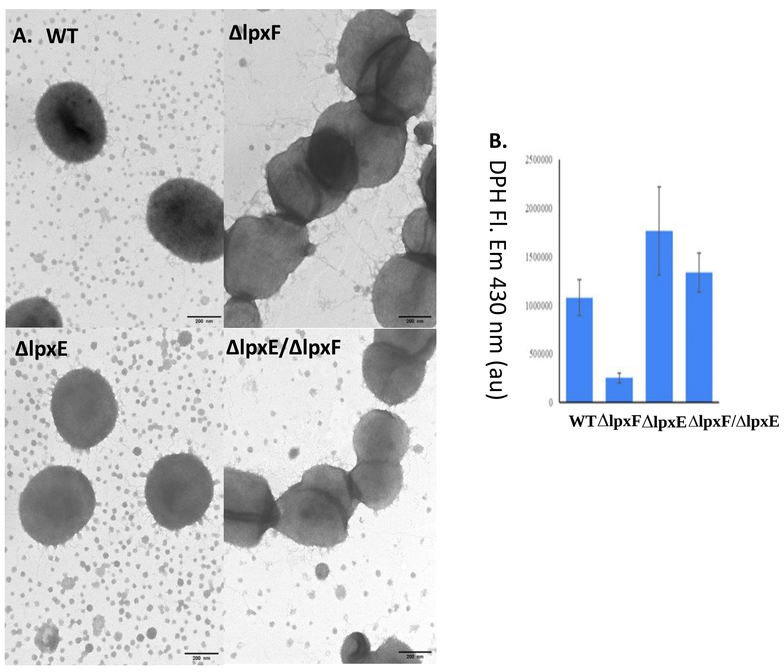


Figure 2. Lipid A phosphorylation influences OMV production. A) Transmission electron microscopy (TEM) was used to view whole bacterial cultures (cells and medium). OMVs can be seen budding off cell surfaces and in the culture medium. **B)** Diphenyl hexatriene (DPH) was used to quantitate relative amounts of OMVs isolated from each strain . Notably, the total biomass of OMVs isolated from ΔlpxF/ΔlpxE did not differ from WT, but TEM images suggest that OMV number may be reduced, and diameter may be more variable for this strain.

Results

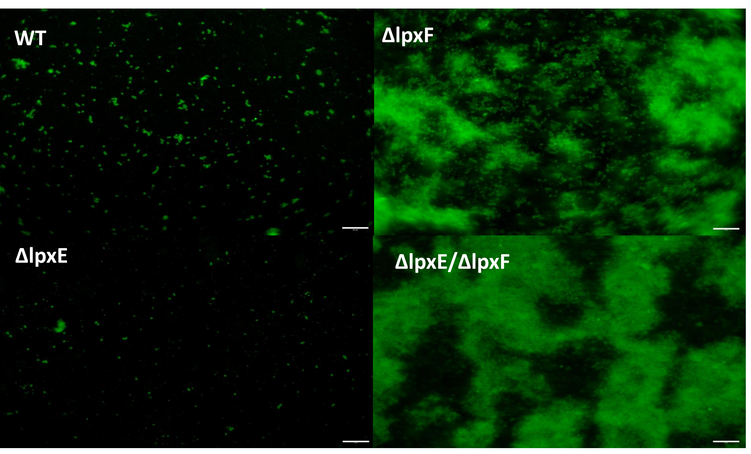


Figure 3. The presence of lipid A 4' phosphatase gene (lpxF) limits microcolony size. The indicated *P. gingivalis* strains formed biofilms on glass coverslips for 48 hours prior to staining with CFSE and fluorescent imaging. Small microcolonies were formed by strains with functional lipid A 4' phosphatase genes (WT and ΔlpxE), whereas strains lacking lipid A 4' phosphatase (ΔlpxF and ΔlpxF/ΔlpxE) formed larger, clumpy microcolonies. Scale bars = 20μm.

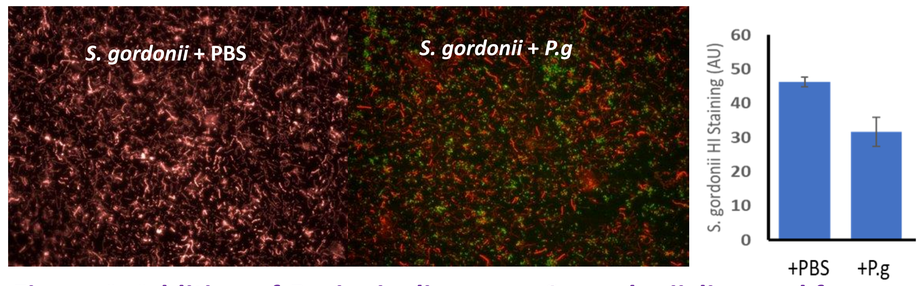


Figure 4. Addition of *P. gingivalis* causes *S. gordonii* dispersal from biofilms. While these bacteria species are generally thought to act synergistically, we observed that the addition of *P. gingivalis* (green) to *S. gordonii* (red) biofilms results in the loss of *S. gordonii* from the biofilm. Scale bars = 20 μm.

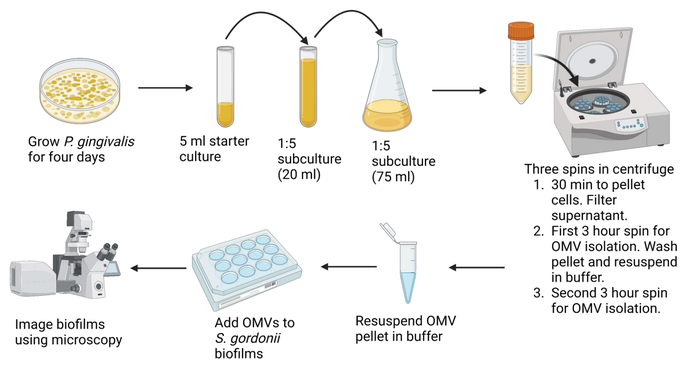


Figure 5. Schematic of methodology for biofilm assays. *P. gingivalis* was struck out and grown for four days, then used for a 5 ml starter culture. After two subsequent subcultures of increasing volume, bacterial cultures were spun down to pellet cells. The supernatant was filtered and spun again for three hours. Resulting OMV pellet was then washed and re-suspended in buffer before undergoing a second three hour spin. OMVs were then added to pre-formed *S. gordonii* biofilms after normalization to the lowest protein concentration. After 24 hours, biofilms were stained and fixed before imaging using confocal microscopy.

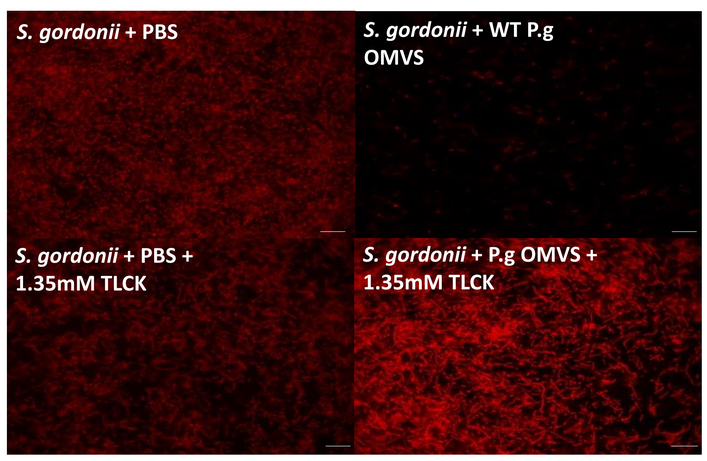


Figure 6. Addition of *P. gingivalis* OMVs causes *S. gordonii* dispersal from biofilms, which depends on cysteine protease activity. After 24 hours of biofilm formation by *S. gordonii*, medium containing planktonic cells was removed and replaced with either PBS (control) or PBS containing the indicated OMVs/protease inhibitors for an additional 24 hours.
Scale bar = 20 μm, TLCK = Cys protease inhibitor, chemical inhibitor of gingipains

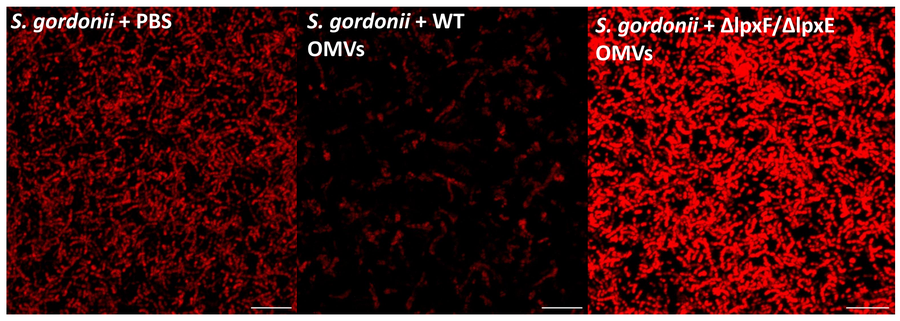


Figure 7. *P. gingivalis* OMVs containing all bis-phosphorylated lipid A do not disperse *S. gordonii* biofilms. After 24 hours of biofilm formation, medium containing planktonic cells was removed and replaced with either PBS or PBS containing normalized amounts (based on protein content) of the indicated OMVs for an additional 24 hours . OMVs from the mutant strain lacking both lipid A phosphatases (ΔlpxF/ΔlpxE) do not exhibit biofilm dispersing activity, suggesting differences in OMV cargo compared to WT. Scale bar = 8 μm.

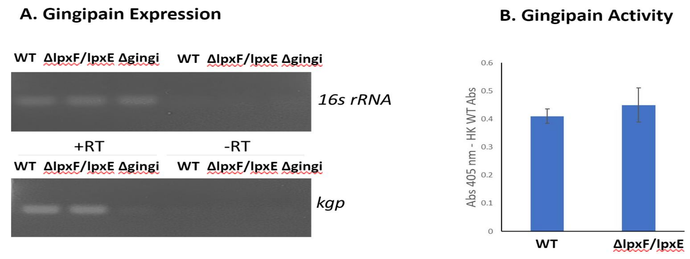


Figure 8. Gingipain proteases are expressed and active in lipid A phosphatase double mutant strain. A) Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) confirmed the expression of the gingipain proteases, which are known to be major OMV cargoes. Representative data for one gingipain gene (*kgp*, Lys specific gingipain) is shown. As expected, the 16s rRNA gene is expressed in all three of the *P. gingivalis* strains. In contrast, the Kgp gingipain protease is expressed only in WT and the ΔlpxF/ΔlpxE strains and not in the gingipain deletion (Δgingi) strain. **B)** Cleavage of Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), a chromogenic substrate for Arg specific gingipains, suggested that differences in whole cell associated gingipain enzymatic activity are unlikely to account for the striking difference in OMV associated biofilm dispersing activity observed for this strain compared to WT.

Lipid A structure as a modulator of OMV cargo loading.

- *P. gingivalis* OMVs containing only bis-phosphorylated penta-acylated lipid A (from strain ΔlpxF/ΔlpxE) do not disperse *S. gordonii* biofilms, whereas WT OMVs with dephosphorylated/deacylated lipid A do disperse *S. gordonii* biofilms.
- There is no significant difference between the total biomass of OMVs produced by WT and ΔlpxF/ΔlpxE strains, but TEM images suggest that there may be differences in OMV size distribution between strains.
- Gingipain proteases are known to be major OMV cargoes, and our preliminary studies suggest that gingipain activity is responsible for the OMV mediated biofilm dispersal reported here.
- Gingipain protease mRNA expression and whole cell gingipain protease activity are not reduced in ΔlpxF/ΔlpxE mutant compared to WT, suggesting that lipid A structure (bis-P) interferes with cargo loading on/in OMVs in the ΔlpxF/ΔlpxE mutant.

Ongoing Studies

- We will confirm that all that all three gingipains (Kgp, RgpA, RgpB) are expressed and active on the cell surface of ΔlpxF/ΔlpxE strains.
- Long-term studies will focus on examining the interactions between LPS and outer membrane proteins that influence the specificity of cargo loading during OMV biogenesis.
- We will also investigate the role of *P. gingivalis* OMV mediated modulation of the dispersion of mature *S. gordonii* biofilms and consider whether the OMV mediated modulation of bacterial adhesion influences pathogenesis.

Acknowledgements

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