

**Anchors Away: Determining the Role of Outer Membrane Proteins
in *Pseudomonas aeruginosa* Vesicle Formation**

Presented by Jean Liew

In partial fulfillment of the requirements for graduation with the
Dean's Scholars Honor's Degree in Biology

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Date

DEDICATION

First of all, I would like to thank Marvin for allowing me, back in January 2008, to work in his lab. This experience has enriched my undergraduate career by bringing textbook concepts to life.



I am also grateful for the opportunity to have met and worked with such a great group of people. Thanks for this project go out to: Aimee Wessel, for her overall guidance and support (and coffee); Aishwarya Korgaonkar, for help with the TEM and MV preps; Holly Huse, for help with MV preps and data analysis; Tina Shay and Jeff Schertzer, for help with the lipid assay; and Greg Palmer, for providing criticism on the first draft of this thesis. Additionally, Stacie Brown has provided me with not only continued support, but also excellent baked goods. I would also like to thank Matt Ramsey and Megan Boulette for their time commitment while working with me on earlier projects.

Lauren Warren, the originator of this project, deserves a big shout-out for her help and support from her far-away post in windy Chicago.

Thanks, also, to my friends (especially Kathryn Busby and Katy Loeffler, who get the honor of being mentioned in both of my theses) and family, for understanding that a tad of madness is a necessary component of the scientific method.

ABSTRACT

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes chronic infections in the lungs of individuals with cystic fibrosis (CF). Like many Gram-negatives, *P. aeruginosa* produces outer membrane vesicles (MVs), which have been shown to package numerous factors including antimicrobial quinolone molecules, toxins, DNA, antibiotic resistance determinants, and cell-cell signaling molecules. The mechanism for the formation of MVs has not been fully elucidated. The Gram-negative outer membrane (OM) contains associated proteins, which anchor it to the peptidoglycan, and keep the OM stable. We hypothesized that peptidoglycan-associated outer membrane lipoproteins OprF, OprL, and OprI contribute to MV formation in *P. aeruginosa*. In this study, we quantified MVs harvested from *oprF*, *oprL*, and *oprI* mutants. The MV levels produced by the *oprL* and *oprI* mutants were not significantly different from those produced by the wild type; however, the *oprF* mutant showed a three-fold increase in MV production. These data indicate that OprF plays a significant role in anchoring the outer membrane to the peptidoglycan and that, in its absence, more MVs are formed.

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BACKGROUND

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen typically found in water, soil, and vegetation, which can cause various chronic and acute infections (35). *P. aeruginosa* pathogenesis is tied to its production of virulence factors, enzymes that perform various functions including allowing adherence to host cells and causing cell and tissue damage (35; 25). *P. aeruginosa* regulates virulence factor production through quorum sensing (QS), a phenomenon that allows bacteria to monitor their cell-population density through a small molecule called an autoinducer, and to alter gene expression accordingly (33; 48). QS interference has been touted as a potential therapeutic approach, through the inhibition of autoinducer biosynthesis (48).

A well-studied *P. aeruginosa* infection site is the lung of individuals with cystic fibrosis (CF) (35). CF is a hereditary disease that affects 1 in 2,500 Caucasian newborns (38). The symptoms of CF are due to a mutation in the CFTR gene, which encodes for an anion channel in exocrine epithelial cell membranes (38). This channel plays a role in mucus secretion, water permeability, and ATP transport. Individuals with CF are unable to effectively rid their lungs of sputum, and the thickened mucus provides a nutrient-rich environment for bacteria (1). >80% of adults with CF have been colonized by *P. aeruginosa*, and the average life expectancy is 37 years (1). Despite aggressive antibiotic treatment, these infections are very difficult to eradicate, due partly to the formation of *P. aeruginosa* biofilms within the CF lung (43). Biofilms, which are a polysaccharide and protein matrix excreted from cells attached to a surface, confer antimicrobial resistance to the

bacteria living within them (10; 45). Resistance to a wide range of antibiotics, including β -lactams and aminoglycosides, is a major problem affecting treatment of *P. aeruginosa* infections (35). Other clinical diseases caused by *P. aeruginosa* include: lower respiratory tract infections, skin infections, especially in burn wounds, urinary tract infections after prolonged in-dwelling urinary catheter usage, external otitis and chronic otitis media, corneal ulcers, bacteremia, and endocarditis (35).

Bacterial Outer Membrane Vesicles

As Gram-negative bacteria, *P. aeruginosa* have a bilayered inner membrane (IM), a gel-like periplasm, a thin layer of rigid peptidoglycan, and an outer membrane (OM). The OM is composed of protein, phospholipid, and lipopolysaccharide (LPS). MVs are spherical secretions 50-250 nm in diameter, which are composed of OM constituents and can also contain compounds found in the periplasm (6). MVs are associated with Gram-negatives growing planktonically, in biofilms, and in laboratory culture media (6). The ability of Gram-negatives to produce and excrete MVs was first identified over 40 years ago (25; 31; 32). Chatterjee and Das (1967) reported on the “excretion of cell wall material” from *Vibrio cholerae* harvested during logarithmic growth, and their TEM images showed the *V. cholerae* membrane bulging out and pinching off (8).

Recent reviews have discussed the myriad functions of MVs that have been identified thus far (25; 31; 32). The most-studied category of MVs is predatory MVs, which contain and concentrate factors which aid in competition with other bacterial species (25). The packaging of these molecules protects them from degradation and

host recognition (32). *P. aeruginosa* predatory MVs contain the autolysin peptidoglycan hydrolase, a periplasmic enzyme that is involved in peptidoglycan turnover, which causes the lysis of other species (20). These MVs break open on the cell wall surface of Gram-positives and hydrolyze the peptidoglycan. In Gram-negatives, the MVs fuse with the OM, release their contents into the periplasm, and cause lysis of the peptidoglycan layer underneath (21; 6).

MVs are involved in interspecies communication, or cell-cell signalling. In the case of *P. aeruginosa*, the QS autoinducer *Pseudomonas* Quinolone Signal (PQS) is packaged within MVs, allowing more efficient signaling than the wasteful alternative of free diffusion into the surrounding environment (29). This phenomenon may even extend to interdomain communication, for example in the hypothesized transfer of Nod factor between *Rhizobium spp.* and soybean roots (31).

DNA transfer of plasmids within species has been shown in *P. aeruginosa* and in other Gram-negative species (22; 13; 20; 26; 40). This method could constitute a fourth mechanism of DNA transfer, in addition to the long-established mechanisms of transformation, conjugation, and transduction (40; 31). Enclosure within MVs affords the DNA protection from exonucleases in the environment, as well as enhancing the efficiency of DNA delivery and uptake (40; 31).

MVs may be utilized in other types of transport. Since it has been found that the antibiotic gentamicin can enter MVs, the therapeutic delivery of antibiotics within MVs may be possible (6). It is proposed that through fusion, these g-MVs can deliver gentamicin to *Pseudomonas* species whose surfaces are otherwise impermeable to this antibiotic, and others of the aminoglycoside family. MVs may also be useful as

vaccine agents due to their species-specific antigenicity, though due to the endotoxicity of the LPS present on the MV surface, they would need to be orally administered (6).

MVs in Bacterial Pathogenesis

In general, pathogenic bacteria produce more MVs than nonpathogenic species (25). For example, *Enterotoxigenic E. coli (ETEC)* produces 10-fold more MVs than nonvirulent *E. coli*, and leukotoxic strains of *Aggregatibacter actinomycetemcomitans*, an oral commensal that is the causative agent of localized aggressive periodontitis, produces 25-fold more MVs than nonleukotoxic strains (23; 25).

P. aeruginosa can utilize MVs for establishing a colonization niche, as well as for modulating host responses (25). Virulence factors enclosed in *P. aeruginosa* MVs include phospholipase C, protease, hemolysin, and alkaline phosphatase, which all cause tissue damage (20; 35). Compounds that are recognized by immune pathways, such as LPS, lipoprotein, and outer membrane proteins are present on the surfaces of MVs (25). *P. aeruginosa* MVs have also been found to stimulate epithelial cells (e.g. in the lung) to secrete IL-8, a chemokine which recruits and activates neutrophils (2). The ability of bacteria to invade tissues is linked to MV binding to host cells (25).

MVs can aid in the delivery of molecules within species, such as the transfer of β -lactamase from β -lactam-resistant strains of *P. aeruginosa* to nonresistant strains (9). The possible utility of excreting MVs to break down β -lactams serves as

an explanation for the lowered drug effectiveness in treating *P. aeruginosa* infections (6). Due to the presence of β -lactamases in MVs, β -lactam antibiotics are predicted to be inactivated upon entry into the vesicles, thus decreasing antibiotic resistance at the infection site.

Mechanism of MV Formation

Though much is now known about the contents of MVs, the mechanism of MV formation has not been fully elucidated. There are currently three proposed models, which are not necessarily mutually exclusive. The first predicts that localized OM detachment from the peptidoglycan is due to decreased lipoprotein linkage in certain areas (19; 31). Wensink and Witholt (1981) originally hypothesized that, since the OM expands faster than the peptidoglycan, it can detach, bulge out, and then invaginate form a vesicle (50). *E. coli* MVs contain very little bound OM lipoprotein, supporting their model. However, the overproduction of the OM relative to the peptidoglycan requires imbalanced growth, whereas MVs may also be produced during balanced growth, casting doubt on the validity of Wensink and Witholt's hypothesis (55).

The second model, proposed by Zhou, et al (1998), predicts that the products of cell wall turnover accumulate in the periplasm, exerting a turgor pressure on the OM, causing it to bulge and bleb out (55). A major building block of peptidoglycan, muramic acid, was detected in MVs, supporting the second model (55).

The third model involves the disruption of the OM caused by changes in the LPS. Kadurugamuwa and Beveridge (1995) found that *P. aeruginosa* MVs are

composed mostly of negatively-charged B-band O-antigen even though the OM contains both B-band and the neutrally-charged A-band O-antigen (20). This evidence suggests that charge-charge repulsion of the LPS in the OM causes membrane instability (20; 6). An investigation by Mashburn and Whiteley (2005) supports this model by showing that PQS engages in ionic interactions with the Mg²⁺ and Ca²⁺ salt bridges that normally stabilize LPS charges (29). These localized areas of the OM then curve out and form MVs. In addition, these studies showed that PQS induces the formation of MVs, as well as its own self-packaging within MVs.

In addition to the role of PQS in causing OM instability the first model, which regards areas of OM with fewer peptidoglycan linkages, is also thought to be relevant for *P. aeruginosa* MV formation. A study done in *Salmonella* by Deatherage, et al (2009) provided quantitative evidence that conserved OM proteins are involved in OM-peptidoglycan and OM-peptidoglycan-IM interactions (11). These proteins help maintain membrane integrity, and play a part in inducing localized MV production through changes in their density and distribution during different cell growth stages.

Peptidoglycan-associated Lipoproteins

Peptidoglycan-associated lipoproteins were first discovered and characterized for the family *Enterobacteriaceae*, but initial studies showed that they did not exist in *P. aeruginosa* (28). However, using the same extraction methods at the lower temperature of 35°C, Mizuno and Kageyama (1979) found that OprF and OprL (then

called Protein F and H, respectively) were associated with peptidoglycan (34). These researchers were also the first to purify the proteins OprF (33,000 kDa), OprI (8,000 kDa), and OprL (18,000 kDa) from *P. aeruginosa*.

P. aeruginosa contains other major OM proteins: Iron repressible outer membrane proteins, receptors which bind iron complexed with siderophores during iron starvation conditions; OprC and OprE, which form channels for trisaccharides; esterase; OprP, a phosphate starvation induced phosphate-specific transport system porin; OprB, a porin involved in glucose uptake; OprD, a porin specific for the β -lactam imipenem; OprG, which is putatively involved in iron uptake; and OprH, which blocks polycationic antibiotic uptake (17). Due to their presence on the cell surface, some Opr proteins are antigenic, as shown by the presence of Opr-specific antibodies in the sera of individuals with previous *P. aeruginosa* exposure (17).

OprF

The function of OprF has been much debated in the literature (17). OprF was originally characterized as a peptidoglycan-associated porin that forms water-filled channels when complexed as trimers (15; 4). To account for the apparent paradox of low OM permeability despite a high porin exclusion limit, the same studies concluded that only about 400 of the 200,000 OprF molecules present per cell actually form large channels (4; 36). These studies hypothesized that low OM permeability also accounts for increased antibiotic resistance in *P. aeruginosa*. Nicas and Hancock (1983) confirmed OprF porin function by showing that *oprF* mutants had lowered permeability (36). These mutants grew poorly without osmotic

stabilizers, such as NaCl, KCl, glucose, sucrose, or potassium succinate (36; 53). Since most OprF channels also appeared to exclude β -lactam antibiotics, Woodruff and Hancock (1988) tested the β -lactam uptake efficiency of OprF (52). Although they only found a low increase in β -lactam resistance for *oprF* mutants in comparison to the wild type, this finding did provide further support that OprF actually exists primarily as small channels.

In the late 1980s, Nakae, et al published results suggesting that OprF was not a porin, thus initiating several years of controversy (54; 14). These conflicting reports were settled by Bellido, et al (1992), with their confirmation that OprF is a porin (3). Sugawara, et al (2006) conclusively resolved the paradox regarding why OprF forms a large channel but yet confers low permeability to the OM (47). They showed that OprF actually exists in two unique conformations: 98% of OprF molecules have a “closed” conformation, while only 2% are in an “open” conformation, corresponding to the large “small” to “large” channel ratio originally proposed by Hancock, et al (4; 36; 52).

The structural role of OprF was hypothesized based upon its association with the peptidoglycan (15; 14). Woodruff and Hancock (1989) investigated this role through comparisons of OprF function with that of its homologue, the *E. coli* major OM protein, OmpA (53). OmpA is a pore-forming protein which exists as an active monomer (46). Woodruff and Hancock’s initial evidence included the shorter and rounder cell morphology of *oprF* mutants (53). They also illustrated that OprF cross-reacts with OmpA, and that *oprF* can complement back wild type morphology in *ompA* mutants. Rawling, et al (1998) showed that the C-terminal 180 amino acids of

OprF are homologous to the C-terminal 168 amino acids of OmpA (39). While the first 154-164 amino acids, which constitute the OprF C-terminal domain, are necessary for the protein's stable expression in the OM, association with the peptidoglycan requires ≥ 215 amino acids (39). Sugawara et al (2006) have proposed that the major function of OprF is actually in OM stabilization, and that its role as a porin is negligible (47).

OprL

OprL has covalently bound fatty acyl chains and is strongly but noncovalently associated with the peptidoglycan (17). Koebnik, et al (1995) proposed that an α -helical motif at the C-terminus interacts with the peptidoglycan (24). OprL is a conserved protein in related *Pseudomonads* (17). The 504 bp open reading frame (ORF) shares 96% sequence homology with the *P. putida* PAL, and 57-64% homology with other PALs, especially at the C-terminal end (27). OprL is required for maintaining OM permeability in *P. putida*, as *oprL* mutants show sensitivity to the presence of SDS, deoxycholate, and EDTA in the medium (41). *E. coli pal* mutants also show increased sensitivity to drugs and detergents, as well as an increased production of MVs (5). Similar to the *oprF* mutant of *P. aeruginosa*, the *P. putida oprL* mutant also does not grow on low osmolarity medium (36; Rodriguez-Herva 1996). The cell morphology of the *P. putida oprL* null mutant also demonstrates a wavy OM (41). This evidence all suggests that *P. aeruginosa* OprL and its homologues have a major role in maintaining OM integrity, possibly through the linkage of the IM and OM.

OprI

Mizuno and Kageyama (1979) isolated OprI from both the OM and the peptidoglycan (34). They provided evidence that OprI is covalently bound to peptidoglycan, but Hancock (1981) found that whether OprI associates covalently or noncovalently with peptidoglycan depends on the strain; in *P. aeruginosa*, OprI is of the latter category (16). OprI is a highly abundant protein that is homologous to the Braun lipoprotein (Lpp), an *E. coli* OM structural component (7; 34). While Braun's lipoprotein has three fatty acid residues, OprI only has one, hexadecanoic acid (34). The *oprI* gene, which is conserved among fluorescent Pseudomonads, was recently tested as a phylogenetic marker for classifying rRNA group I Pseudomonads (12).

Hypothesis

This project concerns the effect of peptidoglycan-associated lipoprotein “anchors” in the OM on MV production. I hypothesize that the absence of OprF, OprL, and OprI in the OM leads to loss of OM integrity, and the increased production of MVs. To test the validity of this MV formation model in *P. aeruginosa*, I generated mutant strains in which the genes encoding the proteins OprF, OprL, and OprI were cleanly deleted in order for their effect on MV production to be examined.

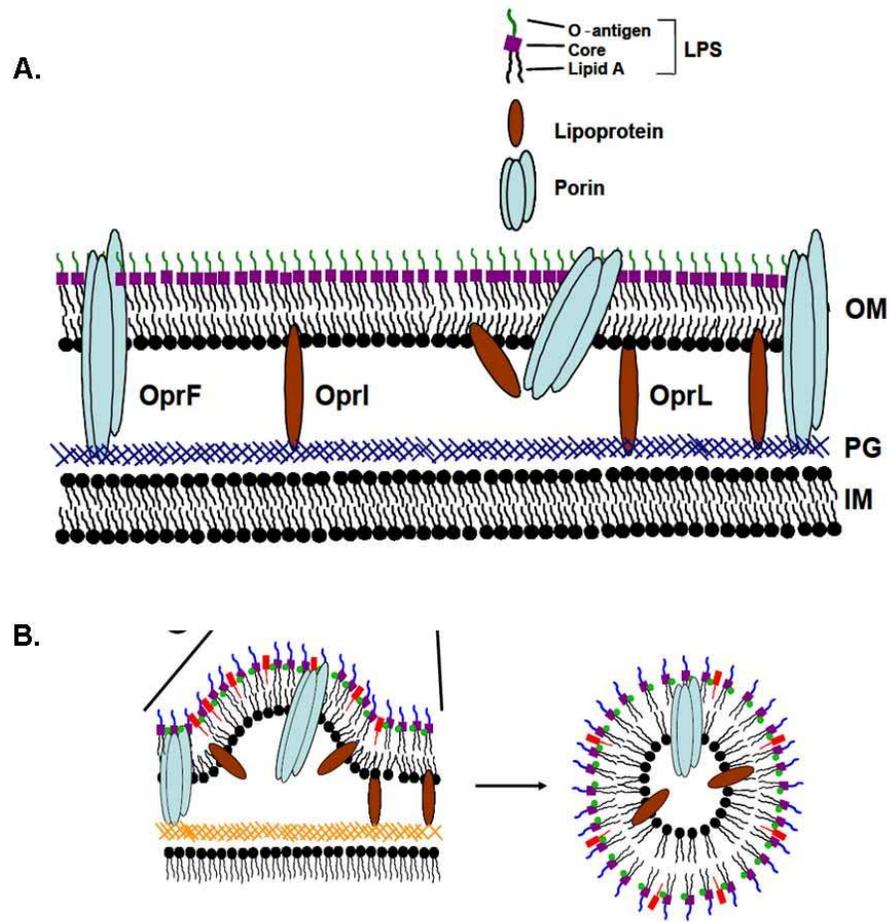


Fig. 1: A. The outer membrane, peptidoglycan, periplasm, and inner membrane of *P. aeruginosa*. The lipoproteins OprF, OprL, and OprI are shown embedded in the OM and associating with the underlying peptidoglycan. **B.** Membrane vesicles form as sections of the OM bulge out and pinch off. I hypothesize that, in the absence of anchorage by peptidoglycan-associated lipoproteins, there is an increased production of MVs. (30)

METHODS

Creation of mutants

Sequences for *oprF*, *oprL* and *oprI* in the *Pseudomonas aeruginosa* strain PA14 were obtained from the *Pseudomonas* Genome Project. To cleanly delete *oprL* and *oprI* from the PA14 chromosome, knockout constructs were first made by PCR-amplifying two 1 kb regions that flank the genes of interest. Four primers were designed for each construct, with BamHI sites on the outermost two primers, P1 and P4, and a region of complementarity between the inner primers, P2 and P3.

<i>oprL</i> primers
P1: 5' CCGGATCCGAGAAGCTCACCGGTATCAAG 3'
P2: 5' GTGCTTGGGCATAACGACTTC CATGTAAC CCTAATGAACCC 3'
P3: 5' GAAGTCGTTATGCCCAAGCAC 3'
P4: 5' CAGGATCCGTAAGTGGGAAATGACCTGCTG 3'
<i>oprI</i> primers
P1: 5' CCGGATCCAGGTAAGTACTCCAGGTTTCAGCCAC 3'
P2: 5' GTTTTCAACAGGTCGTGAGACCGGTGGACA TTTCCATAACAGCAATC 3'
P3: 5' GGTCTCACGACCTGTTGAAAAC 3'
P4: 5' CCGATCCAGGTGATCAAGGCCAAGTAC 3'

Table 1: Primers for the construction of the *oprL* and *oprI* knockout constructs.

The two PCR products for each knockout construct were “sewn” together in a second PCR reaction using the primers P1 and P4.

The resulting 2 kb knockout constructs were TA-cloned into a TOPO TA vector (Invitrogen), and digested using the restriction enzyme BamHI for *oprL*Δ, and EcoRI for *oprI*Δ. The constructs were ligated into the plasmid pEX18TC, which carries a tetracycline resistance cassette, *tet*, and the *Bacillus subtilis* gene, *sacB*, which

encodes for a levansucrase (18). The vector was first transformed into SM10 *Escherichia coli* and then transferred into PA14 via biparental conjugation. pEX18TC contains an *oriT* site, while SM10 carries the mobilization factors *mob* and *tra*, which are all necessary for conjugation to occur. Since pEX18TC is a suicide vector in *P. aeruginosa*, chromosomal integration of the plasmid was selected for using tetracycline. The integration event was verified with PCR using primers designed to amplify the *sacB* open reading frame (5-*sacB*: 5' GCAACAGTATTAACCTTTAC 3' and 3-*sacB*: 5' GTTAACTGTTAATTGTCCTTG 3').

Resolution of the merodiploid state, which resulted in the deletion of the wild type gene, was positively selected for by plating culture dilutions onto Luria Bertani (LB) agar supplemented with 5% sucrose. True mutants no longer contained *sacB*, which has lethal effects on the cell in the presence of sucrose. Colonies were then patched onto LB agar supplemented with 5% sucrose and LB agar containing 50 µg/mL tetracycline in order to select for sucrose-tolerant, tetracycline-sensitive colonies. Negative selection using tetracycline was necessary due to the high *sacB* mutation rate. Clean deletions of *oprL* and *oprI* were verified by PCR using primers for the regions externally flanking the knockout constructs. The resultant PCR products were then sequenced.

<i>oprL</i> Δ verification
oprL-out-flank-5: 5' GGAAGGCAGTTGGCGTCAAC 3'
oprL-out-flank-3: 5' GTGACGACTGAACAGGTCGTC 3'
oprL-internal-5: 5' GCT ACA CCAACT TCC AGA TC 3'
oprL-internal-3: 5' GGA AATCCGACGGTCGAGGTC 3'
<i>oprI</i> Δ verification
oprI-out-flank-5: 5' GCAACTGCCGATCCATTCCTC 3'
oprI-out-flank-3: 5' CCGTTGTCTCACTGTCTTTTCG 3'
oprI-internal-5: 5' CGGCTAATCAAGTTAACCGG 3'
oprI-internal-3: 5' GTGCGTATCATCAACGAGCC 3'

Table 2: Outer flanking primers used for mutant verification.

To complement the *oprL*Δ and *oprI*Δ mutants, *oprL* and *oprI* were ligated into the arabinose-inducible pJN105 plasmid using XbaI and EcoRI digest sites. Empty pJN105 plasmid was electroporated into PA14 and each mutant strain as a control. Induction with arabinose was not necessary due to the basal level of promoter expression.

Knockout Construction

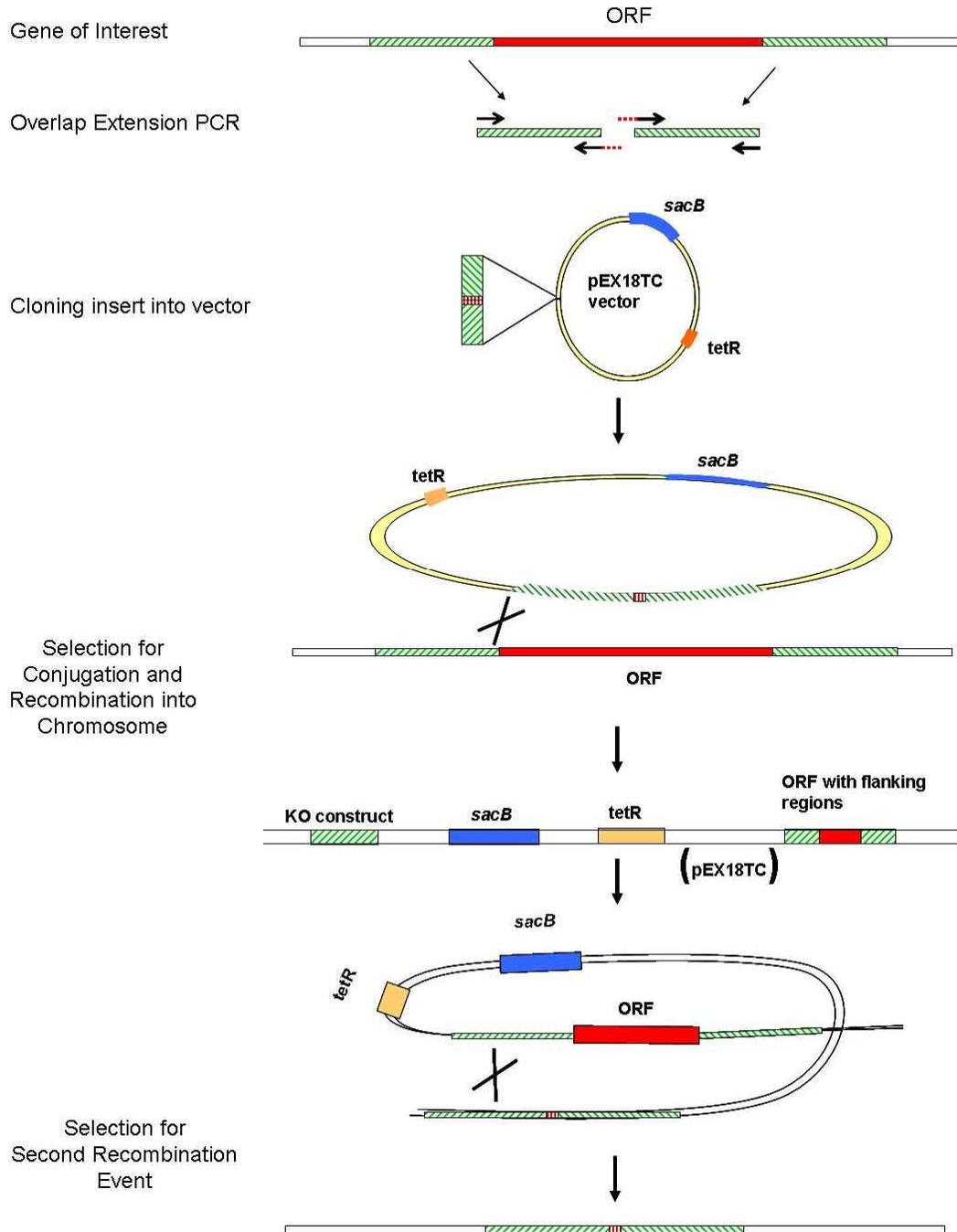


Fig. 2. External regions flanking the genes of interest were amplified and ligated together using PCR to create the knockout construct. The construct was cloned into pEX18TC. The vector was transferred into PA14, where it recombined into the chromosome. Clean deletions of the wild type genes were selected for using positive and counter-selection.

A clean deletion of *oprF* was attempted but was unsuccessful. While the pEX18TC-*oprF* Δ merodiploid was obtained, no knockouts could be generated. In order to generate the mutant, the growth conditions were modified by either supplementing LB with NaCl, or using Mueller Hinton broth. Over sixty colonies, which grew on LB (or Mueller Hinton) agar supplemented with 5% sucrose but not on LB (or Mueller Hinton) agar containing 50 μ g/mL tetracycline, were tested. PCR using primers for regions flanking the knockout constructs (*oprF*-out-flank-5: 5' GTTGGCCTCGAATACGCGAAC 3' and *oprF*-out-flank-3: 5' GTTGAAAGAACTCTGGCTTAC 3') showed that these strains had all crossed back to wild type.

Instead, a PA14 *oprF*- strain was taken from the Non-Redundant Transposon Insertion Mutant Set from the Ausubel Laboratory at Harvard Medical School. The mutant contains a MAR2xT7 transposon insertion, which includes a gentamicin-resistance cassette. Since the insertion is upstream of amino acid 215, the resulting truncated protein cannot associate with the peptidoglycan (39), and thus should not be able to tether the OM to the peptidoglycan.

To complement *oprF*-, the ApR/CbR plasmid pEX1.8, which has an IPTG-inducible *ptac* promoter (a combination of the tryptophan and lactose promoters) was used. *oprF* was TA cloned into the pGEM T-easy vector (Promega) and the gene was digested out using a PstI cut site from the vector backbone, along with a HindIII site that was added onto the reverse primer used to PCR-amplify the gene (5' CCCAAGCTTTTTTCCTTAGAGGCTCA 3'). These sites corresponded with the 5' PstI and 3' HindIII sites downstream of the *ptac* promoter in pEX1.8. The insert was

ligated into pEX1.8, and the vector was electroporated into *oprF*⁻. Empty pEX1.8 plasmid was electroporated into both PA14 and *oprF*⁻ as a control. The vector was induced using 500 µM IPTG.

The doubling time for *oprF*⁻ + pEX1.8 in BHI containing 50 µg/mL carbenicillin was 42 min, as compared to 30 min for both wild type + pEX1.8 and the mutant complemented. The doubling time for *oprL*Δ + pJN105 in BHI containing 25 µg/mL gentamicin was 52 min as compared to 37 min for both wild type + pJN105 and the mutant complemented.

Preparation of Membrane Vesicles

The strains were grown overnight in 25 mL Brain Heart Infusion (BHI) broth at 37°C shaking with the appropriate antibiotics (25 µg/mL gentamicin for strains containing pJN105, and 50 µg/mL carbenicillin for strains containing pEX1.8). Stationary stage cells were pelleted by centrifugation at 5000 xg for 15 min at room temperature. The supernatant containing the MVs was filtered through a .45 µm pore syringe filter (Pall), and 3 mL were removed for lipid quantification. Vesicles from the remaining filtered supernatant were pelleted by ultracentrifugation (Beckman-Coulter Optima L-100K) at 60,000 xg for 1 hr 6 min at 15°C, along with a sterile BHI control of equal volume. The supernatant was removed and the vesicle pellet was resuspended in MV buffer (1.514 g Tris, .073 g NaCl, .030g MgSO₄, suspended in 250 mL deionized distilled water at a pH of 7.2-7.5). The resuspended pellet was then spun again in the ultracentrifuge at 60,000 xg for 1 hr 6 min at 15°C. The pellet was then resuspended in 500 µL MV buffer and filtered through a 300K Nanosep

centrifugal device (Pall Life Sciences) using a tabletop centrifuge, at 8000 xg for 5 min. Vesicles were resuspended in a 50 μ L final volume of MV buffer.

Lipid Quantification Assays

Lipid assays were performed to quantitate the amount of lipid harvested from the MV preps. The assay was adapted from a method by Stewart (1979) (44). The color change is produced by phospholipids complexing with ammonium ferrothiocyanate. The colored complex partitions into the chloroform phase and is easily removed for an absorbance reading, since ammonium ferrothiocyanate is insoluble in chloroform, while lipid is soluble in chloroform.

The protocol is as follows: The 3 mL of filtered supernatant taken from the original filtered culture was transferred into a 20 mL glass vial. 3 mL chloroform was added to each sample, which was vortexed. Samples were centrifuged at 2000 xg for 2 min. The bottom chloroform layer (about 2400 μ L) was transferred to a new 20 mL glass vial and 2400 μ L ammonium ferrothiocyanate (stock solution of 27.03 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 30.4 g NH_4SCN dissolved in 1L deionized distilled water) was added, and the sample was vortexed. The sample was centrifuged at 2000 xg for 5 min. The bottom layer (about 1800 μ L) was moved into a 4 mL glass vial and dried down under nitrogen. The dried sample was resuspended in 1000 μ L chloroform and its absorbance was read at 470 nm. A standard curve was calculated using a 1 mg/mL stock of phospholipid suspended in chloroform.

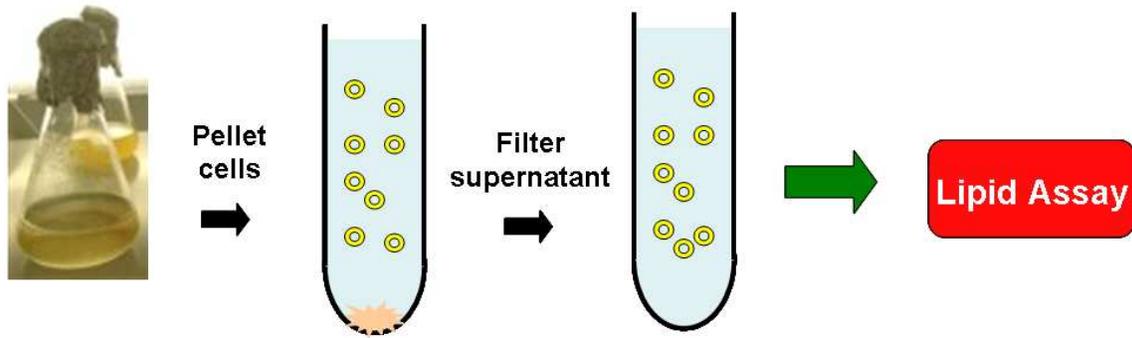


Fig. 3: Steps preceding lipid quantification. Cultures are pelleted by centrifugation, and the filtered supernatant is used in the lipid assay.

Protein Quantification Assays

Total protein from vesicle preparations was quantified using the Coomassie Plus (Bradford) Assay Kit (Thermo Scientific), using a 25 μ L aliquot of the MV prep. Protein concentration was determined by the color change caused by Coomassie Blue binding to proteins in the sample. Absorbance was read spectrophotometrically at a wavelength of 595 nm. A standard curve was calculated using dilutions of a 2 mg/mL bovine serum albumin (BSA) stock (Thermo Scientific).

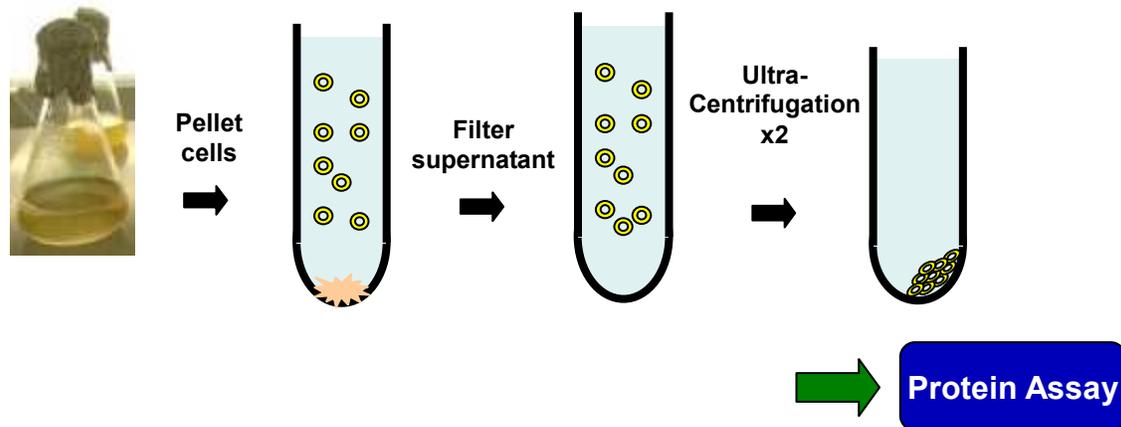


Fig. 4: Steps preceding protein quantification. Cultures are pelleted by centrifugation and supernatant is filtered. Vesicles are pelleted by ultracentrifugation and washed. A Bradford protein assay is then performed.

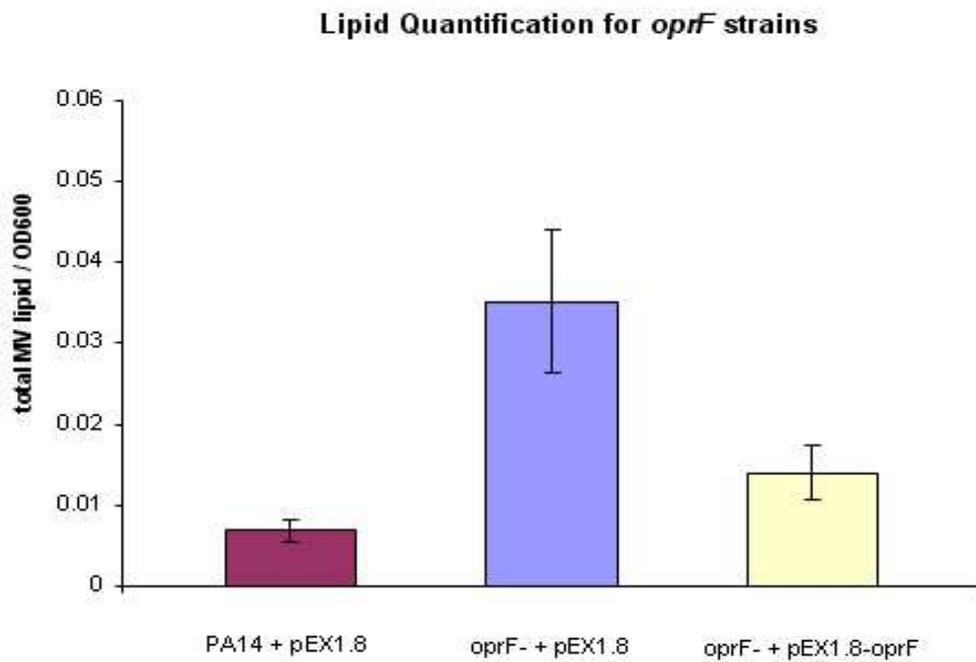
Electron Microscopy

Vesicles were fixed with 3% glutaraldehyde and stored at 4°C for Transmitting Electron Microscope (TEM) imaging. 400 nm copper mesh grids were coated with Formvar and allowed them to dry overnight. For negative staining, fixed vesicles were placed on the grids with a 1:1 ratio of 2% uranyl acetate. Images were visualized with a FEI Tecnai TEM, equipped with an AMT Advantage HR 1kx1k digital camera.

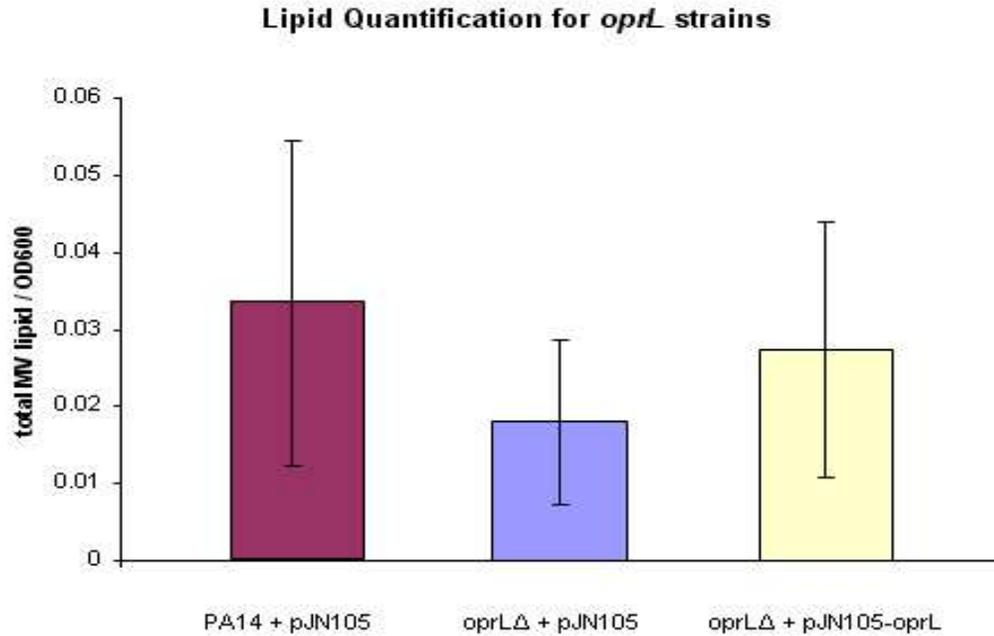
RESULTS

The concentrations of protein ($\mu\text{m}/\text{mL}$) and lipid (mg/mL) were determined from standard curves performed on the same day as the assays. Normalized values were obtained by dividing total protein and lipid over the OD_{600} of the starting cultures. Four experimental replicates were performed for each mutant.

A.



B.



C.

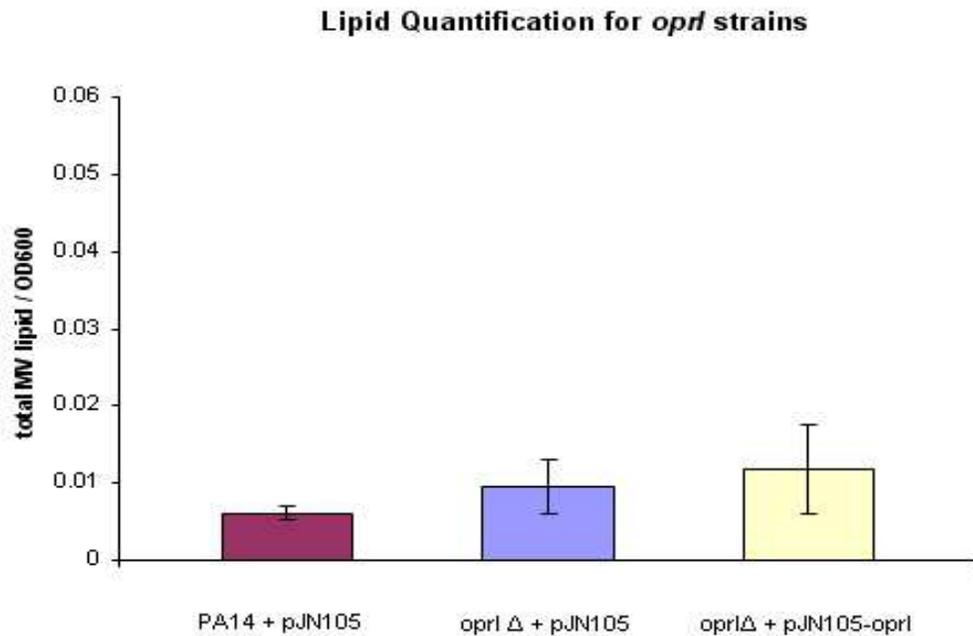
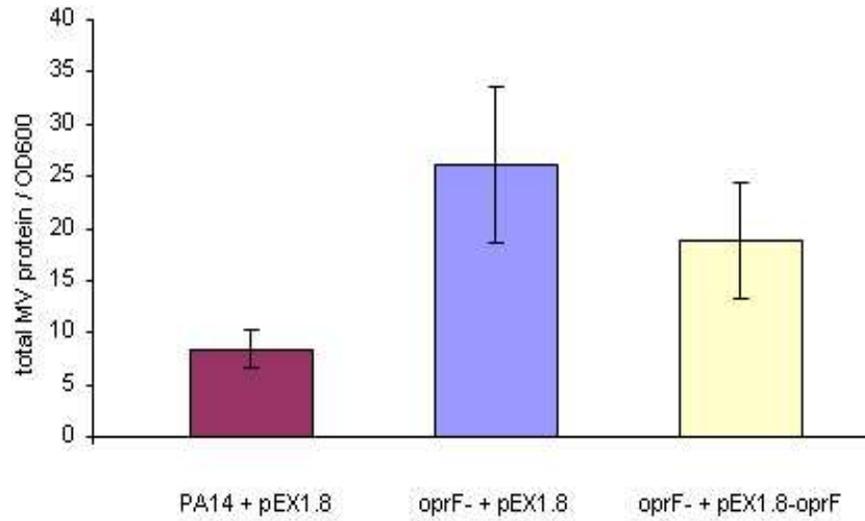


Fig. 5: **A.** Lipid quantification of MV preps for wild type, *oprF* mutant, and *oprF* mutant complemented with the wild type gene. **B.** Lipid quantification of MV preps for wild type, *oprL* mutant, and *oprL* mutant complemented with the wild type gene. **C.** Lipid quantification of MV preps for wild type, *oprI* mutant, and *oprI* mutant complemented with the wild type gene.

Error bars represent the standard error of the mean.

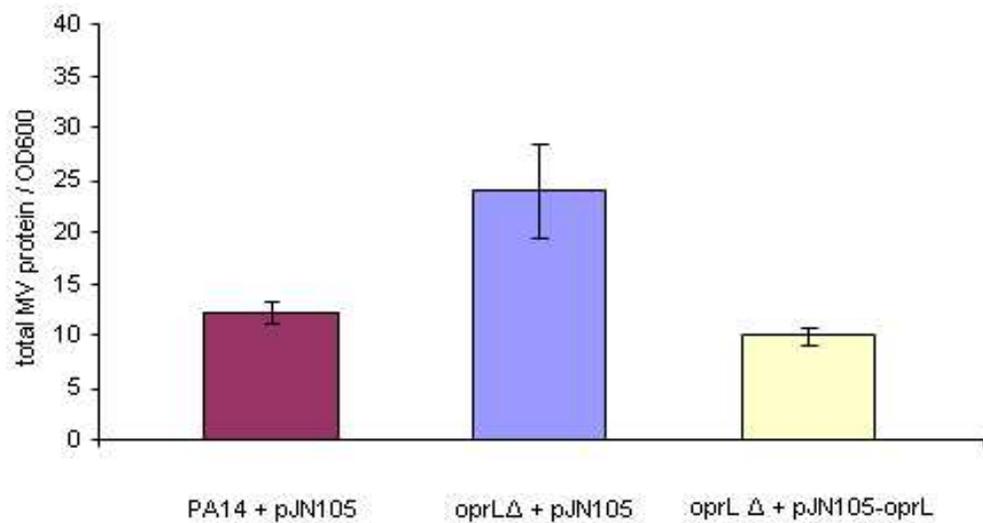
A.

Protein Quantification for *oprF* strains



B.

Protein Quantification for *oprL* strains



C.

Protein Quantification for *oprI* strains

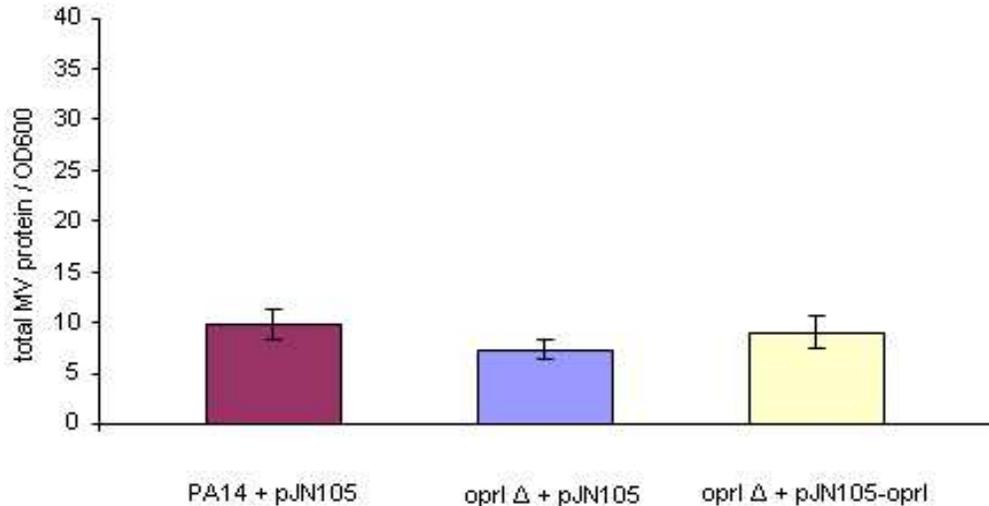


Fig. 6: **A.** Protein quantification of MV preps for wild type, *oprF* mutant, and *oprF* mutant complemented with the wild type gene. **B.** Protein quantification of MV preps for wild type, *oprL* mutant, and *oprL* mutant complemented with the wild type gene. **C.** Protein quantification of MV preps for wild type, *oprI* mutant, and *oprI* mutant complemented with the wild type gene.

Error bars represent the standard error of the mean.

I hypothesized that the OM proteins OprF, OprL, and OprI act as anchors, tethering the OM to the peptidoglycan. The absence of these proteins causes increased MV production as the destabilized OM blebs off. In support of the model, the data from both lipid and protein quantification assays show a three-fold increase in MV production of the *oprF* mutant compared to the wild type. Lipid quantification assays indicate that there is no significant difference in MV production between the *oprL* and *oprI* mutants and the wild type.

Preliminary TEM images show that the average diameter of MVs produced by *oprF*- is 72 nm. Diameters were measured using ImageJ, with n = 67. These MVs

fall within the reported range of 50 – 150 nm for wild type *P. aeruginosa* (20).

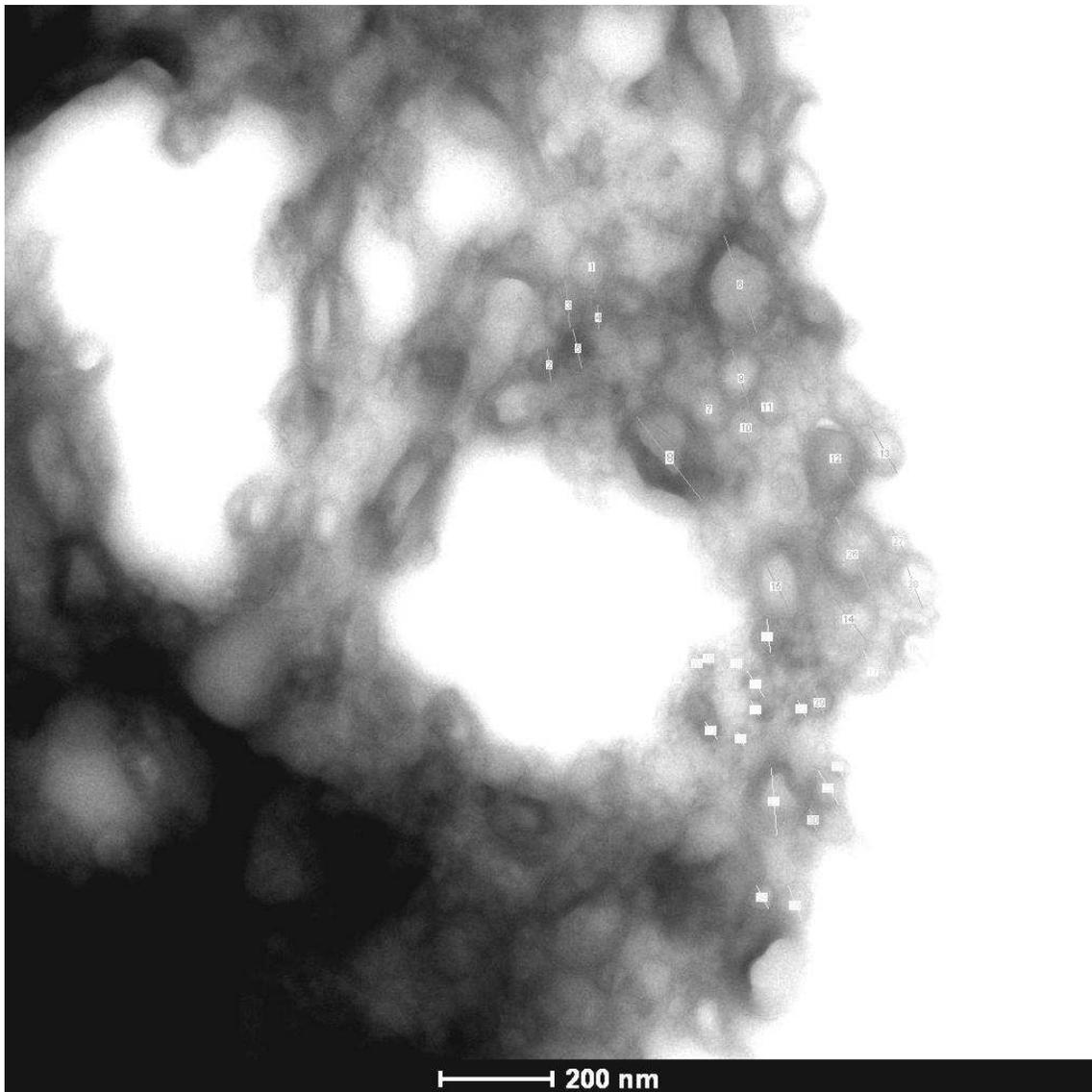


Fig. 7. TEM image of *oprF*- MVs, with measured diameters.

DISCUSSION

The data show that *Pseudomonas aeruginosa oprF* mutants produce three-fold more membrane vesicles than the wild type. These results further support the role of OprF in providing stability to the OM, through its association with the underlying peptidoglycan. OprL and OprI do not appear to have a major role as membrane anchors. The difference between these two proteins and OprF may be due to the latter's larger size and greater frequency within the OM.

These results give weight to the first model of MV formation, which hypothesizes that areas of OM containing fewer protein connections to the underlying peptidoglycan undergo increased MV formation. The utility of the third model, which proposes that changes in the LPS cause the ensuing destabilization of the OM, has already been shown for *P. aeruginosa* (29). The results shown in this study support for the conclusion made by Deatherage, et al (2009) that conserved OM proteins act as potentially unifying factors in MV release (11). Here, the relevant *P. aeruginosa* OM proteins have been tested to ascertain the validity of that assertion.

In a broader view, these studies on particular OM-peptidoglycan connecting proteins provide more background for *P. aeruginosa* MV formation. The ability of *P. aeruginosa* to release MVs is widely-studied phenomenon, as this process shows how these bacteria are able to “talk to its friends and attack its enemies,” as one reviewer so deftly put it (51).

The data presented here also shed more light on the role of OprF in stabilizing the OM, which has been speculated upon since the protein's discovery in

the 1970s. Interest in the structural role of OprF has been renewed by more recent investigations that concentrate on the importance of the protein for structuring the OM, rather than in regulating OM permeability.

My future work will include the construction of double mutants (*oprF-oprL* Δ , *oprF-oprI* Δ , and *oprL* Δ *oprI* Δ), and determining MV production for each. It is expected that the *oprF-oprL* Δ and *oprF-oprI* Δ mutants will show increased MV production as compared to the wild type, following the findings for the *oprF*- single mutant. It would also be interesting to examine the effects an *oprL* Δ *oprI* Δ double mutant would have on MV production, since the *oprL* Δ and *oprI* Δ single mutants do not have a significant effect.

Further TEM imaging will be performed in order to compare the diameters of MVs produced by each mutant to those produced by the wild type. This analysis will show whether MVs produced by mutants differ in size, and whether increased MV produced results in irregularly-shaped MVs.

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