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Role of *Pseudomonas aeruginosa* Peptidoglycan-Associated Outer Membrane Proteins in Vesicle Formation

Aimee K. Wessel,^a Jean Liew,^a Taejoon Kwon,^{b,c} Edward M. Marcotte,^{b,c,d} Marvin Whiteley^{a,b,c}

Section of Molecular Genetics and Microbiology, Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, Texas, USA^a; Center for Systems and Synthetic Biology, Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, Texas, USA^b; Cellular and Molecular Biology Program, Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, Texas, USA^c; Department of Chemistry and Biochemistry, University of Texas at Austin, Austin, Texas, USA^d

Gram-negative bacteria produce outer membrane vesicles (OMVs) that package and deliver proteins, small molecules, and DNA to prokaryotic and eukaryotic cells. The molecular details of OMV biogenesis have not been fully elucidated, but peptidoglycan-associated outer membrane proteins that tether the outer membrane to the underlying peptidoglycan have been shown to be critical for OMV formation in multiple *Enterobacteriaceae*. In this study, we demonstrate that the peptidoglycan-associated outer membrane proteins OprF and OprI, but not OprL, impact production of OMVs by the opportunistic pathogen *Pseudomonas aeruginosa*. Interestingly, OprF does not appear to be important for tethering the outer membrane to peptidoglycan but instead impacts OMV formation through modulation of the levels of the *Pseudomonas* quinolone signal (PQS), a quorum signal previously shown by our laboratory to be critical for OMV formation. Thus, the mechanism by which OprF impacts OMV formation is distinct from that for other peptidoglycan-associated outer membrane proteins, including OprI.

Vesiculation is a highly conserved process occurring in all domains of life (1–4). Among prokaryotes, vesicle formation has been reported in both Gram-negative and Gram-positive bacteria (1, 2, 4, 5). Gram-negative bacteria produce spherical, bilayered vesicles derived from the outer membrane that range in size from 20 to 500 nm (6–10). Similar to the outer membrane, outer membrane vesicles (OMVs) possess an outer leaflet of lipopolysaccharide (LPS) and an inner leaflet of phospholipid (11–15). OMVs also contain outer membrane proteins and entrap periplasmic components as they are released (16–18). OMVs have been found to be associated with Gram-negative bacteria growing planktonically and in surface-attached biofilm communities as well as natural environments (11, 19–21).

Despite their biological importance, the molecular mechanism of OMV formation has not been fully elucidated, though multiple factors have been reported to affect the process (13, 22–28) and numerous models encompassing these factors have been proposed (9, 19, 28–31). A primary hurdle to elucidating the mechanism of OMV formation has been the inability to identify factors that contribute to OMV production. Using the model opportunistic pathogen *Pseudomonas aeruginosa*, our laboratory demonstrated that the quorum-sensing signal 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal [PQS]) stimulates *P. aeruginosa* OMV biogenesis (10, 32). Surprisingly, signaling by PQS was not required for OMV formation (10); instead, OMV formation proceeds through direct interaction of PQS with the LPS component of the outer membrane (29). Based on these results, we recently proposed a detailed *P. aeruginosa* OMV biogenesis model, dubbed the bilayer-couple model, in which PQS induces membrane curvature by stably inserting and expanding the outer leaflet of the outer membrane relative to the inner leaflet (31), resulting in localized membrane curvature and ultimately vesiculation.

One question regarding the bilayer-couple model that remains is the role that peptidoglycan-associated outer membrane proteins play in *P. aeruginosa* OMV biogenesis. Multiple studies in bacteria other than *P. aeruginosa* have suggested that OMV for-

mation is localized to regions of the outer membrane not tethered to the underlying peptidoglycan layer (6, 8, 33). Loss of the peptidoglycan-associated outer membrane protein OmpA, Pal, or Lpp significantly increases OMV formation in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Vibrio cholerae* (6, 34–37). Homologs of OmpA, Pal, and Lpp exist in *P. aeruginosa*, although their involvement in OMV biogenesis is not known (38). OprF is a 38-kDa OmpA homolog that serves both as a porin and as a tether that noncovalently links the outer membrane to peptidoglycan (39). OprF exists in two conformations: when closed, the C terminus anchors the outer membrane to the peptidoglycan layer, and when open, the C terminus inserts into the outer membrane, forming a functional porin (40). OprL is an 18-kDa Pal homolog that also tethers the outer membrane to peptidoglycan (41–44). Finally, OprI is an 8-kDa homolog of Braun's lipoprotein (Lpp) and is proposed to covalently interact with the peptidoglycan layer (45), though this interaction has been reported to differ among *P. aeruginosa* strains (39, 46). OprI is highly abundant in the outer membrane (46) and, similar to *E. coli* Lpp, can exist in a free and a peptidoglycan-bound form (46).

The goal of this study was to assess the involvement of these three peptidoglycan-associated outer membrane proteins in *P. aeruginosa* OMV biogenesis. Here we demonstrate that deletion of *oprF* and *oprI* induces *P. aeruginosa* vesiculation through two distinct mechanisms. The absence of OprF increases OMV production via increased PQS production, while loss of OprI presumably

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Address correspondence to Marvin Whiteley, mwhiteley@mail.utexas.edu.

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TABLE 1 Strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
<i>E. coli</i>		
DH5 α	<i>endA1 hsdR17 supE44 thi-1 recA1 Δ(lacZYA-argF)U169 deoR ϕ80dlacZΔM15</i>	47
SM10	<i>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu</i> (Km ^r)	48
<i>P. aeruginosa</i>		
PA14	Wild type	49
<i>oprL</i> mutant	PA14 Δ <i>oprL</i>	This study
<i>oprI</i> mutant	PA14 Δ <i>oprI</i>	This study
<i>oprF</i> mutant	PA14 <i>oprF::Mar2XT7</i> (Gm ^r)	49
<i>pqsH</i> mutant	PA14 Δ <i>pqsH</i>	This study
<i>oprF pqsH</i> mutant	PA14 <i>oprF::Mar2XT7 ΔpqsH</i> (Gm ^r)	This study
<i>oprI pqsH</i> mutant	PA14 Δ <i>oprI ΔpqsH</i>	This study
Plasmids		
pGEM-T Easy	Sequencing vector	Promega
pEX18Tc	Gene replacement vector (<i>oriT</i> ⁺ <i>sacB</i> ⁺ Tc ^r)	50
pEX18Tc- <i>oprL</i>	pEX18Tc containing 1-kb sequences flanking <i>oprL</i>	This study
pEX18Tc- <i>oprI</i>	pEX18Tc containing 1-kb sequences flanking <i>oprI</i>	This study
pEX18Tc- <i>pqsH</i>	pEX18Tc containing 1-kb sequences flanking <i>pqsH</i>	This study
pEX1.8	Broad-host-range expression vector, IPTG inducible (Ap ^r)	51
pEX1.8- <i>oprF</i>	pEX1.8 carrying <i>oprF</i>	This study
pEX1.8- <i>oprI</i>	pEX1.8 carrying <i>oprI</i>	This study

decreases tethering of the outer membrane to peptidoglycan. These findings are presented in the context of the membrane bilayer-couple model to provide a working model for *P. aeruginosa* OMV biogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or tryptic soy broth (TSB) with ampicillin (100 μ g/ml) or tetracycline (10 μ g/ml), when appropriate. *P. aeruginosa* strains were grown in brain heart infusion (BHI) broth with carbenicillin (150 μ g/ml), gentamicin (50 μ g/ml), or tetracycline (50 μ g/ml), when appropriate.

DNA manipulations. DNA manipulations were performed using standard procedures (52). PCR was performed using an Expand Long Template PCR system (Roche). A QIAprep spin miniprep kit (Qiagen) or GeneJET plasmid miniprep kit (Fermentas) was used for plasmid purification. Restriction endonucleases and buffers were purchased from New England BioLabs or Fermentas Life Sciences. A DNeasy tissue kit (Qiagen) was used to extract chromosomal DNA. DNA sequencing was performed at the DNA Core Facility at the University of Texas Institute for Cell and Molecular Biology.

Construction of *P. aeruginosa* deletion strains. Unmarked deletions in *oprL*, *oprI*, and *pqsH* were made via allelic exchange as previously described (50) with some modifications. Deletion plasmids were constructed using the primer pairs listed in Table 2. The two amplicons were combined using overlap extension PCR and digested using BamHI (for *oprL* and *oprI* deletions) or EcoRI and XbaI (for *pqsH* deletion) and ligated into pEX18Tc. Each deletion plasmid was transformed into *E. coli* SM10 and conjugated into *P. aeruginosa* PA14. For the *P. aeruginosa oprF pqsH* double mutant, the *pqsH* deletion plasmid was conjugated into the *oprF* mutant. Mutant selection was performed as previously described (50), with some modifications. To select the pEX18Tc-*oprL*, pEX18Tc-*oprI*, and pEX18Tc-*pqsH* transconjugants, conjugations were spread onto LB plates with 50 μ g/ml tetracycline and 25 μ g/ml nalidixic acid. To select for the pEX18Tc-*pqsH* transconjugant in the *oprF* mutant background, conjugations were spread on a morpholinepropanesulfonic acid (MOPS)-

buffered defined medium (25 mM MOPS [pH 7.2], 93 mM NH₄Cl, 43 mM NaCl, 3.7 mM KH₂PO₄, 1 mM MgSO₄, 3.5 μ M FeSO₄ · 7H₂O) supplemented with ~1.25% agarose, 20 mM succinate, and 20 μ g/ml tetracycline. To select for the *oprF pqsH* double mutant, transconjugants

TABLE 2 Primer sequences

Name	Sequence ^a
<i>oprL</i> -flanking regions	
<i>oprL</i> -P1	CCGGATCCGAGAAGCTCACCGGTATCAAG
<i>oprL</i> -P2	GTGCTTGGGCATAACGACTTCCATGTAATC CTAATGAACCC
<i>oprL</i> -P3	GAAGTCGTTATGCCCAAGCAC
<i>oprL</i> -P4	CAGGATCCGTAAGTGGAAATGACCTGCTG
<i>oprI</i> -flanking regions	
<i>oprI</i> -P1	5'-CCGGATCCAGGTAAGTCCAGGTTCCAGCCAC
<i>oprI</i> -P2	5'-GTTTTCAACAGGTCGTGAGACCGGTGGA CATTTCCATAACAGCAATC
<i>oprI</i> -P3	5'-GGTCTCACGACCTGTTGAAAAC-3'
<i>oprI</i> -P4	5'-CCGGATCC AGGTGATCAAGGCCAAGTAC-3'
<i>pqsH</i> -flanking regions	
<i>pqsH</i> -P1	5'-CTGAATTCCTTGTCCCTGCAGGTCGATATC-3'
<i>pqsH</i> -P2	5'-CATCGCCGAAGTCCGAAAACAGGATAAGAA CGGTCATCCGGTTC-3'
<i>pqsH</i> -P3	5'-GCAACGGATGACCGTTCTTATCCTGTTTT CGAGTTCGGCGATG-3'
<i>pqsH</i> -P4	5'-CTTCTAGAGATTGCTACAGGTAGCGAGG-3'
Complementation	
<i>oprF</i> -for	5'-CTAACTGACCATCAAGATGGG-3'
<i>oprF</i> -rev	5'-CCC <u>AAGCTT</u> TTTTCTTAGAGGCTCA-3'
<i>oprI</i> -for	5'-CGGAATTCGTTCCACCTTAAGGGGAAC-3'
<i>oprI</i> -rev	5'-CCC <u>AAGCTT</u> CAGGTCGTGAGACCTAT-3'

^a Underlined sequences represent recognition sites for restriction endonucleases.

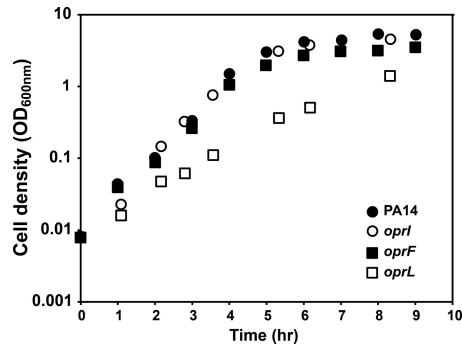


FIG 1 Growth characteristics of wt *P. aeruginosa* and the *oprI*, *oprF*, and *oprL* mutants. Bacteria were grown with shaking (250 rpm) at 37°C in BHI. Representative growth curves are shown.

were grown overnight in MOPS supplemented with 20 mM succinate and 20 μ g/ml tetracycline, diluted into antibiotic-free medium, and spread onto LB plates supplemented with 10% sucrose. Mutants were confirmed by PCR and sequencing.

Complementation of the *P. aeruginosa* *oprF* and *oprI* mutants. *oprF* and *oprI* were PCR amplified from PA14 chromosomal DNA using the primer pairs indicated in Table 2. The *oprF* PCR product was cloned into the pGEM-T Easy vector (Promega) and digested with PstI and HindIII. The *oprI* PCR product was purified and digested using EcoRI and HindIII. Purified digested products were separately ligated into PstI/HindIII- or EcoRI/HindIII-digested pEX1.8, and the resulting plasmids (pEX1.8-*oprF* and pEX1.8-*oprI*) were verified via DNA sequencing. It should be noted that the *oprI* gene amplified and cloned in this study contained 3 base pair differences from the published PA14 genome, resulting in codon changes H36D, X47E, and K79N. Plasmids were electroporated into the *oprF* mutant and *oprI* mutant (52). Isopropyl- β -D-1-thiogalactopyranoside (IPTG) was added to cultures at 500 μ M to induce gene expression.

OMV preparation. For OMV preparation, *P. aeruginosa* overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.001 to 0.05 in BHI broth. Cells were grown to an OD_{600} of 2.7 to 3.9 with shaking at 250 rpm in a 1:10 culture volume/flask volume ratio. When adding exogenous PQS, synthetic PQS resuspended in 500 μ l methanol was added to 25 ml BHI before adding cells, such that the final concentrations of PQS in the culture were 0.5, 10, 20, and 40 μ M. OMVs were purified using methods described previously (13). Briefly, cells were removed by centrifugation (5,000 \times g for 15 min), and the resulting supernatant was filtered through a 0.45- μ m-pore-size membrane (Whatman PuraDisc 25-mm-diameter syringe filters, polyethersulfone [PES]). OMVs were pelleted from cell-free supernatants using an ultracentrifuge with a Beckman 70Ti rotor at 265,000 \times g for 1 h and resuspended in MV buffer (50 mM Tris, 5 mM NaCl, 1 mM $MgSO_4$, pH 7.4).

OMV quantification. OMV production was quantified using a previously described phospholipid assay of purified vesicles (53, 54) with some modifications. Purified OMV pellets were extracted with 2 volumes of chloroform, dried under N_2 gas, and resuspended in chloroform (500 μ l or 1 ml chloroform). The absorbance was measured at 470 nm and normalized by the OD_{600} of the extracted culture. To determine the linear range of detection for the assay, commercially available phosphatidylethanolamine (PE; Fluka Biochemika) was used to generate a standard curve with concentrations ranging from 7.8 to 250 μ g/ml. Measurements made below the limit of detection were assigned a value equal to the lowest limit of the standard curve.

PQS and HHQ extraction and quantification. PQS and HHQ were extracted from cultures using 2 volumes of acidified ethyl acetate (acidified with 0.1 ml acetic acid/liter ethyl acetate). The organic phase was removed and dried under a continuous stream of N_2 gas. PQS was quantified using thin-layer chromatography (TLC) (53). For TLC, dried sam-

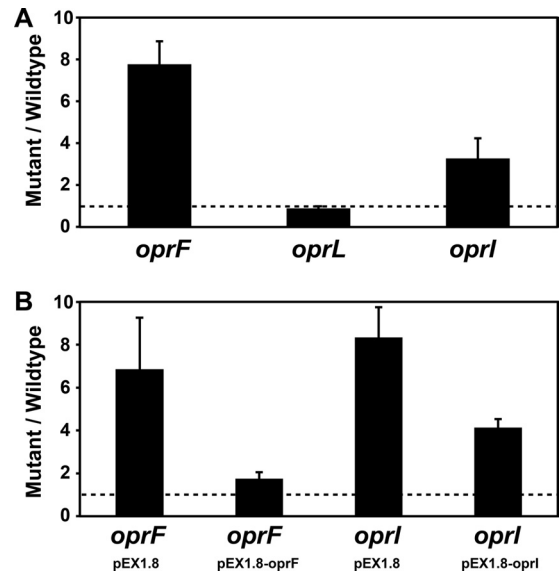


FIG 2 Inactivation of *oprF* and *oprI* increases *P. aeruginosa* OMV production. (A) Fold change in OMV production by the *P. aeruginosa* *oprF*, *oprL*, and *oprI* mutants. Bacteria were grown with shaking (250 rpm) at 37°C to an OD_{600} of \sim 3.5, and OMVs were quantified by measuring OMV lipid. All lipid measurements were normalized to cell number. For each replicate, the fold change in OMV production was calculated by dividing mutant lipid levels by wt lipid levels. The dotted line represents no change in OMV production. Error bars represent standard errors of the means ($n \geq 4$). (B) Complementation of the *oprF* and *oprI* mutants. The fold change in OMV production by the *oprF* and *oprI* mutants carrying either vector alone (pEX1.8) or the complementation plasmids (pEX1.8-*oprF* or pEX1.8-*oprI*) is shown. Bacteria were grown with shaking (250 rpm) at 37°C to an OD_{600} of \sim 3.5 with 500 μ M IPTG. OMVs were quantified and compared to those of wt *P. aeruginosa* carrying pEX1.8 as described for panel A. Error bars represent standard errors of the means ($n \geq 4$).

ples were resuspended in methanol (Optima grade; Fisher), and 5 μ l was spotted onto a dried straight-phase phosphate-impregnated TLC plate. Samples were separated using a 95:5 dichloromethane-methanol mobile phase. Synthetic PQS standards were used to generate a standard curve. PQS spots were measured via photography with excitation by long-wave UV light. HHQ was quantified using high-performance liquid chromatography (HPLC) as previously described (66).

Proteomics. Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) was performed as described previously (55). Briefly, OMVs were isolated as described above and resuspended in lysis buffer (25 mM Tris-HCl [pH 7.5], 5 mM dithiothreitol [DTT], 1.0 mM EDTA, 1 \times Calbiochem protease inhibitor cocktail [CPICPS]). Fifty microliters of diluted OMV lysate was incubated at 55°C for 45 min with 50 μ l of trifluoroethanol (TFE) and 15 mM DTT, followed by incubation with 55 mM iodoacetamide (IAM) in the dark for 30 min. The sample volume was adjusted to 1 ml with buffer (50 mM Tris, pH 8.0), followed by a 1:50 (wt/wt) trypsin digestion for 4.5 h. The reaction was halted by adding 2% (vol/vol; 20 μ l) formic acid. The sample was lyophilized, resuspended with buffer C (95% H_2O , 5% acetonitrile, 0.01% formic acid), and cleaned using a C_{18} tip (Thermo Fisher Scientific). The eluted sample was again lyophilized, resuspended with 120 μ l buffer C, and filtered through an Amicon Ultra-0.5 filter (for 12 min at 14,000 \times g at 4°C). Each sample was injected 2 times into an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific), and data were collected in a 0 to 90% acetonitrile gradient over 5 h. The raw files from LC-MS/MS experiments are available at http://www.marcottelab.org/index.php/PSEAE_oprF_2012.

LC-MS/MS raw files were searched against the *P. aeruginosa* PA14

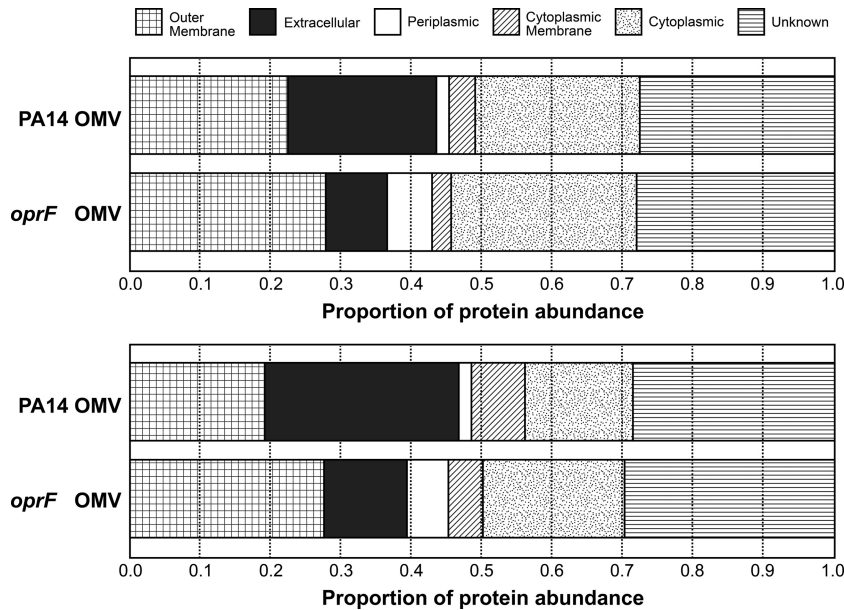


FIG 3 OMVs from the *P. aeruginosa oprF* mutant are not enriched for cytoplasmic membrane proteins. Two biological replicates displaying the proportion of protein abundance from each cellular compartment in wt *P. aeruginosa* OMVs and *P. aeruginosa oprF* mutant OMVs. The protein abundance of each compartment was estimated by dividing the sum of APEX scores of proteins identified in each compartment by the total APEX score for each sample. Protein localization predictions were obtained from the *Pseudomonas* Genome Database. In wt PA14 samples, 159 (first replicate) and 533 (second replicate) proteins were identified. In the *oprF* mutant samples, 504 (first replicate) and 1,140 (second replicate) proteins were identified.

protein sequence database (downloaded from the PseudoCAP database, 23 November 2009 version) (56) with randomly shuffled protein sequences as a decoy. Four different search engines, Crux (57), X!Tandem with *k* score (58, 59), InsPecT (60), and MS-GFDB (61), were used with default options. The results were then integrated with the MSblender program (55). APEX scores (62, 63) estimating absolute protein abundance were calculated using the number of peptide-spectrum matches assigned by MSblender with a false discovery rate with a cutoff of <0.01 and APEX observability score (O_i values) trained by whole-cell lysate proteomics data. Protein localization information was also downloaded from PseudoCAP (56). To simplify the localization data, cellular compartments were prioritized in the following order: outer membrane, extracellular, periplasmic, cytoplasmic membrane, and cytoplasmic. For example, a protein annotated as both periplasmic and cytoplasmic would be considered a periplasmic protein in this analysis. Proteins not localized to one of these five compartments on the basis of annotation were considered unknown. All search results and detailed parameters are also available at http://www.marcottelab.org/index.php/PSEAE_oprF.2012. A summary of the results is available in Table S1 in the supplemental material.

RESULTS AND DISCUSSION

Several OMV biogenesis models hypothesize that loss of outer membrane connections to the underlying peptidoglycan is required for OMV release (6, 8, 33). Supporting this model, deletion of the peptidoglycan-associated outer membrane proteins OmpA, Pal, and Lpp has been shown to significantly increase OMV formation in *E. coli*, *S. Typhimurium*, and *V. cholerae* (6, 34–37). Based on these findings, we predicted that inactivation of peptidoglycan-associated outer membrane proteins in *P. aeruginosa* would increase OMV formation. To test this hypothesis, OMV formation of the *P. aeruginosa* PA14 *oprF*, *oprI*, and *oprL* mutants was assessed as previously described using a spectrophotometric lipid assay (31). It is important to note that in strain PA14, *oprI* is reported to have a premature stop codon (TAA) at position +139

relative to the ATG start codon (64); however, when we sequenced *oprI* from *P. aeruginosa* PA14, it was found that the codon encompassing position +139 instead encodes glutamic acid (139T → G), indicating that the open reading frame is intact. This was confirmed by LC-MS/MS data, which showed that OprI is translated and encodes glutamic acid at amino acid 47.

While the *oprI* and *oprF* mutants grew at rates equivalent to the rate of wild-type (wt) *P. aeruginosa* (Fig. 1), they produced ~3-fold and ~8-fold more OMVs, respectively (Fig. 2A). Expression of *oprI* and *oprF* in *trans* in the corresponding mutants reduced OMV levels (Fig. 2B), indicating that increased OMV production was due to the loss of OprF and OprI. The *oprL* mutant showed a slight decrease in growth rate and growth yield (Fig. 1), although it produced OMVs at levels equivalent to those for the wt (Fig. 2A). While the growth rates of wt *P. aeruginosa* and the *oprF* mutant were equivalent, the *oprF* mutant reached slightly lower cell yields (Fig. 1). On the basis of these lower cell yields (OD_{600} of ~5 for wt *P. aeruginosa* and ~3.5 for the *oprF* mutant) and the observation that *P. aeruginosa* can undergo autolysis (65), it was possible that the increase in OMV production in the *oprF* mutant was due to the presence of cytoplasmic membrane components (arising from lysis) in our OMV preparations. While we did not think that this was likely since the growth yield differences were small, it was critical to examine this possibility experimentally.

If the OMV preparations from the *oprF* mutant were contaminated with cytoplasmic membranes, we reasoned that these preparations would be enriched in cytoplasmic membrane proteins. To examine this, the proteome of OMV samples from wt *P. aeruginosa* and the *oprF* mutant were determined using LC-MS/MS. Raw files, results, and details of the analyses are available at http://www.marcottelab.org/index.php/PSEAE_oprF.2012 and in Table S1 in the supplemental material. As was observed by several

other groups, OMV preparations in wt *P. aeruginosa* are enriched for outer membrane and periplasmic proteins, although some cytoplasmic and cytoplasmic membrane proteins are also present. The relative abundances indicate that the *oprF* mutant OMV sample was also enriched for outer membrane proteins and not inner membrane proteins (Fig. 3), indicating that the increase in OMV production in the *oprF* mutant is not due to cell lysis and contamination by cytoplasmic membrane proteins.

Based on work in other bacteria, the increase in OMV levels in the *oprF* and *oprI* mutants was presumably due to detachment of the outer membrane from the underlying peptidoglycan layer (6, 34–37). However, another possibility is that inactivation of these proteins altered the levels of PQS, thus leading to increased OMVs. To test whether production of PQS and its direct precursor, 2-heptyl-4-quinolone (HHQ), were affected in the *oprF* and *oprI* mutants, PQS was measured using TLC and HHQ was measured using high-performance liquid chromatography (66). The *oprF* mutant produced ~4-fold more PQS and ~1.5-fold more HHQ than wt *P. aeruginosa* (Fig. 4A and C), while the *oprI* mutant produced PQS levels equivalent to those for the wt (Fig. 4A). Importantly, PQS and HHQ production could be genetically complemented by expression of *oprF* in *trans* in the *oprF* mutant (Fig. 4B and C). Recent evidence partially conflicts with these results, determining that a *P. aeruginosa* *oprF* mutant produces lower levels of PQS (67). Our study likely contradicts this study due to the fact that different quantification methods were used: Fito-Boncompagni et al. (67) used an LC/MS method (68) to quantify PQS, and this study used TLC. In contrast to Fito-Boncompagni et al., we and others have found that in the absence of a chelator in the mobile phase, PQS is difficult to quantify using liquid chromatography (66, 69) due to poor peak resolution; thus, TLC provides a more quantifiable approach.

To determine if the increase in OMV production by the *P. aeruginosa* *oprF* mutant was due to increased PQS production, the gene (*pqsH*) encoding the enzyme responsible for the terminal step in PQS production was deleted in the *P. aeruginosa* *oprF* mutant. Since this strain is unable to produce PQS, assessment of OMV formation by this strain allows the determination of the importance of PQS for enhanced OMV formation in the *oprF* mutant. The *P. aeruginosa* *oprF pqsH* double mutant grew similarly to wt *P. aeruginosa* (Fig. 5A) and produced extremely low levels of OMVs (Fig. 5B). In fact, OMVs were not detectable in over half of the OMV preparations. These data support the hypothesis that increased OMV production in the *oprF* mutant is a result of increased PQS production. To further test this hypothesis, we examined OMV production by the *P. aeruginosa* *oprF pqsH* double mutant following supplementation with increasing amounts of PQS (Fig. 5C). OMV production in this strain increased with increasing amounts of PQS (Fig. 5C). Interestingly, addition of PQS at levels produced by the *P. aeruginosa* *oprF* mutant (40 μ M) resulted in production of very high levels of OMVs equivalent to those observed in the *oprF* mutant (Fig. 2A and 5B and C). In addition, the PQS-induced OMV production by the *P. aeruginosa* *oprF pqsH* double mutant was similar to that observed upon addition of PQS to the *P. aeruginosa* *pqsH* mutant (Fig. 5C). These data again support a model in which the increase in PQS production and not simply the lack of OprF is responsible for the increase in OMV formation by the *P. aeruginosa* *oprF* mutant.

The *oprI* mutant produced more OMVs than the wt; however, unlike the *oprF* mutant, it produced wt levels of PQS (Fig. 4A). For

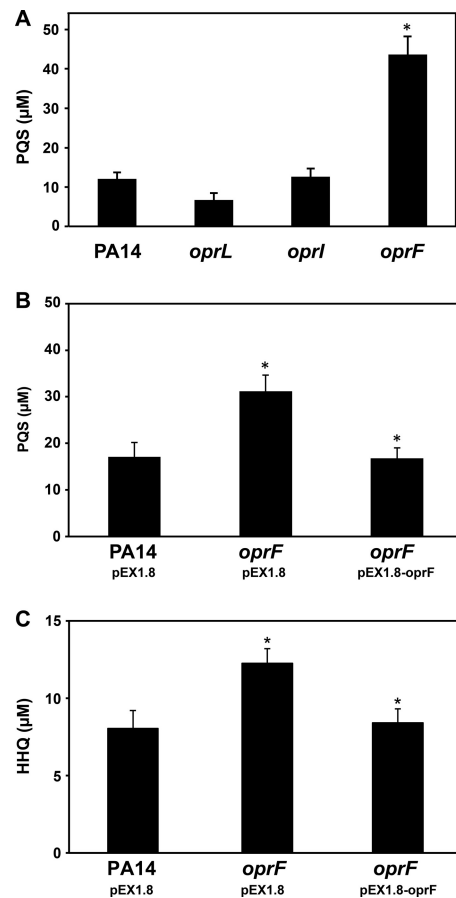


FIG 4 PQS production by wt *P. aeruginosa* and the *oprL*, *oprI*, and *oprF* mutants. (A) PQS was extracted from whole cultures and quantified using TLC. The *oprF* mutant produces ~4-fold more PQS than wt. (B) Complementation of the *P. aeruginosa* *oprF* mutant with pEX1.8-*oprF* restores PQS to wt levels. (C) The *oprF* mutant produces slightly more HHQ than the wt, and complementation of the *oprF* mutant restores HHQ to wt levels. *, $P < 0.02$ via two-tailed Student's *t* test, assuming equal variance ($n \geq 4$).

this reason, we hypothesized that the increased OMV production by the *oprI* mutant was not due to increased PQS production but instead was due to loss of peptidoglycan tethering. To test this hypothesis, we constructed an *oprI pqsH* double mutant and examined OMV production in the presence and absence of exogenous PQS. Similar to the *pqsH* mutant, the *oprI pqsH* double mutant did not produce detectable levels of OMVs (Fig. 5B); however, the *oprI pqsH* double mutant produced 2-fold more OMVs than the *pqsH* mutant upon addition of exogenous PQS (Fig. 5C). These experiments indicate that PQS is necessary for production of detectable OMVs in the absence of OprI; however, loss of OprI leads to increased production of OMVs in the presence of PQS. These data, combined with the fact that OprI is the only *P. aeruginosa* outer membrane protein known to covalently bind to peptidoglycan, suggest that this protein limits PQS-mediated production of OMVs through tethering to peptidoglycan.

This work provides additional insight into the mechanism of OMV formation in *P. aeruginosa*. While the absence of the OmpA homolog OprF increases OMV production, we showed that unlike other bacterial species, this increase is not directly attributable to the loss of peptidoglycan binding but instead is attributable to

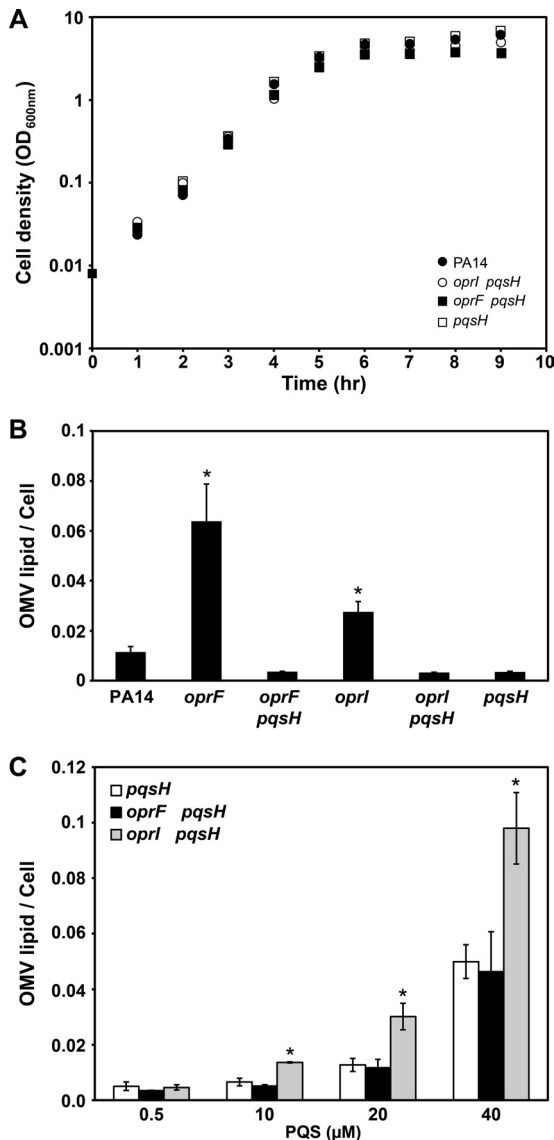


FIG 5 Enhanced OMV production by the *oprF* mutant, but not the *oprI* mutant, is due to increased PQS production. (A) Representative growth curves of wt *P. aeruginosa* (PA14), the *oprI pqsH* double mutant, the *oprF pqsH* double mutant, and the *pqsH* mutant (*pqsH*) grown with shaking (250 rpm) at 37°C in BHI. (B) OMV production by wt *P. aeruginosa*, the *oprF* mutant, the *oprF pqsH* double mutant, the *oprI* mutant, the *oprI pqsH* double mutant, and the *pqsH* mutant. The majority of samples from strains lacking *pqsH* did not produce detectable amounts of OMVs. (C) OMV production upon addition of increasing levels of PQS. Synthetic PQS was added exogenously to cultures to a final concentration of 0.5, 10, 20, or 40 μM, and OMV levels were quantified. All cultures were grown with shaking (250 rpm) at 37°C to an OD₆₀₀ of ~3.5, and OMVs were quantified using the lipid assay. *, $P \leq 0.01$ compared to wild type (B) or the *pqsH* mutant (C) via two-tailed Student's *t* test, assuming equal variance ($n \geq 3$).

increased production of PQS. As demonstrated for many other bacterial species (6, 8, 33), deletion of Braun's lipoprotein homolog *oprI* resulted in an increase in OMV production most likely through the loss of the major peptidoglycan-associated lipoprotein. Several models, which are not necessarily mutually exclusive, describe the molecular mechanisms of OMV formation (8, 13, 26–29, 31, 33), but few studies have clarified which models apply to different species and/or growth conditions. These data have

allowed us to refine the *P. aeruginosa* bilayer-couple model (31) for OMV biogenesis through demonstration that OprI reduces PQS-mediated OMV formation.

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