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 formation 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...
TABLE 1 Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>endA1 hsdR17 supE44 thi-1 recA1 Δ(lacZYA-argF)U169 deoR80d-lacZΔM15</td>
<td>47</td>
</tr>
<tr>
<td>SM10</td>
<td>thi-1 thr leu tonaA lacY supE recA::RP4-2-Tc::Mum (Km')</td>
<td>48</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>PA14</td>
<td>Wild type</td>
</tr>
<tr>
<td>oprL mutant</td>
<td>PA14 ΔoprL</td>
<td>This study</td>
</tr>
<tr>
<td>oprI mutant</td>
<td>PA14 ΔoprI</td>
<td>This study</td>
</tr>
<tr>
<td>oprF mutant</td>
<td>PA14 oprF::Mar2XT7 (Gm')</td>
<td>49</td>
</tr>
<tr>
<td>pqsH mutant</td>
<td>PA14 ΔpqsH</td>
<td>This study</td>
</tr>
<tr>
<td>oprF pqsH mutant</td>
<td>PA14 oprF::Mar2XT7 ΔpqsH (Gm')</td>
<td>This study</td>
</tr>
<tr>
<td>oprI pqsH mutant</td>
<td>PA14 ΔoprI ΔpqsH</td>
<td>This study</td>
</tr>
</tbody>
</table>

Plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-T Easy</td>
<td>Sequencing vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pEX18Tc-oprL</td>
<td>Gene replacement vector (oriT ' srcB' Te')</td>
<td>50</td>
</tr>
<tr>
<td>pEX18Tc-oprL</td>
<td>pEX18Tc containing 1-kb sequences flanking oprL</td>
<td>This study</td>
</tr>
<tr>
<td>pEX18Tc-pqsH</td>
<td>pEX18Tc containing 1-kb sequences flanking pqsH</td>
<td>This study</td>
</tr>
<tr>
<td>pEX1.8</td>
<td>Broad-host-range expression vector, IPTG inducible (Ap')</td>
<td>51</td>
</tr>
<tr>
<td>pEX1.8-oprF</td>
<td>pEX1.8 carrying oprF</td>
<td>This study</td>
</tr>
<tr>
<td>pEX1.8-oprI</td>
<td>pEX1.8 carrying oprI</td>
<td>This study</td>
</tr>
</tbody>
</table>

TABLE 2 Primer sequences

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>oprL-flanking regions</td>
<td>oprL-P1 = CCAGATCCGAGAAGCTCACGGATATCAAG</td>
</tr>
<tr>
<td>oprL-P2 = GTGCTTGCCATAACGATCCATGTAACTC</td>
<td></td>
</tr>
<tr>
<td>oprL-P3 = GAAATGCAGATGGGAAATGCGGTACCC</td>
<td></td>
</tr>
<tr>
<td>oprL-P4 = CAGGATGTACTGGGAAATGACCTGCTG</td>
<td></td>
</tr>
<tr>
<td>oprL-flanking regions</td>
<td>oprL-P1 = 5'-CCGATCCGAGAAGCTCACGGATATCAAG</td>
</tr>
<tr>
<td>oprL-P2 = 5'-GTGCTTGCCATAACGATCCATGTAACTC</td>
<td></td>
</tr>
<tr>
<td>oprL-P3 = 5'-GAAATGCAGATGGGAAATGCGGTACCC</td>
<td></td>
</tr>
<tr>
<td>oprL-P4 = 5'-CAGGATGTACTGGGAAATGACCTGCTG</td>
<td></td>
</tr>
<tr>
<td>pqsH-flanking regions</td>
<td>pqsH-P1 = 5'-CTGTTTCAACAGTGTGAGAAGCGGGTGG</td>
</tr>
<tr>
<td>pqsH-P2 = 5'-CATCGCCGGAACCTGAAAAACAGGATTAAA</td>
<td></td>
</tr>
<tr>
<td>pqsH-P3 = 5'-CAGGATGTACTGGGAAATGACCTGCTG</td>
<td></td>
</tr>
<tr>
<td>pqsH-P4 = 5'-ATGTCAGATGTCGGAAGCGGGTGG</td>
<td></td>
</tr>
</tbody>
</table>

Complementation

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>oprF-for</td>
<td>5'-CTGTTTCAACAGTGTGAGAAGCGGGTGG</td>
</tr>
<tr>
<td>oprF-rev</td>
<td>5'-CATCGCCGGAACCTGAAAAACAGGATTAAA</td>
</tr>
<tr>
<td>oprF-for</td>
<td>5'-CAGGATGTACTGGGAAATGACCTGCTG</td>
</tr>
<tr>
<td>oprF-rev</td>
<td>5'-ATGTCAGATGTCGGAAGCGGGTGG</td>
</tr>
</tbody>
</table>

a Underlined sequences represent recognition sites for restriction endonucleases.
were grown overnight in MOPS supplemented with 20 mM succinate and 10% sucrose. Mutants were confirmed by PCR and sequencing.

**Complementation of the *P. aeruginosa* oprF and oprI mutants.** oprF and oprI were 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 oprF 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 oprF 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-thiogalactopyranoside (IPTG) was added to cultures at 500 μM to induce gene expression.

**OMV preparation.** For OMV preparation, *P. aeruginosa* overnight cultures were diluted to an optical density at 600 nm (OD600) of 0.001 to 0.05 in BHI broth. Cells were grown to an OD600 of 2.7 to 3.9 with shaking at 250 rpm in a 1:10 culture volume/flask volume ratio. When adding side (IPTG) was added to cultures at 500 μM. OMVs were purified using methods described previously (13). Bacteria were grown with shaking (250 rpm) at 37°C in BHI. Replicate cultures were grown overnight in MOPS supplemented with 20 mM succinate and 20 μg/ml tetracycline, diluted into antibiotic-free medium, and spread onto LB plates supplemented with 10% sucrose. Mutants were confirmed by PCR and sequencing.

**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 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 N2 gas. PQS was quantified using thin-layer chromatography (TLC) (53). For TLC, dried samples were resuspended in methanol (Optima grade; Fisher), and 5 μl 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-wavelength 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-Cl [pH 7.5], 5 mM dithiothreitol [DTT], 1.0 mM EDTA, 1X Calbiochem protease inhibitor cocktail [CPIPCS]). Fifty microliters of diluted OMV lysate was incubated at 55°C for 45 min with 50 μl 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 μl) formic acid. The sample was lyophilized, resuspended with buffer C (95% H2O, 5% acetonitrile, 0.01% formic acid), and cleaned using a C18 tip (Thermo Fisher Scientific). The eluted sample was again lyophilized, resuspended with 120 μl buffer C, and filtered through an Amicon Ultra-0.5 filter (for 12 min at 14,000 × 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

LC-MS/MS raw files were searched against the *P. aeruginosa* PA14...
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) 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 (31). It is important to note that in strain PA14, oprl is reported to have a premature stop codon (TAA) at position +139 relative to the ATG start codon (64); however, when we sequenced oprl 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 Oprl is translated and encodes glutamic acid at amino acid 47.

While the oprl 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 oprl 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 (OD600 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-Boncompte et al. (67) used an LC/MS method (68) to quantify PQS, and this study used TLC. In contrast to Fito-Boncompte 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 μM) 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 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 is instead attributable to

---

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 (ν ∼ 4).
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.

REFERENCES


26. Hancock RE, Siehnel R, Martin N. Yem DW, Wu HC.
27. Mizuno T, Kageyama M.
28. Sugawara E, Nestorovich EM, Bezrukov SM, Nikaido H.
29. Sambrook J, Fritsch EF, Maniatis T.
31. Mizuno T.
32. Sambrook J, Fritsch EF, Maniatis T.
33. Vibrio cholerae
39. Pseudomonas aeruginosa
60. Stewart JC. 1980. Colorimetric determination of phospholipids with am-
62. Kwon T, Choi H, Vogel C, Nesvizhskii AI, Marcotte EM. 2011. MS- 
63. flics: a probabilistic approach for identifying peptide identifications from multiple database search engines. J. Proteome Res. 10:2949–2958.