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The role of *Pseudomonas aeruginosa* peptidoglycan-associated outer membrane proteins in vesicle formation

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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 other peptidoglycan-associated outer membrane proteins including OprI.

Vesiculation is a highly conserved process occurring in all domains of life (14, 28, 64, 68). Among prokaryotes, vesicle formation has been reported in both Gram-negative and Gram-positive bacteria (14, 28, 53, 68). Gram-negative bacteria produce spherical, bilayered vesicles derived from the outer membrane that range in size from 20–500 nm (13, 15, 21, 31, 39). Similar to the outer membrane, outer membrane vesicles (OMVs) possess an outer leaflet of lipopolysaccharide (LPS) and an inner leaflet of phospholipid (3, 7, 23, 24, 52). OMVs also contain outer membrane proteins and entrap periplasmic components as they are released (8, 9, 26). OMVs have been found associated with Gram-negative bacteria growing planktonically and in surface-attached biofilm communities as well as natural environments (3, 30, 57, 58).

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 (4, 5, 22, 23, 35, 43, 44, 63), and numerous models encompassing these factors have been proposed (30, 31, 41, 42, 56, 63). 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 (39, 40). Surprisingly PQS signaling was not required for OMV formation (39), instead OMV formation proceeds through direct interaction of PQS with the LPS component of the outer membrane (41). 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 (56), resulting in localized membrane curvature and ultimately vesiculation.

One question that remains regarding the bilayer-couple model 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 (13, 21, 66). Loss of the peptidoglycan-associated outer membrane proteins OmpA, Pal, or Lpp significantly increases OMV formation in *Escherichia coli*, *Salmonella enterica serovar Typhimurium*, and *Vibrio cholerae* (2, 6, 13, 59, 69). Homologs of OmpA, Pal, and Lpp exist in *P. aeruginosa* although their involvement in OMV biogenesis is not known (19). OprF is a 38 kDa OmpA homolog that serves both as a porin and as a tether that non-covalently links the outer membrane to peptidoglycan (18). 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 (61). OprL is an 18 kDa Pal homolog that also tethers the outer membrane to peptidoglycan (29, 37, 45, 46). Finally, OprI is an 8 kDa homolog of Braun’s lipoprotein (Lpp) and is proposed to covalently interact with the peptidoglycan layer (16) though this interaction has been reported to differ among *P. aeruginosa* strains (18, 47). OprI is highly abundant in the outer membrane (47), and similar to *E. coli* Lpp can exist in a free and a peptidoglycan-bound form (47).

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 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) or Tryptic Soy Broth (TSB) with ampicillin (100 µg/mL) or tetracycline (10 µg/mL) when appropriate. *P. aeruginosa strains* were grown in Brain Heart Infusion (BHI) broth with carbenicillin (150 µg/mL), gentamicin (50 µg/mL), or tetracycline (50 µg/mL) when appropriate.

DNA manipulations. DNA manipulations were performed using standard procedures (1). PCR was performed using an Expand Long Template PCR system (Roche). QIAprep spin miniprep kit (Qiagen) or GeneJET plasmid miniprep kit (Fermentas) were used for plasmid purification. Restriction endonucleases and buffers were purchased from New England BioLabs or Fermentas Life Sciences. 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 (20) 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 (20) with some modifications. To select the pEX18Tc-oprL, pEX18Tc-oprI, and pEX18Tc-pqsH transconjugants, conjugations were spread onto LB plates with 50 µg/mL tetracycline and 25 µg/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 NH_4Cl, 43 mM NaCl, 3.7 mM KH_2PO_4, 1 mM $MgSO_4$, 3.5 µM $FeSO_4 \bullet 7H_2O$) supplemented with ~1.25% agarose, 20 mM succinate and 20 µg/mL tetracycline. To select for the *oprF pqsH* double mutant, transconjugants were grown overnight in MOPS supplemented with 20 mM succinate and 20 µg/mL tetracycline, diluted into antibiotic-free media, 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 (1). Isopropyl β-D-1-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 OD_{600} 0.001 – 0.05 in BHI broth. Cells were grown to an OD_{600} 2.7 - 3.9 with shaking at 250 rpm in a 1:10 culture volume:flask volume ratio. When adding exogenous PQS, synthetic PQS re-suspended in 500 µL methanol was added to 25 mL BHI before adding cells, such that final concentrations of PQS in culture were 0.5, 10, 20, and 40 µM. OMVs were purified using methods described previously (23). Briefly, cells were removed by centrifugation (5000 x g for 15 min), and the resulting supernatant was filtered through a 0.45 µm membrane (Whatman PuraDisc 25mm Syringe Filters, PES). OMVs were pelleted from cell-free supernatants using an

ultracentrifuge with a Beckman 70Ti rotor at 265,000 × g for 1 hr 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 (55, 60) with some modifications. Purified OMV pellets were extracted with two volumes of chloroform, dried under N_2 gas, and resuspended in chloroform (500 µL or 1 mL chloroform). The absorbance was measured at 470 nm and normalized by 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, ranging from 7.8 to 250 µg/mL. Measurements made below the limit of detection were assigned a value equal to the lowest limit of the standard curve.

PQS extraction and quantification. PQS was extracted from cultures using two volumes of acidified ethyl acetate (acidified with 0.1 mL acetic acid / L ethyl acetate). The organic phase was removed and dried under a continuous stream of N_2 gas and quantified using Thin-layer chromatography (TLC) (55). For TLC, dried samples were re-suspended 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-wave UV light.

Proteomics. Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) was performed as described (32). Briefly, OMVs were isolated as described above and resuspended in Lysis Buffer (25 mM Tris-HCl (pH 7.5), 5 mM DTT, 1.0 mM EDTA and 1x CPICPS (Calbiochem protease inhibitor cocktail)). 50 µL of diluted OMV lysate were incubated at 55 °C for 45 min with 50 µL of trifluoroethanol (TFE) and 15 mM dithiothreitol (DTT), followed by

incubation with 55 mM iodoacetamide (IAM) in the dark for 30 min. Sample volume was adjusted to 1 mL with buffer (50mM Tris, pH 8.0), followed by a 1:50 w/w trypsin digestion for 4.5 hrs. The reaction was halted by adding 2% v/v (20 µL) of formic acid. The sample was lyophilized, re-suspended with buffer C (95% H_2O, 5% acetonitrile, 0.01% formic acid), and cleaned using a C18 tip (Thermo Fisher Scientific). The eluted sample was again lyophilized, re-suspended 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 (Thermo Fisher Scientific) mass spectrometer and data was collected in a 0 to 90% acetonitrile gradient over five hours. 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 protein sequence database (downloaded from PseudoCAP database, 2009-Nov-23 version) (67) with randomly shuffled protein sequences as a decoy. Four different search engines were used: Crux (50), X!Tandem with k-score (10, 25), InsPecT (62), and MS-GFDB (27) with default options. The results were then integrated with MSblender (32). APEX scores (38, 65) estimating absolute protein abundance were calculated using the number of peptide-spectrum matches assigned by MSblender with FDR < 0.01 cutoff and O_i values trained by whole cell lysate proteomics data. Protein localization information was also downloaded from PseudoCAP (67). To simplify localization data, cellular compartments were prioritized in the following order: outer membrane, extracellular, periplasmic, cytoplasmic membrane, 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 based on 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 are available in Supplemental Table 1.

168

169 **RESULTS AND DISCUSSION**

170 Several OMV biogenesis models hypothesize that loss of outer membrane connections to the
171 underlying peptidoglycan is required for OMV release (13, 21, 66). Supporting this model,
172 deletion of the peptidoglycan-associated outer membrane proteins OmpA, Pal, and Lpp have
173 been shown to significantly increase OMV formation in *E. coli*, *S. Typhimurium*, and *V. cholerae*
174 (2, 6, 13, 59, 69). Based on these findings, we predicted that inactivation of peptidoglycan-
175 associated outer membrane proteins in *P. aeruginosa* would increase OMV formation. To test
176 this hypothesis, OMV formation of the *P. aeruginosa* PA14 *oprF*, *oprI*, and *oprL* mutants was
177 assessed as previously described using a spectrophotometric lipid assay (56). It is important to
178 note that in strain PA14, *oprI* is reported to have a premature stop codon (TAA) at position +139
179 relative to the ATG start codon (33); however when we sequenced *oprI* from *P. aeruginosa*
180 PA14, it was found that the codon encompassing position +139 instead encodes glutamic acid
181 (139T→G), indicating that the open reading frame is intact. This was confirmed by LC-MS/MS
182 data, which showed that OprI is translated, and encodes for glutamic acid at amino acid 47.

183

184 While the *oprI* and *oprF* mutants grew at rates equivalent to wt *P. aeruginosa* (Fig. 1) they
185 produced ~3 fold and ~8 fold more OMVs respectively (Fig. 2A). Expression of *oprI* and *oprF in*
186 *trans* in the corresponding mutants reduced OMV levels (Fig. 2B), indicating that increased
187 OMV production was due to the loss of OprF and OprI. The *oprL* mutant showed a slight
188 decrease in growth rate and growth yield (Fig. 1) although it produced OMVs at levels
189 equivalent to the wt (Fig. 2A). While the growth rate of wt *P. aeruginosa* and the *oprF* mutant
190 were equivalent, the *oprF* mutant reached slightly lower cell yields (Fig. 1). Based on these
191 lower cell yields (OD_{600} of ~5 for wt *P. aeruginosa* and ~3.5 for the *oprF* mutant) and the
192 observation that *P. aeruginosa* has been shown to autolyse (11), it was possible that the
193 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 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 liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). Raw files, results, and details of the analyses are available at http://www.marcottelab.org/index.php/PSEAE_*oprF*.2012 and Table S1. As 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 OM from the underlying peptidoglycan layer (2, 6, 13, 59, 69). 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 using HPLC (49). The *oprF* mutant produced ~4-fold more PQS and ~1.5-fold more HHQ than wt *P. aeruginosa* (Fig. 4A, 4C) while the *oprI* mutant produced PQS levels equivalent to 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-C). Recent evidence

partially conflicts with these results, determining that a *P. aeruginosa oprF* mutant produces lower levels of PQS (17). Our study likely contradicts this study due to the fact that different quantification methods were used: Fito-Boncompte et al. used an LC/MS method (34) 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 (48, 49) 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 for the determination of the importance of PQS for enhanced OMV formation in the *oprF* mutant. The *P. aeruginosa oprF pqsH* double mutant grew similar 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 (Figs. 2A and 5C). In addition, 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 wt; however unlike the *oprF* mutant, it also 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 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 compared to the p*qsH* 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 loss of peptidoglycan binding but instead by increased production of PQS. As demonstrated for many other bacterial species (13, 21, 66), deletion of the 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 (21, 23, 41, 43, 44, 56, 63, 66), but few studies have clarified which models apply to different species and/or growth conditions. This data has allowed us to refine the *P. aeruginosa* bilayer-couple model (56) for OMV biogenesis through demonstration that OprI reduces PQS-mediated OMV formation.

272

FIGURE LEGEND

Fig. 1. Growth characteristics of wt *P. aeruginosa* and the *oprI, oprF,* and *oprL* mutants. Representative growth curves for wt *P. aeruginosa* PA14 and the *oprI*, *oprF,* and *oprL* mutants grown shaking (250 rpm) at 37°C in BHI.

Fig. 2. Inactivation of *oprF* and *oprI* increase *P. aeruginosa* OMV production. A. Fold change in OMV production by the *P. aeruginosa oprF* (*oprF*$^-$), *oprL* (*oprL*$^-$), and *oprI* (*oprI*$^-$) mutants. Bacteria were grown shaking (250 rpm) at 37°C to OD_{600} ~3.5, and OMVs were quantified by measuring OMV total 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 error of the mean, n ≥ 4. **B.** Complementation of the *oprF* and *oprI* mutants. 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). Bacteria were grown shaking (250 rpm) at 37°C to OD_{600} ~3.5 with 500 µM IPTG. OMVs were quantified and compared to wt *P. aeruginosa* carrying pEX1.8 as described in part A. Error bars represent standard error of the mean, n ≥ 4.

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 identified proteins in each compartment by the total APEX score for each sample. Protein localization predictions were obtained from www.pseudomonas.com. In wt PA14 samples, 159 (first replicate) and 533 (second replicate) proteins were identified. In the *oprF* mutant samples, 504 (first replicate) and 1140 (second replicate) proteins were identified.

299

300 **Fig. 4. PQS production by wt *P. aeruginosa* and the *oprL*, *oprI,* and *oprF* mutants. A.** PQS
301 was extracted from whole cultures and quantified using TLC. The *oprF* mutant (*oprF*$^-$) produces
302 ~4-fold more PQS than wt. **B.** Complementation of the *P. aeruginosa oprF* mutant with pEX1.8-
303 oprF restores PQS to wt levels. **C.** The *oprF* mutant produces slightly more HHQ than wt, and
304 complementation of the *oprF* mutant restores HHQ to wt levels. * $P < 0.02$ via 2-tailed Student's
305 *t* test, assuming equal variance, n ≥ 4.

306

307 **Fig. 5. Enhanced OMV production by the *oprF* mutant, but not the *oprI* mutant, is due to**
308 **increased PQS production. A.** Representative growth curves of wt *P. aeruginosa* (PA14), the
309 *oprI pqsH* double mutant (*oprI*$^-$ *pqsH*$^-$), the *oprF pqsH* double mutant (*oprF*$^-$ *pqsH*$^-$), and the
310 *pqsH* mutant (*pqsH*) grown shaking (250 rpm) at 37^{o}C in BHI. **B.** OMV production by wt *P.*
311 *aeruginosa*, the *oprF* mutant, the *oprF pqsH* double mutant, the *oprI* mutant, the *oprI pqsH*
312 double mutant, and the *pqsH* mutant. The majority of samples from strains lacking *pqsH* did not
313 produce detectable amounts of OMVs. **C.** OMV production upon addition of increasing levels of
314 PQS. Synthetic PQS was added exogenously to cultures to a final concentration of 0.5, 10, 20,
315 or 40 μM, and OMV levels quantified. All cultures were grown shaking (250 rpm) at 37°C to
316 OD_{600} ~3.5 and OMVs quantified using the lipid assay. * $P \leq 0.01$ compared to wild type (B) or
317 the *pqsH* mutant (C) via 2-tailed Student's *t* test, assuming equal variance, n ≥ 3.

318

319 **Table 1. Strains and plasmids.**

Strain or plasmid	Description	Source
Strains		
E. coli		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 Δ(lacZYA-argF)U169 deoR [Φ 80dlac Δ(lacZ)*M15]	(54)
SM10	*thi*-1 *thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Km^R)	(12)
P. aeruginosa		
PA14	Wild-type	(36)
oprL mutant	PA14 Δ*oprL*	This study
oprI mutant	PA14 Δ*oprI*	This study
oprF mutant	PA14 *oprF*::Mar2XT7 (Gm^R)	(36)
pqsH mutant	PA14 Δ*pqsH*	This study
oprF pqsH mutant	PA14 *oprF*::Mar2XT7, Δ*pqsH* (Gm^R)	This study
oprI pqsH mutant	PA14 Δ*oprI*, Δ*pqsH*	This study
Plasmids		
pGEMTeasy	Sequencing vector	Promega
pEX18Tc	gene replacement vector (*oriT*$^+$, *sacB*$^+$, Tc^R)	(20)
pEX18Tc-oprL	pEX18Tc containing 1kb sequences flanking *oprL*	This study
pEX18Tc-oprI	pEX18Tc containing 1kb sequences flanking *oprI*	This study
pEX18Tc-pqsH	pEX18Tc containing 1kb sequences flanking *pqsH*	This study
pEX1.8	Broad-host-range expression vector, IPTG inducible (Ap^R)	(51)
pEX1.8-oprF	pEX1.8 carrying *oprF*	This study
pEX1.8-oprI	pEX1.8 carrying *oprI*	This study

320

Table 2. Primer sequences

Name	Sequence[a]
***oprL* flanking regions**	
oprL-P1	CCGGATCCGAGAAGCTCACCGGTATCAAG
oprL-P2	GTGCTTGGGCATAACGACTTCCATGTAACTCCTAATGAACCC
oprL-P3	GAAGTCGTTATGCCCAAGCAC
oprL-P4	CAGGATCCGTACTGGGAAATGACCTGCTG
***oprI* flanking regions**	
oprI-P1	5'–CCGGATCCAGGTACTCCAGGTTCAGCCAC
oprI-P2	5'–GTTTTCAACAGGTCGTGAGACCGGTGGACATTTCCATAACAGCAATC
oprI-P3	5'–GGTCTCACGACCTGTTGAAAAC–3'
oprI-P4	5'–CCGGATCC AGGTGATCAAGGCCAAGTAC–3'
***pqsH* flanking regions**	
pqsH-P1	5'-CTGAATTCCTTGTCCTGCAGGTCGATATC-3'
pqsH-P2	5'-CATCGCCGAACTCGAAAACAGGATAAGAACGGTCATCCGTTGC-3'
pqsH-P3	5'-GCAACGGATGACCGTTCTTATCCTGTTTTCGAGTTCGGCGATG-3'
pqsH-P4	5'-CTTCTAGAGATTGCTACAGGTAGCGAGG-3'
Complementation	
oprF-for	5'-CTAACTGACCATCAAGATGGG-3'
oprF-rev	5'-CCCAAGCTTTTTTCCTTAGAGGCTCA-3'
oprI-for	5'-CGGAATTCGTCCACCTTAAGGGGAAC-3'
oprI-rev	5'-CCCAAGCTTCAGGTCGTGAGACCTAT-3'

[a] Underlined sequences represent recognition sites for restriction endonucleases.

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