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Purification of outer membrane vesicles from *Pseudomonas aeruginosa* and their activation of an IL-8 response

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Abstract

Considerable lung injury results from the inflammatory response to *Pseudomonas aeruginosa* infections in patients with cystic fibrosis (CF). The *P. aeruginosa* laboratory strain PAO1, an environmental isolate, and isolates from CF patients were cultured in vitro and outer membrane vesicles from those cultures were quantitated, purified, and characterized. Vesicles were produced throughout the growth phases of the culture and vesicle yield was strain-independent. Strain-dependent differences in the protein composition of vesicles were quantitated and identified. The aminopeptidase PaAP (PA2939) was highly enriched in vesicles from CF isolates. Vesicles from all strains elicited IL-8 secretion by lung epithelial cells. These results suggest that *P. aeruginosa* colonizing the CF lung may produce vesicles with a particular composition and that the vesicles could contribute to inflammation.

Keywords

Pseudomonas aeruginosa; Outer membrane; Vesicles; Cystic Fibrosis; Aminopeptidase; Pathogenesis

1. Introduction

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen that typically lives in water and soil habitats. Due to its resistance to antibiotics and disinfectants, *P. aeruginosa* flourishes in hospitals as one of the major causes of nosocomial pneumonia and other hospital-acquired infections in immunocompromised patients [1]. Patients afflicted with cystic fibrosis (CF) are particularly vulnerable to chronic *P. aeruginosa* lung infections. These infections are the primary cause of death associated with CF [1,2].

P. aeruginosa produces outer membrane vesicles, which are defined as released, spherical portions of the outer membrane. Vesicles are produced by both pathogenic and non-pathogenic bacteria [3,4]. The vesicle membrane consists of characteristic outer membrane constituents such as lipopolysaccharide (LPS), glycerophospholipids, and outer membrane proteins (OMPs) while the vesicle lumen carries entrapped periplasmic components [4]. Active toxins and other virulence factors have been discovered to be associated with vesicles produced by pathogenic bacteria [3]. *P. aeruginosa* vesicles have been shown to contain the virulence factors pro-elastase, hemolysin, phospholipase C, and alkaline phosphatase, as well as the penicillin-degrading enzyme β -lactamase [3,5–7]. *P. aeruginosa* vesicles have also recently been found to contain the quorum-sensing signalling molecule 2-heptyl-3-

hydroxy-4-quinolone (pseudomonas quinolone signal; PQS) as well as antimicrobial quinolones [8]. These data indicate that the vesicles may play a role in pathogenesis.

Current research suggests that *P. aeruginosa* live anaerobically in the mucus layer of the CF lung and are rarely found in contact with epithelial cells [9]. In the absence of direct cell-to-cell contact, extracellular products of *P. aeruginosa* have been proposed to be the actual provocateurs of airway inflammation [10]. Lung tissue damage caused by persistent inflammation is considered to be a major cause of mortality in CF patients [11]. Vesicles could contribute to the lethal inflammation in the lungs of patients infected with *P. aeruginosa* by stimulating an inflammatory response at a distance.

In this study, we purified and characterized vesicles from several *P. aeruginosa* strains – Fresh clinical isolates CF2 and S470 obtained from the sputum cultures of CF patients at Duke University Hospital and minimally passaged to obtain non-mucoid colonies; ATCC 14886 (“soil”), an environmental isolate that possesses many characteristics associated with pathogenic strains, including multidrug efflux systems, the type III secretion system, *rhl* quorum-sensing system, and proteolytic and hemolytic activities [12]; and PAO1, a sequenced laboratory strain that was originally a blood isolate.

2. Materials and methods

2.1. Bacterial strains and reagents

P. aeruginosa strains used were the laboratory strain PAO1 (Pf1 phage-cured from our lab collection), the soil isolate ATCC 14886 (American Type Culture Collection), and CF clinical isolates CF2 and S470 (Duke University Hospital). Unless indicated, reagents were purchased from VWR.

2.2. Vesicle isolation and purification

Vesicles were purified from a method adapted from Horstman and Kuehn [13]. Bacteria (3–12 l) were grown in LB broth overnight to early stationary phase. Cells were removed by pelleting ($10,000 \times g$, 10 min). Supernatants were concentrated via a 100-kDa tangential filtration concentration unit (Pall-Gellman) to approximately 500 ml. The retentate was centrifuged ($6000 \times g$, 10 min) and filtered through a $0.45 \mu\text{m}$ Durapore PVDF filter (Millipore) to remove remaining bacteria. Vesicles were obtained from the cell-free supernatant by one of two methods. In the first method, the vesicles were pelleted ($39,000 \times g$, 1 h), resuspended in 50 mM HEPES, pH 6.8 (HEPES), and adjusted to 45% Optiprep (Greiner) in 10 mM HEPES/0.85% NaCl, pH 7.4 (HEPES-NaCl) (weight/volume). In the second method, the vesicles were precipitated with 71% or 75% ammonium sulfate (4°C , for at least 3 h), pelleted ($10,000 \times g$, 20 min), dialyzed overnight with HEPES, concentrated (50 kDa MWCO Centrplus, Millipore), and adjusted to 45% Optiprep/HEPES-NaCl. Optiprep gradients were layered over the 2 ml crude vesicle samples as follows: For PAO1: 2 ml 40%, 2 ml 35%, 3 ml 30%, 2 ml 25%, 1 ml 20%; for Soil: 2 ml 40%, 2 ml 35%, 2 ml 30%, 2 ml 25%, 2 ml 20%; for CF isolates: 2 ml 40%, 2 ml 35%, 4 ml 30%, 2 ml 20% Optiprep/HEPES-NaCl by weight. Differences in the gradients reflect optimization in separating flagella and other soluble material from the vesicles for each strain. Gradients were centrifuged ($100,000 \times g$, 16 h) and 1 ml fractions were removed from the top. A portion of each fraction was precipitated with 20% trichloroacetic acid (TCA) and visualized by 15% SDS-PAGE and Coomassie staining. Pure vesicles were recovered from pooled peak fractions by diluting in or dialyzing overnight against HEPES and pelleting ($150,000 \times g$, 1 h). Vesicles (5–50 μl) were checked for sterility and refiltered if necessary. Vesicles obtained by both purification methods were used in experiments shown in Figs. 2, 3, and 5.

Vesicles purified by the ammonium sulfate method were used for the 2-D-DIGE analysis. Enzymatic activities were measured according to [13].

2.3. Vesicle production

To investigate strain-dependent differences in vesicle production, 10 ml cultures were grown in LB to an OD₆₀₀ of 1.0. Filtered supernatant (1 ml) and 1 ml of the original bacterial culture were each TCA precipitated and resuspended in a final volume of 50 µl with HEPES. Culture lysate precipitates were diluted 10-fold. Samples (20 µl) were immunoblotted with either mouse anti-OprF or anti-outer membrane proteins antisera (anti-OMP) (Biogenesis, Poole, England). Blots were developed with ECL (GE Healthcare) and analyzed by densitometry using NIH Image or ImageJ software.

To investigate vesicle production during culture growth, PAO1 overnight cultures were diluted 1:50 or 1:100 into 200 ml LB. At each time point, OD₆₀₀ was measured, and filtered cell-free supernatants (1 ml) were collected, TCA precipitated, and resuspended in 50 µl SDS-PAGE loading buffer. Samples (20 µl) were immunoblotted with anti-OMP and analyzed by densitometry.

2.4. Electron microscopy (EM)

Samples (in Optiprep/HEPES-NaCl, pH 7.4 or 50 mM HEPES, pH 6.8) were applied to 400-mesh carbon-coated copper grids (Electron Microscopy Sciences), stained with aqueous 2% uranyl acetate, air dried, and visualized on a Philips 301 electron microscope operating at 80 kV.

2.5. Membrane purification

Inner and outer membranes from *P. aeruginosa* grown in LB to an OD₆₀₀ of 1.0 were purified as described [14]. The highest density protein peak was used as purified outer membranes, and the lowest density protein peak was used as purified inner membranes. Purified membranes were TCA precipitated.

2.6. Protein identification

2.6.1. Mass spectrometry—Purified vesicle preparations (15–30 µg) were applied to 12.5% or 15% SDS-PAGE, transferred to PVDF, and sequenced by proteolytic digestion and mass spectrometry (John Leszyk, University of Massachusetts Medical School, Shrewsbury, MA). Proteins were identified through MALDI analysis of tryptic peptides and/or MS/MS analysis of selected peptides using MS-Fit and MS-Tag software, respectively.

2.6.2. Two-dimensional differential gel electrophoresis (2-D DIGE)—Purified PAO1 and S470 vesicles (~750 µg) were phenolextracted to remove LPS [15] prior to 2-D DIGE analysis. This procedure should not affect protein composition, as no proteins enter the aqueous phase [14]. 2-D DIGE was performed by the Duke Neuroproteomics Facility. Cy-dye labeling, 2D gel electrophoresis, imaging, and analysis were performed as described [16]. PAO1 and S470 vesicles were compared by 2-D DIGE twice. Significant differences between PAO1 and S470 spots were determined using a threshold of two standard deviations of the mean volume ratios (95th percentile confidence, 3.3 and 3.165 for the two analyses). Spots of interest from the 2-D DIGE were sent to the Michael Hooker Proteomics Core Facility (UNC-CH, NC) where proteins were identified through MALDI analysis of tryptic peptides and/or MS/MS analysis of selected peptides using the MASCOT search engine.

2.7. IL-8 assay

A549 human lung epithelia carcinoma cells were grown in Kaighn's F-12K media containing 10% fetal bovine serum plus penicillin/streptomycin/fungizone (Gibco). Human bronchial epithelial (HBE) cells derived from healthy human volunteers were maintained in Bronchial Epithelial Cell Growth Media supplemented with thyroid extract. Fluorescently labeled vesicles (2.5 µg per well) were incubated with confluent A549 monolayers (approx. 5×10^4 cells per well) or confluent monolayers of HBE cells in serum-free media in 96-well plates (Costar) for 24 h at 37 °C. The amount of IL-8 in supernatants was quantitated using an OptiEA Human IL-8 ELISA kit (Pharmingen) following the manufacturer's instructions. Statistics were calculated using single-factor ANOVA.

3. Results

3.1. Vesicle production

The amount of vesicles in the cell-free supernatant of *P. aeruginosa* cultures was assayed by detecting vesicle proteins using antibodies to either a mixture of outer membrane proteins (anti-OMP) or to OprF, a specific outer membrane protein. Vesicles represented between 0.75% and 2.5% of the total outer membrane in all four strains (Fig. 1A). Production of vesicles was investigated by sampling the cell-free supernatant during lag, log and stationary phase of growth of PAO1. Vesicle production was analyzed by measuring the amount of OMPs present in cell-free culture supernatants (Fig. 1B).

3.2. Purification of *P. aeruginosa* vesicles

To obtain highly purified, native vesicles, we developed a purification method based on previously described methods using a density gradient [13,17]. For each strain, gradient fractions were analyzed for macromolecular composition by EM and for protein composition by TCA precipitation and SDS-PAGE (Fig. 2). For light density fractions, SDS-PAGE analysis revealed bands corresponding to the molecular weights of known OMPs (e.g. OprF, 38 kDa; OprD, 48 kDa; and OprH, 21 kDa) (Fig. 2, lanes 2–4) and by EM, vesicles were the only structure found (Fig. 2, Light). Higher density fractions, by contrast, contained abundant proteins corresponding to the molecular weights of flagellin (53 kDa), pilin (19 kDa) and R2-type pyocins (36 kDa sheath protein and 19 kDa tail protein) (Fig. 2, lanes 6–12) and by EM, these fractions contained structures other than vesicles such as pili, flagella, and phage tail-like R-type pyocins (Fig. 2, Heavy). Based on the electron micrographs and the predicted outer membrane protein bands, fractions containing pure vesicles were pooled.

Vesicles isolated with and without ammonium sulfate were indistinguishable by electron microscopy and SDS-PAGE (data not shown), however the ammonium sulfate procedure significantly increased the yield of purified vesicles by increasing the recovery of secreted material prior to the density gradient separation step. The vesicles from the four strains differed in size and morphology. Vesicles from the CF isolates CF2 (diameter average = 43 nm, mode = 32 nm, $n = 53$) and S470 (diameter average = 37, mode = 38 nm, $n = 38$) were smaller and more regularly round than vesicles from PAO1 (diameter average 96 nm, modes = 63 and 88, $n = 33$) and Soil (diameter average = 90 nm, mode = 76 nm, $n = 31$). The density of vesicles was determined by calculating the average density of the peak vesicle fractions. For all four strains, vesicles had lower densities (<1.136 g/ml) than purified outer membranes (1.282–1.289 g/ml).

Alkaline phosphatase activity was measurable in vesicle preparations from all four strains (303–1220 SU/ng protein), verifying that the vesicles contained periplasmic material and that vesicles were not disrupted during purification.

P. aeruginosa begin to secrete elastase during early stationary phase. We discovered that vesicle proteins were degraded in ammonium sulfate-concentrated supernatants and that degradation was prevented when the elastase inhibitor phosphoramidon was added to stationary phase cultures prior to concentration (data not shown). The elastase concentration present in the growth culture without ammonium sulfate concentration is apparently insufficient to cause significant vesicle protein degradation. To avoid elastase degradation, vesicles were routinely purified from concentrated supernatants of cultures harvested prior to their entry into stationary phase.

3.3. Vesicle protein identification and comparison

Vesicle proteins were compared with proteins in inner and outer membrane preparations (Fig. 3). For all strains, vesicle protein profiles were markedly different from purified inner membranes. The absence of measurable NADH oxidase activity confirmed that cytoplasmic membrane was not included or associated with vesicles (data not shown). By contrast, vesicle protein profiles were very similar to purified outer membranes. Mass spectrometry was used to identify six major vesicle protein bands from a one-dimensional SDS-PAGE separation of vesicles (Fig. 3, arrows). Five of the protein bands were abundant and common to vesicles from all of the strains. These bands were identified from PAO1 vesicles as the major outer membrane proteins OprD, OprF, OprG, and OprH, and a putative outer membrane protein encoded by open reading frame (ORF) PA1288. The amino acid sequence derived from PA1288 contains the Pfam domain Toluene X, which indicates that it is a member of the OMPP1/FadL/TodX family of outer membrane protein transport proteins. Characterized members of this family are involved in aromatic hydrocarbon degradation and long-chain fatty acid transport across the outer membrane. The sixth vesicle protein identified was from the 57.5 kDa band that was only visible in CF2 and S470 vesicles. The protein was identified from CF2 vesicles as PA2939, an extracellular aminopeptidase designated PaAP [18]. PaAP was also identified as a major protein in vesicles from S470 and three other CF isolates by immunoblot (data not shown).

Two-dimensional differential gel electrophoresis (2-D DIGE) was used to detect more subtle differences in protein profiles of vesicles from different strains and to quantitate protein differences. Fluorescently labeled vesicles from PAO1 and S470 were mixed together and run on the same gel (Fig. 4A). The 2-D DIGE was repeated and 621 spots were matched between the two gels. A two-standard deviation threshold was used to identify spots that were differentially expressed between PAO1 and S470 vesicles. This corresponded to approximately a 3-fold change in spot volume. Of the 58 spots that qualified as being differentially expressed on both gels, 45 were more abundant in S470 vesicles and 13 were more abundant in PAO1 vesicles (Fig. 4B). The spot corresponding to PaAP (#26) was enriched at least 65-fold in vesicles derived from S470.

Mass spectrometry was used to identify 18 spots in regions predicted not to yield PaAP (Table 1, Figs. 4B–D). Nevertheless, four spots enriched in S470 (#5, 6, 18, 25) and one spot (#38) enriched in PAO1 were identified as PaAP. Low molecular weight spots which were more abundant in S470 were identified to be the protein expressed by the ORF PA0070 (#57, 58). PA0070 is an ORF with no predicted signal sequence or homology with characterized proteins, however it is 98% identical to ORF PA1400820 in *P. aeruginosa* PA14 and 71% homologous to ORF PFL6074 in *P. fluorescens* Pf-5. Several spots were identified to be OprE; some of the OprE species were more abundant in S470 vesicles (#39, 40), and others were more abundant in PAO1 vesicles (#29, 30, 53). Two isoforms of OprF were also more abundant in PAO1 vesicles (#31, 54). Two spots (#34, 35) contained peptides that matched multiple *P. aeruginosa* proteins, thus, the proteins responsible for the differences in spot abundance could not be determined. Spot 35 contained MexA, the periplasmic component of the MexA,B-OprM multi-drug efflux pump [19], and spot 34

contained flagellin. Flagellin associated with the vesicles is not likely part of a fully polymerized structure since no flagella were seen by EM in the light-density preparative fractions that were used for these vesicle protein analyses (Figs. 2A,D, Light).

3.4. *P. aeruginosa* vesicles stimulate IL-8 production by epithelial cells

The immunological response to *P. aeruginosa* vesicles was investigated by incubating vesicles with A549 cells and assaying the supernatants from the incubations for the proinflammatory chemokine IL-8. Purified vesicles from all of the strains demonstrated a significant increase in IL-8 secretion by A549 cells, with S470 vesicles stimulating significantly more IL-8 ($p < 0.01$) than a similar amount of vesicles from the other strains (Fig. 5A). Primary HBE cells showed a higher background level of IL-8 production, but vesicles from CF isolates and PAO1 stimulated IL-8 secretion significantly over background levels, and S470 vesicles stimulated significantly more IL-8 ($p < 0.01$) than a similar amount of vesicles from the other three strains (Fig. 5B).

4. Discussion

Outer membrane vesicles have been observed in cultures of *P. aeruginosa* and many other Gram-negative pathogens [20]. Our data demonstrate that, under standard laboratory growth conditions, vesicles are a measurable component of *P. aeruginosa* cultures, vesicle production occurs mainly during exponential growth, and vesicle yield is strain-independent (Fig. 1). We calculated that vesicles account for 0.75%–2.5% of the total outer membrane in a late-log phase culture, which is consistent with other reports [21].

We determined that density gradient centrifugation best separated the lipid-containing vesicles away from contaminating proteins and structures (e.g. pili, flagella, and R-type pyocins) that were abundant and visible by electron microscopy and SDS-PAGE in the crude, pelleted cell-free supernatant. In view of the fact that *P. aeruginosa* cell-free supernatants contain a myriad of structures besides vesicles, we caution that native *P. aeruginosa* vesicles used in several previous studies probably also included other secreted material.

While developing a vesicle purification scheme, we learned that secreted elastase was capable of degrading vesicle proteins in concentrated supernatants. Although this information is most useful for developing vesicle purification strategies for bacteria that secrete proteases, it is also interesting to consider that *P. aeruginosa* is capable of digesting the vesicles it produces.

The compositional data for purified vesicles in this study support the general current model of OM vesicle formation [7]: vesicles originate from the OM, encapsulate periplasmic material, and are released intact. The compositional data confirm that vesicles are not simply lysed portions of the cell, since the pelletable material from a cell lysate would contain cytoplasmic membrane as well as outer membrane components. Cell lysis contamination of the vesicle preparations also would have resulted in increasing amounts of material in the cell-free supernatants of cultures during stationary phase, and this was not observed (Fig. 1B). In fact, protein enrichment/exclusion in vesicles and the lighter density of vesicles compared with outer membrane preparations actually point to a domain- or component-dependent vesicle production mechanism. Differences between the protein composition of vesicles and outer membranes were also observed for enterotoxigenic *E. coli* and other bacterial species [4,13], and thus may reflect a common, selective mechanism of outer membrane vesiculation.

We hypothesized that, compared to laboratory or environmental strains, *P. aeruginosa* clinical isolates would either make increased numbers of vesicles or vesicles with distinct characteristics. The yield of *P. aeruginosa* vesicles does not appear to depend on the origin of the strain (Fig. 1A), however, SDS-PAGE and 2-D DIGE analyses suggested that vesicle protein content is strain-dependent. Most notably, PaAP was significantly enriched in purified vesicles from cultures of CF isolates. The correlation of PaAP enrichment with vesicles and CF suggests that the CF lung environment contributes to differences in vesicle composition. As vesicle composition is expected to play a role in their interaction with host cells, it should be considered in further studies to elucidate the physiological roles of *P. aeruginosa* vesicles.

PaAP has been characterized recently as a zinc-dependent leucine aminopeptidase that is regulated in part by the *las* quorum-sensing system and found in the supernatants of both lab and CF strains of *P. aeruginosa* [18,22]. Because vesicles are generally less than 200 nm in diameter and pass through most filters used to sterilize culture supernatants, the PaAP studied previously may be vesicle-associated [18,22,23]. Alternatively, PaAP may be secreted in both soluble and vesicle-associated forms. Interestingly, two PaAP spots (#5, 6, Fig. 4C) were substantially higher than the molecular weight of PaAP, suggesting that PaAP associated with vesicles may be multimeric.

In addition to differences in protein abundance, the conformation of the same protein within different vesicles appears to depend on the strain. Differences in the abundance of OprE isoforms in PAO1 and S470 vesicles (# 29, 30, 39, 40, 53, Fig. 4, Table 1) suggest strain-dependent variations in post-translational modifications or cleavage. Two glycosylated isoforms of OprE are known to exist in PAO1 [23]. Whether the observed strain-specific modifications take place before or after vesiculation is not known.

Together, the microscopy and protein composition data suggest that differences in composition may cause variations in vesicle morphology. The average size of the purified vesicles was consistent with vesicle sizes reported for *P. aeruginosa* and other bacteria [4]. We observed that vesicles originating from CF strains were smaller and more spherical in appearance than vesicles from PAO1 or soil strains. The differences in size and shape may be due to differences in the relative ratio of LPS-to-protein or type of LPS in the vesicles (Bauman and Kuehn, unpublished data). The presence of a protein (namely, ClyA) has been reported to impart morphological differences between vesicles from different *E. coli* strains [21]. Similarly, the morphology of *P. aeruginosa* vesicles from CF isolates also may depend on their major component, PaAP.

Neutrophil recruitment and activation characteristically accompany *P. aeruginosa* infection in the CF lung and these are primarily controlled by the presence of the chemokine IL-8 [24,25]. Our data demonstrate that human lung cells secrete a proinflammatory chemokine response to vesicles from a variety of *P. aeruginosa* strains. Similarly, vesicles from *H. pylori* have been reported to stimulate IL-8 by gastric epithelial cells [26]. Several outer membrane proteins, periplasmic proteins, and secreted products of *P. aeruginosa* are known to modulate the release of cytokines and chemokines [27,28]. One of the major vesicle components is LPS, and *P. aeruginosa* LPS has been shown to induce the release of proinflammatory modulators, including IL-8 [29]. Flagellin is a component of purified vesicles, as discussed above, and is known to stimulate IL-8 [28]. Purified nitrite reductase, a periplasmic enzyme, stimulates IL-8 in a variety of respiratory and immune cell types [28]. Variations in the IL-8 response to vesicles may be accounted for by strain-dependent differences in LPS and/or protein components.

The high load (10^8 to 10^{10} CFU/ml) of *P. aeruginosa* in CF sputum [30] indicates that the bacteria undergo substantial cycles of replication during CF infections. Considering that we found *P. aeruginosa* primarily produces vesicles during exponential growth, we predict that they produce a substantial number of vesicles within the lungs of infected individuals with CF. Vesicle production during bacterial infections has been observed for several other species [3]. Our data also show that for *P. aeruginosa*, vesicle yield may be strain-independent, but vesicle composition is likely to be influenced by the host environment. Furthermore, vesicles activate a significant IL-8 proinflammatory response in lung epithelial cells. These results highlight the potential inflammatory consequences of these miniature capsules of bacterial envelope released by *P. aeruginosa* colonizing an infected lung.

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Abbreviations

2D-DIGE	two-dimensional differential gel electrophoresis
ANOVA	analysis of variance
CF	Cystic Fibrosis
EM	electron microscopy
HBE cells	human bronchial epithelial cells
LB broth	Luria-Bertani broth
LPS	lipopolysaccharide
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
OM	outer membrane
OMP	outer membrane protein
ORF	open reading frame
PVDF	polyvinylidene fluoride
SDS-PAGE	sodium dodecylsulfate polyacrylamide electrophoresis
TCA	trichloroacetic acid

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Fig. 1.

Quantitative analyses of vesicle production. A. Vesicle yield for strains S470 (S4), CF2 (C), Soil (So), and PAO1 (P) was determined by immunoblotting cell-free culture supernatants and whole culture lysates for outer membrane proteins (anti-OMP) or for OprF alone (anti-OprF) and dividing the amount in the cell-free supernatant with the amount in the culture lysate. Samples were analyzed in duplicate for each experiment. The average and SEM are shown; $n = 4$ for anti-OMP experiments and $n = 2$ for anti-OprF experiments. B. The vesicle production of PAO1 during the growth of a culture was monitored by collecting cell-free supernatants at the indicated times and immunoblotting with anti-OMP (diamonds). For each experiment, values were compared to the amount of OMPs detected at the final time point (590 min) which was set to 100%. The average and SEM are shown; $n = 3$. The OD₆₀₀ of the culture is shown (open squares).

Fig. 2.

Protein profiles and electron micrographs of density gradient fractions. Ammonium sulfate-precipitated material from culture supernatants was loaded on the bottom of density gradients and centrifuged overnight. Fractions removed sequentially from the top of each gradient were TCA-precipitated and analyzed by Coomassie-stained SDS-PAGE (Fraction 1 = lightest, Fraction 12 = heaviest). Bracketed fractions indicate the pure vesicle-containing fractions that were pooled and further characterized from strains S470 (A), CF2 (B), soil (C), and PAO1 (D). The migration of molecular weight standards are indicated (kDa). Right panels: electron micrographs representative of pooled upper one-third (light) and lowest one-third (heavy) fractions of the vesicle purification gradients for each strain. Samples were negatively stained with uranyl acetate and visualized at 15,000–91,000 × magnification. Arrows indicate pyocins (nail-like structures) and pili or flagella. Bar = 100 nm in all panels.

Fig. 3.

Vesicle protein profiles resemble purified outer membranes and PaAP is enriched in vesicles from CF isolates. Samples (20 μ g) of inner membrane (IM), outer membrane (OM), and vesicles (V) from each strain were TCA-precipitated and visualized by Coomassie-stained SDS-PAGE. (+), proteins enriched in OM over vesicles; (*), proteins enriched in vesicles over OM; arrows, proteins that were sequenced from PAO1 vesicles, except PaAP (PA2939), which was sequenced from CF2 vesicles. Molecular weight standards are indicated (kDa). [†]OprD migrates at a lower molecular weight in PAO1 than it does in other strains, as noted previously [31].

Fig. 4. Different protein compositions of PAO1 and S470 vesicle proteins revealed by 2-D DIGE. A. Fluorescent image overlay showing 2-D DIGE of PAO1 vesicles (150 μ g) labeled with Cy 3 (green) and S470 vesicles (150 μ g) labeled with Cy 5 (red). Green spots indicate polypeptides that are more abundant in PAO1 vesicles, red spots indicate a higher abundance in S470 vesicles, and yellow spots indicate polypeptides found in equal abundance in both vesicle preparations. B. Positive changes in abundance (grey bars) indicate proteins that were more abundant in S470 vesicles. Negative changes in abundance (open bars) indicate proteins that were more abundant in PAO1 vesicles. Bar numbers correspond to spot numbers given in C and D. Values are the average of two 2-D DIGE gels, error bars indicate SEM. Grey-scale image of Cy 5 (S470) image (C), and Cy3 (PAO1) image (D). Size and pH axes are indicated on gel images. Arrows in B, C, and D indicate spots that were sequenced and are color coded by protein: Red, PaAP; Green, OprF; Black, OprE; Blue, PA0070. (*), spots that matched with more than one *P. aeruginosa* protein sequence.

Fig. 5. Vesicles induce dose-dependent IL-8 production in human airway cells that exceeds LPS response. The concentration of IL-8 was measured by ELISA in supernatants removed from A549 cells (A) or HBE cells (B) incubated without vesicles (CTRL) or with 2.5 μ g vesicles (+VES) for 24 h. Triplicate incubations were analyzed by ELISA in duplicate, and SEM is indicated for 2 to 7 separate experiments; *, $p < 0.005$ compared to control incubations.

Table 1

Proteins identified as differentially expressed in PAO1 and S470 vesicles

Spot #	Average change in abundance ^a	PA #	Gene name	Protein name	MW (kDa) ^b	pI ^b	# of peptides ^c (match score) ^d	Ion score ^e
5	17.1	2939		Probable aminopeptidase PaAP	57.5	4.8	14 (280)	147
6	11.6	2939		Probable aminopeptidase PaAP	57.5	4.8	16 (439)	321
7	-12.4			No Significant Hits				
13	12.7			No Significant Hits				
18	27.5	2939		Probable aminopeptidase PaAP	57.5	4.8	9 (246)	157
25	14.6	2939		Probable aminopeptidase PaAP	57.5	4.8	21 (618)	449
29	-20.5	0291	<i>oprE</i>	Outer membrane porin OprE	49.6	9.1	16 (659)	545
30	-17.6	0291	<i>oprE</i>	Outer membrane porin OprE	49.6	9.1	21 (682)	513
31	-7.7	1777	<i>oprF</i>	Outer membrane protein F	37.6	5.0	9 (330)	269
34	19.3	1777	<i>oprF</i>	Outer membrane protein F	37.6	5.0	10 (202)	141
34		2939		Probable aminopeptidase PaAP	57.5	4.8	15 (175)	92
34		1092	<i>flhC</i>	Flagellin type B	49.4	5.2	12 (165)	101
35	-7.1	0291	<i>oprE</i>	Outer membrane porin OprE	49.6	9.2	22 (758)	575
35		0425	<i>mexA</i>	Multidrug efflux pump membrane fusion protein A	41.1	9.2	9 (72)	28
38	-4.9	2939		Probable aminopeptidase PaAP	57.5	4.8	21 (717)	535
39	17.4	0291	<i>oprE</i>	Outer membrane porin OprE	49.6	9.1	18 (397)	269
40	15.7	0291	<i>oprE</i>	Outer membrane porin OprE	49.6	9.1	27 (1110)	848
53	-5.5	0291	<i>oprE</i>	Outer membrane porin OprE	49.6	9.1	11 (288)	200
54	-6.1	1777	<i>oprF</i>	OprF (Fragment). – <i>Pseudomonas sp. MFY72</i>	34.5		7 (165)	102
57	7.4	0070		Hypothetical protein	31.7	8.9	20 (555)	272
58	7.5	0070		Hypothetical protein	31.7	8.9	11 (222)	57

^aCalculated from two 2D-DIGE gels. Positive numbers indicate spots that were more abundant in S470 vesicles, and negative numbers indicate spots that were more abundant in PAO1 vesicles. See Fig. 4.^bTheoretical molecular weights and pIs.^cNumber of peptides that match the theoretical digest of the primary protein identified.^dScore of the quality of the peptide-mass fingerprint match and the quality of the MS/MS peptide fragment ion matches (if MS/MS data was generated). Scores of 95 or greater are considered significant.^eScore of the quality of the MS/MS peptide fragment ion matches only. Scores of 20 or greater are considered significant.