Proteome Profiles of Outer Membrane Vesicles and Extracellular Matrix of *Pseudomonas aeruginosa* Biofilms

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Experimental Section

E1 - Isolation and purification of the matrix and outer membrane vesicles from P. aeruginosa PAO1 biofilms

Biofilms were scraped from the surface, resuspended in 0.9% (w/v) NaCl and homogenised by vortexing during 3 min. The homogenate was centrifuged at 5 °C with a centrifugal force of 12,000 × *g* during 20 min. The supernatant was retained on ice and the pellet was resuspended in the same volume of 0.9% (w/v) NaCl solution and was centrifuged again. This wash was repeated for a total of three times. The pooled supernatant was further cleared by centrifugation (12,000 x *g*, 20 min, 5 °C) to remove cell debris. The collected supernatant was sequentially filtered through a 1.2, 0.45 and 0.22 µm cellulose acetate filters and the filtrate, essentially isolated matrix material, was collected. After filtration, the absence of cells was verified by transmission electron microscopy (TEM), and also by plating triplicate 100 µL aliquots onto TSA plates and incubating during 18 h at 37 °C.

At this point, one of two pathways was followed. For matrix collection and characterisation, the final filtrate was dialysed (Spectra/Por regenerated cellulose dialysis membranes, 3 kDa MWCO; Fisher Scientific, Loughborough, UK) to remove pigments and low molecular weight components. In total, six changes of water were made, with a sample:water ratio of at least 1:20 (v/v) maintained during dialysis (24 h, 4 ˚C).

When the matrix was processed for outer membrane vesicles (OMVs) isolation, the particulate components were harvested from matrix material by ultracentrifugation at 125,000 × *g* for 1.5 h at 5 ˚C (Beckman Ti45 rotor). The

efficient removal of OMVs from the matrix was also verified by TEM of whole mount preparations.

OMVs were separated from the crude particulate pellet by isopycnic density gradient centrifugation on a Beckman SW28.1 rotor at 100,000 × *g* for 16 h at 5 ˚C. The pellets were resuspended in 30% (v/v) Optiprep /10 mM HEPES (pH 7.4), 0.85% (w/v) NaCl and particulate components separated on a Optiprep gradient consisting of: 0% (1 mL), 18% (1 mL), 20% (1 mL), 22.5% (3 mL), 25% (3 mL), 27.5% (3 mL), 30% (3 mL) and 50% (1 mL). All solutions, were made as volume ratios, contained a final concentration of 10 mM HEPES, 0.85% (w/v) NaCl (pH 7.4). After centrifugation, 200 µL aliquots were collected and assessed by TEM to establish the location of OMVs and whether separation had been successful. OMV-containing fractions were combined and washed twice in 50 mM HEPES buffer and centrifuged on a Beckman Ti45 rotor at 125,000 × *g* for 1.5 h at 5 ˚C. Purified OMVs were resuspended in water and kept at -20 °C. Biological replicates were prepared in triplicate and, for each biological replicate, both the matrix and the OMVs were derived from a single technical replicate. The protein content of the isolated fractions was quantified using micro-bicinchoninic acid protein assay kit (Pierce Bioassay) using BSA as the standard.

E2 - Transmission electron microscopy

Transmission electron microscopy was performed on all matrix and OMV samples. This allowed the verification of sample cleanliness of the OMV fraction from other particulates; it also verified the absence of cells or lysed cells in either fraction. Negatively stained whole mounts were prepared as follows. A Formvar carbon-coated copper grid (200-mesh; Marivac) was floated, film-side down, on

20 µL of sample for 20 s. The grid was removed and the edge gently touched to filter Whatman No.1 paper to wick-off excess sample. The grid, (sample side), was then washed by floating on 50 µL of mili-Q water, blotted, floated on 10 µL of 2% (w/v) uranyl acetate for 10-20 s and blotted dry.

A Philips CM10 transmission electron microscope was used to examine samples. The instrument was operating at an acceleration voltage of 80 kV under standard operating conditions. Images were archived using the iTEM program version 5.0 (Soft Imaging Systems, Münster, Germany).

E3 - Delipidation of samples

Delipidation was performed by preparing an aqueous solution of 1-2 mg protein in 10 mL sterile milli-Q water. An equal volume of Tris-buffered phenol was added and the mixture vortexed followed by heating at 70 °C with continuous mixing during 10 minutes. The samples were left to cool on ice, then centrifuged at 5,000 × *g* for 10 min at 4 °C. The aqueous phase was discarded and an equal volume of mili-Q water was added and phenol extraction was repeated.

Ice-cold acetone was added to the phenol phase (2 volumes), mixed and centrifuged at 5,000 × *g* for 10 min at 4 °C. The obtained pellet was resuspended by adding 2 volumes of ice-cold acetone and the solution centrifuged again using the same settings. The pellet was briefly left to air dry to remove traces of solvent, resuspended in mili-Q water and stored at -20 °C until used. Protein content was estimated by the bicinchoninic acid (BCA) assay; assays were supplemented with 0.01 % SDS to solubilise proteins. These OMVs and matrix protein extracts were used in all subsequent proteomics analysis.

E4 - Two-dimensional gel electrophoresis

OMV-enriched or whole matrix protein extracts (200 µg) were solubilised in 185 µL sample buffer (1% ASB-14, 2 mM tributyl phosphine, 7 M urea, 2 M thiourea, 0.5% (w/v) carrier ampholytes, 0.001% (w/v) bromophenol blue, 40 mM Tris-HCl) and centrifuged at 10,000 × *g* for a period of 10 min.

Samples were applied to the lanes of an isoelectric focussing (IEF) tray and pre-absorbed into 11 cm non-linear pH 3-10 ReadyStrip immobilised pH gradient (IPG) strips for 1 h at room temperature. After this time period, pre-moistened electrode wicks were inserted at each electrode and the strips were overlaid with mineral oil to minimise sample evaporation.

Active rehydration was performed at 50 V for 12 h at 20 °C immediately followed by IEF performed on a PROTEAN IEF cell. Program parameters were set as 250 V (20 min), 8000 V (2.5 h), and 8000 V for 20 kVh to a total of 30 kVh.

On completion of IEF, the IPG strips were gently blotted on pre-moistened blotting paper and equilibrated in buffer (6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 5 mM tributyl phosphine, 2.5 % acrylamide, 375 mM Tris-HCl) for 45 min at room temperature or frozen at -80 °C for future analysis. Equilibrated IPG strips were rinsed in running buffer (0.3% (w/v) Tris-HCl, 1.44% (w/v) glycine, 0.1% (w/v) SDS) and then embedded onto a 14% acrylamide/piperazine bisacrylamide (37.5:1) polyacrylamide electrophoresis gel using ReadyPrep overlay agarose. The second dimension was run at 120 V with cooling. The presence of proteins within gels was detected by staining with Coomassie blue R-250 solution (0.1% (w/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid) for 2 h, followed by multiple changes of destain solution (6.7% (v/v) methanol, 13.3% (v/v) acetic acid) until a clear background was obtained. A

molecular weight marker (Sigma Marker, High Molecular Weight Range) was used in the second dimension. Digital images of gels and processed IPG strips were made with a Canon D60 DSLR and archived as Canon RAW files. Triplicate biological replicates were assessed.

E5 – Reverse phase chromatography

A flow rate of 300 nL min^{-1} was used on the LC system. A 75 µm x 15 cm C18 analytical column (LC Packings, CA, USA) preceded by a C18 trap column (LC Packings, CA, USA) were used for peptide separation at 30 °C. Buffer A (98% (v/v) HPLC water, 2 % (v/v) HPLC acetonitrile with 0.1% (v/v) formic acid (FA)) and buffer B (98% (v/v) HPLC acetonitrile, 2% (v/v) HPLC water with 0.1% (v/v) FA) were used to perform a 65 min gradient. The separation performance was accomplished via Chromeleon software (UV-Vis detection) as well as mass spectrometry (total ion chromatogram). With SCX fractions, a 30 minutes wash with buffer A was performed prior to start the 65 min gradient in order to remove the salt content from the samples.

E6 - In-gel digestion and LC-MS/MS

Gel pieces were washes with 100 mM ammonium bicarbonate 200 µL and de-stained with 50:50 (v/v) HPLC water/ HPLC acetonitrile and the last procedure repeated until gels were clear. Once clear, dehydration was performed by addition 200 µL of acetonitrile and the process repeated until gel pieces were white. Reduction was performed in the presence of 100 µL of 10 mM DTT at 50 °C for 30 min and alkylation was performed in the dark for 60 minutes upon addition of 100 µL of 55 mM iodoacetamide. Trypsin was added (0.2 µg) and

samples were left for 30 min at room temperature to allow absorption of trypsin. More ammonium bicarbonate solution was added to completely cover the gels pieces and the samples were incubated overnight at 37 °C.

After digestion, peptides were extracted to clean Eppendorf tubes after 10 minutes incubation with 100 µL of 5% acetonitrile 0.1% (v/v) formic acid (FA) and a further 10 mins with 100 µL of 50% acetonitrile 0.1% (v/v) FA. Each supernatant was carefully collected and combined with the first peptide fraction. The sample volume was reduced, in order to remove volatile solvents and to concentrate the peptides, to < 10 µL in a vacuum centrifuge at ambient temperature and samples were stored at – 20 °C until required for mass spectrometric analysis.

Results Section

Figure S1

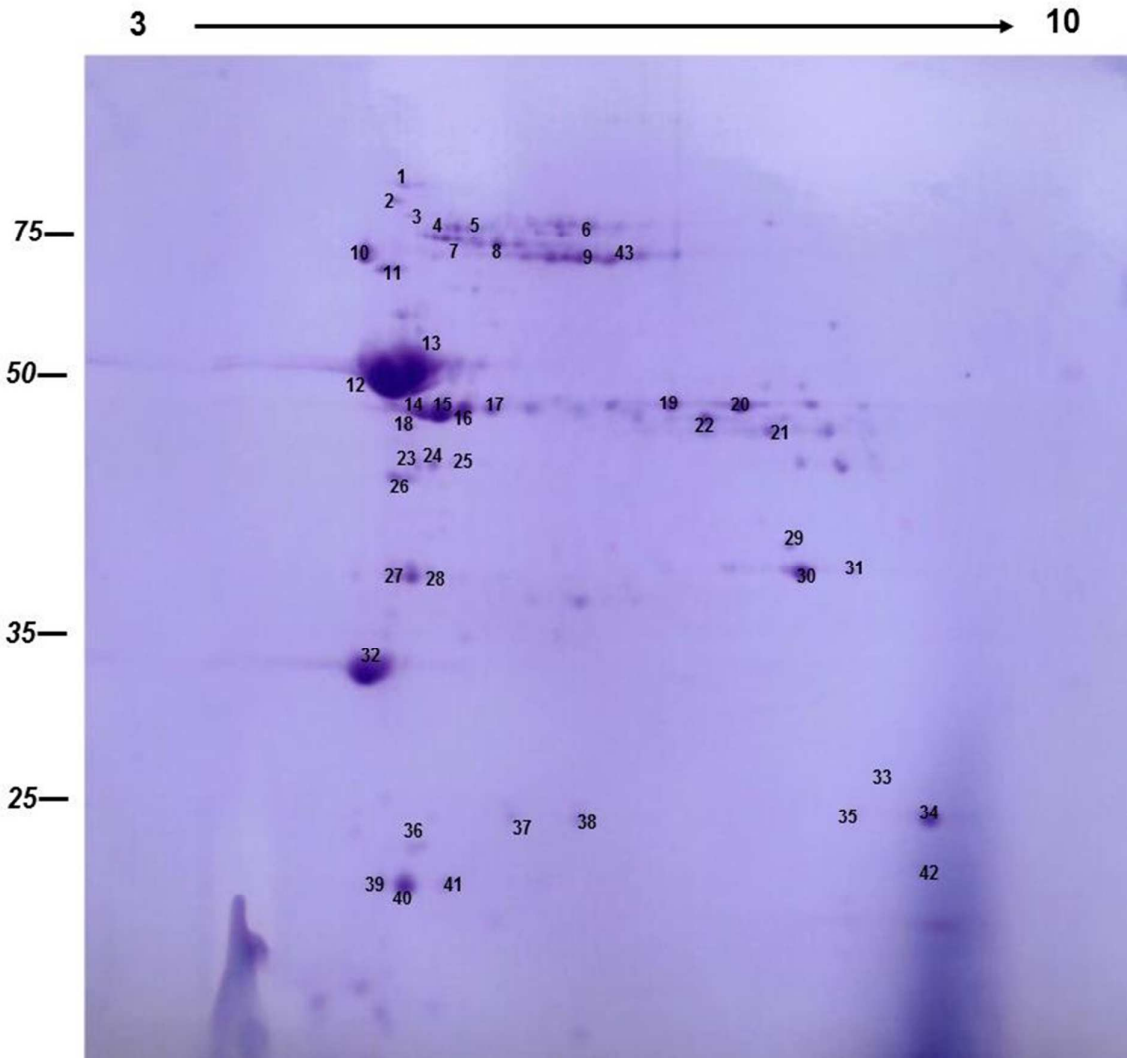

Figure S1 – Coomassie stained 2D gel from OMVs proteins from *P. aeruginosa* PAO1 biofilms. OMVs proteins (200 µg) were focused using a pH 3-10 immobilised pH gradient (IPG) strip and separated by 14% sodium dodecyl polyacrylamide gel electrophoreses (SDS-PAGE). Representative spots (43) were selected for further mass spectrometric analysis after in-gel digestion.

Figure S2

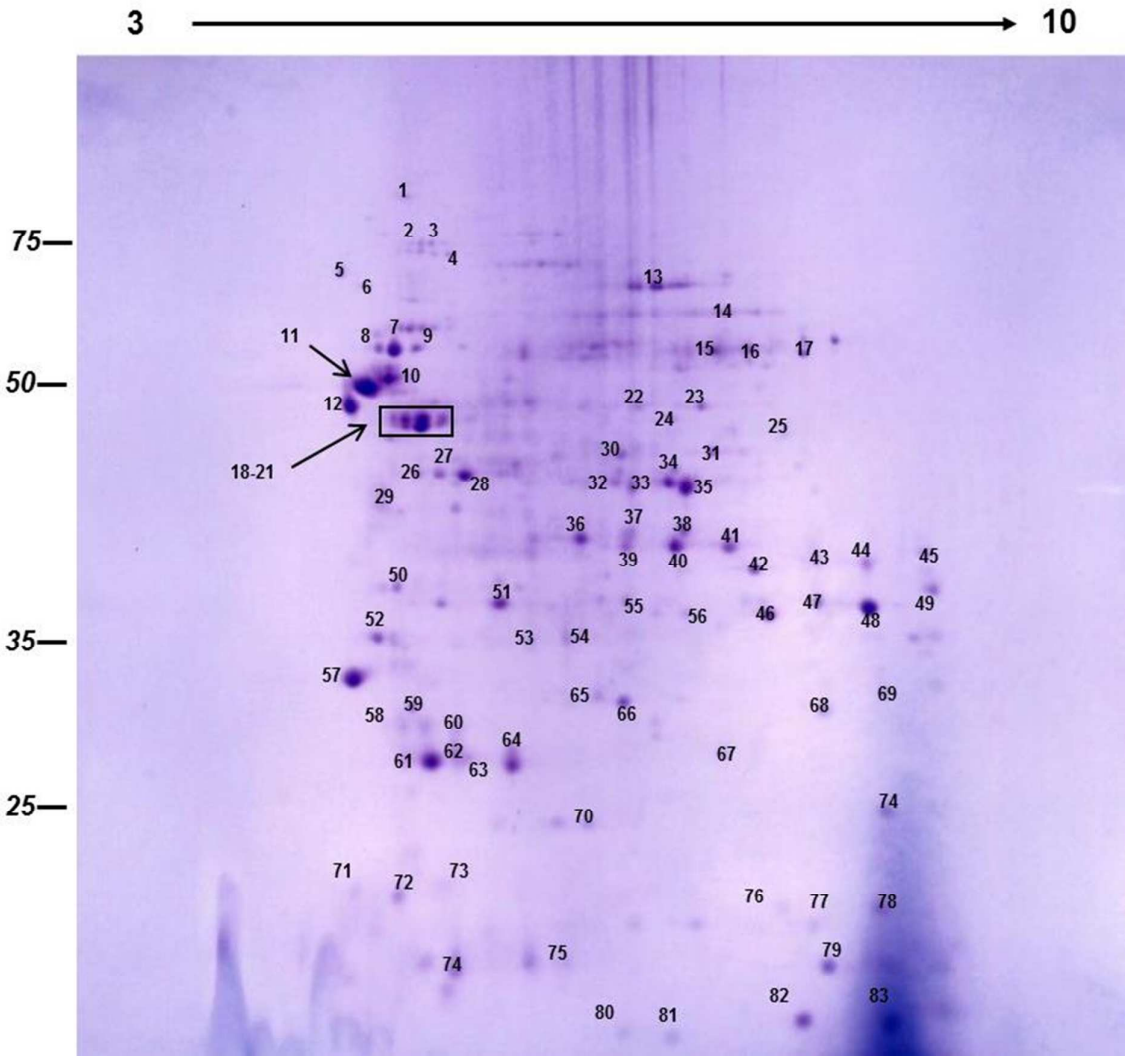

Figure S2 – Coomassie stained 2D gel from the whole matrix proteins from *P. aeruginosa* PAO1 biofilms. Proteins extracted from the whole matrix (200 µg) were focused by isoelectric focusing pH 3-10 IPG strips followed by separation on a 14% SDS-PAGE. Representative spots (83) were selected for further mass spectrometric analysis after in-gel digestion.

Figure S3

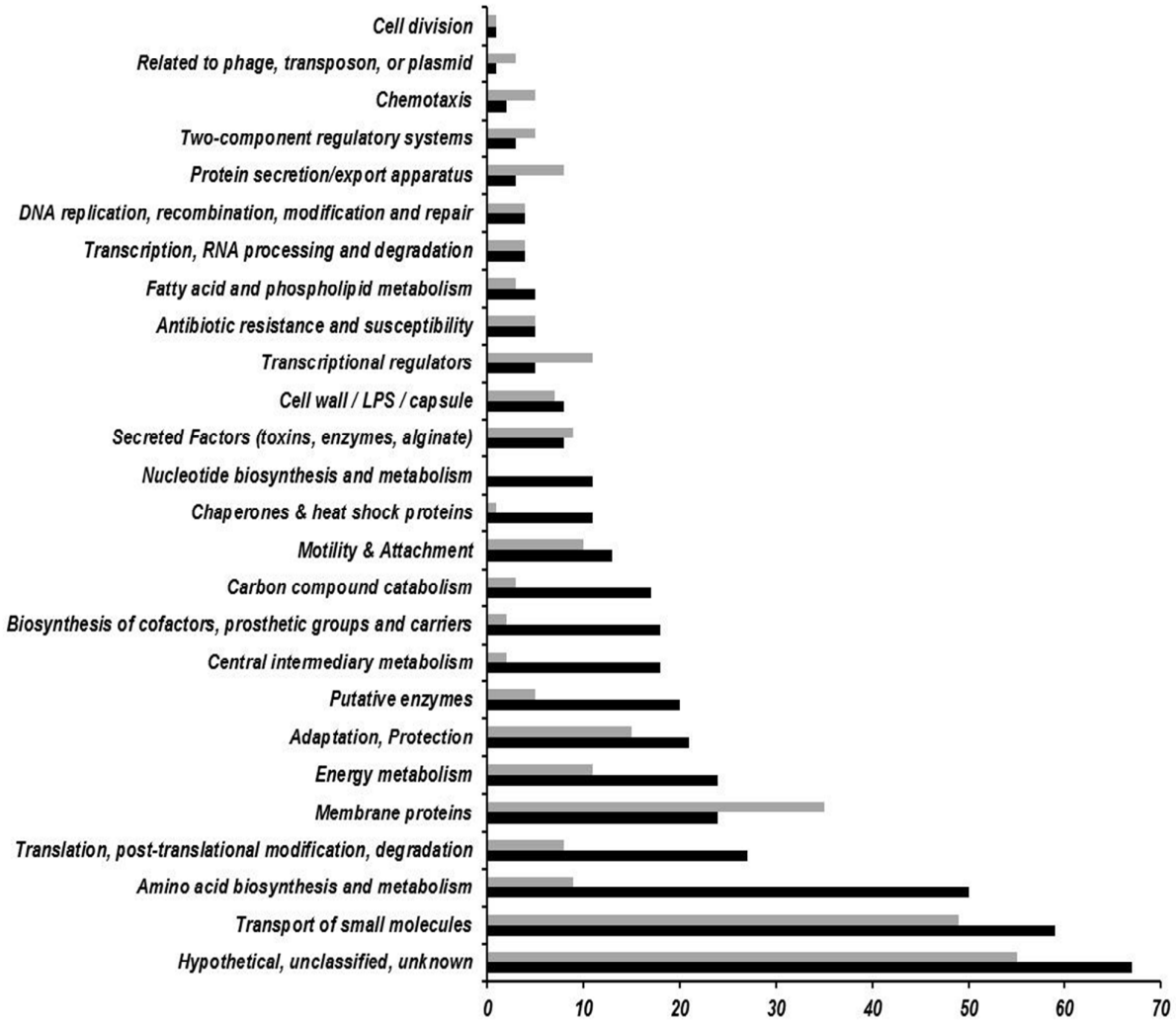

Figure S3 – Functional categories of both OMVs and whole matrix proteins (grey bars and black bars respectively). All identified proteins were grouped in 26 functional categories according *P. aeruginosa* database. On the x and y axis, the number of proteins and functional categories are respectively indicated.

Table S1 – Most hydrophilic (negative GRAVY values) and hydrophobic (positive GRAVY values) proteins from both OMVs and matrix proteomes of *P. aeruginosa* PAO1 biofilms. These GRAVY values are predicted based on amino acid sequence. In addition, predicted pI and MW are also shown as well as subcellular location and functional class according *P. aeruginosa* database.

Locus number	Gene name	Protein name	Subcellular location*	Functional class	pI	Mw	GRAVY	Sample
PA1775	cmpX	Conserved cytoplasmic membrane protein, CmpX protein	OMVs (1); CM (3)	Membrane proteins	8.80	29018.50	0.98	OMVs
PA3109		Colicin V production family protein	CM (3)	Adaptation, Protection	7.52	19245.80	0.98	OMVs
PA5287	amtB	Ammonium transporter AmtB	CM (3)	Membrane proteins; Transport of small molecules	5.58	45835.70	0.93	OMVs
PA5341		LysE type translocator family protein	CM (3)	Hypothetical, unclassified, unknown	11.80	22338.40	0.77	OMVs
PA3641	agcS	Amino acid carrier family protein	OMVs (1); CM (3)	Transport of small molecules	8.30	50326.80	0.75	OMVs
PA4053	ribE	6,7-dimethyl-8-ribityllumazine synthase	Cy (1); Cy (3)	Biosynthesis of cofactors, prosthetic groups and carriers	5.75	16412.90	0.45	OMVs
PA2221		Helix-turn-helix domain protein	Cy (3)	Hypothetical, unclassified, unknown	10.75	45116.90	-0.69	OMVs
PA2457		RHS Repeat family protein	Un (3)	Hypothetical, unclassified, unknown	8.97	36102.60	-0.70	OMVs
PA5120		Hypothetical protein	Un (3)	Hypothetical, unclassified, unknown	8.27	15555.40	-0.78	OMVs
PA4282		Probable exonuclease	Ex (3); Un (3)	DNA replication, recombination, modification and repair	5.50	138959.30	-0.88	OMVs
PA4315	mvaT	Transcriptional regulator MvaT, P16 subunit	Cy (1); Cy (3)	Transcriptional regulators	10.14	14180.20	-0.94	OMVs
PA2146		Conserved hypothetical protein	Un (3)	Hypothetical, unclassified, unknown	10.91	5580.00	-1.59	OMVs
PA3109		Colicin V production family protein	CM (3)	Adaptation, Protection	7.52	19245.80	0.98	Matrix
PA4053	ribE	6,7-dimethyl-8-ribityllumazine synthase	Cy (1); Cy (3)	Biosynthesis of cofactors, prosthetic groups and carriers	5.75	16412.90	0.45	Matrix
PA2478		Thiol:disulfide interchange protein DsbD	CM (2); CM (3)	Membrane proteins; Putative enzymes; Chaperones & heat shock proteins	7.14	62092.70	0.41	Matrix
PA2527	muxB	AcrB/AcrD/AcrF family protein	CM (2); CM (3)	Membrane proteins; Transport of small molecules; Antibiotic resistance and susceptibility	5.07	112313.20	0.35	Matrix
PA4301	tadB	Type II secretion system (T2SS), F family protein	CM (3)	Membrane proteins; Motility & Attachment	10.74	32406.90	0.34	Matrix
PA5262	fimS	Alginate biosynthesis protein AlgZ/FimS	Cy (1); CM (3)	Two-component regulatory systems; Motility & Attachment	7.44	40111.40	0.24	Matrix
PA2911		Probable TonB-dependent receptor	OM (2); OM (3)	Membrane proteins; Transport of small molecules	6.69	80289.30	-0.58	Matrix
PA4710	phuR	Heme/Hemoglobin uptake outer membrane receptor PhuR precursor	OM (1); OM (3)	Transport of small molecules	5.94	84724.30	-0.58	Matrix
PA4514		Probable outer membrane receptor for iron transport	OMVs (1); OM (2); OM (3)	Transport of small molecules	5.90	82336.00	-0.59	Matrix

PA0595	*ostA*	*Organic solvent tolerance protein OstA precursor*	*OMVs (1); OM (3)*	*Adaptation, Protection*	*5.29*	*104271.10*	*-0.60*	*Matrix*
PA4236	*katA*	*Catalase*	*Pe (1); OMVs (1); Un (3)*	*Adaptation, Protection*	*6.69*	*55588.80*	*-0.66*	*Matrix*
PA4232	*ssb*	*Single-stranded DNA-binding protein*	*Cy (3)*	*DNA replication, recombination, modification and repair*	*5.28*	*18557.40*	*-1.03*	*Matrix*

* - OMVs stands for outer membrane vesicles; OM stands for outer membrane, CM stands for cytoplasmic, Pe stands for periplasmic, Cy stands for cytosolic, Ex stands for extracellular and Un stands for unknown. Numbers 1, 2, and 3 stands for level of confidence, where 1 means experimentally proven location in *P. aeruginosa* PAO1, 2 means experimentally proved location in other organisms and 3 means computationally predicated.

Table S2 – Periplasmic proteins identified in the OMVs. Only proteins which have been experimentally identified in the periplasm of *P. aeruginosa* (level 1) or other organisms (level 2) are indicated.

Locus number	*Gene name*	*Protein name*	*Subcellular location**
PA4423		*LppC lipofamily protein*	*OMVs (1); Pe (1)*
PA0972	*tolB*	*Protein TolB*	*OMVs (1); Pe (2)*
PA1777	*oprF*	*Outer membrane porin F*	*OM (1); OMVs (1); Pe (1)*
PA1092	*fliC*	*B-type flagellin*	*Pe (1); OMVs (1)*
PA0766	*mucD*	*Serine protease MucD*	*Pe (1); OMVs (1)*
PA4366	*sodB*	*Superoxide dismutase*	*Pe (1)*
PA1032	*quiP*	*Acyl-homoserine lactone acylase quiP*	*Pe (1)*
PA4579		*Uncharacterized protein*	*Pe (1)*
PA3296	*phoA*	*Alkaline phosphatase H*	*Pe (2)*
PA5167	*dctP*	*TRAP transporter solute receptor, DctP family protein*	*Pe (2)*

* - OMVs stands for outer membrane vesicles and Pe stands for periplasmic. Numbers 1 and 2 stands for level of confidence, where 1 means experimentally proven location in *P. aeruginosa* PAO1 and 2 means experimentally proved location in other organisms.

Table S3 – Extracellular proteins identified in the OMVs. Only proteins which have been experimentally identified as extracellular in *P. aeruginosa* (level 1) or other organisms (level 2) are indicated.

Locus number	*Gene name*	*Protein name*	*Subcellular location**
PA1871	*lasA*	*Protease lasA*	*Ex (1); OMVs (1)*
PA0423	*pasP*	*PasP*	*Ex (1); OMVs (1)*
PA0962	*dps*	*Ferritin-like domain protein*	*Ex (1)*
PA2939		*Peptidase M28 family protein*	*Ex (1)*
PA0041		*Uncharacterized protein*	*Ex (1)*
PA2862	*lipA*	*Lactonizing lipase*	*Ex (1)*
PA1844	*tse1*	*Uncharacterized protein*	*Ex (1)*
PA1245	*aprX*	*Uncharacterized protein*	*Ex (2)*

* - OMVs stands for outer membrane vesicles and Ex stands for extracellular. Numbers 1 and 2 stands for level of confidence, where 1 means experimentally proven location in *P. aeruginosa* PAO1 and 2 means experimentally proved location in other organisms.

Table S4 – Cytoplasmic proteins identified in the OMVs. Only proteins which have been experimentally identified in the cytoplasm of *P. aeruginosa* (level 1) or other organisms (level 2) are indicated.

Locus number	*Gene name*	*Protein name*	*Subcellular location**
PA0516	*nirF*	*Lactonase, 7-bladed beta-propeller family protein*	*Cy (1); OMVs (1)*
PA1804	*hupB*	*DNA-binding protein HU-beta*	*Cy (1); OMVs (1)*
PA5171	*arcA*	*Arginine deiminase*	*Cy (1); OMVs (1)*
PA4385	*groEL*	*60 kDa chaperonin*	*OMVs (1); Cy (2)*
PA4053	*ribE*	*6,7-dimethyl-8-ribityllumazine synthase*	*Cy (1)*
PA4315	*mvaT*	*Putative mvaV*	*Cy (1)*
PA0779	*asrA*	*Lon protease*	*Cy (2)*
PA1781	*nirB*	*Nitrite reductase [NAD(P)H], large subunit*	*Cy (2)*
PA1803	*lon*	*Lon protease*	*Cy (2)*
PA3924		*AMP-binding enzyme family protein*	*Cy (2)*
PA4265	*tufA*	*Elongation factor Tu*	*Cy (2)*
PA4414	*murD*	*UDP-N-acetylmuramoylalanine--D-glutamate ligase*	*Cy (2)*
PA0176	*aer2*	*Methyl-accepting chemotaxis (MCP) signaling domain protein*	*Cy (2)*

* - OMVs stands for outer membrane vesicles and Cy stands for cytoplasmic. Numbers 1 and 2 stands for level of confidence, where 1 means experimentally proven location in *P. aeruginosa* PAO1 and 2 means experimentally proved location in other organisms.

Table S5 – Cytoplasmic membrane proteins identified in the OMVs. Only proteins which have been experimentally identified in the cytoplasmic membrane of *P. aeruginosa* (level 1) or other organisms (level 2) are indicated.

Locus number	*Gene name*	*Protein name*	*Subcellular location**
PA1807	*nikE*	*Nickel import ATP-binding protein NikE*	*OMVs (1); CM (2)*
PA2687	*pfeS*	*Sensor protein pfeS*	*CM (1)*
PA3101	*xcpT*	*Type II secretion system protein G*	*CM (1)*
PA5361	*phoR*	*Phosphate regulon sensor kinase PhoR*	*CM (2)*
PA5512	*mifS*	*His Kinase A domain protein*	*CM (2)*

* - OMVs stands for outer membrane vesicles and CM stands for cytoplasmic membrane. Numbers 1 and 2 stands for level of confidence, where 1 means experimentally proven location in *P. aeruginosa* PAO1 and 2 means experimentally proved location in other organisms.

Table S6 – Extracellular proteins identified in the whole matrix. Only proteins which have been experimentally identified as released on the extracellular region by *P. aeruginosa* (level 1) or other organisms (level 2) are indicated.

Locus number	*Gene name*	*Protein name*	*Subcellular location**
PA3807	*ndk*	*Nucleoside diphosphate kinase*	*Cy (1); Ex (1)*
PA0962	*dps*	*Ferritin-like domain protein*	*Ex (1);*
PA0852	*cbpD*	*Chitin binding domain protein*	*Ex (1)*
PA2939		*Peptidase M28 family protein*	*Ex (1)*
PA3724	*lasB*	*elastase LasB*	*Ex (1)*
PA0423	*pasP*	*PasP*	*Ex (1); OMVs (1)*
PA2862	*lipA*	*Lactonizing lipase*	*Ex (1)*

* - OMVs stands for outer membrane vesicles and Ex stands for extracellular. Numbers 1 and 2 stands for level of confidence, where 1 means experimentally proven location in *P. aeruginosa* PAO1 and 2 means experimentally proved location in other organisms.