SI Material and Methods

Sample preparation, whole cell processing, and reagents. In the absence of an established standard for comparable biofilms and planktonic controls, growth conditions were carefully considered and tested prior to sample preparation. They were matched for all possible variables (except surface attachment), including the nutrient composition and volume of media, vessel material (glass), surface area for gas exchange, and aeration (static). The cultures were monitored for cell density (i.e. OD_{600nm}) and the corresponding readings are reported in Figure S2. Six biological replicates were inoculated with a 5 ml aliquot of an overnight *Pseudomonas aeruginosa* PAO1 culture normalized to an optical density at 600 nm (OD_{600nm}) of 3.0 at each time point (24, 48, and 96 h). Three planktonic replicates were grown in a static incubator at 37°C in 400 ml of tryptic soy broth (TSB) (BD, Franklin Lakes, NJ, USA). Following even surface distribution of the inoculum, three biofilm replicates were grown at 37°C on 400 ml of tryptic soy agar (TSA) (BD) solidified in a glass dish (190 × 100 mm; Corning, Tewksbury, MA, USA). At the end of each incubation period biofilm samples were scraped off the surface using a sterile scoopula and dispersed by mixing in 400 ml of fresh TSB per sample. The optical density of the planktonic cultures and dispersed biofilms was measured by spectrometry (OD_{600nm}) (Fig. S2), and then each sample was diluted to an OD_{600} of 1.0 with fresh TSB. The whole cells were pelleted via centrifugation at 12,000 × *g,* 10 min, 4°C (Beckman Coulter Avanti J-E centrifuge, JA-10 rotor, Mississauga, ON, Canada), and the resulting supernatant was set aside for OMV isolation (see below). The whole cells were washed once in Tris-HCl (pH 8.3) and stored at -20°C until they were lysed using three rounds of liquid nitrogen freeze/room temperature thaw, followed by 4 × 30 s of sonication on ice (setting 3, Ultrasonic Processor XL, Misonix Inc., Farmingdale, NY, USA). Samples were cooled for 60 s on ice between sonication sessions. Cellular debris was removed from the whole cell lysate by centrifugation in an Avanti J-E

centrifuge (6000 × *g*, 10 min, 4°C) (Beckman Coulter), and an aliquot of the supernatants was collected, treated with a protease inhibitor complex (Roche Diagnostics, Indianapolis, IN, USA), and stored at -20°C for further processing. All reagents, unless otherwise stated, were obtained from Sigma-Aldrich Canada Co. (Mississauga, ON, Canada).

OMV isolation and processing. Growing the biofilms on a solid agar surface, rather than in a continuous flow system, allowed for the collection of OMVs without sample mixing or loss. The supernatant from above (i.e. normalized cultures with whole cells removed) were subjected to five rounds of ultracentrifugation at 150,000 × *g*, 1.5 h, 4°C (Beckman L8-55M ultracentrifuge, Ti45 rotor). The resulting pellet containing the OMV was gently resuspended in Tris-HCl (pH 8.3), filtered through a 0.45 μm cellulose acetate membrane syringe filter (Thermo Scientific, Ottawa, ON, Canada), and then sedimented by centrifugation at 21,000 × *g*, 30 min, 4°C using a microcentrifuge (Eppendorf 5424 microcentrifuge, FA-45-24 rotor, Mississauga, ON, Canada) as previously described[2]. The final pellet was resuspended in 500 μl of Tris-EDTA (pH 8.3) and treated with a protease inhibitor complex (Roche) as above. Samples were examined at each step of the isolation by transmission electron microscopy (see details below) and SDS-PAGE (Fig. S3). The final OMV suspension was sonicated on ice (4 x 15 s, setting 3, Ultrasonic Processor XL, Misonix Inc., Farmingdale, NY, USA) with 60 s cooling periods between sonication sessions. Sample were stored at -20°C for further processing.

Imaging. Agar biofilms were imaged using scanning electron microscopy (SEM, Figures 2A, Figure S2). The biofilm samples were harvested from a replicate of the TSA cultures used for

proteome analysis (described above in Sample preparation, whole cell processing, and reagents) and stored in buffer containing equal parts of 0.07 M sodium phosphate dibasic and potassium phosphate (pH 6.8). Samples were fixed using 2% glutaraldehyde followed by 4% osmium tetraoxide and dehydrated with ethanol prior to critical point drying. Finally, samples were coated with gold and imaged using a Hitachi S-570 scanning electron microscope (Hitachi High Technologies America Inc., Schaumburg, IL, USA). For TEM imaging of biofilm whole cells (Figure 2B only), a sample was harvested from a drip-reactor in order to reduce artifacts from sample preparation. All whole cell and OMV samples imaged by TEM (Figure 2B,C and Figure S3) were placed on carbon-coated copper grids and stained with 1% uranyl acetate. Samples were imaged using a FEI Tecnai G2 F20 (FEI, Hillsboro, OR, USA)."

Quantification and in-solution digestion of proteins. The concentration of protein in the lysates was measured using a Micro BCA protein assay kit as per the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Protein (15 μg) was digested as previously described [1,2]. Briefly, proteins were treated with denaturation (6 M urea/2 M thiourea in 10 mM HEPES, pH 8.0), reduction (10 mM dithiothritol in a 50 mM ammonium bicarbonate [ABC] buffer, 30 min incubation), and alkylation (55 mM iodoacetamide in 50 mM ABC, 20 min incubation) buffers. Digestion was completed using 0.3 μg Lys C enzyme per sample (3 h) followed by 0.3 μg trypsin (16 h) (Princeton Separations, Adelphia, NJ, USA). The digestion was stopped by adding 40 μl of 0.1% trifluoroacetic acid for every 100 μl of digestion solution. The resulting peptides were desalted and concentrated using MonoSpin™ C18 microcolumns as per the manufacturer's instructions (GL Sciences, Torrance, CA, USA), and then lyophilized

using a speed vacuum concentrator (Savant Instruments, Holbrook, NY, USA). The samples were reconstituted in 0.1% formic acid in water prior to analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS). Five microliters of reconstituted, digested protein was injected via online partial-loop into an EASY-Spray ES801 column (75 μm × 50 cm) containing PepMap RSLC C18 (2 μm) stationary phase (Thermo Fisher Scientific). The sample was separated on an EASY-nLC 1000 chromatography system (Thermo Fisher Scientific, reverse phase mode, 0.1% formic acid as the mobile phase). A 120 min run was completed for each sample, including a pre-run equilibration and a post-run wash. Samples were run at 40°C, with a 0 to 30% acetonitrile gradient, at a rate of 250 nL/min. Eluted peptides were pumped through an EASY-Spray integrated emitter (Thermo Fisher Scientific) into a Q Exactive mass spectrometer (Thermo Fisher Scientific), and ionized using electrospray ionization. Parent Mass was measured in the Q Exactive Orbitrap, and subsequent fragmentation occurred in a nitrogen-filled higher-energy dissociation (HCD) collision cell, after which the MS scans were acquired with an Orbitrap mass analyzer. Spectrum and peak list generation were performed using Q Exactive 2.2 and Xcalibur 2.2 (Thermo Fisher Scientific) with the following acquisition parameters: MS resolution 70,000 FWHM, MS/MS resolution 17,500 FWHM, target 1 x 10^e6 ions, 10 MS/MS scans/cycle, 15 s dynamic exclusion. Raw data files were extracted and searched against the UniProtKB *P. aeruginosa* ATCC15692 database (5564 entries) using MaxQuant [3] quantitative proteomics software (version 1.4.0.5, Max Planck Institute of Biochemistry, Martinsried, Germany) with the following settings: label-free quantification (LFQ), Trypsin/P digestion, maximum of two missed cleavages and five modifications per peptide, 0.02 Da fragment ion mass tolerance, and 4.5 and 10.0 PPM parent ion tolerance for the

first search and main search, respectively. Variable peptide modification included deamidation of asparagine and glutamine plus oxidation of methionine. Fixed peptide modifications included carbamidomethyl alkylation of cysteine. Modifications used in protein quantification included acetylation of the n-terminus, and oxidation of methionine. A false disovery rate (FDR) of 1% was applied for both peptides and proteins using decoys generated with a reverse ATCC15692 database. The information provided herein is compliant with the Minimum Information about a Proteomics Experiment (MIAPE) Mass Spectrometry Informatics (MIAPE-MSI) guidelines[4]. Potential differences in protein loading are accounted for by an internal standardization calculation in MaxQuant[14]. LFQ intensities were not corrected for the number of theoretically observable peptides (i.e. iBAQ correction). A second pooled analysis with the iBAQ option selected showed minimal changes (data not shown).

Data Analysis.

Annotations, and functional grouping of whole cell proteins. A CSV file of the complete list of annotations for *Pseudomonas aeruginosa* PAO1 were obtained from the Pseudomonas Genome Database[5] and manually matched to the protein list generated by MaxQuant. Whole cell results were sorted by PseudoCAP function, and then organized into subcategories based on inclusion in either KEGG [6] or PseudoCAP pathways. Proteins with more than one PseudoCAP function were placed under the most relevant heading. Next, the proteins were separated into two groups: i) those with PseudoCAP function related to metabolism, and ii) "Other". The LFQ intensities (i.e. protein expression levels) for the proteins in the second group were summed within each subcategory, and then expressed as a fold difference between biofilm and planktonic samples at each time point. The fold difference for each PseudoCAP function was determined by taking the

115 average difference for all of the pathways listed under each functional class. Separately, the
116 proteins ascribed to metabolic pathways were organized under their respective PseudoCAP
117 functional classes. The total LFQ intensity per pathway, per sample group (i.e. three biological
118 replicates) was then expressed as a percentage of the total LFQ intensity for all samples groups
119 within that pathway.
120
121 ***Statistical Analysis of WC and OMV proteins***. Manual annotations (described above) and LFQ
122 intensities (generated in MaxQuant) were loaded into Perseus freeware (version 1.4.0.11, Max
123 Planck Institute of Biochemistry, Martinsried, Germany) for additional bioinformatic and
124 statistical analysis. Imputed LFQ intensity values were used for a single replicate in the 24-BV
125 group to accommodate for contamination and achieve the minimum group size required for
126 statistical analysis (n=3). A transformation of $\log_2(x)$ was applied to all LFQ intensities to satisfy
127 the distribution assumptions of the chosen statistical tests [7]. Two-sample t-tests were used to
128 identify significantly increased or decreased proteins, with cut-offs determined using a
129 permutation-based FDR of 0.05 based on 250 re-sampling iterations. Enrichment or depletion of
130 categorical variables (e.g. PseudoCAP and KEGG pathways) within the separate groups of
131 significant proteins were identified by completing Fisher exact tests, with the threshold set at p
132 <0.05. The diagram of differentially abundant OMV proteins was generated using Wordle™
133 advanced (www.wordle.net).
134
135 ***Whole cell pathway analysis***. A list of proteins involved in phenazine biosynthesis was exported
136 using search field "pathways", keyword "phenazine biosynthesis". Of the 18 exported proteins,
137 13 were identified in the MaxQuant results. Those not identified in the latter group included:

qscR (PA1898), a transcriptional regulator, and phzC2-G2 (PA1901-1905), which are homologous to the phzC1-G1 (PA4212-4216) proteins identified[8]. The LFQ intensities from the filtered proteins were loaded into Perseus (Max Planck Institute of Biochemistry), transformed as above, and used to generate heat maps that represent the average abundance of each protein in each whole cell replicate group. The individual sections of each heatmap corresponding to various portions of the pathways were manually aligned in Illustrator (Adobe Systems Incorporated, San Jose, CA, USA). Additional information included in the phenazine diagram was obtained from various sources [9-12].

Other analyses. Structural feature annotations were manually curated from the Pseudomonas Genome Database[5]. Pie charts, bar graphs, and Student's t-test (2-tailed, homoscedastic) for outer membrane (OM) proteins were generated using Microsoft Excel (Microsoft Corporation, WA, USA) or Prism (GraphPad Software, CA, USA).

Endogenous PYO measurement. Endogenous PYO was measured in cell-free supernatant (CFS) (normalized to whole cell density) or OMV samples (normalized to protein) by LC-MS within the Advanced Analysis Centre at the University of Guelph. Samples were analyzed using a Dionex UHPLC UltiMate 3000 liquid chromatograph interfaced to an amaZon SL ion trap mass spectrometer (Bruker Daltonics, MA, USA). A Poroshell 120 EC- C18 column (2.7 micron particle size, 150 mm x 4.6 mm) was used for chromatographic separation (Agilent Technologies, CA, USA). The initial mobile phase conditions were 98 % water (0.1 % formic acid) and 2% acetonitrile (0.1% formic acid) then a single step gradient to 100% acetonitrile (0.1 % formic acid) in 40 min. The flow rate was maintained at 0.4 mL/min. The mass spectrometer

electrospray capillary voltage was maintained at 4.5 kV and the drying gas temperature of 280 °C with a flow rate of 10 L/min. Nebulizer pressure was 40 psi. Nitrogen was used as both the nebulizing and drying gases, and helium was used as collision gas at 60 psi. Optimal fragmentation conditions for pyocyanin were determined using a standard solution (pyocyanin P0046, Sigma-Aldrich, MO, USA). MRM acquisition mode was used to select the parent ion at 211 m/z with 4 m/z isolation width and 183 m/z product ion was monitored for quantitation. The mass spectrometer was set on enhanced resolution positive-ion mode. The instrument was externally calibrated with the ESI TuneMix (Agilent). Quantitation of pyocyanin was determined using the QuantAnalysis software (Bruker Daltonics). Calibration curves were created with serial dilutions of the standard pyocyanin described above, in solution, and the concentration of unknowns was determined from the calibration function.

Planktonic growth and biofilm formation in the presence of exogenous PYO. The Microtiter Dish Biofilm Formation Assay[13] was completed in a 96-well plate with TSB supplemented with PYO (Sigma, 500-2.0 ng/mL range) or media alone (0 ng/mL). The samples were grown in a static incubator at 37°C for 24 hours and the non-adherent portion of the sample was transferred to a sterile 96-well plate for measurement of planktonic growth (OD600nm). The adherent biofilm was washed with PBS, air dried, and then stained with crystal violet (CV). The CV was solubilized using 30% acetic acid and the absorbance of the samples was measured at a wavelength of 600nm.

Epithelial responses to exogenous PYO**.** Human bronchial epithelial (16HBE14o-) were grown in LHC-8 media (Life Technologies, ON, CA) at 37°C with 5% CO_2 in a collagen-coated 96-well plate until confluence. The spent culture media was replaced with LHC-8 supplemented

with PYO (Sigma, range 4200-66 ng/mL) or media alone (0 ng/mL) and incubated for 1 h as above. The supernatant was collected, filtered with a 0.45 μM cellulose acetate membrane syringe filter (Thermo Scientific), and stored at -20°C until further processing. Commercially available enzyme-linked immunosorbant assays (ELISAs) were used to measure Interleukin-6 (IL-6) (BioLegend, CA, USA) as per the manufacture's instructions.

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