A Temporal Examination of the Planktonic and Biofilm Proteome of Whole Cell
*Pseudomonas aeruginosa* PAO1 using Quantitative Mass Spectrometry

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Running Title: Whole-cell proteomics of *Pseudomonas aeruginosa* PAO1

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Abbreviations

a.s.c. – average spectral counts
AUC – area under the curve
CF – cystic fibrosis
Bf – biofilm
Pt – planktonic
Abstract

Chronic polymicrobial lung infections are the chief complication in patients with cystic fibrosis. The dominant pathogen in late-stage disease is *Pseudomonas aeruginosa*, which forms recalcitrant, structured communities known as biofilms. Many aspects of biofilm biology are poorly understood; consequently, effective treatment of these infections is limited, and cystic fibrosis remains fatal. Here we combine in-solution protein digestion of triplicate growth-matched samples with a high-performance mass spectrometry platform to provide the most comprehensive proteomic dataset known to date for whole cell *P. aeruginosa* PAO1 grown in biofilm cultures. Our analysis includes protein-protein interaction networks and PseudoCAP functional information for unique and significantly modulated proteins at three different time points. Secondary analysis of a subgroup of proteins using extracted ion currents validates the spectral counting data of 1884 high confidence proteins. We demonstrate a greater representation of proteins related to metabolism, DNA stability, and molecular activity in planktonically grown *P. aeruginosa* PAO1. In addition, several virulence-related proteins were increased during planktonic growth, including multiple proteins encoded by the pyoverdine locus, uncharacterized proteins with sequence similarity to mammalian cell entry protein, and a member of the hemagglutinin family of adhesins, HecA. Conversely, biofilm samples contained an uncharacterized protein with sequence similarity to an adhesion protein with self-association characteristics (AidA). Increased levels of several phenazine biosynthetic proteins, an uncharacterized protein with sequence similarity to a metallo-beta-lactamase, and lower levels of the drug target gyrA, support the putative characteristics of *in situ* *P. aeruginosa* infections, including competitive fitness and antibiotic resistance. This
quantitative whole cell approach advances the existing *P. aeruginosa* subproteomes, and provides a framework for identifying and studying entire pathways critical to biofilm biology in this model pathogenic organism. The identification of novel protein-targets could contribute to the development of much needed antimicrobial therapies to treat the chronic infections found in patients with cystic fibrosis.
**Introduction**

*Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen that is a significant cause of morbidity and mortality in immunocompromised individuals, including those with cystic fibrosis (CF). Late-stage polymicrobial infections in CF patients are dominated by *P. aeruginosa*\(^1\), where this highly adaptable bacterium contributes to excessive pulmonary inflammation, subsequent destruction of lung tissue, and decreased lung function\(^2-4\). The ubiquity and persistence of this pathogen is due to a combination of virulence and resistance mechanisms, which allow it to adapt to environmental changes and colonize the CF host\(^5\). A key component of its adaptation and persistence is the transition from free-living (planktonic) bacteria into organized, surface-attached communities known as biofilms\(^6\). Importantly, biofilms are a barrier to host defenses and reduce the efficacy of anti-microbial therapies\(^7,8\). Attempts to understand the biochemical basis of biofilm development and resilience have consistently shown differences in protein composition in the model organism *P. aeruginosa* PAO\(^1\) (vs. their planktonic counterparts). However, technical limitations of these studies have left much of the biofilm proteome unresolved. Accordingly, many aspects of biofilm structure and function are still poorly understood.

In this study, we aimed to establish a quantitative proteomic framework to describe the similarities and differences between *P. aeruginosa* cells as they progress through planktonic (Pt) and biofilm (Bf) lifestyles. To achieve this, we performed an in-solution digest of triplicate whole cell lysates from growth condition-matched planktonic and biofilm cultures collected over three different time points. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed using a high-performance
quadrupole Orbitrap mass spectrometer (Q-Exactive), which resulted in the high-confidence identification of 1884 proteins. We identified proteins unique to each growth mode, and those that were differentially regulated, and assigned functions according to the *P. aeruginosa* Community Annotation Project (PseudoCAP)\(^{10}\). This work advances the growing body of *P. aeruginosa* planktonic subproteomes\(^{11}\) and provides new knowledge about the influence of growth mode on the protein composition of both planktonic and biofilm *P. aeruginosa* over time. A better understanding of the key aspects of *P. aeruginosa* biofilm biology has the potential to reveal novel targets for antimicrobial therapies and vaccine development for cystic fibrosis patients\(^{12}\).
Experimental Procedures

Study design

This study examined the protein content of whole cell samples collected from the biofilm and planktonic growth modes, at three different time points (24, 48, and 96 h). Three replicate cultures for each growth mode and time point were inoculated with aliquots of the same overnight *P. aeruginosa* culture grown to early-logarithmic phase. Growth conditions were closely matched for planktonic and biofilm samples, including media composition and volume, vessel material (glass), and aeration (static). Each of the six samples for a single time point (i.e. two growth modes with three biological replicates each) was carefully handled after harvesting to ensure equivalent post-incubation processing. An in-solution digestion of the whole cell lysates was performed to remove sample bias introduced by the use of 2D gel-based segregation and isolation. Protein content of the digested lysates were separated and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Bacterial strains, media and reagents

The parental strain for all studies was *P. aeruginosa* PAO1. All samples were inoculated with 5 ml of overnight culture normalized to an optical density at 600 nm (OD$_{600}$) of 3.0. Planktonic samples were grown at 37°C in a static culture containing 400 ml of tryptic soy broth (TSB) (BD, Franklin Lakes, NJ, USA). Following even surface distribution of the inoculum, the biofilm samples were grown at 37°C on 400 ml of tryptic soy agar (BD) solidified in a glass dish (190 × 100 mm; Corning, Tewksbury, MA, USA). At the end of each time point (24, 48 and 96 h) biofilm samples were scraped off the surface using a
sterile scoopula and dispersed in 400 ml of fresh TSB per sample. Triplicate cultures were normalized to an OD_{600} of 1.0 before further processing. All reagents, unless otherwise stated, were obtained from Sigma-Aldrich Canada Co. (Mississauga, ON, Canada).

**Preparation of whole cell samples**

Equal volumes of normalized biological replicates were centrifuged in an Avanti J-E centrifuge (12,000 × g, 10 min, 4°C) (Beckman Coulter, Pasadena, CA, USA). The cell pellets were washed once in Tris-HCl (pH 8.3) and then frozen at -20°C. Cells 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) with 60 s of cooling time between sonication sessions. Cellular debris was removed by centrifugation in an Avanti J-E centrifuge (6000 × g, 10 min, 4°C) (Beckman Coulter), and the supernatants were collected, treated with a protease inhibitor complex (Roche Diagnostics, Indianapolis, IN, USA), and stored at -20°C for further processing.

**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^{14}. Briefly, proteins were extracted using a denaturation buffer (6 M urea/2 M thiourea in 10 mM HEPES, pH 8.0), incubated at room temperature in reduction (10 mM dithiothritol in a 50 mM ammonium bicarbonate [ABC] buffer) and alkylation (55 mM iodoacetamide in 50 mM ABC)
buffers for 30 and 20 min, respectively. The digestion solutions were then treated with 0.3 µg Lys C enzyme per sample for 3 h, and incubated overnight with 0.3 µg trypsin (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. Finally, the peptides were desalted and concentrated using MonoSpin™ C18 microcolumns as per the manufacturer’s instructions (GL Sciences, Torrance, CA, USA). In order to accommodate ambient temperature shipping, the samples were 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 in reverse phase mode on an EASY-nLC 1000 chromatography system (Thermo Fisher Scientific) using 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) for fed nano-electrospray ionization (ESI) using a Q Exactive mass spectrometer. 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 was 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 10^6 ions, 10 MS/MS scans/cycle, 15 s dynamic exclusion. A spectral library was created using Proteome Discoverer 1.4 (Thermo Fisher Scientific). Sequest (XCorr Only) version 1.4.0.288 (Thermo Fisher Scientific) and X!Tandem version CYCLONE (2010.12.01.1) (The Global Proteome Machine Organization, thegpm.org) were used to analyze the spectra using the UniProtKB\(^{15}\) –*P. aeruginosa*–ATCC15692 database (5564 entries) and the Pseudomonas Genome Database\(^{10}\) (5560 entries). The tandem mass spectra were matched to amino acid sequences using a 0.02 Da and 10.0 PPM fragment ion mass and parent ion tolerance, respectively, and the following variable modifications: deamidation of asparagine and glutamine, oxidation of methionine and carbamidomethyl alkylation of cysteine (for SEQUEST), Glu$\rightarrow$pyro-Glu of the n-terminus, ammonia-loss of the n-terminus, gln$\rightarrow$pyro-Glu of the n-terminus, deamidation of asparagine and glutamine, and oxidation of methionine and carbamidomethyl of cysteine (for X!Tandem). The information provided herein is compliant with the Minimum Information about a Proteomics Experiment (MIAPE) Mass Spectrometry Informatics (MIAPE-MSI) guidelines\(^{16}\).

**Initial data validation and analysis**

Peptide and protein identifications generated from the amino acid sequences were validated using Scaffold (version 4.0.5, Proteome Software Inc., Portland, OR, USA). Peptides and proteins were accepted if they could be established at >95% probability using the Peptide\(^{17}\) and Protein\(^{18}\) Prophet algorithms respectively, with Scaffold delta
mass-correction, and at least two identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The false detection rate (FDR) was determined by searching the MS/MS spectra against a nonsense database (i.e. versions of the databases described above with the sequences reversed). Statistical analysis of proteins detected in both planktonic and biofilm samples was completed using the average number of spectral counts (a.s.c., calculated from the triplicate samples), which have been shown to accurately reflect the relative abundance of a protein in a sample\textsuperscript{19}. Proteins with a.s.c. values \textless 4 were not included in the analysis because low abundance proteins can fall outside the linear dynamic range of the relationship described above\textsuperscript{19}, and therefore were not deemed suitable for statistical testing. Statistical analysis of the semi-quantitative data was determined using an unpaired, two-tailed, student’s T-test with alpha set at 0.001 to ensure a high level of significance. Welch’s correction was applied to all calculations to add rigor and to account for the majority of the data showing unequal variance (i.e. statistically significant differences in standard deviations). Functional information for the highlighted proteins was gathered from the Pseudomonas Genome Database\textsuperscript{10}. The basic local alignment search tool (BLAST)\textsuperscript{20} was used to search for sequence similarities to hypothetical or unknown proteins using UniProt accession numbers, the non-redundant protein sequences database, and the blastp algorithm. Protein-protein interaction network were built using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, version 9.05) with a medium confidence level (0.4) and all available prediction methods\textsuperscript{21}.
Quantification of select proteins using extracted ion chromatograms

A subgroup of proteins was chosen for manual interpretation and quantitation measurement in order to support the spectral count analysis. Briefly, a spectral library was generated from a representative run of each biological condition. Extracted ion currents (XICs) were measured for a minimum of two representative peptides per sample using Skyline\textsuperscript{22} and Pinpoint (Thermo Fisher Scientific) software. The area under the curve (AUC) measurement, which represents the total signal of a peptide\textsuperscript{23}, was normalized to the AUC of combined XICs from two outer membrane (OM) proteins, BamB and OprL. These two proteins were chosen because they are core to the OM structure. The data was tested using a two-tailed, unpaired student’s T-test. Variances were assessed with an F-Test, and Welch’s correction was applied when the data showed significantly different standard deviations.
Results and Discussion

The objective of this study was to establish a quantitative proteomic framework to describe the similarities and differences between *P. aeruginosa* cells as they progress through planktonic and biofilm lifestyles. Performing in-solution digestion allowed us to generate highly complex samples that could be analyzed with a high performance MS platform. As a result, 1884 protein clusters were detected in the whole cell lysates with >95% probability, and a minimum of two identified peptides (Table S1). This exceeds previous *P. aeruginosa* PAO1 proteomic studies including: 991 proteins detected in the planktonic inner membrane (with a minimum of 1 identified peptide); 338 in planktonic outer membrane vesicles; 395 in the planktonic periplasm; and ~700 from total cell extracts of biofilm and planktonically grown whole cells. Our false discovery rate was 0.1%, identifying three decoys within the detected proteins, all of which were only found in a single replicate with minimal spectral counts (<3). For our initial semi-quantitative analysis of the large dataset, we segregated the 163 proteins with average spectral counts of ≥4 that were either: i) unique to planktonic growth bacteria – those that were detected in planktonic samples and absent in biofilm samples (Table S2); ii) unique to biofilm growth bacteria – those that were detected in biofilm samples and absent in planktonic samples (Table S3); or iii) modulated conserved protein clusters – found in both planktonic and biofilm growth bacteria at significantly different levels (Table S4). The remaining proteins were either present in both growth modes with p values > 0.001, or had average spectral counts < 4 at all three time points. Although we did not consider data for proteins with a.s.c. <4 for quantitative analysis (representing less than 20% of the
dataset), this portion of the data still provides useful qualitative information about the whole cell proteome of PAO1.

Next, protein-protein interaction networks were identified for: i) proteins that were unique or significantly higher in planktonic cultures (Fig 1); and ii) proteins that were unique or significantly higher in biofilm cultures (Fig 2). There were fewer connections between the biofilm proteins (vs. planktonic), suggesting a smaller number of biological interactions between the identified proteins in the biofilm cells. Conversely, since the protein networks are based on a combination of genomic context, high-throughput experiments, co-expression, and previous knowledge, it could also reflect a generalized lack of biofilm-sourced proteins due to the common practice of studying P. aeruginosa sourced from planktonically grown cultures. The latter scenario reiterates the importance of studies, such as this one, which examine the key differences between planktonic- and biofilm-grown bacteria.

The cultures were grown and standardized to ensure that cell densities were closely matched at all time points. The average cell densities for the biofilm samples were recorded at OD$_{600}$ (± SD) of 1.85 (± 0.06), 1.47 (± 0.15) and 2.4 (± 0.14) for 24, 48, and 96 h, respectively. Similarly, the average cell densities for the planktonic samples were recorded at OD$_{600}$ of 1.94 (± 0.1), 1.2 (± 0.1) and 1.3 (± 0.1) for the same matching time points. From this, we concluded that we had comparable samples that were representative of two populations of P. aeruginosa that differed only in their growth mode (free-living planktonic vs. solid surface biofilm). Negative results obtained from immunoassays using specific anti-RNA polymerase antibodies to detect the presence of
RNA polymerase in the cell-free supernatants allowed us to rule out cell lysis as a significant concern in the aged cultures (data not shown).

Proteins unique to planktonically grown *P. aeruginosa* PAO1 cells

From the 163 segregated proteins, we found 68 that were exclusively detected in planktonic samples in at least one time point (Table S2). The greatest number of assigned PseudoCAP functions belong to the categories of “putative enzymes”, “carbon compound metabolism”, and “transcription, RNA processing, and degradation” at 48 h, and “transport of small molecules” and “adaptation and protection” at 96 h (Fig. 3A). Three proteins were identified that were deemed unique to the planktonic samples at all time points examined including: PA3195 (gapA), PA3450 (a probable antioxidant protein), and PA2462. PA2462 is a hypothetical protein found in the outer membrane (OM) and OM vesicles\(^10\) that has a 98% sequence identity to the adhesin HecA. Interestingly, PA2462 ion observed in MS increased in a progressive manner from 4.0, to 10.7, to 20.3 a.s.c. at the 24, 48, and 96 h time points, respectively. HecA mutants of the plant pathogen *Erwinia chrysanthemi* have reduced surface attachment, aggregate formation, and virulence\(^26\). This gradual increase suggests that in the late-stage planktonic culture, in which the bacteria may be reaching saturation, a phenotypic change may be occurring that favors surface attachment, perhaps as a survival mechanism or as a prelude to pellicle/floc formation. Additionally, six proteins encoded by the pyoverdine locus were all identified as unique to the 96-h planktonic samples (Fig 1). These proteins included: PA2385 (pvdQ), PA2386 (pvdA), PA2397 (pvdE), PA2398 (fpvA), PA2402 (pvdI) and PA2424 (pvdL), suggesting higher levels of iron-scavenging capabilities via increased
siderophore biosynthesis and transport\textsuperscript{27} in later-stage planktonic cultures. This is consistent with previous studies which demonstrated \textit{pvd} gene upregulation in planktonic \textit{P. aeruginosa} PA14 during growth in CF sputum\textsuperscript{28}, and mixed-species biofilms\textsuperscript{29}. In contrast, pyoverdine deficiency\textsuperscript{29}, or pyoverdine-deficient subpopulations\textsuperscript{30} within single species \textit{P. aeruginosa} biofilms, have been noted. Moreover, accumulation of pyoverdine-negative strains has been seen to increase as a function of colonization time in some CF patients\textsuperscript{31}. As pyoverdine-deficient PAO1 mutants display reduced virulence in multiple animal models of infection\textsuperscript{32,33}, it is possible that this siderophore is required during the transition from planktonic to biofilm (i.e. early colonization), but becomes less important in mature biofilms (i.e. established infection). This concept is supported by the observed levels in both growth modes studied within; however, further studies are required to determine the relative importance of this and other iron-scavenging compounds in planktonic and biofilm modes of growth.

Other proteins unique to the 96-h planktonic samples include PA0085 (hcp1), whose gene is encoded by the HSI-I virulence locus\textsuperscript{34}, and PA4689, which has 99\% sequence identity with mammalian cell entry (MCE) protein in \textit{P. aeruginosa}. The MCE protein family is best studied in the pathogen \textit{Mycobacterium tuberculosis}. It has been shown to be involved in the invasion of macrophages and other non-phagocytic cells, a key determinant of this pathogen’s ability to evade host responses and cause disease\textsuperscript{35}.

**Proteins unique to \textit{P. aeruginosa} PAO1 cells growing in a biofilm**

Fifty-eight proteins with a.s.c. $\geq$4 were exclusively identified in at least one biofilm sample time point (Table S3). The greatest number of assigned PseudoCAP functions
belonged to the categories of “putative enzymes”, “secreted factors”, and “transcriptional regulators” at 24 h, while “transport of small molecules” was increased at all three time points (Fig. 3B). Four proteins unique to biofilm samples were detected at all three time points: PA1946 (rbsB, binding protein component of ABC ribose transporter), PA3236 (a probable glycine betaine-binding protein), PA3923 and PA3922. PA3923 has 77% sequence identity to the adhesion AidA, while PA3922 only showed sequence similarity to other hypothetical proteins in a variety of *Pseudomonas* species. The consistent detection of PA3923 in the biofilm samples may be biologically relevant in light of studies in pathogenic *Escherichia coli* strains that showed that AidA was able to bind to itself and mediate autoaggregation and biofilm formation. Additional studies have shown that this adhesion can be inhibited by purified AidA and sodium deoxycholate (bile salt). High levels of this protein in our sample could promote cell-cell interactions conducive to growth in a biofilm. In addition, because AidA can bind to itself and other related proteins (e.g. Antigen 43), it could potentially play a role in influencing the composition of mixed species infections, promoting aggregation to other members of the same or different species, thus maximizing virulence potential. Fittingly, an AidA mutant of another CF pathogen, *Burkholderia cenocepacia*, showed deceased accumulation and virulence in a nematode host. The observation that it is sensitive to environmental influences and can be inhibited by purified AidA makes it an attractive target for therapeutic intervention in CF infections.

PA1899 (phzA2), a protein involved in the biosynthesis of phenazine, was exclusively found in the biofilm samples at 24 h. A related protein, PA0051 (phzH), a potential phenazine-modifying enzyme had one of the highest a.s.c. in all of the biofilm
samples and was also unique to biofilm at the 24-h time point. Phenazines play a variety of roles in \textit{P. aeruginosa} virulence including: inhibiting adherence and biofilm development of a competing organism (\textit{Candida albicans})\textsuperscript{39}; enhancing the release of pro-biofilm forming extracellular DNA\textsuperscript{40}; and interfering with host respiratory epithelium via modulation of cellular functions and innate immune responses (for review see\textsuperscript{41}). Interestingly, a study of the redundant operons phz1 and phz2 showed that phz2 was specifically required for phenazine production in \textit{P. aeruginosa} PA14 biofilms (while both contribute to production in planktonic cultures), as well as lung colonization in a mouse model of infection\textsuperscript{42}, further supporting the influence of growth mode on this pathway. Although not exclusive to biofilm samples, a third protein involved in the phenazine biosynthetic process, PA420 (phzM), which converts phenazine-1-carboxylic acid to pyocyanin\textsuperscript{43}, was significantly increased at 48 h in biofilm samples. Increased abundance of multiple proteins in this pathway (Fig 2), at several time points, supports earlier work highlighting the importance of phenazines and their derivatives in biofilm biology and \textit{P. aeruginosa} virulence.

Out of the six sample groups, the quorum sensing regulated protease LasA was only detected in the 24-h biofilm samples. This protein, a putative elastase\textsuperscript{44}, has been shown to facilitate invasion of epithelial cells by \textit{P. aeruginosa} PAO1 \textit{in vitro}\textsuperscript{45}. In addition, LasA is able to lyse staphylococci\textsuperscript{46,47} and therefore could potentially play a role in modulating the prominence of the CF pathogen \textit{Staphlococcus aureus} in mixed-species biofilms. The detection of bacteriolytic proteins in the biofilm samples supports the clinical observation that \textit{P. aeruginosa} is able to outcompete other CF pathogens and eventually become the dominant infecting bacteria in late-stage disease. The inability to
detect LasA at the later time points could be related to the observation that some virulence mechanisms, like drug resistance, can come at the cost of other mechanisms such as invasion\textsuperscript{48}. Accordingly, the uncharacterized protein PA2915 was only detected in 48-h biofilm samples. Further examination showed that this protein has orthologs in a variety of pathogens including \textit{S. aureus}, \textit{Acinetobacter baumannii}, and \textit{Legionella pneumophila}\textsuperscript{49}; sequence analysis showed a 99\% similarity to a beta-lactamase family protein. Production of beta-lactamases by \textit{P. aeruginosa} confers resistance to beta-lactam antibiotics\textsuperscript{50}, and is a significant source of recalcitrance in biofilm-dominated chronic lung infections in CF\textsuperscript{8}.

**Proteins common to both planktonic and biofilm \textit{P. aeruginosa} PAO1 cells**

Thirty-seven proteins (a.s.c. \(\geq 4\)) were detected in both planktonic and biofilm samples at significantly different levels at at least one time point (\(p \leq 0.001\), Table S4). This includes six proteins that were higher in biofilm samples at each of the 24, 48, and 96 h time points (vs. planktonic samples at the same time point). Conversely, 1, 16 and 3 proteins were decreased at the 24, 48, and 96 h time points, respectively. The assigned PseudoCAP functions for the increased proteins showed an even distribution (Fig. 4A), whereas the assigned PseudoCAP functions for the decreased proteins were highest in the categories of “translation, post-translational modification, degradation”, “amino acid biosynthesis”, and “energy metabolism” (Fig. 4B). This supports the general view of biofilms as slow growing, metabolically lethargic communities. One of the modulated proteins, PA3692 (lptF), an outer membrane protein belonging to the OmpA protein family\textsuperscript{10}, was significantly increased in biofilm samples at 24 h, present at high levels at
48 h, and absent in the 96-h biofilm samples. A study by Damron et al. found increased levels of LptF in an alginate-over producing mutant (PAO1kinB::aacC1), as well as several CF isolates grown on a solid surface\textsuperscript{51}. The authors went on to show that this outer membrane lipoprotein\textsuperscript{10} contributed to adhesion to lung epithelial cells \textit{in vitro}\textsuperscript{51}. Another modulated protein of note is PA3168 (gyrA), DNA gyrase subunit A, which was significantly decreased in the 48-h biofilm samples. This protein is the target of fluoroquinolone antibiotics\textsuperscript{52}, and consequently mutations are often found in quinolone-resistant strains of \textit{P. aeruginosa}\textsuperscript{53}. Studies that have examined the experimental adaptation of \textit{P. aeruginosa} to conditions that mimic the CF lung have shown an increased resistance with exposure to artificial sputum medium plus mucin, which was further exacerbated with the addition of an antibiotic; whole-genome sequencing of the adapted pathogens revealed that 4 of the 24 evolved genotypes had \textit{gyrA} mutations\textsuperscript{54}. Decreased levels of this protein in the biofilm samples align with its sensitivity to environmental influences, and would correspond with the putative increase in antibiotic resistance seen in bacteria growing as a part of a biofilm.

\textbf{Quantification of ion currents for select proteins supports spectral counting analysis}

Manual identification of the ion currents and quantitative measurement of extracted ion chromatograms was completed in order to support the spectral data discussed above. Measurement of extracted ion currents (XICs) more accurately represents the quantity of a peptide in a sample\textsuperscript{55}. Consequently, we used this method to substantiate the results of spectral counting within our experiment. In general, the area under the curve (AUC) measurements of the XICs showed a strong concordance with the observations made.
from the spectral count analysis (Fig. 5). Increased accuracy of the AUC measurements of XIC (vs. spectral counting) allowed us to set alpha at 0.05 in order to test our data for statistical significance. In doing so, we were able to confirm most of the significant time points identified in the previous analysis of the selected proteins (Table S4), and add several more. In general, the Pvd family of proteins retained their significant increase in the 96-h time point samples, however, the increased sensitivity of the XIC measurement was able to detect the presence of equal amounts of all four of the proteins in both growth modes (i.e. planktonic and biofilm) at the other two time points as well. These observations further support a decreased iron-scavenging potential in our late-stage biofilm samples. Hcp1 also showed confirmation of decreased levels in biofilm samples at 96 h; an observation that was extended to 48 h with the increased sensitivity of XIC measurement. PA2915 gained significance at 24 and 96 h in the biofilm samples (vs. 48 h only with a.s.c.), and significantly increased levels of the AidA-like protein (PA3923) was confirmed to be significantly higher at all three time points. Furthermore, the more detailed XIC analysis showed an interesting pattern in the 96 h time point. Two of the proteins, PhzA2, PhzH, were confirmed to be higher in biofilm samples at the 24 and 48 h time points, however, a more accurate reading of the MS data (via XIC) revealed that this pattern was reversed for both of these proteins at the 96 h time point (vs. a.s.c. that were either zero or below threshold, Table S2). This supports previous observations of increased spectral counts for virulence-related proteins such as the Pvd-family, PA2462 (similar to HecA), hcp1, and PA4689 (similar to MCE), in our late-stage planktonic samples. This secondary evaluation supported our initial spectral counting analysis and added additional information about more subtle changes within the sample groups. Future
experiments using complementary methods, such as selected reaction monitoring (SRM), have the potential to reveal additional complexities of the biofilm lifestyle.

**Concluding Remarks**

To the best of our knowledge, this study provides the most in-depth body of work to date examining the influence of growth mode on the protein content of whole cell *P. aeruginosa*. In addition, it provides a unique view of both planktonic and biofilm proteomes as they transition through various stages of development. In general, the profiles support many accepted notions of biofilm biology, including increased antibiotic resistance and decreased molecular and metabolic activity.

In addition to the above observations, this work identified multiple highly prominent hypothetical/unknown proteins that could play a significant role in biofilm structure or development. Further examination of the role of unique biofilm proteins with high sequence similarities to the adhesin AidA and a known metallo-beta-lactamase may provide novel targets for the treatment of refractory *P. aeruginosa* infections in CF patients. Of equal interest are proteins that were absent from the biofilm sample. The identification of a protein with 99% identity to mammalian cell entry (MCE) protein in mature planktonic samples warrants further investigation. In addition to MCE, other virulence proteins were prominent in the 96-h planktonic sample including multiple proteins encoded by the pyoverdine locus, and one protein encoded by the IAHP-related virulence locus; both of these paired with high levels of a protein with 98% sequence identity to the adhesin HecA. Further studies are required to determine if this mature planktonic sample, with high virulence potential and surface-attachment abilities,
represents a transitory *P. aeruginosa* population potentially involved in initial biofilm formation. The identification of two putative proteins with sequence similarity to known adhesions (AidA in planktonic samples and HecA in biofilm samples), reiterates the potential importance of these structures and also warrants further investigation.

The work presented here provides a framework and a reference collection for studying the proteins of entire pathways that may be integral to biofilm biology. This approach will become more relevant with the inevitable increase in multi-drug resistant strains of *P. aeruginosa* and the subsequent demand for multi-target antibiotic strategies.
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Figure Legends

Figure 1. Protein interactions for unique and significantly increased proteins in planktonic samples. Interactions were determined using STRING 9.05 (Search Tool for the Retrieval of Interacting Genes/Proteins)\(^2\). One hundred and ninety one interactions were observed between 83 proteins. Lines indicate known or predicted protein-protein interactions, with thicker lines indicating higher levels of confidence. Symbols, description\(^1\): small red squares, proteins involved in DNA replication, recombination, modification and repair (hupB, gyrA, uvrB); red dashed ovals, ribosomal proteins (rpsR, rpmD, rplX); small cyan squares, involved in cell envelope biogenesis (murG, PA4457 a.k.a. KdsD); cyan circles, secretion system component (hcp1, xcpQ). Note, gyrA and rplX are known drug targets\(^1\). Other proteins groups of interest include those involved in iron scavenging and transport (top left corner), oxidative phosphorylation (*) and sulfate metabolism (#).

Figure 2. Protein interactions for unique and significantly increased proteins in biofilm samples. Interactions were determined using STRING 9.05 (Search Tool for the Retrieval of Interacting Genes/Proteins)\(^2\). Sixty interactions were observed between 72 proteins. Lines indicate known or predicted protein-protein interactions, with thicker lines indicating higher levels of confidence. Symbols, description\(^1\): large red circle, phenazine biosynthesis (phzM, phzH, phzA2); cyan squares, propanoate metabolism (PA1737, PA0130, PA3568, prpC); small red squares, transport of small molecules (PA4496/7, PA3236, rbsB); red dashed ovals, energy metabolism (exaA, adhC, PA3416/7). Other
proteins groups of interest include those involved in chemotaxis (*), nitrogen metabolism (‡), and tyrosine metabolism (#).

Figure 3. The exclusive subproteomes of *P. aeruginosa* grown in planktonic and biofilm cultures show functional diversity. PseudoCAP functions of known proteins detected exclusively in (A) planktonic and (B) biofilm samples over time show distinct patterns. Planktonic cells at 48 h had higher levels of proteins involved in transcription, catabolism and metabolism, while later samples (96 h) showed peaks in adaptation and protection. Conversely, biofilms showed peaks in secreted factors, and transcriptional regulators at 24 h. Both samples have a considerable number of putative enzymes and proteins involved in the transport of small molecules, however the pools are comprised of distinct sets of proteins. Thirty percent of the unique proteins (with a.s.c. ≥4) in biofilm samples had unknown function (vs. 20% in planktonic). Abbreviations: aa, amino acid; hsp, heat shock protein; PTM, post-translational modification. Units of measurement indicate the number of significant proteins belonging to a PseudoCAP functional class.

Figure 4. The majority of proteins are decreased in biofilm samples in the shared subproteome of *P. aeruginosa*. Distribution of known PseudoCAP functions for significantly increased (A) and decreased (B) proteins in biofilm samples over time. Significantly decreased proteins (with a.s.c. ≥4, p ≤0.001) in biofilm samples belong to the categories of translation, post-translational modification, degradation, amino acid biosynthesis, and energy metabolism. Abbreviations: aa, amino acid; hsp, heat shock
protein; PTM, post-translational modification. Units of measurement indicate the number of significant proteins belonging to a PseudoCAP functional class.

Figure 5. Secondary validation of MS data using XIC measurement shows strong concordance with the initial analysis. A selection of proteins was manually validated and XICs were measured for further statistical analysis and confirmation of spectral counting data. Y-axis values represent the AUC of the XIC. Asterisks indicate statistically significant differences (p < 0.05). Abbreviations: Bf, biofilm; Pt, planktonic.
Iron scavenging and transport
Park et al., Figure 1
Park et al., Figure 2
Park et al., Figure 4
Park et al., Figure 5