Published in final edited form as:

J Proteome Res. 2019 June 07; 18(6): 2601–2612. doi:10.1021/acs.jproteome.9b00122.

***In vivo* proteome of *Pseudomonas aeruginosa* in airways of cystic fibrosis patients**

Xia Wu¹, Richard J Siehnel², Jayanthi Garudathri², Benjamin J. Staudinger³, Katherine B. Hisert³, Egon A. Ozer⁶, Alan R. Hauser^{5,6}, Jimmy K. Eng¹, Colin Manoil¹, Pradeep K. Singh^{2,3}, James E. Bruce^{1,4,*}

¹Department of Genome Sciences, University of Washington, Seattle, WA, USA.

²Department of Microbiology, University of Washington, Seattle, WA, USA.

³Department of Medicine, University of Washington, Seattle, WA, USA.

⁴Department of Chemistry, University of Washington, Seattle, WA, USA.

⁵Department of Microbiology-Immunology, Northwestern University, Chicago, Illinois, USA.

⁶Department of Medicine, Northwestern University, Chicago, Illinois, USA.

Abstract

Chronic airway infection with *P. aeruginosa* (PA) is a hallmark of cystic fibrosis (CF) disease. The mechanisms producing PA persistence in CF therapies remain poorly understood. To gain insight on PA physiology in patient airways and better understand how *in vivo* bacterial functioning differs from *in vitro* conditions, we investigated the *in vivo* proteomes of PA in 35 sputum samples from 11 CF patients. We developed a novel bacterial-enrichment method that relies on differential centrifugation and detergent treatment to enrich for bacteria to improve identification of PA proteome with CF sputum samples. Using two non-redundant peptides as a cutoff, a total of 1304 PA proteins were identified directly from CF sputum samples. The *in vivo* PA proteomes were compared with the proteomes of *ex vivo*-grown PA populations from the same patient sample. Label-free quantitation and proteome comparison revealed the *in vivo* up-regulation of siderophore TonB-dependent receptors, remodeling in central carbon metabolism including glyoxylate cycle and lactate utilization, and alginate overproduction. Knowledge of these *in vivo* proteome differences or others derived using the presented methodology could lead to future treatment strategies aimed at altering PA physiology *in vivo* to compromise infectivity or improve antibiotic efficacy.

Graphical Abstract

*To whom correspondence should be addressed: James E. Bruce, Department of Genome Sciences, University of Washington, 850 Republican Street, Seattle, WA 98109. Tel: (206)543-0220; Fax: (206) 616-0008; jimbruce@u.washington.edu.

SUPPORTING INFORMATION

The following supporting information is available free of charge at ACS website <http://pubs.acs.org>

Keywords

Pseudomonas aeruginosa; cystic fibrosis; quantitative proteomics; bacterial population proteomes

INTRODUCTION

The Gram-negative bacterium *Pseudomonas aeruginosa* (PA) a major pathogen causing chronic airway infections in people with cystic fibrosis (CF), and infection with PA is associated with increased morbidity and mortality (1). Once established, PA infections in CF airways are typically permanent, and the infecting PA populations cannot be eradicated, even when the bacteria are antibiotic sensitive when tested *ex vivo* (2). Mechanisms producing the *in vivo* persistence of PA in CF airways are incompletely understood; however recent work implicates two key factors. First, PA isolates are thought to adopt a physiological state(s) in CF airways that makes them highly resistant to killing. Second, PA isolates evolve inheritable traits *in vivo* that may enhance their ability to evade host immune defenses and resist antibiotic treatment (1, 3, 4).

There are several examples of physiologic and genetic adaptations in PA that may promote chronic infection. PA in CF airways are typically found living in multicellular bacterial aggregates (5), and aggregated growth can markedly increase resistance to host defenses and antibiotic killing (6, 7). PA may also grow slowly in CF sputum (8), and exhibit metabolic profiles different from *in vitro* grown cells (9–12). This is important because bacterial starvation responses can promote antibiotic tolerance (13). Many CF PA isolates evolve gene mutations that cause overproduction of the exopolysaccharide alginate (the mucoid phenotype) (14) that may also contribute to persistence within the lung (15). In addition, CF PA often evolve loss of function mutations that produce the noninvasive and nonmotile phenotypes (1, 6) that could reduce the immunogenicity of PA isolates and produce resistance to phagocytosis (16, 17). Finally, whole-genome sequencing studies reveal that CF PA evolve extensive genetic diversity during infection. As a result, CF airways contain populations of functionally-diverse, but clonally-related PA genetic variants (3, 18–20) and this diversity may enhance the ability of PA to resist stress and adapt to changing environmental conditions (21).

Proteins carry out the large majority of function in all living systems. Large-scale quantitative measurements of PA proteins by mass spectrometry and their changes

associated with nutrient supply (22, 23), antibiotic exposure (24–26) and biofilm development (27), provide unique insight of protein *in vivo* function. However, understanding PA functioning in the lungs of people with CF has been hindered by several factors. A key limitation is that host proteins in infected airway secretions (and other bodily fluids) are generally far more abundant than bacterial proteins. This disparity in abundance levels combined with limited mass spectrometer dynamic range, where highly abundant host peptides obscure detection of less abundant bacterial peptides, make the characterization of *in vivo* PA proteome difficult (Fig. S1). In addition, human samples like sputum can vary markedly in the number and identity of bacteria present, and the presence of inhibitory substances like proteases and aggregating factors.

Here we report the development of methods that markedly enrich bacterial proteins enabling robust and reproducible identification of the PA proteome directly in CF sputum. Using these enrichment methods, we present an initial view of the CF *in vivo* PA proteome that includes identification of 1304 bacterial proteins. The sputum proteome provides new insights about *in vivo* PA physiology, and the bacterial functions that may promote pathogenesis and persistence in CF airways. Moreover, we show how identification of PA peptides in enriched sputum can enable targeted quantitative *in vivo* measurements in sputum, in situations when bacterial enrichment is not possible.

MATERIALS AND METHODS

Culturing *P. aeruginosa* (PA) from CF sputum samples

The fresh CF sputum (non-frozen) was homogenized by mixing with 5 volumes of PBS (pH 7.4) containing 0.1% dithiothreitol on ice for 30 min. The homogenized sputum was plated on MacConkey agar plates, and grown for 2 days at 37°C. Colony counts were recorded, and 96 PA colonies were randomly selected from each CF sputum sample, and cryopreserved in 20% glycerol as previously described (18, 28). This study was approved by the University of Washington Institutional Review Board (#50542).

PA is the dominant species in CF adult patients (29) and all CF patients in this study were adults (>21 years of age). Another common bacterial species *Staphylococcus aureus* is a Gram-positive species. MacConkey agar is reasonably selective for CF infections because it eliminates gram positives and differentiates between lactose and non-lactose fermenters; PA does not usually ferment lactose. In our previous study (18), we used PCR to confirm the colonies using the method to select are truly PA.

For proteome analysis, PA populations (96 PA isolates) were inoculated by transferring frozen stock cultures into individual wells of 96-well plates containing LB broth, and grown with shaking at 37°C for 24 h. Five microliters of cell cultures were spotted on LB agar in a 96-well array format, and individual colonies were grown for 24 h at 37°C. Cell population mixtures were harvested by flushing the plate with 10 ml sterile PBS. Bacteria were pelleted at 3000 × g at 4°C for 15 min.

Bacterial enrichment procedures with CF sputum samples

Spontaneously expectorated CF sputum was homogenized by mixing with 5 volumes of PBS (pH 7.4) containing 0.1% dithiothreitol on ice for 30 min as described above. The homogenized sputum was digested with Hyclone trypsin (0.25%, Fisher Scientific, Hampton, NH, USA), incubated at 37°C for 20 min, and then centrifuged at $100 \times g$ for 10 min at 4°C. The supernatant from this slow spin was recovered and centrifuged at $10,000 \times g$ for 20 min at 4°C. The resultant pellet was resuspended with ice-cold PBS (pH 7.4) containing Triton X-100 0.1% (v/v). Pipetting is required to fully loosen up the pellet. The resuspended mixture was further incubated on ice for 5 min, and then centrifuged at $10,000 \times g$ for 20 min at 4°C. The pellet was collected, and was resuspended with ice-cold PBS containing Brij 58 0.1% (v/v) (Sigma-Aldrich, St. Louis, MO, USA). The resuspension mixture was incubated on ice for 5 min before centrifugation at $10,000 \times g$ for 25 min at 4°C. The pellet was collected as samples enriched with bacteria for proteomics analysis.

Proteome sample preparation

Identical procedures were used for protein extraction for CF sputum samples and PA *ex vivo* culture cell pellets. Samples were dissolved with extraction buffer [100 mM Tris (pH 8.0), 4 % SDS, 10 mM DTT], and were incubated at 95 °C with a heating block for 5 min. The extraction mixtures were vortexed for 30 s, and were sonicated with a GE-130 ultrasonic processor using settings of 50% amplitude, 30 s duration for two cycles. SDS was removed through buffer exchange with Amicon filters (10 KDa MWCO) (Millipore, Billerica, MA, USA), and proteins were eluted to the urea buffer (8 M urea and 50 mM NH_4HCO_3) (30, 31). Protein alkylation was done with 50 mM iodoacetamide for 30 min in the dark prior to the elution with the Amicon filters (30). Protein concentrations of the eluted proteins were quantified with Bradford assays (Thermo Fisher Scientific, Waltham, MA). 100 µg total proteins were used for trypsin digestion. Urea concentration of the proteins was diluted to less than 1.5 M prior to the digestion with digestion buffer [40 mM NH_4HCO_3 (pH 8.5) and 5% acetonitrile] to reduce the urea inhibition effect of trypsin activity. Digestion was done with sequencing grade trypsin (Promega, Madison, WI, USA), using the substrate to enzyme ratio of 50:1 (w/w) at 37°C and overnight incubation (16–18 h). After the digestion, the mixtures were acidified with 1% trifluoroacetic acid to pH of 3.5, and peptides were purified with C18 Sep-Pak columns (Waters, Milford, MA, USA). The purified peptides were resuspended with 0.1% formic acid for mass spectrometry analysis.

LC-MS/MS analysis

Proteome samples were analyzed with a VELOS-FTICR system (32) coupled to a Waters nanoAcquity UPLC, or with a Q Exactive Plus system coupled to a Thermo EASY-nLC 1000 (Thermo Fisher Scientific, Waltham, MA, USA). The C18 columns of a 3 cm trap column (5 µm, 200 Å) and a 60 cm analytical column (5 µm, 100 Å) (Bruker, Billerica, MA, USA) were used for both systems. For VELOS-FT analysis, the LC separation gradients were 5 % - 35 % acetonitrile at flow rate of 300 nL/min for 120 min. MS¹ scan was acquired at 50,000 resolution, and the top 20 most abundant peaks were selected for tandem mass spectrometry analysis with the ion-trap VELOS. VELOS-FT MS² settings included isolation width of 2.0, activation type of CID, and normalized collision energy of 35, activation Q of

0.25 and activation time of 10 ms. AGC target value was 5×10^5 for FT MS¹, and 1×10^4 for ion trap MS². Charge state exclusion was applied to singly charged ions and those with the undetermined charge states. Dynamic exclusion was enabled, including the settings of exclusion window of 0.5 m/z low to 1.5 m/z high, exclusion duration 30 s, list size of 500, and repeat count of 1.

For QE plus analysis, the LC separation gradients were 2 % - 10 % acetonitrile for 1 min, and 10 % - 30 % acetonitrile for 89 min, at flow rate of 300 nL/min. QE plus settings include 70,000 resolution for MS¹ scan, and 17,500 resolution for MS² scans. AGC target value was 1×10^6 for MS¹, and 5×10^4 for MS². The top 20 most abundant ions were selected for fragmentation analysis, using 1.6 m/z isolation width, 25 NCE. Charge exclusion was applied to ions of undetermined charges, and ions with charges of 1, 6–8 and > 8. The dynamic exclusion was set to 30 s.

PA protein identification with database searches

The raw data was converted to mzXML files with ReAdW (version 4.2.1). The mzXML files were searched with Comet (33) (version 2015.01 rev.02) against the concatenated database containing forward and reverse protein sequences of Human genome, PAO1 core genome, and PA accessory genome (34) tries, downloaded from Uniprot (35) and Pseudomonas Genome Database (36) on April 20, 2015). The core genome sequence of PA was determined from 12 reference genomes using Spine v0.1.2 with a core genome definition of sequences present in at least 11 of the 12 references as previously described (34). Using this core genome sequence and the software package AGEnt v0.1.2 (34), the accessory genome portions of 100 PA isolates obtained from patients with bloodstream infections were determined from their draft genome assemblies. Accession numbers of genome sequences are provided in Supplemental Material. Unique accessory element sequences, defined as those sequences at least 200 bp in length with at least 85% nucleotide similarity, were determined using ClustAGE software v0.4 (37).

The search settings for VELOS-FT data included 20 ppm precursor mass tolerance, ¹³C offsets (−1/0/1/2/3) enabled, fragment bin tolerance 1.0005, fragment bin offset 0.4, variable modifications of methionine oxidation (15.9949 Da), fixed modification of cysteine carbamidomethylation (57.021464 Da). Only fully tryptic peptide sequences were considered and up to 2 missed cleavages were allowed. The Comet search for the QE plus data was performed with the same parameter settings as VELOS-FT data, except for that fragment bin tolerance 0.01 and fragment bin offset 0 were used for QE plus data. The identified peptides were sorted with the Comet expectation score. A false discovery rate (FDR) of 1% was used as a cutoff for acceptable peptide identification score.

PA proteome quantitation with CF sputum samples

Typically, two micrograms of total proteins of bacterial-enriched CF sputum samples were analyzed in each LC-MS/MS run. However, because the fraction of PA proteins present cannot be accurately measured, the true amount of PA proteins that were analyzed in each LC-MS/MS run is not known. We did not rely on the information of cell number or biomass for the proteome quantitation. Instead, we assume that the majority of the PA proteins

maintain similar abundance in PA cells whether they are in CF sputum or *in vitro* growth. Hence, when the equal amount of PA proteins is analyzed, the ratios of the majority of the proteins of *in vivo* abundance versus *in vitro* growth are expected to center at 1, i.e. $\log_2(\text{ratio}) = 0$. Thus, each distribution of measured $\log_2$ ratios was centered at $\log_2=0$. Proteins that did not follow the general trend to be centered at $\log_2\text{ratio} = 0$ could be identified as up- or down- regulated proteins in CF sputum.

Label-free quantitation for MS¹ extracted ion chromatograms was performed with XPRESS (38–40). In the first step, peptide chromatograms were extracted for each identified peptide. A minimum number of chromatographic quantitation points of 5 was required. Mass measurement tolerance for peptide precursor masses of 0.01 Da was used. Peptide chromatograms from technical replicate analyses were averaged to yield representative intensity values. The relative peptide abundance ratios in *in vivo* CF sputum samples versus the *ex vivo* LB samples were obtained for each peptide, based on measured peptide chromatographic peak areas. The median of all measured peptide ratios for each pairwise proteome comparison was centered at $\log_2(\text{ratio}) = 0$. $\log_2$ (ratio) values of multiple peptides derived from the same protein were further averaged to yield the $\log_2$ (protein ratios) (Fig. 4A). $\log_2$ (protein ratio) values from each CF sputum sample were averaged to yield the summarized $\log_2$ (protein ratios) for the 35 CF sputum samples (Fig. 4B).

The statistical significance of protein fold changes was determined with analysis of variance (ANOVA). Protein ratios measured with each CF sputum sample were considered as a biological replicate. Biological replicate values across 35 sputum samples were compared to the corresponding *ex vivo* values. Statistical significance $P = 0.05$ was used as the cutoff.

In addition, a subset of PA proteins were only detected with the *in vivo* samples, while they were not detected with the *ex vivo* conditions in any of the three technical replicates. In this case, these proteins were required to be identified with three or more non-redundant peptides *in vivo*, and if so, these proteins were also considered as up-regulated in a CF sputum sample.

Parallel reaction monitoring (PRM) assays

PRM analysis for label-free peptide quantitation was performed with a Q Exactive Plus system coupled to a Thermo EASY-nLC 1000. Identical LC columns and gradient settings as DDA analysis were used for PRM analysis. Mass spectrometer QE plus settings include 17,500 resolution for MS scans, AGC target value 5×10^4 , isolation window 1.6 m/z, maximum ion time 50 ms, fragmentation energy 25 NCE. The peptide target masses and the scheduled retention time were determined according to the peptide identification results with DDA analysis. The PRM data were analyzed with Skyline (version 3.5.0) (41). Peak selection was manually verified. Peptide identification was confirmed with *in vitro* positive control samples based on peptide elution and fragmentation profiles.

Data Availability

Proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE (42) and PASSSEL (43) with the dataset identifier PXD011792 and PASS01300.

RESULTS

Bacterial enrichment with CF sputum samples

CF sputum is composed of human cells (mainly neutrophils, macrophages, squamous epithelial cells) (44), bacterial cells (often predominantly PA in established disease) (1), and soluble host and bacterial proteins, along with other molecules. We reasoned that two fundamental differences between human and PA cells could be exploited for bacterial enrichment; (1) human cells are bigger than PA cells; and (2) the cell envelope of PA (and other Gram negative bacteria) resists lysis during centrifugation and detergent treatment because of the peptidoglycan wall positioned between cytoplasmic and outer membrane. We utilized these differences to enrich bacterial cells from CF sputum samples to enable *in vivo* PA population proteome analyses (Fig. 1).

We developed an enrichment process that involves several steps. To produce a sample suitable for centrifugal fractionation, we homogenized CF sputum with 5 volumes of PBS containing 0.1 % DTT on ice for 30 min. DTT reduces mucus disulfide bonds and decreases mucus viscosity. To reduce bacterial adherence to host components mediated by protein interactions (45), we added 0.25 % Hyclone trypsin at 37°C for 20 min. We then used a two-step differential centrifugation protocol to separate cells based on size. Low speed centrifugation ($100 \times g$ at 4°C for 10 min) was used to remove human cells, and subsequent high-speed centrifugation ($10,000 \times g$ at 4°C for 20 min) was used to separate bacterial cells from the supernatant containing secreted proteins. We next attempted to preferentially lyse human cells (keeping PA intact) by incubating pellets in ice-cold PBS containing 0.1% Triton X-100 (46, 47). As an additional step to eliminate the residual host cytosolic proteins, sputum pellets were washed with ice-cold PBS containing the detergent Brij 58 (0.1%), which can invert plasma membrane vesicles and release the entrapped soluble proteins (48, 49).

We performed several control experiments to gauge the effectiveness of the protocol. We investigated the possibility that the treatment steps might inadvertently lyse PA. However, we found that 0.1% Triton X-100 or 0.1% Brij 58 did not lyse viable PA reference strain PAO1 strain, a *mucA* mutant of PAO1 exhibiting the mucoid phenotype (50) (Fig. S2A), or CF clinical isolates (Fig. S2B). We also considered the possibility the enrichment protocol could alter the PA proteome. All but one enrichment step was performed at 4°C to limit *ex vivo* proteome changes. However, the 20 min trypsin incubation was performed at 37°C to enhance enzymatic activity. To determine if proteome measurements were altered by enrichment, we measured the relative abundance of PA proteins that could be quantified in sputum with and without enrichment. As shown in Fig. S3A, measurements performed on aliquots from the same sputum sample that were and were not subjected to the enrichment protocol revealed similar protein levels (relative to PA cultured *in vitro*, see below). Thus, the enrichment protocol did not appear to introduce any obvious marked changes in the PA proteome. Future studies with this method will benefit from simultaneous enrichment of both airway and *ex vivo* cultured bacteria to further reduce any minor proteome changes.

We measured the degree of enrichment achieved by the protocol by comparing the number of PA peptides identified in un-enriched and enriched sputum. Fig. 2A shows that the

bacterial-enrichment procedures significantly improve the detection of PA peptides in CF sputum ($P < 0.001$ for all five samples tested), with up to a 3-fold increase in the number of identified PA peptides (for example, 320 vs. 1372 peptides after enrichment). To independently measure the effect of enrichment, we used anti-TufA and anti-RpoA antibodies to measure the PA proteins elongation factor EF-Tu (TufA), and RNA polymerase (RpoA). Fig. 2B shows that both TufA and RpoA proteins were more abundant in bacteria-enriched sputum fractions compared with the sputum aliquot without bacterial enrichment (raw sputum), or samples of intermediate steps in the protocol (supernatants, and $100 \times$ g CF sputum pellets). In comparison, the abundance of soluble human protein GAPDH decreased markedly in the bacterial-enriched sputum fractions, indicating that the protocol also effectively depleted host proteins, consistent with results of mass spectrometry spectral counting analysis (Fig S3B).

Label-free quantitation of PA *in vivo* proteome

We applied the bacterial-enrichment protocol to 35 sputum samples collected from 11 CF patients to examine the *in vivo* PA proteomes (Table S1). For each sample, an aliquot was used for analysis of colony forming units (CFU/ml) to estimate the abundance of PA in the sputum samples, and to collect individual PA isolates for *ex vivo* proteome comparisons (see below). We observed that the number of PA proteins detected were positively correlated with the bacterial cell count ($R^2=0.59$) (Fig. 3). The sputum sample with the greatest PA proteome identification was of 4127 PA peptides, representing 1062 PA proteins. Seven of the analyzed sputum samples achieved the identification of greater than 2000 PA peptides and 550 PA proteins (Table S1).

Relative quantitation of *in vivo* PA proteome levels requires comparison to reference levels, and two issues are introduced regarding the choice of appropriate reference. First, while PA infections in CF are caused by the continuous persistence of single lineages, recent work by our group and others indicate that genetic diversity evolves within infecting PA. Diversification is likely a consequence of the continuous replication of infecting bacteria under strong and diverse selective pressures, and the regional isolation of organisms in different lung regions (3, 18, 20). Thus, the PA bacteria infecting CF patients consist of heterogeneous populations of clonally-related genetic variants (3, 18), and the *in vivo* PA proteome will be comprised of proteins contributed by the population of clonally-related isolates that co-exist in the sputum.

To address this issue, we developed methods using a set of 96 PA isolates from each individual sputum sample where each isolate was independently cultured under identical conditions, and then pooled for proteomic analysis to serve as a matched reference proteome for the individual sputum sample from which the isolates were derived (Fig. 1A). The pooling step enables generation of population proteome data that comprehensively represents sputum PA populations to allow comparison with proteome levels within bacteria enriched directly from sputum. We cultured and pooled 96 PA isolates from each sample as our previous work indicated that this number was sufficient to resolve proteome level lung-regional specificity of PA populations (18).

A second consideration relates the *ex vivo* medium used to grow isolate populations *in vivo*. Luria-Bertani (LB) medium was chosen for initial studies as it is among the most widely used for both laboratory strains and clinical isolates of PA. Furthermore, LB is a nutrient-rich medium that permits the growth of auxotrophic PA isolates that are commonly found in sputum samples (51). Thus, LB medium provides a reasonable proof of principal reference to which *in vivo* proteomic profiles could be compared. Future efforts could utilize the methods developed here to identify *in vitro* growth conditions that best replicate *in vivo* PA proteomes to provide increased insight on more subtle PA proteome changes in the CF lung.

A first question we sought to answer was, do the *in vivo-in vitro* PA proteome measurements provide reliable quantitative information? PA proteomes from enriched sputum and matching cultured isolate pool samples were measured and quantified using MS¹ signal intensities (see methods) to identify relative PA proteins levels in the two conditions. A representative histogram of measured Log₂ PA protein ratios for a single sputum compared against its matched cultured isolates is shown in Fig. 4A, and the average of all protein ratios (sputum relative to cultured matching isolates) for all 35 sputum samples is shown in Fig. 4B. Although these distributions require centering since the total bacterial protein amounts in sputum and cultured pool samples cannot readily be normalized, both appear as normal distributions indicating that most protein levels do not change in sputum relative to cultured isolates as expected. Moreover, the observed distribution normality is supportive of the reliability of this method for relative PA proteome quantitation. The reproducibility of quantified changes in the *in vivo* proteome was assessed with technical replicates which revealed high-quality consistency in measured protein ratios. Even more importantly, analyses of two CF sputum samples that were collected on consecutive days from the same patient in the absence of apparent clinical changes also showed strong correlation of *in vivo* PA proteome results ($R^2=0.79$) (Fig. 4C) relative to the *ex vivo* populations from each day. Taken together, these data illustrate that relative quantitation of *in vivo* and matched pooled isolate proteomes can provide reliable and reproducible measurements that can inform on metabolic and other pathways important for PA growth in CF lung.

The *in vivo* proteome of PA in CF airways

A total of 1304 PA proteins (1235 of which were encoded by core genes and 69 encoded by accessory genes (34)) were identified with two or more non-redundant PA peptides with the entire dataset. Another 82 PA proteins with single peptide ID were repeatedly identified in 3 or more sputum samples and identified with multiple peptides from *ex vivo* samples (Table S2).

We used several criteria to identify a set of PA proteins that exhibited consistent expression differences in CF sputum as compared to *ex vivo* growth (Fig. 5). Proteins in this group had to meet all following criteria: (1) proteins must exhibit a two- or greater-fold expression level difference in enriched sputum verses *ex vivo* growth in at least five sputum samples; (2) proteins must show a two-fold or greater average expression level difference in all measured enriched sputum samples as compared to *ex vivo*-grown PA; and (3) the average expression level difference in enriched sputum verses *ex vivo*-grown conditions had to reach statistical significance ($P<0.05$) by one-way ANOVA.

Sixty-seven PA proteins met the criteria for increased levels in CF sputum *in vivo*. Likewise, 117 PA proteins met the criteria for decreased level *in vivo* compared with the *ex vivo* condition (Fig. S4AB). The enrichment in gene ontology (Fig. 6) and the functional specificity of these proteins are further discussed below.

Outer membrane porins involved in iron and phosphate uptake

PA possesses a diverse group of outer membrane porins (52). Our analysis identified twenty-one outer membrane porins that were expressed at least 2-fold higher in sputum (one-way ANOVA, $P < 0.05$). The most notable were TonB-dependent ferrichrome receptors, which function to transport siderophore molecules (such as pyoverdine, pyochelin) in and out of bacterial cells to facilitate iron acquisition. The well-characterized ferric-pyoverdine receptor FpvB, ferric-pyochelin receptor FptA, and ferrous-heme receptor PhuR were all expressed at higher levels in sputum as compared to *ex vivo*. In addition, TonB-dependent receptors for xenosiderophores (53) (siderophore molecules produced by other bacteria rather than PA), including ferric-enterobactin (PfeA, PirA), ferric-citrate (FecA), ferrioxamine (FoxA), and ferric-mycobactin (FemA) were also increased in sputum. These results are consistent with previous work indicating that bioavailable iron is scarce *in vivo* (54–56), and suggests that PA may use multiple uptake systems to scavenge iron in CF airways. Consistent with the up-regulation of iron uptake in PA in sputum, HitA, a ferric iron-binding periplasmic protein involved in iron transport across the inner cell membrane, was also significantly increased (Fig. S4A). This result is also notable because the HitAB uptake systems imports gallium, a novel anti-pseudomonal antimicrobial in development for CF infections (56, 57). Thus, the observation of HitA upregulation and identified HitA peptides can serve as future targets to quantify *in vivo* during Gallium treatment to provide new insight on Gallium resistance.

In addition to ferrichrome receptors, PA porins increased in sputum include OprO (phosphate uptake (58)), PA0781 (homologous to *Neisseria meningitidis* Zn-transporter ZnuD (59)), OprD and OpdD (uptake for basic amino acids, short peptides, small organic compounds (60, 61)), PA1288 (homologous to *E. coli* FadL (62), long chain fatty-acid uptake), AlgE (alginate transport (63)) and OprH (involved in antibiotic resistance (64)). Consistent with the increases in phosphate and zinc uptake systems, the ABC phosphate transporter components PstA-B-C-S, ABC phosphonate transporter component PA3383 (PhnD) (65), and phosphate uptake regulatory proteins PhoU and PhoB, and the Zn^{2+} ABC transporter components ZnuA (66), were increased in sputum.

Carbon metabolic remodeling of PA in CF airways

We found that PA carbon metabolic enzymes in the TCA cycle, fatty acid biosynthesis, and purine and pyrimidine biosynthetic processes were consistently repressed in CF sputum (Figs. 5, S5A–C), consistent with the slow PA growth rates identified in CF sputum (8). However, five carbon metabolic enzymes exhibited similar protein relative abundance levels in sputum and in *ex-vivo* culture, with several showing significant increases. These include two enzymes in the glyoxylate cycle, isocitrate lyase (AceA) (increased in sputum) and malate synthase (GlcB) (similar level in sputum and culture); and two enzymes for lactate utilization, lactate dehydrogenase (LldD) (similar levels) and L-lactate permease (LldP)

(increased in sputum). In addition, fumarate hydratase (FumC1), which mediates the interconversion of fumarate and malate and plays a role in redox balance regulation (67), was also increased in sputum. All of these five genes were previously shown to be critical for infection and pathogenesis (68–72). Recent transcriptome analyses with CF sputum samples also reported similar transcriptional regulation of carbon metabolic enzymes to favor glyoxylate shunt and lactate utilization (11, 12) (Table S2).

Alginate production and transport

Overproduction of alginate (an anionic polysaccharide) by PA confers the mucoid phenotype, and has been implicated in PA persistence in CF airways (1, 73). Eleven alginate biosynthetic enzymes in PA were detected with CF sputum (Fig. S5D). Using targeted proteome measurements with Parallel Reaction Monitoring (PRM) assays, 30 out of the 35 CF sputum samples (10 of the 11 patients) were confirmed with the expression of alginate proteins in PA in CF airways. In particular, two of these members critical for alginate production AlgD and alginate extracellular transport AlgE in PA were significantly increased in CF sputum compared with *ex vivo* PA proteomes in LB. The RNA-level increases of AlgD and AlgE were also found previously (11, 12). AlgD has been proposed as the rate-limiting step in alginate biosynthesis (74, 75). Increased protein levels of AlgD and AlgE suggest that alginate production and export may be increased in sputum.

Two pathways in PA can promote the expression of AlgD, which involve AlgU mediated AmrZ transcriptional activation (76), or the AlgU-independent AlgB/RpoN activity (77, 78). We found that proteins in the AlgU pathway (including AlgW, AlgR, AmrZ) were modestly down-regulated in CF sputum. Such change may result from a general decreased protein metabolic activity in PA associated with decreased growth rate of PA in sputum. In contrast, the protein levels of AlgB and RpoN did not show a downward trend, with AlgB modestly increased in CF sputum, which suggests that AlgB/RpoN system may serve to promote AlgD up-regulation in the CF sputum samples that were analyzed. Another transcriptional factor AlgP was significantly down-regulated in CF sputum. However, previous work shows that the down-regulation of AlgP did not prevent alginate overproduction (79).

Proteins encoded by accessory genes

The PA genome is composed of generally conserved genomic sequences (called the core genome), interspersed with additional sequences (~ 10–15 % of the genes in a given strain), that are not generally shared by all strains. This additional genetic material is called the accessory genome (80). Accessory gene functions are thought to be important for bacterial survival in harsh environments and infection pathogenesis, as virulence and antibiotic resistance functions are often encoded by accessory genes (80). Seventy proteins encoded by accessory genes were detected in CF sputum samples, and 33 of those were also detected *ex vivo* with LB-cultured populations (Table S2). We used protein BLAST and phyre2 homology modeling (81) to obtain function predictions of these proteins. The greatest increased accessory gene in CF sputum is Gene3548, which was predicted as a homolog of ferrichrome receptor (Phyre2 alignment coverage 94%, sequence identity 23%, confidence score 100%). These results indicate that PA may respond with accessory gene regulation to adapt to *in vivo* growth conditions, as previous reports have shown (34, 82).

DISCUSSION

While progress has been made in studying pathogenesis using *in vitro* and animal models, understanding bacterial functioning at sites of human infection remains elusive. This is particularly true for chronic infection, as representative animal models of chronic infection have been extremely difficult to develop. In the present study, we developed a novel bacterial-enrichment method useful for CF sputum samples that identified PA proteome profiles expressed in CF sputum, which included 1304 PA proteins that were verified with two or more peptide IDs. Importantly our methods involved direct measurements on sputum after enriching for bacterial proteins and were compared to a reference that accounted for the genetic diversity that evolves in CF PA populations. Comparisons of *in vivo* proteomes with *ex vivo* population proteomes of PA isolate pools from the same patient samples (18) reveal key and consistent proteome differences that may be important for *in vivo* PA physiology in CF airways.

For instance, protein increases in iron sequestration and iron metabolic processes of PA suggest iron may be a growth-limiting nutrient in CF sputum. Disruption of bacterial iron metabolism by substituting iron with the metal gallium could reduce survival of PA in CF airways. In a phase 1 clinical trial, gallium had therapeutic effects without toxicity in cystic fibrosis patients infected with PA (56), indicating that disrupting the iron metabolism in PA holds a great promise to reduce PA infection in CF airways. On the other hand, general down-regulation of genes in transcription and translation processes is in line with a decreased growth rate of PA in the CF airway (8). The stability of CF patient airway proteome indicates that PA shares a common expression program associated with the lungs environment, consistent with recent transcriptome findings (11, 12).

Three novel technical aspects are included in this study. First, the enrichment approach we developed enables the *in vivo* proteomes to be measured on freshly expectorated sputum, without the use of culturing steps that would change bacterial protein expression profiles. This is important because very few proteins could be measured on un-enriched samples. Second, the *in vivo* PA proteomes were determined by comparing the data obtained with freshly expectorated sputum to *ex vivo* proteomes of a large pool of PA isolates from the same sputum sample. This is important because lineages of PA genetically diversify during the course of infection and the evolved differences can markedly affect bacterial protein expression (18). Thus, the reference we used likely represents the PA causing infection far better than a single (or few) isolates picked randomly. In addition, the population proteome approach has the advantage of focusing on *in vivo* proteome features in the populations that are of sufficient magnitude to be apparent in a pool of isolates. Therefore, detected protein-level changes are either very large changes within a small subset of the population or more moderate changes consistent among a larger subset of the population. In either scenario, these changes are likely important for function. Third, the identification of these *in vivo* PA proteome markers in CF airways enabled development of PRM assays for targeted detection and quantitation of these proteins with CF sputum samples. Using PRM assays, we were able to directly detect porin proteins (FptA, PhuR, PA4837, Gene3548, OprD, PA1288, OprH), phosphate transporter components (PhuT, PstS), glyoxylate cycle mediating enzymes (AceA, FumC1), and alginate biosynthesis proteins (AlgD, AlgE) in CF sputum samples

without bacterial-enrichment steps (Fig. S6). These targeted PRM assays increase detection sensitivity compared with the conventional DDA approach, and will be useful for future longitudinal proteome quantitation studies of PA with CF sputum samples, or with retrospect frozen sputum samples in which bacterial enrichment are not suitable.

There are also limitations in the current study. First, only one *ex vivo* growth condition (i.e. LB medium) was used to generate the reference proteome to identify the proteins that were differentially expressed in sputum. Future work could use additional *ex vivo* growth conditions that attempted to model CF lung condition more closely, for example using CF sputum as a medium (9, 83). However, a key object of this study was to compare PA protein expression in sputum to the *ex vivo* conditions commonly used in laboratory experiments. Second, because the *ex vivo* proteome is obtained with PA isolates that survived *ex vivo* cultures, isolates that did not grow in the LB condition may not be included in the proteome reference. LB media is complex and thus it enables the growth of most types of auxotrophs. However, it remains possible that some of the observed sputum versus *ex vivo* proteome differences were a consequence of population composition changes caused by culturing the *ex vivo* reference. Third, longitudinal samples were collected and analyzed from several CF patients, including before and after clinical treatment. However, due to the detection sensitivity, low number of PA proteins were detected with CF sputum post clinical treatment with the low PA cell counts. This technical challenge limited our ability to obtain reliable PA proteome changes in response to drug treatment *in vivo*. Future studies with PRM measurements based on the identified PA peptides in this work could improve the protein quantitation with CF sputum of low PA cell counts.

PA infection is strongly associated with lung function decline in CF patients (84, 85). Thus, future application with the methodology could help identify PA protein markers that associate with host clinical status, including lung function, acute exacerbations, antibiotic intervention, or therapies targeting the basic CF defect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank members in the Bruce, Singh and Manoil laboratories for their advice and helpful discussions. The research is supported by NIH grants R01HL110879, R01AI101307, U19AI107775, R01GM086688, and 7S10RR025107, P30 DK089507, and CF Foundation Research Development Program, and the University of Washington's Proteomics Resource (UWPR95794).

Abbreviations

PA	<i>Pseudomonas aeruginosa</i>
CF	Cystic Fibrosis
PRM	Parallel reaction monitoring

REFERENCES

1. Hauser AR; Jain M; Bar-Meir M; McColley SA, Clinical significance of microbial infection and adaptation in cystic fibrosis. *Clin Microbiol Rev* 2011, 24, (1), 29–70. [PubMed: 21233507]
2. Emerson J; McNamara S; Buccat AM; Worrell K; Burns JL, Changes in cystic fibrosis sputum microbiology in the United States between 1995 and 2008. *Pediatr Pulmonol* 2010, 45, (4), 363–70. [PubMed: 20232473]
3. Smith EE; Buckley DG; Wu Z; Saenphimmachak C; Hoffman LR; D'Argenio DA; Miller SI; Ramsey BW; Speert DP; Moskowitz SM; Burns JL; Kaul R; Olson MV, Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. *Proc Natl Acad Sci U S A* 2006, 103, (22), 8487–92. [PubMed: 16687478]
4. Opperman MJ; Shachar-Hill Y, Metabolic flux analyses of *Pseudomonas aeruginosa* cystic fibrosis isolates. *Metab Eng* 2016, 38, 251–263. [PubMed: 27637318]
5. Singh PK; Schaefer AL; Parsek MR; Moninger TO; Welsh MJ; Greenberg EP, Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* 2000, 407, (6805), 762–4. [PubMed: 11048725]
6. Staudinger BJ; Muller JF; Halldorsson S; Boles B; Angermeyer A; Nguyen D; Rosen H; Baldursson O; Gottfrethsson M; Guethmundsson GH; Singh PK, Conditions associated with the cystic fibrosis defect promote chronic *Pseudomonas aeruginosa* infection. *Am J Respir Crit Care Med* 2014, 189, (7), 812–24. [PubMed: 24467627]
7. Tseng BS; Zhang W; Harrison JJ; Quach TP; Song JL; Penterman J; Singh PK; Chopp DL; Packman AI; Parsek MR, The extracellular matrix protects *Pseudomonas aeruginosa* biofilms by limiting the penetration of tobramycin. *Environ Microbiol* 2013, 15, (10), 2865–2878. [PubMed: 23751003]
8. Yang L; Haagenen JA; Jelsbak L; Johansen HK; Sternberg C; Hoiby N; Molin S, In situ growth rates and biofilm development of *Pseudomonas aeruginosa* populations in chronic lung infections. *J Bacteriol* 2008, 190, (8), 2767–76. [PubMed: 18156255]
9. Palmer KL; Mashburn LM; Singh PK; Whiteley M, Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *Journal of Bacteriology* 2005, 187, (15), 5267–77. [PubMed: 16030221]
10. Son MS; Matthews WJ Jr.; Kang Y; Nguyen DT; Hoang TT, In vivo evidence of *Pseudomonas aeruginosa* nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. *Infect Immun* 2007, 75, (11), 5313–24. [PubMed: 17724070]
11. Rossi E; Falcone M; Molin S; Johansen HK, High-resolution in situ transcriptomics of *Pseudomonas aeruginosa* unveils genotype independent patho-phenotypes in cystic fibrosis lungs. *Nat Commun* 2018, 9, (1), 3459. [PubMed: 30150613]
12. Cornforth DM; Dees JL; Ibberson CB; Huse HK; Mathiesen IH; Kirketerp-Moller K; Wolcott RD; Rumbaugh KP; Bjarnsholt T; Whiteley M, *Pseudomonas aeruginosa* transcriptome during human infection. *Proc Natl Acad Sci U S A* 2018, 115, (22), E5125–E5134. [PubMed: 29760087]
13. Nguyen D; Joshi-Datar A; Lepine F; Bauerle E; Olakanmi O; Beer K; McKay G; Siehnel R; Schafhauser J; Wang Y; Britigan BE; Singh PK, Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science* 2011, 334, (6058), 982–6. [PubMed: 22096200]
14. Burns JL; Gibson RL; McNamara S; Yim D; Emerson J; Rosenfeld M; Hiatt P; McCoy K; Castile R; Smith AL; Ramsey BW, Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. *J Infect Dis* 2001, 183, (3), 444–52. [PubMed: 11133376]
15. Hentzer M; Teitzel GM; Balzer GJ; Heydorn A; Molin S; Givskov M; Parsek MR, Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J Bacteriol* 2001, 183, (18), 5395–401. [PubMed: 11514525]
16. Mahenthiralingam E; Campbell ME; Speert DP, Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect Immun* 1994, 62, (2), 596–605. [PubMed: 8300217]

17. Zhang Z; Louboutin JP; Weiner DJ; Goldberg JB; Wilson JM, Human airway epithelial cells sense *Pseudomonas aeruginosa* infection via recognition of flagellin by Toll-like receptor 5. *Infect Immun* 2005, 73, (11), 7151–60. [PubMed: 16239509]
18. Jorth P; Staudinger, Benjamin J; Wu X; Hisert KB; Hayden H; Garudathri J; Harding, Christopher L; Radey, Matthew C; Rezayat A; Bautista G; Berrington, William R; Goddard, Amanda F; Zheng C; Angermeyer A; Brittnacher, Mitchell J; Kitzman J; Shendure J; Fligner, Corinne L; Mittler J; Aitken, Moira L; Manoil C; Bruce, James E; Yahr, Timothy L; Singh, Pradeep K, Regional Isolation Drives Bacterial Diversification within Cystic Fibrosis Lungs. *Cell Host & Microbe* 2015, 18, (3), 307–19. [PubMed: 26299432]
19. Marvig RL; Sommer LM; Molin S; Johansen HK, Convergent evolution and adaptation of *Pseudomonas aeruginosa* within patients with cystic fibrosis. *Nat Genet* 2015, 47, (1), 57–64. [PubMed: 25401299]
20. Yang L; Jelsbak L; Marvig RL; Damkiaer S; Workman CT; Rau MH; Hansen SK; Folkesson A; Johansen HK; Ciofu O; Hoiby N; Sommer MO; Molin S, Evolutionary dynamics of bacteria in a human host environment. *Proc Natl Acad Sci U S A* 2011, 108, (18), 7481–6. [PubMed: 21518885]
21. Boles BR; Thoendel M; Singh PK, Self-generated diversity produces “insurance effects” in biofilm communities. *Proc Natl Acad Sci U S A* 2004, 101, (47), 16630–5. [PubMed: 15546998]
22. Hare NJ; Soe CZ; Rose B; Harbour C; Codd R; Manos J; Cordwell SJ, Proteomics of *Pseudomonas aeruginosa* Australian epidemic strain 1 (AES-1) cultured under conditions mimicking the cystic fibrosis lung reveals increased iron acquisition via the siderophore pyochelin. *J Proteome Res* 2012, 11, (2), 776–95. [PubMed: 22054071]
23. Guina T; Wu M; Miller SI; Purvine SO; Yi EC; Eng J; Goodlett DR; Aebersold R; Ernst RK; Lee KA, Proteomic analysis of *Pseudomonas aeruginosa* grown under magnesium limitation. *J Am Soc Mass Spectrom* 2003, 14, (7), 742–51. [PubMed: 12837596]
24. Wu X; Held K; Zheng C; Staudinger BJ; Chavez JD; Weisbrod CR; Eng JK; Singh PK; Manoil C; Bruce JE, Dynamic Proteome Response of *Pseudomonas aeruginosa* to Tobramycin Antibiotic Treatment. *Mol Cell Proteomics* 2015, 14, (8), 2126–37. [PubMed: 26018413]
25. Peng J; Cao J; Ng FM; Hill J, *Pseudomonas aeruginosa* develops Ciprofloxacin resistance from low to high level with distinctive proteome changes. *J Proteomics* 2017, 152, 75–87. [PubMed: 27771372]
26. Swatton JE; Davenport PW; Maunders EA; Griffin JL; Lilley KS; Welch M, Impact of Azithromycin on the Quorum Sensing-Controlled Proteome of *Pseudomonas aeruginosa*. *PLoS One* 2016, 11, (1), e0147698. [PubMed: 26808156]
27. Park AJ; Murphy K; Krieger JR; Brewer D; Taylor P; Habash M; Khursigara CM, A temporal examination of the planktonic and biofilm proteome of whole cell *Pseudomonas aeruginosa* PAO1 using quantitative mass spectrometry. *Mol Cell Proteomics* 2014, 13, (4), 1095–105. [PubMed: 24532839]
28. Hisert KB; Heltshe SL; Pope C; Jorth P; Wu X; Edwards RM; Radey M; Accurso FJ; Wolter DJ; Cooke G; Adam RJ; Carter S; Grogan B; Launspach JL; Donnelly SC; Gallagher CG; Bruce JE; Stoltz DA; Welsh MJ; Hoffman LR; McKone EF; Singh PK, Restoring Cystic Fibrosis Transmembrane Conductance Regulator Function Reduces Airway Bacteria and Inflammation in People with Cystic Fibrosis and Chronic Lung Infections. *Am J Respir Crit Care Med* 2017, 195, (12), 1617–1628. [PubMed: 28222269]
29. Goss CH; Burns JL, Exacerbations in cystic fibrosis. 1: Epidemiology and pathogenesis. *Thorax* 2007, 62, (4), 360–7. [PubMed: 17387214]
30. Wisniewski JR; Ostasiewicz P; Mann M, High recovery FASP applied to the proteomic analysis of microdissected formalin fixed paraffin embedded cancer tissues retrieves known colon cancer markers. *J Proteome Res* 2011, 10, (7), 3040–9. [PubMed: 21526778]
31. Wu X; Chavez JD; Schweppe DK; Zheng C; Weisbrod CR; Eng JK; Murali A; Lee SA; Ramage E; Gallagher LA; Kulasekara HD; Edrozo ME; Kamischke CN; Brittnacher MJ; Miller SI; Singh PK; Manoil C; Bruce JE, In vivo protein interaction network analysis reveals porin-localized antibiotic inactivation in *Acinetobacter baumannii* strain AB5075. *Nat Commun* 2016, 7, 13414. [PubMed: 27834373]

32. Weisbrod CR; Hoopmann MR; Senko MW; Bruce JE, Performance evaluation of a dual linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer for proteomics research. *J Proteomics* 2013.
33. Eng JK; Jahan TA; Hoopmann MR, Comet: an open-source MS/MS sequence database search tool. *Proteomics* 2013, 13, (1), 22–4. [PubMed: 23148064]
34. Ozer EA; Allen JP; Hauser AR, Characterization of the core and accessory genomes of *Pseudomonas aeruginosa* using bioinformatic tools Spine and AGEnt. *BMC Genomics* 2014, 15, 737. [PubMed: 25168460]
35. Consortium Uniprot., Activities at the Universal Protein Resource (UniProt). *Nucleic Acids Res* 2014, 42, (11), 7486.
36. Winsor GL; Lam DK; Fleming L; Lo R; Whiteside MD; Yu NY; Hancock RE; Brinkman FS, *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. *Nucleic Acids Res* 2011, 39, (Database issue), D596–600. [PubMed: 20929876]
37. Ozer EA, ClustAGE: a tool for clustering and distribution analysis of bacterial accessory genomic elements. *BMC Bioinformatics* 2018, 19, (1), 150. [PubMed: 29678129]
38. Deutsch EW; Mendoza L; Shteynberg D; Farrah T; Lam H; Tasman N; Sun Z; Nilsson E; Pratt B; Prazen B; Eng JK; Martin DB; Nesvizhskii AI; Aebersold R, A guided tour of the Trans-Proteomic Pipeline. *Proteomics* 2010, 10, (6), 1150–1159. [PubMed: 20101611]
39. Han DK; Eng J; Zhou H; Aebersold R, Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. *Nat Biotechnol* 2001, 19, (10), 946–51. [PubMed: 11581660]
40. Kaufman DM; Wu X; Scott BA; Itani OA; Van Gilst MR; Bruce JE; Michael Crowder C, Ageing and hypoxia cause protein aggregation in mitochondria. *Cell Death Differ* 2017, 24, (10), 1730–1738. [PubMed: 28644434]
41. MacLean B; Tomazela DM; Shulman N; Chambers M; Finney GL; Frewen B; Kern R; Tabb DL; Liebler DC; MacCoss MJ, Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* 2010, 26, (7), 966–968. [PubMed: 20147306]
42. Vizcaino JA; Csordas A; del-Toro N; Dienes JA; Griss J; Lavidas I; Mayer G; Perez-Riverol Y; Reisinger F; Ternent T; Xu QW; Wang R; Hermjakob H, 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res* 2016, 44, (D1), D447–56. [PubMed: 26527722]
43. Farrah T; Deutsch EW; Kreisberg R; Sun Z; Campbell DS; Mendoza L; Kusebauch U; Brusniak MY; Huttenhain R; Schiess R; Selevsek N; Aebersold R; Moritz RL, PASSSEL: the PeptideAtlas SRMexperiment library. *Proteomics* 2012, 12, (8), 1170–5. [PubMed: 22318887]
44. Reinhardt N; Chen CI; Loppow D; Schink T; Kleinau I; Jorres RA; Wahn U; Magnussen H; Paul KP, Cellular profiles of induced sputum in children with stable cystic fibrosis: comparison with BAL. *Eur Respir J* 2003, 22, (3), 497–502. [PubMed: 14516141]
45. Scheid P; Kempster L; Griesenbach U; Davies JC; Dewar A; Weber PP; Colledge WH; Evans MJ; Geddes DM; Alton EW, Inflammation in cystic fibrosis airways: relationship to increased bacterial adherence. *Eur Respir J* 2001, 17, (1), 27–35. [PubMed: 11307750]
46. Amiel E; Lovewell RR; O'Toole GA; Hogan DA; Berwin B, *Pseudomonas aeruginosa* evasion of phagocytosis is mediated by loss of swimming motility and is independent of flagellum expression. *Infect Immun* 2010, 78, (7), 2937–45. [PubMed: 20457788]
47. Schweppe DK; Harding C; Chavez JD; Wu X; Ramage E; Singh PK; Manoil C; Bruce JE, Host-Microbe Protein Interactions during Bacterial Infection. *Chem Biol* 2015, 22, (11), 1521–1530. [PubMed: 26548613]
48. Johansson F; Olbe M; Sommarin M; Larsson C, Brij 58, a polyoxyethylene acyl ether, creates membrane vesicles of uniform sidedness. A new tool to obtain inside-out (cytoplasmic side-out) plasma membrane vesicles. *Plant J* 1995, 7, (1), 165–73. [PubMed: 7894507]
49. Hardin SC; Duncan KA; Huber SC, Determination of structural requirements and probable regulatory effectors for membrane association of maize sucrose synthase 1. *Plant Physiol* 2006, 141, (3), 1106–19. [PubMed: 16698903]
50. Mathee K; Ciofu O; Sternberg C; Lindum PW; Campbell JI; Jensen P; Johnsen AH; Givskov M; Ohman DE; Molin S; Hoiby N; Kharazmi A, Mucoid conversion of *Pseudomonas aeruginosa* by

hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology* 1999, 145 (Pt 6), 1349–57. [PubMed: 10411261]

51. Thomas SR; Ray A; Hodson ME; Pitt TL, Increased sputum amino acid concentrations and auxotrophy of *Pseudomonas aeruginosa* in severe cystic fibrosis lung disease. *Thorax* 2000, 55, (9), 795–7. [PubMed: 10950901]
52. Hancock REW; Tamber S, Porins of the Outer Membrane of *Pseudomonas aeruginosa* In *Bacterial and Eukaryotic Porins*, Wiley-VCH Verlag GmbH & Co. KGaA: 2005; pp 61–77.
53. Cornelis P; Dingemans J, *Pseudomonas aeruginosa* adapts its iron uptake strategies in function of the type of infections. *Front Cell Infect Microbiol* 2013, 3, 75. [PubMed: 24294593]
54. Bullen JJ; Rogers HJ; Spalding PB; Ward CG, Iron and infection: the heart of the matter. *FEMS Immunol Med Microbiol* 2005, 43, (3), 325–30. [PubMed: 15708305]
55. Khirya H; Turner AM, The role of iron in pulmonary pathology. *Multidiscip Respir Med* 2015, 10, 34. [PubMed: 26629341]
56. Goss CH; Kaneko Y; Khuu L; Anderson GD; Ravishankar S; Aitken ML; Lechtzin N; Zhou G; Czyz DM; McLean K; Olakanmi O; Shuman HA; Teresi M; Wilhelm E; Caldwell E; Salipante SJ; Hornick DB; Siehnel RJ; Becker L; Britigan BE; Singh PK, Gallium disrupts bacterial iron metabolism and has therapeutic effects in mice and humans with lung infections. *Sci Transl Med* 2018, 10, (460).
57. Garcia-Contreras R; Lira-Silva E; Jasso-Chavez R; Hernandez-Gonzalez IL; Maeda T; Hashimoto T; Boogerd FC; Sheng L; Wood TK; Moreno-Sanchez R, Isolation and characterization of gallium resistant *Pseudomonas aeruginosa* mutants. *Int J Med Microbiol* 2013, 303, (8), 574–82. [PubMed: 23992635]
58. Siehnel RJ; Egli C; Hancock RE, Polyphosphate-selective porin OprO of *Pseudomonas aeruginosa*: expression, purification and sequence. *Mol Microbiol* 1992, 6, (16), 2319–26. [PubMed: 1406271]
59. Calmettes C; Ing C; Buckwalter CM; El Bakkouri M; Chieh-Lin Lai C; Pogoutse A; Gray-Owen SD; Pomes R; Moraes TF, The molecular mechanism of Zinc acquisition by the neisserial outer-membrane transporter ZnuD. *Nat Commun* 2015, 6, 7996. [PubMed: 26282243]
60. Tamber S; Ochs MM; Hancock RE, Role of the novel OprD family of porins in nutrient uptake in *Pseudomonas aeruginosa*. *J Bacteriol* 2006, 188, (1), 45–54. [PubMed: 16352820]
61. Eren E; Vijayaraghavan J; Liu J; Cheneke BR; Touw DS; Lepore BW; Indic M; Movileanu L; van den Berg B, Substrate specificity within a family of outer membrane carboxylate channels. *PLoS Biol* 2012, 10, (1), e1001242. [PubMed: 22272184]
62. van den Berg B; Black PN; Clemons WM Jr.; Rapoport TA, Crystal structure of the long-chain fatty acid transporter FadL. *Science* 2004, 304, (5676), 1506–9. [PubMed: 15178802]
63. Whitney JC; Hay ID; Li C; Eckford PD; Robinson H; Amaya MF; Wood LF; Ohman DE; Bear CE; Rehm BH; Howell PL, Structural basis for alginate secretion across the bacterial outer membrane. *Proc Natl Acad Sci U S A* 2011, 108, (32), 13083–8. [PubMed: 21778407]
64. Nicas TI; Hancock RE, Outer membrane protein H1 of *Pseudomonas aeruginosa*: involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B, and gentamicin. *J Bacteriol* 1980, 143, (2), 872–8. [PubMed: 6259125]
65. Bains M; Fernandez L; Hancock RE, Phosphate starvation promotes swarming motility and cytotoxicity of *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 2012, 78, (18), 6762–8. [PubMed: 22773629]
66. Pederick VG; Eijkelkamp BA; Begg SL; Ween MP; McAllister LJ; Paton JC; McDevitt CA, ZnuA and zinc homeostasis in *Pseudomonas aeruginosa*. *Sci Rep* 2015, 5, 13139. [PubMed: 26290475]
67. Zheng L; Cardaci S; Jerby L; MacKenzie ED; Sciacovelli M; Johnson TI; Gaude E; King A; Leach JD; Edrada-Ebel R; Hedley A; Morrice NA; Kalna G; Blyth K; Ruppin E; Frezza C; Gottlieb E, Fumarate induces redox-dependent senescence by modifying glutathione metabolism. *Nat Commun* 2015, 6, 6001. [PubMed: 25613188]
68. Lindsey TL; Hagins JM; Sokol PA; Silo-Suh LA, Virulence determinants from a cystic fibrosis isolate of *Pseudomonas aeruginosa* include isocitrate lyase. *Microbiology* 2008, 154, (Pt 6), 1616–27. [PubMed: 18524916]

69. Dunn MF; Ramirez-Trujillo JA; Hernandez-Lucas I, Major roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis. *Microbiology* 2009, 155, (Pt 10), 3166–75. [PubMed: 19684068]
70. Richardson AR; Libby SJ; Fang FC, A nitric oxide-inducible lactate dehydrogenase enables *Staphylococcus aureus* to resist innate immunity. *Science* 2008, 319, (5870), 1672–6. [PubMed: 18356528]
71. Exley RM; Wu H; Shaw J; Schneider MC; Smith H; Jerse AE; Tang CM, Lactate acquisition promotes successful colonization of the murine genital tract by *Neisseria gonorrhoeae*. *Infect Immun* 2007, 75, (3), 1318–24. [PubMed: 17158905]
72. Weingarten RA; Taveirne ME; Olson JW, The dual-functioning fumarate reductase is the sole succinate:quinone reductase in *Campylobacter jejuni* and is required for full host colonization. *J Bacteriol* 2009, 191, (16), 5293–300. [PubMed: 19525346]
73. Govan JR; Deretic V, Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. *Microbiol Rev* 1996, 60, (3), 539–74. [PubMed: 8840786]
74. Tatnell PJ; Russell NJ; Gacesa P, GDP-mannose dehydrogenase is the key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa*: evidence from metabolite studies. *Microbiology* 1994, 140 (Pt 7), 1745–54. [PubMed: 7521247]
75. Remminghorst U; Rehm BHA, Bacterial alginates: from biosynthesis to applications. *Biotechnology Letters* 2006, 28, (21), 1701–1712. [PubMed: 16912921]
76. Xu B; Soukup RJ; Jones CJ; Fishel R; Wozniak DJ, *Pseudomonas aeruginosa* AmrZ Binds to Four Sites in the algD Promoter, Inducing DNA-AmrZ Complex Formation and Transcriptional Activation. *J Bacteriol* 2016, 198, (19), 2673–81. [PubMed: 27185826]
77. Boucher JC; Schurr MJ; Deretic V, Dual regulation of mucoidy in *Pseudomonas aeruginosa* and sigma factor antagonism. *Mol Microbiol* 2000, 36, (2), 341–51. [PubMed: 10792721]
78. Leech AJ; Sprinkle A; Wood L; Wozniak DJ; Ohman DE, The NtrC family regulator AlgB, which controls alginate biosynthesis in mucoid *Pseudomonas aeruginosa*, binds directly to the algD promoter. *J Bacteriol* 2008, 190, (2), 581–9. [PubMed: 17981963]
79. Firoved AM; Deretic V, Microarray analysis of global gene expression in mucoid *Pseudomonas aeruginosa*. *J Bacteriol* 2003, 185, (3), 1071–81. [PubMed: 12533483]
80. Kung VL; Ozer EA; Hauser AR, The accessory genome of *Pseudomonas aeruginosa*. *Microbiol Mol Biol Rev* 2010, 74, (4), 621–41. [PubMed: 21119020]
81. Kelley LA; Mezulis S; Yates CM; Wass MN; Sternberg MJ, The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc* 2015, 10, (6), 845–58. [PubMed: 25950237]
82. Lee SA; Gallagher LA; Thongdee M; Staudinger BJ; Lippman S; Singh PK; Manoil C, General and condition-specific essential functions of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 2015, 112, (16), 5189–94. [PubMed: 25848053]
83. Palmer KL; Aye LM; Whiteley M, Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. *J Bacteriol* 2007, 189, (22), 8079–87. [PubMed: 17873029]
84. Regelman WE; Elliott GR; Warwick WJ; Clawson CC, Reduction of sputum *Pseudomonas aeruginosa* density by antibiotics improves lung function in cystic fibrosis more than do bronchodilators and chest physiotherapy alone. *Am Rev Respir Dis* 1990, 141, (4 Pt 1), 914–21. [PubMed: 2109558]
85. Zemanick ET; Harris JK; Wagner BD; Robertson CE; Sagel SD; Stevens MJ; Accurso FJ; Laguna TA, Inflammation and airway microbiota during cystic fibrosis pulmonary exacerbations. *PLoS One* 2013, 8, (4), e62917. [PubMed: 23646159]
86. Eisen MB; Spellman PT; Brown PO; Botstein D, Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* 1998, 95, (25), 14863–8. [PubMed: 9843981]
87. Franceschini A; Szklarczyk D; Frankild S; Kuhn M; Simonovic M; Roth A; Lin J; Minguez P; Bork P; von Mering C; Jensen LJ, STRING v9.1: protein-protein interaction networks, with increased coverage and integration. *Nucleic Acids Res* 2013, 41, (Database issue), D808–15. [PubMed: 23203871]

Figure 1. Overview of experimental procedures of PA proteome analysis with CF sputum samples.

A) Each CF sputum sample was liquefied and diluted with five volumes of PBS that contains 0.1 % dithiothreitol on ice, and then was divided into two portions for *in vivo* or *ex vivo* PA population proteome analyses. For *ex vivo* proteome analysis, the liquefied CF sputum sample was plated on MacConkey agar plates to select PA isolates. Ninety-six PA isolates were randomly collected, cultured independently on LB medium and then pooled for *ex vivo* PA population proteome analysis. For *in vivo* proteome analysis, the liquefied CF sputum

sample was performed with bacterial enrichment procedures as detailed in B). The obtained *in vivo* and *ex vivo* PA proteome profiles are further compared, as illustrated in Fig. 4.

Figure 2. Improved detection of PA peptides/proteins in CF sputum samples with bacterial-enrichment procedures.

A) The number of non-redundant PA peptides detected with CF sputum samples are improved with the bacterial-enrichment method. bacterial-enrichment method. Examples of five CF sputum samples collected from three CF patients are shown. ***, one-way ANOVA, $P < 0.001$. Error bars indicate the standard deviation of PA peptides detected with different LC-MS/MS analyses. B) Immunoblots showing the improved bacterial-protein detection of TufA and RpoA, and the decrease of human protein GAPDH in bacterial-enriched CF sputum samples. Results acquired with different Triton X-100 concentration (0%, 0.1%,

0.5% or 1.0%) during resuspension of the CF sputum pellet are shown. Coomassie protein stain shows equal total protein loading.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3. Identification of PA proteins with the 35 CF sputum samples.

Thirty-five CF sputum samples were processed with the bacterial-enrichment method. X-axis indicates the $\log_{10}$ of the number of the identified PA proteins with 2-peptide identification rule. Y-axis indicates the $\log_{10}$ of the amount of PA cells in the CF sputum samples based on colony forming units per ml (CFU/ml). The orange-color marker indicates the analysis of a CF sputum sample collected from a PA negative patient, and no PA proteins were identified in that sample. Linear regression analysis shows positive correlation of the amount of PA cells in CF sputum samples and the number of PA peptides detected with CF sputum samples.

Figure 4. Proteome quantitation of PA with CF sputum samples.

A) Histogram showing log₂(protein fold change) in a CF sputum sample versus the reference proteome of PA population grown in LB medium. B) Histogram showing the log₂(protein fold change) for all the quantified PA proteins summarized from the 35 CF sputum sample analyses. C) Reproducibility of the quantified *in vivo* PA proteome abundance versus the *ex vivo* in two CF sputum samples. Patient 3 sputum samples 5 and 6 were collected on consecutive days in the absence of patient's clinical changes. *Ex vivo* PA populations were collected independently from these two CF sputum samples. The log₂(protein fold change)

for *in vivo* versus *ex vivo* for commonly quantified proteins were plotted for each sample, showing strong correlation of the quantified protein fold changes. Proteins which were identified as up- or down-regulated proteins through the analysis of the 35 CF sputum samples are highlighted with red or green. The top five proteins of largest fold-change are labeled.

Figure 5. Heat map and clustering analysis of PA *in vivo* proteomes with the 35 CF sputum samples.

Hierarchical clustering with the $\log_2(\text{protein fold change})$ was analyzed with Cluster 3.0 (86). The identity of the sputum samples is explained in Table S1. The main protein clusters for the up-regulation (purple) or down-regulation (green) are indicated. The bacterial density in terms of measured colony forming units (CFU/ml) for each sputum sample is also shown.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 6. Functional enrichment of PA proteins increased or decreased in CF airways. The 67 increased and 117 decreased PA proteins are subjected to gene ontology analysis, using STRING (version 10.0) (87). Significance levels were determined with hypergeometric test with Benjamini-Hochberg FDR correction. The color darkness indicates the P value significance of functional enrichment. Five major functional changes are indicated.