Functional annotation of *Pseudomonas aeruginosa* membrane proteins in response to micronutrients

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Declaration

The work presented in this thesis has not been submitted for a higher degree to any other university or institution other than Macquarie University, North Ryde, Australia.

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Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* PAO1 is well known for its environmental and metabolic versatility, and large genome of 6.3Mbp. Studying the proteome is key to providing accurate, and informative genome annotation. Past studies of the proteome of *P. aeruginosa* have provided proteomic profiles and information supportive for the existence of hypothetical proteins, but many have failed to provide anything more, and so functional annotation has lagged behind.

In this study, 2000 non-redundant proteins from membrane and soluble fractions of PAO1 were quantifiably identified using a targeted proteomics approach after growth with the addition of copper or cobalamin to the growth medium. From this, eight differentially expressed but poorly annotated proteins were chosen for further functional annotation work. A knockout study was performed using transposon insertion mutants of the genes for the eight candidate proteins and growth assays were performed to provide support for their putative functions. A highlight from this group was candidate protein PA3920, which was identified in this study as a P-type ATPase for Cu^{2+}. This work jointly provides evidence for the function of a number of putative proteins and acts as a stepping-stone for further annotation work with the large list of proteins identified.

Abbreviations

Abbreviation	Term
2-DE	2-D electrophoresis
2-DLC	2-D liquid chromatography
2D-DIGE	2-D difference in-gel electrophoresis
BCA	Bicinchoninic acid assay
CF	Cystic fibrosis
COG	Clusters of orthologous groups
DDA	Data dependent acquisition
DIA	Data independent acquisition
DTT	Dithiothreitol
ESI	Electrospray ionization
EVOH	Ethylene-vinyl alcohol
GABA	Gamma-aminobutyric acid
GO	Gene ontology
IEF	Isoelectric focusing
LC	Liquid chromatography
m/z	*mass-to-charge*
MALDI	Matrix assisted laser desorption ionization
MOPS	3-(N-morpholino)propanesulfonic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NMR	Nuclear magnetic resonance
ORF	Open reading frame
PA14	*Pseudomonas aeruginosa* PA14
PAGE	Polyacrylamide gel electrophoresis
PAO1	*Pseudomonas aeruginosa* PAO1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGD	*Pseudomonas* genome database
PseudoCAP	*Pseudomonas aeruginosa* community annotation project
RPM	Revolutions per minute
SCX	Strong cation exchange
SDS	Sodium dodecyl sulfate
SRM/MRM	Selected/Multiple reaction monitoring
SWATH-MS	Sequential windowed acquisition of all theoretical fragment ion mass spectra
TBDT	TonB dependent transporter
TEAB	triethlyammonium bicarbonate buffer
TOF	Time of flight
ZSB	Zinc schiff-base complex (bis-(N-allylsalicylideneiminato)-zinc)

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Chapter 1: Introduction

1.1. *Pseudomonas spp.*

The *Pseudomonas* genus contains 202 species that have been classified based on 16S rRNA, cellular fatty acid analysis, and physiological and biochemical analyses (1). Morphologically they display the general characteristics of being Gram negative, non-spore forming bacilli that exhibit the metabolic versatility to inhabit a wide range of ecological niches, from soil, to marine and freshwater environments (2). An interesting characteristic that many of the *Pseudomonas* genus exhibit is the production of the fluorescent siderophore, pyoverdin (2). The pseudomonads that can produce pyoverdin are aptly named the fluorescent pseudomonads. The fluorescent pseudomonads group houses a number of *Pseudomonas* species that have been of considerable interest to the medical microbiological community for many years due to their involvement as infectious agents. Some examples include: *Pseudomonas oryzihabitans* (3), *Pseudomonas fluorescens* (4), *Pseudomonas putida* (5), and the most commonly identified and the species of highest clinical significance from within this group, *Pseudomonas aeruginosa* (6).

1.2. *Pseudomonas aeruginosa* – an opportunistic pathogen

P. aeruginosa is an opportunistic pathogen that is able to grow between the temperature ranges of 4°C to 42°C displaying optimal growth at 37°C (7). It is the leading cause of nosocomial respiratory tract infections (8), as well as also being an isolate in 10% of all recorded hospital acquired infections (9, 10). To expand on this further, *P. aeruginosa* has been isolated from wound and burn infections (11), implicated in otitis media (12, 13), and is a common isolate in bacteremia cases (12). However, it is probably most noted for being the bearer of increased morbidity and mortality amongst cystic fibrosis (CF) patients. Individuals with CF are incredibly prone to chronic infections of *P. aeruginosa*, (amongst other pathogens) in the respiratory tract that can be attributed to a number of reasons, which include: the pathophysiology of CF (e.g. reduced

mucociliary clearance (14)), the organism's intrinsic and growing resistance to antibiotics (15, 16), and the prevalence of the organism throughout the natural and hospital environment (17). To understand the severity of infection a study released by Emerson, *et al.* (2002) found that young children with CF and an accompanying *P. aeruginosa* infection had a 2.6 times higher risk of death over the 8-year study. Similarly a study by Li, *et al*. (2005) found that the median age of acquisition of non-mucoid *P. aeruginosa* was 1 years of age, but it wasn't until the development of the mucoid phenotype at the median age of 13 years, that a significant deterioration of pulmonary function occurs, thus reaffirming the need to prevent the progression into chronic infection. Once a chronic *P. aeruginosa* infection has been established in CF patients, elimination of the infection is practically impossible, leaving only the early, acute stages of the infection as the target period through aggressive treatment (20).

1.3. Genome project

In response to the increasing need to understand *P. aeruginosa* environmental and infectious versatility, it was one of the earliest microbes to undergo complete genome sequencing (August 2000). Using the archetypal strain PAO1, Stover, *et al.* (2000) reported a large 6.3 million base pairs (Mbp) genome containing 5570 open reading frames (ORFs), with what they conclude as containing the genetic complexity necessary to permit its wide metabolic and environmental adaptability. *P. aeruginosa* PAO1 (will be referred to as PAO1), the reference strain, is a chloramphenicol resistant mutant of a strain of *P. aeruginosa* termed PAO that was isolated from a patient suffering a wound infection in 1954 (21), and since has been widely used as a lab model to represent the species.

There are 26 complete *P. aeruginosa* genome sequences deposited in the NCBI genome database (as of August 2015). 20 of these are clinical isolates, providing an opportunity to compare and contrast genomic potential within these strains.

Table 1: Strains of *P. aeruginosa* with sequenced genomes.

Strain	Genome size (Mbp)	Release date (DD/MM/YYYY)	Isolate	Genbank sequence (journal reference)
PAO1	6.26	16/05/2000	Wound	AE004091.2

				(22)
UCBPP-PA14	6.54	06/10/2007	Burn wound	CP000438.1 (23)
PA7	6.59	05/07/2007	Non-respiratory	CP000744.1 (24)
LESB58	6.60	24/12/2008	CF isolate	FM209186.1 (25)
PACS2	6.49	05/06/2006	CF isolate	AAQW01000001.1 (unpublished)
19BR	6.74	17/08/2011	Clinical isolate	AFXJ01000001.1 (26)
213BR	6.72	17/08/2011	Clinical isolate	AFXK01000001.1 (26)
M18	6.33	19/09/2011	Sweet melon rhizosphere isolate	CP002496.1 (27)
DK2	6.40	20/06/2012	CF isolate	CP003149.1 (28)
NCGM2.S1	6.76	30/09/2011	Urinary tract isolate	AP012280.1 (29)
B136-33	6.42	09/04/2013	Isolate from an infant with community acquired diarrhoea	CP004061.1 (unpublished)
RP73	6.34	24/06/2013	CF isolate	CP006245.1 (30)
VRFPA04	6.82	27/06/2014	Isolate form human corneal button	CP008739.2 (unpublished)
PA1	6.53	12/11/2013	Oil production wastewater isolate	CP004054.1 (unpublished)
PA1R	6.31	12/11/2013	PA1 mutant	CP004055.1 (unpublished)
MTB-1	6.58	05/12/2013	Microbial community isolate of hexachloro-cyclohexane	CP006853.1 (31)
LES431	6.55	17/12/2013	CF isolate	CP006937.1

				(unpublished)
SCV20265	6.73	18/12/2013	CF isolate	CP006931.1 (32)
YL84	6.43	30/01/2014	Compost isolate	CP007147.1 (33)
F22031	6.60	09/01/2015	Clinical isolate	CP007399.1 (unpublished)
NCGM 1984	6.85	18/09/2014	Clinical isolate	AP014646.1 (unpublished)
NCGM 1900	6.81	01/08/2014	Clinical isolate	AP014622.1 (unpublished)
FRD1	6.71	29/01/2015	CF isolate	CP010555.1 (34)
Carb01 63	7.50	29/04/2015	Rectal swab isolate	CP011317.1 (unpublished)
DSM 50071	6.32	02/07/2015	N/A	CP012001.1 (unpublished)
F9676	6.37	13/07/2015	Diseased rice isolate	CP012066.1 (unpublished)

Note: Some genome projects were not published in journals, but those that have been have been acknowledged. The Genbank sequence accession code has been listed and can be used to access information about the authors responsible for the direct submission of the complete genome sequence (as well as other details of interest).

1.4. Genome annotation

In order to better understand *P. aeruginosa* pathobiology, the putative functions of polypeptides encoded by their respective genes needs to be elucidated in order to give meaning to the information obtained through sequencing the genome. Describing an organism's proteome (or annotating the genome) is in a sense a much larger project than the sequencing of a genome as it involves many focused studies aimed at deriving specific information from proteins/genes then collating this information into a proteome map. Having the knowledge of a proteome of an organism is invaluable for any researcher as proteins are the functional elements of cells, and gaining an insight into the identities and quantity of proteins expressed at any one time under a set of specific conditions allows a deeper understanding of the biological system in question.

In terms of PAO1, at the time of its sequencing and subsequent analysis, Stover, *et al.* (2000) provided genome annotation to 54.2% (3039) of ORFs with varying levels of confidence. The 3039 ORFs were assigned into one of 25 functional classes ((e.g. cell division, or chemotaxis) (Not shown, available in Stover, *et al.* 2000)) originally used for the functional characterization of *Escherichia coli* K12 ORFs by Blattner, *et al*. (1997). Furthermore, 372 (6.7%) ORFs had their function demonstrated in *P. aeruginosa* and therefore held the strictest level of confidence towards functional assignment. Similarly, other genes were categorized with lesser confidence levels (2, 3, or 4) based primarily upon their level of sequence identity towards genes characterized in other organisms, as well as on the presence of any conserved amino acid motifs or structural features of an experimentally studied gene. Figure 1 represents PAO1 ORFs grouped according to assigned confidence levels (Levels 1-4, 1 being the highest). 45.8% (2549) of ORFs had confidence level 4 assignments (figure 1), which is the lowest level possible accounting for purely hypothetical proteins, and clearly demonstrating the continued need for greater functional annotation.

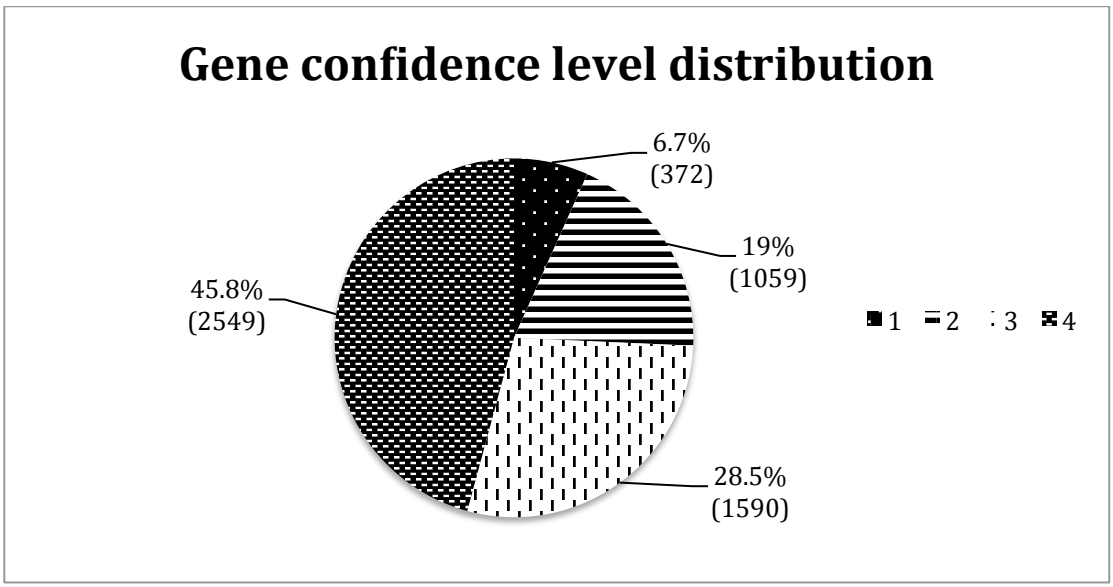


Figure 1: Open reading frame confidence levels 1, 2, 3, and 4 for PAO1. Data and confidence level descriptions derived from Stover, *et al* (2000). "1" refers to genes with their function being of the highest possible confidence level. Confidence level 1-grouped genes have had their function demonstrated in *P. aeruginosa*. "2" (confidence level 2) contained genes with strong sequence identity to genes with a demonstrated function in other organisms. "3" (confidence level 3): Functions proposed based upon the presence of conserved amino acid motifs, structural or limited sequence similarity to an

experimentally studied gene. Finally, “4” (confidence level 4) contained genes with either no sequence similarity to any reported sequence, or were homologs to genes with unknown functions. The values in the parenthesis outline the number of ORFs under each confidence level.

1.5. *Pseudomonas aeruginosa* community annotation project (PseudoCAP)

In response to the sequencing project of the PAO1 genome and the need for critical, peer-reviewed and cost-effective genome annotation, a project termed PseudoCAP, the *P. aeruginosa* Community Annotation Project, was formed in 1997 (36). The project aimed to gather the expertise, and hands-on support of volunteer scientists to be a part of annotating the PAO1 genome. The project was met with enthusiasm and by the publication of the PAO1 genome sequence in 2000, 61 researchers had submitted 1741 annotations (22, 37). This project was remarkable in that all communication and community-based annotations were performed exclusively through the Internet, which was novel at the time, and was granted a huge success.

In the advent of the PAO1 genome publication and the success of the PseudoCAP project the development of the *Pseudomonas* Genome Database (Pseudomonas.com, herein referred to as PGD) ensued. PGD was developed originally to facilitate continually updated community based genome annotation (38) for *P. aeruginosa* but in the years subsequent to the PAO1 genome release there has been much work on sequencing and annotating other *Pseudomonas* strains (as evidenced by table 1) and species, and because of this the PGD expanded in 2005 to include data on other *Pseudomonas* species and strain genomes including *Pseudomonas fluorescens* and *P. aeruginosa* PA14 (herein referred to as PA14).

1.6. PAO1 proteome

Proteomic mapping of PAO1, that is, the process of identification and validation of proteins predicted to be expressed through proteomic profiling experiments, has been key in providing researchers valuable confirmation of expression and potential protein targets for further characterization. There has been much work in proteomic mapping for

PAO1 over the years, the success of which has been primarily dictated by the advancement of mass spectrometry instruments and workflows. The progress over the years in PAO1 proteome mapping can be observed in table 2, whereby the early years of PAO1 profiling was almost solely governed by the tedious 2-D gel-based visualization, extraction, and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) workflows that only led to the identification of a fraction of proteins of the PAO1 proteome (typically no more than a couple hundred proteins). Examples of such studies from table 2 include: Nouwens, *et al*. (2000) - 189, Nalca, *et al*. (2006) - 45, and Imperi, *et al*. (2009) - 395.

The development of shotgun proteomics and liquid chromatography – tandem mass spectrometry (LC-MS/MS) analysis and workflows enabled complex proteomic samples to by-pass traditional (2D-) gel separations, and instead online liquid chromatography fractionation became the more dominant workflow, and coupled to MS/MS enabled much deeper protein coverage. This transition can be observed in table 2 whereby many studies where reporting far higher numbers of protein identifications with LC-MS/MS, two of particular note being the studies by Herbst, *et al.* (2015) with 2801 identifications, and Kumari, *et al.* (2014) with 2965 identifications. The combination of the results from all these studies allows the development and annotation of genome/proteome databases (such as PGD) to become more comprehensive and reliable, and allows researchers to move onto more complex protein functional studies.

Table 2: PAO1 proteomic studies since the year 2000.

Authors (PMID)	Year	PAO1 Proteome (Condition)*	Proteins identified¶	Method of identification
Nouwens et al. (11271498)	2000	Whole membrane	189	2-DE and MALDI-TOF MS
Malhotra et al. (11092861)	2000	Soluble extract: PAO1 wild-type and PAO1 with *mucA22* allele	4	2-DE and amino-terminal sequence analysis
Nouwens et al. (12362351)	2002	Outer membrane	32	2-DE, MALDI-TOF MS and ESI-MS/MS
Nouwens et al. (12362351)	2002	Extracellular	166	2-DE and MALDI-TOF MS and ESI-MS/MS
Nouwens et al. (12724392)	2003	Extracellular: PAO1 wild-type and PAO1	31	2-DE and MALDI-TOF MS

		quorum sensing mutants		
Heim et al. (14641572)	2003	Soluble extract (with and without iron-limitation)	25[§]	2-DE and MALDI TOF
Blonder et al. (15253424)	2004	Whole membrane	707	LC-MS/MS
Kim et al. (15817780)	2005	Extracellular (iron-limiting and iron-rich conditions)	26	2-DE and MALDI-TOF MS
Nalca et al. (16641435)	2006	Soluble extract (Exposure and non-exposure to azithromycin)	43	2-DE and MALDI-TOF
Diab et al. (16622056)	2006	Soluble extract (Grown with glucose or succinate, with or without glycine betaine)	21	2-DE and MALDI-TOF
Patrauchan, Sarkisova & Franklin (17975093)	2007	Cytoplasm ($CaCl_2.2H_2O$ – biofilm and planktonic cultures)	146	2-DE and MALDI-TOF/TOF
Imperi et al. (19333994)	2009	Periplasm	395	2-DE and MALDI-TOF/TOF
Choi et al. (21751344)	2011	Outer membrane vesicles	338	LC-MS/MS
Damron et al. (21631603)	2011	Soluble extract (ammonium metavanadate exposure and non-exposure)	17[§]	2-DLC-MALDI-TOF/TOF
Lecoutere et al. (22950023)	2012	Cytoplasm	159	2-DE and MALDI-TOF
Toyofuku et al. (22909304)	2012	Soluble extract and biofilm matrix	942	SDS-PAGE and LC-MS/MS
Damron et al. (22210761)	2012	Soluble extract: PAO1 wild-type, PAO1 *kinB* and PAO1 *kinB* Δ*rpoN*	121	MALDI-TOF/TOF
Massier et al. (22188372)	2012	Soluble extract (Pulsed light-induced stress exposure and non-exposure)	15	2-DE and MALDI-TOF/TOF

Hare et al. (22264352)	2012	Soluble extract	1700	2-DE, MALDI-TOF MS and 2-DLC-MS/MS
Casabona et al. (23744604)	2013	Cytoplasmic membrane	991	SDS-PAGE and LC-MS/MS
Cierniak et al. (23869205)	2013	Soluble extract (ZSB adapted and ZSB non-adapted)	8	2D-DIGE and LC-MS/MS
Dagorn et al. (23154974)	2013	Soluble extract (GABA exposure and non-exposure)	7	2-DE and MALDI-TOF/TOF
Kumari et al. (24291602)	2014	Soluble extract: PAO1 wild-type, and PAO1 *ampR* deletion mutant (benzyl penicillin exposure and non-exposure)	2965	SDS-PAGE and LC-MS/MS
Park, Surette & Khursigara (25232353)	2014	Soluble extract and outer membrane vesicles resistance sub-proteome (biofilm and planktonic cultures at three time points)	50[§]	LC-MS/MS
Sarkisova et al. (24918783)	2014	Soluble extract: PAO1 *efhP* deletion mutant ($CaCl_2.2H_2O$ – biofilm and planktonic cultures)	15[§]	2-DE and MALDI-TOF/TOF
Kwon et al. (24742327)	2014	Soluble extract	1692	LC-MS/MS
Kubacka et al. (24549289)	2014	Soluble extract (TiO_2 EVOH particles photo-catalysis)	1137	LC-MS/MS
Park et al. (24532839)	2014	Soluble extract (biofilm and planktonic cultures at three time points)	1884	LC-MS/MS
Herbst et al. (25317949)	2015	Whole cell - soluble and insoluble fraction: PAO1 wild-type, and PAO1 Fap overexpressing derivative	2801	SDS-PAGE and LC-MS/MS

Note: Care has been taken to identify as many studies as possible to highlight the full extent of PAO1 proteome profiling within the literature. Many of these studies will overlap in the identities of the proteins identified, and most (but not all) do not attempt to quantify nor attempt to characterize the proteins identified to any great depth.

*: The condition, if applicable (denoted in parenthesis), describes any notable conditions (other than growth medium) PAO1 was subjected to prior to proteome profiling.

¶: The proteins that were identified by the authors varied in their level of pre-annotation.

§: The reported proteins were only the proteins of interest, or the proteins differentially regulated.

1.7. Mass spectrometry to study proteins

Mass spectrometry is a quintessential approach for modern proteomics, which has allowed researchers to reliably structurally characterize and even quantify large biomolecules such as proteins from purified or complex samples. Modern workflows involving complex proteomic samples most commonly involve an in-solution proteolytic digestion (as opposed to in-gel digestion) followed by separation of peptides using nanoLC-MS/MS. Information dependent analysis is commonly used which relies on the mass spectrometer to select a variable number of the most intense precursor ions for subsequent tandem MS analysis. As this selection process is stochastic, run-to-run differences in precursor ion selection is common, requiring replicate analyses to ensure high sample to sample profile coverage.

Currently, new techniques are being introduced that potentially allow for the complete identification of all proteins within complex proteomic samples. Of recent, Gillet, *et al.* (2012) introduced a mass spectrometry workflow titled SWATH-MS that has received a lot of attention for its ability to quantify all proteins within a complex sample. Workflows such as this demonstrate the increasing power, and practicality of mass spectrometry to facilitate the generation of greater information for biomolecules.

1.7.1 SWATH-MS

Sequential Windowed Acquisition of all THeoretical fragment ion mass spectra or SWATH-MS as it is more simply known, is a data-independent acquisition (DIA) method used in tandem mass spectrometry (61). It combines the reproducibility and quantitative power of targeted proteomics (e.g. selected reaction monitoring (SRM)), with that of discovery

proteomics' (e.g. shotgun proteomics) ability to identify large amounts of proteins in any one or more samples (61), and therefore has cemented itself as an acquisition method of enormous potential in the area of proteomics, and was the primary mass spectrometry method used for this study.

Data-dependent acquisition (DDA) (or information-dependent acquisition (IDA)), of which SRM/MRM and shotgun proteomics fall under, rely on either the knowledge or detection of precursors ions to trigger the acquisition of fragment ion spectra over the chromatographic series (61), which is in contrast to SWATH's DIA. So for example in a simple discovery based strategy, the digested protein sample will be eluted across a chromatographic series, ionization will occur and the charged ions (termed precursor ions) will have their *mass-to-charge* (*m/z*) ratios determined by the first mass analyser (MS^1). A common discovery based strategy will be configured in product-ion scan mode, which means that at each point over the chromatographic elution time typically the "top-20" most abundant precursor ions will be selected for fragmentation and MS^2 analysis. SWATH-MS on the other hand does not rely on the knowledge or detection of precursor ions to trigger acquisition and instead will fragment all precursor ions across the entire *m/z* range within user-defined mass and retention time windows. This is visualized in figure 2. The result is complex fragment ion maps, so complex in fact that in order to extract the information to identify peptides, a targeted data extraction strategy to 'mine' the fragment ion maps was required and a novel method was developed (61). The extraction method required the use of spectral libraries, whereby fragment ion signals, their intensities, and chromatographic occurrence (all information contained within the library) are used to mine the fragment ion maps so that the identities and quantities of peptides from the SWATH-MS run can be searched. In most cases a spectral library would not be available for the proteomics sample you are working with, and was not the case for PAO1, so generation of a spectral library is then necessary via an IDA run. However, you can still perform the SWATH acquisition before a spectral library is available since it is only required for the data mining step. One can also mine the fragment ion maps with a simplified or less comprehensive spectral library, and at a later date re-mine the data using more comprehensive spectral

libraries. Figure 3 represents SWATH data extraction using IDA data with Peakview v2.1 with SWATH Micro-App v2.0 software (SCIEX). Figure 4 represents a typical simplified workflow for a SWATH proteomics experiment.

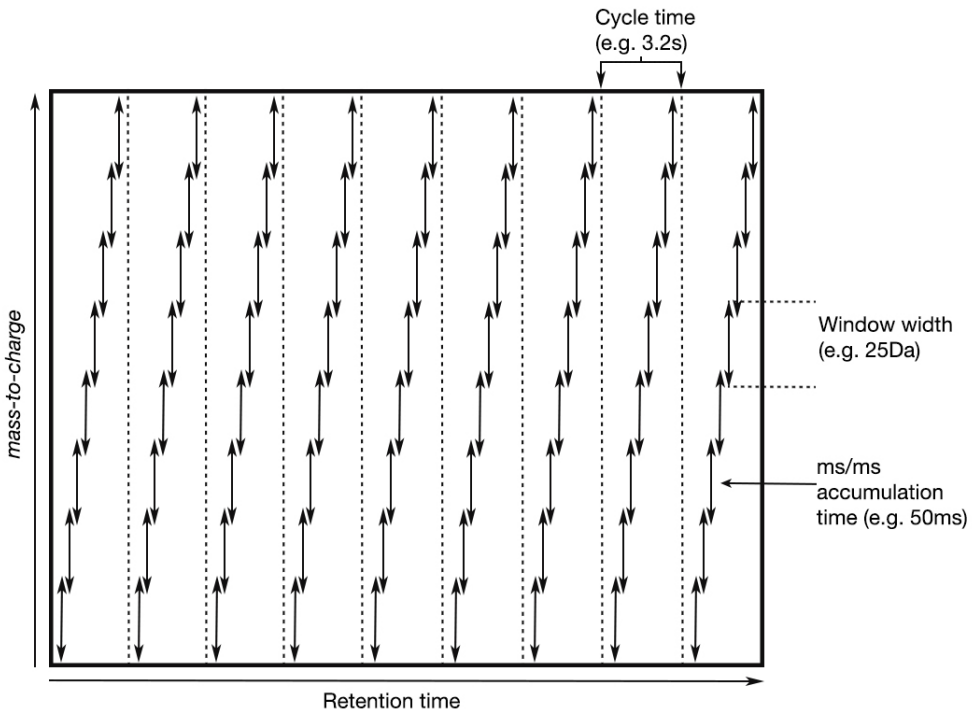


Figure 2: The SWATH-MS DIA method (figure adapted and recreated from Johansen, Van Soest, Mollah, Seymour and Hunter (62)). Each double arrow represents one precursor isolation window (swath) of pre-defined *m/z* width. The *m/z* range typically between 400-1200 *m/z* and composed of 32 isolation windows, is cycled across the entire chromatographic series (retention time) until completion.

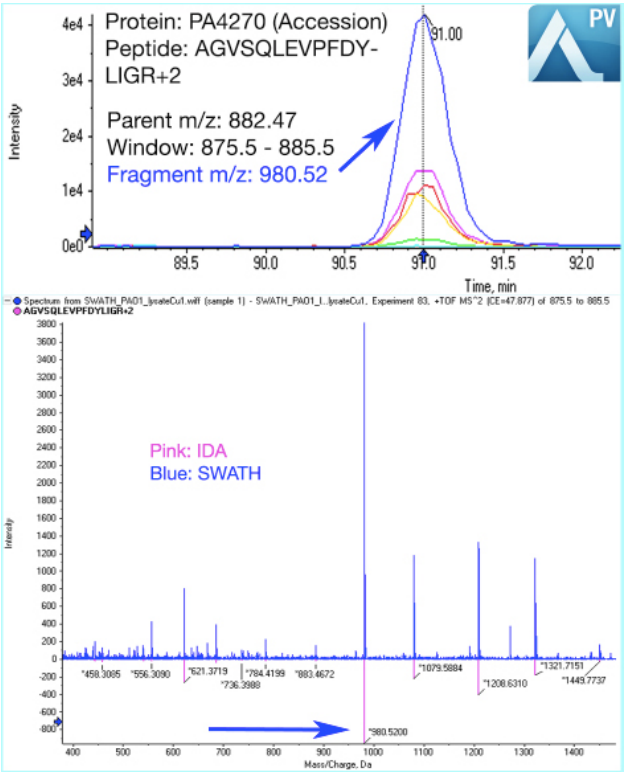


Figure 3: SWATH-MS targeted data analysis of peptide AGVSQLEVPFDYLIGR+2 using PeakView v2.1 with SWATH Micro-App v2.0 (SCIEX). For any particular peptide, fragment ion information is extracted from the spectral library. The fragment ion traces are overlaid with the appropriate swath window (in this case 875.5-885.5) and the peak group (of the swath data) displaying the best matching characteristics with that of the extracted fragment ion traces (980.52) will be used for identification and quantification of the peptide.

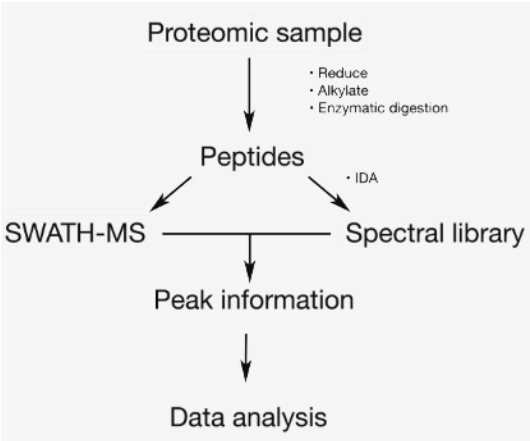


Figure 4: Simplified proteomics workflow for a SWATH-MS experiment. A protein sample is prepared as usual through the reduction and alkylation of the cysteine residues, followed by digestion into peptides. The peptides then undergo further sample preparation to remove contaminants. Ideally, the peptides are split into two aliquots, one for generation of a spectral library and the other for SWATH-MS acquisition; the data is generated and then mined using the spectral library. Data analysis will vary according to requirements.

1.8. The membrane proteome

To start out, the attention of this study was to be on the membrane proteome of PAO1, with special emphases on the proteomic profiles of the expressed membrane proteome under separate copper and cobalamin (vitamin B_{12}) conditions – which will be spoken about in more depth in later sections, but for the moment it is important to highlight the specifics of undertaking a membrane proteomic study.

In the past, proteomic studies of membrane proteins have been challenging because of two reasons primarily. The first is due to the difficulty in removing them from the membrane and the second is due to their hydrophobic nature making them difficult to solubilize for down-stream analysis such as electrophoresis (63). Conventional two-dimensional gel electrophoresis (2-DE) (isoelectric focusing (IEF) first dimension, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) second dimension) can be used for proteins that show limited hydrophobicity, however proteins that are highly hydrophobic are generally not compatible with IEF (64).

Studying the membranes that make up the cell envelope is very important, as they do not just serve as passives barriers, but rather they hold proteins that display a wide range of physiological functions that enable them to communicate and interact with the environment. Such functions can include: passive and active transport, receptors, virulence, and roles in chemotaxis, attachment and motility (63, 65) all of which contribute to pathogenicity. In terms of PAO1 three studies to date have specifically surveyed the outer membrane, periplasmic, and cytoplasmic (inner) membranes. The outer membrane (and extracellular) proteins were surveyed by Nouwens, Willcox, Walsh and Cordwell (66) and compared to the cytotoxic *P. aeruginosa* strain 6206. The study was a follow-up investigation to the study in 2000 by Nouwens, *et al*, whereby the whole cell membrane was investigated. Imperi*, et al.* (67) analysed the periplasmic proteome of PAO1 by 2-DE and MALDI-TOF/TOF, and identified 395 unique proteins, 76 of which were classified as periplasmic proteins. Finally in the analysis of the cytoplasmic membrane, Casabona, Vandenbrouck, Attree and Couté (68) identified 991 non-redundant proteins in PAO1. These studies described attempts to identify as many proteins as possible from these subcellular locations so that a proteome

map of the cell envelope for PAO1 can be further developed. There was minimal study into any specific proteins identified from the group (the proteins varied in the level of pre-annotated annotations), but their work has provided the opportunity for any researchers interested in these subcellular locations to study any of the experimentally confirmed proteins in further detail.

1.8.1. Studying the *P. aeruginosa* membrane proteome

At the current time point it would be recommended that any membrane protein study of *P. aeruginosa* be conducted with at least the sequenced PAO1 strain. The most appropriate methodology would be to use the reference strains (PAO1 and/or PA14) to demonstrate similarities and differences on the molecular scale between one or more strains. Before initiating a proteomic study in *P. aeruginosa* a literature and database search is crucial to ensure relevancy, and to source the most up-to-date proteomic information. PGD (Pseudomonas.com) would be database of first call primarily for two reasons; the first is that it is regularly updated in annotations for PAO1, keeping up-to-date with published material - for example an update on July the 11th 2015 touched on work performed by Kumari*, et al.* (54) in which evidence was produced for the translation of 2965 PAO1 proteins from LC-MS work, and secondly, the database allows comparative analysis for a number of species and strains of which it holds annotations for (69).

On the experimental side of things, the modified carbonate extraction protocol of Molloy (70) allows researchers to efficiently isolate the membrane proteins of Gram-negative bacteria. Numerous studies since have employed this method to extract membrane proteins (71-73), and this method would seem to be the most appropriate method to use in isolating the membrane proteins of *P. aeruginosa.* As mentioned earlier more modern studies are electing to follow the methodology of in-solution tryptic digestion of proteins followed by LC-MS/MS analysis of the peptides for protein identification, and the avoidance of gels is ideal when studying samples of a highly hydrophobic nature.

1.9. Micronutrients

1.9.1. Copper

Copper (Cu) is an essential trace element for all organisms, with bacteria requiring it as a co-factor to many enzymes (74, 75), however at higher concentrations copper will become toxic to the organism, and the toxicity of copper has been researched and exploited as an antimicrobial agent over the years (76-78). Copper toxicity in bacteria is the result of the free ionic form Cu^{2+} that can interfere via interactions with nucleic acids and proteins by way of disrupting structure or displacing metal cofactors (79), oxidation of membrane components (80), and the generation of reactive oxygen species (79).

The most well known copper resistance mechanism found in some species of *Pseudomonas* is the *copABCD* operon, a copper binding and sequestering system homologous to that of the *pcoABCD* operon of *Escherichia coli*. The *copABCD* operon had been first been extensively characterized in *Pseudomonas syringae* (81) where it was shown that strains containing these four genes accumulated copper in the periplasm and outer membrane as a mechanism to prevent it entering the cytoplasm (82). Furthermore, after the identification of the *copABCD* operon, a two-component regulatory system for the *cop* operon was described by (74) as *copRS* that was expressed in the same orientation but under a separate promoter. A *copABCD* system has also been described in *Pseudomonas* putida (75), and a *copABCD/RS* system in *P. fluorescens* (83) after sequence homologues were identified in genome sequencing. In *P. aereuginosa* the *copAB* genes are described as class 2 copper binding proteins, and similarly the regulatory proteins CopR and CopS have been described in detail and are classified as class 1 proteins in the PGD, but there is no evidence of *CopCD* genes in *P. aeruginosa* (79).

P. aeruginosa exposure to (and even absence) copper undoubtedly effects the expression of other lesser known and studied transport, and resistance proteins, such as active efflux systems or binding proteins, that may even share roles with other heavy metals. To date the most extensive study of copper response in *P. aeruginosa* is by (79), whereby a number of genes were highlighted as being differentially expressed under copper ‘adapted’ and copper ‘shocked’ conditions, such as the probable P-type ATPase PA3920.

1.9.2. Cobalamin (vitamin B_{12})

Most prokaryotes require the structurally complex cobalamin (vitamin B_{12}) as a cofactor for a variety of enzymes important in the metabolism of amino acids and nucleotides (84). However despite its importance, only some bacterial species are able to synthesize the compound (85), while many others rely on obtaining it from the environment through cellular membrane transport, such as *E. coli* . *P. aeruginosa* is one organism that has both the ability to take up cobalamin from the environment via the active TonB transport system, while also having the ability to synthesize the compound through a complex aerobic pathway involving around 30 enzymes (86), most of which have not been characterized in detail and can be viewed in the PGD and the accompanying PseudoCyc pathway viewer (87).

Outer membrane porins are inadequate for the facilitation of transport of bulky molecules that are present at low concentrations in the environment (88) (such as cobalamins), and unlike the passive diffusion mechanism of porins, TonB-dependent transporters (TBDTs) are active transporters that promote the transport of rare compounds, and are especially known for their high affinity uptake of iron complexes, and Vitamin B_{12} (89). TBDTs are found expressed throughout Gram-negative bacteria, and as such a number have been characterized in detail (90-92). Not all bacteria express the same TBDTs and this is evident with the comparison of the eight that are encoded in *E. coli* (seven for ferric chelators and one for Vitamin B_{12} (90)), and the 34 that are expressed in *P. aeruginosa* (93). The TBDT of particular interest is the transporter BtuB that is a known transporter of vitamin B_{12} and has been characterized very well in *E. coli* (84, 88, 94), and is highly conserved across Gram-negative bacteria (85). In *P. aeruginosa* the protein PA1271 is a class 3 protein in the PGD and is described as a probable vitamin B_{12} transporter through gene ontology (GO) descriptions. When the protein sequence of PA1271 is BLAST aligned with the *E. coli* Btub protein sequence, 32% sequence identity is achieved carrying an expect value of 4e-77, indicating a high confidence sequence match. PA1271 is therefore a protein of interest in any vitamin B_{12} studies in *P. aeruginosa.*

1.10. Hypotheses and aims

The research presented in this thesis tests the hypothesis that upon exposure to elevated levels of copper or cobalamin micronutrients, *P. aeruginosa* gene expression will change resulting in the differential expression of the proteome, especially the membrane proteome. The proteomic data developed from this project will contribute towards the functional characterization of poorly annotated proteins.

The specific aims of the project are:

1. Characterise the proteomic profile of the membrane sub-proteome of *Pseudomonas aeruginosa* PAO1 using mass spectrometry.
2. Identify proteins that are regulated by growth in high levels of copper and vitamin B_{12}.
3. Contribute data towards the functional annotation of poorly characterized or hypothetically labeled *P. aeruginosa* PAO1 proteins.

Chapter 2: Materials and Methods

2.1. Strains

The strains of *Pseudomonas aeruginosa* used in this study were as follows: PAO1, PA14, and the following 8 strains derived from the PA14 non-redundant transposon insertion mutant set (Harvard Medical School, Boston, MA. (95)): PA14_62390, PA14_17000, PA14_37790, PA14_32270, PA14_03700, PA14_13170, PA14_19490, and PA14_27840.

2.2. Cultivation

All equipment, media, and water (milliQ) were subjected to sterilization by autoclaving (121ºC, and 15psi for 20 minutes) prior to experimental work.

The *P. aeruginosa* strains were grown on Luria Broth (LB) agar plates (NaCl 10g/L, tryptone 10g/L, yeast extract 2.5g/L, and agar 15g/L), and in M9 liquid medium (11.28g/L 1x M9 salts (Sigma Aldrich), 0.24g/L $MgSO_4$, 0.01g/L $CaCl_2$, and 3.6g/L). Cultures were taken from -80ºC stock storage and depending on requirements were either streaked for isolated colonies onto LB plates and grown for 18-24 hours at 37ºC, or cultured into 10mL of M9 and grown for 14-16 hours at 37ºC and 200rpm agitation. These cultures were used for the basis of further experimental work.

2.3. Growth assay

2.3.1. Growth curves

The flask growth study using PAO1 was conducted in 250mL conical flasks with 50mL M9 cultures (1/100 dilution) in triplicate with three conditions: 50µM $CuSO_4$ ($CuSO_4.5H_2O$ ≥98%, Sigma Aldrich), 1µM vitamin B_{12} (≥98%, Sigma Aldrich), or M9 control. Cultures were subjected to 37ºC and 150rpm agitation, with optical density readings (OD_{600}) taking hourly from the fourth hour of growth until the twenty-first hour of growth.

The $CuSO_4$ PA14, and PA14 mutant growth study was conducted in a 340µL deep-well microtitre plate with the strains PA14, PA14_32270, PA14_17000, PA14_37790, PA14_03700, PA14_19490, PA14_27840, and PA14_62390 grown in triplicate (1/100 dilution) in 100µL M9 and

$CuSO_4$ at 50µM, as well as controls for each strain without the $CuSO_4$. Incubation was conducted under 37ºC at 200rpm agitation and optical density (OD_{600}) readings were taken every 15 minutes for 24 hours.

2.3.2. Disc diffusion assay

Transposon insertion mutants PA14_62390, PA14_17000, PA14_37790, PA14_32270, PA14_03700, PA14_13170, PA14_19490, and PA14_27840, and reference strains PAO1, and PA14 were subjected to a $CuSO_4$ disc diffusion plate assay. Bio-Rad filter papers were cut into circular discs (12.0mm x 3.0mm) and were soaked in $CuSO_4$ concentrations of 1.5mM, 2.5mM, and 50mM for 1 hour. Spread plates were prepared as described by (96) for each strain in triplicate. Each plate was dispensed a filter disc of 1.5mM, 2.5mM, and 50mM, and the plates were placed into 37ºC for 24 hours. Annular radii of inhibition surrounding each disc was measured and recorded for each plate.

2.4. Protein concentration – bicinchoninic acid assay

Bicinchoninic acid assay (BCA) protein assay reagent kit supplied by Pierce™ (cat: 23225) was used to quantitate protein concentrations of the proteomic samples. Bovine serum albumin protein standards in the concentration ranges of 0-2mg/mL were used for the generation of standard curves of which sample protein concentrations were calculated from. Protein standards, blanks, and samples were assayed in duplicate.

2.5. SDS-PAGE

1D-SDS-PAGE gels were used to separate proteins based upon molecular weight. 17µL of protein samples suspended in triethylammonium bicarbonate buffer (TEAB) + 1% sodium deoxycholate, 2µL loading buffer (NuPAGE® LDS sample buffer 4x), and 1µL (500µM) dithiothreitol (DTT) were loaded into NuPAGE® 12-well 4-12% Bis-Tris protein gels, and were run at 200V for 30 minutes with 1x 3-(N-morpholino)propanesulfonic acid (MOPS) running buffer. Molecular marker used was the Precision Plus Protein™ standard (Bio-Rad).

Post-gel staining involved washing the gel with fixing solution (50% methanol, 10% acetic acid) for 5 minutes, followed by staining overnight

(6-8 hours) with coomassie 4:1 coomassie diluent solution. Following the overnight staining the gel was incubated with fixing solution for 15 minutes and lastly washed with milliQ water for 5 minutes.

2.6. Sample preparation

Sample preparation was adapted from the methods of Molloy (2008). Growth of PAO1 was conducted in 400mL (1/100 dilution) of M9 in 2L flasks at 37ºC and 150rpm. PAO1 was grown under one of the following three conditions each in triplicate: 50µM $CuSO_4$, 1µM vitamin B_{12}, or M9 control.

Each culture was harvested at mid-log growth by pelleting the medium via centrifugation at 3500g at 4ºC for eight minutes. The supernatant was discarded and the pellet was washed with 1x phosphate buffered saline (PBS), followed by centrifugation at 3900g at 4ºC for eight minutes, and pellet collection in a 2mL screw cap tube. Ice-cold 1x PBS was added to the tubes (500-700µL), as well as protease inhibitor (Roche), and benzonase (Sigma Aldrich). Three cycles of bead beating was performed to lyse the cells using acid-washed 180µm (16-25 U.S. sieve) glass beads (Sigma Aldrich) at intensity 4.5 for 10 seconds with intermittent cooling on ice for 10 minutes between each cycle. Centrifugation of samples was then performed at 2500g at 4ºC for eight minutes and a supernatant from each was collected and retained.

Sodium carbonate based membrane protein extraction was performed on a portion of supernatant from each of the samples. The supernatants to be used were pooled for each of the triplicates and were added to 33mL of ice-cold 100mM sodium carbonate and were placed on a rocker in 4ºC for one hour. The solutions then underwent ultracentrifugation at 115,000g at 4ºC for one hour, after which the supernatant was discarded. 1x PBS was then added to the tubes and the ultracentrifugation procedure was repeated. Reconstitution of the pellet was performed with 60µL of 100mM TEAB buffer with 1% sodium deoxycholate followed by three cycles of vortex and sonication in a water bath for 15 minutes at 4ºC, putting the tubes on ice intermittently. The solution was then placed into 2mL protein low-bind tubes.

In-solution digestion of proteins was performed for membrane and soluble fractions. Reduction and alkylation of cysteine residues was carried out by

incubation with 10mM of DTT at 60ºC for 30 minutes, followed by incubation with 20mM of Iodoacetamide at room temperature, in the dark for 20 minutes. Trypsin was added at a 1:50 ratio of trypsin to protein and left to incubate at 37ºC overnight. 100% formic acid is then added to the samples at a final concentration of 2% formic acid to precipitate out the sodium deoxycholate, that is then subsequently removed, and finally the samples are freeze-dried at -20ºC for mass spectrometry.

2.7. Mass spectrometry

All mass spectrometry runs were performed using a TripleTOF 6600 mass spectrometer with an ekspert™ nanoLC400 system with cHipLC system (SCIEX). Peptide retention occurred through a reverse phase 200µm x 0.5mm cHiPLC® trap column (3µm, 120Å pore size C18-CL), and a 15cm x 200µm cHiPLC® analytical column (3µm, 120Å pore size C18-CL). Freeze-dried tryptic peptide samples were re-suspended in solvent A (2% acetonitrile, 0.1% formic acid). Re-suspension in solvent A allows the hydrophobic sections of the peptides to bind to a reverse-phase column. 3µg (10µL) of sample was injected onto the trap using an auto-sampler, and peptide elution from the reverse-phase column was achieved with solution A, and solution B (95% acetonitrile, 0.1% formic acid) 600µL/min elution gradient over a 140-minute chromatographic runtime. The elution gradient was as follows: from 5% solvent B to 40% solvent B within 120 min, from 40% B to 85% B within 2 min, 85% for 5 min and 5% B for 10 min. Electrospray ionization was used at a spray voltage of 2500V to produce positively charged ions for MS/MS.

2.7.1. IDA

Spectra were collected for the 20 most intense precursor ions (with charge state +2 to +4) across the *m/z* range of 350-1500 (accumulation time 250ms). To reduce redundant precursor selection, a dynamic exclusion of 30 seconds was used. MS/MS spectra were collected from 100-1800 *m/z* with 100ms accumulation time.

Spectra were searched against a *P. aeruginosa* whole protein dataset derived from the PGD (12-08-14) using the ProteinPilot™ software 5.0 (SCIEX). Proteins and peptides were accepted with a protein false discovery rate of <1% . Carbamidomethyl was set as a fixed modification.

2.7.2. SWATH-MS

All precursor ions were selected for fragmentation within each of 100 variable windows (figure 5) covering a *m/z* range of 400-1250 within a total cycle time of 3.1 seconds. Collision energies were calculated for a doubly (+2) charged species with a m/z of lowest mass in window + 10% window size. MS/MS spectra were collected across the *m/z* range of 350-1500 with a 35ms accumulation time

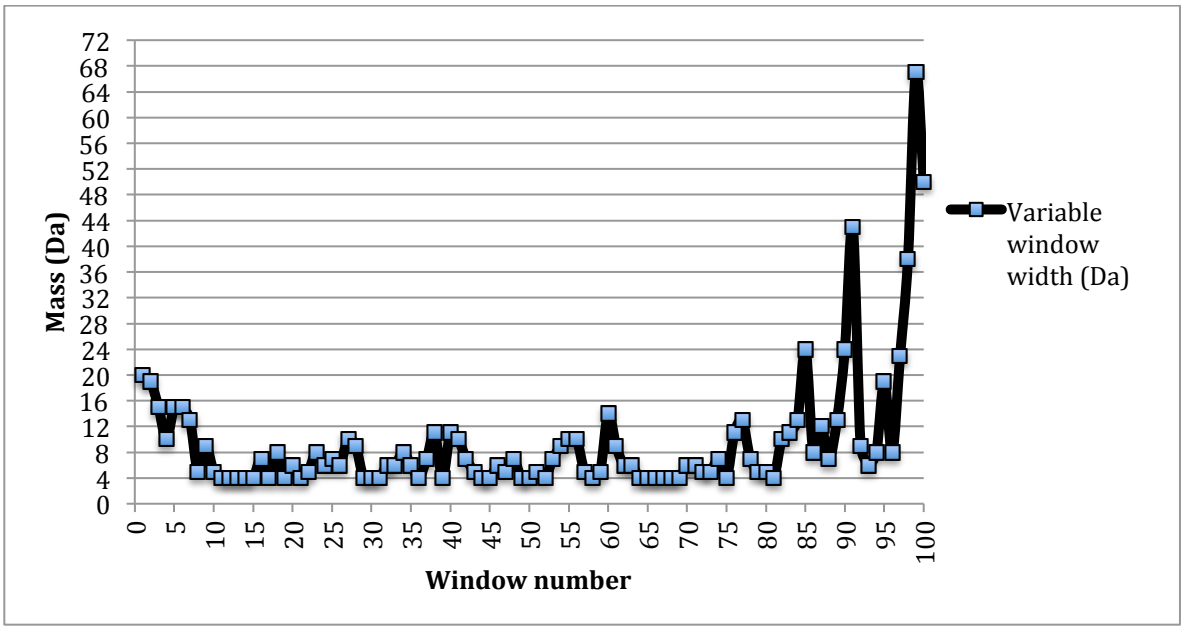


Figure 5: SWATH-MS variable windows (100) across the 400-1250 *m/z* range.

2.8. Data analysis

SWATH-MS data was extracted using Peakview v2.1 with SWATH Micro-App v2.0 (SCIEX). A spectral library generated from the membrane, and soluble fraction IDA runs was used to extract information from the SWATH-MS runs. Data processing in Peakview v2.1 was as follows: number of peptides per protein – max. 100, number of transitions per peptide – 6, peptide confidence level – 99%, transition false discovery rate <1%, 10-minute extraction window and fragment extraction tolerance of 75ppm.

Peakview v2.1 results were loaded into and processed in Perseus v1.5.2.6 (Max Planck Institute of Biochemistry). Peak area data (area under the curve) across all samples and proteins were normalized (division with grouping's calculated median), and $\log_2$ transformed. Student's t-test (two-tailed, homoscedastic) was performed in Perseus to obtain a p-value to determine statistical significance. Peak area was averaged between

triplicates for individual proteins, and conditions (M9, $CuSO_4$, and vitamin B_{12}) and fold change of proteins were calculated between vitamin B_{12} and M9, and $CuSO_4$ and M9 conditions. Additional analysis was performed in Microsoft Excel (Microsoft) using data from the *Pseudomonas* genome database and data exported from the Perseus analysis, which included clusters of orthologous groups, product class and subcellular localization analysis.

2.9. Polymerase chain reaction confirmation of transposon insertion sites

Transposon insertion confirmation was performed on each of the eight transposon deletion mutants and PA14 wild-type by way of arbitrary polymerase chain reaction (PCR) (see figure 6) as described and recommended by the creators of the PA14 mutant library (95) (see: http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/productionmethods.cgi). The primers were obtained from Integrated DNA Technologies (IA, USA) and were as follows:

ARB1D (5'-GGCCAGGCCTGCAGATGATGNNNNNNNNNNGTAT-3')

PMFLGM.GB-3a, (5'-TACAGTTTACGAACCGAACAGGC-3')

ARB2A (5'-GGCCAGGCCTGCAGATGATG-3')

PMFLGM.GB-2a (5'-TGTCAACTGGGTTCGTGCCTTCATCCG-3')

PMFLGM.GB-4a (5'-GACCGAGATAGGGTTGAGTG-3')

3µL of cell lysis used as template for PCR. 1st round PCR (1ng/µL ARB1D, 1ng/µL PMFLGM.GB-3a, 1.25U *Taq* polymerase, 1x *Taq* buffer, and 2.5µM dNTPs)- 95ºC for 30 seconds (x30), followed by 47ºC for 45 seconds (x30), then 72ºC for one minute (x30), with a five minute plate/tube incubation at 72ºC for extension of PCR products at the end of the procedure. 2nd round PCR (1ng/µL ARB2A, 1ng/µL PMFLGM.GB-2a, 1x *Taq* buffer, and 2.5µM dNTPs) - 95ºC for 30 seconds (x40), followed by 45ºC for 30 seconds (x40), then 72ºC for one minute (x40), with a five minute plate/tube incubation at 72ºC for extension of PCR products at the end of the procedure. PCR reagents: *Taq* DNA polymerase with standard *Taq* buffer, and dNTP solution mix were obtained from New England Biolabs (MA, USA).

The procedure was attempted on three occasions, the first following the methods described above (derived from *Liberati, et al*). The second attempt at the procedure involved commencing the first PCR reaction with 3µL of cell suspension (not cell lysis), and the third attempt going a step further by using a higher concentration of primers: ARB1D 0.5µM, PMFLGM.GB-3a 0.3µM, ARB2A 0.3µM, and PMFLGM.GB-2a 0.3µM.

PCR product clean-up was performed with the ISOLATE II PCR and gel kit (Bioline) on the second PCR reaction products. Products stored from PCR reaction one, and the cleaned-up PCR products of reaction two were run on a 1% agarose gel (2µL GelRed for 50µL agarose gel) for one hour at 100V. Cleaned-up PCR final products were quantified using the NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific). Finally the PMFLGM.GB-4a sequencing primer was added to the final products and then sent for sequencing at the Australian Genome Research Facility (Melbourne, Australia).

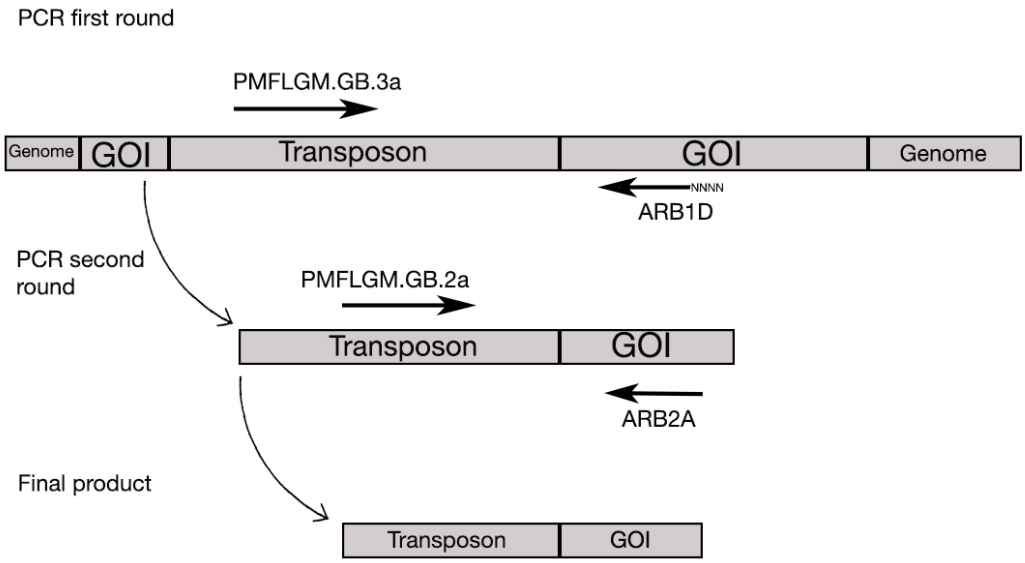


Figure 6: PA14 mutant Arbitrary PCR. The gene of interest (GOI) is interrupted by the transposon (MAR2xT7). In the 1st PCR reaction, ARB1D (arbitrary primer) anneals to many sites along the genome with the help of a low annealing temperature (97). PMFLGM.GB-3a primer anneals specifically to the transposon. The 1st round products are used as templates for the 2nd round of PCR with the aim of amplifying the GOI/transposon hybrid. The product of the 2nd round is used for DNA sequencing in order to determine the GOI.

Chapter 3: Results

In the 15 years since sequencing the *P. aeruginosa* PAO1 genome, there has been a significant increase in the number of genes annotated as product confidence level class 1 and class 2, with the gene products in the class 1 group increasing by 683, and class 2 by 131 (figure 7). However, figure 7 also highlights that many gene products are yet to undergo the same level of annotation, many of which may be of biological and clinical importance and remain functionally uncharacterized. Class 4 is illustrative of this as the gene products with this annotation represent the largest group (39% of all expected gene products) and the proteins are all functionally uncharacterized, being hypothetical or with no known similarity by sequence.

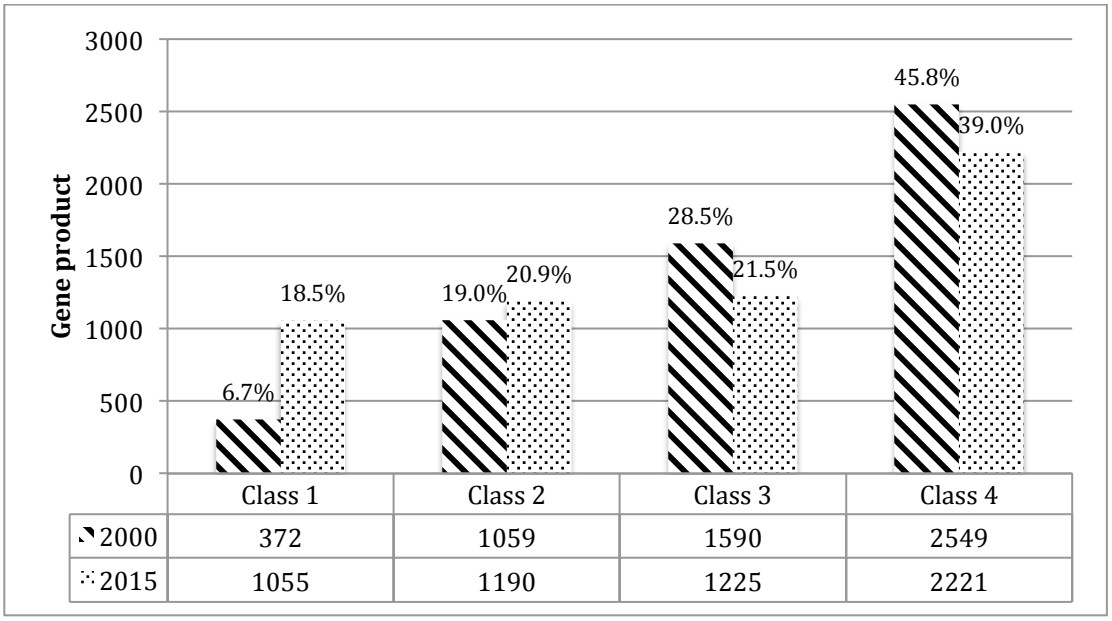


Figure 7: Gene product confidence levels in 2000 (data published by Stover, *et al* (2000)) to the levels now observed in 2015 (data extracted from http://beta.pseudomonas.com (69) (7th of August 2015)). Product name confidence surmises the degree of confidence in the function of a particular protein (38). Pseudomonas.com defines the classes as follows: class 1 proteins have been functionally demonstrated in *P. aeruginosa*, class 2 – function of a highly similar gene demonstrated in another organism, class 3 – function proposed based on amino acid or structural motif, and class 4 relates to previously reported genes of unknown function, or no similarity. The percentage at the top of each bar refer to the proportion of the genome that each class comprises for the described year.

3.1. PAO1 flask growth study

PAO1 was grown in M9 minimal media in three independent experiments to examine the growth response to copper and vitamin B_{12} (Figure 8). The MIC could not be determined using the plate based assay as the high copper concentrations were poorly soluble. Instead, an elevated copper sulfate concentration of 50 μM was used as this did not cause cell death, yet was sufficiently increased which would lead to altered expression of copper regulated genes.

The 50μM copper sulfate supplemented growth study exhibited a statistically significant (p-value <0.05) lag in hourly growth compared to M9 control from hour four until hour 14, whereas the 1μM vitamin B_{12} supplemented growth study experienced a statistically significant (p-value <0.05) increase in hourly growth compared to the M9 growth study from approximately hour 14 until hour 21 (table 8 supplementary information).

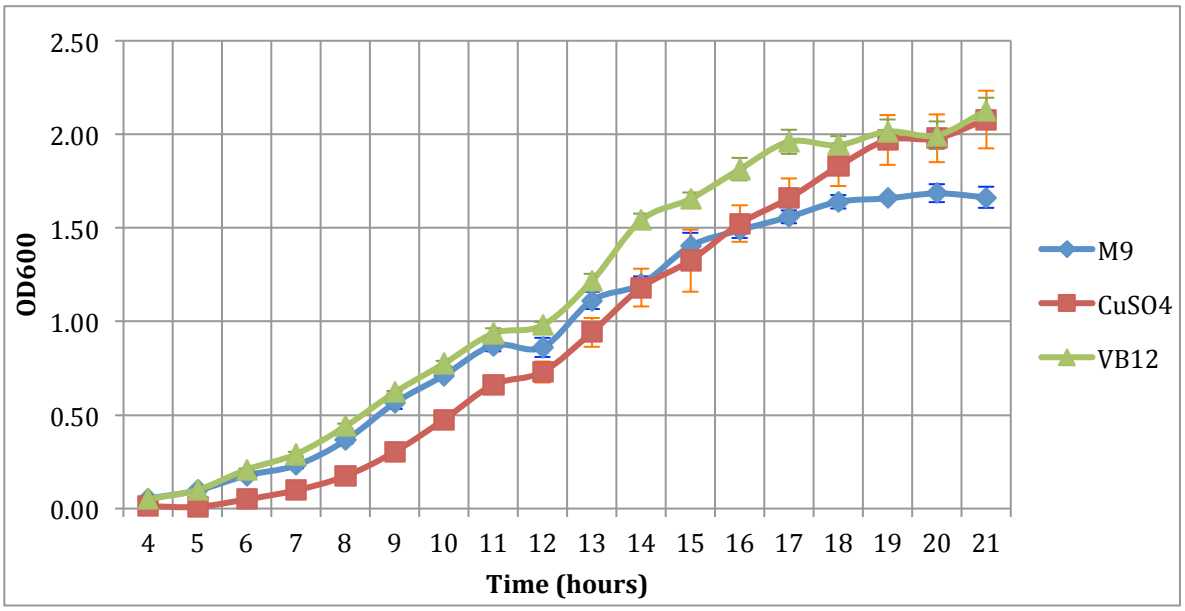


Figure 8: PAO1 growth in $CuSO_4$ and vitamin B_{12} (VB12). The growth study involved PAO1 grown in three different conditions: M9 medium, M9 medium with 50μM $CuSO_4$ addition, M9 medium with 1μM vitamin B_{12} addition. OD_{600} spectrophotometric readings were taken hourly from hour 4 of growth until hour 21.

3.2. PAO1 Proteomics experiment ($CuSO_4$, Vitamin B_{12}, and Wild-type)

3.2.1. TEAB and SDS membrane extraction gel, and membrane protein BCA assay

The M9, $CuSO_4$, and vitamin B_{12} whole cell lysate samples had the membrane proteins isolated via the sodium carbonate extraction protocol. Optimal re-suspension of the membrane pellet had to be achieved and therefore two detergent based re-suspension solutions were tested (TEAB + 1% sodium deoxycholate, and TEAB + 10% SDS). The re-suspended membrane pellets and whole cell lysates were visualized after 1D-SDS-PAGE separation (figure 9).

The TEAB + 1% sodium deoxycholate membrane protein suspension lane (lane 2) displayed the clearer more defined bands over the 10% SDS membrane protein suspension lane (lane “SDS”), and the whole cell lysate samples (lanes “Cu”, “M9”, and “Vb”) also displayed clear and defined separation. A BCA assay was performed on the TEAB + 1%DC and the 10% SDS membrane protein suspension, with TEAB + 1%DC recording a protein concentration of 0.304µg/µL, and the 10% SDS sample recording 0.302µg/µL.

After the pellets were re-suspended, a BCA assay was performed on the membrane protein samples to quantitate and assess protein recovery from the whole cell lysates. These are displayed in tables 9 and 10, (supplementary information) which indicate successful membrane protein extraction across all conditions and replicates with microgram (µg) amounts well sufficient for multiple IDA and SWATH-MS mass spectrometry analyses.

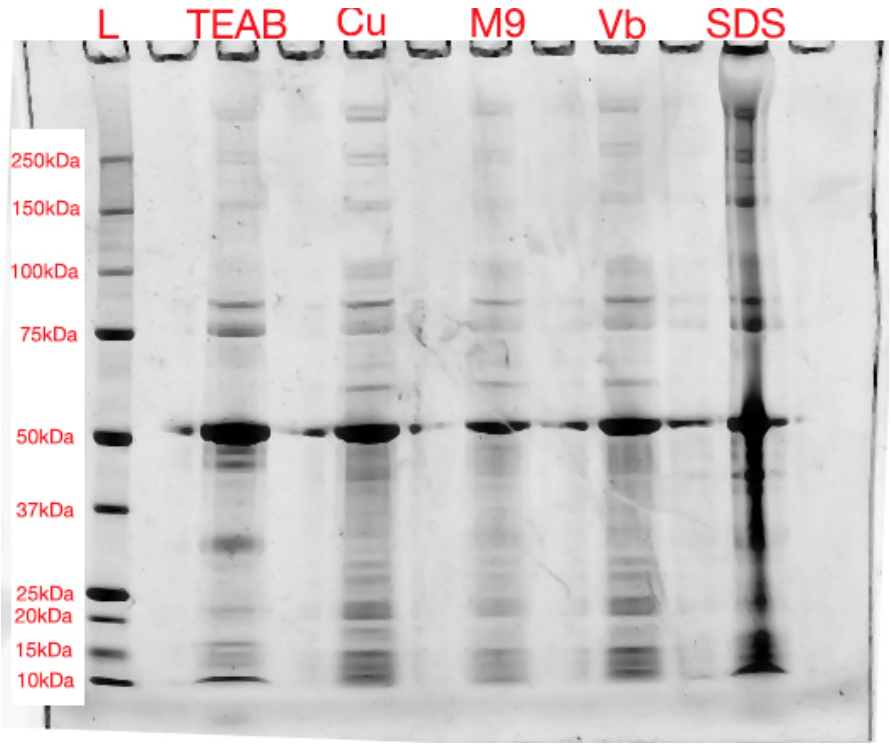


Figure 9: 1D-SDS-PAGE gel used for the assessment of the effectiveness of membrane pellet re-suspension, and for assessment of whole cell lysate protein sample quality. "L" contains the Precision Plus Protein™ standard marker (Bio-Rad), "TEAB" contains TEAB + 1% sodium deoxycholate membrane protein suspension, "Cu", "M9", and "Vb" are representative samples of $CuSO_4$, M9, and vitamin B_{12} whole cell lysate protein samples, and "SDS" contains 10% SDS membrane protein suspension.

3.2.2. IDA (spectral library): membrane fractions

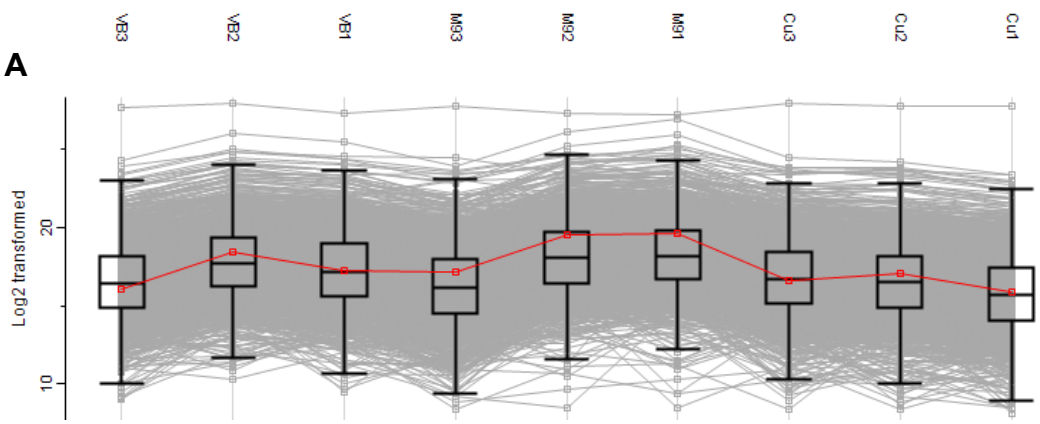
140 minute IDA runs performed on a nanoLC-MS/MS TripleTOF 6600 mass spectrometer across the membrane fractions: M9 (triplicate), $CuSO_4$ (triplicate), and vitamin B_{12} (triplicate) samples provided a combined identification of 1304 proteins at a <1% false discovery rate (FDR), and 11793 distinct peptides at a <1% FDR.

Using the PAO1 protein localization information contained within the PGD (as of May 2015), 40.0% (522) of the 1304 protein groups identified for the spectral library had either been predicted or confirmed to be localized within the periplasmic, outer, or cytoplasmic membranes, or had an unknown localization (could not be predicted by PsortB3.0 – a subcellular localization prediction tool used by the PGD (98)). When excluding the proteins with an unknown localization, 26.8% (349) of the 1304 protein groups have been predicted or confirmed to have localization within the membranes.

3.2.3. SWATH-MS: membrane fractions

To compare protein abundance levels across samples (M9, $CuSO_4$, and vitamin B_{12}) SWATH-MS was carried out. The datasets were mined using the membrane fraction spectral library resulting in the identification of 1216 unique proteins. Protein expression was normalized and log2 transformed as shown in figure 10.

Protein expression levels of the M9 fraction were compared to the copper sulfate, and vitamin B_{12} fractions. 104 proteins in the $CuSO_4$ fraction displayed differing abundance levels when compared to the M9 fraction with a p-value cutoff of 0.05. 57 proteins in the vitamin B_{12} fraction displayed differing expression when compared to the M9 fraction with a p-value cutoff of 0.05, and similarly when the stringency was lowered to a p-value cutoff of 0.01, 32 proteins in the $CuSO_4$ fraction and 10 proteins in the vitamin B_{12} fraction with statistically significant differential expression was observed. These statistically significant differentially expressed proteins (p-values <0.05 and <0.01) are visualized in figures 11-12 for vitamin B_{12} and the $CuSO_4$ samples. These protein lists were refined further when protein fold change was restricted to 1.5 or greater, p-value to <0.01, and localization within the membrane (either predicted or confirmed according to PGD). This reduced the $CuSO_4$ list to 15 proteins, and the vitamin B_{12} list to three proteins. These proteins are tabulated in table 3.



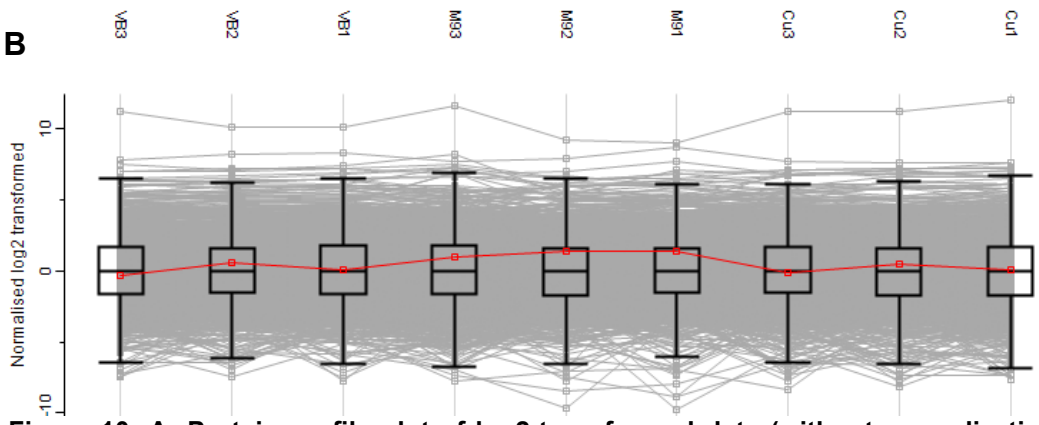


Figure 10: A: Protein profile plot of log2-transformed data (without normalization) across all samples, and B: Protein profile plot of normalized log2-transformed data across all samples. VB1, 2, 3 are vitamin B_{12} replicates 1, 2, and 3, M91, 2, 3 are M9 replicates 1, 2, and 3, and Cu1, 2, 3 are $CuSO_4$ replicates 1, 2, and 3. Each grey line represents one protein and can be tracked across all the samples. The red line is a protein (PA5312) that has been highlighted as an example. The box plots are used as a visual tool to assess the distribution of the data across all samples.

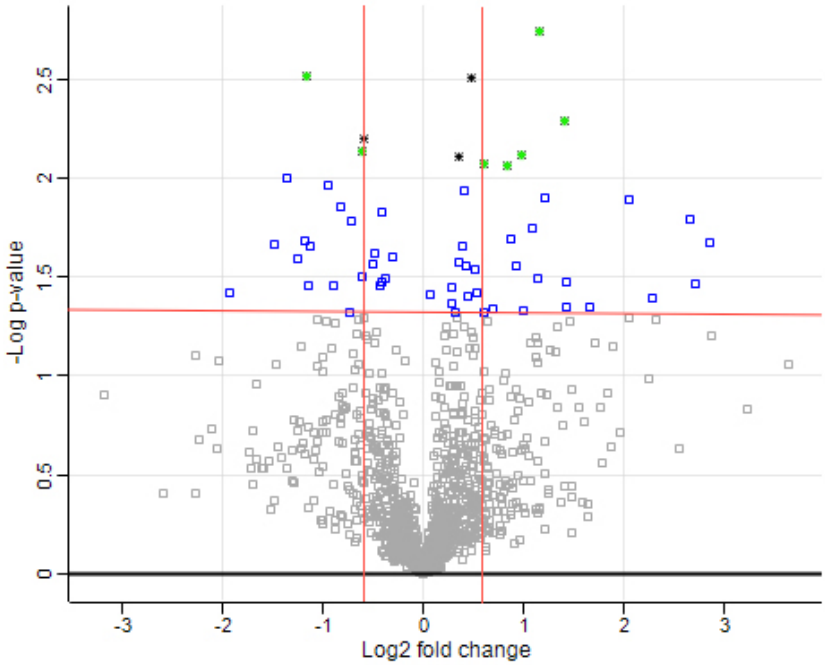


Figure 11: Volcano plot of the –log p-value versus the log2 fold change of the vitamin B_{12}/M9 samples. Each square or star represents one of the 1216 proteins. Blue squares represent proteins that are below the 0.05 p-value threshold, and the black stars represent proteins that are below the 0.01 p-value threshold. Green star indicate proteins that are below the 0.01 p-value threshold and have a fold change >+/-1.5. The horizontal red line indicates the 0.05 p-value cutoff point, and vertical red lines indicate 1.5 fold change.

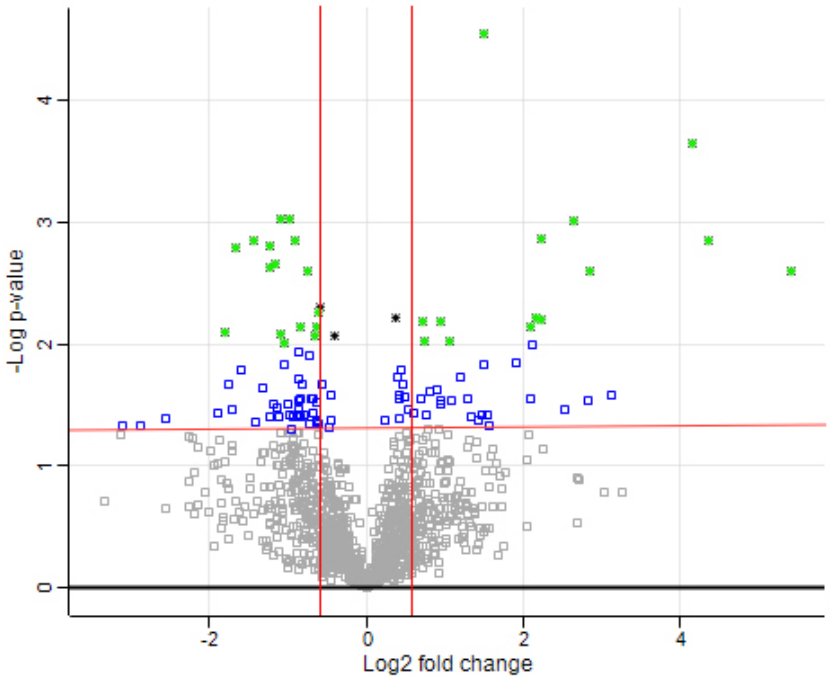


Figure 12: Volcano plot of the –log p-value versus the log2 fold change of the $CuSO_4$/M9 samples. Each square or star represents one of the 1216 proteins. Blue squares represent proteins that are below the 0.05 p-value threshold, and the black stars represent proteins that are below the 0.01 p-value threshold. Green star indicate proteins that are below the 0.01 p-value threshold and have a fold change >+/-1.5. The horizontal red line indicates the 0.05 p-value cutoff point, and vertical red lines indicates 1.5 fold change.

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Table 3: SWATH-MS acquired membrane proteins for the membrane fractions of $CuSO_4$, vitamin B_{12}, and M9 samples. Proteins listed here had a fold change >+/- 1.5 and a p-value of <0.01 with accompanying unknown or membrane localization (predicted or confirmed according to PGD). Proteins expressed in the $CuSO_4$ or vitamin B_{12} samples were compared against the M9 control to determine fold changes for each protein.

Protein	Fold change (+/-)	p-value	Peptides	Product Name	Subcellular Localization (localization [confidence code])¶	Product name confidence
PA3351	20.66	0.001	1	FlgM	Extracellular [Class 3]	Class 1
PA2505	17 .85	0.000	13	tyrosine porin OpdT	Outer Membrane [Class 2]; Outer Membrane Vesicle [Class 1]	Class 1
PA4492	4.67	0.006	1	MagA	Unknown [Class 3]; Cytoplasmic Membrane [Class 1]	Class 1
PA2064	7.21	0.003	9	copper resistance protein B precursor	Outer Membrane [Class 3]; Outer Membrane Vesicle [Class 1]	Class 2
PA2520	4.26	0.007	1	Resistance-Nodulation-Cell Division (RND) divalent metal cation efflux transporter CzcA	Cytoplasmic Membrane [Class 2]	Class 2
PA3746	-2.7	0.001	2	signal recognition particle protein Ffh	Cytoplasmic Membrane [Class 3]	Class 2
PA3920	6.22	0.001	12	probable metal transporting P-type ATPase	Cytoplasmic Membrane [Class 3]	Class 3
PA5021	2.83	0.000	1	probable sodium/hydrogen antiporter	Cytoplasmic Membrane [Class 3]	Class 3
PA3042	-2.14	0.001	3	hypothetical protein	Unknown [Class 3]	Class 4
PA2542	42.58	0.003	1	conserved hypothetical protein	Outer Membrane [Class 3]	Class 4
PA3412	4.47	0.006	1	hypothetical protein	Unknown [Class 3]	Class 4
PA2345	-2.35	0.002	6	conserved hypothetical protein	Unknown (This protein may have multiple localization sites) [Class 3]	Class 4
PA3952	-2.35	0.002	2	hypothetical protein	Unknown [Class 3]	Class 4
PA3040	-2.21	0.002	4	conserved hypothetical protein	Unknown [Class 3]; Outer Membrane Vesicle [Class 1]	Class 4
PA3730	1.67	0.009	1	hypothetical protein	Cytoplasmic Membrane [Class 3]	Class 4
PA2647*	1.98	0.008	1	NADH dehydrogenase I chain L	Cytoplasmic Membrane [Class 3]	Class 2
PA4431*	2.65	0.005	3	probable iron-sulfur protein	Cytoplasmic Membrane [Class 3]; Outer Membrane Vesicle [Class 1]	Class 3
PA4320*	1.52	0.008	3	hypothetical protein	Cytoplasmic Membrane [Class 3]	Class 4

* Vitamin B_{12} differentially regulated proteins.

¶: Class 1**:** Subcellular localization experimentally demonstrated in *Pseudomonas aeruginosa*. Class 2**:** Subcellular localization of highly similar gene experimentally demonstrated in another organism OR to a paralog experimentally demonstrated in the same organism. BLAST expect value of 10e-10 for query within 80-120% of subject length. Class 3**:** Subcellular localization computationally predicted by PSORTb (98).

3.2.4. IDA (spectral library): soluble fraction

140 minute IDA runs performed on a nanoLC-MS/MS tripleTOF 6600 across the soluble fractions: M9 (triplicate), $CuSO_4$ (triplicate), and vitamin B_{12} (triplicate) samples provided at a combined identification of 1731 proteins, and 17549 distinct peptides both at a FDR of <1%.

3.2.5. SWATH-MS: soluble fraction

Identification and relative quantitation of 1591 proteins from the PAO1 soluble fraction was achieved using SWATH acquisition and data extraction using the PAO1 whole cell spectral library generated in the IDA run prior. 17 proteins were identified as differentially expressed in the vitamin B_{12} sample when compared to the M9 control at a significance level of <0.01, and a fold change exclusion of >1.5. Similarly, 57 proteins were differentially expressed in the $CuSO_4$ sample when compared to the M9 control also at a significance of <0.01, and fold change exclusion of >1.5 (data not displayed).

3.2.6 Membrane fraction and soluble fraction results comparison

The membrane fraction and soluble fraction proteomics results were compared and contrasted, which provided additional information to confidently select interesting candidate proteins for further functional analysis.

Proteins identified from the membrane and soluble fractions were compared to a list of 1690 proteins obtained from the PGD (14-08-15) that comprised of proteins that have either confirmed or predicted sub-cellular localization in *P. aeruginosa* membranes. Further comparisons of the membrane and soluble fractions included identifying the number of shared and unique proteins between both sample sets and assigning of cluster of orthologous groups (COG) functional annotations.

Comparing both sample sets identified 807 proteins shared between the two, leaving 409 proteins unique to the membrane fraction and 784 unique to the soluble fraction (figure 13 and table 4) and leaving the combined number of unique proteins identified in both SWATH-MS runs to be exactly 2000. The membrane fraction contained a comparatively larger amount of membrane proteins than that of the soluble fraction (38.9% compared to 20.3%) (table 4). COG category assignment and assessment was performed on each protein and visualization of COG category distribution

can be seen in figure 14. The most striking differences in COG function distribution are seen in the unique membrane and unique soluble proteins lists. Finally, the 2000 non-redundant proteins identified from both SWATH-MS experiments were analyzed for their respective class assignments (per the PGD). Results are tabulated in table 5.

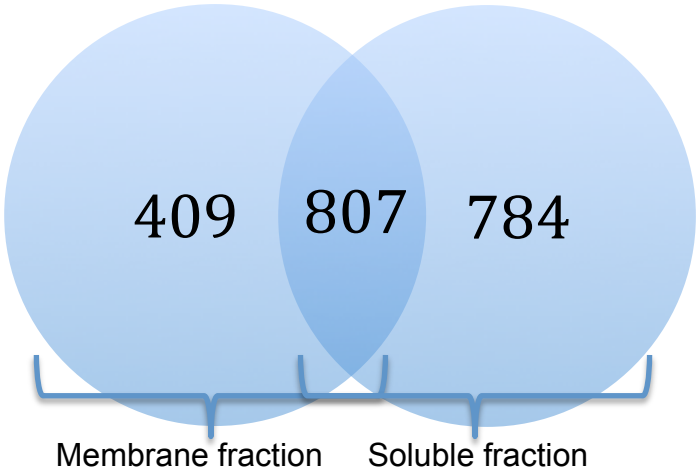

Figure 13: Venn diagram of shared and unique proteins for the soluble and membrane fractions. The membrane fraction had a sum of 1216 proteins whereas the soluble fraction had a sum of 1591 proteins, with a combined unique protein list of 2000.

Table 4: Shared, unique, and membrane proteins of the soluble and membrane fractions. 1591 and 1216 were the total number of quantifiable proteins identified in the soluble and membrane fractions respectively. There were 590 non-redundant membrane proteins identified.

	Soluble fraction	Membrane fraction
Proteins	1591	1216
Mem. Proteins	323 (20.3%)†	473 (38.9%)†
Shared	807 (50.7%)*	807 (66.4%)*
Mem. Proteins	206 (25.5%)†	206 (25.5%)†
Unique	784 (49.3%)*	409 (33.6%)*
Mem. Proteins	117 (14.9%)†	267 (65.3%)†

†: Percentage of membrane proteins found in that specific grouping.

*: Percentage of proteins to that of the soluble or membrane fraction.

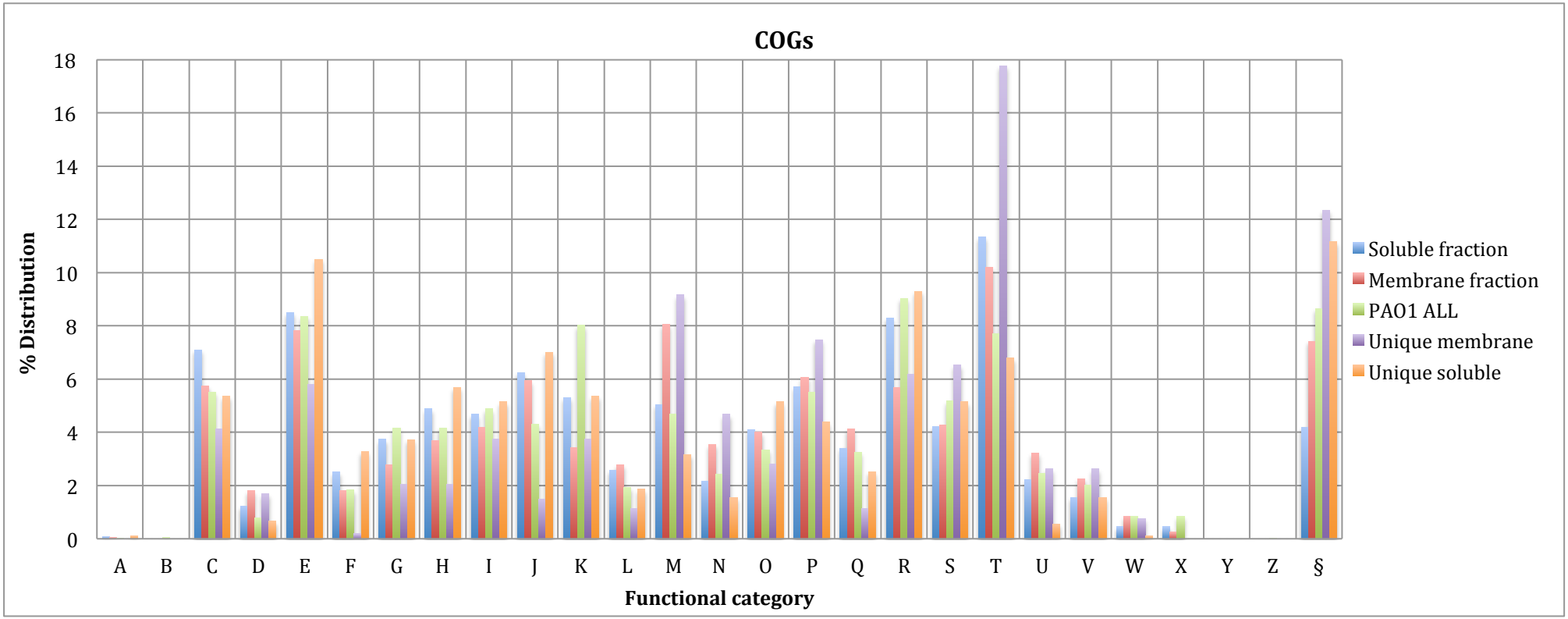

Figure 14: **COG functional categories used to represent major biological functions. This distribution map highlights the COG % distribution for all proteins in the soluble and membrane fractions and their respective unique proteins (unique soluble, and unique membrane), and also contains the % distribution for 6519 proteins of PAO1 (derived from the PGD).** The COG functional categories are: **A** – RNA processing and modification, **B** – chromatin structure and dynamics, **C** – energy production and dynamics, **D** – cell cycle control, cell division, and chromatin partitioning, **E** – amino acid transport and metabolism, **F** – nucleotide transport and metabolism, **G** – carbohydrate transport and metabolism, **H** – coenzyme transport and metabolism, **I** – lipid transport and metabolism, **J** – translation, ribosomal structure, and biogenesis, **K** – transcription, **L** – replication, repair, and recombination, **M** – cell wall/membrane/envelope biogenesis, **N** – cell motility, **O** – post-translational modification, protein turnover, and chaperones, **P** – inorganic ion transport, **Q** – secondary metabolite biosynthesis, transport, and catabolism, **R** – general function prediction only, **S** – function unknown, **T** – signal transduction mechanisms, **U** – intracellular trafficking, secretion, and vesicular transport, **V** – defense mechanisms, **W** – extracellular structures, **X** – mobilome: prophages, and transposons, **Y** – nuclear structure, **Z** – cytoskeleton, and **§** – no COG assignment.

Table 5: Product confidence level (class) groupings for the non-redundant SWATH-MS protein list. Classes are as follows as described by the PGD: class 1 proteins have been functionally demonstrated in *P. aeruginosa*, class 2 – function of a highly similar gene demonstrated in another organism, class 3 – function proposed based on amino acid or structural motif, and class 4 relates to previously reported genes of unknown function, or no similarity.

Class	Proteins
1	449
2	590
3	315
4	644
Not assigned	2
Total	2000

3.3. Proteins of interest

Based on analysis of the SWATH-MS data from cells grown in vitamin B_{12} and $CuSO_4$, 15 differentially expressed proteins were selected for further study. These 15 proteins were chosen because they had one (or more) of the following characteristics: a large abundance fold change, unusual or interesting putative function, predicted to have a function related to $CuSO_4$ or vitamin B_{12}, or was part of a group of proteins whose genes are of the p-value <0.01. Table 6 contains the list of 15 proteins. Proteins from this list were ultimately the targets for functional annotation, and eight were chosen on the basis of transposon deletion mutant availability, and are listed in table 6. Extracted ion chromatograms (XICs) for the eight proteins for the copper and M9 samples are shown in the supplementary information (figure 19) where selectivity, and intensity changes can be visualized.

Table 6: List of 15 proteins from both membrane and soluble fractions that are desirable candidates for further study. The table is separated into two sections: the first for proteins found in the membrane fraction, and the second for proteins found in the soluble fraction. * Indicates proteins found in both fractions. All proteins were found differentially expressed in $CuSO_4$ compared to the M9 control unless indicated with §, whereby these proteins were differentially expressed in vitamin B_{12}.

Membrane	Peptides	Fold change (+/-)	Subcellular Localization (localization [confidence code])	Product name confidence	Description
PA2505*¶	13	17.0	Outer Membrane [Class 3]; Outer Membrane Vesicle [Class 1]	Class 1	opdT - Tyrosine porin
PA2064*	9	7.2	Outer Membrane [Class 3]; Outer Membrane Vesicle [Class 1]	Class 2	copB - copper resistance protein precursor B
PA3920*¶	12	6.2	Cytoplasmic Membrane [Class 3]	Class 3	probable metal transporting P-type ATPase
PA2542	1	42.0	Outer Membrane [Class 3]	Class 4	hypothetical protein
PA3040	4	-2.2	Unknown [Class 3]; Outer Membrane Vesicle [Class 1]	Class 4	hypothetical protein
PA3042	3	-2.1	Unknown [Class 3]	Class 4	hypothetical protein
Soluble	**Peptides**	**Fold change (+/-)**	**Subcellular Localization (localization [confidence code])**	**Product name confidence**	**Description**
PA2505*¶	10	25.0	Outer Membrane [Class 3]; Outer Membrane Vesicle [Class 1]	Class 1	opdT - Tyrosine porin
PA2064*	6	10.0	Outer Membrane [Class 3]; Outer Membrane Vesicle [Class 1]	Class 2	copB - copper resistance protein precursor B
PA1272§	1	-6.3	Cytoplasmic [Class 2]	Class 2	cob(I)alamin adenosyltransferase
PA2065¶	8	4.4	Periplasmic [Class 2]; Periplasmic [Class 3]; Outer Membrane Vesicle [Class 1]	Class 2	copA - copper resistance protein precursor A
PA0283¶	10	3.0	Periplasmic [Class 2]; Periplasmic [Class 3]	Class 2	sulfate binding protein precursor
PA3450¶	6	3.0	Cytoplasmic [Class 3]	Class 3	probable antioxidant protein
PA3920*¶	2	4.0	Cytoplasmic Membrane [Class 3]	Class 3	probable metal transporting P-type ATPase
PA3661¶	2	62.9	Unknown [Class 3]	Class 4	hypothetical protein
PA4714¶	5	16.0	Unknown (This protein may have multiple localization sites) [Class 3]; Unknown (This protein may have multiple localization sites) [Class 3]	Class 4	hypothetical protein
PA2807¶	4	10.0	Unknown [Class 3]	Class 4	hypothetical protein
PA3445	3	6.2	Unknown (This protein may have multiple localization sites) [Class 3]; Unknown (This protein may have multiple localization sites) [Class 3]	Class 4	hypothetical protein
PA2204§	18	3.0	Periplasmic [Class 2]; Periplasmic [Class 3]	Class 4	hypothetical protein

¶: Indicates a protein chosen for further functional annotation.

The (eight) proteins in **bold** were proteins of interest elected as candidates for the functional characterization studies.

3.4. Protein functional annotations

Functional characterization of the eight $CuSO_4$ proteins of interest (table 6) was attempted through a growth assay, and a disc diffusion assay both conducted with *P. aeruginosa* PA14. The reason for conducting these functional characterization studies in PA14 as opposed to PAO1 is simply because the PAO1 transposon insertion mutant library was not available at the time, PA14 was, and because of the very close genetic relatedness of PA14 to PAO1 (>95% similar (95)), most genes between PAO1 and PA14 are close orthologs.

3.4.1. PA14 copper sulfate growth study

A growth assay was conducted, with 7 of the 8 PA14 transposon deletion mutants being able to grow in the M9 control growth medium ($CuSO_4$ absence). Results were obtained for the PA14 wild-type, and mutants PA14_32270 (PA2505), PA14_17000 (PA3661), PA14_37790 (PA2065), PA14_03700 (PA0283), PA14_19490 (PA3450), PA14_27840 (PA2807), and PA14_62390 (PA4714) (growth was retarded/very poor in the PA14_13170 (PA3920) M9 inoculating culture and was subsequently not used). At mid-log growth the 5-point smoothed averages (five adjacent means averaged to give one value) of each deletion strain and the wild-type were compared for abundance differences. The wild-type displayed no statistically significant difference in growth at mid-log when exposed to 50μM $CuSO_4$ (p-value 0.929) (figure 15). A similar trend was seen across most deletion mutants when measuring their growth in M9 with copper stress versus growth in M9, as well as when compared to the wild-type strain results. However one deletion mutant, PA14_17000 (PA3661), a hypothetical protein, provided unusual results when at mid-log, that is, the mutant produced the same markedly increased growth when copper stressed and when grown in just M9, when compared to that of the wild type in both conditions (p-values <0.05). Figure 16 shows the PA14_17000 (PA3661) PA14 mutant growth curve. Standard error is reported on figures 15 and 16, and as seen in the $CuSO_4$ growth assays there was large fluctuations in standard error indicating poor precision in the estimation of the sample's mean, and this is seen in $CuSO_4$ growth assays for the other 6 mutants studied (supplementary information). It was

found that these fluctuations were attributed to the unavoidable interactions the $CuSO_4$ was producing when added in M9 producing small precipitate crystals. Agitation of the solution at 150-200rpm re-dissolves the precipitate at larger solution volumes, however with the scale needed to facilitate this, and the large number of strains, the resources required could not be sourced in the time-frame.

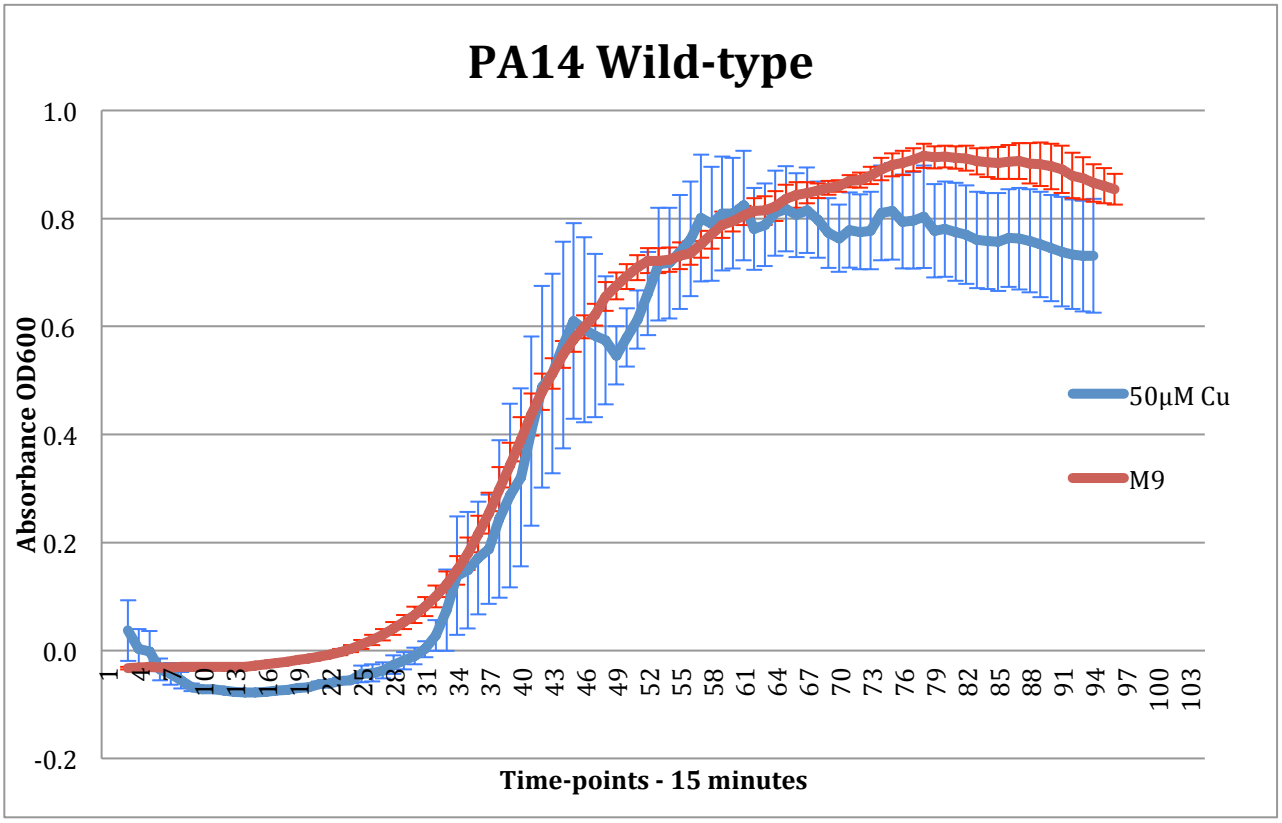

Figure 15: PA14 wild-type 24-hour growth assay. The two conditions measured were the PA14 wild-type in M9 medium, and PA14 wild-type in M9 medium with 50µM $CuSO_4$. A reading was taken every 15 minutes for 24 hours (96 in total) at OD_{600}, and the data was 5 mean-smoothed, resulting in the first 2 data points being absent.

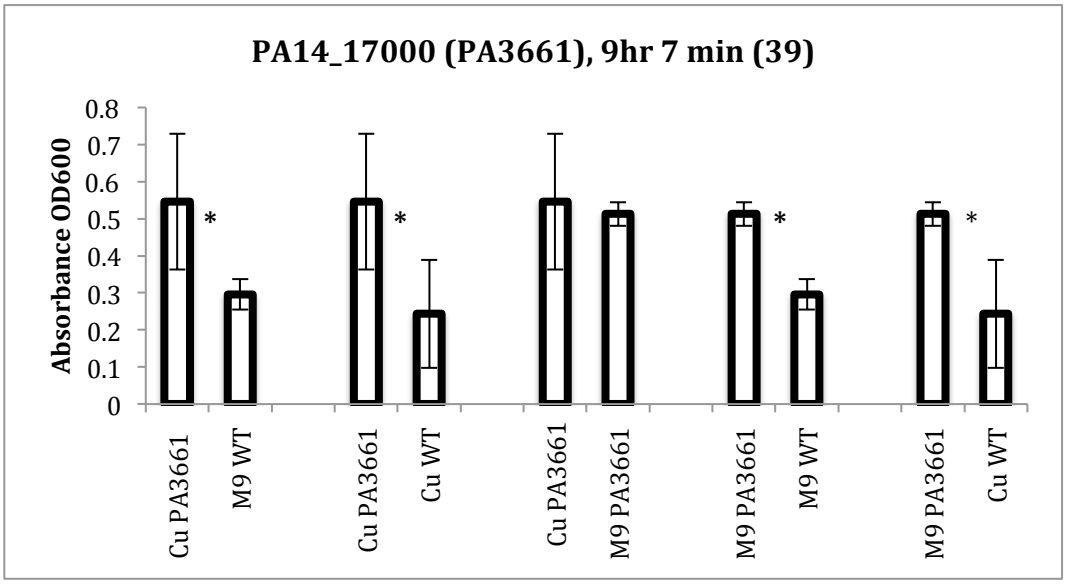

Figure 16: PA14 mutant PA14_17000 (PA3661) mid-log growth absorbance readings comparisons. The growth assays of PA14_17000 (PA3661) in $CuSO_4$, and M9; and PA14 wild-type in M9, and $CuSO_4$ (data not shown) mid-log growth at time-point 39 (9 hours and 7 minutes) was visually and statistically compared. Statistical significance (p-value <0.05) is indicated by "*".

3.4.2. PA14 copper sulfate disc diffusion assay

All eight of the deletion mutants (PA14_13170 (PA3920), PA14_32270 (PA2505), PA14_17000 (PA3661), PA14_37790 (PA2065), PA14_03700 (PA0283), PA14_19490 (PA3450), PA14_27840 (2807), and PA14_62390 (PA4714), as well as PAO1 and PA14 were subjected to a disc diffusion assay and exhibited some level of growth inhibition on exposure to 1mM, 2.5mM, and 50mM $CuSO_4$ (table 7). Three of the mutant strains, PA14_37790 (PA2065), PA14_32270 (PA2505), and PA14_27840 (PA2807), exhibited a statistically significant (p-value <0.05) decrease in growth in the 50mM $CuSO_4$ growth assay when compared to PAO1 and PA14. On the other hand the mutant PA14_13170 (PA3920) had very large and visible zones of inhibition for all three $CuSO_4$ concentrations (1mM, 2.5mM, and 50mM), evidence of a reduction in ability to grow when exposed to high amounts of $CuSO_4$. Figure 17 depicts PA14_13170 disc diffusion assay, where zones of inhibition are clearly evident for $CuSO_4$ discs 1mM, 2.5mM, and 50mM.

Table 7: Disc diffusion assay results for $CuSO_4$ concentrations of 1mM, 2.5mM, and 50mM. Annular radii of inhibition for each concentration disc of each mutant (triplicate)

were statistically compared to that of PAO1 and PA14. PAO1 orthologous gene product identifier in parenthesis. Strains in **bold** displayed statistical significance (p-value <0.05).

	$CuSO_4$ Conc.				
Strain	1mM (cm)*	2.5mM (cm)*	50mM (cm)*	p-value PAO1$^{\Delta}$	p-value PA14$^{\Delta}$
PAO1	0.000	0.000	1.300	N/A	0.609
PA14	0.000	0.000	1.233	0.609	N/A
PA14_62390 (PA4714)	0.000	0.000	1.033	0.065	0.055
PA14_17000 (PA3661)	0.000	0.000	1.267	0.768	0.678
PA14_37790 (PA2065)	0.000	0.000	0.833	0.018	0.013
PA14_32270 (PA2505)	0.000	0.000	0.867	0.015	0.008
PA14_03700 (PA0293)	0.000	0.000	1.167	0.275	0.422
PA14_13170 (PA3920)	0.967	1.200	1.967	0.003	0.001
PA14_19490 (PA3450)	0.000	0.000	1.267	0.768	0.678
PA14_27840 (PA2807)	0.000	0.000	0.800	0.012	0.008

*: Annular radius (cm) from the edge of each disc was measured up until the edge of confluent growth.
Δ: p-value for the 50mM assays

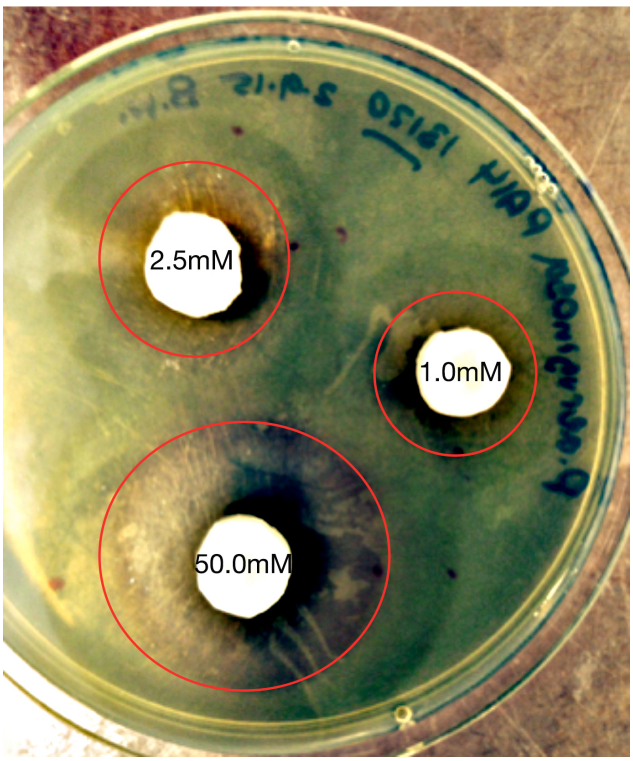

Figure 17: PA14_13170 (PA3920) $CuSO_4$ disc diffusion plate, replicate 1. Zones of inhibition can be seen around all discs. The red circles help show the boundary lines between no growth (within circles) and growth (outside circles).

3.4.3. PA14 mutant PCR confirmation

Attempts of confirmation of transposon insertion through arbitrary PCR was performed on all mutants as described by (95) and modified as per methods on subsequent attempts. Amplification of DNA by PCR was observed through agarose gel electrophoresis and NanoDrop (Thermo Scientific) DNA concentration assay. The agarose gels depicted what seemed to be appropriate amplification of the transposon-gene hybrid DNA products for each of the mutants, but on each occasion the negative control (PA14 wild-type) also showed a similar amplified product. In hindsight, a negative control with no template (just water would have been more appropriate in order to test for PCR amplicon contamination. Figure 18 displays visualization of both rounds of PCR for PA14_13170, PA14_27840, and the negative control – PA14 wild-type. Table 11 displays the results of the NanoDrop 2000 (Thermo Scientific) DNA concentration assay that provided confirmation that significant concentrations of pure DNA were found across all samples (supplementary information). The sequencing results (data not shown) of the cleaned-up round 2 PCR products provided no evidence of the anticipated PCR products for each of the mutants (or the PA14 wild-type) and instead provided non-specific sequences. Further experiments are required here but could not be carried out due to time limitations.

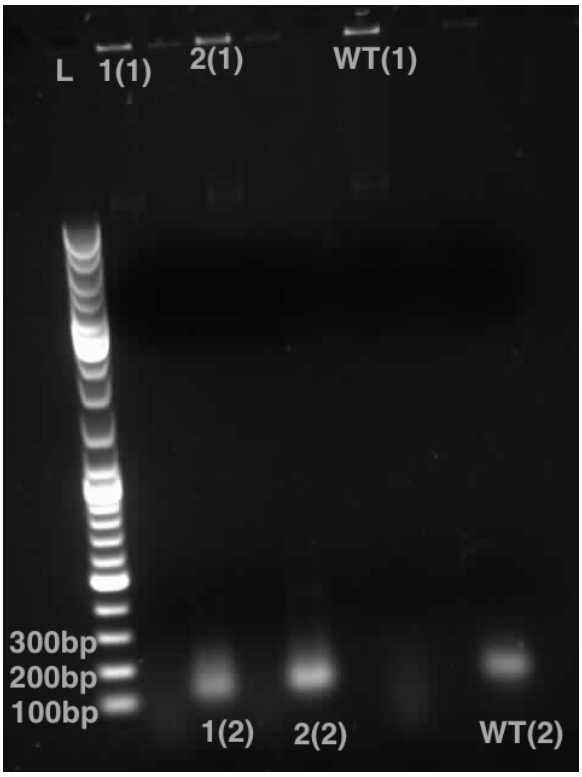

Figure 18: 1% agarose gel of PCR round 1 and 2 products with mutants PA14_13170 and PA14_27840, and PA14 wild-type. L: 1Kb Plus DNA ladder (Thermo Fischer Scientific), 1(1): PA14_13170 PCR round 1 product, 1(2): mutant PA14_13170 PCR round 2 product, 2(1): PA14_27840 PCR round 1 product, 2(2): PA14_27840 PCR round 2 product, WT(1): PA14 wild-type PCR round 1 product, and WT(2): PA14 PCR round 2 product. A product of approximate 150bp can be seen for PCR round 2 products of both mutants, and the wild-type (negative control).

Chapter 4: Discussion

The bioinformatics analysis had revealed a large proportion (81.4% - classes 2, 3, and 4) of PAO1 proteins lacking experimentally validated functional annotations within PAO1 despite the 15 years since the sequencing of the genome (22). This study provided a proteomic map of PAO1 that was created through the quantitative identification of 2000 non-redundant proteins by SWATH-MS analysis that was then narrowed down to eight proteins for functional annotation (PA2505, PA3920, PA2065, PA0283, PA3450, PA3661, PA4714, and PA2807). PA14 gene knockout strains of each protein's gene were employed to study the organism's response to copper using $CuSO_4$ growth and disc diffusion assays. The results enabled the narrowing down of functions for the proteins PA2505, PA2807, PA3920, and PA2065. The strongest results in this study were for the protein PA3920, which was confidently identified as a Cu^{2+} type IB ATPase. Lastly, it is fairly likely this study proved the existence of approximately 341 proteins, which is still yet to be confirmed through deeper analysis with the PGD. However from the list of proteins in table 6, PA3661, PA3445, PA4714, and PA2807 were for the first time confirmed to be translated. The following sections provide deeper explanations, and discussions for the highlighted results.

4.1. Bioinformatic analysis reveals a large number of PAO1 gene products remain poorly described

A defining and important feature to this study was the ability to draw upon readily available, detailed, and up-to-date proteomic and genomic information that was stored within the *Pseudomonas* Genome Database. Extraction and analysis of gene product information of PAO1 contained within the database allowed a complete understanding of the extent to which gene products in PAO1 were annotated.

One aspect of annotation in the PGD is the product name confidence (referred to as class) assignments. Proteins with a class 1 annotation would generally not be of interest in protein identification and function studies, however as in the case of this study, it is still important to record any expression profiles and characteristics of these proteins in case their

recorded annotation does not match or is beyond what is observed in the current study. A mismatch in this sense may indicate that an extension or revision of the protein's current annotation may be in order. The other three classes a protein can be assigned to in the PGD are class 2, 3 or 4. Proteins under any of these classes are clearly lacking in experimentally verified annotation in PAO1 and any PAO1 studies focussing on proteins within these classes would likely be novel and beneficial.

Despite the 15 years since the sequencing of the PAO1 genome (22), there has not been a dramatic shift in the number of class 3 and class 4 proteins that have been functionally categorized (class 3 – 28.5% to 21.5%, and class 4 – 45.8% to 39.0%) and this is highlighted in figure 7. The larger comparative increase in proteins with the class 1 annotation (6.7% to 18.5%) is not representative of the proteomic mapping studies listed in table 2 whose primary aims were to provide evidence of translation rather than detailed characterization. The increase is due to the number of studies published after the sequencing project that were dedicated to functionally annotating proteins of interest identified primarily through sequence similarity to other notable proteins or protein groups across different strains and species. Annotation updates can be found on the protein's descriptions within the PGD.

Creating a proteome map of an organism is important for a number of reasons: the first and most obvious is that it bridges the sequenced genome of an organism to its proteome, secondly it can provide insight into the current biological state of the organism at a cellular or subcellular level, thirdly when coupled with quantitative strategies can provide greater understanding into cellular activity, fourthly conclusions can be drawn comparatively between proteomic maps of different organisms/strains/serotypes of which would have been otherwise difficult or impossible with just genomic information (e.g. expression profiles), and finally proteomic maps can serve as valuable sources of information for identifying candidate proteins for more targeted studies (e.g. functional annotation).

4.2. Growth differences in $CuSO_4$, and vitamin B_{12} supplemented cultures

4.2.1 Vitamin B_{12}

Lee K-M, *et* al. (2012) is the only study found in the literature that has studied the effects or role of vitamin B_{12} in *P. aeruginosa*. Their study focused on the impact of vitamin B_{12} on anaerobic PAO1 growth and biofilm development. They showed that addition of 1μM vitamin B_{12} enabled a dramatic increase in anaerobic growth. This trend was observed, albeit weakly in this aerobic growth study (figure 8, and table 9 (supplementary information)). The *P. aeruginosa* cobalamin biosynthesis pathway is thought to be O_2 dependent for a number of reasons. Firstly, it possesses similar genes to that of *Pseudomonas denitrificans* that are dependent on molecular oxygen (*cobG* (99) and *cobN* (86)) for the *de novo* synthesis of cobalamin, and secondly, Lee K-M, *et al*. (2012), provided results of vitamin B_{12} levels that were quantified in PAO1 under aerobic and anaerobic growth, and found that vitamin B_{12} was produced at very low levels. It can therefore be construed that PAO1 aerobic exposure to exogenous vitamin B_{12} will not have a dramatic impact on the growth of the organism, consistent with the findings in this study.

4.2.2 Copper

In terms of the impact on PAO1 growth under $CuSO_4$ addition, it was known that concentrations of copper above trace level amounts would have an adverse effect on PAO1 growth, and therefore this study needed to be conducted at a concentration of $CuSO_4$ below this threshold. Even though the MIC of $CuSO_4$ could not be determined the arbitrary concentration of 50μM used for this study was adequate enough to provide a good measure of copper-stress response (it has been stated that copper requirements for microorganisms are usually in the range of 1-10μM (80)). At the 50μM $CuSO_4$ concentration used for this study's growth assay a statistically significant (p-value <0.05) lag in growth was observed (Table 8), which is in line with results published by Gordon, *et al*. (1994) where it was shown that when micromolar levels of $CuSO_4$ are added to (exponentially) growing cultures of a number of heterotrophic bacteria

(including *P. aeruginosa*) there is a temporary inhibition of growth followed by recovery.

4.3. A combined total of 2000 non-redundant proteins were identified, many of which may be experimentally confirmed to be expressed for the first time

4.3.1. SWATH-MS, a success

The identification and quantification of 2000 unique proteins (across three conditions) in this study places this research into the top 3 PAO1 protein-mapping studies in terms of number of proteins identified, behind that of Herbst, *et al.* (2015) with 2801 identifications, and Kumari, *et al.* (2014) with 2965 identifications. Additionally, this study, along with the study by Herbst, *et al*. are the two highest ranking PAO1 studies in terms of number of proteins quantified. Considering both are the latest proteomic profiling studies to be released (both 2015) the bar has been set as for what is expected for future studies of this nature.

In this study quantification was performed using extracted ion chromatograms (XICs) derived directly from the mined SWATH maps, examples of which are displayed in figure 19 of the supplementary material. Examples of top ranking peptides from each of the eight candidate proteins (table 6) and their fragment ion traces are shown (figure 19) for comparison across the control (M9), and the copper samples. These highlight the quality of the XICs in their selectivity and intensity changes and ensures quality reporting of quantitative changes from area under the curve measurements.

The 2000 proteins identified in this study equate to approximately 36% of PAO1 total protein potential of which there are 5572 (69), and unlike the studies of Herbst, *et al.* and Kumari, *et al*. this study did not employ gel-fractionation (SDS-PAGE) as part of the sample preparation that would of otherwise enabled a larger group of protein identifications. The tedious nature of gel separation and extraction, as well as the use of SWATH-MS in which a high number of proteins were already expected to be identified are the reasons as to why gel fractionation was not opted for. These results highlight the power of SWATH-MS in enabling deep, label-free

quantitative proteomic coverage, and if available, the use of other (in-solution) fractionation techniques such as MudPIT (multidimensional protein identification technology), which involves separating the peptides via strong cation exchange (SCX) followed by reverse phase chromatography (101) would ideally have been an option in this study to increase the proteomic coverage even further. A study was performed by Krisp, *et al*. (2015) that directly confirms this, and they found that the use of multiphase LC-chips with integrated SCX and reverse phase chromatography systems improved the lower limits of detection, increased reproducibility, and consumed less samples, when used upstream of a targeted mass spectrometry workflow (such as SWATH-MS), over analyses without fractionation (102).

4.3.2. PA3661, PA3445, PA4714, and PA2807 confirmed to be translated

The 2000 unique proteins had been analyzed in regards to their PGD class assignment, and table 5 revealed that this study confirmed the expression of a combined total of 1549 class 2, 3, and 4 proteins. Many of these proteins will have already been experimentally confirmed to exist through the numerous proteomic mapping studies already conducted, however a portion of which for the first will be experimentally confirmed to exist. The significance of this research in confirming the existence of PAO1 proteins cannot be measured at this time as there is currently no function in the PGD to identify how many proteins of PAO1 are theoretical, but on individual protein searches it is noted whether there is evidence for the protein's translation, and therefore the sizable task of individually identifying which proteins from this group have previously been identified is yet to be performed. Searches had been performed of the proteins from table 6 to determine if there was any previous evidence of translation noted, and 4 of the 18 proteins listed there, PA3661, PA3445, PA4714, and PA2807, were all proteins confirmed to exist by this study. Furthermore, considering approximately 22% (4/18) of the proteins from table 6 had no experimental verification for existence, it can be roughly estimated that of the 1549 proteins of class 2, 3, and 4, approximately 341 (22%) proteins from within this group would for the first time be experimentally verified.

4.4. COG analysis of fractions, and the PAO1 proteome revealed expected trends

The COGs database is designed to categorise and group orthologous genes or orthologous groups of paralogs from three or more phylogenetic lineages (103, 104) into a single grouping (COG) representative of their orthologous relationship. There are 4631 COGs as of the 2014 update (105), all of which are classified into 26 (biological) functional classifications, and can be seen in figure 14.

COG analysis was performed (figure 14, table 4) on the membrane, and soluble fraction total proteins lists (1216, and 1591 respectively), as well the proteins classified unique from both fractions (409 from the membrane, and 784 from the soluble), and finally the PAO1 whole proteome. This was performed so that visual identification of protein bias towards certain major biological functions within the fractions could be observed, for example it would be expected in the membrane fraction to see a larger percentage distribution in the functional category of M – cell wall/membrane/envelope biogenesis compared to the soluble fraction and PAO1 whole proteome, and this is exactly what was seen. The inclusion of the soluble and membrane fraction unique proteins was because it was expected that an obvious bias towards certain categories would be observed from these two groups of proteins. This was certainly the case, with some very obvious percentage distribution differences in a number of categories (e.g. T, M, J, and K), allowing further confirmation that the soluble fraction and especially the membrane fraction were getting a good representation of proteins in line with what was expected to be seen.

4.5. SWATH-MS analysis – what was found?

4.5.1. SWATH-MS analysis of the membrane fraction revealed 18 proteins significantly differentially regulated (p-value <0.01, FC>1.5, membrane localization), and the soluble fraction 57 proteins (p-value <0.01, FC>1.5).

The 18 proteins listed in table 3 from the membrane fraction, 15 from the $CuSO_4$ samples, and three from the vitamin B_{12}, had their functional annotations extracted from the PGD to try and ascertain any relationships towards the micronutrients and their described or putative functions, if any

were available. The same was also done with the 57 proteins from the soluble fraction, the data for which were not shown for this study because of the group size, but it is worth to note that any proteins from table 3 that were absent from table 6, were derived from this group of 57 proteins. Functional annotations in the PGD are derived from any combination or availability of Gene Ontology reports (106), manual assignments from PseudoCAP participants, and functional predictions from the InterPro database that utilises 11 source databases (Pfam, PRINTS, PROSITE, ProDom, CATH-Gene3D, HAMAP, PANTHER, PIRSF, SMART, SUPERFAMILY, and TIGRFAMs) (107). These functional annotations can provide essential sources of validation for any results obtained from functional studies, that is, if the observed function of a protein aligns with the predicted functions ascribed from these tools then this adds an extra layer of validation. However for many proteins there lies no available or very basic functional description and are given the label of hypothetical, or conserved hypothetical in the case of those found in several phylogenetic lineages (108), and this can provide a hindrance towards functional annotation efforts. It was noted that many of the proteins from table 3 and table 6 were labeled hypothetical and thus would provide more of a challenge to functionally describe.

4.5.2. Eight proteins from a list of 15 proteins of interest were chosen for functional annotation

The proteins chosen for functional annotation in this study (PA2505, PA3920, PA2065, PA0283, PA3450, PA3661, PA4714, and PA2807) had satisfied the pre-requisite screens for significance, fold change, and in the membrane fraction's case, membrane subcellular localization. This group of eight proteins represented a wide variety of putative functions, as well as representing all of the four product name confidence classes, so the choice of proteins to describe from table 6 came down personal preference. The ideal situation would have been to choose all 15 proteins but the time and resources were not at hand at the time. PA2064, and potentially PA3040 were also strong candidates for functional characterization however the PA14 non-redundant transposon insertion library does not have transposon insertion mutants for these genes. The eight proteins elected for functional annotation assays were also proteins found differentially expressed within the copper supplemented cells only.

4.5.3. A note on the class 1 protein PA2505

The protein PA2505 was the only protein from the list of eight of interest with a product confidence class rating of 1. The functional annotation given to this protein is that of an OpdT tyrosine porin as reported by Tamber, *et al*. (2006). Because this protein was found significantly up regulated in both copper fractions (table 6), it was considered worth investigating, as it was believed its annotated function could be extended.

4.6. PA14 protein functional annotations - disc diffusion assay identified PA3920 as a protein important in copper resistance

The disc diffusion assay was deployed as an alternative functional assay. This assay was modeled after antibiotic susceptibility testing (96) in which diffusion discs carrying known concentrations of antibiotic are placed on a fresh bacterial spread plate and the resultant zones of inhibition around the discs establish the organism's sensitivity.

Inactivation of PA3920 had resulted in an extreme increase in sensitivity towards copper (table 7), a significant result as this protein is currently classified as class 3 by the PGD. The current putative function given to this protein is that it is a metal P-type ATPase, with GO biological processes in metal ion, and cation transport (GO:0030001, and GO:0006812), GO molecular functions in cation transporting ATPase activity, copper ion binding, and metal ion binding (GO:0019829, GO:0005507, and GO:0046872), and numerous functional predictions performed by InterPro highlighting heavy metal associated domains, P-type ATPase domains, and P-type cation transporting ATPase superfamily signatures.

(see:
http://www.pseudomonas.com/feature/show/?id=110660&view=functions)

Furthermore, between the amino acids 221 and 787 TIGRFAM functional prediction provided by the PGD had described the protein as a part of the P-type ATPase subfamily IB (TIGR01525) with a very low expect value of 8.3E-192. The type IB ATPases are known to contain families that actively transport Cu^{2+}, as well as other metal cations such as Cd^{2+}, and Zn^{2+} (110, 111). Additionally, type IB ATPases are described in their function to have two purposes, the first is to provide heavy metals required for cellular

function, and the second is to transport metal ions out of the cells when they reach toxic levels (112). This function of PA3920 can be further supported by the transcriptional profiling study by Tietzel, *et al*. (2006) whereby PA3920 was found to be transcriptionally up regulated in copper sensitive and copper adapted PAO1 cultures. The group also had PAO1 transposon insertion mutants to disrupt the PA3920 gene and when grown in copper the culture was found to be highly sensitive to the copper with respect to the control.

Based on the evidence presented in this study, as well as the study by Tietzel, *et al*. (2006), it would be a valid conclusion that the putative function of PA3920 described within the PGD, that is, its role as a Cu^{2+} type IB ATPase, can at this point be confirmed. In order to further solidify this conclusion in PAO1, studies of structure and copper uptake assays should be performed.

4.6.1. PA2807, PA2065, and PA2505

In regards to the proteins PA2807, and PA2065 whose sensitivity seemed to have decreased in regards to copper exposure (as shown by the reduced annular radii compared to the PAO1 and PA14 controls at 50mM $CuSO_4$ concentration (table 7)), in which both were found to be transcriptionally up-regulated in the presence of copper by Tietzel, *et al* (2006). That group also performed disc diffusion assays, similar to the study performed in this however they used a single disc with a $CuSO_4$ concentration of 500mM, and found that when these proteins are disrupted (via transposon insertion) PAO1 has increased susceptibility towards copper, however they did not see the same result in their MIC analysis, leaving the results unclear.

The hypothetical protein PA2807 is a class 4 22.5kDa protein with GO molecular function descriptions of copper binding and electron carrier activity (GO:0005507, GO:0009055), and in taking a deeper look into PA2065, it is a protein that is strongly suggested to be involved in copper binding and has strong sequence identity (72%) towards the CopA protein of a known copper oxidase and binding protein from *P. syringae* (81). Also PA2064 (downstream of PA2065) was one of the proteins identified as significantly up regulated in copper stressed cultures, and has been strongly inferred to have the same role as the CopB protein in *P. syringae*.

PA2064 also shares strong sequence identity to that of the CopB protein in *P. syringae* at 63%.

So what are we to take away from PA2807, and PA2065 (and PA2064)? Firstly, it is strongly likely that PA2807 has some role in copper binding or activity; therefore it can be deemed that its description as a hypothetical protein can be replaced with a putative function description based on this activity. More importantly there is now proof that PA2807 is translated (no previous indication in the PGD), which is an important verification before any more in-depth studies are conducted. In terms of PA2065 and PA2064 these proteins are currently classified as class 2, this work presented here is not likely to bump them into class 1 as of yet, but it does provide additional evidence to support their hypothesized functions as CopAB copper sequestering proteins, leaving these two proteins as strong candidates for a more personalized examination of function study.

As mentioned earlier, PA2505 was one the eight proteins functionally studied in this project. Its sole function described within the PGD is that of a tyrosine porin, but as it was seen to be strongly up regulated within this study (table 6) when exposed to $CuSO_4$, and therefore there was reason to believe its functional annotation could be extended. The knock-out PA2505 growth assay and disc diffusion did not identify increased susceptibility towards $CuSO_4$, however due to the protein already being identified as a porin, it can be hypothesized that due to the small nature of the copper ion, increased expression of PA2505 porins on the outer membrane help facilitate the diffusion of the copper cations out of the cell, reducing the work-load of other specialized transporters such as the PA3920 P-type ATPase. Further investigation into PA2505 would be ideal such as monitoring its expression under exposure to other metal cations to see if the same trend is observed, as well as further mass spectrometry profiling studies with the gene knock-out.

4.7. Study limitations

4.7.1. Transposon deletion mutant growth study results were unreliable

As mentioned previous (section 3.4.1.) the transposon insertion mutant growth study encountered the problem of precipitates in the medium

brought about by the $CuSO_4$ addition. This caused the results from the growth assays to show large variability, requiring caution needing to be taken in their interpretation. 5-mean smoothing was employed to minimise the variation across the data points, but despite this it remained difficult to identify significant differences between the mutants, and their respective controls that were grown in M9. PA3661 (PA14_17000) was the best growth curve visually (figure 15), and also provided at mid-log growth (9hr 7min) some statistically significant results (p-value <0.05, figure 16).

Little was known about PA3661, a small 12.3kDa hypothetical protein that had yet to be identified as being translated. The protein was identified in the soluble fraction with a very large (62.9) fold change in PAO1 under 50μM $CuSO_4$ addition, and being the largest statistically significant fold change of all proteins it was investigated, with the important finding being that this protein is in fact translated.

Unfortunately one of the proteins of higher focus in this study PA3920 could not be investigated in the growth study as the PA14 transposon insertion mutant PA14_13170 resulted in poor sub-culture's growth.

4.7.2. PCR transposon insertion confirmation troubles and possible solutions

PCR confirmation of transposon insertion in each of the mutants is essential to validating any and all results in the functional assays of this study. The method of arbitrary PCR was the method adapted and recommended by the PA14 mutant library creators (95). The method is quite ingenious in theory in that the use and combination of arbitrary primers and transposon specific primers in two PCR protocols would allow any mutant picked from the library to be screened using the same primers (see figure 6), as opposed to designing and using different primers specific for each gene of interest. The final PCR product would be a hybrid amplicon of a portion of the transposon and a portion of the gene adjacent to it, which only leaves DNA sequencing before the disrupted gene can be identified. Unfortunately this did not play out as planned and repeated attempts yielded what seemed to be good and amplified PCR products (figure 18) but in the end upon sequencing were not. An appearance of a band in the negative control of wild-type PA14 was an indication that there was a problem with the procedure. There is always the possibility that there was in-fact no transposon inserted into the genome, but in the

advent of the PA3920 results, it would be more appropriate to deduce that there was an error with the PCR instead.

There are many things that could have gone wrong in this experiment but the main ones are poor PCR reagent quality, DNA contamination, and arbitrary primers not suitably annealing. The first two potential problems were actively avoided in subsequent attempts, leaving the next most likely problem being with the arbitrary primers. The solution to this would be to either attempt to use different arbitrary primers and/or gradient PCR, before making the move to primers specific for the genes of interest.

4.8. Conclusion

4.8.1. Completion of aims and impact of research

There were 3 aims of this study; the first was to create a proteomic profile of the membrane sub-proteome of PAO1. The identification and quantitation of 590 unique membrane proteins whose sub-cellular localization had been confirmed or predicted (PGD localization ratings of 1, 2, or 3) to be in the membranes would classify as creating a substantial membrane protein profile of PAO1. Additionally many of the 1410 proteins not included in this grouping may in fact be localized within the membranes, but their localization has either not been demonstrated at this point in time or could not be predicted. The second aim was to identify differentially regulated proteins in PAO1 that were grown in copper, and vitamin B_{12}. This aim was also completed, and a sub-selection of these proteins was seen in tables 3 and 6. The final aim was to contribute at some level to the functional annotation of poorly characterized or hypothetical proteins. The measurement of protein expression levels facilitated through targeted mass spectrometry, the use of functional growth assays and the continual consulting with functional predictions available on the PGD has allowed for the confirmation, and narrowing-down of important roles of PA3920, and PA2065 (and PA2064), as well as PA2807, and PA2505 under copper exposure. Importantly, this study has also confirmed the existence of number of previously theoretical PAO1 proteins.

4.8.2. The road ahead

Despite the success of this study in providing new information for PAO1, further work is needed in regards to the functional growth assays with the aim of providing more accurate growth curves, and additionally PCR validation of the transposon insertions needs to be troubleshot and completed, and performing the work with a PAO1 transposon library is something to be looked at. Extending further into the future, there are still many other proteins listed in this study that would be interesting to functionally characterize, especially some proteins within the vitamin B_{12} work. Furthermore in regards to vitamin B_{12} studies, it would be ideal to create a proteomic profile of PAO1 under O_2 limiting, and vitamin B_{12} supplementation conditions to see if there is more of an effect on putative vitamin B_{12} transporters (e.g. PA1271), and the theorized cobalamin biosynthesis pathway in PAO1. Additionally an extension of the copper work could be to investigate other metal cations, one of special interest being iron. Finally, PAO1 despite being the archetypal reference strain is only one strain of many and is a strain of current clinical insignificance; therefore work in clinically relevant strains can be something to be looked at.

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Chapter 6: Supplementary information

6.1. PAO1 growth study

Table 8: PAO1 growth OD_{600} values. The average for the triplicate studies are displayed from hour 4 of growth until hour 21. M9 refers to PAO1 grown in M9 medium, $CuSO_4$ refers to PAO1 grown in M9 medium with addition of 50µM $CuSO_4$, and VB_{12} refers to PAO1 grown in M9 medium with addition of 1µM vitamin B_{12}. p-values for each time point are displayed, whereby a Student's t-test (two-tailed, homoscedastic) was performed to compare the copper sulfate OD_{600} values to the M9 OD_{600} values (M9/$CuSO_4$), and to compare the M9 OD_{600} values to the VB_{12} OD_{600} values (M9/VB_{12}).

	Growth condition			P-value	
Time (hours)	M9 (OD_{600})	$CuSO_4$ (OD_{600})	VB_{12} (OD_{600})	M9/$CuSO_4$	M9/VB_{12}
4	0.0540	0.0120	0.0503	**0.010**	0.691
5	0.0970	0.0093	0.1000	**0.004**	0.857
6	0.1773	0.0493	0.2090	**0.000**	**0.047**
7	0.2310	0.0977	0.2910	**0.003**	0.063
8	0.3640	0.1733	0.4393	**0.001**	**0.045**
9	0.5640	0.3033	0.6207	**0.003**	0.234
10	0.7117	0.4753	0.7747	**0.000**	**0.026**
11	0.8730	0.6617	0.9360	**0.014**	0.300
12	0.8617	0.7303	0.9797	0.093	0.151
13	1.1120	0.9420	1.2160	**0.038**	0.234
14	1.1990	1.1810	1.5407	0.121	**0.007**
15	1.4027	1.3253	1.6553	0.090	0.056
16	1.4880	1.5230	1.8133	0.387	**0.022**
17	1.5590	1.6583	1.9610	0.595	**0.011**
18	1.6397	1.8290	1.9427	0.366	**0.015**
19	1.6580	1.9697	2.0150	**0.001**	**0.012**
20	1.6847	1.9787	1.9953	0.169	**0.045**
21	1.6633	2.0800	2.1280	0.184	**0.013**

6.2. Whole cell lysate BCA assay

Table 9: PAO1 whole cell lysate BCA protein quantitation. The triplicate samples were abbreviated as: vitamin B_{12} replicate 1 – "VB1", vitamin B_{12} replicate 2 – "VB2", vitamin B_{12} replicate 3 – "VB3", $CuSO_4$ replicate 1 – "Cu1", $CuSO_4$ replicate 2 – "Cu2", $CuSO_4$ replicate 3 – "Cu3", M9 replicate 1 – "M91", M9 replicate 2 – "M92", and M9 replicate 3 – "M93". The whole cell lysate of each of the samples (M9, $CuSO_4$, and vitamin B_{12}) that is to be used to isolate the membrane proteins was relatively quantified using a BCA assay to ensure an adequate amount of protein to proceed with.

Sample	Volume (mL)	Concentration (µg/µL)	Sample amount (µg)
VB1	1.0	1.10	1100
VB2	1.0	1.18	1180
VB3	1.0	1.39	1390
Cu1	1.0	1.07	1070
Cu2	1.3	1.55	2015
Cu3	1.0	1.21	1210
M91	1.0	1.31	1310
M92	1.0	2.30	2500
M93	1.3	1.27	1651

6.3. Membrane protein BCA assay

Table 10: PAO1 membrane protein extract BCA assay. The triplicate samples were abbreviated as: vitamin B_{12} replicate 1 – "VB1", vitamin B_{12} replicate 2 – "VB2", vitamin B_{12} replicate 3 – "VB3", $CuSO_4$ replicate 1 – "Cu1", $CuSO_4$ replicate 2 – "Cu2", $CuSO_4$ replicate 3 – "Cu3", M9 replicate 1 – "M91", M9 replicate 2 – "M92", and M9 replicate 3 – "M93". The % recovery column describes the protein amounts (%) recovered from the whole cell lysate samples that were used for the membrane extraction (see table 9).

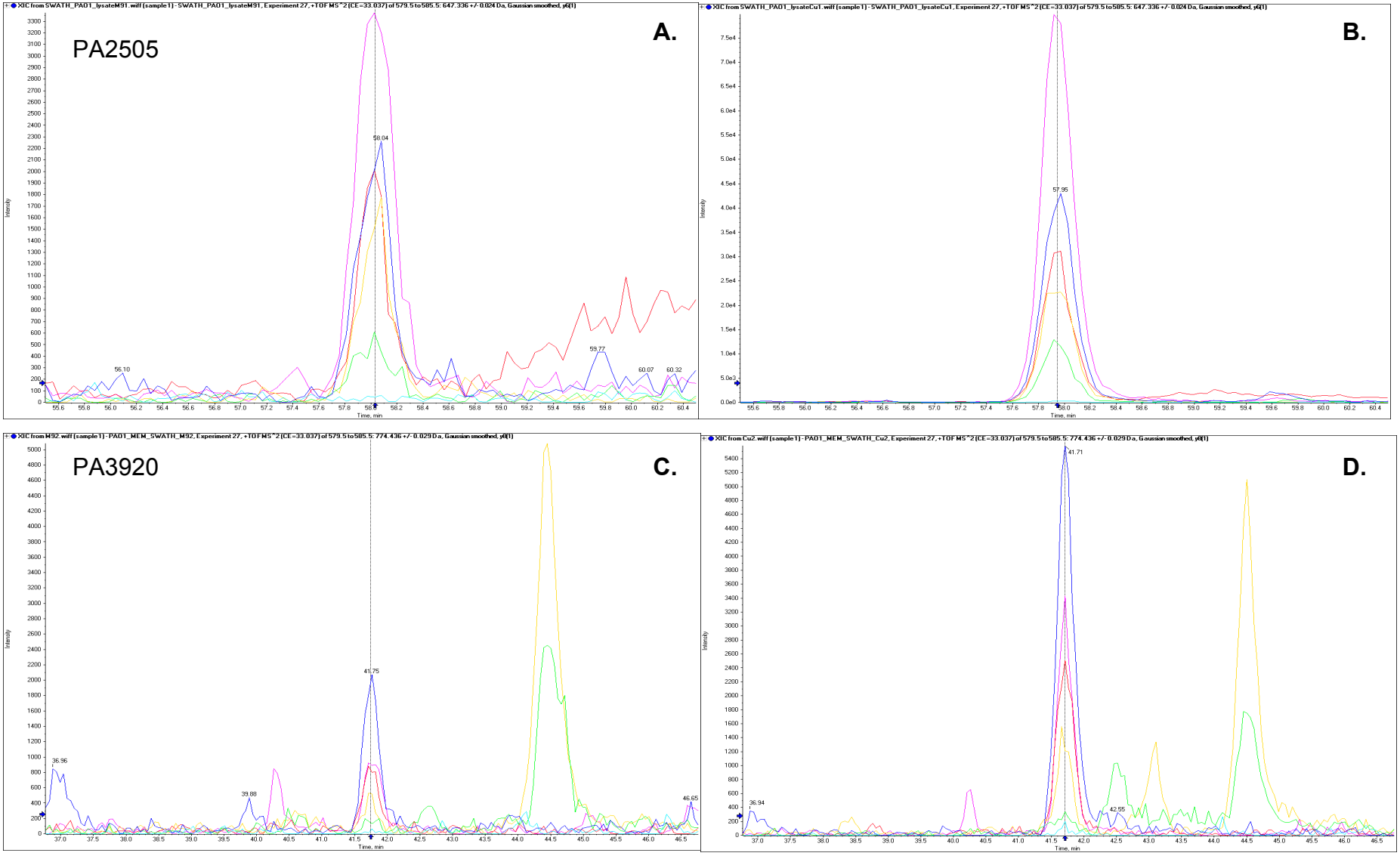
Sample	Volume (mL)	Quantitation (µg/µL)	Sample amount (µg)	% Recovery
VB1	0.5	0.123	61.5	5.6
VB2	0.45	0.121	54.4	4.6
VB3	0.4	0.229	91.6	6.6
Cu1	0.4	0.272	108.8	10.2
Cu2	0.4	0.32	128	6.4
Cu3	0.4	0.238	95.2	7.9
M91	0.45	0.218	98.1	7.5
M92	0.42	0.393	165.1	6.6
M93	0.5	0.161	80.5	4.9

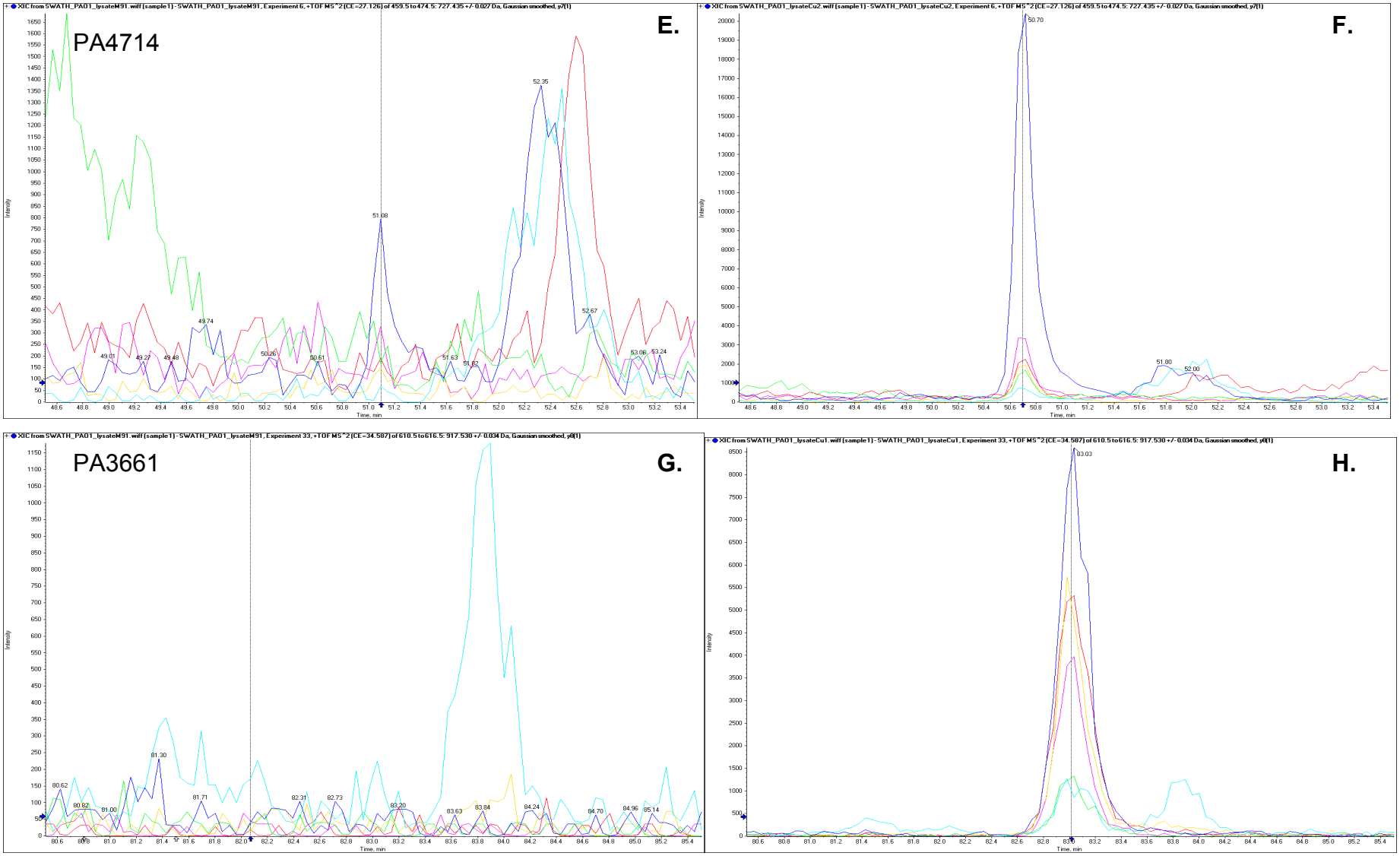
6.4. PA14 mutant PCR confirmation

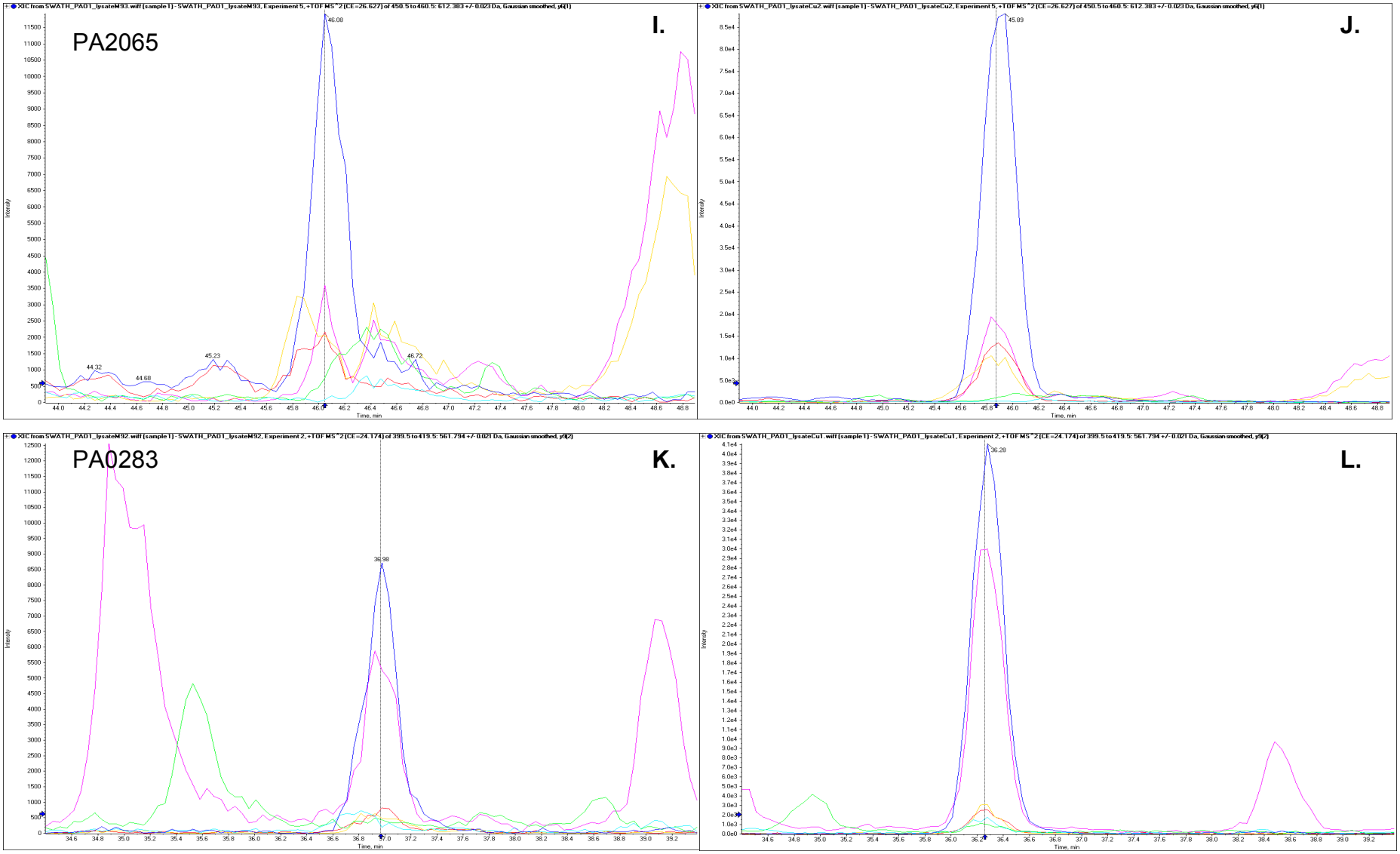
Table 11: NanoDrop DNA concentration assay of PCR round 2 products. The results displayed provide evidence of PCR product amplification across all samples.

Sample	Nucleic Acid Concentration (ng/µL)	A260	A280	260/280	260/230
PA14_13170	56	1.12	0.681	1.64	0.91
PA14_27840	29.5	0.59	0.33	1.79	1.15
PA14_03700	37.5	0.75	0.421	1.78	1.05
PA14_37790	57.5	1.15	0.671	1.71	0.95
PA14_17000	43.8	0.875	0.527	1.66	0.92
PA14_62390	43.9	0.878	0.515	1.7	0.97
PA14_32270	29.3	0.586	0.3	1.95	1.54
PA14_19486	36.7	0.734	0.422	1.74	0.97
PA14 wild-type	48.4	0.968	0.609	1.59	0.83

6.5. Extracted ion chromatograms (XIC) of the eight proteins subjected to functional annotation




PA4714
E.
F.
PA3661
G.
H.
Intensity
Time, min


PA2065
I.
J.
PA0283
K.
L.
Intensity
Time, min

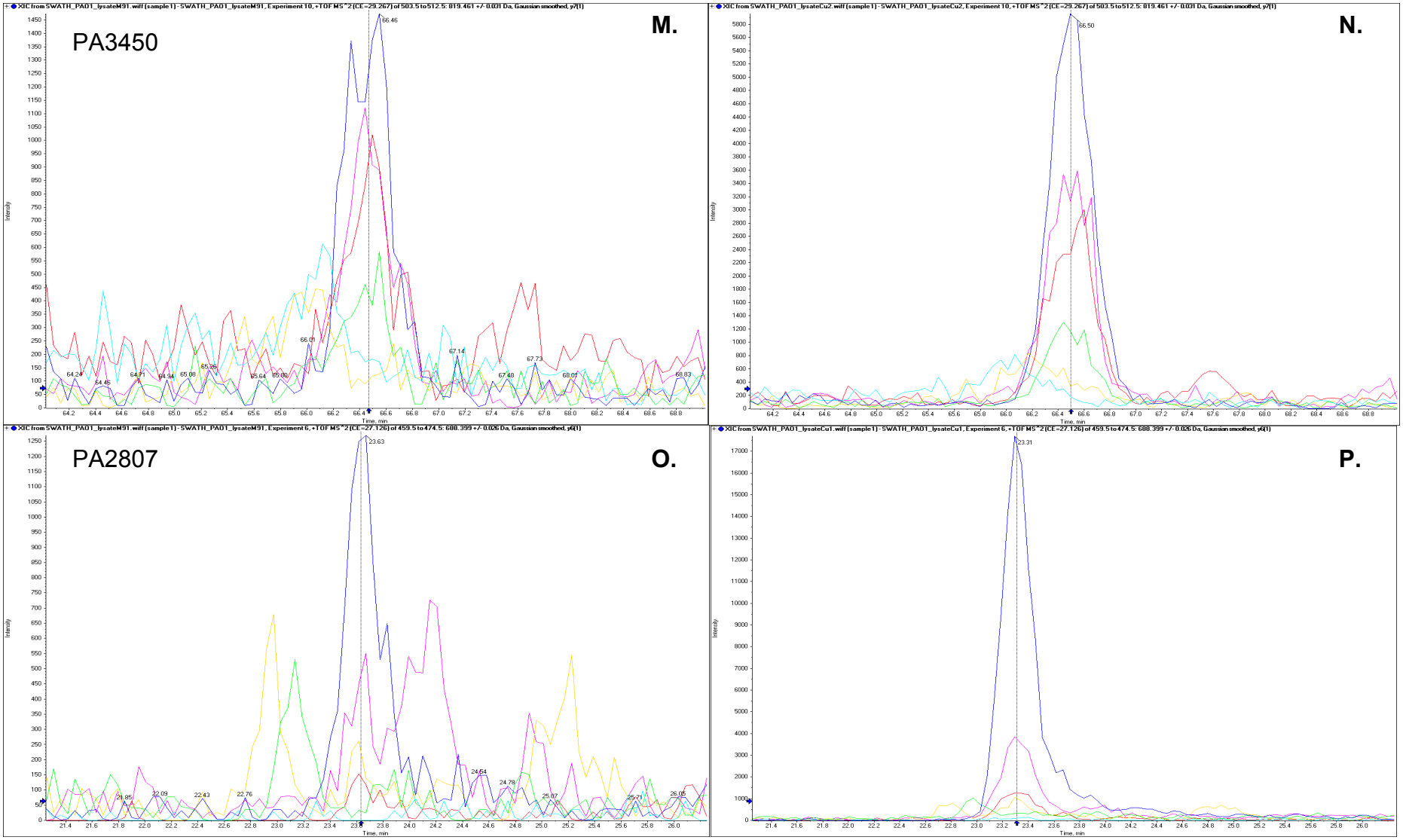


Figure 19: XICs of selected peptide fragment ion traces across all eight proteins. Each protein has an XIC from M9, and Cu samples for comparative purposes. A. PA2505 M9, **B.** PA2505 Cu, **C.** PA3920 M9, **D.** PA3920 Cu, **E.** PA4714 M9, **F.** PA4714 Cu, **G.** PA3661 M9, **H.** PA3661 Cu, **I.** PA2065 M9, **J.** PA2065 Cu, **K.** PA0283 M9, **L.** PA0283 Cu, **M.** PA3450 M9, **N.** PA3450 Cu, **O.** PA2807 M9, **P.** PA2807 Cu.

6.6 Ethics approval

The Biosafety Committee added me to the NLDR 5201401141 on the 29th of July 2015, for the work with the PA14 transposon library. See attached approval letter.

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6 February 2015

Professor Ian Paulsen
Department of Chemistry and Biomolecular Sciences
Faculty of Science and Engineering
Macquarie University

Dear Professor Paulsen,

Re: "Characterizing bacterial transporter, regulator and metabolic genes" (Ref: 5201401141)

NOTIFICATION OF A NOTIFIABLE LOW RISK DEALING (NLRD)

The above application has been reviewed by the Institutional Biosafety Committee (IBC) and has been approved as an NLRD, effective 6 February 2015.

This approval is subject to the following standard conditions:

- The NLRD is conducted by persons with appropriate training and experience, within a facility certified to either Physical Containment level 1 (PC1) or PC2.
- Working requiring Quarantine Containment level 2 (QC2) does not commence until the facility has been certified
- Only persons who have been *assessed by the IBC* as having appropriate training and experience may conduct the dealing. This includes persons involved in all parts of the dealing e.g. researchers, couriers and waste contractors. A copy of the IBC's record of assessment must be retained by the project supervisor.
- NLRDs classified under Part 1 of Schedule 3 of the Regulations must be conducted in a facility (or class of facilities) that is certified to at least a PC1 level **and** that is mentioned in the IBC record of assessment as being appropriate for the dealing;
- NLRDs classified under Part 2.1 of Schedule 3 of the Regulations must be conducted in a facility (or class of facilities) that is certified to at least a PC2 level **and** that is mentioned in the IBC record of assessment as being appropriate for the dealing;
- Any transport of the GMO must be conducted in accordance with the Regulator's *Guidelines for the Transport of GMOs* available at: http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/transport-guide-1
- A copy of the IBC's record of assessment has been attached to this approval.
- The record of assessment must be kept by the person or organisation for 8 years after the date of assessment by the IBC (regulation 13 C of the *Gene Technology Regulations 2001*).
- All NLRDs undertaken by Macquarie University will be reported to the OGTR at the end of every financial year.
- If the dealing involves organisms that may produce disease in humans, the NLRDs must be conducted in accordance with the vaccination requirements set out in the Australian Standard AS/NZS 2243.3:2010

- The Chief Investigator must inform the Biosafety Committee if the work with GMOs is completed or abandoned.

Please note the following standard requirements of approval:

1. Approval will be for a period of 5 years subject to the provision of annual reports. If, at the end of this period the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to submit a Final Report. If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. Please contact the Committee Secretary at biosafety@mq.edu.au for a copy of the annual report.

A Progress/Final Report for this study will be due on: 1 February 2016

2. If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email.

If you need to provide a hard copy letter of approval to an external organisation as evidence that you have approval, please do not hesitate to contact the Committee Secretary at the address below.

Please retain a copy of this email as this is your formal notification of final Biosafety approval.

Yours Sincerely

Associate Professor Subramanyam Vemulpad

Chair, Macquarie University Institutional Biosafety Committee

Encl. Copy of record submitted by Macquarie University to the OGTR.

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