Investigating the interactions of microbial pathogens with membrane glycoproteins

by
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Abstract

Binding of pathogen to host often involves glycans on host epithelial surfaces, a complex process that poses challenges for *in vitro* studies. In this thesis, methods were developed to study the binding of bacteria to human epithelial membrane glycoproteins by *Pseudomonas aeruginosa*, a common respiratory pathogen of individuals with cystic fibrosis (CF). A plate assay was developed and revealed differences between the adhesion of clinical strains isolated from four CF patient sputum samples, and a laboratory prototype of *P. aeruginosa,* to epithelial mucins. Isothermal titration calorimetry was also trialed for measurement of this interaction, but the generation of high background heat compromised the measurement of receptor-binding. Thus the plate assay was used to investigate the role of the glycan component of the glycoproteins in pathogen binding, by assessing bacterial binding before and after treatment of membranes from human epithelial lung cells with glycosidases. All strains increased adhesion following removal of sialic acid, but galactosidase treatment had a varied effect, demonstrating differences between the *P. aeruginosa* strains in their affinity to different glycan substructures. The findings and techniques developed by this thesis will be useful in future studies that seek to understand more about glycan involvement in pathogen to host adhesion. (200 words)

Declaration:

This thesis is a presentation of my original research work carried out as part of the Master of Research program at Macquarie University. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of personal assistance or advice.

Shathili Abdulrahman Mansour

Dedicated to

those CF patients who are in search of hope to live longer...

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Abbreviations

Asn: Asparagine

$CaCl_2$: Calcium chloride

CHAPS: 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

CF: Cystic Fibrosis

CFTR: Cystic fibrosis trans-membrane conductance regular gene

Da: Dalton

DDF: Detergent extraction fractionation

DTT: Dithiothreitol

EDTA: Ethylene-Diamine-Tetra-Acetic acid

ELISA: Enzyme-linked immunosorbent assay

ER: Endoplasmic reticulum

ESI: Electron spray ionisation

FBS: Fetal bovine serum

Gal: Galactose

GalNAc: *N*-acetylgalactosamine

GBPs: Glycan binding proteins

GlcNAc: *N*-acetylglucosamine

Hex: Hexose

ITC: Isothermal titration calorimetry

LB: Luria-Bertani

LC: Liquid chromatography

$MgCl_2$: Magnesium chloride

MS: Mass spectrometry

MUC: Mucin-type

MW: Molecular weight

NaCl: Sodium chloride

NeuAc: *N*-acetylneuraminic acid / sialic acid

NeuGc: N-glycolylneuraminic acid/ sialic acid

OD600: Optical density at 600nm

P. aeruginosa: *Pseudomonas aeruginosa*

PBS: Phosphate buffered saline

PGC: Porous graphitised carbon

PGM: Porcine gastric mucin

Psl: polysaccharide which is synthesized by the polysaccharide synthesis locus (psl)

PTM: Post translational modification

RPMI: Roswell Park Memorial Institute medium

RU: Response unit

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Ser: Serine

SPR: Surface Plasmon resonance

Thr: Threonine

Tris-HCl: Trisaminomethane-hydrocholride

Introduction

Chapter 1

1.1 Introduction to glycobiology

The contribution of post translational modification (PTM) of proteins to the overall functional complexity of organisms is well recognised ([1]). Glycosylation is the most prevalent PTM in which carbohydrates (glycans) are added to the protein backbone. The conjugated glycans play crucial roles in many cellular processes, including intercellular interactions and the adhesion of pathogens to their host ([2]). However, the complexity of glycans and their interactions *in vivo* poses a challenge for research. This is partly because of the large variety of glycan structures that can exist, with differences in the specific monosaccharide components and their linkages. In addition, variations occur in the occupancy (macroheterogeneity) and relative distribution (microheterogeneity) of the glycans on potential glycosylation sites on a protein ([3]). A further challenge for analysis arises from the fact that glycosylation is not template driven but a result of the concerted effort of glycan modifying enzymes, the expression of which may differ according to a variety of factors including cell type or disease state.

The two main types of protein glycosylation linkages found in mammals are *N*-linked and *O*-linked. *N*-glycosylation begins in the endoplasmic reticulum (ER) by adding a precursor glycan to the amide group of the asparagine (Asn) occurring on the amino acid consensus sequence of Asn-X-Serine (Ser)/Threonine (Thr). All *N*-linked glycans contain the same core structure, and after removal or addition of monosaccharides in the Golgi the structures subdivide into high-mannose, hybrid, and complex classifications ([2]) (Figure 1). On the other hand, *O*-linked glycosylation is synthesised entirely in the Golgi apparatus where *N*-acetyl-D-galactosamine (GalNAc) is attached to the hydroxyl group of Ser/Thr residues on the folded protein and is elongated one residue at a time ([4]). *O*-linked glycans can have several types of core structures mainly anchored by GalNAc. Mucin proteins are typically highly *O*-glycosylated with core 1, 2 and 3 structures (Figure 1).

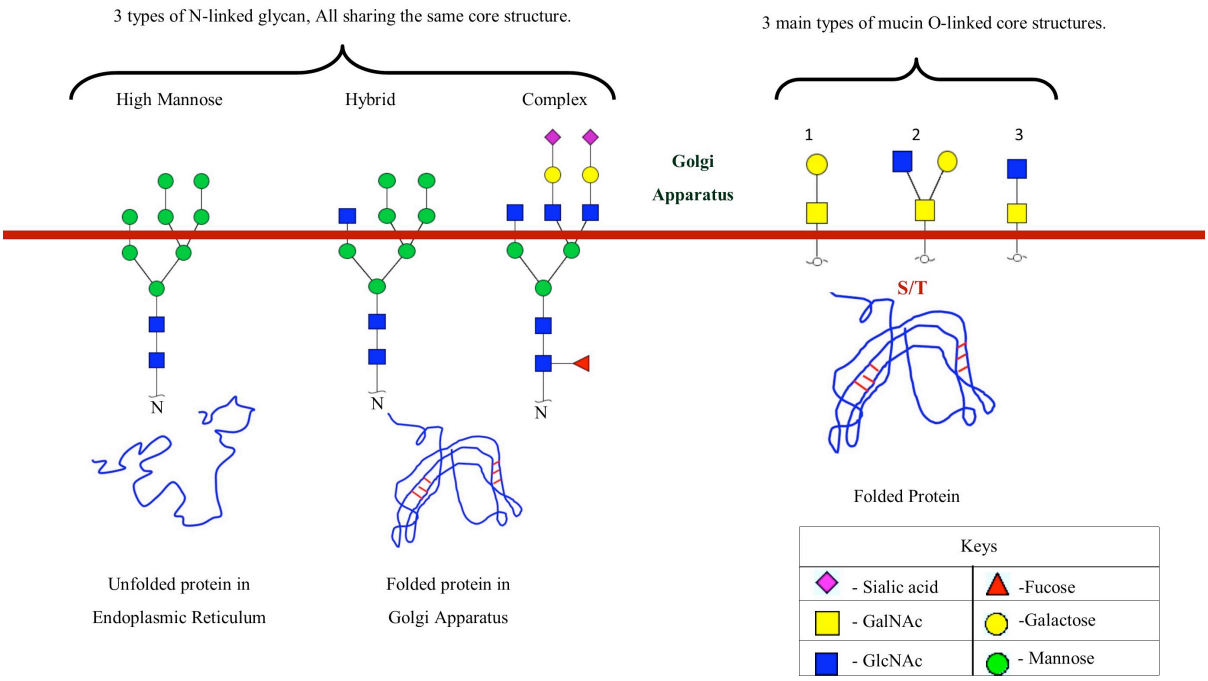

Figure 1: Three types of *N*-linked glycans (high-mannose, hybrid and complex) *N*-linked glycans are added to protein in the endoplasmic reticulum (ER) and undergo further modifications from high mannose to hybrid and complex structures in the Golgi apparatus. *O*-linked glycans are added to serine (S) or threonine (T) residues in the Golgi apparatus. Three *O*-linked core structures that are typically found on the highly glycosylated mucin proteins are shown. GalNAc: *N*-acetylgalactosamine; GlcNAc: *N*-acetylglucosamine.

The great variation in glycan structures allows their involvement in several categories of cellular interactions. Cell surface glycans are greatly involved in the pathogen interaction process ([2]). In this thesis, bacterial exploitation of glycans for attachment to host cell epithelium has been investigated.

1.2 Bacterial binding to host glycans: the first step in infection

Bacterial infection can be divided into several stages, and the initial crucial stage involves adherence to cell surfaces of the host ([5]). The adhesion process involves specific molecular interactions between bacterial adhesins and host receptor molecules such as glycans ([6]). Glycans are ubiquitous on the surface of host epithelial cells in the respiratory, urinary and intestinal tracts and these carbohydrates are recognised by specific glycan binding proteins or lectins of potential pathogens ([2]). For instance, *Pseudomonas aeruginosa* has been shown

to infect the respiratory tract through utilising type IV pili to adhere to *N*-glycans presented in the apical surface of lung epithelial cells ([7]). Furthermore, PapG adhesins located at the tip of uropathogenic *Escherichia coli* P pili adhere to galactose moieties ([8]), whereas strains expressing FimH adhesion bind specifically to mannose epitopes ([9]). Lectins (BabA), expressed by *Helicobacter pylori*, mediate bacterial adhesion to human gastric mucus through binding to glycan configurations characteristic of the Lewis^b blood group antigens, which feature two terminal fucose moieties ([10]).

There are usually multiple adhesins or lectins on the surface of each bacterial cell, and an array of diverse glycans that form potential receptors on host epithelial membranes. Thus, bacterial attachment to host is characterised by multivalent interactions that influence binding affinity ([11]). Single-site interactions between glycans and glycan binding proteins (GBPs) typically have a Kd (equilibrium dissociation constant) in the micro-molar range, whereas affinity is greatly increased in multivalent interactions between complex glycans and multiple GBPs that are typical of bacterial interactions with the epithelium ([11]). In addition, a single lectin may have differential binding affinity to several glycan epitopes, creating an even more complex scenario for adhesion. As a result, a challenge to this research field is the development of *in vitro* methods to accurately mimic *in vivo* conditions (detailed in Section 1.4). In this thesis, methods are developed and optimised to study the adhesion of whole bacteria to a complex mixture of host membrane proteins using the example of *Pseudomonas aeruginosa*, a pathogen that particularly infects the airways of individuals with cystic fibrosis.

1.3 Cystic Fibrosis as a case study

Cystic Fibrosis (CF) is a common inherited disease in Caucasians, occurring in 1 in 2500 live births in Australia, with a potential life expectancy of only 38 years ([12]). CF remains one of the most common genetic killers and this disease is usually associated with a mutation in the Cystic Fibrosis trans-membrane conductance regulator gene (CFTR), resulting in an imbalance of electrolytes over epithelial surfaces ([13]). This is particularly problematic in the respiratory tract where water absorbance increases into the cell and less water is presented in the airway surface liquid resulting in the accumulation of thick, sticky mucus (Figure 2). Overall, the thickened mucus characteristic of CF leads to a high vulnerability for

bacterial infection, which in turn leads to a continuing deterioration of lung function ([14]).

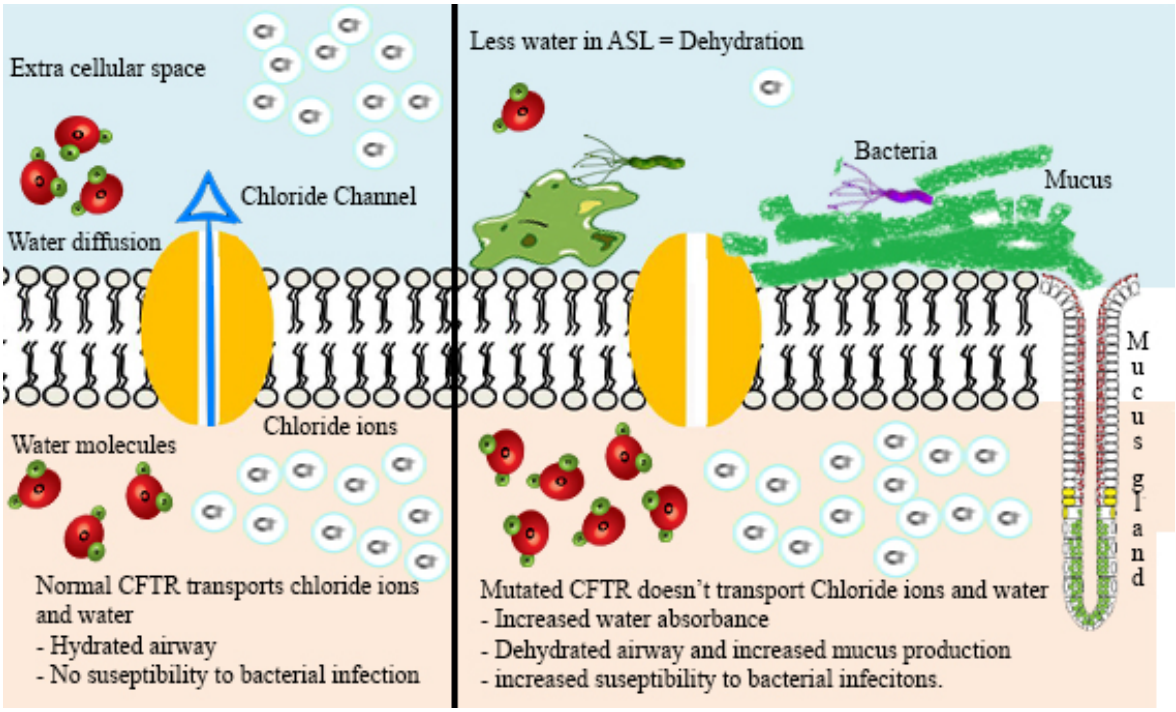

Figure 2: Dehydration of the CF lung is mainly caused by a defective CFTR channel. Bacterial infection is sustained by the increase in water absorption and the accumulation of thick, sticky mucus on the lung epithelial surface. Inspired from [15].

Mucus contains large glycoproteins (mucins) with an abundance of *O*-glycan chains in dense clusters, which provide a niche for bacterial adhesion ([16]). In a well hydrated airway, the secreted mucins MUC5AC and MUC5B assist in the binding and clearance of microbes from the airways ([13]), but dehydrated airways in CF lungs result in mucin immobilisation and microbial colonisation of the lungs. Clinical situation in CF has improved dramatically due to introduction of new treatments, refinement of conventional strategies and acceptance of aggressive management; this has increased the survival age significantly ([12]). Further understanding of the CF microbial infection will streamline development of new treatment strategies.

1.3.1 Microbial infection in CF airways

Colonisation of pathogens in the CF lung tends to be age related, with *Staphylococcus aureus* appearing in infancy followed by *Haemophilus influenzae* in the early years, and *P. aeruginosa* prevalent in adolescence and adulthood ([17]). As an opportunistic microbe

infecting individuals with compromised immune defense, *P. aeruginosa* eventually tends to dominate colonisation in CF due to several factors including resistance to antibiotics, which makes it very hard to treat. According to the national nosocomial infections surveillance system associated with all hospital acquired infections, the resistance of *P. aeruginosa* to antibiotics can be partly attributable to the pathogen's capacity to change into a mucoid form capable of forming biofilms ([17]). Biofilms consist of sticky groups of adherent microorganisms enclosed in a protective extracellular matrix and have been implicated in a wide variety of microbial infections ([18]). Research has shown that initial infection by *P. aeruginosa* may have no significant effect on pulmonary function ([19]), but serious resistant chronic infection may follow as a result of the conversion of the bacterium into a mucoid form (biofilm forming) ([20]). This situation becomes more detrimental with the increase of multi-antibiotic resistant bacteria and the significant decrease in the development of new antibacterial agents ([21]). In light of these issues, new approaches are needed to combat *P. aeruginosa* colonisation of the CF lung. Recent research has been interested in modulating the first step of *P. aeruginosa* infection involving protein-carbohydrate bindings, which may avoid the problem of antibiotic resistance ([22]).

1.3.2 Glycan-dependent adhesins of the pathogen *Pseudomonas aeruginosa*

The binding of *P. aeruginosa* to epithelial cells is strongly reliant on the glycan specificity of its lectins or adhesins (Supplementary Figure 1). Furthermore, a high expression level of *P. aeruginosa* lectins, associated with a significant decline in lung function, has been found in the respiratory tracts of individuals with CF ([23]). There are two main well-studied lectins of *P. aeruginosa*, PA-IL and PA-IIL. PA-IL has a binding preference for galactose (Ka 3.4 $x10^4$ M^{-1} , [24]), and is made up of four subunits that facilitate binding ([25], [26]). In contrast, PA-IIL adheres to both mannose and fucose (Ka 1.6 $x10^6$ M^{-1} , [27]), and shows a particularly high binding affinity to glycan configurations of the $Lewis^x$ and $Lewis^a$ blood group antigens, which contain terminal fucose and terminal galactose. Calcium ions are involved in the binding mechanisms of both lectins via electrostatic interactions between oxygen and calcium as well as hydrogen bonds between lectin and sugar ([28], [29]).

Another major adhesin identified in *P. aeruginosa* is the flagellar cap protein (FLiD). The flagellum is made up of thousands of flagellin subunits capped by FLiD, which forms a

capping structure essential for the initial attachment of this bacterium to the airways of CF patients ([30]; [31];[31]). FliD has been shown to bind to glycan configurations of the blood group antigens Lewis^a, Lewis^y, Lewis^x and sialyl Lewis^x ([32]; [33], [34]). Furthermore, *P. aeruginosa* pili was reported to adhere to sialyl-Lewis^x on glycolipids ([35]) as well as bind to GalNAc1-4 Gal on the gangliosidases asialo-GM1 and asialo-GM2 ([36], [37]). It has also been reported that *P. aeruginosa* produces a sialidase that can enzymatically cleave sialic acid residues from host glycans, exposing underlying sugars which could be more favoured as binding epitopes ([38]).

1.3.3 Aberrant glycosylation of proteins in CF airways

Variations in surface glycans have significant influence in bacterial rate of infection, and it is evident that the distinctive glycosylation patterns found in CF airways are particularly favourable for *P. aeruginosa* adhesion (reviewed in [14]). There has been a particular focus of research on the mucin *O*-glycans, where there is a higher level of sulfation and sialylation in CF secreted airway mucins accompanying a lower level of overall *O*-glycosylation compared to healthy individuals ([39]; [40]). A lower level of sialylation and a higher level of fucosylation has been observed for the membrane-bound mucins in CF airways ([41]), which have been further characterised by an over-representation of various terminal fucosylated and galactosylated epitopes with known affinity to *P. aeruginosa* lectins PA-IL and PA-IIL ([14]).

Deficiency in core *N*-glycan production is another candidate for the increase in *P. aeruginosa* binding to CF lungs. In fact, a strong correlation between *N*-glycosylation deficiency seen in CFTR-defective cells and improvement in *P. aeruginosa* binding has been observed in *in vitro* models ([42]). Recent work in this laboratory by Venkatakrishnan *et al.* (2014) has also demonstrated distinct differences in the *N*-glycan profile of infected CF sputum (including proteins from neutrophils, lung epithelium, goblet cells and others) in comparison to non-infected sputum of non-CF individuals. A truncated glycan structure called a paucimannose was around 30-40% more abundant in infected CF sputum than in non-CF sputum that was pathogen free ([14]). Also, Venkatakrishnan reported a higher level of sialylation and a lower level of bisecting *N*-acetylglucosamine (GlcNAc) in CF sputum compared to non-CF sputum. In an earlier study on glycolipids from primary culture of respiratory epithelial cells derived from CF patients, a decrease in fucosylated and sialylated epitopes was reported ([36]). Such data provides an important background for

studies such as this thesis work that seek to investigate the contribution of glycans to the adhesion of *P. aeruginosa* in the CF airways.

1.3.4 Glycan-focused therapeutics to inhibit *P. aeruginosa* adhesion

The innate antibiotic resistance of *P. aeruginosa* provides great therapeutic challenges. In order to avoid the consequences of antibiotic resistance, the use of glycan decoys to inhibit *P. aeruginosa* adhesion to CF airways has been initiated. Microbial attachment offers an attractive point of intervention in the process of infection, and the utilisation of oligosaccharides to inhibit bacterial attachment has proved successful for a number of pathogens both *in vitro* and *in vivo*, including *Streptococcus pneumonia, H. pylori* and *P. aeruginosa* ([43]; [44]).. Inhalation of both fucose and galactose solutions have been trialed in an attempt to block *P. aeruginosa* lectins PA-IIL and PA-IL in CF patient lungs ([29]). However, significant improvement in lung function was not evident, possibly partly due to the small number of patients involved in the clinical trial, the single valency of the monosaccharide interaction and the short period of time studied ([14]).

Due to their higher binding affinities, large glycan structures that more accurately mimic the actual glycan receptors in CF airways may have better inhibitory effects than monosaccharides alone. In fact, complex oligosaccharide structures purified from human milk were able to inhibit the binding capacity of *P. aeruginosa* PA-IL and PA-IIL lectins *in vitro*. Fucosylated milk glycans were able to significantly inhibit PA-IIL binding to fucose-biotinylated polyacrylamide ([45]), and haemagglutination of PA-IL was greatly inhibited by galactosylated milk glycans ([46]). The importance of multivalent glycan presentation has also been demonstrated in another study where the attachment of fucose and galactose to multivalent dendrimers has provided higher binding affinities than the individual monosaccharides ([47]). In another example, sialylated oligosaccharides provided a 500-fold increase in inhibition of *Streptococcus pneumonia* adhesion to cultured cells when covalently attached to human serum albumin creating multivalent interactions ([48]).

Undoubtedly, the concept of glycan-focused therapeutics for *P. aeruginosa* infection holds potential for CF sufferers, but it is also a considerable way from successful implementation *in vivo*. However, every advance in our understanding of the involvement of glycans in *P.*

aeruginosa adhesion to the respiratory tract could be beneficial in the management and treatment of the disease.

1.4 Techniques for studying the binding of bacteria to glycans

Several techniques have the potential to test the effect of specific sugar structures on the binding of different bacteria; such techniques have evolved rapidly in the past few decades ([11]). The combination of solid-supported binding techniques, such as microarrays, biosensors and plate assays, and in-solution binding techniques, such as Isothermal titration calorimetry (ITC), promise the generation of a more comprehensive understanding of bacterial binding performances. Solid supported binding techniques involving surface presented glycans may be more suitable for representation of protein-glycan interactions occurring on the membrane surface ([49]; [50]). Techniques involving in-solution interactions, such as ITC, may be preferable for demonstrating glycan interactions typical of those occurring in bodily fluids that wash the epithelium; however, the use of ITC for measuring complex glycan interactions is a relatively new research tool requiring further development and optimisation. Solid-based and in-solution techniques for investigating glycan-interactions with bacteria and epithelial cell surfaces are described in more detail below.

1.4.1 Solid-supported techniques

1.4.1.1 Carbohydrate microarrays

Carbohydrate microarrays involve the immobilisation of glycans onto a functionalised slide for probing and affinity analysis of lectins, glycan binding proteins or whole microorganisms. As an extension of both protein microarray and enzyme-linked immunosorbent assay (ELISA), carbohydrate microarrays have the capability of screening many glycan binding interactions simultaneously ([51]), and have thus received the interest of the glyco-scientific community ([52]). However, carbohydrate microarray studies have been limited by the availability of synthetic glycans, which are more typically representative of naturally occurring *N*-glycans or specific epitopes. *O*-glycans and more complex glycoconjugates, such as glycopeptides, are lacking from most types of glycan arrays due to the lack of availability of synthetic representatives ([52]). Furthermore, glycan presentation on microarrays may not effectively mimic the complex glycan presentation *in vivo*, with unknown effects on the binding affinities ([2]).

1.4.1.2 Biosensors

Biosensors based on the principle of Surface Plasmon Resonance (SPR) have been used extensively in the scientific community. Compared to the aforementioned microarray technique, biosensors have the advantage of being label-free; with no need for chemical, fluorescent or radiolabeled tags ([53]). In addition, the relative ease and the real-time measurements provide monitoring of both small and large biochemical interactions ([54]).

SPR relies on the measurement of surface plasmons in the interface between a dielectric medium and a chip. The SPR chip is usually made of gold covalently functionalised with biomolecules placed on top of a prism. This allows for the flow-through technique to measure the surface plasmons that travel parallel to the conducting film ([55]). By maintaining all contributing factors to the specific incident angle constant, it is possible to study small changes in refractive index generated from surface binding. These changes in refractive index correspond to changes in mass concentration ([13]).

For glycan-binding studies, glycans are immobilised on the surface of a SPR imaging chip and then a solution with a lectin is injected over the ligand layer. As the lectins bind the glycan ligand, an increase in SPR signal (expressed in Response Units, RU) is observed. After reaching the desired association, a buffer solution containing no lectins is injected resulting in dissociation of the bound complex and decrease in SPR signal. Through the measurements of these association and dissociation rates, an equilibrium constant can be calculated ([13]).

SPR was shown to be capable of elucidating the binding of whole *Lactobacillus acidophilus* cells to complex colonic mucins ([56]). However, several factors appeared problematic. For instance, the large size of cells resulted in decreased signal ratio, since large cells will not cover the measured area evenly. Also, the low number of bacteria being physically close to the sensor working range contributed to an underestimate of the actual number of binders ([56]).

1.4.1.3 Plate assays

There are various assay techniques that have been used to study glycan-mediated adhesion of microbes in microwell plates without the need for microarray or biosensor technologies.

Traditional plate assay methods have involved radiolabelling ([57]) and haemugluttination-inhibition ([57]), and/or live epithelial cell cultures incorporated into the assay plate ([59]). In addition, time consuming microbial enumeration methods have been employed, involving removal of unbound bacteria from the wells, and re-culturing of a dilution series on agar plates and time consuming colony counts ([60]; [61]).

As a more high-throughput alternative to the traditional methods, a bacterial binding assay was recently developed by this laboratory that involves immobilising glycoproteins onto the PVDF membranes in the wells of a 96 well filter plate. The protein backbone binds to the PVDF through hydrophobic interactions, whereas the glycans are repelled away from the membrane surface. The presented glycans can then be probed with fluorescently labelled bacteria and bacterial binding quantified using a fluorescent plate reader. This technique allows the study of the complex glycoprotein interaction with whole bacteria as well as the *in vivo*-like presentation of the conjugated glycans. In addition, information about the specific glycans involved in the adhesion of bacteria to the epithelial membrane proteins can be demonstrated when pre-incubation of the bacteria with a glycan moiety of interest (*eg*. free sialic acid) results in subsequent inhibition of adhesion to the membrane glycoproteins. This is because the lectins with affinity for the glycan become saturated during the pre-incubation period and have reduced availability for further binding to the immobilized proteins. Alternatively, glycosidases can be used to trim the epithelial cell membrane protein glycans prior to application of the bacteria, revealing the importance of terminal glycan epitopes in bacterial adhesion.

In past applications of this assay in the laboratory, glycosidase treatments of human colon cell epithelial membrane proteins have revealed the importance of terminal fucose and sialic acid moieties in the adhesion of gastrointestinal pathogens. In addition, a fucose polymer called fucoidan from the seaweed *Undaria pinnatifida* was found to be a good inhibitor of adhesion (unpublished data, Robyn Peterson). Fucoidan from *U. pinnatifida* contains fucose (~65 – 80%) and lesser amounts of galactose (~12 – 30%;[62]). The ability of fucoidan to inhibit adhesion of the gut bacteria to the colon epithelial membrane proteins suggested the value of the polymer as a supplement to reduce gastrointestinal infection. In

addition, the inhibitory effects of fucoidan implied a binding affinity of the gut bacteria to fucose and/or galactose.

Since *P. aeruginosa* has lectins known to bind fucose and galactose (Section 1.3.2), the polymer fucoidan was investigated in this thesis for its ability to inhibit adhesion of the pathogen to mucin using an adaptation of the in-house plate assay technique. In addition, glycosidase treatment of human lung epithelial membrane proteins was conducted and the effect on *P. aeruginosa* adhesion was determined.

1.4.2 An in-solution technique: Isothermal Titration Calorimetry (ITC)

Isothermal Titration Calorimetry (ITC) is a technique able to determine the heat released or absorbed from the binding of two molecules in-solution, and various thermodynamic parameters can also be measured such as binding affinity, enthalpy changes, and stoichiometry of the reaction ([63]). This technique involves two identical cell compartments. The reference cell maintains a stationary heat to help keep a constant baseline heat in the sample cell. A ligand is then titrated into the sample cell and the formation of a complex between ligand and receptor results in heat release or absorption. The change in sample cell temperature leads the instrument to alter the heating of the sample cell to keep the two cells at a constant temperature (isothermally). As the concentration of ligands increases in the sample cell, less receptor will be available for binding. Hence, less heat per injection is measured until no additional binding is noted (Figure 3). The generated data can be used to directly define the thermodynamic parameters of binding and calculate the Kd of the carbohydrate-protein interaction of interest ([63]).

ITC has been used in several glycan-binding studies, for example, to investigate the thermodynamics of the binding of *Burkholderia ambifaria* (BambL lectin) to fucose-containing oligosaccharides ([64]). In addition, ITC was used to illustrate the effect of mutating PA-IIL on binding affinity to fucose ([65]). Few ITC studies have been carried out using whole cells or cell extracts due to the complexities of the interactions involved. However, the enthalpy of the binding of a small peptide with whole cells was investigated in two independent experiments ([66], [67]). These studies reflect the potential of using ITC for the study of complex protein-glycan interactions, although the optimisation of the technique for studies of this kind is still lacking. In the work conducted for this thesis, ITC was trialed for

measuring the binding of *P. aeruginosa* membrane proteins to glycoconjugates in solution, with the broad aim of testing the suitability of the technique for future studies of this nature.

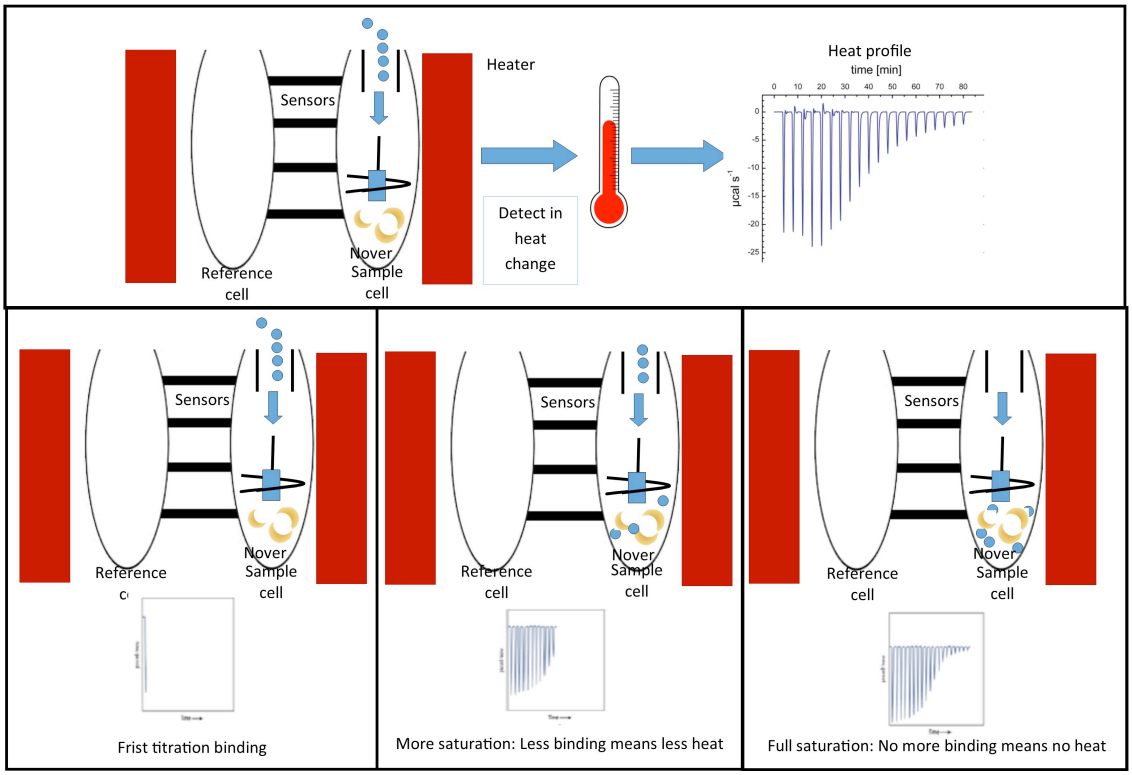

Figure 3: Schematic illustrating the process and interpretation of isothermal titration calorimetry. ITC can detect the heat change in the sample cell, which leads to the generation of a heat profile. The sample cell is made identical to the reference cell. Below, the beginning of titration produces the highest heat generated, followed by less receptor availability (less heat), followed by the saturation of receptors (no heat). Inspired from [68].

1.5 Research interest of this thesis

This thesis is placed in the context of a larger research project at Macquarie University, funded by the Australian Research Council, that is investigating microbial interactions in CF. As part of the project, clinical strains of *Pseudomonas* from sputum were isolated by Venkatakrishnan, unpublished data, from CF patients and subjected to various analyses. These strains have shown differences in use of carbon source, biofilm formation, colony morphology, and virulence (unpublished data, Ani Penesyan). As the first step in understanding microbial colonisation, the ability of these different strains to bind to the respiratory tract of CF patients is also of great interest.

Since *P. aeruginosa* is known to utilise host glycans in adhesion, the work carried out for this thesis aimed to find a sensitive and reproducible method for measuring differential adhesion of the CF *P. aeruginosa* strains to glycoproteins representative of the CF respiratory tract. A hypothesis that the isolated strains may exhibit different overall adherence profiles and/or have different binding affinities to specific glycan moieties could then be tested.

Overall, demonstrating differences in glycan involvement in the adhesion of each strain to glycoproteins typical of the respiratory tract could increase our understanding of *P. aeruginosa* infection process and contribute to the development of new therapeutics to inhibit lung infections in CF patients. Furthermore, the techniques developed in this work may add to the expanding toolbox for future investigations of the contribution of glycan-based interactions in bacterial attachment to the host.

The specific aims were as follows:

1. Adapt and optimise the in-house plate assay protocol for the investigation of the binding affinities of *P. aeruginosa* strains;
2. Investigate ITC for suitability as an in-solution technique for measuring the binding of *P. aeruginosa* membrane proteins to glycans;
3. Compare the binding of *P. aeruginosa* strains to membrane proteins prepared from human lung epithelial cells, and mucin-secreting colon epithelial cells;
4. Determine the terminal glycan epitopes that are important for *P. aeruginosa* strain adhesion by enzymatically altering the surface glycan profiles of epithelial membrane proteins using exoglycosidases.

Materials and Methods

Chapter 2

2.1. Microbial strains

Four of the *P. aeruginosa* strains used in this work were previously isolated from the sputum of patients with CF at Westmead hospital, Sydney as part of the wider ARC Super Science project on CF No FS110200026 at Macquarie University under the MQ Human Research Ethics Committee Approval No 5201100423 and Biosafety approval No VIV030712BHA. In addition, the laboratory reference *P. aeruginosa* strain PAO1 was studied and compared to the CF clinical isolates. PAO1 is a widely used prototype laboratory strain for which a complete genome sequence is available ([69]). The CF strains have been partially characterised and compared to PAO1 by others in the research group. The strains differ in their morphology, degree of mucoidity, and genomic profiles; some major features of interest to this thesis are summarised in Table 1.

Table1: *P. aeruginosa* isolates studied in this thesis, their biological sources, pigments on LB (Luria-Bertani) agar, interesting features and genomic comparisons (Ani Penesyan, unpublished data).

***P. aeruginosa* strain**	**Biological source**	**Pigments on LB agar**	**Interesting features**	**Similarity of genome to PAO1 (%)**
PASS01, from Sydney.	40 year old female CF patient, sputum	Light green colony with greenish halo	Able to form biofilm	89.12
PASS02, from Sydney.	27 year old male patient, Sputum	Orange colony with orange halo	Missing *Psl* (polysaccharide synthesis locus), no biofilm formation.	88.18
PASS03, from Sydney.	23 year old male patient, sputum	Green colony with light green halo	Most mucoid, able to form biofilms, and most alginate expressing strain	88.8
PASS04, from Sydney.	23 year old female patient, sputum	Dark greenish-blue colony with bluish halo	Second most mucoid, able to from biofilms, and can utilise DNA as carbon source.	92.28
PAO1(ATCC 15692).	Wound isolate (1955).	Green with greenish yellow halo	Common laboratory strain, non-mucoid, and able to from biofilm	

2.2. Optimisation of in-house plate assay for *P. aeruginosa* isolates

Although the in-house assay was known to be an effective technique for assessing the binding of bacteria to epithelial membrane proteins (Clara Cheah, PhD thesis), the assay needed to be optimised for the *P. aeruginosa* strains in the context of CF, the focus of this research. To do this, the growth phases of the various *P. aeruginosa* strains in liquid media were characterised, and the optimal concentrations of bacteria and appropriate glycoprotein sample preparation for application to the plate assay were determined.

2.2.1 *P. aeruginosa* growth curve

The growth of PAO1, PASS01, PASS02, PASS03 and PASS04 in 10 ml Luria-Bertani (LB) liquid medium was investigated to determine the timing of the log phase of growth; the phase when bacteria is most viable. The liquid medium was inoculated with one loop of bacteria from an agar plate culture, prior to incubation on a shaker at 185 rpm, 37°C and in the window of 10 hours. Growth was measured by taking Optical density at 600 nm every hour after inoculation.

2.2.2 Determining optimal protein and bacterial concentrations for the assay

The first plate assays were carried out using porcine gastric mucin (PGM, Sigma Aldrich) as the fixed protein source on the 96 well PVDF plate (PALL, # 5026). The PGM, although of different origin, provided a relatively cheap and commercially available representation of mucins that are also characteristic of the lung epithelial surface, and was used for the assay optimisation procedure. A schematic of the assay workflow is shown in Figure 4.

The wells of the PVDF plate were moistened with 50 µl of methanol, then rinsed twice with 100 µl of PBS. PGM (50 µl) at a concentration of 0.5 – 2 mg/ml was applied to each well of the plate and allowed to bind to the PVDF membrane for 30 min at RT, before washing three times with phosphate buffered saline (PBS). PBS was kept in the wells until the bacteria were ready to apply to the PGM. Just prior to application of the bacteria, all PBS was removed. Meanwhile, the *P. aeruginosa* isolates were cultured in 10 ml Luria-Bertani (LB) medium at 37°C for 8 hours. Bacterial cells were pelleted by centrifugation at 3000 × g for 5 min, washed with PBS, and then thoroughly resuspended in 1 ml of PBS with SYBR® Green (0.1%, Life Technologies) for 3 min. Further, bacterial cells were pelleted, washed with PBS and resuspended in PBS to the desired optical density at 600 nm (OD600); only 5% variation was accepted for the desired OD600 value.

The labelled bacterial cells were added to the immobilised PGM in the wells and incubated at RT for 30 mins. This was followed by three washes with PBS, then all PBS was removed. Fluorescence readings for bacterial attachment were measured (Ex 485 nm, Em 520 nm) using a Fluostar Galaxy plate reader (BMG Labtech, Offenburg, Germany). This procedure was carried out using various concentrations of PGM and bacteria to determine the optimal concentrations that enabled full coverage of the PVDF in the wells (to minimise bacterial

binding to PVDF), and fluorescence detection of bacterial adhesion across a linear range that was quantitatively dependent without saturation. Controls were considered as glycoproteins without the addition of fluorescent bacteria. In addition, to measure the uptake of fluorescence by the individual *P. aeruginosa* strains, the fluorescence of 50 µl samples of labelled bacteria at an OD600 of 1 was measured in other wells. Any variation in fluorescent uptake was then taken into account when calculating relative adhesion (Figure 4).

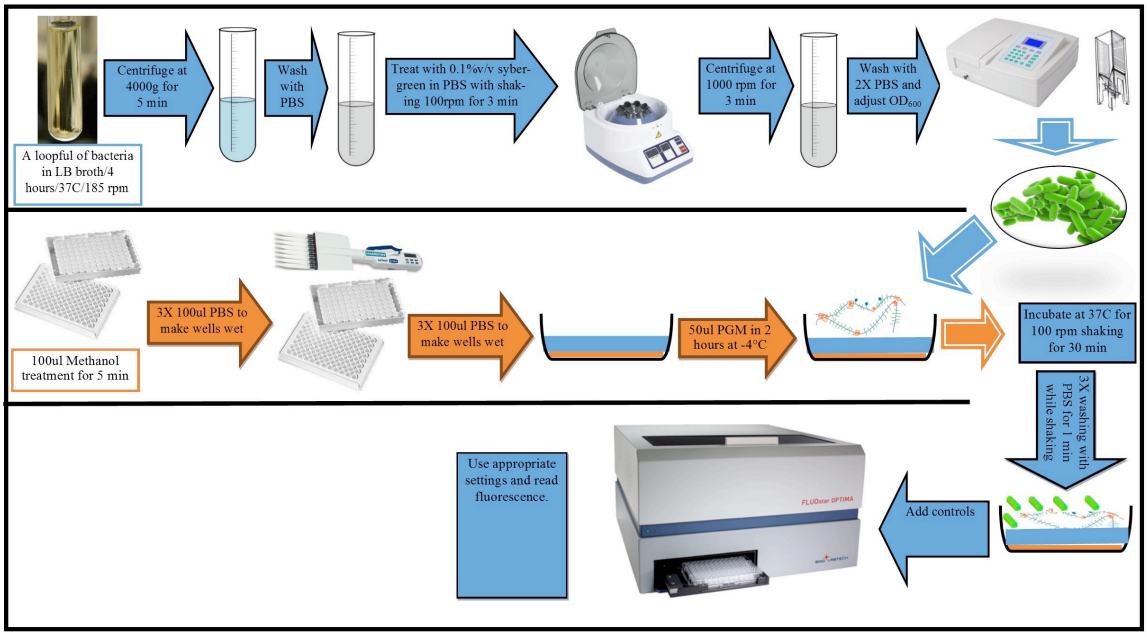

Figure 4: The workflow of the in-house plate assay used to study bacterial binding to immobilised proteins on a PVDF filter plate.

2.2.3 Inhibition assays

Due to the success of the fucose polymer fucoidan in inhibiting the adhesion of human pathogens to epithelial membrane proteins in past research in the laboratory (Section 1.4.1.3), fucoidan was trialed as an inhibitor of *P. aeruginosa* adhesion to PGM. Fluorescently labelled bacteria (final OD600 of 1) were pre-incubated in different concentrations of fucoidan (Depyrogenated *Undaria pinnatifida*, Marinova) prior to application to the PGM on the PVDF plate as described above. Binding was compared to that of bacteria that had been pre-incubated in PBS alone.

2.2.4 Statistical analysis

Paired Student t-tests were used to detect any statistical differences between the adhesion of the *P. aeruginosa* strains, where $p<0.05$ was considered a significant value (*) and $p<0.01$ was considered highly significant (**). Student t-test was performed as two tails and with variance.

2.3 Isothermal titration calorimetry

Following optimisation of the plate assay and establishing preliminary comparison of the binding affinities of the *P. aeruginosa* strains, ITC was also investigated for suitability as an alternate (in-solution) technique to monitor adhesion of *P. aeruginosa* membrane proteins to glycan-containing molecules, using PAO1 membrane proteins, fucoidan and PGM as examples.

2.3.1 Extraction of *P. aeruginosa* (PAO1) membrane proteins for ITC

Membrane proteins were extracted from *P. aeruginosa* strain PA01 as described previously ([70]). PAO1 was grown for 13-15 hours in 300 ml LB medium, while shaking at 185 rpm at 37°C. Cells were collected and washed with a wash buffer (50 mM Tris/HCL buffer, pH 7.5) followed by centrifugation at 2500 x g for 8 mins. Washed cells were then ruptured twice with a French press (French Pressure Cell Press, Thermo Spectronic) at 1400 psi. Centrifugation at 2500 × g pelleted unbroken cells, and supernatant was added to ice-cold sodium carbonate solution (100 mM sodium carbonate) and the mixture was slowly stirred for one hour in an ice bath.

The membrane fraction was collected by ultra-centrifugation (WX Ultra 80, Thermo Scientific) at 115000 rpm for 1 h at 4°C. Residual sodium carbonate solution was removed by washing with PBS. Finally, the membrane pellet was collected from the bottom of the ultra-centrifugation tubes with 500 µl solubilising solution (50 mM Tris/HCl; 15 mM $CaCL_2$; 8M urea; 3% w/v CHAPS). The final concentration of the extracted membrane proteins was estimated with a serial dilution using Bradford assay ([71]).

2.3.2 ITC experimental details

Membrane proteins extracted from PAO1, and ligands, either fucoidan or PGM, were prepared in solubilising buffer (50 mM Tris/HCl; 15 mM $CaCL_2$; 8 M urea; 3% w/v CHAPS).

All samples were dialysed in 1 L Milli-Q water overnight and then sonicated for 5 minutes prior to ITC loading. The sonication step ensures the prevention of aggregation formation and the removal of air bubbles. The sample cell was loaded with 400 µl POA1 membrane proteins, and titrated with 140 µl of ligands, fucoidan or PGM. MicroCalAuto ITC200 was used with the following settings: 19 of 2 µl injections, 25°C, reference power was set to 5 µcal/s, initial delay of 60 s, 1000 rpm stirring speed, high gain, duration of 2 s, 180 s spacing and 5 s filtering period.

2.4 Measuring the adhesion of *P. aeruginosa* strains to human epithelial surfaces

The plate assay technique was used to compare adhesion of the *P. aeruginosa* strains to epithelial cell membrane proteins. The membrane proteins were extracted from two cancer cell lines, which are able to be propagated using standard cell culture techniques. A549 lung epithelial cells and LS174T colon epithelial cells that are known to be high mucin-secreting, were provided by Professor Hazel Mitchell from the Mitchell lab at University of New South Wales.

2.4.1 Cell culture of A549 and LS174T

The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Life Technologies) supplemented with L-glutamine (2 mM, Gibco, Life Technologies), penicillin streptomycin (500 µg and 500 U respectively, Gibco Life Technologies), and 10% fetal bovine serum (FBS, Gibco, Life Technologies) in a humidified 5% CO_2 atmosphere at 37°C. Fresh medium was applied every third day, and upon maximum confluency (~70% growth confluency for LS174T, 90% growth confluency for A549), cells were moved into two to three flasks after trypsinisation. All materials were harvested within four passages. Cells were grown in large culturing plates (100 mm dish) to maximum confluency prior to harvest by manual scraping for membrane extraction.

2.4.2 Extraction of LS174T and A549 membrane proteins by differential detergent fractionation (DDF)

Eukaryotic membrane protein fractions were prepared as described previously ([72]). Briefly, the cells were washed in PBS, re-suspended in 1 ml of cytosolic extraction buffer (0.01% w/v digitonin, 10mM PIPES (pH 6.8), 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 5 mM EDTA, 0.1% w/v protease inhibitor), and placed on ice for 30 min. The extract was

centrifuged at 500 × g for 10 min, and then cytosolic extraction was repeated twice interspersed with 10 min ice incubation. The final pellet was re-suspended in 500 µl membrane extraction buffer (0.5 % v/v Triton X-100, 10 mM PIPES (pH 7.4), 300mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 3 mM EDTA, 0.1% w/v protease inhibitor), and placed on ice for 30 min. The extraction mix was centrifuged at 5000 × g for 10 min. The supernatants containing membrane fraction was collected and protein concentrations were determined using Bradford assay with buffer C and buffer M as controls. Milli-Q water was used to dilute samples to 1 mg/ml.

Finally, 50 µl of A549 and LS174T membrane proteins (1 mg/ml) were applied to the PVDF plate, and used for testing *P. aeruginosa* adherence to human epithelial membrane proteins using the plate assay, as described earlier using PGM.

2.5 Assessing the contribution of terminal glycan epitopes on *P. aeruginosa* adherence

To determine the involvement of the terminal glycans in the binding of *P. aeruginosa* strains, glycosidases were used to remove sialic acid and galactose from the glycans conjugated to the epithelial cell membrane proteins after immobilisation onto the PVDF plate. Treatments were performed at 37°C for overnight incubation.

The glycosidase treatment was first conducted on bovine fetuin (Sigma Aldrich), a well characterised glycoprotein, to optimise the technique. Since the plate assay was optimised with 50 µg of glycoproteins per well, 50 µg fetuin, or epithelial membrane protein, was used for determining the concentration of exoglycosidases required for optimal enzymatic digestion of targeted terminal epitopes. Broad specificity α2-3,6,8 sialidase (480717, MERCK) and β1-4 D-galactosidase (345806, MERCK) were dissolved in 50mM PBS pH 5.3.

2.5.1 Analysis of glycosidase treatment by SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to investigate glycosidase treatment of fetuin that had been incubated overnight in-solution with different sialidase unit concentrations (0.25, 0.5 mU/50 µg fetuin) and different galactosidase unit concentrations (0.25, 0.5 and 1 mU/50 µg fetuin) at 37°C treated sequentially as well as in parallel. A band shift is expected following successful enzymatic

treatment since fetuin is known to contain glycans with terminal sialic acid and subterminal galactose moieties (Green ED 1988). The gel of choice was a NuPAGE® NOVEX® precast 4-12% w/v Bis-Tris gel (Invitrogen). Samples were reduced in NuPAGE® LDS sample loading buffer (Invitrogen) and 50mM DTT at 70°C for 10 mins. Electrophoresis was carried out in MOPS buffer (4.1% w/v 3-morpholinopropanesulphonic acid: 0.68% w/v sodium acetate: 0.5mM EDTA), as specified by manufacturer, at 200 V until the loading dye reached the end of the gel. Gels were then stained with Coomassie Brilliant Blue dye.

2.5.2 Analysis of glycosidase treatment by mass spectrometry

Two overnight enzymatic treatments, sialidase (0.25 mU) only and sialidase (0.25 mU) followed by galactosidase (0.5 mU), were performed on fetuin blotted on a PVDF membrane (Millipore). To confirm the activity of the glycosidases, N-glycans were released from fetuin (50 µg) by an overnight incubation with 2.5 U *N*-glycosidase F (PNGase F, *Flavobacterium meningosepticum*, Roche) at 37°C as decribed previously [73]. Samples were collected in a low-binding Eppendorf tube. This was followed by incubation with 100mM ammonium acetate for 1 hr then drying and 1M sodium borohydride reduction for 3 hr at 50°C. Glacial acetic acid was added to stop the reaction by degradation of the borohydride. Samples were desalted on a strong cation exchange chromatography 20ul-column (Dowex resin AG-50W-X8, Bio-Rad) to remove salts from the glycans. Residual borate was removed by drying the glycans in presence of methanol. Further purification of the reduced glycans was carried out using a porous graphitised carbon (PGC) packing (Extract-clean carbon SPE cartridge, Grace); the C18 reversed phase platform (Thermo Fischer Scientific (analytical scale), strataX, Phenomenex (preparative scale)) maintains PGC packing. The glycans bind to the PGC packing, with residual impurities washed off with 10 bed volumes of water, before eluting the glycans using 3 bed volumes of 40% acetonitrile with 0.1 % v/v trifluoroacetic acid. Eluted glycans were then dried and re-suspended in deionized water.

Analysis of glycan composition was performed by online Porous Graphitised Column-Liquid Chromatography-Electrospray Ionisation-Tandem Mass Spectrometry (PGC-LC-ESI-MS/MS) using a PGC column (5 um particle size, 180 um internal diameter x 10 cm, Hypercarb KAPPA Capillary Column, Thermo Fischer Scientific) on an LC-ESI-MS/MS system (Agilent 1100 Series LC/MSD Trap XCT Plus, Agilent). The column was equilibrated

with 10 mM ammonium bicarbonate (Sigma Aldrich) and samples were separated on a 0-90% acetonitrile v/v in 10 mM ammonium biocarbonate gradient over 45 min, with a flow rate of 2 µl/min. Capillary voltage for ESI was set at 3 kV and mass spectra were acquired in negative ion mode, scanning for ion masses of m/z between 100-2200. Identified glycan masses were searched using GlycoMod (Expasy, http://web.expasy.org/glycomod/) for possible carbohydrate compositions.

Analysis of MS/MS spectra was carried out using DataAnalaysis 4.0 (Bruker Daltonics), and possible glycan structures were identified based on diagnostic fragment ions and fragment ion mass differences (162 Da for hexose, 203 Da for N-acetylhexoseamine (HexNac), 146 Da for fucose, 291 Da for N-acetylneuraminic acid (NeuAc), with a 1 Da allowance) ([73]). Following the optimisation and confirmation of glycosidase treatment of the *N*-glycans from fetuin, the *N*-glycans on the membrane proteins of A549 cell lines were subjected to the optimised glycosidase treatments and MS analysis as described above.

2.5.3 Plate assay with glycosidase treated membrane proteins

The plate assay described in Section 2.3.1/2.3.2 was used to compare the binding of the five *P. aeruginosa* strains (PAO1, PASS01, PASS02, PASS03, and PASS04) to the A549 cell membrane proteins with and without the glycosidase treatments described above. Paired Student t-tests were used to detect any statistical differences in adhesion as a result of removal of terminal binding epitopes.

Results

Chapter 3

3. Results

In this study, the adhesion of *P. aeruginosa* strains to glycoproteins with some similarity to those in human respiratory airways was investigated, with a particular interest in the terminal glycan epitopes that are involved in adhesion, and variations between strains. Two techniques were used: a plate assay that had been established in the laboratory, and ITC, an in-solution technique that held unexplored potential for measuring bacterial glycan interactions. The first experiments were carried out using the established plate assay to optimise the technique for this study and to make a preliminary assessment of differential adhesion of the *P. aeruginosa* strains.

3.1 Optimisation of plate assay for *P. aeruginosa*

The in-house plate assay has been successfully used to study bacterial adhesion to conjugated glycans in previous work in the laboratory, using different bacterial species and immobilised glycoproteins (unpublished data, Robyn Peterson and Clara Cheah). However, several preliminary experiments were required in order to investigate the adhesion of the chosen *P. aeruginosa* strains to glycoproteins typical of respiratory tract to optimize for the different bacterial species. These involved characterisation of the growth phases of the strains in liquid culture, and finding appropriate concentrations of bacteria and glycoprotein for application to the PVDF plate, as described below. The chosen glycoprotein source for this preliminary work was the commercially available porcine gastric mucin (PGM), as an abundant and relatively inexpensive and accessible representation of human respiratory mucins.

3.1.1 Characterisation of the growth of *P. aeruginosa* strains in liquid culture

It had been found in previous work in the laboratory that the viability and adhesion of bacteria used in the plate assay can be influenced by the phase of growth at which they are harvested from pre-cultures. Most consistent results have been achieved when bacteria harvested at the log (exponential) growth phase are used. Therefore, the time required to reach the log growth phase for each *P. aeruginosa* isolate was determined. The growth curves over a 10 hour period, based on OD600, are shown in Figure 5.

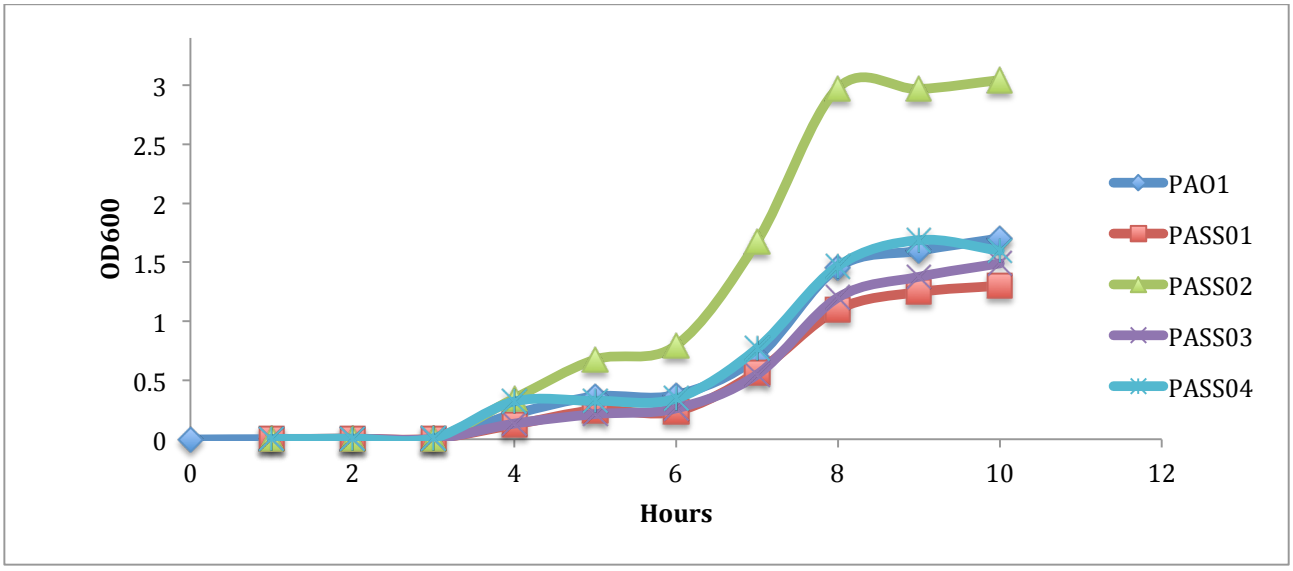

Figure5: Growth curves for four *P. aeruginosa* strains (PAO1, PASS01, PASS02, and PASS03). Optical density at 600 nm was measured every hour after inoculation. The log phase is evident at 6-8 hours for all *P. aeruginosa* strains tested.

As seen in Figure 5, strain PASS2 multiplied faster and reached a substantially higher cell concentration (OD600 >2.5) compared to the other isolates (OD600 ≤1.5) during the 10 h incubation period. However, a common period of log phase growth was observed in all strains after 6-8 h of incubation, and therefore this was chosen as an appropriate time window for harvest prior to use in the assays.

3.1.2 Optimising bacterial and protein concentrations for the assay

In addition to the time of harvest, two parameters also required optimisation in the actual assay procedure, namely the amount of protein to be immobilised in each well of the plate, and the concentration of bacteria to be applied to the immobilised protein to allow measurable adhesion without saturation. Sufficient coverage of the PVDF membrane by the protein of interest was important since many bacteria had been found to adhere directly to the PVDF membrane in past research in the laboratory. It had been previously determined that applying 50 µL of 1 mg/mL protein per well is sufficient to completely block the membrane. Therefore, although PGM had not been used in the assay previously, application of 50 µL of 1 mg/mL PGM per well was chosen initially as an appropriate stable parameter for the protein immobilisation step in the first experiment for this thesis, while the suitability of different concentrations of bacteria was tested (Figure 6). The ability of the 1

mg/ml PGM concentration to sufficiently block the PVDF was then confirmed in a subsequent experiment (Figure 6).

The adhesion of 50 µL of PASS01 (OD600 of 0.5 – 2) to PGM (applied as 50 µL of 1 mg/ml PGM) is shown in Figure 7. A good binding assay must show stable increase with the increase in bacterial concentration, and indeed the results show a linear increase in fluorescence detection reflective of the increase in the number of fluorescent bacteria bound to the PGM. This helped to validate the assay, and that the technique had been adequately mastered. Furthermore, from these results, an OD600 of 1 was chosen as an appropriate bacterial concentration for future assays since it was within the lineal progression of measurable adhesion, may minimise agglutination (in preference to higher concentrations), and was not near saturation levels. The same results were observed with PASS03 (Figure 2 Supplementary data).

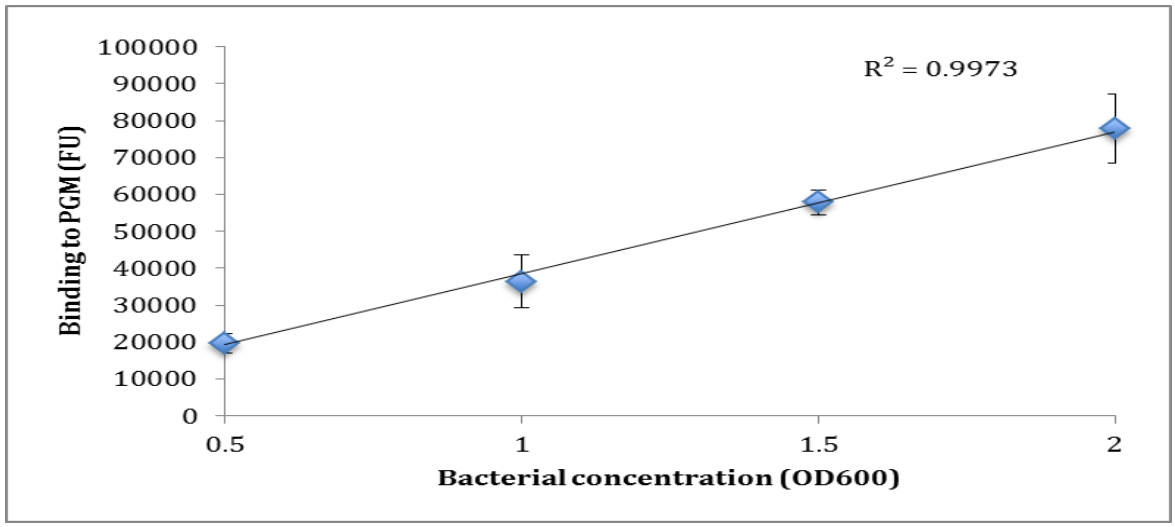

Figure 6: The binding of different concentrations of PASS01 (O.D $_{600nn}$ - 0.5, 1, 1.5, and 2) to PGM. PGM (50 µL) was applied to the PVDF wells at a concentration of 1 mg/ml in the protein immobilisation step of this assay. Experiment was carried out in technical triplicates Error bars indicate one standard deviation (SD) above and below the mean. FU: fluorescence units.

In order to test for the optimal concentration of PGM used in the glycoprotein immobilisation step of the assay (i.e. to block the PVDF membrane and ensure minimal bacteria-PVDF binding occurs), different concentrations of PGM were tested against binding of Pseudomonas strain PAO1 OD_{600nm}=1. A high fluorescence was found with PVDF wells containing no PGM (Figure 7), indicating strong binding of the bacteria to PVDF. A gradual decrease in fluorescence was detected with the increase in PGM concentration, which

reflects gradual blocking of the PVDF with the protein. A gradual increase in fluorescence was then found with 50 µl of 1 mg/ml PGM; this increase then plateaus with no further increase resulting from an increase in PGM concentration indicating that the PVDF had become saturated with protein with any extra PGM being washed away (Figure 7). The same experiment was performed for PASS02 and PASS04 and a similar conclusion was reached (Figure 3, supplementary data).

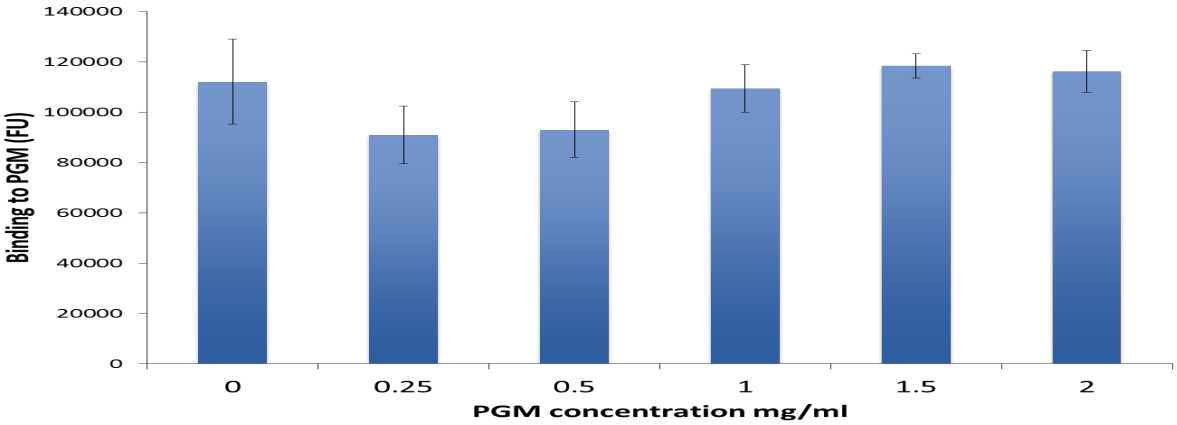

Figure 7: The binding of PAO1 (OD600=1) to PGM, which had been immobilised into the PVDF wells using 50 µl of different PGM concentrations (0-2 mg/ml). The experiment was carried out in technical triplicates, and error bars indicate one standard deviation above and below the mean. FU: fluorescence units.

3.1.3 Comparison of the adhesion of *P. aeruginosa* strains to PGM

Five different *P. aeruginosa* isolates PAO1, PASS1, PASS2, PASS3 and PASS4 were tested for their adherence affinity to PGM, using 50 µL of 1 mg/ml PGM in the protein immobilisation step and a bacterial concentration of OD600=1 for the binding step as optimal parameters. The consideration of each strain having different ability to take up the SYBR®Green dye was accounted for by factoring the fluorescence of 50 µl of each strain at an OD600 of 1 into the calculation of final adherence. Figure 8 is a result of three separate experiments, each of which used samples in triplicate, and shows the relative binding of each strain in comparison to PAO1. Only 2% background fluorescence was seen from the wells containing only PGM without fluorescent bacteria. The binding of strains PASS2, PASS3, was significantly lower than the laboratory strain PAO1, whereas the binding of PASS4 was significantly higher than PAO1.

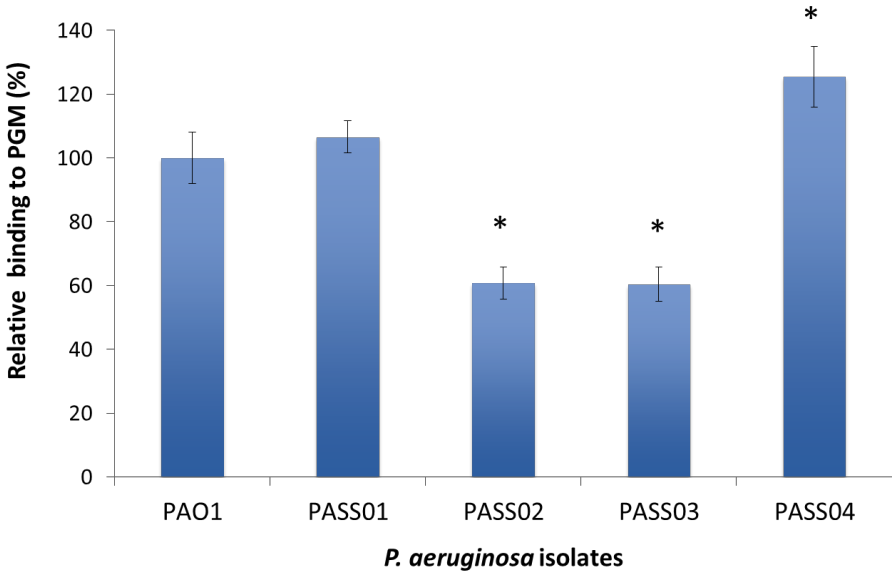

Figure 8: The adherence of five *P. aeruginosa* isolates (PAO1, PASS01, PASS02, PASS03, and PASS04) to PGM. The graph shows the percentage of binding of all strains with clinical isolates compared to the reference strain PAO1 from each experiment, based on biological replicates (n=3) done in technical triplicates. Relative binding is adjusted to account for varied uptake of the fluorescent dye by each strain. Significantly different adhesion relative to PAO1 is indicated by (*).

3.2 Effect of pre-incubation with glycans on bacterial binding

To investigate the ability of glycan-containing compounds to inhibit the adhesion of *P. aeruginosa* to PGM, PAO1 was preincubated in fucoidan before application to the optimised assay procedure as described above. Pre-incubation in 0.1 and 0.25 mg/ml fucoidan significantly inhibited PAO1 binding to PGM (Figure 9).

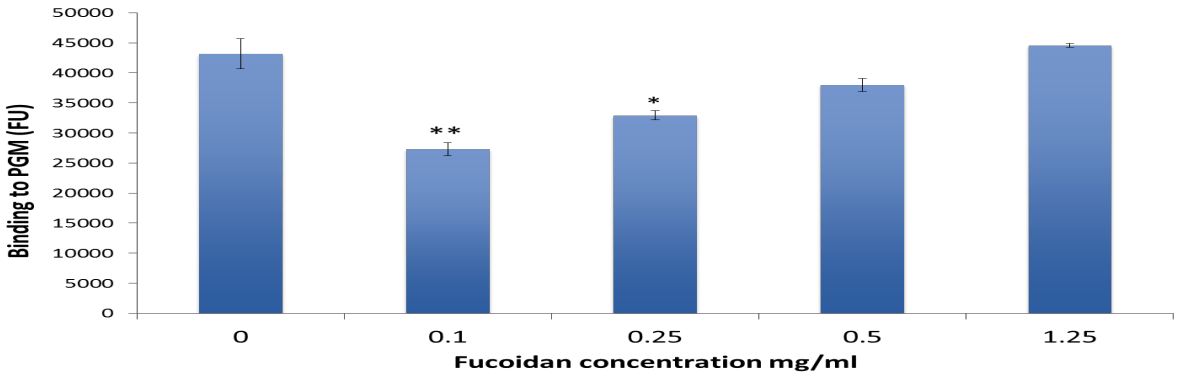

Figure 9: The binding of PAO1 to PGM after pre-incubation of the bacteria with different concentrations of fucoidan (0-1.25 mg/ml), based on biological (n=3) and technical triplicates. Asterisks indicate significant (*, p-value<0.05) or highly significant(, p-value<0.01) differences in *P. aeruginosa* binding compared to the control (no fucoidan).**

Higher concentrations of fucoidan resulted in an actual gradual increasing bacterial binding to PGM. This result seemed, at first, surprising but was actually consistent with previous work in the laboratory which had demonstrated the ability of fucoidan to inhibit bacterial binding at low concentrations, but that the inhibition ceased at higher fucoidan concentrations, and in many cases began to increase the bacterial binding. The reason for this is not known, although it has been speculated that higher concentrations of the polymer might create an agglutination effect. It was considered that ITC might provide greater insight to what was happening in-solution.

3.3 Optimisation of ITC for investigating complex protein-glycan adhesion

MicroCalAuto ITC200 was first calibrated and tested by titrating 120 µl of 5 mM $CaCl_2$ into 0.4 mM EDTA (Figure 4 supplementary data). Good saturation was achieved in this experiment and the molar ratio was 1, which showed the machine was working well. After that, 120 µl of different fucoidan concentrations were titrated into 400 µl 0.4 mg/ml of PAO1 membrane proteins. Since molar concentration is not obtainable from complex mixtures, only the change in enthalpy was measured; the higher the heat released, the higher the binding between protein and ligand is considered to occur. As seen from Figure 10, there was a great increase in the heat profile with an increase in fucoidan concentration. Binding was barely detectable when 1 mg/ml fucoidan was titrated against 0.4 mg/ml PAO1 membrane proteins, and no saturation was seen for values below 1 mg/ml fucoidan concentration. Titrating 0.4 mg/ml PAO1 membranes against 5 mg/ml of fucoidan generated around 0.8 µcal/sec heat, whereas 10 mg/ml fucoidan generated about 1.5 µcal/sec and saturation was observed indicated by the c-shape curve.

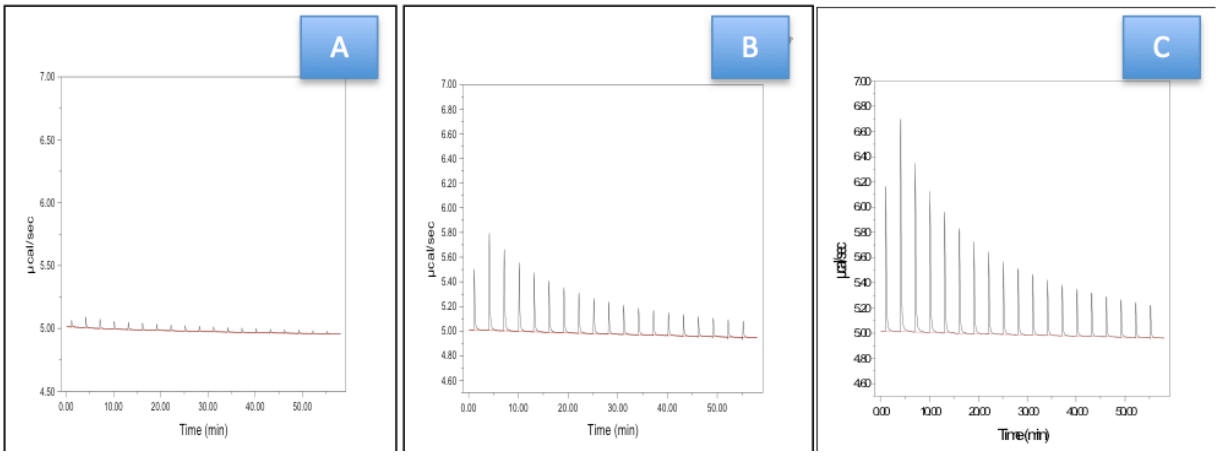

Figure 10: Titration of different fucoidan concentrations into a constant amount of PAO1 extracted membrane proteins (0.4 mg/ml) using ITC technique. (A) 1mg/ml fucoidan (B) 5mg/ml fucoidan (C) 10mg/ml fucoidan. A scale of heat (µcal/sec) generation is plotted against time (min). Saturation was observed at 10mg/ml fucoidan.

To investigate the above interactions further, a number of control experiments were set up; buffer (50 mM Tris/Hcl; 15 mM $CaCl_2$: 3 % w/v CHAPS: 8 M urea pH 7.5) was titrated against itself, fucoidan was titrated against buffer and PAO1 membranes were titrated against buffer (Figure 11). As expected, there was negligible heat released from titrating buffer against buffer. A small amount of heat (0.02 ucal/sec) resulted from the titration of buffer into 0.4 mg/ml PAO1 membrane proteins. However, titration of 0.25 mg/ml fucoidan against buffer generated some heat but increasing the concentration of fucoidan to 10 mg/ml gave a substantial heat generation of 0.55 µcal/sec. Since this did not appear to be a buffer exchange issue as the control of buffer against buffer showed very little heat generation, it was speculated that the heat generated from higher concentrations of fucoidan (without the presence of the bacterial membrane proteins) may have been a result of transient self-aggregation of fucoidan .

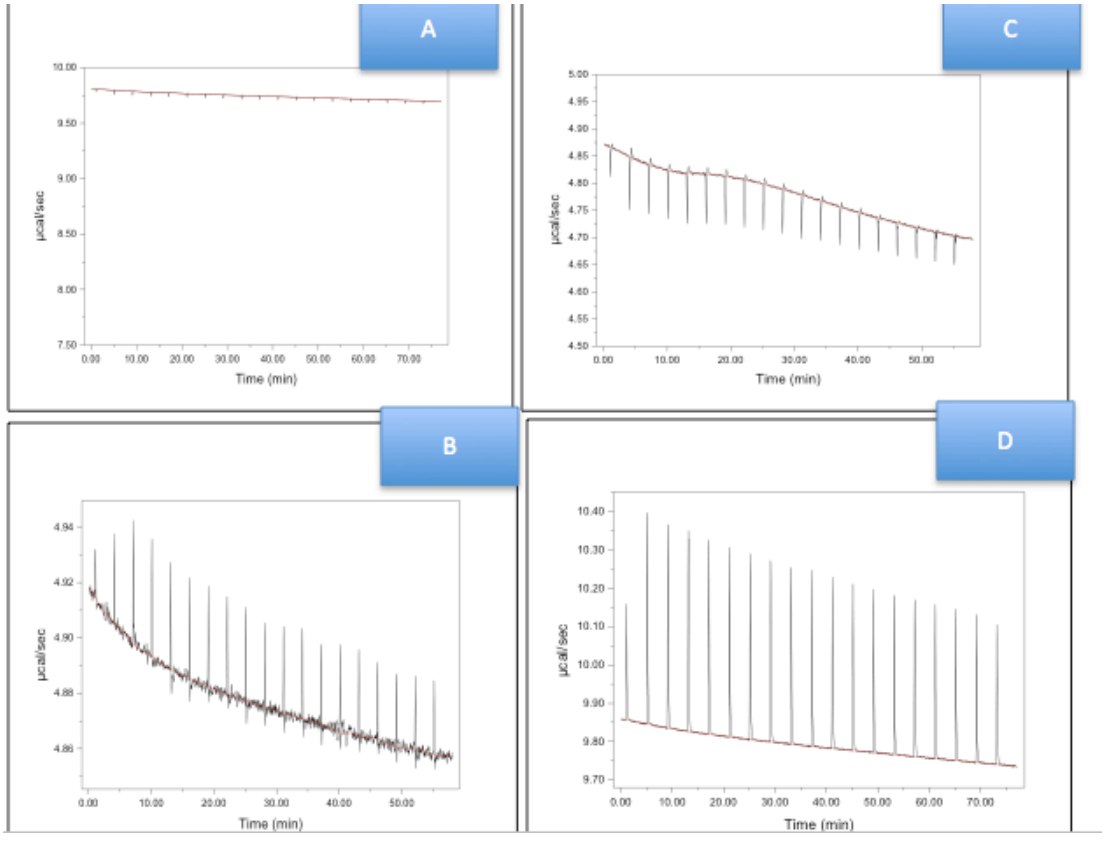

Figure 11: Controls of ITC experiments. Titration of (A) buffer against buffer, (B) buffer against 0.4 mg/ml PAO1 membrane proteins; (C) 0.25mg/ml fucoidan against buffer;, and (D) 10mg/ml fucoidan against buffer. 10 mg/ml fucoidan against buffer generated a high amount of heat (0.55 µcal/sec).

Different concentrations of porcine gastric mucin (PGM, sigma Aldrich) were then titrated against the buffer. There was background heat generation of 0.12 µcal/sec from titrating 120 µl 2.5 mg/ml PGM into buffer, while 5 mg/ml PGM titration produced 0.24 µcal/sec. This again presented unacceptable background heat and suggested the possibility of PGM transiently binding to itself (Figure 12).

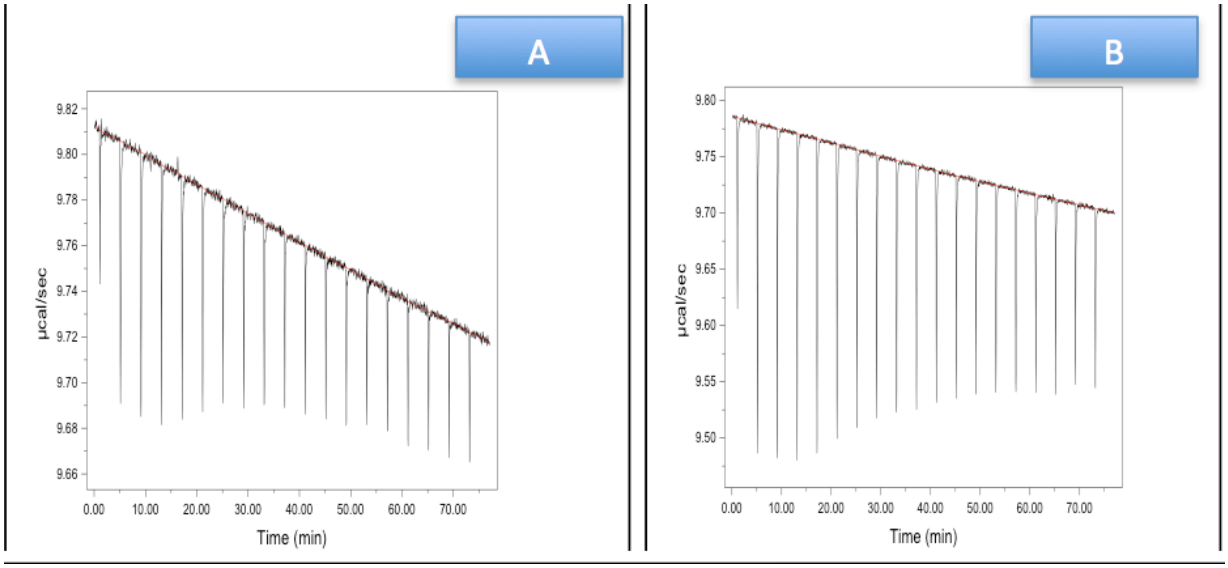

Figure 12: Two different PGM concentrations, (A) 2.5 mg/ml and (B) 5 mg/ml, titrated into buffer, resulting in heat generation of 0.12 µcal/sec and 0.24 µcal/sec, respectively.

In summary, ITC was able to provide in-solution measurements of fucoidan binding to membrane proteins extracted from PAO1. However, several difficulties were faced with the ITC approach. The first issue was the high concentration of glycan ligand (fucoidan) that was needed to reach saturation of the membrane proteins (10 mg/ml). The second issue arose from the high amount of heat generated by fucoidan alone at such a high concentration, possibly due to transient binding of fucoidan to itself. The apparent ability of fucoidan to self-aggregate provided some possible insight into the plate assay results in which although inhibitory when the bacteria were pre-incubated at low fucoidan concentrations, the higher concentrations resulted in increased adhesion of bacteria to PGM (discussed further in Section 4). However, the high background heat generated by both fucoidan and PGM suggested that ITC was not a suitable technique under the tested conditions to elucidate intricate differences in binding of the *P. aeruginosa* isolates that

were the focus of this thesis. On the other hand, the plate assay had been successfully optimised and shown significant and reproducible results of *P. aeruginosa* binding to PGM. As a result, the plate assay was chosen for testing the adherence of the chosen *P. aeruginosa* strains to human epithelial surfaces.

3.4 The adhesion of *P. aeruginosa* strains to human epithelial membrane proteins

All of the five *P. aeruginosa* isolates were tested for their binding to membrane proteins of human lung (A549) and mucin-secreting colon (LS174T) epithelial cells using the optimised plate assay technique. The latter cells were chosen as they are well-described high producers of mucins that are known to coat the surface of epithelial cells *in vivo*. The human cell membrane proteins were immobilised onto the plate using 50 µL of 1 mg/ml protein per well, and bacteria were applied at on OD600 of 1.

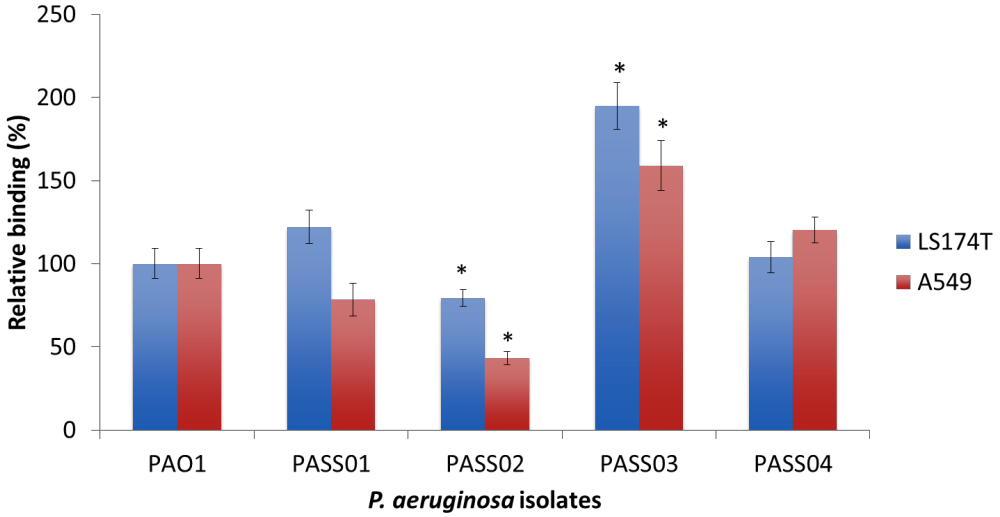

Figure 13: The adherence of *P. aeruginosa* isolates to membrane proteins isolated from human lung (A549) and mucin-secreting human colon (LS174T) epithelial cells. The graph shows the percentage of binding of all strains when compared to PAO1 based on technical and biological triplicates. Data is normalised to account for varied uptake of the dye by the different strains. Significant differences in binding compared to PAO1 is indicated according to Student t-test, p<0.01 (*).

There were no significant differences in adhesion to human lung epithelial cells compared to adhesion to mucin-secreting human colon epithelial cells by any of the *P. aeruginosa* strains. Data was normalised to PAO1 adhesion and compared between strains (Figure 13). There were significant differences in adhesion between the strains with CF isolate PASS02 the weakest binder and CF isolate PASS03 the strongest binder. CF isolates PASS01 and

PASS04 exhibited a similar adhesion as the lab strain PAO1, which was intermediate between PASS02 and PASS03.

3.5 Treating A549 membrane proteins with exoglycosidases and its effect on *P. aeruginosa* adherence

Since *P. aeruginosa* strains exhibited a similar binding profile to A549 lung or mucin secreting LS174T colon epithelial membrane proteins, the lung cell line A549 was chosen to show the effect of exoglycosidase pre-treatment of the human cell membrane proteins on *Pseudomonas* binding. In addition, the lung epithelial cell line was chosen instead of colon epithelial cell line since it gave slightly smaller standard deviation among isolates and since it is a better representation of the respiratory epithelial cells that are the typical site of *P. aeruginosa* infection in CF. Optimisation of glycosidase treatment was carried out using fetuin prior to application of the exoglycosidaes to the epithelial membrane proteins.

3.5.1 Optimisation of sialidase and galactosidase treatment using fetuin

Treatment of fetuin with various concentrations of glycosidases was first investigated using SDS-PAGE (Figure 14). The molecular weight (MW) of fetuin dropped to a similar extent following treatment with 0.25mU sialidase (Gel A; lane 1) as it did with 0.5mU sialidase treatment (Gel A; lane 2) suggesting the same degree of sialic acid cleavage by both enzyme concentrations. When 0.25 mU sialidase and 0.25 mU galactosidase were used simultaneously (Gel B; lane 4), there was a further reduction of fetuin MW, and another shift as galactosidase concentration was increased from 0.25 to 0.5 (Gel B; lanes 5); however, a further reduction in MW was not evident using 1 mU galactosidase (Gel B; lane 6). Sequential treatment with 0.25 mU sialidase (overnight), followed by 0.5 mU galactosidase (overnight), resulted in the same drop in MW as seen by the simultaneous treatment with these enzyme concentrations (Gel B; lane 7). Therefore, 0.25mU sialidase and 0.5 mU galactosidase were chosen as appropriate concentrations for treatment of 50 μg of fetuin, to be examined more closely by mass spectrometry for terminal epitope cleavage.

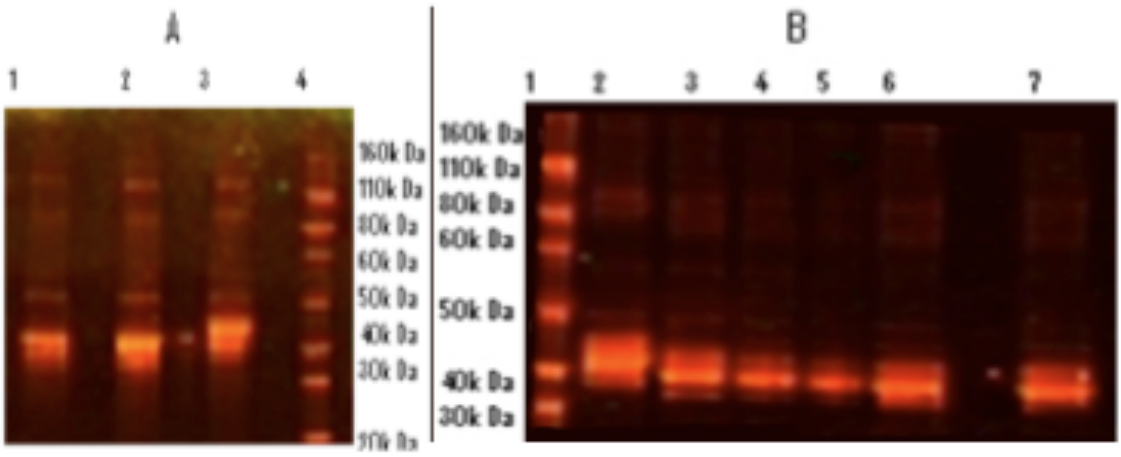

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Figure 14: SDS-PAGE analysis of glycosidase treatment of fetuin. Gel A: Lane 1: 0.25mU sialidase/50 µg fetuin; Lane 2: 0.5mU sialidase/ 50 µg fetuin; Lane 3: fetuin (no glycosidase treatment); Lane 4: MW marker. Gel B: Lane 1: MW marker; Lane 2: fetuin (no glycosidase treatment); Lane 3: 0.25mU sialidase/50 µg fetuin; Lane 4 to Lane 6: 0.25mU sialidase (plus 0.25mU galactosidase (Lane 4), 0.5mU galactosidase (Lane 5), and 1mU galactosidase (Lane 6)); Lane 7: Sequential treatment with 0.25mU sialidase (overnight), followed by 0.5mU galactosidase (overnight).

To determine whether the exoglycosidases were active, the *N*-linked glycans were released from fetuin, with and without glycosidase pre-treatments, and were characterised by mass spectrometry. The successful cleavage of terminal sialic acid by 0.25 mU sialidase was verified by the significant reduction in disialyated biantennary core-fucosylated glycan of *m/z* 1185.0 and corresponding increase of the asialylated version of the same structure at *m/z* 893.9 (Figure 4 5 supplementary data). The additional treatment with 0.5 mU galactosidase significantly reduced the sialidase generated structure of *m/z* 893.9, and increased the two mono-galactosylated biantennary core-fucosylated isomers of *m/z* 812.9; structures which differ only by the location of the one remaining terminal galactose, thus indicating at least partial cleavage of the sialidase-exposed galactose. Therefore, these glycosidase concentrations were considered appropriate for treatment of the A549 lung epithelial membrane proteins, also to be verified by MS.

3.5.2 Verification of glycosidase treatment of lung epithelial membrane proteins by MS

Mass spectrometric analysis of the *N*-linked glycans released from A549 lung epithelial membrane proteins before and after glycosidase treatment(s) revealed the same mass

shifts as seen in the fetuin *N*-linked glycan profile regarding the complex glycan *m/z* 1185 (Figure 15).

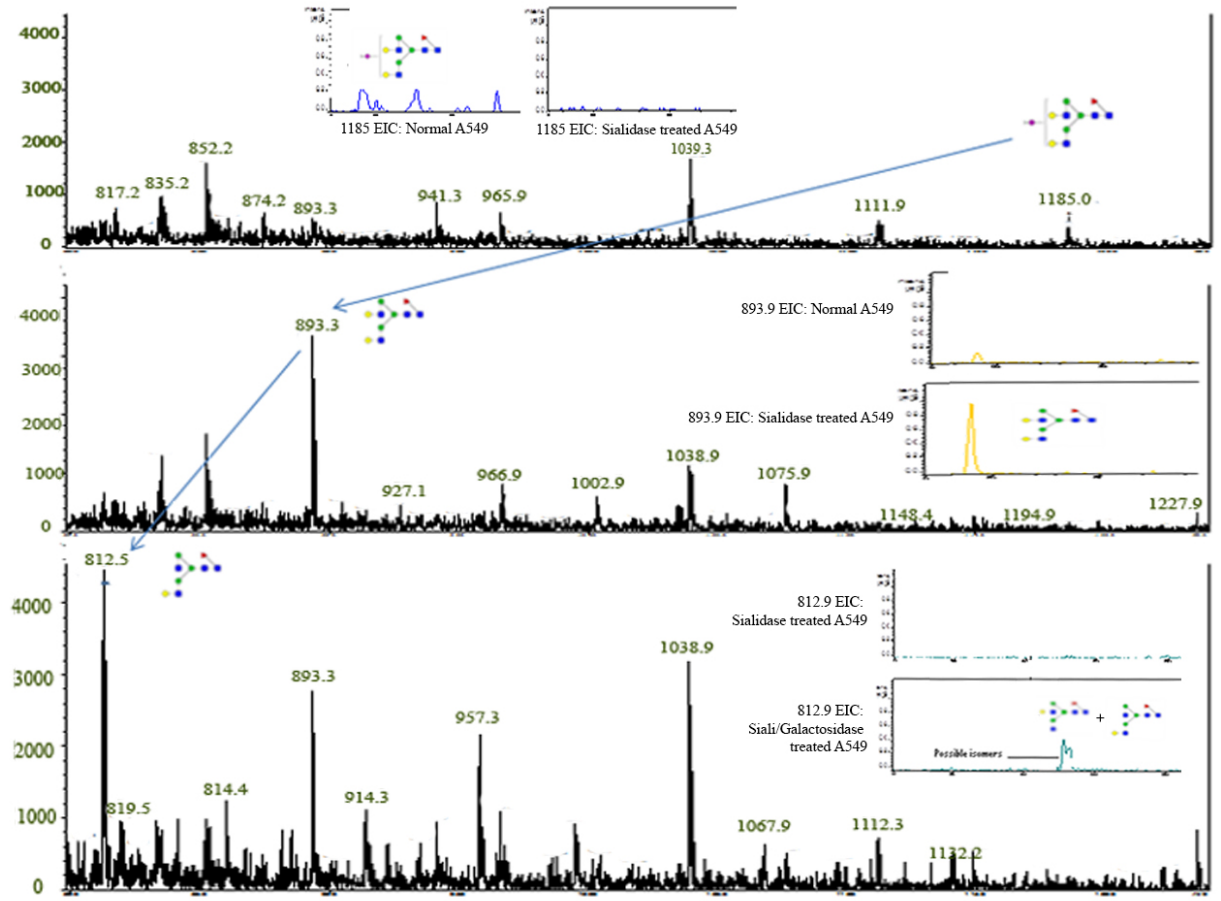


Figure 15: Summed mass spectra from 35-60 mins of *N*-glycans released from A549 lung epithelial cell membrane proteins (top), following sialidase treatment (middle) and sialidase + galactosidase treatment (bottom). Extracted ion chromatograms at particular m/z masses are inset. A significant loss of the disialylated biantennary glycan *m/z* 1185 (doubly charged ion) and a significant gain of completely desialylated biantennary glycan *m/z* 893.9 (doubly charged ion) demonstrates successful sialidase treatment. The addition of galactosidase treatment has resulted in the gain of the mono-galactosylated biantennary glycan of *m/z* 812.9 previously not seen in sample treated with sialidase only, demonstrated successful partial galactosidase treatment.

In addition to the above example, the full analysis of the *N*-linked glycans released from the lung epithelial cells, with and without glycosidase treatments, was carried out (Supplementary table 1). Although three rounds of glycan release and analysis are typically

performed, only one release and analysis was performed in this project due to the limited time available. Distinctive changes in the terminal residues of the *N*-linked glycans were evident, as summarised in Figure 16. As expected, sialidase treatment decreased the amount of terminal sialic acid by more than half, and increased the amount of terminal galactose by 20%; subsequently, upon galactosidase treatment, the exposed terminal galactose then decreased by almost half and the underlying sugar, GlcNAc, appeared as a terminal epitope at around 30% relative abundance. The relative abundance of high mannose glycans was used as a control to normalize the relative percentage abundance, since both of the glycosidases used do not target mannose. High mannose structures remained unchanged across samples with a relative abundance of around 70% (Figure 16).

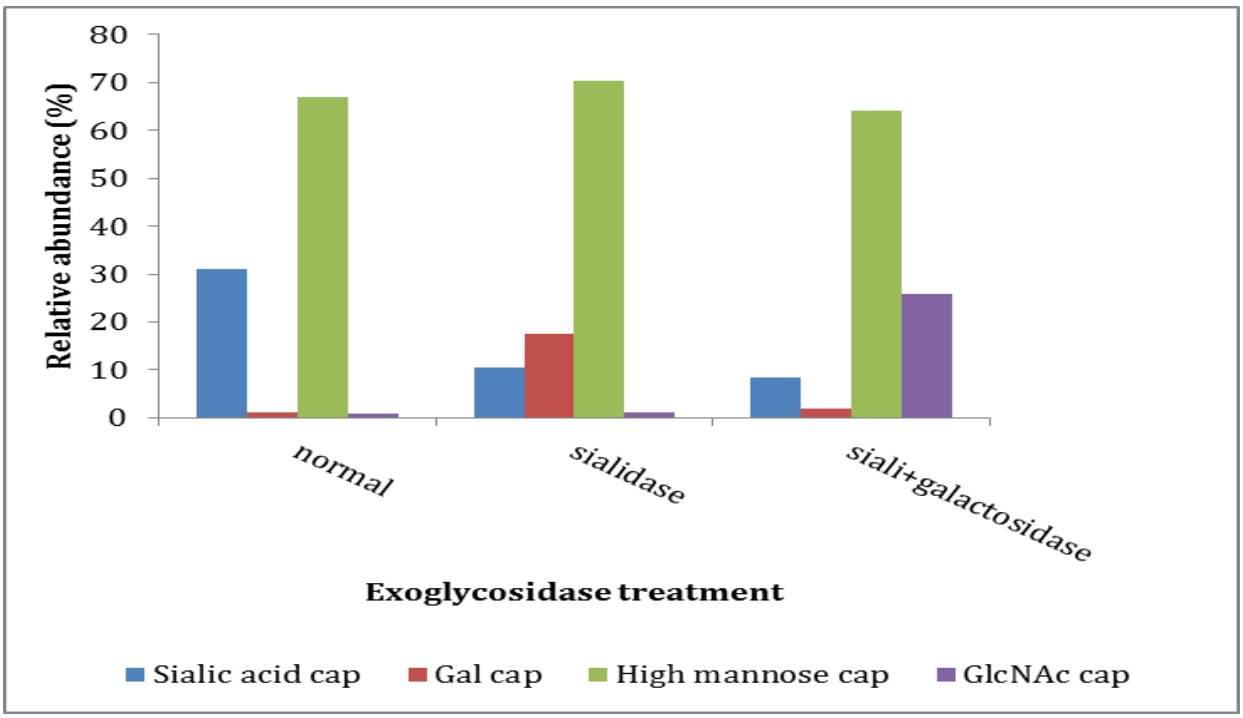


Figure 16: The relative abundances of *N*-linked glycans capped with terminal sialic acid, galactose, mannose (high mannose glycans) or GlcNAc, released from A549 lung epithelial membrane proteins before glycosidase treatment (normal), and following sialidase and sialidase + galactosidase treatment.

3.5.3 The effect of glycosidase treatments on the adhesion of *P. aeruginosa* to A549 lung epithelial membrane proteins

All of the five *P. aeruginosa* isolates (PAO1, PASS01, PASS02, PASS03, and PASS04) were tested for their binding to the A549 lung epithelial membrane proteins, without and with prior glycosidase treatment of the membrane proteins as described above (control, 0.25 mU

sialidase, and 0.25 mU sialidase + 0.5 mU galactosidase). Two independent experiments were performed with triplicates for each strain (Figure 17). Overall, there were significant differences in binding as a result of the enzymatic removal of terminal epitopes. Sialidase treatment of the human lung cell membrane proteins increased the binding of all isolates but, when followed with galactosidase, the adhesion of PAO1 and PASS1 decreased. The binding of PASS02, PASS03 and PASS04 was not significantly affected by the galactosidase treatment.

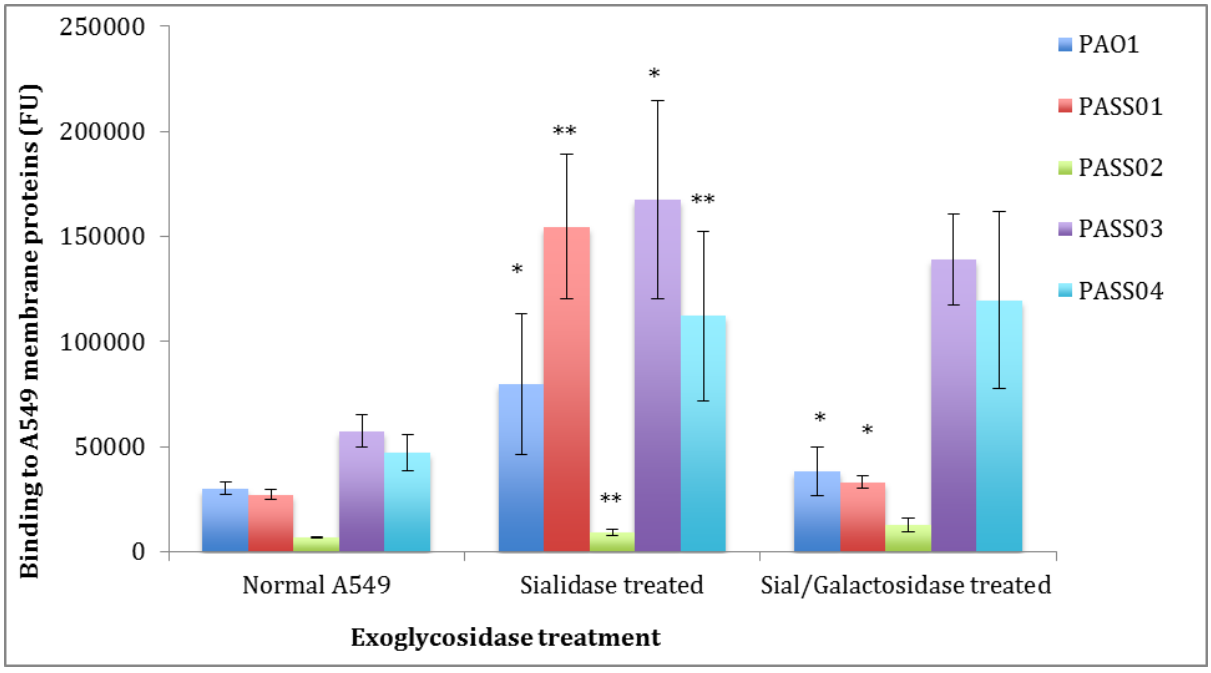


Figure 18: The effect of glycosidase treatment of A549 lung epithelial cell membrane proteins on the adherence of *P. aeruginosa* isolates (PAO1, PASS01, PASS02, PASS03, and PASS04). "Sialidase treated" membrane proteins were subjected to 0.25mU sialidase to remove terminal sialic acid epitopes, whereas "Sial/Galactosidase treated" membrane proteins were subjected to sequential treatments of 0.25mU sialidase and 0.5 mU galactosidase to remove sialic acid and galactose. All *P. aeruginosa* isolates experienced significant (*, p-valuse<0.05) or highly significant (, p-value<0.01) increase in binding upon sialidase treatment, while the addition of galactosidase treatment resulted in a significant decrease in the binding of PAO1 and PASS01 only.**

Discussion

Chapter 4

4. Discussion

In this study, two main techniques were employed to investigate the involvement of glycans in *P. aeruginosa* adhesion to epithelial surfaces. Ligand titration with ITC seemed to be unsatisfactory for accurately measuring the binding of the complex bacterial cell membrane proteins to complex glycoconjugates due to the generation of high background heat from possible self-aggregation of the glycan sources tested (fucoidan and PGM), which compromised the ability to measure the heat released from binding to receptors. On the other hand, the in-house plate assay was successfully optimised with PGM for the investigation of the adhesion of different *P. aeruginosa* clinical isolates, and was subsequently used to measure binding of the bacteria to membrane proteins extracted from human epithelial cell lines. Interestingly, different *P. aeruginosa* clinical isolates were shown to have different adhering abilities and these differed from that of the *P. aeruginosa* standard reference strain. Also, different responses to glycosidase treatments of the epithelial glycans were found among these isolates, indicating diversity in their preferred binding epitopes.

4.1 Intrinsic advantages of the in-house plate assay

Despite the importance of the initial bacterial adhesion stage in the infection process, few approaches are available for such measurements. The plate assay developed in this laboratory relies on SYBR®Green incorporation into bacterial nucleic acid, and subsequent measurement of fluorescence. Theoretically, the incorporation of SYBR®Green into bacterial nucleic acid should not interfere with bacteria surface proteins, which makes this dye suitable for this sort of interaction studies. SYBR®Green is a potentially toxic dye ([74]), but was used at a low concentration in the assay and previous work in the laboratory had demonstrated no loss of cell viability in the time frame of the assay procedure (Robyn Peterson, unpublished data). Also, the incorporation of SYBR®Green, enabling bacterial adhesion enumeration by fluorescence, provides a higher throughput compared to the traditional approaches that required re-culturing and/or colony counting ([60]; [61]). This approach involves whole, live bacterial cells accommodating all their natural surface adhesins and lectins, and maintains an upward orientation of the glycans on proteins hydrophobically bound to the PVDF plate, making the model representative of *in vivo* interactions.

Previously, the plate assay has been used to investigate glycan-mediated adhesion of gut pathogens to human colon epithelial membrane and milk proteins (unpublished data, Robyn Peterson and Clara Cheah). In addition, a modification of the assay has been used to demonstrate the change in the binding of ocular isolates of *P. aeruginosa* to tear fluid glycoproteins, known to carry sialyated and fucosylated structures, after incubation with monosaccharaides or after enzyme modifications of the glycans (Liisa Kautto, unpublished data). As a result, the plate assay seemed to be a good candidate for investigating complex glycan-protein interactions as a model of the lung environment, but required optimisation for the *P. aeruginosa* strains of interest and use of glycoproteins that could be representative of the CF airways.

4.2 Optimisation of the in-house plate assay for this study

Several evaluation and optimisation steps were required to adapt the in-house plate assay for this project. First of all, there was a need to determine the growth curve of the *P. aeruginosa* isolates due to the limited knowledge of their growth behaviour (Figure 5). This was particularly important in the context of the plate assay because previous work in the laboratory had shown an increase in adhesion from bacteria harvested in the log phase, possibly due to the increased cell viability and an increase in cell wall hydrophobicity ([75]). In the log phase, bacteria are multiplying readily due to plentiful resources in the medium, and cell viability is not compromised due to nutrient limitation or accumulated waste as it is in the stationary phase ([76]). All strains exhibited a log phase of growth between 6-8 hours so this was considered an appropriate window of time for harvest for the assay.

It was also necessary to choose a suitable glycoprotein to immobilise to the PVDF membrane of the 96 well plate in order to represent the respiratory airways. For this preliminary work, porcine gastric mucin (PGM) was chosen because it resembles the mucin that is abundant in the CF lungs since it contains abundant Core 1 and Core 2 *O*-glycans (Figure 1) ([77]),, is commercially available at a reasonable cost and is known to have various glycan epitopes shown to be implicated in *P. aeruginosa* adherence to CF epithelium; this includes various siaylated and non-siaylated Lewis structures ([78]). It was necessary to ensure good blocking of the PVDF membrane with the PGM because past research in the laboratory had shown a strong binding of bacteria to the PVDF alone. Findings from this study also demonstrated a strong binding of *P. aeruginosa* to the PVDF. This was not only of

importance for the assay but also implies potential adhesion of the pathogen to synthetic materials in the clinical and hospital settings, which may increase the spread of associated disease. Secondly, there was a need to examine the significance of the relationship between bacterial numbers and fluorescence in the assay; this was confirmed by the gradual increase in fluorescence when increasing the concentration of PASS01 and PASS03 strains applied to the PVDF wells (Figure 6; Figure 1 supplementary). These results demonstrated a reasonable sensitivity for this technique to quantitatively detect bacterial binding to PGM.

After modifying the assay successfully for this study, a preliminary assessment was made of the relative adhesion of the chosen *P. aeruginosa* isolates to the PGM. The in-house clinical isolates (isolated by Vignesh Venkatakrishnan, unpublished data) have been reported with different phenotypic traits, biofilm formation, and level of virulence (unpublished data, Penesyan Ani), and they also exhibited differences in their binding profiles. When comparing clinical isolates to the laboratory reference strain PAO1, PASS3 and PASS2 had a significantly weaker ability to adhere to PGM, whereas PASS01 and PASS04 had a similar binding affinity as PAO1 (discussed further in Section 4.5 below).

4.3 Fucoidan as a potential inhibitor of *P. aeruginosa* adhesion to mucin

Fucoidan is a sulfated fucose polymer derived from brown seaweed that has been found in previous work in the laboratory to inhibit the adhesion of pathogenic bacteria to human epithelial glycoproteins. There are many different forms of fucoidan, depending on the species of seaweed from which they are derived. The fucoidan used in this work was derived from *Undaria pinnatifida* and contains some galactose as well as fucose ([62]; [79]). Interestingly, this same fucoidan, *U. pinnatifida,* have shown antiviral activities against herpes simplex virus (HSV1-2) ([80]). The cell surface of herpes virus-infected cells is reportedly elaborated with various sulphate chains as well as galactose-rich glycoproteins ([81]; [82]), which suggests that the inhibitory effect of fucoidan on the virus may be due to the sulphate and/or galactose presented on its structure. Since both fucose and galactose are known to bind to *P. aeruginosa* lectins, PA-IIL and PA-IL, respectively, it was considered that *U. pinnatifida* fucoidan could provide a good compound to try to competitively inhibit *P. aeruginosa* binding. Also, the increase in fucosylated structures in CF membrane bound

mucins may imply involvement of fucose in *P. aeruginosa* infection and possibly initial adhesion ([41]).

The optimised plate assay was implemented to investigate the ability of fucoidan to inhibit the binding of *P. aeruginosa* to PGM (Figure 9). A 0.1 mg/ml concentration of fucoidan was able to greatly decrease PAO1 binding to PGM, but a gradual increase in PAO1 binding was seen thereafter with the increase in fucoidan concentrations. In fact, 1.25mg/ml of fucoidan showed little change in PAO1 binding from the control (no fucoidan). These results were consistent with previous work in the laboratory that had shown the ability of fucoidan to inhibit bacterial adhesion was optimal at 0.1 mg/ml or less and decreased with increasing concentrations of fucoidan; this continued to the point where an increase in bacterial binding to human epithelial cells was observed after pre-incubation in fucoidan concentrations above 1 mg/ml. A speculated cause of the phenomenon was that the higher concentrations of fucoidan could be causing agglutination of the bacteria and/or the fucose polymer itself. However, the plate assay could not provide insight into the interactions on a molecular level. The recent availability of ITC presented a potentially attractive method to gain greater insight into these bacterial/glycan interactions in-solution.

4.4 Assessing the suitability of ITC for measuring complex interactions between glycans and bacterial membrane proteins

Techniques such as the plate assay are often used for early screening in order to find promising data, and then more rigorous characterisation is performed on those significant indications. Several strategies are available for researchers to detect and rigorously characterise protein –protein interactions but few have the ability to elucidate the often multivalent, complex protein-glycan interactions. Of these techniques, some require genetic manipulation or fluorescent labeling, but in-solution label-free techniques, such as ITC, do not require genetic or chemical alterations to the native molecules keeping them more representative to their *in vivo* state ([83]).

In a previous published study, the *P. aeruginosa* lectin PA-IIL was tested for binding to human milk oligosaccharides, and ITC was able to demonstrate the ability of a fucosylated oligosaccharide (with Lewis^a epitope) to bind to this lectin with a higher affinity than to

individual monosaccharides, including fucose ([45]). There are also a few reported cases where ITC was used to study the binding of whole bacterial cells ([40]; [67]), but these studies were only successful in measuring the binding to small peptides and not complex mixtures of ligands. Also, the enthalpy change of only one ligand injection was measured in these studies.

In light of the above studies, it was considered that ITC could have the capability of measuring change in heat generated from the binding of extracted bacterial membrane proteins to complex glycoproteins, using several injections to obtain a decent saturation curve. As a result, this part of the thesis work was an initiative to test the ability of ITC to investigate complex interactions of this nature, using extracted membrane proteins from PAO1, fucoidan and porcine gastric mucin as examples. The two well-known lectins of *P. aeruginosa* (PA-IL and PA-IIL) are known to coordinate Ca^{2+} ions in their binding to ligands ([28]; [29]). The Ca^{2+} ions are needed within the binding pocket resulting in a higher affinity constant; this was shown with Lewis^a binding to PA-IIL when Ca^{2+} provided higher binding affinity compared to normal protein-carbohydrate interactions ([28]). As a result, $CaCl_2$ was added to the buffer used in ITC experiments.

Saturation was observed when 10mg/ml fucoidan was titrated into 0.4 mg/ml PAO1 membrane proteins, but binding and saturation was not clearly evident at lower concentrations of fucoidan. However, control experiments demonstrated that fucoidan at this same concentration generated heat when titrated only against the buffer (Figure 11). Since the buffer was the same as that used in the fucoidan sample, it did not seem likely that it was the titration into the buffer that caused the heat generation as such. The most likely possibility was that fucoidan was binding to itself as there were no other potential adhesins present. Furthermore, a similar heat generation was also seen from PGM alone, again suggesting self-aggregation of the mucin. Regardless of the cause, the background heat generated by the fucoidan (or PGM) alone, when applied at concentrations at which binding to the membrane proteins could be detected, suggested that the current ITC protocol was not suitable for detecting subtle differences in the adhesion of *P. aeruginosa* membrane proteins to complex glycans of interest to this thesis. Since there was limited time to optimise the technique further, ITC was not continued for this study. On the other hand, ITC was able to provide some insight into a phenomenon that had puzzled the laboratory for

some time, namely that incubation in high concentrations of fucoidan tended to increase the binding of bacteria to immobilised glycoproteins in the plate assay, whereas low concentrations of fucoidan inhibited bacterial adhesion. The binding of fucoidan to itself could result in aggregation and associated agglutination of the bacteria, resulting in the observed increase in the plate assay binding.

4.5 Investigating the adhesion of *P. aeruginosa* strains to human epithelial cells

The in-house plate assay thus appeared to represent the best technique for further study and comparison of the adhesion of the CF *P. aeruginosa* strains to glycoproteins representative of the respiratory airways. The use of PGM in the preliminary assays was appropriate to enable optimisation of the plate assay technique for this study, but for the next stage of the work better models of human epithelium were chosen. The mucin-secreting human colon epithelial cell line LS174T was chosen in order to project the human epithelium aspect as well as the mucin aspect of adherence, which is of heightened importance in CF. The human lung epithelial cell line A549 was more typical of the respiratory tract but without the secreted mucin seen in CF patient lungs.

Membrane proteins from LS174T and A549 cell lines were extracted by the use of differential detergent fractionation (DDF). This technique employs sequential detergent treatments portioning cellular proteins into structurally and functionally distinct fractions, and also is appropriate for limited amount of biomaterials ([84]; [85]). While carbonate extraction relies on size and density to divide membrane proteins, DDF has the advantage of preserving the integrity of sub-cellular membrane proteins such as microvesicles ([72]). LS174T cell lines have been shown to secrete glycoproteins by forming microvesicles, which make DDF more suitable for these eukaryotic cell lines (Moh E Honours thesis, 2013). Finally, DDF is able to recover a large proportion of the eukaryotic membrane proteome making it superior to carbonate extraction for this study ([72]).

The adherence of all of the *P. aeruginosa* isolates to the LS174T mucin-secreting colon epithelial membrane proteins was found to be very similar to their adhesion to the A549 lung epithelial membrane proteins (Figure 13), suggesting the two cell lines present similar binding epitopes to the bacteria. Although LS174T cells are known to secrete mucins, a feature that the A549 cells do not exhibit, it is possible that the secreted mucins may not

have been captured in the membrane preparation procedure. Cell surface bound membrane mucins were probably recovered, but both lung and colon epithelia have been found to contain similar membrane bound mucins, including MUC1, MUC4, and MUC20 ([86]).

The CF isolate PASS02 was again seen as a significantly weaker binder to colon and lung human epithelial membrane proteins, as was observed with PGM. Both PASS01 and PASS04 seemed to bind similarly to the laboratory strain PAO1 as to colon and lung epithelial membrane proteins, while PASS03 was shown to bind significantly more than PAO1. Interestingly, PASS03 had shown a significantly weaker binding to PGM than the control strain (Figure 8), which may be explained by some differences in the epitopes expressed between the human and porcine systems ([87]). For instance, pigs can synthesise a different form of sialic acid (NeuGc , *N*-glycolylneuraminic) that is missing in humans due to an evolutionary genetic mutation ([88]). In addition, sialic acid in the human respiratory tract is predominantly attached by alpha 2-6 linkage, while both alpha 2-6 as well as alpha 2-3 linkaged sialic acid can be found in pigs ([89]).

4.6 Differential adhesion of the *P. aeruginosa* strains in light of their known characteristics

A lot of the published experimental research on *P. aeruginosa* has used the strain PAO1 as a prototype considered relevant to respiratory infection *in vivo*. The PAO1 strain has been fully sequenced ([69]), providing a useful database for identification and genomic and proteomic studies of *P. aeruginosa* clinical strains. However, since PAO1 was originally isolated from a wound, rather than the respiratory tract, and has been kept *in vitro* in culture collections since 1955, its suitability as a model strain for CF studies could be questioned. Observations of this research group in recent years have revealed differences in the glycan-affinities of different bacterial strains of the same species, including lab and clinical strains of *Staphylococcus epidermidis* (Chi-Hung Lin, unpublished data) and ocular and urinary tract isolates of *P. aeruginosa* (Liisa Kautto, unpublished data).

The availability of the annotated PAO1 gene sequence had already prompted use of PAO1 in the wider SuperScience project on CF infection at Macquarie University, and genomic comparisons have been made to the CF strains described in this thesis. A comparison between shared genes between strains shows PASS02 and PASS03 to have the least

similarity with PAO1 (88.18% and 88.80% respectively). These values coincide with both strains having significantly different adherence to human epithelium compared to PAO1. Also, PASS02, being the weakest binder and PASS03, being the strongest, were shown to have the least similarity in gene sequence amongst all other strains (86.40%) (Ani Penesyan, unpublished data; Table 1, Section 2.1).

In the case of PASS02, this strain provided the least adherence to both PGM and human epithelial membrane proteins. Multiple genome alignment with PAO1 revealed an absence of a large cluster of genes in PASS02 that encodes the *Psl* polysaccharide gene responsible for the production of Psl extracellular polysaccharides, which was conserved across all other PASS strains (Ani Penesyan unpublished data). PASS02 does not form biofilms (Ani Penesyan unpublished data), which may be due to the lack of *Psl* ([90]). In contrast, PAO1 is already known as a biofilm-forming strain ([91]), and PASS03 and PASS04 were found to have higher biofilm formation than PAO1 (Ani Penesyan, unpublished data), providing further evidence of the importance of *Psl* in biofilm formation. The plate assay results of this thesis also seem to imply the importance of *Psl* in the initial adhesion of *P. aeruginosa* to the epithelial cell surface since PASS02, that lacks this polysaccharide, has the lowest adherence to the membrane proteins. This is consistent with a previous study where overproduction of *Psl* led to increased *P. aeruginosa* adhesion, and mutation of *Psl* led to decrease in adhesion ([92]; [93]). *Psl* has a helical anchor shape allowing it to promote strong bacterial cell-cell interactions in the early stage of adhesion ([94]). Furthermore, it has been found to have various other functions in signaling, evading immune response, reducing phagocytosis, and antibiotic resistance ([95]; [96]; [97]; [98]).

The CF strain PASS03 was observed to have the highest binding profile to A549 and LS174T human epithelium. During culturing in the laboratory, PASS03 was phenotypically characterised as the most mucoid strain among the CF isolates. Mucoidity is a result of alginate production and is associated with biofilm formation and antibiotic resistance in the CF lung ([99]; [100]; [101]). Other research has also demonstrated a relationship between mucoidity and adhesion; *P. aeruginosa* clinical isolates from sputum of CF patients were tested for binding to tracheal epithelium ([102]), and mucoid strains adhered to ciliated epithelium 10 to 100 fold better than non-mucoid strains. Interestingly, in preliminary proteomics data in our own research group, PASS03 was seen to overexpress several

alginate related proteins compared to all other strains (Karthik Kamath, unpublished data). In comparison, PAO1 has been characterised as a non-mucoid strain ([103]) and had a reduced binding to the epithelial cell membrane proteins in the plate assay in comparison to PASS03. This study suggests an important role played by alginate in *P. aeruginosa* adherence to human lung epithelium.

4.7. The involvement of terminal glycan epitopes in *P. aeruginosa* adhesion

The involvement of glycans in bacterial adhesion to epithelial surfaces has been shown in many independent studies ([9]; [104]), and *P. aeruginosa* has been reported to adhere to various glycan epitopes (Supplementary Figure 5). One speculation for a glycan role in *P. aeruginosa* adherence to CF epithelium involves the aberrant glycosylation found in CF airways, which may result in increased availability of preferred binding epitopes for the pathogen ([32]; [33]; [34]; [35]). Inhibition studies, in which sugar analogues are tested for their ability to inhibit binding, are one way of investigating binding epitopes. A more direct approach is the use of exoglycosidases, which specifically trim terminal monosaccharide components of the structural epitopes, can determine their involvement in the binding. One advantage of using exoglycosidases is that it is not interfering with the basic biosynthetic cellular machinery, but simply eliminating certain epitopes selectively after normal synthesis has been completed.

In this study, there were two main reasons for the suitability of using exoglycosidase treatment for enhancing our understanding of each isolate's glycan binding preference(s). First of all, the monosaccharide components of the terminal binding epitopes that are important for adhesion of each isolate could be determined as the effect of sequential removal of sialic acid and galactose was monitored. Secondly, the *in vitro* sialidase treatment provided a mimic of the action of the natural sialidase produced by *P. aeruginosa*. The pathogen has been shown to encode a sialidase-like enzyme, which catalyses the hydrolysis of sialic acid in various glycoconjugates ([105]). Unlike other microbes such as *E. coli* ([106]), *P. aeruginosa* does not utilise sialic acid as a carbon source ([107]). This was confirmed for all *P. aeruginosa* isolates tested in this work in a Biolog assay carried out in the wider research group, in which none of the strains exhibited respiration on sialic acid ([14]). Instead, the sialidase is thought to assist the pathogen by exposure of the penultimate β-galactose, and asialylated Lewis antigens, which are the preferred binding epitopes of *P.*

aeruginosa lectins ([108]). In fact, this enzyme has been structurally characterised, and the knockout resulted in significant decrease in biofilm formation ([109]).

Since both cell lines gave a similar overall pattern of binding and little significant difference was seen between *P. aeruginosa* binding to colon or lung epithelial cells, one cell line was chosen to show the effect of exoglycosidase treatment (A549 lung). Following optimisation and verification of glycosidase treatment of fetuin, mass spectrometry was also used to verify the action of sialidase and galactosidase on the A549 membrane proteins. In general, the glycan analysis indicated successful, if not complete, enzymatic treatment, as shown by the expected changes in relative abundance of structures with terminal sialic acid, galactose, or GlcNAc moieties (Figure 15; Supplementary Table 1). In addition, the sequential reduction of a specific monosialylated biantennary core-fucosylated structure (*m/z* 1185.0) could be observed, with an increase in the corresponding products (Figure 16). However, one of the detected glycans, with a mass of 1039.4, interpreted initially as a structure containing one core fucose and terminal sialic acid, was not reduced by the sialidase treatment. This indicated that the detected structure may have actually been *m/z* 1040.0, having one core fucose and two terminal fucoses, since this would have been within the expected errors of ion trap mass spectrometric resolution. Only MS/MS level of analysis is able to differentiate the two structures as analysed by this low resolution MS, but unfortunately the only glycan release performed had no good MS/MS of this specific ion and no further investigation was possible on this matter due to lack of time. However, the presence of this ion and its resistance to sialidase treatment suggested the presence of a fucosylated Lewis epitope, of significance to known *P. aeruginosa* binding affinities ([33]; [32]; [34]).

Sialidase treatment resulted in significant increases in the binding of all *P. aeruginosa* strains to A549 lung epithelial membrane proteins, suggesting their preference for the sub-terminal epitope, galactose and/or asialylated Lewis epitopes. This indicated the importance of the natural sialidase produced by *P. aeruginosa* in exposing these epitopes. Sialidase has been observed to cleave sialic acid and increase microbial adhesion for several microbes ([110]; [111]) but the role of the *P. aeruginosa* sialidase in pathogenesis is not yet fully understood. In the context of cystic fibrosis, it is poignant to note that a lower level of

sialylation was evident in the membrane bound mucins and lung epithelial cells of CF patients ([41]; [36]; Section 1.3.3), potentially enabling increased *P. aeruginosa* adhesion.

After the terminal sialic acid had been removed, the subsequent galactosidase treatment resulted in a decrease in the binding of PAO1 and PASS01, affirming their adherence preference for the sub-terminal galactose. The adhesion to galactose may be attributable to the PA-IL lectin, which is known to have galactose affinity ([25]; [26]). Interestingly, there was no change in the binding of PASS02, PASS03 and PASS04 after galactosidase treatment, implying that these strains may bind to epitopes other than galactose, that are also more accessible after the removal of sialic acid. These could include the terminal fucose of Lewis epitopes, or mannose moieties of hybrid glycans. The PA-IIL lectin has reported affinities for both fucose and mannose ([29]) and could perhaps be utilised more by PASS02, PASS03 and PASS04 than by the other strains. However, genomic screening has shown that all the strains have genes encoding both the PA-IL lectin and PA-IIL lectin (Ani Penesylan, unpublished data). Transcriptomic and proteomic studies in progress in the research group may provide more insight into the differential expression of these genes. There may also be several other, previously uncharacterized, adhesins involved that are differentially expressed.

It is notable that all strains continued to adhere to the epithelial membrane proteins following the treatment by the two glycosidases, suggesting that many adherence factors may come into play other than those glycans specifically tapped by this experiment. The high relative abundance of a high mannose glycan in the MS analysis of the A549 cell membrane proteins (Supplementary Table 1) represents many potential sites of attachment for the mannose-binding PA-IIL lectin, which would have been unaffected by the sialidase and galactosidase treatment. Furthermore, multivalent glycan/protein interactions are very likely to be involved since the plate assay is able to present whole live bacteria with many diverse glycans and proteins at the one time, just as would be the case *in vivo* on the epithelial surface of the lungs.

In summary, the involvement of glycans in the adhesion of all *P. aeruginosa* strains to lung epithelial membrane proteins has been demonstrated in this thesis by their significant alteration in binding following exoglycosidase treatment. Although the removal of sialic

acid resulted in a universal advantage for adhesion across all strains, significant strain-specific differences were seen in the particular impact of the further galactosidase treatment on their adhesion, indicating differences in preferences for particular glycan epitopes between strains.

4.8. Overall contributions of this thesis to the research field

This thesis contributes to the notion of glycan involvement in bacterial adhesion to human epithelium using *P. aeruginosa*, a prevalent pathogen in CF, as an example. The work has also validated a new in-house assay for assessing and differentiating glycan-mediated adhesion of *P. aeruginosa* strains to complex samples such as epithelial membrane proteins. Isothermal titration calorimetry was also tested for its suitability for investigating complex bacteria-glycan interactions and, although not able to be optimised in this project, future work might benefit from the data presented here.

Clinical strains of *P. aeruginosa* that had been isolated from CF patients as part of a wider ARC SuperScience project on CF at Macquarie University were investigated for their binding affinities and several significant variations were found amongst them. This work contributes to the overall characterisation of these strains for the wider project, and interesting correlations were revealed. Of particular note, a strain (PASS02) that had been found to lack a gene for the polysaccharide *Psl* in previous genomic studies, was found to be the least adherent strain in this work, and this also correlated with an inability to form biofilms. Furthermore, a comparison of the CF clinical strains to the prototype laboratory strain PAO1 revealed differences in their adhesion to lung epithelia, which suggests PAO1 might not be a suitable model for experimental research in the context of diseases such as CF.

A universal role of sialic acid in moderating *P. aeruginosa* adhesion to human epithelia was demonstrated in this thesis, since removal of the terminal sialic acid moiety resulted in increased adhesion of all strains. This work suggests that the reduced sialylation of respiratory epithelia and membrane mucins observed in CF patients ([36]; [41]) may be particularly advantageous for *P. aeruginosa* colonisation. Further enzymatic treatment suggested that galactose was an important binding epitope for some *P. aeruginosa* strains, including the laboratory strain PAO1, but some of the CF isolates appeared less dependent

on galactose for adhesion. Although the reason for these subtle differences is not yet known, nor their impact upon *P. aeruginosa* colonisation *in vivo* in a disease such as CF, this work sets the stage for future studies, which should take into account the complexities of glycan-adhesion exhibited by this pathogen.

Due to the increase in *P. aeruginosa* antibiotic resistance, the development of other strategies to combat the pathogen would be beneficial, particularly for vulnerable individuals afflicted by conditions such as CF. The findings and methodologies presented in this thesis may contribute towards the development of glycan-focused prevention and treatment of *P. aeruginosa* infection.

4.9. Future studies

This thesis has reported significant differences in the adhesion of *P. aeruginosa* strains isolated from CF patients, adding another piece to a developing picture of the strains in the broader SuperScience study on CF at Macquarie University. Research is continuing at time of writing that will reveal the full proteomic profiles of each isolate, which may contribute further insight into strain-specific mechanisms of adhesion and colonisation. This work could potentially reveal differential expression/function of *P. aeruginosa* lectins (*eg.* PA-IL, PA-IIL) or adhesive factors (*eg. Psl,* alginate), which are not evident at a genome level. Then, placed beside the differential adhesion observed in this project, some inferences could be made into the strain-specific involvement of these factors in glycan interactions and particular focuses of interest identified. This could be followed by more sensitive techniques, such as NMR (nuclear magnetic resonance), SPR (surface plasmon resonance) or ITC, in order to more closely examine binding interactions between specific glycans and an isolated, or recombinant, glycan-binding protein of interest. In this way, information gained about strains from the *in vivo*-like complex glycan/protein interactions observed in the plate assay technique used in this thesis could be selectively channeled into techniques more suited for isolated molecular-level analysis. In addition, Macquarie University has access to a *P. aeruginosa* knockout library which could be investigated for differential binding using the techniques developed in this thesis, leading to the identification of genes and proteins involved in adhesion. Ultimately, it is envisioned that a greater understanding of the role of glycans in the adhesion of pathogens to their hosts could be translated into

effective glycan-focused therapies that could assist in the fight against many pathogenic diseases in a world faced by increasing antibiotic resistance.

References

Chapter 5

1. Mann, M. & Jensen, O. N. (2003). Proteomic analysis of post-translational modifications. *Nat Biotechnol* **21**, 255-61.
2. Stanley, P., Schachter, H. & Taniguchi, N. (2009). N-Glycans. In *Essentials of Glycobiology* 2nd edit. (Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Bertozzi, C. R., Hart, G. W. & Etzler, M. E., eds.), Cold Spring Harbor (NY).
3. Parekh, R. B., Dwek, R. A., Sutton, B. J., Fernandes, D. L., Leung, A., Stanworth, D., Rademacher, T. W., Mizuochi, T., Taniguchi, T., Matsuta, K. & et al. (1985). Association of rheumatoid arthritis and primary osteoarthritis with changes in the glycosylation pattern of total serum IgG. *Nature* **316**, 452-7.
4. Perez-Vilar, J. & Hill, R. L. (1999). The structure and assembly of secreted mucins. *J Biol Chem* **274**, 31751-4.
5. Sharon, N. (2006). Carbohydrates as future anti-adhesion drugs for infectious diseases. *Biochim Biophys Acta* **1760**, 527-37.
6. Soto, G. E. & Hultgren, S. J. (1999). Bacterial adhesins: common themes and variations in architecture and assembly. *J Bacteriol* **181**, 1059-71.
7. Bucior, I., Abbott, J., Song, Y., Matthay, M. A. & Engel, J. N. (2013). Sugar administration is an effective adjunctive therapy in the treatment of *Pseudomonas aeruginosa* pneumonia. *Am J Physiol Lung Cell Mol Physiol* **305**, L352-63.
8. Ohlsson, J., Jass, J., Uhlin, B. E., Kihlberg, J. & Nilsson, U. J. (2002). Discovery of potent inhibitors of PapG adhesins from uropathogenic Escherichia coli through synthesis and evaluation of galabiose derivatives. *Chembiochem* **3**, 772-9.
9. Mulvey, M. A., Lopez-Boado, Y. S., Wilson, C. L., Roth, R., Parks, W. C., Heuser, J. & Hultgren, S. J. (1998). Induction and evasion of host defenses by type 1-piliated uropathogenic Escherichia coli. *Science* **282**, 1494-7.
10. Boren, T., Falk, P., Roth, K. A., Larson, G. & Normark, S. (1993). Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. *Science* **262**, 1892-5.
11. Chabre, Y. M. & Roy, R. (2010). Design and creativity in synthesis of multivalent neoglycoconjugates. *Adv Carbohydr Chem Biochem* **63**, 165-393.
12. Davis, P. B., Drumm, M. & Konstan, M. W. (1996). Cystic fibrosis. *Am J Respir Crit Care Med* **154**, 1229-56.
13. Boucher, R. C. (2007). Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. *Annu Rev Med* **58**, 157-70.
14. Venkatakrishnan, V., Packer, N. H. & Thaysen-Andersen, M. (2013). Host mucin glycosylation plays a role in bacterial adhesion in lungs of individuals with cystic fibrosis. *Expert Rev Respir Med* **7**, 553-76.
15. Cohen, T. S. & Prince, A. (2012). Cystic fibrosis: a mucosal immunodeficiency syndrome. *Nat Med* **18**, 509-19.
16. Strous, G. J. & Dekker, J. (1992). Mucin-type glycoproteins. *Crit Rev Biochem Mol Biol* **27**, 57-92.
17. Fridkin, S. K., Steward, C. D., Edwards, J. R., Pryor, E. R., McGowan, J. E., Jr., Archibald, L. K., Gaynes, R. P. & Tenover, F. C. (1999). Surveillance of antimicrobial use and antimicrobial resistance in United States hospitals: project ICARE phase 2. Project Intensive Care Antimicrobial Resistance Epidemiology (ICARE) hospitals. *Clin Infect Dis* **29**, 245-52.

18. Lewis, K. (2001). Riddle of biofilm resistance. *Antimicrob Agents Chemother* **45**, 999-1007.
19. Kerem, E., Corey, M., Gold, R. & Levison, H. (1990). Pulmonary function and clinical course in patients with cystic fibrosis after pulmonary colonization with *Pseudomonas aeruginosa*. *J Pediatr* **116**, 714-9.
20. Holby, N. & Olling, S. (1977). *Pseudomonas aeruginosa* infection in cystic fibrosis. Bactericidal effect of serum from normal individuals and patients with cystic fibrosis on *P. aeruginosa* strains from patients with cystic fibrosis or other diseases. *Acta Pathol Microbiol Scand C* **85**, 107-14.
21. Nelson, R. (2003). Antibiotic development pipeline runs dry. New drugs to fight resistant organisms are not being developed, experts say. *Lancet* **362**, 1726-7.
22. Dwek, R. A., Butters, T. D., Platt, F. M. & Zitzmann, N. (2002). Targeting glycosylation as a therapeutic approach. *Nat Rev Drug Discov* **1**, 65-75.
23. Hidron, A. I., Edwards, J. R., Patel, J., Horan, T. C., Sievert, D. M., Pollock, D. A., Fridkin, S. K., National Healthcare Safety Network, T. & Participating National Healthcare Safety Network, F. (2008). NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. *Infect Control Hosp Epidemiol* **29**, 996-1011.
24. Avichezer, D., Katcoff, D. J., Garber, N. C. & Gilboa-Garber, N. (1992). Analysis of the amino acid sequence of the *Pseudomonas aeruginosa* galactophilic PA-I lectin. *J Biol Chem* **267**, 23023-7.
25. Lanne, B., Ciopraga, J., Bergstrom, J., Motas, C. & Karlsson, K. A. (1994). Binding of the galactose-specific *Pseudomonas aeruginosa* lectin, PA-I, to glycosphingolipids and other glycoconjugates. *Glycoconj J* **11**, 292-8.
26. Chen, C. P., Song, S. C., Gilboa-Garber, N., Chang, K. S. & Wu, A. M. (1998). Studies on the binding site of the galactose-specific agglutinin PA-IL from *Pseudomonas aeruginosa*. *Glycobiology* **8**, 7-16.
27. Garber, N., Guempel, U., Belz, A., Gilboa-Garber, N. & Doyle, R. J. (1992). On the specificity of the D-galactose-binding lectin (PA-I) of *Pseudomonas aeruginosa* and its strong binding to hydrophobic derivatives of D-galactose and thiogalactose. *Biochim Biophys Acta* **1116**, 331-3.
28. Cioci, G., Mitchell, E. P., Gautier, C., Wimmerova, M., Sudakevitz, D., Perez, S., Gilboa-Garber, N. & Imberty, A. (2003). Structural basis of calcium and galactose recognition by the lectin PA-IL of *Pseudomonas aeruginosa*. *FEBS Lett* **555**, 297-301.
29. Sudakevitz, D., Kostlanova, N., Blatman-Jan, G., Mitchell, E. P., Lerrer, B., Wimmerova, M., Katcoff, D. J., Imberty, A. & Gilboa-Garber, N. (2004). A new Ralstonia solanacearum high-affinity mannose-binding lectin RS-IIL structurally resembling the *Pseudomonas aeruginosa* fucose-specific lectin PA-IIL. *Mol Microbiol* **52**, 691-700.
30. Arora, S. K., Ritchings, B. W., Almira, E. C., Lory, S. & Ramphal, R. (1998). The *Pseudomonas aeruginosa* flagellar cap protein, FliD, is responsible for mucin adhesion. *Infect Immun* **66**, 1000-7.
31. Chevance, F. F. & Hughes, K. T. (2008). Coordinating assembly of a bacterial macromolecular machine. *Nat Rev Microbiol* **6**, 455-65.

32. Scharfman, A., Degroote, S., Beau, J., Lamblin, G., Roussel, P. & Mazurier, J. (1999). *Pseudomonas aeruginosa* binds to neoglycoconjugates bearing mucin carbohydrate determinants and predominantly to sialyl-Lewis x conjugates. *Glycobiology* **9**, 757-64.
33. Scharfman, A., Arora, S. K., Delmotte, P., Van Brussel, E., Mazurier, J., Ramphal, R. & Roussel, P. (2001). Recognition of Lewis x derivatives present on mucins by flagellar components of *Pseudomonas aeruginosa*. *Infect Immun* **69**, 5243-8.
34. Lillehoj, E. P., Kim, B. T. & Kim, K. C. (2002). Identification of *Pseudomonas aeruginosa* flagellin as an adhesin for Muc1 mucin. *Am J Physiol Lung Cell Mol Physiol* **282**, L751-6.
35. Xia, B., Sachdev, G. P. & Cummings, R. D. (2007). *Pseudomonas aeruginosa* mucoid strain 8830 binds glycans containing the sialyl-Lewis x epitope. *Glycoconj J* **24**, 87-95.
36. Saiman, L. & Prince, A. (1993). Pseudomonas aeruginosa pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. *J Clin Invest* **92**, 1875-80.
37. Krivan, H. C., Ginsburg, V. & Roberts, D. D. (1988). *Pseudomonas aeruginosa* and *Pseudomonas cepacia* isolated from cystic fibrosis patients bind specifically to gangliotetraosylceramide (asialo GM1) and gangliotriaosylceramide (asialo GM2). *Arch Biochem Biophys* **260**, 493-6.
38. Cacalano, G., Kays, M., Saiman, L. & Prince, A. (1992). Production of the *Pseudomonas aeruginosa* neuraminidase is increased under hyperosmolar conditions and is regulated by genes involved in alginate expression. *J Clin Invest* **89**, 1866-74.
39. Davril, M., Degroote, S., Humbert, P., Galabert, C., Dumur, V., Lafitte, J. J., Lamblin, G. & Roussel, P. (1999). The sialylation of bronchial mucins secreted by patients suffering from cystic fibrosis or from chronic bronchitis is related to the severity of airway infection. *Glycobiology* **9**, 311-21.
40. Schulz, B. L., Sloane, A. J., Robinson, L. J., Prasad, S. S., Lindner, R. A., Robinson, M., Bye, P. T., Nielson, D. W., Harry, J. L., Packer, N. H. & Karlsson, N. G. (2007). Glycosylation of sputum mucins is altered in cystic fibrosis patients. *Glycobiology* **17**, 698-712.
41. Rhim, A. D., Stoykova, L., Glick, M. C. & Scanlin, T. F. (2001). Terminal glycosylation in cystic fibrosis (CF): a review emphasizing the airway epithelial cell. *Glycoconj J* **18**, 649-59.
42. Martino, A. T. Mueller, C. Braag, S, Cruz, P. E. & Jin, S. (2011). *N*-glycosylation augmentation of the cystic fibrosis epithelium improves *Pseudomanas aeruginosa* clearance. *Am J Respair Cell Mol Biol* **44**, 824-830.
43. Cundell, D. R. & Tuomanen, E. I. (1994). Receptor specificity of adherence of Streptococcus pneumoniae to human type-II pneumocytes and vascular endothelial cells in vitro. *Microb Pathog* **17**, 361-74.
44. Schweizer, F., Jiao, H., Hindsgaul, O., Wong, W. Y. & Irvin, R. T. (1998). Interaction between the pili of *Pseudomonas aeruginosa* PAK and its carbohydrate receptor beta-D-GalNAc(1-->4)beta-D-Gal analogs. *Can J Microbiol* **44**, 307-11.
45. Perret, S., Sabin, C., Dumon, C., Pokorna, M., Gautier, C., Galanina, O., Ilia, S., Bovin, N., Nicaise, M., Desmadril, M., Gilboa-Garber, N., Wimmerova, M., Mitchell, E. P. & Imberty, A. (2005). Structural basis for the interaction between human milk oligosaccharides and the bacterial lectin PA-IIL of *Pseudomonas aeruginosa*. *Biochem J* **389**, 325-32.
46. Lesman-Movshovich, E., Lerrer, B. & Gilboa-Garber, N. (2003). Blocking of *Pseudomonas aeruginosa* lectins by human milk glycans. *Can J Microbiol* **49**, 230-5.

47. Kolomiets, E., Swiderska, M. A., Kadam, R. U., Johansson, E. M., Jaeger, K. E., Darbre, T. & Reymond, J. L. (2009). Glycopeptide dendrimers with high affinity for the fucose-binding lectin LecB from *Pseudomonas aeruginosa*. *ChemMedChem* **4**, 562-9.
48. Barthelson, R., Mobasseri, A., Zopf, D. & Simon, P. (1998). Adherence of *Streptococcus pneumoniae* to respiratory epithelial cells is inhibited by sialylated oligosaccharides. *Infect Immun* **66**, 1439-44.
49. Laurent, N., Voglmeir, J. & Flitsch, S. L. (2008). Glycoarrays--tools for determining protein-carbohydrate interactions and glycoenzyme specificity. *Chem Commun (Camb)*, 4400-12.
50. Page, D. & Roy, R. (1997). Optimizing lectin-carbohydrate interactions: improved binding of divalent alpha-mannosylated ligands towards Concanavalin A. *Glycoconj J* **14**, 345-56.
51. Leid, J. G., Willson, C. J., Shirtliff, M. E., Hassett, D. J., Parsek, M. R. & Jeffers, A. K. (2005). The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN-gamma-mediated macrophage killing. *J Immunol* **175**, 7512-8.
52. Pei, Z., Yu, H., Theurer, M., Walden, A., Nilsson, P., Yan, M. & Ramstrom, O. (2007). Photogenerated carbohydrate microarrays. *Chembiochem* **8**, 166-8.
53. Sagle, L. B., Ruvuna, L. K., Ruemmele, J. A. & Van Duyne, R. P. (2011). Advances in localized surface plasmon resonance spectroscopy biosensing. *Nanomedicine (Lond)* **6**, 1447-62.
54. Soelberg, S. D., Chinowsky, T., Geiss, G., Spinelli, C. B., Stevens, R., Near, S., Kauffman, P., Yee, S. & Furlong, C. E. (2005). A portable surface plasmon resonance sensor system for real-time monitoring of small to large analytes. *J Ind Microbiol Biotechnol* **32**, 669-74.
55. Hahnefeld, C., Drewianka, S. & Herberg, F. W. (2004). Determination of kinetic data using surface plasmon resonance biosensors. *Methods Mol Med* **94**, 299-320.
56. Uchida, H., Fujitani, K., Kawai, Y., Kitazawa, H., Horii, A., Shiiba, K., Saito, K. & Saito, T. (2004). A new assay using surface plasmon resonance (SPR) to determine binding of the Lactobacillus acidophilus group to human colonic mucin. *Biosci Biotechnol Biochem* **68**, 1004-10.
57. Prakobphol, A., Burdsal, C. A. & Fisher, S. J. (1995). Quantifying the strength of bacterial adhesive interactions with salivary glycoproteins. *J Dent Res* **74**, 1212-8.
58. Beachy, J. C., Goldman, D. & Czech, M. P. (1981). Lectins activate lymphocyte pyruvate dehydrogenase by a mechanism sensitive to protease inhibitors. *Proc Natl Acad Sci U S A* **78**, 6256-60.
59. Acord, J., Maskell, J. & Sefton, A. (2005). A rapid microplate method for quantifying inhibition of bacterial adhesion to eukaryotic cells. *J Microbiol Methods* **60**, 55-62.
60. Bermudez, L. E., Young, L. S. & Inderlied, C. B. (1994). Rifabutin and sparfloxacin but not azithromycin inhibit binding of *Mycobacterium avium* complex to HT-29 intestinal mucosal cells. *Antimicrob Agents Chemother* **38**, 1200-2.
61. Tuomola, E. M. & Salminen, S. J. (1998). Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *Int J Food Microbiol* **41**, 45-51.
62. Lee, J. B., Hayashi, K., Hashimoto, M., Nakano, T. & Hayashi, T. (2004). Novel antiviral fucoidan from sporophyll of Undaria pinnatifida (Mekabu). *Chem Pharm Bull (Tokyo)* **52**, 1091-4.
63. Aki, H. & Yamamoto, M. (1994). Thermodynamic characterization of drug binding to human serum albumin by isothermal titration microcalorimetry. *J Pharm Sci* **83**, 1712-6.

64. Richichi, B., Imberty, A., Gillon, E., Bosco, R., Sutkeviciute, I., Fieschi, F. & Nativi, C. (2013). Synthesis of a selective inhibitor of a fucose binding bacterial lectin from *Burkholderia ambifaria*. *Org Biomol Chem* **11**, 4086-94.
65. Wimmerova, M., Mishra, N. K., Pokorna, M. & Koca, J. (2009). Importance of oligomerisation on *Pseudomonas aeruginosa* Lectin-II binding affinity. In silico and in vitro mutagenesis. *J Mol Model* **15**, 673-9.
66. Thornton, D. J., Rousseau, K. & McGuckin, M. A. (2008). Structure and function of the polymeric mucins in airways mucus. *Annu Rev Physiol* **70**, 459-86.
67. Alves, I. D., Bechara, C., Walrant, A., Zaltsman, Y., Jiao, C. Y. & Sagan, S. (2011). Relationships between membrane binding, affinity and cell internalization efficacy of a cell-penetrating peptide: penetratin as a case study. *PLoS One* **6**, e24096.
68. Zhou, X., Kini, R. M. & Sivaraman, J. (2011). Application of isothermal titration calorimetry and column chromatography for identification of biomolecular targets. *Nat Protoc* **6**, 158-65.
69. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S. & Olson, M. V. (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959-64.
70. Molloy, M. P., Herbert, B. R., Slade, M. B., Rabilloud, T., Nouwens, A. S., Williams, K. L. & Gooley, A. A. (2000). Proteomic analysis of the Escherichia coli outer membrane. *Eur J Biochem* **267**, 2871-81.
71. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54.
72. Ann, H. J. Froohlich, J. W. & Lebrilla, C. B. (2009). Determination of glycosylation sites and site-specific heterogenity in glycoproteins. *Curr Opin Chem Biol* **13,** 421-426.
73. Ramsby, M. L. & Makowski, G. S. (1999). Differential detergent fractionation of eukaryotic cells. Analysis by two-dimensional gel electrophoresis. *Methods Mol Biol* **112**, 53-66.
74. Rason, M. A., Randriantsoa, T., Andrianantenaina, H., Ratsimbasoa, A. & Menard, D. (2008). Performance and reliability of the SYBR Green I based assay for the routine monitoring of susceptibility of *Plasmodium falciparum* clinical isolates. *Trans R Soc Trop Med Hyg* **102**, 346-51.
75. van Loosdrecht, M. C., Lyklema, J., Norde, W. & Zehnder, A. J. (1990). Influence of interfaces on microbial activity. *Microbiol Rev* **54**, 75-87.
76. Zwietering, M. H., Jongenburger, I., Rombouts, F. M. & van 't Riet, K. (1990). Modeling of the bacterial growth curve. *Appl Environ Microbiol* **56**, 1875-81.
77. Karlsson, N. G. Nordman, H. Karlsson, H. Carlstedt, I. & Hansson, G. C. (1997). Glycosylation differnces between pig gastric mucin populations a comparative study of the neutral oligosacchrides using mass spectrometry. *Biochem J* **326,** 911-917.

78. Thomsson, K. A., Karlsson, N. G. & Hansson, G. C. (1999). Liquid chromatography-electrospray mass spectrometry as a tool for the analysis of sulfated oligosaccharides from mucin glycoproteins. *J Chromatogr A* **854**, 131-9.
79. Chizhov, A. O., Dell, A., Morris, H. R., Haslam, S. M., McDowell, R. A., Shashkov, A. S., Nifant'ev, N. E., Khatuntseva, E. A. & Usov, A. I. (1999). A study of fucoidan from the brown seaweed Chorda filum. *Carbohydr Res* **320**, 108-19.
80. Adhikari, U., Mateu, C. G., Chattopadhyay, K., Pujol, C. A., Damonte, E. B. & Ray, B. (2006). Structure and antiviral activity of sulfated fucans from *Stoechospermum marginatum*. *Phytochemistry* **67**, 2474-82.
81. Spear, P. G. (2004). Herpes simplex virus: receptors and ligands for cell entry. *Cell Microbiol* **6**, 401-10.
82. Kumarasamy, R. & Blough, H. A. (1985). Galactose-rich glycoproteins are on the cell surface of herpes virus-infected cells. 1. Surface labeling and serial lectin binding studies of Asn-linked oligosaccharides of glycoprotein gC. *Arch Biochem Biophys* **236**, 593-602.
83. Peters, W. B., Frasca, V. & Brown, R. K. (2009). Recent developments in isothermal titration calorimetry label free screening. *Comb Chem High Throughput Screen* **12**, 772-90.
84. Reiter, T., Penman, S. & Capco, D. G. (1985). Shape-dependent regulation of cytoskeletal protein synthesis in anchorage-dependent and anchorage-independent cells. *J Cell Sci* **76**, 17-33.
85. Katsuma, Y., Marceau, N., Ohta, M. & French, S. W. (1988). Cytokeratin intermediate filaments of rat hepatocytes: different cytoskeletal domains and their three-dimensional structure. *Hepatology* **8**, 559-68.
86. Kerschner, J. E. (2007). Mucin gene expression in human middle ear epithelium. *Laryngoscope* **117**, 1666-76.
87. Mizuochi, T., Taniguchi, T., Shimizu, A. & Kobata, A. (1982). Structural and numerical variations of the carbohydrate moiety of immunoglobulin G. *J Immunol* **129**, 2016-20.
88. Irie, A., Koyama, S., Kozutsumi, Y., Kawasaki, T. & Suzuki, A. (1998). The molecular basis for the absence of N-glycolylneuraminic acid in humans. *J Biol Chem* **273**, 15866-71.
89. Sriwilaijaroen, N., Kondo, S., Yagi, H., Takemae, N., Saito, T., Hiramatsu, H., Kato, K. & Suzuki, Y. (2011). N-glycans from porcine trachea and lung: predominant NeuAcalpha2-6Gal could be a selective pressure for influenza variants in favor of human-type receptor. *PLoS One* **6**, e16302.
90. Lau, P. C., Dutcher, J. R., Beveridge, T. J. & Lam, J. S. (2009). Absolute quantitation of bacterial biofilm adhesion and viscoelasticity by microbead force spectroscopy. *Biophys J* **96**, 2935-48.
91. Schleheck, D., Barraud, N., Klebensberger, J., Webb, J. S., McDougald, D., Rice, S. A. & Kjelleberg, S. (2009). *Pseudomonas aeruginosa* PAO1 preferentially grows as aggregates in liquid batch cultures and disperses upon starvation. *PLoS One* **4**, e5513.
92. Ma, L., Jackson, K. D., Landry, R. M., Parsek, M. R. & Wozniak, D. J. (2006). Analysis of *Pseudomonas aeruginosa* conditional psl variants reveals roles for the psl polysaccharide in adhesion and maintaining biofilm structure postattachment. *J Bacteriol* **188**, 8213-21.
93. Colvin, K. M., Irie, Y., Tart, C. S., Urbano, R., Whitney, J. C., Ryder, C., Howell, P. L., Wozniak, D. J. & Parsek, M. R. (2012). The Pel and Psl polysaccharides provide

Pseudomonas aeruginosa structural redundancy within the biofilm matrix. *Environ Microbiol* **14**, 1913-28.
94. Ma, L., Conover, M., Lu, H., Parsek, M. R., Bayles, K. & Wozniak, D. J. (2009). Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathog* **5**, e1000354.
95. Irie, Y., Borlee, B. R., O'Connor, J. R., Hill, P. J., Harwood, C. S., Wozniak, D. J. & Parsek, M. R. (2012). Self-produced exopolysaccharide is a signal that stimulates biofilm formation in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **109**, 20632-6.
96. Byrd, M. S., Pang, B., Mishra, M., Swords, W. E. & Wozniak, D. J. (2010). The *Pseudomonas aeruginosa* exopolysaccharide Psl facilitates surface adherence and NF-kappaB activation in A549 cells. *MBio* **1**.
97. Mishra, M., Byrd, M. S., Sergeant, S., Azad, A. K., Parsek, M. R., McPhail, L., Schlesinger, L. S. & Wozniak, D. J. (2012). *Pseudomonas aeruginosa* Psl polysaccharide reduces neutrophil phagocytosis and the oxidative response by limiting complement-mediated opsonization. *Cell Microbiol* **14**, 95-106.
98. Zegans, M. E., Wozniak, D., Griffin, E., Toutain-Kidd, C. M., Hammond, J. H., Garfoot, A. & Lam, J. S. (2012). *Pseudomonas aeruginosa* exopolysaccharide Psl promotes resistance to the biofilm inhibitor polysorbate 80. *Antimicrob Agents Chemother* **56**, 4112-22.
99. Wozniak, D. J., Wyckoff, T. J., Starkey, M., Keyser, R., Azadi, P., O'Toole, G. A. & Parsek, M. R. (2003). Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci U S A* **100**, 7907-12.
100. Fegan, M., Francis, P., Hayward, A. C., Davis, G. H. & Fuerst, J. A. (1990). Phenotypic conversion of *Pseudomonas aeruginosa* in cystic fibrosis. *J Clin Microbiol* **28**, 1143-6.
101. Lam, J., Chan, R., Lam, K. & Costerton, J. W. (1980). Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect Immun* **28**, 546-56.
102. Marcus, H. & Baker, N. R. (1985). Quantitation of adherence of mucoid and nonmucoid *Pseudomonas aeruginosa* to hamster tracheal epithelium. *Infect Immun* **47**, 723-9.
103. Mathee, K., Ciofu, O., Sternberg, C., Lindum, P. W., Campbell, J. I., Jensen, P., Johnsen, A. H., Givskov, M., Ohman, D. E., Molin, S., Hoiby, N. & Kharazmi, A. (1999). Mucoid conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence activation in the cystic fibrosis lung. *Microbiology* **145 (Pt 6)**, 1349-57.
104. Peterson, R., Cheah, W. Y., Grinyer, J. & Packer, N. (2013). Glycoconjugates in human milk: protecting infants from disease. *Glycobiology* **23**, 1425-38.
105. Leprat, R. & Michel-Briand, Y. (1980). Extracellular neuraminidase production by a strain of *Pseudomonas aeruginosa* isolated from cystic fibrosis. *Ann Microbiol (Paris)* **131B**, 209-22.
106. Vimr, E. R. & Troy, F. A. (1985). Identification of an inducible catabolic system for sialic acids (nan) in Escherichia coli. *J Bacteriol* **164**, 845-53.
107. Knirel Yu, A., Vinogradov, E. V., Kocharova, N. A., Paramonov, N. A., Kochetkov, N. K., Dmitriev, B. A., Stanislavsky, E. S. & Lanyi, B. (1988). The structure of O-specific polysaccharides and serological classification of *Pseudomonas aeruginosa* (a review). *Acta Microbiol Hung* **35**, 3-24.

108. Guzman, C. A., Plate, M. & Pruzzo, C. (1990). Role of neuraminidase-dependent adherence in Bacteroides fragilis attachment to human epithelial cells. *FEMS Microbiol Lett* **59**, 187-92.
109. Xu, G., Ryan, C., Kiefel, M. J., Wilson, J. C. & Taylor, G. L. (2009). Structural studies on the *Pseudomonas aeruginosa* sialidase-like enzyme PA2794 suggest substrate and mechanistic variations. *J Mol Biol* **386**, 828-40.
110. Galen, J. E., Ketley, J. M., Fasano, A., Richardson, S. H., Wasserman, S. S. & Kaper, J. B. (1992). Role of *Vibrio cholerae* neuraminidase in the function of cholera toxin. *Infect Immun* **60**, 406-15.
111. Camara, M., Mitchell, T. J., Andrew, P. W. & Boulnois, G. J. (1991). *Streptococcus pneumoniae* produces at least two distinct enzymes with neuraminidase activity: cloning and expression of a second neuraminidase gene in *Escherichia coli*. *Infect Immun* **59**, 2856-8.

Appendix

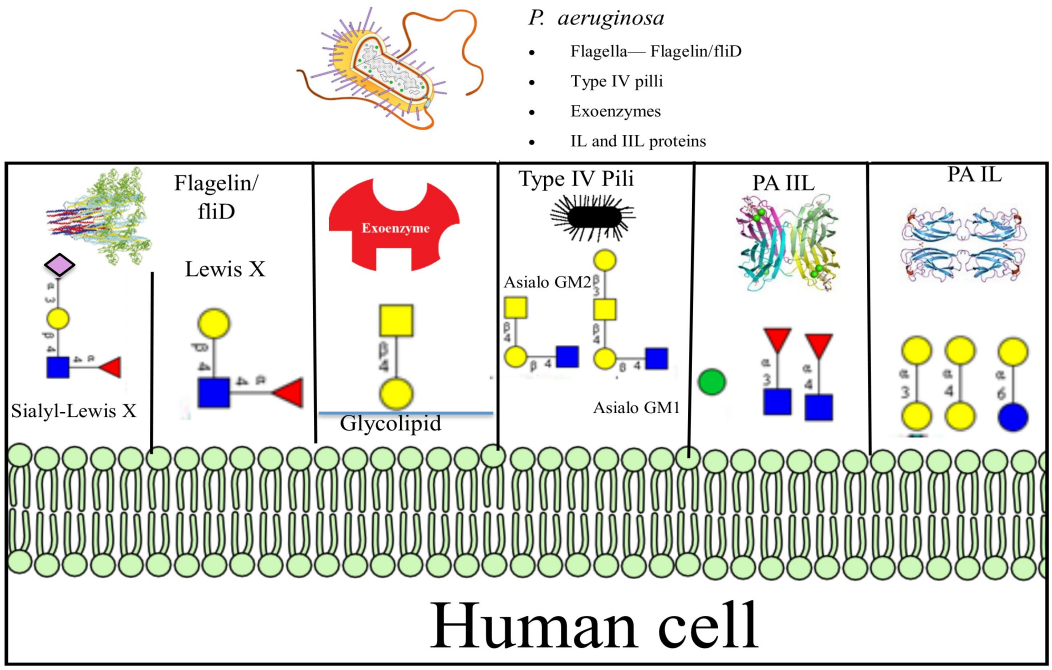


Figure S1: An illustration depicting the glycan-binding preferences of *P. aeruginosa* adhesins/lectins. Developed from [14].

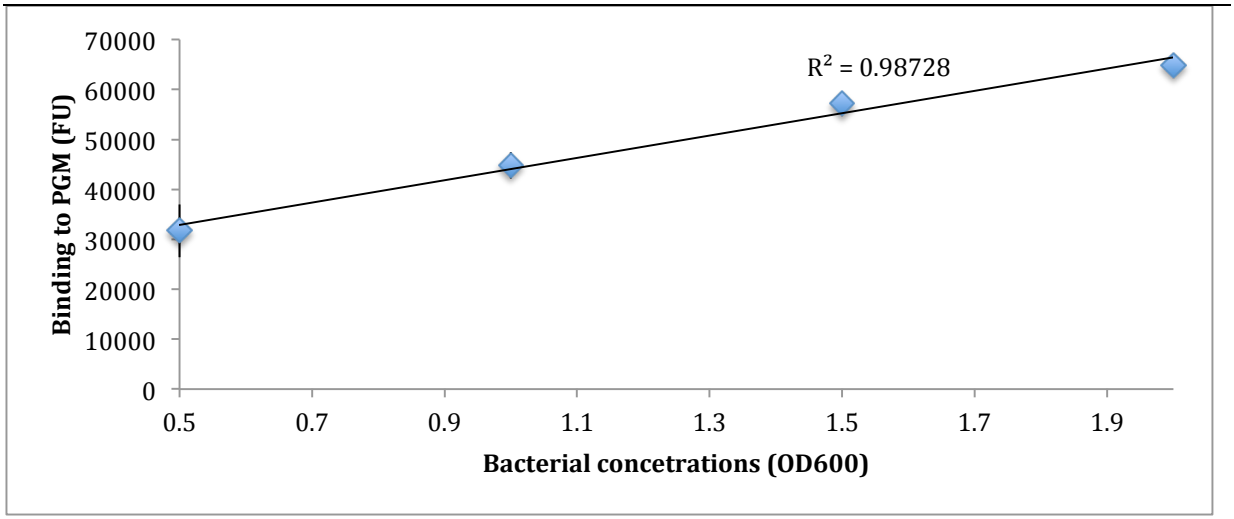


Figure S2: The binding of different concentrations of PASS03 (OD600 of 0.5, 1, 1.5, and 2) to PGM. PGM was immobilised to the wells using 50 µL of 1 mg/ml PGM. Each sample was tested in triplicate. FU: fluorescence units

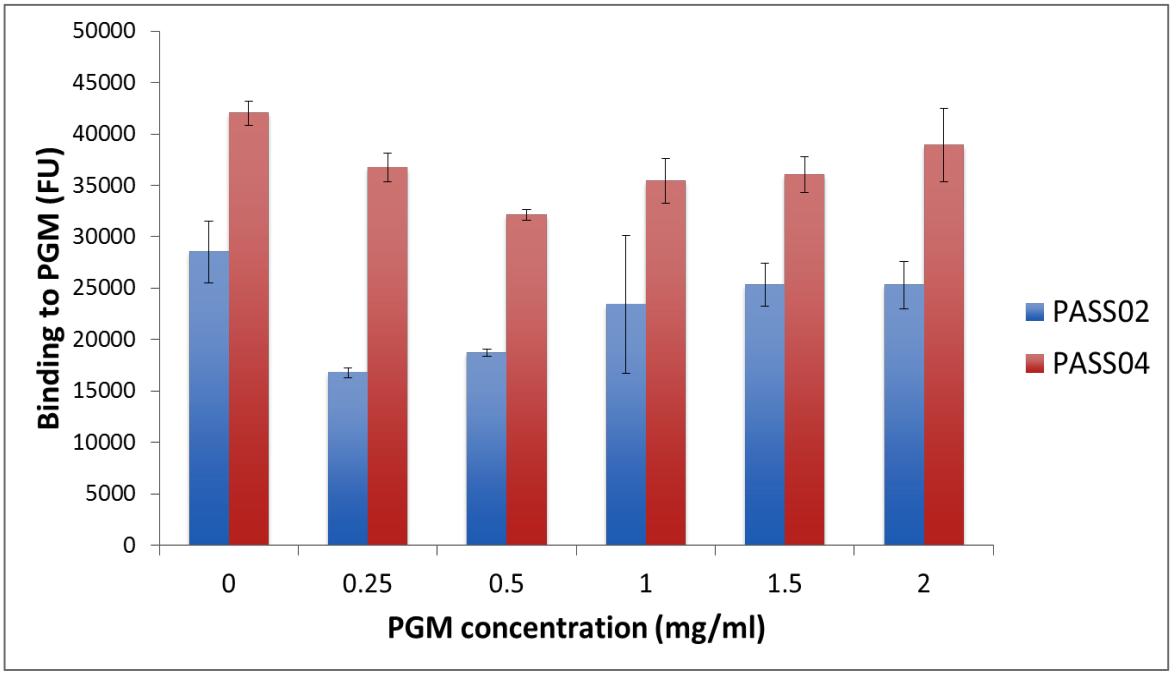


Figure S3: The binding of PASS02 and PASS04 (OD600=1) to PGM, which had been immobilised into the PVDF wells using 50 µl of different PGM concentrations (2, 1.5, 1, 0.5, 0.25, and 0 mg/ml). Samples were tested in triplicate. FU: fluorescence units.

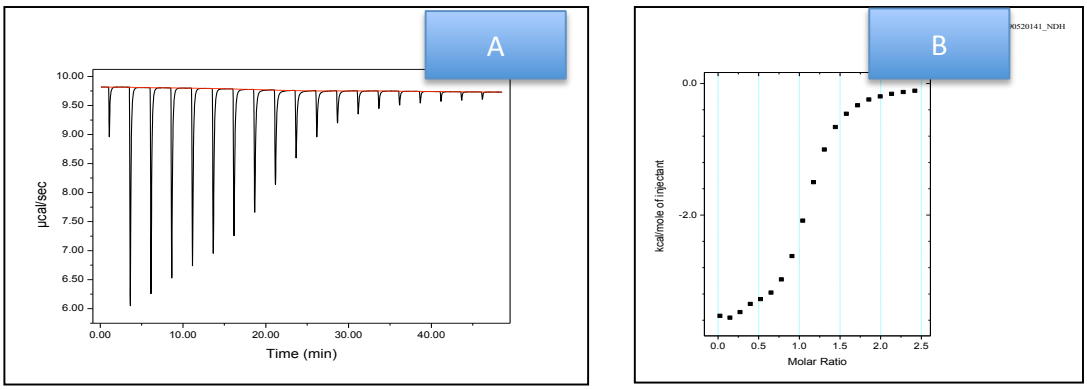


Figure S4: The calibration of ITC machine with 5mM $CaCl_2$ titrated into 0.4mM EDTA. The molar ratio can be depicted from the figure on B, of a value of 1. A clear saturation is achieved in this experiment shown in the c shape curve in A.

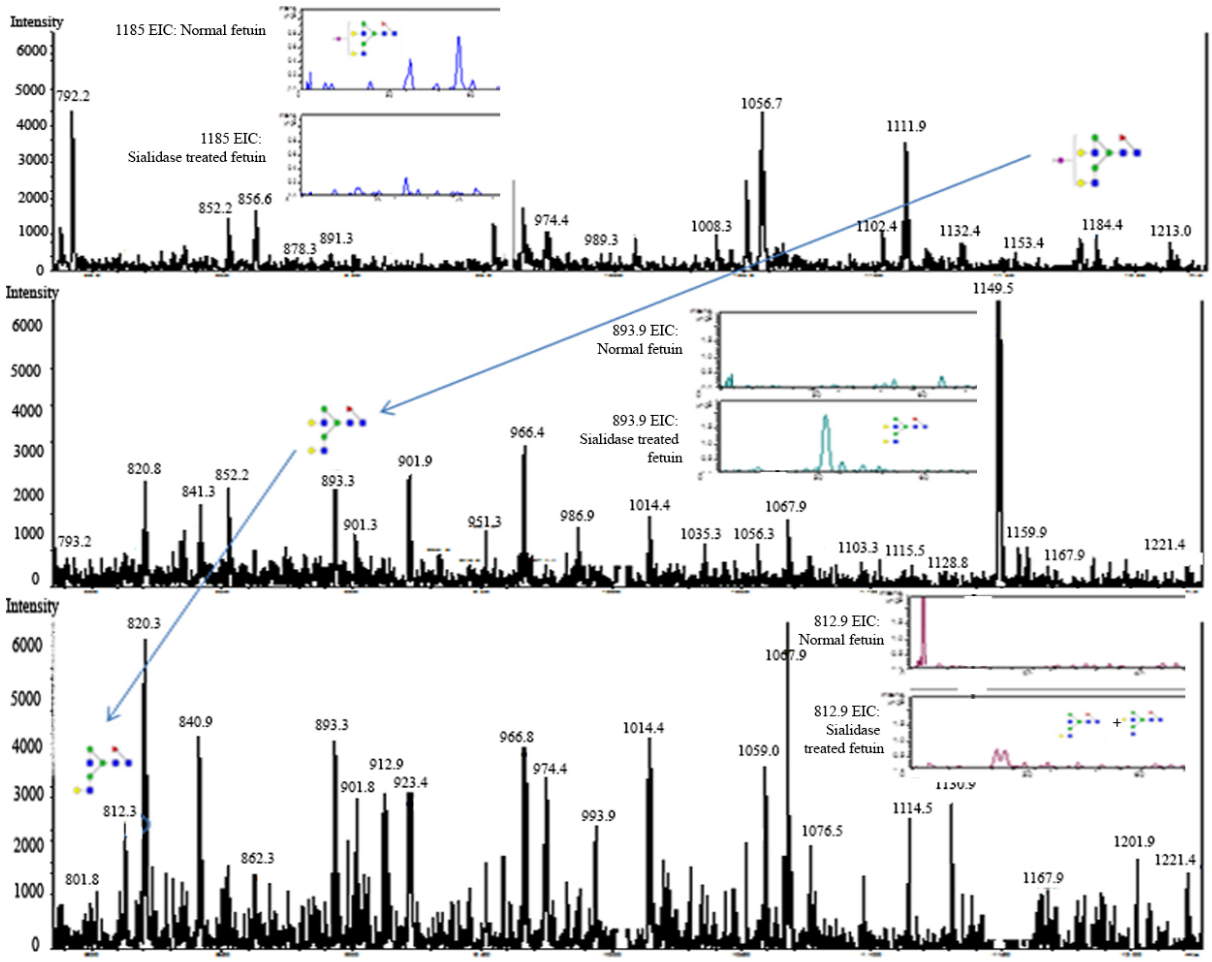
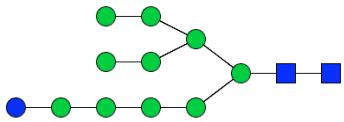
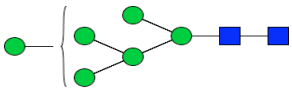
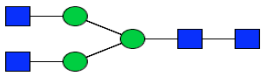
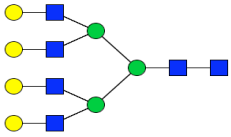
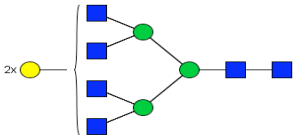
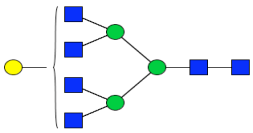
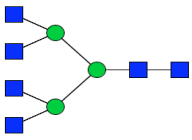
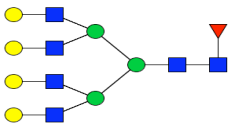
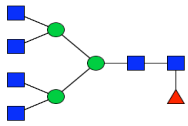
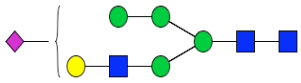
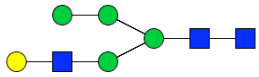
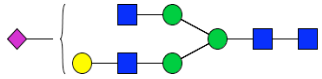
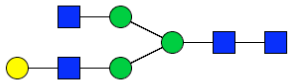
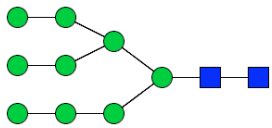
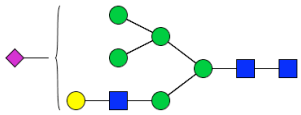
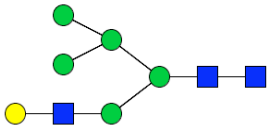
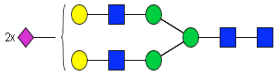
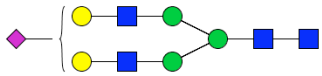
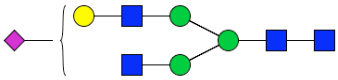
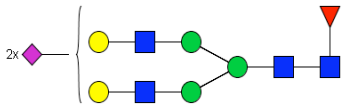
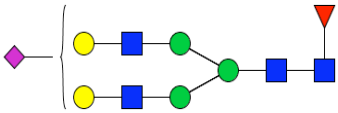
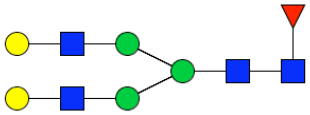
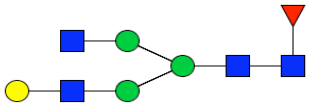
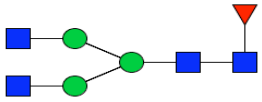


Figure S5: Summed mass spectra from 35-60 mins of *N*-glycans released from fetuin (top), following sialidase treatment (middle) and sialidase + galactosidase treatment (bottom). A significant loss of *m/z* 1185.0 and a significant gain of *m/z* 893.9 demonstrates successful sialidase treatment. The sequential treatment with galactosidase has resulted in the gain of *m/z* 812.9 previously not seen in sample treated with sialidase only, demonstrating successful galactosidase treatment. Insets: extracted ion chromatograms of indicated ions for control fetuin, sialidase treatment, and sialidase + galactosidase treatment.

Table S1: The relative abundances of all ions found in the mass spectrometric analysis of *N*-linked glycans released from A549 human lung epithelial membrane proteins (normal) and following sialidase, and sialidase + galactosidase treatment. Each ion is shown with its corresponding charge, relative abundance, and the suggested composition shown by Glycomod with proposed structure.

Ion mass (Charge)	Normal	Sialidase	Sialidase/Galactosidase	Glycomod suggested structure
1022.9(2)	6.36	8.58	3.18	
1397.5(1)	14.82	15.41	8.3	
659.1(2)	0.9	1.1	0.12	
791.2(3)	0.44	1.8		
683.2(3)			0.01	
629.2(3)			0.02	
575.2(3)			0.12	
840.2(3)	0.55	0.52	0.42	

624.3(3)			0.11	
865.1(2)	0.8			
719.6(2)		0.6	2.01	
886.1(2)	1.14		0.03	
740.6(2)		1.21	0.89	
941.9(2)	45.76	46.28	52.72	
946(2)	2.32	0.82	0.075	
800.5(2)		2.85	0.525	
1111.9(2)	1.33			

966.4(2)		2.03	0.1	
885.4(2)			0.42	
1185(2)	10.2	0.33		
1039.4(2)	15.38	7.28	7.79	
893.9(2)		11.22	0.54	
812.9(2)			9.1	
732(2)			13.52	

Nicki Packer <nicki.packer@mq.edu.au>

External Approval Noted- Packer (5201100423)

Ethics Secretariat <ethics.secretariat@mq.edu.au> **19 April 2011 15:06**
To: Prof Nicolle Packer <nicki.packer@mq.edu.au>
Cc: Prof Helena Nevalainen <helena.nevalainen@mq.edu.au>

Dear Prof Packer

Re: "Interactions of microbial pathogens with sputum from cystic fribrosis patients"

The above application was considered by the Executive of the Human Research Ethics Committee. In accordance with section 5.5 of the National Statement on Ethical Conduct in Human Research (2007) the Executive noted the final approval from the Sydney West Area Health Service and your right to proceed under their authority.

Please do not hesitate to contact the Ethics Secretariat if you have any questions or concerns.

Please do not hesitate to contact the Ethics Secretariat at the address below, if you require a hard copy letter of the above notification.

Please retain a copy of this email as this is your official notification of external approval being noted.

Yours sincerely

Dr Karolyn White
Director of Research Ethics
Chair, Human Research Ethics Committee

SECTION A

MACQUARIE UNIVERSITY

Biohazard Risk Assessment Form – NON GMO

Notification Number:	*VIV030712BHA*

Investigator completing assessment :	**Vignesh Venkatakrishnan**	**Date of assessment:**	**03/07/2012**
Department:	**Chemistry and Biomolecular Science**	**Name of Supervisor submitting this assessment:**	**Prof. Nicolle Packer**
Contact number/email:	**9850-8200/ vignesh.venkatakrishnan@mq.edu.au**		

Reason for this assessment

☒ **New research** ☐ **New information relating to existing research** ☐ **other**_____

Exact location(s) of research:

E8A 109, E8C 320, E8C 326

Control measures: Eliminate risk ☐ **Substitute the hazard** ☐ **Isolate the hazard** ☒ **Implement engineering controls** ☐ **Administration** ☐ (e.g. Training) **PPE**☐

All samples are sealed in a box and carried to cobalt cave for gamma irradiation.

Supporting documents which must be read in conjunction with this assessment. (e.g. Safe Working Procedures, Safety Data Sheets, Guidelines/Protocols)

What is the type of the biological material?

Bacteria ☒ **Fungi** ☒**Virus** ☐ **Cell Line** ☐ **Tissue** ☐ **Parasite** ☐ **Animal** ☐ **Plant** ☐ **Soil** ☐ **Toxin** ☐ **Prions** ☐ **Nucleic Acid**☐ **other** ☐_____

What is the name of the biological agent?

Pseudomonas aeruginosa, Burkholderia cenocepacia **(bacteria) and fungi** ***Aspergillus fumigatus*** **and** ***Scedosporium prolificans*** **that are cultured from the sputum samples.**

List the Personal Protective Equipment required:

Gloves ☒_____(e.g. chemical resistant) **Eye protection** ☒**safety glasses**(e.g. safety glasses/goggles) **Clothing_Coveralls**_____ ☒ (e.g. button up lab coat/coveralls/apron)

Footwear ☒_____(e.g. Enclosed/Gumboots/overshoe covers) **Respiratory Protection** ☐_ (e.g.PF2 face mask) **Other** ☐_____

The University does not hold risk group 4 microorganisms

What are the risks associated with this Biological Agent. (Can be more than one risk group depending on method)			
Risk Group	**Details of Biohazards**	**Biosafety level**	**Risk Reduction Measures** (must be followed by the researcher)
Group 1- Low Individual and community risk (Microorganism that is unlikely to cause human, plant or animal disease)		(e.g.BSL1/PC1)	1 Standard laboratory procedures will be followed in accordance with Laboratory Microbiological Standards AS/NZ 2243:3:2010 and university guidelines (see supporting documents - Section A above) and include spillage and emergency response. 2 Investigator has attended university Biosafety training course (see 3) 3 Supervisor identified in Section A confirms that the investigator has received appropriate training and instruction or has adequate supervision and understands safe laboratory practice according to AS/NZ2243:3:2010 and university guidelines (see supporting documents - Section A above)
Group 2- Moderate individual risk, limited community risk (Microorganism that is unlikely to be a significant risk to laboratory workers, the community/livestock/environment. Laboratory exposures may cause infection but effective treatment and preventative measures are available and the risk of spread is limited).	*Pseudomonas aeruginosa, Aspergillus fumigatus, Scedosporium prolificans, Burkholderia cenocepacia*	PC2 level	1 Standard laboratory procedures will be followed in accordance with Laboratory Microbiological Standards AS/NZ 2243:3:2010 and university guidelines which are appropriate for **Risk Group 2** (see supporting documents - Section A above) and include spillage and emergency response. 2 Investigator has attended university Biosafety training course (see 3) 3 Supervisor identified in Section A confirms that the investigator has received appropriate training and instruction or has adequate supervision and understands safe laboratory practice according to AS/NZ 2243:3:2010 and university guidelines (see supporting documents - Section A above)
Group 3 -High individual risk, limited community risk (Microorganisms that usually causes serious human or animal disease and may present a significant risk to laboratory workers. It could present a limited to moderate risk if spread in the community or the environment, but there are usually effective preventative measures or treatment available).			1 Standard laboratory procedures will be followed in accordance with Laboratory Microbiological Standards AS/NZ 2243:3:2010 and university guidelines which are appropriate for **Risk Group 3** (see supporting documents - Section A above) and include spillage and emergency response. 2 Investigator has attended university Biosafety training course (see 3) 3 Supervisor identified in Section A confirms that the investigator has received appropriate training and instruction or has adequate supervision

			and understands safe laboratory practice according to AS/NZ 243:3:2010 and university guidelines (see supporting documents - Section A above)

Process and equipment to be used	You must include: - 1 Description and quantity of any chemicals, gasses, substances and radiation used. 2 Any aerosols produced and any controls necessary to ensure the health and safety of investigators and others 3 Any alternative and/or additional control measures to those identified above and explain why these are necessary. 4 Safe Work Procedure if there is no existing SWP* 5 Explain why risks cannot be eliminated. 6 Waste disposal method.

1. **Pathogens**: The CF sputum samples are obtained from the Westmead Hospital are transported in a ziplock bag from Westmead to Macquarie using a sealed container with dry ice. The sample is then opened and an aliquot is transferred to Eppendorf tube in the laminar flow hood in the PC2 lab E8A109. The samples are then cultured on to Luria Broth agar plates and Horse blood agar plates to isolate the pathogens *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Aspergillus fumigatus*. The cultured organisms would be added to REVCO media and stored in the freezer. None of the above mentioned work is performed at Westmead Hospital. Everything regarding handling the pathogens are carried out at Macquarie University PC2 laboratory E8A 109.

2. **Radiations used**: The CF sputum samples are sealed in a box and carried to irradiation facility in E8A basement in a sealed container where they are irradiated for 2 hour at measurement of approx 2000 grays in the Cobalt Cave to kill any pathogens present in the sample as tested by lack of growth. This sample would then be brought to E8C 320 laboratory for further work. Proper induction has been obtained for the use of Cobalt cave.

3. It is a safe measure to irradiate the organisms which should completely kill all the pathogens and would be safe to be brought to the laboratory for further work related to glycomics work.

4. Before starting the experiment, the lab bench would be wiped using 4% Pyroneg to remove any spills from bench surface and then sterilized with 70% ethanol. Safety glasses and lab coat will be worn in PC2 lab at all times.

   Once the work is completed, the bench will be cleaned and the bench will be wiped using 4% Pyroneg and 70% ethanol to decontaminate it.

5. The risks involved could well be reduced by following the above mentioned steps and every measure has been taken for the safety of others working in the lab.

6. **Waste disposal method**: Everything that has been in contact with the pathogens (consumables as loops, tips, test tubes) will be thrown in to the bin for autoclaving or sterilizing. The bench will be wiped using 4% pyroneg and 70% ethanol to decontaminate it. All the wastes would be sealed and would be carefully taken for autoclaving and it will be disposed accordingly.

SECTION B	
MACQUARIE UNIVERSITY	**Biohazard Safety Committee – Risk Assessment Decision**
Notification Number:	*VIV030712BHA*

Important Information

For **non GMO** investigations email this assessment to biohazard@mq.edu.au for approval by the **Biohazard** Safety Committee.

Individual Responsibilities

By submitting this assessment the **Supervisor** identified in Section A, confirms that any supporting documents, training, guidance, instruction or protocols issued by the University will been followed so far as reasonably practicable to ensure the work is carried out without risk to health, safety or the environment. The named **Supervisor** confirms that the investigator has received appropriate training and instruction or will have adequate supervision and understands safe laboratory practice according to AS/NZ 2243:3:2010 and university guidelines.

Decision to be completed by the Biohazard Safety Committee:

The Committee has agreed that this risk assessment is sufficient for investigations to commence? **Yes** ☒ **No** ☐ **Further action required** ☒

Further Action/Comments:
- In relation to Point 4 *(Before starting the experiment, the lab bench would be wiped using 4% Pyroneg to remove any spills from bench surface and then sterilized with 70% ethanol. Safety glasses and lab coat will be worn in PC2 lab at all times. Once the work is completed, the bench will be cleaned and the bench will be wiped using 4% Pyroneg and 70% ethanol to decontaminate it.)* – all "work" should be conducted in the Class II cabinets, NOT on the bench, and any spills on the bench should be cleaned up by the previous user with 1% available chlorine.
- Place a 'tick' in Engineering Methods, Administration, and PPE.
- Correct the procedure to match pathogen risk group with the right containment (BSC Class II, PC2 Lab).

Name of Approver (Committee Rep):	Michael Gillings (Chairperson, Biohazard Committee)
Extension and email address:	Michael.gillings@mq.edu.au ext: 8199
Date Approved and submitted to Health and Safety Unit:	4th September, 2012