INVESTIGATING THE INTERPLAY BETWEEN
ASPERGILLUS FUMIGATUS AND AIRWAY
MUCINS IN CYSTIC FIBROSIS

A thesis submitted to The University of Manchester for the
degree of Doctor of Philosophy in the Faculty of Biology,
Medicine and Health School of Biological Sciences

2018

ABIGAIL C COWLEY
Contents

List of figures ................................................................. 3
Abbreviations ................................................................. 8
Abstract ........................................................................... 11
Declaration ........................................................................ 12
Copyright Statement ....................................................... 12
Acknowledgements .......................................................... 13

1. Chapter 1: Introduction .................................................. 14
   1.1 The airway mucus barrier .......................................... 14
   1.2 Mucins: an overview ............................................... 16
      1.2.1 Mucin structure and function ....................... 16
          1.2.1.1 Central domains .................................. 16
          1.2.1.2 N- and C-terminal domains ............... 16
          1.2.1.3 Mucin glycans .................................. 18
      1.2.2 Mucin biosynthesis ........................................ 19
   1.3 From mucins to mucus ............................................ 21
      1.3.1 Mucin expansion and gel formation ........... 21
      1.3.2 Mucin-associated molecules ................... 22
   1.4 Cystic fibrosis: a disease affecting mucus producing organs ...... 25
      1.4.1 CFTR structure and function .................. 25
      1.4.2 CFTR mutations ....................................... 28
      1.4.3 The mucus barrier in CF ......................... 30
      1.4.4 Mucins in CF ........................................ 32
   1.5 Airway infections in CF .......................................... 33
      1.5.1 Bacterial infections in CF airways ............. 33
      1.5.2 Fungal infections in CF airways .............. 34
   1.6 Aspergillus in CF ................................................ 34

Copyright Statement ........................................................ 12
Acknowledgements .......................................................... 13

1. Chapter 1: Introduction .................................................. 14
   1.1 The airway mucus barrier .......................................... 14
   1.2 Mucins: an overview ............................................... 16
      1.2.1 Mucin structure and function ....................... 16
          1.2.1.1 Central domains .................................. 16
          1.2.1.2 N- and C-terminal domains ............... 16
          1.2.1.3 Mucin glycans .................................. 18
      1.2.2 Mucin biosynthesis ........................................ 19
   1.3 From mucins to mucus ............................................ 21
      1.3.1 Mucin expansion and gel formation ........... 21
      1.3.2 Mucin-associated molecules ................... 22
   1.4 Cystic fibrosis: a disease affecting mucus producing organs ...... 25
      1.4.1 CFTR structure and function .................. 25
      1.4.2 CFTR mutations ....................................... 28
      1.4.3 The mucus barrier in CF ......................... 30
      1.4.4 Mucins in CF ........................................ 32
   1.5 Airway infections in CF .......................................... 33
      1.5.1 Bacterial infections in CF airways ............. 33
      1.5.2 Fungal infections in CF airways .............. 34
   1.6 Aspergillus in CF ................................................ 34
1.6.1 Interactions between *A. fumigatus* and the mucus barrier ..............................36

1.6.2 Impact of *A. fumigatus* on airway mucins..................................................38

1.7 Summary and hypothesis.......................................................................................41

1.8 Aims.......................................................................................................................42

2. Chapter 2: Materials and Methods......................................................................43

2.1 Materials.................................................................................................................43

2.2 Methods..................................................................................................................43

2.2.1 Sputum collection and processing.................................................................43

2.2.2 Cell culture.........................................................................................................43

2.2.3 Mucin purification.............................................................................................44

2.2.4 Slot blotting.......................................................................................................44

2.2.5 Periodic Acid Schiff (PAS) staining...............................................................44

2.2.6 Agarose gel electrophoresis............................................................................45

2.2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) .........................................................................................................................45

2.2.8 Silver staining....................................................................................................45

2.2.9 Western blotting...............................................................................................45

2.2.10 Immunodetection............................................................................................45

2.2.11 Antibodies......................................................................................................46

2.2.12 Purification of NT5B and CT5B...................................................................46

2.2.13 Size exclusion chromatography with Multi Angle Laser Light Scattering (SEC-MALLS) ...........................................................................................................47

2.2.14 Tandem mass spectrometry (MS/MS) .........................................................47

2.2.15 Preparation and characterisation of *A. fumigatus* culture filtrates.............48

2.2.15.1 *A. fumigatus* strains..................................................................................48

2.2.15.2 Preparation of *A. fumigatus* spores.........................................................48

2.2.15.3. Preparation of *A. fumigatus* culture filtrates........................................48

2.2.15.4 Size exclusion chromatography...............................................................48
3. Chapter 3: Degradation of airway mucins by *A. fumigatus* ................................................52

3.1 Introduction........................................................................................................52

3.2 Results................................................................................................................53

3.2.1 Purification and characterisation of MUC5B and MUC5AC..........................53

3.2.2 Preparation and characterisation of *A. fumigatus* culture filtrates..............57

3.2.3 *A. fumigatus* degrades MUC5B and MUC5AC........................................59

3.2.4 Mucin-degrading proteases are secreted after 12 hours in liquid culture........62

3.2.5 *A. fumigatus* proteases cleave the N- and C-terminus of MUC5B..............64

3.2.6 Cleavage of mucin glycans by *A. fumigatus*.............................................68

3.3 Discussion.........................................................................................................71

3.4 Conclusion.......................................................................................................73

4. Chapter 4: Identification of mucin degrading proteases and their regulators......74

4.1 Introduction.......................................................................................................74

4.2 Results.............................................................................................................75

4.2.1 Mucin degradation is mediated by distinct classes of proteases..............75

4.2.2 Mucin degradation by *A. fumigatus* is regulated by multiple secreted
products......................................................................................................................77

4.2.3 Mep protease degrades MUC5B and MUC5AC.............................................83

4.2.4 Identification of mucin degrading components of \textit{A. fumigatus} culture
filtrates.........................................................................................................................83

4.2.5 PalH and PacC are regulators of mucin degradation.................................87

4.3 Discussion...........................................................................................................90

4.4 Conclusion........................................................................................................93

5. Clinical implication of \textit{A. fumigatus} in cystic fibrosis...........................................94

5.1 Introduction..........................................................................................................94

5.2 Results................................................................................................................95

5.2.1 Effects of \textit{A. fumigatus} on the biochemical properties of CF mucins......95

5.2.2 \textit{A. fumigatus} reduces mucin viscosity.......................................................100

5.2.3 \textit{A. fumigatus} may induce airway remodelling and mucin secretion.......100

5.2.4 \textit{A. fumigatus} cannot utilise mucins as a sole carbon source..............104

5.3 Discussion...........................................................................................................106

5.4 Conclusion..........................................................................................................108

6. Chapter 6: General Discussion.............................................................................109

6.1 Future work.........................................................................................................112

6.1.1 Identification of proteolytic cleavage sites..............................................112

6.1.2 Identification of other mucin degrading proteases.................................113

6.1.3 Further investigations into the clinical implications of \textit{A. fumigatus} in
cystic fibrosis............................................................................................................113

6.1.4 Investigations into the use of mucins as a sole carbon source by
\textit{A. fumigatus}........................................................................................................114

6.2 Conclusions.......................................................................................................114

References...............................................................................................................115

Word count: 36,487
List of Figures

Figure 1.1 Structure and organisation of the airway surface liquid
Figure 1.2 Mucin domain organisation and glycan structure
Figure 1.3 Mucin intracellular assembly and packaging
Figure 1.4 Mucin secretion within the airways
Figure 1.5 CFTR structure and domain organisation
Figure 1.6 CFTR channel gating and transport
Figure 1.7 Effects of CFTR mutation classes
Figure 1.8 Airway mucus in cystic fibrosis
Figure 1.9 Prevalence of respiratory microorganisms in CF patients
Figure 1.10 *A. fumigatus* life cycle
Figure 1.11 Effects of *A. fumigatus* on the airway mucus barrier
Figure 3.1 Purification of MUC5B and MUC5AC
Figure 3.2 Characterisation of MUC5B and MUC5AC by SEC-MALLS
Figure 3.3 Composition of *A. fumigatus* (CEA10) culture filtrate
Figure 3.4 Degradation of MUC5B and MUC5AC by *A. fumigatus* culture filtrates
Figure 3.5 *A. fumigatus* culture filtrate causes a change in mucin sedimentation
Figure 3.6 Mucin degrading proteases are secreted after 12 hours in liquid culture
Figure 3.7 Purification of MUC5B N-terminus
Figure 3.8 Purification of MUC5B C-terminus
Figure 3.9 *A. fumigatus* culture filtrate degrades the N- and C-termini of MUC5B
Figure 3.10 Cleavage of D1, D3 and CK domains of MUC5B by *A. fumigatus* culture filtrates
Figure 3.11 Cleavage of mucin glycans by *A. fumigatus*
Figure 4.1 Mucin degradation is mediated by distinct classes of proteases
Figure 4.2 Mucin degradation by *A. fumigatus* secreted protein knockout strains (1)
Figure 4.3 Mucin degradation by *A. fumigatus* secreted protein knockout strains (2)
Figure 4.4 Mucin degradation by *A. fumigatus* secreted protein knockout strains (3)
Figure 4.5  Mucin degradation by *A. fumigatus* secreted protein knockout strains (4)
Figure 4.6  Mucin degradation by *A. fumigatus* secreted protein knockout strains (5)
Figure 4.7  Mep protease degrades MUC5B and MUC5AC
Figure 4.8  Purification of *A. fumigatus* culture filtrate
Figure 4.9  Isolation of mucin degrading proteases in *A. fumigatus* culture filtrate
Figure 4.10 Mucin degradation may be regulated by PalH/PacC signalling
Figure 5.1  MUC5B and MUC5AC variation in CF sputum
Figure 5.2  Purification of mucins from CF sputum
Figure 5.3  Biomolecular analysis of CF mucins
Figure 5.4  Effects of *A. fumigatus* on mucin viscosity
Figure 5.5  Effects of *A. fumigatus* on MUC5B and MUC5AC secretion in mouse airways
Figure 5.6  Effects of *A. fumigatus* on intracellular MUC5B levels in mouse airways
Figure 5.7  *A. fumigatus* growth in the presence of different carbon sources

**List of Tables**

Table 1.1  O-glycan core structures
Table 3.1  MS/MS analysis of MUC5B/MUC5AC mucin preparation
Table 3.2  MS/MS analysis of *A. fumigatus* (CEA10) culture filtrate
Table 4.1  Proteomic analysis of active *A. fumigatus* culture filtrate fractions
Table 5.1  Proteomic analysis of CF sputum
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔF508:</td>
<td>CFTR phenylalanine 508 deletion</td>
</tr>
<tr>
<td>Af+:</td>
<td>Positive for <em>Aspergillus fumigatus</em></td>
</tr>
<tr>
<td>Af-:</td>
<td>Negative for <em>Aspergillus fumigatus</em></td>
</tr>
<tr>
<td><em>A. fumigatus</em>:</td>
<td><em>Aspergillus fumigatus</em></td>
</tr>
<tr>
<td>ASL:</td>
<td>Airway surface liquid</td>
</tr>
<tr>
<td><em>C. albicans</em>:</td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>Ca²⁺:</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CF:</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFTR:</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>Cl⁻:</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>CO₂:</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CsCl:</td>
<td>CsCl</td>
</tr>
<tr>
<td>CT5B:</td>
<td>C-terminus of MUC5B</td>
</tr>
<tr>
<td>Cys:</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Cys-domains:</td>
<td>Cysteine-rich subdomains</td>
</tr>
<tr>
<td>Da:</td>
<td>Dalton</td>
</tr>
<tr>
<td>dH₂O:</td>
<td>Distilled water</td>
</tr>
<tr>
<td>ddH₂O:</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DMEM:</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA:</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT:</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA:</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Gal:</td>
<td>Galactose</td>
</tr>
<tr>
<td>GalNAc:</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GlcNAc:</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>g/ml:</td>
<td>Grams per milliliter</td>
</tr>
<tr>
<td>IgG:</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>kDa:</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Mbar:</td>
<td>Millibar</td>
</tr>
<tr>
<td>MCC:</td>
<td>Mucociliary clearance</td>
</tr>
<tr>
<td>MDa:</td>
<td>Medadaltons</td>
</tr>
</tbody>
</table>
mg/ml: Milligrams per milliliter
Mins: Minutes
ml: Millilitre
mPa: Millipascal
MSD: Mean squared displacement
MS/MS: Tandem mass spectrometry
Mw: Molecular weight
Na⁺: Sodium ion
NaCl: Sodium chloride
NeuAc: N-acetyleneuraminic acid
NT5B: N-terminus of MUC5B
OD: Optical density
P. aeruginosa: Pseudomonas aeruginosa
PAS: Periodic acid-Schiffs
PBS: Phosphate buffered saline
PCL: Periciliary layer
PI: Protease inhibitor
RCF: Relative centrifugal force (G-force)
RI: Refractive index
SDS: Sodium dodecyl sulphate
SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC-MALLS: Size exclusion chromatography with multi-angle laser light scattering
Ser: Serine
TBST: Tris-buffered saline Tween
TFF: Trefoil factor
µg: Microgram
µg/ml: Micrograms per millilitre
µl: Microlitre
UV: Ultraviolet
V: Volts
<table>
<thead>
<tr>
<th>v/v:</th>
<th>Volume/volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>w/v:</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>x:</td>
<td>Multiplied by</td>
</tr>
</tbody>
</table>
Abstract

University name: University of Manchester

Candidate name: Abigail C Cowley

Degree title: Doctor of Philosophy

Thesis title: Investigating the interplay between *Aspergillus fumigatus* and airway mucins in cystic fibrosis

Date: 2018

*Aspergillus fumigatus* (*A. fumigatus*) is the most common fungal pathogen found in cystic fibrosis (CF) airways, although its prevalence has until recently been underestimated. Colonisation and infection with *A. fumigatus* is associated with a decline in lung function in CF patients, but it’s precise role in disease progression remains unclear. Airway mucus is the first point of contact for *A. fumigatus* spores following inhalation, providing an important site for host-pathogen interactions. The interplay between *A. fumigatus* and the mucus barrier has only recently begun to be studied, and its effects on the respiratory mucins MUC5B and MUC5AC are yet to be elucidated.

Through the use of agarose gel electrophoresis and rate zonal centrifugation, an in depth analysis of the effects of *A. fumigatus* on purified mucins was performed. Treatment of MUC5B and MUC5AC with *A. fumigatus* culture filtrates led to a reduction in mucin size over time. Recombinantly expressed mucin domains were used to identify potential cleavage sites within MUC5B, identifying the D1 and D3 domains of the N-terminus and CK domain of the C-terminus as regions targeted by secreted proteases. Lectin assays were used to determine the effects of *A. fumigatus* on mucin glycosylation, with data suggesting cleavage of mucin glycans by glycosidase enzymes.

Using protease inhibitors and a panel of *A. fumigatus* secreted protein knockouts, Alp1, signal peptidase I, DppV and Mep were identified as putative mucin-degrading proteases. Knockout strains for the cell-surface receptor PalH and the transcription factor PacC identified these proteins as regulators of mucin degrading proteases, with culture filtrates from mutant strains showing a reduced ability to degrade MUC5B and MUC5AC.

Through the use of purified CF mucins, the potential effects of *A. fumigatus* on CF airways was investigated. Mucins from patients testing positive for *A. fumigatus* appeared smaller than those from *A. fumigatus* negative patients, with mucin degradation correlating with a reduction in mucin viscosity. Using a repeated exposure in mice it was shown that *A. fumigatus* may cause
an increase in intracellular MUC5B levels and extracellular MUC5AC levels. Finally, our data suggest that mucins alone are not sufficient to act as a sole carbon source to support *A. fumigatus* growth.

Overall, these data provide a useful insight into the potential role of *A. fumigatus* in CF, highlighting mucin degradation as a potential mechanism of disease pathogenesis.

**Declaration**

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

**Copyright Statement**

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://documents.manchester.ac.uk/DoculInfo.aspx?DocID=24420), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.library.manchester.ac.uk/about/regulations/) and in The University’s policy on Presentation of Theses.
Acknowledgements

Firstly I would like to thank my supervisors Dave Thornton and Alex Horsley, whose knowledge and guidance have been a great help throughout my PhD. Their support and encouragement have given me the opportunity to attend a number of international conferences, which have been a fantastic source of ideas and a great chance to meet experts within the field. I would also like to thank my co-supervisor David Denning, who has always been on hand to provide help and advice when needed.

I am extremely grateful to Elaine Bignell and all of the members of the Manchester Fungal Infection Group, for welcoming me into their lab and always being happy to help. Elaine has offered a tremendous amount of advice regarding all things Aspergillus, and her discussions have been invaluable in guiding me through the final three years of my PhD.

Thanks to all of the members of the Thornton lab for their technical guidance, and for all of the great days (and nights!) out over the past 4 years. I would also like to thank the North West Lung Charity for their funding, without which this work would not have been possible.

Finally, I would like to say a huge thanks to my family and friends for your encouragement throughout my PhD. I would also like to thank George, who has always been there to cheer me up when things have been tough. I also couldn’t go without mentioning my three rabbits Peanut, Boo and Cliff, who have been a welcome distraction during my time writing up and made the process a lot more bearable!

I would like to dedicate this thesis to my Mum, Dad and brother Chris (with a special mention to our little Darcy). Your endless support throughout my education has been incredible. I wouldn’t have been able to do it without you!
Chapter 1: Introduction

1.1 The airway mucus barrier

The lung environment is constantly exposed to a myriad of environmental challenges including inhaled allergens, pathogens and other foreign particulates. To protect the airways against these challenges, the epithelial surface is coated by a layer of mucus. This complex and dynamic secretion is a key component of the innate immune system, forming a protective barrier against potentially pathogenic insults (Fahy and Dickey, 2010). One of the main functions of mucus is to maintain hydration of mucosal surfaces, allowing them to remain lubricated and avoid mechanical damage. As well as forming a physical barrier, mucus also possesses antimicrobial characteristics, owing to the presence of various host defence factors (Rogan et al, 2006). The precise properties and composition of the mucus barrier varies throughout the body, adapting to the specific needs of each individual tissue. Within the gut for example, mucus forms two distinct layers; an inner adherent layer forms a static barrier that is impenetrable to bacteria, with a loose outer layer providing a more dynamic environment that is highly populated with bacteria (Johansson et al, 2011). Within the airways, the constant influx of foreign particles provides a need for a more dynamic and transportable barrier that allows their removal from the respiratory tract (Verdugo, 2012).

Lining the airways is a protective film known as the airway surface liquid (ASL). Under homeostatic conditions the ASL can be divided into two distinct layers, and this organisation is key to maintaining a healthy airway environment. Mucus forms the outermost layer of the ASL, and is able to trap inhaled pathogens and particulates for their subsequent removal by mucociliary transport. Beneath this layer, and directly above the epithelium, is the periciliary layer (PCL). The properties of the PCL allow efficient ciliary beating such that the overlying mucus can be cleared from the lung, removing pathogenic insults away from the airway surface (Figure 1A). Although the PCL is often viewed as a ‘watery’ layer, it is in fact occupied by large membrane-tethered mucin glycoproteins, which through intramolecular repulsion are able to stabilise the PCL to prevent invasion by the mucus layer and collapse of the two-layer system (Figure 1B) (Button et al, 2012).

The biophysical properties of mucus are essential for its protective function, and maintaining normal lung function. Whilst the viscosity of the barrier facilitates pathogen entrapment, it must also remain transportable to allow their removal by mucociliary clearance (MCC) and/or cough (Cone 2009). Excessive mucus production is a common feature of chronic airway diseases such as cystic fibrosis (CF). Coupled with alterations in mucus rheology, this results in aberrant transport properties and impaired mucus clearance from the lung, leading to infection and inflammation (Ehre et al, 2014).
Figure 1.1: Structure and organisation of the airway surface liquid

A) In healthy airways the airway surface liquid (ASL) is divided into two distinct layers. The periciliary layer (PCL) allows efficient ciliary beating such that inhaled particles and microorganisms can be removed from the lung by MCC. Above the PCL lies the mucus layer, which is secreted from the epithelium by goblet cells, club cells and the submucosal glands (not shown). B) The surface of ciliated epithelia is dominated by the presence of transmembrane mucins and other mucopolysaccharides, which act as a macromolecular mesh that stabilises the PCL.
1.2 Mucins: an overview

Mucus is a heterogeneous mixture composed mainly of mucus, as well as salts, lipids, proteins and nucleic acids. The main macromolecular components of this secretion are the polymeric gel-forming mucins. These O-linked glycoproteins form huge networks that provide the structural framework of mucus gels, and determine many of its functional and biophysical properties (Fahy and Dickey, 2010).

1.2.1 Mucin structure and function

Mucins exist in a variety of different forms, including cell-tethered and secreted mucins which can occur as polymeric and nonpolymeric isoforms. Cell-tethered mucins are an important component of the glycocalyx, a dense carbohydrate-rich layer that forms a protective barrier above the epithelial surface and prevents invasion of the PCL by the mucus layer (Kesimer et al, 2013). In this thesis, we will focus on the secreted gel-forming mucins. Polymeric gel-forming mucins are encoded by MUC genes located at chromosomes 12q12 (MUC19) and 11p15.5 (MUC2, MUC5AC, MUC5B and MUC6) (Chen et al, 2004; Pigny et al, 1996), with the latter sharing a complex multidomain organisation (Figure 1.2) that is thought to have arisen by gene duplication.

1.2.1.1 Central domain

The central region of the MUC genes consists of a single exon encoding several large mucin domains. The length, sequence and number of these domains can vary between different mucin species, although a hallmark feature are their variable number tandem repeat (VNTR) regions. These regions of the mucin polypeptide are rich in serine (Ser), threonine (Thr) and proline (Pro) residues and act as a scaffold for O-glycosylation. This largely expands these regions to give mucins a huge volume occupancy in solution, which is important for mucus gel formation. Mucin domains are also interspersed with cysteine-rich domains known as ‘cys-domains’; these domains share a high degree of sequence similarity, although their number varies between different mucin species. Although the precise function of the cys domains is unclear, it is possible that they add flexibility to the central region, and they are also believed to be involved in mucin-mucin interactions (Thornton et al, 2008; Ambort et al, 2011).

1.2.1.2 N- and C-terminal domains

Flanking the central region are the N- and C-terminal von Willebrand factor (vWF)-like domains: D1, D2, D’ and D3 domains at the N-terminus, and D4, B, C and CK domains at the C-terminus (Figure 1.2) (Thornton et al, 2008). Studies on recombinant N-terminal domains of MUC5B (NT5B) have shown that the N-terminus is approximately 175 kDa in size, and appears to form disulphide-linked dimers via the D3 domain (Ridley et al, 2014). The C-terminus of MUC5B (CT5B) is thought to be slightly smaller at around 150 kDa, and also appears to form homotypic disulphide-linked dimers (Ridley et al, 2016). These cysteine-rich domains are involved in
Figure 1.2: Mucin domain organisation and glycan structure

A) Domain organisation of a mucin monomer, showing the N- and C-terminal VWF-like domains, central mucin domains (MD) and Cys-domains (Cys). B) Structural organisation of the MUC5B and MUC5AC central regions. Mucin domains are punctuated with non-glycosylated Cys-domains, which vary in number between different mucin species. The number, length and sequence of the mucin domains may vary, resulting in mucins that are polydisperse in size. Repetitive (purple striped) and non-repetitive (purple) sequences within the mucin domains have also been highlighted. C) Schematic representation of mucin glycans that decorate the central mucin domains, showing the structural diversity that may arise through variations in monosaccharide composition, length and branching.
disulphide-mediated polymerisation of mucins to form polymers of up to 50 MDa in size and 10 µm in length. Through mucin crosslinking and chain entanglement, these polymers form the huge viscoelastic network that forms the basis of mucus gels (Raynal et al, 2003).

1.2.1.3 Mucin glycans

Mucins are typically over 70% carbohydrate by mass. Their central mucin domains are highly decorated with a huge array of O-glycans, meaning mucins are often likened to a ‘bottle-brush’ structure. There are many ways in which these glycan structures can differ, including length, composition, branching and modifications. At the core of the glycan chains are 8 possible structures, which invariably begin with a single N-acetylgalactosamine (GalNAc) residue linked to the hydroxyl group of Ser/Thr (known as the Tn antigen) (Table 1.1). These core structures can be extended to create highly heterogeneous oligosaccharide chains that typically consist of alternating N-acetylgalactosamine (GlcNAc) and galactose residues, terminating in fucose or sialic acid residues (Figure 1.2). Further modifications of these monosaccharides may also occur in the form of acetylation or sulphation, meaning mucins are polyanionic in nature (Varki et al, 2009). As well influencing mucin conformation, glycosylation is also important for mucin hydration, which is essential for maintaining lubrication of mucosal surfaces (Crouzier et al, 2015). It also protects mucins against proteolytic degradation, and plays a key role in pathogen sequestration (Thornton et al, 2008). In the stomach, MUC5AC (as well as the membrane-bound mucin MUC1) has been shown to bind *Helicobacter pylori* via Leb structures on its glycan chains. As such, these mucins may act as a decoy receptor to prevent access of the bacteria to the epithelial surface and thus prevent infection (Linden et al, 2004). In the same way, *Pseudomonas aeruginosa* and *Aspergillus fumigatus* have been shown to bind to MUC5B via sialic acid- and fucose-specific lectins, which is believed to facilitate removal of these pathogens from the airways (Ramphal and Pyle, 1983).

Table 1.1 O-glycan core structures

<table>
<thead>
<tr>
<th>Core</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tn antigen</td>
<td>GalNAcαSer/Thr</td>
</tr>
<tr>
<td>Core 1</td>
<td>Galβ1-3GalNAcαSer/Thr</td>
</tr>
<tr>
<td>Core 2</td>
<td>GlcNAcβ1-6(Galβ1-3)GalNAcαSer/Thr</td>
</tr>
<tr>
<td>Core 3</td>
<td>GlcNAcβ1-3GalNAcαSer/Thr</td>
</tr>
<tr>
<td>Core 4</td>
<td>GlcNAcβ1-6(GlcNAcβ1-3)GalNAcαSer/Thr</td>
</tr>
<tr>
<td>Core 5</td>
<td>GalNAcα1-3GalNAcαSer/Thr</td>
</tr>
<tr>
<td>Core 6</td>
<td>GalNAcβ1-6GalNAcαSer/Thr</td>
</tr>
<tr>
<td>Core 7</td>
<td>GalNAcα1-6GalNAcαSer/Thr</td>
</tr>
<tr>
<td>Core 8</td>
<td>Galα1-3GalNAcαSer/Thr</td>
</tr>
</tbody>
</table>
1.2.2 Mucin biosynthesis

Mucin secretion occurs both constitutively and in response to a wide variety of stimuli (Thornton et al., 2008). The main polymeric mucins found in airway mucus are MUC5AC and MUC5B. MUC5B exists in both low- and high-charge isoforms, with the former containing more fucose and the latter containing more sulphate (Troxler et al., 1998). MUC5AC is produced by secretory cells at the epithelial surface, whereas MUC5B is produced mainly by mucous cells in the submucosal glands (Hovenberg et al., 1997) (Figure 1.4). Although the reasons for this are not fully understood, this likely represents differential roles of these mucins within the airways. Production at distinct sites may allow the airways to fine-tune mucus composition in response to various environmental challenges, perhaps regulating secretion of each mucin species depending on the nature of the incoming insult. For example, MUC5AC may be involved in more acute responses within the airways. Indeed, Muc5ac production is upregulated dramatically during allergic inflammation (Evans et al., 2015; Zhen et al., 2007; Alevy et al., 2012), and its localisation towards the epithelial surface may allow rapid secretion in response to allergen exposure. De novo Muc5ac expression in the intestine has also been observed during acute Trichuris muris infection (Hasnain et al., 2011), which further supports this hypothesis. MUC5B on the other hand may play a more constitutive role in airway defence and predominate during more chronic insults, with its secretion from the submucosal glands occurring at a constant low level to provide a baseline level of protection (Roy et al., 2014).

Following translation within secretory cells, mucin polypeptides are transported through the endoplasmic reticulum (ER), where they are N-glycosylated and form disulphide-linked dimers via their C-terminal CK domains (Thornton et al., 2008; Ridley et al., 2016). This is followed by O-glycosylation of mucin dimers within the Golgi (Figure 1.3), which as described above (section 1.2.1.3), confers a huge degree of structural heterogeneity to mucins (Sheehan et al., 2004). Although partially dependent on the amino acid sequence of the mucin domain, glycosylation is largely determined by the levels and specificities of glycosyltransferases present throughout the secretory pathway, and this can also vary different between cells and tissues (Linden et al., 2008). O-glycosylation is initiated by polypeptide-GalNAc transferases (ppGalNAcTs), which catalyse the transfer of GalNAc monosaccharides from UDP-GalNAc to serine and threonine residues within the mucin domains (Varki et al., 2009). There are 15 mammalian ppGalNAcTs which vary in their specificities; whilst also catalysing the transfer of GalNAc onto different acceptor substrates, some have been shown to display lectin activity and are therefore influenced by neighbouring glycosylation (Gerken et al., 2006).

Once glycosylated, mucins undergo disulphide-mediated multimerisation via their N-terminal D domains. They are then packaged into secretory granules and stored prior to their release into the extracellular milieu (Ridley et al., 2014) (Figure 1.3). Due to their huge size, mucins must undergo considerable reorganisation in order to be packaged into these granules, the diameter of which is up to 10 times smaller than a full length mucin polymer (Perez-Vilar, 2007). Low pH
Following translation, mucin monomers are transported through the endoplasmic reticulum where they form disulphide-linked dimers via their C-terminal CK domains. As they enter the Golgi, mucin dimers become extensively O-glycosylated within their central mucin domains. Also within the Golgi (or within the secretory granule), mucin dimers polymerise via their N-terminal D3 domains, and Ca^{2+}-mediated non-covalent interactions result in crosslinking of these polymers to allow packaging into the granule prior to exocytosis.
and high calcium concentrations are thought to facilitate mucin packaging within these granules, shielding the negative charges of their glycan chains to prevent expansion (Verdugo et al., 1990). One model of mucin packaging has been proposed by Ambort, suggesting that mucins form concatenated ring structures around their N-terminus, with the C-termini forming dimers. As well as showing calcium-dependence, these interactions also occurred only at low pH, mimicking conditions of the intragranular environment (Ambort et al., 2012). Based on electron microscopy (EM) studies of MUC5B, Kesimer has proposed an alternative model of mucin packaging in which mucin dimers are organised around central proteinaceous nodes via their N- and C-terminus. This EM data suggests the presence of around 50-100 mucin monomers per granule, with dimers organised around 10-15 nodes (Kesimer et al., 2010).

Biophysical studies have demonstrated reversible N-terminal interactions in MUC5B, with four D3 domains associating in a calcium-dependent manner. These have been hypothesised to represent these ‘node’ regions, and after removal of calcium may allow rapid unpacking and expansion of mucins as they are released from the secretory granule (Ridley et al., 2014).

1.3. From mucins to mucus

Once secreted, mucins form an expansive network that acts as a structural and functional scaffold within mucus gels. Due to limitations in studying mucins in their native conformation, the precise details of their structure and interactions within mucus are incompletely understood. Although many aspects of this have been explored, these observations have often been based on mucins that have been purified under highly denaturing conditions (Thornton et al., 1990; Thornton et al., 1991; Davies et al., 1996), and thus may not reflect the true nature of these molecules in vivo. Further studies are needed to elucidate the supramolecular organisation of mucins in airway mucus, and to fully understand the complexity of this network.

1.3.1 Mucin expansion and gel formation

Upon release from the secretory granule, the shielding effects of the granular environment are lost, leading to rapid swelling and expansion of the mucin network to form a functional and transportable gel. This Donnan system involves an influx of Na\(^+\) ions and water through the secretory pore, which replace the intracellular Ca\(^{2+}\) ions and allow electrostatic repulsion between the polyanionic chains, driving a change in mucin conformation (Verdugo et al., 1990). Bicarbonate also appears to be important in this process, functioning as a Ca\(^{2+}\) chelator to enhance swelling and hydration (Chen et al., 2010). According to Kesimer’s model, release from the granule is followed by an expansion of carbohydrate-rich regions around the nodes. This was thought to allow further processing of mucins to form linear polymers, perhaps involving proteolytic cleavage of mucin crosslinks (Kesimer et al., 2010). However, more recently it has been showed that proteolytic processing of MUC5B does not occur during its biogenesis (Ridley et al., 2016). In Ambort’s model of mucin unpacking, N-terminal ring structures are disrupted upon granular release to form a flat net-like architecture made up of N-terminal trimeric regions.
and C-terminal dimeric regions, which are stacked on top of one another to form stratified sheets (Ambort et al, 2012). It is important to note however that there is no evidence that MUC5B or MUC5AC occur in this form.

Based on early studies using spectroscopy, it has been suggested that polymer chain entanglement is important for mucus gel formation. According to this model, mucins form linear structures that can interact with one another via non-covalent bonds between the glycan chains, with entanglement between the mucin polymers creating a loose network structure (Verdugo et al, 1983; Lee et al, 1977; Sellers et al, 1988). The formation of this network also appears to be dependent upon mucin concentration. Using particle tracking microrheology it has been shown that upon reaching a certain threshold concentration, mucin polymers begin to self-assemble to form a semi-dilute network at low pH. Upon reaching a further threshold level, this switches to a highly entangled network with a much higher viscosity (Georgiades et al, 2014). These findings highlights the importance of mucins in determining mucus biophysical properties, and likely explain the increase in mucus viscosity observed in diseases such as cystic fibrosis where mucin concentrations are increased.

1.3.2. Mucin-associated molecules

Although mucins are the predominant molecular constituents of mucus, there are many other molecules present that contribute to its overall structural and functional properties. These include both protein and non-protein molecules, which may be covalently or non-covalently bound within the mucin network in order to maintain their localisation within the mucosal environment.

Trefoil factor peptides (TFFs) are secreted by many mucus-producing cells throughout the body, and are believed to play an important role in mucosal repair (Taupin and Podolsky, 2003). Evidence suggests that TFFs are coexpressed with mucins, and TFF1 and TFF3 have been shown to be cosecreted with MUC5B and MUC5AC in the respiratory tract. TFF2 expression has also been reported in the lung, although at much lower levels (Madsen et al, 2007; Wiede et al, 1999). TFF1 has been shown to interact with MUC5AC via its C-terminal C domain, perhaps in a calcium-dependent manner (Tomasetto et al, 2000; Ruchaud-Sparagano et al, 2004). It has been speculated that these interactions promote stability of the mucus gel matrix to increase its resistance to proteases and mechanical stress (Gajhede et al, 1993). The physiological relevance of these interactions is not fully understood, although studies suggest that TFFs may regulate mucus rheological properties. TFF2 has been shown to cause an increase in mucus viscosity, with data suggesting a physical entanglement between the molecules. This may be of more importance in the gut where the epithelium is exposed to more mechanical stress and acidic pH (Thim et al, 2002; Kjellev et al, 2006), however it may also play a role during allergic
airway disease (AAD). TFF2 expression is upregulated in mice with chronic AAD, with data suggesting it can respond to epithelial injury by reversing airway remodelling changes. Exogenous TFF2 was shown to reduce epithelial thickness and goblet cell hyperplasia (Royce et al., 2013) suggesting a protective role that may also be important during other chronic airway diseases. Galectin-3 has also been shown to interact with MUC5B causing an increase in viscosity, and this is believed to have a stiffening effect by facilitating mucin crosslinking (Radicioni et al., 2016).

Mucins have also been shown to interact with other protective molecules, which may be important for their retention within the extracellular space. For example, salivary mucins appear to form heterotypic complexes with secretory IgA, and this may be important for clearing pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* from the oral cavity to prevent colonisation of the mucosal surface (Biesbrock et al., 1991). In a model of respiratory bacterial infection, data suggested that the protective role of sIgA required binding to a mucin-like secretory component, and this was also dependent on glycosylation of this component (Phalipon et al., 2002). This supports the idea that mucins interact with sIgA via their central glycosylated regions in order to facilitate its role in host defence, although further studies are needed to confirm this hypothesis. DMBT1 is another protein that has been shown to form complexes with respiratory mucins. This glycoprotein plays an important role in innate immunity, serving as a pattern recognition molecule that can bind to a broad range of pathogens as well as other host defence molecules such as IgA (Thornton et al., 2001; Ligtenberg et al., 2010).

Many other protein and non-protein molecules also exist within the mucus barrier, which may interact with mucins in various ways. Although it is likely that the mucin network acts as a scaffold for many of these molecules, they may also influence its structural properties. As discussed in section 1.3.1, bicarbonate appears to play an important role in mucin expansion by displacing Ca$^{2+}$ ions during exocytosis (Chen et al., 2010). Bicarbonate may also be involved in maintaining the buffering capacity of the ASL, which appears to be essential for maintaining normal airway function (Fischer and Widdicombe, 2006). Changes in ASL and mucus pH have been observed during airway diseases such as asthma and cystic fibrosis, and have been linked to increased epithelial stress and susceptibility to respiratory infections (Ricciardolo et al., 2004; Tang et al., 2016; Berkebile and McCray, 2014). This thesis will focus on the mucus barrier in CF, and the potential role of mucins during such infections.
MUC5AC and MUC5B are the main polymeric mucins found in airway mucus. MUC5AC is secreted from goblet cells at the epithelial surface, whereas MUC5B is secreted mainly by mucous cells within the submucosal glands (as well as small amounts produced by goblet cells). Following release from the secretory granule, mucin polymers expand and interact with one another to form a vast structural network that acts as a scaffold within mucus gels. Many other molecules also interact with mucins; these include both protein and non-protein molecules that act as mucin cross-linkers as well as other host defense molecules, which confer a number of protective functions to the mucus barrier.
1.4 Cystic fibrosis: a disease affecting mucus producing organs

CF is the most common autosomal recessive disorder in Caucasian populations, affecting around 1 in every 2500 births. Clinically, CF is characterised by the accumulation of airway secretions and chronic progressive airway infection. The combined effects of these thick luminal secretions along with airway wall inflammation lead to significant remodelling of the airway environment, causing a decline in lung function. CF was originally considered to be a fatal childhood disease, however advances in therapeutic strategies have led to an increase of the average life expectancy of CF patients to 37 years. Nevertheless, the life expectancy of these patients remains significantly reduced, and they continue to suffer from numerous health issues which negatively impact on their quality of life. A deeper understanding of the processes underlying CF pathophysiology will aid the development of novel therapeutic agents (Cystic Fibrosis Trust, 2018).

1.4.1 CFTR structure and function

Cystic fibrosis transmembrane conductance regulator (CFTR) is a 1480-residue membrane protein that is a member of the ATP-binding cassette (ABC) transporter family. It is typically located on the apical surface of epithelial cells, where it is responsible for regulating fluid and electrolyte transport throughout the body (Sheppard and Welsh, 1999). Typical of ABC transporters, the CFTR protein consists of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). Unlike other members of the family, CFTR also possesses N- and C-terminal extensions as well as an additional regulatory (R) domain, which contains multiple phosphorylation sites (Figure 1.5). The TMDs are composed of 6 transmembrane α-helices that are believed to form a pore through which ions are transported. The NBDs on the other hand are responsible for ATP binding and hydrolysis, which is essential for channel gating. One model suggests that ATP binding facilitates channel opening by inducing conformational changes in the TMD, via the formation of NBD head-to-tail heterodimers (Cant et al, 2014). Phosphorylation of the R domain is also necessary for CFTR function; this appears to increase interactions of this disordered region with the C-terminus and decrease interactions with the NBD (Bozoky et al, 2013). In order to conserve cellular ATP levels, the R region then becomes dephosphorylated by intracellular phosphatases in order to return the channel to its inactive stage (Bozoky et al, 2013; Sheppard and Welsh, 1999) (Figure 1.6).
Figure 1.5 CFTR structure and domain organisation

CFTR is composed of 2 transmembrane domains (TMDs) and 2 nucleotide binding domains (NBDs), as well as a regulatory R domain. From N-terminus to C-terminus these are arranged TMD1-NBD1-R-TMD2-NBD2. The 6 transmembrane α-helices of each TMD are connected by 3 extracellular loops (ECLs) and 2 intracellular loops (ICLs), with the 4th ECL modified by N-glycosylation at two residues (894 and 900). The ICLs are believed to act as coupling helices to facilitate interactions between the TMDs and NBDs. Adapted from Cant et al, 2014.
Although CFTR is mainly considered a chloride channel, its pore is not very selective (Linsdell, 2001). As well as directly regulating transepithelial chloride transport, CFTR also directly and indirectly regulates the movement of bicarbonate, sodium, and water across epithelial cell membranes (Quinton, 2008; Choi et al, 2001). Within the airways, this is important for maintaining hydration of the airway surface and the biophysical properties of the mucus barrier. For example, chloride efflux through CFTR promotes water transport into the extracellular environment in order to hydrate the mucosal surface. Bicarbonate is also able to pass through the CFTR channel, although less efficiently than chloride ions. This is believed to facilitate mucin expansion to create a transportable mucus gel, which is essential for maintaining a healthy airway environment (Chen et al, 2010). Defects in CFTR can perturb this balance of ion and water influx/efflux, creating problems such as mucus stagnation and impaired immune responses (Quinton, 2008).

Figure 1.6 CFTR channel gating and transport
A schematic representation of the CFTR transport cycle. TMD1 and TMD2 are shown as blue cylinders, and NBD1 and NBD2 as orange elongated crescents. The blue circle represents the CFTR transporter substrate. 1) Substrate binds to the intracellular side of the TMDs, increasing NBD affinity for ATP and facilitating NBD dimer formation. 2) ATP binding promotes the formation of closed NBD head-to-tail sandwich dimer, driving the switch of the TMDs to an open outward-facing conformation and releasing the substrate into the extracellular space. 3) ATP is hydrolysed by the NBDs, destabilising the NBD dimer. 4) ADP and Pi are released, causing the TMD to revert to an open inward-facing conformation so that the cycle may begin again. Figure adapted from Cant et al, 2014.
1.4.2 CFTR mutations

CF is caused by mutations in the gene encoding the CFTR protein. Many different CFTR mutations are known to cause CF, and these can be classified according to their effects on CFTR biosynthesis, processing and function (Table 1.2) (Figure 1.7). By far the most common of these mutations is ΔF508, accounting for almost two-thirds of cases worldwide (Castellani et al., 2008). Typically, classes I-III result in a more severe disease phenotype, whereas classes IV-VI are associated with milder cases (Marson et al., 2016). Mutations in the CFTR gene do not act alone in determining disease severity however, with other ‘modifier genes’ and environmental factors believed to influence the clinical phenotype of patients with CF. Lack of functional CFTR leads to a thickening of mucus secretions within affected organs, including the lungs, pancreas, intestine and reproductive tract. Of notable significance are the effects of CFTR mutations on the airways, with chronic pulmonary infections being the main cause of morbidity and mortality in CF patients.

Table 1.2: Traditional classification of CFTR mutations (adapted from Schmidt et al., 2016)

<table>
<thead>
<tr>
<th>Class</th>
<th>CFTR Defect</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Lack of functional protein</td>
<td>G542X, W1282X, R553X, 621+1G→T</td>
</tr>
<tr>
<td>II</td>
<td>Trafficking defect</td>
<td>ΔF508, N1303K, ΔI507, R560T</td>
</tr>
<tr>
<td>III</td>
<td>Impaired channel regulation</td>
<td>G551D, G178R, G551S, S549N</td>
</tr>
<tr>
<td>IV</td>
<td>Decreased channel conductance</td>
<td>R117H, R347P, R117C, R334W</td>
</tr>
<tr>
<td>V</td>
<td>Reduced synthesis</td>
<td>3849+10kbC→, 2789+5G→A, 3120+1G→A, 5T</td>
</tr>
<tr>
<td>VI</td>
<td>Decreased stability</td>
<td>4326delTC, Q1412X, 4279insA</td>
</tr>
</tbody>
</table>
Figure 1.7 Effects of CFTR mutation classes

CFTR mutations can be classified according to their effects on CFTR trafficking and function. Class I mutations result in a loss of functional protein that does not reach the cell surface. Class II mutations cause incorrect processing of CFTR, resulting in a loss of total protein. Class III mutations cause defects in channel regulation and ineffective channel regulation. Class IV mutations result in reduced channel conductance. Class V mutations still allow the production of functional protein but result in a reduced rate of synthesis. Class VI mutations lead to reduced levels of expression of mutated CFTR, with accelerated turnover at the apical membrane. (Taken from Banjar and Angyalosi, 2015)
1.4.3 The mucus barrier in CF

As discussed, chronic airway obstruction is a well-recognised feature of CF. Unlike in healthy airways, the ionic imbalance created by CFTR dysfunction causes dehydration of the ASL, leading to an increase in mucus concentration (Henderson et al., 2014; Matsui et al., 1998). As a result, the osmotic pressure of the mucus layer increases to such an extent that it exceeds that of the PCL, leading to a collapse of the two-layer ‘gel-on-brush’ system and compression and invasion of the PCL. Consequently, mucociliary transport ceases, leading to mucus stasis and adherence to the airway surface. Build-up of these thick luminal secretions and lack of MCC prevents efficient removal of airborne pathogens (viral, bacterial and fungal) from the lung (Figure 1.8). This provides an ideal environment for these organisms to flourish, promoting the development of chronic pulmonary infections. It is not yet clear whether these defects in pathogen clearance are a primary cause of CF, or whether they occur secondary to other factors such as excessive airway inflammation. Clinical studies have produced conflicting results; whilst many have provided evidence to suggest a causative role for MCC defects in CF (Hoegger et al., 2014; Sun et al., 2014), it is still unclear whether these defects are present at birth (McShane et al., 2004; Robinson et al., 2002). More recently it has been shown that CFTR-knockout ferrets treated from birth with antibiotics still developed symptoms of CF, including inflammation, mucus accumulation and bronchiectasis. These findings show that even in the absence of infection, mucoinflammatory symptoms of CF can still occur, thus suggesting an underlying non-infectious cause of disease progression (Rosen et al., 2018).

Although mucus dehydration is known to be one of the key factors that contribute to CF lung disease, the potential roles of other mucus abnormalities are not yet fully understood. Studies using the CF pig model have shown that a decrease in pH and increase in calcium concentration negatively impact on mucociliary transport by increasing mucus viscosity (Tang et al., 2016), perhaps due to incomplete mucin expansion post-secretion. This acidification has also been shown to reduce the antimicrobial activity of the ASL, an effect that was rescued upon increasing pH (Pezzulo et al., 2012). As well as predisposing CF airways to infection from the onset, this may also lead to other defects, for example in mucus secretion. Hoegger and colleagues showed that secreted mucus strands exhibit abnormal tethering to the submucosal glands, suggesting that the properties of CF mucus are altered even before it is secreted onto the airway surface. This has been linked to reduced anion secretion into the lumen of the glands, which appears to alter mucus properties in such a way that reduces its ability to detach from the gland as it emerges (Hoegger et al., 2014). These changes in mucus properties impact on its role in airway clearance, exacerbating MCC defects and impairing host defence against invading pathogens.
Figure 1.8 Airway mucus in cystic fibrosis

In CF airways, dehydration of the airway surface coupled with increased mucin secretion leads to a thickened mucus barrier with altered rheological properties. This causes compression of the PCL and impaired MCC, resulting in a build-up of adherent mucus secretions within the lung. This static mucus layer provides an ideal environment for inhaled pathogens, which can no longer be removed from the airways efficiently. This promotes colonisation of the lung by these airborne pathogens, leading to chronic respiratory infections that are characteristic of CF airway disease.
1.4.4 Mucins in CF

Due to their central role in defining mucus properties, it is unsurprising that changes in mucins have been observed in CF. The altered extracellular environment of the CF lung can have significant impacts on mucin structure and function, which in turn can alter mucus rheological properties. Reduced bicarbonate secretion due to CFTR dysfunction may prevent sufficient expansion of mucins post-secretion, which likely results in a more viscous mucus gel with reduced transportability (Chen et al., 2010).

Although it is well recognised that mucins are the main constituent of healthy mucus, their importance in determining CF sputum properties has been subject to some debate (Cowley et al., 2017). Some studies have reported lower levels of MUC5B and MUC5AC in CF sputum as compared with healthy controls, identifying DNA and actin as the main polymeric components of these secretions (presumably due to inflammatory cell necrosis) (Rubin, 2007; Henke et al., 2004). Mucin levels were found to increase during pulmonary exacerbation, although only to levels similar to those of the controls (Henke et al., 2007). Based on these findings it has been suggested that DNA, rather than mucins, is responsible for the viscoelastic properties of CF sputum. It is important to note however that these studies were based predominantly on patients chronically infected with Pseudomonas aeruginosa. Sputum from patients with no history of P. aeruginosa infection appeared to contain similar levels of mucin to those of healthy individuals (Henke et al., 2011), suggesting that these changes may only occur as a result of pulmonary exacerbation. CF airways are however abundant in bacterial and fungal proteases, which may degrade mucins and interfere with their detection. More recently, mass spectrometry studies conducted by Henderson and colleagues have revealed that CF mucins become cleaved at antibody recognition sites (Henderson et al., 2014). This highlights the inaccuracy of immunological assays in measuring mucin concentrations in CF sputum, and may explain the low levels of mucins reported in previous studies.

Many studies have highlighted the importance of mucins in CF sputum. Using size exclusion chromatography/differential refractometry (SEC/dRI) techniques it has been shown that mucin concentrations are in fact higher in CF secretions than in normal secretions (Henderson et al., 2014). Burgel and coworkers found that MUC5AC and particularly MUC5B levels were higher in mucus plugs from CF airways compared with sputum from non-CF controls (Burgel et al., 2007). Quantitative proteomic studies have also shown that CF secretions are enriched in MUC5B and MUC5AC in vitro, and this was apparent even in the absence of infection and inflammation (Peters-Hall et al., 2015). This suggests that increased mucin concentration may be a direct result of CFTR mutation, and supports the hypothesis that mucins play an important role in both early and advanced CF airway disease. Further evidence to support a role for mucins in CF has been provided by Horsley and colleagues, who showed that compared with DNase treatment, reduction of CF sputum using DTT had a much larger impact on sputum elasticity. This
highlights the importance of considering mucolytic therapies when treating patients with CF, as this may facilitate mucin solubilisation and promote airway clearance (Horsley et al, 2014).

CF mucins also appear to have significantly altered glycosylation patterns compared with those from healthy individuals. Mucins from CF sputum have been shown to possess a higher sugar content and more O-glycans than those from control samples. In particular, sulphation levels seem to vary widely in CF mucins, with some studies showing an increase in these modifications whilst others show a decrease compared with non-CF controls. Changes in sulphation and fucosylation levels have also been described in mucins from CF patients (Xia et al, 2005; Schulz et al, 2007). The underlying cause of these changes is currently unclear, however the continuous cycle of inflammation and infection may indeed play a role. Proinflammatory cytokines such as tumour necrosis factor α (TNF-α) are abundant in the CF lung, and have been shown to increase the expression and activity of specific sulpho- and glycosyltransferases (including sialyl- and fucosyl-transferases) (Delmotte et al, 2002).

These altered glycosylation patterns may have significant impacts within the CF lung. As described in section 1.2.1.3, mucin glycans are believed to act as decoy receptors for invading pathogens and may facilitate their removal from the airways. Changes in glycan structure may perturb these host-pathogen interactions, perhaps reducing their ability to sequester pathogens and increasing the bacterial and fungal burden of the CF lung.

1.5 Airway infections in CF

Chronic pulmonary infections are a major complication of CF, and one of the main causes of respiratory failure (Lyczak et al, 2002). These infections are polymicrobial, with many bacterial, fungal and viral pathogens frequently isolated from the airways of CF patients. These microbial communities vary widely from patient to patient, and this complexity brings about many challenges when developing treatment strategies (Filkins and O’Toole, 2015). Chronic infection leads to excessive inflammation within the airways, resulting in tissue damage, airway obstruction and worsened lung function (Döring et al, 2012).

1.5.1 Bacterial infections in CF airways

Bacterial colonisation of the airways is known to occur from an early age in many CF patients, with organisms such as Staphylococcus aureus and Haemophilus influenza commonly identified in sputum samples during the first decade of life. As patients get older these bacteria are eventually replaced with other organisms, most notably P. aeruginosa (Coutinho et al, 2008; Lyczak et al, 2002) (Figure 1.9). The role of both acute and chronic P. aeruginosa infections in CF airway disease has been extensively studied, and these have been linked with increased morbidity and mortality (da Silva et al, 2013). P. aeruginosa has been shown to secrete numerous proteases that can degrade mucins, including human neutrophil elastase (HNE) and
elastase B (LasB) (Henke et al., 2011). Mucin-degrading activity has also been observed in *Burkholderia cepacia* complex (Bcc), another bacterial pathogen associated with CF (Schwab et al., 2014). This may compromise the protective function of the mucus barrier and facilitate tissue invasion, not only by *P. aeruginosa* but also them myriad of other microbes present in the CF lung. Despite the clear importance of bacterial infections in CF airway disease, there is a growing body of evidence to support a role for fungal infections as well.

### 1.5.2. Fungal infections in CF airways

Colonisation of the airways by fungal organisms is a common occurrence in CF, however their prevalence has only more recently been recognised. For many years fungi were merely considered bystanders within the CF lung, and their presence in expectorated sputum samples was often dismissed as oral contamination. Improvements in culture techniques over the past decade has allowed more frequent identification of these organisms in patient samples, and has revealed an association between fungal colonisation and worsened clinical outcomes (Middleton et al., 2013). A variety of yeasts and filamentous fungi may be isolated from CF sputum, including *Candida*, *Scedosporium*, and *Aspergillus* species (Williams et al., 2016). Although our understanding of the role fungal colonisation/infection in CF pathogenesis is relatively limited, our knowledge is steadily improving. It is becoming clear that these pathogens may play a more significant role in CF airways than previously understood, and further studies are needed in order to fully understand the complex details of the CF lung microbiome.

### 1.6 Aspergillus in CF

*Aspergillus* (in particular *A. fumigatus*) is the most common fungal species found in the CF lung, present in up to 60% of patient samples (Mortensen et al., 2011). This filamentous fungus acts as an allergen and opportunistic pathogen in CF airways, and can cause a number of disease states associated with deterioration in lung function (Baxter, 2013).

*A. fumigatus* is a ubiquitous saprophytic fungus that releases airborne conidia into the environment. Although typically found within soil and decaying vegetation, its ability to withstand a diverse range of environmental conditions means it can also act as an opportunistic pathogen in humans (Dagenais and Keller, 2009). The infectious life cycle of *A. fumigatus* begins with the release of asexual conidia into the environment. Due to their small size, these hydrophobic spores are readily dispersed, remaining dormant until they encounter certain conditions that allow their metabolic activation. Once activated, conidia germinate and produce hyphae that protrude into the surrounding environment (McDonagh et al., 2008). During mycelial growth these hyphae also produce fruiting structures known as conidiophores, at the end of which lies
Figure 1.9 Prevalence of respiratory microorganisms in CF patients

The presence of different microorganisms within the CF lung can vary widely between different age groups. In terms of bacterial species, *S. aureus* tends to predominant in younger age groups, whereas in older patients *P. aeruginosa* begins to predominate. Other bacterial pathogens that are more prevalent in older patients include MRSA and *B. cepacia*, whereas others such as *H. influenza* appear to decrease with age. (Figure taken from Cystic Fibrosis Foundation Patient Registry Annual Data report, 2016)
the conidial head. Each conidial head produces thousands of conidia, which are released into the environment allowing the cycle to begin again (Latgé, 1999) (Figure 1.10). Humans inhale many of these conidia each day. In healthy airways this generally poses no threat; spores are readily removed by mechanisms such as MCC, and those that are able to bypass this system will be phagocytosed by alveolar macrophages or epithelial cells. Due to compromises in the immune system, patients with CF are unable to clear these spores efficiently, allowing this highly adaptive fungus to persist within the airways and cause disease (Sugui et al, 2014). Exposure to the increased temperature of the human body triggers conidial swelling and germination, and within 6-8 hours they begin to produce hyphae (McDonagh et al, 2008). Once established within the airways, A. fumigatus can cause a wide range of host responses in these patients, including allergic bronchopulmonary aspergillosis (ABPA-S), aspergillus sensitisation, and aspergillus bronchitis. Other complications may also occur in patients who are severely immunocompromised (for example following lung transplantation), such as invasive aspergillosis and the formation of aspergillomas. This recent classification has improved our understanding of aspergillosis in CF, although evidence suggests that patients may switch between these different disease phenotypes, or even exhibit more than one of these states synchronously (Baxter et al, 2013; Jones et al, 2014). This added level of complexity may increase the difficulty of diagnosing and treating these conditions, as well as establishing their precise role in CF airway disease.

1.6.1 Interactions between A. fumigatus and the mucus barrier

Alterations in mucus properties and impaired ciliary function results in reduced mucus clearance in CF, and this static and adherent barrier promotes airway colonisation by airborne pathogens such as A. fumigatus (Matsui et al, 1998). As the first point of contact for A. fumigatus spores following inhalation, it is not surprising that the mucus barrier appears to be involved in its pathogenicity, although knowledge surrounding this area is relatively limited. In order to become established within the airways, A. fumigatus must first adhere to components of the respiratory mucosa. A number of studies have investigated the adhesion of A. fumigatus to the airway epithelium. This process is believed to involve sialic acid-dependent interactions with laminin and fibrinogen via lectins on the conidial cell wall. Under normal conditions these proteins are not exposed at the epithelial surface. However tissue damage, for example due to the abundance of bacterial products present in the CF lung, may result in exposure of laminin from the basement membrane and deposition of fibrinogen due to subsequent inflammatory responses (Bouchara et al, 1997; Tronchin et al, 2002). It is possible that mucins are also targets for conidial binding, as their glycan chains are often terminated in sialic acid residues (Thornton et al, 2008). This is supported by findings by Tronchin and coworkers, who demonstrated binding of mucins by A.fumigatus conidia via a sialic acid-specific lectin (Tronchin et al, 2002). Sialic acid-dependent interactions with mucins have also been observed in other CF pathogens such as P. aeruginosa (Ramphal and Pyle, 1983), and may thus represent a common mechanism of pathogen adhesion within the airways. More recently,
The infectious life cycle of *A. fumigatus* begins with the release of airborne conidia into the environment. Upon encountering the correct conditions, these conidia will begin to germinate and produce hyphae, which will continue to grow and produce spore-bearing structures known as conidiophores, allowing the cycle to start again. Following inhalation of these spores by immunocompromised patients (such as those with CF), the conditions within the lung are sufficient to initiate germination and produce a host immune response.
a fucose-specific lectin (FleA) has been identified on *A. fumigatus* conidia, and this is believed to be important for host recognition and clearance. FleA has been shown to interact with mucins, and also appears to be involved in binding and phagocytosis by alveolar macrophages (Kerr *et al.*, 2016). A mouse model of *A. fumigatus* pneumonia highlighted the importance of FleA in host defence against infection. Mice that were treated with ΔfleA conidia showed increased lung injury and infection compared with those treated with wild type conidia, suggesting a potential role for this lectin as a pathogen-associated molecular pattern (Kerr *et al.*, 2016). As such, binding to mucins may facilitate fungal clearance under healthy conditions by trapping conidia within the mucus layer, allowing their subsequent removal by MCC. However this may not be the case in CF, where binding to mucins may actually promote fungal colonisation of the airways due to mucus stasis, allowing spores to persist within the lung environment (Cowley *et al.*, 2017). Swollen conidia also secrete gliotoxin, which has been shown to slow ciliary beat frequency and may also influence airway colonisation (Amitani *et al.*, 1995). Furthermore, an *in vitro* study performed by Chaudhary and colleagues showed that CF epithelial cells exhibit defective uptake and killing of *A. fumigatus* conidia, as well as excessive conidia-induced apoptosis (Chaudhary *et al.*, 2012). This may have a significant impact on the integrity of the epithelium, further compromising host defence against the fungus.

1.6.2 Impact of *A. fumigatus* on airway mucins

As well as interacting with the mucus barrier, inhaled *A. fumigatus* conidia may also manipulate mucins in order to establish a suitable niche within the airways. If retained within the airways, these conidia may germinate and release virulence factors and allergens to produce a host immune response (Cowley *et al.*, 2017). *A. fumigatus* secretes a variety of proteases, many of which are believed to act as virulence factors, and the proteolytic activity of different isolates appears to correlate with their ability to invade host tissues (Tomee and Kauffman, 2000). Fungal proteases also appear to play a role in ABPA and pulmonary aspergillosa, with protease-specific IgG antibodies identified in patient sera (Tomee *et al.*, 1994). As major components of airway mucus and important players in host defence, it is likely that mucins may be targeted by these proteases in order to compromise barrier function. Proteolytic degradation of mucins has previously been reported in *P. aeruginosa* and *Candida albicans* (Aristoteli and Wilcox, 2003; Colina *et al.*, 1996; St Leger and Screen, 2000), however these studies were performed using commercially available mucins, which have been shown to contain more impurities and have lower molecular weights than those isolated from fresh mucus secretions (Jumel *et al.*, 1996). *A. fumigatus* also secretes glycosidases, and these too have been shown to exhibit mucin-degrading activity (St Leger and Screen, 2000). There are number of reasons why *A. fumigatus* may degrade mucins. As the main macromolecular constituents of mucus, it is likely that mucin degradation may alter its biophysical properties. This may make the barrier more penetrable to invading pathogens, facilitating access to the underlying epithelium and colonisation of the mucosal environment (Cowley *et al.*, 2017) (Figure 1.11). *A. fumigatus* may also degrade mucins in order to utilise them as a nutrient source to support fungal growth; this
has been seen in a number of bacteria (Salyers et al., 1977), and may thus occur in fungal pathogens as well. Furthermore, cleavage of mucin glycans may serve as a mechanism of evading pathogen entrapment, removing carbohydrate epitopes that would otherwise allow mucins to bind microbes and facilitate their removal from the airways.

Due to the complexity of the CF airway microbiome, it is difficult to determine the precise effects of individual microorganisms. A. fumigatus has been shown to affect other airborne pathogens and vice versa, and this most likely affects their impact within the airways. Studies have shown that patients co-colonised with A. fumigatus and P. aeruginosa have worse lung function than those infected with either organism alone (Reece et al., 2017; Amin et al., 2010), suggesting a synergistic effect between these organisms. In contrast, other studies have demonstrated an antagonistic relationship between the two. P. aeruginosa has been shown to inhibit A. fumigatus biofilm formation in a contact independent manner, with CF isolates found to be more inhibitory than non-CF isolates (Ferreira et al., 2015). This highlights the complexity of intermicrobial interactions that exist within the CF lung, which must be taken into account when considering treatment strategies to target pulmonary infections.

Another way in which A. fumigatus may affect mucins is through altering their gene expression. Chronic exposure to A. fumigatus has been shown to induce goblet cell hyperplasia and increase Muc5ac expression in the lungs of asthmatic rats, alongside an increase in IL-13 (Gao et al., 2012). In another study, MUC5AC expression was shown to be induced by A. fumigatus serine protease activity in airway epithelial cells, through the sequential activation of TNF-α-converting enzyme (TACE), TGF-α and epidermal growth factor receptor (EGFR) (Oguma et al., 2011). As such, MUC5AC may be an important factor involved in the healthy host response against A. fumigatus. Indeed, upregulation of MUC5AC has been observed in other host-pathogen interactions. For example, de novo expression of intestinal Muc5ac occurs in response to acute Trichuris muris infection in mice, and this has been shown to be critical for worm expulsion and preventing chronic infection with the parasite (Hasnain et al., 2011). It is likely that upregulation of Muc5ac alters the structural organisation of mucus in such a way that facilitates pathogen entrapment and removal, as mice that are deficient in Muc5ac have been shown to possess a more porous mucus barrier (Hasnain et al., 2011). MUC5AC may also play a similar role within the airways, regulating host defence against A. fumigatus and facilitating removal of fungal spores from the lung (Cowley et al., 2017). Although these studies suggest a role for MUC5AC within this context, most studies have neglected the potential role of MUC5B, the other major gel-forming mucin found in airway mucus. It has been shown previously that Muc5b is required for defence against bacterial infections in mouse airways, with Muc5b−/− mice displaying a significant reduction in MCC and increased in bacterial burden within the distal airways (Roy et al., 2014). It is likely that MUC5B plays a similar role within human airways, and is protective against fungal pathogens as well. Precisely how mucins are affected by A. fumigatus, and vice versa, must be explored further in order to fully elucidate the mechanisms underlying disease pathogenesis. Understanding these details further may be crucial in developing effective therapeutic agents to treat patients such as those with CF.
Figure 1.11 Effects of *A. fumigatus* on the airway mucus barrier

*A. fumigatus* secretes a vast array of products into the extracellular environment that act as virulence factors to promote survival of the fungus within the host. These secreted products include various proteases and glycosidases, which may degrade mucins by cleaving both their glycan chains and polypeptide backbone. Due to the importance of mucins in determining mucus biophysical properties, it is likely that this compromises its protective function. Mucin degradation may make the mucus barrier more penetrable to conidia, facilitating access to the underlying epithelium. Mucins may also be utilised as a nutrient source, further promoting *Af* survival and pathogenesis within the lung.
1.7 Summary and hypothesis

*A. fumigatus* is a major fungal pathogen found in CF airways. Colonisation and infection with *A. fumigatus* is associated with a decline in the clinical outcome of patients (Middleton *et al*, 2013), however its role in the progression of CF airway disease is not yet fully understood. It has been shown previously that *A. fumigatus* can degrade mucins (St Leger and Screen, 2000). This study was performed using pig gastric mucin and bovine submaxillary mucin and it is thus unclear whether it will have the same impact on human airway mucins. Due to the adaptability of this opportunistic pathogen, we hypothesise that the same effects will be seen with human mucins, and that *A. fumigatus* will also degrade the respiratory mucins MUC5B and MUC5AC.

*A. fumigatus* secretes an array of putative virulence factors, many of which are proteases. This allows the fungus to degrade components of the surrounding environment, invading host tissues and contributing to conditions such as ABPA and pulmonary aspergilloma (Tomee and Kauffman, 2000; Tomee *et al*, 1994). The precise role of each of these proteases have not been fully elucidated, and it is likely that many of them have multiple and overlapping functions. As such, we hypothesise than a number of secreted proteases produced by *A. fumigatus* are involved in degrading mucins, which may alter mucus properties and promote airway colonisation.

Due to the importance of mucins in defining mucus barrier properties, it is likely that *A. fumigatus* degrades these components of the barrier in order to exploit its protective function. One of the characteristics of mucus gels that gives them this protective function is their viscoelastic properties, which allows invading pathogens to become entrapped within these secretions (Cone *et al*, 2009). We thus hypothesise that mucin degradation reduces mucus viscosity, which may make the barrier more penetrable to fungal spores allowing easier access to the underlying epithelium. We also hypothesise that mucins may be degraded by *A. fumigatus* in order to utilise them as a nutrient source, to promote fungal growth and survival within the airways.

Thus, the hypothesis of this thesis is that *A. fumigatus* degrades MUC5B and MUC5AC, altering mucus barrier properties. This may increase the susceptibility of CF airways to fungal colonisation perhaps by making the barrier more penetrable to *A. fumigatus* spores, and may also allow the use of mucins as a nutrient source to promote fungal growth.
1.8 Aims

In order to investigate the interplay between *A. fumigatus* and airway mucins and test this hypothesis, the aims of this thesis are:

- To study the effects of *A. fumigatus* on the size distribution of purified MUC5B and MUC5AC, using agarose gel electrophoresis and rate zonal centrifugation.
- To identify potential cleavage sites of *A. fumigatus* proteases using recombinantly expressed N- and C-terminal domains of MUC5B, through the use of subdomain-specific antibodies.
- To study the effects of *A. fumigatus* culture filtrates on mucin glycosylation using lectin assays.
- To explore the potential role of *A. fumigatus* proteases and other secreted products in regulating mucin degradation, using protease inhibitor studies, secrete protein knockout strains and purified *A. fumigatus* proteases.
- To use purification and proteomic techniques to identify the active mucin degrading components of *A. fumigatus* culture filtrates.
- To identify potential regulators of mucin degrading proteases using knockout strains for specific regulatory proteins.
- To explore the effects of *A. fumigatus* on the size and viscosity of CF mucins using density gradient centrifugation, SEC-MALLS and particle tracking microrheology.
- To study the potential effects of *A. fumigatus* *in vivo*. Through the use of a repeated exposure model in mice, the effects of *A. fumigatus* on mucin secretion will be studied using immunodetection and histology techniques.
- To perform growth assays to determine whether *A. fumigatus* can utilise mucins as a sole carbon source.
Chapter 2: Materials and Methods

2.1 Materials

Dithiothreitol (DTT) and Tris-base were purchased from Melford Laboratories. Ethanol, methanol, ethylenediaminetetraacetic acid (EDTA), glacial acetic acid, sodium chloride (NaCl), sodium citrate, and sodium dodecyl sulphate (SDS) were purchased from Fisher Scientific. Biotinylated lectins were purchased from Vector Labs. All other reagents were purchased from Sigma-Aldrich, unless otherwise stated.

Aspergillus complete medium (ACM) was prepared using 0.00075% (w/v) adenine, 0.01% (w/v) glucose, 0.001% (w/v) yeast extract, 0.002% (w/v) bacteriological peptone, 0.001% (w/v) casamino acids, 0.1% (v/v) vitamin solution*, 0.2% (v/v) Aspergillus salt solution**, 5 mM ammonium tartrate, pH 6.5 with NaOH). * Vitamin solution contained 0.004% (w/v) PABA (4-aminobenzoic acid), 0.0005% (w/v) aneurin (thiamine), 0.00001% (w/v) biotin, 0.24% (w/v) inositol, 1% (w/v) nicotinic acid, 2% (w/v) Panto (DL-phantothenic acid), 2.5% (w/v) pyridoxine, 1% (w/v) riboflavin, 0.014% (w/v) choline chloride. Aspergillus salt solution contains 0.26% w/v potassium chloride, 0.26% w/v MgSO₄.7H₂O (Magnesium dihydrogen heptahydrate), 0.76%, w/v KH₂PO₄ (Potassium dihydrogen orthophosphate), 0.5% Trace elements solution. Trace elements solution contains 0.0004% w/v Na₂B₄O₇.10H₂O (di-sodium tetraborate 10H₂O), 0.004% w/v CuSO₄.5H₂O (cupric sulphate 5H₂O), 0.008% w/v FePO₄.2H₂O (ferric orthophosphate dihydrate), 0.008% MnSO₄.2H₂O (manganese sulphate dihydrate), 0.008% w/v Na₂MoO₄.2H₂O (sodium molybdate 2H₂O), 0.08% w/v ZnSO₄.7H₂O (zinc sulphate 7H₂O).

Aspergillus minimal medium was prepared using 0.2% (v/v) Aspergillus salt solution, 0.2% (v/v) glucose, 5 mM ammonium tartrate, 1 µg/ml biotin.

2.2 Methods

2.2.1 Sputum collection and processing

Sputum samples were collected from adult CF patients testing either positive or negative for *Aspergillus fumigatus* during clinical visits, and stored at 80°C. Samples were thawed prior to processing and diluted 1:5 (by weight) in 8M GuCl. Patients were aged between 24 and 56 years old, with a median age of 40 years old. All patients were chronically infected with *Pseudomonas aeruginosa*.

2.2.2 Cell culture

Capan-1 cells were purchased from ATCC and used as a source of MUC5AC and MUC5B. Cells were cultured in T75 and triple layer cell culture flasks (Corning) and maintained in
Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% (v/v) FBS, 1% L-glutamine and 1% Penicillin-Streptomycin (Sigma Aldrich) at 37°C in 5% CO₂ humidified air.

EBNA-293 cells transfected with the N-terminal domain (NT5B) and C-terminal domain (CT5B) of MUC5B were cultured in T75 and triple layer culture flasks (Corning), and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin-Streptomycin (Sigma-Aldrich) and 0.05% (v/v) puromycin (Thermo Fischer Scientific) at 37°C in 5% CO₂ humidified air.

For all cell lines, media was collected twice weekly and centrifuged in a Sigma 3-16 KL centrifuge (Sigma 11180 rotor) (Sigma-Aldrich) at 3878 RCF for 10 mins to remove any cellular debris. Conditioned media was then concentrated using a Vivaflow 200 ultrafiltration device (Mw cut-off 100 kDa).

2.2.3 Mucin purification

Mucins were isolated using isopycnic density gradient centrifugation, as described previously (Thornton et al. 2008). In order to separate mucins from other proteins, saliva/sputum/conditioned media was subjected to caesium chloride (CsCl) density gradient centrifugation in 0.1M NaCl at a starting density of 1.4 g/ml. Mucin-rich fractions were identified using Periodic Acid Schiff (PAS) staining and immunoblotting, and subsequently pooled. To separate mucins from nucleic acids, pooled fractions were then subjected to a second CsCl density gradient centrifugation in 0.1M NaCl, at a starting density of 1.5 g/ml. Mucin-rich fractions were again identified using PAS staining and immunoblotting and pooled. Centrifugation was carried out at 125,082 RCF for 65 hours using a Beckman Optima L-90K Ultracentrifuge (Beckman Ti45 rotor) (Beckman Coulter) at 15°C.

2.2.4 Slot blotting

Samples were loaded onto pre-wetted nitrocellulose membrane using a Minifold II 72-well slot blot system coupled to a vacuum pump. Blots were then analysed by PAS or immunodetection, as described below.

2.2.5 Periodic Acid Schiff (PAS) staining

Following slot blotting, membranes were rinsed briefly in double-distilled H₂O (ddH₂O), and incubated with 1% (v/v) periodic acid and 3% (v/v) acetic acid. This was followed by two 2 min washes in ddH₂O and two 5 min incubations in sodium metabisulphite (0.1% w/v in 0.01 M HCl). Membranes were subsequently incubated in Schiff’s reagent for <15 mins until bands appeared. Finally, membranes were rinsed using sodium metabisulphite (0.1% w/v in 0.01 M HCl) followed by ddH₂O and dried. Blots were imaged using a ChemiDoc MP system (Bio-Rad) and quantified using ImageLab software (Bio-Rad).
2.2.6 Agarose gel electrophoresis

Prior to electrophoresis, mucins were reduced in 20 mM dithiothreitol (DTT) for 10 mins at 100°C. Reduced mucins were electrophoresed in a 0.7% (w/v) agarose gel at 30 V for 17 hours in TAE buffer (40 mM Tris-acetate, 1 mM EDTA and 0.1% w/v SDS). For analysis of whole mucins, gels were reduced following electrophoresis by treatment with 20 mM DTT for 15 mins. Mucins were then transferred to pre-wetted nitrocellulose membrane by vacuum blotting in 0.6 M NaCl and 0.06 M sodium citrate, using a Pharmacia LKB VacuGeneXL at 50 mbar. Analysis was then carried out by immunodetection.

2.2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using NuPAGE Novex 4-12% Bis-Tris Protein Gels (Thermo Fischer Scientific) in NuPAGE MOPS SDS running buffer (Thermo Fischer Scientific). Samples were mixed with NuPAGE LDS sample buffer (4X) (Thermo Fischer Scientific) and incubated at 100°C for 10 mins, then loaded alongside Precision Plus Protein™ All Blue Prestained Protein Standards (Bio-Rad). Electrophoresis was performed at 200V for 50 mins, in an XCell SureLock Mini-Cell Electrophoresis System (Thermo Fischer Scientific). Gels were then visualised by silver staining or Western blotting.

2.2.8 Silver staining

Following SDS-PAGE, gels were rinsed briefly in dH$_2$O then fixed for 20 mins in 50% MeOH, 5% acetic acid. Gels were then washed for 10 mins in water and sensitised for 1 min in 0.02% sodium thiosulphate, followed by two 1 min washes in dH$_2$O. Next, gels were submerged in chilled AgNO$_3$ for 20 mins, rinsed twice for 1 min in dH$_2$O, then developed using 0.4% (w/v) Na$_2$CO$_3$, 0.1% formaldehyde. Development was stopped once bands appeared using 5% acetic acid, and gels were visualised using a Chemidoc MP system (Bio-Rad).

2.2.9 Western blotting

Following SDS-PAGE, gels were rinsed briefly in dH$_2$O then transferred to pre-wetted nitrocellulose membrane (Thermo Fischer Scientific) using an XCell II Blot Module (Thermo Fischer Scientific), in 1X NuPAGE transfer buffer (Thermo Fischer Scientific) for 1 hour at 30 V. Membranes were then visualised by immunodetection.

2.2.10 Immunodetection

Following slot blotting or western blotting, membranes were rinsed briefly in ddH$_2$O blocked for 1 hour using 5% (w/v) milk in TBST (10 mM Tris pH 7.4, 150 mM NaCl, 0.05% Tween 20). Membranes were subsequently incubated overnight with primary antibody , then for 1 hour with
secondary antibody. Prior to visualisation, membranes were subjected to four 10 min washes in TBST. Between each step, membranes were washed four times for 10 mins in TBST.

2.2.11 Antibodies

MUC5B was targeted using the primary antibody EUMUC5B (1:1000 dilution), a mouse monoclonal antibody raised against the sequence RNREQVGKFKMC which is present in the Cys domains of MUC5B (Rousseau et al, 2003).

MUC5AC was targeted using the primary antibody MAN5ACI (1:2000 dilution), a rabbit polyclonal antibody raised against the sequence RNQDQQGPFKMC which flanks the tandem repeats (Thornton et al, 1996).

NT5B was targeted using the primary antibodies 5BVI and 5BVIII (1:1000 dilution) raised against the D1 and D3 domains of MUC5B, respectively.

CT5B was targeted using the primary antibody CC1 (1:1000 dilution) raised against the CK domains of MUC5B.

Secondary antibodies used were IRDye 680RD Goat Anti-Mouse IgG (1:25,000 dilution) (Li-COR Biosciences), and IRDye 800CW Donkey Anti-Rabbit IgG (1:25,000 dilution) (Li-COR Biosciences)

2.2.12 Purification of NT5B and CT5B

MUC5B N-terminus (NT5B) and C-terminus (CT5B) were isolated from human embryonic kidney (HEK-293-EBNA) cells (ATCC). Cells had been stably transected by Dr Caroline Ridley with a His-tagged N-terminal construct of MUC5B (residues 26-1304) incorporating D1-D2-D'-D3 domains (Ridley et al, 2014). Another cell line had been stably transected in the same way using a His-tagged C-terminal construct of MUC5B (residues 4958-5766) incorporating D4-B-C-CK domains. Cells were cultured as described in section 2.2.2, and media was collected every 3 days. Conditioned media was dialysed into Tris buffer (20 mM Tris-base, 400 mM NaCl, 10 mM imidazole, pH 7.4) and stored at 4°C.

Recombinantly expressed NT5B and CT5B were first purified by nickel-affinity chromatography using a 1 ml HisTrap FF column (GE Healthcare), and elution was carried out using 100 mM imidazole in Tris buffer. NT5B- and CT5B-rich fractions were identified by SDS-PAGE followed by Coomassie blue staining, and pooled for the second purification step. Pooled fractions were then applied to a Superose 6 column (10/300 column) (GE Healthcare) connected to an ÄKTA prime plus purification system and separated by size-exclusion chromatography at a flow rate of 0.5 ml/min, with elution carried out using a 25 mM Tris-HCl, 10 mM NaCl buffer (pH 7.4). The absorbance of each fraction at 280 nm was measured, and NT5B- and CT5B-rich fractions were again identified by SDS-PAGE followed by Coomassie blue staining and pooled. Pooled fractions were then subjected to a final round of purification by anion exchange chromatography at a flow rate of 1 ml/min using a Resource Q column (GE Healthcare) connected to an ÄKTA prime plus purification system, with elution carried out with a
gradient of 0.0-0.5 M NaCl in 25 mM Tris-HCl buffer (pH 7.4). Again, the absorbance of each fraction at 280 nm was measured and NT5B- and CT5B-rich fractions identified by SDS-PAGE followed by Coomassie blue staining. Protein concentration of pooled fractions was then measured using bicinchoninic acid (BCA) assay (Fischer). Purified NT5B and CT5B were then dialysed into 10 mM NaCl, 10 mM Tris-HCl (pH 7.4) for use in future experiments.

2.2.13 Size Exclusion Chromatography with Multi Angle Laser Light Scattering (SEC-MALLS)

SEC-MALLS was used to determine the concentration and size distribution of purified mucins. In preparation, samples were dialysed into light scattering buffer (0.2 M NaCl, 1 mM EDTA, 0.01 M sodium azide). To determine mucin concentration, samples were first reduced in 20 mM DTT for 3 hours at 37°C, then alkylated using 50 mM iodoacetamide for 20 mins at room temperature in the dark. Prior to loading the column, samples were centrifuged at 32367 RCF for 10 mins to remove any aggregates or large particles that may cause column blockage. Samples were then loaded onto a Superose 6 column (10/300 column) (GE Healthcare) and eluted in light scattering buffer at a flow rate of 0.31 ml/min. Light scattering and refractive index (RI) of the column effluents were then measured using an inline DAWN EOS laser photometer and Optilab T-rEX refractometer (Wyatt Technology), respectively. Analysis was performed using ASTRA 6 software (Wyatt).

2.2.14 Tandem Mass Spectrometry (MS/MS)

Samples were prepared for MS/MS analysis by reduction in 20 mM DTT for 3 hours at 37°C, followed by alkylation in 50 mM iodoacetamide for 20 mins at room temperature in the dark. 0.1 M ammonium bicarbonate was then added, followed by trypsin digestion overnight at 37°C. Tryptic peptides were then desalted using a ZipTip (Millipore) and dried using vacuum centrifugation, then resuspended in 50% acetonitrile and 0.1 % formic acid.

Tryptic peptides were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using an UltiMate 3000 Rapid Separation LC coupled to a LTQ Velos Pro mass spectrometer (Thermo Fisher Scientific). Peptides were first concentrated on a 20mm x 180 µm pre-column (Waters), then separated in a gradient ranging from 99% solution A (0.1% formic acid in ddH2O) and 1% solution B (0.1% formic acid in acetonitrile), to 25% solution B (0.025% formic acid in acetonitrile) using a 75 mm x 250 mm 1.7 µM ethylene bridged hybrid (BEH) C18 analytical column (Waters). Peptides were then automatically selected for fragmentation using data-dependent analysis.

MS/MS data were then searched using the MASCOT search engine (Matrix Science) against the desired database. For mucins purified from cell culture, the UniProt database was used (Taxonomy: Homo sapiens); for A. fumigatus culture filtrates, the SwissProt database was used (Taxonomy: Fungi); for CF mucins, the SwissProt database was used (Taxonomy: Homo sapiens). For all searches, the following parameters were used:
2.2.15 Preparation and characterisation of *A. fumigatus* culture filtrates

### 2.2.15.1 *A. fumigatus* strains

To study mucin degradation by *A. fumigatus*, the CEA10 strain was used. This strain was originally isolated from a patient with invasive aspergillosis. For studies into the time course of protease secretion, A1160 (a CEA10-derived strain in which the CTP synthase pyrG has been removed and then reintroduced back into the genome) and A1160ΔprtT strains were also used. To investigate the role of various *A. fumigatus* enzymes, a panel of secreted protein knockout strains were kindly supplied by Elaine Bignell (Manchester Fungal Infection Group), which were generated from the A1160 strain. The role of the PalH cell surface receptor and PacC transcription factor, disruption of *pacC* and *palH* genes was carried out in the ATCC 46645 strain using a pyrithiamine resistance gene (ptrA) and hygromycin B (hygB) cassette, respectively.

### 2.2.15.2 Preparation of *A. fumigatus* spores

*A. fumigatus* was grown on ACM for 48 h at 37°C. Conidia were harvested in 10 ml ddH$_2$O filtered through sterile miracloth (Millipore) to remove any hyphal fragments. The filtrate was then centrifuged for 10 mins at 2332 RCF. The pellet was resuspended in 10 ml ddH$_2$O and centrifuged for a further 10 mins, followed by a further resuspension and centrifugation step. Spores were quantified using a Neubauer haemocytometer (Thermofischer Scientific).

### 2.2.15.3 Preparation of *A. fumigatus* culture filtrates

Flasks containing AMM were inoculated with 1x10$^6$ cfu mL$^{-1}$ and incubated at 37°C in an orbital incubator at 180 rpm. To remove any fungal biomass, culture media was filtered through sterile miracloth. Filtrate was then sterilised using a 0.22 µm syringe filter (Merck Millipore) and stored at 4°C.

### 2.2.15.4 Size Exclusion Chromatography

Proteins were separated by size exclusion chromatography using a Superose 12 column (10/300 column) (GE Healthcare Life Sciences) attached to an ÄKTAprime plus purification system (GE Healthcare Sciences) at a flow rate of 0.5 ml/min. Elution was carried out using a
25 mM Tris-HCl, 10 mM NaCl buffer (pH 7.4). The absorbance of each fraction at 280 nm was measured and proteins were visualised by SDS-PAGE followed by silver staining.

2.2.16 Analysis of mucin degradation

2.2.16.1 Mucin degradation assay

Purified mucins or recombinant mucin domains were incubated with whole or fractionated *A. fumigatus* culture filtrate at 37°C. Reactions were stopped using a protease inhibitor cocktail (Thermo Fischer Scientific) and samples mixed with reducing loading buffer (50% v/v glycerol, 1M NaCl, 0.1 M Tris-HCl, 5 mM EDTA, 6 M urea, 1% w/v SDS, 0.01% v/v bromophenol blue, 20 mM DTT) at 100 °C for 10 mins. Samples were analysed by agarose gel electrophoresis or SDS-PAGE followed by immunoblotting, as well as rate zonal centrifugation. For protease inhibitor studies, filtrates were pre-incubated with protease inhibitors at room temperature for 30 mins prior to incubation with mucins.

2.2.16.2 Rate zonal centrifugation

Samples were loaded on top of a 5-35% sucrose gradient and centrifuged in a Beckman Optima L-90K Ultracentrifuge (Beckman SW40 rotor) at 201,958 RCF for 1.5 hours at 4°C. Following centrifugation, fractions were collected from the top of the tube and the distribution of mucins was analysed by immunoblotting.

2.2.16.3 Lectin assay

Purified mucins were incubated with *A. fumigatus* culture filtrate (pre-incubated for 30 mins with protease inhibitors) at 37°C for 24 hours. Samples were then transferred to pre-wetted nitrocellulose membrane via slot blotting; to allow glycopeptides to adhere to the membrane, 50 µl of poly-L-lysine (Mr 300,000) (Sigma Aldrich) was loaded prior to the samples. Membranes were rinsed briefly in TBST, then incubated with a panel of biotin-labelled lectins (ConA – α-linked mannose, MPL – α-linked GalNAc, MALI - Gal(β-1,4)GlcNAc, MALII - α-2,3-linked sialic acid) at room temperature overnight. This was followed by four 10 min washes in TBST. Membranes were then incubated with IRDye® 800CW Streptavidin (1:25000) (LI-COR Biosciences) for 1 hour at room temperature in the dark, followed by four 10 min washes in TBST. Signals were quantified using a LI-COR Odyssey CLX imaging system (LI-COR Biosciences) and LI-COR Image Studio software.

2.2.17 Particle tracking microrheology

Mucin viscosity was measured using particle tracking microrheology. Following treatment of purified CF mucins with *A. fumigatus* culture filtrate for 24 hours at 37°C, 505nm uncoated polystyrene beads were added to the samples at a 1:600 ratio. Samples were then loaded onto uncoated microscope slides (Thermo-Fischer Scientific) with Secure-seal imaging spacers, and covered with a glass cover slip. Slides were then observed using an Olympus IX-71 inverted
microscope and videos of the Brownian motion of the beads was measured at 100X magnification using a FastCam 1024-PCI fast camera and bright CoolLED light source, at a resolution of 1024 x 512 pixels. Videos were recorded at 500 frames per second for 1000 frames, and 15 videos were taken of each sample. The two-dimensional bead trajectories from each video were acquired and converted into mean squared displacement (MSD) values using PolyParticle Tracker Matlab software, which was developed by Dr Pantelis Georgiades (Georgiades, 2013; Georgiades et al, 2014b; Rogers et al, 2007).

2.2.18 Immunohistochemistry

2.2.18.1 Intranasal challenge of mice with A. fumigatus spores

C57BL/6 mice were maintained in the Biological Services Unit at the University of Manchester. All mice were kept in sterilized, filter-topped cages and fed autoclaved food in the animal facility. Mice were treated with $0.4 \times 10^6$ live A. fumigatus CEA10 spores at d1, 3, 5, 7, 9, 11, 14, 16 and 18. Following anaesthesia using isoflurane mice were challenged intranasally with 50 µl of spores diluted in PBS, and BAL fluid and lung tissue were harvested at d1 and d19. BAL fluid was analysed by slot blotting followed by immunostaining and lung tissue was analysed by immunohistochemistry. All animal work was performed under the animal license of Professor Andrew MacDonald.

2.2.18.2 Tissue processing

Formalin-fixed lungs from A. fumigatus treated mice were kindly provided by Peter Cook (University of Manchester). Tissues were processed using a Microm Spin Tissue processor 120 (Microm UK Ltd) using the following steps: 70% IMS (7 hours), 90% IMS (45 mins), 95% IMS (45 mins), 100% IMS (3 x 45 mins), 100% IMS (3 x 30 mins). Processed tissue was then embedded in paraffin wax blocks and 5 µm sections taken from the left lung using a microtome (Leica Biosystems) and applied to gelatin-coated microscope slides.

2.2.18.3 Slide preparation

In preparation for staining slides were dewaxed 100% xylene, then washed in 100% ethanol twice for 10 mins each. Slides were then boiled in citrate buffer (10 mM citric acid in PBS, pH6) for 3 mins in the microwave, then washed three times for 2 mins each in PBS.

2.2.18.4 Immunostaining

For MUC5B, tissues were then reduced in 10 mM DTT for 30 mins and alkylated in 25 mM iodoacetamide for 30 mins, both at room temperature (this step was omitted for MUC5AC). Slides were subsequently washed 3 times in PBS for 2 mins and blocked in 10% goat serum for 30 mins at 4 °C. This was followed by an overnight incubation in primary antibody (1:100 45M1 antibody for MUC5AC, 1:100 78 antibody for MUC5B, made up in serum), three 2 min washes in PBS, then incubation with secondary antibody (1:800 IRDye 680RD Goat Anti-Mouse IgG for
MUC5AC and 1:800 IRDye 800CW Donkey Anti-Rabbit IgG for MUC5B) (LI-COR Biosciences). Slides were again washed 3 times in PBS then incubated with DAPI (1:1000 in PBS) for 5 mins at room temperature in the dark. Finally, slides were washed twice in PBS and once in water, dried in the dark then mounted in mowiol solution.

2.2.19 *A. fumigatus* growth assay

Growth curves were performed on the CEA10 strain of *A. fumigatus*, in either AMM (-glucose), AMM (+glucose) or AMM (- glucose, + mucin). Each medium was prepared with a final concentration of 0.01% Tween20. The assay was performed in a CytoOne 96-well plate (StarLab), with 2,000 spores per well (5 µl of 4 x 10⁵ spores/ml stock in 195 µl culture media preparation). For each condition, samples were added in replicates of 10. The inter-well spaces of the plate were filled with dH₂O, then the plate covered with a sticky permeable plate cover. Plates were placed in a Powerwave X-1 plate reader and the OD600 measured for 48 hours at 37°C, with data analysed using KC4 3.02 software (Biotek).
Chapter 3: Degradation of airway mucins by *A. fumigatus*

3.1 Introduction

It is well established that mucins and mucus are crucial in protecting the airways against invading pathogens, allowing infectious agents to become trapped and cleared from the lung. The immune response during airway disease has been the subject of much research, and it has been well documented that an increase in goblet cells and mucus secretion occurs in response to infection and inflammation (Cash *et al*, 1979; Blyth *et al*, 1998; Hauber *et al*, 2006).

*A. fumigatus* is known to secrete an arsenal of proteins that act as virulence factors within the host, often taking advantage of already compromised defence systems. Many of these putative virulence factors have been studied extensively, and have been shown to affect processes such as mucociliary clearance and phagocyte function. However, there is little understanding of the effects of *A. fumigatus* and its secreted products on the airway mucus barrier. It has been shown previously that *A. fumigatus* is able to degrade host-derived macromolecules including horse lung polymers and porcine gastric mucin (St Leger and Screen, 2000). Due to the importance of mucins in defining mucus properties, such disruption of the mucin network will likely compromise its protective function. These studies however were performed using commercially available mucins, which have been shown to have a reduced size and contain more impurities as compared with mucins prepared from fresh mucus secretions (Jumel *et al*, 1996). As such, these findings may not be representative of what occurs within the human lung, and further studies are required in order to understand the true effects of *A. fumigatus* on airway mucins. We aimed to address this issue by measuring the ability of *A. fumigatus* to degrade MUC5B and MUC5AC purified from human cell lines, using a variety of biochemical approaches.

Through the use of agarose gel electrophoresis we assessed the ability of *A. fumigatus* culture filtrates to degrade full length purified MUC5B and MUC5AC, and rate zonal centrifugation was used to study their effects on mucin size and sedimentation profile. Using SDS-PAGE analysis and mass spectrometry, we also identified regions targeted by proteases within recombinantly expressed N- and C-terminal domains of MUC5B. Culture filtrates collected at different time points were used to determine when this proteolytic activity is secreted by *A. fumigatus*, and a panel of protease inhibitors allowed us to identify which classes of proteases are involved in this process. Furthermore, lectin binding assays were used to determine whether *A. fumigatus* can also degrade mucin glycans, and which sugars may be cleaved.
3.2. Results

3.2.1 Purification and characterisation of MUC5B and MUC5AC

In order to study the effects of *A. fumigatus* on airway mucins, MUC5B and MUC5AC were co-purified from Capan-1 cell media using caesium chloride (CsCl) density gradient centrifugation under non-denaturing conditions. The first density gradient centrifugation was performed at 1.4 g/ml to separate mucins from other proteins within the media (Davies and Carlstedt, 2000) (Figure 3.1A). To identify the presence of contaminating proteins, the absorbance of each fraction was measured at 280 nm. Mucin-rich fractions were identified by immunoblotting, with a peak identified between densities of 1.33 and 1.46 g/ml. These fractions were pooled and subjected to a second density gradient centrifugation at 1.5 g/ml, to separate mucins from nucleic acids (Davies and Carlstedt, 2000) (Figure 3.1B). Mucins were present between densities of 1.33 and 1.58 g/ml, and these fractions were again pooled for further investigation.

Following purification, mucin-rich fractions were analysed by SEC/MALLS to gather information about the concentration and size distribution of the mucins. Refractive index data was used to measure the concentration of mucins within the sample, showing a yield of 25 µg/ml (Figure 3.2A). Mucins were shown to be polydisperse in size, with an average molecular weight of 11 MDa (± 5.543%) (Figure 3.2B).

To assess the purity of the mucin preparation, conditioned media was analysed by MS/MS (Table 3.1). MUC5B and MUC5AC were identified as the 1st and 7th most abundant proteins within the sample, showing that the purification had been successful. Twenty one other proteins were also identified within the sample.

Together these results show that we have successfully enriched both MUC5B and MUC5AC, and highlight the polydispersity in mucin size.
Figure 3.1: Purification of MUC5B and MUC5AC

Mucins were purified using caesium chloride (CsCl) density gradient centrifugation, and identified via immunoblotting using MUC5B- and MUC5AC-specific antibodies. A) Conditioned media was subjected to CsCl density gradient centrifugation in 0.1 M NaCl, at a starting density of 1.4 g/ml. B) Pooled fractions from the first centrifugation were subjected to a second CsCl density gradient centrifugation in 0.1M NaCl, at a starting density of 1.5 g/ml.
Figure 3.2 Characterisation of MUC5B and MUC5AC by SEC/MALLS

A) SEC/MALLS analysis using a Superose 6 column was used to determine the concentration of MUC5B/MUC5AC present in the purified mucin sample. B) Light scattering data and refractive index data were used to determine the molecular weight distribution of the mucin species within the sample.
Table 3.1 MS/MS analysis of MUC5B/MUC5AC mucin preparation

MS/MS data for peptides generated from the purified mucin preparation were analysed using the MASCOT search engine, performed against the UniProt database (Taxonomy: *Homo sapiens*). For each protein identified, the number of unique peptides was also determined. The table below shows the top 20 hits identified.

<table>
<thead>
<tr>
<th>Proteins identified</th>
<th>Number of unique peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC5AC</td>
<td>35</td>
</tr>
<tr>
<td>DMBT1</td>
<td>9</td>
</tr>
<tr>
<td>Agrin</td>
<td>14</td>
</tr>
<tr>
<td>Ubiquitin conjugation factor E4 B</td>
<td>16</td>
</tr>
<tr>
<td>WD repeat-containing protein 87</td>
<td>28</td>
</tr>
<tr>
<td>Malignant fibrous histiocytoma-amplified sequence 1</td>
<td>7</td>
</tr>
<tr>
<td>MUC5B</td>
<td>26</td>
</tr>
<tr>
<td>Prolyl endopeptidase</td>
<td>6</td>
</tr>
<tr>
<td>Filamin-C</td>
<td>24</td>
</tr>
<tr>
<td>Armadillo repeat protein deleted in velo-cardio-facial syndrome</td>
<td>5</td>
</tr>
<tr>
<td>Neurobeachin</td>
<td>30</td>
</tr>
<tr>
<td>Lipopolysaccharide-responsive and beige-like anchor protein</td>
<td>14</td>
</tr>
<tr>
<td>Tubulin polyglutamylase</td>
<td>13</td>
</tr>
<tr>
<td>Hexokinase-1</td>
<td>9</td>
</tr>
<tr>
<td>Neuroserpin</td>
<td>3</td>
</tr>
<tr>
<td>ADP-ribosylation factor-binding protein GGA3</td>
<td>2</td>
</tr>
<tr>
<td>Myotubulinin-related protein 1</td>
<td>6</td>
</tr>
<tr>
<td>Putative type-1 protein phosphatase inhibitor 4</td>
<td>2</td>
</tr>
<tr>
<td>Disrupted in schizophrenia 1 protein</td>
<td>7</td>
</tr>
<tr>
<td>Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform</td>
<td>4</td>
</tr>
</tbody>
</table>
3.2.2. Preparation and characterisation of *A. fumigatus* culture filtrates

In order to study the effects of *A. fumigatus* secreted products on airway mucins, culture filtrates were collected from the CEA10 strain of *A. fumigatus*. This is a wild-type strain of the fungus, which was originally isolated from a patient with invasive aspergillosis (Aspergillusgenome.org, 2018). For these initial experiments, culture filtrates were collected after 96 hours in liquid culture, which allowed us to obtain secretory products released during different stages of germination.

The composition of the culture filtrate was first analysed using SDS-PAGE and silver staining (Figure 3.3). Five distinct bands were observed, with molecular weights of around 35, 42, 51, 74 and 76 kDa. MS/MS analysis of the culture filtrate identified 14 proteins (Table 3.2); these included proteases (Alp1/AspF13 and neutral protease 2), glycosidases (Alpha-1,2-Mannosidase and EgIC), four uncharacterised proteins as well as other known proteins (Asp-hemolysin, ribonuclease T2, ChiB1 and superoxide dismutase).

These data have highlighted the complex nature of *A. fumigatus* culture filtrates, identifying a number of enzymes that may have the ability to degrade both the protein and carbohydrate components of mucins.

![Silver stained SDS-PAGE gel of CEA10 culture filtrate collected after 96 hours in liquid culture. The culture filtrate sample (CF) was loaded alongside markers (M) of known molecular weight.](image)

**Figure 3.3 Composition of *A. fumigatus* (CEA10) culture filtrate**

Silver stained SDS-PAGE gel of CEA10 culture filtrate collected after 96 hours in liquid culture. The culture filtrate sample (CF) was loaded alongside markers (M) of known molecular weight.
Table 3.2 MS/MS analysis of *A. fumigatus* (CEA10) culture filtrate

MS/MS data for peptides generated from *A. fumigatus* CEA10 culture filtrate were searched against the SwissProt database using the Mascot server, with the limited taxonomy of ‘fungi’. The number of unique peptides represents the number of peptide sequences unique to this protein group.

<table>
<thead>
<tr>
<th>Proteins identified</th>
<th>Number of unique peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncharacterised protein</td>
<td>10</td>
</tr>
<tr>
<td>Asp-hemolysin</td>
<td>8</td>
</tr>
<tr>
<td>Alpha-1,2-Mannosidase</td>
<td>10</td>
</tr>
<tr>
<td>Cell wall protein</td>
<td>7</td>
</tr>
<tr>
<td>GPI anchored cell wall beta 1,3 endoglucanase EglC</td>
<td>5</td>
</tr>
<tr>
<td>Ribonuclease T2, putative</td>
<td>7</td>
</tr>
<tr>
<td>Class V chitinase ChiB1</td>
<td>6</td>
</tr>
<tr>
<td>Alkaline serine protease Alp1</td>
<td>4</td>
</tr>
<tr>
<td>Allergenic cerato-platanin Asp F13</td>
<td>5</td>
</tr>
<tr>
<td>Uncharacterised protein</td>
<td>2</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>4</td>
</tr>
<tr>
<td>Neutral protease 2</td>
<td>4</td>
</tr>
<tr>
<td>Uncharacterised protein</td>
<td>4</td>
</tr>
<tr>
<td>Uncharacterised protein</td>
<td>3</td>
</tr>
</tbody>
</table>
3.2.3. *A. fumigatus* degrades MUC5B and MUC5AC

A previous study by St Leger and Screen showed that *A. fumigatus* can degrade commercial pig gastric mucin (St Leger and Screen, 2000) however the effects of *A. fumigatus* on human mucin species remain to be investigated. To address this, the effects of *A. fumigatus* culture filtrate on the biochemical properties of purified MUC5B and MUC5AC were studied.

MUC5B and MUC5AC were treated with *A. fumigatus* CEA10 96-hour culture filtrate over a time course of 24 hours, and the effects were studied using agarose gel electrophoresis followed by Western blotting (Figure 3.4). A decrease in antibody reactivity was observed for both MUC5B and MUC5AC throughout the time course, with a complete loss of signal after 24 hours. As well as reduction in band intensity, changes in the banding pattern of the mucins were also observed. As expected, mucins in the control samples were observed as a large smear which highlights the heterogeneity in mucin glycosylation, and this was seen for both MUC5B and MUC5AC. Lower intensity bands were also observed towards the top of the gel, representing a smaller population of unreduced mucins of higher molecular weight. Following treatment with the culture filtrate, a reduction in these high molecular weight bands was observed for both mucins. For MUC5AC, smears appeared towards the bottom of the gel suggesting an increase in low molecular weight degraded mucin molecules.

To determine whether these results were due to mucin depolymerisation or simply a loss of antibody epitopes, the effects of *A. fumigatus* on the sedimentation behaviour of unreduced MUC5B and MUC5AC were studied. Mucin sedimentation is related to their size; larger molecules will sediment more rapidly whereas smaller molecules will sediment more slowly (Sheehan and Thornton, 2000), and as such this allowed mucin degradation to be assessed. Following treatment of mucins with the culture filtrate in the presence and absence of proteases, mucin sedimentation was analysed using rate zonal centrifugation in a 5-35% sucrose gradient and the distribution of mucins across the gradient was measured by immunoblotting (Figure 3.5). Treatment of both MUC5B and MUC5AC with the culture filtrate caused a dramatic change in their sedimentation behaviour. In the absence of culture filtrate a sharp peak for both mucins was observed at fraction 23, as well as a much broader and shallower peak between fraction 7 and 19. Treatment with the culture filtrate for 24 hours caused both mucins to sediment more slowly, represented by a shift in the mucin peaks towards lower sucrose concentrations. A large peak for both mucins was observed between fractions 4-16, and a much smaller peak at fraction 23 as compared with the untreated controls. In the presence of a mixture of protease inhibitors, this shift in sedimentation behaviour was much less pronounced. As with the control samples, a sharp mucin peak was observed at fraction 23, with a smaller peak between fractions 6 and 16.

These data provide further evidence that *A. fumigatus* can degrade MUC5B and MUC5AC over time. Results show that treatment with *A. fumigatus* culture filtrate causes a reduction in the size of mucin polymers, and that this process is protease dependent.
Figure 3.4: Degradation of MUC5B and MUC5AC by A. fumigatus culture filtrate

Mucins were treated with CEA10 culture filtrate at 37°C for 24 hours, and samples taken at various time points. In order to measure mucin degradation, samples were reduced and analysed using agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies. For quantification, levels of mucin in the treated samples were normalised to those of the controls at each time point (n=3).
Mucins were treated with CEA10 culture filtrate for 24 hours in the presence or absence of protease inhibitors (PI). The effects on their sedimentation behaviour was analysed by rate zonal centrifugation in a 5-35% sucrose gradient. Following fractionation of the gradient, changes in the sedimentation profile were assessed via slot blotting using MUC5B- and MUC5AC- specific antibodies, whereby a shift to the left-hand side denotes a decrease in size.

Figure 3.5: *A. fumigatus* culture filtrate causes a change in mucin sedimentation
3.2.4 Mucin-degrading proteases are secreted after 12 hours in liquid culture

Proteases are amongst many of the putative virulence factors secreted by *A. fumigatus*, many of which act as allergens within the host environment (Bowyer and Denning, 2007). To determine when these proteases are secreted during germination, the proteolytic effects of culture filtrates collected at different time points during liquid culture were studied.

Culture filtrates were collected from the CEA10 strain of *A. fumigatus* after 4, 8, 12, 24 and 96 hours of growth in liquid medium. The mucin-degrading activity of each culture filtrate was assessed using the optimised degradation assay, whereby mucins were treated with the culture filtrates for 24 hours and analysed using agarose gel electrophoresis followed by Western blotting (Figure 3.6). As shown by immunoblotting, culture filtrates collected after 4, 8 and 12 hours did not degrade either MUC5B and MUC5AC. Those collected at 24 and 96 hours degraded both mucins over the 24 hours, with a complete loss of antibody reactivity observed compared to the controls.

To determine whether this time course of protease secretion was strain specific, culture filtrates were also collected from A1160 (a CEA10-derived strain) and ΔprtT (A1160 background) isolates at the same time points and again tested in the mucin degradation assay (Figure 3.6). Consistent with our findings from the CEA10 strain, culture filtrates collected after 4, 8 and 12 hours were unable to degrade the mucins, whereas those collected at 24 and 96 hours displayed a high level of mucin degrading activity such that no antibody reactivity was observed for either MUC5B or MUC5AC.

These data provide evidence to suggest that mucin degrading proteases are secreted by *A. fumigatus* after 12 hours of growth in liquid medium, and show that this time course of protease secretion is not specific to the CEA10 strain.
Figure 3.6 Mucin degrading proteases are secreted after 12 hours in liquid culture

A. fumigatus culture filtrates were collected from four different isolates (CEA10, A1160-, and ΔprtT) at multiple time points during liquid culture (4, 8, 12, 24 and 96 hours). Following treatment of mucins with the culture filtrates at 37°C for 24 hours, the presence of proteolytic activity was assessed using gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies.
3.2.5 *A. fumigatus* proteases cleave the N- and C-terminus of MUC5B

Results have shown that *A. fumigatus* is able to degrade MUC5B and MUC5AC, and through the use of immunoassays we were able to speculate that cleavage may occur within the N- and C-terminal domains of the mucins leading to depolymerisation. In order gain a deeper insight into this process, further investigations were carried out using recombinantly expressed N- and C-terminal domains of MUC5B.

N-terminal (NT5B) and C-terminal (CT5B) MUC5B domains were purified from conditioned cell media collected from stably transfected 293-EBNA cells. NT5B and CT5B purified by nickel affinity chromatography was kindly provided by Dr Caroline Ridley. Further purification was then carried out using size exclusion chromatography followed by anion exchange chromatography (Figure 3.7-3.8). Mucin-rich fractions were identified at each stage using SDS-PAGE and Coomassie blue staining and pooled for further investigation.

In order to determine whether *A. fumigatus* proteases are able to cleave the MUC5B N- and C-terminus, further degradation studies were carried out using purified NT5B and CT5B. NT5B and CT5B were treated with CEA10 culture filtrate for 24 hours, and degradation was visualised using SDS-PAGE followed by silver staining (Figure 3.9). As expected based on previous studies showing that both NT5B and CT5B are expressed as a monomer and dimer (Ridley et al., 2014; Ridley et al., 2016), NT5B control samples appeared as two main bands; these were observed at ~80 kDa and 160 kDa, representing monomeric and dimeric forms of the domain respectively. When treated with the culture filtrate a reduction in band intensities was observed as well as a shift in the banding pattern to a lower molecular weight, suggesting cleavage of both the monomer and dimer. In the presence of protease inhibitors, no cleavage was observed. Again, as expected, CT5B control samples appeared as two bands representing the monomer and dimer, at ~125 kDa and 250 kDa respectively. A shift in the banding pattern was observed following treatment with the culture filtrate, although this was much less than was seen for NT5B. No changes were seen in the presence of protease inhibitors.
Figure 3.7 Purification of MUC5B N-terminus

MUC5B N-terminus (NT5B) was purified by A) size exclusion using a superose 6 column and B) anion exchange chromatography using a Resource Q column. In order to trace the protein as it was eluted from the column, the absorbance of each fraction at 280 nm was measured. Fractions were then analysed using SDS-PAGE and visualised by Coomassie blue staining.
**Figure 3.8 Purification of MUC5B C-terminus**

MUC5B C-terminus (CT5B) was purified by chromatography using A) Superose 6 and B) Resource Q columns. The absorbance of each fraction at 280 nm was measured as they were eluted from the column. Fractions were then analysed using SDS-PAGE and visualised by Coomassie blue staining.
Figure 3.9 A. fumigatus culture filtrate degrades the N- and C-termini of MUC5B

MUC5B N-terminus (NT5B) and C-terminus (CT5B) were treated with A. fumigatus CEA10 culture filtrate (Af) at 37°C for 24 hours, either in the presence of absence of protease inhibitors (PI). Samples were then analysed by SDS-PAGE and visualised by silver staining.
To identify which regions of the N- and C-terminal domains were being cleaved by these proteases, NT5B and CT5B were again treated with A. fumigatus culture filtrates. As with some of our experiments on whole mucins, culture filtrates were collected at different time points during liquid culture (4, 8, 12, 24 and 96 hours) and from three different strains of A. fumigatus (CEA10, A1160 and ΔprtT). Following treatment of NT5B and CT5B for 24 hours, degradation was visualised by Western blotting using antibodies specific for the D1 and D3 domains of the N-terminus and CK domain of the C-terminus of MUC5B (Figure 3.10). As expected from our previous results, only culture filtrates collected after 12 hours showed proteolytic activity, and this was the case for all three strains. Cleavage of both the D1 and D3 domains of NT5B was observed as well as the CK domain of CT5B, indicated by a shift in banding patterns. The most dramatic effect was observed for the D1 domain, where a significant reduction in signal occurred following treatment with the 24 and 96 hour culture filtrates.

Together these results show that A. fumigatus exhibits proteolytic activity against both the N- and C-terminus of MUC5B, with the N-terminal domain more susceptible to cleavage. Data suggests that proteases within the culture filtrates are able to cleave the D1, D3 and CK domains of this mucin.

3.2.6 Cleavage of mucin glycans by A. fumigatus

A previous study by St Leger and Screen suggested that A. fumigatus is able to cleave mucin carbohydrates as well as mucin protein (St Leger and Screen, 2000). Following our observations that A. fumigatus exhibits proteolytic activity against MUC5B and MUC5AC, the effects of A. fumigatus culture filtrate on sugar content of these purified mucins were studied.

Purified MUC5B and MUC5AC were treated with A. fumigatus culture filtrate for 24 hours; to prevent any interference from the action of proteases, this was performed in the presence of protease inhibitors. The levels of different sugars was then measured using biotinylated lectins with different specificities (Figure 3.11). Results showed almost no different in signal from MPL (α-linked GalNAC), MALI (Gal(β-1,4)GlcNAc) and MALII (α-2,3-linked NeuAc) lectins compared to the untreated controls. For ConA (mannose), a slight increase in signal was observed following treatment with the culture filtrate.

These preliminary results provide evidence to suggest that A. fumigatus may cleave mucin glycans. No cleavage either α-linked GalNac, Gal(β-1,4)GlcNAc or α-2,3-linked sialic acid from MUC5B or MUC5AC was observed, although a slight increase was seen in the levels of mannose, which may suggest the cleavage other sugars to reveal mannose residues towards the polypeptide core.
Figure 3.10 Cleavage of D1, D3 and CK domains of MUC5B by *A. fumigatus* culture filtrates

MUC5B N-terminus (NT5B) and C-terminus (CT5B) were treated with culture filtrates from CEA10, KU80- and ΔprtT strains of *A. fumigatus*, at 37°C for 24 hours. In order to measure degradation, samples were analysed by Western blotting using A) 5BVI (N-terminal D1 domain) B) 5BVIII (N-terminal D3 domain) and C) CCK (C-terminal CK domain) antibodies. Untreated NT5B and CT5B were used as controls (C).
Mucins were treated with CEA10 culture filtrate at 37°C for 24 hours, in the presence of protease inhibitors. Following treatment the levels of different sugars were measured using lectins of different binding specificities (ConA – α-linked mannose, MPL – α-linked GalNAc, MALI - Gal(β-1,4)GlcNAc, MALII - α-2,3-linked sialic acid). Untreated mucins were used as negative controls.
3.3. Discussion

*A. fumigatus* is an opportunistic pathogen found in the airways of many CF patients, contributing to a decline in lung function and a range of clinical outcomes such as ABPA and aspergillus bronchitis. The precise effects of *A. fumigatus* on CF airways remain relatively unclear, but it is becoming increasingly apparent that colonisation and infection with the fungus can contribute to the progression of CF lung disease and a worsened clinical outcome (Middleton *et al.*, 2013). As the first point of contact for *A. fumigatus* spores following inhalation by the host, the mucus barrier provides an important site for host-fungal interactions (Cowley *et al.*, 2017). Until now, the interplay between *A. fumigatus* and airway mucus has not yet been studied in detail, however it is likely that interactions between the two form an important step in fungal colonisation. We here provide evidence that *A. fumigatus* can degrade MUC5B and MUC5AC, the predominant mucin species found within the airways. The initial experiments relied solely on immunological detection, which in some cases may lead to an apparent reduction in mucin concentration due to the cleavage of antibody epitopes (Henderson *et al.*, 2014). However, the change in sedimentation behaviour that occurred following treatment with the culture filtrate confirmed that these observations were not simply due to the cleavage of antibody epitopes, but in fact showed a considerable reduction in mucin size. These findings are in line with data published by St Leger and Screen, who showed that *A. fumigatus* can degrade pig gastric mucin and bovine submaxillary mucin (St Leger and Screen, 2000). As a major component of the extracellular matrix, it is not surprising that mucins may be utilised by opportunistic pathogens such as *A. fumigatus*. By degrading mucins, *A. fumigatus* may be able to utilise them as a source of nutrients to support fungal growth. Mucin degradation may also allow easier penetration of the mucus barrier to facilitate colonisation of the airways and access to the underlying epithelium (Cowley *et al.*, 2017). The effects of fungal pathogens on mucins is an area that has not yet been studied in detail, although mucin degradation by *Candida albicans* has also been reported. Ultrastructural studies have shown that *C. albicans* is able to degrade the intestinal mucus barrier in mice, which more recently has been attributed to the aspartyl proteinase Sap2p (Cole *et al.*, 1991; Colina *et al.*, 1996). Together with the findings reported here, these studies suggest that mucus barrier degradation is a mechanism involved in the pathogenesis of fungal pathogens, facilitating growth and colonisation of mucosal surfaces within the host. Further studies should explore the effects of *A. fumigatus* on MUC5B and MUC5AC in vivo. Using a mouse model of *A. fumigatus* colonisation and infection would allow us to gain a more accurate insight into how the fungus impacts the mucus barrier within the host, and this has been explored in a later chapter.

In healthy individuals the presence of *A. fumigatus* within the lung is relatively short-lived, with inhaled conidia rapidly removed by host defence mechanisms such as mucociliary clearance and cough, as well as phagocytosis by alveolar macrophages. In patients with CF, these innate defence mechanisms are impaired, meaning inhaled pathogens can no longer be removed from
the airways efficiently. This inefficient removal of *A. fumigatus* spores allows them to persist within the airways and undergo germination, accompanied by the release of multiple virulence factors and other secreted products. This will allow the fungus to exploit the mucosal environment in order to promote fungal growth and create a suitable niche in which to flourish (Dagenais and Keller, 2009; Cowley *et al*., 2017). It was found that proteolytic activity against MUC5B and MUC5AC appeared within *A. fumigatus* culture filtrates after 12 hours of growth in liquid medium, which is in line with previous studies by Bignell and colleagues which showed that *A. fumigatus* spores become invasive at this 12 hour time point when grown on A549 cells (Bignell *et al*., University of Manchester, personal communication). It is likely that the secretion of mucin degrading proteases allows the breakdown of the mucin network, making the mucus barrier more penetrable to fungal spores and hyphae and facilitating access to the epithelial surface. Without degrading these mucins, inhaled conidia may remain trapped within the mucus layer, unable to harness nutrients and colonise the airway environment.

Gel forming mucins possess a multidomain organisation consisting of a large central mucin domain that is punctuated with non-O-glycosylated cys domains, and either side of this central region lie the N- and C-terminal globular domains (Thornton *et al*., 2008). Using recombinantly expressed N- and C-terminal domains of MUC5B, it was shown here that both the D1 and D3 domains of the N-terminus and the CK domain of the C-terminus are cleaved by *A. fumigatus* proteases, with the N-terminus more susceptible to such cleavage. The susceptibility of these domains to proteolytic cleavage is not surprising; because the central region of mucins is extensively modified by O-glycosylation it is largely resistant to the action of proteases, however the same is not true of the N- and C-terminal domains (Thornton *et al*., 2008). Studies have shown that the N-terminus of MUC5B is less folded than the C-terminus, which may explain the increased susceptibility of these domains to proteolytic cleavage. Structural analysis of NT5B using transmission electron microscopy have shown that the MUC5B N-terminus forms an extended boomerang-like structure, with dimerisation mediated by the D3 domains. Although the structure of the C-terminus is yet to be defined, these domains are believed to exist in a more compact and folded conformation (Ridley *et al*., 2014; Ridley *et al*., University of Manchester, personal communication). Cleavage of these domains provides a possible explanation for the apparent reduction in mucin size observed in previous experiments; by cleaving the N- and C-terminal domains, it is likely that *A. fumigatus* proteases will induce mucin depolymerisation. Due to the importance of the N- and C-termini in mucin polymerisation, cleavage of these domains will likely disrupt mucin-mucin interactions that form the network structure of the barrier. In the context of airway colonisation, this breakdown of the mucin network may compromise the protective properties of the mucus barrier, providing a more favourable niche environment. The cys domains that intersperse the mucin domains may also be cleaved by *A. fumigatus* proteases, as these too are not protected by extensive glycosylation. Further studies using recombinant MUC5B cys domains would allow us to determine whether this is indeed the case. To gain a more detailed insight into the mechanism of action of *A. fumigatus* proteases, proteomic studies should also be carried out in order to
identify specific cleavage sites within these subdomains. Although it has been shown already which domains of the mucins may be targeted by proteases, it is still not clear where exactly within these domains they cleave.

As discussed above, much of the central region of mucin polypeptides is protected by extensive glycosylation of the mucin domains. As well as conferring protease resistance to this region and allowing mucus gel hydration, this vast array of glycans also provide many epitopes to allow pathogen sequestration within the barrier (Thornton et al, 2008). It has been shown that a fucose-specific lectin on the surface of A. fumigatus (FleA) is able to bind to mucin glycans, and this is believed to be critical for the removal of the fungus from the airways (Kerr et al, 2016). This binding of mucins to A. fumigatus may cause retention of inhaled conidia within the mucin network, allowing them to be cleared through mucociliary clearance. As suggested by St Leger and Screen, A. fumigatus may also degrade these glycan chains through the action of secreted glycosidases (St Leger and Screen, 2000). Data presented here provides evidence to support this hypothesis. Treatment of mucins with A. fumigatus culture filtrates appeared to cause no reduction in GalNAc, Gal(β-1,4)GlcNAc and α-2,3 sialic acid levels. However, mannose levels appeared to increase slightly following treatment. MUC5B and MUC5AC become modified by C-mannosylation within their Cys subdomains (Perez-Vilar, 2004). Under normal circumstances these mannose residues may be not be sufficiently exposed to allow extensive lectin binding; cleavage of other glycans however may lead to exposure of these sugars resulting in a higher level of binding, explaining the increase in signal observed following treatment with A. fumigatus culture filtrate. It is possible that other sugars may be also be cleaved from mucin glycans that were outside the scope of this study. Further investigations must be carried out using additional lectins to examine the effects of A. fumigatus culture filtrate on the levels of other sugars present on mucins, such as galactose, fucose, GlcNAc, and various different linkages. Proteomic analysis would also allow us to develop our knowledge in this area, providing a more detailed view on any structural changes that may occur to these mucin glycans.

3.4. Conclusion

Here we provide novel insights into the effects of A. fumigatus on airway mucins. It has been shown that A. fumigatus secreted products are able to degrade MUC5B and MUC5AC, the main gel forming mucins found in airway mucus. Evidence has been provided to suggest that mucin degrading proteases are secreted after 12 hours of germination, which may coincide with the fungus developing an invasive phenotype within the lung. Further to this, potential cleavage sites for A. fumigatus proteases have been identified within the N- and C-terminus of MUC5B. These data provide us with new insights into a potential mechanism of A. fumigatus pathogenesis, whereby mucins may be degraded by the fungus in order to gain nutrients from the host and facilitate colonisation of the airway environment.
Chapter 4: Identification of mucin degrading proteases and their regulators

4.1 Introduction

_A. fumigatus_ pathogenesis relies on a multitude of factors that allow it to colonise the airways and overcome the host immune system. Many putative virulence factors have been identified in _A. fumigatus_, which have various roles in perturbing the lung's defences (Tomee and Kauffman, 2000). Many of these virulence factors are secreted hydrolytic enzymes such as proteases that have been shown to degrade extracellular molecules in order to acquire nutrients from its surrounding environment. As well as supporting fungal growth, the action of these proteases may also play a role in tissue invasion and thus promote pathogenicity. Thought not believed to be directly responsible for fungal penetration of host tissues, it is likely that proteases are important during the early stages of infection (Tomee and Kauffman, 2000). Mycelial extracts from patients with ABPA have been shown that _A. fumigatus_ protease activity can induce desquamation and inflammation in airway epithelial cells. This may create a niche for _A. fumigatus_ to flourish, creating a cycle of colonisation and inflammation within the lung (Tomee et al., 1997). _A. fumigatus_ protease activity have also been shown to induce the expression and secretion of MUC5AC, which may thus promote the overproduction of airway mucus with altered biophysical properties (Farnell et al., 2012). Although much evidence exists to support a role for these virulence factors during infection and inflammation, these findings are not definitive. Further studies are needed in order to improve our knowledge of these putative virulence factors and their precise mechanism of action.

Protease secretion by _A. fumigatus_ is under the control of various transcription factors and regulatory pathways. For example, PrtT is a transcription factor that regulates the expression of multiple secreted proteases. ΔprtT mutants show a reduced capacity to induce cell detachment and subsequent death in airway epithelial cells, although the virulence of this mutant strain is not attenuated (Sharon et al., 2009). Another transcription factor involved in regulating the expression of proteases and other secreted products is PacC. One study has shown that ΔpacC mutants exhibit a non-invasive phenotype in the mammalian respiratory epithelium, and along with others has identified PacC as a master regulator of _A. fumigatus_ pathogenicity determinants (Bertuzzi et al., 2014; Bignell et al., 2005)

As discussed above, the role of _A. fumigatus_ proteases and their regulators have been studied in a variety of different contexts regarding the pathogenicity of the fungus and host immunological responses. Little is known however about the effects of _A. fumigatus_ proteases on mucins and mucus barrier properties. In the previous chapter it was shown that _A. fumigatus_ culture filtrates can degrade MUC5B and MUC5AC in a protease-dependent manner, however the details of these proteases are as yet unclear. The aim of this chapter was to identify _A. fumigatus_ proteases that can degrade mucins, and potential regulators of these secretory products.
Using protease inhibitor studies we investigated the role of different classes of protease in mucin degradation. To identify proteins that may be involved in degrading MUC5B and MUC5AC, a panel of *A. fumigatus* knockout strains was used, and the effects of their culture filtrates on mucins was assessed. The ability of purified *A. fumigatus* proteases to degrade mucins was also studied. In addition to this, the proteolytically active components of the culture filtrates were isolated and analysed using a proteomics approach. In order to identify potential regulators of mucin degrading proteases, further *A. fumigatus* knockout strains were studied and their effects on MUC5B and MUC5AC degradation was assessed.

4.2. Results

4.2.1 Mucin degradation is mediated by distinct classes of proteases

Our previous results have shown that *A. fumigatus* is able to degrade MUC5B and MUC5AC, suggesting the presence of proteases within the culture filtrate. These experiments were performed using a protease inhibitor cocktail, and thus provided no information regarding the nature of these proteases. This was investigated further using protease inhibitors of various specificities, studying the effects of different inhibitor classes on the proteolytic activity of *A. fumigatus* culture filtrates.

Purified mucins were treated with *A. fumigatus* culture filtrate for 24 hours in the presence or absence of various protease inhibitors. Following treatment, degradation was visualised by agarose gel electrophoresis followed by Western blotting using MUC5B- and MUC5AC-specific antibodies (Figure 4.1). As expected, treatment carried out in the presence of the protease inhibitor mix resulted in no degradation of either MUC5B or MUC5AC. Both mucins were still degraded in the presence of leupeptin, pepstatin A and aprotinin, which inhibit serine and cysteine proteases, aspartic proteases, and serine proteases respectively. In the presence of chymostatin and antipain, (which inhibit chymotrypsin-like and serine proteases, respectively), MUC5B and MUC5AC were no longer fully degraded, although the level of inhibition was not as high as in the presence of the protease inhibitor mix.

Together these results show that mucin degradation by *A. fumigatus* is inhibited by distinct classes of proteases, including serine and chymotrypsin-like proteases. Variation in the levels of inhibition between these different classes suggests that multiple proteases are involved in this process, with these specific subsets resulting in only partial inhibition. Increased inhibition in the presence of the protease inhibitor mix suggests that metalloproteases may also be involved in degrading mucins. Furthermore, our data were consistent between MUC5B and MUC5AC, suggesting that both mucins are equally susceptible to cleavage by *A. fumigatus* proteases.
Figure 4.1 Mucin degradation is mediated by distinct classes of proteases

To assess the protease activity of *A. fumigatus* culture filtrates, mucins were treated with CEA10 culture filtrate in the presence of various protease inhibitors at 37°C for 24 hours (untreated mucins were used as a control). Degradation was visualised using agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies. (n=3).
4.2.2 Mucin degradation by *A. fumigatus* is regulated by multiple secreted products

*A. fumigatus* secretes an array of proteins into its surrounding environment, many of which have been identified as putative virulence factors (Tomee and Kauffman, 2000). We have shown already that *A. fumigatus* can degrade MUC5B and MUC5AC in a protease-dependent manner, identifying the classes of proteases responsible for this degradation process. In order to identify specific proteases and other secreted products that may play a role in this process, the effects of gene disruption on multiple secreted proteins and their potential role in mucin degradation was investigated.

Culture filtrates were collected from a panel of 75 secreted protein knockouts of *A. fumigatus*, as well as A1160, a CEA10-derived strain from which these mutants had been generated. To determine whether any of the target proteins are involved in mucin degradation, each of the culture filtrates collected from these knockouts were tested in the degradation assay. Following treatment of purified mucins with the culture filtrates for 24ours, their proteolytic activity was assessed using agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies (Figure 4.2-4.5). Of the strains tested, culture filtrates from 29 of the knockouts showed a reduced ability to degrade mucins compared to the wild type control. Of the 21 that were enzyme knockouts, those of particular interest included 3 proteases (Alp1, signal peptidase I and DppV), 4 glycosidases (cellulase, XlnA, glycosyl transferase and endo-1,4 beta-glucanase) and 1 amidohydrolase. Other enzymes for which the knockout strain displayed reduced mucin degrading activity included lyases (secreted pectate lyase A and poly(beta-D-mannuronate) lyase), permeases (purine nucleoside permease, amino acid permease and purine nucleoside permease), phytases, chitinases (class V chitinase, class III chitinase ChiA2 and class III chitinase ChiA2), esterases (esterase D and feruloyl esterase), 1 phosphatase (histidine acid phosphatase) and 1 phospholipase (phosphatidylglycerol specific phospholipase). Non-enzyme knockout strains that showed reduced mucin-degrading activity included 1 allergen, 2 hypothetical proteins, 2 cell wall proteins, 2 integral membrane proteins and 1 cell surface protein (Mas1).

These data provide evidence to suggest that mucin degradation may be directly or indirectly regulated by multiple secreted products, and identify Alp1, signal peptidase 1 and DppV as proteases for which the knockout strains lack mucin degrading activity.
Figure 4.2 Mucin degradation by A. fumigatus secreted protein knockout strains (1)

Purified mucins were treated with culture filtrates from a panel of A. fumigatus secreted protein knockouts (KOs) at 37°C for 24 hours (untreated mucins were used as a negative control (C) and mucins treated with the A1160 wild-type culture filtrate were used as a positive control (WT)). Degradation was visualised via agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies. The target protein for each KO along with its Uniprot accession number is shown in the table above, and those showing reduced mucin degrading activity are highlighted in bold.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Accession number</th>
<th>Secreted protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AFUA_4G11800</td>
<td>Alkaline serine protease 1 (Alp1)</td>
</tr>
<tr>
<td>2</td>
<td>AFUA_3G00650</td>
<td>Aminopeptidase Y</td>
</tr>
<tr>
<td>3</td>
<td>AFUA_8G07080</td>
<td>Elastinolytic metalloproteinase (Mep)</td>
</tr>
<tr>
<td>4</td>
<td>AFUA_3G14030</td>
<td>Extracellular phytase</td>
</tr>
<tr>
<td>5</td>
<td>AFUA_8G07090</td>
<td>Extracellular Pro-Ser-rich protein</td>
</tr>
<tr>
<td>6</td>
<td>AFUA_4G09580</td>
<td>Major allergen (Aspf2)</td>
</tr>
</tbody>
</table>
Figure 4.3 Mucin degradation by *A. fumigatus* secreted protein knockout strains (2)
Purified mucins were treated with culture filtrates from a panel of *A. fumigatus* secreted protein knockouts (KOs) at 37°C for 24 hours (untreated mucins were used as a negative control (C) and mucins treated with the A1160 wild-type culture filtrate were used as a positive control (WT)). Degradation was visualised via agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies. The target protein for each KO along with its Uniprot accession number is shown in the table above, and those showing reduced mucin degrading activity are highlighted in bold.
Purified mucins were treated with culture filtrates from a panel of A. fumigatus secreted protein knockouts (KOs) at 37°C for 24 hours (untreated mucins were used as a negative control (C) and mucins treated with the A1160 wild-type culture filtrate were used as a positive control (WT)). Degradation was visualised via agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies. The target protein for each KO along with its Uniprot accession number is shown in the table above, and those showing reduced mucin degrading activity are highlighted in bold.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Accession number</th>
<th>Secreted protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AFUA_6G08700</td>
<td>beta-glucosidase, putative</td>
</tr>
<tr>
<td>2</td>
<td>AFUA_6G06660</td>
<td>beta-galactosidase, putative</td>
</tr>
<tr>
<td>3</td>
<td>AFUA_1G14170</td>
<td>beta-galactosidase, putative</td>
</tr>
<tr>
<td>4</td>
<td>AFUA_5G07080</td>
<td>beta-glucosidase, putative</td>
</tr>
<tr>
<td>5</td>
<td>AFUA_1G06150</td>
<td>L-serine dehydratase, putative</td>
</tr>
<tr>
<td>6</td>
<td>AFUA_2G01240</td>
<td>beta-D-fructofuranoside fructohydrolase</td>
</tr>
<tr>
<td>7</td>
<td>AFUA_1G16700</td>
<td>beta galactosidase, putative</td>
</tr>
<tr>
<td>8</td>
<td>AFUA_2G00810</td>
<td>purine nucleoside permease, putative</td>
</tr>
<tr>
<td>9</td>
<td>AFUA_4G10130</td>
<td>alpha-amylase (Amy1), putative</td>
</tr>
<tr>
<td>10</td>
<td>AFUA_1G01300</td>
<td>GPI anchored protein, predicted poly(beta-D-mannuronate) lyase, putative</td>
</tr>
<tr>
<td>11</td>
<td>AFUA_7G06140</td>
<td>beta-D-glucoside glucohydrolase</td>
</tr>
<tr>
<td>12</td>
<td>AFUA_4G10150</td>
<td>alpha-galactosidase, putative</td>
</tr>
<tr>
<td>13</td>
<td>AFUA_1G15050</td>
<td>Hsp70 chaperone (Orp150), putative</td>
</tr>
<tr>
<td>14</td>
<td>AFUA_1G16250</td>
<td>alpha-glucosidase B</td>
</tr>
<tr>
<td>15</td>
<td>AFUA_7G00800</td>
<td>acid phosphatase, putative</td>
</tr>
<tr>
<td>16</td>
<td>AFUA_8G01710</td>
<td>antigenic thaumatin domain protein, putative</td>
</tr>
<tr>
<td>17</td>
<td>AFUA_4G12900</td>
<td>alpha-1,6-mannosyltransferase subunit (Ecm39), putative</td>
</tr>
<tr>
<td>18</td>
<td>AFUA_2G14490</td>
<td>endoglucanase, putative</td>
</tr>
</tbody>
</table>

**Figure 4.4 Mucin degradation by A. fumigatus secreted protein knockout strains (3)**

Purified mucins were treated with culture filtrates from a panel of A. fumigatus secreted protein knockouts (KOs) at 37°C for 24 hours (untreated mucins were used as a negative control (C) and mucins treated with the A1160 wild-type culture filtrate were used as a positive control (WT)). Degradation was visualised via agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies. The target protein for each KO along with its Uniprot accession number is shown in the table above, and those showing reduced mucin degrading activity are highlighted in bold.
Figure 4.5 Mucin degradation by *A. fumigatus* secreted protein knockout strains (4)

Purified mucins were treated with culture filtrates from a panel of *A. fumigatus* secreted protein knockouts (KOs) at 37°C for 24 hours (untreated mucins were used as a negative control (C) and mucins treated with the A1160 wild-type culture filtrate were used as a positive control (WT)). Degradation was visualised via agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies. The target protein for each KO along with its Uniprot accession number is shown in the table above, and those showing reduced mucin degrading activity are highlighted in bold.
Figure 4.6 Mucin degradation by A. fumigatus secreted protein knockout strains (5)

Purified mucins were treated with culture filtrates from a panel of A. fumigatus secreted protein knockouts (KOs) at 37°C for 24 hours (untreated mucins were used as a negative control (C) and mucins treated with the A1160 wild-type culture filtrate were used as a positive control (WT)). Degradation was visualised via agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies. The target protein for each KO along with its Uniprot accession number is shown in the table above, and those showing reduced mucin degrading activity are highlighted in bold.
4.2.3 Mep protease degrades MUC5B and MUC5AC

Using protease inhibitors and knockout screening we have shown that *A. fumigatus* secretes a specific subset of proteases that degrade mucins. Alongside this approach, in order to gain more insight into the role of specific proteases in this process, the mucin degrading activity of available purified *A. fumigatus* proteases was studied.

MUC5B and MUC5AC were treated with 3 purified proteases from *A. fumigatus* – DppV (dipeptidyl-peptidase), Mep (metalloprotease) and S28 (serine protease). Treatment was carried out at 37°C for 24 hours, and CEA10 culture filtrate was used as a positive control. Degradation was assessed via agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies (Figure 4.6). Treatment with DppV resulted in minimal degradation of the mucins at both 410 µg/ml and 210 µg/ml. The same was observed for S28; although a slight reduction in band intensities was seen, this was negligible as compared with the CEA10 positive control. Mep on the other hand was able to degrade both mucins, and this was observed at both 1240 µg/ml and 210 µg/ml.

These results show that Mep protease alone, but not DppV and S28, is able to degrade MUC5B and MUC5AC.

4.2.4 Identification of mucin degrading components in *A. fumigatus* culture filtrates

In our earlier experiments it was shown that *A. fumigatus* culture filtrates can degrade MUC5B and MUC5AC, and using multiple approaches a number of putative mucin degrading proteases were identified. Following on from this, and in a parallel approach, the aim was to investigate whether these proteases were the same active components present within the culture filtrates.

In order to purify the active mucin degrading components, CEA10 culture filtrate was first fractionated using size exclusion chromatography. In order to trace the sample as it was eluted from the column, the absorbance of each fraction was measured at 280 nm (Figure 4.8A). Three peaks were observed between fractions 12-15, 17-21 and 24-29. A fourth much larger peak was observed between fractions 30-39, followed by two further peaks between fractions 39-42 and 43-47. Column fractions were then analysed by SDS-PAGE, which showed that most proteins within the sample were present between fractions 24-31 (Figure 4.8B).

To determine which of the fractions contained the mucin degrading components, mucins were treated with each of the fractions for 24 hours and degradation visualised via agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies (Figure 4.9). Most of the fractions showed no proteolytic activity against the mucins, with only fractions 27-31 able to degrade MUC5B and MUC5AC. Fraction 29 showed the highest level of activity, resulting in an 88% decrease in signal for MUC5B and 77% decrease in signal for MUC5AC; as the most active mucin degrading fraction, this was then selected for further analysis.
Figure 4.7 Mep protease degrades MUC5B and MUC5AC

Mucins were treated with purified A. fumigatus proteases (DppV, Mep and S28) at 37°C for 24 hours. Following treatment, degradation was measured via agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies.
Figure 4.8 Purification of *A. fumigatus* culture filtrate

A) *A. fumigatus* CEA10 culture filtrate was purified using size exclusion chromatography in 10 mM Tris 0.1 M NaCl buffer using a Superose 12 column. Absorbance of each fraction at 280 nm was measured as they were eluted from the column. B) Following size exclusion chromatography, fractions were separated using SDS-PAGE and proteins visualised by silver staining.
Figure 4.9: Isolation of mucin-degrading proteases in *A. fumigatus* culture filtrates

A) Mucins were treated with *A. fumigatus* culture filtrate fractions at 37°C for 24 hours. To visualise mucin degradation, samples were analysed by agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies. For quantification, relative intensity levels were normalised to the sample that gave the highest signal.
In order to identify putative mucin-degrading components of the culture filtrate, fraction 29 was analysed by tandem mass spectrometry following SDS-PAGE gel lane excision (Table 4.1). Thirty proteins were identified within the sample. Amongst these hits were the proteases Alp1/Asp F13 and neutral protease 2, as well as 3 uncharacterised proteins. A number of glycosidase enzymes were also identified, including alpha-1,2-mannosidase, EglC, endo-1,4 beta glucanase, glucoamylase, arabinogalactan endo-beta-1,4-galactanase, glycosyl hydrolase, 1,3-beta-glucanosyltransferase and endoglucanase.

In these experiments, the mucin degrading components of the *A. fumigatus* culture filtrate were isolated. The serine protease Alp1/Asp F13 and neutral protease 2 were found within the most active fraction of the culture filtrate, identifying these proteases as putative mucin degrading enzymes. A number of carbohydrate cleaving enzymes were also identified within this active part of the culture filtrate, as well as 3 uncharacterised proteins, which may also possess mucin degrading activity.

### 4.2.5 PalH and PacC are regulators of mucin degradation

A number of studies have utilised gene disruption techniques to investigate the role of protease secretion on *A. fumigatus* virulence, highlighting a role for various transcription factors in this process (Bergman *et al*, 2009; Monod *et al*, 1993; Jaton-Ogay *et al*, 1994). It is known that *A. fumigatus* proteases are under the control of multiple signalling pathways within the fungus, with a number of transcription factors identified as key regulators of their expression and secretion. Of note, the PacC transcription factor is involved in pH-responsive signalling in *A. fumigatus*, and plays a role in regulating the expression of multiple secreted proteins (Bignell *et al*, personal communication). Upstream of PacC is a cell-surface receptor called PalH, which along with other members of this signalling pathway relays environmental changes to PacC in order to regulate its target genes (Cornet and Gaillardin, 2014). Due to the wide range of secreted proteins under the control of PalH/PacC signalling, the potential role of this pathway in regulating mucin degradation by *A. fumigatus* was investigated.

Culture filtrates were collected from 3 wild type (CEA10, ATCC and A1160) and 3 mutant (ATCCΔpalH, ATCCΔpacC and A1160ΔpacC) strains of *A. fumigatus*. Purified mucins were treated with each culture filtrate for 24 hours, and degradation was measured via agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies (Figure 4.10). As expected, both mucins were degraded by all wild type culture filtrates, with a higher level of proteolytic activity observed for the CEA10 and A1160 wild type strains. Culture filtrates from the ΔpalH and ΔpacC mutants showed a reduced ability to degrade mucins, with those from the ATCC background showing no apparent proteolytic activity compared to untreated and wild type controls. The A1160ΔpacC culture filtrate showed some ability to degrade mucins, however this activity was still reduced compared to that of the wild type strain.

These data highlight a potential role for PalH and PacC in regulating mucin degradation, suggesting that mucin degrading proteases secreted by *A. fumigatus* are under the control of the PalH/PacC signalling pathway.
Table 4.1: Proteomic analysis of active *A. fumigatus* culture filtrate fractions

Proteomic analysis of the most proteolytically active *A. fumigatus* culture filtrate fraction. Following SDS-PAGE and silver staining, bands were excised and analysed by MS/MS analysis. Data from the peptides generated were searched against the Swissprot database using the Mascot server, with the limited taxonomy of ‘Fungi’.

<table>
<thead>
<tr>
<th>Identified Proteins</th>
<th>Unique peptide count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncharacterized protein OS=Neosartorya fumigata</td>
<td>10</td>
</tr>
<tr>
<td>Asp-hemolysin OS=Neosartorya fumigata</td>
<td>8</td>
</tr>
<tr>
<td>alpha-1,2-Mannosidase OS=Aspergillus fumigatus</td>
<td>10</td>
</tr>
<tr>
<td>Cell wall protein OS=Aspergillus fumigatus</td>
<td>7</td>
</tr>
<tr>
<td>GPI anchored cell wall beta 1,3 endoglucanase EgIC OS=Aspergillus fumigatus</td>
<td>5</td>
</tr>
<tr>
<td>Ribonuclease T2, putative OS=Aspergillus fumigatus</td>
<td>7</td>
</tr>
<tr>
<td>Class V chitinase ChiB1 OS=Aspergillus fumigatus</td>
<td>6</td>
</tr>
<tr>
<td>Alkaline serine protease Alp1 OS=Aspergillus fumigatus</td>
<td>4</td>
</tr>
<tr>
<td>Allergenic cerato-platanin Asp F13 OS=Aspergillus fumigatus</td>
<td>5</td>
</tr>
<tr>
<td>Uncharacterized protein OS=Aspergillus fumigatus</td>
<td>2</td>
</tr>
<tr>
<td>Superoxide dismutase [Cu-Zn] OS=Aspergillus fumigatus</td>
<td>4</td>
</tr>
<tr>
<td><strong>Neutral protease 2</strong> OS=Aspergillus fumigatus</td>
<td>4</td>
</tr>
<tr>
<td>Uncharacterized protein OS=Aspergillus fumigatus</td>
<td>4</td>
</tr>
<tr>
<td>Uncharacterized protein OS=Aspergillus fumigatus</td>
<td>4</td>
</tr>
<tr>
<td>Alpha amylase OS=Aspergillus fumigatus</td>
<td>3</td>
</tr>
<tr>
<td>Endo 1,34 beta glucanase OS=Aspergillus fumigatus</td>
<td>4</td>
</tr>
<tr>
<td>Eno2p OS=Saccharomyces cerevisiae</td>
<td>3</td>
</tr>
<tr>
<td>Glucoamylase OS=Aspergillus fumigatus</td>
<td>3</td>
</tr>
<tr>
<td>Pectate lyase A OS=Aspergillus fumigatus</td>
<td>3</td>
</tr>
<tr>
<td>Cell wall protein PhiA OS=Aspergillus fumigatus</td>
<td>3</td>
</tr>
<tr>
<td>Arabinogalactan endo-beta-1,4-galactanase OS=Aspergillus fumigatus</td>
<td>3</td>
</tr>
<tr>
<td>FAD/FMN-containing isoamyl alcohol oxidase MreA OS=Aspergillus fumigatus</td>
<td>2</td>
</tr>
<tr>
<td>Glycosyl hydrolase OS=Aspergillus fumigatus</td>
<td>2</td>
</tr>
<tr>
<td>Actin (Fragment) OS=Hanseniaspora uvarum</td>
<td>2</td>
</tr>
<tr>
<td>1,3-beta-glucanosyltransferase OS=Aspergillus fumigatus</td>
<td>3</td>
</tr>
<tr>
<td>Pyruvate kinase OS=Saccharomyces cerevisiae</td>
<td>2</td>
</tr>
<tr>
<td>Secretory pathway protein Ssp 120 OS=Aspergillus fumigatus</td>
<td>2</td>
</tr>
<tr>
<td>Endoglucanase OS=Aspergillus fumigatus</td>
<td>2</td>
</tr>
<tr>
<td>Oxygenase FAD dependent OS=Aspergillus fumigatus</td>
<td>2</td>
</tr>
<tr>
<td>Cellulase CelA/allergen Asp F7 OS=Aspergillus</td>
<td>2</td>
</tr>
</tbody>
</table>
To identify potential upstream regulators of mucin degrading proteases, culture filtrates were collected from both wild type (CEA10, ATCC, A1160) and mutant (ATCCΔpalH, ATCCΔpacC, A1160ΔpacC) strains of *A. fumigatus*. Mucins were treated with each culture filtrate at 37°C for 24 hours, and mucin degradation visualised using agarose gel electrophoresis followed by immunoblotting using MUC5B- and MUC5AC-specific antibodies.

**Figure 4.10: Mucin degradation may be regulated by PalH/PacC signalling**
4.3 Discussion

Through the use of multiple biochemical approaches new insights have been provided into the role of *A. fumigatus* proteases in mucin degradation. Using protease inhibitors we were able to confirm that mucin degradation by *A. fumigatus* is a protease-dependent process, and identified which classes of protease are responsible for degrading MUC5B and MUC5AC. Due to the high degree of sequence homology between these mucins (Rousseau *et al*., 2007), it is not surprising that they were equally susceptible to proteolytic cleavage. In line with other studies highlighting a role for serine proteases in inducing epithelial cell damage (Kogan *et al*., 2004), we have shown that such proteases can also degrade mucins and may thus impact on mucus barrier properties. Similarly, serine and matrix metalloproteases have been shown to be upregulated by *A. fumigatus* when grown on mucin based medium. Upregulation of specific subsets of proteases in the presence of mucins may highlight the adaptability of the fungus, whereby it can utilise the resulting peptides as a source of nutrients to promote fungal growth (Farnell *et al*., 2012). It is also possible that the action of these proteases disrupts the structural network formed by mucins so that the barrier becomes more penetrable, allowing easier access to the epithelial surface prior to inducing tissue damage. Indeed, degradation of mucins by serine proteases has previously been shown in the sputum of CF patients, which has been attributed to protease secretion by *P. aeruginosa* (Henke *et al*., 2011). Our findings suggest that this may also be due to *A. fumigatus*, and it is likely that other pathogens present in the CF lung may too have these effects. Disruption of the mucin network by *A. fumigatus* may also facilitate airway colonisation by other pathogens, contributing to the high microbial burden of CF airways.

Metalloproteases have also been shown to cleave mucins. One study has shown that TagA, a metalloprotease secreted by *Vibrio cholera*, is able to cleave mucins, which is believed to promote intestinal colonisation by the bacteria (Szabady *et al*., 2011). *Escherichia coli* has also been shown to secrete a mucin-degrading protease called Yghj, which is able to degrade the intestinal mucins MUC2 and MUC3, suggesting a shared virulence feature between these pathogens (Luo *et al*., 2013). We can therefore assume that these particular classes of protease are involved in a common mechanism of pathogenesis across multiple mucosal pathogens including *A. fumigatus*. By targeting these proteases with specific inhibitors, it may be possible to prevent mucin degradation and restore protection of the airway surface.

By utilising culture filtrates collected from a panel of secreted protein knockout strains, we were able to identify proteases and other secreted proteins involved in mucin degradation. Our results suggest that Alp1, DppV and signal peptidase I are able to degrade MUC5B and MUC5AC. In agreement with these findings, Alp1 (also known as Asp F13) has been shown to be upregulated by *A. fumigatus* in the presence of mucins. When grown on mucin based medium, Alp1 gene expression increased ~7 fold compared with controls grown on minimal medium, and mass spectrometry showed an increase in this protease within the culture supernatant (Farnell *et al*., 2012). Alp1 has also been shown to degrade other extracellular matrix components, including elastin, fibronectin and collagen I-IV, disrupting integrin-mediated...
attachments of airway smooth muscle cells (Iadarola et al, 1998; Balenga et al, 2015). In airway epithelial cells, Alp1 has been shown to upregulate MUC5AC expression through the sequential activation of TNFα-converting enzyme (TACE), TGF-α and epidermal growth factor receptor (EGFR), which may be a key mechanism of A. fumigatus pathogenesis (Oguma et al, 2011). The dipeptidyl-peptidase DppV is another predicted allergen that appears to be responsible for protein degradation of the host cell during invasion. It has been shown by Beauvais and colleagues that expression and secretion of DppV is dependent upon its surrounding environment, with the highest levels of the protease present within media containing only protein or protein hydrolysate (Beauvais et al, 1997). In another study, levels of DppV along with other proteolytic enzymes such as Alp1 were shown to increase in the presence of potential nutrient sources such as elastin, collagen and keratin (Wartenberg et al, 2011). As such, it is likely that these proteases are also upregulated in the presence of mucins, which as discussed above may be utilised as a source of nutrients or degraded in order to facilitate penetration of the mucus barrier. Signal peptidase I is a protein that has not yet been cited in the literature with regards to A. fumigatus and its putative function is based solely on its sequence (Nierman et al, 2005), however this family of enzymes are involved in cleaving signal sequence peptides from secretory proteins in order to convert them into their mature form (Tuteja 2005). As such, it is possible that this protein has an additional or alternative function to the one that has previously been proposed, and we here provide novel evidence to suggest that it may be involved in degrading mucins within the airways. We have also identified a potential role for a number of carbohydrate degrading enzymes. As a saprophytic organism, A. fumigatus secretes enzymes such as cellulase, xylanase, and lyases in order to degrade plants and other decaying matter within the environment, allowing it to obtain nutrients (de Vries and Visser, 2001). Our results suggest that such enzymes may also support fungal growth within the airways by degrading mucins and perhaps other components of the extracellular matrix. As highly glycosylated proteins, mucins are largely protected against proteolytic degradation (Thornton et al, 2008). By cleaving these glycan side chains that decorate mucin molecules, A. fumigatus may be able to utilise these sugars as a nutrient source, as well as exposing the polypeptide core and thus allowing further degradation of the mucin by the action of proteases. As the precise functions of these proteins and many other secreted products remains unknown, further studies are required in order to elucidate their true role in A. fumigatus pathogenesis. Purifying these proteins and studying them in isolation could provide a useful insight into their functional role, in the context of both mucin degradation as well as other stages of airway colonisation.

We also provide evidence to suggest a novel role for the metalloprotease Mep. This is one of the major proteases secreted during A. fumigatus infection, and upregulation and secretion of Mep in pig lung medium has previously been described by Farnell and colleagues (Monod et al, 1999; Farnell et al, 2012). Although we have shown that culture filtrate from a Mep-deficient strain of A. fumigatus displays no reduction in mucin degrading activity, it is likely that there are multiple proteases involved in this process. As such, deletion of only one of these proteases
may only have a small effect when others are still present within the culture filtrate. We have clearly shown that purified Mep is able to degrade MUC5B and MUC5AC at both high and low concentrations, whereas the other proteases tested exhibited no proteolytic activity against either of the mucins. It has been speculated that DppV is involved in extracellular matrix degradation, however we found that purified DppV was unable to degrade MUC5B and MUC5AC. Although we have shown that culture filtrate from a DppV knockout showed a reduced ability to degrade mucins, it is possible that this protease plays a role in generating substrates for other mucin degrading proteases, and may not be sufficient to degrade mucins in isolation. Although not representative of the conditions found within the lung, studying the effects of other purified proteases would be invaluable in determining which of these secreted products may be involved in degrading mucins.

Our studies have allowed us to identify putative mucin degrading proteases as well as other secreted products that may be involved in regulating this process. By purifying the mucin degrading components of the culture filtrates used in our initial degradation studies, we identified Alp1/Asp F13 as one of the proteases present within these active fractions. These findings are in line with our previous results generated using culture filtrates from secreted protein knockouts, with both data sets suggesting that Alp1 is one of the major proteases responsible for mucin degradation by *A. fumigatus*. As we have discussed already, Alp1 is upregulated in mucin-based medium (Farnell *et al.*, 2012), suggesting that secretion of this protease may represent an adaptive mechanism of the fungus. Within the lung, particularly in the context of CF where mucin concentrations are known to be increased (Henderson *et al.*, 2014), *A. fumigatus* may upregulate Alp1 expression and secretion in order to selectively degrade proteins that are abundant within the surrounding environment, allowing it to create a suitable niche in which to flourish. Another secreted product we identified as a putative mucin degrading protease was neutral protease 2. The function of this protease has not yet been described in the literature, and as such it's role in *A. fumigatus* colonisation is currently unknown. We provide evidence to suggest a role for this protease in mucin degradation, due to its presence within the proteolytically active portion of the culture filtrate. Further studies are now required in order to determine the true role of this protease and its importance in *A. fumigatus* virulence. Purification of neutral protease 2 as well as Alp1 would allow us to directly determine whether these proteases can degrade mucins, perhaps revealing important aspects of *A. fumigatus* pathogenesis.

We have also identified potential regulators of mucin degradation. Using culture filtrates from ΔPalH and ΔPacC knockouts we have shown that these proteins may be involved in regulating mucin degrading proteases. PalH is a transmembrane receptor involved in pH signalling in *A. fumigatus*, which upon activation triggers a signalling cascade that leads to cleavage and subsequent activation of the transcription factor PacC. Once activated, PacC is able to translocate to the nucleus where it regulates the expression of multiple genes that promote fungal growth (Grice *et al.*, 2013). PacC null mutants have been shown to display a non-invasive
phenotype in mice, although this is not believed to be due to a lack of protease activity (Bertuzzi et al., 2014). However, the fact that culture filtrates from both PalH and PacC knockout strains are unable to degrade MUC5B and MUC5AC strongly suggests that this signalling pathway is crucial in regulating mucin degradation within the airways, which may be an important step in the early stages of colonisation. By studying the effects of all downstream targets of PacC, we may be able to identify other enzymes involved in mucin degradation. Knockouts of other transcription factors and components of various signalling pathways would also provide a valuable insight into other regulatory mechanisms governing this process.

4.4 Conclusion
Here we have identified potential key regulators of mucin degradation by A. fumigatus. It has been shown that multiple secreted products are involved in regulating mucin degradation, identifying a number of proteases and other enzymes that appear to be crucial for this process. We provide evidence to support a role for Alp1 and Mep as mucin degrading proteases, as well as other secreted enzymes such as and neutral protease 2, signal peptidase 1, DppV. Data has also been presented to suggest that PalH/PacC signalling is a key mechanism involved in regulating mucin degradation, which may drive further studies that will identify more of these mucin degrading enzymes. These findings allow us to bridge some of the gaps in our knowledge regarding the interplay between mucins and A. fumigatus, and the steps that may be involved in airway colonisation.
5.1 Introduction

The importance of mucins in cystic fibrosis has been highlighted by a number of studies. As shown by Horsley and colleagues, mucins have a significant impact on sputum rheology (Horsley et al., 2014). Mucin concentrations are higher in CF airway secretions than in healthy individuals, and along with dehydration of the airway surface this leads to an overproduction of mucus with aberrant transport properties. These alterations in mucus barrier properties promote the development of chronic respiratory infections with organisms such as A. fumigatus, and the role of such pathogens in the progression of CF airway disease is still relatively unclear (Henderson et al., 2014; Cowley et al., 2017). Interactions between A. fumigatus and airway mucins has recently been studies by Kerr and colleagues, who showed that a fucose-specific lectin on the surface of A. fumigatus (FleA) is able to bind fucosylated structures on mucin glycans. This is believed to facilitate entrapment of the fungus within the mucus barrier, allowing subsequent removal by mucociliary clearance. (Kerr et al., 2016). A. fumigatus has also been shown to degrade commercially available mucins (St Leger and Screen, 2000), and these findings have been supported by data presented in earlier chapters of this thesis. Although these findings provide some insight into the potential role of A. fumigatus in CF airway disease, the clinical implications of these changes in mucin properties are not yet understood.

As an opportunistic pathogen, A. fumigatus is able to adapt well to a variety of different environments. The small size and thermotolerance if A. fumigatus spores are important factors in determining its ability to penetrate and survive within the human airways, although other factors are also likely to facilitate this process (St Leger and Screen, 2000). As mucus viscosity is key to its role in host defense, it is possible that A. fumigatus may alter mucus biophysical properties in order to exploit its protective function, however this has not yet been studied to our knowledge. A. fumigatus has been shown to degrade various extracellular macromolecules including horse lung polymer, pig gastric mucin and bovine submaxillary mucin, and this is believed to act as a mechanism of nutrient acquisition within the airways (St Leger and Screen, 2000). There are a number of possible implications of mucin degradation by A. fumigatus that are yet to be explored. We aim to delineate some of these potential roles by studying the effects of A. fumigatus on the biochemical and rheological properties of CF mucins. The effects of A. fumigatus on mucin secretion in vivo will also be studied, as well as the utilisation of mucins as a carbon source to support fungal growth.

To further investigate the role of A. fumigatus in CF airway disease, mucins were purified from the sputum of CF patients testing either positive or negative for A. fumigatus, and the biochemical properties of these mucins were assessed using SEC-MALLS. Particle tracking microrheology was used to determine the effects of A. fumigatus on mucin viscosity. To study the effects of A. fumigatus on mucin gene expression and secretion in vivo, qPCR and immunohistochemistry were used following treatment of mice with A. fumigatus spores using a
repeated exposure model. Finally, growth assays were performed to determine whether *A. fumigatus* is able to utilise mucins as a sole carbon source.

5.2 Results

5.2.1. Effects of *A. fumigatus* on the biochemical properties of CF mucins

To study the potential effects of *A. fumigatus* on CF mucins, sputum was collected from patients testing either negative (*Af*-) or positive (*Af*+) for *A. fumigatus*. In order to observe any differences in MUC5B and MUC5AC levels between the two groups, each *Af*+ sputum sample was analysed by agarose gel electrophoresis followed by immunoblotting, alongside age matched *Af*- controls (Figure 5.1). MUC5AC was detectable in all of the samples tested, however MUC5B was detected in only 2 *Af*+ samples and 4 *Af*- samples. Mucins were then purified from each sputum group using caesium chloride (CsCl) density gradient centrifugation. The first centrifugation step was performed at a starting density of 1.4 g/ml, to separate mucins from other proteins within the sputum (Figure 5.2A). For *A. fumigatus*-negative (*Af*) sputum, mucin-rich peaks were identified between densities of 0.37-1.57 g/ml by both PAS staining and immunoblotting. For *A. fumigatus*-positive (*Af*) sputum, PAS staining and immunoblotting identified mucin-rich peaks between densities of 0.56-1.44 g/ml. Fractions 2-5 of the *Af*- sputum and fractions 4-13 of the *Af*+ sputum were then pooled and subjected to a second density gradient centrifugation at a starting density of 1.5 g/ml, to separate mucins from nucleic acids (Figure 5.2B). For *Af*- sputum, mucin-rich peaks were identified between 0.42-1.50 g/ml by PAS staining and immunoblotting. For *Af*+ sputum peaks were identified between 0.46-0.66 g/ml. Fractions 7-14 of the *Af*- sputum and fractions 10-21 of the *Af*+ sputum were subsequently pooled for further investigation.

To assess the purity of our mucin samples, *Af*- and *Af*+ CF mucins were then analysed by MS/MS (Table 5.1). MUC5B and MUC5AC were identified as the top 2 hits in both *Af*- and *Af*+ samples, thus showing that they were the most abundant proteins following the purification process.

In order to investigate the potential effects of *A. fumigatus* on the biochemical properties of CF mucins, the size distribution of *Af*- and *Af*+ CF mucins was analysed by SEC-MALLS. For both *Af*- and *Af*+ mucins, differential refractive index data revealed a broad peak at 6-9 ml, with a slight shoulder at ~8.3 ml representing a population of smaller mucin molecules. For the *Af*+ mucins an additional peak at 9-10 ml was observed, which again represents a population of mucins that are much smaller in size (Figure 5.3A). Data also showed that the average molecular weight was similar between the two groups, however a loss of high molecular weight mucins was observed in the *Af*+ mucin sample (Figure 5.3B).

Overall these data highlight the high degree of variation in the mucin content of CF sputum, showing no apparent difference in the levels of MUC5B and MUC5AC between *Af*+ and *Af*- patients. Results show that mucins from both patient groups are polydisperse in size, with those from *Af*+ patients appearing smaller on average than those from *Af*- patients.
Figure 5.1 MUC5B and MUC5AC variation in CF sputum

CF sputum samples were collected from patients testing both positive and negative for *A. fumigatus*. Once processed, samples were analysed by agarose gel electrophoresis followed by Western blotting using MUC5B- and MUC5AC-specific antibodies. *Af*+ samples (A) were loaded alongside age-matched *Af*- controls (C). MUC5B is shown in green and MUC5AC is shown in red.
A. *fumigatus* negative

A. *fumigatus* positive

**Figure 5.2 Purification of mucins from CF sputum**

Mucins were isolated from *Af*- and *Af*+ CF sputum using caesium chloride (CsCl) density gradient centrifugation. Mucin-rich fractions were identified by PAS staining and immunoblotting using MUC5B- and MUC5AC-specific antibodies. **A)** Sputum from each patient group was subjected to CsCl density gradient centrifugation at a starting density of 1.4 g/ml. **B)** Pooled fractions from the first centrifugation were subjected to a second CsCl density gradient centrifugation at a starting density of 1.5 g/ml.
Table 5.1 Proteomic analysis of CF sputum

MS/MS data for peptides generated from the purified CF mucins were analysed using the MASCOT search engine, performed against the SwissProt database (Taxonomy: Homo sapiens). For each protein identified, the number of unique peptides was also determined.

**A. fumigatus negative**

<table>
<thead>
<tr>
<th>Proteins identified</th>
<th>Number of unique peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC5B</td>
<td>29</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>25</td>
</tr>
<tr>
<td>Neutrophil defensin 3</td>
<td>2</td>
</tr>
<tr>
<td>Lactotransferrin</td>
<td>14</td>
</tr>
<tr>
<td>Protein S100-A9</td>
<td>6</td>
</tr>
<tr>
<td>Annexin A1</td>
<td>3</td>
</tr>
<tr>
<td>Protein S100-A8</td>
<td>4</td>
</tr>
<tr>
<td>Alpha-amylase 1</td>
<td>2</td>
</tr>
<tr>
<td>WAP four-disulfide core domain protein 2</td>
<td>1</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>5</td>
</tr>
</tbody>
</table>

**A. fumigatus positive**

<table>
<thead>
<tr>
<th>Proteins identified</th>
<th>Number of unique peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC5B</td>
<td>37</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>18</td>
</tr>
<tr>
<td>Neutrophil defensin 3</td>
<td>3</td>
</tr>
<tr>
<td>Protein S100-A9</td>
<td>5</td>
</tr>
<tr>
<td>Lactotransferrin</td>
<td>20</td>
</tr>
<tr>
<td>Deleted in malignant brain tumours 1 protein</td>
<td>8</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>11</td>
</tr>
<tr>
<td>Protein S100-A8</td>
<td>4</td>
</tr>
<tr>
<td>Neutrophil gelatinase-associated lipocalin</td>
<td>6</td>
</tr>
<tr>
<td>Alpha-amylase 1</td>
<td>7</td>
</tr>
</tbody>
</table>
Figure 5.3 Biomolecular analysis of CF mucins

Mucins were purified from CF sputum testing either positive (green) or negative (red) for *A. fumigatus*, and analysed by SEC-MALLS to determine A) Differential refractive index and B) Molar mass.
5.2.2. *A. fumigatus* reduces mucin viscosity

It is well established that mucus secretions have an increased viscosity in CF, although the impact of fungal pathogens such as *A. fumigatus* on their biophysical properties have not yet been studied. In order to investigate this, we studied the effects of *A. fumigatus* culture filtrates on the viscosity of purified CF mucins.

Mucins purified from *Af*- CF sputum were treated with *A. fumigatus* culture filtrate at 37°C for 24 hours, and changes in biophysical properties were measured using particle tracking microrheology. Treatment with the culture filtrate resulted in a reduction in viscosity from 16.93 to 2.99 mPaS (Figure 5.4A). Furthermore the average MSD readings were higher within the treated mucins as compared with the untreated controls, representing an increase in the movement of beads within the sample over time (Figure 5.4B).

These results show that *A. fumigatus* is able to alter the viscoelastic properties of CF mucins, causing a reduction in mucin viscosity over time.

5.2.3. *A. fumigatus* may induce airway remodelling and mucin secretion

*A. fumigatus* secreted proteases have been shown to induce airway inflammation and remodelling in murine airways, and appear to induce MUC5AC expression in airway epithelial cells (Namvar *et al.*, 2015; Oguma *et al.*, 2011). In order to investigate this further, we studied the effects of *A. fumigatus* on MUC5B and MUC5AC secretion using a repeated exposure model in mice.

Mice were administered with 9 doses of *A. fumigatus* spores over 3 weeks, and lung tissue samples collected at day 1 (prior to treatment) and day 19 (1 day after final exposure). In order to investigate any changes in mucin secretion, lungs were subjected to bronchiolar lavage (BAL) and samples were analysed by slot blotting followed by immunostaining (Figure 5.5). BAL samples from d19 mice contained significantly higher MUC5AC levels than d1 controls, whereas MUC5B levels showed no significant difference. Lungs were subsequently fixed in formalin and cross sections of the left lung were analysed by immunofluorescence microscopy (Figure 5.6). At d19 the airways appeared wider than those from d1, and an increase in goblet cell size was observed. Intracellular MUC5B staining was also increased at d19, however MUC5AC staining was unsuccessful (data not shown).

These results provide supportive evidence to suggest that *A. fumigatus* may induce airway remodelling and mucin hypersecretion in mice.
Figure 5.4: Effects of *A. fumigatus* on mucin viscosity

Purified CF mucins (*Af*) were treated with *A. fumigatus* culture filtrate for 24 hours, and the effects on mucin viscosity were measured using particle tracking microrheology. Untreated mucins were used as a negative control. A) The MSD of 500 nm beads was calculated using 15 videos B) The viscosity of each sample was calculated from the MSD values.
Figure 5.5 Effects of *A. fumigatus* on MUC5B and MUC5AC secretion in mouse airways
Changes in MUC5B and MUC5AC levels in the BAL fluid of *A. fumigatus* treated mice were measured via immunoblotting using MUC5B- and MUC5AC-specific antibodies. *** P<0.001, ** P<0.01, * P<0.05 (n=3; two independent experiments)
Figure 5.6 Effects of *A. fumigatus* on intracellular MUC5B levels in mouse airways

Immunofluorescence microscopy of lung tissue sections from *A. fumigatus* treated mice. Tissues from control (d1) and treated (d19) mice were stained for MUC5B (green) and counterstained with DAPI (blue). n=2.
5.2.4. *A. fumigatus* cannot utilise mucins as a sole carbon source

Previous experiments have shown that *A. fumigatus* is able to degrade mucins, and that this can influence mucus rheological properties. It has been postulated that mucin degradation may facilitate nutrient acquisition within the host airways (Cowley et al., 2017), and as such the ability of *A. fumigatus* to utilise MUC5B and MUC5AC as a sole carbon source was investigated.

*A. fumigatus* was grown in minimal medium (AMM) containing either 1% glucose or purified mucins for 48 hours, and growth was measured using the OD600 (Figure 5.7). An increase in absorbance over the time course showed that growth had occurred within the AMM supplemented with glucose. In AMM supplemented with mucins however, no growth was observed over the 48 hours, with absorbance readings similar to those of the AMM only controls.

These data suggest that *A. fumigatus* cannot use mucins as a sole carbon source. Purified MUC5B and MUC5AC were unable to support fungal growth alone, whereas media supplemented with glucose was able to induce *A. fumigatus* growth over 48 hours.
**Figure 5.7 A. fumigatus growth in the presence of different carbon sources**

*Aspergillus fumigatus* growth in *Aspergillus* minimal medium (AMM) containing either glucose (purple line) or mucin (blue line). Growth was monitored by measuring OD600 over 48 hours, and the values plotted represent the average of 10 individual wells within a 96-well plate. AMM containing neither glucose or mucin was used as a negative control (orange line).
5.3 Discussion

*Aspergillus fumigatus* is the most common fungal pathogen found in CF airways and can cause a range of host responses including ABPA, aspergillus bronchitis and aspergillus sensitisation (Baxter *et al*, 2013). Due to the complex nature of the CF lung microbiome, and the many intermicrobial interactions that are likely to exist, the clinical impacts of each specific pathogen are difficult to define. As such, the implications of *A. fumigatus* colonisation and infection on the progression of CF airway disease remain unclear. As the first point of contact following inhalation, the mucus barrier provides a likely site for host-pathogen interactions (Cowley *et al*, 2017), however the interplay between *A. fumigatus* and airway mucus has only recently begun to be investigated.

Some studies had shed light on the potential interactions between the two; *A. fumigatus* protease activity has been shown to upregulate mucin gene expression (in particular MUC5AC) in airway epithelial cells, and the secretion of such proteases appears to be upregulated in the presence of mucin-based medium (Oguma *et al*, 2011; Farnell *et al*, 2012). Others have shown that *A. fumigatus* can degrade mucins (St Leger and Screen, 2000), and we have also provided data to support this. In order to gain a more clinically relevant insight into the effects of *A. fumigatus* on CF mucins, MUC5B and MUC5AC were co-purified from sputum collected from patients testing either negative (*Af*-) or positive (*Af*+) for *A. fumigatus*. Following CsCl density gradient centrifugation, mucins from *Af*+ sputum were on average isolated within lower density fractions than those from *Af* - sputum. Isolation of mucins in this way is based on their high buoyant density (Davies and Carlsedt, 2000), and these observations thus suggest a reduction in mucin size and/or glycosylation. Further to this, SEC-MALLS data also showed a decrease in the size of *Af*+ mucins compared to *Af* - mucins. Although these data show a trend towards a reduction in the size of CF mucins from *Af*+ sputum, these findings aren’t conclusive. Due to the complexity of CF airway disease, it can only be speculated that the changes observed are solely due to the presence of *A. fumigatus*. Although all patients were also colonised with *P. aeruginosa*, we cannot rule out the presence of other pathogens that weren’t common across the cohort that may contribute to these effects. However, together with previous data presented on mucins purified from cell culture, these observations provide a useful insight into the potential role of *A. fumigatus* in CF. Additional approaches may involve analysing the properties of mucins from patients only infected with *A. fumigatus*, however this is unlikely to be possible in the context of CF. The use of animal models such as the CF pig may be a more effective way of exploring these effects; by treating newborn piglets with *A. fumigatus*, airway secretions can then be collected and the properties of mucins assessed.

CF is characterised by the build up of thick mucus secretions within affected organs, which in the lung leads to reduced mucociliary clearance and chronic airway infections (Rowe *et al*, 2005). This increase in mucus viscosity is believed to be in part due to dehydration of the airway surface, as well as defective bicarbonate secretion which results in reduced mucin expansion (Ehre *et al* 2014). Although the viscous properties of mucus are essential to its
protective function, it must remain transportable in order to allow efficient airway clearance. In CF, mucus viscosity increases to such a level that it prevents this from occurring, and instead the now static barrier provides a favourable niche in which inhaled pathogens can flourish (Henderson et al., 2014). It has already been shown that *A. fumigatus* secreted products can degrade MUC5B and MUC5AC, although the consequences of this are not yet clear. As mucins are essential in defining mucus biophysical properties, it was not surprising that treatment of CF mucins with *A. fumigatus* culture filtrate led to a dramatic decrease in viscosity. The mean squared displacement of beads was much higher within the treated samples, which likely represents disruption of the mucin network due to cleavage of the N- and C-terminal polymerising domains. *In vivo*, this reduction in viscosity may make the mucus barrier more penetrable to *A. fumigatus* spores, facilitating access to the epithelial surface and promoting further colonisation and tissue damage. In order to investigate this further, future experiments may study the effects of *A. fumigatus* on unprocessed sputum samples. Although the complexity of CF sputum may result in interference from other fungal/bacterial pathogens, this would provide a useful complementary approach that may generate more clinically relevant data.

It has been shown previously that *A. fumigatus* can induce MUC5AC expression in airway epithelial cells (Oguma et al., 2011), however these findings are based on *in vitro* studies using *A. fumigatus* extracts. In order to investigate this further, preliminary experiments were carried out to study the effects of *A. fumigatus* on mucin secretion *in vivo*, using a murine model of repeated exposure to *A. fumigatus* CEA10 spores. Results showed that the BAL fluid of d19 mice contained less MUC5B than controls, although this change was not significant. MUC5AC levels were around 2 times higher in d19 mice, which is in agreement with the literature. This increase in MUC5AC rather than MUC5B is not surprising; although MUC5B is required for airway defence, it is believed to provide more of a baseline level of protection (Roy et al., 2014). MUC5AC on the other hand is more associated with acute or allergic responses, appearing to be the more reactive of the two. It has been suggested that MUC5AC may play a significant role in mucus obstruction and airway hyperreactivity (AHR), with *Muc5ac*−/− mice displaying reduced mucus obstruction and AHR even in the presence prolonged inflammation (Evans et al., 2015). As such, the apparent increase in MUC5AC secretion in the presence of *A. fumigatus* may be detrimental to the host, contributing to the continuous cycle of infection and inflammation and worsening the clinical outcome of patients. Although no apparent changes in MUC5B secretion were observed in d19 treated mice, histology data suggested an increase in intracellular MUC5B levels. The reasons for this are not yet clear; it is possible that although MUC5B is still expressed during *A. fumigatus* exposure it may not be secreted (or only secreted at low levels), perhaps due to a switch towards MUC5AC secretion. It is also possible that these differences are due to the nature of the samples used. Tissue samples were taken from a small section of the distal airways, and are therefore only representative of changes occurring within this part of the lung. The BAL fluid on the other hand contains secretions from the whole lung, and as such any changes that may occur within the distal airways may not be apparent. Although
tissues were also probed for MUC5AC, the antibody staining was unsuccessful. As such, these experiments should be repeated in order to successfully determine the effects of *A. fumigatus* on MUC5AC expression within the airways. qPCR should also be performed to gain a more accurate measure of how MUC5B and MUC5AC expression in altered during *A. fumigatus* exposure. Overall, this may provide a deeper understanding of the effects of *A. fumigatus* upon the airways, and its potential role in CF lung disease.

As an opportunistic pathogen, *A. fumigatus* is an extremely adaptable organism that is able to exploit its surrounding environment in order to survive. Degradation of mucins by *A. fumigatus* is an example of this, and it has been shown both here and in previous studies that secreted products released by the fungus are able to degrade both MUC5B and MUC5AC (St Leger and Screen, 2000). As discussed above, this appears to affect the rheological properties of CF mucins causing a significant reduction in viscosity, and we have thus speculated that mucin degradation may alter mucus barrier properties in order to facilitate airway colonisation and promote access to the airway epithelium. Another possibility is that mucins are degraded by *A. fumigatus* to provide a source of nutrients that will support fungal growth within the airways. However, our results suggested to mucins may not be sufficient to act as a sole carbon source for *A. fumigatus*, with no growth observed over 48 hours within mucin-containing medium. As only one concentration of mucin was tested in this assay, these data are not sufficient to provide conclusive evidence. It is possible that a higher concentration of mucins may be able to support *A. fumigatus* growth, and this should therefore be tested in future experiments. Furthermore, CF sputum is a complex biological substance, and it is possible that mucins are utilised in combination with other components to provide a sufficient source of nutrients to support growth within the airways.

### 5.4 Conclusion

These data provide insights into the potential impacts of *A. fumigatus* on the mucus barrier in CF. It has been shown that *A. fumigatus* may reduce the size of CF mucins and that this degradation can reduce mucin viscosity. *In vivo* data suggest that repeated exposure to *A. fumigatus* spores increases MUC5AC expression in mice, and may also induce MUC5B expression. Finally, it has been demonstrated that *A. fumigatus* cannot utilise mucins as a sole carbon source at the concentrations used in this study. Alongside previous findings, these results shed light onto the importance of mucins in *A. fumigatus* colonisation and infection, and the potential clinical implications of this pathogen on CF airways.
Chapter 6: General Discussion

*A. fumigatus* is a major opportunistic pathogen found in CF airways, and it is well established that it can lead to a decline in lung function and worse clinical outcome for patients colonised/infected with the fungus (Middleton *et al*, 2013). The wide range of host responses to *A. fumigatus* (*Baxter* *et al*, 2013) mean its precise role in promoting CF disease progression is difficult to define. As the first point of contact following inhalation of *A. fumigatus* spores, it is likely that the airway mucus barrier is a site of important host-pathogen interactions (Cowley *et al*, 2017). Here the gel-forming mucins MUC5B and MUC5AC form a complex and dynamic network that provides many sites for such interactions to occur, and is important for maintaining barrier function. As such, *A. fumigatus* may exploit this mucin network in order to compromise mucus barrier function, in order to facilitate airway colonisation and infection. The aim of this thesis was to explore the interplay between *A. fumigatus* and airway mucins, and elucidate some of the potential mechanisms involved in its pathogenesis. We investigate how *A. fumigatus* affects the biochemical and biophysical properties of MUC5B and MUC5AC, and identify potential regulators of these potentially pathogenic processes.

We have demonstrated here that *A. fumigatus* secreted products degrade MUC5B and MUC5AC. It has been shown previously that *A. fumigatus* can degrade mucins, targeting both the protein and carbohydrate regions of the molecules (St Leger and Screen, 2000). These studies however were performed using commercially available mucins, and the effects of *A. fumigatus* on human airway mucins had until now not been explored. Although it has been shown that *A. fumigatus* can induce MUC5AC expression in airway epithelial cells (Oguma *et al*, 2011), its effects on MUC5B remained unknown. Treatment of purified MUC5B and MUC5AC with *A. fumigatus* culture filtrates resulted in a considerable reduction in mucin size over time. Due to the importance of the N- and C-terminal domains in mucin polymerisation, these findings suggested that secreted proteases may cleave these domains in order to degrade these polymers and disrupt the mucin network. Treatment of recombinantly expressed N- and C-terminal domains of MUC5B with *A. fumigatus* culture filtrates resulted in cleavage of these domains, providing supportive evidence for this hypothesis.

Mucin glycosylation is imperative for mucus gel formation, allowing expansion of mucin molecules and hydration of the barrier. Further to this, these numerous glycan chains provide many microbial binding sites, facilitating pathogen entrapment (Thornton *et al*, 2008). A fucose-specific lection on *A. fumigatus*, FleA, has been shown to bind mucins, in what is believed to be an important mechanism of host defence against the fungus. By binding *A. fumigatus* spores, mucins allow them to become immobilised within the barrier, and subsequently removed by mechanisms such as mucociliary clearance (Kerr *et al*, 2016). This may indeed be the case in healthy airways, where airway mucus forms a transportable barrier that is easily swept from the lung to remove inhaled pathogens. In contrast, airway secretions in CF are extremely thick and tenacious, preventing this efficient mechanism of pathogen removal from occurring (Ehre *et al*, 2016).
2014). Allowed to persist within the airways, *A. fumigatus* may now begin to secrete enzymes that degrade components of its surrounding environment, such as mucins. It has been shown here already that *A. fumigatus* can degrade mucin protein, and St Leger and Screen have also demonstrated degradation of mucin carbohydrate (St Leger and Screen, 2000). In support of this, treatment of MUC5B and MUC5AC with *A. fumigatus* culture filtrate resulted in changes in the levels of different sugars on the mucins. Although these data suggest cleavage of mucin glycans, these experiments were limited by the number of lectins used in the assay. Mucins contain many different sugars that were not all represented in the current study (Varki *et al.*, 2009), and as such this must be explored further. Nevertheless, cleavage of mucin carbohydrates may represent a mechanism whereby *A. fumigatus* escapes entrapment within the mucin network to allow penetration of the mucus barrier, and these sugars may also be utilised as a source of nutrients to support fungal growth.

Protease secretion by *A. fumigatus* is known to be an important part of its pathogenesis, although its involvement in CF disease progression is still under investigation. *A. fumigatus* secretes many proteases that are believed to facilitate fungal adhesion and tissue penetration during early infection, and also appear to induce local inflammation (Tommee and Kauffman, 2000). Data presented here suggests that a number of these proteases may also degrade airway mucins, in particular Alp1, signal peptidase 1 and DppV. *A. fumigatus* has previously been shown to upregulate Alp1 when grown in mucin-based medium (Farnell *et al.*, 2012), suggesting that this protease may be selectively secreted within the airways in order to degrade the mucus barrier. Furthermore, DppV has been shown to degrade other extracellular matrix components such as elastin and collagen (Wartenberg *et al.*, 2011), and it is thus not surprising that it may also degrade MUC5B and MUC5AC. Secretion of these proteases by *A. fumigatus* may be important during the early stages of airway colonisation, whereby they target mucins using an array of extracellular enzymes in order to degrade the mucus barrier and compromise host defences.

*A. fumigatus* proteases are under the control of multiple signalling pathways, which regulate their expression and secretion in response to various extracellular cues. One such pathway is involved in pH-responsive signalling via the transcription factor PacC, which is known to regulate the expression of many proteases and other secreted products (Bignell *et al.*, personal communication). PacC appears to be involved in regulating epithelial entry and tissue invasion during pulmonary aspergillosis, however this is not believed to be a solely protease-dependent process. As such, the role of many of these proteases in *A. fumigatus* pathogenicity remains unclear (Bertuzzi *et al.*, 2014). Through the use of culture filtrates from ΔpacC mutants, we have demonstrated a potential role for PacC in regulating mucin degradation. Similarly, the cell-surface receptor PalH, which lies upstream of PacC in this signalling pathway, also appears to play a role in regulating mucin-degrading proteases. Culture filtrates from both ΔpalH and ΔpacC mutants showed an inability to degrade MUC5B and MUC5AC, providing evidence to suggest that many of the downstream targets of PalH/PacC signalling are responsible for
degrading these mucins. Although a number of extracellular products have been identified as putative mucin-degrading enzymes, there are many other proteases under the control of this signalling pathway that may be involved in this process. In order to investigate this further, the effects of all proteases under PacC control on mucin degradation should now be explored.

As shown here, *A. fumigatus* is able to degrade the MUC5B and MUC5AC, however these studies have been performed *in vitro* using purified mucins. As such, it is not yet clear whether these findings represent the true effects of the fungus *in vivo*. Mucins purified from the sputum of CF patients testing positive for *A. fumigatus* appear to be smaller on average than those from patients testing negative for *A. fumigatus*. This provides evidence to suggest that mucins are indeed degraded within the airways, however due to the complex nature of CF sputum it is not possible to exclude the effects of other pathogens present within the airways. Exploring the effects of anti-fungal treatment on mucin degradation may thus be a useful way of complementing this approach; by purifying mucins at different stages during treatment, it may be possible to see whether the mucin-degrading effects of *A. fumigatus* can be reversed.

Central to the protective properties of mucus are its viscoelastic properties, which allow inhaled pathogens to become trapped within the barrier and removed from the airways to prevent infection. In CF, defects in mucociliary clearance prevents clearance of these pathogens, allowing them to persist within the airways and cause infection (Henderson *et al*, 2014). As an opportunistic pathogen, *A. fumigatus* is likely to exploit the mucus barrier in order to promote its survival and colonise the airways, and mucin degradation may be central to this process. As a key factor in determining mucus biophysical properties, disruption of the mucin network is likely to affect the viscosity of the barrier. Treatment of CF mucins with *A. fumigatus* culture filtrate appears to reduce mucin viscosity and increase the MSD of beads within the sample. *In vivo*, this may represent a mechanism whereby *A. fumigatus* degrades the mucin network in order to promote penetration of the mucus barrier and facilitate access to the underlying epithelium. As these studies were carried out using purified CF mucins, future studies must explore the effects of *A. fumigatus* on the viscosity of unprocessed CF sputum. Polystyrene beads may also be replaced with *A. fumigatus* spores, which may provide a more physiologically relevant view of how secreted products of the fungus may influence the viscosity of CF sputum.

As well as influencing mucin properties, previous studies have shown that *A. fumigatus* protease activity can induce MUC5AC expression in human bronchial epithelial cells (Oguma *et al*, 2011). Its effects of mucin gene expression and secretion *in vivo* however has not yet been studied, in particular its effects on MUC5B. In agreement with these earlier studies, data presented here suggest that repeated exposure to *A. fumigatus* increases MUC5AC secretion in mice, and also increases intracellular MUC5B levels. In order to gain more insight into this process, future studies should also investigate the effects of *A. fumigatus* gene expression through the use of qPCR.
Although evidence strongly suggests that *A. fumigatus* degrades MUC5B and MUC5AC, and that this reduces mucin viscosity, the reasons for this are not yet fully understood. As discussed, it is possible that mucins are degraded in order to facilitate penetration of the mucus barrier and access the epithelial surface. As an opportunistic pathogen, *A. fumigatus* is well adapted to harvest various proteinaceous nutrients from its surrounding environment, and it is thus likely that mucins are also degraded as a nutrient source to support fungal growth and survival within the airways. Preliminary data suggest that MUC5B/MUC5AC alone cannot support *A. fumigatus* growth, and are therefore not sufficient as a sole carbon source. Although mucins alone may not be sufficient to support fungal growth, it is possible that mucins are utilised in combination with other extracellular macromolecules. Furthermore, this experiment was performed using a single mucin concentration. As such, it is possible that a higher concentration of mucins may be able to support fungal growth, and future studies should therefore test a range of mucin concentrations in this growth assay.

### 6.1 Future work

**6.1.1 Identification of proteolytic cleavage sites**

*A. fumigatus* produces many extracellular proteases, a number of which have been identified as putative virulence factors. However, the mechanisms of action of these proteases and their role of *A. fumigatus* pathogenesis are incompletely understood. It has been shown here that *A. fumigatus* secreted proteases cleave MUC5B and MUC5AC, with treatment of the full length mucins with *A. fumigatus* culture filtrates resulting in a considerable reduction in mucin size. Studies on recombinant MUC5B N- and C-terminal domains suggested that the D1 and D3 domains of the N-terminus and the CK domain of the C-terminus are cleaved by these proteases. Although these findings reveal which subdomains of the mucins may be targeted, specific cleavage sites have yet been identified. Further studies should utilise a proteomics approach in order to determine where exactly these proteases cleave mucins. Mass spectrometry is a highly sensitive technique that is often used to identify protease cleavage sites (van den Berg and Tholey, 2012). One approach that could be used would be to use a gel-based assay, whereby SDS-PAGE of *A. fumigatus* treated mucins is followed by mass spectrometry of excised bands in order to determine the sequence of the cleavage products. However, such techniques are limited by their reduced sample recovery rates and gel-to-gel reproducibility, and the use of downstream ‘work-proteases’ such as trypsin means that specific cleavage sites cannot always be determined (van den Berg and Tholey, 2012). Another approach that could be employed here is the peptide-centric ‘proteomic identification of protease cleavage sites’ (PICS) method developed by Schilling and Overall, which utilises LC-MS/MS alongside bioinformatics to identify endoprotease cleavage sites. Analysis of mucins in this way, following treatment with *A. fumigatus* culture filtrates or with specific secreted proteases would determine where exactly these enzymes cleave MUC5B and MUC5AC, and
may allow us to speculate as to what the implications may be on their structural and biophysical properties.

6.1.2 Identification of other mucin degrading proteases

*A. fumigatus* secretes many extracellular proteases that have been identified as putative virulence factors. We have shown that *A. fumigatus* proteases can degrade MUC5B and MUC5AC, however it is not yet known which proteases are involved in this process. Through the use of protease inhibitor studies we were able to show that serine, chymotrypsin-like and matrix metalloproteases may be responsible for degrading mucins. Secreted protein knockout strains allowed the identification of specific proteases that appear to be involved in mucin degradation, in particular Alp1, signal peptidase I and DppV. Although 75 knockout strains of *A. fumigatus* were tested here, a number of other secreted enzymes also exist that are yet to be investigated, and their involvement in mucin degradation must also be explored in future studies. Purified *A. fumigatus* proteases were also investigated in this study, with Mep identified as a mucin degrading protease. Due to the small number of proteases available at the time of this study, only 3 proteases have been investigated in our assay. Future studies should now be carried out using other purified proteases, in order to explore their potential role in mucin degradation.

6.1.3 Further investigations into the clinical implications of *A. fumigatus* in CF

Although *A. fumigatus* is now a well-known coloniser/infector of the CF lung, its impacts on the airways and role in disease progression have yet to be fully elucidated. This is in part due to the wide range of host responses and varying clinical states presented by patients, who may display more than one disease state synchronously or even switch between different phenotypes (Baxter *et al*., 2013). As a key player in the innate immune response, it is important to determine how *A. fumigatus* may influence the mucus barrier and vice versa.

We have shown here that *A. fumigatus* is able to reduce mucin viscosity, however these findings are based on purified CF mucins, and may thus not represent the true effects of the fungus *in vivo*. Through the use of animal models, future studies should investigate the effects of *A. fumigatus* on mucins and the mucus barrier using fresh mucus secretions. Using a CF pig or ferret, airway secretions could be collected from birth and throughout treatment with *A. fumigatus*. This would allow measurements of viscosity and mucin secretion levels to be monitored under more physiologically relevant conditions. By harvesting lung tissue, changes in mucin gene expression could also be measured over time. Another approach would be to carry out a clinical trial of *A. fumigatus*-positive CF patients throughout the course of antifungal treatment. By collecting sputum samples before, during and after treatment, changes in MUC5B and MUC5AC levels and biochemical properties could be identified. However, due to the complexity of CF airway disease, potential interference from other pathogens would need to be taken into account here.
6.1.4 Investigations into the use of mucins as a carbon source by *A. fumigatus*

As an opportunistic pathogen, *A. fumigatus* is able to adapt to a variety of different environments and utilise a diverse range of extracellular macromolecules as nutrient sources to support fungal growth. A previous study has shown that *A. fumigatus* proteases are able to degrade pig gastric mucin and bovine submaxillary mucin (St Leger and Screen, 2000), and it has here been shown that they also degrade MUC5B and MUC5AC isolated from human cell lines. The reasons for degrading airway mucins remain unclear; as well as compromising host defences against the *A. fumigatus*, mucin degradation may also provide a source of nutrients for the fungus.

Our data suggest that MUC5B/MUC5AC cannot be utilised as a sole carbon source to support *A. fumigatus* growth. However, these findings were preliminary and based on experiments that utilised only a single mucin concentration. It is thus possible that at a higher concentration (perhaps similar to that found in the CF lung), these mucins may be sufficient to support fungal growth, and this should thus be addressed in future studies.

6.2 Conclusions

This study has provided new insights into the interplay between *A. fumigatus* and airway mucins. For the first time it has been shown that *A. fumigatus* proteases can degrade MUC5B and MUC5AC, targeting both the protein and carbohydrate regions of the mucins. It has been shown that both the N- and C-terminus of MUC5B are cleaved by *A. fumigatus* proteases, identifying the D1, D3 and CK domains as regions that are targeted by the fungus. Furthermore, the secreted proteases Alp1, signal peptidase 1, DppV and Mep have been identified as putative mucin-degrading enzymes, and this proteolytic activity appears to be under the control of PalH/PacC signalling.

In addition to this, novel data has been presented to suggest that CF mucins from *A. fumigatus* positive patients are reduced in size, and degradation of these mucins appears to correlate with a reduction in mucin viscosity. The effects of *A. fumigatus* on mucin secretion *in vivo* have also been studied, with intracellular MUC5B and extracellular MUC5AC levels appearing higher following repeated exposure to *A. fumigatus* in mice. Finally, it has been shown that mucins alone may not be sufficient to act as a sole carbon source for *A. fumigatus*. Overall these data shed light onto the potential role of *A. fumigatus* in CF, and identify key regulators of mucin degradation. These pathways provide new avenues for future studies to explore, which may ultimately lead to the development of novel therapeutic agents.
References


