Sialic Acid Metabolism in the Opportunistic Fungal Pathogen, *Aspergillus fumigatus*

by

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B.Sc. (Hons.), Simon Fraser University, 2007

Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

in the

Department of Biological Sciences
Faculty of Science

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**Approval**

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Abstract

My research investigated sialic acid metabolism in the opportunistic fungal pathogen, *Aspergillus fumigatus*. The sialic acid, N-acetylneuraminic acid (Neu5Ac), is a sugar found on fungal spore cell surface that mediates adhesion to host proteins and phagocytes. The aims of the thesis were to characterize a novel *A. fumigatus* exo-sialidase (AfS), and to clone and characterize putative *A. fumigatus* nucleotide sugar transporters (AfNSTs) to identify CMP-Neu5Ac or UDP-galactose transporters.

The *A. fumigatus* sialidase gene was expressed in *E. coli* and crystallized; the crystal structure and Michaelis – Menten kinetic analysis revealed that the glycoside of another sialic acid, 2-keto-3-deoxynononic acid (KDN), was a better substrate for the enzyme than glycosides of Neu5Ac. This enzyme represents the first sialidase characterized from the Kingdom Fungi. To better understand why KDN is a better substrate for *AfS* than Neu5Ac, using the enzyme structure as a guide in conjunction with known sialidase structures, a point-mutation (R151L) was introduced in the substrate binding pocket to better accommodate glycans with terminal Neu5Ac. Activity of the R151L mutant was slightly enhanced toward Neu5Ac. Moreover, amino acid sequence comparisons revealed that this amino acid may be a hallmark of KDNases.

In addition, I attempted to identify a CMP-sialic acid transporter in *A. fumigatus*, a type of nucleotide sugar transporter (NST). NSTs mediate nucleotide sugar transport into the endoplasmic reticulum and Golgi complex for subsequent addition to glycoproteins and glycolipids. STD-NMR analysis and $^{14}$C-transport assays were conducted to examine the substrate specificity of four putative *A. fumigatus* NSTs expressed in yeast. Two transporters (*AfNST1* and *AfNST5*) bound UDP-glucose and UDP-galactose, and transported $^{14}$C-UDP-galactose. Epitope maps showed that the UDP-moiety anchored the nucleotide sugar and that sugar structure conferred specificity because not all UDP-sugars bound to the NSTs. No CMP-sialic acid transport was detected. Despite similarities in substrate preference between *AfNST1* and *AfNST5*, growth and morphology of the corresponding knock-out mutants differed; only the *AfΔNST5KO* was compromised when grown on media containing cell wall stressors. Using lectins and flow cytometry, I found that the level of cell surface galactose was significantly reduced in both knockout strains as compared to the wild type; however, sialic acid density on conidia was significantly reduced only in the *AfΔNST5KO* mutant. This research demonstrates for the first time that NSTs are important for the integrity of the fungal cell and may represent novel targets for antifungal agents.
Keywords: fungal pathogen; invasive aspergillosis; sialidase; UDP-sugar; nucleotide sugar transporters; endoplasmic reticulum
This thesis is dedicated to all the people in my life who have journeyed through the graduate school experience with me. Thank you for sticking by me through all the peaks and valleys.
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List of Acronyms

ΔΔC_T  ΔC_T test condition - ΔC_T calibrator condition
ΔC_T  C_T value of gene of interest – C_T value of reference gene
2^−ΔΔC_T  Fold change value
3F-β-KDN  3-fluoro-β-KDN
A_620nm  absorbance detected at 620 nm
ABPA  allergic bronchopulmonary aspergillosis
AfNST  A. fumigatus nucleotide sugar transporter
AfNST1  A. fumigatus nucleotide sugar transporter one
AfNST2  A. fumigatus nucleotide sugar transporter two
AfNST5  A. fumigatus nucleotide sugar transporter five
AfNST6  A. fumigatus nucleotide sugar transporter six
AfS  A. fumigatus exo-sialidase/ KDNase
AfΔnst1KO  A. fumigatus NST1 knockout mutant
AfΔnst5KO  A. fumigatus NST5 knockout mutant
AIDS  acquired immune deficiency syndrome
Ala  alanine
Asp  aspartic acid
ATCC  American Type Culture Collection
BCA  bicinchoninic acid
bodipy-Lac  4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-lactose
BSA  bovine serum albumin
CAN*  Chronic Necrotizing Aspergillosis
CAZy  carbohydrate-active enzyme
CBM  carbohydrate-binding modules
CHO  Chinese hamster ovary cell
CMP  cytidine 5′-monophosphate
CMP-Neu5Ac  cytidine-5′-monophosphate N-acetylneuraminic acid
CMP-Sia-syn  CMP-sialic acid synthetase
CST  CMP-Neu5Ac transporters
C_T  cycle threshold
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<tr>
<td>DHN</td>
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</tr>
<tr>
<td>GDP-Man</td>
<td>GDP-mannose</td>
</tr>
<tr>
<td>GEF</td>
<td>Golgi-enriched fractions</td>
</tr>
<tr>
<td>GH</td>
<td>glycosyl hydrolases</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>Glu</td>
<td>glucose</td>
</tr>
<tr>
<td>HIBM</td>
<td>hereditary inclusion body myopathy</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus high-performance</td>
</tr>
<tr>
<td>IA</td>
<td>invasive aspergillosis</td>
</tr>
<tr>
<td>IC50</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>K</td>
<td>chemically defined fungal media (Kafer's minimal media)</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kcat</td>
<td>Turnover number</td>
</tr>
<tr>
<td>kcat/Km</td>
<td>catalytic efficiency</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>kDA</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>KDN</td>
<td>2-keto-3-deoxy- D-glycero-D-galacto-nonulosonic or 2-keto-3-deoxynononic acid</td>
</tr>
<tr>
<td>KDN2en</td>
<td>2,3-didehydro-2,3-dideoxy-D-glycero-D-galacto-nonulosonic acid</td>
</tr>
<tr>
<td>KDN-Mu</td>
<td>α2-6 isomer of 8-fluoro-methylumbelliferyl α-2-keto-3-deoxy-d-glycero-d-galacto-nononylgalactopyranoside</td>
</tr>
<tr>
<td>KDNα2,6GalβFMU</td>
<td>3-Deoxy-D-manno-oct-2-ulosonic acid</td>
</tr>
<tr>
<td>Ki</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>Km</td>
<td>Apparent dissociation constant or Michaelis constant</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani media</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LED</td>
<td>light-emitting diode</td>
</tr>
<tr>
<td>LFA</td>
<td><em>Limax flavus</em> agglutinin</td>
</tr>
<tr>
<td>LOS</td>
<td>lipooligosaccharide</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharides</td>
</tr>
<tr>
<td>ManNAc</td>
<td>N-acetylmannosamine</td>
</tr>
<tr>
<td>ManNAc-6P</td>
<td>ManNAc-6-phosphate</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential media</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>Mu</td>
<td>4-methylumbelliferone</td>
</tr>
<tr>
<td>MvS</td>
<td><em>Micromonaspora viridifaciens</em> sialidase</td>
</tr>
<tr>
<td>MYPD</td>
<td>Malt, yeast extract, peptone, dextrose media</td>
</tr>
<tr>
<td>NANP</td>
<td>Neu5Ac-9P phosphatase</td>
</tr>
<tr>
<td>NANS</td>
<td>Neu5Ac-9-P synthetase</td>
</tr>
<tr>
<td>Neu5,9Ac2</td>
<td>9-0-acetyl-N-acetylneuraminic acid</td>
</tr>
<tr>
<td>Neu5Ac2en</td>
<td>2-deoxy-2,3-dehydro-N-acetylneuraminic acid</td>
</tr>
<tr>
<td>Neu5Ac-Mu</td>
<td>4-methylumbelliferyl α-D-N-acetylneuraminic acid</td>
</tr>
<tr>
<td>Neu5Gc</td>
<td>N-glycolyneuraminic acid</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>nickel nitrilotriacetic acid agarose</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>nickel nitrilotriacetic acid agarose</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NST</td>
<td>nucleotide sugar transporter</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline (pH 7.4)</td>
</tr>
<tr>
<td>PBS-T</td>
<td>phosphate-buffered saline (pH 7.4)/0.05% Tween 20</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>protein data bank</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>PHYLIP</td>
<td>Phylogeny Inference Package</td>
</tr>
<tr>
<td>PHYRE</td>
<td>protein homology/analogy recognition engine</td>
</tr>
<tr>
<td>PNA</td>
<td>Arachis hypogaea agglutinin</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>R151</td>
<td>arginine 151</td>
</tr>
<tr>
<td>R151L</td>
<td>arginine 151 mutated to leucine</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SC</td>
<td>synthetic complete</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SG</td>
<td>synthetic galactose</td>
</tr>
<tr>
<td>SLC35</td>
<td>solute carriers 35</td>
</tr>
<tr>
<td>Sn1</td>
<td>substitution, nucleophilic, unimolecular reaction</td>
</tr>
<tr>
<td>SNA</td>
<td>Sambucus nigra agglutinin</td>
</tr>
<tr>
<td>SR</td>
<td>synthetic raffinose</td>
</tr>
<tr>
<td>STD NMR</td>
<td>saturated transfer difference nuclear magnetic resonance</td>
</tr>
<tr>
<td>STDD</td>
<td>saturation transfer double difference</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline (pH 7.5) with 0.01% Tween 20</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TcTS</td>
<td>recombinant trans-sialidase from Trypanosoma cruzi</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TIGR</td>
<td>The Institute of Genomic Research</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>UDP</td>
<td>uridine diphosphate</td>
</tr>
<tr>
<td>UDP-Gal</td>
<td>uridine diphosphate galactose</td>
</tr>
<tr>
<td>UDP-Galf</td>
<td>UDP-galactofuranose</td>
</tr>
<tr>
<td>UDP-Glc</td>
<td>UDP-glucose</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>UDP-N-acetylglucosamine</td>
</tr>
<tr>
<td>UDP-GlcUA</td>
<td>UDP-glucuronic acid</td>
</tr>
<tr>
<td>UDP-Rhm</td>
<td>UDP-rhamnose</td>
</tr>
<tr>
<td>Vmax</td>
<td>maximum reaction rate of enzyme</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>Y358</td>
<td>tyrosine 358</td>
</tr>
<tr>
<td>Y358H</td>
<td>tyrosine 358 mutated to a histadine</td>
</tr>
<tr>
<td>YAG</td>
<td>yeast extract-agar-glucose</td>
</tr>
<tr>
<td>YM</td>
<td>yeast malt agar</td>
</tr>
<tr>
<td>α2,3-SMUG</td>
<td>α2,3 isomer of 4-methylumbelliferyl α-D-N-acetylneuraminylgalactopyranoside</td>
</tr>
<tr>
<td>α2,6-SMUG</td>
<td>α2,6 isomer of 4-methylumbelliferyl α-D-N-acetylneuraminylgalactopyranoside</td>
</tr>
</tbody>
</table>
Chapter 1.

Introduction

1.1. Introduction to *Aspergillus fumigatus*

1.1.1. *Aspergillus* species

The fungal genus *Aspergillus* encompasses approximately two hundred species of filamentous fungi some of which are employed for industrial purposes while others are important pathogens of animals and plants (1, 2). This genus of filamentous fungi belongs to the phylum Ascomycota. Industrially important members of this genus include *Aspergillus niger*, used for citric acid production, and *Aspergillus oryzae*, which has a long history of use in the manufacturing of sake, miso, and soy sauce (3). About 20 species are known to be opportunistic pathogens but only a small number are able to cause systemic disease in humans, including *Aspergillus fumigatus*, *Aspergillus nidulans*, *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus* (4, 5). Of these, the majority of human infections are cause by *A. fumigatus* (6).

1.1.2. *Aspergillus fumigatus*

*Aspergillus fumigatus* is ubiquitous, aerobic, filamentous, and saprotrophic and is found in various substrata around the world (7). Primarily found in soil and compost, it plays an important role in decomposition and recycling of environmental organic materials, such as carbon and nitrogen sources (4).

Like other filamentous fungi, *A. fumigatus* grows long branched hyphae that becomes tangled masses of mycelium (Figure 1.1) and grows on its substratum until the conditions of the site are no longer suitable for further growth (1). *A. fumigatus* can
reproduce both sexually and asexually (8, 9). In 2009, sexual reproduction was identified; it has a heterothallic breeding type and hyphal fusion between two mating types leads to the formation of a spherical fruiting body called a cleistothecium (8). Many acsi are contained within these cleistothecia, and each ascus contains eight ascospores. Because ascospores are products of meiosis, some genetic variation is created (8). However, *A. fumigatus* commonly reproduces asexually. Asexual conidiospores (also known as spores) give rise to clones of the parent and thousands of grey-green haploid conidiospores are produced by the conidiophore (Figure 1.1) (4, 10).

![Figure 1.1](image)

**Figure 1.1** Light microscope and scanning electron microscope images of *Aspergillus fumigatus*

The structure of the fungal conidiospore, phialides, and conidiophore are labeled in (A) and the structure of an *A. fumigatus* mycelial mass is shown in (B).

Source: Yeung et al., unpublished

The prolific number of haploid conidia has facilitated the worldwide distribution of *A. fumigatus*. Each conidium ranges from 2-3 µm in diameter, which allows them to be easily dispersed by any environmental disturbance. Once the conidiospores are in the air, they remain buoyant, traveling great distances (10). Asexual conidiospores are present in the atmosphere at concentrations ranging from 1-100 conidia/m³. It has been estimated that we breathe in ~200 spores per day and the small diameter of the spores permits entry into lung alveoli (11).
1.1.3. Host defences against *Aspergillus*

Although we inhale about several hundred conidia per day, normal, healthy individuals rarely experience any adverse consequences of conidial intake due to the defenses of our immune systems (4, 11, 12).

Nonspecific immunological defence via the mucociliary escalator acts before the conidia enters the alveoli, trapping spores and removing them from the upper respiratory tract through ciliary action (11). If the conidia does penetrate to the lower respiratory tract, they are engulfed by epithelial and alveolar macrophages (13). Immune cells of mice and humans have shown that phagocytes launch potent chemical assaults that eradicate the spores from lung tissue and spores are subsequently destroyed within phagolysosomes by reactive oxygen species (ROS), phagolysosomal acidification and calcium fluxes (14, 15).

Neutrophils also play a role in defence against *A. fumigatus*, targeting hyphae from any germinated spores that may have escaped engulfment by macrophages (16). Killing of hyphae is an extracellular process as they are too large to be ingested, though there is evidence that monocytes and macrophages also play a small role in the destruction of hyphae (4, 17). Polymorphonuclear neutrophils attack developing hyphae via enzymatic granule components and reactive oxygen species (18–20).

The innate immune response is thus an essential component for elimination of fungus in the host; therefore, to healthy individuals, *A. fumigatus* is accordingly a weak pathogen because inhalation rarely has adverse effects (21).

**Immunocompromised hosts**

In immunocompromised individuals, conidia and developing hyphae may not be cleared from the respiratory tract, which allows the spores to adhere to pulmonary epithelial cells (Figure 1.2) (11, 22). Serious, life-threatening infections can result due to germination and hyphal extension which leads to tissue destruction, respiratory failure (23) and distribution of the fungus throughout the body further leads to colonisation of other organs (24).
Inhalation by immunosuppressed patients results in conidia binding to lung epithelia, germinating, and two possible outcomes: (1) patients with corticosteroid induced immunosuppression, some neutrophils would be recruited for fungal control with significant inflammation; therefore, excessive inflammation and tissue necrosis would occur before the fungal infection can be cleared or, (2) patients with neutropenia, uncontrolled hyphal growth would result and dissemination through the cardiovascular system.


There are two main groups of immunocompromised patients: first, those who inherit or develop conditions such as neutropenia, leukemia, or granulocytopenia and second, individuals who are administered immune suppressants to prevent rejection after undergoing bone marrow transplants or solid organ transplants (11, 24). In neutropenic patients and animal models of chemotherapy-induced neutropenia with nearly non-existent levels of neutrophils and other leukocytes, there is low levels of inflammation (Figure 1.2). As a consequence, angioinvasion and dissemination of the fungus to other organs via the circulatory system can occur (25, 26). Under corticosteroid immunosuppression, phagocyte functions such as phagocytosis, phagocyte oxidative burst, production of cytokines and chemokines, and cellular migration are impaired (Figure 1.2). Consequently the ability of phagocytes to kill A. fumigatus conidia and hyphae is diminished (27–29). Neutrophils, macrophages, lymphocytes, and other polymorphonuclear and mononuclear cells are recruited to the
site of infection to limit fungal invasion, and often results in tissue injury. The excessive inflammatory response is thought to contribute to the observed mortality by *A. fumigatus* in these patients (27, 28).

Over the past several decades, *A. fumigatus* has an increasingly large role as a human pathogen due to the escalating number of immunosuppressed individuals who are at greatest risk of developing *A. fumigatus*-related conditions (7, 17). The advent of the worldwide HIV/AIDS epidemic, use of intense chemotherapy treatments against a variety of cancers, and the increase in use of immunosuppressive drugs as treatments for various autoimmune disorders have all contributed to this rise in the number of immunosuppressed individuals (30, 31). In addition, patients who have been extensively burned have a high risk of skin colonization and invasion (32).

### 1.1.4. The spectrum of diseases caused by *A. fumigatus*

The term aspergillosis encompasses all fungal diseases caused by the *Aspergillus* genus. Portals of entry include skin wounds, the cornea and the ear; but 80-90% of infections involve the respiratory tract (24). Although about 20 species of *Aspergillus* are known to cause aspergillosis, *A. fumigatus* is the most common isolate from patients with such diseases (2). Heavy exposure to *A. fumigatus* conidia can also cause various allergic conditions, including asthma, allergic sinusitis, and alveolitis. However, these conditions can often be remedied through the removal of the individual from the spore-rich environment (4).

Allergic Bronchopulmonary Aspergillosis (ABPA) may occur due to an excessive inflammatory response to inhaled conidia (33). This allergic reaction to fungal allergens is most often observed in those already suffering from underlying lung conditions such as asthma or cystic fibrosis (34, 35). Symptomatically, it typically presents as asthma but over time progresses to lung fibrosis, proximal bronchiectasis, and respiratory failure (21, 36). If left untreated, ABPA can progress from an initial acute stage to a life-threatening end stage with fibrosis.

Aspergilloma is a non-invasive disease that can arise in patients with pre-existing lung damage due to other diseases such as tuberculosis or sarcoidosis (24). An
Aspergilloma is the development of a mass of fungal hyphae (fungal ball) that colonizes a pre-existing damaged lung cavity. The hyphae are embedded in a proteinaceous matrix of lung cells and continue to sporulate while obstructing the airway (37). In addition to the fungal mycelia, the aspergilloma would also include inflammatory cells, fibrin, mucus, and cell debris (38). The aspergilloma can exist in a patient for years with manageable symptoms, such as chronic cough or dyspnea (38). However, as the fungal mass continues to develop, blood vessels in portions of the lung are disrupted which causes hemoptysis that can lead to internal bleeding and can become fatal (37).

Chronic Necrotizing Aspergillosis (CNA) and Invasive Aspergillosis (IA) occur in immunocompromised hosts who cannot effectively eliminate inhaled spores or destroy the hyphae that grow once the spores have germinated (24). CNA is a semi invasive aspergillosis which leads to lung destruction. It differs from an aspergilloma in that cavities from pre-existing lung damage are not needed for local invasion of lung tissue to occur (39, 40). Compared to invasive aspergillosis it is a slower, chronic process that stays contained within the lungs. It is commonly seen in elderly patients with a history of lung disease or in mildly immunosuppressed patients (40). Some patients may be asymptomatic, but the usual symptoms include fever, cough, sputum production, and weight loss. Treatment consist of IV therapy with an anti-fungal agent or surgical resection (40).

**Invasive aspergillosis (IA)**

The most serious disease caused by *A. fumigatus* is invasive aspergillosis (IA) and *A. fumigatus* is responsible for the majority of cases of IA (Denning 1998; Segal 2009). IA is an invasive fungal infection and initial colonization of the respiratory tract is routinely the portal of entry. Following colonization, the fungus infiltrates the bloodstream and is disseminated to other organs in the body (Figure 1.3) (17, 41). Dissemination to the brain is the most serious and typical of end-stage IA. IA primarily affects immunocompromised individuals; it is a very rare condition in immunocompetent people (17, 30, 41).
As shown in Figure 1.2, IA generally exhibits two forms: neutropenic IA and non-neutropenic IA. Neutropenic patients susceptible to IA include patients undergoing cytotoxic therapy or cyclophosphamide treatment for transplants, HIV patients, and chemotherapy patients (17). Neutropenic hosts allow *A. fumigatus* spores to germinate and hyphal penetration into blood vessels may result in dissemination to other sites of infection. Neutropenic IA is characterized by thrombosis and hemorrhage from extensive hyphal growth (25).

Non-neutropenic IA is typical for patients undergoing high-dose corticosteroid therapies. Other susceptible hosts include patients who have had tuberculosis that resulted in lesions and damaged pulmonary tissues, asthma patients or cystic fibrosis patients (11). Often these patients are combating multiple diseases at once; therefore, the immune condition of these patients is greatly varied and the underlying condition of the host greatly determines the severity of the disease progression. As described in section 1.1.3, the most severe symptom of non-neutropenic IA is excessive inflammation caused by host defense responses to *A. fumigatus* spores and secreted fungal allergens. Typical responses in inflammation that causes tissue damage is a consequence of the phagocytic oxidative burst, production of cytokines and chemokines,
and macrophage cellular migration (42). Excessive inflammatory responses lead to damage to lung tissue cell death and fibrosis.

Treatment of invasive aspergillosis (IA)

Without treatment, mortality rates for IA approach 100% (43). At present, the primary treatment for IA includes oral doses of voriconazole or intravenous administration of amphotericin B (44). It has been shown that patients treated with voriconazole have a greater survival rate than those treated with amphotericin B; therefore, voriconazole is the first-line treatment for IA (45). Unfortunately, these treatments are only successful in 40-50% of patients (46). Surgery may also be done, but the risks involved in the removal of the area of colonization must be taken into consideration (47).

Voriconazole belongs to a class of synthetic azole drugs that inhibit the fungal lanosterol 14α-demethylase, an enzyme in the biosynthesis of ergosterol. Ergosterol is a sterol that is unique to fungal cell membrane; the equivalent molecule in humans is cholesterol. Since ergosterol is not present in humans, this pathway is a very good drug target. The decreased ergosterol concentration and accumulation of the methylated sterol, lanosterol, which accumulates in the membrane, will result in cell membrane disruption. Lanosterol causes the formation of channels that allows small molecules to leak from the inside of the fungal cell and this ultimately causes cell death (48, 49). Amphotericin B belongs to the polyene class of antifungals, it directly binds to ergosterol in the fungal cell membrane which makes the plasma membrane more crystalline by forming membrane-spanning channels. The channels cause leakage of cellular components and osmotic cellular lysis (50).

The limitation of amphotericin B treatment is due to its nephrotoxicity (47). Other drug treatment side effects include reduced renal blood flow, fever, chills, nausea, diarrhea, and hypotension (51). Two lipid-associated formulations of amphotericin B have decreased the risk of nephrotoxicity associated with its use (52).

The fungal cell wall is another major target for antifungal drug development (53, 54). Caspofungin and Anidulafungin belong to a class of semi-synthetic antifungals
known as echinochandins that are β1-3 glucan synthase (Fks1) inhibitors. These inhibit (1,3)-β-glucan synthesis, an essential component of the fungal cell wall (50, 53). Recently, combination therapy of voriconazole and anidulafungin or caspofungin has shown an increase in survival after three months of treatment by approximately 10% when compared to voriconazole therapy alone (55, 56).

Despite some improvement, antifungal drug treatment remains inadequate; detrimental effects such as attenuation of activity, increased resistance or toxicity, and drug-drug interactions are barriers to better treatment outcomes. Consequently, despite treatment, the mortality rate of IA patients continues to be unacceptably high (55, 57). Therefore, there is a need for novel antifungal therapeutic agents and generation of these new agents can be facilitated by a better understanding of the virulence factors and mechanisms of pathogenesis of A. fumigatus.

1.2. Virulence factors of A. fumigatus

The nature of a fungal infection requires the understanding of the molecular nature of the fungi and consideration of host defenses or immunosuppressive therapies. Since A. fumigatus conidia are so ubiquitous, it has not been possible to discriminate between clinical and environmental isolates and every strain identified so far has the capacity to be pathogenic (4). A. fumigatus conidiospores comprise less than 1% of airborne spores, and yet are responsible up to 90% of human infections and almost all of the IA cases (2). In a hospital study, A. niger constituted approximately 50% of the airborne spores but accounted for 17% of patient isolates, while A. fumigatus represented just over 1% of airborne spores but accounted for about 50% of patient isolates (30).

The small spore size and thermotolerance play a large role in A. fumigatus prevalence in IA (12, 58). Conidia of A. fumigatus are small enough (2 -3 µm in diameter) to escape mucociliary clearance and infiltrate the lower respiratory tract (bronchioles and alveoli), which increases likelihood of pathogenicity over other Aspergillus species. The larger conidia of A. flavus and A. niger are cleared by the mucociliary elevator in the tissues of the upper respiratory tract (11).
A. fumigatus is more thermotolerant than other Aspergillus spp.; it has an increased radial growth and germination rate at 37 °C and can grow at temperatures above 50 °C (59). Rapid germination rates were correlated to an increased frequency of pathogenesis over other Aspergillus spp. (60, 61). Three genes have been shown to be necessary for thermotolerant growth, in particular, the ribosome biogenesis protein CrgA was demonstrated to be the most important gene for thermotolerance as demonstrated by the decrease growth and virulence phenotype of the knockout mutant (58).

Many researchers have speculated that A. fumigatus virulence is a multifactorial process and it is important to differentiate that a virulence factor is only essential for virulence, but not required for normal growth (7). Therefore the mutation of a gene that decreases virulence in vivo but causes the fungus to not be able to grow in vitro, is actually an essential gene that encodes for a virulence specific trait and not a true virulence factor (7).

It has been shown that enzymes involved in siderophore biosynthesis by A. fumigatus are virulence factors (62). Siderophores are small secreted peptides that chelate Fe(III) and are taken up by specific ferri-siderophore receptors on the fungal plasma membrane (63). Deletion of a gene in the siderophore biosynthetic pathway, sidA, eliminated virulence in a mouse model of IA (62, 64).

Other virulence determinants that have been identified in A. fumigatus include (i) the biological pathways involved in nutrient sensing/utilization and regulatory systems (10), and (ii) cell wall components or other structures that protects the conidia and promotes the conidia’s adherence to its host (65, 66). Mutants of many genes that have been identified to encode proteins implicated for virulence have been created with promising results. For nutritional components, the genes include pyrG, a decarboxylase for uracil/uridine synthesis (67), pabaA, a synthetase for the final step in folate biosynthesis (68), cpcA, a transcriptional activator that regulates amino acid biosynthesis (69), and lysF, a homoaconitase required in lysine biosynthesis (70, 71).
1.2.1. The structure of the cell wall and its role in virulence

Adherence of *A. fumigatus* spores to airway epithelial cells is a necessary first step for infection and is therefore an important aspect of the virulence of *A. fumigatus* (22). The cell wall of *A. fumigatus* is primarily composed of the polysaccharides α(1,3)-glucan, β(1,3)-glucan, chitin, galactomannan, and β(1,3),(1,4)-glucan (72). However, the structural organization and the concentration of mentioned polysaccharides differ between the conidial and hyphal morphotypes (Figure 1.4) (73). The outermost layer of conidia is the rodlet layer followed by a melanin layer; both components confer hydrophobicity. The rodA gene encodes a protein that forms the uniform rodlet structure on conidia that prevents the recognition of β(1,3)-glucan by immune cells (65, 74). Conidia of a rodA deficient mutant displayed reduced adherence to collagen and albumin *in vitro* (75). However, the *A. fumigatus* rodA mutant did not show attenuated virulence in a murine model of IA, despite exposure of β(1,3)-glucan in the mutant that resulted in immune response not seen with wild type spores (76).
Melanin is an essential component of A. fumigatus conidia conferring their grey-green color, and it was found that a pigmentless-conidium mutant of A. fumigatus had an altered conidial surface structure and reduced virulence in mice (77). Melanin is a polymer formed from the crosslinking of phenolics and A. fumigatus uses the DHN (1,8-dihydroxynapthalene) pathway to produce melanized conidia. The melanin biosynthetic genes is a six gene cluster (pksP, ayg1, arp2, arp1, abr1, and abr2) identified in the A. fumigatus genome and the pigmentless mutant was a consequence of a single deletion
(pksP, ayg1, or arp2) in the initial three steps (78). In contrast to wild-type, it was found that all of the melanin mutant *A. fumigatus* strains (∆pksP, ∆ayg1, or ∆arp2) activated dendritic cells and the subsequent cytokine production. Although melanin is immunologically inert, Bayry et al. (2014) showed that melanin is required for virulence by facilitating proper formation of the *A. fumigatus* conidial surface.

In chronic aspergillosis infections, *A. fumigatus* develops a biofilm characterized by mycelia embedded in a secreted extracellular matrix known as mycetoma (79, 80). The surface of this matrix is rich in polysaccharides, most notably galactomannan, galactosaminogalactan (GAG), and α1,3-glucans (76). Of these, α1,3-glucans and GAG are important for adhesion to host cells (81, 82).

Once the conidium germinates, the rodlet and melanin layers disappear and α1,3-glucan is exposed on surface of the hyphae growing under biofilm conditions (73, 76). The α1,3-glucan is a major adhesive involved in the aggregation of germinating conidia and biofilm. The polysaccharide is synthesized in the cytosol by three α1,3-glucan synthases, *ags1, ags2, ags3*, from the substrate UDP-glucose (83). A triple knockout mutant of *A. fumigatus* (∆ags) was devoid of α-glycan (∆ags) and although a distinct phenotype was not observed *in vitro*, it was less pathogenic than the parental strain *in vivo*. The mutations caused extensive structural modification of the conidial cell wall. In contrast to the parental strain, the ∆ags mutant conidia were more amorphous and hydrophilic due to an additional layer of the glycoproteins, and β(1,3)-glucan and chitin were exposed (83).

Furthermore, an *A. fumigatus* regulatory gene, *medA*, that mediates biofilm biogenesis have been identified (84). It was found that the knockout mutant, ∆medA, had reduced adhesion of conidia to plastic, fibronectin, pulmonary epithelial cells, and endothelial cells. In a murine model of IA, ∆medA was hypovirulent (84). A transcriptome and carbohydrate analysis of the ∆medA strain revealed that MedA governs the expression of GAG through the regulation of a carbohydrate synthetic cluster located on chromosome 3 (22, 84).

The outer layer of the hyphal cell wall contains galactosaminogalactan (GAG) which has been implicated in the pathogenesis of aspergillosis and is absent in the
dormant conidia (73). GAG is a linear heterogeneous polysaccharide chain comprised of α1,4 linked galactose and α1,4 linked N-acetylgalactosamine residues (81, 82). This polysaccharide favors A. fumigatus infections. Demonstrated in vivo, mice dosed with GAG had a lowered immune response: decreased inflammation observed by decreased neutrophil recruitment and higher fungal loads (85). Two UDP-glucose-4-epimerase in A. fumigatus, uge3 and uge5, affect the production of UDP-Gal and UDP-GalNAc which are required for the production of GAG. The double knockout Δuge3Δuge5 A. fumigatus mutant resulted in complete absence of hyphal GAG (Lee et al. 2014); however, the role of GAG-mediated adherence in A. fumigatus virulence is currently unknown.

1.2.2. Adhesion of conidia via sialic acids.

Sialic acids are negatively charged monosaccharides that also have been shown to mediate conidial binding to host basal lamina proteins and epithelial cells of the human host (Figure 1.5) (66, 87, 88). Binding of laminin by A. fumigatus conidial sialic acid residues was identified by both Bouchara et al. (1997) and Wasylnka and Moore (2000) but different mechanisms of adhesion were proposed. Alveolar basal lamina is a specialised extra cellular matrix (ECM), composed of laminin, type IV and V collagen, entactin, chondroitin and heparin sulfate proteoglycan, as well as fibronectin (66, 89). Diseased lungs are documented to have increased amounts of fibronectin and other ECM proteins in order to aid in repair of damaged epithelia (90). Conidia may be able to bind to epithelia more readily as a result, which would contribute to the development of invasive aspergillosis. Negatively charged carbohydrates, dextran sulfate and heparin, were found to inhibit binding of spores, suggesting that negatively charged surface molecules on conidia bind to the positive charged glycosaminoglycan (GAG) binding domain on fibronectin and basal lamina proteins (66). This was confirmed by studies showing that conidia adhered only to the GAG-binding domain of fibronectin and not to the cell-binding domain (Figure 1.5) (66).
Pathogenic *Aspergillus* species were found to have a higher density of sialic acids on the conidial surface than non-pathogenic species (87). Compared to other fungal species, *A. fumigatus* experiences more efficient binding to tissues like laminin (91) and fibrinogen (92), both of which are found in the basal lamina. This suggests that sialic acids may play a role in the pathogenicity and increased efficiency of binding may be associated with increased virulence by pathogenic *Aspergillus* species (87).

The sialic acid, *N*-acetylneuraminic acid (Neu5Ac), on *A. fumigatus* conidia is α2,6-linked to a galactose residue (88). Enzymatic removal of sialic acids resulted in decreased binding of conidia to fibronectin. On the other hand, the loss of surface sialic acids decreased the uptake of conidia by cultured murine macrophages, suggesting that phagocytic uptake is mediated in part by recognition of sialylated glycans on the fungus (88). Based on these results, it is likely that sialic acids do not protect *A. fumigatus* conidia from phagocytosis through masking of molecular targets. In contrast, it has been
proposed that for many microbial pathogens, the cell surface sialylation is important by aiding in host recognition or in the evasion of the host immune response (93). To understand the importance of sialic acids in microbial pathogenesis, the following sections describe the current literature on sialic acid structure and mechanism.

1.3. Sialic Acids

1.3.1. Structure

Sialic acids are the most prevalent monosaccharides occupying the terminal position of cell surface glycoconjugates on the surface of eukaryotic cells (94, 95). The term ‘sialic acid’ refers to a family of greater than 50 substituted derivatives of a 9-carbon, α-keto acidic monosaccharide, neuraminic acid (Figure 1.6). All sialic acids have a distinctive negative charge that comes from the carboxylate group at carbon 1 which is deprotonated at physiological pH; the negative charge strongly influences the chemical properties of this sugar (96). Diversity in sialic acid structure is generated through substitutions at the hydroxyl and amino groups located on carbons 4, 5, 7, 8, and 9. The most important variation on the core structure occurs at carbon 5 where it is either N-acetylated, yielding N-acetylneuraminic acid (Neu5Ac), N-glycolylated, as in N-glycolylneuraminic acid (Neu5Gc), or substituted with a hydroxyl group generating 2-keto-3-deoxynonulosonic acid (KDN). It is less common in nature to find the amino group at carbon 5 unsubstituted; this is the core neuraminic acid structure (95).
The sialic acid family consists of over 50 members. Carbon 5 can be substituted with an N-acetyl group yielding N-acetyl neuraminic acid, an N-glycolyl group yielding N-glycoly neuraminic acid, an unsubstituted amino group yielding neuraminic acid, or the amino group can be replaced with a hydroxyl, generating 2-keto-3-deoxynulosonic acid (KDN); at neutral pH the carboxylate is deprotonated.

Neu5Ac is the best studied and most abundant naturally-occurring sialic acid (95, 97). Neu5Gc occurs frequently in the animal kingdom; however, humans cannot synthesize this sialic acid due to a mutation in the biosynthetic gene, cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) (98). In ferrets, the CMAH gene is deleted and Neu5Gc is not presented on the cell surface (99). In addition, Neu5Gc has not been reported in plants or microbes (100). KDN has been detected in microorganisms, such as Neisseria meningitidis (101) and Escherichia coli K1 (102), and in animals, such as the rainbow trout (103) as well as in human fetal red blood cells and ovarian tumor tissues (104).

Other commonly found modifications that further increase structural diversity of sialic acids include substitutions at the hydroxyl groups on carbon 4, 5, 8, and 9 with acetates, sulphates, phosphates and methyl ethers (105). It is common to have a single acetyl group substitution, especially at O-9; however, acetylation may occur at O-4, O-7, or O-8. It is not unusual to have di- and tri-O-acetylated sialic acids (105). Furthermore, lactyl and phosphoryl groups can occur at O-9, and methyl and sulphate groups are sometimes found at O-8. Some of the above-mentioned substitutions can be combined, thus generating the multitude of sialic acid structures known to exist in nature (106). In addition to substituents at various hydroxyl and amino groups, anhydro and lactone
forms have been identified in biological samples (107, 108). Finally, sialic acids can be linked by sialyltransferases to glycoconjugates via α2,3- or α2,6- linkages to galactose, α2,6- linkages to N-acetylgalactosamine (GlcNAc), or α2,8- or α2,9- linkages to another sialic acid, thereby further increasing the diversity of sialic acid structures in nature (105, 106).

1.4. Biosynthesis and Presentation of Sialic Acid on Cell Surfaces

1.4.1. Biosynthesis

Sialic acid is well distributed and has many different roles in nature; however, it is not synthesized in all domains of life and the biosynthetic pathways of many organisms are still to be discovered (95). In microorganisms, de novo sialic acid synthesis has been detected in pathogenic bacteria, including species such as Escherichia coli K1 (109), and Campylobacter jejuni (110) and in fungi, such as Cryptococcus neoformans (111) and Aspergillus fumigatus (87). The sialic acid biosynthetic pathway has been characterized in mammals and bacteria and the pathways are very similar since enzymes that perform common functions are used (95, 112). At present, the sialic acid biosynthetic pathway used by most eukaryotic organisms, including fungi, are yet to be elucidated; therefore, the following section will focus on genes characterized in mammalian and bacterial species.

The first committed step in sialic acid synthesis is the epimerization of uridine diphosphate linked N-acetylglucosamine (UDP-GlcNAc) by UDP-GlcNAc-2-epimerase to N-acetylmannosamine (ManNAc) (Figure 1.7) (95, 96, 113, 114). In bacteria, UDP-GlcNAc 2-epimerase is encoded by the neuC gene. ManNAc is directly used as a substrate for Neu5Ac synthase (NeuB) in a condensation reaction with phosphoenolpyruvate (PEP) to yield Neu5Ac. In animals, the first step is similar but the ManNAc is first phosphorylated to form ManNAc-6-phosphate (ManNAc-6P) by the kinase activity of the bifunctional UDP-GlcNAc 2-epimerase (105). The UDP-GlcNAc 2-epimerase/ManNAc 6-kinase in the sialic acid biosynthetic pathway of animals is encoded by the GNE gene (115). Condensation of ManNAc-6P with PEP by Neu5Ac-9-
P synthetase (NANS) yields Neu5Ac-9-phosphate, which is then dephosphorylated by Neu5Ac-9P phosphatase (NANP) to yield Neu5Ac.

In prokaryotes and eukaryotes, the reaction by UDP-GlcNAc 2-epimerase is the rate limiting step of the pathway and plays a key role in the biosynthesis of sialic acids (96, 112). Missense mutations in this gene give rise to hereditary inclusion body myopathy (HIBM) in humans and inactivation causes embryonic lethality in mouse (115). Structurally, the N-terminus of the mammalian and bacterial UDP-GlcNAc-2-epimerase is homologous. The ManNAc kinase activity of the mammalian enzyme is on the C-terminus of the UDP-GlcNAc-2-epimerase protein which is also homologous to kinases of the sugar-kinase/Hsp70/actin superfamily (116). The activity of this enzyme is controlled by an allosteric negative feedback loop triggered by CMP-Neu5Ac, the activated sugar nucleotide product of Neu5Ac formed by a subsequent reaction downstream (116).
Figure 1.7  Sialic acid and sialoglycoconjugate biosynthesis in animal and bacterial cells

The sialic acid biosynthetic pathway of mammals (A) and bacteria (B). The cellular localization of enzymes in mammals is noted. Part of Figure (A) is adapted from Keppler et al. 2001.
1.5. **Transport of CMP-sialic acid into the endoplasmic reticulum/ Golgi lumen via nucleotide sugar transporters**

To present sugars within cell-surface glycoconjugates, the monosaccharide is activated to its nucleotide sugar form by the addition of a mono or di-phosphate, such as UDP, GDP, or CMP, to form a nucleotide sugar (117). Nucleotide sugars are synthesized in the cytosol by the sugar specific nucleotide-sugar synthetases; however, in mammals, the synthesis of CMP-sialic acid occurs in the nucleus (118, 119). In *Drosophila melanogaster*, CMP-sialic acid synthase is localized in the Golgi. However, the mechanism by which sialic acid is transported into the *D. melanogaster* Golgi is unknown (120).

CMP-sialic acid synthetase (CMP-Sia-syn) activates sialic acid to cytidine-5’-monophosphate N-acetylneuraminic acid (CMP-Neu5Ac) by facilitating the formation of the high energy phosphate bond between CMP and sialic acid (Figure 1.7). To form glycosidic bonds for the biosynthesis of sialoglycoconjugates, the reaction is driven forward by the hydrolysis of the phosphate ester bond catalyzed by sialyltransferases (121).

In eukaryotes, the activated sugar is transported to the Golgi via CMP-Neu5Ac transporters (CST) and then transferred to glycoconjugates terminating in either galactose (Gal), N-acetylgalactosamine (GalNAc) or another sialic acid by a sialyltransferase(95, 105). The sialylated glycoconjugates are often found on proteins and lipids intended for the cell surface.

1.5.1. **Structure and Function of Nucleotide Sugar Transporters (NST)**

Nucleotide sugar molecules cannot move across the membrane of the endoplasmic reticulum (ER) and Golgi lumen unless facilitated by specific nucleotide sugar transporters (NSTs). These transporters are present in all eukaryotes and have been characterized in many model organisms such as *Saccharomyces cerevisiae*, *Candida* spp., *Leishmania donovani*, and *Drosophila melanogaster* (117).
NSTs are a family of evolutionarily conserved transmembrane proteins that are classified under the solute carriers 35 (SLC35) family of membrane proteins (122). The family is divided into seven subfamilies (SLC35A-G) based on sequence similarities and each NST subfamily is further divided to differentiate the substrate(s) transported (123). Despite high architectural conservation, the amino acid sequence similarity cannot be used to predict function and substrate specificity. For example, over 40 % identity in amino acid sequence of the human CMP-sialic acid and the UDP-galactose / UDP-N-acetylglucosamine transporter was revealed, but these transporters are very specific for their corresponding substrates (124).

Many NSTs have been cloned and important structural and mechanistic features have been elucidated. NSTs are type III transmembrane proteins composed of 6 and 10 membrane spanning domains with N- and C-termini exposed at the cytosolic side of the Golgi apparatus (119, 125). No crystal structure for any NST has been determined since crystallization of membrane proteins for X-ray crystallography remains difficult and therefore specific structural features are not known (123, 126). Secondary structure prediction software allowed the identification of trans-membrane domains; however, prediction of trans-membrane domains is not very accurate and does not take in consideration that the ER/Golgi membrane is thinner than the cell membrane (119).

Nevertheless, biochemical and genetic analysis of NSTs have demonstrated these proteins generally have high substrate specificity and possess highly conserved stretches of amino acids that are essential for function, particularly nucleotide sugar binding (118, 124). An analysis of mutant murine CMP-sialic acid transporters (CSTs) that had either the N-terminal or C-terminal tail removed, it showed that the N-terminus did not affect CST localization in the Golgi and that an ER export motif on the C-terminus was sufficient to mediate ER export of CST to allow localization in the Golgi (127).

As antiporters, NSTs function by exchanging the nucleotide sugar with the corresponding nucleoside-monophosphate generated in the lumen through the action of glycosyltransferases and nucleoside diphosphatases (119, 128). The mechanism of the UDP-galactose (UDP-Gal) transporter is illustrated in Figure 1.8. CST is the specific NST that exclusively transports CMP-sialic acid. Murine CST has been functionally
expressed in yeast and it has been demonstrated in vitro that the transporter was capable of bringing in a molecule of CMP- sialic acid in exchange for a molecule of CMP by using Golgi vesicles loaded with CMP in the lumen (129).

**Figure 1.8  Mechanism of the UDP-Galactose Transporter**
Illustration of the transport of UMP in exchange for UDP-gal into the Golgi lumen. Inside the Golgi lumen, the UDP-gal is covalently linked to a glycoprotein by galactosyl transferase. A phosphate is removed from UDP as it is transformed into UMP by nucleoside diphosphatase. The inorganic phosphate is hypothesized to exit via a phosphate transporter. With the same type of transport system, CMP-sialic acid (cytosol) would exchange for CMP (Golgi). However, there is no phosphatase step following the removal of the sialic acid from the activated sugar. Therefore, an existing concentration of CMP is required.

NSTs have long been believed to have an absolute substrate specificity for one nucleotide sugar; however, recent studies show that NSTs are able to transport multiple substrates. For example a human NST that transports both UDP-glucuronic acid (UDP-GlcUA) and UDP-\(N\)-acetylgalactosamine (UDP-GalNAc) into the Golgi was identified (130). In addition, a human and Drosophila UDP-Gal transporter (DmUGT / hUGTrel7) was found to transport both UDP-GalNAc and UDP-Gal (131). Furthermore, a UDP-Gal/UDP-glucose (UDP-Glc) transporter (AtUTr1) was found in the plant, Arabidopsis thaliana. NSTs that are able to transport more than one nucleotide-sugar, however, are always selective for the same nucleotide (132).
1.6. Mechanism of Sialic Acid Acquisition

Since no sialic acid biosynthetic pathway has been identified in *A. fumigatus*, alternative methods for the biosynthesis of sialic acid may exist, or the fungus may salvage sialic acid from the environment.

Microbes can obtain sialic acids by taking up sialyl precursors from the environment and process them in a truncated biosynthetic pathway (113). Bacteria can secrete sialidases that catalyze a hydrolysis reaction to remove sialic acid from the host cell glycans. The sialic acids are then imported into the cell, or directly linked to cell surface glycoconjugates (113). Initially, it was thought that sialidase activities were only associated with pathogenic bacteria because sialidase activity was found in bacteria isolated from various infections (133). However, it was later discovered that sialidases also had an important role in bacterial nutrition as sialidases were found in many non-pathogenic bacteria (133, 134).

Precursor scavenging has been demonstrated in the bacterium, *Haemophilus influenzae*, which expresses only CMP-sialic acid synthetase (NeuA) and sialyltransferases. This bacterium does not have a sialidase; instead, it imports free Neu5Ac from the host or Neu5Ac made available by a neighboring sialidase-expressing bacteria through a transporter located in the inner membrane (135). This family of transporters binds sialic acid with high affinity and specificity. *Pasteurella multocida*, *Haemophilus ducreyi*, and *Escherichia coli* K-12 also use a similar transport system (113).

Several *Neisseria gonorrhoea* strains and *Neisseria meningitidis* have eliminated the need for uptake by expressing an extracellular sialyltransferase (*Neisseria* LPS sialyltransferase) that is able to bind free CMP-sialic acid present in human secretions and transfer it to lipopolysaccharides (LPS) (113, 136). A similar strategy is also used by *Trypanosoma cruzi* and related protozoan pathogens, which require surface sialylation for host-cell infection but are unable to synthesize sialic acids de novo (137). These parasites express a surface-located *trans*-sialidase that functions to transfer sialic acid residues directly from one glycan to another, thereby avoiding the need to synthesize
CMP-sialic acid as an intermediate (137). In this transglycosylation process, sialic acids that are α2,3-linked to galactose are reversibly transferred from the host cell to another galactose residue on the protozoan cell surface. This reaction retains the original α anomeric configuration of the transferred sialic acid residue and yields α2,3-sialyllactosyl residue on the surface of the parasite (138).

1.7. Utilization of Sialo-glycoconjugates in Microbial Pathogenesis

Carbohydrates added to the cell surface of proteins and lipids provide major contact and communication elements for the cell (139). Due to the ubiquity and importance of sialic acids, many pathogenic microbes have evolved to use this molecule as a source of nutrition or as a tool in their pathogenesis (106). Successful microbes obtain sialic acid either by synthesizing them de novo or by acquiring them from their environment. The expression of surface sialic acid residues allow microbes to adhere or to mimic the host and avoid recognition by the host immune system (93).

Bacteria

For bacterial pathogens, adhesion of bacterial cells to host cells mediated by bacterial cell surface receptors or sialic acid receptors is one way to initiate infection. For example, Helicobacter pylori uses the sialic acid binding motif, 3'-sialyllactose, as an adherence ligand to bind gastric epithelial cells (140, 141). In the non-typeable (non-capsulated) Haemophilus influenzae, a terminal sialic acid is found on the lipooligosaccharide (LOS) and sialylation of LOS promotes resistance to serum killing and plays a role in formation of biofilms (142). The bacterial biofilm is a protective and highly organized matrix for the bacterial colony that allows it to adhere to surfaces and facilitate pathogenesis (143). PolyKDN has been found on the cell wall polymers of Streptomyces spp. that are known plant pathogens. Approximately 20% of their cell wall is made up of KDN and the sugar is thought to mediate attachment to host plant cells (144, 145). Moreover, KDN was present only on lipopolysaccharide of the thymine autotrophic mutant of Sinorhizobium fredii, whereas 3-deoxy-d-manno-oct-2-ulosonic acid (KDO) was present on the wild type (146). KDO is not a member of the sialic acid
family, but it is a monosaccharide containing eight carbon atoms including a carboxylic acid group where numbering of the structure begins. KDO also has a hydroxyl group at carbon five as in KDN. The main difference between KDO and KDN is the configuration of both molecules, and the ninth carbon and its hydroxyl substituent found in KDN (103). KDO is used by bacteria in the synthesis of lipopolysaccharides and the biosynthetic pathways of KDN and KDO are similar (103, 144). This gram-negative rhizobial bacterial species symbiotically forms nitrogen-fixing nodules in the root of leguminous plant hosts and cell surface polysaccharide is known to play important roles in the bacterium-plant relationship as a factor that triggers infection. Interestingly, the change from KDO to KDN altered the host range of the bacterium (146).

Several species of bacteria, including pathogenic *Escherichia coli*, *Neisseria gonorrhoeae*, and *Haemophilus influenza*, have sialic acids on their cell surface, which is thought to offer a form of protection from the host immune response by inhibiting activation of the alternate complement pathway (148, 149). It was discovered that a porin, Por1B, in the outer membrane of *N. gonorrhoea* is the site of Factor H binding and that sialylation of lipo-polysaccharide (LOS) in the outer membrane enhances Factor H binding to Por1B (150). Factor H is a mammalian glycoprotein that functions in the negative regulation of the alternate complement pathway by binding to complement factor C3b, thereby preventing C3b binding to microbes and avoiding opsonization of the bacterial cell (151–153). Furthermore, on the human respiratory pathogen, *Klebsiella ozaenae* serotype K4, a KDN containing pentasacharide was isolated from its capsule (154). The capsular polysaccharide of different bacterial pathogens are very much strain dependant, and the capsule is considered to be the dominant virulence property. These polysaccharides contribute by facilitating resistance to phagocytosis and killing by host serum as well as protection against desiccation and attack from phages (155).

**Protozoa**

Sialic acids are also important in protozoan pathogenesis. Neu5Ac has been found on the surface of *Trypanosoma cruzi*, *Trypanosoma brucei*, and *Trypanosoma congolense*, the causative agents of Chagas’ disease (156, 157). The sialic acids on the protozoan surface are ligated to the cell surface by trans-sialidases secreted by the protist. Sialic acid masks the underlying galactose residue which helps the pathogen
resist the host’s complement system (158). Meanwhile, the removal of host sialic acids by the trans-sialidase can cause apoptosis of the host’s immune cells located in the spleen, thymus, and peripheral ganglia thereby weakening the host immune system which will further the parasite’s pathogenesis (156). Finally, invasion of red blood cells by the merozoite stage of the malaria parasites Plasmodium falciparum and Plasmodium reichenowi is mostly sialic acid-dependent (159). Both parasites are not sialylated, but they utilize their erythrocyte-binding antigen 175 (EBA-175) proteins to bind and invade host red blood cells (160). The recognition by P. falciparum EBA-175 is specific for Neu5Ac whereas P. reichenowi EBA-175 recognizes only Neu5Gc. Since primates express both Neu5Gc and Neu5Ac, and humans only express Neu5Ac, humans are susceptible to P. falciparum whereas primates are infected by P. reichenowi (161).

**Fungi**

Sialic acids have been found on the surface several fungal species in addition to A. fumigatus; these include Fonsecaea pedrosoi (Souza et al. 1986; Alviano et al. 2004), Cryptococcus neoformans (111), Paracoccidioides brasiliensis (164), Candida albicans (165), Sporothrix schenckii (166), and Pneumocystis carinii (167). However, the role of sialic acid in pathogenesis has only been investigated in a few species. Neu5Ac and its 9-O-acetylated derivative, Neu5,9Ac2 were found on the surface of C. neoformans when it was grown in sialic acid free media (111). The removal of sialic acid from C. neoformans by treatment with sialidase resulted in increased phagocytosis of the sialidase-treated cells by mouse peritoneal macrophages suggesting that sialic acid protects the fungus from host immune responses (111). Moreover, sialic acids are found on the conidial and mycelial surface of Fonsecaea pedrosoi but not on the parasitic form of the fungus known as sclerotic cells (162, 163). Removal of sialic acid by sialidase treatment resulted in 50% less attachment or internalization by neutrophils compared to controls. Therefore, sialic acids are likely to play a role in protecting F. pedrosoi conidia against phagocytosis by human neutrophils and protects the fungus from host cells until the fungus differentiates into the sclerotic form.

The role of sialic acids in the adhesion of Aspergillus fumigatus was presented in section 1.2.2.
1.8. Sialidases in Microbial Pathogenesis and Nutrition

Exo-sialidases are glycosyl hydrolases (GH) enzymes that remove terminal sialic acid from glycan chains (168). The main characteristics of sialidases in pathogenic bacteria are high levels of activity, high specificity, and extracellular activity. There are at least three characteristics of sialidases that can influence the disease process of pathogenic bacteria. These include: (1) inducibility of enzyme activity at the site of infection, (2) substrate specificity, which also reflects the site of colonization, and (3) the presence of additional sialic acid metabolic enzymes, such as the sialic acid permease to ensure that the sialic acid is transported into the bacteria (133).

Many strains of *Clostridium perfringens*, the causitive agent of a range of histotoxic infections, such as gas gangrene, enteritis or enterotoxemia, produce three different sialidases: NanI, NanJ, and NanH (169). NanI is primarily involved with cleavage of oligosaccharides and gangliosides for nutritional purposes; more recently, NanI was found to facilitate adherence of the bacteria to enterocyte-like Caco-2 cells and to increase binding of *C. perfringens* toxins to host cells, thus increasing cytotoxicity (169). Similarly, pathogenic strains of *Vibrio cholerae* excrete a sialidase that increases the severity of the infection by increasing the binding and penetration of the cholera toxin to enterocytes (170, 171).

The respiratory pathogen, *Streptococcus pneumoniae*, is responsible for severe infections such as meningitis and pneumonia. A large scale screen of the *S. pneumoniae* genome revealed that many carbohydrate enzymes, including the sialidase (NanA), are virulence factors (172). NanA is a cell-wall attached sialidase that works together with two other cell surface-bound enzymes, the exo-β-D-N-acetylglucosaminidase (StrH) and the β-galactosidase (BgaA), to degrade the nonreducing terminal arms of complex N-glycans and to resist opsonophagocytic killing by human neutrophils (173, 174). In addition, NanA has roles in host colonization, biofilm formation, and modification of the surface of other competing bacteria (135, 175).

Some pathogenic and non-pathogenic bacteria can utilize sialic acid as a sole carbon and nitrogen source and as a source of amino sugars (GlcNAc and glucosamines) for cell wall biosynthesis (113). The pathway by which this occurs uses
the N-acetyleneuraminic aldolase (NanA) that cleaves Neu5Ac to ManNAc and pyruvate. ManNAc is enzymatically converted to fructose 6-phosphate that enters central metabolism. There is evidence that sialic acid catabolism competes with sialic acid presentation on the cell surface: it was found that feeding *N. meningitidis* and *E.coli* with ManNAc induced the synthesis of GlcNAc-6P 2-epimerase, a key enzyme in sialic acid catabolism, but at the cost of the production of capsular polysialic acid (176). For non-typeable *H. influenzae*, if sialic acid transport into the cell is reduced, sialylation of the lipo-oligosaccharide was decreased, and therefore reduction to infectivity. However, an upregulation of sialic acid catabolic genes was observed which suggests the reduction in cell surface sialylation was secondary to the reduction of sialic acid available for nutrition (177).

**KDNase**

The sialidases discussed so far are known to cleave the most common sialic acid, Neu5Ac, from glycoconjugates. Another class of sialidase cleaves KDN in a similar fashion and they are referred to as KDNases. The KDNase enzyme was first identified in the bacterium *Sphingobacterium multivorum*, which was found in sewage ponds of a trout hatchery (178). The location was important because KDN was first discovered in the cortical alveolar polysialoglyco-protein (PSGP) of rainbow trout eggs (179). The bacterial KDNase was localized in the periplasm and had optimal activity at neutral pH (178, 180). *S. multivorum* was able to use KDN glycoconjugates as a sole carbon source, but not Neu5Ac or Neu5Ac oligomers. These data suggest that the enzyme plays a role in nutrient acquisition (177). Furthermore, the KDNase showed broad linkage specificity and was capable of hydrolyzing both α2,6 and α2,8 linkages. The specific activity was 300-fold higher on synthetic KDN substrates than on Neu5Ac substrates (177, 179).

It was recently found that KDN can also be cleaved from glycoconjugates by the bacterial sialidase from *Micromonospora viridifaciens* (183, 184). Because KDNases are thought to be less abundant than sialidases, Angata et al. (1994) speculated that the presence of KDN was to protect ovulated trout eggs and embryos from bacterial attack. Since KDN-glycoconjugates were more abundant on trout gamete cells (ovarian fluid, vitelline envelopes, and sperm membrane) than on other organs of the fish, KDN has
also been implicated in developmental functions (179, 183). Except *Sphingobacterium multivorum* and *Micromonospora viridifaciens*, KDNase activity has not been detected in other bacterial species; however, it has been found in the liver of the fish, *Misgurnus fossilis* (187), the star fish, *Asterina pectinifera* (188), and the oyster, *Crassostrea virginica* (189).

1.9. Structure and Mechanism of Sialidases

Sialidases are divided into two main categories: *exo*-sialidases that catalyze the removal of terminal, α-ketosidically linked sialic acid, and *endo*-sialidases that break internal glycosidic bonds in polysialic acids. In this thesis, only *exo*-sialidases will be discussed.

**Exo-sialidases**

*Exo*-sialidases are further categorized into different to carbohydrate-active enzyme (CAZy) families: GH 33 (bacterial and eukaryotic enzymes), GH34 (sialidases from influenza virus strains), and GH38 (other viral sialidases). There are low sequence identities between the families, however, they are structurally similar (96). The catalytic domain has the signature six-bladed-β-propeller fold dictated by conserved amino acid repeats known as Asp boxes ((S/T)XD(X)GXT(W/F)) and the structure of the catalytic center is preserved with a set of key conserved amino acids. In addition, there are species specific carbohydrate binding domains within the sialidase which have been proposed to increase catalytic efficiency. Although all *exo*-sialidases share a common overall structure and catalytic centre, each enzyme is highly specific to one or a few substrates and has different enzyme kinetics (96). Most sialidases exhibit substrate specificity regarding the sialic acid linkage; generally more enzymes that cleave α2,3 linkages than α2,6 linkages have been identified. In addition, O-methylation and O-acetylation of sialic acids can hinder the hydrolysis of the glycosidic bond (190).

*Exo*-sialidases are retaining hydrolases meaning that the sialidase hydrolyzes terminal sialic acids from glycoconjugates with retention of configuration at the anomeric centre (190, 191). This is achieved by a two step double-displacement mechanism in
which a covalent intermediate enzyme is formed before the free enzyme is regenerated (Figure 1.9) (190). The anomeric carbon is C-2 in sialic acid. Cyclic monosaccharides can exist in two possible stereoisomers, known as anomers, α and β. The two configurations are due to the ability of the anomeric hydroxy group to assume two possible orientations when the monosaccharide is initially cyclized (94). Most other retaining hydrolases require substrate assisted catalysis: a protein carboxylate nucleophile and a general acid/base catalyst in the catalytic site, which are essential in generating a covalent glycosyl-enzyme intermediate (192). However, structures of sialidases showed that no carboxylate group was favorably positioned in the active site to generate such an intermediate. Instead, a tyrosine is in the correct position and it is activated by a nearby glutamate so that the tyrosine can act as a nucleophile (Figure 1.9) (193, 194). It has been argued that tyrosine is a better nucleophile because the use of a negatively charged carboxylate group, as in other retaining glycoside hydrolases, would interfere due to charge repulsion with the negatively-charged sialic acid (195). Alternatively, tyrosine is viewed as a less reactive leaving group that is required to compensate for the higher reactivity of sialosides compared with other glycosides (194).
Figure 1.9  Mechanism of Exosialidase (Retaining Glycoside Hydrolase Families 33 and 34)

Tyrosine is the catalytic nucleophile that is activated by an adjacent base residue. In the first step, the activated nucleophile attacks the anomeric centre to displace the aglycon and form a glycosyl-enzyme intermediate. At the same time, the residue on the opposite face of the anomeromic carbon, typically an aspartate, functions as a general acid and protonates the glycosidic oxygen as the bond cleaves.

In the second step, the glycosyl-enzyme intermediate is hydrolyzed by water. The aspartate residue is now acting as a general base and it deprotonates the water as the water molecule attacks the anomeromic carbon. The tyrosine becomes the leaving group and the glycan bond between the nucleophile and the anomeromic carbon is eliminated. Subsequently, the tyrosine is reprotonated. As shown in the figure, each step passes through an oxocarbenium ion-like transition state.


1.10. Aims of thesis research

Aspergillus fumigatus is an opportunistic fungal pathogen that causes a range of diseases that primarily affects immunocompromised individuals. The most severe disease caused by A. fumigatus is invasive aspergillosis (IA). Cell wall glycans and metabolic enzymes involved with glycan presentation have been attributed to the pathogenesis of numerous microbial pathogens. Many A. fumigatus cell wall components have been identified to be important virulence factors. The sialic acid, N-acetylneuraminic acid, was found on the fungal cell wall surface and previous studies
have indicated that sialic acid is involved with pathogen-host interactions. However, to date, no sialic acid biosynthesis genes have been identified in any fungus. In spite of this, sialic acids were detected even when *A. fumigatus* was grown in a chemically-defined medium lacking sialic acid sources, suggesting that sialic acids were synthesized *de novo* (87, 196). Therefore, enzymes that mediate metabolism of sialic acid in *A. fumigatus* could be important components to the pathogenicity of the fungi.

The overall aim of this work is to investigate how *A. fumigatus* metabolises sialic acid to better understand the role of sialic acids in *A. fumigatus* pathogenesis. My research first focused on two aspects of sialobiology: first, to characterize the putative *A. fumigatus* sialidase (*Af*S), and second, to study four putative *A. fumigatus* nucleotide sugar transporters (*AfNSTs*).

Chapter 2 describes the characterization of the *A. fumigatus* sialidase (*AfS*) (Afu4g13800). The enzyme was cloned and expressed as a recombinant protein which allowed for biochemical analysis using both synthetic and natural sialylated substrates. In addition, the expression levels of this enzyme in *A. fumigatus* and how the *AfS* is evolutionarily related to other microbial sialidases was studied.

In Chapters 3 and 4, I investigated the structure and function of the recombinant *AfS*. The crystal structure of the sialidase was solved. Structural and mechanistic mutations were made and the biochemical analysis with wild type *AfS* and mutant *AfS* were completed to elucidate effects on enzyme stability and kinetics of the enzyme. The three mutant *AfS* were also crystallized. In addition, AfS was localized in *A. fumigatus*.

In chapter 5, studies on four *A. fumigatus* NSTs are presented (NST1, Afu6g13073; NST2, Afu8g02090; NST5, Afu1g05440; NST6, Afua_1g06050). Since all glycans presented on the cell surface must be added in the ER/Golgi to a growing glycosidic chain on a lipid or a protein, I postulated that one or more of the NSTs could influence sialic acid presentation on *A. fumigatus*. Biochemical analyses were completed on purified recombinant NSTs expressed in yeast and the biological role of the transporters in the presentation of sialic acid on *A. fumigatus* were evaluated in two NST knockout strains.
Chapter 6 contains a general discussion of the results from this thesis and suggestions for further research.
Chapter 2.

Characterization of the *Aspergillus fumigatus* sialidase


Author contributions: This project was designed in collaboration with Prof. Margo Moore, Mark Warwas and the author of this thesis. I performed the qPCR experiments, protein purification, trans-sialidase activity assay, sialidase activity assays on biological substrates, preferred carbon source utilization experiment, and lectin binding experiments. Mark Warwas performed the computational analysis, cloned the sialidase gene, protein purification, kinetic analysis, and pH profile analysis. Deepani Indurugalla synthesized α2,3-SMUG and α2,6-SMUG, and assisted with the kinetic analysis. Phylogenetic analysis was completed by Prof. Margo Moore and Prof. Arne Mooers.

2.1. Abstract

A gene encoding a putative sialidase was identified in the genome of the opportunistic fungal pathogen, *Aspergillus fumigatus*. Computational analysis showed that this protein has Asp box and FRIP domains, it was predicted to have an extracellular localization, and a mass of 42kDa, all of which are characteristics of sialidases. Structural modeling predicted a canonical 6-bladed β-propeller structure with the model’s highly conserved catalytic residues aligning well with those of an experimentally determined sialidase structure. The gene encoding the putative A.
*fumigatus* sialidase (AfS) was cloned and expressed in *Escherichia coli*. Enzymatic characterization found that the enzyme was able to cleave the synthetic sialic acid substrate, 4-methylumbelliferyl α-D-N-acetylneuraminic acid (Neu5Ac-Mu), and had a pH optimum of 3.5. Further kinetic characterization using 4-methylumbelliferyl α-D-N-acetylneuraminylgalactopyranoside revealed that AfS preferred α2,3-linked sialic acids over the α2,6 isomers. No *trans*-sialidase activity was detected. qPCR studies showed that exposure to MEM plus human serum induced expression. Purified AfS released sialic acid from diverse substrates such as mucin, fetuin, epithelial cell glycans and colominic acid, though *A. fumigatus* was unable to use either sialic acid or colominic acid as a sole source of carbon. Phylogenetic analysis revealed that the fungal sialidases were more closely related to those of bacteria than to sialidases from other eukaryotes.

2.2. Introduction

*Aspergillus* species are saprophytic fungi that are found in soil, water and decaying organic matter. There are greater than 200 species of *Aspergillus*; however, only *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans* and *Aspergillus terreus* cause invasive disease (invasive aspergillosis) in immunocompromised humans (4). Although it is not the most prevalent *Aspergillus* species, *A. fumigatus* accounts for 53-68% of all *Aspergillus* infections (197) suggesting that it possesses specific virulence factors that promote host colonization. Immunocompromised patients such as those undergoing bone marrow and solid organ transplant, patients with hematological malignancies, HIV/AIDS patients and patients with chronic granulomatous disease, are most at risk of developing invasive aspergillosis (5, 198, 199). Antifungal drugs are used to treat invasive aspergillosis; however, the current success rate averages only 53% (200) with mortality rates ranging between 20% and 84% for hematopoetic stem cell and solid organ transplant recipients, respectively (201). Invasive aspergillosis is now a principal cause of death in medical centers worldwide (202). Due to the high incidence and severity of infection and lack of adequate antifungal therapy, understanding the mechanisms underlying pathogenesis by *A. fumigatus* is crucial.
Infection by *A. fumigatus* occurs primarily via inhalation of airborne conidiospores (conidia). As the first step in fungal colonization is thought to be the adhesion of conidia to lung tissue, several studies have attempted to identify adhesins present on the spore surface. Work from our laboratory has shown that *A. fumigatus* has surface sialic acids which may mediate adhesion to basal lamina proteins (66). Furthermore, pathogenic species of *Aspergillus* were shown to have a higher density of sialic acids compared to non-pathogenic species (87). More recently, we have demonstrated that sialoglycoconjugates on the conidial surface mediate the uptake of spores into human Type II pneumocytes and cultured murine macrophages (88). Although no sialic acid biosynthetic genes have been identified to date in the genome sequence of *Aspergillus*, sialic acid biosynthesis has been reported to occur de novo (87). An alternative pathway for sialic acid acquisition in microbes is the uptake of pre-formed sialic acid via plasma membrane permeases (113).

Sialic acids are released from available glycans by the action of sialidase enzymes (*N*-acetylneuraminic acid hydrolase, EC 3.2.1.18); a family of exo-glycosidases that catalyze the hydrolytic cleavage of sialic acid residues in glyconjugates. Sialidases are widespread in nature and to date, they have been identified in viruses, bacteria and animals (203). Sialidases play important roles in diverse biological processes such as lysosomal catabolism (204), muscle cell differentiation (205), cancer invasion and metastasis (Miyagi *et al.*, 2004), nutrition and microbial pathogenesis (133). Viral sialidases (neuraminidases) are important in the release of newly formed virus particles from the host cell thereby playing an important role in the life cycle of the virus (206). In the bacterial pathogen *Pseudomonas aeruginosa*, sialidase was found to play a significant role in biofilm production leading pneumonia and bacteremia in a mouse model of the disease (207). In another bacterial pathogen, *Vibrio cholerae*, sialidase is thought to convert complex gangliosides to GM1, the receptor for cholera toxin, thereby increasing binding of the cholera toxin to host cells expressing the receptor and contributing to the pathogenicity of this organism (208). Finally, *Streptococcus pneumoniae* uses two sialidases along with other exoglycosidases to obtain carbohydrates from host glycoproteins that are utilized as carbon sources required for growth (209).
We have recently discovered a gene encoding a putative sialidase in the genome sequence of *Aspergillus fumigatus* Af293. Here, we report the first successful cloning and expression of a fungal sialidase. Because of the importance of sialidases in microbial pathogenesis, the specific aims of the current research were: to clone and characterize the *A. fumigatus* sialidase (*AfS*) and assess its substrate specificity and kinetic parameters, to monitor gene expression in various growth media, to determine the substrate specificity of the purified enzyme, to determine whether sialic acids are used for nutrition in *A. fumigatus*, and to determine the phylogenetic relationship of this enzyme with other bacterial and animal sialidases.

### 2.3. Materials and Methods

#### 2.3.1. Computational analyses

A 1221 base pair gene encoding a putative sialidase was identified in the *A. fumigatus* Af293 genome database available at The Institute of Genomic Research (TIGR) (http://www.tigr.org) (now the J. Craig Venter Institute) using a keyword search. The intron/exon structure of the gene was determined using the program GlimmerM (http://www.tigr.org/tdb/glimmerm/glmr_form.html) optimized for *A. fumigatus*. The derived protein sequence was then analyzed using the programs iPSORT (http://hc.ims.u-tokyo.ac.jp/ipsort/) (210), SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) (211) and NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) to determine its localization, presence and length of signal peptide, and to identify potential N-glycosylation sites. Sequence alignments were performed using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Finally, the derived protein sequence was submitted to the PHYRE protein homology/analogy recognition engine (Imperial College of London, http://www.sbg.bio.ic.ac.uk/phyre/) (212), to generate a tertiary structural model using the sialidase from *Salmonella typhimurium* (Protein Data Bank (PDB) file: 3sil) as a template. Model quality was assessed using DeepView Swiss-PDB Viewer (http://ca.expasy.org/spdbv/) (213) and images prepared using the molecular visualization system, PyMol (DeLano Scientific LLC, Palo Alto, CA).
A 1020 bp upstream fragment of the putative sialidase was analyzed for regulatory sequences in the untranslated region both manually and with the program Match - 1.0 Public, which uses a library of mononucleotide weight matrices from TRANSFAC 6.0 (http://www.gene-regulation.com) (214).

2.3.2. Phylogenetic analysis

The phylogeny of selected sialidases was inferred using both distance and maximum likelihood techniques in PHYLIP (Phylogeny Inference Package) version 3.67 (distributed by the author, Department of Genome Sciences, University of Washington, Seattle) (215). Data was gathered by submitting the predicted amino acid sequence of the putative A. fumigatus sialidase to Blastp (216). Twenty-one bacterial, fungal or vertebrate sialidases with E values less than 4e-07 were selected for further analysis. The sequences used were as follows: XP_001266669.1, conserved hypothetical protein [Neosartorya fischeri NRRL 181]; XP_001214142.1, predicted protein [Aspergillus terreus NIH2624]; XP_001226947.1, hypothetical protein CHGG_09020 [Chaetomium globosum CBS 148.51]; YP_001107328.1, neuraminidase [Saccharopolyspora erythraea NRRL 2338]; NP_827111.1, neuraminidase [Streptomyces avermitilis MA-4680]; NP_630638.1, neuraminidase (secreted protein) [Streptomyces coelicolor A3(2)]; ZP_02071388.1, hypothetical protein BACUNI_02826 [Bacteroides uniformis ATCC 8492]; YP_213298.1, putative sialidase [Bacteroides fragilis NCTC 9343]; Q02834.1, NANNH_MICVI Sialidase precursor (Neuraminidase)(Micromonospora viridifaciens); AAI52169.1, Zgc:100806 [Danio rerio]; NP_001103203.1, hypothetical protein LOC100000684 [Danio rerio 2]; ABO30984.1, Neu3.1 [Danio rerio 3]; YP_001304276.1, glycoside hydrolase family 33, candidate sialidase [Parabacteroides distasonis ATCC 8503]; YP_001138502.1, hypothetical protein cgR_1608 [Corynebacterium glutamicum]; NP_776547.1, sialidase 3 (membrane sialidase) [Bos taurus]; XP_001366978.1, PREDICTED: similar to sialidase 3 [Monodelphis domestica](Opposum); YP_001301367.1, glycoside hydrolase family 33, candidate sialidase [Bacteroides vulgatus ATCC 8482]; Q9UQ49.1, NEUR3_HUMAN Sialidase-3 (Membrane sialidase) (Ganglioside sialidase) (N-acetyl-alpha-neuraminidase 3); NP_057929.1, neuraminidase 3 [Mus musculus](Mouse1); BAE26342.1, unnamed protein product [Mus musculus](Mouse2); and NP_446462.1, neuraminidase 3 [Rattus norvegicus].
Sequences were aligned and formatted for PHYLIP analysis using Clustal X2.0. Maximum-Likelihood and Neighbour-Joining algorithms were employed using the Jones-Taylor-Thornton matrix and 100 replicates were used for bootstrapping. Unrooted bootstrap consensus trees were compiled and the output files visualized using the program, Dendroscope v1.2.4 (217). Statistical tests of user trees was done in PHYLIP using the Kishino-Hasegawa test as implemented in proml (218).

The AfS sequence was also used to search all the predicted protein sequences for the fungi that have been sequenced or are being sequenced at the Broad Institute (http://www.broad.mit.edu/cgi-bin/annotation/cgi/blast_page.cgi) and by the Concordia University Fungal Genomics Project (https://fungalgenomics.concordia.ca/). Codon usage was analyzed by the Graphical Codon Usage Analyzer v2, Oct 2006 (219).

2.3.3. Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) was used to assess expression of the sialidase in *A. fumigatus*. To accomplish this, DNase-free total RNA was prepared from *A. fumigatus* mycelia cultured at 37°C for 6 hours in minimal essential medium (MEM) (Life Technologies) supplemented with 10% (v/v) human serum (male) (Sigma) and 50 μM FeCl₃ using the Qiagen RNeasy kit (Qiagen, Mississauga, ON) and used as the template for first strand cDNA synthesis. First strand cDNA was synthesized by adding 4.5 μg RNA, 2 pmol oligo-dT primer, 10 mM dNTPs (Fermentas, Burlington, ON), 0.1 M DTT, 40 units RNaseOUT ribonuclease inhibitor, 4 μl 5 x first strand buffer, 200 units Superscript II RNA polymerase (Invitrogen, Burlington, ON) and ribonuclease-free H₂O (Ambion, Austin, TX) for a total volume of 20 μl. The resulting solution was incubated for 60 minutes at 42 °C. The cDNA product (2 μl) was then used as a template for PCR amplification by adding it to a solution consisting of 15 mM dNTPs, 10 pmol forward primer (5'-ATCAACGACCCGGCC (Afsialcdna-F)) and reverse primer (5'-CTAATTGTAGGACCATTTCAGGATC (Afsialcdna-R)), 50 mM MgSO₄, 5 μl 10 x Platinum Pfx PCR buffer, 1.25 units Platinum Pfx DNA polymerase (Invitrogen) and H₂O for a total volume of 50 μl.
2.3.4. Cloning and expression of the *Aspergillus fumigatus* sialidase in *Escherichia coli*

Standard molecular biology methods were performed as described by Sambrook *et al.* (1989) (220). Plasmids were prepared using the Plasmid Mini kit according to the manufacturer's directions (Qiagen). Genomic DNA was obtained from *A. fumigatus* strain ATCC 13073 mycelia grown overnight in MYPD liquid broth (0.3%, w/v, yeast extract and malt extract; 0.5%, w/v, peptone; and 1%, w/v, glucose) using the phenol/chloroform method and was used as a template for polymerase chain reaction amplification of the putative sialidase gene. PCR amplification of the putative sialidase gene was accomplished using the following PCR reagents: 15 pmol forward and reverse primers, 10 mM dNTPs, 2.5 units Taq DNA polymerase (Fermentas), 2 µg *A. fumigatus* genomic DNA, 4% DMSO, 5 µl 10X Taq buffer and H₂O for a total volume of 50 µl. Forward (5`-GGCATTACGGCTAGCATCAACGACCCGGCC (Afsial-F)) and reverse (5`-TACGCACCAGCGGCCGCCTAATTGTTAGGACCATTCCAGGATC (Afsial-R)) primers (Invitrogen) were designed to amplify a fragment beginning 60 base pairs downstream of the transcription start site (to prevent amplification of the predicted signal peptide) and ending at the stop codon and included *NheI* and *NotI* restriction enzyme recognition sites (underlined) to facilitate cloning into the protein expression vector pET28A+ (EMD Chemicals Inc., San Diego, CA). Following PCR amplification, the entire reaction volume was run on an agarose gel and the correct PCR product was excised and purified using the QIAquick gel extraction kit (Qiagen).

The PCR-amplified sialidase gene was treated with restriction enzymes (*NheI*/*NotI* (Fermentas)) and ligated (with the addition of 5% DMSO to the ligation reaction) into the *NheI*/*NotI* site of pET28A+, in-frame with an N-terminal 6xHis tag creating the plasmid, pAfS. The recombinant plasmid was used to transform *Escherichia coli* DH5α (Life Technologies, Gaithersburg, MD) on Luria-Bertani (LB) media supplemented with kanamycin (30 µl ml⁻¹) (BioShop Canada Inc., Burlington, ON) and kanamycin-resistant transformants screened for the presence of pAfS by colony PCR using primers targeting the T7 promoter and terminator (Invitrogen), which flank the multiple cloning site of pET28A+. Correct gene amplification and insertion was verified in several clones by restriction analysis and DNA sequencing (Macrogen, Korea).
pAfS containing a complete and correct in-frame sequence of the putative sialidase was isolated from the appropriate clones and used to transform the protein expression host *E. coli* Tuner (DE3) (EMD Chemicals Inc.). Protein expression in transformed host cells was accomplished by culturing *E.coli* Tuner (DE3) in 250 ml LB-kanamycin broth at 37 °C with shaking at 200 rpm to an OD$_{600}$ of 0.8. This was followed by inducing protein expression with isopropyl β-D-thiogalactopyranoside (IPTG) (BioShop Canada Inc.) at concentrations of 0.2 mM, 0.4 mM, or 1 mM at room temperature, 30 °C or 37 °C with shaking at 200 rpm for 6 or 24 hours. Protein concentration was determined using the method of Bradford (1976).

### 2.3.5. Protein purification

After protein induction, bacterial cells containing the expressed sialidase were cooled on ice and pelleted by centrifugation at 3000 g for 20 minutes at 4 °C. The pelleted cells were then resuspended in lysis buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM imidazole, pH 8.0) at 2 ml/gram bacterial pellet (wet weight). Lysozyme (1 mg ml$^{-1}$) (BioShop Canada Inc.) was added to the bacterial suspension, which was then incubated on ice for 30 minutes. Following lysozyme treatment, the suspension was sonicated with four 10-second bursts at 200 W with 1 minute cooling periods between bursts. The lysate was then centrifuged at 18000 g for 30 minutes at 4 °C to clear cellular debris. The cleared lysate containing the expressed 6xHis-tagged sialidase was added to 1 ml nickel nitritotriacetic acid agarose (Ni-NTA) (Qiagen) equilibrated with lysis buffer and allowed to bind while shaking on a rotary shaker for 60 minutes at 4 °C. Following binding, bound sialidase were eluted from Ni-NTA beads by batch purification; the bound beads were washed with 5 mL of wash buffer #1 (50 mM Tris-HCl, 100 mM NaCl, 20 mM imidazole, pH 8.0) twice and 5 mL of wash buffer #2 (50 mM Tris-HCl, 100 mM NaCl, 50 mM imidazole, pH 8.0) once, with each wash followed by centrifugation at 800 g for 3 minutes at 4 °C. Sialidase is eluted in 5 ml elution buffer (50 mM Tris-HCl, 100 mM NaCl, 250 mM imidazole, pH 8.0), followed by centrifugation at 800 g for 3 minutes at 4°C, and the resultant is collected. Residual Ni-NTA beads are removed by filtering the resultant through a molecular biology column at 4 °C. Imidazole and non-specific proteins were removed by gel filtration chromatography (ÄKTAfPLC, GE Healthcare) with the clean-up buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) at 4 °C.
Fractions collected were analyzed by 12% SDS-PAGE gel. Selected fractions containing only sialidase were concentrated by loading the fraction into a 30,000 molecular weight cut-off centrifugal filter (Millipore, Billerica, MA) and centrifuging at 3000 g for 30 minutes at 4 ºC. The concentrated protein solution was frozen using liquid nitrogen and stored at -80 ºC.

2.3.6. Sialidase activity assays

The activity of enzyme preparations was determined by measuring cleavage of the synthetic sialic acid substrate, 4-methylumbelliferyl α-D-N-acetylneuraminic acid (Neu5Ac-Mu) (Sigma, Oakville, ON), as described previously (221). Briefly, reactions were set up in 96-well plates by adding 200 µM Neu5Ac-Mu, crude cell lysate or purified sialidase and 40 mM sodium formate, pH 3.5 for a total volume of 100 µl. Plates were incubated at 37 ºC for 15 minutes followed by the addition of 200 µl cold stop solution (0.1 M glycine, 0.014 M NaCl, 25% ethanol, pH 10.7). Following the addition of stop solution, the amount of 4-methylumbelliferone released from Neu5Ac-Mu was determined using a Cary Eclipse fluorescence spectrophotometer at excitation and emission wavelengths of 365 and 450 nm, respectively. Fluorescence produced during the course of the reaction was related to the concentration of Neu5Ac-Mu cleaved with a standard curve of 4-methylumbelliferone (Sigma).

To determine the A. fumigatus sialidase activity on biological substrates, the sialidase was incubated with either heat-inactivated A549 cells (a human Type II pneumocyte cell line) (ATCC), bovine mucin (Sigma), colominic acid (Sigma), fetuin (Sigma), and asialofetuin (Sigma) for 3 hours in 40 mM sodium formate, pH 3.5, or in 16 mM sodium tartrate, pH 5.2. To quantify the amount of sialic acid released, the method described by Kolisis (1986) (222) and Simpsons et al. (1993) (223) were used with some modifications and adapted for 96 well plates. The amount sialic acid released from each sialidase reaction was tested in triplicate and each test consisted of 90 µL of sample, 1M Tris-HCl, 0.4 mM NADH, 0.1 units sialic acid aldolase, and 0.2 units lactate dehydrogenase (LDH). Prior to the addition sialic acid aldolase and LDH, the initial A₃₄₀ was recorded spectrophotometrically. The tests were incubated for 1 hour at 37 ºC and the final A₃₄₀ was recorded. Quantification of the total sialic acid released was calculated
as described by Kolisis (1986). The positive control used in these experiments was the 
MvS (Bennet Laboratory, Simon Fraser University); this is a well characterized sialidase 
and the optimal pH of this enzyme is 5.2 (194).

2.3.7. pH rate profile

Optimal pH of the purified enzyme was determined by measuring the specific 
activity of the sialidase towards Neu5Ac-Mu in buffers of varying pH. Buffers used were 
40 mM sodium formate (pH 3.5), 50 mM sodium acetate (pH 4.5) and 32.5 mM 2-(N-
morpholino)ethanesulfonic acid (pH 6) (Sigma). The ionic strength of all buffers was 
maintained at 100 mM with NaCl and all buffers were prepared for use at 37 °C.

2.3.8. Quantitative RT-PCR

Total RNA was obtained from A. fumigatus grown in MYPD for 24 hrs, two 
chemically defined media (Kafer’s and Neilands’) for 24 hrs, and MEM + 10% human 
serum (Sigma) for 2 hrs, 4 hrs, 6 hrs, and 24 hrs at 37 °C. RNA samples were isolated 
using the RNeasy Plant RNA minikit (QIAGEN) and quantified via the NanoDrop ND-
1000 Spectrophotometer. To remove any possible genomic DNA contamination, RNA 
used for cDNA first-strand synthesis was treated with DNase1 (Fermentas). Total first-
strand cDNA was made using qScript cDNA Supermix (Quanta Bioscience) with random 
primers. β-tubulin forward primer (5’-TTC CGC AAT GGA CGT TAC CT) and reverse 
primer (5’-ACA GAG CGG TCT GGA TCT TGT), and sialidase forward primer (5’-CGG 
ACG CGA AGA AGT TCA AC) and reverse primer (5’-TGC CAG TGC CAT CAT TGA 
AG) (Invitrogen) were to design to amplify 148 bp and 153 bp fragments, respectively. 
The expected qPCR products were blasted against the A. fumigatus genome and other 
fungal genomes using WU-Blast2 (www.yeastgenome.org) to ensure sequence 
specificity. Quantitative real-time PCR (qPCR) was completed by using PerfeCTa SYBR 
Green SuperMix (Quanta Bioscience). To validate the use of the 2^−ΔΔCT method (224), an 
efficiency test was completed by performing qPCR on a dilution series of cDNA from A. 
fumigatus grown in MYPD at 37 °C for 24 hrs. The ΔCT (CΔsialidase – CΔβ-tubulin) was 
calculated for each cDNA dilution. A plot of the log cDNA dilution versus ΔCT was made 
and the absolute value of the slope was less than 1 (data not shown). This indicated that
the efficiencies of the target and reference genes were similar and ∆∆CT calculation for the relative quantification of the sialidase gene could be used.

2.3.9. Kinetic analyses

Michaelis-Menten parameters were measured using Neu5Ac-Mu and an α2,3 isomer of 4-methylumbelliferyl α-D-N-acetylneuraminylgalactopyranoside (α2,3-SMUG) (225). Each 400 µl reaction was performed at 37 °C by equilibrating the buffer, substrate (and for the α2,3-SMUG kinetic determination, Aspergillus oryzae galactosidase (Sigma)) for 5 minutes in a heat block prior to addition of 9.6 µg purified sialidase. The progress of the reaction was continuously monitored for 10-15 minutes using a Cary Eclipse fluorescence spectrophotometer equipped with a Peltier temperature controller set to 37 °C and excitation and emission wavelengths of 365 and 450 nm, respectively. For Neu5Ac-Mu, kinetic parameters were determined from 21 initial rate measurements using a substrate concentration range of 500 µM to 4000 µM. For α2,3-SMUG, kinetic parameters were determined from 5 initial rate measurements using a substrate concentration range of 250 µM to 4000 µM. The rate versus substrate concentration data were fitted to the Michaelis-Menten equation using GraphPad (GraphPad Software Inc., San Diego, CA).

2.3.10. Trans-sialidase Activity Assay

The assay used to detect for possible trans-sialidase activity was previously described in Aharoni et al. (2006) (226) and its supplementary materials. Briefly, A. fumigatus (45 µg) or Trypanosoma cruzi trans-sialidase (0.6 µg) were incubated with either 4'-methyl-umbelliferyl linked N-acetyl-neuraminic acid (Neu5Ac-Mu) (Rose Scientific) alone, or Neu5Ac-Mu and bodipy-lactose in 100 µL of 40 mM sodium formate, pH 3.5 for 30s. Bodipy-lactose and Trypanosoma cruzi trans-sialidase were gifts from Dr. Steven Withers’ laboratory (University of British Columbia). Determined in this study, the optimal pH for A. fumigatus sialidase was pH 3.5, and the $K_m$ of A. fumigatus on Mu-Neu5Ac was of 3300 ± 380 µM and the catalytic efficiency ($k_{cat}/K_m$) was 22.3 ± 3.0 M$^{-1}$ s$^{-1}$. Therefore, it is known that N-acetylneuraminic acid (Neu5Ac) can be released from Neu5Ac-Mu by the A. fumigatus sialidase. The products from the trans-sialidase
experiment were analyzed by TLC using a ethyl-acetate:methanol:H2O (7:2:1 v/v/v) mobile phase and visualized under UV at 350 nm. The TLC image was obtained using a PowerShot A720IS digital camera (Canon).

### 2.3.11. Preferred Carbon Source Utilization of *A. fumigatus*

To determine the preferred carbon source use by *A. fumigatus*, 10⁶ *A. fumigatus* spores were inoculated per well in 96-well plates containing chemically defined media with either glucose, colominic acid, or sialic acid as a carbon source, or no carbon source. All carbon sources were tested in triplicate. Media were adjusted to ~pH 6 using K₂HPO₄ prior to inoculation. A parafilm overlay was added to each well to prevent cross contamination of spores from the different wells of the plate. Plates were placed in a moist chamber and incubated at 37 °C and growth of the fungus was monitored for 87 hours spectrophotometrically at A₆₂₀nm (Expert Plus Microplate Reader, Biochrom Asys).

### 2.3.12. Sialic acid detection by lectin binding

*A. fumigatus* conidia were inoculated into minimal media and grown for two weeks at 37 °C. During the two week period, the cultures were replaced with fresh media every three days by first centrifuging the cultures, removing the supernatant and resuspending the mycelia in fresh minimal media. Spores were also inoculated into MYPD broth and incubated for 24 hours at 37 °C. At the end of the incubation period, mycelia were harvested by filtration, washed and incubated with the sialic acid-specific lectin, *Limax flavus* agglutinin (LFA) conjugated to Texas Red (EY Laboratories) as described previously by Wasylinka *et al.* (2001) (87). Controls were incubated in the same way except that the LFA was omitted from the incubation. Fungi were viewed under 400X magnification with a fluorescence microscope (Zeiss) equipped with an LED lightsource (Colibri) with an excitation wavelength of 590 nm. Images were captured with the Zeiss Axiocam MRm.
2.4. Results

2.4.1. Computational analyses

Analysis of the intron/exon structure of the putative sialidase gene using GlimmerM revealed no potential introns in this sialidase. Examination of the amino acid sequence revealed that the sialidase has the canonical FRIP sequence at the N-terminal region, one Asp box (SXDXGXXT/W) and a predicted mass of 42 kDa. Further analysis of the protein sequence with iPSORT, SignalP and NetNGlyc revealed that this putative sialidase has an extracellular localization, a 20 amino acid signal peptide and one low scoring N-glycosylation site at Asn 215. The protein sequence (without the 20 amino acid signal peptide) was then submitted to the PHYRE protein homology/analogy recognition engine to generate a three dimensional model of this sialidase using the sialidase from *S. typhimurium* as a template. The model generated displayed the canonical 6-bladed β-propeller structure consistent with sialidases. Moreover, structural alignment using the sialidases from *S. typhimurium* and *M. viridifaciens* as templates resulted in good overall structural homology with root mean square deviations of only 0.58 Å and 1.42 Å, respectively. An analysis of the Ramachandran plot of the model revealed that only 8 amino acids fell out of the allowed range of phi and psi angles (data not shown). Finally, structural and sequence alignments of the model with other bacterial and animal sialidases showed that catalytic amino acids Asp 92, Glu 260 and Tyr 370 (*M. viridifaciens* numbering) as well as the highly conserved arginine triad of the model align well with those of other sialidases (Figure 2.1).
Figure 2.1 **Structural modeling and sequence alignment of the AsS.**

The putative sialidase was predicted by PHYRE to fold into a 6-bladed β-propeller. (B) Structural superposition of the putative AsS (black) on the sialidase from *M. viridifaciens* (grey) (PDB: 1EUS). (C) Superposition of the *A. fumigatus* model active site on to that of *M. viridifaciens*. It can be seen that catalytic residues (Asp 92, Glu 260 and Tyr 370) as well as the arginine triad (Arg 68, Arg 276 and Arg 342) (*M. viridifaciens* numbering) align well with those of the AsS model. (D) Sequence alignment of the *A. fumigatus* sialidase with sialidase from *M. viridifaciens* (Mv), *Dan rerio* (Dr), *Bos taurus* (Bt), *Homo sapiens* (Hs), *Rattus norvegicus* (Rn) and *Mus musculus* (Mm). Catalytic residues (Asp 92, Glu 260 and Tyr 370), marked with *, and the arginine triad (Arg 68, Arg 276 and Arg 342), marked with ■, are well conserved among these sialidases. The conserved Asp box (SXDXGXT) is outlined.
Regulatory sequences were identified in the promoter sequence of the A. fumigatus sialidase (Figure 2). The eukaryotic consensus sequence GGNCAATCT (ggcccaatct) is located 669 bp upstream of a TATAAA box. Analysis by the Match1.0 program also revealed a STRE motif (agggga) 62 bp upstream of the CAAT box. STRE has been shown to bind C2H2 transcription factors that regulate gene expression in response to stress (227). The STRE-binding transcription factor Seb1 is a C2H2 transcription factor in filamentous fungi that regulates response to osmotic stress (228). A Seb1-like transcription factor has been identified in the genome sequence of A. fumigatus suggesting that the sialidase may be upregulated under stress.

Finally, a single GATA element was found in the upstream region of the sialidase. Trans-acting zinc-finger DNA binding proteins (the GATA proteins) regulate gene expression by binding to these elements in responsive genes; however, binding is significantly weaker when only one GATA element is present (229) suggesting that this element may have only a minor role in controlling gene expression.

2.4.2. Gene expression, sialidase cloning and protein expression

RT-PCR was used to determine whether the putative sialidase gene in A. fumigatus was expressed. Using a total RNA extract as a template, RT-PCR amplified an 1161 bp cDNA product consistent with the expected size of the A. fumigatus cDNA
product thus providing evidence that this sialidase is expressed in *A. fumigatus* (Figure 2.3A). To amplify the *AfS* gene for cloning into a protein expression vector, primers were designed to amplify the gene beginning 60 base pairs downstream of the gene start site to avoid amplification of the signal peptide, which, along with the N-terminal 6xHis tag, may have been cleaved after extracellular secretion by the protein expression host. The amplified fragment was cloned into pET28A+, which was used to transform *E. coli* DH5α (Figure 2.3B). Restriction digest and sequencing were used to verify correct amplification and insertion of the gene into pET28A+ (data not shown). Sequencing revealed a single synonymous base difference T$_{996}$ to C$_{996}$ (maintaining an aspartic acid at this position) between the cloned gene and the gene sequence available for strain 293 in the *A. fumigatus* genome database. The recombinant plasmid was then used to transform the protein expression host, *E. coli* Tuner.
Figure 2.3 Cloning and expression of AfS and activity of lysates against sialic acid substrates

A) RT-PCR using total RNA from A. fumigatus as a template yielded an 1161 bp cDNA band consistent with the sialidase gene product indicating the sialidase was expressed in A. fumigatus under experimental conditions. Lane 1 represents the DNA ladder, lane 2 is the AfS RT-PCR product and lane 3 is the negative RT-PCR control. B) Colony PCR of transformed E. coli yields a 1475 bp band (1161 bp (sialidase gene) + 314 bp (flanking region)) showing that the AfS gene is successfully cloned into the pET28A+ protein expression vector. Lane 1 represents the DNA ladder and lanes 2 – 6 are the colony PCR products of the 5 selected kanamycin-resistant clones. C) Transformation of the E. coli Tuner protein expression strain with pAfS and induction by IPTG (0.2 mM – 1.0 mM) produced a 42 kDa band consistent with the predicted mass of the AfS. Lane 1 represents the protein ladder, lane 2 is the lysate from untransformed E. coli Tuner, lane 3 is the lysate from mock (pET28A+) transformed cells, lane 4 is the lysate from transformed but uninduced cells and lanes 5 – 7 represent cell lysates from E. coli Tuner transformed with pAfS and induced with varying concentrations of IPTG. D) Crude bacterial cell lysates transformed with pAfS and induced with IPTG have activity toward the synthetic sialic acid substrate, Neu5Ac-Mu. Importantly, untransformed, mock transformed and uninduced E. coli Tuner controls do not express a 42 kDa band or have activity toward Neu5Ac-Mu.

Successful transformation was detected by colony PCR (data not shown) and by monitoring the expression of the putative sialidase by SDS-PAGE and by assessing the activity of the crude bacterial cell lysate toward the sialidase substrate, Neu5Ac-Mu. Transformed E. coli Tuner induced with IPTG produced a protein with a molecular mass of 42 kDa, consistent with the predicted mass of this sialidase, and the crude lysate had activity toward Neu5Ac-Mu. The protein expression and enzyme activity were solely attributable to the cloned A. fumigatus gene because untransformed, mock (pET28A+) transformed and transformed but uninduced E. coli Tuner crude lysates did not produce 42 kDa bands and were not able to cleave MUN (Figure 2.3c and 2.3d). Several expression conditions were assessed to maximize protein expression and enzymatic activity and it was found that inducing protein expression with 1 mM IPTG for 24 hours at 30 °C was optimal (data not shown). Furthermore, the addition of bovine serum albumin, CaCl₂, MgCl₂ or MnCl₂ did not increase enzyme activity in the MUN activity assay (data not shown). Finally, the optimal pH of this enzyme was determined using affinity purified sialidase to cleave MUN in buffers ranging in pH from 3 to 6. The data obtained indicated that the AfS has a pH optimum of 3.5 (Figure 2.4).
The purified AfS has a pH optimum of 3.5.

To measure the optimum pH of the sialidase, activity toward MUN was determined at 37°C in buffers ranging in pH from 3 – 6. Buffers used were 40 mM sodium formate (pH 3-3.5), 50 mM sodium acetate (pH 4-5.5) and 32.5 mM 2-(N-morpholino)ethanesulfonic acid (pH 6). Ionic strength of the buffers was maintained at 100 mM with NaCl.

2.4.3. Quantitative RT-PCR

Expression of the AfS was quantified by qPCR of RNA extracted from different conditions. Mean of triplicate tests were conducted for each sample and the data is presented in Table 2.1. The normalized expression level of sialidase in MYPD and in two nutritionally defined fungal minimal media (Kafer and Neiland) was approximately the same. In contrast, when conidia were grown in MEM plus 10% human serum, sialidase gene expression increased 3-5 fold within the first 4 hours of incubation, a period which corresponds to conidial swelling and germination. Thereafter, expression levels decreased almost 20-fold. Agarose gels of the qPCR reactions demonstrated that there were no DNA contamination in these reactions and no non-specific sequences were amplified, therefore the results are indicative to revealing the AfS gene expression (data not shown).
Table 2.1  Relative AfS gene expression values under different growth conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Sialidase Avg. C_T</th>
<th>β-tubulin Avg. C_T</th>
<th>ΔC_T</th>
<th>ΔΔC_T</th>
<th>Normalized sialidase amount relative to β-tubulin (2^ΔΔCT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYPD, 24 hrs</td>
<td>26.18</td>
<td>19.52</td>
<td>6.67</td>
<td>0</td>
<td>1.00 (0.78 - 1.27)</td>
</tr>
<tr>
<td>Kafer’s minimal media, 24 hrs</td>
<td>28.36</td>
<td>21.73</td>
<td>6.63</td>
<td>-0.03</td>
<td>1.02 (0.79 - 1.33)</td>
</tr>
<tr>
<td>Neilands’ minimal media, 24 hrs</td>
<td>26.31</td>
<td>19.41</td>
<td>6.89</td>
<td>0.24</td>
<td>0.86 (0.32 - 2.29)</td>
</tr>
<tr>
<td>MEM + human serum, 2 hrs</td>
<td>32.03</td>
<td>26.91</td>
<td>5.12</td>
<td>-1.54</td>
<td>2.93 (1.75 - 4.93)</td>
</tr>
<tr>
<td>MEM + human serum, 4 hrs</td>
<td>28.23</td>
<td>23.83</td>
<td>4.4</td>
<td>-2.26</td>
<td>4.80 (2.76 - 8.34)</td>
</tr>
<tr>
<td>MEM + human serum, 6 hrs</td>
<td>31.75</td>
<td>22.22</td>
<td>9.52</td>
<td>2.87</td>
<td>0.14 (0.08 - 0.24)</td>
</tr>
<tr>
<td>MEM + human serum, 24 hrs</td>
<td>32.46</td>
<td>22.68</td>
<td>9.78</td>
<td>3.12</td>
<td>0.12 (0.10 - 0.13)</td>
</tr>
</tbody>
</table>

Data reported are the mean of triplicate runs conducted for each sample of RNA from the different growth conditions. A. fumigatus conidia were grown in MYPD for 24 hrs, two chemically defined media (Kafer’s and Neilands’) for 24 hrs, and MEM + 10% human serum for 2 hrs, 4 hrs, 6 hrs, and 24 hrs at 37°C. β-tubulin was used as a reference gene to normalize data. ΔC_T = C_T value of gene of interest (sialidase) – C_T value of reference gene (β-tubulin). ΔΔC_T = ΔC_T test condition - ΔC_T calibrator condition. The calibrator condition is MYPD for 24 hrs at 37°C. Fold change value is calculated as 2^ΔΔCT according to Livak & Schmittgen, 2001.

2.4.4. Enzyme kinetics

Michaelis-Menten kinetic parameters of the AfS were measured using Neu5Ac-Mu and an α2,3 isomer of 4-methylumbelliferylα-D-N-acetylneuraminylgalactopyranoside (α2,3-SMUG) as substrates (225). The AfS had a K_m of 3300 ± 380 µM and a catalytic efficiency (k_{cat}/K_m) of 22.3 ± 3.0 M^{-1} s^{-1} for Neu5Ac-Mu. The α2,3 linkage was preferentially cleaved over substrates with an α2,6 linkage; the AfS had a K_m of 3100 ± 780 µM and catalytic efficiency (k_{cat}/K_m) of 0.144 ± 0.001 M^{-1} s^{-1} toward α2,3-SMUG (Table 2.2).
### Table 2.2  Kinetic parameters of selected sialidases

<table>
<thead>
<tr>
<th>Sialidase</th>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
<th>Specific activity (nmol mg protein$^{-1}$ h$^{-1}$)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. fumigatus</em></td>
<td>Neu5Ac-Mu</td>
<td>3300 ± 380</td>
<td>0.0743 ± 0.0050</td>
<td>22.3 ± 3.0</td>
<td>383.7 ± 19.1$^a$</td>
<td>Warwas et al., 2010</td>
</tr>
<tr>
<td></td>
<td>α2,3-SMUG</td>
<td>3100 ± 380</td>
<td>0.0004 ± 0.0001</td>
<td>0.144 ± 0.001</td>
<td>6.7 ± 2.1$^d$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α2,6-SMUG</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>2.2 ± 0.4$^d$</td>
<td></td>
</tr>
<tr>
<td><em>HsNeu2</em></td>
<td>Neu5Ac-Mu</td>
<td>440</td>
<td></td>
<td></td>
<td>3060$^{a,c}$</td>
<td>Monti et al., 1999;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chavas et al., 2005</td>
</tr>
<tr>
<td><em>Neu3</em></td>
<td>Neu5Ac-Mu</td>
<td>140</td>
<td></td>
<td></td>
<td>250.7$^a$</td>
<td>Ha et al., 2004; Monti et</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>al., 2000</td>
</tr>
<tr>
<td><em>C. perfringens</em></td>
<td>Neu5Ac-Mu</td>
<td>165 ± 0.024</td>
<td></td>
<td>1.7 X 10$^7$</td>
<td></td>
<td>Kruse et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Neu5Ac-Mu</td>
<td>35 ± 6</td>
<td>44.0 ± 0.1</td>
<td>(1.3 ± 0.2) X 10$^6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. viridifaciens</em></td>
<td>Neu5Ac-Mu</td>
<td>380 ± 70</td>
<td>0.20 ± 0.02</td>
<td>530 ± 150</td>
<td></td>
<td>Watson et al., 2003;</td>
</tr>
<tr>
<td></td>
<td>α2,3-SMUG</td>
<td>380 ± 70</td>
<td>0.20 ± 0.02</td>
<td>530 ± 150</td>
<td></td>
<td>Indurugalla et al., 2006</td>
</tr>
<tr>
<td></td>
<td>α2,6-SMUG</td>
<td>240 ± 30</td>
<td>0.069 ± 0.003</td>
<td>290 ± 50</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Assays were conducted using 200 μM Neu5Ac-Mu
$^b$ Assay was conducted using 1 mM Neu5Ac-Mu
$^c$ Enzyme expressed and purified from Cos7 cells
$^d$ Assays were conducted using 500 uM α2,3-SMUG or α2,6-SMUG substrate
n.d. denotes not determined

#### 2.4.5. Substrate profile

AfS was able to release sialic acids from a wide variety of natural substrates including bovine salivary mucin, colominic acid (a homopolymer of sialic acid with α2,8-ketosidic linkages), bovine fetuin, a serum glycoprotein containing both α2,6- and α2,3-linkages in a ratio of ~31:19 (230), and glycoproteins and glycolipids from thermally-denatured human lung epithelial cells (A549 pneumocyte cell line, containing both α2,3-
and α2,6-linkages) (Figure 2.5). Two pH conditions were tested because the optimal pH for AfS was pH 3.5 and the optimal pH for the positive control, *Micromonospora viridifaciens* sialidase (MvS), was pH 5.2.

![Bar chart showing relative activity of AfS with various substrates at pH 3.5.](chart)

**Figure 2.5**  **AfS activity with various sialylated substrates at pH 3.5.**

Enzymatic activity of AfS was measured with heat-inactivated A549 cells (a human pneumocyte cell line), bovine mucin, colominic acid, fetuin, and asialofetuin. All substrates were tested at the pH optimum for the enzyme as described in Materials and Methods. The specific activity of each enzyme was measured against the specific activity against fetuin to calculate the relative activity of the enzyme towards each biological substrate.

In both pH conditions, the asialofetuin control contained no released sialic acids, as expected. The substrate profiles at pH 3.5 is shown in Figure 5. At pH 5.2, activity was higher with A549 cells and fetuin (data not shown) suggesting that, even though the enzyme activity is lower at pH 5.2, the substrate was more accessible. MvS ranged from five times to over 200 times more effective in removing sialic acid from all of the substrates at both pH compared to the fungal enzyme (Table 2.3). Despite the ability of the sialidase to release sialic acid from these substrates, *A. fumigatus* was unable to use either colominic acid (poly α2,8 sialic acid) or sialic acid as a sole source of nutrition (Figure 2.6).
Table 2.3  *AfS* activity compared to the activity of *MvS*

<table>
<thead>
<tr>
<th></th>
<th><em>A. fumigatus</em></th>
<th><em>M. viridifaciens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 5.2</td>
<td>Specific Activity (nmol/mg protein/hr)</td>
<td>Specific Activity (nmol/mg protein/hr)</td>
</tr>
<tr>
<td>A549 cells</td>
<td>11.7</td>
<td>69.7</td>
</tr>
<tr>
<td>Mucin</td>
<td>11.2</td>
<td>2736.6</td>
</tr>
<tr>
<td>Colominic Acid</td>
<td>17.0</td>
<td>3906.8</td>
</tr>
<tr>
<td>Fetuin</td>
<td>54.9</td>
<td>4072.9</td>
</tr>
<tr>
<td>Asialofetuin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pH 3.5</td>
<td>2.7</td>
<td>385.7</td>
</tr>
<tr>
<td>22.4</td>
<td>5907.1</td>
<td></td>
</tr>
<tr>
<td>38.1</td>
<td>5324.6</td>
<td></td>
</tr>
<tr>
<td>41.8</td>
<td>2381.1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.6  Growth of *A. fumigatus* on different nutritional sources.
K indicates the defined medium (Kafer's) with the additional 1% carbon source supplemented as indicated; where Glu = glucose, Sialic Acid= Neu5Ac.
2.4.6. *Trans*-sialidase activity

*Trans*-sialidase activity was not detected with the AfS. Figure 2.6 shows that incubation of AfS with bodipy-lac, a fluorescent substrate for *trans*-sialylation, and Neu5Ac-Mu generated Mu but produced no sialylated derivative (bodipy-Lac-SA). These data indicate that AfS was able to remove terminal Neu5Ac residues but was unable to catalyze their transfer to the acceptor substrate. The positive control, recombinant *trans*-sialidase from *Trypanosoma cruzi* (*TcTS*), produced the bodipy-Lac-SA derivative, as expected.

![Figure 2.7](image)

**Figure 2.7** *Trans*-sialidase activity analysis of AfS

AfS was incubated with Neu5Ac-Mu, or with Neu5Ac-Mu and bodipy-lactose. Sialidase activity cleaves the sialic acid from MUN releasing the fluorescent product 4-methylumbelliferone (Mu). *Trans*-sialidase activity results in the addition of sialic acid to the acceptor, bodipy-lactose (B-Lac) forming the more polar product, bodipy-lactose-sialic acid (B-Lac-SA). Recombinant TcTS formed B-Lac-SA (and some Mu as expected); however, no B-Lac-SA was formed by AfS. The faint band in the AfS lane that runs slightly higher than B-Lac-SA is Neu5Ac-Mu, confirmed by its purple fluorescence. SA = Neu5Ac.
2.4.7. *De novo* synthesis of sialic acids

In a previous study, we reported that *A. fumigatus* was able to synthesize sialic acids when grown on solid media containing no sialic acid, suggesting that sialic acid biosynthesis occurred *de novo* (87). To confirm this finding, we used synthetic liquid media to ensure that no sialylated compounds could have contributed to sialic acids in the fungus. Figure 2.7 shows that *A. fumigatus* mycelia bind the sialic acid-specific lectin, LFA, when grown in liquid minimal medium containing only glucose as a carbon source. Similar results were obtained with growth in complex medium, MYPD. Staining intensity in MYPD was greater, confirming our previous finding that sialic acid density is enhanced when the fungus is grown in this medium (87). Thus, *de novo* biosynthesis of sialic acids occurs in *A. fumigatus*. 
Figure 2.8  Fluorescence imaging of lectin labelling of *A. fumigatus* grown in broth media. *A. fumigatus* was grown in minimal media where glucose was the only carbon source (A - B) and in MYPD (E - F). After washing, mycelia were incubated with LFA bound to Texas Red and processed for fluorescence microscopy as described in Materials and Methods. Controls were treated identically except that LFA-TR was omitted (minimal media, C – D; MYPD, G – H).
2.4.8. Phylogenetic analysis

The phylogeny of the fungal sialidase was investigated using both Neighbour-Joining distances inferred from protdist and Maximum Likelihood analysis of protein evolution; both resulted in the same placement of the sialidase from A. fumigatus. The consensus tree generated from the maximum likelihood analysis is shown in Figure 2.8. Many studies have shown that the Fungi are the sister group to animals and form part of the crown group of eukaryotes along with plants and animals (231, 232). The four fungal species formed a separate cluster from both the animal and bacterial sialidases. The closest relative to A. fumigatus was N. fischeri; this reflects the known close phylogenetic relationship between these organisms (233). However, our analyses also revealed that the fungal sialidases are more closely related to sialidases from Actinobacteria belonging to the order Actinomycetales, in particular, Streptomyces coelicolor, Streptomyces avermitilis and Saccharopolyspora erythraea. Although it is in a different genus, S. erythraea biology closely resembles that of these streptomycetes (234). Interestingly, sialidases from two other Actinobacteria, M. viridifaciens and C. glutamicum, clustered separately with other Bacteria, the Bacteroidetes (B. vulgatus, P. distasoni, B fragilis and B. uniformis) (Fig. 2.8) and the Planctomycetes (data not shown). To determine whether the data statistically supported this separation of Actinomycetales, we performed a Kishino-Hasegawa test for two trees; the original tree versus one in which the S. avermitilis, S. coelicolor and S. erythraea sialidases were constrained to be in a clade with M. viridifaciens and C. glutamicum. The results showed that this tree was significantly worse (Diff logL = -12) than the tree shown in Figure 2.8. Consistent with the finding that the fungal sialidase has no trans-sialidase activity, the trans-sialidase protein of Trypanosoma cruzi formed a unique branch that diverged from the Bacteroides spp., distinct from the C. glutamicum/M. viridifaciens sialidases (data not shown).

Only three other species of Fungi, A. terreus, C. globosum and N. fischeri (shown on tree) gave significant blastp hits when searched against the A. fumigatus sialidase. The candidate pool included fungi being sequenced by both the Broad Institute and the Concordia University Fungal Genomics project (a total of 40 species representing zygomycetes, chytridiomycetes, ascomycetes and basidiomycetes). In addition to N.
*fischeri*, the genomes of several other *Aspergillus* species have been sequenced; however, other than *A. terreus*, we found no significant matches, including from *Aspergillus nidulans* (data not shown). The *A. nidulans* genome has two proteins that have sialidase domains and one protein has two Asp boxes. However, these genes are unlikely to encode sialidases: no FRIP sequences are present, and the top models generated by PHYRE did not align well to the *M. viridifaciens* sialidase as only 189 and 304 atoms were aligned in each protein (compared to thousands of atoms in the *AfS*) and there was no alignment of catalytic residues (data not shown). Many glycoside hydrolases as well as other non-homologous proteins possess Asp-box repeats (235) and these sequences may encode other glycoside hydrolases.

![Figure 2.9](Image)

**Figure 2.9** Unrooted bootstrap consensus tree from maximum-likelihood analysis of the amino acid sequences of 22 sialidases from Bacteria, Animalia and Fungi.

The mammalian and fish sialidases (all sequences on the right split at 100% bootstrap) form a cluster distinct from the Bacterial and Fungal sialidases. Despite the fact that Fungi are more closely related to animals than bacteria, the fungal sialidases (*N. fischeri, A. fumigatus, A. terreus* and *C. globosum*) are more closely related to a subset of bacterial sialidases from the Actinobacteria, *S. avermitilis, S. erythraea* and *S. coelicolor*. 
2.5. Discussion

We have successfully cloned and expressed a sialidase from *Aspergillus fumigatus* which represents the first cloning of a fungal sialidase. Initial computational analysis indicated a protein mass of 42 kDa, the presence of conserved FRIP and Asp-box domains and folding into the canonical 6-bladed β-propeller, characteristics which are present in all known sialidases (236). Molecular modeling revealed that the active site of the AfS aligned well with that of the experimentally determined structure of the sialidase from the bacterium, *M. viridifaciens* (pdb 1EUS). Tyr 370, Asp 92 and Glu 260 of the MvS, which function as the nucleophile, general base and general acid, respectively, correspond with Tyr 338, Asp 64 and Glu 229 of the AfS model. Furthermore, the highly conserved arginine triad, which functions to stabilize the sialic acid carboxylic acid is also conserved further supporting the quality of this model (236). The fungal sialidase was enzymatically active when tested using standard substrates.

The kinetic parameters $K_m$ and $k_{cat}$ and specific activity of the AfS toward Neu5Ac-Mu are lower than those reported for the bacterial sialidases from *C. perfringens* and *M. viridifaciens* (Table 2.2) (225, 237); however, the specific activity of the AfS is similar to that of the human ganglioside sialidase Neu3 (204, 238). One possible explanation for the lower activity of the fungal sialidase compared to bacterial sialidases could be that the substrate (Neu5Ac-Mu) used for these assays is not the natural substrate for this enzyme. For example, human ganglioside sialidase, Neu3, preferentially acts on the ganglioside GD1a with a lower $K_m$ value (98 µM for GD1a compared to 143 µM for Neu5Ac-Mu) and a five fold greater specific activity (120 nmol mg protein$^{-1}$ h$^{-1}$ for Neu5Ac-Mu compared to 600 nmol mg protein$^{-1}$ h$^{-1}$ for GD1a) than it does for Neu5Ac-Mu (237). Furthermore, Neu3 preferentially cleaves terminal α2,8-linked sialic acids from GD3 over GD1b despite their similar terminal trisaccharides (Neu5Ac(α2,8)Neu5Ac(α2,3)Gal), illustrating the important role that the entire substrate molecule plays in the recognition process (204). Crystallization of the AfS and structural analysis will likely provide more information regarding substrate recognition and catalytic mechanism. Alternatively, the decreased activity may be due to a lack of proper posttranslational modification due to the expression of the eukaryotic AfS in bacteria. There is a potential, albeit low scoring, N-glycosylation site at Asn 215; glycosylation at
this site may be important for proper enzyme folding and function. No N-glycosylation has been detected in the human cytosolic sialidase, Neu2 (239); however, elimination of glycosylation at one of the four glycosylation sites in the neuraminidase from the Newcastle disease virus was found to significantly decrease neuraminidase activity and this corresponded to a decreased virulence of the virus (240).

Bacterial sialidases have an important role in nutrition by supplying the bacterium with both carbon and nitrogen. The AfS is able to remove sialic acid from four characterized sialylated biological substrates found in nature. It was most efficient at removing sialic acid from bovine fetuin, but least efficient at removing sialic acid from thermally-denatured human lung epithelial cells (A549 cells) and bovine salivary mucin. Although all three substrates have α2,3 and α2,6 linked sialic acids, the exact amount of α2,3 linked sialic acid on the substrates are unknown. However, the fungal enzyme was not as efficient as the MvS. Moreover, of the sialic acid substrates tested, only fetuin and asialofetuin were nutritional sources for the fungus showing similar growth after 80 hours (Figure 2.6). Therefore, it is likely that A. fumigatus is utilizing the proteins as a source of amino acids for nutrition. The fungal sialidase was also able to cleave sialic acid from colominic acid, a homopolymer of sialic acid with α2,8-ketosidic linkages, indicating that it has ability to cleave polysialic acid. Polysialic acids are found on the capsule of many bacteria, such as E. coli K1 and N. meningitidis; these microbes are found either in the soil which is the natural habitat of A. fumigatus or the respiratory tract of mammalian hosts which is the site of infection for A. fumigatus (241, 242). Similar to C. perfringens and S. pneumoniae, it's possible that A. fumigatus employs its sialidase to modify glycan on other bacteria (135, 243). Because we have shown that A. fumigatus cannot use sialic acid as a sole source of carbon, the sialidase is likely to play a more important role in removal of terminal sialic acid from glycoproteins and glycolipids.

Sialidases have been isolated and characterized from a wide variety of eukaryotes and prokaryotes. As far as we are aware, sialidase activity of cells or culture filtrates has been reported for only a handful of fungi including Sporothrix schenckii (244) and its teleomorph, Ophiostoma stenocera (245), as well as Fonsecaea pedrosoi (Alviano et al. 2004). Saito and Yu (1995) (247) summarized the characteristics of microbial sialidases and included fungal sialidases in the title; however, no true fungi
were included in their study. Another study indicated that *Candida albicans* possessed sialidase activity (248); however, this was later refuted by others (245). In support of this, we did not identify a sialidase gene in the *C. albicans* genome in our study. Unfortunately, genome sequences for *Ophiostoma sternocera* and *Fonsecaea pedrosoi* are not yet available so we could not include these in our phylogenetic analysis. In our study, fungal sialidases form a unique cluster more closely related to a subset of prokaryotic sialidases than to enzymes from other eukaryotes. Furthermore, sialidases were only detected in a few fungi from diverse lineages suggesting that these genes were acquired horizontally or have been lost from, or changed significantly in, all but a few ascomycete fungi. A possible bacterial origin of the fungal sialidases is suggested by our analyses; while the majority of *A. fumigatus* genes (77%) contain introns with an average of 1.8 per gene (249), the *A. fumigatus* sialidase does not contain any introns; the mean difference in codon usage between the fungal sialidase and other *A. fumigatus* genes was greater than the difference between the sialidase codon usage and all *S. avermitilis* genes (24.3% versus 10.4%, respectively). The % G+C value of the *A/š* (64.7%) was intermediate between the average % G+C for *A. fumigatus* exons (54%) (233) and the average for streptomycetes (approximately 70%). Finally, the Actinobacteria and the fungi that contain sialidase share similar habitats which increases the possibility of gene transfer; all are filamentous, soil-dwelling saprobes that secrete hydrolytic enzymes to break down naturally-occurring polymers as a source of carbon and energy. Nevertheless, these data do not rule out the possibility that the gene was acquired by a common ancestor of the Pezizomycotina (which includes all of the fungal species with putative sialidases) (250) but then retained in only a few species.

Sialidase expression in *A. fumigatus* was upregulated during germination in MEM containing 10% human serum. Using microarray analysis, we have found that serum exposure induces stress response genes (unpublished data) and mediators of the stress response may activate *A. fumigatus* gene expression through the STRE motif or via other uncharacterized mechanisms. Alternatively, sialidase expression may be developmentally regulated in *A. fumigatus*.

The role of the *A/š* in the life cycle of the fungus or in pathogenesis is currently unclear. Recently, we have shown that α2,6-linked unsubstituted sialic acids are present
on the surface of *A. fumigatus* conidia and that these sugars play important roles in mediating the interaction between conidia and mouse macrophages and human type II epithelial cells in vitro (251). *AfS* had no trans-sialidase activity; therefore, sialic acids on the conidial surface must have been synthesized *de novo*, yet the mechanisms are unknown. In other microorganisms, several pathways have been elucidated for the biosynthesis of sialic acid and its display on the cell surface. For example, some bacteria have a complete biosynthetic pathway while others have only a few enzymes and rely on environmental sources for sialic acid or its precursors (Vimr et al. 2004). Even though our results indicate that sialic acid is synthesized *de novo* in *A. fumigatus*, work in our laboratory and by others (106, 196) has failed to identify orthologues of known sialic acid biosynthetic genes in the genomes of *A. fumigatus* and other fungi. The *A. fumigatus* genome does contain genes encoding several nucleotide-sugar transporters and glycosyltransferases, which together may function to sialylate the surface of *A. fumigatus* conidia. Furthermore, Rodrigues et al. (2003) have partially purified a sialyltransferase from another fungal pathogen, *Cryptococcus neoformans* although the gene has not yet been identified. Nevertheless, supplementation of growth media with sialic acid or N-acetylmannosamine (a sialic acid precursor in other organisms) did not enhance sialic acid levels in *A. fumigatus* (196). Alpha-2,3-linked sialic acids are undetectable in *A. fumigatus* (87, 251) and the preference for this substrate suggests that the *A. fumigatus* sialidase is likely not involved in glycan remodelling.

Thus, sialic acid metabolism in fungi has many intriguing findings but the full picture of sialobiology in this Kingdom has yet to emerge.

To further characterize the *AfS*, we collaborated with Judith Telford and Garry Taylor at St. Andrews University. The investigation of the *A. fumigatus* sialidase crystal structure and the mechanism of the enzyme will be discussed in the following two chapters.
Chapter 3.

The *Aspergillus fumigatus* sialidase is a 3-Deoxy-D-glycero-D-galacto-2-nonulsonic Acid Hydrolase (KDNase)

This chapter is a modified form of the paper originally published in the Journal of Biological Chemistry, published by The American Society for Biochemistry and Molecular Biology, Inc. (c) 2011, with permission, of Telford JC, Yeung JHF, Xu G, Kiefel MJ, Watts AG, Hader S, Chan J, Bennet AJ, Moore MM, and Taylor GL. The *Aspergillus fumigatus* sialidase is a 3-deoxy-D-glycero-D-galacto-2-nonulosonic acid hydrolase (KDNase): structural and mechanistic insights. The Journal of Biological Chemistry. 286(12):10782-10792. In addition, some contents of this chapter form part of an ongoing study by the Moore Laboratory.

Author contributions: The project was designed by Prof. Margo Moore, Prof. Andrew Bennet, Prof. Garry Taylor, Dr. Judith Telford and the author of this thesis. Juliana Yeung performed the kinetic analysis and examined the effects of inhibitors, Neu5Ac2en and KDN2en, on the *A. fumigatus* sialidase (AfS). In addition, Juliana Yeung completed the biological assessments: (1) effects of Neu5Ac2en on *A. fumigatus* growth, (2) carbon source utilization, (3) localization of AfS in *A. fumigatus*, (4) phenotype experiments on the *A. fumigatus* sialidase knockout mutant. Drs. Judith Telford, Guogang Xu, Andrew Watts, Stefan Hader (St. Andrews University) crystallized and resolved the different AfS structures. KDN2en and 2,3F-KDN were synthesized by Dr. Milton Kiefel (Griffith University) and KDN-MU and KDN were synthesized by Dr. Jefferson Chan (SFU Chemistry). NMR analysis was completed collaboratively by Dr. Jefferson Chan and Juliana Yeung.
3.1. Abstract

*Aspergillus fumigatus* is a filamentous fungus that can cause severe respiratory disease in immunocompromised individuals. A putative sialidase from *A. fumigatus* was cloned and shown to be relatively poor in cleaving N-acetylneuraminic acid (Neu5Ac) in comparison to bacterial sialidases. The *A. fumigatus* sialidase discussed here is the first crystal structure of a fungal sialidase and the first structure of a KDNase. When the apo structure was compared to bacterial sialidase structures, the active site of the *Aspergillus* enzyme suggested that Neu5Ac would be a poor substrate due to a smaller pocket that normally accommodates the acetamido group of Neu5Ac in sialidases. A sialic acid with a hydroxyl in place of an acetamido group is 2-keto-3-deoxynononic acid (KDN). We show that KDN is the preferred substrate for the *A. fumigatus* sialidase and that *A. fumigatus* can utilise KDN as a sole carbon source. The enzyme was not inhibited by the sialidase transition state analogue-like compound, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (Neu5Ac2en), but it was inhibited by the related KDN2en. In addition, KDN2en bound to the enzyme in a crystal structure. These data along with NMR experiments and further analysis of the AfS crystal structure, revealed that *A. fumigatus* sialidase is therefore a KDNase with a similar catalytic mechanism to Neu5Ac exo-sialidases. AfS was found inside or bound to the fungi and only a small amount was present in culture supernatant.

3.2. Introduction

Sialic acids comprise the most chemically and structurally diverse carbohydrate family. Over 50 naturally occurring analogues exist, the majority based on 5-acetamido-2-keto-3,5-dideoxy-D-glycero-D-galacto-nonulosonic (*N*-acetylneuraminic acid, Neu5Ac) and 2-keto-3-deoxy-D-glycero-D-galacto-nonulosonic (2-keto-3-deoxynononic acid, KDN) that only differ at the C5 position (103). KDN, like Neu5Ac, occurs widely in bacteria and vertebrates, is found in almost all types of glycoconjugates including glycolipids, glycoproteins and capsular polysaccharides, and can be linked α2,3-, α2,4-, α2,6- or α2,8- to other carbohydrates (183). KDN was first discovered in the cortical alveolar polysialoglycoprotein of rainbow trout eggs as the capping carbohydrate on
polysialic acid chains that were resistant to bacterial sialidases (179). In mammals, KDN was first identified in various tissues including human lung carcinoma cells but at a much lower abundances than Neu5Ac (252), and was subsequently found on human red blood cells and ovarian cancer cells (253). The development of linkage specific KDN antibodies (253, 254) has allowed the identification of α2,8-linked polyKDN in many mammalian tissues (255), including the human lung (256).

Sialidases, or neuraminidases, catalyse the removal of terminal sialic acids from a variety of glycoconjugates and play an important role in pathogenesis, bacterial nutrition and cellular interactions. Crystal structures of a growing number of exosialidases that cleave Neu5Ac are available from bacteria (257–260), viruses (261–263), trypanosomes (137, 264), leech (265) and man (266). All sialidases share the same six-bladed β-propeller fold for their catalytic domains, with conservation of key catalytic amino acids (267). The non-viral sialidases have an R-I/L-P motif containing one of the three active site arginines, and also have up to five bacterial neuraminidase repeats (BNRs) or Asp-boxes (S/T-X-D-[X]-G-X-T-W/F). The BNRs occur at topologically identical positions in the β-propeller fold, remote from the active site, but any function beyond dictating a structural fold is unknown. Like many glycoside hydrolases, sialidases can possess carbohydrate-binding modules (CBMs) in addition to the catalytic domain, placed upstream, downstream or even inserted within the β-propeller domain. It has been suggested that the presence of these carbohydrate-binding modules (CBMs) increases the catalytic efficiency of the sialidases, particularly in the presence of polysaccharide substrates (268). In the case of sialidases having CBMs, these often recognise sialic acid as has been shown for the sialidases from Vibrio cholerae (14), Clostridium perfringens (269) and Streptococcus pneumonia (270). Nearly all Neu5Ac-specific sialidases are inhibited by 2-deoxy-2,3-dehydro-N-acetyl-neuraminic acid (Neu5Ac2en, 3), a putative transition-state analogue.

A sialidase specific for KDN ketosidic linkages was first discovered in the bacterium Sphingobacterium multivorum, and this so-called KDNase released KDN from naturally occurring substrates, including KDNα2-3Gal, KDNα2-6GalNAc, and KDNα2-8KDN linkages, but was not inhibited by Neu5Ac2en (178, 180). The KDNase was however inhibited by 2,3-didehydro-2,3-dideoxy-D-glycero-D-galacto-nonulosonic acid
Further analysis of this enzyme using synthetic KDN analogues suggested that the hydroxyl group at C5 was important for recognition of the inhibitor by the enzyme, and that like the Neu5Ac sialidases, the thermodynamically less stable α-form of the product is the first product of cleavage, suggesting a similar catalytic mechanism to the Neu5Ac exosialidases (271, 272).

*Aspergillus fumigatus* is a common soil fungus and is the major species of *Aspergillus* that causes invasive aspergillosis in immunocompromised humans (4). Infection by *A. fumigatus* is mainly through inhalation of airborne conidiospores (conidia) that adhere to lung tissue. It has been shown that *A. fumigatus* conidia have surface sialic acids that may adhere to basal lamina proteins (66), and that pathogenic species of *Aspergillus* have a higher density compared to non-pathogenic species (87). The origin of these sialic acids remains a mystery, as although sialic acid biosynthesis has reported to occur *de novo* in *A. fumigatus* (87), its genome appears to lack the known Neu5Ac biosynthetic enzymes. In addition, *A. fumigatus* is incapable of utilising or incorporating exogenous Neu5Ac or ManNAc (196). Nevertheless, *A. fumigatus* does encode a sialidase that has recently been cloned and characterised (88).

The *A. fumigatus* sialidase has a 20-amino acid signal peptide, an RIP motif, one BNR, and shares 30% sequence identity with the bacterial sialidase from *Micromonospora viridifaciens* whose structure is known (273). The sequence of the *A. fumigatus* sialidase appears to possess the key active site residues of a sialidase: an arginine triad that interacts with the carboxylic acid group of sialic acids, a nucleophilic tyrosine (Tyr<sup>358</sup>), its associated general acid (Glu<sup>249</sup>), and an acid/base (Asp<sup>84</sup>) (274).

This chapter will focus on the biochemical analysis of the *A. fumigatus* sialidase (AfS), and the localization of the enzyme in *A. fumigatus*. The crystal structures of the recombinant AfS protein will be discussed. With the AfS apo structure, a comparison of the active site with the *M. viridifaciens* sialidase (MvS) was possible and suggested that KDN might be a better substrate than Neu5Ac for the *A. fumigatus* enzyme. It was shown that this is the case using kinetic analyses with the fluorescent substrate 4-methylumbelliferyl3-deoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid (KDN-MU). NMR was used to show that catalysis of KDN-MU by AfS proceeds with retention of
configuration at the anomeric carbon. AfS was not inhibited by Neu5Ac2en, but by the KDN-related KDN2en; this was demonstrated both biochemically and as a crystal with KDN2en in the active site of AfS. Similarly, Neu5ac2en did not affect *A. fumigatus* growth and *A. fumigatus* can utilise KDN, and not Neu5Ac, as an effective sole carbon source. By Western blot and indirect immunofluorescence, AfS was found to be ubiquitously distributed throughout the cell, though a higher amount of the protein was found around the periphery of the fungi. These studies suggest a nutritional role for AfS in the lifecycle of *A. fumigatus*, but also stimulate further studies into the potential role of KDN in the pathogenesis of the organism.

3.3. Materials and Methods

3.3.1. Expression and purification of AfS for structural studies and biochemical analysis

The AfS gene from the *A. fumigatus* clinical isolate Af293 was amplified and ligated into the pET28A+ (EMD Chemicals Inc., San Diego, CA) vector and expressed in *Escherichia coli* (DE3) cells as previously described (274). Briefly, *E. coli* expressing the AfS/PET28A+ vector were grown in Luria Broth with 50 µg/ml kanamycin at 37 °C, shaking at 220 rpm. Once the culture OD$_{600}$ had reached 0.9 AfS expression was induced by 0.5 mM IPTG (Isopropyl thio-β-D-galactopyranoside) and incubated overnight. Cells were then harvested by centrifugation at 16,780 g for 25 mins and re-suspended in phosphate buffered saline (50mM NaH$_2$PO$_4$, 300 mM NaCl, pH8) with EDTA-free Protease inhibitor cocktail (one tablet per 25 ml of solution; Roche Diagnostics) and DNase I (Sigma, final concentration 20 µg/ ml) and sonicated 5 times for 30 s. The sonicated culture was then centrifuged to remove insoluble cell debris at 37,000 g for 30 mins. The supernatant was then filtered through a 0.22 µm pore syringe driven filter before being loaded onto a 5ml HisTrap column (GE Healthcare) equilibrated with PBS. The bound protein was eluted with 5 column volumes of 300 mM imidazole in PBS. The eluted fraction was then subjected to size exclusion chromatography by loading onto a 120 mL Sephacryl S-200 column (GE Healthcare). Fractions containing AfS were identified and purity assessed by SDS-PAGE. AfS intended for crystallization was further assessed by matrix-assisted laser desorption/ionisation time-of-flight mass
spectrometry (MALDI-TOF MS). Fractions containing high purity AfS were then pooled, concentrated and diluted into 50 mM Tris-HCl, 100 mM NaCl, pH 8, then stored at -20 °C.

3.3.2. Crystallization of AfS

Details of the crystallization of AfS and the crystal structure of AfS in complex with KDN, KDN2en and 2,3 difluoro-KDN can be found in Appendix A of this chapter.

3.3.3. Sialidase and KDNase activity assays

The activity of enzyme preparations was determined by measuring cleavage of the synthetic sialic acid substrate, 4-methylumbelliferylα-D-N-acetylneuraminic acid (Neu5Ac-MU, 6) (Sigma, Oakville, ON), or KDN-MU. Briefly, reactions were set up in 96-well plates by adding 200 µM Neu5Ac-MU or KDN-MU, purified recombinant enzyme and the specific reaction buffer for a total volume of 100 µl. Plates were incubated at 37 ºC for 10 minutes followed by the addition of 200 µl cold stop solution (0.1 M glycine, 0.014 M NaCl, 25% ethanol, pH 10.7). Following the addition of stop solution, the amount of 4-methylumbelliferone released from Neu5Ac-MU or KDN-MU was determined using a fluorescence spectrophotometer at excitation and emission wavelengths of 365 and 450 nm, respectively. Fluorescence produced during the course of the reaction was related to the concentration of Neu5Ac-MU or KDN-MU cleaved by comparison with a standard curve of 4-methylumbelliferone (Sigma). Each reaction was performed in triplicate.

3.3.4. Kinetic assay

Michaelis-Menten parameters for the AfS and MvS were measured using Neu5Ac-MU or KDN-MU. Each 100µl reaction mixture was incubated at 37 ºC for 1 minute prior to addition of either purified sialidase. The progress of the reaction was continuously monitored for 20 minutes using a fluorescence spectrophotometer equipped with a temperature controller set to 37 ºC and excitation and emission wavelengths of 365 and 450 nm, respectively. For Neu5Ac-MU, kinetic parameters were
determined from 19 initial rate measurements using a substrate concentration range of 50 µM to 5000 µM. For KDN-MU, kinetic parameters were determined from 22 initial rate measurements using a substrate concentration range of 10 µM to 800 µM. The rate versus substrate concentration data were fitted to the Michaelis-Menten equation using GraphPad (GraphPad Software Inc., San Diego, CA).

3.3.5. pH profile

The optimal pH of the purified enzyme was determined by measuring its specific activity (nmol mg⁻¹ min⁻¹) towards KDN-MU in buffers of varying pH. The buffers used were 40 mM sodium formate (pH 3-3.5), 50 mM sodium acetate (pH 4–5), 16 mM sodium tartrate (pH 5.2), 32.5 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6–7), 40 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 6.5), and 50 mM Tris-HCl (pH 7.5–9). The ionic strength of all buffers was maintained at 100 mM with NaCl and all buffers were prepared for use at 37 ºC.

3.3.6. Temperature profile

*A. fumigatus* sialidase activity towards Neu5Ac-MU or KDN-MU was measured in 40 mM sodium acetate at pH 4.0. The activity of the enzyme was evaluated at 10, 15, 20, 25, 30, 37, 40, 45, 50 and 60 ºC. Each reaction mixture was incubated for 10 min and the amount of 4-MU released was determined as described above.

3.3.7. NMR experiments

¹H NMR spectroscopy was used to examine product formation from the hydrolysis of KDN-MU by AfS and to test for *trans*-sialidase activity. Reaction mixtures included 15 µL 23 mg/ml AfS, 15 µL 33 mg/ml KDN in ²H₂O. In the case of the *trans*-sialidase assay, 15 µL 20 mg/ml galactose or N-acetylgalactosamine in ²H₂O was also added. The reaction mixture was then made up to 600 µl with PBS pH 7 in ²H₂O and placed in a 5 mm NMR tube. Reaction mixtures were incubated at room temperature and spectra recorded at 0, 0.5, 1, 5, 12 and 24 hours. All NMR spectra were recorded at 298 K using a 500 MHz Bruker spectrometer taking 64 scans with a 2 s relaxation delay.
During the relaxation delay the remaining water signal was suppressed by continuous low power irradiation.

### 3.3.8. Inhibition experiments with Neu5Ac2en and KDN2en

The ability of two sialidase reaction intermediates, Neu5Ac2en and KDN2en to inhibit *A. fumigatus* sialidase (AfS) and *Micromonospora viridifaciens* sialidase (MvS) (a gift from Dr. Andrew Bennet) was examined. The concentration of enzyme (Table 3.1) used in each experiment varied based on the amount of enzyme available and previous experimental data of the relative activity for each enzyme to the different substrates to ensure that the reaction would not proceed too quickly for observations. The catalytic efficiency ($k_{cat}/K_m$) for *M. viridifaciens* sialidase was $801.57/\text{M}*\text{s}$ for KDN-Mu and $1.5 \times 10^6/\text{M}*\text{s}$ for Neu5Ac-Mu, and for *A. fumigatus* KDNase $1.85 \times 10^5/\text{M}*\text{s}$ for KDN-Mu and $22.29/\text{M}*\text{s}$ for Neu5Ac-Mu as determined from the kinetic experiments performed in the previous section.

**Table 3.1 Concentration of AfS or MvS used in each inhibition experiment**

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>substrate</th>
<th>AfKDNase (µM)</th>
<th>MvSialidase (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5Ac2en</td>
<td>Neu5Ac-Mu</td>
<td>2030</td>
<td>180</td>
</tr>
<tr>
<td>KDN2en</td>
<td>KDN-Mu</td>
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<td>Neu5Ac-Mu</td>
<td></td>
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<td>KDN-Mu</td>
<td>56.9</td>
<td>2.4</td>
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</tbody>
</table>

The substrate concentration used were 200 µM for both Neu5Ac-Mu and KDN-Mu. To determine the IC50 and Ki of KDN2en on AfKDNase, 50 µM of KDN-Mu was used since it was necessary to use a concentration much less than the Km of that enzyme for the substrate. Neu5Ac-Mu was commercially purchased from Rose Scientific (Edmonton, Albt) and KDN-Mu was from the Bennet Laboratory (SFU). The inhibitors: Neu5Ac2en, a well characterized inhibitor of sialidases, was made by Dr. Fahimeh Shimoossavee (Bennet Laboratory, SFU) and KDN2en, the KDN form of the inhibitor, was made by Dr. Milton Kiefel (Griffith University, Australia). Both inhibitors were tested at 0.001, 0.01, 0.1, 1, and 10 mM concentrations. Experiments for each sialidase were conducted at the optimal pH for each of the enzymes (AfS (pH 4.0; sodium acetate buffer) and MvS (pH 5.2, sodium tartrate)). The enzyme and inhibitor
were first incubated in the reaction buffer for 30 min at room temperature follow by the addition of the substrate and the reaction was monitored for 60 min at 37 °C. A similar experiment with no inhibitor was performed to monitor the activity of the positive control. The progress of the reaction was continuously monitored for 30 minutes at 37 °C using a fluorescence spectrophotometer at excitation and emission wavelengths of 365 and 450 nm, respectively. The rate versus substrate concentration data were fitted to the Michaelis-Menten equation using GraphPad (GraphPad Software Inc., San Diego, CA). Subsequently, the dose-dependent sigmoidal curve for the IC\textsubscript{50} was calculated using GraphPad. Since KDN2en is a competitive inhibitor of KDN-Mu in the enzymatic reaction with AfKDNase, the \( K_i \) was calculated using this formula: \( K_i = IC_{50}/(K_m/S+1) \). \( K_m \) was determined as described in the earlier section of this chapter in kinetic analysis.

### 3.3.9. Preferred Carbon Source Utilization of \textit{A. fumigatus}

To determine the preferred carbon source use by \textit{A. fumigatus}, \( 10^4 \) \textit{A. fumigatus} spores were inoculated per well in 96-well plates containing chemically defined fungal media (Kafer’s minimal media, K) with 25 mM carbon source of glucose, mannose, KDN, or sialic acid, or with no carbon source. All carbon sources were tested in triplicate. The KDN contained a maximum of 5% mannose; therefore, a K(+0.05MAN) sample, which contains 1.25 mM mannose, was tested to ensure that the small amount of mannose in the KDN sample did not contribute significantly to fungal growth. All media were adjusted to \( \sim \) pH 6.5 prior to inoculation. A paraffin overlay (50 \( \mu l \)) was added to each well to prevent cross contamination of spores between wells. All plates were placed in a moist chamber and incubated at 37 °C. Growth of the fungus was monitored spectrophotometrically at OD 620nm (Expert Plus Microplate Reader, Biochrom Asys).

### 3.3.10. Localization of AfS in \textit{A. fumigatus}

To determine whether AfS is cell associated or secreted, total protein was concentrated from \textit{A. fumigatus} grown in MYPD or Kafer’s minimal media with glucose or KDN and from the corresponding culture supernatant.
**Concentration of Total Protein from A. fumigatus**

To collect total protein from the fungus, it was first necessary to remove the cell wall, generating protoplasts, in order to lyse the cell and concentrate its total protein. The fungus was grown in 100 mL of liquid media overnight at 37 °C and shaken at 200 rpm.

Mycelia were harvested by filtering with a Buckner funnel lined with sterile Miracloth. The mycelia were washed with 10 mL of NCC buffer (0.8 M NaCl and 50 mM CaCl\(_2\)) and approximately 3 grams of slightly wet mycelia were transferred to sterile 125 mL flasks and 10 mL of Vinotaste buffer (140 mg/mL Vinotaste (Gusmer Enterprises) in NCC buffer) was added to each flask. Clumps of mycelia were broken by vigorous pipetting using a 10 mL sterile pipette. The flasks containing the mycelia and Vinotaste buffer was incubated at 30 °C with gentle shaking at 100 rpm for approximately 2.5 hrs, and vigorous pipetting using a 10 mL pipette after 1 and 2 hrs of incubation was needed to release protoplasts. Protoplast development was monitored microscopically. When the production of protoplast was complete as evident by broken and collapsed hyphal fragments, the suspension was filtered over a funnel containing a thin layer of glass wool. Protoplasts were pelleted at 2000 g for 10 min and re-suspended in 5 mL of STC buffer (1.2 M Sorbitol 10 mM Tris, 50 mM CaCl\(_2\)). After two washes with this buffer, the protoplasts were spun down and re-suspended in 1 mL of STC buffer.

Total protein was then isolated and concentrated by trichloroacetic acid (TCA) precipitations as outlined in the protocol in Keogh et al. (2006). The protoplasts were washed three times with 20% TCA and centrifuged for 5 min at 4000 g. The pellet was then resuspended in 250 µL of 20% TCA with 250 µL of glass beads (0.5 mm) in a 1.5 mL microcentrifuge tube and chilled for 5 min. The sample was then vortexed at maximum speed using 3, 1 minute pulses, with 1 min on Ice in between. To obtain the lysate, the bottom of the tube was pierced with a hot 22 gauge needle and placed into a 2 mL microcentrifuge tube. Both microcentrifuge tubes were spun together at 6000 g for 2 min at 4 °C. The beads were washed with an additional 300 µL of 5% TCA, centrifuged, and the supernatant was pooled together with the first spin. An additional 700 µL of 5% TCA is added and mixed with pipetting, and the mixture was spun down at max speed for 10 min at 4 °C. The pellet collected was washed with 750 µL of 100% ethanol (-20C). Liquid was removed and the pellet was resuspended in 40 µL of 1M Tris,
pH 8, and a small portion of this mixture was removed for protein quantification by Bradford Assay (Biorad). The remainder of the mixture was added to 80 µL of 2 x SDS-loading dye (60mM Tris, pH6.8, 2% SDS, 10% glycerol, 0.2% bromophenol blue, and 100 mM DTT) and boiled for 5 min at 95 °C. Then it was centrifuged at max speed for 5 min at room temperature and the supernatant was collected for immunoblotting.

**Concentration of Total Protein from Culture Supernatant**

Culture supernatant (100 mL) was collected after *A. fumigatus* grown in MYPD and Kafer’s minimal media with glucose or KDN was grown overnight at 37 °C and shaken in liquid media at 200 rpm. The culture supernatant was transferred into dialysis tubing (Fisher Scientific) with a molecular weight cut off at 30 kDa and the media was exchanged with 50 mM Tris, pH 8 buffer, at 4 °C, stirring overnight. The total protein from the media, now in 50mM Tris buffer, was concentrated by Amicon centrifuge filters (30 kDa molecular weight cut-off) to 500 µL. The Bradford assay (Biorad) was used to determine the protein concentration. 2 x Laemmli buffer was added to each sample and boiled for 5 min at 95°C before immunoblotting.

**Immunoblotting**

Concentrated protein collected from *A. fumigatus* protoplasts or culture supernatant was analyzed by immunoblotting. Due to the low protein concentration of samples, 5 µg of total protein from *A. fumigatus* and 1 µg of total protein from culture supernatant were analyzed. Purified recombinant *A. fumigatus* sialidase was used as the positive control. The samples were separated on a 10% SDS-PAGE gel, transferred onto a nitrocellulose membrane (GE bioscience) and blocked overnight in 3% BSA diluted in Tris-buffered saline with Tween-20 (TBST) (12.1g Tris base, 8.8g NaCl, pH 7.5, 0.1% Tween 20, 1 L H₂O). Membranes were then incubated overnight with rabbit polyclonal anti-recombinant *A.fumigatus* sialidase antibody (prepared by Pacific Immunology), diluted 1:1000 in TBST with BSA. Membranes were washed 3 times, 5 min each, in TBST before the addition of goat anti-rabbit antibodies conjugated to HRP (Jackson ImmunoResearch), diluted 1:50,000 in TBST with BSA and incubated for 2 hours. The membranes were washed 4 times, 10 min each, in TBST before the addition of the chemiluminescence substrate (Thermo Scientific SuperSignal West Pico), and then exposed to film (Kodak BioMax Light Film).
**Indirect Immunofluorescence Microscopy of AfS in A. fumigatus Wild-type**

Wild-type *A. fumigatus* conidia were seeded onto sterile coverslips in 5 mL MYPD liquid medium and grown to germlings at room temperature for 20 hours. All coverslips were processed according to Kaminskyj (2001) using a PIPES \([\text{piperazine}-N,N=\text{bis}(2\text{-ethanesulfonic acid})]\)-based buffer containing EGTA to preserve microtubule ultrastructure (275). Briefly, cells were fixed in freshly prepared 3.7% paraformaldehyde for 40 min and washed 3 times with buffer. The cell walls were partially solubilized with 50 mg/ml Vinotaste (Gusmer Enterprises) containing 50 mg/ml BSA for 15 min at 30°C. The cell membranes were permeabilized with 0.01% Triton X-100 and blocked with 5% BSA plus 2% normal goat serum plus 0.1% Tween 20 in buffer. Primary antibody, rabbit polyclonal anti-recombinant *A. fumigatus* sialidase antibody, was diluted 1:500 in blocking buffer and incubated with the cells for 2 hours at room temperature, and the coverslips were washed in buffer containing 0.1% Tween 20. Rabbit anti-sera from pre-immune bleeds was used as the primary antibody for the negative control, and these samples were treated the same as the experimental sample. The secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen), was used at a dilution of 1:300 in 10% blocking buffer. Coverslips were mounted in Prolong Gold (Invitrogen) and stored in the dark at 4 °C. Slides were examined using a Quorum Angstrom structured illumination device (590 nm) attached to a Leica DMI 4000B microscope fitted with a Hamamatsu Orca R2 CCD camera controlled by Metamorph v7.8.3 software. Images were processed using ImageJ and Photoshop without altering the integrity of the data.

3.4. Results

3.4.1. Structure of AfS

The *A. fumigatus* sialidase (AfS) was crystallized and solved by SAD phasing (PDB 2xzi). Resolution of the AfS apo structure was 1.45 Å. The closest structural homologue to AfS is MvS (PDB 1eus). AfS has the canonical, six-bladed \(\beta\)-propeller fold of a sialidase with an excursion between strands 2 and 3 of the second propeller blade, involving residues 142-172, that forms a cap region above the active site. Crystallization data can be found in Appendix A.
A superposition of AfS onto MvS-Neu5Ac2en complex (PDB 1esus) showed both structures were very similar. There is a conservation of key residues in the AfS active site: the arginine triad, the nucleophilic tyrosine and its associated glutamic acid, and the acid/base aspartic acid. More importantly, in the hydrophobic pocket that usually accommodates the acetamido group of Neu5Ac2en, in AfS, Arg^{171} is protruding into the pocket while in MvS, Leu^{169} is found at a similar position (Figure 3.1). This suggested that AfS recognises a sialic acid with a smaller group at C5. The only sialic acids with a smaller group at C5 are neuraminic acid, that has an amino group at C5 but does not occur naturally, and KDN that has a hydroxyl at C5. This may explain why Neu5Ac2en would not bind to AfS and it is not an inhibitor of AfS activity.

![Figure 3.1](image-url)
Complex with KDN

The structure derived from a crystal of AfS soaked in KDN-MU revealed two αKDN molecules bound per monomer (Figure 3.2 A, B). One KDN occupies the active site and is in a boat conformation, the second occupies the adjacent site in a chair conformation. The distance from the anomeric oxygen of the active site KDN is only 6.4Å from C9 and 7.7Å from C8 of the second KDN.

Figure 3.2  Crystal structure of the A. fumigatus KDNase

The crystal structure of A. fumigatus sialidase as (A) a ribbon model with KDN in the enzyme active site and (B) the hydration model of the enzyme active site with KDN. As predicted from the crystal structure overlay of the bacterial and fungal enzymes, KDN is the better sialic acid substrate due to the smaller substitution at the fifth carbon of the hexose ring, a hydroxyl versus an N-acetyl group for Neu5Ac. Two adjoining KDN binding sites were found in the enzyme, but only one catalytic site was found when the protein crystal was soaked in a KDN solution. Neu5Ac could not be soaked into the crystal.

As observed in the superimposed structures of AfS and MvS-Neu5Ac2en complex, the conserved key features of the sialidase active site were again observed when AfS was complexed with KDN. These include a tri-arginal cluster (Arg59, Arg265, Arg332) that interacts with the carboxylate group of Neu5Ac. The position of the first arginine (Arg59) is stabilised by a conserved glutamic acid (Glu374). A tyrosine (Tyr358) and a glutamic acid (Glu249) hydrogen bond with each other and sit beneath and close to the C1-C2 bond of the substrate. A conserved feature of sialidase active sites is the acid/base catalyst, Asp94.
All active sites of sialidases that hydrolyse Neu5Ac have a hydrophobic pocket to accommodate the N-acetyl group of the substrate, but the exact residues that form this pocket are generally not conserved. In AfS, although the pocket around the C5 hydroxyl of KDN has two hydrophobic residues lining it, Trp^{202} and Ala^{204}, the pocket is predominantly polar and filled with water molecules. O5 of KDN does not make direct contact with AfS, but interacts via water molecules. At its closes approach, Arg^{171} is only ~4.5Å from O5 of KDN.

When the structure of the *C. perfringens* NanI sialidase in complex with B_{2,5} αNeu5Ac (PDB 2bf6) is superimposed on AfS, the acetamido methyl group of Neu5Ac would be <2.5Å from Arg^{171} (data not shown). Therefore, Arg^{171} appears to be a major determinant of AfS being a KDNase. Arg^{171} packs on one side of the indole ring of Trp^{202}, with Gln^{148} packing on the other side, and together may define a KDN-recognition sequence motif.

In the second KDN site, this KDN makes only 4 direct hydrogen bonds with AfS. It is unlikely that this second site could bind Neu5Ac as the pocket around O5 is polar and in particular the presence of Arg^{388} would preclude the binding of the acetamido group of Neu5Ac. This hypothesis is supported by repeated failed attempts to visualize Neu5Ac in either KDN binding site.

**Complex with KDN2en and covalent intermediate (3F-β-KDN)**

KDN2en bound to the active site of AfS and a 1.84 Å resolution structure was resolved. All interactions with AfS, both direct and water-mediated were identical in the two sites. In addition, 3-fluoro-β-KDN (3F-β-KDN) bound to the AfS active site and it was covalently linked to Tyr^{358} similar to what has been observed in the *T. cruzi* trans-sialidase (193), *T. rangeli* sialidase (276) and *C. perfringens* NanI (277) covalent complexes. The presence of the fluorine at C3, and its close proximity (2.7Å) to the acid/base Asp^{84}, appears to lead to a dual conformation being observed for the side chain of this residue. The crystallography data for AfS complex with KDN2en and 3F-β-KDN is in Appendix A.
3.4.2. KDNase activity of the *A. fumigatus* sialidase

Kinetic analysis with Michaelis-Menten parameters showed that AfS prefers KDN over Neu5Ac when Neu5Ac-MU or KDN-MU were used as substrates. AfS had a $K_m$ of 5.8 ± 1.7 mM and a catalytic efficiency ($k_{cat}/K_m$) of 0.86 M$^{-1}$s$^{-1}$ for Neu5Ac-MU; whereas for KDN-MU, it had a $K_m$ of 0.23 ± 0.02 mM and a catalytic efficiency of $(1.82 ± 0.09) \times 10^5$ M$^{-1}$s$^{-1}$. MvS has a reported $k_{cat}/K_m$ of $(7.23 ± 0.04) \times 10^6$ M$^{-1}$s$^{-1}$ for Neu5Ac-Mu (278) and it was determined that the bacterial sialidase has a $k_{cat}/K_m$ of $(1.03 ± 0.36) \times 10^3$ M$^{-1}$s$^{-1}$ for KDN-Mu (Figure 3.3). In other words, AfS has a similar catalytic efficiency with KDN-Mu as MvS has with Neu5Ac-Mu. Relative to other published KDNase enzymes, AfS has a $K_m$ that is approximately 5 to 15 fold higher than other characterized KDNases: with KDN-Mu as the substrate, the $K_m$ was 42 μM for *S. multivorum* (271), 16 μM for oyster KDN-sialidase (279), 50 μM for starfish KDN-sialidase (188) and 70 μM for loach KDN-sialidase (187).

![Figure 3.3 Comparison of the Catalytic Efficiency of AfS and MvS](image)

The catalytic efficiency of AfKDNase (AfS) and MvSialidase (MvS) using two synthetic substrates were determined by Michaelis-Menten kinetic analysis.
AfS had a pH optimum of between 3.5 and 4.5 with KDN-Mu, similar to its optimum with Neu5Ac-Mu. The optimal temperature range of AfS with KDN-Mu was from 30 to 37 °C (Figure 3.4). The pH and temperature optimum of AfS is similar to KDNases characterized from other eukaryotes, such as rainbow trout (179), loach (187), star fish (188) and oyster (279). However, it differs from the KDNase of the bacterium *S. multivorum*, which had optimal activity at 25 °C, and pH 5.7 – 6.0 (178).
Figure 3.4 Activity profiles of AfS on KDN-Mu and Neu5Ac-Mu as substrates

The optimal pH (A) and optimal temperature (B) for AfS activity for KDN-Mu and Neu5Ac-Mu; relative fluorescence units (RFU) were detected spectrophotometrically. The optimal pH for enzyme activity on KDN-Mu was pH 4 and for pH 3.5 for Neu5Ac-Mu. Meanwhile, the optimal temperature for AfS on both KDN-Mu and Neu5Ac-Mu is ~37°C.
3.4.3. NMR monitoring of KDN-Mu hydration by AfS

The hydrolysis of KDN-Mu to form KDN was monitored using $^1$H NMR spectroscopy in deuterated buffer at a pD of 3.6 (Figure 3.5). The $\mathrm{H}_3^{\text{eq}}$ proton of $\alpha$KDN-Mu has a characteristic doublet of doublets ($\delta = 2.75$ ppm, $J_{3e,3a} = 12.7$ Hz, $J_{3e,4} = 4.7$ Hz). With the addition of AfS, cleavage of the umbelliferyl group leads to a shift of the $\mathrm{H}_3^{\text{eq}}$ resonance to $\delta = 2.60$ ppm of the $\alpha$KDN product. Over time $\alpha$KDN mutarotates to the thermodynamically more stable $\beta$KDN with the appearance of $\mathrm{H}_3^{\text{eq}}$ resonance at $\delta = 2.10$ ppm.

Figure 3.5  
The $^1$H NMR spectrum showing hydration of KDN-MU by AfS.
The spectra presented were recorded at time ~ 0 hr, 0.5 hr, 1 hr, 5 hr and 12 hrs (bottom to top).

3.4.4. Inhibitory effects of Neu5Ac2en and KDN2en on AfS

Neu5Ac2en and KDN2en were examined for their ability to inhibit AfS (Figure 3.6). Both compounds are analogues of the transition state like structures that can inhibit either a sialidase or KDNase reaction (280). MvS was examined concurrently as a control because the inhibitory effect of Neu5Ac2en on MvS has been well documented (281).
Figure 3.6  Structures of (A) Neu5Ac2en and (B) KDN2en

Neu5Ac2en was examined over a range of concentrations (0.1 to 10 mM) as a direct competitor against the substrate Neu5Ac-Mu. In this study, 100 µM of Neu5Ac2en inhibited approximately half of the bacterial enzyme activity and at 1 mM, almost all activity was inhibited. In contrast, even with 10 mM of inhibitor, the fungal enzyme was not inhibited (Figure 3.7A).

When KDN-Mu was used as the substrate and Neu5Ac2en as the inhibitor, a similar result was observed (Table 3.2); MvS activity was inhibited by over 90% whereas no inhibition of AfS was observed at any concentration tested. Although KDN-Mu is not the direct competitor of Neu5Ac2en and KDN-Mu is not a primary substrate for MvS, it is clear that Neu5Ac2en is an effective inhibitor of the bacterial sialidase but not the fungal sialidase.

KDN2en inhibited AfS when KDN-Mu was the substrate. With 1 mM KDN2en, 30% of the fungal enzyme activity was inhibited and at 10 mM, only 14% of the activity remained. In contrast, MvS activity was inhibited by 50% using 1mM KDN2en (Figure 3.7B). However, when Neu5Ac-Mu was the substrate, KDN2en did not inhibit MvS (Table 1). Since it was established earlier that Neu5Ac-Mu is not a good substrate for AfS, the inhibitory effect of KDN2en on this reaction was not tested.
Dose dependent inhibitory effects of Neu5Ac2en and KDN2en were tested on AfS (■) and MvS (♦). The inhibitors were assessed as a direct competitor against their respective substrates. (A) Neu5Ac2en was tested on both enzymes and Neu5Ac-Mu was the substrate. (B) KDN2en, the KDN equivalent of the reaction intermediate, was tested on both enzymes with KDN-Mu was the substrate. Triplicate data from one experiment is presented.

Figure 3.7   Inhibitory Effects of Neu5Ac2en and KDN2en on AfS and MvS
Table 3.2  Assessment of inhibition by Neu5Ac2en and KDN2EN on AfS and MvS when Neu5Ac-Mu and KDN-Mu are substrates

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>AfS (µM)</th>
<th>MvS (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5Ac2en</td>
<td>Neu5Ac-Mu</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>Neu5Ac2en</td>
<td>KDN-Mu</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>KDN2en</td>
<td>KDN-Mu</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KDN2en</td>
<td>Neu5Ac-Mu</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

Inhibition (++): > 30% inhibition with ≤100 µM of inhibitor, some inhibition (+): > 30% inhibition with ≤1 mM of inhibitor, no inhibition (0), not tested (-)

To further examine the inhibitory effects of KDN2en on AfS when the substrate was KDN-Mu, a sigmoidal dose response curve was generated and the IC$_{50}$ and $K_i$ were calculated based on this curve (Figure 3.8). The IC$_{50}$ was determined to be 566 µM and by calculation, the $K_i$ was 102 µM. KDN2en has been shown to be more effective on other KDNases than on AfS: the $K_i$ values were 7.7 µM for S. multivorum KDNase (271), 7 µM for Oyster KDNase-sialidase (279), and 40 µM for star fish KDNase-sialidase (188).
Figure 3.8  Sigmoidal dose response curve of KDN2en on the activity of *A. fumigatus* KDNase

The sigmoidal dose response curve as calculated by GraphPad to determine the IC50 and the inhibition constant (Ki) of KDN2en on *Af* when KDN-Mu was the substrate.

3.4.5.  *A. fumigatus* metabolism of different carbon sources

To determine whether KDN could be a carbon source for *A. fumigatus*, we measured growth in defined media supplemented with various carbon sources (Figure 3.9). The data indicate that *A. fumigatus* is unable to use Neu5Ac as the sole source of carbon; however, it was able to use KDN though less efficiently on a molar basis compared to glucose or mannose. There was also a short lag in growth suggesting that KDN catabolic enzymes must be induced prior to utilization of this substrate.
Figure 3.9  KDN but not Neu5Ac is a carbon source for A. fumigatus
Growth of A. fumigatus on different carbon sources. Carbon sources were added to the Kafer’s defined medium where Glu = glucose, Man = mannose, SA = Neu5Ac. All carbon sources were 25mM except for (+0.05MAN) that contained 1.25 mM mannose. Values represent the mean of triplicate cultures.

3.4.6. Localization of AfS in A. fumigatus: Western Blot analysis and Indirect Immunofluorescence Detection

Total protein extracted from A. fumigatus was concentrated and KDNase was found to be expressed by the fungus when grown in rich fungal media (MYPD) or defined media supplemented with either KDN or glucose (Figure 3.10A). The culture supernatant from the same fungal cultures were concentrated and AfKDNase was also detected by Western blot. However, little AfS was detected in the culture supernatant than in the total cell lysate even though an equal amount of protein was loaded in each sample lane for the Western blot (Figure 3.10B). The recombinant AfS was used as the positive control.

Indirect immunofluorescence using anti-AfKDNase antisera was used to determine where AfKDNase is localized in A. fumigatus. As with the Western blot, AfKDNase was found to be evenly distributed throughout the cell; however, a higher
concentration was observed on the cell periphery though whether this is the plasma membrane or cell wall cannot be established from these images (Figure 3.11). In contrast, when pre-immune antisera was used, AfKDNase was not detected. DAPI staining of the nucleus was completed as a positive control to demonstrate that the cells were permeabilized in both treatment groups.

**Figure 3.10 Western blot analysis to identify location of AfS in A. fumigatus grown in different carbon sources**

AfS is primarily found associated with the fungal cell (A) and only small amounts were found in concentrated culture supernatant (B). *A. fumigatus* was grown in Kafer’s minimal media supplemented with either 1% glucose (Glu) or 1% KDN (KDN) as the carbon source, and in rich fungal media (MYPD). A 43 kDa protein was detected using anti-recombinant AfS antisera. Recombinant AfS was the positive control.
**Figure 3.11** Indirect immunofluorescence to localize of AfS in *A. fumigatus*

AfS was found to be uniformly distributed throughout *A. fumigatus*, but a higher concentration is observed at the envelope (cell membrane or cell wall). Anti-recombinant AfS antisera, followed by a fluorescent stain (Alexa fluor 568) conjugated to a goat anti-rabbit antibody, was used to detect AfKDNase. DAPI was a positive control stain for the nucleus inside the fungi. Pre-immune rabbit antiserum was used as the negative control.
3.5. Discussion

The AfS structure presented in this chapter is the first structure of a fungal sialidase, and the first structure of a KDN-specific sialidase, or KDNase. The solved crystal structure of the enzyme allowed us to better analyze the physical structure of the enzyme instead of solely relying on computer models. Although essential structural components and the catalytic site of AfS is similar to the crystal structure of sialidases from other microbes, the differences made the fungal enzyme a much better KDNase than a sialidase. Neu5Ac specific sialidases have a hydrophobic pocket to accommodate the acetamido group at C5; in contrast, AfS has a water-filled, polar pocket where modelling shows that Arg\textsuperscript{171} would clash with acetamido group of Neu5Ac. The equivalent residue occupying the same structural position is a serine in \textit{C. perfringens} NanI (277) and \textit{S. pneumonia} NanA/ NanB (259, 270) or leucine in \textit{MvS} (273) and the residue forms part of the larger, more hydrophobic pocket accommodating the acetamido group of Neu5Ac. The hydroxyl at C5 of KDN does not interact directly with AfS, but makes several water-mediated interactions. Arg\textsuperscript{171}, the second arginine of an EGRR motif, sits one side of Trp\textsuperscript{202}, part of a WD motif, on the other side of which is Gln\textsuperscript{148} that interacts with the O7 and O9 hydroxyls of KDN. With a KDN substrate, it is likely that catalysis would proceed with the release of the aglycone moiety as described for other sialidases (Newstead et al. 2008). This similarity between the mechanism of AfS and other sialidases was supported by NMR data that showed that catalysis of KDN-Mu by AfS proceeds with retention of configuration at the anomeric carbon.

Consistent with the kinetic analyses completed, the catalytic efficiency of AfS in cleaving the 4-methylumbelliferyl leaving group from KDN-Mu was $10^5$ times greater than from MuNeu5Ac. MvS was only $10^3$ times more efficient at cleaving Neu5Ac-Mu than KDN-Mu. It is clear that the primary substrate for AfS is KDN-Mu while Neu5Ac-Mu is the substrate for MvS. Although MvS is a better KDNase than AfS is a sialidase, we predict that mutagenesis of Arg\textsuperscript{171} in AfS may modulate KDN binding. This will be addressed in the next chapter.

In addition, AfS was not inhibited by Neu5Ac2en, a transition state analogue like substrate that has been shown to inhibit Neu5Ac-hydrolysing sialidases (282, 283).
observation is not surprising since it was not possible to co-crystallize AfS with Neu5Ac2en which was largely due to the poor fit of the inhibitor in the enzyme active site. As expected, KDN2en inhibited AfS KDNase activity, though less effectively compared to inhibition observed with other KDNases (188, 271, 279). Even though MvS can cleave KDN-Mu, it is not the primary substrate for the enzyme since unlike Neu5Ac, KDN does not fit well in the catalytic site. Our observations in this chapter were similar to those reported by Terada et al. (1997) using the S. multivorum KDNse. This enzyme could only be inhibited by KDN2en, but not by Neu5Ac2en, and KDN2en could not inhibit the sialidase activity of Arthrobacter ureafaciens or C. perfringens sialidases. To improve the inhibitory activity of the structure, KDN2en may be modified by the addition of different functional groups to find the form with maximum inhibitory potency (279).

The carbon source utilization experiment revealed that A. fumigatus uses KDN but not sialic acid as a carbon source. This clearly demonstrates that KDN is nutritionally more important sialic acid for A. fumigatus. Furthermore, AfS was found distributed throughout the fungal cell and secreted into the culture supernatant, albeit to only a small extent, using indirect immunofluorescence, the enzyme was found to be concentrated at the cell periphery. This suggests that the fungus cleaves surrounding KDN-linked glyconconjugates and transports the KDN into the cell as a carbon source.

It is well established that bacterial sialidases have an important role in nutrition by supplying the bacterium with both carbon and nitrogen (113, 284). The few reports on the occurrence of KDN in bacteria show KDN to be part of the capsules and cell envelopes of pathogenic bacteria (186). KDN was found to be a non-terminal part of complex surface oligosaccharides in Klebsiella ozaenae serotype K4, Sinorhizobium fredii strain SVQ293 and Streptomyces sp. MB-8 (144–146, 154). A polyKDN polymer, with the KDN molecules linked β2-4 to each other, was reported in the cell wall of the plant pathogen Streptomyces sp. VKM Ac-2124 (144). Although there is little evidence to date, it may be possible that A. fumigatus finds sources of KDN in its natural ecological niche in soil where is survives and grows on organic debris.

The AfS crystal structure revealed a second KDN binding site adjacent to the active site which is an unusual finding for an exo-sialidase. Many bacterial and parasite
sialidases or trans-sialidases possess additional carbohydrate-binding modules (CBMs) that often recognise sialic acid (14, 259, 269). The presence of these modules increases catalytic efficiency of the enzyme by targeting the sialidase to appropriate substrates (268). Intriguingly, polyKDN has been found in the human lung, as well as in other tissues, identified through the use of a monoclonal antibody recognising α2,8-linked KDN molecules (252, 256). PolyKDN in the lung is a relatively minor component of glycoproteins and glycolipids compared to Neu5Ac, and is reported to be associated with a developmentally-regulated 150kDa glycoprotein not present in adults, but present in various types of human lung tumours (256).

The biochemical analysis of the AfS presented in this chapter, and the structural analysis of AfS completed with our collaborators at St. Andrew’s University is the first demonstration that the naturally occurring sialic acid, KDN, is involved with fungal metabolism. AfS is a unique enzyme since it has a high preference for KDN linked substrate over Neu5Ac linked substrates though the catalytic site structure is very similar to characterized bacterial sialidases. Modelling with the crystal structure of AfS and Neu5Ac suggests that the water-filled, polar pocket containing Arg171 is the defining feature of the fungal KDNase. Meanwhile, the data presented in this chapter suggests that AfS has a similar catalytic mechanism as other sialidases; however, to confirm this, additional analytical studies are required using AfS enzymes that have key catalytic amino acids mutated. The next chapter of this thesis presents an in depth investigation of three AfS catalytic site mutant enzymes using biochemical and structural analysis.
Chapter 4.

Kinetic and Structural Evaluation of Selected Active Site Mutants of the *Aspergillus fumigatus* KDNase (Sialidase)

This chapter is a modified form of the article published in Biochemistry, published by The American Chemical Society, Inc. (c) 2013, by permission under the authorship of Yeung JHF, Telford JC, Shidmoossavee FS, Bennet AJ, and Moore MM. Kinetic and structural evaluation of selected active site mutants of the *Aspergillus fumigatus* KDNase (sialidase). Biochemistry. 52(51): 9177-9186.

Authors Contributions: The author of this thesis designed the project, expressed the proteins, performed the kinetic measurements and completed the PyMol modeling. Dr. Judith Telford performed the site-directed mutagenesis on the pAfS plasmid and the crystallography work. Dr. Fahimeh S. Shidmoossavee synthesized the KDNα2,6GalβFMU substrate and performed the ¹H NMR analysis.

4.1. Abstract

We previously cloned and characterized an exo-sialidase from the opportunistic fungal pathogen, *Aspergillus fumigatus*, and showed that the preferred 2-keto-3-deoxynononic acid (KDN) over *N*-acetylneuraminic acid (Neu5Ac) as a substrate. Hence, the enzyme is a KDNase. The purpose of the current study was to investigate the structure and function relationships of critical catalytic site residues in the *A. fumigatus* KDNase. Site-directed mutagenesis was used to create three recombinant mutant enzymes: the catalytic nucleophile (Y358H), the general acid/base catalyst (D84A), and an enlargement of the binding pocket to attempt to accommodate the *N*-acetyl group of Neu5Ac (R171L). Crystal structures for all enzymes were determined.
The D84A mutant had a greater effect on decreasing the activity of AfS compared to the same mutation in the structurally similar sialidase from the bacterium, *Micromonospora viridifaciens*. These data suggest that the catalytic acid is more important in the reaction of AfS and that catalysis is less dependent on nucleophilic or electrostatic stabilization of the developing positive charge at the transition state for hydrolysis. Removal of the catalytic nucleophile (Y358H) significantly lowered the activity of the enzyme but this mutant remained a retaining glycosidase as demonstrated by NMR spectroscopic analysis. This is a novel finding that has not been shown with other sialidases. Kinetic activity measured at pH 5.2 revealed that R171L had higher activity on a Neu5Ac-based substrate compared to wild type KDNase; hence, leucine in place of arginine in the binding pocket improved catalysis towards Neu5Ac substrates. Hence, whether a sialidase is primarily a KDNase or a neuraminidase may be due in part to the presence of an amino acid that creates a steric clash with the N-acetyl group.

4.2. Introduction

*Aspergillus* species are filamentous fungi that have emerged as a cause of serious disease in patients with underlying medical conditions such as cystic fibrosis or immune suppression (10). *Aspergillus fumigatus* is the most prevalent airborne fungal pathogen in developed countries and it is responsible for approximately 90% of life-threatening cases of invasive pulmonary aspergillosis in immunocompromised patients (7). The first steps of *A. fumigatus* pathogenesis is inhalation of airborne conidiospores (conidia) by a susceptible individual followed by attachment of conidia to lung tissue proteins (11). Glycans on the surface of *A. fumigatus* conidia contain terminal N-acetylneuraminic acids (Neu5Ac) that are α2,6-linked to underlying galactose residues. These sialic acids are responsible in part for mediating conidial attachment to host lung cells and basal lamina proteins such as fibronectin (87, 251). The removal of conidial sialic acids significantly decreased spore uptake by cultured murine macrophages and Type 2 pneumocytes compared to controls(251). These data indicate that sialylated molecules on the conidial surface act as ligands for both professional and non-professional phagocytes.
Although sialic acids are mostly found in higher eukaryotes, they are also found in many species of bacteria (93, 285, 286), fungi (87, 111, 166, 287), and protozoans (285, 288, 289). These microorganisms, both pathogenic and non-pathogenic, have evolved to synthesize these negatively charged sugars *de novo* or capture them from the environment for use as a carbon/nitrogen source. Some pathogenic microorganisms have evolved to use sialic acid by coating themselves in sialic acid to resist components of the host innate immune response (177, 285, 289). In addition, certain pathogenic bacteria utilize cell surface sialic acids to interact specifically with different host-cell surface receptors (93); such as *Campylobacter jejuni*, the bacterium that cause Guillain-Barré syndrome, interacts with host macrophages by binding to sialoadhesins (Siglec-1)(290).

*A. fumigatus* grown in chemically-defined media lacking sources of sialic acid displayed *N*-acetylneuraminic acid (Neu5Ac) on the surface of conidia (87, 251) suggesting that *de novo* synthesis of sialic acid occurs in this fungus. However, sialic acid biosynthetic genes have not yet been identified by us or others in any of the *A. fumigatus* strains sequenced to date (196). A gene encoding a sialidase (EC 3.2.1.18), a glycosyl hydrolase that cleaves glycosidic linkages of sialic acid, was identified in the genome sequence of *A. fumigatus*. Subsequent studies revealed that the *A. fumigatus* sialidase was a functional protein that structurally resembled bacterial sialidases (274).

Many microbial pathogens secrete sialidases, enzymes that hydrolyze sialic acid from oligosaccharides. The released sialic acids are then imported into the cell, or directly linked to cell surface glycoconjugates (285). Sialidases also have an important role in bacterial nutrition (133, 291). Sialidase activity has been identified in other pathogenic fungi such as *Fonsecaea pedrosoi* and *Sporothrix schenckii* (166, 244, 292). Compared to bacterial sialidases, the *A. fumigatus* sialidase (*AfS*) had lower activity in cleaving *N*-acetylneuraminic acid (Neu5Ac) from glycan substrates. Kinetic analysis of the recombinant *AfS* revealed that its preferred substrate is not Neu5Ac but rather 2-keto-3-deoxy-β-glycero-δ-galacto-nononic acid (KDN), another naturally occurring sialic acid that has an –OH group in place of the *N*-acetyl group at carbon 5 (282). *A. fumigatus* was shown to use KDN but not Neu5Ac as a carbon source (282). KDN is found in almost all types of glycoconjugates where KDN residues are often in place of
Neu5Ac and frequently found among lower vertebrates and bacteria (183). Very little is known about the catabolism of KDN; however, it was shown that the KDNase-producing bacterium *Sphingobacterium multivorum* used KDN as a sole carbon source (177). KDNase has also been identified in several species of mollusks and in rainbow trout (183). To date, the catalytic mechanism of KDNases has not been fully investigated.

The active sites of sialidases and *trans*-sialidases found in viruses, bacteria and eukaryotes all possess several key features essential for catalysis: a strictly conserved cluster of three arginines that bind the carboxylate group of the substrate via electrostatic interactions; a distal glutamate residue which forms a salt bridge with one of the conserved arginine residues (96); a tyrosine-glutamic acid dyad and an aspartic acid that act as the general acid/base catalyst. All known natural sialidases catalyze the hydrolysis of substrate with retention of anomeric configuration, an observation that is compatible with this family of enzymes operating via a standard double displacement mechanism (96, 193, 194).

Though all sialidases have a similar tertiary structure, members of this family of enzymes share low sequence identity. All active sites of sialidases that hydrolyze Neu5Ac have a hydrophobic pocket to accommodate the *N*-acetyl group of the substrate but the exact residues that form this pocket are generally not conserved. The crystal structure of the recombinant *Af*S showed that the arginine pocket accommodates the smaller hydroxyl group at C-5 in KDN (282). Comparison of the crystal structure of *Af*S with *Micromonospora viridifaciens* sialidase (*Mv*S) indicated that the presence of arginine 171 (not part of the tri-arginine cluster) in place of leucine in this pocket - as in MvS - may prevent recombinant *Af*S from holding Neu5Ac substrate in place for efficient catalysis.

Previous mutagenesis work on the MvS found that mutating the critical nucleophilic tyrosine in the active site substantially altered the mechanism of the enzyme by changing it from a retaining to an inverting glycoside hydrolase (194). Similarly, it has been shown that a natural nucleophilic variant of the *Trypanosoma cruzi* *trans*-sialidase, which is inactive, has a tyrosine to histidine substitution (293). Interestingly, a mutation of the general acid/base catalyst in *Mv*S from an aspartic acid to a glycine did not affect
the function of the enzyme significantly (278). In the present study, we examined the effect of selected active site mutations on the structure and activity of the Afs. Specifically, we examined a mutation of the active site nucleophile (Y358H) and an acid catalyst mutant (D84A) (Figure 4.1). In addition, we studied the kinetics and structure of the R171L mutant of AfKDNase to determine whether modifying the pocket to resemble that of the MvS, resulted in greater activity in cleaving Neu5Ac compared to KDN.

Figure 4.1  Amino acids in the Afs catalytic domain targeted for site-directed mutagenesis
Two amino acids (Y358 and D84) are believed to be the catalytic nucleophile and the general acid, respectively, that are essential for the hydrolase reaction. The arginine residue (R171) is believed to be the key amino acid reducing the efficiency of Neu5Ac cleavage by Afs. The KDN analogue in the figure is KDN2en.

4.3. Materials and Methods
All chemicals were of analytical grade or better and were purchased from Sigma-Aldrich unless noted otherwise. 8-fluoro-4-methylumbelliferyl β-D-galactopyranoside (Gal β-FM, 3, Scheme 1 and Appendix B) was made by following a published procedure (294). Cytidine 5’-triphosphate disodium salt was purchased from 3B Scientific Corp. Pyrophosphatase from Baker’s Yeast (S. cerevisiae) was purchased from Sigma-Aldrich. E.Coli Neu5Ac aldolase was purchased from Codexis, and CMP- Neu5Ac synthase from Neisseria meningitidis was expressed according to the literature (not purified)(295).
Photobacterium sp. JT-ISH-224 α-2,6-sialyl transferase was a kind donation from M. Gilbert (National Research Council, Ottawa). ^1^H- and ^13^C-NMR spectra were acquired on a Bruker 600 MHz spectrometer, and chemical shifts are listed in parts per million and coupling constants (J) are given in hertz. Melting points were determined on a MPA100—automated melting point system. Optical rotations were measured on a Perkin-Elmer 341 polarimeter and are reported in units of g per 100 mL.

4.3.1. Expression and purification of AfS mutants for kinetic and structural studies

The KDNase gene from the A. fumigatus clinical isolate Af293 was originally cloned into pET28A+ vector (EMD chemicals Inc., San Diego, CA) and expressed in Escherichia coli (DE3) cells(274). Point mutations were made with site-directed mutagenesis to generate the Y358H, R171L and D84A mutants using the QuikChange Lightning mutagenesis kit (Agilent Technologies). Briefly, E. coli expressing KDNase in a pET28A+ vector were grown in Luria Broth with 50 µg/mL kanamycin at 37 °C, shaking at 220 rpm. Once the culture OD₆₀₀ had reached 0.5, protein expression was induced by 0.5 mM IPTG (Isopropyl β-D-1-thio-galactopyranoside) and incubated overnight at room temperature.

Cells were then harvested as previously described (274). Briefly, E. coli cultures were collected by centrifugation at 3000 g for 20 min at 4 °C. Following lysozyme treatment and sonication, the suspension was centrifuged. The lysate containing the expressed 6XHis-tagged KDNase was added to 1 mL nickel nitritotriacetic acid agarose (Ni-NTA) (Qiagen) and the KDNase was eluted in 5 mL elution buffer (50 mM Tris-HCl, 100 mM NaCl, 20 mM imidazole, pH 8.0). Imidazole and non-specific proteins were removed by gel filtration chromatography (ÄTKAFPLC, GE Healthcare, with Hi-Prep 26/60 Sephacryl S-100H resin and column size of 320 mL) with clean-up buffer (50 mM Tris-HCl, 100 mM NaCl, pH 8.0) at 4 °C. Fractions collected were analyzed by 12% SDS-PAGE gel. Selected fractions containing only KDNase were concentrated by using a 30,000 molecular weight cut-off centrifugal filter (Millipore, Billerica, MA). The concentrated protein solution was frozen using liquid nitrogen and stored at -80 °C.
4.3.2. **Determination of the pH optimum for each mutant**

The optimal pH of the purified enzymes were determined by the amount of 4-methylumbelliferone released from KDN-Mu and Neu5Ac-Mu, respectively, by AfS in buffers of varying pH. The buffers used were 40 mM sodium formate (pH 3.0 - 3.5), 50 mM sodium acetate (pH 4 – 5), 16 mM sodium tartrate (pH 5.2), 32.5 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6 – 7), 40 mM 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 6.5), and 50 mM Tris-HCl (pH 7.5 – 9.0). The ionic strength of all buffers was maintained at 100 mM with NaCl and all buffers were prepared for use at 37 °C. The amount of 4-methylumbelliferone released during the enzyme reaction was detected by a fluorescence microplate reader (SpectraMax M2e, Molecular Devices) at excitation and emission wavelengths of 365nm and 450nm, respectively. The amount of fluorescence detected was adjusted to reflect the molar concentration of substrate cleaved using standard curves generated with 4-methylumbelliferone (Fluka) over a range of concentrations (1µM to 150 µM) in the different aforementioned pH buffers.

4.3.3. **Kinetic Activity Assays**

The activity of enzyme preparations were determined by the cleavage of the synthetic sialic acid substrate, 4-methylumbelliferylα-D-N-acetylneuraminic acid (Neu5Ac-Mu) (Rose Scientific, Edmonton, AB), 4-methylumbelliferyl α-2-keto-3-deoxynononic acid (KDN-Mu) and the α2-6 isomer of 8-fluoro-methylumbelliferyl α-2-keto-3-deoxy-D-glycero-D-galacto-nononylgalactopyranoside (KDNα2,6GalβFMU).

Reactions with Neu5Ac-Mu and KDN-Mu were set up in 96-well plates by adding the substrate in a range of concentrations (50 µM to 800 µM), purified recombinant enzyme, and the specific reaction buffer for a total volume of 100 µL. In reactions with KDNα2,6GalβFMU, 200 µM of substrate was used. Aspergillus oryzae β-galactosidase (Sigma) (0.1%) was added to each reaction. Plates were incubated at 37 °C for 45 minutes and the amount of 4-methylumbelliferone released was determined using a fluorescence microplate reader (SpectraMax M2e, Molecular Devices) at excitation and emission wavelengths of 365nm and 450nm, respectively.
Extended kinetic activity experiments were carried out up to 600 minutes for reactions with R171L or D84A and KDNα2,6GalβFMU to ensure that enzymatic activity was observed to completion. Each enzyme reaction had a total volume of 500 µL; each was set up in a quartz cuvette that contained 200 µM of substrate plus 0.1% *Aspergillus oryzae* β-galactosidase (Sigma) in the appropriate reaction buffer. The fluorescence over the course of the reaction was measured using a Cary Eclipse fluorescence spectrophotometer. All kinetic experiments were performed in triplicate. A 50 mM sodium acetate/100 mM sodium chloride buffer was used for kinetic experiments at pH 4.0, and a 16 mM sodium tartrate/100 mM sodium chloride buffer was used for experiments at pH 5.2.

Fluorescence produced during the course of the reaction was related to the concentration of Neu5Ac-Mu, KDN-Mu, or KDNα2,6GalβFMU cleaved by comparison with a standard curve of 4-methylumbelliferone (Fluka) or 8-fluoro-4-methylumbelliferone (synthesized starting from 2,3,4-trifluoronitrobenzene purchased from TCI America) (294).

Standard curves generated with 4-methylumbelliferone over a range of concentrations (1 µM to 150 µM) were made using pH 4.0 and 5.2 buffers, and the standard curve made with 8-fluoro-4-methylumbelliferone of concentrations between 5 µM to 400 µM was made using pH 4.0 buffer. To ensure the amount of *A. oryzae* β-galactosidase used was sufficient and that the reaction was a zero order reaction, 0.1% *A. oryzae* β-galactosidase was incubated with 150 µM and 350 µM 8-fluoro-4-methylumbelliferyl β-D-galactopyranoside (Gal β-FM) and the activity was observed by fluorescence spectrometry using excitation and emission wavelengths of 365 nm and 450 nm, respectively. The observed results from these experiments did exhibit zero order enzyme kinetic activity (data not shown); therefore, it was assumed that the reaction with *A. oryzae* β-galactosidase and GalβFMU would not interfere with the reaction between the mutant KDNases and KDNα2,6GalβFMU.
4.3.4. **Determination of retaining or inverting activity of the Y358H mutant by $^1$H NMR**

$^1$H NMR spectroscopy was used to examine product formation from the hydrolysis of KDN-MU by the Y358H. NMR spectra were acquired on a 600 MHz Bruker spectrometer. The reaction mixture consisted of 0.34 µg of Y358H mutant AfKDNase, 0.01% bovine serum albumin (BSA, BioShop Canada), 0.5 mg KDN-MU, made up to 600 µL with a solution of 20 mM homopiperazine-1,4-bis(2-ethanesulfonic acid) (Santa Cruz Biotechnology, Inc.) pH 4.0, and placed in a 5 mm NMR tube. Prior to adding the enzyme, the magnetic field was manually shimmed and a $^1$H-NMR spectrum was acquired. The magnetic field re-shimmed and re-tuned after adding the enzyme in order to improve signal to noise. Shimming took about 15 min during which the enzyme reaction started. The reaction mixture was incubated at 296K and all NMR spectra were acquired continuously over a period of 2 h (16 scans and 2 minute per spectrum).

4.3.5. **Crystal structure determination of mutants**

Crystals of the mutant proteins were formed and cryoprotected for freezing and X-ray data collection as described previously for the wild type AfS (296). All data were collected at 100 K in house (Rigaku-MSC Micromax-007 X-ray generator and Saturn 944+ CCD detector). MOSFLM (297) was used to integrate the data; the crystals of the mutant proteins were isomorphous with the wildtype, and belong to space group P2$_1$. The apo-model, PDB code 2XCY, was refined in REFMAC5 (298) against data collected for each of the mutant structures AfS$_{Y358H}$, AfS$_{D84A}$ and AfS$_{R171L}$. For the R171L mutant, crystals were soaked in 500 mM of the sialidase transition state analogue 2-deoxy-2,3-dehydro- $N$-acetylneuraminic acid (Neu5Ac2en) for 20 minutes prior to data collection. Modelling of the protein and the soaked ligands were carried out in Coot (299) and further refined with REFMAC5. The resulting models were validated by Coot and MolProbity (300). Data collection and refinement statistics are given in Appendix B (Table B1).
4.3.6. Analysis of putative KDNases using PyMol and ClustalW2

Three-dimensional structures of putative sialidases: *Aspergillus terreus* (NCBI XP_001214142.1), *Trichophyton rubrum* (NCBI XP_003239017.1), *Trichophyton equinum* (NCBI EGE01838.1), *Sphingobacterium* sp.21 (NCBI YP_004319774.1) and *Streptomyces avermitilis* (NCBI NP_827111.1), were generated using Protein Homology/analogy Recognition Engine V 2.0 (Phyre2) (Imperial College of London, http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Kelley et al., 2015) with their sequences found in the NCBI database. The top model generated by Phyre2 for each sequence of the above organisms was selected: putative sialidases for *A. terreus*, *T. rubrum*, and *T. equinum* were modelled to the crystal structure of *A. fumigatus* KDNase (template c2xcyA), and for *Sphingobacterium* sp. 21 and *S. avermitilis* were modelled to the crystal structure of sialidase from *Parabacteroides distasonis* (template cjf46c). The Phyre generated structures of each putative sialidase were compared with the crystal structures of the catalytic domains of known sialidases: *Micromonaspora viridifaciens* (PDB 1EUS), *Streptococcus pneumoniae* NanA (PDB 2VVZ), *Clostridium perfringens* Nanl (PDB 2BF6), and of the *A. fumigatus* KDNase (PDB 2XZI). The structures were aligned based on sequence homology using PyMol (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC.). The placement of Neu5Ac2en is based on the co-crystal structures of *M. viridifaciens* sialidase and Neu5Ac2en (PDB 1EUS) and for KDN2en, the co-crystal structure of *A. fumigatus* KDNase and KDN2en (PDB 2XZI). The same sequences above were aligned using Clustal W2 (EMBL-EBI, http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Larkin et al., 2007)

4.4. Results and Discussion

To investigate the structural and functional significance of the key amino acid residues in the active site of the *AfS*, three mutant enzymes, Y358H, D84A and R171 were constructed and expressed in *E. coli*, and their crystal structures resolved. Kinetic analysis was carried out for all enzymes and the results were compared to both wild type *AfS* as well as *MvS*. 

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4.4.1. pH Optima of R171L, Y358H, and D84A

Before determining the kinetic parameters of the mutant enzymes, the pH optimum for each of the enzymes was determined and the results are shown in Figure 4.2. With 4-methylumbelliferyl α-D-KDN (KDN-Mu) (pKa ~1.58) as the substrate, the pH optimum of all mutant enzymes was pH 4.0, the same as for wild type AfS. The wild type enzyme exhibited some activity at pH 5.2 ($k_{cat}/K_m = 47.7 \text{ M}^{-1}\text{s}^{-1}$) but the nucleophile mutant (Y358H) and the general acid/base mutant (D84A) were essentially non-functional at this pH. R171L was the only mutant enzyme that exhibited any activity at pH 5.2 ($k_{cat}/K_m = 0.43 \text{ M}^{-1}\text{s}^{-1}$). In contrast, the pH optimum was pH 5.2 for all mutant KDNase enzymes with 4-methylumbelliferyl α-D-Neu5Ac (Neu5Ac-Mu) (pKa ~1.55) as the substrate, whereas the pH optimum was 3.5 for wild type (274). Unlike the wild type enzyme where some activity could be detected between pH 3.0 to pH 5.2, enzyme activity was only observed at pH 5.2 for the mutant enzymes.

![Figure 4.2](image)

**Figure 4.2** pH profile for R171L, D84A, and Y358H with either KDN-Mu or Neu5Ac-Mu as substrate

pH profile R171L (●), D84A (▲), Y358H (■). The pH optimum was different when KDN-Mu or Neu5Ac-Mu was the substrate; all mutant enzymes had a pH optimum of 4.0 with KDN-Mu (A) and with Neu5Ac-Mu (B) activity was present only at pH 5.2. The Y-axis represents the amount of 4-methylumbelliferone (µM) released after 20 min as described in the Experimental Section. The pH optimum of the wild type AfS with KDN-Mu is pH 4.0 (data not shown) and as determined previously in Telford et al. 2011.
4.4.2. Kinetic analysis of the R171L, Y358H, and D84A mutants of *A. fumigatus* sialidase/KDNase

Michaelis-Menten parameters for R171L, Y358H, and D84A were measured with the following substrates: KDN-MU, Neu5Ac-MU, or KDNα2,6GalβFMU (4; synthesis outlined in Appendix B, Scheme 1). The catalytic efficiency ($k_{cat}/K_m$) was measured for all three mutants using the three different substrates and the data is presented in Table 4.1.

**Table 4.1** Kinetic parameters of *A. fumigatus* sialidase/KDNase wild type enzyme and the three mutant enzymes generated in this study.

<table>
<thead>
<tr>
<th>Sialidase</th>
<th>Substrate</th>
<th>$k_{cat}/K_m$ (M⁻¹s⁻¹)</th>
<th>Substrate</th>
<th>$k_{cat}/K_m$ (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Af KDNase</td>
<td>KDN-MU</td>
<td>1.82 ± 0.09 x 10⁵*</td>
<td>Neu5Ac-MU</td>
<td>22.3 ± 3.0 *(ref 22)</td>
</tr>
<tr>
<td></td>
<td>KDN-MU*</td>
<td>47.7 ± 3.6</td>
<td>Neu5Ac -MU (pH 5.2)</td>
<td>53.3 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>KDNα2,6GalβFMU</td>
<td>8.0 ± 0.6 x 10³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R171L</td>
<td>KDN-MU</td>
<td>75.2 ± 7.7</td>
<td>Neu5Ac -MU</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>KDN-MU*</td>
<td>0.33 ± 0.02</td>
<td>Neu5Ac -MU*</td>
<td>74.2 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>KDNα2,6GalβFMU</td>
<td>1.62 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y358H</td>
<td>KDN-MU</td>
<td>26.8 ± 0.9</td>
<td>Neu5Ac -MU*</td>
<td>14.5 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>KDNα2,6GalβFMU</td>
<td>8.9 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D84A</td>
<td>KDN-MU</td>
<td>11.6 ± 0.6</td>
<td>Neu5Ac -MU*</td>
<td>19.0 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>KDNα2,6GalβFMU</td>
<td>0.2 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mv Sialidase</td>
<td>KDN-Mu</td>
<td>(1.03 ± 0.36) x 10³</td>
<td>Neu5Ac -MU</td>
<td>(7.2 ± 1.4) x 10⁵*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*(ref 29)</td>
</tr>
</tbody>
</table>

Values obtained with a bacterial sialidase (Micromonospora viridifaciens (Mv) sialidase under the same conditions are show for comparison. * Kinetic experiments from this study were performed at pH 5.2 using 16 mM sodium tartrate (pH 5.2) buffer. All other experiments were completed using 50 mM sodium acetate (pH 4.0) buffer, or pH 3.5 (ref 17)

With KDN-Mu as a substrate, R171L had a catalytic efficiency of 75.2 M⁻¹s⁻¹ at pH 4.0; however, at pH 5.2, the catalytic efficiency dropped more than two orders of magnitude to 0.33 M⁻¹s⁻¹. The catalytic efficiencies of Y358H and D84A were 26.8 and
11.6 M⁻¹s⁻¹, respectively. At pH 5.2, Y358H and D84A had no detectable catalytic activity on KDN-Mu. The catalytic efficiencies of the mutant enzymes on KDN-Mu were significantly lower compared to the wild type enzyme ($k_{\text{cat}}/K_m$ of $(1.82 \pm 0.09) \times 10^5$ M⁻¹s⁻¹) (296). However, at pH 5.2, the wild type enzyme has a similar catalytic efficiency ($k_{\text{cat}}/K_m$ of 47.7 M⁻¹s⁻¹) to R171L at pH 4.0.

At pH values greater or lesser than 0.5 pH units from pH 5.2, the mutant enzymes cleaved Neu5Ac-Mu either at a very low catalytic efficiency or at levels that were below detectable limits: at pH 4.0, the $k_{\text{cat}}/K_m$ was 2.3 M⁻¹s⁻¹ for R171L and undetectable for D84A and Y358H. In contrast, the wild type enzyme was able to cleave Neu5Ac-Mu with similar kinetic efficiency at pH 3.5 ($k_{\text{cat}}/K_m = 22.3$ M⁻¹s⁻¹) (274) and at pH 5.2 ($k_{\text{cat}}/K_m = 53.3$ M⁻¹s⁻¹). However, at the pH optimum of 5.2, the catalytic efficiency for the mutants with Neu5Ac-Mu was within an order of magnitude of that of the wild type enzyme: R171L ($k_{\text{cat}}/K_m = 74.2$ M⁻¹s⁻¹), Y358H ($k_{\text{cat}}/K_m = 14.5$ M⁻¹s⁻¹), and D84A ($k_{\text{cat}}/K_m = 19.0$ M⁻¹s⁻¹) (Table 4.1).

With the natural substrate analogue, KDNα2,6GalβFMU at pH 4.0, the activity of all mutant enzymes was ~1000-fold lower than the wild type AfS. Wild type AfS had a $k_{\text{cat}}/K_m$ of 8000 M⁻¹s⁻¹. Although, R171L and Y358H had similar catalytic efficiency to each other, $k_{\text{cat}}/K_m$ of 1.6 M⁻¹s⁻¹ and $k_{\text{cat}}/K_m$ of 8.9 M⁻¹s⁻¹, respectively, D84A had even lower catalytic efficiency on KDNα2,6GalβFMU ($k_{\text{cat}}/K_m$ of 0.2 M⁻¹s⁻¹). In this coupled enzyme reaction, the release of 8-fluoro-4-methylumbelliferone by β-galactosidase was not rate determining for KDNα2,6GalβFMU because the concentration of β-galactosidase did not affect the rate (data not shown). We compared the ratios of the catalytic efficiencies of KDN-Mu/KDNα2,6GalβFMU for the wild-type enzyme and the mutant enzymes: the wild-type enzyme was 22.8 ± 2.0 and the ratios for the mutant enzymes were 46.4 ± 4.9 (R171L), 3.01 ± 0.14 (Y358H), and 58 ± 29 (D84A). These observations rule out contamination of mutant enzymes by wild type because mutant enzymes containing a small amount of wild type would yield the same ratio as the wild type.
4.4.3. Crystal structures of R151L, Y358H, and D84A

The three dimensional structure of the three mutants determined by X-ray crystallography to a resolution of 1.84Å, shows that the changes made to either the tyrosine or the aspartic acid in the active site, and the replacement of the arginine to a leucine in the C-5 hydroxyl group binding pocket did not affect the overall structure of the enzyme. All three mutant enzymes were expressed, purified and crystallized with the same procedures as those used for the wild type KDNase (296).

4.4.4. R171L mutant KDNase

Analysis of the AfKDNase crystal structure suggested that modification of arginine 171 to a leucine should accommodate the N-acetyl group of Neu5Ac. A crystal structure of the mutant in the presence of the Neu5Ac-related tight binding inhibitor, Neu5Ac2en, shows that this is indeed the case (Figure 4.3), but the ligand was only present in one of the monomers in the asymmetric unit of the crystal, and had a low occupancy (0.6) in the other. Nevertheless, expanding the binding pocket in R171L resulted in an enzyme that was a 100-fold more active sialidase than a KDNase at pH 5.2, while AfS had a similar level of activity with both substrates (Table 4.2). At the pH optimum of 4.0, the wild type enzyme had 8000-fold higher activity on KDN-Mu compared to R171L. Therefore, converting the arginine to a leucine made AfS a relatively less active KDNase. These data indicate that the close interaction between the arginine in the binding pocket and the C5 hydroxyl group of the sugar is essential for catalysis.

Table 4.2 Ratio of the catalytic efficiency of the wild type KDNase and the R171L mutant using either KDN-MU or Neu5Ac-MU as the substrate

<table>
<thead>
<tr>
<th></th>
<th>k_{cat}/K_m (KDN-MU)</th>
<th>k_{cat}/K_m (Neu5Ac-MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>8160</td>
<td>0.89</td>
</tr>
<tr>
<td>R151L</td>
<td>33.3</td>
<td>0.01</td>
</tr>
<tr>
<td>pH 5.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.3  Crystallographic analysis of the R171L mutant in complex with Neu5Ac2en.

Top, a stereo 1.84Å Fo-Fc electron density contoured at 1.8σ for the ligand bound in monomer A of the asymmetric unit. Bottom, a stereo image showing interactions of Neu5Ac2en with the active site of monomer A.

Previous phylogenetic analysis revealed that the *A. fumigatus* KDNase is shares a common ancestor with bacterial sialidases in the phyla Actinobacteria and Bacteroidetes (274). Previous work has shown that the sialidase from *Sphingobacterium multivorum*, a member of Bacteroidetes is also a KDNase (177). We predicted that other KDNases would have a steric clash with the N-acetyl group of Neu5Ac. We modelled the three-dimensional structures of selected known sialidases (*Clostridium perfringens, Streptococcus pneumoniae, M. viridifaciens*) as well as putative KDNases (*A. terreus, Trichophyton rubrum* and *T. equinum, Sphingobacterium* sp.21 and *Streptomyces avermitilis*) with AF5 (Figure 4.4). From the structures, arginine was found in all of the
putative KDNases at the same position in the active site as R171 in \textit{AfS} whereas in the known sialidases, the amino acid residue interacting with the \textit{N}-acetyl group was serine. ClustalW2 alignments showed that in each case, the putative KDNases had an arginine at the same position in the sequence (Figure 4.5). Experimental validation will be required to confirm whether KDN-containing glycans are the preferred substrate of these enzymes.

<table>
<thead>
<tr>
<th>Substrates Placed in Enzyme Active Sites</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Neu5Ac2EN</td>
<td>KDN2EN</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aspergillus fumigatus (\textit{AfS})</th>
<th>\begin{itemize} \item R171 \item 1.7 \end{itemize}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micromonospora viridifaciens (\textit{MvS})</td>
<td>\begin{itemize} \item L169 \item 4.6 \end{itemize}</td>
</tr>
<tr>
<td>Aspergillus terreus</td>
<td>\begin{itemize} \item R167 \item 4.5 \end{itemize}</td>
</tr>
</tbody>
</table>
Modeling of Neu5Ac2EN or KDN2EN C5 substituent interaction with the enzyme active site of putative microbial sialidase/KDNase.

Alignment of the modelled catalytic site allowed for a direct comparison of how Neu5Ac2EN or KDN2EN would be positioned in the enzyme active site of putative sialidase/KDNase. The interaction between the C5 substituent of either Neu5Ac2EN or KDN2EN, and the amino acid located at a similar position to Afs Arg171 or Mvs Leu169 in the putative enzymes were measured (Å). The clash observed between the –N-acetyl group of Neu5Ac2en Afs Arg171 is also seen for the putative sialidases found in A. terreus, Sphingobacterium sp21 and T. rubrum. The catalytic domains of the determined structures of C. perfringens and S. pneumonae sialidases were aligned to the putative sialidase/ KDNase structures using PyMol and provided similar results (data not shown).
Table 4.5 Clustal W2 alignment of selected bacterial and fungal sialidases

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
<th>Sialidase Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cperfringens</td>
<td>YLCLYDSSGK-ETFVRENS-VVYKDS--NKITEY----TINALGDL 164</td>
<td></td>
</tr>
<tr>
<td>Spneumoniae</td>
<td>YQILYREKGAYTRENGTYYITDP--KADTYRVDQVPDFVAYASDKDGL 190</td>
<td></td>
</tr>
<tr>
<td>Afumigatus</td>
<td>WEPRLVGGSTAVGNTIFVPPDNN--TYYLFLSNGGAYZSGQGDV 153</td>
<td></td>
</tr>
<tr>
<td>Aterreus</td>
<td>WESLRETVGSGGNTIFVPPDNN--TYYLFLSNGGAYZSGQGDV 149</td>
<td></td>
</tr>
<tr>
<td>Trubrum</td>
<td>WESLRETVGSGGNTIFVPPDNN--TYYLFLSNGGAYZSGQGDV 137</td>
<td></td>
</tr>
<tr>
<td>Tequnim</td>
<td>WESLRETVGSGGNTIFVPPDNN--TYYLFLSNGGAYZSGQGDV 155</td>
<td></td>
</tr>
<tr>
<td>Sphingobacterium sp. 21</td>
<td>WSALGVEVGKNGNTIFVPPDNN--TYYLFLSNGGAYZSGQGDV 154</td>
<td></td>
</tr>
<tr>
<td>Savermitilis</td>
<td>WGLPQAVNANGLNFAFPVLDTG--BRVLLVHVAAGA 100</td>
<td></td>
</tr>
<tr>
<td>Mviridificiens</td>
<td>QVSVASQITAPIFGSDPSVYLDEERG--TIFNHVYVSRQA 111</td>
<td></td>
</tr>
<tr>
<td>Cperfringens</td>
<td>FONKIKIINNS----APKAWGSRKSLVINYSDQDKWGQKNSPGQFQV 211</td>
<td></td>
</tr>
<tr>
<td>Spneumoniae</td>
<td>YKGNQLOLRLNFTQFKITSPRRAKSLWNYSDQDKWQKNSPGQFQV 240</td>
<td></td>
</tr>
<tr>
<td>Afumigatus</td>
<td>LDPDPVTRKIDSTKE---GPQHLTSTDDGNSKTVLKLK 195</td>
<td></td>
</tr>
<tr>
<td>Aterreus</td>
<td>LDPDPVTRKIDSTKE---GPQHLITSTDDGNSKTVLKLK 191</td>
<td></td>
</tr>
<tr>
<td>Trubrum</td>
<td>LDPDPVTRKIDSTKE---GPQHLITSTDDGNSKTVLKLK 179</td>
<td></td>
</tr>
<tr>
<td>Tequnim</td>
<td>LDPDPVTRKIDSTKE---GPQHLITSTDDGNSKTVLKLK 177</td>
<td></td>
</tr>
<tr>
<td>Sphingobacterium sp. 21</td>
<td>--DEEHNTSTDQFAW-----IGHWYTSDDHATGNDSDVPTDRGTL 194</td>
<td></td>
</tr>
<tr>
<td>Savermitilis</td>
<td>SEDA1TRDQGKLGSSNLSQGGLQITDRQGTL 140</td>
<td></td>
</tr>
<tr>
<td>Mviridificiens</td>
<td>GFA0GRFQTDAPDN--VHLANVSDTGGGSLTHRCITADIT 152</td>
<td></td>
</tr>
</tbody>
</table>

Clustal W2 alignment of selected bacterial and fungal sialidases

The arginine residue in the active site corresponding to R171 in the A. fumigatus KD NaSe is boxed for all sialidases except M. viridifaciens. In the M. viridifaciens sialidase, the leucine at this site in the crystal structure corresponds to the boxed L169 on the figure. Using Phyre2 modelling (www.sbg.bio.ic.ac.uk/phyre2), all boxed R amino acids were confirmed to align to the active site crystal structures of known sialidases: either Afs (R171) (A. terreus (R167), T. rubrum (R 173) and T. equinum (R155)), or the bacterial sialidase from Parabacteriodes distasonis (Sphingobacterium sp. 21 (R170) and Streptomyces avermitilis (R116)). ClustalW2 can be accessed at http://www.ebi.ac.uk/Tools/msa/clustalw2/.
The cavity around the N-acetyl group of Neu5Ac2en is certainly larger in the R171L mutant compared to wild-type (Figure 4.6 A,B); however, the indole ring of W202 is only 3Å from the Neu5Ac2en methyl group, and this suggests that mutation of W202 to a smaller hydrophobic amino acid might open up the cavity further.

![Figure 4.6](image)

**Figure 4.6** Effects of R151L mutation on cavity around C5 of ligand

In both images, Neu5Ac2en is modeled from the superposition of an MvS-Neu5Ac2en structure on AfS. A. Wild type AfS showing the clash with R171. B. Structure of the R171L mutant showing a slightly larger cavity.

### 4.4.5. Y358H mutant KDNase

The removal of the catalytic nucleophile reduced the activity of AfS by more than 1000-fold compared to wild type (Table 4.1). This result is similar to that found with the MvS tyrosine mutants (Y370A, Y370D, and Y370G); in the MvS mutants removal of the nucleophile resulted in a $10^2$-fold decrease in activity. A more profound decline ($10^5$-fold) in kinetic activity was found in the Y347F *Clostridium perfringens* small sialidase when compared to wild type (301), and in the *T. cruzi* Y342F and Y342T mutants in which activity was eliminated (293).

The replacement of the nucleophilic tyrosine in the MvS active site with a small negatively charged or hydrophobic side chain altered the mechanism of the enzyme by turning it into an inverting sialidase (194). In contrast, the *A. fumigatus* Y358H mutant
enzyme remained an active retaining KDNase (Figure 4.7). The hydrolysis of KDN-Mu to form KDN was monitored as a time course reaction using $^1$H NMR spectroscopy in deuterated buffer at a pD of 4.0. The H3$_{eq}$ proton of $\alpha$KDN-Mu showed the characteristic doublet of doublets ($\delta = 2.82$ ppm, $J_{3e,3a} = 12.7$ Hz, $J_{3e,4} = 4.7$ Hz). As seen in the first spectrum recorded after the addition of enzyme, an upfield chemical shift occurs for the H3$_{eq}$ resonance of the initial hydrolysis product ($\delta = 2.68$ ppm) resulting from cleavage of the umbelliferyl leaving group (Figure 4.7). Over time, $\alpha$KDN mutarotates to the equilibrium mixture that favours the thermodynamically more stable $\beta$KDN with the appearance of the H3$_{eq}$ resonance at $\delta = 2.20$ ppm. The structure of the Y358H mutant showed little change from the wild type (Figure 4.8A, B). As can be clearly seen in the crystal structure, the replacement of the tyrosine with a histidine residue results in substitution of the nucleophilic oxygen with a more distal nitrogen atom (Figure 5D). Although histidine may act as a nucleophile, in the deglycosylation step (hydrolysis of the enzyme-bound intermediate) for the wild type enzyme the tyrosinyl leaving group (with catalysis by the glutamate) is a good leaving group whereas the histidinyl (even when protonated) is a much worse leaving group. Only extensive isotope studies would reveal whether His is acting as a nucleophile or whether the reaction proceeds via a carbocation. This result, along with the decrease in activity of Y358H mutant (6300 fold for KDN-Mu) suggests that some transition state stabilizing for glycosylation by AfS results from weak nucleophilic attack occurring on the anomeric centre of the KDN substrate by the tyrosine but likely none by the histidine residue. In other words, moving the nucleophilic group to a greater distance likely changed the mechanism to a dissociative $S_N$1 reaction. It is known that the lifetime of a N-acetyleneuraminylium ion, which is $> 3 \times 10^{-11}$ s$^{-1}$, (sialic acid cation) (302) is at least ten-fold longer than that for a glucopyranosylum ion (303) and that a glucopyranosylum ion is a viable intermediate in non-nucleophilic environments (304, 305). As a result, we conclude that the Y358H mutant AfS could operate via a dissociative $S_N$1 mechanism in a similar fashion to the MvS Y370F mutant (281). Alternatively, it is possible that the glutamate residue changes its function from acid/base to nucleophilic catalysis. Again detailed kinetic isotope effect studies would be required to confirm this point.
Figure 4.7 The Y358H mutant of the AfS is a retaining sialidase as determined by \textsuperscript{1}H-NMR.

Hydrolysis of KDN-MU to form KDN was monitored in a time course. The spectra reveal the gradual depletion of the substrate followed by the production of \(\alpha\)-KDN. The accumulation of \(\beta\)-KDN at 135 min results from mutarotation of \(\alpha\)-KDN to yield the equilibrium mixture that favours the more stable \(\beta\)-anomer of the sugar.

4.4.6. D84A mutant KDNase

The third catalytic residue in the enzyme active site is the conserved aspartic acid residue that acts as a general acid/base catalyst. Mutation of this amino acid to alanine in AfS decreased its activity by 15,000 - 50,000 fold. The effect of altering the acid/base catalyst was much more pronounced in the case of AfS than it was for similar mutations made in MvS (D92G, D92A and D92S), which only resulted in ~10-20-fold decline in activity (278), and the T. cruzi trans-sialidase (D59A) which showed \(k_{cat}/K_m\) values similar to those of the wild type enzyme (138). As can be seen, the D84A mutation did not cause significant rearrangement of the active site of the AfS (Fig. 4.8 A, C, E), a conclusion that is similar to that for the D92G mutation in MvS (278). Together, these data suggest that the general acid/base is more important catalytically in the reactions of the AfS than it is for MvS. It is possible that this phenomenon is related to
the lower pH optimum of the *A. fumigatus* enzyme pH 4.0 where the general acid residue will have a greater degree of protonation relative to the sialidase from *M. viridifaciens* (pH 5.2) (194) and the trans-sialidase from *T. cruzi* (pH 7.6) (138).
Figure 4.8  Crystal structures of the Y358H and D84A mutants.

(A). Overlay of the two molecules in the asymmetric unit of AfSY358H (green), AfSD84A (blue) and the wild type (yellow). There is no significant difference in the active site residues in the active site of either AfSY358H (B) or AfSD84A (C). There are dual conformations seen for Arg265 in the AfSY358H (B) and Arg171 in AfSD84A (C) neither of which are likely to affect the substrate binding. (D) The imidazole ring of His358 of AfSY358H superimposes over the hydroxyphenyl ring of Tyr358 of the wild type enzyme. (E) The overlay of AfSD84A with the wild type enzyme focusing on the mutation.
4.5. Conclusions

Structure can often help predict function. A single amino acid change to enlarge the binding pocket in R171L mutant converted the AfS into a more efficient sialidase. However, the activity of the R171L mutant was still significantly less with sialic acid as a substrate compared to KDN; therefore, additional amino acid changes will be necessary to accommodate the N-acetyl group of sialic acid. We hypothesize that an additional mutation to reduce the size of tryptophan 202 would most likely improve the sialidase activity of the AfS (Figure 4.6). Despite the close structural similarity of the active sites of the A. fumigatus KDNase with the bacterial sialidase from Micromonospora viridifaciens, mutants of two active site residues generated different results with the two enzymes. For example, unlike the M. viridifaciens sialidase and T. cruzi trans-sialidase in which mutation of the active site tyrosine changed the enzyme from a retaining to an inverting sialidase, the Y358H mutant of AfS remained a retaining sialidase. In addition, the Y358H mutant retained appreciable enzymatic activity unlike the protozoan enzyme where the mutation of the nucleophile eliminated catalytic activity. Since the optimal pH of AfS is 4.0, lower than other known sialidases, it was not unexpected that mutation of the acid in the catalytic centre (D84A) caused a greater decrease in the activity of AfS compared to aspartate mutations in the M. viridifaciens sialidase or T. cruzi trans-sialidase. These data represent the first detailed kinetic analysis of any KDNase and expand our understanding of the influences that active site structure have on sialidase activity and substrate selectivity.
Chapter 5.

UDP-Sugar Transporters in the Fungal Pathogen, *Aspergillus fumigatus*

This chapter is a modified form of the manuscript in preparation for publication under the authorship of Juliana Yeung, Mark Warwas, Brandon Kwok, Lindsay Woof, Helen Croft, Amrit Bath, Tysha Donnelly, Linda Pinto, Joe Tiralongo*, Thomas Haselhorst*, and Margo M. Moore* 2015. UDP-Sugar Transporters in the Fungal Pathogen, *Aspergillus fumigatus*.

Author contributions: This project was designed by Prof. Margo Moore and Juliana Yeung. JY performed all the experimental work in cloning, expressing, and isolation of the AfNSTs, conducting the radiolabeled transport assys and STD-NMR experiments, creating of the *AfΔnst5KO*, and phenotype analysis of the knockout fungi strains. Mark Warwas and Linda Pinto created the *AfΔnst1KO*. Undergraduate students: Brandon Kwok, Helen Croft and Amrit Bath assisted with the creation of the *AfΔnst5KO*, Tysha Donnelly assisted with the optimization of AfNST expression, and Lindsay Woof helped with the phenotype analysis of the knockout mutants. Dr. Joe Tiralongo collaborated on the STD-NMR experiments. Dr. Thomas Haselhorst optimized the pulse sequences used for the STD-NMR experiments and trained Juliana Yeung how to operate the NMR spectrometer and to use the Top-Spin program to analyse the results.

5.1. Preamble

The in-depth characterization of the *A. fumigatus* sialidase (KDNase) addressed in the previous chapters revealed that *A. fumigatus* has the ability to metabolize KDN. However, we still wished to investigate how Neu5Ac was presented on the fungal cell surface with the ultimate goal of creating a sialic acid-deficient mutant. This chapter will
address how we investigated this aspect of sialic acid metabolism in *A. fumigatus*: characterization of four putative nucleotide sugar transporters (NSTs) and creation of *A. fumigatus* NST knockout mutants to study the biological role of these NSTs.

5.2. Abstract

*Aspergillus fumigatus* is a pathogenic mould that is the primary cause of invasive aspergillosis, a systemic infection that affects immunocompromised patients. The fungal cell wall is a unique network of complex carbohydrates that is a primary target for the development of antifungal agents. Presentation of sugars on the cell surface is mediated by nucleotide sugar transporters (NSTs) that import activated sugars into the ER/Golgi lumen for addition to growing glycosidic chains. We characterized four *A. fumigatus* NSTs (*Af*NSTs) to determine their substrate specificity and functional importance. Using Golgi-enriched fractions made from yeast overexpressing *Af*NST1 and *Af*NST5, we found that both NSTs transported UDP-14C-galactose (Gal). Saturation transfer difference (STD) NMR experiments revealed that UDP-Gal and UDP-Glu bound to *Af*NST1 and *Af*NST5, while UDP-GalNAc bound to *Af*NST1 and *Af*NST6. Knockout (KO) mutants of *Af*NST1 and *Af*NST5 displayed significant growth differences on defined media supplemented with the polysaccharide-binding dye, Congo Red. Unlike wild type or the *AfΔnst1KO, AfΔnst5KO* displayed severe hyphal deformities on medium containing Congo Red. Flow cytometry analysis revealed that both knockout mutants had lower amounts of galactose on the conidial surface but only *AfΔnst5KO* had reduced amounts of sialic acid. Our study reveals that although both NST1 and NST5 were able to transport UDP-Gal, the knockout phenotypes revealed that these proteins have profoundly different effects on morphology. The finding that *A. fumigatus* NSTs have compensatory mechanisms for activated sugar transport has implications for cell wall biosynthesis as a target for antifungal drug development.

5.3. Introduction

*Aspergillus fumigatus* is a filamentous fungus that is the most common cause of a life-threatening systemic mycosis called invasive aspergillosis that primarily infects
immunocompromised hosts (17). The small size of *A. fumigatus* asexual spores (conidia) allows their deep infiltration into alveolar tissue, resulting in adhesion to pulmonary epithelial cells or basal lamina (22, 66). Germination and hyphal growth of *A. fumigatus* results in invasion of deeper tissues, and hyphae that have invaded the vasculature may disseminate and cause infection in other organs such as the brain (11).

The cell wall of *Aspergillus fumigatus* is composed of glycoproteins as well as the structural polysaccharides, chitin (poly- β-1,4-N-acetylglucosamine), β-1,3/β1,4-glucans and β-1,3/β1,6-glucans (306). The glucans and chitin are synthesized by specific plasma membrane synthases that polymerize activated sugars present in the cytosol (307, 308). Galactosaminogalactan (GAG) is a heterogenous linear polymer consisting of α1-4 linked galactose and N-acetylglactosamine (82) and GAG biosynthetic machinery in *A. fumigatus* has been localized to the plasma membrane (309). In addition, the cell wall contains galactomannan (GM), a linear polymer of α1,2/α1,6-mannan with short side chains of β-1,5-galactofuran, (4, 85). Unlike other cell wall polysaccharides, have shown that galactomannan is synthesized in the Golgi apparatus and requires functional GDP-mannose and UDP-galactofuranose transporters (310). Both GAG and GM form part of the soluble fraction of the cell wall, play a role in biofilm formation and are found in patient blood (82). The presence of galactomannan in patient blood or bronchoalveolar lavage fluid is detected by the monoclonal antibody EB-A2 for clinical diagnosis of invasive aspergillosis (311, 312). Galactosaminogalactan polymers on *A. fumigatus* are also antigenic and antibodies to galactosaminogalactans antibodies have been detected in human serum, even in the absence of infection by *Aspergillus* species (85). Galactosaminogalactan has also been shown to participate in the adhesion of *A. fumigatus* to the cell surface (82).

Glycoproteins are important for fungal development as well as cell wall synthesis and integrity. For example, α-1,3-glucanosyltransferases require proper N-glycosylation to catalyze the synthesis of α-1,3-glucan at the plasma membrane (313). Global repression of N-glycosylation in *A. fumigatus* resulted in upregulation of the unfolded protein response and a severe growth defect and compromised cell wall integrity (314). The biosynthesis and modulation of glycoproteins destined for the cell surface in *A. fumigatus* occurs primarily in the endoplasmic reticulum (ER) or Golgi complex (315,
Glycoproteins and glycolipids are formed in these compartments from activated nucleotide sugars such as UDP-galactose (UDP-Gal) or UDP-N-acetylgalactosamine (UDP-GalNAc). In mammals, N- and O-glycans may be terminated with members of the sialic acid family; 9-carbon, negatively-charged monosaccharides. Although the enzymes responsible for their biosynthesis have not yet been identified, *A. fumigatus* conidia also possess sialic acids and these have been shown to play a role in fungal pathogenesis. In *A. fumigatus*, ο2,6-linkages join sialic acids with the underlying galactose residues in glycan chains (88) and pathogenic Aspergillus species were found to have a higher density of sialic acids on the conidial surface than non-pathogenic species (87). Binding of *A. fumigatus* conidia to epithelial cells (304) and to the basal lamina (66, 88) was shown to be mediated in part by sialic acids on the fungal spore. Finally, removal of sialic acids decreased conidial uptake by cultured murine macrophages and Type 2 pneumocytes (88) suggesting that ligands with sialylated glycans were recognized by these professional phagocytes.

Nucleotide sugar transporters (NSTs) are multi-pass membrane proteins in the Golgi and ER that mediate the transfer of the nucleotide sugars from the cytosol into the ER or Golgi lumen where the nucleotide sugars are transferred to growing glycan chains by sugar transferases (317). An NST can be specific for a single nucleotide sugar, as has been shown in the murine CMP-Neu5Ac transporter (129) and the Chinese hamster ovary cell (CHO) UDP-Gal transporter (318). Other NSTs are able to transport more than one nucleotide-sugar but these transporters are always selective for the same nucleotide; examples include the family of six *Arabidopsis* UDP-rhamnose (UDP-Rhm)/UDP-Gal transporters (319) and the three *Caenorhabditis elegans* NSTs that transport UDP-glucose (UDP-Glc), UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-Gal, and UDP-GalNAc (320). To date, only the UDP-galactofuranose transporter and the GDP-mannose transporter have been characterized in *A. fumigatus* (310, 321).

Through a bioinformatics search of the *A. fumigatus* genome in the NCBI database, six putative *AfNSTs* were identified. Later, 10 additional NSTs were annotated by a different study (310). By characterizing these sugar transporters, we aim to gain a better understanding of glycan processing in fungi. Details of the initial bioinformatics analysis of the characterized transporters are discussed and presented in Table C3.
Specifically, the purpose of this study was to investigate four of the six \textit{A. fumigatus} NSTs we identified on the NCBI database by (A) cloning four genes encoding those proteins and expressing them in yeast, (B) enriching the AfNSTs in endoplasmic reticulum (ER) and Golgi enriched fractions from transformed yeast, (C) characterizing ligand specificity by measuring transport activity of radiolabelled nucleotide sugars, and analyzing nucleotide sugar binding to NSTs by saturated transfer difference (STD) NMR spectroscopy. In addition, we created NST knockout strains in \textit{A. fumigatus} of two NST and compared their morphology and glycan composition to wild type under conditions of cell wall stress.

5.4. Materials and Methods

5.4.1. \textit{A. fumigatus} strains and growth conditions

The clinical isolate of \textit{A. fumigatus} (strain ATCC 13073) was used for all studies. Unless otherwise specified, the fungus was grown on MYPD agar, and conidia were harvested with PBS containing 0.05% Tween 20 and washed twice with PBS. The accession numbers for the \textit{A. fumigatus} putative nucleotide sugar transporter (\textit{nst}) genes examined in this study were Afu6g13073 (\textit{Afnst1}), Afu8g02090 (\textit{Afnst2}), Afu105440 (\textit{Afnst5}), and Afua_1G06050 (\textit{Afnst6}).

5.4.2. Bioinformatic analyses

Bioinformatic analyses were conducted using the genomic sequence for the \textit{A. fumigatus nst} genes from sequencing of the \textit{A. fumigatus} ATCC 13073 strain. The Af293 genomic sequence was obtained from the Aspergillus Genome Database (www.aspgd.org). ProtParam was used to estimate the molecular mass. HMMTOP 2.0 (322, 323) (http://www.enzim.hu/hmmtop/) was used to predict the transmembrane domains and Topo2 (324) (http://www.sacs.ucsf.edu/cgi-bin/open-topo2.py) was used to illustrate the 2D prediction of the fungal NST proteins. WolF PSORT (325) (http://wolfpsort.seq.cbrc.jp/) was used to predict the cellular localization of the \textit{A. fumigatus} nst.
5.4.3. Cloning and overexpression of Afnst genes in *Saccharomyces cerevisiae*

RNA from *A. fumigatus* was extracted as described in the QiAgen RNeasy Plant Mini Kit (QiAgen, Mississauga, ON) with an additional on-column DNase digestion step to remove all traces of genomic DNA with the RNase-Free DNase Set (QiAgen, Mississauga, ON). First-strand cDNA was generated by SuperScript II RT (Invitrogen) using gene specific reverse primers. Primers were designed based on the annotation by the *A. fumigatus* genome project (www.aspgd.org) and Table C1 includes the list of primers used. Platinum *Pfx* DNA Polymerase (Invitrogen) was used in the PCR reactions with gene-specific primers to generate *Afnst1* and *Afnst5* for cloning into the multiple cloning site of pESC-URA (Agilent Technologies) with a FLAG tag at the C-terminus. *Afnst6* was cloned into pESC-URA with a MYC tag at the C-terminus and *Afnst2* was cloned into pRS313 (a gift from Dr. Christopher Beh). The plasmids were transformed into *E. coli* DH5α, and sequenced to confirm that correct gene was inserted.

Subsequently, all pESC-URA-NST plasmids were separately transformed into *Saccharomyces cerevisiae* (YPH500) by the lithium acetate method as per the manufacturer’s directions (Agilent Technologies). Empty pESC-URA plasmid was transformed into the same yeast strain as a negative control.

In pESC-URA, the expression of *Afnst1*, *Afnst5* and *Afnst6* genes in *S. cerevisiae* was controlled by the GAL promoter. The yeast strains were maintained on synthetic raffinose (SR) media (0.67% yeast nitrogen base without amino acids, 2% raffinose, 1.3 g amino acid drop out powder without uracil, 1 litre water). Overnight pre-cultures were grown in SR broth and induction of each protein was accomplished by growing yeast containing p*Afnst1* for 12 hrs, yeast transformed with p*Afnst5* for 20 hrs, or yeast transformed with p*Afnst6* for 16 hr in synthetic galactose (SG) broth (per litre: 0.67% yeast nitrogen base without amino acids, 2% galactose, 1.3 g amino acid drop-out powder without uracil). For *Afnst2*, expression was controlled by the Met3 promoter; hence, the yeast were maintained on synthetic complete (SC) media (0.67% yeast nitrogen base without amino acids, 2% glucose, 2.0 g amino acid drop out powder without histidine, and 1 mg/mL methionine). As a negative control, methionine was added to the cultures to suppress the expression of *Afnst2*. Overnight pre-cultures were
grown in SC broth and induction of *Afnst2* was achieved when the yeast were grown in media without methionine.

### 5.4.4. Isolation of Endoplasmic Reticulum (ER) and Golgi-enriched fractions (ERGEF) from yeast

Yeast transformed with *pAfnst1*, *pAfnst5*, and *pESC-URA* grown in both uninduced (SR media) and induced (SG media) conditions, were used to prepare Golgi-enriched fractions. Cells were pelleted by centrifugation at 4000 *g* for 15 min at 4 °C. Pellets were resuspended in lysis buffer (1 M sorbitol, 10 mM HEPES-Tris, pH 7.4, 1 mM EDTA, and 20 mM KCl) containing protease inhibitor cocktail (GoldBio Corp) and 0.5 mg of zymolyase (US Biologicals) per gram of pellet (wet weight) was added. After incubation for 40 min on ice, Acid-washed 1-mm glass beads were added to each solution (1:4 v/v) and the suspension was vortexed vigorously for 10 min in 30-second intervals with cooling on ice in between. Cells were pelleted at 4000 *g* for 10 min at 4 °C and the supernatant collected by centrifugation at 10,000 *g* for 10 min at 4 °C in Oakridge tubes. The supernatant was then centrifuged at 100,000 *g* for 60 min at 4 °C. The pellets collected after the 100,000 *g* spin have microsomes made from ER and Golgi membranes (326–329); henceforth the pellet will be refer to as ERGEF. These pellets were re-suspended in ice-cold re-suspension buffer (0.8 M sorbitol, 10 mM HEPES-Tris, pH 7.4, 1 mM EDTA, 20 mM KCl, 1 mM MgSO₄) containing protease inhibitor cocktail. Pellets were stored on ice and used for transport assays, or snap frozen in liquid nitrogen and stored at -80 °C until used in STD NMR experiments.

### 5.4.5. Western blot analysis

Total protein from the ER/Golgi-enriched fractions was quantified by the bicinchoninic acid (BCA) method (Thermo Scientific). Protein from each strain (75 µg) was loaded on 8.5 M urea SDS-PAGE gels using loading buffer containing 2 M urea. The expression of FLAG-tagged *Afnst1* and *Afnst5* were detected by Western blotting following transfer to a nitrocellulose membrane (Amersham Hybond-ECL) using a mouse monoclonal anti-FLAG antibody (1:1000 dilution, LifeTein) followed by incubation with goat anti-mouse antibody conjugated to HRP (1:10,000, Jackson ImmunoResearch).
The anti-FLAG antibody was pre-sorbed with whole cell lysate of untransformed yeast (YPH500) to eliminate nonspecific antibody binding. Bands were detected by chemiluminescence (Amersham ECL Prime Western Blotting Detection) and the digital image was captured with the FujiFilm LAS-4000 Chemi-luminescent Scanner-T.

5.4.6. Radiolabelled nucleotide sugar transport assays

Fresh ER/Golgi-enriched fractions (ERGEF) containing 200 µg of total protein were incubated in the reaction buffer (20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 5.0 mM MgCl₂, 1.0 mM MnCl₂, and 10 mM 2-mercaptoethanol) with 9 µM of ¹⁴C-labelled UDP-galactose (55 mCi/mmol) [galactose ¹⁴C-1] or ¹⁴C-CMP-sialic acid (55 mCi/mmol) [sialic acid ¹⁴C₆] American Radiolabeled Chemicals, Inc. for 5 min at 30 °C. The reaction was stopped by adding 1 mL of cold stop buffer (20 mM Tris-HCl (pH 7.5), 0.25 sucrose, 5.0 mM MgCl₂), and the radioactivity associated with the ERGEF was trapped with 0.45 µm filter (Millipore). Filters were then washed with 10 mL of stop buffer. The amount of radioactivity on the filter was measured by liquid scintillation counting (Beckman Coulter model LS6500). A known anion transporter inhibitor (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt (DIDS), 200 mM (Sigma) was used as a negative control (330, 331). DIDS was incubated with the induced samples for 5 min at 30 °C prior to the transport assay experiment. A second negative control was prepared using ERGEF made from uninduced yeast grown in SR medium. To determine the net radioactivity in each sample, a background value was obtained for each ERGEF in which 1 mL of stop buffer was added immediately after the radiolabelled sugar was added to the ERGEF mixture. This background was subtracted to obtain net radioactivity associated with transport. Statistical analysis was completed using GraphPad Prism 5.

5.4.7. Saturated Transfer Difference (STD) - Nuclear Magnetic Resonance (NMR)

All STD NMR spectra were obtained using a Bruker 600 MHz ultrashield NMR apparatus at 283 K equipped with standard triple resonance CryoProbe as described in Maggioni et al. (2008). ER/Golgi-enriched fractions (ERGEF) from various yeast strains containing 200 µg of total protein were first exchanged into 200 µL of deuterated 20 mM...
Tris buffer, supplemented with 2 mM MgCl₂, (in D₂O), pH 7.5. STD spectra were acquired using 200 µg of total protein from the ERGEF and a protein: ligand ratio of 1:100 (mol/mol). The ERGEF/ligand mixture (250 µL) was placed in Shigemi NMR tubes (Shigemi Co.) for all acquisitions. Protein saturation was achieved using a Gaussian pulse train cascade for 3 sec at the on-resonance frequency of -1 ppm and at the off-resonance frequency of 300 ppm. To suppress residual HDO signals, water suppression by gradient tailored excitation (WATERGATE) sequence was used. A total of 512 scans were acquired for each sample. The on- and off-resonance spectra FIDs were stored and processed separately and the subtraction of the on-resonance and the off-resonance spectrum resulted in the STD NMR spectrum. To determine specific binding of ligands to the overexpressed proteins, saturation transfer double difference (STDD) spectra were calculated. In the STDD spectrum, the STD spectrum of ERGEF from yeast transformed with an empty pESC vector was subtracted from the STD spectra obtained with ERGEF of yeast expressing a NST. For mapping of the binding epitope of a ligand to an NST, the relative STD NMR effects were calculated according to the formula $A_{STD} = (I_0 - I_{sat})/I_0 = I_{STD}/I_0$ using the H1 proton of the Ribose (H1 Rib) to 100%.

5.4.8. Construction of the A. fumigatus Δnst1 (AfΔnst1) and A. fumigatus Δnst5 (AfΔnst5) knockout strains

Homologous recombination was used to create the AfΔnst1 and AfΔnst5 strains. The hygromycin phosphotransferase (hph) cassette, encoding resistance to hygromycin, was used for AfΔnst1 and the ble cassette encoding the phleomycin resistance gene was used in the generation of AfΔnst5 (Figure C1). The list of primers used is on Table C2. Briefly, 1000 bp flanking regions from nst1 and nst5 were amplified from A. fumigatus genomic DNA, and the hph cassette was amplified from pID620 (a gift from Dr. D. Holden, based on the plasmid Bluescript SK+), and the ble cassette was amplified from pBCphleo (Silar 1995) using the Q5 High Fidelity DNA polymerase (New England Biolabs). Fusion PCR using the same enzyme was used to join the three components together and the amplicon was cloned into pJET (Thermo Scientific) for sequencing prior to cloning in pCambia. Transformation of A. fumigatus ATCC 13073 with the fusion PCR product was accomplished using Agrobacterium-mediated transformation (AMT) as described in Sugui et al. (2005) and Michielse et al. (2005 and 2008). A. fumigatus
transformants were selected for on selective minimal media: MEA (2% malt extract, 0.1% peptone, 2% glucose/dextrose, 15% agar), supplemented with Rose Bengal (100 µg/mL) and dichloran (8µg/mL), and hygromycin (200 µg/mL) for *AfΔnst1* or phleomycine (100 µg/mL) for *AfΔnst5*. Genomic DNA was extracted from the selected colonies by the CTAB method (Carlson et al. 1991) and successful knockout was confirmed by sequencing and Southern blot analysis (Roche DIG High Prime DNA Labeling and Detection Kit) (Figure C2).

5.4.9. Phenotype analysis of nst knockout strains

Growth of parental, *AfΔnst1*, and *AfΔnst5* strains under various growth conditions –1.5x10⁵ conidia of each strain were inoculated to solid media and the growth of the fungus recorded over 96 hrs. Images of growth on plates were captured with a digital camera (Cannon). Nutrient-rich media tested were yeast malt (YM) agar (Bacto), YM supplemented with 200 µg/ml hygromycin, YM plus 100 µg/mL phleomycin, yeast extract-agar-glucose (YAG). YAG media supplemented with 50µg/mL sodium dodecyl sulfate (SDS), 0.5 mg/mL Congo Red, or 1.0 M sorbitol were also tested as well as YAG not supplemented with minerals or vitamins. The minimal medium (180 mL agar and water, autoclaved; 72.9 mL sterile water; 15 mL 20 x salt (1.5 mL; 200 x MgCl₂; 0.3 mL vitamin solution; 0.3 mL trace elements) was used as is or supplemented with 1% carbon source including glucose, *N*-acetylneuraminic acid, 2-keto-3-deoxy-d-glycero-d-galacto-nononic acid (KDN), pyruvate, or mannose. Slide cultures were prepared using the same media. Fungal morphology was observed using a Zeiss Axioskop 2-plus microscope. Statistical analysis was completed using GraphPad Prism 5.

5.4.10. Analysis of surface glycans on conidia by lectin binding

Harvested conidia (10⁸ spores per mL) were washed three times with PBS. Conidiospores treated with *Micromonospora viridifaciens* sialidase (gift from Dr. A. Bennet, Simon Fraser University) were incubated with 3 µg of sialidase in sialidase buffer (16 mM sodium tartrate and 100 mM sodium chloride at pH 5.2) for 2.5 hours at 37 °C. The spores were washed three times with PBS after sialidase treatment. Both sialidase treated and untreated spores were blocked in 60 µL PBS + 10% goat serum for
1 h at room temperature. After blocking, each sample was split in half and buffer containing 200 μg/ml of *Sambucus nigra* agglutinin (SNA) conjugated to FITC (SNA-FITC) or Peanut agglutinin (PNA-FITC) (EY Lectins) was added. Control samples received buffer only (0.02 M sodium bicarbonate, pH 9.0-9.5). Samples were incubated overnight at room temperature and conidia were washed three times to remove unbound lectin. Conidia were then transferred into 5-ml polypropylene tubes at 1x10⁶ conidia per ml PBS to analyze bound lectin by flow cytometry (BD Jazz Fluorescence Activated Cell Sorter). Remaining conidia were resuspended in PBS and mounted onto a glass slide for bright field microscopy and fluorescence microscopy using a Zeiss- Axioskop 2-plus microscope equipped with Colibri light-emitting diode (LED) fluorescent light source using wavelengths of 494nm (excitation) and 518nm (emission). Images were recorded on AxioCam MRm camera using AxioVision 4.7.1 software. Statistical analysis was completed using GraphPad Prism 5.

5.5. Results

5.5.1. Bioinformatic analyses of *A. fumigatus* nst genes

Six genes in the *A. fumigatus* genome transporters originally annotated in NCBI (www.pubmed.com) and AspGD (www.aspgd.org) as nucleotide sugar transporters (NST); we labeled these *Afnst1*-*Afnst6*. The results of bioinformatic analyses of the four expressed *Afnst* genes are shown in Table C3. All of the transporters with the exception of *Afnst1* were predicted by HMMTOP Transmembrane prediction page (322, 323) (http://www.sacs.ucsf.edu/cgi-bin/hmmtop.py) to have an even number of transmembrane domains where both the N- and C- termini are facing the lumen, similar to models of other characterized NSTs such as the human CMP-sialic acid transporter (334) and the human UDP-galactose transporter (335, 336). *Afnst1* was predicted to have an odd number of transmembrane domains as observed in the *A. fumigatus* UDP-galactofuranose transporter that was predicted to have 11 TM domains (321). Graphical representation of *Afnst1* and *Afnst5* were made based on the predictions made by HMMTOP and generated using TOPO2: Transmembrane Protein Display (Figure C3). According to WoLF PSORT (protein subcellular localization prediction) (325), all four
AfNSTs were predicted to be in the endoplasmic reticulum where a majority of glycan remodeling of cell surface glycosidic chains takes place.

5.5.2. Expression of four putative nucleotide sugar transporter genes in yeast

Four genes, *Afnst1*, *Afnst2*, *Afnst5* and *Afnst6* were cloned into an *S. cerevisiae* expression vector and ER/Golgi-enriched fractions (ERGF) were analyzed by Western blotting to determine expression levels under induced and non-induced growth conditions (Figure C4). Molecular masses of the recombinant AfNST proteins matched their predicted molecular weights from ProtParam (337) (ExPASy; http://web.expasy.org/protparam/). Expression of the AfNST proteins in the induced condition was significantly higher in all samples (Figure C4). Low expression of AfNST2 and AfNST6 was observed in ERGF grown under non-inducing conditions (Figure C4) even though their promoters (Gal1 promoter for NST6 or Met3 promoter for NST2) were shown to be leaky in other studies (338, 339).

5.5.3. Transport assays with radiolabeled $^{14}$C UDP-galactose and $^{14}$C CMP-sialic acid

To determine which of the four AfNST proteins was capable of transporting UDP-galactose or CMP-sialic acid, transport assays using radiolabeled nucleotide sugars were performed using freshly-prepared ERGF from yeast grown under inducing conditions. Negative controls were ERGF from yeast carrying an empty vector, or yeast transformed with an *Afnst* gene grown in non-inducing conditions. The results showed that ERGF expressing either NST1 or NST5 transported $^{14}$C-UDP-galactose (Figure 5.1A). Addition of the anion transport inhibitor, DIDS, inhibited the transport of $^{14}$C-UDP-galactose. Similar results were obtained in previous studies of the human CMP-sialic acid transporter (340) and the *Leishmania* GDP-mannose transporter (341). Our results confirm the annotations in the *Aspergillus* genome database (AspGD): AfNST1 was predicted to be a UDP-galactose transporter and AfNST5 a UDP-glucose/UDP-galactose transporter. Neither AfNST1 nor AfNST5 transported $^{14}$CMP-sialic acid, and no transport of either nucleotide sugar was observed with ERGF from yeast transformed with *Afnst2* or *Afnst6* (Figure 5.1B).
Figure 5.1  Nucleotide sugar transport of A\(\text{NSTs}\) expressed in yeast
Transport of \(^{14}\text{C}-\text{UDP-galactose}\) (A) and \(^{14}\text{C}-\text{CMP-sialic acid}\) (B) by \(A.\text{fumigatus}\) nucleotide sugar transporters expressed in yeast. Golgi-enriched fractions from induced yeast expressing various nst genes were tested for their ability to transport the radiolabelled nucleotide sugars. Controls were ERGEF from transformed yeast grown without inducer, and GEF from induced yeast pretreated with the anion channel inhibitor, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS).The results are from three independent experiments; t-tests were completed to evaluate the significance in the difference of the substrate transported by each \(A.\text{fumigatus}\) transporter compared to the yeast background. *Significantly different (t-test at \(P<0.05\)).
5.5.4. **Substrate binding of A\textit{f}NSTs determined by STD NMR spectroscopy**

Early studies of NST suggested that each NST transported only a single substrate (117, 124); however, more recent studies have shown that certain NST proteins are capable of transporting more than one nucleotide sugar (319, 320). We used STD NMR spectroscopy to screen ERGEFs expressing \textit{A. fumigatus} NST proteins for their ability to bind six different nucleotide-sugars. Selected STD NMR spectra are shown in Figures 5.2-5.4. The nucleotide sugars used in the experiments and the results for three \textit{A. fumigatus} NSTs are summarized in Table 5.1; A\textit{f}NST2 was not included because no binding was detected using any of the nucleotide sugars employed. Consistent with the results from our $^{14}$C-nucleotide sugar transport assays, STD NMR experiments revealed that UDP-galactose bound to A\textit{f}NST1 and A\textit{f}NST5 but not to A\textit{f}NST2 or A\textit{f}NST6 (Figure 5.2). CMP-sialic acid did not show any STD NMR effects suggesting that it did not bind to any of the A\textit{f}NSTs (Figure 5.5). Additional STD NMR experiments further revealed that UDP-glucose bound to A\textit{f}NST1 and A\textit{f}NST5 (Figure 5.3) and UDP-$N$-acetylgalactosamine bound to A\textit{f}NST1 and A\textit{f}NST6 (Figure 5.4). STD NMR effects of the binding nucleotide sugars were determined quantitatively and epitope maps were calculated. This analysis showed that the highest degree of saturation of the nucleotide sugars bound to A\textit{f}NST1, A\textit{f}NST5, and A\textit{f}NST6 could be determined for the nucleotide moiety. In particular, the protons H$^6$\textit{Ura}, H$^1$\textit{Rib}, and H$^5$\textit{Ura} received the highest degree of saturation from the protein. For the protons of the sugar moiety only medium or weak STD NMR effects could be detected leading to the assumption that the main site of binding is the nucleotide. These results are consistent with the model proposed by Capasso et al. (1984) in which the nucleotide moiety acts as an anchor to facilitate binding on the protein while the sugar portion of the molecule dictates NST specificity.
Table 5.1  Nucleotide sugar binding to 3 recombinant AfNST proteins expressed in yeast.

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<th>NST1</th>
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<tr>
<td>UDP-galactose*</td>
<td>+</td>
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<tr>
<td>CMP-sialic acid*</td>
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<td>UDP-glucose</td>
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<td>UDP-N-acetylgalactosamine</td>
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<td>UDP-N-acetylglucosamine</td>
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<tr>
<td>UDP-glucuronic acid</td>
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These data are based on STD-NMR analysis of Golgi-enriched membrane fractions. Data that has been corroborated by radiolabelled sugar uptake experiments is noted with a *.
Figure 5.2  STD NMR spectra of UDP-Galactose (UDP-Gal), in the presence of ER/Golgi-enriched fractions overexpressing A. fumigatus nucleotide sugar transporters

(A) STD NMR spectra of UDP-Gal, in the presence of ER/Golgi-enriched fractions overexpressing A. fumigatus nucleotide sugar transporters AfNST1, AfNST2, AfNST5, AfNST6, and 1H NMR spectra of UDP-galactose. All spectra were recorded at 283 K, 600 MHz in deuterated Tris buffer (10 mM, pH 7.5) and MgCl2 (2 mM). The relative binding intensities of UDP-Gal protons on (B) AfNST1 and (C) AfNST5 are highlighted.
Figure 5.3  STD NMR spectra of UDP-Glucose (UDP-Glu), in the presence of ER/Golgi-enriched fractions overexpressing *A. fumigatus* nucleotide sugar transporters

(A) STD NMR spectra of UDP-Glu in the presence of ER/Golgi-enriched fractions overexpressing *A. fumigatus* nucleotide sugar transporters *AfNST1*, *AfNST2*, *AfNST5*, *AfNST6*, and "H NMR spectra of UDP-Glu. All spectra were recorded as described for experiments with UDP-Gal. The residual water signal was removed by applying a WATERGATE sequence. The relative binding intensities of UDP-Glu protons on (B) *AfNST1* and (C) *AfNST5* are highlighted.
Figure 5.4  STD NMR spectra of UDP-Gal, in the presence of ER/Golgi-enriched fractions overexpressing *A. fumigatus* nucleotide sugar transporters

(A) STD NMR spectra of UDP-GalNAc in the presence of ER/Golgi-enriched fractions overexpressing *A. fumigatus* nucleotide sugar transporters *AfNST1*, *AfNST2*, *AfNST5*, *AfNST6*, and ¹H NMR spectra of UDP-GalNAc. All spectra were recorded as described for experiments with UDP-Gal. The relative binding intensities of UDP-Glu protons on (B) *AfNST1* and (C) *AfNST6* are highlighted.
5.5.5. **Morphological examination of *A. fumigatus* NST1 and NST5 knockout mutants grown on different media conditions**

To assess the biological significance of UDP-galactose transporters in *A. fumigatus*, knockout mutants of *AfNST1* and *AfNST5* were constructed. The *AfNST1* knockout strain revealed no significant difference in growth compared to WT on YAG medium with or without 1 M sorbitol, an osmotic stressor, or minimal media with or without mannose.

Growth of strains on YAG supplemented with the dye Congo Red revealed significant differences in phenotype. Congo Red binds to chitin chains and inhibits the activity of enzymes that connect β 1,3-glucan and β 1,6-glucan, thus weakening the cell wall (342). It has been used to detect fungal cell wall mutants. On agar plates supplemented with 375 µg/mL of Congo Red, the growth of the *Af∆nst5KO* mutant was impaired whereas *Af∆nst1KO* grew better than wild type (Figure 5.5). Microscopically, the hyphae of the *Af∆nst5KO* mutant were stunted and swollen (Figure 5.6). In addition, the majority of *AfΔN nst5KO* conidophores and spores retained the red dye while wild type and *AfΔnst1KO* exhibited less Congo Red staining (data not shown). Conidia of the *AfΔnst5KO* strain had a significantly greater diameter than wild type or *AfΔnst1KO* conidia (2.2 ± 0.3 µm for wild type, 2.3 ± 0.3 µm for *AfΔnst1KO*, and 4.1 ± 0.7 µm for *AfΔnst5KO* strain, n = 30 spores, p<0.05). These data indicate that alterations in the cell wall were not restricted to hyphae but also affected conidial integrity in *AfΔnst5KO*.

![Figure 5.5](image1.png)

**Figure 5.5** **Morphological Observations of *A. fumigatus* Mutant and Wild type Grown on Congo red media**

Growth of *A. fumigatus* after 72 hours (A) wild type, (B) *AfΔnst5KO*, and (C) *AfΔnst1KO* in rich fungal media (YAG) supplemented with 375 µg/mL Congo red. Scale bar indicates 5 mm.
Figure 5.6  Microscopic morphological Observations of *A. fumigatus* Wild type and NST5 knockout mutant

Morphology of *A. fumigatus* wild type strain (A, C) compared to AfΔnst5KO (B, D) after 96 hrs of growth in YAG supplemented with 150 µg/mL Congo Red and observed after 96 hrs. Inset shows conidia of each strain.

5.5.6.  Galactose and sialic acid on the conidia of NST1 and NST5 knockout mutants

The level of sialic acid and galactose on the surface of *A. fumigatus* was affected by NST deletion (Figure 5.7). Flow cytometric analysis of sialic acid-specific lectin (SNA) binding to conidia indicated that AfΔnst5KO had significantly less sialic acid (a reduction of ~50%) on the spore surface compared to wild type and AfΔnst1KO (Figure 5.7). Levels of the terminal galactose were determined by incubation of conidia with the galactose-specific lectin, PNA before and after sialidase treatment. Though AfΔnst5KO had less surface sialic acid compared to AfΔnst1KO, after sialidase treatment, a similar level of galactose was detected in the two strains (Figure 5.7). Both knockout strains had significantly less galactose compared to wild type. All control samples, whether treated with sialidase or not, showed no significant fluorescence (data not shown).
AfNST knockout affects glycan composition on conidial surface. Conidia untreated or pretreated with sialidase from *M. viridifaciens* were incubated with either the sialic acid binding lectin, SNA-FITC (top), or the galactose-binding lectin, PNA-FITC (bottom). The amount of lectin bound was quantified by flow cytometry with a fluorescence detector set at 488 nm. The data show the mean of 3 independent experiments ± S.D.
5.6. Discussion

This report describes the first identification of two UDP-galactose transporters (called AfNST1 and AfNST5) in the fungal pathogen, *A. fumigatus*. Using STD NMR spectroscopy we showed that both *A. fumigatus* NSTs (AfNST1 and AfNST5) bound UDP-galactopyranose (UDP-gal) and UDP-glucose whereas AfNST1 and AfNST6 bound to UDP-GalNAc. These results are the first demonstration of biochemical equivalence and the possibility of multi-substrate specificity of nucleotide sugar transporters in Fungi. Interestingly, despite the finding that both AfNST1 and AfNST5 transported UDP-gal, only AfNST5 but not AfNST1 knockout mutants showed evidence of compromised cell wall integrity. In addition, AfΔnst5KO had significant reduction in sialic acid levels on the conidial surface whereas AfΔnst1KO was similar to wild type. Knockout of either AfNST1 or AfNST5 lowered but did not eliminate galactose expression on the spore surface. The phenotypes of the NST mutants are consistent with the results of our functional studies.

Functional redundancy of multiple nucleotide sugar transporters has been previously reported in two human UDP-galactose transporter isoforms, UGT1 and UGT2 (335, 336). These isoforms were found to be equally important for the glycosylation of proteins in mammalian cells (343). In the protozoan parasite, *Leishmania major*, the two UDP-gal transporters, LPG5A and LPG5B, were identified as functionally equivalent, but knockout of either gene produced mutants that were phenotypically different (344). Similarly, we found that AfNST1 and AfNST5 are both UDP-gal transporters; however, the AfNST1 and AfNST5 knockout strains had different phenotypes on growth medium containing Congo Red, a dye that binds to cellulose and chitin (344).

Multi-substrate nucleotide sugar transporters have been identified in both nematodes and plants, such as the 4-substrate NST in *C. elegans* (320) and more recently, 6 UDP-rhamnose/ UDP-gal transporters were identified in *Arabidopsis* (319). For both organisms, the authors suggested that substrate redundancy is an important aspect for maintaining a consistent cell wall glycan structure. Since multiple UDP-sugars could bind to the AfNSTs examined in this study, it is likely that those transporters would exhibit multi-substrate specificity. In *A. fumigatus*, the cell wall structure is not yet entirely described; however, several components of the cell wall have been identified such as
chitin, various α- and β-glucans, galactomannan and galactosaminogalactan. These glycans are composed of the monosaccharides N-acetylglucosamine, glucose, mannose, galactofuranose, and N-acetylgalactosamine (315). To date, only the UDP-galactofuranose transporter (321) and the GDP-mannose transporter (310) have been identified in A. fumigatus and these transporters exhibited the ability to transport a single monosaccharide. A total of sixteen putative nucleotide sugar transporters have been found by us through an NCBI database search and others (321). Therefore, the possibility for AfNSTs to be functionally redundant is not unexpected and our results show that the fungus possesses compensatory mechanisms to ensure the structural integrity of the cell wall.

We overexpressed the A. fumigatus UDP-galactose transporters in Saccharomyces cerevisiae by transforming a protein expression vector encoded with the NST gene and studied their properties in ER/Golgi-enriched vesicles isolated from yeast grown with inducing agents. The microsome fraction collected at 100,000g used for analysis is primarily composed of ER/ Golgi membrane (326, 328, 329), however, this subcellular fraction still a crude mixture of membranes, including the plasma membrane, vacuolar membranes, and various endosomal vesicles and compartments (326, 327). As demonstrated in the Western blot data (Figure C4), the microsome fractions collected for further biochemical analysis are the fractions that are enriched with our protein of interest. UDP-gal transport has previously been reported in the yeast, Saccharomyces cerevisiae when the gene HUT1 was overexpressed (345). Furthermore, in Schizosaccharomyces pombe, the UDP-gal transporter Gms1p has been functionally characterized (346). However, the UDP-gal transporter (HUT1) was not essential in S. pombe and in S. cerevisiae: it was expressed at very low levels under normal culture conditions and disruption of the gene did not alter glycosylation (347). In our study, S. cerevisiae transformed with an empty protein expression vector grown with inducing agents was used as the negative control and the results showed that native UDP-gal transporter activity exhibited by the yeast did not affect the results.

STD NMR spectroscopy results supported the findings of 14C-nucleotide sugar transport assays and provided evidence that suggests both AfNST1 and AfNST5 can transport multiple UDP-sugars. Finally, the data indicates that AfNST6 is likely to be a
UDP-GalNAc transporter, though transport assays are necessary to confirm this. Epitope mapping demonstrated the protons of the nucleotide residue exhibited a close contact to \( \text{AfNST} \). The determination of an epitope map by STD NMR spectroscopy can provide reliable data on how a ligand binds to the receptor proteins, including transmembrane proteins (348–352).

Results from our study are consistent with the observations obtained for the binding of CMP-sialic acid to the mammalian CMP-sialic acid transporter (348) and the binding of GDP-mannose to the \( A. \text{fumigatus} \) GDP-mannose transporter (353). A study by Chiaramonte et al. (2001) found that modification of the 2’ hydroxyl on the ribose in CMP prevented binding to the mammalian CMP-sialic acid transporter. For the \( \text{AfNSTs} \) examined, we showed that the interaction with the nucleotide sugar was mediated by a medium level of proton binding at the 2’ and 3’ hydroxyl of the ribose on the nucleotide moiety.

UDP-Gal is an important nucleotide sugar in \( A. \text{fumigatus} \): it is necessary for the biosynthesis of the virulence factor, galactosaminogalactan (6, 54) and it is essential for the production of UDP-galactofuranose, an important component of galactomanan (310). UDP-Gal\(f\) is enzymatically produced by UDP-galactopyranose mutase from UDP-Gal (354). The enzyme has been identified but not localized in \( A. \text{fumigatus} \), however in the related fungal specie, \( \text{Aspergillus nidulans} \), it the mutase is localized in the cytosol (344) Furthermore, as a subunit in a glycosidic chain, galactopyranose was identified as the subterminal sugar for the presentation of sialic acids on \( A. \text{fumigatus} \) conidiospores (88). Both \( \text{AfNST1} \) and \( \text{AfNST5} \) transported UDP-gal and STD-NMR studies revealed that UDP-glucose could bind to both transporters consistent with the current annotation of NST5 as a UDP-gal/UDP-glu transporter (Table C3). UDP-glu is required for the production of \( \beta 1,3\)-glucan and \( \alpha 1,3\)-glucan for the \( A. \text{fumigatus} \) cell wall. Both polysaccharides are major components of the cell wall making up close to 46% of the total mass of the \( A. \text{fumigatus} \) cell wall and have important roles in cell wall strength and adhesion (53, 83).

\( \text{AfNST1} \) but not \( \text{AfNST5} \) bound UDP- \( N \)-acetylgalactosamine. Galactosaminogalactan is a linear heterogenous polysaccharide chain of \( \alpha 1,4\)-galactose
linked to α1,4- N-acetylgalactosamine. The biosynthetic pathways of galactosaminogalactan, is not well understood. Galactosaminogalactan is immunosuppressive yet the knockout mutants were hypervirulent (85). Interestingly, the AfNST1 knockout was not significantly different from wild type in either spore size or sialic acid levels, though more subtle changes in charge or biofilm production were not investigated.

AfΔnst1KO and AfΔnst5KO had different phenotypes on growth medium containing Congo Red, a dye that bind to chitin (356). Exposure to Congo Red activates the fungal cell wall stress response by activating genes encoding proteins that have cell wall-reinforcing functions (43). Some cell wall mutants show increased resistance to Congo Red such as S. cerevisiae mutants that lack a major chitin synthase, chs3 (357, 358); however, most cell wall mutants display an increased sensitivity to Congo Red due to the activation of the cell wall stress response (357, 359). In our study, the AfΔnst1KO mutant was relatively resistant to Congo Red, whereas AfΔnst5KO showed increased sensitivity. This suggests that different cell stress response pathway could have been activated or that the depletion of specific sugars changed the integrity of the cell wall (342).

Knockout of A. fumigatus NST5 decreased the levels of sialic acid and both knockout strains had reduced galactose on the surface of fungal conidia though residual glycans remained on the fungal surface suggesting that compensatory mechanisms exist. Compensatory pathways in fungal carbohydrate metabolism have been described; studies on three α1,3 glucan synthases, AGS1, AGS2, AGS3 (83) and two UDP-glucose 4-epimerases, Uge3 and Uge5 (86) have demonstrated that knocking out individual genes was not sufficient to reveal a phenotype in which a particular glycan was absent from the A. fumigatus cell wall.

The present study broadens our understanding of NST and their role in the metabolism of carbohydrates in fungi. We initially hypothesized that deletion of the UDP-galactose transporter or more directly, a CMP-sialic acid transporter, would allow us to create a sialic acid deficient strain of A. fumigatus in the same manner as the CHO Lec8 strain (318) or in the S. pombe Δgsm1 mutant (346). The NST5 knockout in A. fumigatus
reduced sialic acid by ~50% compared to wild type but did not completely eliminate sialic acid from conidia. If the elusive sialic acid biosynthetic pathway remains unidentified in A. fumigatus, creating multiple nucleotide sugar transporter knockouts in A. fumigatus may reveal the importance of this sugar in A. fumigatus adhesion and virulence.
Chapter 6.

Concluding Remarks

Aspergillus fumigatus is an important airborne fungal pathogen that causes a range of diseases from allergic reactions to severe invasive infections in immunocompromised patients (11). The primary ecological niche is soil and its main role is carbon and nitrogen recycling. The fungus sporulates abundantly and the small conidia disperses easily making it one of the most ubiquitous fungi with airborne conidia (1). The first step of an infection by a pathogen is by attachment to the host (360). On the surface of host cells, sialic acids are found on the terminal position of cell surface glycans that are used for many essential host cellular processes and microbial pathogens have evolved to use these molecules beneficially during an infection (105, 284).

Previous studies in our laboratory have found sialic acids α 2,6- linked to an underlying galactose residue on the surface of A. fumigatus and there were shown to be involved with the adhesion of A. fumigatus conidia to epithelial cells of the human host (87, 251). The removal of sialic acid from A. fumigatus spores decreased uptake of conidia by cultured murine macrophages and Type 2 pneumocytes, suggesting sialic acid on conidia partly mediates immune recognition by the host (251). Growing A. fumigatus on minimal media without sources of sialic acid revealed that this sugar was found to be synthesized de novo by the fungi (87, 196, 361). However, no known sialic acid biosynthetic genes have been found in the genome of A. fumigatus (196, 251) and sialic acid metabolism is not well characterized in fungi (113). In my study, I identified several sialic acid metabolic genes through a database search of the annotated A. fumigatus genome. My research has contributed to the knowledge of sialic acid metabolism in A. fumigatus through characterization of five novel proteins, a sialidase/KDNase (AFS) and four novel nucleotide sugar transporters (ANSTs).
6.1. *A. fumigatus* sialidase/ KDNase (*AfS*)

To characterize the novel sialidase from *A. fumigatus*, the *AfS* gene was cloned and expressed as a recombinant enzyme for analysis as a purified protein. Kinetic analysis and substrate specificity experiments revealed that the enzyme has a preference for α2,3 linked sialic acid substrates and can cleave sialic acid from a range of biological substrates including human Type II pneumocyte (A549) cells. Although *AfS* was able to cleave sialylated substrates, the fungal enzyme was not as efficient as other characterized microbial sialidases, such as ones from *Micromonospora viridifaciens* or *Clostridium perfringens* (194, 225, 237).

Neu5Ac, KDN and Neu5Gc are the three major naturally occurring sialic acids (105) and up to this point, only Neu5Ac has been investigated since it is the most common and most important in biology. When the recombinant *AfS* was crystallized, the solved structure revealed that the pocket which normally accommodates the *N*-acetyl group, located at C5 on Neu5Ac, was smaller compared to other crystallized sialidases. A crystal could not be generated with *AfS* and Neu5Ac or Neu5Ac2en; however, it was possible with the (structurally) smaller sialic acid, KDN, and its corresponding inhibitor analogue, KDN2en. I showed that *AfS* is the first enzyme that is highly specific for cleaving KDN-linked substrates found in the fungal kingdom, and is the first KDNase to be crystallized.

With the exception of the binding pocket for C5 of the substrate, structurally and mechanistically, I found that *AfS* is very similar to *MvS*. When the crystal structure of *AfS* was overlaid on to the crystal structure of *MvS* (PDB code 1eus), the enzyme active site was located in a similar position and the mechanistically important amino acids, the general acid/ base (D84 *AfS*/ D92 *MvS*) and nucleophile (Y358 *AfS*/ Y370 *MvS*), were aligned at almost identical positions. Likewise, the KDN bound to the active site of *AfS* in a similar conformation as Neu5Ac would bind to *MvS* and both enzymes would proceed with a retaining mechanism. Confirmed by kinetic analysis using two synthetic KDN substrates, KDN-Mu and KDNa2,6GalβFMu, *AfS* was more effective at cleaving the two synthetic KDN substrates than Neu5Ac-Mu by 10^5 and 10^3 orders of magnitude,
respectively. The KDNase activity of AfS was comparable to the effectiveness of MvS as a sialidase (194, 362).

To probe the catalytic activity of AfS, mutant AfS with either the nucleophilic tyrosine changed to a histidine (Y358H AfS) or the general acid/base changed to an alanine (D84A AfS) were tested to see how structure and function correlated between AfS and MvS. In another study, mutation of the Trypanosoma cruzi trans-sialidase tyrosine nucleophile to a histidine (Y342H) destroyed normal catalytic activity (293). If the Y358H mutation in AfS had had attenuated activity, yet retained good binding, further development would have been warranted to make this AfS mutant into a lectin that is highly selective for KDN. Although there is no published example where an experimentally introduced mutation lead to the creation of a lectin, in Trypanosoma cruzi, the parasite’s genome encodes for many proteins that are inactive forms of the trans-sialidase and those proteins have been purified for use as lectins in in vitro studies (363). The sequence of the inactive enzymes are >95% identical to the active trans-sialidase and in many cases, the inactivation is invariably due to a mutation of the catalytic tyrosine to a histidine (364). However, the residual activity precludes the use of the AfS Y358H in this manner. Y358H AfS was similar to the nucleophilic mutation in MvS (Y370H) (281): Y358H AfS remained an active enzyme and continued to perform catalysis with a retaining mechanism. Although it was not determined if Y370H MvS is a retaining sialidase, MvS mutants with phenylalanine or glutamic acid in place of the tyrosine (Y370F and Y370E) continued to have a retaining mechanism even though it did not have the customary nucleophilic oxygen atom (281). Only when Y370 in MvS was mutated to a small negatively charged or hydrophobic side chain where it displayed an inverting mechanism (194, 365). Because both AfS and MvS structures are very similar, it is likely that similar mechanistic changes will be observed in AfS if similar mutations were made to Y358 in AfS.

However, when the general acid/base was changed from an aspartic acid to an alanine in AfS (D84A), enzyme activity was almost completely attenuated; there was a 10^4 fold decrease in activity compared to wild type AfS. In contrast, similar mutations in both MvS (D92A) and T. cruzi trans-sialidase (D59A) did not result in a significant decrease in enzyme activity compared to wild type (138, 194). Because AfS has a lower
pH optimum compared to the other two enzymes, we proposed that the general acid/base is likely to be catalytically more important in the AfS reaction. Therefore, it is also important to consider the environment in which the enzyme operates in predicting the effects of structural changes of an enzyme.

In this direct comparison of two structurally and biochemically similar sialidases, I demonstrated that the fit of the substrate in the active site influenced the preference for KDN over Neu5Ac as a substrate. Neu5Ac-specific sialidases have a hydrophobic pocket to accommodate the acetamido group at C5; in contrast, AfS has a water-filled, polar pocket where modelling shows that Arg^{171} would have <2.5 Å from the acetamido group of Neu5Ac. In many biological interactions, including substrate-enzyme interactions, most hydrogen bonds observed are weak hydrogen bonds and they require at least an average of 2.5 Å distance between the two atoms (366, 367). Therefore, Arg^{171} in AfS would consequently clash with the C5 group of Neu5Ac. To address this, I created the R171L AfS mutant to mimic the structure of the C5 binding pocket in MvS. Even though the mutation only slightly improved the catalytic efficiency of the enzyme when Neu5Ac-Mu was the substrate, R171L AfS was successful in providing a larger pocket to accommodate Neu5Ac2en for co-crystallization. It is possible that the fit within the catalytic site can be further optimized within R171L AfS for Neu5Ac substrates. On either side of Arg^{171} are Trp^{202} and Gln^{148} that contribute to the polar environment in the binding pocket; therefore, modification of either amino acid may have also been necessary.

Modelling of three-dimensional structures of putative KDNases from Aspergillus terreus, Trichophyton rubrum, and Sphingobacterium sp21 (Chapter 4) revealed that in these enzymes, an arginine residue was present in a similar position to Arg^{171} in AfS and Leu^{169} in MvS. When Neu5Ac2En was modelled in their enzyme active sites, a steric clash similar to the one when evaluating the fit of Neu5Ac2En in the three-dimensional model of AfS was observed. In contrast, when KDN2En was placed in the same position, a space between ~4.5 - 10 Å was predicted between the C5 hydroxyl of KDN and the arginine residue in all putative KDNases. Examination of the crystal structures of characterized sialidases, such as MvS (PDB 1eus), C. perfringens Nanl (PDB 2bf6), and S. pneumoniae NanA (PDB 2vvz), ~ 4.6 - 9.7 Å was found between the acetamido group
of Neu5Ac and the amino acid at a similar position of the equivalent binding pocket. Structurally, the only difference between the three most common naturally occurring sialic acids is the substitution at C5. The structural and biochemical evidence presented in this thesis suggests that >2.5 Å space between the C5 substituent of sialic acid and A/S is essential, and an arginine in the C5 pocket is one of, if not the only, amino acid that defines the difference between a sialidase and a KDNase.

In A/S, as observed in the crystal structure of A/S and KDN, Arg\textsuperscript{171} is the second arginine of an EGR\textsubscript{R} motif that sits one side of Trp\textsuperscript{202}, part of a WD motif, on the other side of which is Gln\textsuperscript{148} that interacts with the O7 and O9 hydroxyls of KDN via hydrogen bonds. Extending a BLASTP search of the protein database with A/S, shows this Q-[-24aa]-EGR\textsubscript{R}[-30aa]-WD motif to be conserved in the fungi Neosartorya fischeri, A. terreus, Arthroderma gypseum, Trichophyton verrucosum, Microsporum canis, Chaetomium globosum and Nectria haematococca, all of which share at least 72% sequence identity with A/S (282). Furthermore, a degenerate form of this motif, DGR\textsubscript{R}[-30aa]-W, occurs in proteins annotated as sialidases from the Actinobacteria, Streptomyces bingchenggensis and Streptomyces avermitilis (287). It is possible that together with Arg\textsuperscript{171}, together these three residues define a KDN-recognition motif. Further experimental validation is necessary to prove this theory.

KDNase activity was first shown in the bacterium, Sphingobacterium multivorum, isolated from the sludge of a sewage pond of a rainbow trout hatchery by the same research group that first identified KDN on the cortical alveolar polysialoglycoprotein of rainbow trout eggs (178, 179). To date, only a limited number of organisms possess a KDNase or sialidase/KDNase. In trout, KDNase activity was found in the kidney, spleen, and ovaries; with the highest activity detected in ovulatory follicles at the time of natural effacement (182). In other animals, KDNase activity were found in the tissue of loach (187), star fish (188), and oyster (189). In microbes, KDNase activity has only been found in S. multivorum (178), A. fumigatus (A/S), and M. viridifaciens (MvS) (184, 282), but given the analysis presented above, we believe that it is present in other microbes.

In 2010, A/S was found to share a common ancestor with bacterial sialidases in the phyla Actinobacteria and Bacteroidetes and only be closely related to three other
putative fungal sialidases from *A. terreus*, *Chaetomium globosum* and *Neosartorya fischeri* presented in Chapter 2. I repeated the BLASTP search of the protein database with *AfS* in 2014 and identified ten other putative fungal sialidase genes: four *Trichophyton* species (*T. rubrum*, *T. tonsurans*, *T. verrucosum*, and *T. equinum*), *Myceliophthora thermophila*, three *Arthroderma* species (*A. benhamiae*, *A. gypseum*, and *A. otae*), *Nectria haematococca*, and two *Metarhizium* species (*M. acridum* and *M. anisopliae*). Compared to the number of sequenced fungal genomes available, 13 putative sialidases/ KDNases in the Fungal kingdom is still a small number; as of 2014, 356 complete fungal genomes have been sequenced out of 100,000 identified fungal species (1000 Fungal Genomes Project, Joint Genome Institute, United States Department of Energy). It is likely that as more fungal genomes are sequenced and annotated, more sialidases/ KDNases will be identified in the Kingdom Fungi. In addition, the fungal species identified to have a putative sialidase/KDnase are all pathogenic; for example: the *Trichophyton* species identified causes athlete’s foot, ringworm and other cutaneous fungal infections in humans and animals (368), and the *Metahizium* species cause infections in insects (369). It is possible that there is a link between the presence of a KDNase/ sialidase and the pathogenicity of the fungi through the bias of sequencing species that are important in human medicine/ agriculture, may cause these species to be overrepresented in the list of sequenced fungi. Nevertheless, it remains a subject for future research. Furthermore, with the solved structure of *AfS* and Arg^{171} demonstrated to be an important amino acid for KDNase activity, it maybe possible to use *AfS* as a model, as well as the DGRR-30aa-W motif, to identify more KDNases in other sequence organisms in nature.

Why does *A. fumigatus* need a KDNase? From the research presented in this thesis, KDN but not Neu5Ac is a carbon source for *A. fumigatus*, suggesting that the fungus uses *AfS* to remove KDN from its surrounding environment for nutrition. Although *A. fumigatus* is an opportunistic pathogen that primarily infects from the host’s lungs, its natural habitat is in soil, compost heaps and decaying matter (11). In bacteria, KDN has been isolated from the cell wall or capsule of *Klebsiella ozaenae* serotype K4 (154), *Sinorhizobium fredii* (146), several *Streptomyces* species (144, 145, 370), *Pseudomonas corrugate* (371), *Bacteroides thetaiotaomicron* (372), and *Escherichia coli* K-12 (373). With the exception of *B. thetaiotaomicron*, which is a human gut symbiont, all other
bacterial species with KDN are either plant or human pathogens that inhibit a similar ecological niche to A. fumigatus. By Western blot and indirect immunofluorescence, I found that AfS was primarily associated with the total cellular protein fraction and AfS to be localized mostly around the periphery of the fungus. Therefore, AfS is primarily localized in the cell membrane/cell wall where it may be able to access KDN-containing glycoconjugates.

KDN has also been isolated from numerous animals, from amphibians and fish to mammals, including humans (183). KDN was found distributed throughout the organisms examined as a terminal sugar in place of Neu5Ac; however, higher concentrations of KDN were found in the ovaries or testes, on cells from early development, or on cancerous cells (186, 374–376). Particularly for fish and amphibians, KDN was found primarily on the egg jelly coats or the skin mucous (375, 377–379). Since another natural reservoir for A. fumigatus is decaying organic matter, these may represent additional sources of KDN for the fungus. Even in a live immunocompromised host, A. fumigatus can potentially use the exposed KDN for nutrition. From my research, it was found that AfS expression was upregulated when the fungus was grown in MEM + serum, a nutrient poor condition that mimics the environment in human lungs (Chapter 2). Upregulation of KDNase may promote the survival of A. fumigatus in numerous environments. Alternatively, the increased expression of KDNase may be triggered by some other stress response, but further research is necessary to determine why KDNase is upregulated.

In addition to A. fumigatus, E. coli K-12 has also been shown to use free KDN as a carbon source (373). S. multivorum can use KDN-glycoside as a carbon source, but not free KDN (380). It was found that the E. coli K-12 sialic acid transporter (NanT) was able to transport KDN, Neu5Ac, and Neu5Gc; all of which are carbon sources for the bacterium. In addition, through complementation studies with the E.coli K-12 ∆nanT knockout strain, the siaPQM TRAP transporter from Haemophilis influenza, and the STM1128 SSS transporter from Streptomyces enterica ssp. Typhimurium were found to be able to transport KDN (373, 381). The sialic acid aldolase (NanA) is another gene that is important for KDN catabolism in E. coli K-12: NanA is responsible for the cleavage of KDN to pyruvate and D-mannose and it is the first step in the KDN catabolic
process (373, 382, 383). The *E. coli* K-12 ΔnanA knockout was not viable in media where KDN was the sole carbon source, however, *E. coli* K-12 mutants with downstream sialic acid catabolic genes knocked out were able to grow on the same media (373). A Blastp search of these important KDN catabolic genes with the *A. fumigatus* genome did not reveal a similar transporter. However a reverse blastp search revealed a putative protein (XP_746979) in *A. fumigatus* with 97% sequence coverage and 31% sequence identity to *E. coli* K-12 NanA. This putative *A. fumigatus* protein belongs to the dihydrodipicolinate synthetase family, which includes several types of enzymes that are structurally and mechanistically similar: examples include the sialic acid aldolase/lyase, dihydrodipicolinate synthetase (involved in the biosynthesis of lysine) and D-5-keto-4-deoxyglucarate dehydratase (involved in gluconate metabolism) (384, 385). I had also cloned and expressed this *A. fumigatus* protein (XP_746979) that is potentially an aldolase/lyase. Preliminary work revealed that the protein was not able to breakdown Neu5Ac or form KDN; however, it was possible that the experimental conditions were not optimized (unpublished data). Since *A. fumigatus* cannot use Neu5Ac as a carbon source, it’s not surprising that the fungus does not possess an aldolase that can break down this sugar. A study of the *E. coli* K-12 NanA crystal structure showed that point mutations in the protein can completely change the substrate specificity of the enzyme (386). It is possible that the putative *A. fumigatus* aldolase/lyase is specific for KDN and further work should be done to assess the function of this putative protein.

The biological importance of AfS can only be probed by making knockout mutants. An *A. fumigatus* KDNase knockout mutant has been created by Jason Nesbitt from the Moore Laboratory. A distinct phenotype compared to wild type was observed when the mutant strain was grown in minimal fungal media supplemented with various carbon sources, and the mutant was more susceptible to osmotic stress (Jason Nesbitt, unpublished data). It is possible that the functions of AfS in *A. fumigatus* is important to nutrition and other vital biological processes. Investigation of this mutant *A. fumigatus* strain is currently ongoing.

KDN has only been detected in a relatively small number of organisms. However, it is likely that KDN is more widespread in organisms; but due to the methods commonly used, KDN may not be detected. On *A. fumigatus*, Neu5Ac has been detected on the
cell surface even when the fungus was grown on minimal media without a source of sialic acid (87, 361). However, it is currently unknown if KDN is also present on the surface of *A. fumigatus*. Although the specific role of KDN in microbes have not been demonstrated, it was suggested by the studies where KDN was found on the organisms’ surface that the sugar plays a similar role in those organisms as Neu5Ac (106, 183). In pathogenic microbes, Neu5Ac on the cell surface allows the microbe to adhere or to mimic the host and avoid recognition by the host immune system (284).

Recently, in *Streptomyces coelicolor*, three KDN biosynthetic genes were characterized: the UDP-N-acetylglucosamine 2-epimerase (sco4879), CMP-Neu5Ac synthetase, NeuA (sco4880), and Neu5Ac synthetase, NeuB (sco4881). The removal of any one of these genes prevented KDN from being present on cell surface of the mutant bacteria as determined by $^{13}$C NMR analysis of the cell wall (370). When all three genes were present, determined by complementation with a plasmid replacing the missing gene, the production of KDN was restored. In *B. thetaiotaomicron*, other KDN biosynthetic genes have been identified: KDN-9P synthase (BT1714), KDN-9P phosphatase (BT1713), CMP-KDN synthetase (BT1715). These enzymes were found to be highly specific for CMP-KDN synthesis since the purified enzymes catalyzed the reactions with KDN substrates at least 100 fold more effectively than with Neu5Ac substrates (387). For both *S. coelicolor* and *B. thetaiotaomicron*, the biosynthetic genes were found as a series of three genes that formed part of a cluster of other putative sialic acid metabolic genes. Ostash et al. (2014) and Wang et al. (2008) identified these biosynthetic genes as a cluster and were able to identify similar genes *in silico* in other bacteria. Unfortunately, similar KDN biosynthetic genes from *S. coelicolor* and *B. thetaiotaomicron* were not be found in a Blastp search with the *A. fumigatus* genome (J. Yeung, unpublished data). Alternatively, it has been suggested that KDN could also be formed directly from Neu5Ac by deacylation and deamination; however, no enzymes that would facilitate the direct conversion of Neu5Ac to KDN has been found in any organism examined to date (183, 370, 372, 388). Therefore, the series of sialic acid biosynthetic genes used by *A. fumigatus* must be unlike any ones currently characterized in bacteria or mammals, and the sialic acid biosynthetic pathway in *A. fumigatus* remains a mystery.
6.2. *A. fumigatus* nucleotide sugar transporters (*AfNSTs*)

Despite the elusive mechanism used by *A. fumigatus* to synthesize sialic acid de novo, monosaccharides must be activated to its nucleotide sugar form and transported into the ER or Golgi lumen by nucleotide sugar transporters (NSTs) for presentation on the terminus of cell-surface glycoconjugates. Since sialic acid on the surface of *A. fumigatus* is linked α2,6 to an underlying galactose residue (251), it was originally hypothesized that through the removal of the CMP-Neu5Ac transporter, or the UDP-Gal transporter it would be possible to generate a sialic acid deficient *A. fumigatus* strain in the same manner as the CHO Lec8 (318) and Lec2 (389) strains. In the CHO cells, knockout of the CMP-sialic acid transporter (Lec2) or the UDP-galactose transporter (Lec8) resulted in sialic acid deficient cell lines (318, 389). If we could construct a sialic acid deficient strain, it would be possible to evaluate the role of sialic acid in the virulence of *A. fumigatus*. Four novel *A. fumigatus* nucleotide sugar transporters (*AfNSTs*): *AfNST1*, *AfNST2*, *AfNST5*, *AfNST6* were cloned and expressed in yeast and isolated in Golgi-enriched fractions were studied. Using radiolabelled UDP-Gal and CMP-Neu5Ac, it was found that *AfNST1* and *AfNST5* were able to transport $^{14}$UDP-Gal, but unable to transport $^{14}$CMP-Neu5Ac. Similar results were revealed by STD NMR analysis of the *AfNSTs* with a library of nucleotide sugars. In addition to UDP-Gal binding to *AfNST1* and *AfNST5*, it was found that UDP-Glc could bind to *AfNST1* and *AfNST5* and UDP-GalNAc could bind to *AfNST1* and *AfNST6*.

Early work on NSTs suggested a one transporter-one substrate model (119, 125, 129, 318). More recently, the ability of NSTs to transport multiple substrates has been shown in studies of NSTs from numerous organisms. NSTs are highly conserved transmembrane proteins and the NSTs that are able to transport more than one nucleotide-sugar are always selective for the same nucleotide (132). For example a human NST, hUGTrell7, transports UDP-glucuronic acid (UDP-GlcUA)/ UDP-N-acetylgalactosamine (UDP-GalNAc) into the Golgi was identified (130). A similar transporter in *Drosophila* was identified by sequence homology (DmUGT) was found to be multi-specific for UDP-GalNAc and UDP-Gal (131). Furthermore, AtUTr1 was originally annotated as a putative UDP-Gal transporter in *Arabidopsis thaliana* and was later found to be capable of transporting both UDP-Gal and UDP-Glc (132). Recently, it
was found that AtUTr1 actually belonged to a family of six other sugar transporters in *A. thaliana* that were characterized to be UDP-rhamnose (UDP-Rha)/ UDP-Gal transporters (319). In the nematode, *Caenorhabditis elegans*, four of the 18 putative NSTs have been characterized and all transports multiple UDP sugars, with the most recent NST characterized to be capable of transporting four substrates (320, 390–392). In addition, the four *C. elegans* NSTs had redundant substrate specificity; this was likely and necessary since the *C. elegans* glycome only has seven different monosaccharides and 18 possible NSTs (320). Similar to the UDP-sugar NSTs characterized in other organisms, I observed functional redundancy for the *A. fumigatus* AfNST1 and AfNST5 for UDP-Gal transport. Moreover, STD-NMR analysis showed that partial substrate overlap were observed only for UDP-sugars for AfNST1, AfNST5 and AfNST6, and the greatest binding intensities were observed between the nucleotide moiety of the nucleotide sugar and the AfNSTs. This observation further confirms that selectivity of multi-substrate transporters lies primarily in the nucleotide recognition and that the sugar moiety provides additional selectivity.

To determine the biological role of the two transporters capable of transporting UDP-Gal, two *A. fumigatus* knockout mutants were created, AfΔnst1KO and AfΔNST5KO. In spite of the fact that both were UDP-Gal transporters, AfΔnst1KO and AfΔNST5KO were morphologically distinct when grown in a high stress conditions; in particular, AfNST5 knockout had a greater adverse impact on fungal growth. NST knockout mutants from plants, nematodes and protozoa have also shown that similar *in vitro* activities of NSTs does not equate to similar *in vivo* phenotypes when the different transporters are knocked out. For example, double knockout mutants of two UDP-Rha/UDP-Gal transporters in *A. thaliana* did not eliminate the presence of Rha or Gal. However, the single knockout of URGT1, reduced the amount of Gal on the cell wall, whereas knockout of URGT2, reduced the amount of Rha on the seed coat (319). This is in spite of the functional redundancy of six similar transporters present in the *A. thaliana* genome that would compensate for the function of the missing transporter. Likewise, in *C. elegans*, two transporters that were demonstrated to have partial overlap in substrate specificity: SRF-3 transports UDP-Gal and UDP-GlcNAc (392) and CO3H5.2 transports UDP-GlcNAc and UDP-GalNAc (391). When CO3H5.2 was knocked-down in the SRF-3 deficient *C. elegans* mutant, abnormal morphology was observed in the worm. However,
these defects were not detectable in the SRF-3 deficient mutant or in wild type worms that had the same RNAi treatment (320).

Another similar finding was obtained with two UDP-Gal transporters, LPG5A and LPG5B, characterized in the protozoan parasite, *Leishmania major*. A variety of secreted and surface glycoconjugates on the parasite contain phosphoglycan polymers (PG) that are primarily composed of galactose and mannose residues (393, 394). Both LPG5A and LPG5B were demonstrated to be UDP-Gal transporters through complementation studies with Lec8 cells by rescuing the galactose deficient phenotype on the CHO cell surface (393). The single LPG5A or LPG5B gene knockout mutants in *L. major* still made phosphoglycan polymers (PG), but the LPG5A knockout mutation caused a significant change to the PG composition that was not observed in the LPG5B knockout mutant or wild type *L. major*. Only when both genes were knocked-out, it was possible to prevent PG presentation on the surface and the phenotype was rescued when either one or both genes were reintroduced (393).

Together, these data and our findings show that compensatory mechanisms in the control of glycan presentation on cell surface is observed across different organisms. The glycome of *A. fumigatus* has not been solved, but from the evidence available, *A. fumigatus* possesses at least seven different monosaccharides that make-up the main components of the cell wall (N-acetylglucosamine, glucose, mannose, galactofuranose, and N-acetylgalactosamine) (73, 310, 315) and the two terminal sugars found on spore surface (galactose and Neu5Ac) (251). To date, the GDP-mannose (Man) and the UDP-galactofuranose (Gal\(\text{f}\)) transporters have been identified in *A. fumigatus* and these transporters were demonstrated to be specific for the one nucleotide-sugar substrate (321, 353). In particular, Engel et al. (2012) found that deletion of the UDP-Gal\(\text{f}\) transporter from the *A. fumigatus* genome lead to an absence of galactofuran, and deletion of the UDP-Man transporter resulted in a strain devoid of galactomannan. Both galactofuranose and mannose are the primary components of galactomannan which is an essential component of the fungal cell wall (315, 395). In contrast, other studies of *A. fumigatus* cell wall metabolic proteins, such as the α1,3 glucan synthases (83) and UDP-glucose 4-epimerases (396), found that fungal carbohydrate metabolism has numerous compensatory pathways and mutli-gene knockout mutants are necessary to eliminate
their presence on the cell surface. From a genome search in the NCBI database, 16 putative *A. fumigatus* NSTs have been found by us and others (321). With the exception of the CMP-Neu5Ac transporter, the remaining 11 uncharacterized putative *A. fumigatus* NSTs are likely to have overlapping substrate specificities since they will most likely be UDP-sugar transporters and there are only five other monosaccharides used by *A. fumigatus*. To most effectively create a sialic acid deficient strain of *A. fumigatus*, the CMP-Neu5Ac transporter will need to be identified. If that is not possible, then a multi-NST gene knockout *A. fumigatus* strain will need to be created assuming the mutations are not lethal and the knockout strain is viable for phenotype analysis.

Although the removal of *Af*NST5 from the *A. fumigatus* genome did not eliminate sialic acid from the surface of the fungus, it significantly reduced the amount of sialic acid present on the spore surface. These *Af*Δnst5KO spores bound ~ 60% less to fibronectin compared to wild type spores (Lindsay Woof, personal communication). Consistent with previous findings by Warwas et al. (2007) with *MvS* treated wild type *A. fumigatus* spores, the reduction of sialic acid resulted in decreased binding of *Af*Δnst5KO to fibronectin. Since fibronectin and other extracellular matrix proteins are more prevalent in diseased lungs (397), it is possible that *Af*Δnst5KO will be less infectious than wild type *A. fumigatus* because the efficiency in spore binding will be reduced. Conidia uptake experiments by immune cells will show if the reduction in sialic acid affects phagocytosis. The critical test will be to compare the growth and virulence of the WT and the *Af*Δnst5KO in a murine aspergillosis model to determine whether sialic acid is truly important in adhesion to host tissues.
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through a covalent sialyl-enzyme intermediate: tyrosine is the catalytic

of *Aspergillus fumigatus* conidia binding to extracellular matrix proteins by sialic


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Appendix A.

Supplementary Materials

Chapter 3: The Aspergillus fumigatus sialidase is a 3-Deoxy-D-glycero-D-galacto-2-nonulsonic Acid Hydrolase (KDNase)

Crystalization of AfS: Selenomethionine incorporation

_E. coli_ expressing the AfS/PET28A+ vector were grown in 150 ml LB with 50 μg/ml at 37 °C, shaking at 220 rpm overnight. Cells were harvested by centrifugation at 16,800 g for 20 mins at 4 °C. The supernatant was discarded and the pellet was re-suspended in 100 ml PBS and re-centrifuged. Supernatant was discarded and the pellet re-suspended in 4 ml PBS and added to 1 L sterile Selenomethionine (SeMet) media (17 mM NH₄Cl, 22 mM KH₂PO₄, 6 mM Na₂HPO₄*7H₂O, 25 μM Fe₄(SO₄)₃ and 30 μM thiamine, 20% w/v glucose, 0.3% w/v MgSO₄). Addition of 50mg of selenomethionine and kanamycin (50μg/ml) was then made to the selenomethionine minimal media. The culture was incubated at 37 °C shaking at 200 rpm until OD₆₀₀ reached 0.9 when AfS expression was induced by 0.5 mM IPTG and incubated overnight. Cells were harvested and AfS extracted and purified as above.

Protein crystallization

The optimal AfS concentration for crystal trials was determined as 13.7 mg/ml by pre-crystallization assay (Hampton Research). Sitting-drop vapour diffusion plates, held at 20 °C were used for all crystallization experiments. To ascertain crystallization conditions for AfS, commercial screens Cryo I and II (Emerald Biosystems), Crystal screens I and II and Index (Hampton Research), PEG screen (Qiagen) and Wizard (Emerald Biosystems) were used. Purified AfS was added to each screen condition in a 1:1 ratio by the high throughput Rhombix Screen robot. Crystals from the PEG screen (Qiagen), condition 20% (w/v) polyethylene glycol (PEG) 3350 and 0.2 M sodium nitrate, appeared after four days and did not require any optimisation. The AfS structure with selenomethionine incorporated crystallised in identical conditions. The structures of AfS
in complex with KDN, KDN2en and 2,3F-KDN were achieved by soaking AfS crystals by addition of 1 μl of 70 mM KDN-MU, 100 mM KDN2en (398) or 500mM 2,3F-KDN, respectively, to 4 μl well containing 1:1 ratio of 13.7 mg/ml AfS and 20% (w/v) PEG 3350 and 0.2 M sodium nitrate and leaving for 20 mins. The synthesis of KDN-MU and 2,3F-KDN is described in the supplementary information.

Data collection

Crystals were cryoprotected by transfer to 20% (w/v) glycerol in crystallization buffer for a few seconds prior to placement in liquid nitrogen. Native data were collected at 100 K in house (Rigaku-MSC Micromax-007 X-ray generator and Saturn 944+ CCD detector). Data at the selenium absorption edge were collected on beamline ID23-1, and the covalent intermediate data on beamline ID14-1 at the European Synchrotron Radiation Facility (ESRF), Grenoble. MOSFLM (297) and the CCP4 suite (399) were used to integrate and analyse the diffraction data. Crystals of AfS belong to space group P21.

Structure determination and refinement

The structure of AfS was solved by SAD (single wavelength anomalous diffraction) using data collected at the selenium absorption peak. The selenium peak itself was 0.9794Å, as determined by a crystal florescence scan. The selenium sites were determined using SHELXD (400) from the anomalous differences. SHELXE (400) was used to resolve the phase ambiguity and to apply density modification, resulting in an electron density map with a clear protein/solvent boundary and a polyalanine interpretation of the backbone of a large part of the structure. The phases from SHELXE were used in Phenix (401), together with the amino acid sequence to automatically build an initial structure at 2.15Å. The model of the apo structure was refined in REFMAC5 (298) using in-house data collected from a SeMet crystal data to 1.84Å and manually fitted/improved with Coot (299) and validated by Coot and MolProbity (300). The structures of AfS in complex with ligands were refined similarly. Data collection and refinement statistics, together with PDB codes of the deposited structures are given in Table A1.
Table A1  Data collection and refinement statistics. Numbers in parentheses refer to the highest resolution shell.

\[ R_{\text{merge}} = \frac{\sum_{hkl} \sum_{i} |I_{hkl,i} - \langle I_{hkl} \rangle|}{\sum_{hkl} \langle I_{hkl} \rangle} \]

R-factor and R-free = \((\sum |F_o| - |F_c|)/(|F_o|)/(|F_c|)\)

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Appendix B.

Supplementary Materials

Chapter 4: Kinetic and Structural Evaluation of Selected Active Site Mutants of the *Aspergillus fumigatus* KDNase (Sialidase)

Structural determination of *AfS* mutants

Table B1  X-ray data collection and refinement statistics

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</tr>
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<td><em>No. observations</em></td>
<td>273,633</td>
<td>274,759</td>
<td>302,861</td>
</tr>
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<td>2.0 (1.8)</td>
<td>2.2 (1.9)</td>
<td>2.2 (2.0)</td>
</tr>
<tr>
<td><em>Completeness (%)</em></td>
<td>98.9 (94.4)</td>
<td>98.5 (92.0)</td>
<td>99.2 (96.9)</td>
</tr>
<tr>
<td>*R&lt;sub&gt;merge&lt;/sub&gt; (%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.5 (14.2)</td>
<td>8.8 (21.2)</td>
<td>8.6 (27.8)</td>
</tr>
<tr>
<td><em>I/σ(I)</em></td>
<td>18.2 (7.5)</td>
<td>11.0 (5.0)</td>
<td>13.8 (4.2)</td>
</tr>
<tr>
<td><strong>Refinement</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Reflections used</em></td>
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<td>66,869</td>
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<td><em>N. atoms</em></td>
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<tr>
<td><em>Number of protein atoms</em></td>
<td>6,039</td>
<td>5,981</td>
<td>6,010</td>
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<tr>
<td><em>Number of water</em></td>
<td>1,162</td>
<td>1,165</td>
<td>1,396</td>
</tr>
<tr>
<td><em>molecules</em></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Number of ligand atoms</em></td>
<td>N/A</td>
<td>N/A</td>
<td>20</td>
</tr>
<tr>
<td>Average B-factor (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>9.9</td>
<td>9.9</td>
<td>12.1</td>
</tr>
<tr>
<td></td>
<td>AFS_{D54A}</td>
<td>AFS_{Y358H}</td>
<td>AFS_{R171L}</td>
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<tr>
<td>------------------</td>
<td>------------</td>
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<tr>
<td><strong>Data collection</strong></td>
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<td><strong>Protein</strong></td>
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<td>7.6</td>
<td>9.5</td>
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<td><strong>Water</strong></td>
<td>22.4</td>
<td>21.8</td>
<td>28.9</td>
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<tr>
<td><strong>Ligand</strong></td>
<td>N/A</td>
<td>N/A</td>
<td>13.5</td>
</tr>
<tr>
<td><strong>R-factor</strong></td>
<td>0.143</td>
<td>0.173</td>
<td>0.155</td>
</tr>
<tr>
<td><strong>R-free</strong></td>
<td>0.178</td>
<td>0.224</td>
<td>0.194</td>
</tr>
<tr>
<td><strong>Root mean square deviation in bond length (Å)</strong></td>
<td>0.009</td>
<td>0.010</td>
<td>0.009</td>
</tr>
<tr>
<td><strong>Root mean square deviation in bond angles (°)</strong></td>
<td>1.14</td>
<td>1.24</td>
<td>1.19</td>
</tr>
</tbody>
</table>

\(a\) = Values in parentheses correspond to the highest resolution shell.

\(b\) = \(R_{\text{merge}} = \frac{\sum_{hkil} |I_{hkil} - \langle I_{hkil} \rangle|}{\sum_{hkil} |I_{hkil}|} / \frac{\sum_{hkil} |I_{hkil}|}{\sum_{hkil} |I_{hkil}|} \)

\(c, d\) = R-factor and R-free = \(\frac{\sum |F_o - |F_c| |}{\sum |F_o|} / \frac{\sum |F_o|}{\sum |F_o|} \)

were the \(R_{\text{free}}\) was calculated from 5% of reflections excluded from refinement.

**Synthesis of 8-fluoro-methylumbelliferyl α-2-keto-3-deoxy-D-glycero-D-galacto-nononylgalactopyranoside (KDNα2,6GalβFMU)**

![Chemical diagram showing the synthesis of KDNα2,6GalβFMU]

**Scheme B1  Chemo-enzymatic synthesis of KDN α 2,6Gal β FMU (4)**
To a solution of sodium pyruvate (50 mg, 0.45 mmol) and mannose (400 mg, 2.3 mmol) in deionized water (1 mL) was added a solution of 0.2 mM aldolase (50 μL) containing 0.05 mM bovine serum albumin at pH = 7.75. After incubating for 3 days at 37 °C, the mixture was heated to 70 °C for 20 min. The resultant mixture was then cooled to room temperature and centrifuged (3500 RPM) for 30 min. The supernatant was subsequently concentrated and purified by anion exchange column (formate form) chromatography eluting initially with water to remove excess mannose and then 2 M formic Acid. Fractions containing the ketodeoxynonulosonic acid (3-deoxy-D-glycero-D-galacto-non-2-ulosyranosonic acid, Kdn, 1) were combined and freeze dried to give 100 mg of white powder (87% yield). The so-obtained Kdn (1, 100 mg, 0.37 mmol) was dissolved in H₂O (2 mL) and converted into the corresponding activated donor CMP-Kdn (2) by incubation with CMP-Neu5Ac synthase (1 mL, crude expression supernatant) in the presence of cytidine 5'-triphosphate disodium salt (285 mg, 0.56 mmol) in a solution containing Tris (1 M; 3.3 mL), MgCl₂ (1 M; 1.3 mL), pyrophosphatase (100 μL, 0.1 U/mL) at 37 °C and pH = 8 (the pH was adjusted by adding NaOH prior to addition of enzyme) for 3 h. After centrifuging the reaction mixture (15 min, 3500 rpm), the supernatant was reacted with β-galactoside 3 (150 mg, 0.42 mmol) in the presence of α-2,6-sialyltransferase (250 μL, 1 U/mL) by incubating the mixture at 37 °C overnight. After the reaction was complete, as judged by acquisition of a ¹H NMR spectrum on a small portion of the mixture, the reaction media was centrifuged and the supernatant was purified on a reversed-phase C18 sep-pack cartridge that was eluted successively with water and 1:20 MeCN/H₂O (v/v). Fractions containing the product were lyophilized to give Kdnα-2,6-Galβ-FMU (4) as white powder (50 mg, 20% yield); Mpt = 208–209 °C,
[α]$_{D}^{20}$ = −23.0 (c = 0.1, H$_2$O); $^1$H NMR (600 MHz, D$_2$O) δ: 1.62 (t, 1 H, $J_{3a,4} = 12.3$, H-3$^II_{ax}$), 2.51 (s, 3H, CH$_3$), 2.72 (dd, 1 H, $J_{3e,3a} = 12.5$, $J_{3e,4} = 4.8$, H-3$^II_{eq}$), 3.46 (t, 1 H, $J_{5,4}$ = $J_{5,6} = 9.4$, H-5$I$), 3.65 (m, 3 H, H-4$^II$, H-6$^II$, H-9$^a_{II}$, H-6$^a$), 3.83 (m, 2 H, H-3$I$ and H-5$I$), 3.90 (m, 3 H, H-7$^II$, H-2$I$, H-6$^b$I), 4.02 (m, 3 H, H-8$^II$, H-9$^b_{II}$, H-4$^I$), 5.22 (d, 1 H, $J_{1,2} = 7.8$, H-1$I$), 6.38 (s, 1 H, H-3), 7.36 (t, 1 H, $J_{6,5} = J_{6,F} = 8.1$, H-6), 7.63 (d, 1 H, $J_{5,6}$ = 9.2, H-5$I$); $^{13}$C NMR (150 MHz, D$_2$O) 17.60 (NHCOC$_2$H$_3$), 39.37 (C-3$^II$), 62.20 (C-6$^I$), 62.56 (C-9$^II$), 67.36 (C-5$^I$ or C-3$^I$), 67.92 (C-4$^I$), 69.42 (C-5$^II$), 69.63 (C-4$^II$), 69.83 (C-2$I$), 71.60 (C-7$^II$), 71.88 (C-5$^I$ or C-3$^I$), 73.07 (C-6$^II$), 73.66 (C-8$^II$), 99.74 (C-2$^I$), 100.38 (C-1$I$), 111.61 (C-5), 112.31 (C-2), 116.04 (Ar), 119.99 (d, $J_{C,F} = 4.3$ Hz, C-5), 139.04 (d, $J_{C,F} = 248.3$, C-8), 141.58 (d, $J_{C,F} = 9.1$ Hz, Ar), 146.46 (d, $J_{C,F} = 7.7$ Hz, Ar), 155.62 (Ar), 162.50 (Ar), 173.08 (C-1$^{II}$). HRMS-FAB (m/z): [M–H$^+$] calcd for C$_{25}$H$_{30}$FO$_{16}$, 605.1523; found, 605.1535.
Appendix C.

Supplementary Materials

Chapter 5: UDP-Sugar Transporters in the Fungal Pathogen, *Aspergillus fumigatus*

Table C1  List of primers for plasmid construction and gene expression experiments

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Target</th>
<th>Sequence</th>
<th>plasmid/ tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>AfNST1cDNARev</td>
<td>NST1 3' flanking sequence</td>
<td>CTCGTCCCCGTGACTGCTTAT</td>
<td>pESC-URA/ FLAG</td>
</tr>
<tr>
<td>AfNST1 Fwd</td>
<td>NST1 5' flanking sequence w/ cut site</td>
<td>ATTGC GGCGCCATGCCTCC ACTGGAAGTA</td>
<td>pESC-URA/ FLAG</td>
</tr>
<tr>
<td>AfNST1 Rev</td>
<td>NST1 3' flanking sequence w/ cut site</td>
<td>CCGACTAGTGCTCCTCCG TGACTGCTTAT</td>
<td>pESC-URA/ FLAG</td>
</tr>
<tr>
<td>AfNST2cDNARev</td>
<td>NST2 3' flanking sequence</td>
<td>TACTCTGCCCCTGCGAAGG</td>
<td>pESC-URA/ FLAG</td>
</tr>
<tr>
<td>AfNST2 fwd</td>
<td>NST2 5' flanking sequence w/ cut site</td>
<td>ATTGC GGCGCCATGCTTCTGA CTGATGAACATATA</td>
<td>pESC-URA/ FLAG</td>
</tr>
<tr>
<td>AfNST2 Rev</td>
<td>NST2 3' flanking sequence w/ cut site</td>
<td>CCGACTAGTGCTACTCTGCCCTGCGAAGG</td>
<td>pESC-URA/ FLAG</td>
</tr>
<tr>
<td>AfNST2 fwd_HA</td>
<td>NST2 5' flanking sequence w/ cut site</td>
<td>AGCTCGAATTCATGCTTGAG TGATGAACATATA</td>
<td>pRS313/ HA</td>
</tr>
<tr>
<td>AfNST2 Rev_HA</td>
<td>NST2 3' flanking sequence w/ cut site</td>
<td>GAGCTACTAGTGCTCAGATA GCGTAATCTGGTACGCTGTTAT</td>
<td>pRS313/HA</td>
</tr>
<tr>
<td>AfNST5cDNARev</td>
<td>NST5 3' flanking sequence</td>
<td>CTCTCTTCTTCCAGTTAATGCT</td>
<td>pESC-URA/ FLAG</td>
</tr>
<tr>
<td>AfNST5 fwd</td>
<td>NST5 5' flanking sequence w/ cut site</td>
<td>AT GTGCGCCGATGCACCTT GTGCCCTGAG</td>
<td>pESC-URA/ FLAG</td>
</tr>
<tr>
<td>AfNST5 Rev</td>
<td>NST5 3' flanking sequence w/ cut site</td>
<td>CCGACTAGTGCTCCTTTCTTCCAGTTAATGCT</td>
<td>pESC-URA/ FLAG</td>
</tr>
<tr>
<td>AfNST6cDNARev</td>
<td>NST6 3' flanking sequence</td>
<td>TATGTGCTCAATGGGCTCC</td>
<td>pESC-URA/</td>
</tr>
<tr>
<td>AfNST6 fwd</td>
<td>NST6 5' flanking sequence w/ cut site</td>
<td>AGCTCGCCGGGCTATGGCT ACTAGGGCTGG</td>
<td>pESC-URA/ FLAG</td>
</tr>
<tr>
<td>AfNST6 Rev</td>
<td>NST6 3' flanking sequence w/ cut site</td>
<td>GAGCTGTCGACTATGTGCTCA ATGGGCTCC</td>
<td>pESC-URA/ FLAG</td>
</tr>
</tbody>
</table>
Figure C1  Design for the fusion construct showing replacement of the Afnst5 gene (grey) by the ble cassette. Primers targeted to 1200 bp upstream and downstream (1a and 4a) and 1000 bp upstream and downstream (1 and 4) of the gene were designed to generate the entire fusion construct. All primers used are referred to by number in table s3 and are shown in colored boxes. The nst5 gene is 1311bp and the ble cassette is 1468bp.

Table C2  Sequences of the primers ordered for fusion PCR according to the design in Figure 3. Endonuclease sites are underlined.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gta tcc tgc aga ctc cat gat tgc ccg tga</td>
</tr>
<tr>
<td>1a</td>
<td>gta tcc tgc agt cga tca acc atg gcc aa</td>
</tr>
<tr>
<td>2</td>
<td>att cag aag aag tgg ccc gtt</td>
</tr>
<tr>
<td>3</td>
<td>tgg tct gtt cga ccc tta tt</td>
</tr>
<tr>
<td>4</td>
<td>atc gcc tgc aga tca tag agt tgt cct gtc tg</td>
</tr>
<tr>
<td>4a</td>
<td>atc gcc tgc aga cca gca tca gtc tca ttg ga</td>
</tr>
<tr>
<td>5</td>
<td>aac ggg cca ctt ctt ctg aat cgg aga ata tgg agc ttc atc</td>
</tr>
<tr>
<td>6</td>
<td>aat aac ggt cga aca gag cca att aac gcc ttc gag cgt cc</td>
</tr>
</tbody>
</table>
Figure C2   Southern Blot analysis of *A. fumigatus* NST5 knockout mutants
Genomic DNA of each *AfnST5* knockout strains and wild type *A. fumigatus* were digested with endonucleases to confirm that the NST5 gene was knockout by homologous recombination. (A) *AfnST5* knockout genomic DNA were digested with Nru1 and Sac1, and (B) the digested DNA was subsequently treated with Nar1, only one band at ~3027 bp is detected. This demonstrates that only one copy of the knockout cassette was inserted into the genome by homologous recombination, since Nar1 doesn’t cut within the ble sequence. Contrast, when wild type genomic DNA was treated with both Nru1 and Nar1, the *Afnst5* gene is cut which generates a ~954 bp fragment as seen on blot A.
### Table C3  *Aspergillus fumigatus* Nucleotide Sugar Transporters Investigated in this study

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Annotation*</th>
<th>Locus (AspGD)</th>
<th>Accession (NCBI)</th>
<th>Amino Seq length</th>
<th>TMD (HMMTOP®)</th>
<th>Localization(WoLF®)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AfNST1</td>
<td>UDP-galactose transporter activity</td>
<td>Afu6g13070</td>
<td>XP_751184</td>
<td>423</td>
<td>7</td>
<td>Integral membrane protein: ER</td>
</tr>
<tr>
<td>AfNST2</td>
<td>sugar: hydrogen symporter activity; carbohydrate transporter; Golgi transmembrane</td>
<td>Afu8g02090</td>
<td>XP_746997</td>
<td>354</td>
<td>10</td>
<td>Integral membrane protein: ER</td>
</tr>
<tr>
<td>AfNST5</td>
<td>UDP-galactose/ UDP-glucose transporter activity</td>
<td>Afu1g05440</td>
<td>XP_750293</td>
<td>415</td>
<td>8</td>
<td>Integral membrane protein: Vac/ PM/ ER</td>
</tr>
<tr>
<td>AfNST6</td>
<td>sugar: hydrogen symporter activity; carbohydrate transport; Golgi membrane</td>
<td>Afua_1G06050</td>
<td>XP_750355</td>
<td>435</td>
<td>8</td>
<td>Integral membrane protein: PM/ ER</td>
</tr>
</tbody>
</table>

*Schematic drawings of the *A. fumigatus* nucleotide sugar transporters made with the bioinformatic program Topo2 (http://www.sacs.ucsf.edu/TOPO2/) based on the amino acid sequence of the nucleotide sugar transporters from Af13073 and the transmembrane domain prediction tool HMMTOP (http://www.enzim.hu/hmmtop/index.php).

*Gene ontology (GO) annotations retrieved from AspGD (www.aspgd.org)

*Severs used to predict transmembrane topology were HMMTOP (Tusnday and Simon, 2001) Abreviation used: Transmembrane domain (TMD)

*Intracellular localization was predicted using WoLF PSORT: protein localization predictor (Horton et al., 2006) (www.wolfpsort.org). Abreviations used: Plasma membrane (PM), Endoplasmic reticulum (ER), Vacuolar (Vac)
Figure C3  Predicted Topology of (A) AfNST1 (XP_751184) and (B) AfNST5 (XP_750293)
The expression of all four NSTs in Golgi-enriched fractions was determined by Western blot under denaturing conditions and the approximate molecular weights (kDa) were determined by comparing to molecular size markers. Molecular masses identified were similar to prediction by Protpram (http://web.expasy.org/protparam/). Immunological markers used to identify the NSTs were (A) FLAG (NST1 and NST5), (B) MyC (NST6) and (C) HA (NST2). + indicates that yeast were grown in inducing conditions and - indicates non-inducing conditions.
Figure C5   STDD NMR spectra of ER/Golgi enriched fractions in the presence of CMP-sialic acid

(A) STD NMR spectra of CMP-Neu5Ac in the presence of ER/Golgi-enriched fractions overexpressing A. fumigatus nucleotide sugar transporters AnNST1, AnNST2, AnNST5, AnNST6, and 1H NMR spectra of CMP-Neu5Ac. All spectra were recorded as described for experiments with UDP-Gal. The residual water signal was removed by applying a WATERGATE sequence. (B) Labeled structure of CMP-Neu5Ac corresponding to the spectrum.
Table C4  Binding intensity of UDP-Galactose to AfNST1 and AfNST5 as determined by STD NMR analysis

<table>
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<tr>
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<th>AFNST1</th>
<th></th>
<th>AFNST5</th>
<th></th>
</tr>
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<tbody>
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<td>ppm</td>
<td>ASTD</td>
<td>% level</td>
<td>ASTD</td>
<td>% level</td>
</tr>
<tr>
<td>URA H6</td>
<td>7.72</td>
<td>0.0047</td>
<td>31 l</td>
<td>0.0058</td>
</tr>
<tr>
<td>RIB H1</td>
<td>5.74</td>
<td>0.0154</td>
<td>100 h</td>
<td>0.0149</td>
</tr>
<tr>
<td>URA H5</td>
<td>5.73</td>
<td>0.0133</td>
<td>86 h</td>
<td>0.0131</td>
</tr>
<tr>
<td>GAL H1</td>
<td>5.39</td>
<td>0.0066</td>
<td>43 m</td>
<td>0.0078</td>
</tr>
<tr>
<td>RIB H2&amp;H3</td>
<td>4.12</td>
<td>0.0078</td>
<td>51 m</td>
<td>0.0069</td>
</tr>
<tr>
<td>RIB H4</td>
<td>4.03</td>
<td>0.0123</td>
<td>80 h</td>
<td>0.0071</td>
</tr>
<tr>
<td>GAL H4</td>
<td>3.76</td>
<td>0.0066</td>
<td>43 m</td>
<td>0.0066</td>
</tr>
<tr>
<td>GAL H3</td>
<td>3.65</td>
<td>0.0102</td>
<td>66 h</td>
<td>0.0118</td>
</tr>
<tr>
<td>GAL H2</td>
<td>3.53</td>
<td>0.0088</td>
<td>57 m</td>
<td>0.0094</td>
</tr>
<tr>
<td>Rib H5</td>
<td>4.01 &amp; 3.93</td>
<td>0.0049</td>
<td>32 l</td>
<td>-</td>
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Table C5  Binding intensity of UDP-Glucose to AfNST1 and AfNST5 as determined by STD NMR analysis

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<tr>
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<th>AFNST1</th>
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<th>AFNST5</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>ppm</td>
<td>ASTD</td>
<td>% level</td>
<td>ASTD</td>
<td>% level</td>
</tr>
<tr>
<td>URA H6</td>
<td>7.72</td>
<td>0.0058</td>
<td>36 l</td>
<td>0.0075</td>
</tr>
<tr>
<td>RIB H1</td>
<td>5.74</td>
<td>0.016</td>
<td>100 h</td>
<td>0.0154</td>
</tr>
<tr>
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<td>84 h</td>
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<tr>
<td>GLU H1</td>
<td>5.39</td>
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<tr>
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<td>0.0068</td>
<td>43 m</td>
<td>0.0088</td>
</tr>
<tr>
<td>RIB H4</td>
<td>4.03</td>
<td>0.0092</td>
<td>58 m</td>
<td>0.0088</td>
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<tr>
<td>RIB H5</td>
<td>3.97</td>
<td>0.0041</td>
<td>26 l</td>
<td>0.0059</td>
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<tr>
<td>GLU H5</td>
<td>3.65</td>
<td>0.006</td>
<td>38 l</td>
<td>0.0082</td>
</tr>
<tr>
<td>GLU H6</td>
<td>3.58 &amp; 3.52</td>
<td>0.006</td>
<td>38 l</td>
<td>0.0068</td>
</tr>
<tr>
<td>GLU H3</td>
<td>3.5</td>
<td>-</td>
<td>-</td>
<td>0.009</td>
</tr>
<tr>
<td>GLU H2</td>
<td>3.27</td>
<td>0.0051</td>
<td>32 l</td>
<td>0.0085</td>
</tr>
<tr>
<td>GLU H4</td>
<td>3.2</td>
<td>0.0051</td>
<td>32 l</td>
<td>0.008</td>
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Table C6  Binding intensity of UDP-N-acetylgalactosamine to AfNST1 and AfNST6 as determined by STD NMR analysis

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<th>ppm</th>
<th>ASTD %</th>
<th>level</th>
<th></th>
<th>ppm</th>
<th>ASTD %</th>
<th>level</th>
</tr>
</thead>
<tbody>
<tr>
<td>URA H6</td>
<td>7.72</td>
<td>0.005</td>
<td>35</td>
<td>l</td>
<td>0.0034</td>
<td>43</td>
<td>m</td>
</tr>
<tr>
<td>RIB H1</td>
<td>5.74</td>
<td>0.0143</td>
<td>100</td>
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Tables C3 – C5 list the details of the binding intensities for each nucleotide sugar to the respective AfNSTs as presented in figures 2 – 4. The STD NR spectrum of the Golgi enriched fraction induced with the specific AfNST (GeF-I): nucleotide sugar (NS) complex was used to calculate the relative STD NMR effects. Golgi enriched fractions carrying an empty plasmid (GeF-E) was the control spectrum. A STDD spectrum (I_{STD}=GeF-I:NS – GeF-E:NS) and the off-resonance spectrum (I_o) of the STD NMR were used to calculate relative STD MR effects for each nucleotide sugar. All STD NMR effects were calculated according to the formula A_{STD} = (I_o x I_{SAT})/I_o = I_{STD}/I_o using the Rib H1 proton to 100%.
A

Wild type       Af\textsuperscript{nst}5KO       Af\textsuperscript{nst}1KO

DIC

Fluorescence

B

Wild type       Af\textsuperscript{nst}5KO       Af\textsuperscript{nst}1KO

DIC

Fluorescence
C

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DIC

Fluorescence

D

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DIC

Fluorescence
Figure C6  DIC and fluorescent images of *A. fumigatus* wild type, AfΔnst5KO, and AfΔnst1KO spores bound to FITC labeled sialic acid specific (SNA) and galactose specific (PNA) lectins. DIC and fluorescent images of *A. fumigatus* wild type, AfΔnst5KO, and AfΔnst1KO spores bound to FITC labeled sialic acid specific (SNA) and galactose specific (PNA) lectins. Spores were not treated (A) and not treated (B) with *M. viridifaciens* sialidase prior to SNA binding. Spores were treated (C) and not treated (D) with *M. viridifaciens* sialidase prior to PNA binding. Lectin binding to wild type and NST mutant strains shows that sialic acids and the underlying galactose are still present on the spore surface.