
**SIALIC ACIDS AS POTENTIAL VIRULENCE FACTORS
OF THE FUNGAL PATHOGEN, *ASPERGILLUS
FUMIGATUS***

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ABSTRACT

Aspergillus fumigatus is an opportunistic fungal pathogen that causes invasive disease in immunosuppressed individuals. Sialic acids have been identified on *A. fumigatus*, but neither their structure nor biological significance had been studied in detail. Sialic acids are a family of 9-carbon monosaccharides, which are important in microbial pathogenesis. The objectives of this study were: 1) to identify the linkage, sub-terminal monosaccharide and structure of *A. fumigatus* sialic acids, 2) to determine the role of sialic acids in adhesion to the extracellular matrix protein, fibronectin, and 3) to determine the role of conidiospore sialic acids in phagocytosis by cultured cells. Unsubstituted sialic acid linked α 2,6 to galactose was identified on the surface of *A. fumigatus* conidia. Removal of conidial sialic acids decreased binding to fibronectin and uptake by cultured cells. The data indicate that sialylated glycans on *A. fumigatus* mediate both adhesion and uptake of this fungus by host cells.

Keywords: Sialic acid; *Aspergillus fumigatus*; phagocytosis; adhesion; high-performance liquid chromatography

This thesis is dedicated to
my wife, *Monica Gonzalez*;
my parents,
Zygmunt and Krystyna Warwas;
and
my brothers,
Adam and Thomas Warwas.

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LIST OF ABBREVIATIONS

ABPA	allergic bronchopulmonary aspergillosis
AIDS	acquired immune deficiency syndrome
ATCC	American Type Culture Collection
BSA	bovine serum albumin
BSM	bovine submaxillary mucin
CMP	cytidine 5`-monophosphate
DMB	1,2-diamino-4,5-methylenedioxybenzene dihydrochloride
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FCS	fetal calf serum
GFP	green fluorescent protein
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HSCT	hematopoietic stem cell transplant
IA	invasive aspergillosis
Kb	kilobase
KDN	2-keto-3-deoxy-nonulosonic acid
MAA	<i>Maakia amurensis</i> agglutinin
MALDI	matrix-assisted laser desorption ionization
Neu5Ac	N-acetylneuraminic acid
Neu5,9Ac ₂	9-O-acetyl-N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
PBS	phosphate-buffered saline (pH 7.4)
PBS-T	phosphate-buffered saline (pH 7.4)/0.05% Tween 20
PCR	polymerase chain reaction
PNA	<i>Arachis hypogaea</i> agglutinin
Siglec	sialic acid-binding immunoglobulin-like lectin
SNA	<i>Sambucus nigra</i> agglutinin
TOF	time of flight
UDP	uridine diphosphate

1 INTRODUCTION

1.1 Introduction to *Aspergillus fumigatus*

1.1.1 Biology of fungi and *Aspergillus fumigatus*

Fungi are eukaryotic, heterotrophic, non-phototrophic, spore-forming organisms that obtain nutrients by absorption through the plasma membrane. Most are saprophytes, obtaining nutrients from decomposing organic matter; however, some fungi are pathogenic. They are able to reproduce by sexual and/or asexual means. Fungi grow by producing branched filaments called hyphae with the entire mass of hyphae called the mycelium. The hyphae of some fungi are divided by walls called septa. These septa allow cytosol along with nutrients to flow between cells; however, these fungi are not considered multicellular organisms.

Fungi belong to the kingdom *Fungi* and are members of the domain *Eucarya*. The kingdom *Fungi* consists of a monophyletic group known as the eumycota or true fungi. Traditional fungal taxonomy classified fungi on the basis of their mode of reproduction. The major phyla based on this phenotypic classification were Zygomycota, Ascomycota, Basidiomycota and Deuteromycota. More recent molecular classification using 18S rRNA and examination of mitochondrial cristae has placed the members of Deuteromycota into either Ascomycota or Basidiomycota (Guarro *et al.*, 1999; Prescott *et al.*, 2002). Molecular classification using 18S rRNA has also revealed a more

ancient group of fungi, the Chytridiomycota. Water molds and slime molds resemble fungi in their appearance and life-style, however, based on molecular characterization they are phylogenetically distinct from the true fungi (Prescott *et al.*, 2002).

Zygomycota are terrestrial fungi, which grow as haploid mycelium spread by airborne sporangiospores. Strains are designated plus or minus and when a plus strain grows in close proximity to a minus strain they form projections called gametangia that fuse leading to the formation of a highly resistant zygospore, which, upon favourable conditions, undergoes meiosis and germinates into a haploid zygosporangium (Prescott *et al.*, 2002).

Sexual reproduction in Ascomycota begins with the formation of a sac-like ascus, which contains two or more haploid ascospores. Ascospores are formed when hyphal tips located on the same (homothallic) or different (heterothallic) thallus and their nuclei fuse to form a diploid zygote, which then undergoes meiosis resulting in the production of haploid ascospores. These ascospores are released from the ascus and germinate to produce haploid mycelia, which are able to reproduce asexually by forming conidiospores (Prescott *et al.*, 2002)

Basidiomycota form sexual spores called basidiospores that are produced by the basidium, a club-shaped structure produced at the hyphal tip.

Basidiospores germinate forming mycelium, which is then able to fuse forming dikaryotic mycelium. Pairs of nuclei within the dikaryon then fuse to form diploid mycelia, which immediately undergoes meiosis to produce four basidiospores.

Mature spores are eventually released into the atmosphere and germinate.

Finally, the Chytridiomycota are distinguished by their production of an asexual zoospore, which is motile by the way of a single posterior flagellum (Bauman *et al.*, 2004).

Fungi are extremely important organisms as they both benefit mankind and also represent a major cause of plant and human disease. They degrade a diverse variety of complex organic materials to release carbon, nitrogen, phosphorus and other components back into the environment making these nutrients available for use by other organisms. Fungi are important in the food industry, such as in the production of wine, beer, bread, cheeses and soy sauce. The commercial production of certain drugs and industrial materials, such as antibiotics and organic acids is also accomplished using fungi. Finally, fungi are important in research as they are used as models of fundamental biological processes; for example, the yeast *Saccharomyces cerevisiae* is often used as a model eukaryotic cell.

Fungi are important plant pathogens: 12/19 of the most threatening plant pathogens are fungi (Maor & Shirasu, 2005). In contrast, of the greater than 100,000 fungal species, relatively few have been implicated in human disease, and most of these cause superficial or subcutaneous mycoses (Bauman *et al.*, 2004). Even fewer species can cause systemic mycoses, which are fungal infections deep within the body that are not restricted to specific tissues or organs. These infections often begin in the lungs and then spread throughout the body. Human fungal pathogens can be divided into two types: primary pathogens and opportunistic pathogens. A primary pathogen is able to cause

disease in an immunocompetent individual. An example of a primary pathogen is *Histoplasma capsulatum*, which causes histoplasmosis in people exposed to contaminated soil as well as bird or bat guano (Woods, 2002; Woods, 2003). An opportunistic pathogen can only cause disease in hosts that are immunocompromised. *Candida albicans*, an opportunistic yeast pathogen, affects bone marrow and solid organ transplant patients (Trullas *et al.*, 2005), patients with hematological malignancies (Herbrecht *et al.*, 2005), HIV/AIDS patients (Ruhnke, 2004) and patients with chronic granulomatous disease (Rosenzweig & Holland, 2004). All of the above patient groups have some type of immunosuppression that makes them susceptible to *C. albicans*.

Opportunistic fungi have little effect on immunocompetent hosts, but can lead to life-threatening invasive mycoses in immunocompromised individuals (Prescott *et al.*, 2002). Of all the opportunistic fungal pathogens, the *Aspergillus* species are the most widely distributed in nature and are the major airborne fungi causing disease in immunocompromised patients (Shelton *et al.*, 2002; Herbarth *et al.*, 2003; Mezzari *et al.*, 2003; Cetinkaya *et al.*, 2005; Fang *et al.*, 2005). However, of the greater than 200 *Aspergillus* species that exist, only *A. fumigatus*, *A. flavus*, *A. terreus*, *A. niger* and *A. nidulans* are associated with disease (Minari *et al.*, 2002; Morgan *et al.*, 2005; Zander, 2005). Furthermore, even though it is present at lower amounts in the environment compared to other *Aspergillus* species (Shelton *et al.*, 2002), *A. fumigatus* is responsible for approximately 53-68% of all *Aspergillus* infections (Minari *et al.*, 2002; Morgan *et al.*, 2005; Pagano *et al.*, 2006). Therefore, the frequency of *A. fumigatus*

infections is unrelated to their prevalence in the environment suggesting that this organism possesses unique virulence factors.

Aspergillus fumigatus is a filamentous, saprophytic fungus classified in the phylum Ascomycota. It is found in soil and obtains nutrients from decomposing organic material, and, therefore, like many fungi, *A. fumigatus* plays an important role in the environment recycling carbon and nitrogen (Rhodes, 2006). *A. fumigatus* is found worldwide though, as stated above, it is not the most prevalent in nature (Shelton *et al.*, 2002). Reproduction occurs through the formation of asexual conidiospores and a sexual stage has not yet been identified. However, sequence data from the *A. fumigatus* genome sequencing project revealed the presence of genes involved in sexual reproduction (Nierman *et al.*, 2005) and recent studies have provided evidence for a sexual cycle (Paoletti *et al.*, 2005). Figure 1.1 shows the structure of *A. fumigatus* including the conidiophore, a specialized hypha that forms conidiogenic structures and the phialides, which produce chains of mitotic conidiospores. Each conidiophore can release thousands of conidiospores into the air and these conidiospores are the primary infectious agents (Latge, 1999). Once in the air, the small size (2-3 μm) of conidia allows them to remain airborne for extended periods and disperse in the environment (Latge, 1999). *A. fumigatus* is classified as a thermophile as it can grow at temperatures up to 55°C and is able to survive at temperatures up to 70°C (Greaves, 1975).

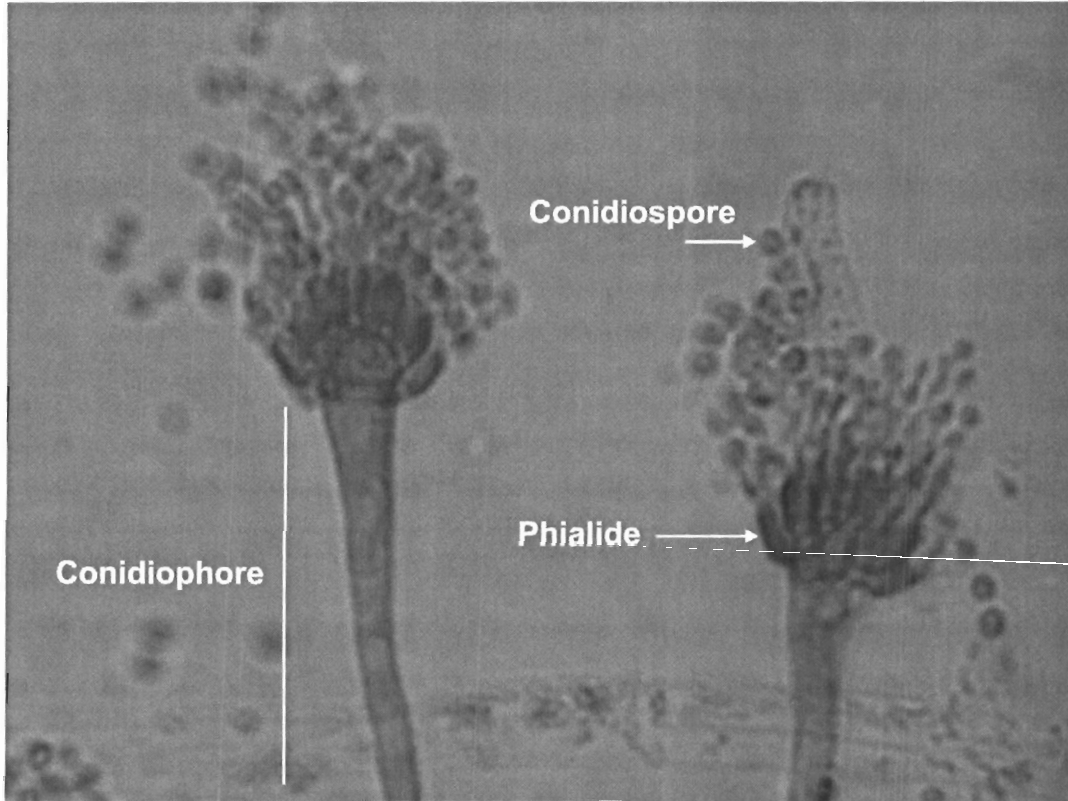


Figure 1.1: Light microscopic image of *Aspergillus fumigatus* courtesy of the Fungal Research Trust (www.fungalresearchtrust.org) with permission.

1.1.2 Diseases caused by *Aspergillus fumigatus*

A. fumigatus conidia are ubiquitously dispersed in the atmosphere. In fact, it is estimated that there are between 1 and 16 colony forming units per cubic meter of air (Leenders *et al.*, 1999). Therefore, the main portal of entry of *A. fumigatus* conidia is by inhalation into the respiratory tract. The small size of the conidia allow them to penetrate deep into the alveoli of the lung, which can lead to the development of several types of pulmonary *Aspergillus* infections (Hori *et al.*, 2002). *Aspergillus* infections may also occur at other sites, such as the skin (Klein & Blackwood, 2006), the eye (Callanan *et al.*, 2006), the gastrointestinal tract (Jayshree *et al.*, 2006) and bone (Mouas *et al.*, 2005), but

these are less frequent and will not be discussed further here. The type of disease caused by *A. fumigatus* is largely dependant on the immunological status of the host. *Aspergillus* infections are classified into three main types: 1) allergic diseases, such as asthma, allergic sinusitis, alveolitis and allergic bronchopulmonary aspergillosis (ABPA); 2) non-invasive disease, such as aspergilloma; and, 3) invasive disease, such as invasive aspergillosis (IA). Unlike asthma, allergic sinusitis and alveolitis, where removal of the patient from the source of allergy leads to recovery of the patient (Latge, 1999), ABPA, aspergilloma and invasive aspergillosis involve mycelial growth in the lung and medical treatment is required for improvement (Agarwal *et al.*, 2006; Demir *et al.*, 2006; Fluckiger *et al.*, 2006). These conditions are discussed in greater detail in the following paragraphs.

ABPA occurs in immunocompetent individuals whose polymorphonuclear leukocytes and alveolar macrophages actively participate in a heightened immune response to *Aspergillus*. ABPA begins with the inhalation of airborne conidia, which germinate into hyphae and mycelia in the mucus of the bronchi eventually penetrating the airway epithelial barrier. Upon penetration, *Aspergillus* antigens are released and are presented to T-cells, leading to the production of *A. fumigatus* CD4+ T-cells (Stevens *et al.*, 2003) and cytokines (Kauffman, 2003). In fact, animal models of ABPA have revealed that CD4+ T-cells are essential in airway hyperactivity in ABPA (Corry *et al.*, 1998). ABPA occurs in 5-22% of asthmatic patients (Eaton *et al.*, 2000; Agarwal *et al.*, 2006) and in 2-8% of cystic fibrosis patients (Geller *et al.*, 1999; Mastella *et al.*, 2000). In asthmatic

patients, ABPA onset usually occurs in young adults along with other allergic conditions and after the onset of asthma. In cystic fibrosis patients, ABPA is common in patients greater than 6-years of age and with higher microbial colonization and poor nutritional status (Tillie-Leblond & Tonnel, 2005). Ultimately, patients may develop a corticosteroid-dependent asthma phase, characterized by persistent and severe asthma followed by bronchiectasis and fibrosis (Tillie-Leblond & Tonnel, 2005). Treatment of ABPA is usually with antiinflammatory agents such as glucocorticosteroids which suppress the immune response (Greenberger, 2002). Antifungal agents such as itraconazole have been shown to be effective in asthmatic ABPA patients (Wark *et al.*, 2003); however, the efficacy of itraconazole in cystic fibrosis ABPA patients is not yet clear as there is a lack of studies in this group of patients. However, one small study found that in ABPA patients with cystic fibrosis, symptoms did improve after administration of itraconazole (de Almeida *et al.*, 2006).

Aspergilloma is another serious disease caused by *A. fumigatus* and occurs in immunocompetent individuals with pre-existing cavities in their lungs secondary to tuberculosis, sarcoidosis or in chronically obstructed paranasal passages. In 45-89% of aspergilloma patients, tuberculosis was found to be the underlying pulmonary disease (Park & Jheon, 2002; Akbari *et al.*, 2005). Aspergilloma consists of a mass of growing hyphae in the lung cavity with sporulating conidiophores at the surface of the mass. The cavity often becomes thickened with fibrotic tissue and because it has a rich blood supply, bleeding may occur (Franquet *et al.*, 2001). If blood vessel walls are breached by the

fungus, the resulting hemoptysis can be massive and fatal (Demir *et al.*, 2006). Aspergillomas may also occur when patients who became infected by *Aspergillus* during treatment with immunosuppressive agents recover and their anti-*Aspergillus* antibody titres increase. The aspergilloma may remain stable; however, if the individual's underlying disease relapses, and immunosuppression is re-instated or recurs, an invasive disease can result (Latge, 1999). Antifungal treatment seems to have little benefit likely because the drugs do not penetrate the fungal mass (Kawamura *et al.*, 2000). Hence, treatment for aspergilloma is usually surgical removal of the fungal mass (Corr, 2006) though this surgery is associated with a mortality rate of 0.9-3.3% (Park & Jheon, 2002; Akbari *et al.*, 2005).

Finally, invasive aspergillosis (IA) is the most serious systemic infection caused by *A. fumigatus*. IA has become a leading cause of death in immunocompromised individuals worldwide (Marr *et al.*, 2004). Individuals most at risk include acute leukemia patients after hematopoietic stem cell transplantation (HSCT) or cytotoxic treatment (Pagano *et al.*, 2006), solid organ transplant patients, especially lung transplantation (Minari *et al.*, 2002), patients with chronic granulomatous disease (Winkelstein *et al.*, 2000) and patients with HIV/AIDS (Markowitz *et al.*, 1996). There are four main types of IA: 1) acute and chronic aspergillosis, 2) tracheobronchitis and obstructive bronchial disease, 3) acute invasive rhinosinusitis; and 4) disseminated disease (Latge, 1999).

In acute aspergillosis, symptoms are non-specific and include fever, chest pain, weight loss and a general feeling of unwellness. Acute infection occurs in

the most severely immunosuppressed patients and the disease progresses rapidly (Denning, 1998). In chronic IA, the symptoms are prominent and occur in less severely immunosuppressed patients, such as in those with diabetes mellitus, chronic granulomatous disease, HIV/AIDS and alcoholic patients (Denning, 1998). Tracheobronchitis and obstructive bronchial disease is common in HIV/AIDS patients (Kemper *et al.*, 1993) and lung transplant patients (Kramer *et al.*, 1991). This form of IA is characterized by excess mucus production, inflammation and pseudomembrane production. Eventually, the airways become obstructed by fungal material and the disease can disseminate (Kemper *et al.*, 1993).

In acute invasive rhinosinusitis, *Aspergillus* invades sinus mucosa or surrounding bone; symptoms include neurological impairment and is associated with a high mortality rate (18%) (Parikh *et al.*, 2004). Finally, disseminated disease affects other organs and tissues, such as the brain, kidneys, central nervous system and the upper and lower gastrointestinal tract (Hori *et al.*, 2002). Symptoms of disseminated cerebral disease include neurological defects, seizures, coma and death (Hori *et al.*, 2002). Antifungal drugs are used to treat invasive aspergillosis; however, the current success rate of treatment is approximately 53% (Herbrecht *et al.*, 2002) with mortality ranging between 42% and 76% in HSCT patients (Cornet *et al.*, 2002; Morgan *et al.*, 2005) and between 50% and 84% in solid organ transplant recipients depending on the transplantation type (Minari *et al.*, 2002; Gavalda *et al.*, 2005). Spread of IA to the CNS is associated with very high mortality rates (100%) (Jantunen *et al.*,

2003; Trullas *et al.*, 2005). The diseases caused by *A. fumigatus* are summarized in Table 1.1.

Table 1.1: Incidence and mortality of diseases caused by *Aspergillus fumigatus*

Disease	Patient population	Incidence (%)	Mortality (%)	References
ABPA	Cystic fibrosis	2 - 8	-**	Geller, D., et al. 1999; Mastella, G., et al. 2000
	Asthma	5 - 22	-**	Eaton, T., et al. 2000; Agarwal, R., et al. 2006
Aspergilloma	Tuberculosis	45 - 89*	1 - 3	Akbari, J., et al. 2005; Park, C., et al. 2002
IA	Lung transplant	13 - 33	50 - 78	Minari, A., et al. 2002; Sole, A., et al. 2005
	Heart	1 - 2	66 - 67	Gavalda, J., et al. 2005; Morgan, J., et al. 2005
	Liver	0 - 2	67 - 84	Gavalda, J., et al. 2005; Morgan, J., et al. 2005
	Acute leukemia	8 - 14	39 - 60	Cornet, M., et al. 2002; Pagano, L., et al. 2006
	Allogenic HSCT	3 - 13	71 - 76	Cornet, M., et al. 2002; Morgan, J., et al. 2005
	Autologous HSCT	0 - 1	42 - 54	Cornet, M., et al. 2002; Morgan, J., et al. 2005
	HIV/AIDS	0 - 5	57	Cornet, M., et al. 2002; Markowitz, G., et al. 1996
	Chronic granulomatous disease	26 - 33	19	Winkelstein, J., et al. 2000; Liese, J., et al. 2000

* This rate does not represent an incidence of aspergilloma in tuberculosis cases, rather it is the percentage of patients with aspergilloma with tuberculosis as their underlying lung disease.
 **Mortality rates are difficult to establish in ABPA because it is difficult to distinguish between death due to cystic fibrosis or asthma and ABPA.

1.1.3 Treatment of invasive aspergillosis

Until recently, treatment of invasive aspergillosis was accomplished using monotherapy consisting of either amphotericin B deoxycholate or itraconazole (Marr *et al.*, 2004). Unfortunately, the mortality rate of therapy using amphotericin B as a first-line therapy ranges between 62% for leukemia/lymphoma patients undergoing chemotherapy and 92% in solid organ

transplant patients (Lin *et al.*, 2001) with 30% and 88% mortality in localized pulmonary infection and disseminated IA, respectively (Lin *et al.*, 2001).

Inadequate dosing and disruption of treatment due to nephrotoxicity, a common side effect of amphotericin B (Pathak *et al.*, 1998), decreases the efficacy of treatment (Wingard *et al.*, 1999). Lipid formulations of amphotericin B are better tolerated by patients; however, their effectiveness is equal to amphotericin B deoxycholate (Bowden *et al.*, 2002) even when administered at a higher dosage (Ellis *et al.*, 1998). The azole drug, itraconazole, when used as a first-line anti-*Aspergillus fumigatus* drug resulted in a response rate of between 39% (Denning *et al.*, 1994) and 48% (Caillot *et al.*, 2001), similar to the response rate observed for amphotericin B deoxycholate. Even though their mechanisms of action are different, the success rate achieved by combining amphotericin B with itraconazole is generally not improved over monotherapy with either drug (Steinbach & Stevens, 2003). Recently, newer antifungals have been developed and approved for treatment, such as the azole, voriconazole and the echinocandin, caspofungin. First-line treatment of patients with allogeneic HSCT, acute leukemia, or other hematologic diseases with voriconazole resulted in a 12-week survival rate of 71% versus 58% for patients treated with amphotericin B for the same period (Herbrecht *et al.*, 2002). Voriconazole is now the recommended first-line treatment for IA (Marr *et al.*, 2004). More recently, combination therapy using voriconazole and caspofungin was associated with increased 90-day survival and lower mortality in patients with hematologic malignancies or undergoing hematopoietic stem cell transplant compared to

patients treated with voriconazole alone (63% versus 32%) (Marr *et al.*, 2004). Similarly, an increased 90-day survival rate was observed with combination therapy of voriconazole and caspofungin in solid organ transplant patients compared to a liposomal amphotericin B treated control group (Singh *et al.*, 2006). Several other drugs are currently in clinical trials for the treatment of IA including the azoles, posaconazole and ravuconazole, as well as the echinocandins, anidulafungin and micafungin (Steinbach & Stevens, 2003). A recent study found that posaconazole used as salvage therapy when initial antifungal drug treatment was unsuccessful resulted in a 42% success rate (Kauffman, 2006). Micafungin was assessed as a primary therapy for IA as well as in patients who were unresponsive to prior antifungal therapy. It was revealed that treatment with micafungin resulted in a 60% response rate in both groups of patients (Kohno *et al.*, 2004). Although there have been recent improvements in the chemotherapy of IA, the mortality rate is still unacceptably high and we still do not know whether the new drug regimens will show significantly greater efficacy in the longer term (> 90 days). Further work must be conducted to better understand the properties of *A. fumigatus* that allow it to successfully colonize the immunosuppressed host.

1.1.4 Host defences against *Aspergillus*

The primary lines of defence against *Aspergillus* infection in the lungs are the alveolar macrophages, neutrophils and dendritic cells. Hence, disorders of phagocytic function, either genetic, such as in chronic granulomatous disease, or induced, such as in corticosteroid-induced immunosuppression, are risk factors

of invasive aspergillosis. In immunocompetent individuals, inhaled conidia that reach the lung are phagocytosed by macrophages before they have a chance to germinate (Philippe *et al.*, 2003). Killing of conidia by macrophages is accomplished by the acidification of the phagolysosome (Ibrahim-Granet *et al.*, 2003) as well as by the release of reactive oxygen intermediates (ROIs), which destroy the conidia (Philippe *et al.*, 2003). Killing of conidia by macrophages is very efficient: a study in our laboratory found that after 12 hours, greater than 99% of conidia coincubated with J774 cells (a murine macrophage cell line) either did not bind and were washed off or were killed by this cell line (Wasylnka & Moore, 2003). Although macrophages are able to efficiently kill conidia, some conidia evade killing by macrophages and rapidly begin to swell and germinate (Ibrahim-Granet *et al.*, 2003). Defense against hyphal invasion of the host is carried out by neutrophil recruitment and activation (Schaffner *et al.*, 1982). Since hyphae are too large to internalize, neutrophils damage hyphae extracellularly by binding to hyphae and releasing lysozyme, cationic peptides as well as through oxidative killing mechanisms (Washburn *et al.*, 1987).

In addition to directly killing *Aspergillus*, macrophages also activate host production of specific cytokines, including tumor necrosis factor alpha (TNF α), which is activated through Toll-like receptor signalling (Braedel *et al.*, 2004). In turn, these cytokines stimulate the production of chemokines, such as macrophage inflammatory protein-2 and macrophage inflammatory protein-1 α , which act to recruit neutrophils and monocytes to infected tissues thereby enhancing the host response against the pathogen (Mehrad *et al.*, 1999).

Furthermore, it is thought that in addition to aiding in neutrophil recruitment, TNF α also enhances the ability of these cells to kill *A. fumigatus* hyphae, likely by enhancing superoxide production (Roilides *et al.*, 1998).

Finally, dendritic cells (DCs), which are located in the respiratory tract, are also involved in first-line defense against *Aspergillus* by acting as both effectors and activators of the host immune response (Bellocchio *et al.*, 2005). Exposure of DCs to *Aspergillus* antigens induces their activation, maturation and migration to lymph nodes and the spleen where they induce T-cell responses against *Aspergillus* (Bozza *et al.*, 2002). These T-cell responses can either be Th1 or Th2 CD4 T-cell responses. Th1 responses are protective through the production of inflammatory cytokines, such as IFN- γ , IL-1, IL-2, IL-6, IL-12 and IL-18 as well as by the stimulation of effector macrophages and neutrophils. On the other hand, Th2 responses are associated with progressive infection through the depression of effector cell activity, by decreasing IFN- γ production and increasing levels of IL-4 and IL-10 (Cenci *et al.*, 1998; Cenci *et al.*, 1999; Del Sero *et al.*, 1999; Cenci *et al.*, 2001; Shoham & Levitz, 2005). Therefore, DCs represent an important link between the innate and adaptive immune response towards *Aspergillus*.

In conclusion, macrophages, neutrophils and DCs represent the primary immune defense mechanism against *Aspergillus*. Although humoral responses, such as the complement cascade, also participate in the immune response against this organism, the primary underlying condition in patients that develop IA

is phagocytic dysfunction thereby illustrating the importance of these cells in the immune response to *Aspergillus*.

1.1.5 Virulence factors of *A. fumigatus*

As stated above, *A. fumigatus* is present at lower levels in the environment than other fungi (Shelton *et al.*, 2002) yet this species is responsible for 53-68% of all *Aspergillus* infections (Minari *et al.*, 2002; Morgan *et al.*, 2005; Pagano *et al.*, 2006). Therefore, *A. fumigatus* must possess unique virulence factors that aid it in establishing infection in immunocompromised hosts. A virulence factor is essential for virulence; however, is not required for normal growth *in vitro* (Latge, 1999; Latge, 2001). To date, several genes have been identified that encode proteins implicated in the virulence of *A. fumigatus*. For an extensive review, refer to Rementeria *et al.*, 2005. Most of the genes implicated in *A. fumigatus* virulence are genes involved in obtaining or synthesizing essential nutrients. These genes include the following: *pabaA*, which encodes para-aminobenzoic acid synthetase responsible for catalyzing the final step in folate biosynthesis (Brown *et al.*, 2000); *pyrG*, which encodes orotidine-5'-phosphate decarboxylase important in the synthesis of uracil or uridine (D'Enfert *et al.*, 1996); *cpcA*, which encodes a transcriptional activator that regulates amino acid biosynthesis (Krappmann *et al.*, 2004); and, *lysF*, which encodes homoaconitase which is required in lysine biosynthesis (Liebmann *et al.*, 2004). Mutations in the above-described genes resulted in decreased virulence *in vivo*; however, without the addition of the compounds synthesized by the respective proteins or their pathways, *A. fumigatus* was unable to grow *in vitro*. For

example, the *pabaA* mutant of *A. fumigatus* was avirulent in the mouse model of IA; however, it required the addition of folate to the growth media in order to support growth (Brown *et al.*, 2000). Therefore, by definition, this gene is not a true virulence factor of *A. fumigatus*. Latgé has proposed that virulence in *A. fumigatus* is multifactorial and that true virulence factors do not exist (Latgé, 2001). Recently, Schrettl *et al.* (Schrettl *et al.*, 2004) and Hissen *et al.* (Hissen *et al.*, 2005) (refer to Appendix 1 for more detail) have shown that siderophore biosynthesis is essential for *A. fumigatus* virulence. Siderophore-mediated iron acquisition was found to be the primary mechanism used in *A. fumigatus* to acquire iron in vivo. It was also revealed that the *sidA*-deficient strain of *A. fumigatus* was still able to grow on media containing FeCl₃ (Hissen *et al.*, 2005), indicating that other iron assimilation pathways exist in *A. fumigatus*. Thus, *sidA* may represent a true virulence factor of *A. fumigatus*.

Many microbial pathogens are sialylated on their cell surface and it has been found that surface sialylation is important in microbial pathogenesis by aiding in host recognition or in the evasion of the host immune response (Smith *et al.*, 1995; Shinya *et al.*, 2006). Sialic acids have been found on other fungal pathogens as well as on *A. fumigatus*. Additionally, it was found that virulent strains of *A. fumigatus* are sialylated to a greater extent than non-virulent strains (Wasylnka *et al.*, 2001). Therefore, the objective of this thesis research is to establish the role of sialic acids in the virulence of *A. fumigatus*. A review of sialic acid structure and their involvement in microbial pathogenesis is presented in more detail below.

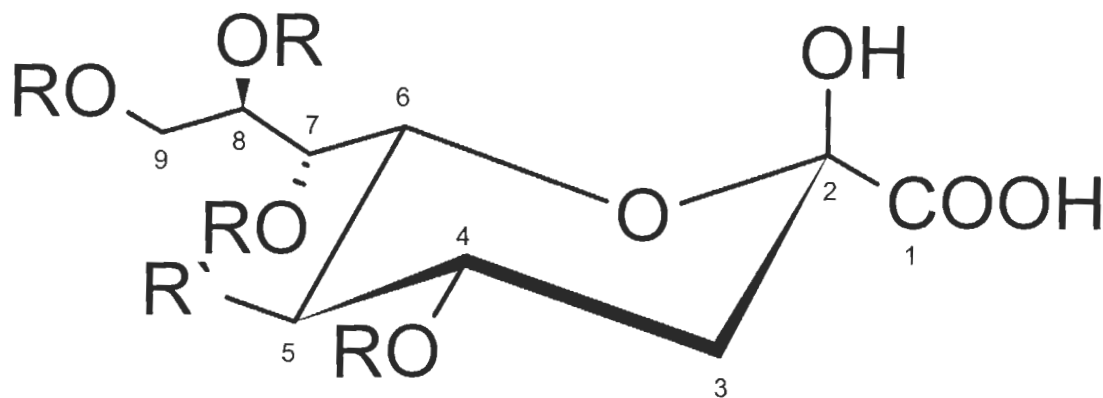
1.2 Introduction to sialic acids

1.2.1 The structure of sialic acids

Sialic acids are a family of greater than 50 substituted derivatives of a 9-carbon monosaccharide, neuraminic acid (Klein *et al.*, 1997). The expression of sialic acids occurs without exception in deuterostomes, and to a lesser extent in other life forms. Sialic acids occur in several Gram-negative bacteria, pathogenic fungi and protozoans. Sialic acid biosynthetic genes have also been predicted from DNA sequences in Gram-positive bacteria as well as in cyanobacteria, spirochetes and in an archaeal organism. To date, there have been no reports on the occurrence of sialic acids in plants (Angata & Varki, 2002).

Diversity in sialic acid structure is generated through substitutions at the hydroxyl and amino groups located on carbons 4, 5, 7, 8 and 9 (Fig. 1.2) (Angata & Varki, 2002; Schauer, 2004). The amino group on carbon 5 can be acetylated or glycolylated yielding N-acetylneuraminic acid (Neu5Ac) or N-glycolylneuraminic acid (Neu5Gc), respectively. Alternatively, carbon 5 can have a hydroxyl group instead of an amino group yielding 2-keto-3-deoxy-nonulosonic acid (KDN) (Nadano *et al.*, 1986). Uncommonly, the amino group at carbon 5 is unsubstituted yielding neuraminic acid (Manzi *et al.*, 1990). Structural diversity is further generated by substitutions at the hydroxyl groups located on carbons 4, 7, 8 and 9. The most common substitution is a single acetyl group, but it is not unusual to have di- and tri-O-acetylated sialic acids (Varki, 1992; Klein *et al.*, 1997). Furthermore, methyl, lactyl, sulfate and phosphoryl groups also occur (Varki, 1992). Acetyl groups most often occur at O-9; however, they are also

found at O-4, O-7 and O-8 (Klein *et al.*, 1997). Lactyl and phosphoryl groups occur at O-9 and methyl and sulphate groups are found at O-8 (Klein *et al.*, 1997). All of the above listed substitutions can be combined thereby yielding the multitude of sialic acid structures known to exist in nature (Klein *et al.*, 1997). In addition to substituents at various hydroxyl and amino groups, anhydro and lactone forms have been identified in biological samples (Kamerling & Vliegthart, 1975; Mitsuoka *et al.*, 1999; Cebo *et al.*, 2001). With the exception of unsaturated forms (Kamerling & Vliegthart, 1975) and 9-O-phosphorylated Neu5Ac, which occurs as a biosynthetic intermediate (Lawrence *et al.*, 2000), sialic acids occur glycosidically linked in glycoproteins and glycolipids and are often positioned as terminal monosaccharides in these glycoconjugates (Schauer, 2004). Finally, sialic acids can be linked by sialyltransferases to glycoconjugates in α 2,3 or α 2,6 linkages to galactose, in α 2,6 linkages to N-acetylgalactosamine, or in α 2,8 or α 2,9 linkages to another sialic acid thereby further increasing the diversity of sialic acid structures in nature (Harduin-Lepers *et al.*, 2001).



R = H-, Ac-, Lt-, Me-, P-, S-

R' = AcNH-, GcNH-, HO-, NH₂-

Figure 1.2: Structural diversity in the sialic acids.

The sialic acid family consists of over 50 members. Carbon 5 can be substituted with an N-acetyl group (AcNH-) yielding N-acetylneuraminic acid, an N-glycolyl group (GcNH-) yielding N-glycolylneuraminic acid, an unsubstituted amino group (NH₂-) yielding neuraminic acid, or the amino group can be replaced with a hydroxyl (HO-) generating KDN. Further diversity is generated by substitutions at the hydroxyl groups located on carbons 4, 7, 8 and 9. These hydroxyls can be unsubstituted or can be substituted by acetyl groups (Ac-), lactyl groups (Lt-), methyl groups (Me-), sulphate groups (S-), or phosphate groups (P-). One or more of these substitutions can be combined in a single molecule thereby yielding the great diversity of sialic acid structures.

Because few studies are available on fungal sialic acid biosynthesis, we base our studies on mammalian sialic acid biosynthesis. Mammalian sialic acid biosynthesis begins with conversion of uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) to N-acetylmannosamine (ManNAc) by the enzyme UDP-GlcNAc 2-epimerase (Fig. 1.3), and then ManNAc is phosphorylated to ManNAc-6-phosphate by the kinase activity of the bifunctional UDP-GlcNAc 2-epimerase (Hinderlich *et al.*, 1997; Stasche *et al.*, 1997). These first two steps of sialic acid biosynthesis are the rate limiting steps of the pathway. The next step is the condensation reaction catalyzed by Neu5Ac-9-phosphate synthetase between

the 6-carbon ManNAc-6-phosphate and the 3-carbon phosphoenolpyruvate (PEP) to yield the 9-carbon Neu5Ac-9-phosphate (Lawrence *et al.*, 2000; Nakata *et al.*, 2000). Neu5Ac-9-phosphate is then dephosphorylated by Neu5Ac-9-phosphate phosphatase to yield Neu5Ac; however, the phosphatase responsible for this reaction has not yet been identified (Angata & Varki, 2002). Neu5Ac is then the substrate for CMP-Neu5Ac synthetase, an enzyme localized in the nucleus which converts Neu5Ac to the sugar nucleotide donor form, CMP-Neu5Ac (Munster *et al.*, 1998). CMP-activation is required for the following reasons: the hydrolysis of the nucleotide-sugar releases the free energy required for the conjugation reaction; sialyltransferases that incorporate sugars into glycans are localized in the Golgi and only CMP-Neu5Ac, but not free Neu5Ac, is transported into the Golgi via nucleotide-sugar transporters in exchange for free nucleosides (Eckhardt *et al.*, 1996); CMP-sialic acid is the substrate for the sialic acid modifying enzyme, CMP-Neu5Ac hydroxylase, which generates Neu5Gc; and, CMP-sialic acid in the cytosol acts as a negative inhibitor for UDP-GlcNAc 2-epimerase thereby regulating sialic acid biosynthesis (Munster *et al.*, 1998). Once in the Golgi lumen, CMP-Neu5Ac is incorporated into glycoconjugates by sialyltransferases (Harduin-Lepers *et al.*, 2001).

To date, the gene encoding CMP-Neu5Ac hydroxylase is the only gene to be cloned that is involved in sialic acid modification in mammals. CMP-Neu5Gc is synthesized from CMP-Neu5Ac in the cytosol by the action of CMP-Neu5Ac hydroxylase (Kawano *et al.*, 1995). This gene is present, but non-functional, in humans, hence, Neu5Gc is not synthesized in humans (Muchmore *et al.*, 1998).

Acetylations, methylations, lactylations, sulfations and phosphorylations are presumed to occur in the Golgi lumen after incorporation of the sialic acid into the glycoconjugate, but, to date, only O-acetyltransferase *activity* has been identified in mammals, and no genes have been cloned for these enzymes (Diaz *et al.*, 1989; Butor *et al.*, 1993). Therefore, further work is needed to characterize the genes and enzymes responsible for mammalian sialic acid modification.

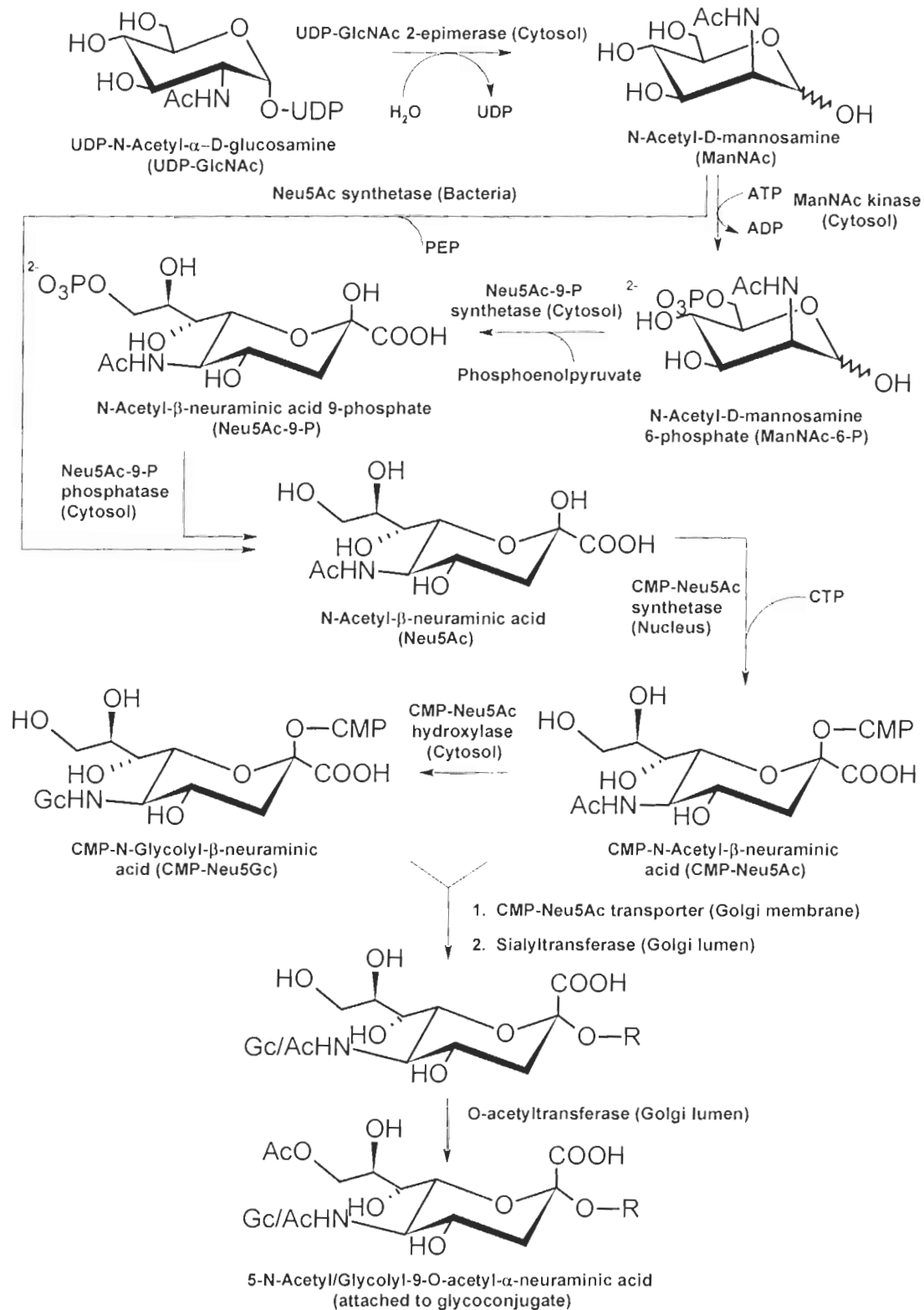


Figure 1.3: Sialic acid biosynthesis in mammals and bacteria.

The cellular localization of enzymes in mammals is noted in parenthesis. Modified from Tanner, 2005 (Tanner, 2005) and Vimr *et al.* 2004 (Vimr *et al.*, 2004).

Several bacterial species also possess sialic acids on their surface. Three mechanisms are employed by bacteria to obtain sialic acid: these include *de novo* biosynthesis, donor scavenging, and precursor scavenging. *De novo* sialic acid biosynthesis is accomplished by the sialic acid biosynthetic enzymes encoded by the *neuCBASEOD* gene cluster (Table 1.2). Biosynthesis in bacteria is similar to that of mammalian sialic acid biosynthesis with some exceptions. GlcNAc 2-epimerase synthesizes ManNAc without phosphorylation of ManNAc to ManNAc-6-P and ManNAc is directly used as the substrate for Neu5Ac synthetase yielding Neu5Ac (Vann *et al.*, 1997; Vann *et al.*, 2004). Neu5Ac is then activated to its sugar-nucleotide form by CMP-Neu5Ac synthetase (Zapata *et al.*, 1989; Tullius *et al.*, 1996) and transferred to glycoconjugates by sialyltransferase enzymes (Steenbergen & Vimr, 1990; Gilbert *et al.*, 2000). Recently, in Group B *Streptococcus*, the *neuD* gene was found to encode an O-acetyltransferase, which is responsible for the O-acetylation of Neu5Ac (Lewis *et al.*, 2006). This represents the first identification of a gene involved in modifying sialic acid hydroxyl groups located on carbons 4,7,8 and 9. Bioinformatic analysis in the same study identified *neuD* homologues in the sialic acid biosynthetic gene clusters of several other bacteria, among these are *Campylobacter jejuni* and *Pseudomonas aeruginosa*. Additionally, the *neuO* gene in *E. coli* K1 has been found to encode an O-acetyltransferase important in the O-acetylation of polymeric sialic acid (Steenbergen *et al.*, 2006).

Table 1.2: Sialic acid biosynthetic genes in bacteria

Gene	Enzyme encoded	Bacterial species	References
<i>neuC</i>	UDP-GlcNAc 2-epimerase	<i>E. coli</i> K1	Vann,W., <i>et al.</i> 2004
<i>neuB</i>	Neu5Ac synthetase	<i>E. coli</i> K1	Vann,W., <i>et al.</i> 1997
<i>neuA</i>	CMP-Neu5Ac synthetase	<i>E. coli</i> K1; <i>Haemophilus ducreyi</i>	Zapata,G., <i>et al.</i> 1989; Tullius, M., <i>et al.</i> 1996
<i>neuS</i>	sialyltransferase	<i>E. coli</i> K1; <i>Campylobacter jejuni</i>	Steenbergen,S., <i>et al.</i> 1990; Gilbert,M., <i>et al.</i> 2000
<i>neuE</i>	unknown	-	-
<i>neuO</i>	O-acetyltransferase for poly sialic acid	<i>E. coli</i> K1	Steenbergen,S., <i>et al.</i> 2006
<i>neuD</i>	O-acetyltransferase	Group B <i>Streptococcus</i>	Lewis,A., <i>et al.</i> 2006

Donor scavenging is employed by several *Neisseria gonorrhoea* strains. *N. gonorrhoea* does not have sialic acid biosynthetic enzymes, but expresses a sialyltransferase (NeuS) that is able to bind free CMP-Neu5Ac present in small amounts in human secretions and transfer it to an oligosaccharide (Smith *et al.*, 1995; Vimr *et al.*, 2004).

Precursor scavenging is another method used to sialylate the cell surface of bacteria (Vimr *et al.*, 2004). This has been demonstrated in *Haemophilus influenzae*, which expresses only CMP-Neu5Ac synthetase (NeuA) and sialyltransferases (Bouchet *et al.*, 2003; Jurgisek *et al.*, 2005). *Haemophilus influenzae* uses free Neu5Ac from the host by importing it through a tripartite ATP-independent periplasmic transporter system (Severi *et al.*, 2005) and then activating it to its nucleotide-sugar donor form using NeuA. CMP-Neu5Ac is then transferred to the *H. influenzae* lipo-oligosaccharide by sialyltransferases (Jurgisek *et al.*, 2005).

KDN is a structurally related α -ketoacid found in vertebrates, such as the rainbow trout (Nadano *et al.*, 1986) and in some bacteria (Knirel *et al.*, 1989; Gil-Serrano *et al.*, 1998). KDN is synthesized through the condensation of mannose-6-phosphate (Man-6-phosphate) with PEP catalyzed by KDN-9-phosphate synthetase, which is specific for Man-6-phosphate and not ManNAc-6-phosphate (Angata *et al.*, 1999). KDN-9-phosphate is then dephosphorylated by KDN phosphatase to yield KDN, which can then undergo nucleotide activation, transport into the Golgi, incorporation into glycoconjugates and modification in a similar fashion as Neu5Ac (Angata *et al.*, 1999). The source of Man-6-P for KDN biosynthesis has been debated, but it is now clear that free Man is phosphorylated by 6-O-phosphokinase to yield Man-6-phosphate, which is then utilized for glycoconjugate biosynthesis (Alton *et al.*, 1998).

1.2.2 Sialic acids in microbial pathogenesis

Because sialic acids occupy the terminal position in glycoconjugates and are negatively charged at physiological pH, they confer a negative charge to the cell surface (Varki, 1997; Schauer, 2004). These properties allow sialic acids to play important and diverse roles in many biological processes. For example, Stocker & Bennett, 2006 have shown that the regulation of voltage-gated Na⁺ and K⁺ channels is mediated by the negative charge conferred to the cell surface by sialic acid. Sialic acids have also been implicated in the initial extravasion of metastatic tumor cells from a primary tumor, and subsequently, in the specific adherence of these cells to a secondary site (Lin *et al.*, 2002). Sialic acids are important in immune system modulation via interactions with sialic acid-binding

immunoglobulin-like lectins (siglecs) (Crocker, 2005). Finally, the adherence and invasion of host cells as well as immune evasion by diverse microbes has been shown to involve sialic acids. These interactions are described in more detail below.

1.2.2.1 Viruses

Sialic acids play important roles in the pathogenesis of many microbes including viruses, bacteria, protozoa and fungi. Viruses are not themselves sialylated, but specifically bind to sialylated receptors on the host cell surface using viral hemagglutinin (Olofsson & Bergstrom, 2005; Shinya *et al.*, 2006). The binding of influenza A and B viruses to host cells is accomplished by the specific recognition of the host sialic acid linkage by the virus (Suzuki *et al.*, 1985, 1986, 1992, and 2005). Recognition of specific sialic acid linkages can influence viral host range. For example, human influenza A viruses bind α 2,6-linked sialic acids while avian influenza A viruses bind α 2,3-linked sialic acids (Shinya & Kawaoka, 2006). Because avian influenza viruses recognize α 2,3-linked sialic acid and since cells expressing these structures are found primarily in the lower respiratory tract, to replicate efficiently, the virus particle must penetrate deep into the alveoli where this linkage is expressed. This occurs infrequently; therefore, infection of human hosts by the current avian influenza viral strains rarely occurs. Human viruses, on the other hand, recognize α 2,6-linked sialic acids located in the epithelia of the upper respiratory tract. Thus, these viruses are easily spread by coughing and sneezing (Shinya *et al.*, 2006).

In addition to recognizing the sialic acid linkage, viruses belonging to the family *Orthomyxoviridae* and *Coronaviridae* are able to specifically bind O-acetylated sialic acids (Strasser *et al.*, 2004). Viruses recognizing 9-O-acetylated sialic acids include the following: Influenza C, human coronavirus, bovine coronavirus, swine hemagglutinating encephalomyelitis virus and porcine torovirus (Rogers *et al.*, 1986; Vlasak *et al.*, 1988; Schultze *et al.*, 1991; Smits *et al.*, 2005). Viruses recognizing 4-O-acetylated sialic acids include infectious salmon anemia virus, mouse hepatitis virus, puffinosis coronavirus, and sialodacryoadenitis virus (Wurzer *et al.*, 2002; Hellebo *et al.*, 2004; Strasser *et al.*, 2004). Finally, the bovine torovirus recognizes di-O-acetylated sialic acid on host cells (Smits *et al.*, 2005). Binding of these viruses to host O-acetylated sialic acids via viral hemagglutinin has been shown to be important though not absolutely required for infection. Specifically, some mouse hepatitis virus strains that do not express hemagglutinin-esterase were able to infect mouse fibroblast cells *in vitro* by recognizing host glycoprotein S receptor via their viral glycoprotein S (Gagneten *et al.*, 1995). It was proposed that receptors recognizing O-acetylated sialic acids may serve to modulate tissue tropism or may act as alternate receptors in cells devoid of the primary receptors (Gagneten *et al.*, 1995; Regl *et al.*, 1999). Finally, in addition to mediating host cell surface binding and host specificity, recognition of host sialic acid linkages and O-acetylations is important in viral replication. These structures are recognized and cleaved by viral sialidases and O-acetylestherases thereby preventing viral recapture by host sialic acids. Therefore, this plays a crucial role in the viral life

cycle by allowing the newly formed viral particle to be released from the cell surface (Palese *et al.*, 1974; Suzuki, 2005; Ohuchi *et al.*, 2006).

1.2.2.2 Bacteria

Unlike viruses, several species of bacteria have sialic acids on their cell surface. Bacterial cell surface sialic acids have been shown to play important roles in pathogenesis. One role is the protection of the bacterium from the host immune response by inhibiting the alternate complement pathway. Factor H is a glycoprotein that functions in the negative regulation of the alternate complement pathway by binding C3b and converting it to inactive C3b (Ram *et al.*, 1998). The interaction between factor H and C3b leading to complement inhibition is enhanced by sialylated cells thereby preventing destruction of self cells. For example, *Neisseria gonorrhoea* contains sialylated lipo-polysaccharide, which results in enhanced binding of Factor H to C3b ultimately leading to an inhibition of the alternate complement pathway (Ram *et al.*, 1998). Another role for sialic acids in bacterial pathogenesis is the adhesion of the bacteria to host cells. Several studies have shown that adherence of bacterial cells to host cells is mediated by bacterial cell surface receptors that recognize host cell sialic acids rather like the recognition by viruses of sialylated host cell receptors (Sakarya & Oncu, 2003). Sialic acid-binding motifs have been identified in the adhesins of *E. coli* and *Helicobacter pylori* (Jacobs *et al.*, 1986; Evans *et al.*, 1993). Sialic acids have also been implicated in the formation of biofilms of non-typeable *Haemophilus influenzae* (Jurcisek *et al.*, 2005). Biofilms play important roles in pathogenesis by providing protection from toxins and from the host immune

response as well as in obtaining nutrients (Sutherland, 2001; Dunne, 2002). Finally, sialic acids have been implicated in the development of autoimmune syndromes secondary to bacterial infection. For example, the structural similarity between the sialylated lipo-oligosaccharide of *Campylobacter jejuni* and ganglioside oligosaccharides in humans is thought to induce the expression of cross-reactive anti-ganglioside antibodies, which then attack host peripheral nervous tissue leading to paralysis (Yuki *et al.*, 2004).

1.2.2.3 Protozoans

Sialic acids are also widespread and important in the pathogenesis of protozoan parasites. Neu5Ac has been found on the surface of *Trypanosoma cruzi* (Souto-Padron *et al.*, 1984), *T. brucei* (Engstler *et al.*, 1993), *T. congolense* (Mutharia & Steele, 1995), *Entamoeba histolytica* (Chayen *et al.*, 1988), *E. invadens* (Ribeiro *et al.*, 1989), *Trichomonas vaginalis* (Dias Filho *et al.*, 1992), *T. foetus* (Dias Filho *et al.*, 1988) and *Toxoplasma gondii* (Gross *et al.*, 1993). In addition to Neu5Ac, Neu5,9Ac₂ has been detected on the cell surface of *Leishmania donovani* (Chatterjee *et al.*, 2003) and *Crithidia fasciculata* (do Valle Matta *et al.*, 1999). Finally, although *Plasmodium falciparum* and *P. reichenowi* are not sialylated, they recognize host cell surface sialic acids and use them to invade host cells (Martin *et al.*, 2005).

Sialic acids are acquired through three main mechanisms in protozoa. First, *T. cruzi*, *T. brucei* and *T. congolense*, possess a trans-sialidase enzyme (Martin *et al.*, 2005), a modified sialidase that is able to transfer a host sialic acid residue to a galactose instead of H₂O (Buschiazzo *et al.*, 2000). This results in

the transfer of the sialic acid from the host cell surface to the surface of the parasite. In *T. gondii* and *L. donovani*, neither trans-sialidase activity nor sialic acid biosynthetic activity have been detected (Odenthal-Schnittler *et al.*, 1993; Chatterjee *et al.*, 2003; Chava *et al.*, 2004). Sialic acids on the surface of these organisms are presumed to be obtained through adsorption, likely from the host extracellular environment. In *L. donovani* binding of sialic acid-specific lectins to the parasite surface decreased when this organism was cultured in decreasing concentrations of sialic acid-containing fetal calf serum (Chatterjee *et al.*, 2003). Finally, *E. histolytica* (Chayen *et al.*, 1988) and *C. fasciulate* (do Valle Matta *et al.*, 1999) obtain their sialic acids through *de novo* biosynthesis, which was demonstrated from metabolic labelling experiments using H³-glucosamine, a precursor to the sialic acid biosynthetic pathway.

The role of sialic acids in protozoans is best characterized in the *Trypanosomes*. Sialic acids acquired by trans-sialidase form a negatively charged coat that protects the parasite from recognition by human anti-galactosyl antibody-mediated cytolysis by masking exposed galactose residues located on O-linked oligosaccharides on the trypanosomal surface (Pereira-Chioccola *et al.*, 2000). Trans-sialidase-mediated removal of cell surface sialic acids from host immune cells located in the spleen, thymus, and peripheral ganglia can induce apoptosis in those cells thereby weakening the host immune response, and, consequently, indirectly promoting the evasion of host defences by the parasite (Leguizamon *et al.*, 1999). It has been proposed that platelets could induce parasite lysis (Umekita & Mota, 1989) and enhance parasite clearance in vivo

(Tribulatti *et al.*, 2005). Trans-sialidase has been shown to mark platelets for clearance by removing their surface sialic acids resulting in thrombocytopenia (Tribulatti *et al.*, 2005). Lowering the platelet count by removing surface sialic acids represents a further mechanism of immune evasion.

P. falciparum and *P. reichenowi* are genetically and morphologically very similar, but their recognition of sialic acids drastically alters their host specificity. Both protozoan parasites bind and invade host cells using their respective erythrocyte-binding antigen 175 (EBA-175) proteins. *P. falciparum* EBA-175 recognizes Neu5Ac specifically (and its binding is inhibited in the presence of Neu5Gc), and *P. reichenowi* EBA-175 recognizes Neu5Gc exclusively. Therefore, since humans express only Neu5Ac while chimps and great apes express both Neu5Ac and Neu5Gc, humans are infected with *P. falciparum* and not *P. reichenowi* whereas chimps and apes are susceptible to *P. reichenowi* (Martin *et al.*, 2005).

1.2.2.4 Fungi

Several fungal species have also been found to express sialic acids on their cell surface. These include *Fonsecaea pedrosoi* (Souza *et al.*, 1986; Alviano *et al.*, 2004), *Cryptococcus neoformans* (Rodrigues *et al.*, 1997), *Paracoccidioides brasiliensis* (Soares *et al.*, 1993; Soares *et al.*, 1998), *Candida albicans* (Soares *et al.*, 2000), *Sporothrix schenckii* (Alviano *et al.*, 1982; Oda *et al.*, 1983), *Pneumocystis carinii* (De Stefano *et al.*, 1990) and several *Aspergillus* species including *Aspergillus fumigatus* (Wasylnka *et al.*, 2001). Neu5Ac was the most common sialic acid derivative encountered; however, Neu5,9Ac₂ was identified on the surface on *C. neoformans* and *F. pedrosoi* (Rodrigues *et al.*, 1997; Alviano *et al.*, 2004). Although sialic acids have been detected on the surface of fungal cells, the biosynthetic machinery responsible for this expression has not yet been identified. In *P. brasiliensis*, *C. neoformans*, *F. pedrosoi*, and *A. fumigatus* sialic acids were detected even when these organisms were grown in chemically-defined medium lacking sialic acid sources, indicating that sialic acids were synthesised *de novo*. CMP-Neu5Ac sialyltransferase activity was demonstrated in *C. neoformans* representing, for the first time, evidence for the existence of sialylation machinery in fungi (Rodrigues *et al.*, 2002).

It has been proposed that sialic acids in fungi play a role in the protection of fungal cells against phagocytosis by the host immune system. Phagocytes have receptors for galactose or N-acetylgalactose residues, which are usually the sub-terminal monosaccharides to which sialic acids are linked (Linehan *et al.*, 2000). Removal of cell surface sialic acids from *S. schenckii* and *C. neoformans*

using sialidase resulted in increased phagocytosis of the sialidase-treated cells by mouse peritoneal macrophages (Oda *et al.*, 1983; Rodrigues *et al.*, 1997). Additionally, sialidase treatment of *F. pedrosoi* conidia resulted in a greater number of interactions between conidia and neutrophils (Alviano *et al.*, 2004). For *C. neoformans* and *F. pedrosoi*, it was proposed that sialic acids may protect the organism during the early stages of infection until the organism can reach a mature and highly-resistant stage. In *C. neoformans*, a highly-resistant stage is achieved by synthesizing a capsular polysaccharide, and, in *F. pedrosoi*, this is achieved by differentiating into sclerotic cells (Rodrigues *et al.*, 1997; Alviano *et al.*, 2004). Interestingly, sialic acids were not detected on the surface of sclerotic cells, further suggesting that, in *F. pedrosoi*, sialic acids play a role in immune evasion early in infection until the organism has a chance to adapt and differentiate into a more mature and fully resistant form (Alviano *et al.*, 2004).

1.3 Aims of current research

Given the potential importance of sialic acids and their modifications in fungal pathogenesis, the specific aims of the current research were as follows: 1) to characterize the linkage, sub-terminal monosaccharide and structure of terminal sialic acids on the surface of *A. fumigatus* conidia, 2) to determine whether sialic acids on the surface of *A. fumigatus* conidia are important in the binding of conidia to the extracellular matrix protein, fibronectin, 3) to establish whether sialic acids reduce the extent of phagocytosis by cultured mouse macrophages and by cultured human Type II pneumocytes, and 4) to generate a sialic acid-deficient mutant strain of *A. fumigatus* and compare its virulence to

wild type and rescue strains in a mouse model of IA. To analyze the linkage and sub-terminal sugar, sialic acids were released from conidia using mild acid or enzymatic hydrolysis and analyzed by lectin binding. Released sialic acids were derivatized and their structure determined by reverse phase high-pressure liquid chromatography (HPLC) and matrix-assisted laser desorption ionization (MALDI) mass spectroscopy. The importance of sialic acids in the binding to fibronectin was assessed by measuring the binding of sialidase-treated or untreated conidia to fibronectin-coated wells. The importance of sialic acids in phagocytosis was assessed using fluorescence microscopy. Finally, a mutant strain of *A. fumigatus* was constructed by insertional disruption of the gene encoding the putative CMP-sialic acid transporter.

2 THE STRUCTURE OF SIALIC ACIDS OF *ASPERGILLUS FUMIGATUS* CONIDIOSPORES

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2.1 Introduction

Previous work in our laboratory identified negatively-charged carbohydrates on the surface of *A. fumigatus* conidia that were involved in adhesion to basal lamina proteins (Wasylnka & Moore, 2000). Subsequent analysis by lectin binding, thin layer chromatography and mass spectroscopy revealed that *A. fumigatus* possessed sialic acids on the conidial and hyphal surfaces. Hence, this was indirect evidence that sialic acids may mediate adhesion to host proteins. Neu5Ac was the only sialic acid species identified, and it was found that conidia did not bind to the sialic acid-specific lectins, *Maackia amurensis* agglutinin (MAA) or *Sambucus nigra* agglutinin (SNA), which recognize α 2,3 and α 2,6-linked sialic acids, respectively (Shibuya *et al.*, 1987; Knibbs *et al.*, 1991). This lack of reactivity with SNA and MAA suggested that Neu5Ac was not present in either α 2,6 or α 2,3 linkages (Wasylnka *et al.*, 2001). The absence of O-acetylated sialic acids was surprising given that these derivatives have been found in other fungal genera (Alviano *et al.*, 1982; Soares *et al.*, 1993; Rodrigues *et al.*, 1997; Soares *et al.*, 2000; Alviano *et al.*, 2004).

However, the absence of these derivatives might have been a consequence of the original conditions used to isolate sialic acids from *A. fumigatus*. In earlier studies, strongly acidic conditions were used to release sialic acids from the conidial surface. In addition, subsequent purification steps utilized strong base to adjust pH and used a strongly basic anion exchange resin to purify sialic acids from the crude acid hydrolysate. Both of these processes are known to release labile O-acetyl groups from sialic acids (Varki & Diaz, 1984). Furthermore, hydrolysis was carried out for shorter periods of time at a lower temperature than the current study, which may not have been enough time to release O-acetyl derivatives from conidia, as these have been found to require longer hydrolysis times and higher temperatures for release (Varki & Diaz, 1984). Finally, the absence of modified sialic acids may have been a result of the relatively low sensitivity of the techniques utilized to detect sialic acid derivatives, which may be present in low amounts (Hara *et al.*, 1989). Given the occurrence of Neu5,9Ac₂ on the cell surface of other fungi (Rodrigues *et al.*, 1997; Alviano *et al.*, 2004) and the importance of sialic acids and their modifications in the pathogenesis of other microbes including fungi, the goal of this study was to determine the structure of sialic acids on terminal glycans of *A. fumigatus*.

The aim of the research presented in this chapter was to characterize the linkage, underlying monosaccharide and structure of terminal sialic acids released from the surface of *A. fumigatus* conidia. To characterize the sialic acid linkage and to identify the underlying monosaccharide in *A. fumigatus*, lectin binding analysis was performed using carbohydrate-specific lectins. To establish

the structure of *A. fumigatus* sialic acids, sialic acids were released from conidia using mild acid, covalently derivatized with a sialic acid-specific fluorescent compound and analysed by reverse phase high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization (MALDI) time of flight (TOF) mass spectroscopy.

2.2 Materials and methods

***A. fumigatus* strains and growth conditions**

A. fumigatus strain ATCC 13073 (American Type Culture Collection, Manassas, VA) was used in this study. Fungi were grown on MYPD agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 0.5% dextrose) for 3 days at 37°C or 5 days at 28°C until conidia were fully mature. Conidia were harvested by adding phosphate-buffered saline, pH 7.4/0.05% Tween 20 (PBS-T) directly onto the plate and scraping with a sterile cotton swab until conidia were in suspension. The conidial suspension was then vortexed vigorously to break apart chains of conidia and filtered through sterile glass wool to remove hyphae. Conidia were then washed three times with phosphate-buffered saline (PBS), resuspended in PBS and counted with a haemocytometer.

Sialidase-treatment of *A. fumigatus* conidia

Harvested *A. fumigatus* conidia (1×10^8) were washed once with 16 mM sodium tartrate buffer, pH 5.2, and resuspended in 25 μ l of 16 mM sodium tartrate buffer supplemented with 0.1% bovine serum albumin (BSA) (Sigma, Oakville, ON). *Micromonospora viridifaciens* sialidase (74 μ g) (a generous gift

from Dr. J. Watson and Dr. A. Bennet, Department of Chemistry, Simon Fraser University) was added to conidia and allowed to incubate between 2 and 3 hours at 50°C. After sialidase treatment, conidia were washed three times with PBS and stored at 4°C overnight until analysis. The supernatant was clarified by centrifugation at 3000 times g for 5 minutes and filtered through a 10000 molecular weight cut-off filter (Millipore, Billerica, MA) and stored at -20°C for further analysis.

Mild acid hydrolysis of *A. fumigatus* conidia to release surface sialic acids

A. fumigatus conidia (1×10^{11}) were washed twice with sterile water and 1×10^{10} conidia were added to 50 ml plastic tubes. To each tube, 35 ml of 2.5 M acetic acid was added and suspension was incubated for 5.5 hours at 90°C with shaking by hand every half hour. After incubation, tubes were immediately cooled on ice, clarified by centrifugation at 3000 times g for 10 minutes and lyophilized. Dried hydrolysates were resuspended in 500 µl sterile water, filtered through a 10000 molecular weight cut-off filter and stored at -20°C.

Lectin binding assays

To assess the extent of removal of surface sialic acids by sialidase treatment or mild acid treatment, lectin binding was used. Conidia were incubated with biotinylated lectins and the extent of binding was determined by fluorescence microscopy and/or flow cytometry. *Arachis hypogaea* agglutinin (PNA), *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA) (all from Sigma) were biotinylated by incubating 1 mg of lectin (in 1 ml

PBS) with 300 µg of N-hydroxysuccinimidobiotin (long chain (Pierce Biotechnology, Inc., Rockford, IL) in 15 µl dimethylformamide) for 1 hour at room temperature. Biotinylated lectins (for simplicity, from this point forward biotinylated lectins will be referred to as SNA, MAA and PNA or lectins) were then dialysed against PBS in 5000 molecular weight cut-off centrifugal filters (Millipore) and resuspended in 500 µl PBS for a final stock concentration of 2000 µg/ml for each lectin.

Sialidase-treated or untreated and mild acid treated or untreated *A. fumigatus* conidia (1×10^7) were blocked with 45 µl PBS/10% goat serum for 1 hour at room temperature. After 1 hour, 5 µl of SNA stock solution was added directly to the blocking reaction for a final lectin concentration of 200 µg/ml and allowed to incubate for 1 hour at room temperature. Conidia were then washed 3 times with PBS to remove unbound lectin and bound lectin was detected by incubation for 1 hour at room temperature in the dark with 25 µl of 1% Streptavidin-Oregon green (Molecular Probes, Burlington, ON) in PBS/10% goat serum. After incubation, samples were washed 3 times with PBS, resuspended in an appropriate amount of PBS and mounted on glass slides. Samples were viewed with an Olympus VANOX AHBS3 microscope (Olympus America, Inc., Center Valley, PA) equipped with epifluorescence at 1000X magnification. Bright field and fluorescence images were captured with a Sony 950 camera using Northern Eclipse imaging software (Empix Imaging Inc., Mississauga, ON). The extent of sialic acid removal was also assessed by flow cytometry. Flow cytometry was performed on a Coulter EPICS Elite Esp flow cytometer

(Beckman-Coulter Inc., Mississauga, ON) using a 488 nm laser for excitation energy and a 550 nm dichroic filter capturing an emission band at 525 nm. 10000 events were captured for each sample.

To determine the linkage of surface sialic acids to the underlying carbohydrate, *A. fumigatus* conidia (1×10^7) were incubated with SNA, MAA or PNA and visualized with Streptavidin-Oregon green using fluorescence microscopy as described above.

For competition experiments, conidia were incubated with SNA, as described previously, with the exception that 38 mM α 2,6-sialyllactose (Sigma) was added along with the lectin to compete with conidial sialic acids for SNA.

To identify the second terminal residue, sialidase-treated or untreated *A. fumigatus* conidia were incubated with PNA and visualized with Streptavidin-Oregon green using fluorescence microscopy as described above.

Identification of surface sialic acids from *A. fumigatus* by fluorometric high-pressure liquid chromatography (HPLC) and matrix-assisted laser desorption ionization (MALDI) mass spectroscopy

Dried mild acid or enzyme hydrolysates of *A. fumigatus* were derivatized with 1,2-diaminomethylenedioxybenzene (DMB) (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) by resuspending the dried hydrolysates in 100 μ l H₂O and adding 25 μ l of this solution to 100 μ l of DMB solution (7 mM DMB, 0.75 M β -mercaptoethanol and 18 mM sodium hydrosulfite in 1.4 M acetic acid). DMB is a compound that reacts specifically with α,β -dicarbonyl

compounds to produce 3-substituted 6,7-methylene dioxy-2-(1H)-quinoxalinone derivatives, which are highly fluorescent and thereby allow for the detection of femto molar quantities of sialic acids and their derivatives in biological mixtures (Figure 2.1) (Hara *et al.*, 1989). This solution was heated for 2.5 hours at 56°C in the dark. After 2.5 hours, the derivatization reaction was stopped by cooling on ice.

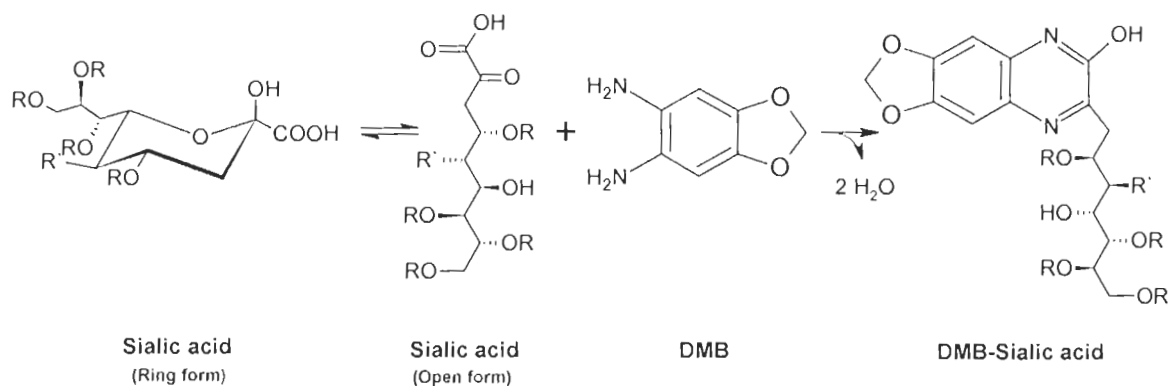


Figure 2.1: Derivatization of sialic acids with 1,2-diamino-4,5-methylenedioxybenzene, dihydrochloride (DMB) yielding a 3-substituted 6,7-methylene dioxy-2-(1H)-quinoxalinone derivative (DMB-Sialic acid).

Refer to the caption for Figure 1.2 to define R and R'.

DMB-derivatized samples were directly separated using a Waters 600E HPLC system (Waters Corporation, Mississauga, ON) with a reverse phase C-18 column (250 x 4.6 mm, 5µm particle size, Phenomenex, Torrance, CA) using isocratic elution with a solvent system of H₂O/acetonitrile/methanol (84/9/7, v/v/v) at a flow rate of 0.9 ml/min. DMB-derivatized sialic acids were detected with a Hewlett Packard 1046A programmable fluorescence detector (Hewlett-Packard Development Company, L.P., Palo Alto, CA) using an excitation wavelength of 246 nm and emission wavelength of 442 nm. Authentic N-acetylneuraminic acid (Sigma) and mild acid hydrolysates of bovine submaxillary mucin (BSM) (Sigma),

which is a source of Neu5Ac, Neu5Gc and O-acetylated derivatives, with the exception of the Neu4,5Ac₂ derivative (Reuter *et al.*, 1983) and horse serum (HS) (Sigma), which is a source of Neu4,5Ac₂ (Manzi *et al.*, 1990), were used as standards.

To confirm O-acetylation, sialic acids were de-O-acetylated by treating with 0.2 M NaOH for 30 minutes on ice (Varki & Diaz, 1984). However, for the 4-O-acetylated derivative these conditions were not sufficient; therefore, to de-O-acetylate this derivative 0.2 M NaOH was used for 45 minutes at 37°C as previously described by Tiralongo *et al.*, 2000. O-acetylated sialic acids were confirmed based on their relative retention times, decrease of the O-acetylated peak and increase in size of the parent sialic acid molecule peak. The *A. fumigatus* hydrolysate was treated using both conditions to ensure the release of the O-acetyl groups that may be present on the sialic acid.

For MALDI-TOF mass spectroscopy, several fractions corresponding to peaks of interest were collected in plastic tubes, dried, resuspended in a minimal volume of distilled deionized water and pooled. Samples were mixed with an equal volume of 2,5-dihydroxybenzoic acid matrix solution (10 mg/ml in H₂O/acetonitrile, 50/50, v/v) (Sigma). Mass spectra were collected with a PerSeptive Biosystems MALDI-TOF mass spectrometer (Applied Biosystems, Streetsville, ON) in the positive ion mode using the average of 100 laser shots, an accelerating voltage of 20000 V, mass range of between 0 and 4000 daltons and a delay time of 150 nsec.

2.3 Results

Surface sialic acids in *A. fumigatus* are α 2,6-linked to the sub-terminal carbohydrate

Sialoglycoconjugates were previously identified by our laboratory on the surface of *A. fumigatus* conidia (Wasylnka *et al.*, 2001). To identify the linkage of these surface sialic acids and the sub-terminal carbohydrate, a lectin-binding assay was performed. Biotinylated SNA, which binds specifically with α 2,6-linked sialic acids (Shibuya *et al.*, 1987) or biotinylated MAA, which binds with α -2,3-linked sialic acids (Knibbs *et al.*, 1991) were incubated with conidia and visualized with a secondary fluorophore, Streptavidin-Oregon green. Conidia were also incubated with biotinylated PNA, a lectin specific for galactose bound to GlcNAc (Lotan *et al.*, 1975). Figure 2.2 shows that only SNA was able to bind to conidia to a significant extent indicating that surface sialic acids were primarily linked to the underlying carbohydrate by α 2,6 linkages. Furthermore, the analysis revealed that there were no α 2,3-linked sialic acids or exposed galactose residues present.

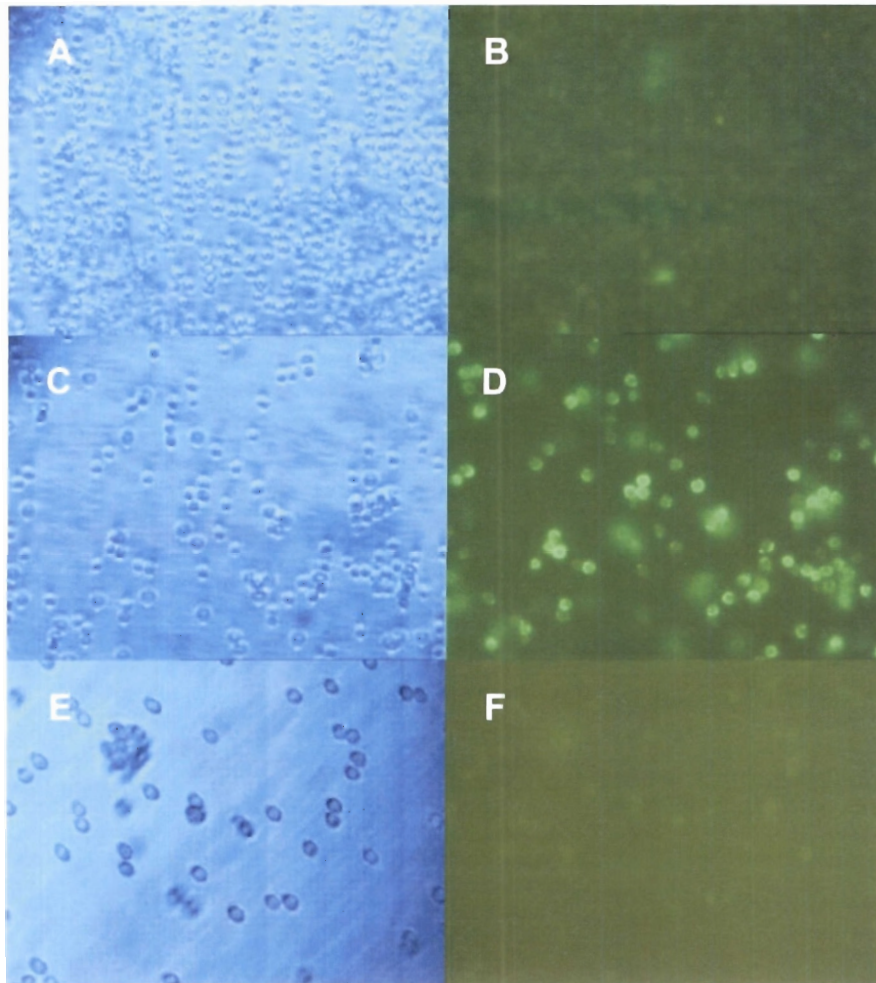


Figure 2.2: Lectin binding reveals that *A. fumigatus* surface sialic acids are primarily linked in α 2,6-linkages to sub-terminal carbohydrates.

To determine the linkage of surface sialic acid to the underlying sugar, *A. fumigatus* conidia were incubated with biotinylated lectins and visualized using a secondary fluorophore, Streptavidin Oregon green, and fluorescence microscopy. Lectins used in the study were MAA, which is specific for α 2,3-linked sialic acids; PNA, which is specific for galactose residues; and SNA, which is specific for α 2,6-linked sialic acids. A, C and E represent bright field images and B, D and F represent fluorescent images. A and B represent the lectin binding results using MAA. C and D represent the lectin binding results using SNA. E and F represent the lectin binding results using PNA. Results indicate that *A. fumigatus* surface sialic acids are primarily linked in α 2,6 linkages and that no terminal galactose residues are exposed.

In the present study, we used 4 times more SNA (200 μg vs 50 μg) compared to a previous study conducted in our laboratory (Wasylnka et al., 2001). In the previous study, we concluded that *A. fumigatus* sialic acids were not linked in $\alpha 2,3$ or $\alpha 2,6$ linkages because conidia were unreactive to both MAA and SNA at those concentrations. To confirm that the binding of SNA to conidial sialic acids in the present study was specific, lectin binding and visualization was performed as described above except that 38 mM $\alpha 2,6$ -sialyllactose (Sigma) was added to the incubation as a competitive inhibitor. The results showed that $\alpha 2,6$ -sialyllactose was able to efficiently compete with conidial sialic acids for SNA, suggesting that the fluorescence observed using SNA was the result of SNA binding to conidia via sialic acids rather than non-specific interactions (Figure 2.3). Increased concentrations of MAA were also employed (50, 200 and 800 $\mu\text{g}/\text{ml}$); however, fluorescence microscopy did not show any increase in lectin binding at these elevated concentrations. Thus, $\alpha 2,3$ -linked sialic acids are either not present or are present below detection limits on *A. fumigatus* conidia.

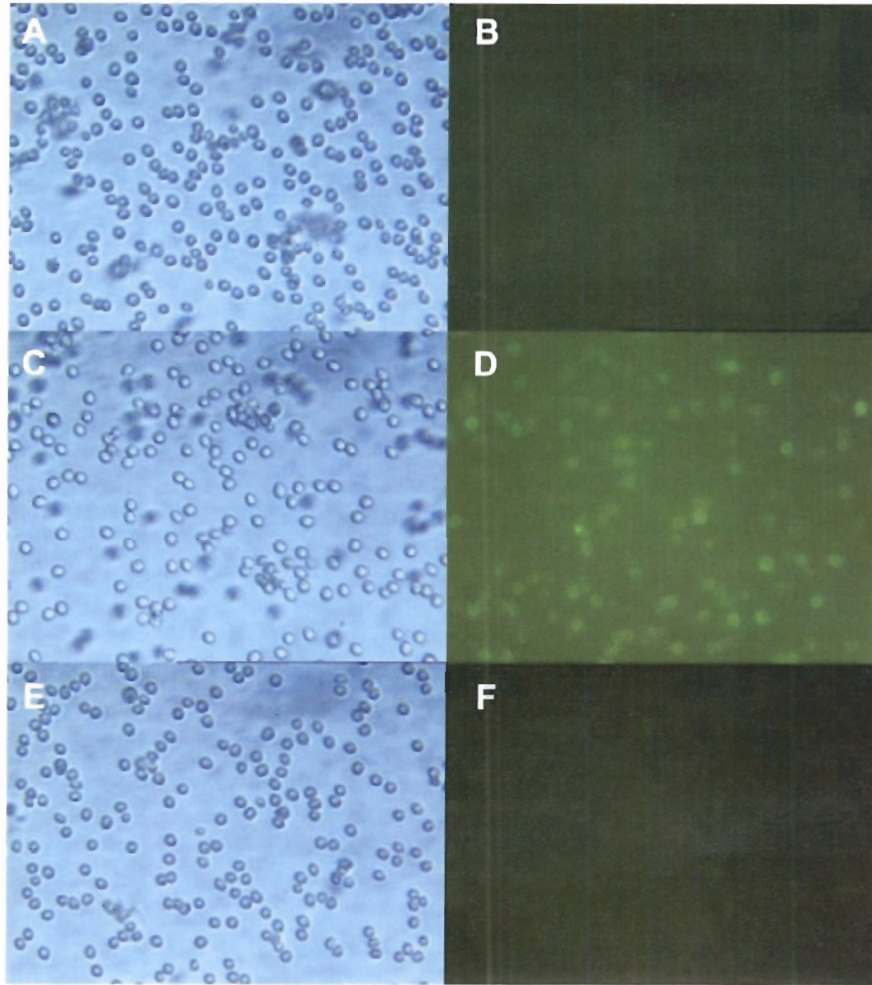


Figure 2.3: SNA binding to *A. fumigatus* conidia is specific for sialic acids.

A. fumigatus conidia were incubated with SNA in the presence of 38 mM α 2,6-sialyllactose. Panels A, C and E represent bright field images and panels B, D and F represent fluorescent images. A and B represent the negative control (- SNA, - sialyllactose), C and D represent the positive control (+ SNA, - sialyllactose), and E and F represent the competition experiment (+ SNA, + sialyllactose).

Removal of sialic acid using *Micromonospora viridifaciens* sialidase

To further establish that binding of SNA to conidia was specific and to assess the extent of sialic acid removal by *Micromonospora viridifaciens* sialidase, conidia were either treated or untreated with *Micromonospora viridifaciens* sialidase and the extent of sialic acid release was assessed by lectin binding followed by fluorescence microscopy or flow cytometry. Fluorescence microscopy (Figure 2.4) qualitatively showed that *M. viridifaciens* sialidase efficiently removed surface sialic acids from *A. fumigatus* conidia. Quantitative analysis using flow cytometry established that approximately 90% of surface sialic acids were removed by *Micromonospora viridifaciens* sialidase (Figure 2.5). Furthermore, these studies confirmed the specificity of SNA binding because enzymatic removal of conidial sialic acids eliminated SNA binding.

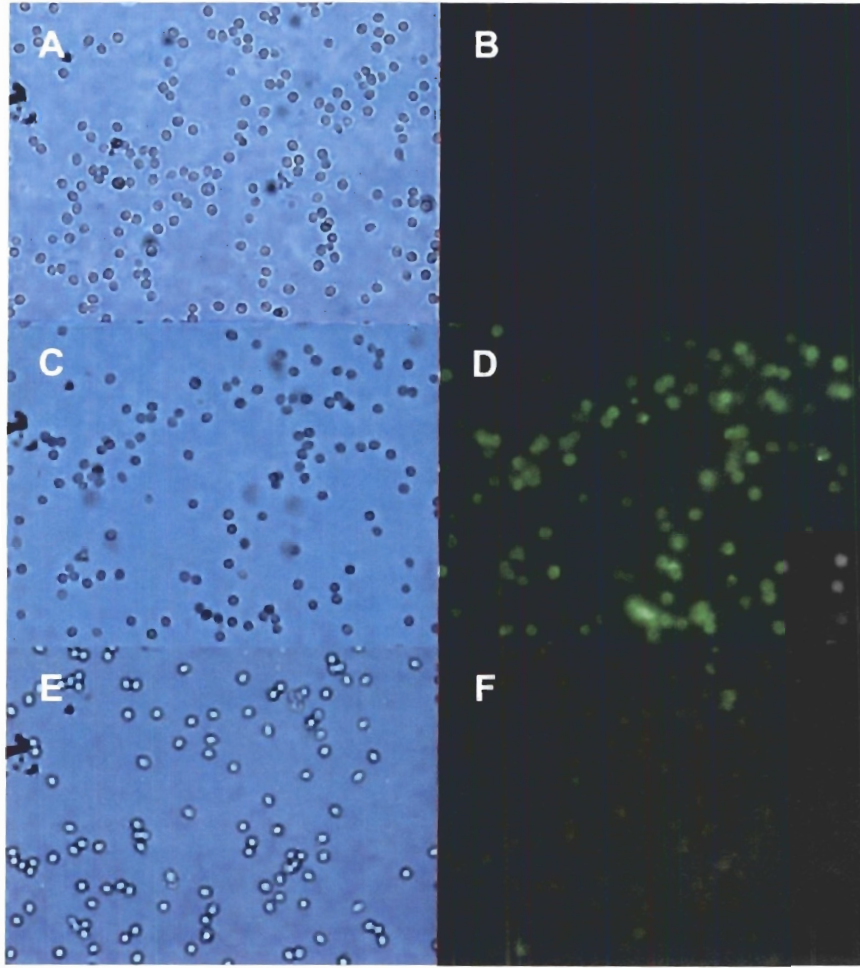
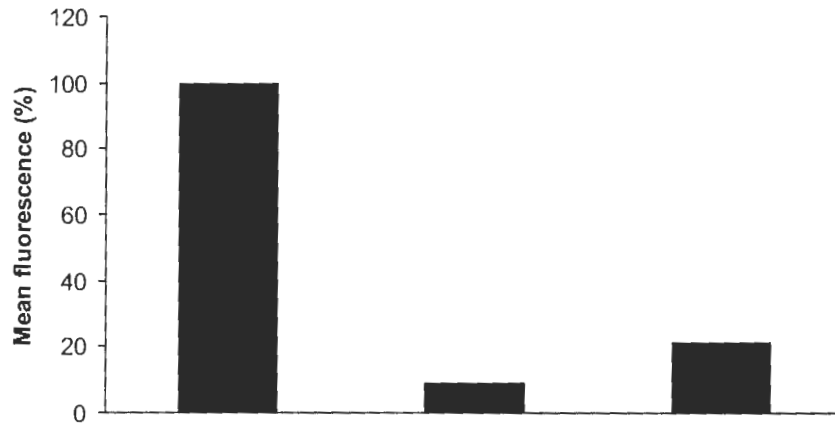
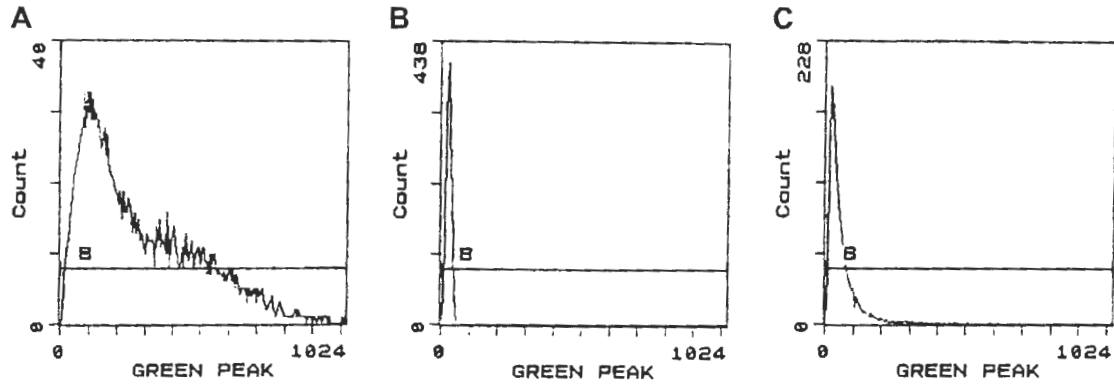


Figure 2.4: *M. viridifaciens* sialidase efficiently released surface sialic acids from *A. fumigatus* conidia.

To assess the extent of sialic acid removal and to determine whether SNA was binding specifically to conidial sialic acids, SNA_b binding and fluorescence microscopy were used. Panels A, C and E represent the bright field images and panels B, D and F represent fluorescent images. A and B represent the negative control (- SNA, - sialidase), C and D represent the positive control (+ SNA, - sialidase), and E and F represent sialidase treated conidia (+ SNA, + sialidase).



Sialidase treatment	-	-	+
SNA	+	-	+

Figure 2.5: Flow cytometry reveals that SNA binding is decreased by 90% after sialidase treatment of conidia.

Sialidase treated or untreated conidia were reacted with SNA and Streptavidin Oregon green and the differences in fluorescence were measured by flow cytometry. (Top) A) Represents untreated conidia reacted with biotinylated SNA and S-OG (-sialidase, +SNA) (positive control). B) Represents untreated conidia reacted with S-OG only (-sialidase -SNA) (negative control). C) Represents sialidase treated conidia reacted with SNA and S-OG (+sialidase, +SNA). (Bottom) Summary of the data from flow cytometry where percent mean fluorescence is directly correlated with sialic acid density. The positive control was arbitrarily set to 100%.

The sub-terminal monosaccharide on the glycan chain is galactose

In mammalian cells, sialylated glycans often have a galactose residue at the sub-terminal position (Varki, 1998). To determine whether the underlying monosaccharide is galactose in *A. fumigatus*, sialidase treated *A. fumigatus* conidia were assessed by lectin binding using PNA, a lectin that binds specifically to exposed galactose residues linked to GlcNAc (Lotan *et al.*, 1975) and visualized with Streptavidin-Oregon green and fluorescence microscopy. PNA bound to conidia only after sialidase treatment suggesting that the underlying sugar is galactose (Figure 2.6).

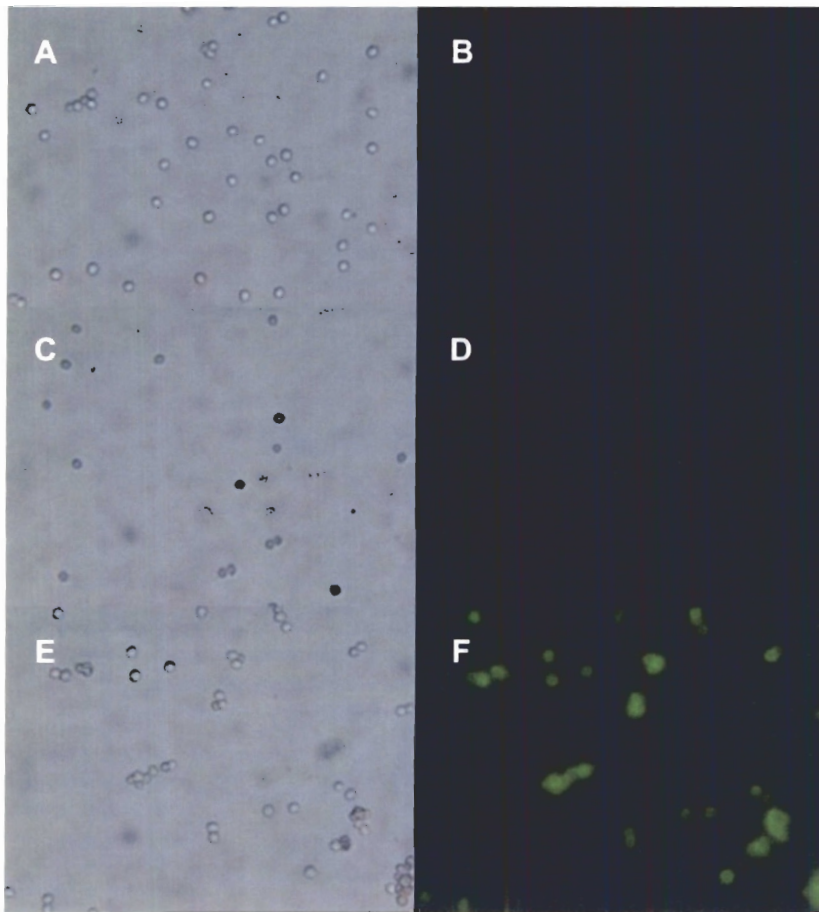


Figure 2.6: PNA binding reveals that the sub-terminal carbohydrate is galactose.

Sialidase treated conidia were subjected to lectin binding with PNA. Panels A, C and E represent the bright field images and panels B, D and F represent fluorescent images. A and B are the negative control (+ PNA, - sialidase), C and D are another negative control (- PNA, + sialidase), and E and F show sialidase treated conidia (+ PNA, + sialidase).

HPLC and MALDI mass spectroscopy reveal that Neu5Ac is the major sialic acid on the surface of *A. fumigatus* conidia

To determine the type of sialic acid present on the surface of *A. fumigatus* conidia, HPLC and MALDI-TOF mass spectroscopy were used. First, mild acid treatment was used to release terminal sialic acids from conidia. This treatment releases terminal sialic acids from glycoconjugates without destroying

substitutions that may be present on the sialic acid molecule (Varki & Diaz, 1984). Released sialic acids were derivatized with the fluorescent compound, DMB, and analyzed by HPLC. HPLC revealed three DMB-reactive compounds in the mild acid hydrolysate of *A. fumigatus* conidia; however, only one of these peaks eluted with a relative retention time that corresponded to peaks in the bovine submaxillary mucin (BSM) or horse serum (HS) standards. This compound had a retention time that corresponded to that of Neu5Ac in both sets of standards (Figure 2.7). Nevertheless, fractions corresponding to each of the three peaks were collected, dried and pooled for mass spectroscopic analysis. MALDI-TOF mass spectroscopy confirmed that peak 1 was unsubstituted Neu5Ac by the presence of ions at $m/z = 426 [M+H]^+$, $m/z = 448 [M+Na]^+$ and $m/z = 464 [M+K]^+$, the resulting masses of DMB-derivatized Neu5Ac and its sodium and potassium adducts, respectively (Figure 2.8). Peaks 2 and 3 did not elute with relative retention times corresponding with those of the standards (Figure 2.7), and MALDI mass spectroscopic data was inconsistent with that of any known sialic acid derivative (Figures 2.9 and 2.10). In fact, mass spectral data revealed that peaks 2 and 3 were of lower mass (peak 2 $m/z = 394 [M+H]^+$, peak 3 $m/z = 387 [M+H]^+$) than Neu5Ac. KDN is a structurally related α -keto acid that is also found in glycoconjugates (Nadano *et al.*, 1986; Knirel *et al.*, 1989; Gil-Serrano *et al.*, 1998). However, KDN would elute before Neu5Ac under the conditions employed in this study and KDN has a DMB-derivatized mass of $m/z = 385 [M+H]^+$ (Klein *et al.*, 1997). In addition, alkaline hydrolysis neither eliminated peak 3 nor increased the size of the Neu5Ac peak, indicating that peak 3 is not

likely to be an O-acetylated sialic acid derivative. Peak 2 was extremely unstable, in fact, the peak disappeared even under control conditions. However, there was no corresponding increase in the Neu5Ac peak thereby indicating that this peak was not likely an O-acetylated sialic acid (Figures 2.11-2.13). Peak 3 has been identified by other researchers in the sialic acid field as a reagent peak though its chemical structure is not known (Hara *et al.*, 1989; Chatterjee *et al.*, 2003; Lewis *et al.*, 2006). Hence, Neu5Ac was established as the predominant sialic acid on the surface of *A. fumigatus* conidia.

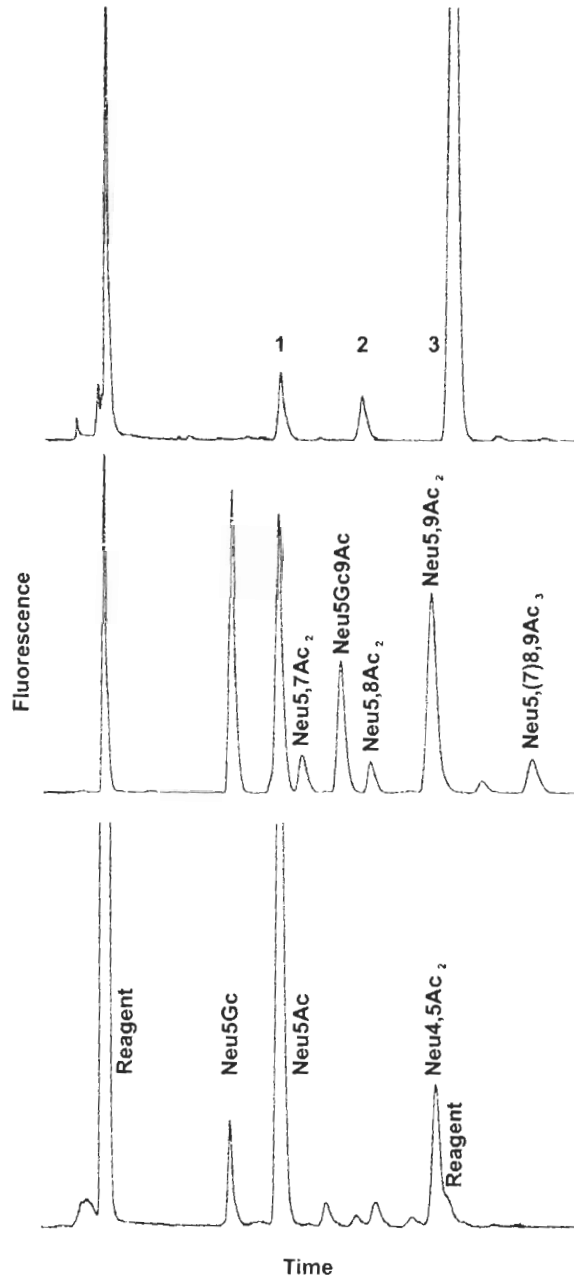


Figure 2.7: HPLC reveals three DMB-reactive compounds in the mild acid hydrolysate of *A. fumigatus* conidia.

Sialic acids from *A. fumigatus* conidia (top) were released by mild acid hydrolysis, derivatized with DMB and analyzed by HPLC compared to standard sialic acids obtained from a mild acid hydrolysate of bovine submaxillary mucin (middle), and horse serum (bottom). Only one compound (peak 1) from *A. fumigatus* had a relative retention time that corresponded to Neu5Ac in the standards. Because the concentration of BSM was high, the reagent peak did not appear in this run.

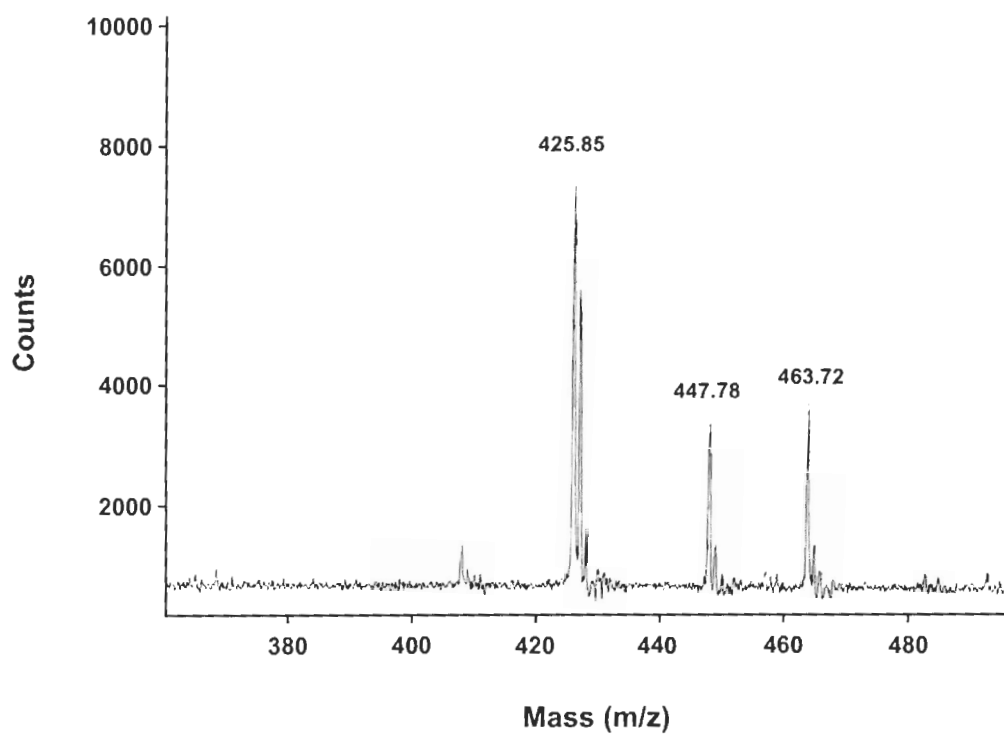


Figure 2.8: MALDI mass spectroscopy reveals that peak 1 is Neu5Ac.

MALDI mass spectroscopy of peak 1 revealed that this compound is in fact Neu5Ac by the presence of the molecular ion peak at $m/z = 426 [M+H]^+$, which corresponds to protonated Neu5Ac-DMB as well as sodium $[M+Na]^+$ and potassium $[M+K]^+$ adducts at $m/z = 448$ and $m/z = 464$, respectively.

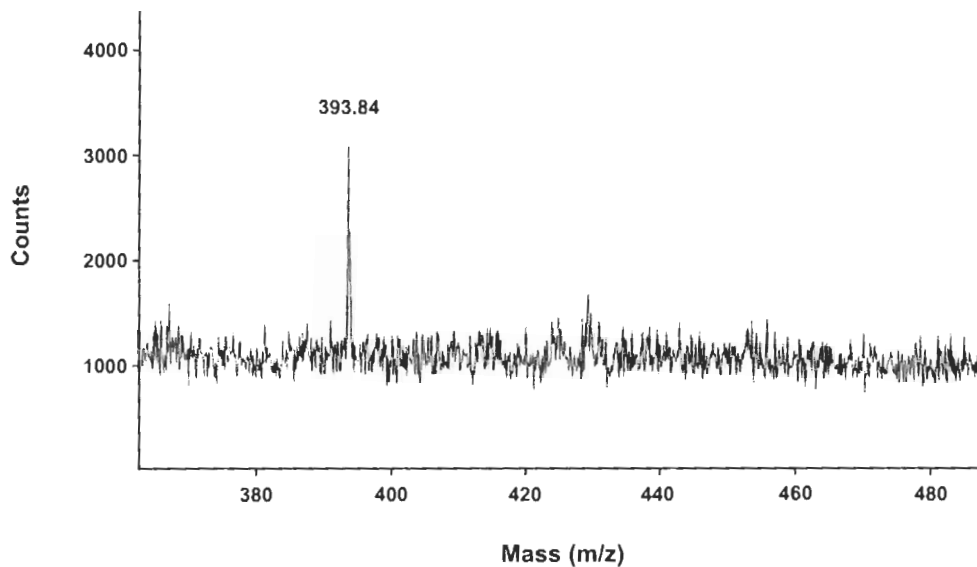


Figure 2.9: MALDI mass spectroscopy of peak 2.

Mass spectral data of peak 2, $m/z = 393.64 [M+H]^+$, is inconsistent with that of any known sialic acid derivative.

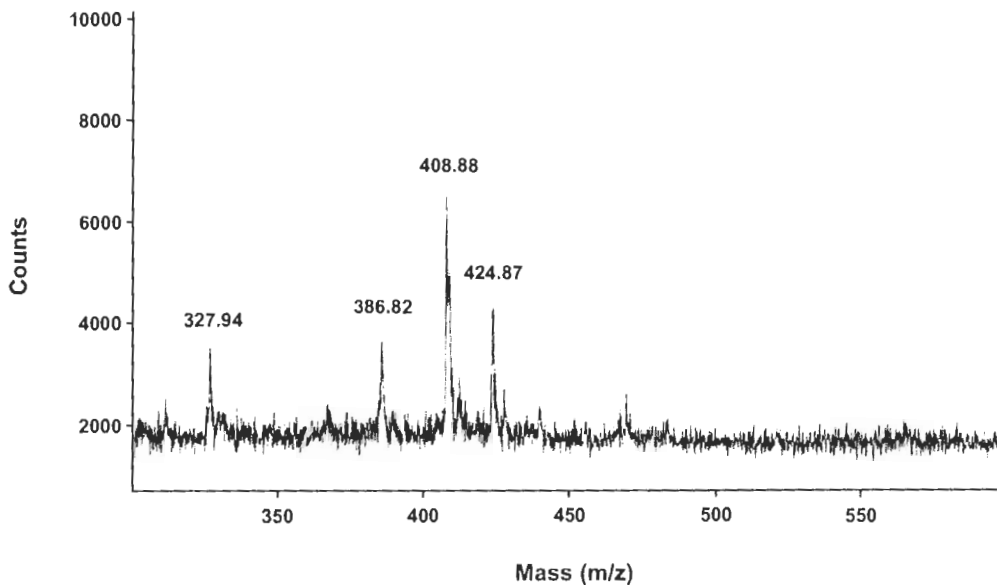


Figure 2.10: MALDI mass spectroscopy of peak 3.

Mass spectral data of peak 3, $m/z = 386.82 [M+H]^+$, is inconsistent with that of any known sialic acid derivative.

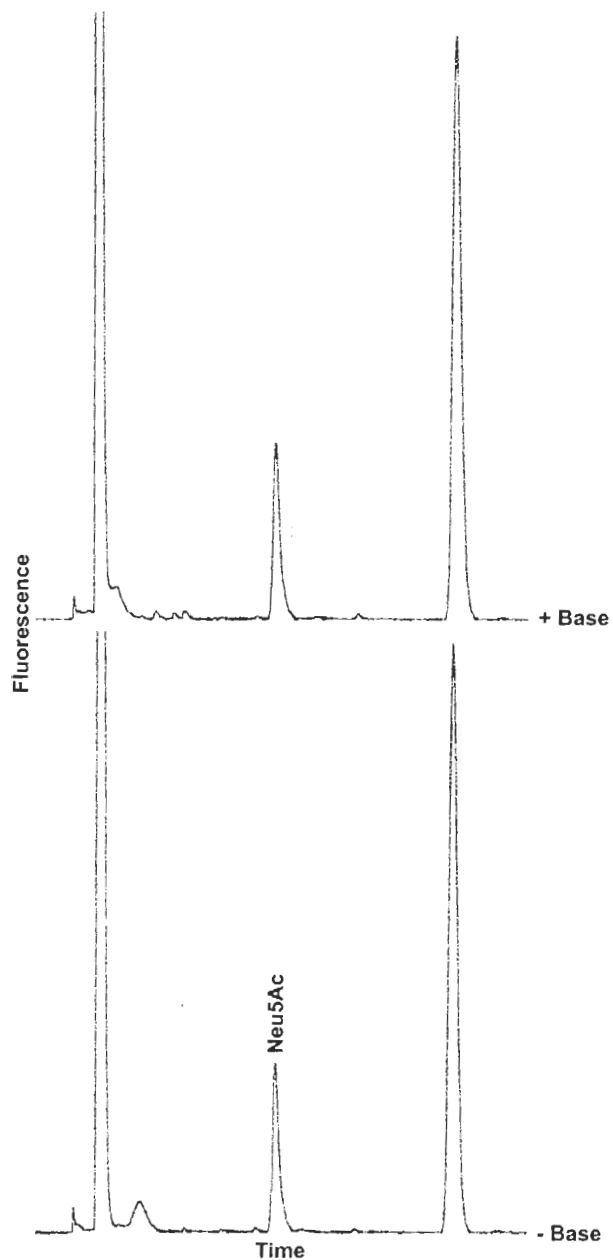


Figure 2.11: Alkaline treatment of *A. fumigatus* mild acid hydrolysate.

Results indicate that peak 3 is not an O-acetylated sialic acid as it was not eliminated by alkaline hydrolysis. Peak 2 was eliminated even under control conditions; however, there was no corresponding increase in the size of the Neu5Ac thereby indicating that this peak is unlikely to represent an O-acetylated sialic acid. The top figure shows the HPLC chromatogram after NaOH treatment of *A. fumigatus* mild acid hydrolysate. The bottom chromatogram represents the control sample.

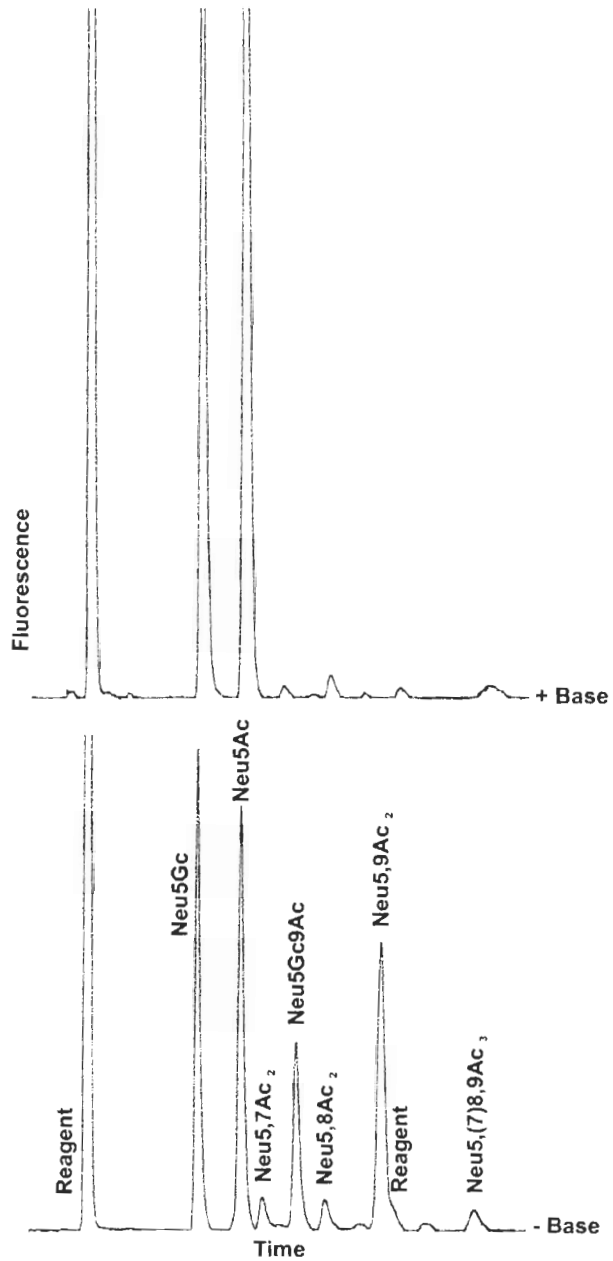


Figure 2.12: Alkaline treatment of BSM mild acid hydrolysate.

Alkaline treatment of the acid hydrolysate of BSM eliminated O-acetylated sialic acids with a corresponding increase in the parent sialic acids, Neu5Ac and Neu5Gc. The bottom chromatogram shows the retention time of sialic acids present in BSM.

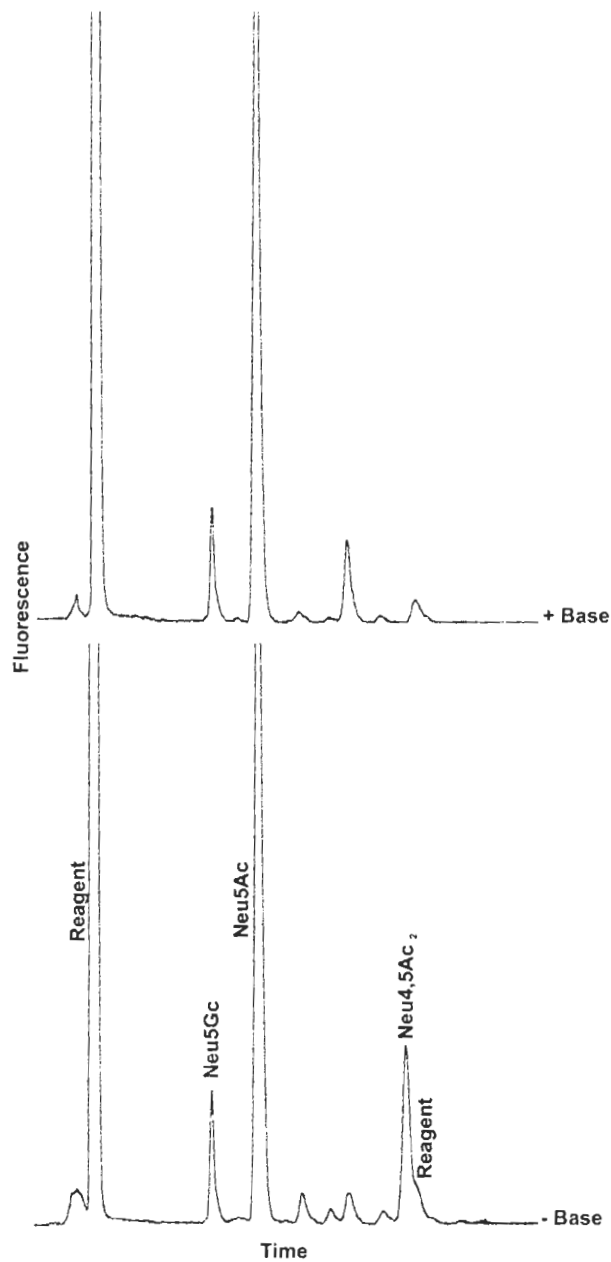


Figure 2.13: Alkaline treatment of HS mild acid hydrolysate.

Alkaline treatment of the acid hydrolysate of HS (top) eliminated Neu4,5Ac₂ with a corresponding increase in the parent sialic acid, Neu5Ac. Sialic acids present in horse serum are shown in the bottom (control) chromatogram.

2.4 Discussion

Lectin binding was used to determine the linkage of *A. fumigatus* surface sialic acids to the underlying sugar. It was found that biotinylated SNA, a lectin specific for α 2,6 linkages, bound *A. fumigatus* conidia but only with a 4-fold greater amount of SNA than was used in previous studies conducted in our laboratory (Wasylnka *et al.*, 2001). SNA binding to conidia was specific since 1) it was eliminated upon the addition of α 2,6-sialyllactose, and 2) sialidase treatment abolished SNA binding. These data indicated that conidial sialic acids in *A. fumigatus* are primarily linked in α 2,6 linkages. The lack of binding by MAA and PNA indicate that α 2,3-linked sialic acids and exposed galactose residues are at levels below the detection limit of our method. Furthermore, PNA was reactive only with sialidase-treated conidia, revealing that the sub-terminal sugar is galactose. Previous work in our laboratory using lower concentrations of SNA and MAA led to the interpretation that *A. fumigatus* sialic acids may be linked by an α 2,8 or α 2,9 linkage to an underlying sugar, most likely another sialic acid (Wasylnka *et al.*, 2001). However, evidence from the present study indicates that sialic acid is linked to an underlying galactose via an α 2,6 linkage. Interestingly, sialic acids present on the surface of other fungi are also found predominantly in α 2,6-linkages and always linked to galactose (Rodrigues *et al.*, 2002; Alviano *et al.*, 2004); however, α 2,3-linked sialic acids have also been detected. For example, in *C. albicans* it was found that both MAA and SNA bound indicating that sialic acids are present in both linkages (Soares *et al.*, 2000).

Previous attempts to remove sialic acids from *A. fumigatus* conidia using commercially-available *Clostridium perfringens* and *Arthrobacter ureafaciens* sialidases removed only half of the sialic acids (Wasylnka *et al.*, 2001). *Micromonospora viridifaciens* sialidase has been shown to effectively cleave sialic acids in α 2,3, α 2,6 and α 2,8 linkages (Sakurada *et al.*, 1992; Watson *et al.*, 2003). Therefore, conidia were treated with *M. viridifaciens* sialidase to remove surface sialic acids. A possible explanation for the inefficient binding of both SNA and *Clostridium perfringens* and *Arthrobacter ureafaciens* sialidases leading to their inefficient cleavage of surface sialic acids is that perhaps *A. fumigatus* sialic acids exist as disialylated structures attached to galactose, with one sialic acid being linked in an α 2,6-linkage and the other in an α 2,3-linkage. This steric hindrance could lead to inefficient lectin and enzyme binding. Because *M. viridifaciens* sialidase has a shallower active site compared to that of *Clostridium perfringens* and *Arthrobacter ureafaciens* sialidases (J. N. Watson, direct communication), it is possible that the presence of such disialylated structures would be less likely interfere with *M. viridifaciens* binding and result in the increased release of sialic acid from *A. fumigatus* conidia. This hypothesis has not yet been tested.

To determine the structure of sialic acids on the surface of *A. fumigatus* conidia, sialic acids were released from the conidial surface by mild acid hydrolysis, derivatized with DMB, which reacts specifically with α,β -dicarbonyl compounds, and analyzed by reverse phase HPLC comparing to standards consisting of authentic Neu5Ac and mild acid hydrolysates of BSM and HS.

HPLC revealed that the mild acid hydrolysate of *A. fumigatus* conidia contained three DMB-reactive compounds, one of which was confirmed to be Neu5Ac. The relative retention times of the other two peaks as well as their molecular masses did not correspond to those of any known sialic acids. Moreover, alkaline treatment of the *A. fumigatus* mild acid hydrolysate did not abolish peaks 2 or 3 causing a corresponding increase in the parental sialic acid peak (either Neu5Ac or Neu5Gc), indicating that these peaks are unlikely to represent O-acetylated sialic acid derivatives. Furthermore, studies have shown that DMB does not react with glucose, galactose, fructose, glucosamine, galactosamine, maltose, cellobiose, gentiobiose, lactose, fucose, ribose, deoxyribose and acidic sugars, such as the uronic acids (Hara *et al.*, 1989). DMB reacts with α,β -dicarbonyl compounds, which are abundant and play important roles in metabolism (Hara *et al.*, 1989). Finally, several published studies also report an unidentified reagent peak with eluting between Neu5,9Ac₂ and Neu5,7(8),9Ac₃ (Hara *et al.*, 1989; Chatterjee *et al.*, 2003; Lewis *et al.*, 2006). Taken together and with the absence of significant evidence that peaks 2 and 3 represent cell surface carbohydrates of interest, it is likely that they are artefacts of the conditions of this study, and, therefore, Neu5Ac is the major sialic acid on the surface of *A. fumigatus* conidia. The finding that Neu5Ac is the cell surface sialic acid in *A. fumigatus* is interesting since other pathogenic fungi such as *C. neoformans*, and *F. pedrosoi* were found to have O-acetylated sialic acids on their surface. There are examples, however, of pathogenic fungi with only unsubstituted Neu5Ac on their surface, such as *C. albicans* and *P. brasiliensis* (Soares *et al.*, 1993; Soares *et*

al., 2000). The reasons behind these differences may become more clear when the function of these cell surface sialic acids in fungi is better characterized.

3 ROLE OF SIALIC ACID ON THE SURFACE OF *ASPERGILLUS FUMIGATUS* CONIDIA IN BINDING TO FIBRONECTIN AND UPTAKE BY LUNG EPITHELIAL AND MACROPHAGE CELL LINES

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3.1 Introduction

Sialic acids have been shown to be important in microbial pathogenesis, often playing recognition or anti-recognition roles (Estabrook *et al.*, 1997; Schauer, 2004; Shinya *et al.*, 2006). Previous work in our laboratory found that pathogenic *Aspergillus* species had greater amounts of sialic acids on their surface than non-pathogenic *Aspergillus* species; however, the role of these sialic acids in *A. fumigatus* pathogenesis was unclear (Wasylnka *et al.*, 2001). The initial step of infection is thought to be the adhesion of *A. fumigatus* conidia to host lung cells or to components of the lung extracellular matrix (ECM) (Bouchara *et al.*, 1994). The extracellular matrix of the basal lamina, which is secreted by epithelial cells, is composed of laminin, type IV collagen, type V collagen, entactin, chondroitin sulphate proteoglycan, heparin sulphate and fibronectin (Sannes & Wang, 1997). It is not fully understood how fibronectin becomes deposited in the basal lamina of the lungs; however, the basal lamina of fibrotic lungs caused by microscopic injury have increased levels of deposited

fibronectin (Torikata *et al.*, 1985). Increased levels of fibronectin are thought to aid in tissue repair by facilitating the attachment of cells required for reepithelialization to occur (Clark *et al.*, 1982). The binding of *A. fumigatus* conidia to isolated ECM proteins, such as fibronectin, laminin and type IV collagen has been studied and two different mechanisms of binding have been proposed. Early studies suggested that the cell binding domain of fibronectin, containing the RGD sequence, is recognized by an integrin-like molecule on the conidial surface. Support for this mechanism came from competition experiments in which binding of conidia to fibronectin was decreased by the addition of a synthetic GRGDS peptide (Bromley & Donaldson, 1996; Gil *et al.*, 1996). In contrast, this was not found to be the case with laminin where competition with GRGDS peptide did not have an effect on conidial binding (Bouchara *et al.*, 1997). Instead, addition of sialic acid or sialyllactose in these competition experiments inhibited the adhesion of conidia to laminin. From those results, the authors proposed that sialic acids on laminin mediate the binding between *A. fumigatus* conidia and laminin through a sialic acid-specific lectin located on the surface of *A. fumigatus*. Subsequent research in our laboratory showed that binding of conidia to fibronectin was not mediated by binding to RGD or to oligosaccharides located on the fibronectin or laminin glycoproteins (Wasylnka & Moore, 2000). Rather, conidia specifically bound to the positively-charged glycosaminoglycan (GAG) binding domain of fibronectin, which has no oligosaccharide attached, and not to the RGD cell-binding domain or the collagen-binding domain. Finally, it was shown that binding to the GAG domain

was inhibited by negatively-charged sugars. Together, this suggested the possibility that negatively-charged sugars mediated binding to fibronectin (Wasylnka & Moore, 2000). Later studies confirmed the presence of sialic acids on conidia (Wasylnka *et al.*, 2001). In addition, pathogenic *Aspergillus* species possessed higher levels of surface sialylation than non-pathogenic *Aspergillus* species (Wasylnka *et al.*, 2001). This difference in sialic acid density correlated with differences in fibronectin binding ability between pathogenic and non-pathogenic *Aspergillus* (Wasylnka *et al.*, 2001). Therefore, it was hypothesized that adhesion of *A. fumigatus* to fibronectin is mediated by surface sialic acids (Wasylnka *et al.*, 2001).

In addition to a potential adhesion role in pathogenesis, sialic acids have also been implicated in protecting pathogenic fungi from the host immune response. For example, in *Sporothrix schenkii* and *Cryptococcus neoformans*, removal of surface sialic acids using sialidase increased the level of phagocytosis by mouse peritoneal macrophages 8- and 2-fold, respectively (Oda *et al.*, 1983; Rodrigues *et al.*, 1997). Similarly, removing surface sialic acids from *Fonsecaea pedrosoi* increased the level of association between the fungus and neutrophils (Alviano *et al.*, 2004). Previous work in our laboratory found that *A. fumigatus* conidia are efficiently internalized by cultured type II pneumocytes and by murine macrophages (Wasylnka & Moore, 2002); however, the identity of the fungal ligands is not known.

The aims of the research outlined in this chapter are to determine whether sialic acids on the surface of *A. fumigatus* conidia mediate the binding between

conidia and the extracellular matrix protein, fibronectin, and to assess whether sialic acids confer anti-phagocytic properties to *A. fumigatus* conidia.

3.2 Materials and methods

***A. fumigatus* strains and growth conditions**

Two strains of *A. fumigatus* were used in this study: *A. fumigatus* ATCC 13073 (American Type Culture Collection) and the GFP-expressing strain of *A. fumigatus* ATCC 13073 (gGFP) developed previously in our laboratory (Wasylnka & Moore, 2002). Both strains were grown on MYPD agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 0.5% dextrose) for 3 days at 37°C or 5 days at 28°C until conidia were fully mature. Fully mature conidia were harvested by adding PBS-T directly onto the plate and scraping with a sterile cotton swab until conidia were in suspension. The conidial suspension was then vortexed vigorously to break apart chains of conidia and filtered through sterile glass wool to remove hyphae. Conidia were then washed 3 times with PBS, resuspended in PBS and counted with a haemocytometer.

Metabolic inactivation and sialidase treatment of conidia

Aspergillus fumigatus conidia (1×10^8) were harvested as described above and were treated with 100 μ M carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma) dissolved in 99% ethanol to metabolically inactivate conidia by uncoupling oxidative phosphorylation, and thus preventing the resynthesis of sialic acids. Pilot studies had confirmed that this CCCP concentration effectively prevented *A. fumigatus* growth when re-plated (data not shown). Previous work

in our laboratory indicated that there was no significant difference in the extent of uptake between viable and non-viable conidia by cultured lung epithelial cells (A549) and mouse macrophages (J774) (Wasylnka & Moore, 2002). After CCCP treatment, conidia were washed three times with PBS and once with 16 mM sodium tartrate buffer, pH 5.2, and resuspended in 25 μ l of 16 mM sodium tartrate buffer supplemented with 0.1% bovine serum albumin (BSA) (Sigma). *Micromonospora viridifaciens* sialidase (74 μ g) (a generous gift from Dr. J. Watson and Dr. A. Bennet, Department of Chemistry, Simon Fraser University) was added to conidia and allowed to incubate for 1 hour at 50°C. After 1 hour, a second aliquot of 37 μ g sialidase was added with 12.5 μ l of 16 mM sodium tartrate buffer and allowed to incubate for one hour more. After sialidase treatment, conidia were washed three times with PBS and stored at 4°C overnight until assayed.

Fibronectin adherence assay

Fibronectin adherence assays were performed in 8-chamber glass slides by coating the slides with 300 μ l of 50 μ g/ml fibronectin (Sigma) in PBS and incubating at 37°C for 1 hour and then at 4°C overnight. The following day, slides were blocked with 500 μ l/well PBS/0.1% BSA for 1 hour at 37°C. After 1 hour, 1×10^7 sialidase-treated or sham-treated *A. fumigatus* 13073 conidia were added to the wells and incubated at 37°C for 1 hour. Nonadherent cells were removed by washing three times with 200 μ l PBS-T. After washing, cells were fixed with 300 μ l in PBS/2.5% glutaraldehyde at room temperature for 1 hour. Cells were visualized by light microscopy using an Olympus VANOX AHBS3

microscope at 1000X magnification. Bright field images were captured with a Sony 950 camera using Northern Eclipse imaging software. Five random fields for each well were counted. Each condition was performed in triplicate and three independent experiments were performed. Reported results are the mean \pm standard deviation of the three independent experiments. The Student t-test was used to assess the difference in binding of sialidase-treated and untreated conidia to fibronectin-coated wells.

Cell culture of A549 lung epithelial and J774 macrophage cell lines

The mouse macrophage (J774) and type II pneumocyte (A549) cell lines were obtained from ATCC and maintained on RPMI 1640 medium containing 10% fetal calf serum (FCS) (v/v) (Canadian Life Technologies, Burlington, ON) at 37°C in a humidified 5% CO₂ incubator. A549 cells were passaged by removing media from the plate, washing once with PBS, incubating with 2.5% trypsin-EDTA for 5 minutes at 37°C followed by centrifugation and resuspension of the pellet in fresh media. J774 cells were passaged by removing media from the plate, adding PBS, scraping the plate with a rubber-tipped glass rod followed by centrifugation and resuspension of the pellet in fresh RPMI 1640/10% FCS. Cells were usually diluted 1/10 to 1/20 during passages, which were performed three times per week.

Uptake assay

A549 cells were seeded at 2.5×10^5 cells/well in 1 ml of RPMI 1640/10% FCS on 12 mm number 1 coverslips in 24-well plates (Fisher Scientific, Ottawa,

ON) and grown for 16 hours at 37°C. J774 cells were seeded at 2.5×10^5 cells/well for 2 hours at 37°C in the same media. After growth, cells were blocked with 1 ml RPMI 1640/10% FCS/0.5% BSA at 37°C for 1 hour. After blocking, cells were infected with 2×10^6 CCCP-inactivated, GFP-expressing *A. fumigatus* 13073 conidia either treated or untreated with sialidase (as described above) in 1 ml RPMI 1640/10% FCS and allowed to incubate at 37°C for 1 or 3 hours for J774 and A549 cells, respectively. After incubation, plates were put on ice to stop phagocytosis and nonadherent conidia were removed by washing the wells 3 times with 1 ml PBS-T. Extracellular spores were labelled using a rabbit anti-*Aspergillus* primary antibody raised against *A. fumigatus* cell wall components, which was previously developed in our laboratory (Wasylnka & Moore, 2002). Primary antibody was diluted 1:75 in PBS/10% goat serum and 1 ml was added to each well and allowed to incubate on ice for 1 hour. After 1 hour, wells were washed 3 times with 1 ml PBS and cells were incubated with 1 ml goat anti-rabbit secondary antibody conjugated with Alexa Fluor 594 (Molecular Probes) diluted 1:575 in PBS/10% goat serum on ice for 45 minutes. After that, cells were washed 3 times with 1 ml PBS and fixed with 500 μ l PBS/4% paraformaldehyde on ice for 1 hour. After 1 hour, coverslips with cells were once again washed 3 times with 1 ml PBS, mounted onto slides with ProLong Antifade mounting media (Molecular Probes), sealed and stored at 5°C until analysis. Samples were analysed with a Zeiss Axioplan 2 microscope (Carl Zeiss, Inc, Thornwood, NY) equipped with epifluorescence filters using a 63X magnification lens. Images were captured with a Sony DXC-950P 3CCD camera

using Eclipse Imaging software and the extent of uptake was assessed by comparing the numbers of extracellular conidia (green and red) to intracellular conidia (green) after merging the green and red channels in Adobe Photoshop (Adobe, Inc., San Jose, CA) (Figure 3.1). Eight random fields for each coverslip were counted. Each condition for each cell type was performed in triplicate and reported as the mean \pm the standard deviation of three independent experiments. The Student t-test was used to assess the difference in uptake between the sialidase treated and untreated conditions. Confocal images were obtained on a Zeiss LSM 410 confocal microscope equipped with a Krypton/Argon laser 488 nm, 568 nm and 647 nm lines. Confocal images were processed using ImageJ software (Abramoff *et al.*, 2004).

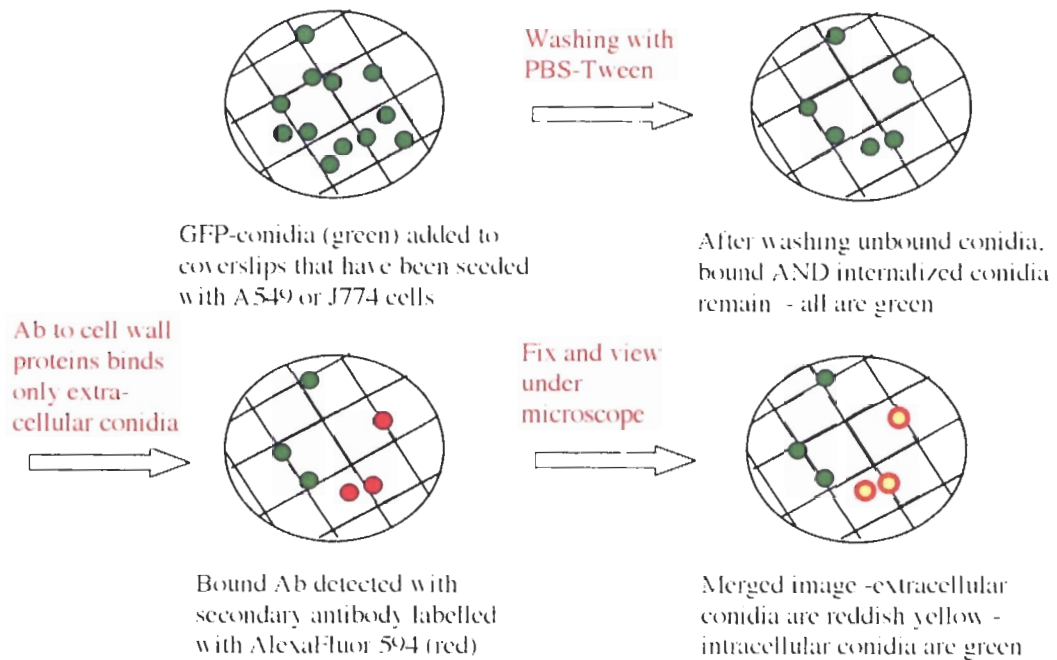


Figure 3.1: Phagocytosis assay method.

Coverslips were seeded with cultured macrophages or type II pneumocytes and infected with either sialidase treated or untreated GFP-expressing conidia. Nonadherent conidia were removed by washing with PBS-T. Next, an antibody against components of the *A. fumigatus* cell wall was added which labeled extracellular spores only. This anti-*A. fumigatus* antibody was then detected with a secondary antibody conjugated with Alexafluor594; therefore, extracellular spores are labelled red. In the merged image, extracellular conidia appear yellow.

3.3 Results

Fibronectin adhesion assay

To evaluate whether sialic acids are important in mediating the binding between *A. fumigatus* conidia and the extracellular matrix protein, fibronectin, sialidase-treated or untreated conidia were incubated in fibronectin-coated wells and the difference in the levels of adherent conidia was assessed by light microscopy. Sialidase treatment of conidia reduced the level of binding of conidia to fibronectin-coated wells by greater than 65% compared to untreated spores. This indicated that adhesion of *A. fumigatus* conidia is mediated

primarily by the binding of conidial sialic acid to fibronectin, likely to the GAG-binding domain of fibronectin (Wasylnka & Moore, 2000) (Figure 3.2).

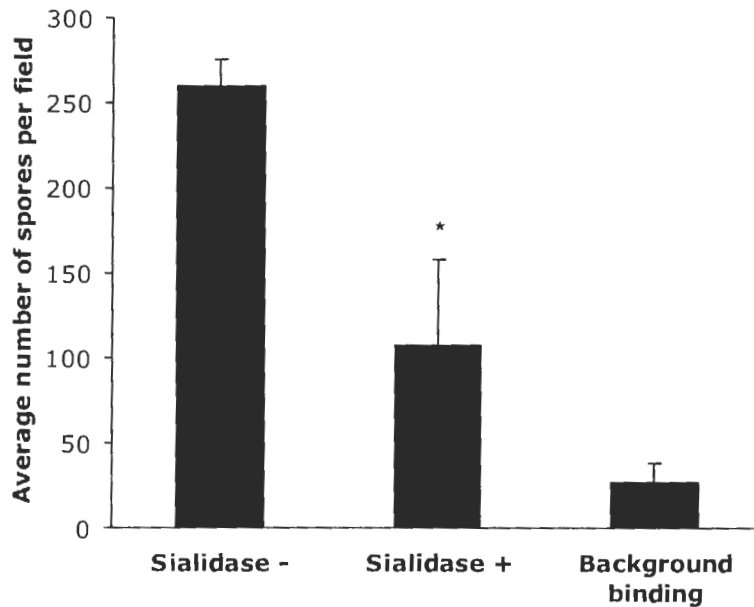


Figure 3.2: Adhesion of conidia to fibronectin is decreased by 65% after sialidase treatment.

Sialidase-treated and sham-treated conidia were incubated in fibronectin-coated wells and the difference in the number of conidia binding to the wells was compared. Background binding was determined in wells that contained no fibronectin, but were otherwise treated identically to the other samples. The decrease in adherent conidia in the sialidase-treated sample compared to control was significant at $p < 0.001$ *. Data represent the mean \pm standard deviation of three independent experiments.

Phagocytosis assays

To assess whether conidial sialic acids are important in the uptake of conidia by cultured cells, sialidase-treated or untreated GFP-expressing *A. fumigatus* conidia were incubated with either a murine macrophage cell line (J774) or a human lung epithelial cell line (A549) and phagocytosis was allowed to proceed. Figure 3.3 shows representative micrographs of the uptake assay. In the merged images, it is apparent that sialidase treatment reduced

internalization of conidia in both cell lines. The data from the three independent experiments was collected and converted to percent uptake and this is shown in Figure 3.4. Enzymatic removal of sialic acids from conidia significantly reduced their internalization by both J774 cells (by 33%) and A549 cells (by 53%).

Interestingly, although conidiospore uptake was reduced with sialidase treatment, the number of adherent conidia binding to J774 cells was not significantly different (Figure 3.5). On the other hand, although uptake was significantly decreased by sialidase treatment in A549 cells, the number of adherent conidia significantly *increased* when they were treated with sialidase (Figure 3.5)

Therefore, no correlation was found between the extent of binding of conidia and their internalization by cultured pneumocytes. These data suggest that sialylated ligands on *A. fumigatus* are involved in endocytosis, but that adhesion to host cells involves other fungal molecules as well.

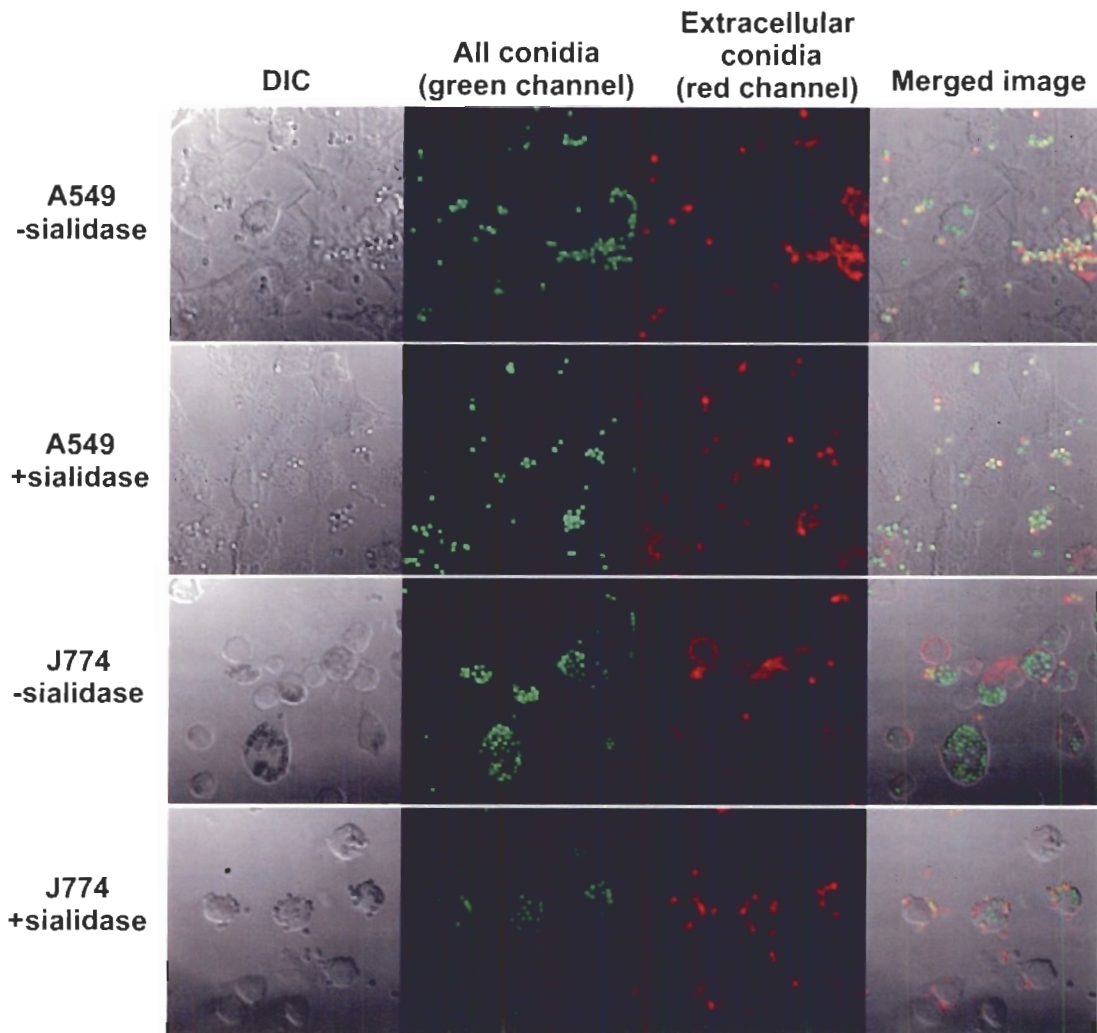


Figure 3.3: Level of phagocytosis by A549 and J774 cells is decreased upon sialidase treatment of conidia.

Sialidase-treated or untreated GFP-expressing *A. fumigatus* conidia were incubated with either a lung epithelial cell line (A549) or a macrophage cell line (J774) and phagocytosis was allowed to proceed for 1 and 3 hours, respectively. Extracellular conidia were then labelled with an anti-*A. fumigatus* antibody and visualized by a secondary antibody conjugated with Alexa Fluor 594. Intracellular spores appear green only, due to GFP fluorescence, whereas, extracellular spores appear yellowish red in the merged image. Level of uptake was determined by subtracting the number of extracellular conidia (green and red (yellowish red)) from the total conidia (green only). Differences in uptake were determined by comparing the amount of uptake in the sialidase treated condition compared to the untreated condition for each cell line. From left to right in each panel are the differential interference contrast microscopy images, green channel image, red channel image and overlay of the three.

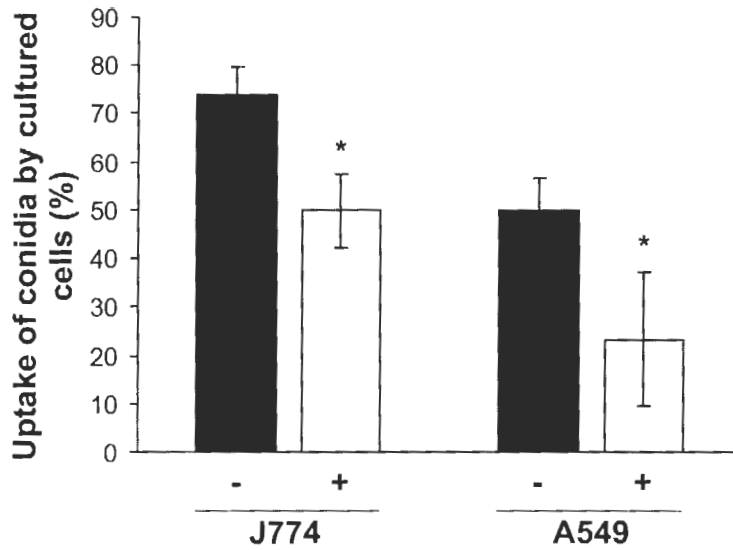


Figure 3.4: Uptake of *A. fumigatus* conidia by cultured cells is decreased upon sialidase treatment.

Conidial uptake for each cell type with (+, white bar) or without (-, black bar) sialidase treatment was assessed microscopically by subtracting extracellular conidia from total conidia in 8 fields in triplicate. The percent uptake from three independent experiments \pm standard deviation is shown. Sialidase-mediated removal of conidial sialic acids significantly decreased internalization by both J774 and A549 cells compared to the untreated control at $p < 0.05$ (*).

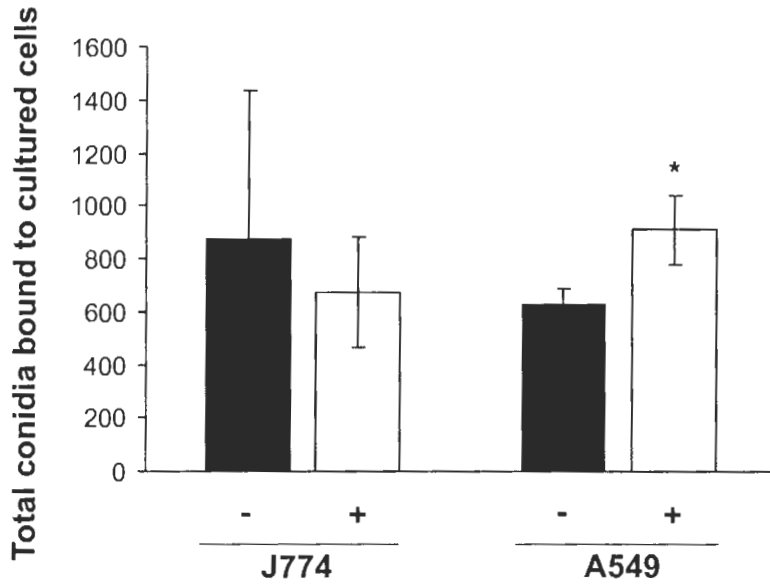


Figure 3.5: Total *A. fumigatus* conidia bound to cultured cells.

Total bound conidia to each cell type with (+, white bar) or without (-, black bar) sialidase treatment was assessed microscopically in 8 fields per experiment in triplicate. Total bound conidia from three independent experiments \pm standard deviation is shown. Binding of *A. fumigatus* conidia to A549 cells was significantly increased after sialidase treatment at $p < 0.05$ (*).

3.4 Discussion

Microbial infection is a complex process involving a multitude of factors. It has been divided into four stages: adhesion of the microbe to the host, invasion of the host, growth and multiplication within the organism, interference with host defence mechanisms and damage to host tissue (Sakarya & Oncu, 2003). The initial contact between the host cell and the microbe was found to involve biophysical and biochemical interaction mediated by host cell receptors recognized by microbial adhesion molecules (Finlay & Falkow, 1997). Adhesion in bacteria is well characterized; however, little is known about this process in fungi. In the present study, sialidase-treatment reduced binding of *A. fumigatus* conidia to fibronectin by greater than 65%, confirming our hypothesis that sialic

acids mediate the adhesion to fibronectin. Conidia likely bound to the positively-charged GAG-binding domain of fibronectin since this domain was previously found to play an important role in conidial binding (Wasylnka & Moore, 2000). Given that there are increased levels of fibronectin in lung tissue upon injury (Torikata *et al.*, 1985), and that lung injury is a known risk factor associated with IA (Bodey & Vartivarian, 1989), conidial surface sialic acids may play important roles during the initial adhesion step of infection *in vivo*.

Phagocytosis assays revealed that sialidase-treatment of *A. fumigatus*-GFP conidia decreased uptake by both the J774 macrophage and A549 lung epithelial cell lines by 33% and 53%, respectively. This was unexpected because research on other fungal species, namely *C. neoformans*, *S. schenckii* and *F. pedrosoi*, has shown that removal of fungal sialic acids *increased* their uptake or association with phagocytes. Increased uptake was attributed to the exposure of galactose, the sub-terminal carbohydrate that is recognized by galactose receptors on the phagocyte surface (Oda *et al.*, 1983; Rodrigues *et al.*, 1997; Alviano *et al.*, 2004). In contrast, our results suggest that cell surface sialic acids on *A. fumigatus* may not be involved in evading host innate immune responses as has been proposed for other fungal pathogens. In addition to galactose receptors, macrophages have many other receptors on their cell surface that mediate cell-cell interactions (Linehan *et al.*, 2000). Among these is sialoadhesin (Linehan *et al.*, 2000). Sialoadhesin is part of a family of sialic acid-binding immunoglobulin-like lectins (siglecs) and is found exclusively on macrophages (Angata & Brinkman-Van der Linden, 2002). Sialoadhesin

specifically recognizes α 2,3- and α 2,6-linked Neu5Ac and to a lesser extent, α 2,8-linked Neu5Ac (Angata & Brinkman-Van der Linden, 2002). The molecular basis for sialic acid recognition has been elucidated by X-ray crystallography and nuclear magnetic resonance spectroscopy. Sialoadhesin forms important hydrogen bonds with the glycerol side chain of Neu5Ac, specifically, with the hydroxyl groups on carbons 7, 8 and 9 (May *et al.*, 1998), consequently, sialoadhesin does not recognize 9-O-acetylated sialic acids (Kelm *et al.*, 1994; Shi *et al.*, 1996; Kelm *et al.*, 1998). Further support for the importance of the glycerol side chain was obtained from experiments that shortened the glycerol side chain using mild periodate treatment (Reuter *et al.*, 1989). This treatment prevented sialic acid binding to sialoadhesin thereby demonstrating the importance of the glycerol side chain in the binding of sialic acid by sialoadhesin (Collins *et al.*, 1997). Moreover, sialoadhesin does not recognize Neu5Gc, a common sialic acid derivative in which the N-acetyl group located on carbon 5 is hydroxylated (Kelm *et al.*, 1994; Kelm *et al.*, 1998). Previous studies on *C. neoformans*, *S. schenckii* and *F. pedrosoi* found that all of these fungi possessed modified sialic acids on their surface: *S. schenckii* was found to have Neu5Gc (Oda *et al.*, 1983) and both *C. neoformans* and *F. pedrosoi* were found to have Neu5,9Ac₂ on their surface (Rodrigues *et al.*, 1997; Alviano *et al.*, 2004). Hence, binding of these fungi to macrophage sialoadhesin would be expected to be negligible, and their recognition by macrophages must be mediated by ligands other than sialic acid. In other words, the modified sialic acids reduced the extent of phagocytosis thereby pointing to a role for sialic acid O-acetylation in

pathogenesis in these organisms. In *A. fumigatus*, sialidase-treatment also exposes galactose residues that are potentially recognized by macrophages via their galactose receptors (Linehan *et al.*, 2000); however, it appears that sialic acid recognition and uptake mediated by sialoadhesin dominated the interaction between conidia and macrophages. Consequently, removal of conidial sialic acids reduced uptake.

Pathogens have developed many strategies to survive and thrive in the host. One strategy is to invade host cells, which is believed to aid in the evasion of host defenses. For example, *Listeria monocytogenes* is phagocytosed by macrophages. After internalization, *L. monocytogenes* degrades the phagosome and has access to the cytosol, which is rich in nutrients (Meresse *et al.*, 1999). The yeast pathogen, *Candida albicans* is taken up into phagolysosomes of macrophages, *C. albicans* develops germ tubes, escapes and destroys the cell (Kaposzta *et al.*, 1999). In contrast, in the brain, *C. albicans* invades and escapes endothelial cells by transcytosis thereby leaving the cell intact (Jong *et al.*, 2001). Previous findings in our laboratory indicate that *A. fumigatus* is able to survive and germinate in the phagolysosomes of A549 cells thereby perhaps representing a strategy to evade the host immune response (Wasylnka & Moore, 2003). Furthermore, we found that this uptake of conidia was specific as latex beads were not internalized. Moreover, the extent of uptake was not significantly different between live conidia and heat-killed conidia, suggesting that the process is mediated through heat-stable ligands on the fungal surface (such as a carbohydrate) and does not require metabolic activity on the part of *A. fumigatus*.

Removing conidial sialic acids significantly decreased internalization of conidia by A549 cells, suggesting that sialylated adhesins on conidia are recognized by host cell receptors, which then endocytose the conidium. Such internalization may represent a strategy to evade the host immune response. However, a receptor for sialic acid on epithelial cells has not yet been identified. Additionally, we found that we had increased binding of conidia to A549 cells upon sialidase treatment, perhaps because of less repulsion without the negative charge conferred by sialic acid. We therefore conclude that sialylated ligands are specifically recognized by A549 receptors which then mediate conidial uptake, and that the extent of uptake is independent of the overall number of conidia bound to the cell.

4 CONSTRUCTING A SIALIC ACID-DEFICIENT MUTANT OF *ASPERGILLUS FUMIGATUS*

4.1 Introduction

In the foregoing chapters, we have shown that unsubstituted sialic acids are present on the surface of *A. fumigatus* conidiospores and that sialic acids are important in their adhesion to fibronectin, and in their uptake by lung epithelial and macrophage cell lines. In these experiments, sialic acids were removed enzymatically and resynthesis was inhibited by treating conidia with a metabolic inhibitor. To assess the importance of sialic acids in *A. fumigatus* virulence *in vivo*, a viable sialic acid-deficient mutant must be constructed because we have previously shown that *A. fumigatus* has the ability to synthesis sialic acid *de novo* (Wasylnka *et al.*, 2001).

Previous work in our laboratory attempted to identify genes in *A. fumigatus* using the *A. fumigatus* genome database (TIGR: <http://www.tigr.org>) that are homologous to known sialic acid biosynthetic genes in mammals and bacteria. These genes included UDP-GlcNAc 2-epimerase, ManNAc kinase, Neu5Ac-9-P synthetase and CMP-Neu5Ac synthetase. Unfortunately, none of these genes matched sequences in the *A. fumigatus* genome. However, we did identify a sequence in the *A. fumigatus* genome that was homologous to the mammalian CMP-sialic acid transporter (CST). CST functions to transport CMP-sialic acid from the cytosol to the Golgi for incorporation into glycoconjugates (Eckhardt *et*

al., 1998). This sequence also shares sequence similarity to the UDP-galactose transporter (UGT), which transports UDP-galactose from the cytosol to the Golgi (Hirschberg *et al.*, 1998), and in subsequent annotation of the *A. fumigatus* genome, this sequence has been labelled as UGT (Preliminary sequence data was obtained from The Institute for Genomic Research website at <http://www.tigr.org>). Strains of both mammalian and bacterial cells with defective CST and UGT have been studied. UGT-deficient Chinese hamster ovary (CHO) cells were unable to transport UDP-galactose into the Golgi and consequently incorporation of galactose into glycoconjugates was blocked (Deutscher & Hirschberg, 1986). Because glycosylation occurs in a sequential fashion with the product of one step serving as the substrate for the next step (Alberts *et al.*, 2002), and, since sialic acid is usually linked to a sub-terminal galactose (Varki, 1998), sialic acid was not added to those glycoconjugates (Deutscher & Hirschberg, 1986). CST-deficient CHO cell mutants have also been constructed and these were found to be deficient in surface sialylation (Deutscher *et al.*, 1984). Transport of other nucleotide sugars was unaffected in the UGT- and CST-deficient mutants, sialic acid biosynthesis was intact and these mutants were viable. Together, this data indicated that deletion of the gene encoding either the CST or UGT in *A. fumigatus* would lead to a viable mutant that was deficient in cell surface sialylation. Therefore, the objective of this research was to create a sialic acid-deficient mutant of *A. fumigatus* by disrupting the CMP-sialic acid/UDP-galactose transporter by insertional mutagenesis, and to examine the phenotype of putative transformants. The

ultimate goal is to evaluate the mutant alongside the parental and a rescued strain in a mouse model of invasive aspergillosis.

4.2 Materials and methods

Strains and growth conditions

A. fumigatus strain ATCC 13073 (American Type Culture Collection) was used in this study. Fungi were grown on MYPD agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 0.5% dextrose) for 3 days at 37°C or 5 days at 28°C until conidia were fully mature. Once fully mature, conidia were harvested by adding PBS-T directly onto the plate and scraping with a sterile cotton swab until conidia were in suspension. The conidial suspension was then vortexed vigorously to break apart chains of conidia and filtered through sterile glass wool to remove hyphae. Conidia were then washed 3 times with PBS, resuspended in PBS and counted with a haemocytometer.

Construction of the transformation plasmid, pMW

Standard molecular biology techniques were performed as described by Sambrook et al. (Sambrook *et al.*, 1989). Plasmids were propagated in *E. coli* DH5 α (Life Technologies, Gaithersburg, MD). Preparation of genomic DNA from *A. fumigatus* was performed using the phenol-chloroform method (Sambrook *et al.*, 1989) or using the Genomic DNA preparation kit from Fermentas (Burlington, ON). Plasmid preparation was performed using the Plasmid Maxi kit according to the manufacturers directions (Qiagen, Mississauga, ON). PCR amplification of upstream and downstream flanking fragments of the *A. fumigatus* *cst* gene was

accomplished using the following PCR reagents: 100 pmol SAT primers (described below), 2 mM dNTPs (Invitrogen, Burlington, ON), 2.5 units Taq DNA polymerase (Qiagen), 10 µg *A. fumigatus* genomic DNA, 5 µl 10X Taq buffer in a total reaction volume of 50 µl. The following temperature program for amplification was used: a pre-dwell period at 94°C for 2 minutes followed by 30 cycles of template denaturing at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 90 seconds, followed by a post-dwell period at 72°C for 10 minutes. PCR product clean-up was performed using the Qiaquick PCR purification kit (Qiagen) and agarose gel extraction of DNA was performed using the Qiaquick gel extraction kit (Qiagen). Custom primers were produced by Invitrogen.

The putative CMP-sialic acid transporter gene (*cst*) sequence was obtained from the *Aspergillus fumigatus* genome project (http://www.sanger.ac.uk/Projects/A_fumigatus/) funded by The Wellcome Trust Sanger Institute (Cambridge, UK). Based on this sequence, one set of primers was designed to amplify an 1150 bp fragment (F1) upstream of the *cst* gene and another set of primers was designed to amplify a fragment consisting of the final 1000 bp of the 3' end of the gene and 800 bp downstream of the gene (F2). The primers 5'-CTCGAGCTCGAGCCATGCTCCACTGCAAGAT (SAT1) and 5'-ATCGATATCGATAACAGGAAAGGGGAAAGCTC (SAT2) were used to amplify the 1150 bp fragment (F1) and included restriction enzyme recognition sites (underlined) for *Xho*I and *Cla*I, respectively. The primers 5'-CTGCAGCTGCAGATTCTCGATAAAGCTGCCGA (SAT3) and 5'-

GGATCCGGATCCCATGCTGTCCCACCTTCTCTGC (SAT4) were used to amplify a 1820 bp fragment (F2) and had restriction sites (underlined) for *PstI* and *BamHI*, respectively. F1 was cut with *XhoI* and *Clal* and ligated into pID620 yielding pMW-1. pID620 was a kind gift from Dr. D. Holden and is based on the plasmid Bluescript SK+ with an *E. coli hygromycin phosphotransferase (hph)* cassette, encoding resistance to the antibiotic hygromycin, flanked by the *gpdA* promoter and the *trpC* terminator (both from *A. nidulans*) inserted into the *EcoRI* restriction site of SK+. F2 was cut with *PstI* and *BamHI* and ligated into pMW-1, generating pMW. Correct insertion of the 1150 bp and 1820 bp fragments into pID620 flanking the *hph* gene was confirmed by agarose gel electrophoresis and DNA sequencing (The CMMT / CFRI DNA Sequencing Core Facility, University of British Columbia, Vancouver, BC) (data not shown). Plasmid construction is diagrammed in Figure 4.1

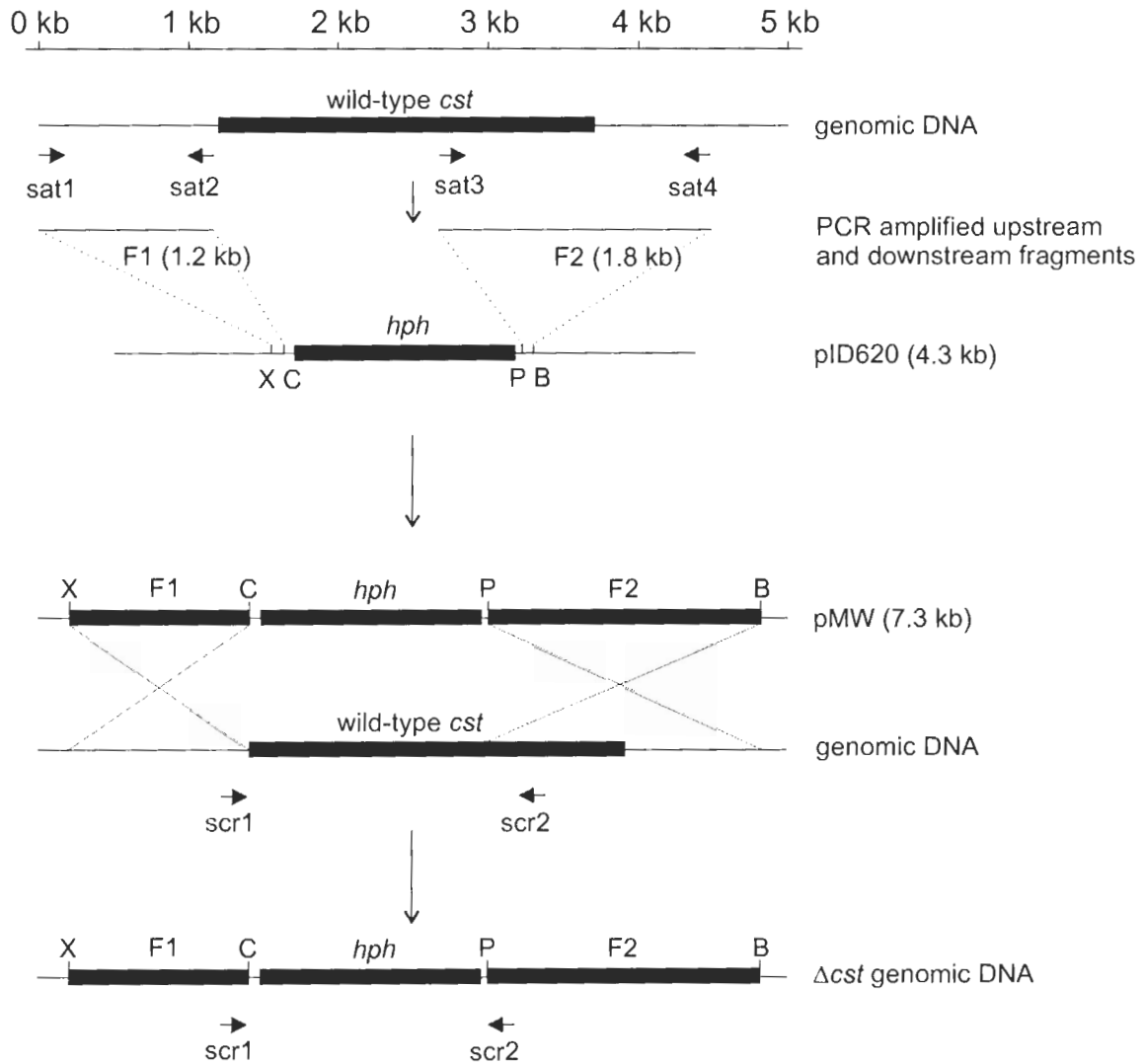


Figure 4.1: Disruption of the *cst* gene by homologous recombination.

Map shows the binding sites of the various primers and where the amplified fragments are inserted into pID620 to yield pMW. pMW is then used to replace the *cst* gene by homologous recombination to yield the Δ *cst* strain. X, C, P and B represents XhoI, Clal, PstI and BamHI restriction sites, respectively. Note that the map shows that F1 and F2 were ligated into pID620 in one step; however, this was actually a 2-step process with F1 ligated before F2. The binding sites of scr1 and scr2 in the wild type and Δ *cst* mutant strains are shown. These primers were used to determine whether successful replacement of the wild type gene by the transformation construct occurred.

Transformation of *A. fumigatus* conidia with pMW by electroporation

A. fumigatus conidia were harvested as described above and 5×10^9 conidia were used to inoculate 250 ml YG media (0.5% yeast extract, 2% D-glucose). Conidia were allowed to swell overnight on a rotary shaker at 100 rpm at room temperature. After 16 to 20 hours, the appearance of irregular-shaped conidia, which was assessed by light microscopy, indicated that conidia were sufficiently swollen and ready for electroporation. Electrocompetent conidia were then collected by centrifugation at 4°C, washed with 200 ml ice-cold sterile water and resuspended in 20 ml YED media, pH 8 (1% yeast extract, 1% glucose, 20 mM HEPES) and incubated for 1 hour on a rotary shaker at 100 rpm at 30°C. Spores were collected by centrifugation and resuspended in 800 µl ice-cold electroporation buffer (EB) (10 mM Tris-HCl pH 7.5, 270 mM sucrose, 1 mM lithium-acetate) and kept on ice. A 50 µl aliquot of conidia was mixed with 1-2 µg of *NotI*-linearized pMW DNA in a total volume of 10 µl, transferred to a 0.2 cm electroporation cuvette (BioRad Laboratories, Inc., Mississauga, ON) and kept on ice for 15 minutes, after which a pulse was applied with a BioRad Gene Pulser electroporation device (settings 1 kV, 400 W, 25 µF). Following the pulse, 1 ml ice-cold YED was added to the conidial suspension and the solution was transferred to a 15 ml plastic tube and kept on ice for an additional 15 minutes. Conidia were then allowed to recover by incubating them for 90 minutes on a rotary shaker at 100 rpm at 30°C. Conidia were then plated on MYPD agar plates, left overnight at room temperature and overlaid the next day with 10 ml MYPD agar supplemented with 200 µg/ml hygromycin B (Invivogen, San Diego,

CA). Hygromycin-resistant transformants were observed after 3 days at 37°C and colonies were transferred aseptically to individual plates of MYPD agar using a sterile inoculating loop.

PCR screening to analyze transformants

Selected hygromycin-resistant transformants were screened by PCR. Screening primers 5'-CCTGACGTTCCGGATCAAAC (scr1) and 5'-GAAGTCGGTAGGCTCACTCG (scr2) were designed to bind the end of F1 and beginning of F2 producing a 1.8 kb fragment if the wild-type gene is present and a 1.6 kb fragment if the transforming DNA had replaced the wild-type gene by double crossover at the *csf* locus. Both fragments would be generated if the transforming DNA integrated ectopically into the genomic DNA (Figure 4.1). PCR screening was accomplished using the following PCR reagents: 100 pmol screening primers, 2 mM dNTPs (Invitrogen), 2.5 units Taq DNA polymerase (Qiagen), 10 µg mutant genomic DNA, 5 µl 10X Taq buffer in a total reaction volume of 50 µl. The following temperature program for amplification was used: a pre-dwell period at 94°C for 2 minutes followed by 30 cycles of template denaturing at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 90 seconds, followed by a post-dwell period at 72°C for 10 minutes.

Southern blot (performed by Linda Pinto)

Southern analysis was carried out on genomic DNA extracted from both the wild type and 212A mutant strains of *A. fumigatus* to confirm insertional disruption of

the putative *cst* gene. Genomic DNA was completely digested with the restriction enzymes *Clal* and *XhoI* and separated by electrophoresis on a 0.8% agarose gel. Separated restriction digested DNA was then transferred to a Hybond XL membrane (Amersham, Baie d'Urfe, QB) using standard techniques (Koetsier *et al.*, 1993). Probes to the disrupted *cst* gene were constructed by PCR using primers 5'-GATGTAGGAGGGCGTGGATA (*hph*probefor) and 5'-ATAGGTCAGGCTCTCGCTGA (*hph*proberev), which amplify a 150 bp fragment to probe the *hph* gene, and 5'-CAAGTGGCGGTTATTTTCGAT (*frag2105for*) and 5'-CGACGTCAAGCTCGTTCATA (*frag2149rev*), which amplify a 310 bp fragment to probe the 5' end of F2. These probes were ³²P labelled using deoxycytidine 5'-[α-³²P]triphosphate in the dNTP mix during PCR amplification of the probes.

Sequencing of putative transformants

Transformants that produced a single band of 1.6 kb after amplification using screening primers, *scr1* and *scr2*, were sequenced (Macrogen DNA Sequencing, Seoul, Korea) to confirm that wild type DNA was successfully replaced. For sequencing, a 6272 bp fragment, which included the disrupted *cst* gene, was amplified using primers *scrAfor* and *scrBrev*. The following PCR reagents were used in the amplification of the 6272 bp fragment that was sent for sequencing: 2 µl dimethylsulfoxide, 10 mM dNTPs, 260 ng mutant genomic DNA, 200 ng of both forward and reverse primers, 5 units of Pfu ultra high fidelity DNA polymerase (Stratagene, La Jolla, CA), 7.5 µl Pfu Ultra 10X buffer in a total volume of 75 µl. The following temperature program was used for amplification:

a pre-dwell of 92°C for minutes followed by 9 cycles consisting of denaturing at 92°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 68°C for 12 minutes. After the initial 9 cycles, the following temperature program was used: denaturing at 92°C for 10 seconds, annealing at 60°C for 30 seconds and extension at 68°C for 12 minutes plus 10 seconds per cycle for 19 cycles. Once the 6272 bp fragment was amplified, it was sent for sequencing along with several primers spanning the entire fragment to ensure adequate coverage of the sequence. The primers and their binding sites are listed in Table 4.1.

Table 4.1: Primers used in sequencing analysis of mutant genomic DNA

Primer name	Primer sequence	Location relative to <i>cst</i> gene
scafor	5'-ctgaccaggggttcaatgctt	1965 bp upstream
sat1	5'-cgatgctccactgcaagat	1312 bp upstream
561for	5'-gcaaaggttgctttccatgt	758 bp upstream
screen1	5'-ggactgcaaggcctagttg	213 bp upstream
screen2	5'-gaagtcggtaggctcactcg	within gene 855 bp from 3' end
frag2p1	5'-attctcgataaagctgccga	in <i>hph</i> cassette
833for	5'-agcttggtgatccggagttg	within gene 157 bp from 3' end
84for	5'-gcgcttcaaggtcgctctatc	640 bp downstream
sat4	5'-atgctgtcccacttctctgg	815 bp downstream
scribrev*	5'-gtggtcgatcaggttgaggt	1806 bp downstream

*Used only for amplification of the fragment containing disrupted *cst*

Lectin binding assay to assess the phenotype of putative transformants

Lectin binding assays using the sialic acid-specific lectin, *Sambucus nigra* agglutinin (SNA) were performed to determine whether surface sialic acids were present on the *A. fumigatus* hygromycin-resistant transformants. Conidia (1×10^7) from hygromycin-resistant *A. fumigatus* transformants and wild type were blocked with 45 μ l PBS/10% goat serum for 1 hour at room temperature. After 1 hour, 5 μ l SNA was added for a final lectin concentration of 200 μ g/ml and

allowed to incubate for 1 hour at room temperature. Conidia were then washed 3 times with PBS and bound lectin was detected by incubation with 25 μ l of 1% Streptavidin-Oregon green (Molecular Probes) in PBS/10% goat serum for 1 hour at room temperature. After incubation, samples were washed 3 times with PBS, resuspended in an appropriate amount of PBS and mounted on glass slides. Samples were viewed with an Olympus VANOX AHBS3 microscope equipped with epifluorescence at 1000X magnification. Bright field and fluorescence images were captured with a Sony 950 camera using Northern Eclipse imaging software.

HPLC analysis to assess transformants

Hygromycin-resistant transformants were also assessed by HPLC analysis to quantify the amount of conidial sialic acids that were released by mild acid treatment. *A. fumigatus* wild-type, and mutants 21-2A and 21-3D were grown on MYPD and minimal media, the conidia were harvested in PBS-T, washed 3 times in PBS and 5×10^9 conidia in PBS were transferred to 50 ml plastic vials. Conidia were centrifuged to remove PBS and resuspended in 30 ml of 2.5 M acetic acid to release terminal sialic acids, as described in chapter 2. After hydrolysis for 5 hours at 90°C, 25 ml of supernatant was collected and freeze-dried to remove acetic acid. Dried hydrolysate was resuspended in 80 μ l H₂O and 25 μ l of that solution was derivatized with 1,2-diaminomethylenedioxybenzene (DMB) and analyzed by HPLC, as described in Chapter 2.

4.3 Results

Constructing the transformation plasmid pMW

To disrupt the *cst* gene in *A. fumigatus*, the transformation plasmid pMW was constructed by amplifying the sequences upstream and downstream of the *cst* gene and inserting those two fragments into pID620 flanking the *hph* gene. Successful amplifications and insertions were confirmed by gel electrophoresis (Figure 4.2) and by sequencing (data not shown).

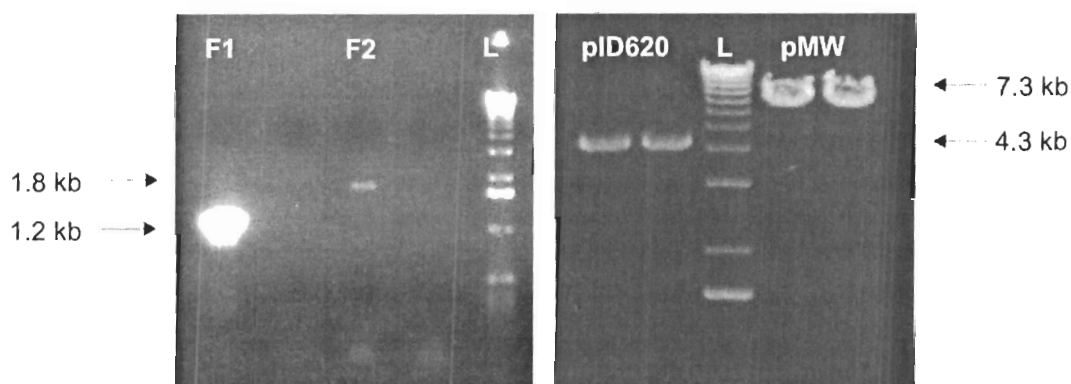


Figure 4.2: Confirmation of correct insertion of fragments F1 and F2 to create pMW.

To assess correct amplification of F1 and F2 fragments and correct insertion into pID620, agarose gels were run to ensure that DNA fragments of the correct size were present. On the left, the presence of bands at 1.2 kb and 1.8 kb corresponding to F1 and F2, respectively, indicated that the primers sat1, 2, 3, and 4 correctly amplified F1 and F2, respectively. On the right, insertion of F1 and F2 into pID620 was confirmed by the presence of a band at 7.3 kb, corresponding to the size of pID620 (4.3 kb) with F1 (1.2 kb) and F2 (1.8 kb) inserted. L represents the DNA ladder, with the brightest band in the ladder representing the 1.6 kb marker.

Transformation of *A. fumigatus* conidia by pMW

A. fumigatus was transformed by electroporation using pMW. Many hygromycin-resistant transformants were identified and were further evaluated by lectin binding, PCR and HPLC. The hygromycin-resistant transformants that emerged were divided into two groups; hygromycin-resistant transformants obtained in initial transformation experiments were allocated to group 1, and transformants obtained while characterizing group 1 were allocated to group 2. Group 1 was assessed for the presence of surface sialic acids using SNA binding and it was found that one mutant, 8dr1, displayed lower fluorescence than the wild-type control (compare panels B and F in Figure 4.3).

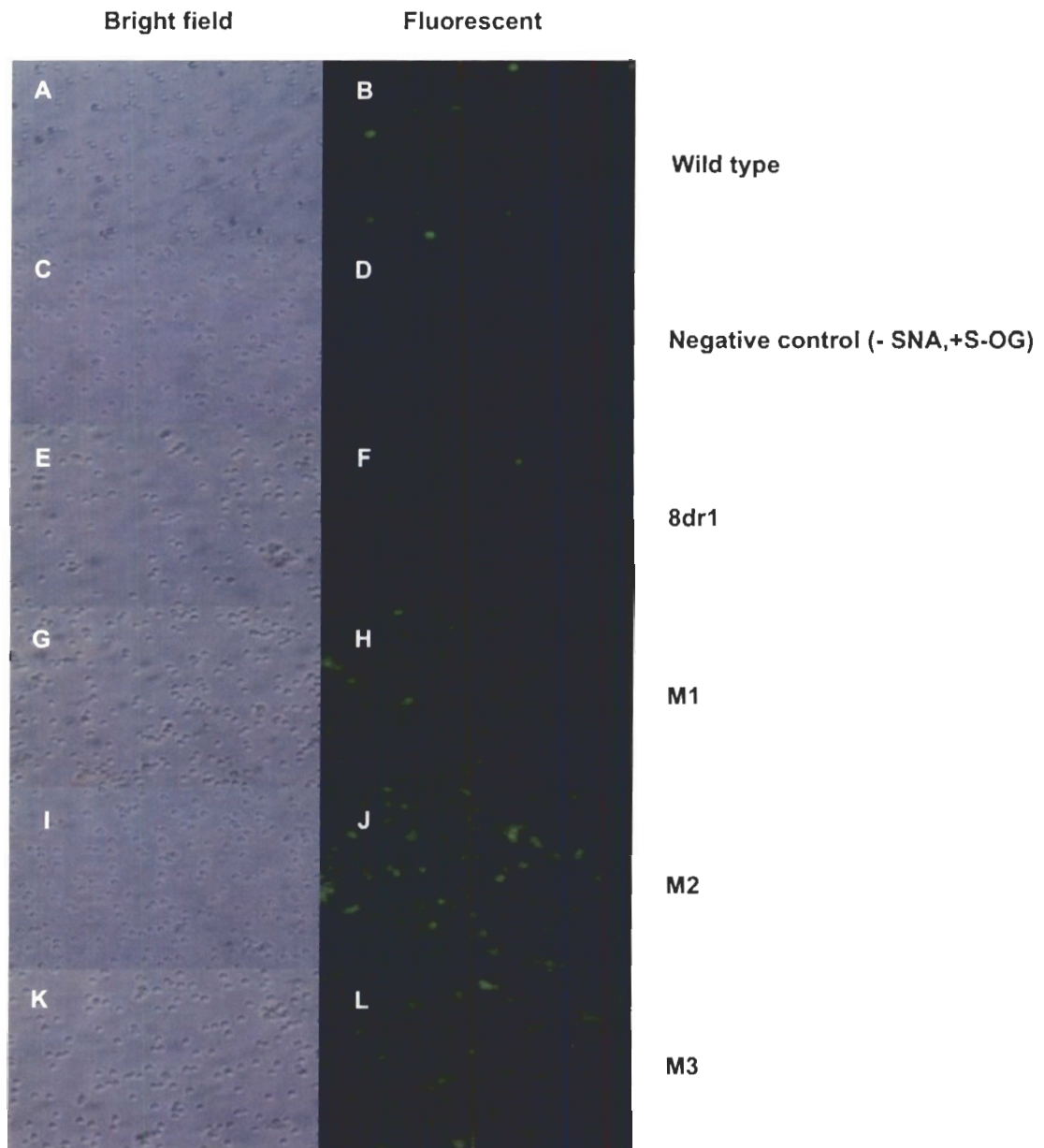


Figure 4.3: Lectin-binding of group 1 mutants.

To evaluate whether hygromycin-resistant transformants obtained by electroporation were deficient in surface sialic acids, a lectin binding assay using SNA was performed. A and B represent the positive control (+ SNA). C and D represent the negative control (- SNA). The bottom 8 panels show hygromycin-resistant transformants. Lectin binding revealed that the transformant in panel E and F was less reactive with SNA compared to wild-type. This transformant was named 8dr1 and was subjected to further PCR analysis. M1, M2 and M3 are transformants obtained that did not display lowered fluorescence and were later found to be ectopic mutants.

Mutant 8dr1 was further analyzed using PCR and screening primers, screen 1 and 2, as described in Methods and Materials. Briefly, the forward primer was designed to bind just upstream of the *cst* gene and the reverse primer bound within the *cst* gene about 1 kb before the 3' end of the gene. If the wild-type gene was present, PCR amplification would produce a 1.8 kb fragment. If the transforming DNA replaced the wild-type *cst* gene by double crossover, PCR amplification would yield a 1.6 kb fragment. Finally, if an ectopic integration of the transforming DNA occurred, PCR amplification would produce both fragments. Unfortunately, PCR of genomic DNA obtained from three colonies of 8dr1 revealed that all isolates were the result of ectopic integration (Figure 4.4). At present, it is not clear why this strain had apparently less SNA-reactive sites on conidia. The biotinylated SNA and S-OG were likely outdated as the wild type fluorescence was relatively weak and the lack of fluorescence of 8dr1 may have been an artefact.

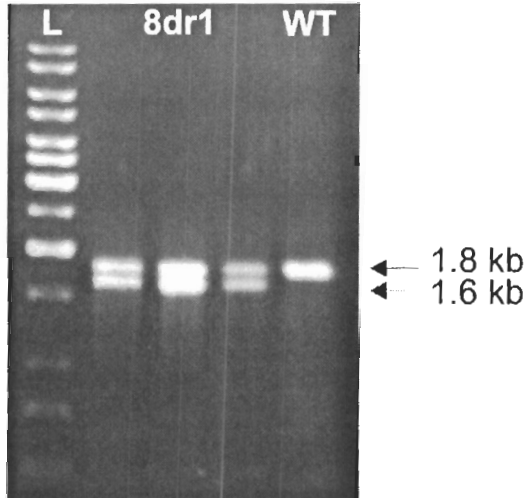


Figure 4.4: 8dr1 is an ectopic transformant.

PCR screening showed that all three isolates of the putative transformant, 8dr1, are the result of ectopic integration. All three isolates produced the wild type band at 1.8 kb as well as the 1.6 kb band characteristic of the integrated pMW. L represents the DNA ladder, WT represents wild-type genomic DNA amplified with screening primers.

Group 2 hygromycin resistant transformants were also assessed for the replacement of the wild type *cst* gene by the transformation construct by PCR using screening primers as described earlier. PCR amplification using screening primers scr1 and scr2 produced a single 1.6 kb band in two hygromycin-resistant transformants (212A and 213D), suggesting that the wild-type *cst* gene was replaced by the transformation construct (Figure 4.5). Furthermore, Southern blot analysis of wild type and 212A confirmed that the *cst* gene was disrupted (data not shown). Finally, sequencing of a 6272 bp fragment and alignment of the mutant and wild type DNA, which included the disrupted *cst* gene as well as flanking regions, confirmed that almost the entire wild type gene was replaced by the transformation construct (Figure 4.6).

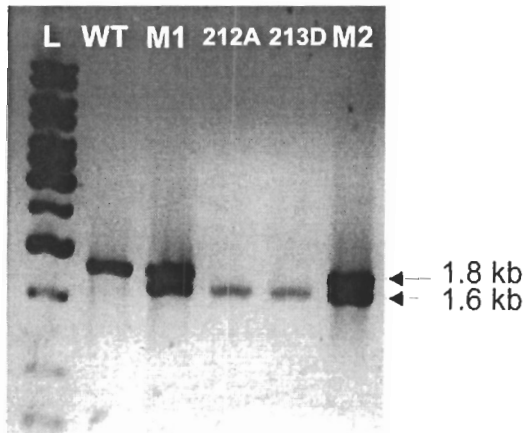


Figure 4.5: PCR screening of group 2 transformants.

Hygromycin-resistant transformants from group 2 were screened by PCR as before. Two transformants, 212A and 213D, both produced single bands of 1.6 kb, consistent with a targeted replacement of the *cst* gene by the transformation construct. L represents the DNA ladder, WT represents wild-type genomic DNA amplified with screening primers, M1 and M2 represent ectopic mutants obtained in the same transformation experiment.

Transformants 212A and 213D were further evaluated by quantifying the amount of surface sialic acids using HPLC. Surprisingly, no significant differences in the level of surface sialylation between the wild type and the *cst* mutants was observed. Peak areas corresponding to the Neu5Ac peak were 1.68×10^6 , 1.64×10^6 and 1.14×10^6 arbitrary fluorescence units for 212A, 213D and wild type, respectively (Figure 4.7). This corresponds to approximately 2×10^5 sialic acid molecules per conidium. This result indicated that either the putative *cst/ugt* gene product does not play a role in the sialylation pathway, or that an alternative pathway exists in *A. fumigatus* to sialylate glycoconjugates.

Figure 4.6: Alignment of the wild type *A. fumigatus* *cst* gene with the disrupted *cst* from mutant 212A.

Alignment of the wild type *cst* gene (**afwt**) \pm 1000 base pairs with the first 5715 base pairs from the 6272 bp fragment amplified from 212A (**mutant**) genomic DNA reveals that almost the entire *cst* coding region is replaced by the transformation construct DNA. The top underline highlights the entire *cst* gene sequence (introns and exons) while the bottom underline represents only the coding region of this gene. Stars represent identical nucleotide bases. F1 and F2 represent fragments 1 and 2, respectively, and *hph* is the hygromycin resistance cassette.

```

mutant      GGCTCGCACACCTTCTTGGGATTAAGTCCGGGGCTGCCACCTCAGCGTCCGTACGTGCA 60
afwt      -----

mutant      GATCAAGGTGCAACCTACCATGACAAGGACGGTACTATTCCTCAGGGTGCTGCTTCTGAT 120
afwt      -----

mutant      GCTGAACAGAAAAACGATCAACTCAAATCGCGCAAGCGTGGCTACTCTGAAAGCCTACCA 180
afwt      -----

mutant      TCCCCCGTACCAGCGGGCATCACCGCTGACCCCGACACTGCCTATACCCGATTCTCA 240
afwt      -----

mutant      CCCCTTCCATCACAGAAACCGCCCCACATCGCCCTCCTCCCGCAGTGCCTCACGCTCAG 300
afwt      -----

mutant      AGAAGGCACTCCAGAAGACGCACAGATAGCAGCGAGGCGATTGCCTTAGACGCAACTGAA 360
afwt      -----

mutant      GAGTCGGGAGAGGCAAATGAGTCCCAATCTGGGCACTTGACTGGAACCAAGCCAGTCGC 420
afwt      -----

mutant      AGTTTAGCTGCTGTACACTAGATAGGTGGTGACATTTGTTGATTTCTTCCCTTATCTT 480
afwt      -----

mutant      GTATCTCGACATCTGGAGCTGCCACTCACTCATGATCTCTCATTACTTTGTTCTTGTTT 540
afwt      -----

mutant      ATATCATGTTTCTCCACGTCATCCCTCCACACCAGGCGGATCATCTCTTATGTCAGAG 600
afwt      -----

mutant      | F1 begin
afwt      CATTGTCGATGCTCACTGCAAGATTGCAACATATACCACATGACTTAAAGTATATTTCT 660
afwt      -----

mutant      TTGAATCTGACAATCCCTTGATACAAGGCATATCATCTCAACATTCACCTGCAATACCC 720
afwt      -----

mutant      CATATAAGGAAGGCAACGACAAGTCTACATTGAATTACGCTGGTTTAGTGACTCTCTTA 780
afwt      -----

mutant      CTTCAGATCTAGACAATTTTGCAACCTAAGTACTACTCTGTGTACAAAGCCGATCGTGT 840
afwt      -----

mutant      ATCACCTAAGGCTCCAAGTCAAGCAAAGCAAGCAATGCATTCACTGGTGGGAAACTCG 900
afwt      -----

mutant      TTCAGTACGCGATTTAATCAGGGTCAGTGTGTAGAACTTCTGTTAAGGATAGTGCACA 960
afwt      -----TCAGGGTCAGTGTGTAGAACTTCTGTTAAGGATAGTGCACA 42
          *****

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F1

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mutant      GGCCACGATCCAGGCTGTACTGCTAGGGTGATGTGGCTTGCCAGCTTGCCAGCGAAGG 1020
afwt       GGCCACGATCCAGGCTGTACTGCTAGGGTGATGTGGCTTGCCAGCTTGCCAGCGAAGG 102
*****

mutant      AGCACCTTCAGACTAAAGTTGGAGGCAGGAGGACCCGACCCATTGTGAGACGAGACTGG 1080
afwt       AGCACCTTCAGACTAAAGTTGGAGGCAGGAGGACCCGACCCATTGTGAGACGAGACTGG 162
*****

mutant      GCTCTGACGTCAGACCCTAAGGTC AAGTCAAGGATTTCAAGTAACAAATTAACGGATA 1140
afwt       GCTCTGACGTCAGACCCTAAGGTC AAGTCAAGGATTTCAAGTAACAAATTAACGGATA 222
*****

mutant      ACAACTAGGTAATAATAATGGCAAAGTTGCTTTCATGTAGTTGATCAATCATGTTAAC 1200
afwt       ACAACTAGGTAATAATAATGGCAAAGTTGCTTTCATGTAGTTGATCAATCATGTTAAC 282
*****

mutant      TGATTCATTGGACTATTTTTCTTTGTGGCTCGTCCAAAAAACTGGTGAGCTGGTGGACT 1260
afwt       TGATTCATTGGACTATTTTTCTTTGTGGCTCGTCCAAAAAACTGGTGAGCTGGTGGACT 342
*****

mutant      TCGGATCATCAGTATTCTGTACTGGCGATCGGTGGACAAGGGGGAGTCAAGGCTCAAGG 1320
afwt       TCGGATCATCAGTATTCTGTACTGGCGATCGGTGGACAAGGGGGAGTCAAGGCTCAAGG 402
*****

mutant      CTCAGTATTATTACCATAGACTTGCAGAGTAGGTAGACCTCTCCGTGGCCCGCACACCCG 1380
afwt       CTCAGTATTATTACCATAGACTTGCAGAGTAGGTAGACCTCTCCGTGGCCCGCACACCCG 462
*****

mutant      CCCAGCTGGTAATTTGGTACCCAGAATCCACACAGAATCAGTGATTGCTCCCGGT 1440
afwt       CCCAGCTGGTAATTTGGTACCCAGAATCCACACAGAATCAGTGATTGCTCCCGGT 522
*****

mutant      GGACTCCTTGCTGAATCTTGAAGTACTCATCGCAGTGGAGGACTCTATTTATGCTGA 1500
afwt      GGACTCCTTGCTGAATCTTGAAGTACTCATCGCAGTGGAGGACTCTATTTATGCTGA 582
*****

mutant      GAACCGCTTGCTGGCCAGGCCATTTGATTCTTGGTCTTCGCTTTTATCTACGCTATCT 1560
afwt      GAACCGCTTGCTGGCCAGGCCATTTGATTCTTGGTCTTCGCTTTTATCTACGCTATCT 642
*****

mutant      GACGTCGTCGTTTTATTCCATCTGCGCCCTTGTGCAGTGTACCCCGGTTTTTCTTT 1620
afwt      GACGTCGTCGTTTTATTCCATCTGCGCCCTTGTGCAGTGTACCCCGGTTTTTCTTT 702
*****

mutant      TAAAATACGCGCTACGAGGGGCTTTTGTCTAGCACGCTGAGGACAAACCGCGTTTTTGT 1680
afwt      TAAAATACGCGCTACGAGGGGCTTTTGTCTAGCACGCTGAGGACAAACCGCGTTTTTGT 762
*****

mutant      CCTGCCCCAGCGGCAGTTTGTGGCGACTGCAAGGCCTAGTTTGAAAAACCGTGAGAGC 1740
afwt      CCTGCCCCAGCGGCAGTTTGTGGCGACTGCAAGGCCTAGTTTGAAAAACCGTGAGAGC 822
*****

mutant      TTTCCCTTTCTGTT-ATCGATAAGCTTGATATCGAATTCGTCGACGTTAACTGGTCC 1799
afwt      TTTCCCTTTCTGTTATCGGCACGCGACAACCTATTTCCGACCTCGGCATTTCT
*****

mutant      CGGTCGGCATCTACTCTAATCCTTTGCCCTCGGACGAGTGTGGGGCGTCGGTTTCACT 1859
afwt      CAACGTCCTTTCTCCGAATAAGAAAACAGGCCAAAATCAGCAACAAC--CTACATCT 940
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *

mutant      ATCGGCGAGTACTTCTAC--ACAGCCATC-GGTCCAGACGGCCGCGCTTCTGCGG--GCG 1914
afwt      TCCCGTGGGTTGCGTTCGGAACACTTGTACGGGCTGGCACCGCATAGTGTTCACA 1000
*   *   *   *   *   *   *   *   *   *   *   *   *   *

mutant      ATTTGTGTACGCCGACAGTCCCGCTCCGGATCGGACGATTGCGTCG---CATCGACCC 1971
afwt      ATGGGGAAACACACTCGGGGCCAGTCTTCAGGGAAGGGAGGTGTTTTGAGACGTTGTTCT 1060
** * * * * * * * * * * * * * * * * * * * * * *

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F1

End F1 | Begin *hph*

hph

mutant	TGCGCCCAAGCTG--CATCATGAAATTGCC-----GTCAACCAAGCTCTGATAGAGTT	2023
afwt	TGGATATTGGTTAGTCTTCTCCTCAGGGCCTCTTGGTTGGACTGCGTGTTCTTTTGA	1120
	** *	
mutant	GGTCAAGACCAATGCGGAGCATATACGCCCGAGGCGCGGCGATCTGCAAGCTCCGGAT	2083
afwt	TCACCGAGTCAGAACTTGAGGTTTAAGTTACTATTACTGACTTCTCCTTTGTAGCTGAC	1180
	* *	
mutant	GCCTC-----CGCTCGAAGTAGCGGCTGCTGCTCCATACAAGCCAACACGGCC	2134
afwt	GGTCCAATATACGGCGTTTGTACTGGTAAGTTT-TTCTTCGACTTGGGGCAAGAGTGACA	1239
	* *	
mutant	TCCAGAAGAGGATG--TTGGCGACCTCGTATTGGGAATCCCCGAACATCGCTCGCTCCA	2192
afwt	CCCAGCTAAACTCTACTCTATAACAGCTTTTGCAATTATCCCGAGTCATGCCTCCCACTG	1299
	**** *	
mutant	GTC AATGACCGCTGTATGCGGCCATTGTCGTCAGGACATTGTTGGAGCCGAAATCCGC	2252
afwt	GA-GGTAAGCGATACCTCAGCTTACCAGCAGTCTTCTCAACGAAGTCACCAAGCTTGGC	1358
	* *	
mutant	ATGCAAGGAGTGCAGGACTTCCGGGCGAGTCTCGGCCAAAGCATCAGCTCATCGAGAGC	2312
afwt	ATTTCTTGACGGTGGCCCTTACGAAAGTTTCGAGATCA--GCTCC--CCCATCGAT-GC	1413
	** *	
mutant	CTGCGGACGGACGCACTGACGGTGTCTGTCATCACAGTTTCCAGTGATACATGGGG	2372
afwt	CTGCA--ACGTGCTCTCTCTTCTGCTGACCGGTACTGTGTTCTCCGGTGACAGTTGGAA	1471
	**** *	
mutant	ATCAGCAATC-----GCGCATATGAAATCACG----CCATGTAGTGTATTGACCGATT	2421
afwt	ACTTGCCATTCTCTGCCAGCTTATACACACTCGCGAACTCTTGAATATATTGCACTTTC	1531
	* *	
mutant	C--CTTGGGTCGAAATGGGCGAACCCTGCTGCTGCTAAGATC--GGCCGACGCGA	2476
afwt	CAACTTGACGGCCGAAACTTCCAGGTGATGTACCAGTTGAAACTCCTCGTGGCTCCAT	1591
	* *	
mutant	TCGCATCCATGGCCTCCGCGACCG-GCTGGAGAACAGCGGGCAGTTCGGTTTCAGGCAGG	2535
afwt	TGTGGCCTTGTCTCTGAAGAGAGCTATACCGCTGCTGAAATGGGGTTTCTGATTCT	1651
	* *	
mutant	TCTTGCAAC--GTGAC-ACCCTGTGCA--CGGCGGAGATGCAATAGGTAGGCTCTCGC	2590
afwt	TCTTCTGCTCGGCGTCTATCTTGTGCAAAATGCCAGATGGAGCCACCAGTAAATCTCACT	1711
	*** *	
mutant	TGAACTCCC AATGTCAAGCACTTCCGGAATCGGGAGCGCGCCGATGCAAAAG-TGCC-G	2648
afwt	TGATCACGAGCCGTGCATCACTTTCCCCCGCTATTGAAAGATGAAAGCTGTAG	1771
	*** *	
mutant	ATAAACATAACGATCTTTGTAGAAACCATCGGCGCAGCTATTTACCCGAGGACAT-ATC	2707
afwt	AGGAAAACGAGCCAATTTGCACAAGCGTTACGCA-ACCTACGAGGGCATCGAAGAGGACC	1830
	* *	
mutant	CACGCCCTCTACATCGAAGCTGAA-AGCACGA--GATTCTTCGCCCTC--CGAGAGCT	2761
afwt	TATTGACGGCTATGCCGCGATTGAACAGCACAGTTGGTCTGCTGGCCACGATTGGGGCT	1890
	* *	
mutant	GCATCAGGTCGGAGA-CGCTGTCGAACTTTTCGATCAGAACTTCTCGACAG-----	2812
afwt	CGCTCGCTTCGGGGCTCGCGGGGTATACTTTGAAAAAGTACTAAAAGACAGCGTAAAA	1950
	** *	
mutant	--ACGTCGGGTGAGTTCAGGCTTTTTCATT----TGGATGCTGGGTAGAATAGGTAA	2865
afwt	CCACCTCTTTGTGGGTGCGTAATGTGCAGTTGCCGTATATTCTTTGTTCCAGCCCTCT	2010
	* *	
mutant	GT-CAGATTGAATCTGAAATAAAGGGAGGAAGG----GCGAAC-----TTAAGAAGGT	2913
afwt	TCATCGGTGCTCTTCTCGATGGGAAAAGGTGCGCCGAAACGGCTTCTTTGAGGGCT	2070
	* *	

hph

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mutant      ATGACCGGTCGT-----CCTTACCTTGCT-----TGACAAACGCACCAAGTTAT 2960
afwt       ATAATTGGGTTGTTGGTCCACTATCATGGTTCAGGCTATTGGGGGAATCACGACCTTT 2130
          ** * **** * *      * * * * *      * *      * * * * * * *
          -----

mutant      CGTGACCAAGCA-GCAGATGATAAT--AATGTCCTCGTTCCTGTCTGTAATAAGAGTC 3017
afwt       TCTGTATCAGCCATGCATACAAGGACGCAAGAAACGTGGCCACTGCTATTAGCATTGTC 2190
          ** * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      ---ACACTTCGAGCGCC--GCCGCTACT--GCTACAAGT----GGGGCTG----ATCT 3060
afwt       TCAGCACTTTGGGGAGTATGTGGTTGTGTGGTTTGAACCTCACAGGGAACGTATGTATCT 2250
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      GACCAGTTGCCTAAATGAACCATCTTGCA--AACGACACAAATTTTGCTCACCGCCT 3118
afwt       CACTCAATCACGAACCTTCGCACATGGTACTGATGTTGACTCCTTCAGTTCATCCTTG 2310
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      GGACG--ACTAAACCAAATAGGCATTATTGTTGAC--CTCCA-----CTAGCT 3164
afwt       GGACTTTCCGGTGTCTTGCCACATTCCTCTATGAGGATTCAGCTTAGATTCCAGCT 2370
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      CCAGCCAAGCCCAAAAAATGCT---CCTTCAAT---ATC-----AG 3199
afwt       CTACTAAAGCTCACAGCATCCGTCCGCCTCAAATTCGGATCGAGCGGTTTGAGAAGGAAG 2430
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      TTAACGTCGACGAA--TTC-----CTGCAG-----ATTCTCGATAAAGCTGC 3239
afwt       CCAGAGCGACGAGACTTCACCTGTCGAGGTCCGACGACGAATTCGATAAAGCTGC 2490
          ** * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      CGAGCACCCCATTCCTGTCTCAAGCTGGGTTGTCCACCTCCAGACCCACGTCCAGGCG 3299
afwt       CGAGCACCCCATTCCTGTCTCAAGCTGGGTTGTCCACCTCCAGACCCACGTCCAGGCG 2550
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      TTGCTAGAATCAGCTCTGGTGCAGTCAAGTGGCGGTTATTTGATAAGCAGTCACGGG 3359
afwt       TTGCTAGAATCAGCTCTGGTGCAGTCAAGTGGCGGTTATTTGATAAGCAGTCACGGG 2610
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      ACGAGTGAGCCTACCGACTTCCAACAAATCCCGACTTCTCCTGTCAATATTCAGCGTT 3419
afwt       ACGAGTGAGCCTACCGACTTCCAACAAATCCCGACTTCTCCTGTCAATATTCAGCGTT 2670
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      GATCCGGTATCCGTGATATCGAGAAGTAATAAGAGTGCCTGACTCCGAGTCATTGCATT 3479
afwt       GATCCGGTATCCGTGATATCGAGAAGTAATAAGAGTGCCTGACTCCGAGTCATTGCATT 2730
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      TCTTTTGGAGGAAAGTGTAGCGAAGTCTTGTACTCTATACCTTTTGTGACCATGGTTG 3539
afwt       TCTTTTGGAGGAAAGTGTAGCGAAGTCTTGTACTCTATACCTTTTGTGACCATGGTTG 2790
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      ATTTTGGTTATTATATCTTCATATAGCGTGAGTCATGGGGGATGCTCCTGATGATGAA 3599
afwt       ATTTTGGTTATTATATCTTCATATAGCGTGAGTCATGGGGGATGCTCCTGATGATGAA 2850
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      TGTACAAGTTTGAATGAATGGATATGAACGAGCTTGACGTCGAGCTTATCGTCCCAGGA 3659
afwt       TGTACAAGTTTGAATGAATGGATATGAACGAGCTTGACGTCGAGCTTATCGTCCCAGGA 2910
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      TATTAACAGCACTTGATTCAATAATTATTTGTTGACTCCTCTGTATCAAGTTTCCCA 3719
afwt       TATTAACAGCACTTGATTCAATAATTATTTGTTGACTCCTCTGTATCAAGTTTCCCA 2970
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      CGTGCTCTTCTCGCTGGGGCATCTATACCATCCGCTGCCCCCTCCCTATTCCGGG 3779
afwt       CGTGCTCTTCTCGCTGGGGCATCTATACCATCCGCTGCCCCCTCCCTATTCCGGG 3030
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

mutant      CTGCTGTTGTTCCACTGAATCTCTTGCACTCATCTCCACGGTTAAAGCTTCTCGGAGC 3839
afwt       CTGCTGTTGTTCCACTGAATCTCTTGCACTCATCTCCACGGTTAAAGCTTCTCGGAGC 3090
          * * * * * * * * * * * * * * * * * * * * * * * * * * *
          -----

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hph

End *hph* | F2 begin

F2

mutant	TTAGCACTGTCTGTCCTTTATCGATCATTATGGGGCGCAAATGCCACCAGCCCTTCTCGA	3899
afwt	TTAGCACTGTCTGTCCTTTATCGATCATTATGGGGCGCAAATGCCACCAGCCCTTCTCGA	3150

mutant	TATCACAGCATAGTGCTTCCAATCAAAGGCCGATGGGTGCAAGGCGGCCCAACTTGGGC	3959
afwt	TATCACAGCATAGTGCTTCCAATCAAAGGCCGATGGGTGCAAGGCGGCCCAACTTGGGC	3210

mutant	TGCCGGCATCAAAGGCAATCATCAATCAGAGACTTATTGAAGGCGCTCAGATCCCTCAGC	4019
afwt	TGCCGGCATCAAAGGCAATCATCAATCAGAGACTTATTGAAGGCGCTCAGATCCCTCAGC	3270

mutant	CTTGGAACAGACCTCGAGGATATGGATCTGGGAACGTCGAGCCCCATAGCCAGCTTGTG	4079
afwt	CTTGGAACAGACCTCGAGGATATGGATCTGGGAACGTCGAGCCCCATAGCCAGCTTGTG	3330

mutant	ATCCGGAGTTGCAATCCTCCGTAGTGATGATCATGAGCTGGACGCCCTTGGTGAGTGGC	4139
afwt	ATCCGGAGTTGCAATCCTCCGTAGTGATGATCATGAGCTGGACGCCCTTGGTGAGTGGC	3390

mutant	TCCGAGTGCACGAGTTCAATACAATGGCGCCGTGAATCAAGTTGACTGAAAGATTCCAG	4199
afwt	TCCGAGTGCACGAGTTCAATACAATGGCGCCGTGAATCAAGTTGACTGAAAGATTCCAG	3450

mutant	ACCTCGAACTACTTGCTCAGTCAGATGAAAGCAACCAGAACCCACTGAGGCAACCGGCTA	4259
afwt	ACCTCGAACTACTTGCTCAGTCAGATGAAAGCAACCAGAACCCACTGAGGCAACCGGCTA	3510

mutant	AGTTAACTTTGCATCAGTCCAGCCTAGAAATGATAGCACGAAGCAGGTATCCCATT	4319
afwt	AGTTAACTTTGCATCAGTCCAGCCTAGAAATGATAGCACGAAGCAGGTATCCCATT	3570

mutant	TCCAGAGTACGCTCATGCCTCGCTATCCTGTATAAGAAGCTAATGAACGCAGCGAGCTC	4379
afwt	TCCAGAGTACGCTCATGCCTCGCTATCCTGTATAAGAAGCTAATGAACGCAGCGAGCTC	3630

mutant	CAATATAACGCCACTCCGAAGCTTAGATTCTAGCTCATCAGATGTAGGCCTAGCGTCGCC	4439
afwt	CAATATAACGCCACTCCGAAGCTTAGATTCTAGCTCATCAGATGTAGGCCTAGCGTCGCC	3690

mutant	TTCTAGTCCACTCAGTCGATTGCAGCCCGGAAAGACGATTATGACACGTCGGGAGCAAGA	4499
afwt	TTCTAGTCCACTCAGTCGATTGCAGCCCGGAAAGACGATTATGACACGTCGGGAGCAAGA	3750

mutant	GTGTCAAGCCACCGGGCGTTGAGACAGTACCACGAAGACCAATTATCGCTTGAGGAAGA	4559
afwt	GTGTCAAGCCACCGGGCGTTGAGACAGTACCACGAAGACCAATTATCGCTTGAGGAAGA	3810

mutant	ATCGCCATCGCACAGAAGTTCTACAGTATCAATTGGTGCAGCACAATGTAAGAAAGCCC	4619
afwt	ATCGCCATCGCACAGAAGTTCTACAGTATCAATTGGTGCAGCACAATGTAAGAAAGCCC	3870

mutant	CTTGA AAAACATCCCCATGTCAGTTAGAGGAATTGTCCTTGTCTCAGTACATGAGCTCCC	4679
afwt	CTTGA AAAACATCCCCATGTCAGTTAGAGGAATTGTCCTTGTCTCAGTACATGAGCTCCC	3930

mutant	GGAAAGCTACAGTCCATCTTCAATTCCCCGTATTCAATGCTGTACAGTCAAAATGTTT	4739
afwt	GGAAAGCTACAGTCCATCTTCAATTCCCCGTATTCAATGCTGTACAGTCAAAATGTTT	3990

mutant	TCAGAGTGTCTACAAGACTGACGACAATATTGTGCTGTCAGCTCCTACTGGAAGTGGTAA	4799
afwt	TCAGAGTGTCTACAAGACTGACGACAATATTGTGCTGTCAGCTCCTACTGGAAGTGGTAA	4050

mutant	GACTGTCATCATGGAATTGGCGATCTGCTGCTGCTCAGCACCTTGAAGATGAGCGCTT	4859
afwt	GACTGTCATCATGGAATTGGCGATCTGCTGCTGCTCAGCACCTTGAAGATGAGCGCTT	4110

F2


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mutant      CAAGGTCGTCATCAGGCGCCGACCAAATCCCTCTGTTCCGAGCGATTTCCGGACTGGAG 4919
afwt       CAAGGTCGTCATCAGGCGCCGACCAAATCCCTCTGTTCCGAGCGATTTCCGGACTGGAG 4170
*****

mutant      CAGGAAGTTCATGTCGTTGGGACTCCAGTGTGCGGAGCTAACTGGCGACACTGATCATA 4979
afwt       CAGGAAGTTCATGTCGTTGGGACTCCAGTGTGCGGAGCTAACTGGCGACACTGATCATA 4230
*****

mutant      CCAGCTCAGAAGTGTTCAGAACAGTCAGATCATCGTTACTACACCAGAGAAGTGGGACAG 5039
afwt       CCAGCTCAGAAGTGTTCAGAACAGTCAGATCATCGTTACTACACCAGAGAAGTGGGACAG 4290
*****

|End F2
mutant      CATGACTCGGAAGTGAAAGATCATGCACGCTTGATGCAATTGGTAAAGCTCTTCTGAT 5099
afwt       CATGACTCGGAAGTGAAAGATCATGCACGCTTGATGCAATTGGTAAAGCTCTTCTGAT 4350
*****

mutant      TGATGAGGTTACATTCTCAAGGAGGCTCGTGGTGCACGCTGGAAGCGGTTGTGTCGAG 5159
afwt       TGATGAGGTTACATTCTCAAGGAGGCTCGTGGTGCACGCTGGAAGCGGTTGTGTCGAG 4410
*****

mutant      AATGAAGACATTCGGGTCGAATGTCCGCTTCGTCGCTCTGAGTGCTACGGTCCCAAAC 5219
afwt       AATGAAGACATTCGGGTCGAATGTCCGCTTCGTCGCTCTGAGTGCTACGGTCCCAAAC 4470
*****

mutant      GGAGGATATTGCGTGTGCTCGGTCGGTAAAGATGCAATGAATCAACACGTACCAGCACCCG 5279
afwt       GGAGGATA----- 4478
*****

mutant      AGAGCACTTTGGGGAGGATTTTCGTCCCGTCAAGCTCCAGAAGTTTGTGTACGGTTATCA 5339
afwt       -----

mutant      GTCATACAGCAATGATTTTGCCTTCGACAAGTTATGCGGTTCGAAGTAAGCACGCAAATA 5399
afwt       -----

mutant      TCGTCCTGTGCGGATGTTTACTGAGGTTTCATAGGCTTCCCGATGTTCTAGGTACGCACT 5459
afwt       -----

mutant      CATGTAGGAAGCCAATCATGATTTTCTGTTGTACTAGGAATTCGTGCGTCGCTACCGCAA 5519
afwt       -----

mutant      AAGAGCTTGCTCGTCTATGGTCCATGACCAACCTCCGGCAAGGTTATGGAAGCATCTG 5579
afwt       -----

mutant      CGAAGCATCTCGAAGCCACAATGCAGACCTCAGAAGTATGCACTGATCTCGAGTTCATG 5639
afwt       -----

mutant      GTTACGGAGCTAACTGGAGGCGACAGCCACGCTGATGGCAGGAGTCGATTTTCATCACGC 5699
afwt       -----

mutant      CGGCCTCGACCCTGCT 5715
afwt       -----

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F2

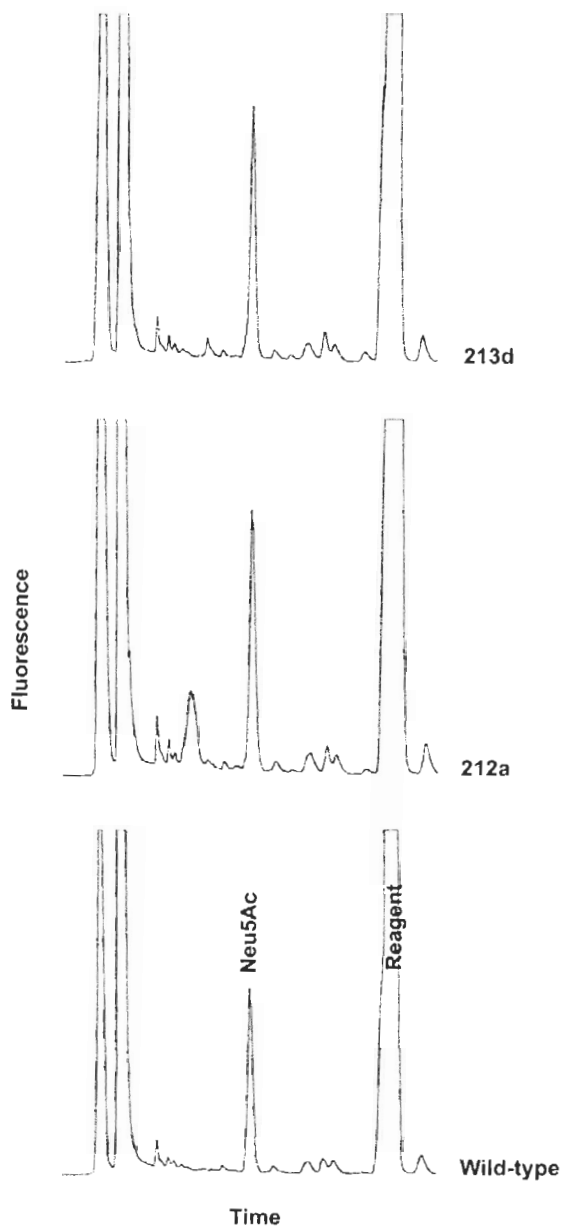


Figure 4.7: HPLC analysis of mild acid hydrolysates of wild type and putative sialic acid-deficient mutant *A. fumigatus* strains revealed that surface sialic acids were present on *A. fumigatus* transformants.

Conidia from transformants 212A and 213D and from wild type (5×10^9) were treated with mild acid to release surface sialic acids, derivatized with DMB, and aliquots of the same volume were analyzed by HPLC. A comparison of peak areas revealed that the level of surface sialylation was not decreased in the mutants. The elution position of Neu5Ac standard is indicated. Peak areas corresponding to the Neu5Ac peak were 1.68×10^6 , 1.64×10^6 and 1.14×10^6 arbitrary fluorescence units for 212A, 213D and wild type, respectively.

4.4 Discussion

In the previous chapters, we described the structure of sialic acids on the surface of *A. fumigatus* and showed that sialylation is important both in binding to fibronectin and in the uptake of conidia by cultured human epithelial cells and murine macrophages. To test the importance of surface sialylation in *A. fumigatus*, we attempted to construct a sialic acid-deficient mutant by disrupting the putative CMP-sialic acid transporter in *A. fumigatus*. We have previously identified a sequence in the *A. fumigatus* genome that is homologous to the CMP-sialic acid transporter in mammals, which functions to transport CMP-sialic acid from the cytosol into the Golgi for further incorporation into glycoconjugates and is present as a single copy in the *A. fumigatus* genome. Alternatively, this sequence could represent the UDP-galactose transporter, but based on evidence from UGT-deficient CHO cells, disruption of this gene was expected to lead to a sialic acid-deficient mutant of *A. fumigatus*. Therefore, our aim was to disrupt this gene by insertion of the *hph* gene, which encodes hygromycin resistance, thereby creating a sialic acid-deficient mutant of *A. fumigatus*. Two mutants were identified using PCR screening that had the interrupted *cst* gene in place of the wild type gene. Correct insertion and disruption was confirmed by southern blot and sequencing. Although the *cst/ugt* gene was successfully disrupted, assessment of surface sialylation revealed that the mutants had equivalent levels of sialic acid on their surface as the wild type. Therefore, despite the successful disruption of the putative *cst/ugt* gene, cell surface sialylation remained intact in *A. fumigatus*.

Glycoproteins are synthesised on the ribosome and are translocated into the lumen of the endoplasmic reticulum (ER) where initial glycosylation of the nascent polypeptide occurs (Palade, 1975). Sugars required for glycosylation in the ER enter the ER primarily via the dolichol phosphate shuttle (Abeijon & Hirschberg, 1992). Following initial glycosylation in the ER, proteins move into the Golgi where they are further modified by several glycosyltransferases, which add sugars, and glycosylhydrolases, which remove sugars from the oligosaccharide. The sugars required for glycosylation in the Golgi are imported from the cytosol through nucleotide-sugar transporters (NSTs) (Capasso & Hirschberg, 1984a; Hirschberg *et al.*, 1998). NSTs recognize monosaccharides which are activated into their nucleotide-sugar forms by nucleotide-sugar synthetases located in the cytosol (Coates *et al.*, 1980), with the exception of the activation of sialic acid into CMP-sialic acid, which occurs in the nucleus (Munster *et al.*, 1998). Molecular cloning of NSTs did not begin until 1996; however, since then, many NSTs have been cloned and important mechanistic and structural features have been obtained. Based on hydrophobicity analysis, it was found that NSTs are type III transmembrane proteins composed of between 6 and 10 membrane spanning domains with the N- and C-termini exposed at the cytosolic side of the Golgi apparatus (Hirschberg *et al.*, 1998; Eckhardt *et al.*, 1999; Gao & Dean, 2000). NSTs are thought to function as homodimers (Puglielli *et al.*, 1999a; Puglielli *et al.*, 1999b; Gao & Dean, 2000) and are antiporters that transport one nucleotide-sugar molecule into the Golgi lumen while transporting the corresponding nucleoside monophosphate from the Golgi

to the cytosol (Waldman & Rudnick, 1990). Nucleoside mono- or di- phosphates act to inhibit glycosyltransferases, so their removal by this mechanism allows for efficient glycosylation to occur. The primary sequence is not an indicator of transporter specificity. For example, the UDP-*N*-acetylglucosamine transporter from mammals is only 22% identical to that of yeast (Guillen *et al.*, 1998), whereas the mammalian CMP-sialic acid transporter is between 40% and 50% identical to mammalian UDP-galactose transporter or the UDP-*N*-acetylglucosamine transporter, respectively.

To date, the structural features critical for recognition of substrate and transport are not clear. It was found that the transport of nucleotide-sugars by NSTs is inhibited by the corresponding nucleoside mono- or diphosphate, suggesting that the nucleoside moiety is important for recognition by the transporter (Capasso & Hirschberg, 1984b). Several important amino acids have been identified in a variety of NSTs; however, because these are invariant across all NSTs studied to date they likely do not play a role in specific substrate recognition (Eckhardt *et al.*, 1998; Oelmann *et al.*, 2001). In one report, it was found that helices 1 and 8 of UGT are required for UDP-galactose transport, and, for CST, helices 2, 3 and 7 are indispensable for CMP-sialic acid recognition and transport (Aoki *et al.*, 2001). Additionally, since NSTs are highly hydrophobic membrane proteins, there are no crystal structures available for any NST. Many recent reports have suggested that NSTs may be able to transport multiple substrates (Hong *et al.*, 2000; Muraoka *et al.*, 2001); however, in those studies, the NSTs were expressed in *Saccharomyces cerevisiae* Golgi in the context of

other Golgi proteins. To address whether the background in which NSTs are expressed contributes to substrate specificity, Tiralongo *et al.* (Tiralongo *et al.*, 2006) have recently expressed mouse CST in *E. coli* and reconstituted this transporter into zero background artificial liposomes. This study showed that murine CST is monospecific for CMP-sialic acid suggesting that other NSTs which were found to be able to transport more than one type of nucleotide-sugar may also be monospecific, and that the experimental conditions used may have erroneously indicated that particular transporters can transport >1 nucleotide sugar. These results also confirm the results of early studies that showed that NSTs are monospecific. Given the specificity of these transporters and the critical role they play in glycosylation, disruption of the genes encoding these proteins may efficiently disrupt glycosylation as was the case with CHO cells where disruption of either CST or UGT greatly reduced surface sialylation (Deutscher *et al.*, 1984; Deutscher & Hirschberg, 1986).

A recent homology search using BLAST revealed that the putative *A. fumigatus* *cst* gene sequence was recently annotated in the *Aspergillus fumigatus* genome (TIGR: <http://www.tigr.org>) as the UDP-galactose transporter. Moreover, this gene sequence is highly homologous (42% identity) to the *Schizosaccharomyces pombe* *gms1+* gene, which encodes the UDP-galactose transporter in this organism (Tabuchi *et al.*, 1997). Therefore, it is very likely that this transporter is indeed the UGT. The possibility of this gene encoding a UDP-galactose transporter was anticipated; however, as was shown with CHO cells (Stanley, 1985) if no terminal galactose were present due to a defective UGT, no

incorporation of sialic acid should occur. Further biochemical assessment will be conducted to determine whether galactose is present on the surface glycoconjugates of these mutants. Specifically, the mutants will be treated with sialidase to remove the terminal sialic acids and lectin binding using PNA will be performed to determine if galactose is incorporated into mutant glycoconjugates as is the case in the wild type.

One reason for the continued sialylation of conidia despite successful gene disruption could be that the remaining sequence encoding the UDP-galactose transporter is sufficient to bind and transport UDP-galactose. Correspondingly, Aoki *et al.* (Aoki *et al.*, 2001) have found that helices 9 and 10 as well as the C-terminus are not required for UGT activity and can be replaced with the corresponding sequences in CST or be deleted. Furthermore, when internal sequences (helices 2 through 7) were replaced with the corresponding CST sequences, the transporter was still able to efficiently transport UDP-galactose. In our study, alignment of the disrupted gene with the wild type gene revealed that almost the entire gene coding sequence was disrupted; therefore, the possibility of a functional protein being produced by this mutant is unlikely. Only biochemical assays to measure UDP-galactose or CMP-sialic acid uptake into isolated Golgi vesicles will resolve this issue.

The sialyltransferase family is a large family of glycosyltransferases that transfer CMP-sialic acid onto glycoconjugates (Harduin-Lepers *et al.*, 2001). Many sialyltransferases have been identified to date and these account for the great variety of sialylated structures that exist in nature. Each sialyltransferase is

specific in terms of the glycan structure recognized and the type of sialylated structure that is formed (Harduin-Lepers *et al.*, 2001). Some members of the sialyltransferase family have strict substrate specificity while others have a broader substrate specificity. For example, STGal-I specifically adds sialic acid to Gal β 1-4GlcNAc in an α 2,6 linkage, but is unable to add sialic acid to either Gal β 1-3GlcNAc or Gal β 1-3GalNAc. On the other hand, ST6GalNAc-I is able to add sialic acids to either GalNAc α -O-Ser/Thr or to Gal β 1-3GalNAc α -O-Ser/Thr (Harduin-Lepers *et al.*, 2001). Therefore, the presence of multiple sialyltransferases or a single sialyltransferases with broad substrate specificity may have led in continued sialylation despite elimination of the subterminal galactose. More work must be conducted in order to characterize the sialic acid biosynthetic pathway in *A. fumigatus* in order to identify suitable candidates for gene disruption.

5 GENERAL DISCUSSION

A. fumigatus is a filamentous, saprophytic fungus that causes invasive disease in immunosuppressed individuals (Latge, 1999). Although *Aspergillus* species are not the most prevalent fungal organisms, they are the most wide spread in the environment (Shelton *et al.*, 2002). Although there are greater than 200 *Aspergillus* species, *A. fumigatus* is responsible for approximately 53-68% of all *Aspergillus* infections (Minari *et al.*, 2002; Morgan *et al.*, 2005; Pagano *et al.*, 2006).. Therefore, *A. fumigatus* must possess unique virulence factors that allow it to colonize the host.

Sialic acids have been found on *C albicans* (Soares *et al.*, 2000), *P. brasiliensis* (Soares *et al.*, 1993), *S. schenckii* (Alviano *et al.*, 1982), *C. neoformans* (Rodrigues *et al.*, 1997) and *F. pedrosoi* (Alviano *et al.*, 2004). In *C. neoformans* and *F. pedrosoi*, the presence of 9-O-acetylated derivative of Neu5Ac was demonstrated by the binding of influenza C virus, which is specific for 9-O-acetylated sialic acids (Rodrigues *et al.*, 1997; Alviano *et al.*, 2004). In *S. schenckii*, the N-glycolylated derivative was found (Alviano *et al.*, 1982); however, in *C. albicans* and *P. brasiliensis*, only unsubstituted Neu5Ac was encountered (Soares *et al.*, 1993; Soares *et al.*, 2000). The absence of O-acetylated sialic acid derivatives in *C. albicans* may have been due to the conditions employed in that analysis. Specifically, the determination of sialic acids in *C. albicans* utilized strong acid (HCl) in the separation and this treatment

is known to de-O-acetylate sialic acids (Varki & Diaz, 1984). In addition, to isolate sialic acids, the authors employed a mild acid hydrolysis for only one hour at 70°C, which may not have been sufficient time for the release of O-acetylated sialic acids as these are known to be more resistant to acid hydrolysis than unsubstituted sialic acid (Varki & Diaz, 1984). For *P. brasiliensis*, the authors did not assess the nature of the sialic acids on the cell surface. For *A. fumigatus*, we employed mild acid conditions known to preserve sialic acid O-acetylation in both isolation and analysis steps, and we have convincingly demonstrated that unsubstituted Neu5Ac is present on the conidial surface of *Aspergillus fumigatus*. In addition to the sialic acid structure, we also studied the sialic acid linkage and underlying monosaccharide. Consistent with the findings in other fungi, we found that *A. fumigatus* sialic acids are linked in α 2,6 linkages to galactose. However, in *F. pedrosoi*, *C. albicans* and *P. brasiliensis*, α 2,3 linked sialic acids linked to galactose were identified (see references provided above). Finally, work conducted on other fungi revealed that the sialic acid density ranges between 10^5 and 10^6 sialic acid molecules per cell. Accordingly, we calculated a density of 2×10^5 sialic acids per conidium.

Sialic acids have been suggested to play important roles in microbial pathogenesis by helping the microbe avoid immune detection and by aiding in adhesion to host cells. The present research confirmed earlier work conducted in our laboratory which showed that *A. fumigatus* conidia are efficiently internalized by the J774 cell line (Wasylnka & Moore, 2002). It was also shown that greater than 99% of conidia internalized by macrophages were killed

(Wasylnka & Moore, 2003). The primary defence mechanism thought to be involved in the killing of *A. fumigatus* conidia by macrophages is through the production of reactive oxygen intermediates (ROIs) (Philippe *et al.*, 2003). The importance of ROIs in conidial killing is suggested by the finding that invasive aspergillosis occurs in patients with chronic granulomatous disease who have a genetic defect in the production of ROI (Almyroudis *et al.*, 2005). Furthermore, there is increased ROI production upon internalization of conidia by macrophages (Nessa *et al.*, 1997). Moreover, the amount of ROI produced by macrophages obtained from cortisone acetate-treated mice was significantly decreased and this was correlated with a 60% decrease in the killing of *A. fumigatus* conidia by these macrophages (level of uptake was the same), implicating ROI in conidial killing by macrophages (Philippe *et al.*, 2003). Although macrophages are able to efficiently internalize and kill conidia in the immunocompetent host, in immunocompromised patients with deficient ROI production due to corticosteroid treatment, such as in individuals with solid organ transplants or haemopoetic stem cell recipients, or in individuals with genetic defects, such as chronic granulomatous disease (Duncan & Wilkes, 2005), *A. fumigatus* may survive and thrive in this environment leading to invasive disease. It has also been shown that conidia are able to produce compounds that inhibit host production of ROIs (Slight *et al.*, 1996); further enhancing the ability of conidia to survive and grow in macrophages. Perhaps the internal environment of the immunosuppressed host is more favourable for *A. fumigatus* growth and

dissemination then the extracellular environment; therefore, invasion of macrophages by *A. fumigatus* may aid in initially establishing infection.

Conidial sialic acids may be important in internalization of conidia by macrophages since we found that removing surface sialic acids from *A. fumigatus* conidia reduced uptake by murine J774 cells. Uptake by J774 cells may be mediated by sialoadhesin, which is a sialic acid-binding immunoglobulin-like lectin found exclusively on macrophages that recognizes Neu5Ac (Kelm *et al.*, 1994). Recently, it was found that sialoadhesin can mediate the phagocytosis of small bacteria and fungi (Jones *et al.*, 2003). Alternatively, it was proposed that sialoadhesin together with another macrophage cell surface receptor may accomplish internalization of microbes (Jones *et al.*, 2003). Our data support this view as sialidase treatment reduced uptake by only 30%. One such receptor may be the scavenger receptor A (SR-A), which is found on macrophages. It was found that phagocytosis of bacterial pathogens by macrophages obtained from SR-A knock-out mice was reduced by 40% *in vitro* suggesting a possible role for this receptor in bacterial phagocytosis (Peiser *et al.*, 2000; Thomas *et al.*, 2000). The role of SR-A in fungal phagocytosis has not yet been established.

Cultured human type II pneumocytes (A549 cells) were also previously shown to be able to efficiently internalize *A. fumigatus* conidia (Wasylnka & Moore, 2002). This activity was confirmed in the present study. Unlike macrophages, significantly more conidia internalized by A549 cells survived and germinated within the phagolysosomal compartment, ultimately breaching the

plasma membrane and growing extracellularly (Wasylnka & Moore, 2003). A recent study focused on the changes that occur upon *A. fumigatus* uptake into A549 cells (Kogan *et al.*, 2004). After infection of A549 cells by *A. fumigatus*, membranes began to bleb; the actin cytoskeleton underwent major structural changes; and there was a loss of focal contacts between A549 cells and ECM. Heat-killed conidia were unable to induce the same cytological changes, which were later found to be due to secreted serine and cysteine proteases.

Membrane blebbing and actin reorganization are signs of cell stress and the beginning of apoptosis. Additionally, the loss of focal contacts is important since this may enable hyphae to penetrate between and underneath the cells (Kogan *et al.*, 2004). Together, these changes are thought to aid in the initial colonization of the host (Kogan *et al.*, 2004). Although the changes in membrane shape after phagocytosis were mediated by secreted proteases, a heat-stable ligand on the surface of conidia was hypothesized to be responsible for their internalization as heat-killed conidia were also internalized. This confirms the results from our laboratory research described in this thesis, which found that removal of surface sialic acids from the surface of *A. fumigatus* conidia reduced their internalization by A549 cells by 50% suggesting that sialic acids are also important for uptake by this cell line. Unlike sialoadhesin, which is found on macrophages and is known to bind Neu5Ac, sialic acid-binding molecules have not yet been identified on the surface of epithelial cells.

Although removing surface sialic acids from *A. fumigatus* significantly reduced the level of uptake of conidia by both murine macrophages and lung

epithelial cells, phagocytosis of conidia still occurred and a significant amount of sialidase-treated conidia were internalized, particularly with J774 cells.

Therefore, alternative phagocytic mechanisms must exist that are responsible for phagocytosis of *A. fumigatus* conidia. One alternative mechanism of phagocytosis of conidia by J774 and A549 cells maybe through toll-like receptor signalling. Toll-like receptors (TLRs) are involved in regulating both innate and adaptive immunity and have been found to be important in phagocytosis (Pandey & Agrawal, 2006). TLRs are a family consisting of 13 members (TLR 1-13) and are type I integral membrane proteins composed of a pathogen-binding ectodomain, a transmembrane helix and a cytosolic signalling domain (Martin & Wesche, 2002). TLRs are expressed on diverse cell types, including airway epithelial cells (Homma *et al.*, 2004; Sha *et al.*, 2004) and macrophages (Zhang *et al.*, 2005). TLRs are known as pattern recognition receptors and are able to bind ligands, such as proteins (Okamura *et al.*, 2001), nucleic acids (Alexopoulou *et al.*, 2001; Kariko *et al.*, 2004) and polysaccharides (Termeer *et al.*, 2002) from bacterial, viral, protozoal and fungal origin (Pandey & Agrawal, 2006). TLRs are thought to function in cooperation with other receptors to internalize pathogens. One such receptor is dectin-1, a C-type lectin that recognizes the fungal cell component, β 1,3 glucan (Brown & Gordon, 2001; Kataoka *et al.*, 2002). Luther *et al.*, 2006 showed that the addition of monoclonal antibodies toward dectin-1 and TLR 2 decreased the level of phagocytosis of *A. fumigatus* conidia by macrophages. Furthermore, transfecting either TLR 2 or dectin-1 genes into human embryonic kidney cells (HEK) revealed that dectin-1, but not TLR 2, is

important in conidial binding to these cells, however, both receptors are required for phagocytosis. Finally, although phagocytosis was decreased by monoclonal antibody binding to either dectin-1 or TLR 2, significant phagocytosis still occurred, which the authors suggested may be due to the presence of other conidial ligands important in phagocytosis. TLRs have also been found on A549 cells and have been implicated indirectly in the binding and uptake of *A. fumigatus* conidia (Tsutsumi-Ishii & Nagaoka, 2003; Sha *et al.*, 2004). However, no conclusive evidence for the role of TLRs in binding and uptake of conidia by A549 cells has been obtained.

We have successfully disrupted the *cst/ugt* gene in *A. fumigatus*, which was confirmed by PCR and sequencing. However, despite this successful disruption, sialic acid levels on the surface of mutant conidia were equivalent to those of the wild type. As described in Chapter 1, microbes employ a variety of strategies to sialylate their cell surface, such as the use of trans-sialidase, *de novo* biosynthesis, precursor scavenging and donor scavenging. *A. fumigatus*, like some other fungal pathogens, such as *C. neoformans*, *C. albicans* and *P. brasiliensis*, is able to produce sialic acids in chemically defined media with no added sialic acid, suggesting that a *de novo* sialic acid biosynthesis pathway exists in this organism. Therefore, future work in obtaining a sialic acid-deficient strain of *A. fumigatus* should focus on characterizing the sialic acid biosynthesis pathway in *A. fumigatus*. A starting point may be to identify which components of the sialylation machinery are present in *A. fumigatus* through metabolic labelling experiments where *A. fumigatus* is grown in the presence of labelled substrates

that are known to be utilized at particular stages of the sialic acid biosynthesis pathway thereby identifying the presence of the corresponding enzymes. Homology searching may also help to identify suitable targets for disruption; however, since the bulk of information on the genes and enzymes involved in sialic acid biosynthesis is derived from mammalian biosynthesis, this may be difficult since the protein similarities may be low and therefore missed. For example, the UDP-N-acetylglucosamine transporter of mammalian cells and yeast have only 22% identity despite carrying out the same function (Guillen *et al.*, 1998). Therefore, it is important to identify important structural features of these enzymes that are unlikely to diverge over time, such as substrate binding sites, and search the databases using those sequences. As more information emerges on the various enzymes involved in sialic acid biosynthesis, this task will likely become easier.

6 APPENDIX

6.1 The *Aspergillus fumigatus* siderophore biosynthetic gene *sidA*, encoding L-ornithine N5-oxygenase, is required for virulence: construction of a rescue strain of Δ *sidA* and assessment *in vitro* and *in vivo*

The construction of a siderophore-deficient *A. fumigatus* strain and characterization of this mutant strain *in vitro* and *in vivo* was part of Anna Hissen's doctoral thesis research. To complete this body of work, we constructed a rescued strain of the siderophore-deficient *A. fumigatus* strain. We characterized this mutant genotypically as well as its ability to grow in various media. Finally, we compared the relative virulence of the wild-type, siderophore-deficient and rescued strains *in vivo* in a mouse model of invasive aspergillosis.

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6.1.1 Introduction

Iron is an important element for eukaryotic cells as it is required for processes such as DNA synthesis, DNA repair, respiration and removal of free radicals in cells (Crichton & Ward, 1992; Crichton & Ward, 1998). Iron is abundant in the environment; however, its bioavailability is poor. This is because of the low solubility of iron in aerobic environments at neutral pH (Hissen *et al.*,

2005). Many microbial organisms have adapted to low iron bioavailability by producing iron-binding siderophores, which are low molecular weight peptides capable of specifically chelating iron (Neilands, 1995). Additionally, siderophores play important roles in iron storage (Matzanke *et al.*, 1987).

Host animals can limit the growth of pathogenic microbes by drastically reducing free iron levels. This is accomplished by the production of high-affinity iron-binding molecules, such as transferrin, lactoferrin, heme and ferritin, which are able to suppress free iron levels to 10^{-18} M (Bullen, 1981). Transferrin is the primary iron-binding molecule in plasma, and, in response to infection, host defences increase iron acquisition by transferrin, which further lowers the free iron concentration (Bullen, 1981).

Previous work in our laboratory has shown that *A. fumigatus* produces siderophores that are able to acquire transferrin-bound iron. Consequently, *A. fumigatus* is able to grow in media containing concentrations of serum that are inhibitory to other microbes. *A. fumigatus* is able to produce several types of siderophores; however, recent work has shown that *A. fumigatus* produces N', N'', N'''-triacetylfusarinine C (TAF) and ferricrocin in the greatest amounts (Nilius & Farmer, 1990; Hissen *et al.*, 2004), especially in iron-deficient media or in media containing serum (Nilius & Farmer, 1990; Hissen *et al.*, 2004). Both TAF and ferricrocin have higher affinities for iron than does transferrin, which explains why these siderophores are able to acquire iron that is bound by transferrin. Furthermore, a kinetic analysis revealed that TAF and ferricrocin

preferentially remove the C-terminal iron of transferrin, and that iron removal is cooperative (Hissen & Moore, 2005).

Siderophore production is a virulence factor in several bacterial pathogens, including *Pseudomonas aeruginosa* (Meyer *et al.*, 1996; Takase *et al.*, 2000), *Vibrio* species (Litwin *et al.*, 1996; Wertheimer *et al.*, 1999) and *Burkholderia cepacia* (Sokol *et al.*, 1999). Genes involved in siderophore biosynthesis have been identified. In *A. fumigatus*, it was found that *sidA* encodes L-ornithine N⁵-oxygenase, which catalyses the first step in siderophore biosynthesis, the hydroxylation of the N-terminal amino group of L-ornithine producing N⁵-hydroxy-L-ornithine. N⁵-hydroxy-L-ornithine is later acylated to form a hydroxamate group, which is responsible for iron chelation – a key feature of hydroxamate siderophores (Hissen *et al.*, 2005). Furthermore, it was found that *A. fumigatus* SidA was 75% identical to the L-ornithine N⁵-oxygenases of *A. nidulans* and *A. oryzae*. Moreover, this protein was 50% identical to that of Sid1 from *Ustilago maydis*.

To assess the importance of siderophore biosynthesis in the virulence of *A. fumigatus*, previous work focussed on constructing a siderophore-deficient mutant and evaluating its ability to synthesize siderophores with the ultimate goal to assess the virulence of the *sidA* mutant *in vivo*. To accomplish this task and to complete this project, a rescue mutant was constructed by ectopically inserting the wild type *sidA* gene into the genomic DNA of the *sidA*-deficient mutant strain. The relative virulence of the wild type, *sidA*-deficient and rescue strains was compared in a mouse model of invasive aspergillosis. Therefore, the specific

aims were to construct a rescue mutant of the siderophore-deficient strain of *A. fumigatus*, to test this rescued strain along with the wild type and siderophore-deficient strains for their ability to grow in iron-limited media and to produce siderophores, and to compare the relative virulence of the three strains *in vivo* in a mouse model of invasive aspergillosis.

6.1.2 Materials and methods

***A. fumigatus* strains and growth conditions**

A. fumigatus 13073 (*hph*⁻, *sidA*⁺), Δ *sidA* (*hph*⁺, *sidA*⁻) and Δ *sidA*^R (*hph*⁺, *sidA*⁺) were used in this study and were grown and harvested as previously described on MYPD plates with or without 200 μ l hygromycin B (Hoffmann - La Roche, Mississauga, ON).

Construction of rescue transformation plasmid pComp2

Standard molecular biology techniques were performed as described by Sambrook et al. (Sambrook *et al.*, 1989). Plasmids were propagated in *E. coli* DH5 α (Life Technologies). Genomic DNA preparation was performed using the phenol-chloroform method (Sambrook *et al.*, 1989) or using a genomic DNA preparation kit (Fermentas). Plasmid preparation was performed using the Plasmid Maxi kit (Qiagen), PCR product clean up was performed using the Qiaquick PCR purification kit (Qiagen) and agarose gel extraction of DNA was performed using the Qiaquick gel extraction kit (Qiagen). Custom primers were produced by Invitrogen.

The *A. fumigatus sidA* gene was PCR amplified from wild-type *A. fumigatus* genomic DNA using primers flanking the wild-type gene. The primers 5'-GAATTCGAATTCTGTCCAAGAGCACCCACACCTC (comp1) and 5'-GAATTCGAATTCCCATCAGATAACGCGTGAAA (comp2) were designed to amplify a fragment beginning 505 bp upstream of the *sidA* gene extending to 560 bp downstream of the gene. These primers were designed with *EcoR1* restriction sites (underlined) to facilitate ligation into the transformation plasmid. The following PCR reagents were used in the amplification of the intact *sidA* gene and flanking sequences: 2 mM dNTPs, 2 µg *A. fumigatus* genomic DNA, 100 pmol of each comp1 and comp2 primers, 5 units Pfu Ultra high fidelity DNA polymerase (Stratagene), 5 µl Pfu Ultra 10X buffer in a total volume of 50 µl. The following temperature program was used for amplification: a pre-dwell period of 95°C for 2 minutes followed by 30 cycles consisting of denaturing at 95°C for 30 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 8 minutes. Following the 30 cycles, there was a post-dwell period of 10 minutes at 72°C. The plasmid Bluescript SK+ was cut with *EcoR1* (Invitrogen), dephosphorylated with Apex alkaline phosphatase (Epicenter, Madison, WI) and gel purified. The PCR amplified *sidA* gene was ligated with pBluescript yielding pComp2 and used to transform *E. coli* DH5α. Successful plasmid construction was confirmed by restriction analysis using the restriction enzymes *PstI*, *HindIII*, *XbaI*, *EcoR1* and *BamHI* as well as by sequencing.

Construction of the *A. fumigatus* rescue strain

A. fumigatus Δ *sidA* strain was grown on minimal media agar plates (10 g glucose, 0.85 g NaNO₃, 0.52 g KCl, 0.52 g MgSO₄·7H₂O, 1.52 g KH₂PO₄, 40 µg Na₂B₄O₇·10H₂O, 0.4 mg CuSO₄·5H₂O, 1 g FePO₄·4H₂O, 0.6 mg MnSO₄·H₂O, 0.8 mg Na₂MoO₄·2H₂O, 8 mg ZnSO₄·7H₂O, 1 mg nicotinic acid, 2.5 mg riboflavin, 2 mg pantothenic acid, 0.5 mg pyridoxine, 10 µg biotin, 0.2 mg p-aminobenzoic acid and 10 µM TAF in 1 L H₂O, pH 6.5) and 5 x 10⁹ conidia were used to inoculate 250 ml YG media as described in chapter 3 with the exception that 10 µmol TAF and ferricrocin were added to help with swelling of spores. Conidia were electroporated with 0 to 2 µg *Xba*I-linearized pComp2 and transformants selected on Grimm-Allen (GA) media (1 g KHSO₄, 3 g K₂HPO₄, 3 g (NH₄)₂SO₄, 20 g sucrose, 2 mg thiamine, 20 µg CuSO₄, 1 mg MnSO₄, 5.5 mg ZnSO₄, 810 mg MgSO₄ in 1 L H₂O, pH 6.9) supplemented with 150 µM dipyriddy. Dipyriddy is an iron chelator therefore only transformants with an intact *sidA* gene would be able to obtain iron and grow on this media. Three colonies that grew on Grimm-Allen agar supplemented with dipyriddy were further assessed for growth on minimal media supplemented with hygromycin to ensure that wild type *A. fumigatus* was not present. It was found that these three transformants were hygromycin-resistant and were further assessed for successful ectopic integration of the wild type *sidA* gene using PCR and for their ability to produce siderophores.

Screening of potential rescue mutants

Three transformants emerged that were able to grow on Grimm-Allen media supplemented with dipyriddy and on minimal media with hygromycin.

These mutants were further examined for their ability to produce siderophores. Siderophore production was detected in these mutants by inoculating 5 ml MYPD liquid media with 5×10^6 conidia and allowing them to grow at 37°C overnight. The next day, the presence of siderophores was detected by addition of FeSO₄, which produced the characteristic brick-red colour of ferrated siderophores.

Successful ectopic integration of the PCR amplified *sidA* gene was assessed by PCR using the primers 5`-TTGAACGGAAGTCAGAATCG (oof) and 5`-ACAGGTTCCCTCATGTCTGC (oor), which flank the *sidA* gene. If only the interrupted gene was present (Δ *sidA* strain), PCR amplification with oof and oor would produce a 2.6 kb fragment; however, if both the interrupted *sidA* gene and the ectopically integrated *sidA* gene was present (Δ *sidA*^R strain), PCR amplification would produce both the 2.6 kb and 1.4 kb fragments. PCR screening with oof and oor was accomplished using the following PCR reagents: 100 pmol oof and oor primers, 2 mM dNTPs (Invitrogen), 2.5 units Taq DNA polymerase, 10 µg transformant genomic DNA, 5 µl 10X Taq buffer in a total reaction volume of 50 µl. The following temperature program for amplification was used: a pre-dwell period at 94°C for 2 minutes followed by 30 cycles of template denaturing at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 90 seconds, followed by a post-dwell period at 72°C for 10 minutes.

Growth in iron-limited media

The ability of the three *A. fumigatus* strains (wild type, Δ *sidA* and Δ *sidA*^R) to grow in different media was assessed. Conidia (1×10^6) were added into 1 ml

volumes of MYPD or Grimm-Allen media. In some samples, GA was supplemented with 5 μ M FeCl₃, 10 μ M TAF and/or 10% human serum (Sigma). The extent of growth was assessed by measuring the dry weights of *A. fumigatus* mycelia. The entire contents of the tube containing the growing culture were filtered onto a Whatman number 1 filter, the mycelia were washed with distilled water and oven dried until a constant weight was obtained.

Mouse model of invasive aspergillosis

A. fumigatus was grown and harvested as described previously with the exception that conidia were resuspended in sterile saline instead of PBS. Female BALB/c mice weighing between 18 and 22 g (Charles River Laboratories, Wilmington, MA) were given 0.5 mg/ml tetracycline in their drinking water and were immunosuppressed by subcutaneous injections of 200 mg/kg cortisone acetate (Baxter Medical, Mississauga, ON) in sterile saline on days -3, 0, 2 and 4 of the study. Cortisone acetate was prepared as a 30 mg/kg suspension in sterile saline. Mice were randomly assigned to one of four treatment groups. These included the parental strain (n = 10), the Δ *sidA* strain (n = 10), the Δ *sidA*^R strain (n = 10) and the saline control group (n = 5). On day 0, immunosuppressed mice were anesthetized with isoflurane and intranasally inoculated with a 20 μ l volume of saline containing 5 x 10⁶ conidia of either of the various strains or saline for control mice ensuring entire inoculum was inhaled. Mice were monitored for 14 days to observe clinical symptoms and were deemed to have reached endpoint if they displayed ruffled fur and one of the following: 1) laboured breathing, hunching and decreased movement; or 2) disorientation and

loss of balance. Mice displaying these symptoms were euthanized. Additionally, at the end of the 14 day period, surviving mice were also euthanized. At the end-point or at the end of the 14 day period, lungs of the euthanized animals were fixed by opening the chest, isolating the trachea and perfusing the lungs with 10% formalin in PBS. After 2 minutes, lungs were entirely removed and further fixed in 10% formalin in PBS overnight at room temperature. After fixation, lungs were paraffin embedded, sectioned and stained with hematoxylin and eosin for imaging. Images were obtained on a Zeiss LSM10 confocal microscope equipped with a QImaging 10-bit camera (QImaging Inc., Burnaby, BC).

Statistics

Mouse survival data was analyzed using log rank analysis using Prism 4.0 software (GraphPad, San Diego, CA).

6.1.3 Results

Construction of the *A. fumigatus* rescue strain

To construct a rescue strain of *A. fumigatus* $\Delta sidA$, the wild-type *sidA* gene was PCR amplified and ligated into the EcoR1 restriction site of pBluescript generating pComp2 (Figure 6.1). *A. fumigatus* $\Delta sidA$ was then transformed by electroporation using pComp2 yielding $\Delta sidA^R$. Correct ectopic integration was assessed by the ability of the transformants to produce siderophores by visually determining ferrated siderophore production by the presence of a brick-red colour after addition of $FeSO_4$. Three transformants were identified that were able to synthesize siderophores (data not shown). Transformants producing

siderophores were further assessed by PCR. Screening primers were designed to amplify the entire *sidA* gene; therefore, ectopic integration of *sidA* into the genomic DNA of the Δ *sidA* strain would yield two bands, a 2.6 kb band corresponding to *sidA* interrupted by the hygromycin resistance gene, and a 1.4 kb band corresponding to the uninterrupted *sidA*. All three transformants that produced siderophores showed ectopic integration of the *sidA* gene (Figure 6.2). R3 produced the most siderophores and was denoted the rescue strain.

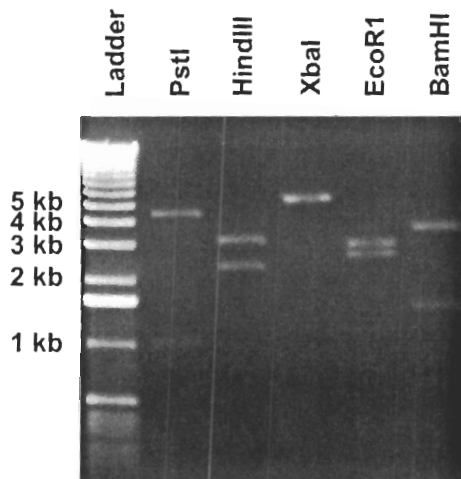


Figure 6.1: Restriction analysis of pComp2 confirms correct insertion of *sidA* into pBluescript SK+.

Digestion of pComp2 with various restriction enzymes was expected to produce the following band sizes if the PCR-amplified *sidA* gene was correctly inserted into pBluescript SK+: *PstI*, 1 kb and 4.5 kb; *HindIII*, 2.3 kb and 3.2 kb; *XbaI*, 5.5 kb; *EcoRI*, 2.6 kb and 2.9 kb; and *BamHI*, 1.5 kb and 4 kb. Restriction digests revealed that *sidA* was correctly inserted into pBluescript SK+ to yield pComp2.

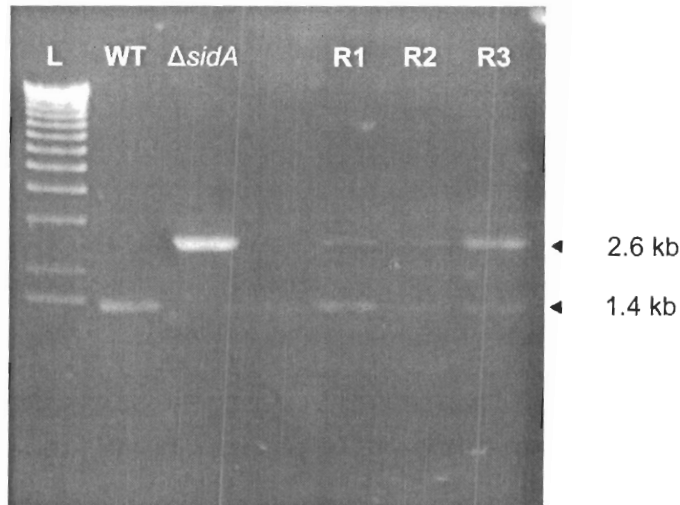


Figure 6.2: PCR screening of rescue transformants of $\Delta sidA$.

Transformation of the $\Delta sidA$ strain with the transformation plasmid pComp2 produced three transformants that could grow on media containing dipyriddy, and on media containing hygromycin. They were also able to produce detectable levels of siderophores. These transformants were further assessed by PCR using screening primers that bind to the ends of the wild-type *sidA* gene to determine if the *sidA* gene was ectopically integrated. If only the wild-type gene was present, PCR amplification would produce a 1.4 kb fragment. If the *sidA* gene was interrupted by *hph*, PCR amplification would produce a 2.6 kb fragment. Finally, if an ectopic integration of the *sidA* gene occurred, PCR amplification would yield two bands (the interrupted *sidA* gene and wild type *sidA* gene). PCR screening revealed that all three transformants (R1, R2 and R3) contain the wild type *sidA* gene as well as the disrupted gene.

Growth of *A. fumigatus* strains in various media

In rich media, all three strains (wild type, $\Delta sidA$ and $\Delta sidA^R$) were able to grow well. However, in iron-limiting (GA) media, the $\Delta sidA$ was unable to grow efficiently whereas the wild type and rescue strains, were able to efficiently grow. Addition, of 5 μM FeCl_3 to the growth media (GA + 5 μM FeCl_3) partially restored growth of the $\Delta sidA$ strain. The addition of 10% human serum to the media (GA + 5 μM FeCl_3 + 10% human serum), which effectively sequesters free iron, completely inhibited growth of the siderophore-deficient strain and this effect was reversed to wild type levels by the addition of 50 μM TAF or ferricrocin, indicating

that siderophores are important in obtaining serum-bound iron. Finally, the importance of siderophores in iron acquisition for *A. fumigatus* growth was further confirmed by the ability of the rescue strain, $\Delta sidA^R$, to grow as well as the wild type strain in GA + 5 μM FeCl_3 + 10% serum (Table 6.1).

Table 6.1: Growth of *A. fumigatus* strains on different media

Medium	Strain	Growth (mg [dry wt]) ^a
YM	Wild type	3.9 ± 0.2
	$\Delta sidA^R$	5.0 ± 0.4
GA	Wild type	3.9 ± 0.7
	$\Delta sidA$	0.3 ± 0.5 ^c
	$\Delta sidA^R$	7.6 ± 1.6
GA + FeCl_3 (5 μM)	Wild type	6.5 ± 0.4
	$\Delta sidA$	1.4 ± 1.1 ^c
	$\Delta sidA^R$	7.5 ± 0.3
GA + TAF (ferrated, 10 μM)	Wild type	6.2 ± 0.5
	$\Delta sidA$	4.4 ± 1.5
	$\Delta sidA^R$	6.0 ± 0.1
GA + FeCl_3 + serum ^b	Wild type	9.4 ± 0.7
	$\Delta sidA$	0.0 ± 0.0 ^c
	$\Delta sidA^R$	9.5 ± 0.8
GA + FeCl_3 + serum + desferri-TAF (50 μM)	Wild type	10.1 ± 0.4
	$\Delta sidA$	12.3 ± 1.2
	$\Delta sidA^R$	11.3 ± 0.3
GA + FeCl_3 + serum + desferriferricrocin (50 μM)	Wild type	9.7 ± 2.0
	$\Delta sidA$	11.1 ± 1.1
	$\Delta sidA^R$	11.0 ± 1.2

^a Data presented are averages ± standard deviations of triplicate values. This experiment was performed three times with similar results.

^b 10% (vol/vol) human serum.

^c Significant growth inhibition relative to wild type ($P < 0.03$ by analysis of variance with Dunnett's multiple-comparison test).

The *sidA* gene is important in the virulence of *A. fumigatus* in a mouse model of invasive aspergillosis

To evaluate whether the *sidA* gene is important in the virulence of *A. fumigatus*, the relative virulence of the wild type, $\Delta sidA$ and $\Delta sidA^R$ strains were compared in vivo in a mouse model of invasive aspergillosis. Mice were immunosuppressed with cortisone acetate and intranasally inoculated with 5×10^6 conidia of either of the three strains or a saline control. The four treatment groups had 10 mice each with the exception of the control group that had 5 mice. Mice challenged with the wild type or rescue strains all reached end-point (see Materials and Methods) by day 4 whereas the 10 mice challenged with the $\Delta sidA$ strain and 5 mice inoculated with saline survived to day 14, the end of the study (Figure 6.2). This clearly indicates that the *sidA* gene is a virulence factor of *A. fumigatus*.

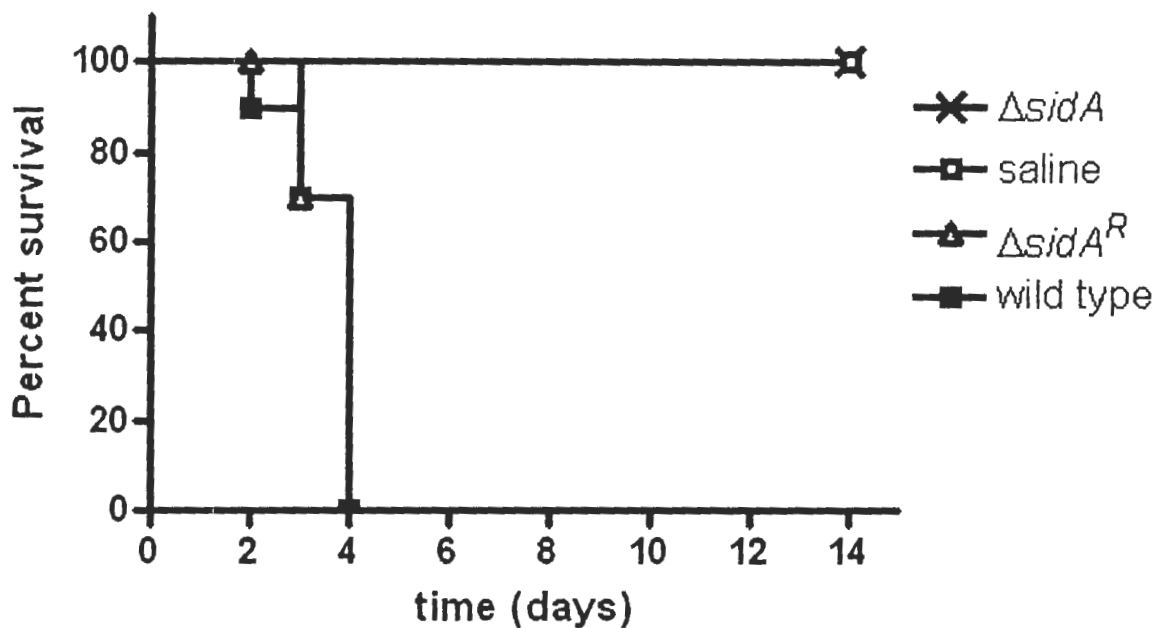


Figure 6.3: Survival curve for female BALB/c mice infected with *A. fumigatus* wild type, $\Delta sidA$, $\Delta sidA^R$ or saline.

Mice were immunosuppressed with cortisone acetate and inoculated with 5×10^6 conidia of either *A. fumigatus* wild-type, $\Delta sidA$, $\Delta sidA^R$ strains or a saline control and monitored for 14 days. End-point was reached if the mice displayed ruffled fur and one of the following: 1) laboured breathing, hunching and decreased movement; or 2) disorientation and loss of balance. Results revealed that mice infected with *A. fumigatus* wild type and $\Delta sidA^R$ strains reached end-point within 4 days while mice infected with $\Delta sidA$ or a saline control survived until the end of the study. Survival curves of the wild type and $\Delta sidA^R$ strains compared to the $\Delta sidA$ strain are significantly different ($P < 0.0001$) as assessed by log rank analysis.

6.1.4 Discussion

Previous work in our laboratory identified the gene encoding L-ornithine-*N*⁵-monooxygenase (*sidA*) in *A. fumigatus* and a *sidA*-deficient mutant, Δ *sidA*, was created by Anna Hissen, a doctoral student in our laboratory. To complete this project, the current work focussed on constructing a rescue strain that was able to synthesize siderophores by ectopically inserting the intact wild type *sidA* gene into the genome of the Δ *sidA* strain. We assessed the ability of the wild type, Δ *sidA* and Δ *sidA*^R strains to produce siderophores indirectly by the formation of a red colour upon the addition of FeSO₄, and unlike the Δ *sidA* strain, the wild type and the rescue strains produced siderophores.

The growth of the various *A. fumigatus* strains on different media was assessed. There was very minimal growth of the Δ *sidA* strain in low-iron (GA) media, which was partially overcome by the addition of 5 μ M FeCl₃ to the media (GA + 5 μ M FeCl₃), indicating that siderophores are required for growth in low-iron environments. Because addition of 5 μ M FeCl₃ promoted growth of the Δ *sidA* strain, we concluded that *A. fumigatus* possesses mechanisms in addition to siderophore-mediated iron uptake to access iron. One such mechanism could be through the reduction of ferric iron to ferrous iron, for which host transferrin molecules have low affinity (Harris, 1986), followed by uptake from the extracellular environment by ferrous iron transporters. Ferric reductases have been identified in several microbial organisms, for example *C. albicans* (Knight *et al.*, 2002), *C. neoformans* (Jacobson *et al.*, 1998) and *H. capsulatum* (Timmerman & Woods, 2001; Foster, 2002). Furthermore, a gene in *A. nidulans*

homologous to the *S. cerevisiae freA* gene, which encodes a metalloreductase and would function to reduce ferric iron, has been discovered in *A. nidulans* (Oberegger *et al.*, 2001). In addition, both the wild type and the $\Delta sidA$ *A. fumigatus* strains had ferric reductase activity (Hissen *et al.*, 2005). Therefore, it was proposed that the $\Delta sidA$ strain may have increased reductase activity in response to the loss of siderophore-mediated iron uptake. However, there was no significant difference in the level of ferric reductase activity between the wild type and the siderophore-deficient strain suggesting that siderophore-mediated iron uptake is the primary iron uptake mechanism in *A. fumigatus*. Several other lines of evidence demonstrate the importance of the siderophore-mediated iron uptake mechanism, as opposed to the reductive iron assimilation strategy, to obtain iron in *A. fumigatus*. First, *in vitro* growth of $\Delta sidA$ in iron-deficient conditions was less than the growth of wild type strain, indicating that ferric reductases did not fully compensate for the loss of iron due to disruption of siderophore biosynthesis. Furthermore, it was found that the poor growth of $\Delta sidA$ in low iron media and media containing human serum was fully rescued by the addition of 10 μM TAF or 50 μM desferri-TAF, respectively, suggesting that the poor growth under these conditions was solely due to the lack of siderophore production and secretion by this strain. Moreover, $\Delta sidA$ was unable to remove iron bound by transferrin (Hissen *et al.*, 2005), which further confirmed that siderophores, and not low molecular weight ferric reductases, were responsible for iron removal from transferrin.

The importance of siderophores in bacterial pathogenesis has been demonstrated in several bacterial pathogens, including *Pseudomonas aeruginosa* (Meyer *et al.*, 1996; Takase *et al.*, 2000), *Burkholderia cepacia* (Sokol *et al.*, 1999), *E. coli* (de Lorenzo & Martinez, 1988) and in *Vibrio* species (Litwin *et al.*, 1996; Wertheimer *et al.*, 1999). However, the importance of siderophores in the pathogenesis of fungi is less well understood. We assessed the virulence of the wild type, $\Delta sidA$ and $\Delta sidA^R$ strains in a murine model of IA and found that the wild type and rescue strains were able to effectively colonize the host while the siderophore-deficient $\Delta sidA$ strain was completely avirulent. Therefore, we concluded that siderophore biosynthesis is crucial to *A. fumigatus* virulence and the *sidA* gene represents a true virulence factor in this organism. This was also shown by Schrettl *et al.* (Schrettl *et al.*, 2004) who also found that siderophore-deficient *A. fumigatus* was avirulent in a mouse model of the disease. Schrettl *et al.* also showed that disruption of reductive iron assimilation through the disruption of the iron permease gene in *A. fumigatus* (*itrA*) was not important in the virulence of *A. fumigatus* in a mouse model of IA. Hence, siderophore-mediated iron acquisition alone is critical for the growth of *A. fumigatus* *in vitro* and *in vivo*.

In conclusion, *A. fumigatus* uses a siderophore-mediated iron acquisition strategy *in vivo* and this system is crucial to the virulence of this fungal pathogen in a mouse model of IA. Since mammals lack siderophore biosynthetic genes, this pathway represents a good target for the development of novel antifungal

agents. Given the importance of this pathway in iron acquisition and in virulence, this may lead to an effective treatment for IA.

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