THE ROLE OF SIDEROPHORES IN IRON UPTAKE AND VIRULENCE OF THE OPPORTUNISTIC FUNGAL PATHOGEN, *ASPERGILLUS FUMIGATUS*

by

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ABSTRACT

Aspergillus fumigatus is an opportunistic fungal pathogen that can cause life-threatening invasive aspergillosis and is a leading cause of mortality in bone marrow and lung transplant recipients. Currently, the mortality rate of invasive aspergillosis is approximately 50%. The virulence factors of *Aspergillus* species remain largely unknown. The purpose of this research was to determine the mechanism(s) by which *A. fumigatus* survives within the iron-limiting environment of the host.

A. fumigatus conidia germinated and grew extremely well in the presence of human serum, which is normally fungistatic due to the presence of the iron-binding protein, transferrin. This result implied that *A. fumigatus* possesses highly effective mechanisms for iron uptake. *A. fumigatus* was able to remove transferrin-bound iron across a dialysis membrane, indicating that molecules <10 kDa were responsible for iron acquisition. Siderophores, which are small iron-binding molecules, were detected during the first 8 hours of culture. Several hydroxamate siderophores were purified from *A. fumigatus* cultures, and the most abundant were identified as N'N''N'''-triacetylfusarinine C (TAF) and ferricrocin.

The individual microscopic rate constants for removal of iron from the two iron-binding sites on human transferrin were calculated for both TAF and ferricrocin. Both *A. fumigatus* siderophores could remove iron from all transferrin species, but preferentially removed C-terminal iron. The rates of iron removal from diferric transferrin were 2.4 ±

0.2 $M^{-1}min^{-1}$ for TAF and 1.6 ± 0.3 $M^{-1}min^{-1}$ for ferricrocin. These rates were similar to those of bacterial siderophores which are known virulence factors.

A siderophore secretion mutant was constructed in *A. fumigatus* by deletion of *sidA*, which encodes L-ornithine N^5-oxygenase, the first committed step in siderophore biosynthesis. This mutant was unable to grow in low iron medium, or in medium containing serum. It was also unable to remove iron from human diferric transferrin. The *sidA* mutant showed significantly reduced virulence in a mouse model of invasive aspergillosis.

In conclusion, hydroxamate siderophores produced by *A. fumigatus* play an important role in iron uptake in vivo. Therefore, siderophores represent one of the few virulence factors identified in *A. fumigatus*. The siderophore-iron uptake pathway represents a potential target for anti-*Aspergillus* drug development.

DEDICATION

This thesis is dedicated to my children Max and Sophie Hissen, who bring smiles and laughter to every day.

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CONTRIBUTIONS OF OTHER AUTHORS TO THIS WORK

Several other authors contributed to the work presented in this thesis. Jodie Klippenstein performed the metabolic labelling of basal lamina proteins and measured the rate of hydrolysis of these proteins by *Aspergillus fumigatus* proteinases (Figure 2-3). Linda Pinto purified siderophores from *A. fumigatus* culture (Table 3-2) and carried out the mass spectrometry (Table 3-3) with the assistance of Blair Johnston. Linda Pinto also assisted with the NMR spectra which were run by Marcy Tracey and interpreted by Dr. Alan Tracey (Tables 3-4, 3-5 and 3-6). The gene deletion vector pGAW2 (Figure 5-4) was constructed by Adrian Wan. Jurgen Hissen integrated the differential equations in Chapter 4. Figure 5-14, showing lung sections from infected mice, was prepared by Dr. Margo Moore and interpreted by Dr. Clive Roberts of the University of British Columbia.

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CHAPTER ONE: INTRODUCTION

Fungi

Fungi are ubiquitous heterotrophic eukaryotic organisms, inhabiting a wide range of environments. They can grow either as single-celled yeast or as chains of cells known as hyphae. Most have complicated life cycles including both sexual and asexual reproduction. Many fungi are saprophytes, obtaining their nutrition from dead organic material, which is absorbed across the cytoplasmic membrane. Because of their mode of nutrition, they play extremely important ecological roles. In the environment, fungi are efficient decomposers of dead material and in the process make nutrients available to many other organisms (Bougher and Tommerup, 2000). They are capable of secreting a great variety of hydrolytic enzymes which can break down many natural polymeric substrates such as lignin, cellulose and keratin.

Fungi impact human life in several important ways. Fungi are important in the production of many foods including cheeses, breads, beer and wine (Bougher and Tommerup, 2000) and the variety of hydrolytic enzymes produced by fungi has made them important in many industrial processes. Fungi are important in the manufacture of certain drugs and antibiotics which have greatly improved our ability to treat infections. Unfortunately, many fungi are pathogens of commercially important plants (Martinez-Espinoza *et al.*, 2002), as well as agents of food spoilage (Tournas, 1994). Some fungi cause superficial infections in otherwise healthy individuals, while several species are

capable of causing serious and fatal infections, particularly amongst immunocompromised populations (Groll and Walsh, 2001).

Human fungal pathogens

Of over 200,000 known fungal species, only 270 have been described to cause illness in humans (Perfect, 1996). This group of pathogenic fungi is responsible for a wide range of infections, ranging from superficial colonization to invasive disseminated disease. There is great taxonomic diversity in the fungal pathogens, which are members of many fungal divisions. The pathogens may grow as yeasts, as hyphae, or as both yeast and filamentous forms in vivo. Though several fungal species are primary pathogens of humans (Table 1-1), the severe and invasive fungal infections most often affect the immunocompromised. The recent rise in rates of serious fungal infections is related to the increasing numbers of susceptible individuals, including bone marrow and organ transplant recipients, cancer patients being treated with chemotherapy, people with human immunodeficiency virus (HIV) infection, the critically ill and very low birth weight infants (Dixon *et al.*, 1996). The opportunistic fungal pathogens are summarized in Table 1-2.

Table 1-1: Some common primary fungal pathogens

Source: Hogan *et al.*(1996)

Species	Taxonomy (division; order)	Geographical distribution	Illness
Blastomyces dermatitidis	Ascomycota; Onygenales	American midwest	acute or chronic pneumonia, cutaneous or disseminated infection
Coccidioides immitis	Ascomycota; Onygenales	semiarid areas of United States and Mexico	coccidioidomycosis (San Joaquin Valley Fever), asymptomatic infection, pneumonitis or disseminated infection
Histoplasma capsulatum	Ascomycota; Onygenales	North and Latin America, acquired immune deficiency syndrome (AIDS) a risk factor	mild respiratory infections
Paracoccidioides brasiliensis	Ascomycota; Onygenales	subtropical forests of South America	mucosal or skin lesions, pulmonary infections
Sporothrix schenckii	Ascomycota; Ophiostomatales	world-wide, traumatic implantation of mycelia or conidia from soil	non-systemic, suppurative and granulomatous cutaneous nodules in lymphatic system

Table 1-2: Some common opportunistic fungal pathogens

Source: Hogan *et al.*(1996)

Species	Taxonomy (division; class)	Risk factors	Illness
Aspergillus fumigatus	Ascomycota; Eurotiomycetes	neutropenia, high doses of corticosteroids, late-stage AIDS	allergic aspergillosis, aspergilloma, invasive aspergillosis
Candida albicans	Ascomycota; Saccharomycetes	neutropenia, AIDS	systemic infection, oral (thrush), vaginitis
Cryptococcus neoformans	Basidiomycota; Heterobasidiomycetes	Impaired cell-mediated immunity, such as AIDS, lymphoma, corticosteroid therapy	meningoencephalitis
Mucor spp.	Zygomycota; Zygomycetes	diabetic ketoacidosis, neutropenia, deferoxamine therapy, malnutrition	sinusitis, rhinocerebral infection, pneumonitis
Penicillium marneffei	Ascomycota; Eurotiomycetes	AIDS	fever, anaemia, lymphadenopathy, hepatomegaly
Pneumocystis carinii	Ascomycota; Pneumocystidomycetes	AIDS	pneumonia

Aspergillus fumigatus

Aspergillus fumigatus is the most common cause of mould infections worldwide (Latgé, 2001). The genus *Aspergillus* is classified within the division Ascomycota and contains roughly 200 species. Several species can cause disease in humans and animals, though *A. fumigatus* is responsible for approximately 90% of human *Aspergillus* infections (Latgé, 2001). *A. flavus* is also a significant pathogen whereas other species, such as *A. niger*, *A. nidulans* and *A. terreus*, more rarely cause disease (Denning, 1998).

Aspergilli are filamentous fungi whose natural niche is the soil. They obtain nutrients by decomposing organic matter such as compost, hay, and other plant material; thereby playing an important role in recycling carbon and nitrogen. *A. fumigatus* is a particularly thermotolerant species, capable of growth at temperatures up to 55°C and producing conidia that can survive temperatures of up to 70°C (Raper and Fennell, 1965). Thermotolerance may have evolved in *A. fumigatus* because of the high temperatures that occur in decomposing organic matter. *A. fumigatus* is ubiquitous worldwide and reproduces by production of abundant asexual conidia (Figure 1-1).

A. fumigatus has a haploid genome of roughly 32 million base pairs, the sequencing of which is mostly complete (Denning *et al.*, 2002). A sexual cycle has not been observed in *A. fumigatus*; however parasexuality was described in *A. fumigatus* in the early 1960s (Stromnaes and Garber, 1963). Parasexuality is a phenomenon whereby two fungal strains growing in close proximity can be induced to form a heterokaryon, resulting in stable diploid nuclei (Brakhage and Langfelder, 2002).

Techniques involving transformation of protoplasts or electroporation of swollen conidia are commonly used in *A. fumigatus*. The recent genome sequencing combined

with development of efficient transformation systems have greatly facilitated the study of individual genes in this species.

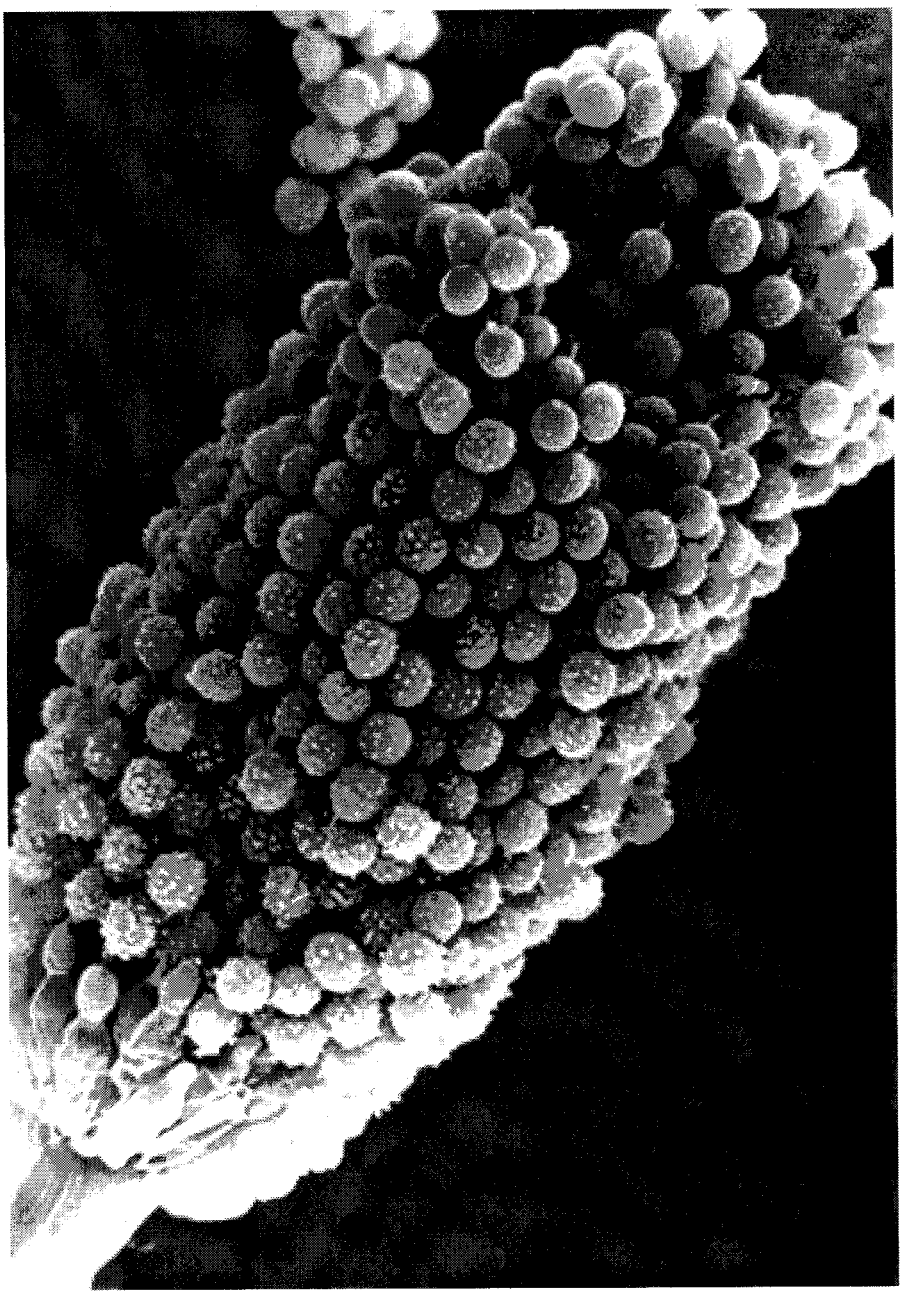


Figure 1-1: Electron micrograph of an *A. fumigatus* conidiophore

Scale: 1 cm = 9 microns. © Fungal Research Trust, by permission.

A. fumigatus conidia are only 2.5-3.0 μM in diameter and are therefore small enough to penetrate into lung alveoli upon inhalation (Latgé, 2001). Studies estimate that all humans inhale at least several hundred *A. fumigatus* conidia daily (Hospenthal *et al.*, 1998). Immunocompetent animals challenged with high doses of *A. fumigatus* conidia clear the inoculum from their lungs within a matter of hours. Phagocytic cells, particularly alveolar macrophages and neutrophils ingest conidia which are deposited in the lungs. Neutrophils also adhere to hyphae, which they kill by secreting reactive oxygen intermediates (Diamond *et al.*, 1983). However, in the absence of effective phagocytic cells, such as in neutropenic patients or patients treated with corticosteroids, conidia can germinate and produce hyphae that invade tissues. In addition, *A. fumigatus* can colonize damaged or diseased tissues in immunocompetent individuals.

Diseases caused by *Aspergillus* species

The severity of aspergillosis is generally determined by the state of the immune system of the patient. Diseases caused by *A. fumigatus* can be divided into two broad categories. Allergic reactions and non-invasive colonization are observed in immunocompetent individuals, while systemic infections with high mortality rates most often affect the immunocompromised. In almost all cases, the route of entry for the infectious conidia is the respiratory tract or damaged tissues. The first documented case of human *Aspergillus* infection was an aspergilloma, or colonization of pre-existing lung cavity, reported in 1842 (Cawley, 1947). Early cases of aspergillosis were often associated with certain occupations with exposures to high concentrations of conidia, such as pigeon-crammers, hair combers, farmers, feed-mill workers, threshers and fur cleaners (Cawley, 1947). It was not until the advent of corticosteroids and cytotoxic

chemotherapy in the 1950s that the number of cases of invasive pulmonary aspergillosis began to rise dramatically.

Non-invasive *Aspergillus* diseases

In addition to aspergillomas, *Aspergillus* species cause several different non-invasive infections of the immunocompetent, including sinusitis, allergic bronchopulmonary aspergillosis, keratitis and postoperative infections.

Aspergilloma is a surface colonization of preexisting lung cavities that were caused by tuberculosis, sarcoidosis, bullous lung disorders or in chronically obstructed paranasal sinuses. Aspergillomas consist of spherical masses of hyphae with sporulating structures at the periphery. The fungal growth occurs within the cavity and is non-invasive and often asymptomatic (Latgé, 1999). Symptoms may be caused by toxins and allergens secreted by the growing fungi. These symptoms can include weight loss, cough, and hemoptysis, which can be massive and even fatal (Chen *et al.*, 1997). Internal bleeding can also result. Prior to the increase in immunocompromised populations starting in the early 1950s, aspergilloma was the typical *Aspergillus* infection. In the United Kingdom, studies have shown that pulmonary cavities of 2 cm or larger develop aspergilloma in 15-20% of patients and the 5 year survival of patients with aspergilloma is about 40% (Denning, 2001).

Allergic diseases including farmer's lung, asthma, allergic sinusitis and alveolitis can occur following repeated exposure to *Aspergillus* conidia or antigens and do not involve colonization of the affected individual. Removal of the environmental source of the conidia generally results in clinical improvement. Patients with asthma and cystic fibrosis can develop a severe allergic pulmonary complication, allergic broncho-

pulmonary aspergillosis (ABPA) which is a hypersensitive reaction to *A. fumigatus*. These susceptible groups produce viscous secretions in the respiratory tract which can trap conidia, which then germinate and release fungal toxins and antigens (Bodey and Vartivarian, 1989). This condition manifests itself as a bronchial asthma and can often be treated by management of asthma and corticosteroid treatment (Bodey and Vartivarian, 1989). Left untreated, ABPA can lead to pulmonary fibrosis resulting in a progressive and ultimately fatal loss of lung function (Denning, 2001). Though it is a very difficult syndrome to diagnose, it is estimated that ABPA occurs in 1 to 2% of asthmatic patients and 7 to 35% of cystic fibrosis patients (Latgé, 1999).

A. fumigatus can also colonize damaged tissues. Keratitis is an infection of the eye, usually associated with traumatic injury, and is an important cause of blindness in the developing world. *Aspergillus* species have been shown to be responsible for about 25% of cases of fungal keratitis in studies in Nigeria (Gugnani *et al.*, 1978) and Bangladesh (Williams *et al.*, 1987). *A. fumigatus* can also cause cutaneous infections. Burn-associated *A. fumigatus* cutaneous infections progress rapidly and are very difficult to treat, resulting in very high amputation and mortality rates (Becker *et al.*, 1991). *A. fumigatus* can infect operative sites and foreign body (eg catheter) insertion sites, particularly in neutropenic patients (Denning, 1998). Cutaneous infections are also observed in premature neonates (Rowen *et al.*, 1995) and may occur in children with acquired immune deficiency syndrome (AIDS) (Shetty *et al.*, 1997).

Invasive aspergillosis

The increase in number of immunosuppressed patients and success of modern immunosuppressive therapies has lead to increasing rates of invasive aspergillosis.

Aspergillosis invasive disease increased 14-fold over the 12 years from 1980 to 1992 (Groll *et al.*, 1996). Since there is a lack of effective prophylactic treatment for fungal infections, and an increasing size of the immunocompromised population, fungal infection rates are likely to continue to rise. Antibacterial and antiviral prophylaxis and therapies have improved to the extent that opportunistic fungal infections have emerged as a principal cause of mortality in solid organ transplant recipients (Singh, 2003) and leukemia patients (de Pauw and Meunier, 1999). *Aspergillus* species cause the most common fungal infection in lung transplants (Kubak, 2002) and have become the most common pulmonary infection in bone marrow/stem cell transplant recipients (Walsh and Groll, 1999). Early treatment is critical for successful treatment of invasive aspergillosis, however there is currently a lack of effective diagnostic tests. Risk groups for invasive aspergillosis are summarized in Table 1-3.

Invasive aspergillosis is difficult to diagnose with certainty and can have an extremely rapid progression, with death occurring within one to two weeks of the onset of the disease (Latgé, 1999). Four types of invasive aspergillosis have been described, each affecting different tissues. The first and most common type is acute and chronic pulmonary aspergillosis. Symptoms of pulmonary aspergillosis are non-specific and variable and may include fever, chest pain, cough, malaise, and weight loss. Acute pulmonary aspergillosis occurs in the most immunocompromised patients, who are also the least likely to display symptoms and who exhibit the fastest progression of disease (Denning, 1998). At a much lower frequency, chronic pulmonary aspergillosis can develop in individuals with less severe immunosuppression such as diabetes mellitus,

chronic granulomatous disease, AIDS, and alcoholism. In chronic disease, symptoms are more prominent and develop over weeks or months (Denning, 1998).

Table 1-3: Risk groups for invasive aspergillosis

patient group	average incidence of invasive aspergillosis	references
lung transplant recipients	12-26%	(Grossi *et al.*, 2000; Helmi *et al.*, 2003; Minari *et al.*, 2002)
acute leukemia	5-25%	(de Pauw and Meunier, 1999)
allogenic bone marrow transplant	5-10%	(Denning *et al.*, 1997a)
advanced AIDS	1-12%	(Latgé, 2001; Shetty *et al.*, 1997; Wallace *et al.*, 1998)
chronic granulomatous disease	25-40% (lifetime incidence)	(Denning, 1998)
heart-lung transplant recipients	19-26%	(Grossi *et al.*, 2000)
other solid organ transplant recipients	0.4-10%	(Brown *et al.*, 1996; Denning *et al.*, 1997a; Minari *et al.*, 2002; Patel and Paya, 1997)
heart transplant recipients	2-13%	(Utili *et al.*, 2000)

The second type of invasive aspergillosis, tracheobronchitis and obstructive bronchial disease, is more common in AIDS patients (Kemper *et al.*, 1993) and lung transplant recipients (Kramer *et al.*, 1991), though about 25% of patients with this condition are apparently immunocompetent (Kemper *et al.*, 1993). This condition involves various degrees of fungal invasion of the mucosa characterized by excess mucous production, inflammation and pseudomembrane formation. The airway eventually becomes obstructed with necrotic and fungal material and the disease can disseminate (Kemper *et al.*, 1993).

Acute and chronic invasive sinusitis involves *Aspergillus* invasion of sinus mucosa and/or the bone and may occur concurrently with pulmonary aspergillosis. Acute sinusitis is particularly common in neutropenic bone marrow transplant recipients (Drakos *et al.*, 1993), while chronic sinusitis appears in the mildly immunocompromised or in otherwise healthy patients (Denning and Stevens, 1990; Washburn *et al.*, 1988). Symptoms of invasive sinusitis include headache, visual impairment, nasal stuffiness, and impairment of smell (Denning, 1998). *Aspergillus* sinusitis is not easily distinguished from infection by bacteria or other fungi and is therefore difficult to diagnose. Uncontrolled *Aspergillus* sinusitis often leads to extension to the palate, facial skin, orbit or brain (Denning, 1998). Chronicity and relapse are common.

The fourth category of invasive aspergillosis is disseminated disease. Dissemination is often suspected in patients with invasive aspergillosis, but is rarely proven before autopsy (Denning, 1998). The brain is the most commonly affected organ, but the skin, kidneys, heart and eyes may also be affected (Latgé, 1999). Cerebral aspergillosis occurs in 10-20% of all invasive aspergillosis cases and the brain is rarely the sole site of infection (Denning, 1998; Hagensee *et al.*, 1994). Symptoms include alterations in mental status and seizures shortly before death in immunocompromised patients. The less immunocompromised may experience headaches and occasionally fever. Cerebral lesions appear on computerized tomography scans but definitive diagnosis of cerebral aspergillosis requires biopsy; however, the lesions are often deep-seated and difficult to access surgically (Denning, 1998).

Lastly, several *Aspergillus* species are important primary pathogens of animals. Some important veterinary diseases include pulmonary infections of birds, especially

turkeys (Peden and Rhoades, 1992), mastitis and abortion in cattle (Chihaya *et al.*, 1992), and guttural pouch aspergillosis in horses (Guillot *et al.*, 1997).

Treatment of invasive aspergillosis

Until recently, only itraconazole and amphotericin B were licenced for treatment of invasive aspergillosis. The crude mortality from invasive aspergillosis is around 100% in the absence of treatment (Denning, 1996), unless it is possible to permanently remove the immunocompromising factors. The mortality rate of invasive aspergillosis drops to approximately 50% with anti-fungal treatment (Latgé, 2001). Success of treatment is highly dependant on timing of diagnosis, the type of invasive aspergillosis and the immune status of the patient. Recently, resistance to some of the common anti-fungal drugs, particularly itraconazole, has been reported.

Itraconazole has been in use since 1990. It can be delivered orally, and is generally used on patients who cannot tolerate amphotericin B. Azole drugs, including itraconazole, work by inhibiting a cytochrome P-450 involved in ergosterol biosynthesis. Ergosterol is a sterol which forms an important component of the fungal cellular membrane. Itraconazole resistance appears to occur via two mechanisms. One is a change in the P-450 target of the drug, and the other is a probable drug efflux mechanism (Nascimento *et al.*, 2003). Resistant strains of *A. fumigatus* have been isolated from patients in several European countries and in the United States (Denning *et al.*, 1997b; Verweij *et al.*, 2002). In the UK, 6% of *A. fumigatus* isolates were found to be resistant to itraconazole (Denning *et al.*, 1997b).

Amphotericin B has been in use for the last 40 years. It has a very wide range of activity and is active against most fungal pathogens. It is a polyene macrolide antibiotic

which binds to sterols, particularly ergosterol, disrupting the integrity of fungal membranes. The main drawbacks to the use of amphotericin B are its poor solubility and high renal toxicity. The prevalence of *A. fumigatus* strains with resistance to amphotericin B is thought to be low, though there is some evidence that strains resistant to amphotericin B are associated with poor treatment outcomes (Lass-Florl *et al.*, 1998). The success rate of amphotericin B therapy is around 34%, though this varies widely depending on the nature of the infection and the immune status of the host (Denning, 1998).

New drugs in development show some promise for improved therapeutic outcomes. There are several novel azole drugs in development, including voriconazole, posaconazole and ravuconazole. These newer azole drugs have improved bioavailability, half-lives and safety profiles (Tkacz and DiDomenico, 2001). Voriconazole has been shown to be effective in vitro against itraconazole-resistant strains of *A. fumigatus* (Arikan *et al.*, 1999). Clinical use of voriconazole was approved in the United States in May 2002, posaconazole is undergoing Phase III clinical trials, and ravuconazole is in Phase II clinical trials (Anaissie *et al.*, 2004).

Another new type of antifungal agent is the echinocandins, a group of glucan synthesis inhibitors including caspofungin, anidulafungin and micafungin. These molecules are large lipopeptides that inhibit beta-(1,3)-glucan synthesis, damaging fungal cell walls. In vitro and in vivo, echinocandins are fungistatic against *Aspergillus* species (Denning, 2003). Caspofungin appears to be better tolerated than amphotericin B. In immunocompromised adults with invasive aspergillosis who did not respond to or tolerate other antifungal agents, 41% showed a complete or partial response to

caspofungin (Keating and Jarvis, 2001). Caspofungin was approved for clinical use in the United States in January 2001, while micafungin and anidulafungin are currently in Phase II/III clinical trials (Anaissie *et al.*, 2004). Caspofungin is also approved for use in Canada (Health Canada, 2004).

These new drugs offer some potential for improved therapy for aspergillosis patients, though none promise a dramatic improvement in treatment rates. They are all active against only a few targets: glucan synthesis (cell wall), ergosterol biosynthesis and ergosterol disruption (cell membrane). In order to improve the survival rates of patients with invasive aspergillosis, it is necessary to develop new drug strategies. One such strategy is to search for novel targets which are specific to the fungal cells and essential for virulence, then develop drugs which are active against these targets.

Virulence factors in fungi

For fungi to be capable of survival and growth inside the hostile environment of a host organism, they must possess certain virulence factors that allow them to overcome host defenses and cause disease. Many of the fungi that infect humans are primarily free-living saprophytes, and some would argue that what are termed "virulence factors" are simply traits that evolved as adaptations to a saprophytic life. That these factors also allow growth within a human or animal host is an unfortunate coincidence. Fungal infections are rarely transmitted from person to person, further evidence that few fungi have evolved specifically as pathogens. However, the 270 fungal species that cause infection in humans must possess some virulence factors that are absent in most fungi.

Obvious virulence factors include the ability to grow at physiological temperature and pH. For instance, strains of the fungal pathogen *Sporothrix schenckii* with lowered

thermotolerance are less able to disseminate in a mouse model and are only able to form cutaneous lesions (Hogan, 1996). Route of entry is also important. Species which access host tissues via airborne spores require that the infectious particle is small enough to be inhaled deep into the lungs. Many fungal pathogens are opportunistic and only infect the immunocompromised; therefore, virulence depends upon the type and extent of immunosuppression in the host.

A good test of virulence factors is the construction of isogenic strains that differ only in one gene. This concept is summarized in the Molecular Koch's Postulates as defined by S. Falkow (1988), and is applied to examine the roles of individual genes and their products in contributing to virulence of an organism. Briefly, the postulates state that the phenotype of the property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species; that specific inactivation of a gene or genes should attenuate the virulence of the organism, and restoration of the mutated gene(s) should return pathogenicity to the organism.

Recently, many potential fungal virulence genes have been cloned, allowing specific knockout mutations to be performed or allowing the expression of the gene in an avirulent species. The study of virulence factors in fungi has been hampered by some inherent difficulties, namely the lack of a sexual cycle for many important pathogens, including *A. fumigatus*, which precludes the use of genetic studies. However, *A. fumigatus* is amenable to transformation, which makes the study of specific molecular targets possible.

Virulence factors of *A. fumigatus*

The search for virulence factors of *A. fumigatus* has been underway for many years. Several putative virulence genes have been disrupted, and these studies are summarized below. Analysis of these studies is complicated by the use of different models to evaluate virulence. These models differ in the species of animal used, the nature of immunosuppressive regimen and the route of infection. The comparison of virulence factors would benefit from development of a standard virulence test.

Early studies focussed on toxins as potential virulence factors. *A. fumigatus* produces several toxins, including gliotoxin, restrictocin, ribonucleotoxin, and phospholipases. The cytotoxin restrictocin is an inhibitor of translation which acts by inactivating eukaryotic ribosomes. However, mutants in restrictocin production retained full virulence in a neutropenic mouse model, indicating that restrictocin is not required for pathogenesis (Smith *et al.*, 1993). The *aspfI* gene encoding a ribonucleotoxin has also been disrupted by gene deletion, but studies in mice demonstrated that the *aspfI* gene product did not contribute to the virulence of *A. fumigatus* (Paris *et al.*, 1993). There is, however, some evidence of a role for gliotoxin in virulence. Gliotoxin has been demonstrated to have immunosuppressive activity in vitro (Mullbacher and Eichner, 1984) and in vivo (Sutton *et al.*, 1994). Injection of high concentrations of gliotoxin makes mice more susceptible to subsequent challenge with *A. fumigatus* conidia (Sutton *et al.*, 1996). Furthermore, animals infected with a non-gliotoxin producing strain survived significantly longer than those infected with a gliotoxin producer (Sutton *et al.*, 1996).

A. fumigatus secretes a variety of proteases which could allow dissemination by degrading protein barriers within the host. Elastinolytic proteinases produced by *A. fumigatus* could allow it to degrade lung tissues, which contain high concentrations of elastin. A study by Kothary *et al.* (1984) using immunocompromised mice found decreased virulence of *A. fumigatus* strains that had low elastinolytic activity. Several *A. fumigatus* proteases have since been characterized, including the alkaline proteinase Alp, a metalloproteinase Mep and an aspartic proteinase Pep. To date, no link has been found between any proteinases and virulence of *A. fumigatus*. Mutants in all the individual proteases, including an Alp Mep double mutant, retained complete virulence in immunosuppressed mice (Jaton-Ogay *et al.*, 1994; Monod *et al.*, 1993b; Reichard *et al.*, 1997; Tang *et al.*, 1993).

Antioxidants, such as catalase and dihydroxynaphthalene(DHN)-melanin have also received some attention as possible virulence factors because they offer protection against reactive oxygen species (ROS) produced by phagocytic cells. DHN-melanin is deposited in the conidial wall (Langfelder *et al.*, 1998) resulting in the characteristic grey-green colour of the conidia. It is an excellent scavenger of free radicals and also prevents binding of complement C3 to *A. fumigatus* conidia. The inhibition of C3 binding slows the rate of phagocytosis of melanized cells by alveolar macrophages (Jahn *et al.*, 2000) and neutrophils (Tsai *et al.*, 1998).

Disruption of *pksP*, a polyketide synthase necessary for melanin biosynthesis, reduced mortality rates from 100% to between 10 and 40% in mice injected intravenously with *A. fumigatus* conidia (Jahn *et al.*, 2002; Langfelder *et al.*, 1998; Tsai *et al.*, 1998). Melanin mutants are white and have been shown to have increased sensitivity to ROS

(Langfelder *et al.*, 1998). Additionally, the *pksP* mutants were more rapidly killed by monocyte-derived macrophages, which more rapidly formed phagolysosomes following phagocytosis of *pksP* mutants (Jahn *et al.*, 2002). However, strains lacking other enzymes of the melanin biosynthetic pathway such as AYG1 or ARP2, which catalyse the second and fourth steps, respectively, did not show attenuated virulence in a murine model (Tsai *et al.*, 1999). These data suggest that either intermediates in the melanin biosynthesis pathway are more important for virulence than melanin itself, or that *pksP* itself is involved in synthesis of toxic or immunosuppressive compounds other than melanin (Langfelder *et al.*, 2003). In addition, the protective role of melanin is limited to the initial colonization of the host, because while the infectious conidia are heavily melanized, mycelia of *A. fumigatus* are not pigmented.

A. fumigatus produces three different catalases: two mycelial catalases and one conidial catalase. Mutants deficient in a single mycelial catalase retain full virulence (Calera *et al.*, 1997), while a strain which was deficient in both of the mycelial catalases exhibited delayed infection in a rat model. Lesions appeared much more slowly in the lungs of rats infected with the mycelial catalase-deficient strain. The lesions observed 13-days post-infection in mice infected with the mutant strain were similar to those observed in the lungs of wild type-infected mice five days post-infection (Paris *et al.*, 2003). In addition, the most severe pulmonary lesions were only observed in the lungs of mice infected with the wild type strain, indicating that mycelial catalases offer transient protection to *A. fumigatus* mycelia in vivo (Paris *et al.*, 2003).

Several nutritional mutations with attenuated virulence have been described. Disruption of the p-aminobenzoic acid (PABA) synthesis pathway blocked folate

biosynthesis and resulted in *A. fumigatus* strains that were completely avirulent in a mouse model of invasive aspergillosis (Brown *et al.*, 2000). *lysF*, a gene involved in the lysine biosynthesis pathway, was demonstrated to be essential for survival of *A. fumigatus* in vivo in a low-dose mouse model of invasive aspergillosis (Liebmann *et al.*, 2004). A *pyrG* mutant was auxotrophic for uridine and uracil and was unable to germinate in vivo in the absence of uridine and uracil and was completely avirulent in a murine model of invasive aspergillosis (D'Enfert *et al.*, 1996).

The *A. fumigatus cpcA* gene encodes the transcriptional activator of the cross-pathway control system of amino acid biosynthesis. Mutants defective in this pathway showed decreased virulence in a murine model of invasive aspergillosis. The Δ*cpcA* strain caused 30% mortality compared to 90% mortality with the wild type (Krappmann *et al.*, 2004). *rhbA* is a ras-related protein which appears to play a role in nutrient sensing. Disruption of *rhbA* significantly reduced virulence of *A. fumigatus*, from 90% to about 50% mortality in a mouse model (Panepinto *et al.*, 2003). The *areA* gene of *A. fumigatus* is a transcription factor required for expression of genes involved in nitrogen metabolism, particularly the use of nitrogen sources other than ammonium or glutamine. Though the mortality was ultimately the same for both wild type and Δ*areaA* strains, the illness was delayed. 50% mortality was observed after 5 days for wild type, but not until 9 days for the Δ*areaA* strain (Hensel *et al.*, 1998). Histidine kinases play a role in osmoregularity and cell wall assembly. A putative two-component histidine kinase, *fos-1*, has been shown to contribute to virulence of *A. fumigatus* when injected intravenously in mice (Clemons *et al.*, 2002). Mice infected with the Δ*fos-1* strain survived twice as long as those infected with the wild type strain.

In summary, the only mutations found to date which completely abrogate virulence of *A. fumigatus* have been nutritional mutations preventing PABA, lysine, uridine and uracil biosynthesis. These mutations generate auxotrophic mutants whose viability is also compromised in vitro. Thus these genes are not virulence factors in the traditional sense. Mutants in *pksP*, a polyketide synthase, showed reduced virulence in some models of invasive aspergillosis; however, the exact mechanism by which *pksP* contributes to virulence is unknown. Gliotoxin, catalase, *areA*, *rhbA*, *fos1* and *cpcA* contribute to virulence of *A. fumigatus*, but are not absolutely required for pathogenicity.

A greater understanding of the limiting nutrients in the lung would aid in the identification of more virulence factors in *A. fumigatus*. Minimum levels of all the essential nutrients required by a particular pathogenic organism must be present at the site of infection in order for the pathogen to grow and cause damage to the host. The studies showing avirulence of PABA, lysine, uridine and uracil auxotrophic mutants has demonstrated that these compounds are not available within the lung in sufficient quantities to support growth of *A. fumigatus*. In addition to these molecules, some trace elements are suspected to be present in very low quantities in vivo. Iron, in particular, is known to be a very difficult element to obtain in vivo, and iron uptake presents a challenge to all pathogenic microorganisms.

Iron in biological systems

With the exception of some *Lactobacillus* species (Pandey, 1994; Posey and Gherardini, 2000), iron is required for the growth and reproduction of all organisms. *Borellia* species, which are the causative agents of Lyme disease and the syphilis pathogen, *Treponema pallidum* also lack requirements for iron (Posey and Gherardini,

2000). However, both *Borellia burgdorferi* and *T. pallidum* are obligate intracellular parasites with minimal genomes and though they may not require iron directly, they require the iron-dependant metabolic products of their hosts for energy and biosynthesis (Posey and Gherardini, 2000).

Iron has two stable oxidation states that can be manipulated to catalyze a wide variety of essential biochemical reactions. Iron is involved in such important biological processes as photosynthesis, N_2 fixation, methanogenesis, H_2 production and consumption, respiration, the trichloroacetic acid (TCA) cycle, oxygen transport, gene regulation, DNA biosynthesis and repair and detoxification of free radicals (Andrews *et al.*, 2003; Crichton and Ward, 1992, 1998). The biological role of iron requires it to be incorporated into proteins, or in complexes forming part of iron–sulfur clusters or heme groups. Incorporating iron into proteins allows for control of the local redox potential (ranging from -300 to +700 mV), geometry and spin state of the iron atoms, such that they can fulfil their necessary biological function (Andrews *et al.*, 2003).

Iron is abundant in the Earth's crust, but is poorly bioavailable due to its low solubility in aerobic environments at neutral pH. In the presence of oxygen, ferrous iron is readily oxidized to ferric iron, which forms highly insoluble ferric hydroxides at neutral pH (Guerinot, 1994). Conversely, excess cellular iron is damaging because iron can catalyse formation of deleterious hydroxyl free radicals by Fenton Chemistry. The mechanisms for hydroxyl and superoxide formation proposed by Haber and Weiss (1934) are now generally accepted:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^{\cdot} \quad \text{(initiation step)}$$

$$OH^{\cdot} + H_2O_2 \rightarrow H_2O + O_2^{\cdot -} + H^+ \quad \text{(chain reaction)}$$

$$O_2^{\cdot -} + H^+ + H_2O_2 \rightarrow O_2 + OH^{\cdot} + H_2O \quad \text{(chain reaction)}$$

$$Fe^{2+} + OH^{\cdot} + H^+ \rightarrow Fe^{3+} + H_2O \quad \text{(termination step)}$$

Hydroxyl radicals can cause serious damage to DNA and other cellular macromolecules (Byers and Arceneaux, 1998). Thus in aerobic environments, iron is both poorly available and potentially toxic. Conversely, since iron is a key component of antioxidant enzymes such as catalases, peroxidases and superoxide dismutases (Byers and Arceneaux, 1998) insufficient iron levels will not only compromise cell metabolism and growth but also depress the oxidative defences and lead to oxidative injury. For these reasons, all organisms require carefully controlled systems for the uptake and storage of iron.

Iron-binding molecules in the host

Since pathogenic microorganisms have an absolute requirement for iron, host animals can limit microbial growth by withholding iron in vivo. High affinity iron-binding molecules such as transferrin, lactoferrin, heme and ferritin maintain free iron levels in tissues at concentrations of approximately 10^{-18} M (Bullen, 1981), far too low to support microbial growth. Transferrins (Figure 1-2) are glycoproteins with molecular weights of 75,000 to 80,000 Da, and are the predominant iron-binding molecules in plasma. Lactoferrin is structurally very similar to transferrin and is found in neutrophils and in various secretions such as tears, saliva, nasal secretions and milk. Transferrin is

also present in airway surface fluids (Govindaraju *et al.*, 1998) where it would be encountered by inhaled *A. fumigatus* conidia. Transferrin plays an important role in efficient and selective iron transport in vertebrates, and because of its ability to chelate free ferric iron, transferrin also has antioxidant, bacteriostatic and fungistatic properties. Transferrin folds into a bilobal tertiary structure, in which each lobe contains a single site where iron is bound in association with a bicarbonate ion. The two lobes, shown in Figure 1-2, are structurally different and are not chemically equivalent (Baker *et al.*, 2002).

Transferrin is only about 30% saturated in healthy individuals. In response to infection, non-specific host defences decrease the level of transferrin saturation even further by releasing lactoferrin from neutrophils and increasing synthesis of ferritin (Bullen, 1981). This excess iron-binding capacity in circulating plasma and lymph makes free iron highly unavailable to potential pathogens in the extracellular environment.

Iron uptake by microbial pathogens

Iron uptake is critically important to the growth of pathogenic microorganisms in vivo; therefore they have evolved several different strategies to access iron from host iron-binding molecules. Extracellular pathogens are limited to transferrin and lactoferrin, as these are the only iron-containing molecules found extracellularly. Transferrin, heme and ferritin are all potential sources of iron to intracellular pathogens and hemolytic pathogens.

Figure 1-2: Ribbon diagram showing the tertiary structure of transferrins

Visible is the characteristic bilobal structure, with the N-lobe on the left and the C-lobe on the right. The C-terminal helix in purple may play a role in communication between lobes. In the cleft between the two domains of each lobe, a red iron atom can be seen bound in combination with a carbonate molecule. From Baker *et al.*(2003).

Transferrin receptors and heme

Some bacterial pathogens, such as *Staphylococcus* species (Modun *et al.*, 1998), *Neisseria* and *Haemophilus* species (Byers and Arceneaux, 1998) express transferrin receptors or lactoferrin receptors. These receptors are located at the outer membrane surface and are induced by iron starvation (Furrer *et al.*, 2002). Transferrin and lactoferrin receptors have not been described in fungi.

Heme is the most abundant source of iron within the host, and is therefore an attractive target for pathogens. Heme is usually bound within erythrocytes, but can be released by hemolysis. Once released from cells, heme is rapidly scavenged by the host proteins hemopexin and albumin, or it can be directly taken up by microbes. Many bacteria can use heme, hemoglobin or the hemopexin–heme complex as direct sources of iron. These products are bound by specific cell-surface receptors in some bacteria, and are transported across the cell membranes (Furrer *et al.*, 2002). Hemolysis spontaneously occurs in sickle cell anemia, malaria and trauma, and these conditions tend to predispose patients to infections (Bullen, 1985). Pathogenic fungi can also cause hemolysis. For example, the fungus *Candida albicans* produces the hemolytic toxin, mannan, which lyses red blood cells and releases heme into the bloodstream (Watanabe *et al.*, 1999). Heme is assimilated via an independent hemin-uptake system (Santos *et al.*, 2003).

Ferric reductases

Under aerobic conditions, iron is spontaneously oxidized to the ferric form (FeIII) which is insoluble at neutral pH. Aerobic organisms often express membrane-bound ferric reductases, which reduce ferric iron to the more soluble ferrous iron (FeII). The

ferric reductase is usually coupled with a ferrous iron transporter to move iron into the cell. Ferric reductase systems have been well characterized in the yeast, *Saccharomyces cerevisiae*. This species has a two-component complex consisting of a multicopper iron oxidase coupled with a permease (Askwith *et al.*, 1996). The ferrous iron permease of *C. albicans* has been found to be essential for virulence (Ramanan and Wang, 2000). Transferrin has a very low affinity for ferrous iron; therefore reduction of transferrin-bound ferric iron could be used to access this iron source. The ferric reductases of the fungus *Histoplasma capsulatum* have been reported to remove iron from both hemin and transferrin (Timmerman and Woods, 2001).

Proteinases

There is also evidence that proteinases can contribute to iron uptake by pathogens. Proteolysis of iron-binding molecules could release iron into solution, which would then be available to microbes. For example, *Porphyromonas gingivalis*, which causes adult periodontitis, does not express any the typical iron uptake pathways including ferric reductase or transferrin-binding activities. However, *P. gingivalis* can degrade transferrin, and was found to require proteinases for growth in media containing transferrin as the sole iron source (Brochu *et al.*, 2001). In another example, an alkaline protease of *Pseudomonas aeruginosa* increased its growth rate in media containing transferrin as the sole iron source (Shigematsu *et al.*, 2001). A different proteinase produced by *P. aeruginosa* was shown to increase the rate of transfer of iron from transferrin to the siderophore pyoverdin (Doring *et al.*, 1988).

pH

pH is an important consideration in iron uptake because transferrin releases bound iron at pH values below 6 (Baker *et al.*, 2002). *H. capsulatum* is an intracellular pathogen that regulates phagolysosomal pH, maintaining it near 6.6. A slightly acidic pH releases one of the two iron atoms bound to transferrin, but does not allow the proper activation of lysosomal hydrolases. Artificially raising the pH by addition of chloroquine greatly decreased the survival of intracellular *H. capsulatum*, and this effect was countered by addition of iron nitriloacetate, from which iron is available at neutral and alkaline pH (Newman *et al.*, 1994). Thus, the careful control of phagolysosomal pH by this pathogen is an important strategy in maintaining iron availability.

Similarly, diabetic ketoacidosis is a risk factor for mucormycoses, invasive fungal infections caused by zygomycetes. Acidosis lowers the serum pH, which diminishes the ability of transferrin to chelate iron, allowing the free iron concentration in serum to rise (Jurado, 1997). Abe *et al.* (1986) have postulated that the effect of raising the free iron concentration in the serum could be responsible for the *Mucor* infections associated with diabetic ketoacidosis.

Siderophores

Production of siderophores is a very common and efficient method of iron uptake employed by microorganisms and monocotyledonous plants. Siderophores are low molecular weight, high-affinity iron chelators (Neilands, 1995), secreted in response to iron deprivation. Most fungi produce several siderophores, and some soil fungi have been shown to produce 10 or more different siderophores in culture (Jalal *et al.*, 1984). Siderophores have two functions. The first is to supply the cells with iron, and the

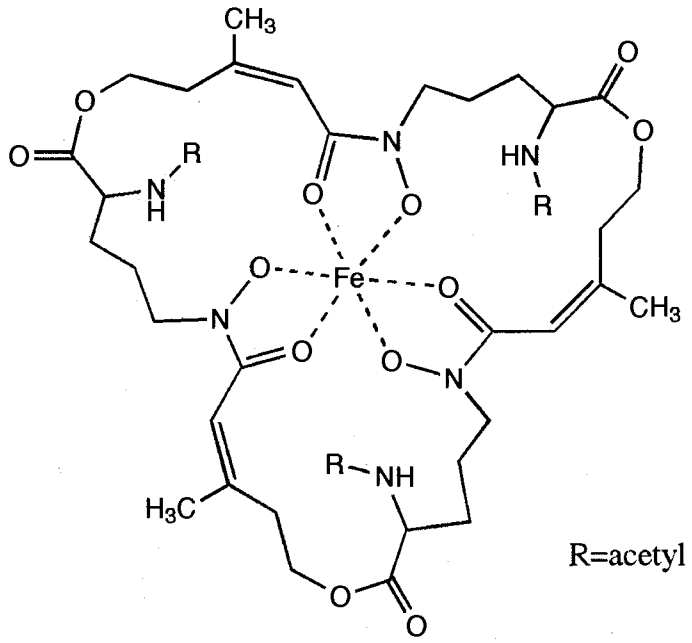
second is to function as intracellular iron storage molecules (Matzanke *et al.*, 1988). Many fungi and bacteria are able to use exogenous siderophores for their iron supplies by expressing receptors for a variety of siderophores. For example, though the fungus *S. cerevisiae* does not produce siderophores, it expresses receptors for ferrioxamine B, ferricrocin and rhodotorulic acid (Howard, 1999). This ability offers a competitive advantage during growth in iron-limiting conditions in the presence of many species. However, since many of the well-studied fungal pathogens such as *C. albicans* and *Cryptococcus neoformans* do not secrete siderophores, fungal siderophores have not been as well-studied as bacterial siderophores. Siderophores are known to be produced by a few fungal pathogens, including *Aspergillus* species (Diekmann and Krezdorn, 1975), *H. capsulatum* (Howard, 2000) and the plant pathogen *Ustilago maydis* (Ardon *et al.*, 1998).

Classes of siderophores and their structures

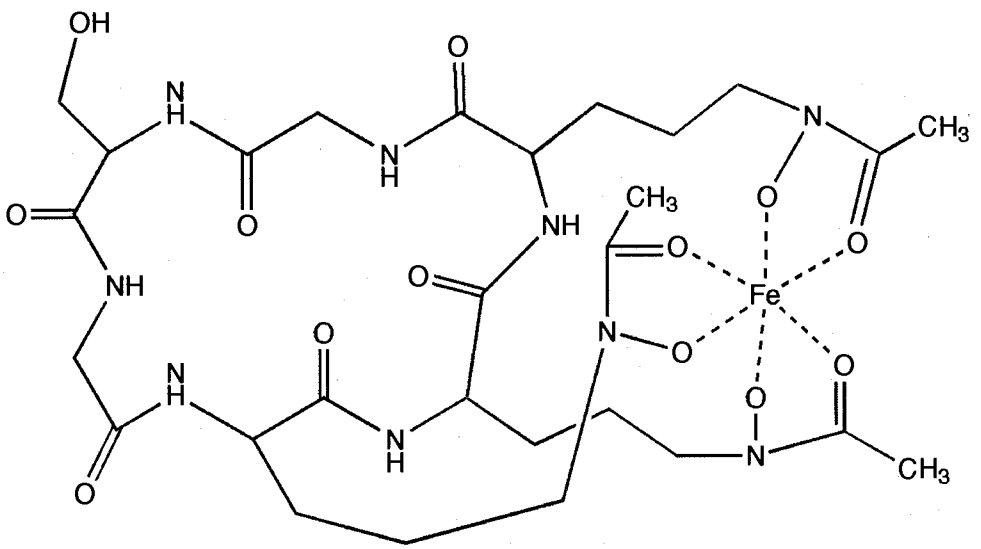
Siderophores are classified into three chemical classes based on their iron chelating groups: catechols, hydroxamates, and carboxylates. Bacteria produce all three classes of siderophores. Fungi produce predominantly hydroxamate siderophores, though carboxylates are also produced by some basidiomycetes (Howard, 1999). *A. fumigatus* has been reported to produce several hydroxamate siderophores including ferricrocin, ferrichrome, ferrichrome C and N'N''N'''-triacetylfusarinine C (TAF) (Diekmann and Krezdorn, 1975).

Ferricrocin, ferrichrome and ferrichrome C are all members of the ferrichrome class of siderophores, while TAF is a hydroxamate siderophore of the fusarinine class. TAF is composed of three N-δ-acyl-N-δ-hydroxyornithine units joined head-to-tail (Figure 1-2A). It is produced by *Aspergillus* species and a few other fungi (Adjimani and

Emery, 1987; Moore and Emery, 1976). Ferrichromes are composed of cyclic X-X-X-Orn-Orn-Orn residues, where Orn represents N-δ-acyl-N-δ-hydroxyornithyl residues, and X can be any of glycine, alanine or serine residues (Howard, 1999). The structure of ferricrocin (Gly-Ser-Gly-Orn-Orn-Orn) is shown in Figure 1-2B. In addition to being secreted by many species (Bartholdy *et al.*, 2001; Bentley *et al.*, 1986; Frederick *et al.*, 1981; Haselwandter and Winkelmann, 2002; Hordt *et al.*, 2000; Matzanke *et al.*, 1988; Ohra *et al.*, 1995), ferricrocin is the main intracellular iron storage molecule in *Neurospora crassa* (Matzanke *et al.*, 1988) and *Aspergillus nidulans* (Eisendle *et al.*, 2003).



A



B

Figure 1-3: Chemical structures of the *A. fumigatus* siderophores N'N''N'''-triacetylfusarinine C (TAF) (A) and ferricrocin (B)

Objectives

At the outset of this research, little was known about the mechanisms of iron acquisition by *A. fumigatus*. It had long been known that *A. fumigatus* produces hydroxamate siderophores (Diekmann and Krezdorn, 1975); however, little was known about iron uptake by *A. fumigatus* in vivo or in vitro. It was also not known whether any of the aforementioned siderophores contributed to the virulence of *A. fumigatus*.

This research was undertaken with two main goals. The first objective was to determine the mechanism by which *A. fumigatus* obtained iron from biological sources. The second was to determine if any of the *A. fumigatus* iron uptake pathways were required for virulence.

To achieve these goals we first examined the ability of *A. fumigatus* to grow in the presence of human serum and human serum transferrin, to determine whether transferrin was an iron source for *A. fumigatus*. Next, since it was well known that *A. fumigatus* produces siderophores and proteinases, we examined the roles of these compounds in obtaining iron from serum transferrin. Two *A. fumigatus* siderophores were purified and we assessed their abilities to remove iron from transferrin and measured the kinetic rates of iron transfer from transferrin to *A. fumigatus* siderophores. Our final objective was to create a siderophore secretion mutant to examine the role of siderophores in iron uptake from transferrin in vitro, as well as to assess the role of siderophores in virulence of *A. fumigatus*. These studies have shed some light on the methods of iron uptake by *A. fumigatus* in vivo, and may contribute towards the ultimate aim of finding new drug targets against this pathogen.

CHAPTER TWO: SERUM STIMULATES GROWTH OF AND PROTEINASE SECRETION BY *ASPERGILLUS FUMIGATUS*

Summary

Serum contains iron-binding proteins which inhibit the growth of most pathogenic microorganisms including fungi. The purpose of this research was to investigate the effect of serum on growth of the opportunistic fungal pathogen *Aspergillus fumigatus*. Supplementing minimal essential medium (MEM) with up to 80% of either human or fetal bovine serum (FBS) stimulated growth, increasing the amount of *A. fumigatus* dry biomass approximately 4-fold. In addition, a 100-fold increase in proteinase secretion, as measured by azocasein hydrolysis, was observed when either human serum or FBS was added to MEM at a concentration of 10%. The fungal proteinases secreted in serum-containing media were shown to degrade ^{3}H-labeled basal lamina proteins. The factor in serum that stimulated proteinase secretion had a mass greater than 10 kDa and was 85% inactivated by heating serum for 30 minutes at 66°C. The proportion of proteinases of each catalytic class secreted by *A. fumigatus* differed in serum compared to those secreted in media containing single proteins. Proteinase secretion did not result from increased protein concentration in the medium *per se* because BSA alone, in equal protein concentration to serum, produced only 20% of the proteinase activity per mg dry weight when compared to FBS. Addition of BSA plus 100 μM $FeCl_3$ to MEM promoted the same degree of growth as serum, indicating that a combination of nutritional factors in serum may stimulate growth. However, proteinase secretion was still only 30% of the

level observed with FBS. These data indicate that in contrast to most fungal pathogens, serum was not inhibitory to the growth of *A. fumigatus*. Furthermore, the nutrients in serum stimulated high levels of proteinase secretion, potentially increasing the invasiveness of this species.

Introduction

Serum inhibits the growth of microorganisms

Mammalian serum is inhibitory to the growth of many microbes, including some of the most common fungal pathogens. Fetal bovine serum has been shown to inhibit the growth of *Penicillium marneffei* (Taramelli *et al.*, 2000), while the growth of *Candida albicans* (Minn *et al.*, 1997) and *Cryptococcus neoformans* (Granger *et al.*, 1986; Nassar *et al.*, 1995) is inhibited by human serum. Other fungal pathogens whose growth is inhibited by normal human serum include *Histoplasma capsulatum* (Sutcliffe *et al.*, 1980), members of the genera *Cunninghamella* and *Absidia* (Eng *et al.*, 1981), *Rhizopus oryzae* (Artis *et al.*, 1982) and *R. microsporus* var. *rhizopodiformis* (Verdonck *et al.*, 1993).

The inhibitory action of serum is thought to be due to its ability to chelate iron, depriving invading pathogens of this essential nutrient (Ratledge and Dover, 2000). The role of iron in fungal pathogenesis has been recently reviewed (Howard, 1999; Weinberg, 1999). Transferrin in serum binds iron with high affinity, and apotransferrin is inhibitory to the growth of *C. neoformans* (Sridhar *et al.*, 2000), *C. albicans* (Howard, 1999), *H. capsulatum* (Sutcliffe *et al.*, 1980), and *Rhizopus* species (Artis *et al.*, 1982). Human serum contains approximately 0.2 mg/ml transferrin, which is only about 30% iron-

saturated, therefore high concentrations of the growth inhibitory iron-free transferrin (apo-transferrin) and monoferric-transferrins are found in vivo.

Studies have shown that increased iron burden is a risk factor for infection by many different fungal pathogens. This finding is consistent with the hypothesis that iron chelation is responsible for the growth inhibitory effects of serum. Supplementing serum with iron reverses its fungistatic effect for *C. neoformans* (Sridhar *et al.*, 2000), *P. marneffei* (Taramelli *et al.*, 2000), *H. capsulatum* (Sutcliffe *et al.*, 1980), and *C. albicans* (Radke *et al.*, 1994). Moreover, treatment of dialysis patients with the iron-chelating drug desferrioxamine B is a risk factor for zygomycoses because iron bound to desferrioxamine is efficiently used as an iron source by some species of zygomycetes (Abe *et al.*, 1990; de Locht *et al.*, 1994). Iron overload generally increases the risk of infections by saturating iron-binding molecules and making iron available to pathogens (Sunder-Plassmann *et al.*, 1999).

Iron uptake by *A. fumigatus* has not been thoroughly studied, though several different siderophores have been characterized in *A. fumigatus*, and as a saprophyte it is an abundant producer of proteinases. Either or both of these factors could contribute to iron uptake by *A. fumigatus* in vivo.

Proteinases of *A. fumigatus*

Proteinases, also known as endopeptidases, are enzymes that degrade proteins by hydrolysing internal peptide bonds. Proteinases can be divided into four different categories: aspartic, metallo-, serine and cysteine proteinases (Hooper, 2002). These proteinases differ in their catalytic mechanisms. Serine proteinases, such as chymotrypsin and subtilisin contain histidine, aspartate and serine residues at the active

site. The family of cysteine proteinases, of which papain is the best characterized member, similarly contain histidine and aspartate residues at the active site, but the nucleophile is the thiolate ion of cysteine rather than the hydroxyl group of serine. Pepsin is the typical aspartic proteinase, a class of enzymes which contain two aspartates at the catalytic site. Metalloproteinases, a very old class of enzymes, typically contain a zinc atom, or more rarely cobalt or nickel at the catalytic site (Hooper, 2002). Different inhibitors can be used to distinguish between the different classes of proteinases.

A. fumigatus, like most fungi, has been found to secrete a variety of different proteinases. These include a serine proteinase, Alp, which undergoes post-translational modification resulting in up to six different forms which can be resolved by isoelectric focussing (Kunert, 2001). A metalloproteinase, Mep, is also produced (Jaton-Ogay *et al.*, 1994). One or possibly two aspartic proteinases, Pep and Pep2, have also been described (Reichard *et al.*, 1997). Proteinase secretion is inducible in *A. fumigatus* and occurs in response to the presence of proteins or protein hydrolysate (Bouchara *et al.*, 1993).

The role of proteinases in virulence of *A. fumigatus* is unclear, but secretion of proteinases is thought to be necessary to break down protein barriers in the host. Several types of protein barriers are present in the lung. The lung contains two adjacent layers of basal lamina, secreted by and separating the epithelial and vascular endothelial cells. The basal laminae are composed mainly of laminin, collagen IV, and heparan-sulfate proteoglycans, organized into a complex supramolecular structure (Kleinman *et al.*, 1982). Because the basal lamina is located directly under the alveolar epithelium, it is one of the first barriers to dissemination of *A. fumigatus*. In addition, lung tissue is very high in protein, particularly elastin and collagen (Monod *et al.*, 1995). Proteinases

secreted by pathogens could play a role in penetration of the basal lamina and decomposition of other lung constituents such as elastin and collagen.

Infections due to *Aspergillus* are characterized by degradation of lung parenchyma (Monod *et al.*, 1995), and some studies have shown a correlation between elastase production and virulence in a mouse model of aspergillosis (Kothary *et al.*, 1984). However, single gene disruption experiments have failed to show any role for individual proteinases in virulence of *A. fumigatus*. A double mutant in Alp and Mep did not show any reduction in virulence (Jaton-Ogay *et al.*, 1994), nor did a Pep mutant that secreted no measurable aspartic proteinase activity (Reichard *et al.*, 1997).

Objectives

Little is known about the effects of serum on the growth of *A. fumigatus*. In this report, we studied the effect of serum on the growth of *A. fumigatus* and on the pattern of proteinase secretion *in vitro*. We demonstrated that, unlike most other common fungal pathogens, neither fetal bovine serum nor human serum inhibited the growth of *A. fumigatus*, but both in fact stimulated growth when present in concentrations up to 80%. In addition, both human and fetal bovine sera stimulated very high levels of serine and metallo-proteinase secretion in *A. fumigatus*. An attempt was made to identify the serum factor responsible for stimulating such high levels of proteinase secretion.

Materials and Methods

Strains and growth conditions

Aspergillus fumigatus (ATCC 13073) was originally isolated from a human pulmonary lesion and was obtained from the American Type Culture Collection (ATCC) and maintained on YM slants (0.3% malt extract, 0.3% yeast extract, 0.5% peptone and

0.5% glucose) at 4°C. *A. fumigatus* was cultured on YM plates at 28°C for 5-10 days, until fully conidiated. Conidia were harvested by flooding the culture plate with phosphate buffered saline (PBS) containing 0.05% Tween-20, and swabbing with a sterile cotton swab. The conidia were then agitated, filtered through a plug of sterile glass wool to remove hyphae, and resuspended in PBS. Concentrations of conidia were determined by counting in a hemacytometer.

For most experiments, *A. fumigatus* was cultured in minimal essential medium (MEM) (Life Technologies, Burlington, Ontario) containing the stated concentration of serum or other medium components in a total volume of 5 ml in 25 ml culture flasks. For iron-depletion experiments, glassware was treated overnight in 1 mM EDTA, followed by 2 hours in 0.5 M HCl, and then rinsed six times with deionized water. MEM contains 1 mg/ml glucose, amino acids, vitamins and salts, but no iron (Eagle, 1959). Trace levels of iron and other metal contaminants were removed from MEM by stirring overnight with 6% w/v Chelex 100 (Sigma, Oakville, Ontario). After Chelex treatment, 2 g/L $CaCl_2$ and 0.98 g/L $MgSO_4$ were added to MEM. Media containing high serum concentrations were prepared using a 10x stock of MEM to ensure that the MEM concentration remained constant. Conidia were added to media at a final concentration of $1x10^6$/ml, and flasks were incubated at 36°C and 150 rpm. Dry weights were measured by filtering the entire contents of each flask through Miracloth (Calbiochem, La Jolla, California) and rinsing thoroughly with dH_2O to remove all traces of culture medium. Mycelia were then transferred to pre-weighed microcentrifuge tubes, lyophilized overnight and weighed.

The Type II pneumocyte human cell line A549 was obtained from ATCC (CCL-185) and maintained in RPMI 1640 (Life Technologies, Burlington, Ontario) containing 10% fetal bovine serum (Life Technologies, Burlington, Ontario), 2 g/L sodium bicarbonate, 0.1 mg/ml streptomycin and 25 units/ml penicillin G. For culture of *A. fumigatus* on A549 cells, the medium was aspirated from a confluent plate, the plate was washed three times with PBS, then 10 ml MEM containing 10^6 conidia per ml was added.

Serum and other reagents

Fetal bovine serum (FBS) was obtained from Life Technologies (Burlington, Ontario). Human serum (male) was obtained from Sigma (Oakville, Ontario). All sera were stored in 10 ml or 100 ml aliquots at -20°C until use. Serum was thawed in a 37°C waterbath and processed as noted below.

Serum was heat-treated in a Braun Thermomix 1441 11L waterbath for 30 minutes at the specified temperature (56 or 66°C) or treated at 100°C in a boiling water bath for 30 minutes. Serum was separated into high and low molecular weight fractions by centrifugation at 3000g for 1 hour at 0°C through an Ultrafree 10,000 molecular weight cutoff filter (Millipore, Bedford, Massachusetts). The retained material was dialyzed against three changes of water at 4°C through a membrane with 10,000-12,000 molecular weight cut-off to thoroughly remove low molecular weight components.

Iron was removed from serum as described by Wilson *et al.* (50). Serum was filtered through an Ultrafree 10,000 molecular weight cutoff filter (Millipore, Bedford, Massachusetts), and the filtrate stored at 4°C. The protein-containing fraction was lowered to pH 4.0 with 1M HCl, and EDTA was added to a final concentration of 10 mM. The fraction was dialyzed overnight against PBS containing 6% w/v Chelex 100

(Sigma, Oakville, Ontario) before being recombined with the low-molecular weight components. The iron content of the treated serum was determined by digesting the samples in boiling nitric acid, making appropriate dilutions in iron-free water and measuring the iron concentrations by atomic absorption spectrometry using a Perkin Elmer AAnalyst 100.

Matrigel™ was obtained from Becton Dickinson Labware, Bedford, Massachusetts. Matrigel™ was added to MEM at a concentration of 6% (v/v).

Azocasein assay

Proteinase secretion was quantified using the azocasein assay as outlined by Reichard *et al.* (1990). Azocasein (Sigma, Oakville, Ontario) was dissolved at 5 mg/ml in assay buffer containing 50 mM Tris, pH 7.5, 0.2M NaCl, 5mM $CaCl_2$, 0.05% Brij 35, and 0.01% sodium azide. Media from *Aspergillus* cultures were removed at various time points and centrifuged to pellet cells. Azocasein solution (400µl) was mixed with 100µl supernatant from *Aspergillus* cultures and incubated in a 37°C waterbath for 90 minutes. Reactions were stopped by addition of 150 µl 20% trichloroacetic acid, and allowed to stand at ambient temperature for 30 minutes. Tubes were then centrifuged for 3 minutes at 8,000g and 500µl of the supernatant was added to 500 µl 1M NaOH. The absorbance of released azo dye was read on a spectrophotometer at 436 nm. For inhibitor studies, 400 mM stocks of phenylmethylsulfonylfluoride (PMSF) and 1,10-phenanthroline (both from Sigma, Oakville, Ontario) were prepared in ethanol. Culture supernatants were pre-incubated with 4 mM inhibitor for 15 minutes at 37°C, then assay buffer containing 4 mM inhibitor was added.

Azocoll assay for collagenase activity

Azocoll substrate (Calbiochem, La Jolla, California, >100 mesh) was used to measure collagenase activity. Azocoll is composed of insoluble particles of collagen to which a bright-red azo dye has been attached. The Azocoll assay was based on the method of Chavira *et al.* (1984). Azocoll was suspended in a buffer containing 50 mM Tris, pH 7.5, 1mM $CaCl_2$ and 0.01% sodium azide. Azocoll suspension (800 µl) was incubated with 5 µl supernatant from *Aspergillus* cultures for 3 hours at 37°C on a rotator. The tubes were centrifuged at 8000 g for 3 minutes and the release of azo dye was determined by measuring the absorbance of the supernatant at 520 nm.

Preparation of radioactively labeled extracellular matrix

Preparation of labeled extracellular matrix was modified from the method of Morschhaeusser *et al.* (1997). A549 cells were seeded onto 24-well plates and incubated in RPMI 1640, 10% serum, for 48 hours in a 5% CO_2 atmosphere until they had reached sub-confluence. The medium was removed and replaced with glucose-free RPMI containing 1% FBS for 1 hour. The medium was then replaced by fresh glucose-free RPMI containing 1% FBS and 2.5 µCi/ml D-[6-^{3}H]-mannose (American Radiolabeled Chemicals Inc., St. Louis, Missouri) and incubated for 48 hours. Cells were lysed by deoxycholate treatment as described by Hedman *et al.* (1979). The confluent cells were rinsed three times with 1 ml PBS and then incubated 3 times for 10 minutes in 1 ml 10mM Tris-Cl pH 8.0, 0.5% sodium deoxycholate and 1 mM PMSF with slow shaking at 4°C. Wells were then washed 4 times for 5 minutes at 4°C with 2 mM Tris-Cl, pH 8.0 with slow shaking. The absence of cells after this treatment was confirmed by microscopy. The incorporation of ^{3}H-mannose into the extracellular matrix was

estimated by subtracting the combined radioactivity remaining in the supernatant, cell lysate and washes from the total radioactivity initially added.

Extracellular matrix degradation assays

Samples of culture medium were withdrawn from a 48 hour culture of *A. fumigatus* in MEM containing 10% serum or MEM alone. Control samples were prepared that contained fresh MEM or fresh MEM containing 10% serum. Accumax (Innovative Cell Technologies, San Diego, California), containing trypsin, collagenases, and other proteinases, was used as a positive control for basal lamina hydrolysis. Culture media and control media were added in 1 ml volumes to the wells containing the labeled extracellular matrix and the plates were incubated at 37°C. Samples (50 µl) were withdrawn from each well after 0, 0.5, 1.0 and 3.5 hours and the radioactivity released was measured by liquid scintillation counting (LSC) in Biodegradable Count Scintillant (Amersham, Piscataway, New Jersey) on a Beckman LS6500 multi-purpose scintillation counter.

Statistics

Data analysis was performed using the Student's t-test or analysis of variance followed by a Tukey's multiple comparison procedure.

Results

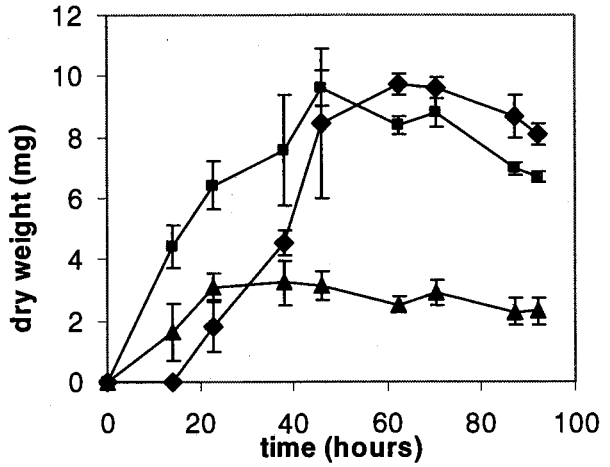
Fetal bovine and human serum stimulate growth and proteinase secretion in *A. fumigatus*

Incubation of *A. fumigatus* in MEM containing 10% fetal bovine serum (FBS) resulted in a faster rate of growth and a higher total biomass as compared to the same medium lacking serum (Figure 2-1A). Growth was delayed by about 20 hours in media

containing human serum, but the same maximum growth levels were reached after 48 hours. In serum-containing media, cultures reached stationary phase after roughly 50 hours of incubation, and the fungal dry weight in the presence of 10% serum was approximately four-fold greater than that of fungi cultured in serum-free MEM (Figure 2-1A).

Proteinase secretion by *A. fumigatus* was monitored using azocasein hydrolysis. The presence of either 10% FBS or 10% human serum greatly stimulated proteinase secretion (Figure 2-1B). In MEM alone, proteinase secretion by *A. fumigatus* was negligible during the 4 day incubation period. In MEM containing 10% serum, proteinase secretion peaked between 40 and 60 hours of culture, as late log phase was reached. Proteinase activity in the culture medium then decreased gradually over time as the cultures aged. Levels of proteinase secretion were similar with both human serum and FBS, though maximum levels of proteinase secretion were reached during an earlier stage of growth with human serum. The pH of the media was 7.2 at the start of the incubation, and rose steadily over the course of the incubation to a maximum of 8.9. The pH was never observed to drop below the initial level of 7.2.

A



B

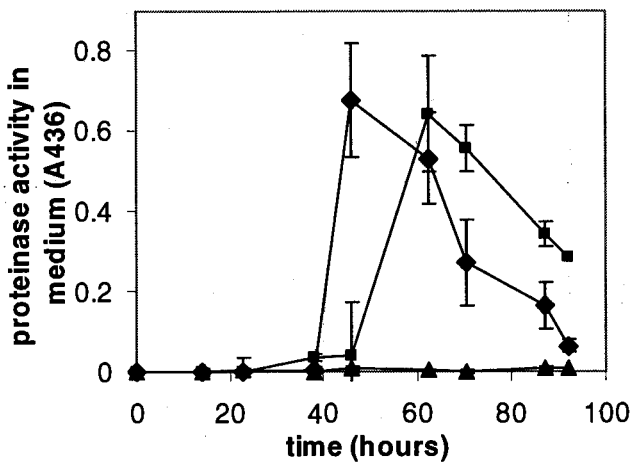


Figure 2-1: Growth curve (A) and proteinase secretion (B) for *A. fumigatus* in 10 ml MEM with or without 10% FBS or 10% human serum

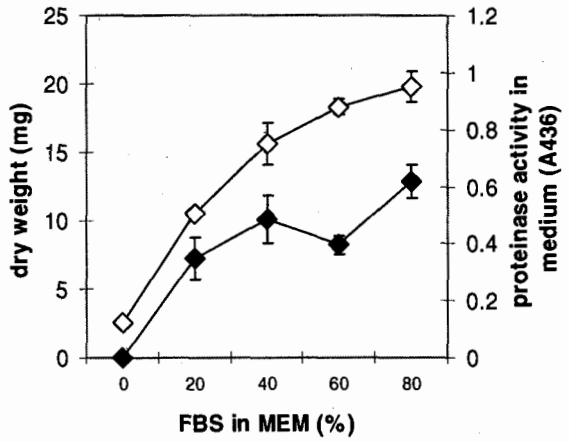
A. fumigatus was cultured in MEM alone (triangles), MEM containing 10% FBS (squares) or MEM containing 10% human serum (diamonds) as described in Materials and Methods. Flasks were removed from incubation at several time points and the contents of the whole culture flasks were filtered through Miracloth and mycelia transferred to pre-weighed microcentrifuge tubes. Tubes were lyophilized overnight and weighed to determine dry weights (A). Proteinase activity in the supernatant (B) was measured by the azocasein assay as described in Materials and Methods. The data shown are the mean ± standard deviations for three replicates and are representative of three independent experiments.

Serum contains many proteinases including matrix metalloproteinases (MMPs). The serum MMPS are unable to hydrolyze casein (unpublished observations), therefore all azocasein hydrolysis is catalysed by fungal proteinases. To exclude the possibility that serum MMPs were simply activating latent fungal proteinases, proteinases secreted in media containing boiled serum were incubated with 10% fresh serum. Proteinase activity, as measured by the azocasein assay, decreased after 30 minutes incubation of fungal proteinases with fresh serum (data not shown). Thus, it is unlikely that the increased fungal proteinase activity in the presence of serum is caused by activation of latent fungal proteinases by serum proteinases.

Serum concentrations up to 80% support increased growth and proteinase secretion

Previous work with *Rhizopus rhizopodiformis* showed a stimulation of fungal growth by human serum at concentrations up to 20%, but inhibition of growth at all higher human serum concentrations (Verdonck *et al.*, 1993). Therefore, the ability of *A. fumigatus* to grow in higher concentrations of fetal bovine and human sera was evaluated. The growth of *A. fumigatus* was found to increase with increasing serum concentrations, and no evidence of growth inhibition by FBS was observed at concentrations up to 80% (Figure 2-2A). Human serum also supported increased growth of *A. fumigatus* at concentrations up to 80% (Figure 2-2B).

A



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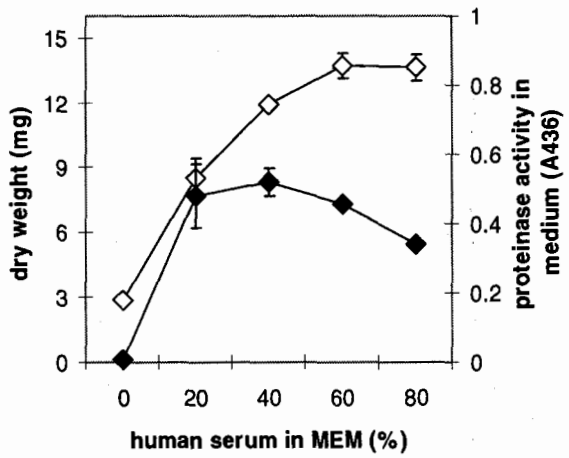


Figure 2-2: Growth of and proteinase secretion by *A. fumigatus* in MEM containing high concentrations of fetal bovine serum (A) or human serum (B)

A. fumigatus (10^6 conidia/ml) was cultured in 5 ml MEM containing fetal bovine serum (A) or human serum (B) concentrations ranging from 0 to 80%. The MEM concentration was held constant. Flasks (25 ml) were incubated for 50 hours at 37°C and 150 rpm. Dry weights were measured and azocasein assays performed as described in Materials and Methods. The data shown are the mean ± standard deviations for triplicate values. (A) is representative of three independent experiments and (B) is representative of two independent experiments. Open symbols show dry weights, while black symbols represent proteinase activity.

Proteinase secretion by *A. fumigatus* also increased steadily with increasing fetal bovine serum concentrations. Human serum also stimulated proteinase secretion, but the effect reached a maximum at 40% serum and declined slightly at higher concentrations.

The proteinases secreted by *A. fumigatus* are able to hydrolyze basal lamina proteins

To determine whether the proteinases secreted by *A. fumigatus* in serum-containing medium could hydrolyze proteins that might be encountered during infection, including those found in basal lamina, two assays were conducted. Since lung tissue is very high in collagen, the hydrolysis of Azocoll (an insoluble collagen) by supernatants from *A. fumigatus* cultured in MEM with or without serum was measured. Additionally, the release of proteins from radioactively labeled basal lamina by *A. fumigatus* supernatants was determined. Azocoll was efficiently hydrolyzed by the *A. fumigatus* secreted proteinases (Table 2-1) and the pattern of relative hydrolysis rates when grown in the presence of fetal bovine or human serum was similar to that observed with azocasein (Figure 2-1B). *A. fumigatus* produced proteinases capable of Azocoll hydrolysis when grown in MEM without serum but only at 10% the level observed when serum was present (Table 2-1).

Table 2-1: Azocoll hydrolysis by *A. fumigatus* proteinases secreted in serum-containing media

	A_{520} values[a]		
	No conidia	46 hours[b]	69 hours[b]
Fetal bovine serum	0.00 ± 0.01	0.07 ± 0.07	0.50 ± 0.15 *
Human serum	0.01 ± 0.00	0.60 ± 0.28 *	0.23 ± 0.08 *
No serum	0.00 ± 0.00	0.06 ± 0.05	0.02 ± 0.00

[a] Values are means of triplicates ± standard deviations of A_{520} values obtained after incubation of 5µl culture supernatants with 800 µl 5 mg/ml Azocoll suspension for 3 hours at 37°C.
[b] Length of *A. fumigatus* incubation
* Absorbance values are significantly greater than in media cultured without *A. fumigatus* ($p < 0.05$)

We next investigated if the serum-enhanced proteinase secretion could contribute to the invasiveness of *A. fumigatus* by aiding in breakdown of human pulmonary basal lamina. This protein barrier must be breached by pathogens to disseminate from the airways into the lung tissue, vasculature and other organs. Radiolabeled basal lamina was prepared using the human type II pneumocyte cell line A549, and the basal lamina glycoproteins were labeled using ^{3}H-mannose. The proteinases secreted by *A. fumigatus* in a medium containing serum resulted in a faster rate of hydrolysis of basal lamina proteins compared to a medium lacking serum (Figure 2-3). In agreement with the Azocoll assay results, low levels of basal lamina hydrolysis were observed from *A. fumigatus* cultured in MEM alone. Hydrolysis of basal lamina proteins by fungal proteinases was rapid, occurring mostly during the first 30 minutes of incubation. After 200 minutes, the fungal proteinases from serum-containing media and Accumax, the mixture of trypsin and collagenases, had released equivalent DPM from basal lamina.

Controls containing fresh media in which fungi had not been cultured caused only a small baseline release of ^{3}H-mannose during the 200 minute sampling period (Figure 2-3).

The pattern of proteinase secretion depends upon the composition of the growth medium

The molecular weights and catalytic classes of the proteinases induced by serum were compared to those induced by other proteins. *A. fumigatus* was cultured in MEM containing either 8 mg/ml gelatin, 10% FBS, 10% human serum, MEM alone, 8 mg/ml BSA, 8 mg/ml BSA and 100 μM $FeCl_3$, A549 cells or 6% Matrigel. Matrigel is isolated from the EHS mouse sarcoma and is composed of the basal lamina proteins: laminin, collagen IV, entactin and heparan sulfate proteoglycan (Kleinman *et al.*, 1982). Culture supernatant was withdrawn and analyzed by azocasein assay with proteinase inhibitors.

The proportion of proteinases in the serine and metalloproteinase catalytic classes was highly dependent on protein substrate (Figure 2-4). For example, in MEM containing gelatin, serine proteinases were secreted almost exclusively; whereas in media containing BSA, roughly equal amounts of serine and metalloproteinases were secreted. In media containing either human or fetal bovine serum, the proportion of proteinase activity was roughly 2/3 serine proteinases and 1/3 metalloproteinases. No inhibition was detected with inhibitors of cysteine or aspartic proteinases in any of the samples (data not shown).

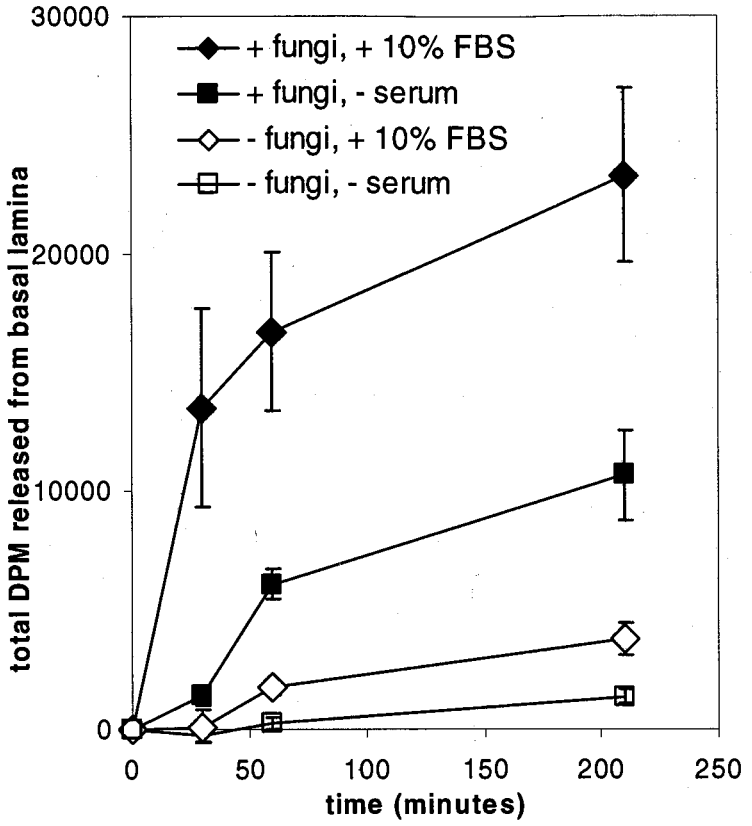


Figure 2-3: Release of ^{3}H-mannose from radiolabeled basal lamina by *A. fumigatus* proteinases

Basal lamina was metabolically labeled with ^{3}H-mannose by A549 cells as described in Materials and Methods. Media from *A. fumigatus* cultures grown in MEM with FBS or MEM alone was withdrawn after 48 hours and applied to the radiolabeled basal lamina at time 0. Hydrolysis of basal lamina proteins could be detected by monitoring release of ^{3}H into solution by liquid scintillation counting (LSC). Control media were prepared that contained MEM with serum or MEM alone, but no *A. fumigatus*. Counts are reported as mean total dpm released into solution ± standard deviations of three replicates. Data are representative of three independent experiments. Total DPM released from basal lamina by Accumax after 200 minutes was 29,800 ± 6,400 DPM. At 210 minutes, the DPM released by the fungi + serum supernatant was significantly higher than the DPM released by the supernatant which had contained fungi but no serum ($p < 0.025$).

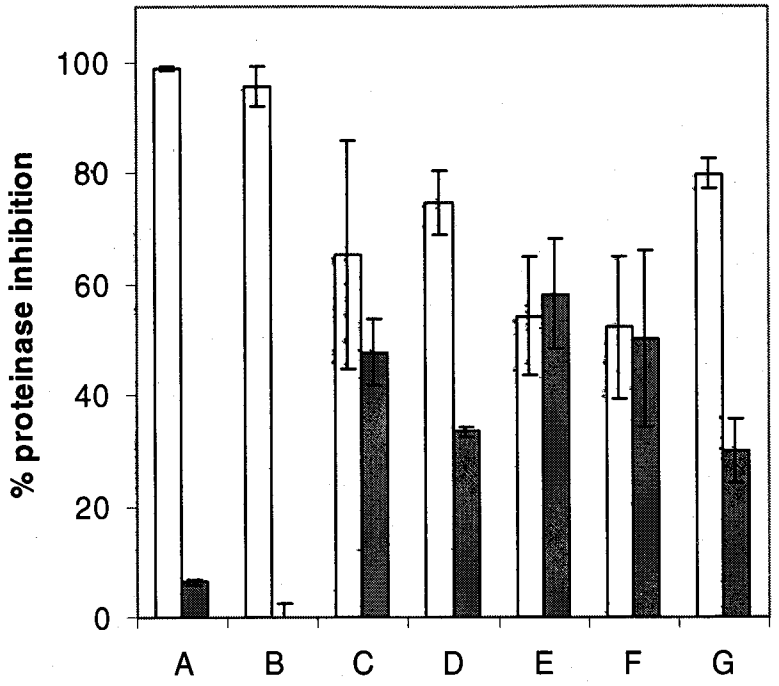


Figure 2-4: Catalytic classes of *A. fumigatus* secreted proteinases

A. fumigatus was cultured in MEM containing (A) 6% Matrigel, (B) 8 mg/ml gelatin, (C) 10% human serum, (D) 10% FBS, (E) 8 mg/ml BSA, (F) 8 mg/ml BSA and 100 μM $FeCl_3$, or (G) A549 cells. Supernatants from the cultures were incubated with 5 mg/ml azocasein in buffer containing either a serine proteinase inhibitor, 4 mM PMSF, or a metalloproteinase inhibitor, 4 mM 1,10-phenanthroline. An equivalent volume (1%) of ethanol was used as control. Azocasein activities (A_{436}) without inhibitors were (A) 0.34 ± 0.01, (B) 0.20 ± 0.02, (C) 0.46 ± 0.18, (D) 0.37 ± 0.07, (E) 0.08 ± 0.02, (F) 0.15 ± 0.06, (G) 0.63 ± 0.12. Data for each inhibitor are reported as percent activity in the presence of that inhibitor relative to activity in the absence of inhibitor, and are the means of three replicates ± standard deviations. White bars show percent inhibition by 4 mM PMSF (= percent serine proteinases), while dark bars show percent inhibition by 4 mM 1,10-phenanthroline (= percent metalloproteinases). No inhibition was detected with inhibitors of cysteine or aspartic proteinases in any of the samples (data not shown).

The factor in FBS that stimulates proteinase secretion in *A. fumigatus* is larger than 10 kDa, and is partially heat-labile

In an effort to identify whether a particular serum component stimulated proteinase secretion, FBS was passed through a 10 kDa molecular weight cut-off filter, and the retained material was dialyzed against distilled water, separating serum into high and low molecular weight fractions. The volume of the high molecular weight fraction was adjusted to restore the original serum protein concentrations. Upon incubation of *A. fumigatus* with the two fractions, proteinase secretion could only be observed in the presence of the high molecular weight fraction, and levels were similar to those stimulated by whole serum (Table 2-2). *A. fumigatus* cultured in media containing the low molecular weight fraction and containing MEM alone secreted very low levels of proteinases (Table 2-2). Thus, proteinase secretion is induced by a serum component greater than 10 kDa in size.

Table 2-2: Comparing the growth and proteinase secretion by *A. fumigatus* in MEM containing serum, dialysed serum, or low molecular weight serum components

MEM plus:	Proteinase secretion[a]	Dry weight (mg)[b]
10% FBS	0.70 ± 0.07	6.9 ± 1.0
10% dialysed serum (>10 kDa)	0.51 ± 0.12	8.9 ± 0.5
10% serum filtrate (< 10 kDa)	0.05 ± 0.04 *	3.7 ± 0.3 *
MEM alone	0.01 ± 0.00 *	1.9 ± 0.2 *

[a] Proteinase secretion was measured using azocasein hydrolysis. Values are means of triplicate measurements ± standard deviations for proteinase secretion (A_{436}) by *A. fumigatus* after 72 hours incubation in MEM containing 10% serum or 10% high or low molecular weight serum fractions, prepared as described in Materials and Methods.
[b] Dry weights are mean values ± standard deviations for total dry mycelial mass from 5 ml cultures after 72 hours incubation.
* Values are significantly different lower than those observed with 10% FBS ($p < 0.025$).

FBS was also heat-treated to determine if the serum factor that induces proteinase secretion in *A. fumigatus* is heat-labile. It was found that heating serum to 56°C did little to change the growth of *A. fumigatus* or its level of proteinase secretion. However, heating serum to 66°C or 100°C caused proteinase secretion to be decreased by about 85% (Table 2-3). In contrast, the growth of *A. fumigatus* was unaffected by heat-treatment of serum (Table 2-3).

Table 2-3: Comparing the growth of and proteinase secretion by *A. fumigatus* in MEM containing FBS or heat-treated FBS

Serum treatment:	Proteinase secretion[a]	Dry weight (mg)[b]
Unheated	0.84 ± 0.21	8.5 ± 1.0
56°C	0.87 ± 0.05	9.1 ± 0.2
66°C	0.11 ± 0.02 *	8.5 ± 0.2
100°C	0.09 ± 0.02 *	9.3 ± 0.5

[a] Proteinase secretion was measured using the azocasein assay. Values are means of triplicate measurements ± standard deviations for proteinase secretion (A_{436}) by *A. fumigatus* after 72 hours incubation in MEM containing FBS that had been heated to 56, 66 or 100°C for 30 minutes.
[b] Dry weights are mean values ± standard deviations for total dry mycelial mass from 5 ml cultures after 72 hours incubation.
* Values are significantly different than those observed with unheated serum ($p < 0.025$)

The stimulation of growth by serum can be mimicked by BSA plus iron

Proteinase secretion in *A. fumigatus* can be induced by the presence of protein or protein hydrolysate in the medium (Bouchara *et al.*, 1993), thus we tested the ability of one serum protein, bovine serum albumin (BSA) to stimulate growth and proteinase secretion. A BSA stock solution of 80 mg/ml, roughly equivalent to the protein concentration in serum, was prepared and added to the culture medium to give a final

concentration of 8 mg/ml. Addition of BSA to MEM significantly stimulated the growth of *A. fumigatus*, though growth was still only 80% of that found in the presence of FBS (Figure 2-5). Proteinase secretion per mg dry weight was significantly increased when BSA was added to MEM. Nevertheless, the levels of proteinase secretion in BSA were only 20% of those observed when cultures were grown in 10% FBS (Figure 2-5).

Serum can be a source of iron if a microorganism possesses a mechanism to remove iron bound to transferrin or other iron-binding proteins (Weinberg, 1999). Using atomic absorption spectrometry, we found that the iron levels of the sera used in our experiments were as follows: FBS was 4.7 ppm (84 μM) and human serum was 1.1 ppm (20 μM) whereas the level in MEM was below detection limits (0.1 ppm). We therefore postulated that the presence of iron in serum might be responsible for its growth-enhancing effect on *A. fumigatus*. Addition of 100 μM $FeCl_3$ to MEM had no effect on either growth or proteinase secretion by *A. fumigatus*, while addition of 100 μM $FeCl_3$ and 8 mg/ml BSA together resulted in the same amount of growth as seen with 10% FBS (Figure 2-5). The combination of $FeCl_3$ and BSA, while significantly improving growth, had only a small effect on proteinase secretion compared to BSA alone: levels of proteinase secretion with BSA plus iron remained at 30% of the levels found with FBS (Figure 2-5).

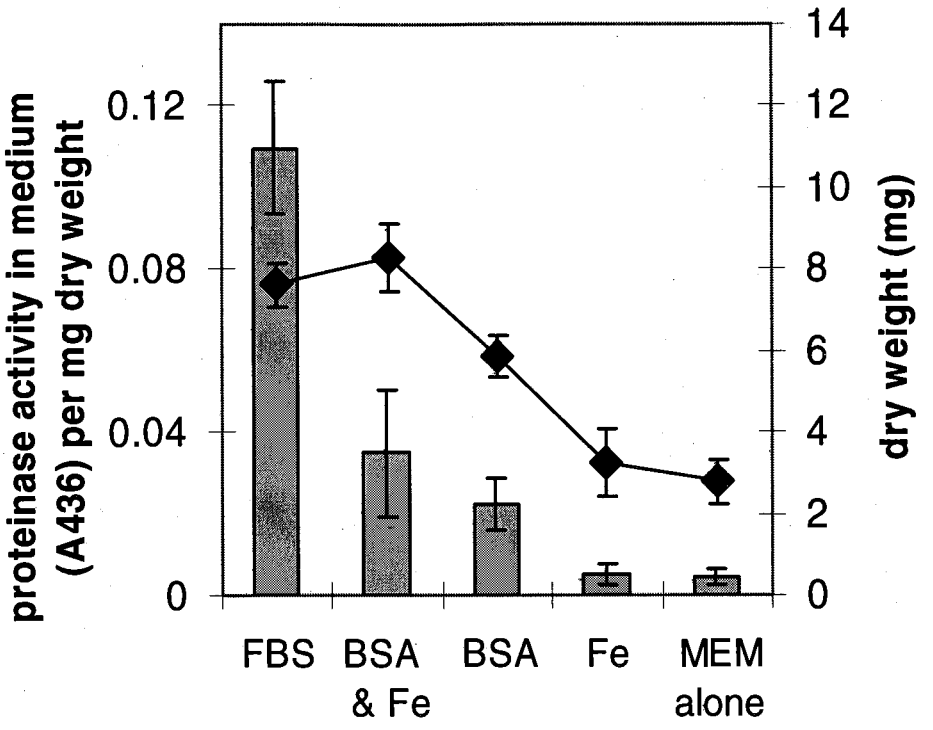


Figure 2-5: Growth of and proteinase secretion by *A. fumigatus* in MEM containing BSA and iron

MEM (5 ml) containing 10% FBS, 8mg/ml BSA, 100 μM $FeCl_3$, 8mg/ml BSA and 100 μM $FeCl_3$, or MEM alone were inoculated with 10^6 *A. fumigatus* conidia per ml and incubated at 37°C and 150 rpm. Proteinase secretion per mg dry weight (bars) and dry weight (diamonds) were measured as described in Materials and Methods. Data shown are means of five independent experiments ± standard deviations. Statistical analysis of the growth data revealed that FBS and BSA plus iron were both significantly greater from BSA alone ($p < 0.01$) which in turn, was significantly greater than iron plus MEM and MEM alone ($p < 0.01$). For the proteinase secretion data, FBS was significantly greater than BSA and BSA plus iron, and these two groups were different from MEM plus iron and MEM alone ($p < 0.01$).

We also attempted to remove iron from serum by treating serum with Chelex beads as described by Wilson *et al.* (50). However, treatment of either FBS or human serum resulted in removal of only 50% of the total iron (data not shown). This level of iron was sufficient to maintain the same level of growth of *A. fumigatus* as found in untreated sera: after 66 h of incubation, the biomass of cultures with Chelex-treated human serum was 6.84 ± 0.46 mg whereas in untreated sera, the biomass was 5.81 ± 0.91 mg (n=3). No effect on proteinase secretion was observed: proteinase secretion per mg dry weight from Chelex-treated human sera was 0.05 ± 0.01, and untreated human sera was 0.07 ± 0.01. Supplementing samples containing Chelex-treated serum with 10 μM iron restored the level of proteinase secretion to 0.07 ± 0.01. These data indicate that removal of up to 50% of the iron present in human sera (final iron content of 10 μM) did not inhibit growth of *A. fumigatus* and had no significant effect on proteinase secretion by this fungus.

Discussion

Growth of *A. fumigatus* in serum

Serum plays an important role in the defense against microbial growth. In extracellular fluids, iron-chelating proteins maintain the free iron concentration at about 10^{-18} M (Bullen, 1981), too low to support the growth of many microbes including most pathogenic fungi. Transferrin is considered to be a major antifungal agent in serum, while lactoferrin is present in other bodily fluids and is responsible for the antifungal properties of milk (Andersson *et al.*, 2000). Pathogenic fungi employ several different strategies for iron acquisition from biological sources such as transferrin, including

reduction of ferric to ferrous iron and production of various siderophores (Howard, 1999).

The purpose of the current study was to determine whether serum is inhibitory to the growth of the fungal pathogen *A. fumigatus*. Two interesting observations were made while studying the growth of *A. fumigatus* in serum. First, *A. fumigatus*, unlike other fungal pathogens, was able to thrive in an iron-limiting environment and grew in concentrations of FBS and human serum as high as 80%. Second, serum stimulated high levels of proteinase secretion by *A. fumigatus*. We hypothesize that *A. fumigatus* is able to access iron in serum, and the combination of available iron and protein in serum permits abundant growth of *A. fumigatus*. It should be noted that other studies have shown that growth of *A. fumigatus* is inhibited by serum (Chiller *et al.*, 2000). These authors measured growth after 24 hours incubation at 37°C, whereas we measured growth after 50 hours. Since growth was delayed in the presence of serum, this may explain the difference in the results.

Iron was not released by changes in pH in the batch cultures because the pH of the culture medium remained neutral to alkaline. Ideally, a better buffered medium should have been used to maintain a more neutral pH. Alkaline pH does not affect the binding of iron to transferrin, but might affect other aspects of iron flux or gene expression by *A. fumigatus*.

The basal medium used throughout the study was MEM, which contains glucose, amino acids, vitamins, and salts (Eagle, 1959). Though it does not contain added iron, trace levels present in the MEM were sufficient to permit some growth of *A. fumigatus*. The addition of FBS or human serum to MEM greatly increased the growth of *A.*

fumigatus, even at serum concentrations up to 80%. To our knowledge, *A. fumigatus* is the only fungal pathogen able to tolerate and thrive in such high serum concentrations. Human serum at a concentration of 1% in RPMI inhibited the growth of *C. albicans* by 98% (Minn *et al.*, 1997). The growth of *Cryptococcus neoformans* was inhibited by 76% in RPMI containing 5% human serum, but was not inhibited by fetal bovine serum (Granger *et al.*, 1986; Nassar *et al.*, 1995). Human serum was inhibitory to the growth of *R. rhizopodiformis*, but only at concentrations greater than 20% (Verdonck *et al.*, 1993).

Though fetal and adult sera contain roughly equal transferrin concentrations, fetal transferrin is more highly iron saturated. Transferrin in FBS is present at 1.2 to 1.8 mg/ml and is 55-92% iron saturated (Kakuta *et al.*, 1997), while transferrin in human serum is usually 25-35% saturated (Weinberg, 1977). FBS therefore has less potential to chelate free iron in the medium, which could explain its inability to inhibit growth of pathogens such as *C. neoformans*. Our study showed that human serum stimulated growth and proteinase secretion of *A. fumigatus* to the same extent as FBS, despite its greater theoretical iron chelating potential. Nevertheless, the observed delay in growth in human serum compared to FBS may have been caused by the lower iron availability in human serum.

Supplementing MEM with 100 μM $FeCl_3$ alone did not increase growth, indicating that iron levels in MEM were not growth-limiting for *A. fumigatus*. Furthermore, iron was not limiting for *A. fumigatus* in MEM plus 10% FBS because addition of 100 μM $FeCl_3$ did not result in increased growth and proteinase secretion (data not shown). However, addition of BSA and 100 μM $FeCl_3$ to MEM supported growth levels equivalent to those observed in serum-containing MEM. These results

show that growth of *A. fumigatus* in media containing 8 mg/ml BSA was limited by iron availability. Since iron was not growth-limiting in media containing serum, *A. fumigatus* must be able to access the iron in serum. Dialysis of serum against water did not result in the loss of growth-promoting activity, therefore the source of iron accessed by *A. fumigatus* was likely molecules larger than 10 kDa; probably transferrin-bound iron.

The mechanism by which *A. fumigatus* obtains iron in serum-containing media is unknown. It is thought that *A. fumigatus* can produce at least six siderophores, the most prominent of which have been tentatively identified as N,N',N"-triacetylfusarinine C, and ferricrocin (Nilius and Farmer, 1990). These siderophores have the potential to help scavenge iron in serum-containing media, but their role in virulence has not been examined.

Role of proteinases in growth of *A. fumigatus* in serum-containing media

The role of proteinases in virulence of *A. fumigatus* has been extensively studied and at least three secreted proteinases have been described; however, *A. fumigatus* strains deficient in one or more of these proteinases have been shown to completely retain virulence (Jaton-Ogay *et al.*, 1994; Monod *et al.*, 1993b; Reichard *et al.*, 1997; Tang *et al.*, 1993). All three known *A. fumigatus* secreted proteinases are secreted in vivo (Lee and Kolattukudy, 1995; Markaryan *et al.*, 1994; Reichard *et al.*, 1990), but their role in vivo is unknown, nor is it known if other proteinases are also secreted in vivo. It is not known which, if any, of the described proteinases are induced by serum. Serum also contains many inhibitors of proteinases, such as α_2-macroglobulin and α_1-antichymotrypsin. Our results show that the proteinases produced by *A. fumigatus* are able to evade or overwhelm the proteinase inhibitors in serum.

The results presented in this study suggest a complex regulation of proteinase secretion by *A. fumigatus*. Patterns of proteinase secretion were highly dependent on the composition of the medium. For example, gelatin and Matrigel stimulated production of serine proteinases exclusively, while serum, BSA and A549 cells induced secretion of both serine and metalloproteinases. Aspartic proteinases have been described in *A. fumigatus* (Reichard *et al.*, 1997); however these were not detected in this study.

Hydrolysis of transferrin is one potential means of iron acquisition in serum. High levels of proteinase secretion could allow *A. fumigatus* to degrade iron-binding proteins and release iron required for growth. However, hydrolysis of iron-binding proteins is not likely to be the only mechanism for iron acquisition by *A. fumigatus* because proteinase secretion was not detected until cultures have reached late log phase. Thus, most of the growth, and need for iron, had already occurred before high levels of proteinases were produced.

The proteinases secreted in response to the presence of serum have the potential to contribute to the virulence of *A. fumigatus*. They efficiently cleaved Azocoll, a general proteinase substrate derived from bovine collagen. The secreted proteinases also degraded human pulmonary basal lamina proteins in intact basal lamina. Earlier studies have shown that *A. fumigatus* can produce proteinases capable of hydrolyzing basal lamina components. For example, Tronchin *et al.* (1993) reported a loss of laminin epitopes from kidney and lung tissue slices incubated with a crude protease extract from *A. fumigatus* grown in nitrogen-restricted Czapek-Dox medium. Increased secretion of basal lamina-degrading proteinases in vivo could result in increased invasiveness of this species.

Unfortunately, the identities of the serine and metallo proteinases induced by serum are not known. It is clear that the *A. fumigatus* proteinases Alp and Mep are not required for virulence (Jaton-Ogay *et al.*, 1994). However, the regulation of proteinase secretion by *A. fumigatus* appears to be highly dependent on the growth environment. Alp and Mep were detected in media containing a soluble collagen as the sole carbon and nitrogen source (Monod *et al.*, 1991; Monod *et al.*, 1993a). Growth of *A. fumigatus* in media containing a single protein may induce very different patterns of proteinase secretion than did growth on complex substrates such as A549 pulmonary cells or serum. The recent sequencing of the *A. fumigatus* genome should facilitate expression studies to determine if the alkaline proteinase secreted in response to serum and A549 cells is Alp, or another proteinase altogether.

The serum factor inducing proteinase secretion is not merely protein in the medium, because BSA alone did not induce high levels of proteinase secretion. Furthermore, while heat-treatment of serum did not affect the growth yield, it did decrease its ability to induce proteinase secretion by *A. fumigatus*. This suggests that the serum stimulates growth by acting as a nutrient source (particularly protein and iron), whereas stimulation of proteinase secretion requires non-denatured serum protein(s). Proteinases are secreted earlier in the growth phase of *A. fumigatus* in media containing BSA and $FeCl_3$ than in serum-containing media. The delay in proteinase secretion in media containing serum may be related to the inaccessibility of iron as accessing iron bound to serum proteins would be expected to take longer than accessing free iron in solution. Identification of the mechanism by which *A. fumigatus* obtains iron from

serum, and exploration of the possible role of siderophores in growth in serum-containing media are the subjects of Chapter 3.

CHAPTER THREE: SURVIVAL OF *ASPERGILLUS FUMIGATUS* IN SERUM INVOLVES REMOVAL OF IRON FROM TRANSFERRIN: THE ROLE OF SIDEROPHORES

Summary

Aspergillus fumigatus can grow in medium containing up to 80% human serum, indicating it possesses an effective mechanism for obtaining iron from serum transferrin. The purpose of the work described in this chapter was to examine the mechanisms of iron uptake by *A. fumigatus*, and to determine if *A. fumigatus* was able to access transferrin-bound iron. In iron-depleted MEM, *A. fumigatus* growth was supported by addition of diferric-transferrin or $FeCl_3$ but not by apo-transferrin. Proteolytic degradation of transferrin by *A. fumigatus* occurred in MEM/serum; however, transferrin degradation did not occur until late logarithmic phase. Moreover, transferrin was not degraded by *A. fumigatus* incubated in MEM/diferric-transferrin. Urea-PAGE showed that in MEM/diferric-transferrin medium, diferric-transferrin was completely converted to apo-transferrin by *A. fumigatus*. In human serum, all of the monoferric transferrin was converted to apo-transferrin within 8 hours. Siderophores were secreted by *A. fumigatus* after 8 hours of growth in MEM/serum, and 12 hours in MEM/diferric-transferrin. The involvement of small molecules in iron acquisition was confirmed by the fact that transferrin was deferrated by *A. fumigatus* even when transferrin was physically separated by a membrane with a 12 kDa molecular weight cut-off. Five siderophores were purified from *A. fumigatus* culture medium and the two major siderophores were identified as N'N''N'''-triacetylfusarinine C (TAF) and ferricrocin. Both TAF and ferricrocin

removed iron from diferric-transferrin with an affinity comparable to another well-characterized siderophore, ferrichrome. These data indicate that *A. fumigatus* survival in human serum in vitro involves siderophore-mediated removal of iron from transferrin. Proteolytic degradation of transferrin may play a secondary role in iron acquisition.

Introduction

The importance of iron in infection

Serum is inhibitory to the growth of many microorganisms, including most fungi, because free iron concentrations in serum are too low to support growth. Nevertheless, the studies described in Chapter 2 show that *Aspergillus fumigatus* can grow in media containing up to 80% human serum, indicating that it possesses an effective mechanism for acquiring iron from serum constituents. Transferrin is the main iron-binding molecule in serum, therefore transferrin is a likely source of iron for *A. fumigatus* growing in media containing high serum concentrations.

Iron binding molecules are potential sources of iron for pathogens. Intracellular iron-binding molecules include ferritin and heme compounds, whereas transferrin and lactoferrin are the main source of iron in extracellular fluids (Byers and Arceneaux, 1998; Ratledge and Dover, 2000). There is some indirect evidence that iron plays a role in virulence of fungi. The risk of invasive aspergillosis has been indirectly linked to serum iron levels in a study by Iglesias-Osma *et al.* (1995). These researchers showed that the iron saturation of transferrin increased 1.6-fold during neoplastic episodes and that this corresponded to an increased risk of aspergillosis. Another study demonstrated that deferoxamine, a hydroxamate siderophore produced by actinomycetes, stimulated the growth of *Rhizopus* spp. in iron-loaded patients (Boelaert *et al.*, 1993). Deferoxamine

chelates transferrin-bound iron, and the ferrated siderophore can then support the growth of *Rhizopus* in vivo.

Possible mechanisms of iron uptake by *A. fumigatus*

Several mechanisms have been described by which pathogenic microorganisms access transferrin-bound iron. These include the expression of transferrin receptors, enzymatic reduction of ferric iron at the cell surface by ferric reductases, secretion of siderophores, manipulation of pH and production of proteinases. Transferrin and lactoferrin receptors are expressed by several bacterial species (Byers and Arceneaux, 1998; Furrer *et al.*, 2002; Ratledge and Dover, 2000), but these have never been described in fungi and are unlikely to be present in *A. fumigatus*. In contrast, ferric reductases are very common in both bacteria and fungi, and have been characterized in all of the major yeast pathogens, including *Candida albicans* (Morrissey *et al.*, 1996), *Histoplasma capsulatum* (Timmerman and Woods, 1999), and *Cryptococcus neoformans* (Jacobson *et al.*, 1998; Nyhus *et al.*, 1997).

Many microorganisms, including many filamentous fungi, produce siderophores in response to a low concentration of free iron (Dave and Dube, 2000). Siderophores bind iron with constants (pM) in the range of 22 to 50, sufficiently strong to remove iron attached to molecules such as transferrin, which has a pM value for complexation with iron of 23.6 (Harris *et al.*, 1979). *A. fumigatus* is known to secrete several hydroxamate siderophores, including N'N''N'''-triacetylfusarinine C (TAF) and ferricrocin (Diekmann and Krezdorn, 1975; Nilius and Farmer, 1990; Wiebe and Winkelmann, 1975); however, there is no evidence that siderophores produced by *A. fumigatus* are involved in iron uptake in vivo.

Proteinases can play a role in iron uptake by hydrolysing iron-binding proteins such as transferrin and lactoferrin (Brochu *et al.*, 2001; Doring *et al.*, 1988; Shigematsu *et al.*, 2001). Fungi such as *A. fumigatus* produce a wide range of proteinases. *A. fumigatus* produces one or more proteinases from the serine proteinase, metalloproteinase and aspartic proteinase families. As was demonstrated in Chapter 2, serum induces secretion of high concentrations of proteinases, which could then degrade serum proteins. Fungal proteinases are very diverse and can hydrolyse a variety of substrates; however, it is not known if any of the *A. fumigatus* proteases hydrolyse transferrin or play a significant role in iron uptake.

Objectives

Since *A. fumigatus* can flourish in media containing high concentrations of human serum, the purpose of this study was to determine whether the growth of *A. fumigatus* in human serum was the result of its ability to remove iron from transferrin. The specific objectives were to determine the extent to which *A. fumigatus* was able to grow in the presence of transferrin as the sole iron source and to examine whether transferrin was deferrated during incubation with *A. fumigatus*. Because *A. fumigatus* is an abundant producer of proteinases, the possibility that proteolytic degradation of transferrin was releasing free iron into solution was also investigated. Finally, siderophore secretion was quantified, siderophores were purified from *A. fumigatus* cultures, and their ability to remove transferrin-bound iron was measured.

Materials and Methods

Strains and growth conditions

Aspergillus fumigatus (ATCC 13073) was obtained from the American Type Culture Collection and maintained, cultured and harvested as described in Chapter 2.

A. fumigatus was cultured in minimal essential medium (MEM) (Life Technologies, Burlington, Ontario), pH 7.4, in 5 ml volumes in 25 ml culture flasks. *A. fumigatus* conidia were added to media at a final concentration of $1x10^6$/ml, and flasks were incubated at 37°C and 150 rpm for the times indicated. Traces of iron were removed from glassware by overnight treatment with 1 mM EDTA, followed by 18 hours in 5% HCl and thorough rinsing with deionized water. The iron-chelating agent, 2,2'-dipyridyl (ICN Biomedicals Inc., Aurora, Ohio) was filter-sterilized and added to MEM at a concentration of 250 μM, a concentration empirically determined to inhibit growth of *A. fumigatus*. Human diferric-transferrin and apo-transferrin were obtained from Sigma (Oakville, Ontario). Human serum (male) was obtained from Sigma (Oakville, Ontario), stored frozen until use, and added to MEM at a concentration of 10%. Dry weights of *A. fumigatus* cultures were measured as described in Chapter 2.

Electrophoresis and Western blot analysis of transferrin

Proteolytic cleavage of transferrin during the growth of *A. fumigatus* was monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), on 10% acrylamide gels. Media were withdrawn from *A. fumigatus* cultures, and electrophoresed according to the procedure of Laemmli (1970). Gels were silver stained, or transferred to PVDF membrane (BioRad), blocked with 5% BSA, probed with a rabbit IgG fraction of anti-human transferrin (1:1000 dilution, Rockland Inc., Gilbertsville, PA),

and treated with goat-anti-rabbit horseradish peroxidase. Bands were visualized by adding the diaminobenzidine substrate.

Urea-polyacrylamide gel electrophoresis

Urea-polyacrylamide gel electrophoresis (urea-PAGE) is a technique that can resolve the four different molecular forms of transferrin: diferric transferrin (Fe_N-Tf-Fe_C), N-terminal monoferric transferrin (Fe_N-Tf), C-terminal monoferric transferrin (Tf-Fe_C) and deferrated apotransferrin (apo-Tf) based on the relative resistance of each form to denaturation by urea (Makey and Seal, 1976). Diferric transferrin is more resistant to denaturation than apo-transferrin, and therefore maintains its native conformation and runs faster through the gel. Because the two iron binding lobes of transferrin are not chemically equivalent (Baker *et al.*, 2002), the resistance to denaturation of N-terminal and C-terminal monoferric transferrins are different, allowing the two forms to be resolved by urea-PAGE.

Urea-PAGE was carried out as described by Wolz *et al.* (1994) in a Protean II xi cell (Bio-Rad). Human transferrin (10 μg) in a 10μl volume was loaded in each lane of the gel. Gels were run at 4°C for 18-20 hours, then rinsed for 30 minutes in 0.05% SDS and stained with SYPRO Orange (Molecular Probes, Eugene OR). Bands were visualized by scanning on a Typhoon 9410 Imager (Amersham).

Quantification of siderophores

Total siderophore concentrations were measured using the chrome azurol S (CAS) assay shuttle solution (Schwyn and Neilands, 1987). CAS is a molecule which changes colour from orange to blue upon binding iron. It can be used to detect the presence of iron chelating molecules, which remove iron from the CAS molecule and cause the blue

colour to disappear. Culture supernatants from *A. fumigatus* were diluted and mixed with an equal volume of the CAS shuttle solution and the absorbance at 630 nm was measured. Dilutions of desferri-ferrichrome (Sigma) were used to generate a standard curve.

Removal of iron from transferrin sequestered within dialysis membrane

Human diferric-transferrin (25 μM) was dissolved in MEM and sealed within a dialysis bag with a 12 to 14 kDa nominal molecular weight cut-off (Fisher). The dialysis bag was then immersed in 25 ml of MEM contained in a 125 ml flask. The medium in the flask was inoculated with 2.5 x 10^7 conidia and incubated at 37°C with slow shaking for 48 hours. As a control, an uninoculated flask was maintained under the same conditions.

Siderophore purification

Hydroxamate siderophores were purified from the culture supernatant of *A. fumigatus* using a modification of the method described by Payne (1994). *A. fumigatus* was cultured in acid-washed flasks containing 4L modified Grimm-Allen medium (1g/L $KHSO_4$, 3g/L K_2HPO_4, 3g/L $(NH_4)_2SO_4$, 20 g/L sucrose, 1 g/L citric acid, 2mg/L thiamine, 20 μg/L $CuSO_4$, 1mg/L $MnSO_4$, 5.5 mg/L $ZnSO_4$, 810 mg/L $MgSO_4$, pH 6.9). This medium was inoculated with 4 x 10^9 conidia, and the flasks incubated at 150 rpm and 37°C for 72 hours. The culture was filtered through Miracloth to remove mycelia, and the filtrate concentrated under vacuum to 350 ml. Ammonium sulfate (50% saturation) and 5g/L $FeCl_3$ were added and the solution was stirred at 4°C for 16 hours. The concentrated filtrate was filtered through Whatman paper and extracted 5 times with 50 ml benzyl alcohol. Anhydrous ethyl ether (750 ml) was added to the combined benzyl alcohol fractions, and the siderophores extracted 8 times into 15 ml ddH_2O. The aqueous

layer was washed with diethyl ether and lyophilized to dryness. Siderophores were purified by flash column chromatography, using dichloromethane and methanol. The separation of siderophores was confirmed by thin layer chromatography.

Desferri-TAF was extracted from media using the identical procedure, without addition of $FeCl_3$. Purification was achieved by preparative thin layer chromatography on pre-cleaned silica gel 60 F_{254} plates run with 1-butanol:ethanol:water 5:3:2. The bands were visualized under UV light and iron-reactive layers were scraped, extracted with water and lyophilized.

Iron was removed from ferricrocin by treatment with 8-hydroxyquinoline. Ferricrocin was dissolved in slightly alkaline water (10 mg/ml) and a 10-fold (w/w) excess of 8-hydroxyquinoline was added. This mixture was heated for 30 minutes at 60°C and left to stand overnight at room temperature. Most of the 8-hydroxyquinoline was removed by centrifugation, and the remainder was removed from the supernatant by five extractions with chloroform.

Siderophore identification

MALDI-TOF mass spectra were obtained for samples dispersed in an α-cyano-4-hydroxycinnamic acid matrix (TAF and desferri-TAF) or a 2,5-dihydroxybenzoic acid matrix (ferricrocin and desferri-ferricrocin) using a PerSeptive Biosystems Voyager-DE instrument.

^{1}H and ^{13}C NMR spectra were recorded at 293K on a Bruker AMX-400 NMR spectrometer or a Varian Inova 500 MHz NMR spectrometer. All chemical shifts are reported relative to TMS. Correlation spectroscopic (COSY, TOCSY, NOESY, and

ROESY) studies of desferri-ferricrocin allowed assignment of all signals to specific amino acid residues.

Incubation of *A. fumigatus* desferri-siderophores with diferric-transferrin

Desferri-TAF, desferri-ferricrocin, and desferri-ferrichrome were diluted to concentrations ranging from 5mM to 5 μM and incubated with diferric-transferrin (25 μM) in buffer (50 mM Tris, 150 mM NaCl, 20 mM $NaHCO_3$, pH 7.4) for 16 hours at 37°C. The extent of iron saturation of transferrin at the end of the incubation period was determined by resolving the different transferrin species on urea-PAGE gels.

Statistics

The Student t-test was used for statistical analysis of data.

Results

Diferric-transferrin but not apo-transferrin supports the growth of *Aspergillus fumigatus* in iron-depleted medium

MEM was made iron-limiting by addition of the iron-chelator 2,2'-dipyridyl. A concentration of 250 μM 2,2'-dipyridyl was empirically determined to be the minimum inhibitory concentration for *A. fumigatus* ATCC 13073 (data not shown). The addition of apo-transferrin to MEM containing 2,2'-dipyridyl did not support growth of *A. fumigatus*, whereas the addition of either 25 μM diferric-transferrin or 50 μM $FeCl_3$ to the iron-limited MEM promoted statistically significant growth of *A. fumigatus* (Table 3-1). These data indicate that transferrin-bound iron is available to *A. fumigatus*.

Table 3-1: Growth of *A. fumigatus* in MEM (5 ml) containing 250 μM of the iron chelator, 2,2'-dipyridyl, and supplemented with either diferric-transferrin, apo-transferrin, or $FeCl_3$

Iron source	Mycelial dry weight after 96 hours (mg)
none (MEM and 2,2'-dipyridyl alone)	0.13 ± 0.07
diferric-transferrin (25 μM)	1.30 ± 0.90 *
apo-transferrin (25 μM)	0.02 ± 0.05
$FeCl_3$ (50 μM)	0.80 ± 0.40 *

* growth was significantly greater than in the absence of any iron source (none) ($p<0.05$). Data shown are means ± standard deviations of triplicate measurements.

Serum transferrin is not degraded during the growth of *A. fumigatus*

Like many fungi, *A. fumigatus* is a prolific producer of proteinases. The results presented in Chapter 2 showed that growth of *A. fumigatus* in human serum, an iron-deficient medium, was accompanied by increased secretion of fungal proteinases. In theory, these proteinases could degrade human transferrin and release iron which would then be available for uptake by fungal cells. Degradation of human transferrin was monitored by culturing *A. fumigatus* in MEM containing either 2.5 μM human diferric-transferrin or 10% human serum. As expected, the addition of serum to MEM stimulated proteinase secretion as evidenced by the degradation of serum transferrin beginning at 46 hours of culture. However, transferrin was stable for at least the first 22 hours of incubation. There was a small decrease in the amount of transferrin at 46 hours, and considerable degradation was observed at all later timepoints (Figure 3-1A). Stationary phase was reached between 22 and 46 hours of growth (Figure 2-1A), therefore significant serum transferrin degradation by *A. fumigatus* did not occur until after the

beginning of stationary phase. In contrast, transferrin was stable in serum for at least 286 hours in control flasks which contained no *A. fumigatus* (Figure 3-1, lane C).

When diferric-transferrin alone was added to MEM it was not degraded by *A. fumigatus* after 286 hours of incubation (Figure 3-1B). Because transferrin degradation was not observed in either medium during the early growth of *A. fumigatus* when the organism has the greatest requirement for iron, it is unlikely that transferrin proteolysis is the primary mechanism of iron acquisition by *A. fumigatus*.

A. fumigatus can remove iron from diferric-transferrin

The ability of *A. fumigatus* cultures to remove iron from diferric-transferrin was investigated using urea-PAGE. *A. fumigatus* was cultured in MEM containing either 10% human serum or 2.5 μM human diferric-transferrin. In media containing 2.5 μM diferric-transferrin, the diferric-transferrin decreased in relative amount and apo-transferrin was detected within 8 hours (Figure 3-2A). Human serum contained a mixture of apo-transferrin and monoferric-transferrin but within 8 hours of incubation with *A. fumigatus*, the monoferric transferrin was no longer detected in human serum, and only apo-transferrin was present (Figure 3-2B). Since transferrin was not degraded until more than 22 hours of culture (Figure 3-1), these data indicate that *A. fumigatus* was able to remove iron from intact diferric-transferrin.

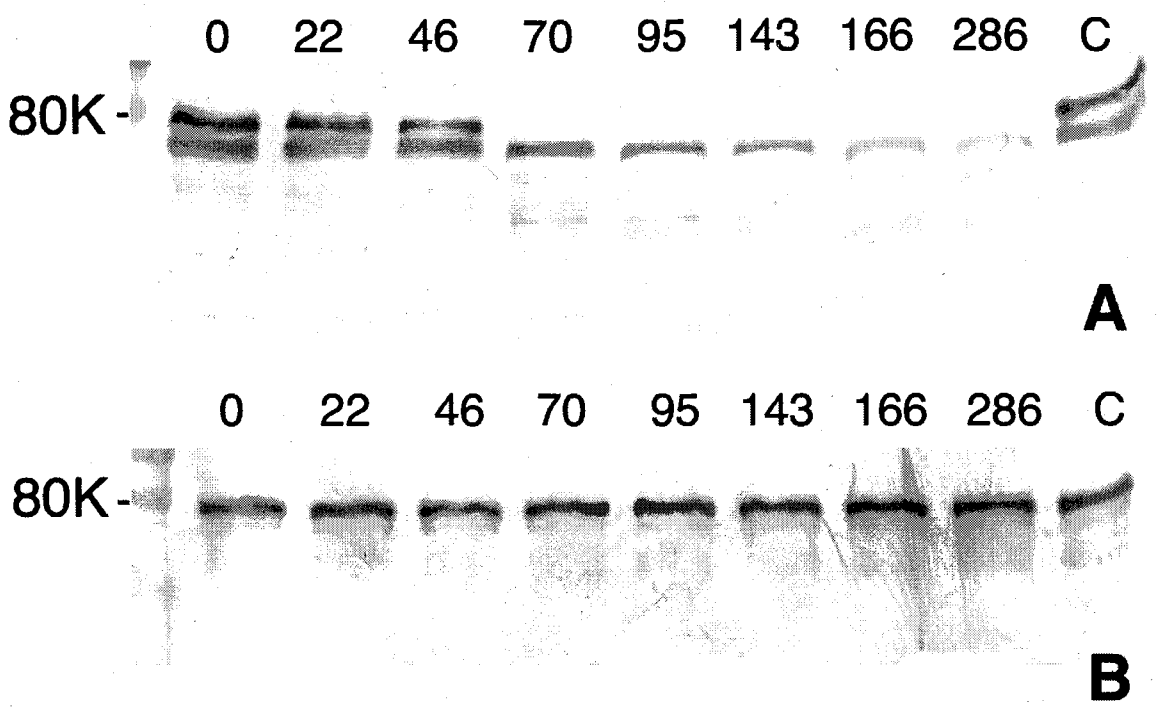


Figure 3-1: Degradation of transferrin by *A. fumigatus* in liquid culture

A. fumigatus was incubated in MEM containing 10% human serum (A) or 2.5 μM diferric-transferrin (B). Supernatants were withdrawn after the stated number of hours from the cultures and the presence of transferrin was determined by Western blotting following SDS-PAGE. Controls (C) were uninoculated samples incubated for 286 hours. The band underneath transferrin in panel A is another serum protein that cross-reacted with the polyclonal anti-transferrin antibody. 80K indicates the position of an 80 kDa standard.

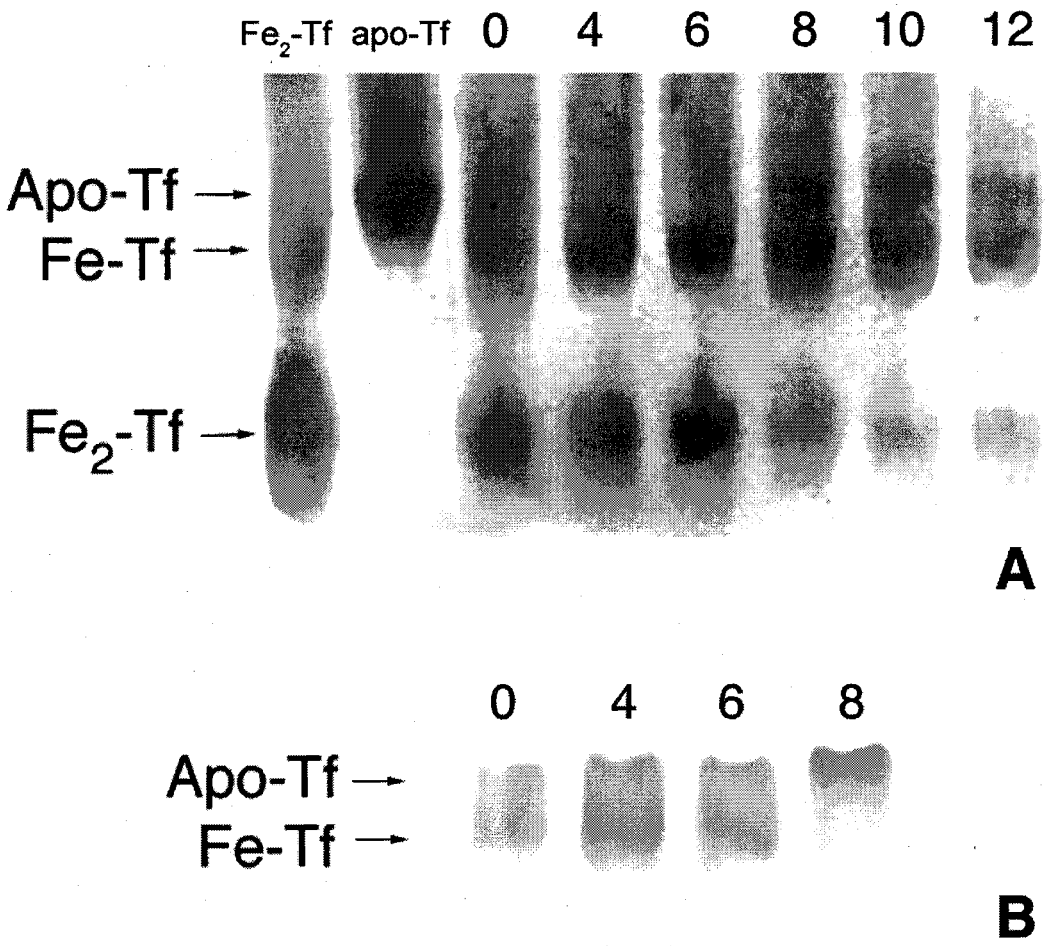


Figure 3-2: Iron removal from transferrin by *A. fumigatus*

A. fumigatus was cultured in MEM containing 2.5 μM purified human diferric-transferrin (A) or 10% human serum (B). Culture media were withdrawn and the iron saturation of transferrin analyzed by urea-PAGE. Transferrin was visualized by silver staining (A) or by Western blotting (B). The numbers above the lanes indicate the length of incubation with *A. fumigatus* in hours. The diferric transferrin standard (Fe_2-Tf) contained both monoferric and diferric transferrin. Apo-Tf represents an apo-transferrin standard.

A. fumigatus secretes siderophores in the early phase of growth in transferrin-containing media

Total siderophore secretion by *A. fumigatus* was quantified with the CAS shuttle solution, using desferri-ferrichrome to generate a standard curve. Cultures were monitored during the early phases of growth to determine if siderophore secretion could be responsible for removal of iron from transferrin observed in the first 8 hours of culture. In MEM containing 10% human serum, significant levels of siderophores were first detected by the CAS assay at 8 hours ($p < 0.05$), which was about the time conidia germinated. In MEM supplemented with 2.5 μM diferric-transferrin, significant levels of siderophore secretion were observed at 10 hours ($p < 0.05$) (Figure 3-3). Thus, siderophore secretion occurred after 8 to 12 hours incubation, which was soon after germination of *A. fumigatus* conidia. The detection of siderophores coincided with the observed removal of transferrin-bound iron at 8 hours of incubation (Figure 3-2).

A. fumigatus can remove iron from transferrin across a dialysis membrane

Diferric-transferrin was contained within dialysis tubing to determine whether small molecules produced by *A. fumigatus*, such as siderophores, were responsible for removal of iron from transferrin. Diferric-transferrin (25 μM) was sealed within a dialysis bag with a molecular weight cut-off of 12-14 kDa. Most known fungal siderophores are smaller than 1000 Da and should therefore readily pass through the dialysis membrane. The dialysis bag was suspended in MEM inoculated with *A. fumigatus* and incubated at 37°C for 48 hours. The iron-binding state of the transferrin was monitored by urea-PAGE. Despite physical separation from *A. fumigatus*, diferric-transferrin contained within the dialysis bag was almost completely deferrated during incubation with *A. fumigatus* (Figure 3-4). In the uninoculated control, no iron removal

from transferrin was observed. Acid production did not cause the release of iron from transferrin because the pH of the MEM did not drop below 7.0 during the incubation period (data not shown).

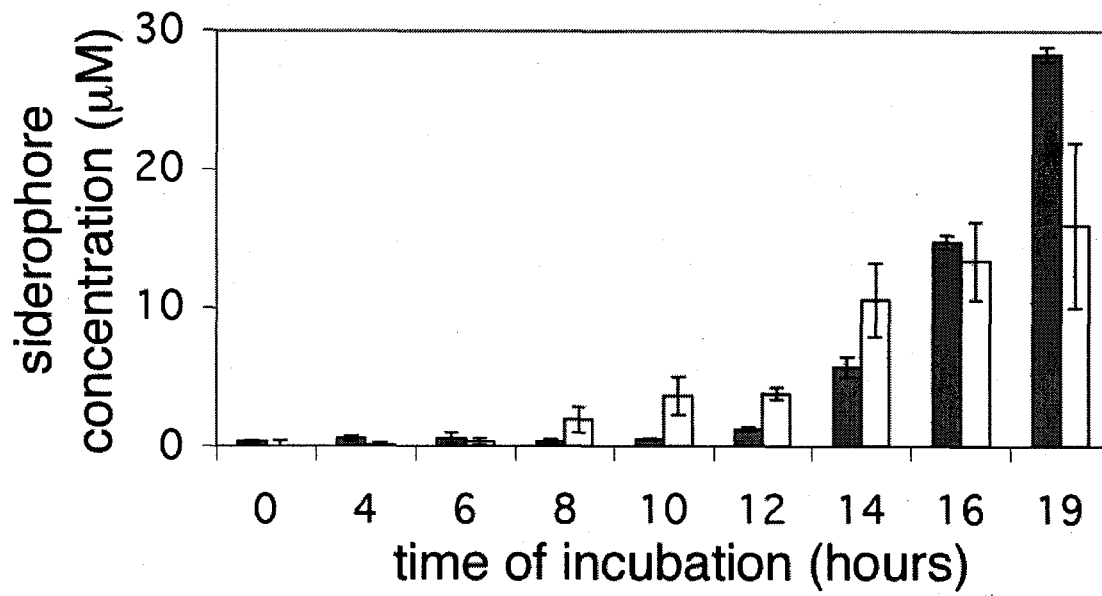


Figure 3-3: Siderophore secretion by *A. fumigatus* in MEM containing diferric-transferrin or human serum

A. fumigatus spores (10^6/ml) were added to MEM containing 2.5 μM diferric-transferrin (dark bars) or 10% human serum (light bars) at time 0. The cultures were incubated at 37°C and 150 rpm. The siderophore concentrations in the culture supernatants were determined using the CAS assay. Data show means ± standard deviations of triplicate measurements. Values are significantly greater than 0 after 8 hours for serum, and after 10 hours for diferric-transferrin ($p < 0.05$).

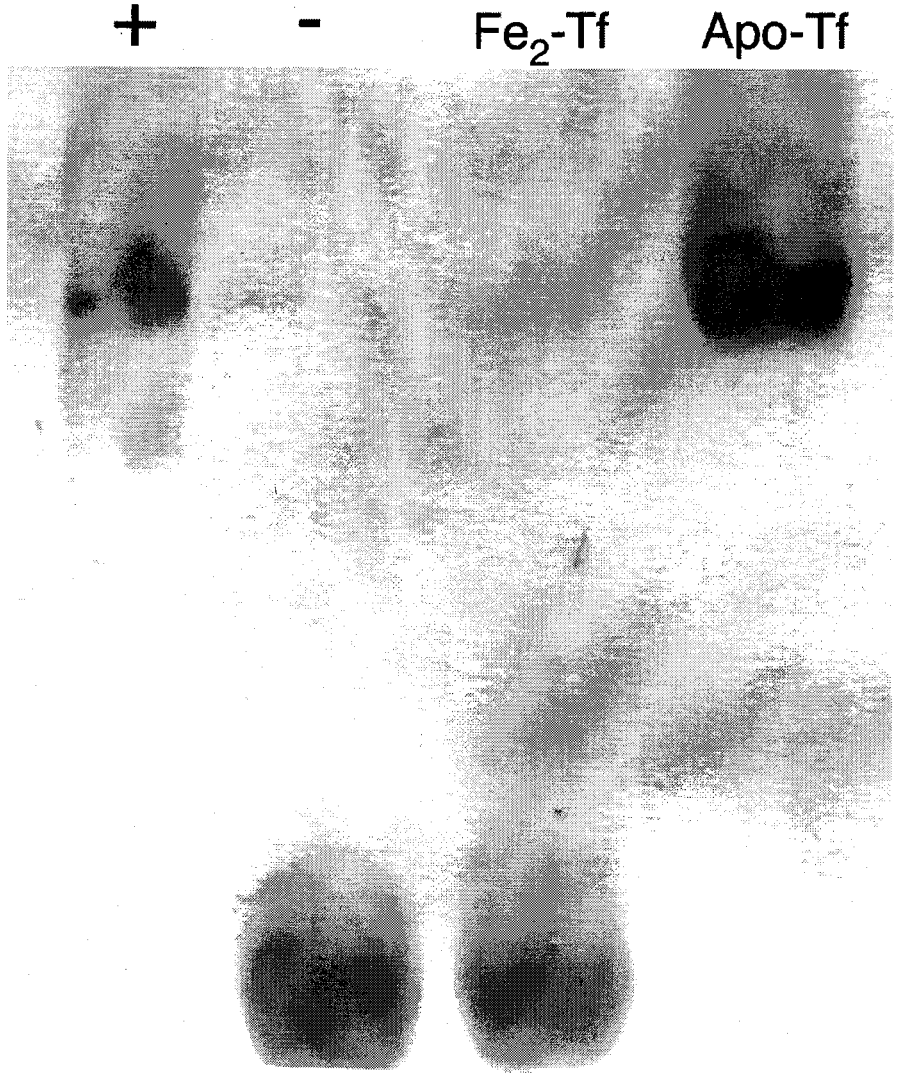


Figure 3-4: *A. fumigatus* can transport iron from transferrin across a dialysis membrane

A. fumigatus was inoculated into MEM in which a dialysis bag containing diferric-transferrin (25 μM) was suspended. *A. fumigatus* was incubated for 48 hours, then transferrin was withdrawn from the dialysis bag and analyzed by urea-PAGE (+). An uninoculated control flask containing MEM plus transferrin in a dialysis bag was also examined (-). Pure diferric- and apo-transferrin standards were also run (Fe_2-Tf and Apo-Tf, respectively).

Purification of siderophores from *A. fumigatus* culture medium

Siderophores were purified from *A. fumigatus* culture medium as described in Materials and Methods. Extraction of the culture medium with benzyl alcohol revealed five different iron-binding compounds, which could be distinguished by their R_f values after thin layer chromatography (Table 3-2). These compounds were purified by flash column chromatography. The red, orange and yellow compounds lost their colour when treated with the iron-chelator 8-hydroxyquinoline. The majority of siderophores produced under these culture conditions were contained in fraction 3, while fractions 1, 2 and 5 were produced in much smaller amounts (Table 3-2). Fractions 3 and 4 were also the most abundant siderophores produced by *A. fumigatus* grown in MEM containing 10% human serum (data not shown).

Table 3-2: Siderophores produced by *A. fumigatus* ATCC 13073

Fraction	R_f [a]	Yield[b] (mg)
1	0.57	} 15
2	0.51	
3	0.43	450
4	0.31	23
5	0.09	13

[a] solvent was chloroform:benzyl alcohol:methanol (2:1:1 v/v/v) except for Fraction 5 for which the solvent ratio was 2:1:2.
[b] Dry weight of pure compound isolated per litre of culture medium. *A. fumigatus* was inoculated into Grimm-Allen medium and grown for 72 hours at 37°C and 150 rpm. Siderophore purification is described in Materials and Methods.

Identification of siderophores

MALDI-MS, ^{1}H-NMR and ^{13}C-NMR were used to identify the two most abundant *A. fumigatus* siderophores as N'N''N'''-triacetylfusarinine C (TAF) and ferricrocin.

MALDI-MS spectra were run on both the ferrated and desferri-forms of both siderophores. The mass ions observed (Table 3-3) are in agreement with the identification of the siderophores as TAF ($C_{39}O_{15}N_6H_{57}Fe$, 905.78) and ferricrocin ($C_{28}O_{13}N_9H_{44}Fe$, 770.58).

Table 3-3: Calculated and measured mass ions for the ferrated and desferri-forms of Fractions 3 and 4

	Calculated values TAF	**Observed Fraction 3**	Calculated values ferricrocin	**Observed Fraction 4**
Ferrated MH^+	906.79	**906.77**	771.59	**771.37**
Ferrated MNa^+	928.79	**928.76**	793.57	**793.38**
Desferri MH^+	853.97	**853.74**	718.76	**718.33**
Desferri MNa^+	875.97	**875.67**	740.75	**740.37**

^{1}H-NMR and ^{13}C-NMR spectra of the deferrated siderophores (Tables 3-4, 3-5 and 3-6) were in agreement with published spectra of desferri-TAF (Jalal and van der Helm, 1991) and desferri-ferricrocin (Haselwandter and Winkelmann, 2002; Llinas *et al.*, 1972; Llinas *et al.*, 1976). The ^{1}H NMR spectrum of desferri-ferricrocin has not been fully reported, therefore detailed proton assignments were determined by using a combination of COSY, TOCSY, NOESY and ROESY NMR spectra obtained in DMSO-d_6 solvent at 293K (Table 3-5).

Table 3-4: NMR chemical shifts observed for purified desferri-fraction 3 at 293K confirm its identity as desferri-TAF

Structural group	^{1}H NMR in DMSO-d_6		^{13}C NMR in $CDCl_3$	
	Literature[a]	Fraction 3	Literature[a]	Fraction 3
αCH	4.18 (m)	4.06-4.23 (m)	52.5	52.6
βCH_2	1.62 (m)	1.44-1.73 (m)	29.3	28.9
γCH_2	1.62 (m)	1.44-1.73 (m)	23.3	23.1
δCH_2	3.48 (m)	3.50 (m)	48.1	47.1
-COO-	-	-	170.9	171.2
N^{δ}-Acyl-CH=	6.22 (s)	6.31 (s)	118.4	119.0
N^{δ}-Acyl-CH_2-	2.64 (t)	2.66 (m)	32.4	31.7
N^{δ}-Acyl-CH_2O-	4.18 (m)	4.06-4.23 (m)	62.9	62.7
N^{δ}-Acyl-CH_3	1.87 (s)	1.86 (s)	24.4	24.4
N^{δ}-Acyl=C<	-	-	149.1	149.0
N^{α}-Acetyl CH_3	1.84 (s)	1.83 (s)	22.9	22.8
N^{α}-Acetyl >C=O	-	-	172.0	171.2*
hydroxamic >C=O	-	-	172.0	172.1*
N^{δ}-OH	-	9.74		
N^{α}H	-	8.21		

* assignments may be reversed
[a] values from Jalal *et al.* (1991)

Table 3-5: ^{1}H- NMR chemical shifts for desferri-fraction 4 observed at 293K in DMSO-d_6 confirms its identity as desferri-ferricrocin

residue	Glycine	Serine	Glycine	N^5-acetyl-N^5-hydroxyl-L-ornithine		
	1	2	3	4	5	6
NH	8.54	8.08	8.35	8.08	7.91	7.84
αCH	3.90, 3.41	4.19	3.77, 3.69	3.97	4.17	4.00
βCH	-	3.66, 3.55	-	1.69, 1.56	1.67, 1.57	1.62, 1.49
δCH	-	-	-	1.50, 1.50	1.52, 1.52	1.53, 1.43
γCH	-	-	-	3.45, 3.45	3.41, 3.49	3.44, 3.44
CH_3	-	-	-	1.96	1.96	1.93
OH	-	3.50	-	9.83[a]		

[a] broad NMR signal centred at 9.83 ppm and compatible with ornithine OH

Table 3-6: ^{13}C- NMR assignments for desferri-fraction 4 (desferri-ferricrocin) observed at 293K in D_2O

residue	glycine	serine	N^5-acetyl-N^5-hydroxyl-L-ornithine
αCH	43.6, 43.8	56.2	54.8, 54.8, 55.0
βCH	-	61.3	28.4, 29.2, 29.5
δCH	-	-	23.2, 23.3, 23.4
γCH	-	-	48.2, 48.3, 48.3
Acetyl CH_3	-	-	20.2
C=O	172.3, 172.6	172.9	174.5, 174.7, 174.9
Hydroxamic C=O	-	-	174.1

Incubation of *A. fumigatus* siderophores with diferric-transferrin

To determine whether individual *A. fumigatus* siderophores were able to compete for transferrin-bound iron, diferric-transferrin was incubated with dilutions of purified desferri-TAF and desferri-ferricrocin. Diferric-transferrin was also incubated with commercially-available desferri-ferrichrome for comparison. The iron saturation of transferrin was monitored by urea-PAGE. Results were similar for all three desferri-siderophores. Desferri-TAF, desferri-ferricrocin and desferri-ferrichrome were all able to remove some iron from diferric-transferrin (25 μM) when present at 500 μM, though complete absence of diferric-transferrin was not observed until the desferri-siderophore concentration was 5 mM (Figure 3-5). Siderophore concentrations in culture reach approximately 50 μM, lower than the concentrations required for removal of iron from transferrin by purified siderophores. However, the equilibrium is altered in culture by the presence of cells which act as a sink for iron removed from transferrin, which could allow lower concentrations of siderophores to remove all iron from transferrin. These results confirm that at least two of the *A. fumigatus* siderophores have potential to remove iron from human transferrin.

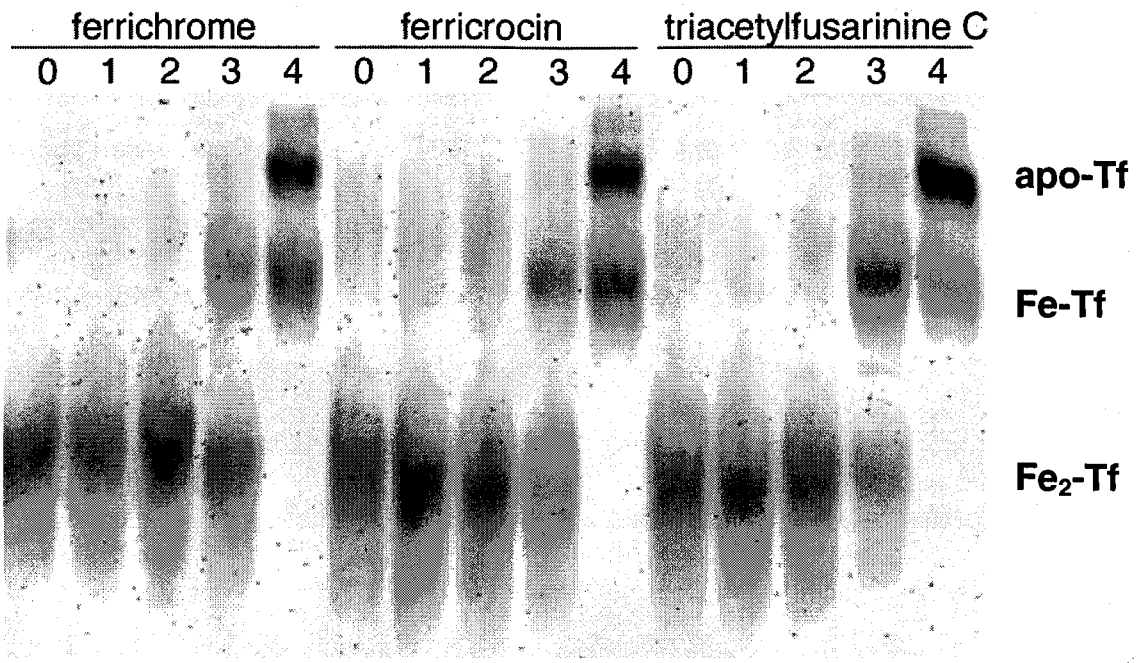


Figure 3-5: Iron saturation of transferrin following incubation with *A. fumigatus* siderophores

Purified desferri-siderophores were incubated with diferric-transferrin (25 μM) at 37°C for 16 hours, then analysed by urea-PAGE as described in Materials and Methods. Desferri-ferrichrome, desferri-ferricrocin and desferri-TAF were serially diluted to final concentrations, 5 μM (lanes 1), 50 μM (lanes 2), 500 μM (lanes 3) and 5 mM (lanes 4). Controls containing diferric-transferrin alone were also run (lanes 0).

Discussion

Siderophore production by microorganisms allows their growth in environments where iron is poorly available. In aerobic environments, ferric ions are abundant but are largely bound up as insoluble ferric hydroxides (Neilands, 1995). At neutral pH, the equilibrium concentration of aquated Fe^{3+} cannot exceed 10^{-17}M (Aisen, 1998) yet iron is required in micromolar amounts for fungal growth (Winkelmann, 1992). Therefore, soil microorganisms such as *A. fumigatus* have adapted to iron-limited environments by producing a variety of siderophores. Organisms that secrete siderophores to access iron from environmental ferric hydroxides may also effectively scavenge transferrin-bound iron in vivo.

The work described in Chapter 2 demonstrated that *A. fumigatus* can grow in human serum, and the results from this chapter indicate that *A. fumigatus* overcomes the iron limitation of serum by secreting hydroxamate siderophores which remove iron from serum transferrin. Proteolytic cleavage of transferrin by *A. fumigatus* is a secondary mechanism by which it can obtain iron.

A. fumigatus uses siderophores to remove iron from human diferric-transferrin

In this study, diferric-transferrin, but not apo-transferrin, promoted growth of *A. fumigatus* in iron-deficient media indicating that *A. fumigatus* can use diferric-transferrin as an iron source. Urea-PAGE can distinguish between the four different forms of transferrin (Fe_2(diferric)-transferrin, Fe_C-transferrin, transferrin-Fe_N, and apo-transferrin) based upon their different resistances to denaturation in 6M urea. Diferric-transferrin incubated with *A. fumigatus* conidia was converted to apo-transferrin within 8 hours, approximately the same time that siderophores were detected by the CAS assay, and very

soon after germination of conidia. Unlike proteinase secretion (Chapter 2), siderophore production occurred early in the growth of *A. fumigatus*. These data suggest that siderophore-mediated removal of iron from human transferrin is important in the growth of *A. fumigatus*. The removal of iron from transferrin across a dialysis membrane further supported the hypothesis that *A. fumigatus* siderophores mediated the removal of iron from transferrin, as opposed to expression of transferrin receptors or enzymatic ferric reductases.

Siderophores of *A. fumigatus*

Five siderophores were purified from *A. fumigatus* culture, and the two major siderophores were identified as TAF and ferricrocin. TAF and ferricrocin were also the major siderophores produced in MEM containing 10% human serum (data not shown). To date, all characterized *A. fumigatus* siderophores belong to the hydroxamate class of siderophores, including TAF and ferricrocin. Nilius and Farmer (1990) reported production of six siderophores by *A. fumigatus*, with TAF the most prominent, followed by ferricrocin. Other studies have detected ferricrocin and ferrirubin in *A. fumigatus* cultures (Wiebe and Winkelmann, 1975), though the type and ratio of siderophores produced appears to vary from strain to strain (Diekmann and Krezdorn, 1975).

Siderophores observed in other *Aspergillus* species include ferrichrome, fusigen, ferrichrysin, ferrirhodin, and ferrirubin (Nilius and Farmer, 1990) and the asperchromes (Jalal *et al.*, 1984). *Aspergillus nidulans* also produces TAF and ferricrocin as the two major siderophores. Eisendle *et al.* (2003) have shown that a mutant of *A. nidulans* deficient in hydroxamate siderophore production was unable to grow unless the medium was supplemented with siderophores or very high concentrations of ferrous iron.

TAF is produced by several *Aspergillus* species (Diekmann and Krezdorn, 1975; Eisendle *et al.*, 2003), while ferricrocin is an important intracellular iron storage compound in fungi such as *A. nidulans* (Eisendle *et al.*, 2003) and *Neurospora crassa* (Matzanke *et al.*, 1987). Ferricrocin is produced by a wide variety of other fungi including *Cenococcum geophilum* (Haselwandter and Winkelmann, 2002), *Phialocephala fortinii* (Bartholdy *et al.*, 2001) and *Colletotrichum gloeosporioides* (Ohra *et al.*, 1995).

TAF has been reported to bind iron with a pM of 31.8 in phosphate buffer, pH 6.8 and 30°C (Adjimani and Emery, 1987), and ferricrocin and ferrichrome have reported pM values of 26.5 (Wong *et al.*, 1983) and 25.2 (Dhungana *et al.*, 2003), respectively. The high iron affinities of these compounds theoretically enable these siderophores to remove iron from ferrated transferrin. This was confirmed in our study as all three siderophores removed transferrin-bound iron. A few hydroxamate siderophores have been reported to remove iron from transferrin in other studies. These include rhodotorulic acid (Timmerman and Woods, 2001), a dihydroxamate siderophore with a pM of 21.9 (Harris *et al.*, 1979) and aerobactin (Konopka *et al.*, 1982), a bacterial siderophore with a prototypical hydroxamic-citrate structure and a pM of 23.3 (Harris *et al.*, 1979). *E. coli* strains bearing the plasmid for aerobactin production can grow in the presence of transferrin and virulence is associated with the synthesis of aerobactin (Williams and Warner, 1980).

Role of proteinases in iron uptake

Microorganisms probably employ several mechanisms to ensure a continual supply of iron for growth. We therefore postulated that *A. fumigatus* may possess more

than one mechanism to obtain iron from serum. As described in Chapter 2, *A. fumigatus* produces proteinases when cultured in serum-containing media and these proteinases could allow iron release from transferrin. In the present study, a small amount of transferrin degradation was apparent after 46 hours growth in 10% human serum and transferrin was completely degraded within 70 hours. When grown in MEM containing human serum, *A. fumigatus* reached stationary phase after approximately 25 hours whereas the peak of proteinase production occurs after 40-48 hours (Chapter 2). The fact that transferrin was not hydrolyzed until late logarithmic phase may also be related to the relative resistance of diferric-transferrin to proteolytic cleavage when compared to apo-transferrin (Esparza and Brock, 1980). Because the demand for iron is highest during logarithmic phase when active growth is occurring, proteolytic degradation of transferrin is unlikely to be the primary mechanism by which *A. fumigatus* obtains iron. This was further supported by the results that showed that *A. fumigatus* was able to grow in MEM containing pure transferrin (no serum) without any degradation of transferrin.

The relative importance of hydrolysis of iron-binding proteins and siderophore secretion has been evaluated in some bacterial pathogens. Okujo et al. (1996) created a mutant of *Vibrio vulnificus* using chemical mutagenesis that was deficient in secretion of an extracellular protease (VVP) but was still able to secrete the siderophore, vulnibactin. They compared the growth in diferric-transferrin of this mutant with a VVP-secreting strain that produced only low amounts of the siderophore. Their results indicated that siderophore production rather than VVP secretion was necessary for growth when ferrated transferrin was the sole iron source. In another study, Wolz *et al.* (1994) created a strain of *Pseudomonas aeruginosa* that was unable to produce the elastase LasB. LasB

mutants were still able to remove iron from transferrin using the *P. aeruginosa* siderophore, pyoverdin, suggesting that siderophore production alone was sufficient to obtain iron. However, when pyoverdin and transferrin were present in equimolar concentrations, iron exchange was enhanced by proteolytic degradation of transferrin by LasB (Wolz *et al.*, 1994). Mutants in AP, another proteinase produced by *P. aeruginosa*, grew much more slowly than the parental strain in media containing transferrin as the sole iron source (Shigematsu *et al.*, 2001). These data suggest that proteolytic degradation of transferrin contributes to iron uptake in *P. aeruginosa*. A similar scenario could be envisioned for *A. fumigatus* growing in serum during late logarithmic phase when protease secretion is maximal.

We have provided evidence that the growth of *A. fumigatus* in human serum in vitro is supported by the production of siderophores. However, we cannot exclude the possibility that low molecular weight reductants also participate in iron acquisition, as has been shown in *H. capsulatum* (Timmerman and Woods, 2001) and *C. neoformans* (Nyhus *et al.*, 1997). Direct evidence for a role of *A. fumigatus* siderophores in virulence awaits the study of siderophore-negative mutant strains, which are described in Chapter 5.

CHAPTER FOUR: SITE-SPECIFIC RATE CONSTANTS FOR IRON ACQUISITION FROM TRANSFERRIN BY THE *ASPERGILLUS FUMIGATUS* SIDEROPHORES N'N"N"'-TRIACETYLFUSARININE C AND FERRICROCIN

Summary

Despite low levels of free iron, *Aspergillus fumigatus* grows in the presence of human serum in part because it produces high concentrations of siderophores. The most abundant siderophores produced by *A. fumigatus* are N'N''N'''-triacetylfusarinine C (TAF) and ferricrocin, both of which have thermodynamic iron binding constants that theoretically allow them to remove transferrin-bound iron. The rate of removal of iron from diferric-transferrin (Tf) by both siderophores was measured, as were the individual microscopic rates of iron removal from each transferrin species (diferric-Tf, N-terminal monoferric-Tf and C-terminal monoferric-Tf) by both TAF and ferricrocin. TAF removed iron from all transferrin species at a faster rate than ferricrocin. Both siderophores showed a preference for removing C-terminal iron, evidenced by the fact that k_{1C} and k_{2C} were much larger than k_{1N} and k_{2N}. Cooperativity in iron binding was observed with TAF, as the C-terminal iron was removed by TAF much faster from monoferric- than from diferric-transferrin. With both siderophores, C-terminal monoferric-transferrin concentrations remained below measurable levels during incubations. This indicates that k_{2C} and k_{1C} are much larger than k_{1N}, which made it impossible to measure k_{1N}. TAF and ferricrocin both removed transferrin-bound iron with second order rate constants that were comparable to several bacterial siderophores,

indicating they may play a role in iron uptake in vivo and therefore contribute to the virulence of *A. fumigatus*.

Introduction

Transferrin

Transferrin is an iron-binding glycoprotein with a molecular weight of 80,000 Da. It plays an important role in efficient and selective iron transport in vertebrates, and because of its ability to chelate free Fe (III), transferrin also has antioxidant, bacteriostatic and fungistatic properties. It is found primarily in plasma, but is also present in body fluids, including airway surface fluids (Govindaraju *et al.*, 1998). Transferrin folds into a bilobal tertiary structure, in which each lobe contains a single site where iron is bound in association with a bicarbonate ion (Figure 1-2). The two lobes are structurally different and are not chemically equivalent (Baker *et al.*, 2002), and there is evidence of cooperativity in iron binding between the two lobes (Baker *et al.*, 2002).

In vivo, the presence of transferrin and other iron-binding proteins such as lactoferrin reduces free iron to concentrations far below those required to support microbial growth, resulting in what is termed nutritional immunity (Byers and Arceneaux, 1998; Ratledge and Dover, 2000). In healthy individuals, transferrin is only partially saturated (~30%), and during infections, non-specific host defences decrease the level of transferrin saturation even further by increasing ferritin synthesis and releasing lactoferrin from neutrophils (Bullen, 1981).

Iron uptake by siderophores

Several studies have shown that siderophore-mediated iron acquisition is important in the colonization and persistence of bacterial pathogens in vivo, including

Escherichia coli (Torres *et al.*, 2001), *Vibrio* species (Litwin *et al.*, 1996; Wertheimer *et al.*, 1999), *Burkholderia cepacia* (Sokol *et al.*, 1999) and *Pseudomonas aeruginosa* (Meyer *et al.*, 1996). *Aspergillus fumigatus* produces several hydroxamate siderophores; however, the role of siderophores in virulence of fungi is unknown.

TAF is the most abundant siderophore purified from *A. fumigatus* cultures, amounting to approximately 90% (w/w) of the total siderophores isolated (Chapter 3). TAF is a hydroxamate siderophore of the fusarinine class, and is composed of three N-δ-acyl-N-δ-hydroxyornithine units joined head-to-tail with three units of anhydromevalonic acid (Figure 1-3A). Ferricrocin, the second most abundant secreted siderophore of *A. fumigatus*, is a hydroxamate of the ferrichrome class (Figure 1-3B). Preliminary results presented in Chapter 3 indicate that both TAF and ferricrocin can remove iron from human diferric-transferrin.

Siderophores must be able to compete both thermodynamically and kinetically with transferrin for iron if they are to be effective for iron uptake in vivo. Thermodynamic iron binding affinities (pM) are measured as the negative log concentration of free iron in a solution containing 1 μM Fe^{3+} and 10 μM chelator at pH 7.4. pMs are known for many siderophores (Table 4-1) and are useful for comparing iron affinities; however, pM values do not always correlate with kinetic rates for iron removal from transferrin. For instance aerobactin, with a pM of 23.3, can efficiently remove iron from transferrin (Konopka *et al.*, 1982), even though transferrin has a higher pM value of 23.6 (Harris *et al.*, 1979). Conversely, desferrioxamine B competes poorly for transferrin-bound iron in vitro (Pollack *et al.*, 1977) despite its pM value of 26.6.

TAF and ferricrocin possess pM values of 31.8 (Adjimani and Emery, 1987) and 26.5 (Wong *et al.*, 1983), respectively, both of which are higher than that of transferrin (Table 4-1), indicating that these siderophores can theoretically remove iron from transferrin. However, since high pM values do not necessarily indicate a fast kinetic rate of iron removal by the siderophore, it is necessary to measure the rate of iron removal from transferrin by both TAF and ferricrocin to determine their potential for iron uptake in vivo.

Table 4-1: Published iron affinity values (pM) for bacterial and fungal siderophores

Siderophore	pM	reference
bacterial siderophores		
aerobactin	23.3	(Harris *et al.*, 1979)
enterobactin	35.6	(Harris *et al.*, 1979)
pyoverdin	32	(Wendenbaum *et al.*, 1983)
desferrioxamine B	26.6	(Harris *et al.*, 1979)
fungal siderophores		
rhodotorulic acid	21.9	(Harris *et al.*, 1979)
ferrichrome	25.2	(Harris *et al.*, 1979)
ferricrocin	26.5	(Wong *et al.*, 1983)
TAF[a]	31.8	(Adjimani and Emery, 1987)
ferrichrysin	25.8	(Harris *et al.*, 1979)

[a] pM measured at pH 6.8.
Transferrin has a pM value of 23.6 (Harris *et al.*, 1979).

Kinetic analysis

Iron removal from transferrin can be studied using high ligand concentrations to generate pseudo-first-order conditions. The observed rate constants can then be used to calculate second order rates. Iron removal from transferrin can be described by the following scheme:

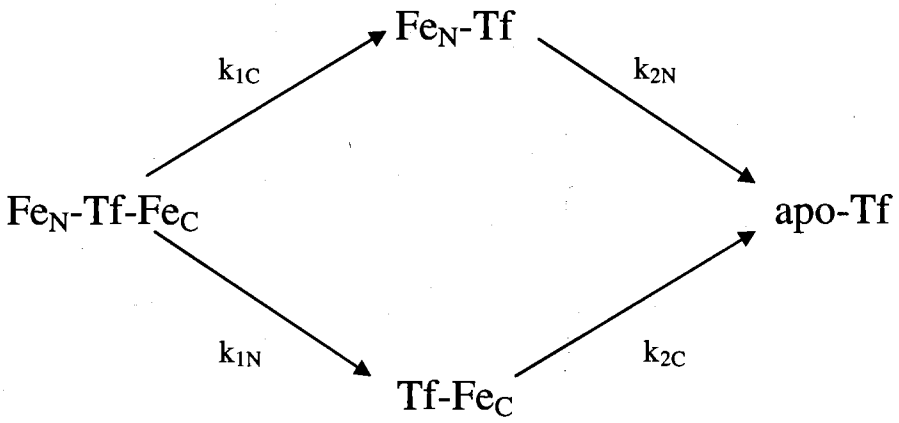


Figure 4-1: The four microscopic rate constants describing iron removal from transferrin

Objectives

Ferrated transferrin was incubated with the *A. fumigatus* siderophores, TAF and ferricrocin, and the concentrations of each molecular form of transferrin were measured over time. This information was used to calculate the microscopic rate constants for iron removal from each position of transferrin by each siderophore. These are the first measurements of microscopic rate constants for fungal siderophores, therefore the rates were compared to those of iron chelators produced by other pathogens. An

understanding of the kinetic ability of *A. fumigatus* siderophores to remove transferrin-bound iron may explain the resistance of this species to the antifungal properties of serum.

Materials and Methods

Strains and growth conditions

Aspergillus fumigatus (ATCC 13073) and was obtained from the American Type Culture Collection and maintained, cultured and harvested as described in Chapter 2.

Purification of *A. fumigatus* siderophores

Hydroxamate siderophores were purified from the culture supernatant of *A. fumigatus* as described in Chapter 3. Iron was removed from TAF and ferricrocin by treatment with 8-hydroxyquinoline as described in Chapter 3.

Preparation of Fe_N-Tf-Fe_C

Human diferric-transferrin (Fe_N-Tf-Fe_C) was purchased from Sigma and was iron saturated as described by Turcot *et al.* (2000) and Bali and Harris (1990). Iron nitrilotriacetate was prepared by dissolving 15 μmol $FeCl_3$ in a solution of 6M HCl and 30 μmol nitrilotriacetic acid. The pH was adjusted to 4.0 with KOH, then water was added to 10 ml. Transferrin (200 μM) in kinetics buffer (250 mM Tris, 100 mM NaCl, 50 mM $NaHCO_3$, pH 7.4) was saturated by addition of 400 μM freshly prepared iron nitrilotriacetate and incubated for 2 hours at 37°C. The transferrin solution was concentrated 10-fold in a Centricon filter (10,000 Da molecular weight cut-off, Millipore) and reconstituted to its original volume with fresh kinetics buffer. This wash was performed 6 times to eliminate unbound iron. Successful ferration of Tf was confirmed by urea-PAGE, and transferrin was diluted to 250 μM prior to use.

Preparation of Tf-Fe_C

C-terminal monoferric-transferrin was produced by incubation of human apo-transferrin (apo-Tf, Sigma) with exactly one equivalent freshly prepared iron nitrilotriacetate. Apo-transferrin (200 μM) was incubated with 200 μM iron nitrilotriacetate and incubated for 2 hours at 37°C. The transferrin solution was dialysed in a Centricon filter (10,000 Da molecular weight cut-off, Millipore) as described for Fe_N-Tf-Fe_C in order to eliminate nitrilotriacetate. Successful preparation of C-terminal monoferric-transferrin was confirmed by urea-PAGE, and transferrin was diluted to 250 μM prior to use.

Preparation of Fe_N-Tf

N-terminal monoferric-transferrin was prepared by the method of Baldwin and de Sousa (1981). This method employs an ethylenediaminetetracetic acid (EDTA) solution in the presence of high concentrations of sodium perchlorate to selectively remove the C-terminal iron from diferric-transferrin. The ClO_4^- accelerates iron removal from the C-terminal site of transferrin such that the rate is 260-times faster than removal from the N-terminal site (Baldwin and de Sousa, 1981). Diferric-transferrin (250 μM) was incubated with 100 mM EDTA in a 0.1M Hepes buffer (pH 7.4) that also contained 2.67 M $NaClO_4$ for 1 hour at 37°C. The sample was then dialysed in a Centricon filter (10,000 Da molecular weight cut-off, Millipore) as described for Fe_N-Tf-Fe_C to eliminate EDTA and $NaClO_4$. Successful preparation of N-terminal monoferric-transferrin was confirmed by urea-PAGE, and transferrin was diluted to 250 μM prior to use.

Kinetics of iron removal from transferrin

The 10x transferrin stock (250 μM) was warmed to 37°C, then diluted 1:10 with buffer to give a final reaction concentration of 25 μM (approximately 2 mg/ml). Desferri-siderophores were prepared in 50 mM stock solutions in kinetics buffer, then diluted to concentrations ranging from 1 to 20 mM with kinetics buffer and warmed to 37°C before mixing with transferrin. These siderophore concentrations resulted in a 40- to 600-fold excess of siderophore to transferrin. Control reactions contained transferrin but no siderophore and were incubated under identical conditions. Reactions were sampled by removing 10 μl aliquots, mixing with 10 μl urea polyacrylamide gel electrophoresis (urea-PAGE) loading buffer (1X TBE, 10% glycerol and 0.2% bromphenol blue), and quick freezing in a dry ice / ethanol bath. The high pH of the loading buffer stops the iron transfer reaction (Stintzi and Raymond, 2000). Samples were stored at -30°C until urea-PAGE analysis was carried out.

Urea polyacrylamide gel electrophoresis and kinetic data analysis

Urea- PAGE was carried out as described by Wolz *et al.* (1994) in a Protean II xi cell (Bio-Rad). Transferrin (10 μg) in a 10μl volume was loaded in each lane of the gel. Gels were run at 210 V and 4°C for 20 hours, then rinsed for 30 minutes in 0.05% SDS and stained with SYPRO Orange (Molecular Probes, Eugene OR). Bands were visualized by scanning on a Typhoon 9410 Imager (Amersham).

Gels were analysed using ImageQuant 5.2 (Molecular Dynamics). The percent transferrin present in each band of each sample was calculated, allowing the percentage transferrin found in each of its four states to be calculated at all time points. The data shown were combined from at least three independent experiments. Non-linear

regression analysis was performed using Prism 4 (GraphPad) and values shown are best-fits ± standard errors.

Results

Iron removal by TAF and ferricrocin

Diferric transferrin was incubated at 37°C with increasing concentrations of either TAF or ferricrocin and urea-PAGE analysis (Figure 4-2) was used to resolve the concentrations of the four possible transferrin species over time. Each lane of the gel was analysed using ImageQuant software and the total peak area for each lane was normalized to 100%. The relative fraction areas of each transferrin species during a typical incubation with either TAF or ferricrocin are plotted in Figure 4-3. Control reactions containing ferrated transferrin but no siderophore showed no change in distribution of transferrin species over time (data not shown).

During incubation with either TAF or ferricrocin, the concentration of Fe_N-Tf-Fe_C decreased exponentially until it was completely consumed. With both siderophores, the concentration of Fe_N-Tf increased to approximately 50% of the total transferrin, and then it was slowly consumed. Interestingly, only very low levels of C-terminal monoferric-transferrin were observed, indicating that k_{1N} was smaller than the rate k_{1C} for both TAF and ferricrocin. The proportion of apo-Tf rose continuously as the concentration of N-terminal monoferric-transferrin decreased.

Because C-terminal monoferric-transferrin was not formed in large quantities in these reactions, the rate k_{2C} was measured in separate experiments, using C-terminal monoferric-transferrin as the starting material.

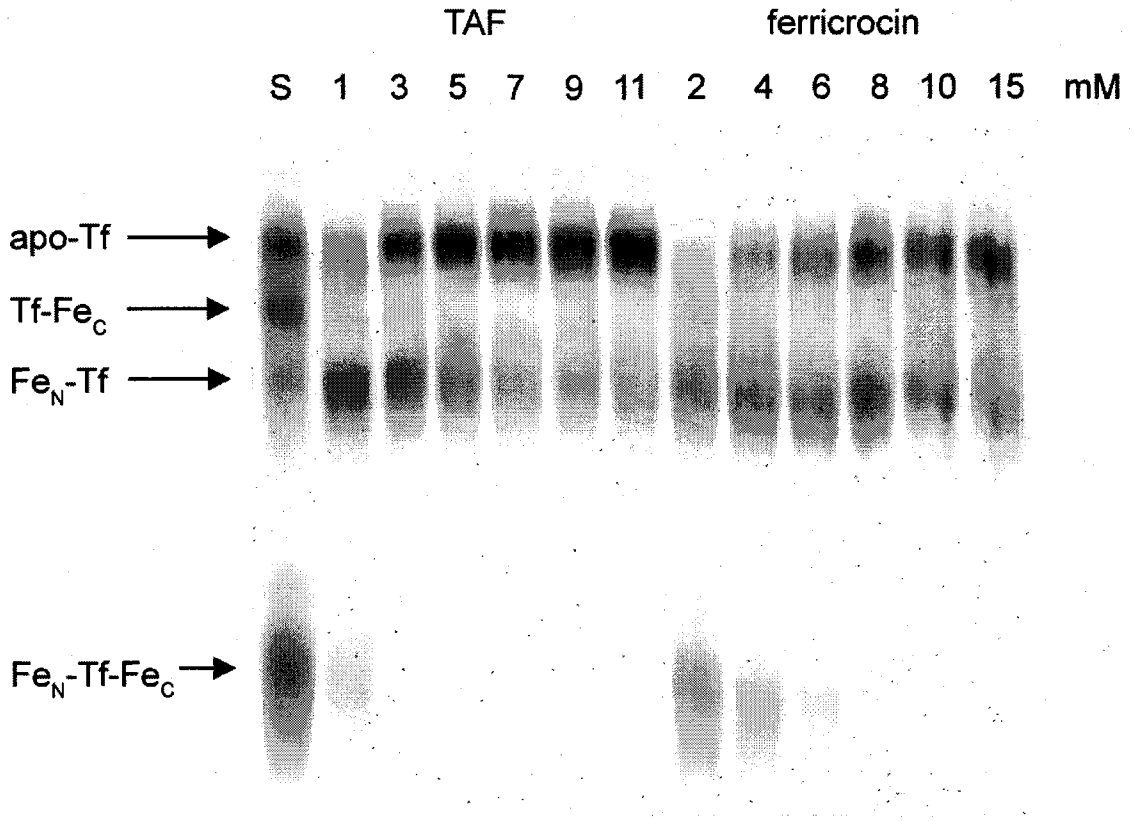
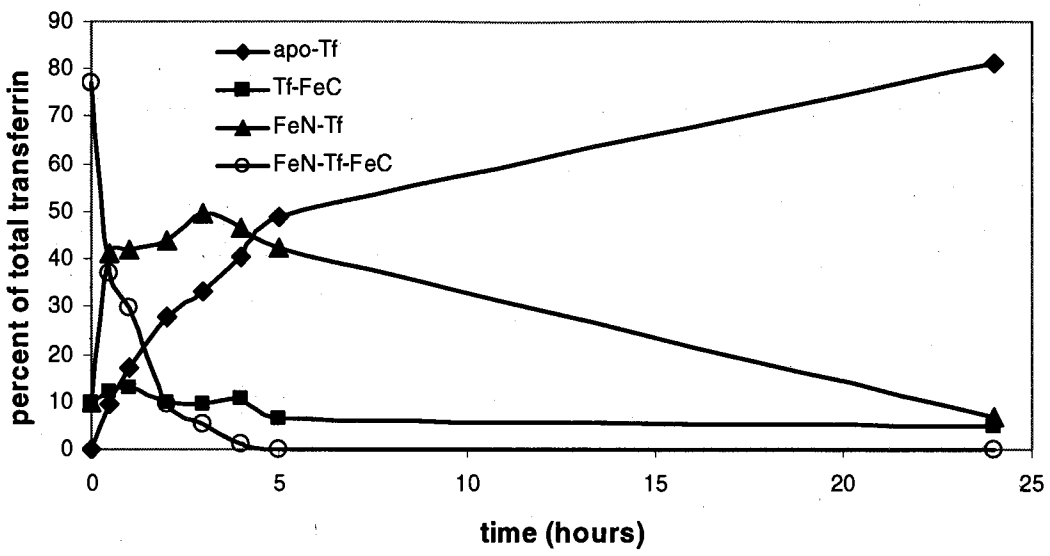
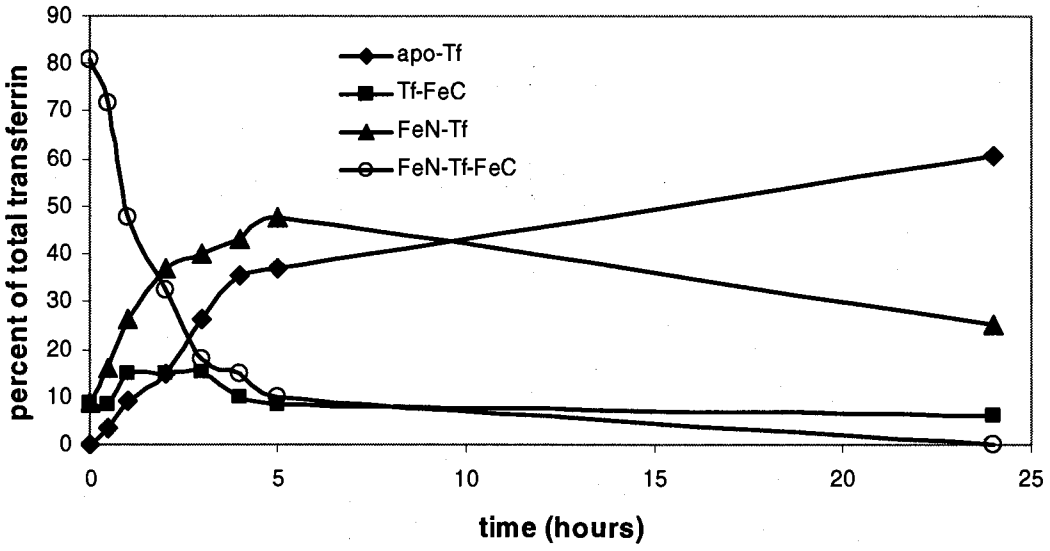


Figure 4-2: Representative urea-PAGE gels displaying transferrin species present after incubation for 5 hours with increasing concentrations of TAF or ferricrocin

Human diferric-transferrin (25 μM) was incubated with siderophores at 37°C in 250 mM Tris, 150 mM NaCl, 20 mM $NaHCO_3$, pH 7.4. Samples were removed after 5 hours incubation and analyzed by urea PAGE. Apo-Tf = apotransferrin, Tf-Fe_C = C-terminal monoferric-transferrin, Fe_N-Tf = N-terminal monoferric-transferrin, and Fe_N-Tf-Fe_C = diferric-transferrin. S denotes a lane of standards containing all four transferrin species. The numbers at the top of the gel indicate siderophore concentrations in mM.



A



B

Figure 4-3: The distribution of transferrin species during incubation with 3 mM TAF (A) or 6 mM ferricrocin (B) for 24 hours at 37°C

Human diferric-transferrin (25 μM) was incubated with siderophores at 37°C. Samples were removed after various intervals and analyzed by urea PAGE. Gels were scanned and bands quantified as described in Materials and Methods. Apo-Tf = apotransferrin, Tf-Fe_C = C-terminal monoferric-transferrin, Fe_N-Tf = N-terminal monoferric-transferrin, and Fe_N-Tf-Fe_C = diferric-transferrin.

Kinetics (k_{obs})

Equations 1-4 relate the concentrations of each transferrin species to the rate constants defined in Scheme 1.

equation

(1) $$\frac{d[Fe_N - Tf - Fe_C]}{dt} = -(k_{1C} + k_{1N})[Fe_N - Tf - Fe_C]$$

(2) $$\frac{d[Fe_N - Tf]}{dt} = k_{1C}[Fe_N - Tf - Fe_C] - k_{2N}[Tf - Fe_N]$$

(3) $$\frac{d[Tf - Fe_C]}{dt} = k_{1N}[Fe_N - Tf - Fe_C] - k_{2C}[Fe_C - Tf]$$

(4) $$\frac{d[apo - Tf]}{dt} = k_{2C}[Tf - Fe_C] + k_{2N}[Fe_N - Tf]$$

Equations 1-3 were solved to give expressions for the concentration of the four transferrin species over time (equations 5 through 7).

(5) $$[Fe_N - Tf - Fe_C] = [Fe_N - Tf - Fe_C]_o - (k_{1C} + k_{1N})[Fe_N - Tf - Fe_C]_o e^{-(k_{1N}+k_{1C})t}$$

(6) $$[Fe_N - Tf] = [Fe_N - Tf]_o - \frac{k_{2N}(k_{1N}[Fe_N - Tf - Fe_C]_o - [Fe_N - Tf]_o k_{2N} + [Fe_N - Tf]_o k_{1C} + [Fe_N - Tf]_o k_{1N})}{-k_{2N} + k_{1C} + k_{1N}} e^{-k_H t}$$

$$+ \frac{k_{1C}[Fe_N - Tf - Fe_C]_o (k_{1C} + k_{1N})}{-k_{2C} + k_{1C} - k_{1N}} e^{-(k_{1C}+k_{1N})t}$$

(7) $$[Tf - Fe_C] = [Tf - Fe_C]_o - \frac{k_{2C}(k_{1N}[Fe_N - Tf - Fe_C]_o - [Tf - Fe_C]_o k_{2C} + [Tf - Fe_C]_o k_{1C} + [Tf - Fe_C]_o k_{1N})}{-k_{2N} + k_{1C} + k_{1N}} e^{-k_{2C}t}$$

$$+ \frac{k_{1N}[Fe_N - Tf - Fe_C]_o (k_{1C} + k_{1N})}{-k_{2C} + k_{1C} + k_{1N}} e^{-(k_{1C}+k_{1N})t}$$

Microscopic rate constants k_{1C} and k_{1N}

The pseudo-first order rate constants for iron removal from Fe_N-Tf-Fe_C were determined for both siderophores by fitting the concentrations of Fe_N-Tf-Fe_C to the exponential decay curve in equation 5 (Figure 4-4). This equation gave the rate of disappearance of Fe_N-Tf-Fe_C, which is the sum of k_{1C} and k_{1N}. Exponential decreases in concentration of Fe_N-Tf-Fe_C were observed for all concentrations of TAF and ferricrocin.

A plot showing the formation and consumption of Fe_N-Tf was used to determine the pseudo-first order rate constants for k_{1C}. The concentrations of Fe_N-Tf were fit to equation 6 (Figure 4-5). Unfortunately, it was not possible to calculate an accurate value for k_{1N}, because the change in concentration of Tf-Fe_C was very small at all siderophore concentrations. The concentration of Tf-Fe_C remained small because k_{1N} is much smaller than k_{1C} and k_{2C} for both siderophores. These values indicate a marked preference for removal of the C-terminal iron from diferric-transferrin by both TAF and ferricrocin.

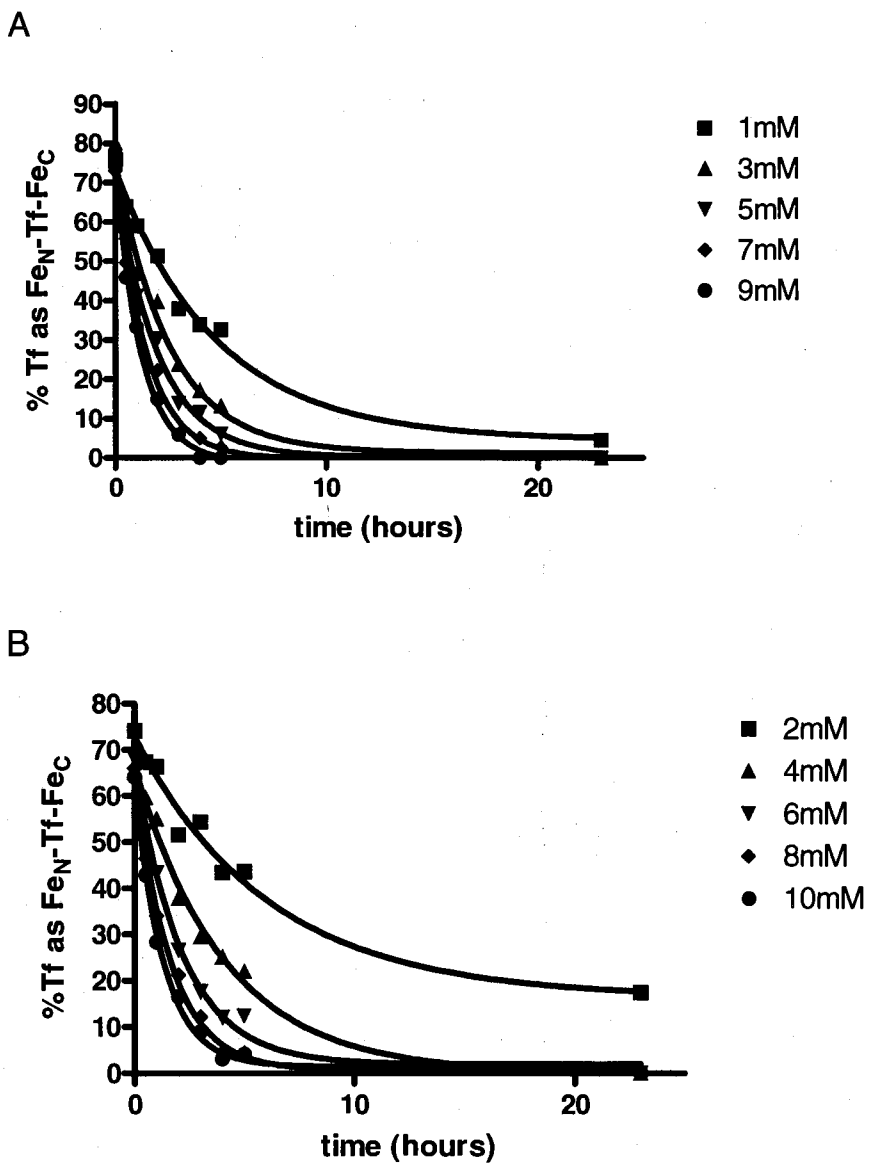


Figure 4-4: Exponential decay plot for the concentration of diferric-Tf (Fe_N-Tf-Fe_C) as a percent of total transferrin during incubation with increasing concentrations of TAF (A) or ferricrocin (B)

Concentrations of Fe_N-Tf-Fe_C were determined by scanning urea-PAGE gels on a Typhoon 9410 Imager and quantifying the Fe_N-Tf-Fe_C bands using ImageQuant software. These data were fit to equation 5 to calculate the pseudo first order rate constant, $k_{1N} + k_{1C}$.

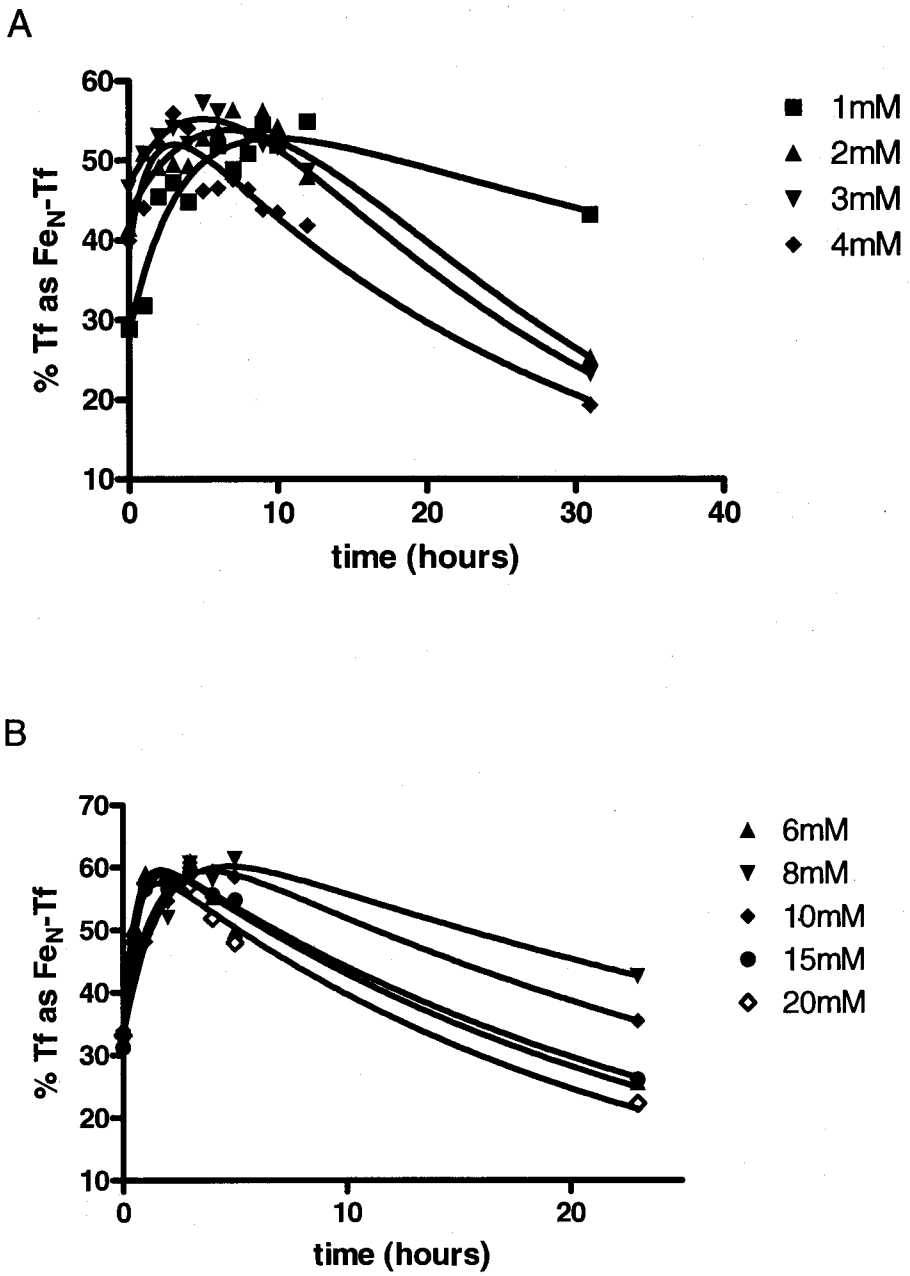


Figure 4-5: The proportion of total transferrin present as N-terminal monoferric-transferrin (Fe_N-Tf) during incubation of Fe_N-Tf-Fe_C with TAF (A) or ferricrocin (B)

These data were fit to equation 6 to calculate the pseudo first order rate constants, k_{1C} and k_{2N}.

Microscopic rate constants k_{2C} and k_{2N}

The rates of removal of iron from the monoferric transferrins Tf-Fe_C, k_{2C}, and Fe_N-Tf, k_{2N}, were also determined. k_{2N} was calculated from the rate of decrease of Fe_N-Tf concentration shown in Figure 4-5. The data were fit to equation 6 while holding constant all the other previously calculated rates to determine observed values for k_{2N} for each siderophore.

The values for k_{2C} were determined in an independent experiment starting with C-terminal monoferric-transferrin. Tf-Fe_C concentrations decreased exponentially (Figure 4-6), and the concentrations were fit to an exponential decay curve to determine k_{obs} values for k_{2C}.

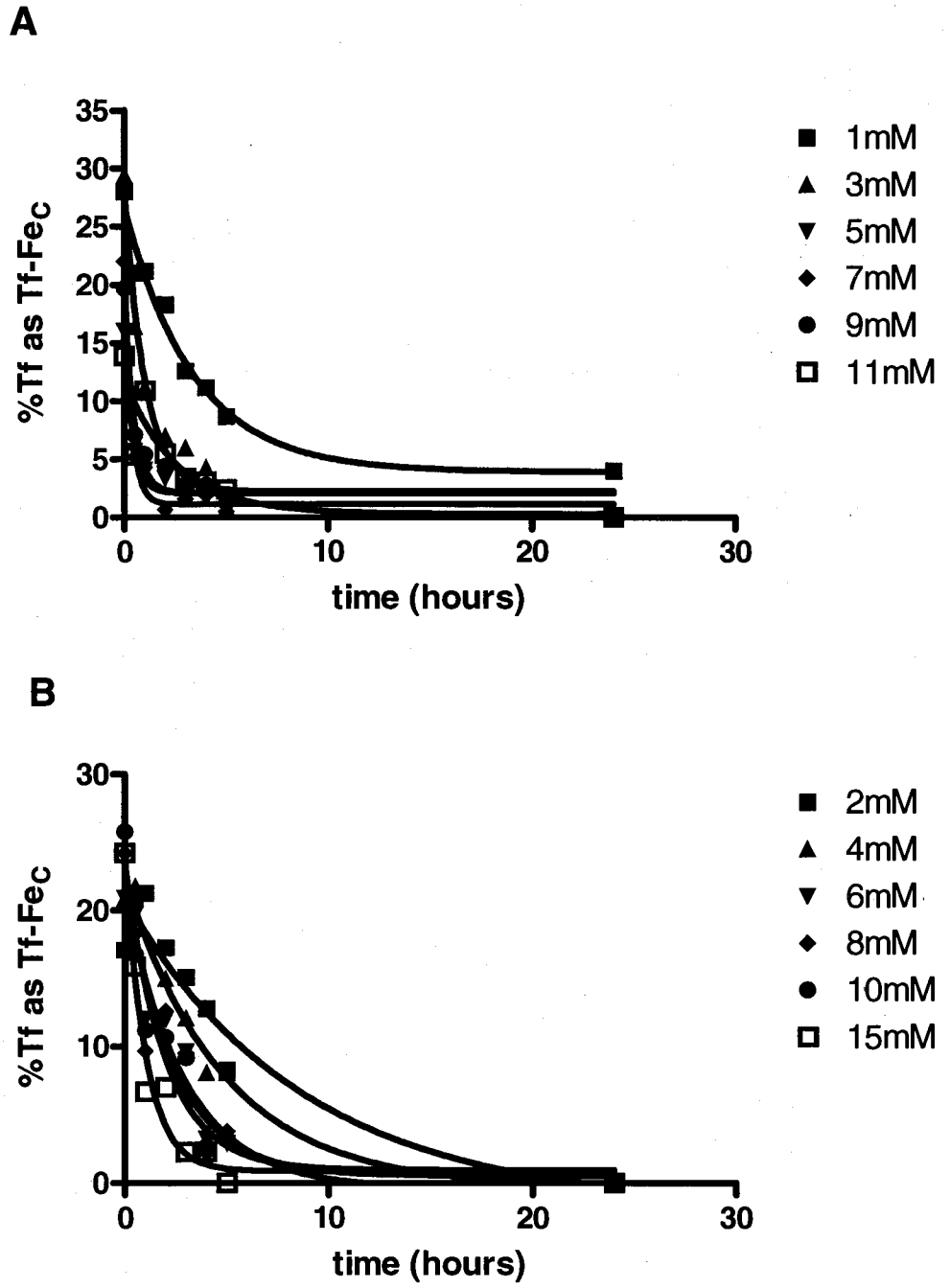


Figure 4-6: The proportion of total transferrin present as C-terminal monoferric-transferrin (Tf-Fe_C) during incubation with TAF (A) or ferricrocin (B)

These data were fit to equation 7 to calculate the pseudo first order rate constant k_{2C}.

Second order kinetics (k' and k_{max})

The pseudo first order rate constants calculated above were used to determine the second-order rate constant k' (rate = k'[siderophore][Tf]). The pseudo first order rate constants for formation or depletion of Fe_N-Tf-Fe_C, Tf-Fe_C, and Fe_N-Tf all showed a hyperbolic relationship with k_{obs} for each siderophore, demonstrating that the rates show saturation behaviour with respect to ligand concentration. The second order rate constant k' predicts the rate of reaction at low ligand concentration while k_{max} is the maximum rate constant at ligand saturation. Both k' and k_{max} were determined by plotting the pseudo first order rate constants (k_{obs}) obtained for $k_{1N} + k_{1C}$ (Figure 4-7), k_{1C}, k_{2N} and k_{2C} (Figure 4-8) against siderophore concentration and fitting the data to equation 8 (Stintzi and Raymond, 2000):

$$(8) \qquad k_{obs} = \frac{k'[\text{siderophore}]}{1 + (k'/k_{max})[\text{siderophore}]}$$

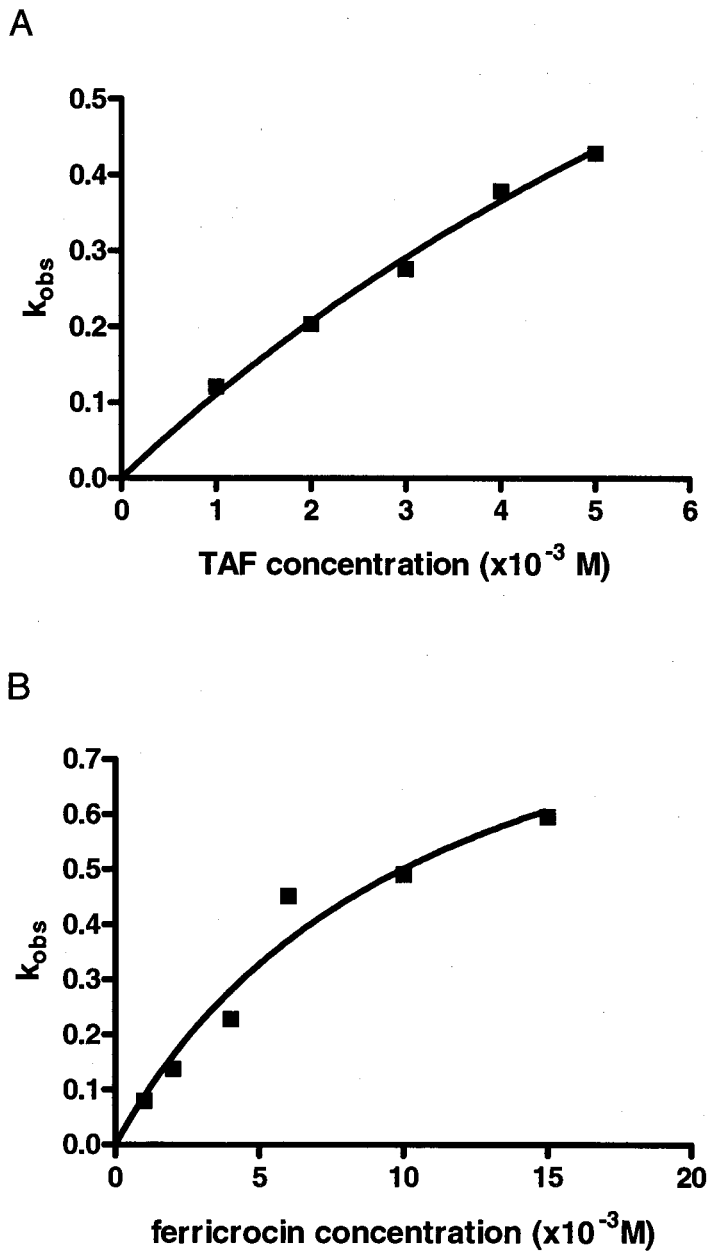


Figure 4-7: The observed rate constants (k_{obs}) for iron removal from Fe_N-Tf-Fe_C as a function of TAF (A) or ferricrocin (B) concentration

Second order rates were calculated by fitting data to the hyperbolic function $k_{obs}=k'[siderophore]/(1+(k'/k_{max})[siderophore])$, where k' is the second order rate constant and k_{max} is the maximum rate at high siderophore concentration.

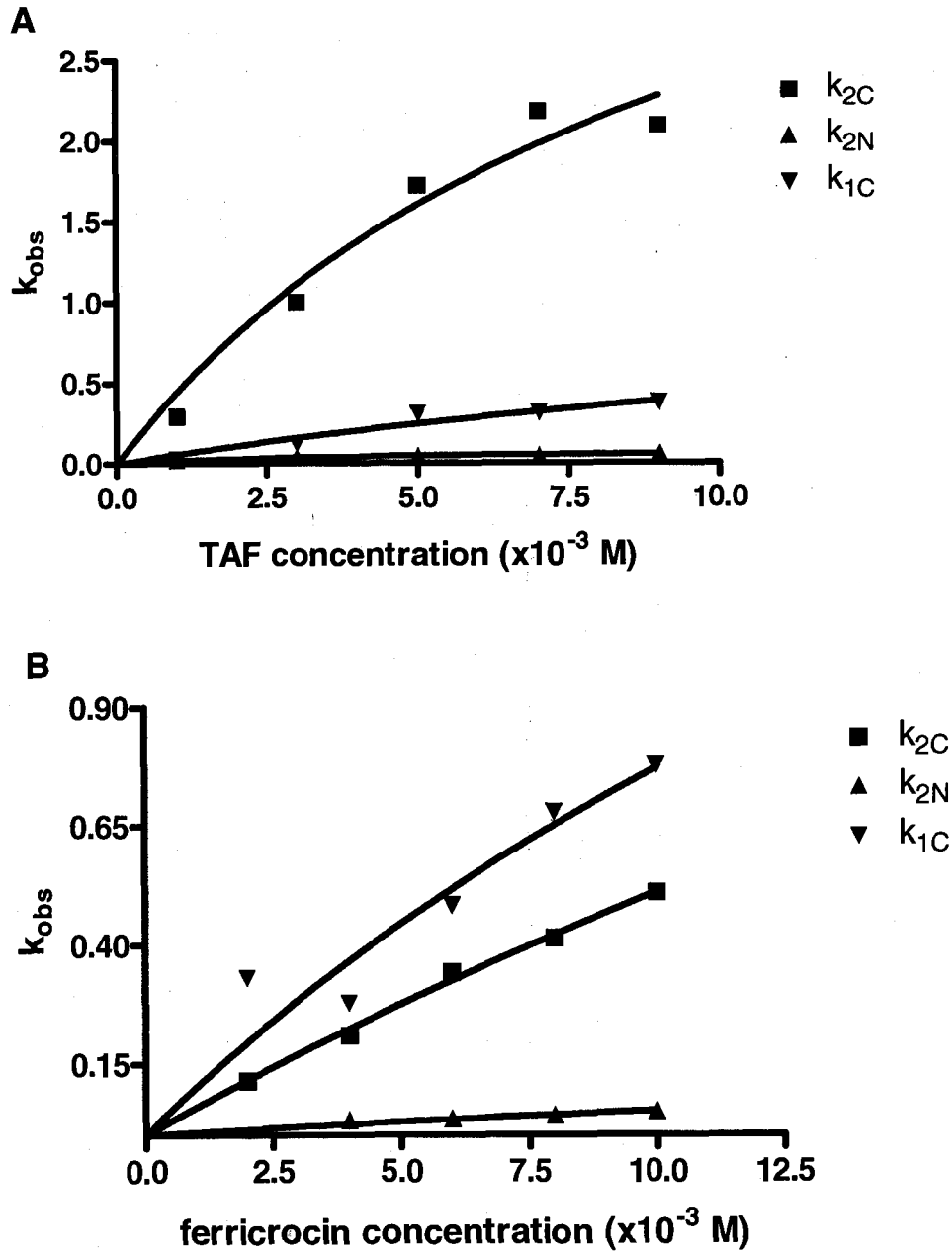


Figure 4-8: The observed rate constants (k_{obs}) as a function of TAF (A) or ferricrocin (B) concentration

Second order microscopic rate constants were calculated for k_{1C}, k_{2C} and k_{2N} by fitting data to the hyperbolic function $k=k'[siderophore]/(1+(k'/k_{max})[siderophore])$, where k' is the second order rate constant and k_{max} is the maximum rate at high siderophore concentration.

For the removal of the first iron from Fe_N-Tf-Fe_C, a k' of 2.37 ± 0.23 $M^{-1}min^{-1}$ and k_{max} of 0.13 ± 0.04 min^{-1} for TAF were determined. For ferricrocin k' was 1.57 ± 0.32 $M^{-1}min^{-1}$ and k_{max} 0.021 ± 0.005 min^{-1}. Removal of the C-terminal iron occurred much more quickly than removal of the N-terminal iron. Table 4-2 summarizes the rate constants for k_H, the sum of k_{1C} and k_{1N}, which is a measure of the rate of iron removal from diferric transferrin. These data can be used to compare k_H values for *A. fumigatus* siderophores to those of siderophores produced by other microbial pathogens. The individual microscopic rate constants, k_{1C}, k_{2N} and k_{2C} are compared in Table 4-3.

Table 4-2: Second order rate constants for removal of iron from diferric transferrin (k_{1N} +k_{1C}) by TAF and ferricrocin compared with the published values for other iron chelators

Pseudo first order rates (k_{obs}) were calculated by fitting the concentration of Fe_N-Tf-Fe_C to the exponential decay function, equation 5. Second order rates were then calculated from fits of the hyperbolic function $k_{obs}=k'[siderophore]/(1+(k'/k_{max})[siderophore])$, where k' is the second order rate constant and k_{max} is the maximum rate at high siderophore concentration. Values shown represent the best-fits ± standard error for TAF and ferricrocin.

ligand	Second order rate constant k' ($M^{-1}min^{-1}$)	Maximum rate k_{max} (x 10^{-2} min^{-1})	Reference
TAF	2.4 ± 0.2	12.7 ± 4.0	this study
ferricrocin	1.6 ± 0.3	2.1 ± 0.5	this study
amonabactin	78	4.5	(Stintzi and Raymond, 2000)
Pyrophosphate	2.88	18.4	(Stintzi and Raymond, 2000)
NTP	--	4.3	(Bali and Harris, 1990)
enterobactin	8.1	2.1	(Harris *et al.*, 1979)
aerobactin	3.44	0.27	(Konopka *et al.*, 1982)

Table 4-3: Comparison of microscopic rate constants k_{1C}, k_{1N}, k_{2C} and k_{2N} for TAF and ferricrocin with published values for other iron chelators

Pseudo first order rate constants were calculated by fitting concentrations of Fe_N-Tf to equation 6 (k_{1C} and k_{2N}) or by fitting concentrations of Tf-Fe_C to equation 7 (k_{1N}). Second order rates were calculated as described in the text.

ligand	Second order rate constant k' ($M^{-1}min^{-1}$)				Maximum rate k_{max} ($x\ 10^{-2}\ min^{-1}$)				Reference
	k_{1C}	k_{2C}	k_{1N}	k_{2N}	k_{1C}	k_{2C}	k_{1N}	k_{2N}	
TAF	2.0±0.6	10.7±4.1	ND[a]	0.35±0.08	8.6±1.6	8.0±3.7	ND[a]	0.13±0.02	this study
ferricrocin	1.73±0.04	0.98±0.07	ND[a]	0.29±0.05	1.7±0.4	6.0±2.0	ND[a]	0.11±0.02	this study
amonabactin	19	14	59	$<10^{-2}$	1.7	1.3	2.8	0	(Stintzi and Raymond, 2000)
Ppi	0.84	1.0	2.8	2.4	2.1	0.77	9.2	9.0	(Stintzi and Raymond, 2000)
citrate	ND	.0887	ND	ND	ND	0.403	ND	3.71	(Harris *et al.*, 2003)

[a]ND = not determined

Discussion

Many siderophores, including those of *A. fumigatus*, likely evolved to acquire soil-bound iron from the environment and their ability to remove iron from human iron-binding molecules is an unfortunate consequence. The two most abundant siderophores of *A. fumigatus*, TAF and ferricrocin, have a high affinity for iron (pM values of 31.8 (Adjimani and Emery, 1987) and 25.6 (Wong *et al.*, 1983), respectively), and therefore can compete thermodynamically for iron with host iron binding compounds such as transferrin, which has a pM value of 23.6 (Harris *et al.*, 1979).

We have measured the site-specific rate constants for iron acquisition from diferric-transferrin by each of these siderophores. The iron uptake rates for TAF were similar to those of pyrophosphate (Ppi) with k' values of 2.4 $M^{-1}min^{-1}$ vs 2.88 $M^{-1}min^{-1}$, respectively. The k_{max} values for TAF and Ppi were also similar. k_{max} was 0.127 min^{-1} for TAF and 0.184 min^{-1} for Ppi. Ferricrocin acquired iron from diferric transferrin more slowly: k' was 1.57 $M^{-1}min^{-1}$ and k_{max} was 0.021 min^{-1}.

k' allows direct comparison between the efficiency of ligands to remove iron from transferrin at low concentrations. The k' values show that at low siderophore concentrations, TAF and ferricrocin are slightly slower at removing iron from Fe_N-Tf-Fe_C than the bacterial siderophores amonabactin, aerobactin and enterobactin (Table 4-2); nevertheless, the k' values were of a similar order of magnitude to those of aerobactin and enterobactin. This is notable because aerobactin is known to contribute to the virulence of *E. coli* (Torres *et al.*, 2001).

There was a large difference between k_{max} values of TAF and ferricrocin. At very high siderophore concentrations, TAF can remove transferrin-bound iron 5-times faster

than ferricrocin (k_{max} of 0.127 min^{-1} vs 0.021 min^{-1}). Both these k_{max} values are much higher than k_{max} reported for aerobactin. Since TAF binds iron with a larger thermodynamic iron binding constant (pM) than ferricrocin, it was not surprising that TAF removed transferrin-bound iron faster than ferricrocin.

We also compared the rates of iron removal from the N-terminal and C-terminal binding sites of Fe_N-Tf-Fe_C. Both *A. fumigatus* siderophores showed a definite preference for the C-terminal iron of diferric transferrin (Table 4-3). Most siderophores display a preference for one site; the bacterial siderophores enterochelin (Ford *et al.*, 1988) and amonabactin (Stintzi and Raymond, 2000) preferentially removed N-lobe-bound iron while aerobactin showed a preference for the C-terminal site of Fe_N-Tf-Fe_C (Ford *et al.*, 1988). In addition, the rate of iron removal from C-terminal monoferric transferrin was much higher than the rate of removal of iron from the N-terminal monoferric transferrin. Thus in both monoferric and diferric transferrins, the C-terminal iron was always removed more rapidly than N-terminal iron by both ferricrocin and TAF. This preference is interesting because the C-terminal iron is considered to be bound to transferrin with higher affinity than the N-terminal iron (Leibman and Aisen, 1979). Hydroxamate siderophores might have structural characteristics which allow them easier access to the C-terminal iron binding site.

The impact of cooperativity in iron binding by transferrin was evident in this study. With TAF, the C-terminal iron was much more rapidly removed from monoferric transferrin than from diferric transferrin (k' values of 10.8 $M^{-1}min^{-1}$ for k_{2C} vs 2.3 $M^{-1}min^{-1}$ for k_{1C}). This effect was not observed with ferricrocin, where the second order rate constants for k_{1C} and k_{2C} were of similar magnitudes. It is thought that the iron status

of one lobe can influence binding or release from the other. Iron binding is known to induce a conformational change in Tf from an open (apo) to closed (diferric) form (Figure 4-9), and this change is communicated between lobes (reviewed by Baker *et al.* (2002)).

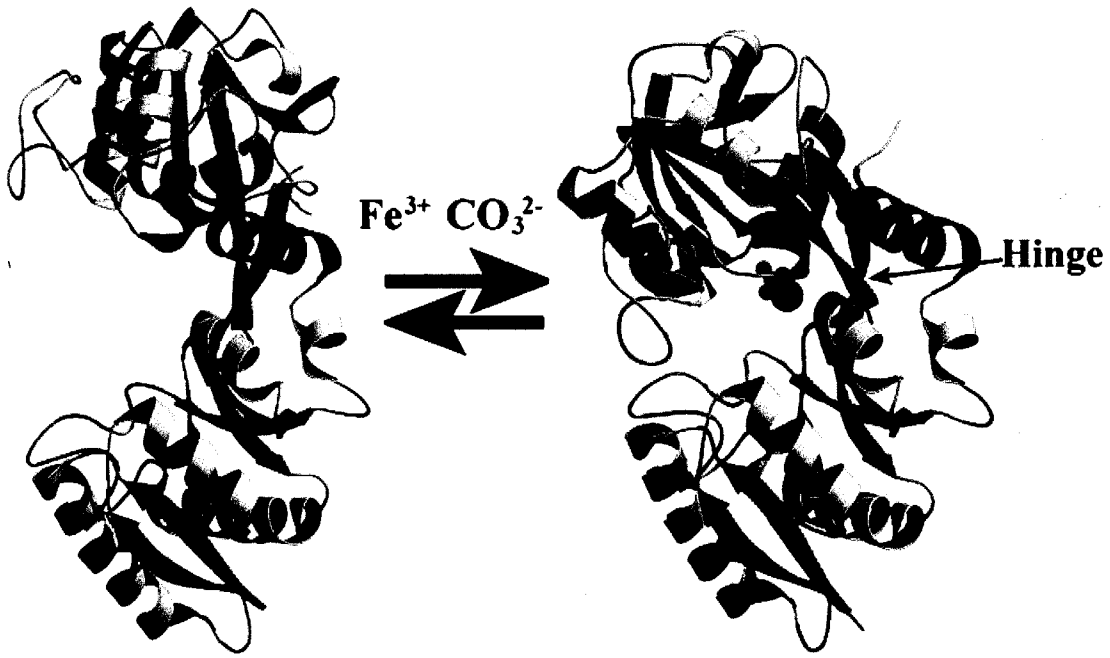


Figure 4-9: Change in conformation of human transferrin upon iron binding
From Baker *et al.* (2003). © 2003 National Academy of Sciences, U.S.A., by permission.

Cooperativity has been observed with other iron chelators, for example, amonabactin demonstrated cooperativity at the N-terminal site and little cooperativity at the C-terminal (Stintzi and Raymond, 2000). Conversely, pyrophosphate displayed little

difference in the rate of removal of the first and second irons from each site of Tf (Stintzi and Raymond, 2000) (Table 4-3).

In human plasma, transferrin is only about 30% saturated with iron, with most of the iron occupying the more weakly binding N-lobe (Leibman and Aisen, 1979). For this reason, it is interesting to examine k_{2N}, since it is the rate of iron removal from Fe_N-Tf, the most abundant transferrin species in vivo. The rates of k_{2N} for TAF and ferricrocin were very similar, approximately 0.3 $M^{-1}min^{-1}$ which is slower than the rate of iron removal from Tf-Fe_C for both siderophores The rates of iron removal from Fe_N-Tf were slow compared to Ppi or citrate, but significantly faster than amonabactin. However, low concentrations of Fe_N-Tf-Fe_C and Tf-Fe_C are also present in the blood, so the other rates for iron removal may also be relevant in vivo.

Some caution must be used in extrapolating in vitro rates of iron uptake to in vivo rates. For example, serum has been shown to reduce the rate of iron transfer from transferrin to siderophores (Konopka and Neilands, 1984). *E. coli* produces two siderophores, enterochelin and aerobactin. Enterochelin, a catechol, has a far higher affinity for iron than aerobactin (pM of 35.6 vs 23.3); however, serum inhibited the rate of transfer of transferrin-bound iron to enterobactin whereas it had no effect on the same transfer reactions to aerobactin (Konopka and Neilands, 1984). Since it is the production of aerobactin, rather than enterobactin, which is associated with invasiveness (Warner *et al.*, 1981), aerobactin must possess structural features that enhance its ability to remove transferrin-bound iron beyond its thermodynamic iron binding affinity.

Another example is the siderophore desferrioxamine B, which removes transferrin-bound iron only slowly in vitro. Nevertheless, desferroxamine still promotes

growth of fungi, particularly *Rhizopus* species, in serum (Boelaert *et al.*, 1993). These examples demonstrate that many factors can influence the iron exchange kinetics in vivo and caution must be used in relating kinetic rates in vitro to the importance of siderophores in virulence.

In conclusion, we have provided evidence that the *A. fumigatus* siderophores, TAF and ferricrocin, can both remove iron from Fe_N-Tf-Fe_C at rates similar to the siderophores of bacterial pathogens. With both siderophores, C-terminal iron is removed preferentially from transferrin. In addition, both siderophores were able to remove iron from Fe_N-Tf, the predominant form of Tf in human plasma.

CHAPTER FIVE: SIDEROPHORES ARE REQUIRED FOR VIRULENCE OF *ASPERGILLUS FUMIGATUS*: CHARACTERIZATION OF *SIDA*, A GENE ENCODING L-ORNITHINE N^5-OXYGENASE

Summary

We have identified and deleted the *sidA* gene of *Aspergillus fumigatus*, which encodes L-ornithine N^5-oxygenase, the first committed step in hydroxamate siderophore biosynthesis. *AfusidA* codes for a protein of 501 amino acids with significant homology to L-ornithine N^5-oxygenases produced by *A. nidulans* and *A. oryzae*. A stable Δ*sidA* strain was created by deletion of *AfusidA*. This strain was unable to synthesize any siderophores, including N'N''N'''-triacetylfusarinine C (TAF) and ferricrocin. Growth of Δ*sidA* was the same as wild type in rich media; however, Δ*sidA* was unable to grow in low-iron defined media or media containing 10% human serum unless supplemented with TAF or ferricrocin. No significant differences in ferric reductase activities were observed in the parental strain and Δ*sidA*, indicating that blocking siderophore secretion did not result in upregulation of this pathway. Unlike the parental strain, Δ*sidA* was unable to remove iron from human transferrin. The Δ*sidA* strain was avirulent in a mouse model of invasive aspergillosis, indicating that siderophore production is critical for *A. fumigatus* iron uptake in vivo. Siderophore biosynthesis represents one of the few pathways identified in *A. fumigatus* that is absolutely required for virulence.

Introduction

Siderophore biosynthesis

The synthesis of both N'N''N'''-triacetylfusarinine C (TAF) and ferricrocin starts with ornithine, an amino acid that does not occur in proteins but is important in the formation of urea and other polyamines. Ornithine is similar in structure to lysine, and contains an amino group at the terminus of a three-carbon side chain. The first step in hydroxamate siderophore biosynthesis is catalysed by L-ornithine N^5-oxygenases, and involves N-hydroxylation at the terminal amino group of ornithine (Figure 5-1) to form an unusual N-O bond. The N-O functional group is later acylated to form the iron-chelating hydroxamate functional group, an essential feature of all hydroxamate siderophores (shown in Figure 1-3).

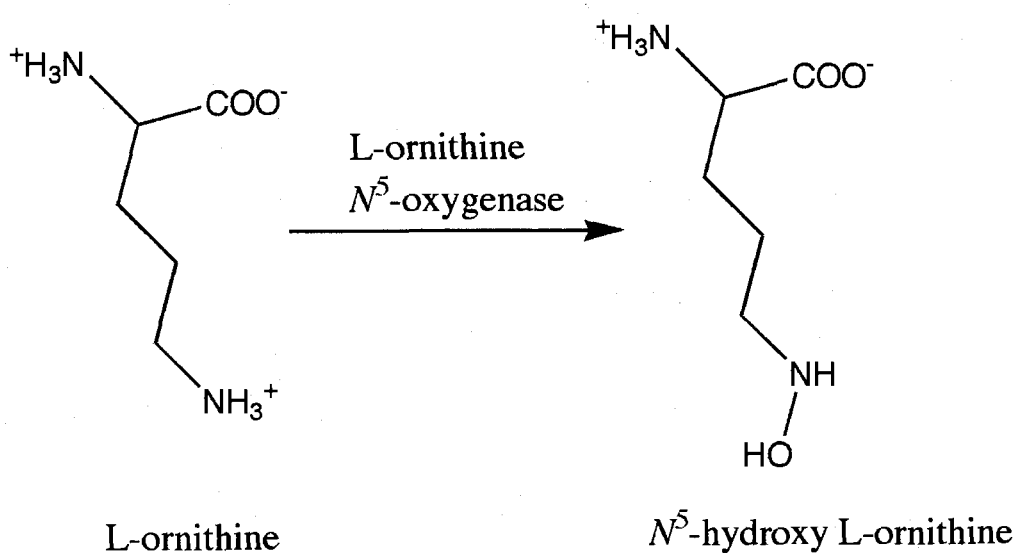


Figure 5-1: Reaction catalysed by L-ornithine N^5-oxygenase

Some of the genes involved in synthesis of hydroxamate siderophores have been characterized. These include *sidA* in *Aspergillus nidulans* (Eisendle *et al.*, 2003), *dffl* in *Aspergillus oryzae* (Yamada *et al.*, 2003), *pvdA* in *Burkholderia cepacia* (Sokol *et al.*, 1999) and *Pseudomonas aeruginosa* (Visca *et al.*, 1994), and *sidl* in *Ustilago maydis* (Mei *et al.*, 1993) all of which encode L-ornithine N^5-oxygenases (Figure 5-2). Genes coding for L-lysine N^6-oxygenases, such as *iucD*, and which are also involved in siderophore biosynthesis, have been characterized in bacteria such as *E. coli* (de Lorenzo *et al.*, 1986). Several non-ribosomal peptide synthetases (Figure 5-2) have also been characterized, including *sidC* which catalyses the final step in ferricrocin synthesis in *A. nidulans* (Eisendle *et al.*, 2003) and *sid2* which completes the synthesis of ferrichrome in *U. maydis* (Yuan *et al.*, 2001).

L-ornithine N^5-oxygenases all share common features. They are FAD-dependent enzymes that catalyse N-hydroxylation of ornithine at the expense of NADPH (Stehr *et al.*, 1998). The binding sites for NADPH, FAD and substrate have been identified. FAD-dependent enzymes generally contain the typical fingerprint sequence, GXGXXG for FAD binding. Hydroxylases involved in siderophore biosynthesis, such as *iucD*, *pvdA* and *sidl*, typically exhibit an exchange of the last glycine to proline (Stehr *et al.*, 1998). In ornithine hydroxylases, the final glycine of the NADPH binding site, GXGXXG, is also typically not conserved. The C-terminal region of the proteins contains a substrate binding site which starts with a highly conserved aspartate, followed by eight hydrophobic amino acids, the core region of which consists of the sequence L/FATGY. This binding site typically ends with a proline after four more variable amino acids, however with the siderophore biosynthesis enzymes, *pvdA* and *sidl*, the last

proline is not conserved (Stehr *et al.*, 1998). Siderophore biosynthesis pathways are absent in human cells, therefore these pathways represent potential new targets for antimicrobial chemotherapy.

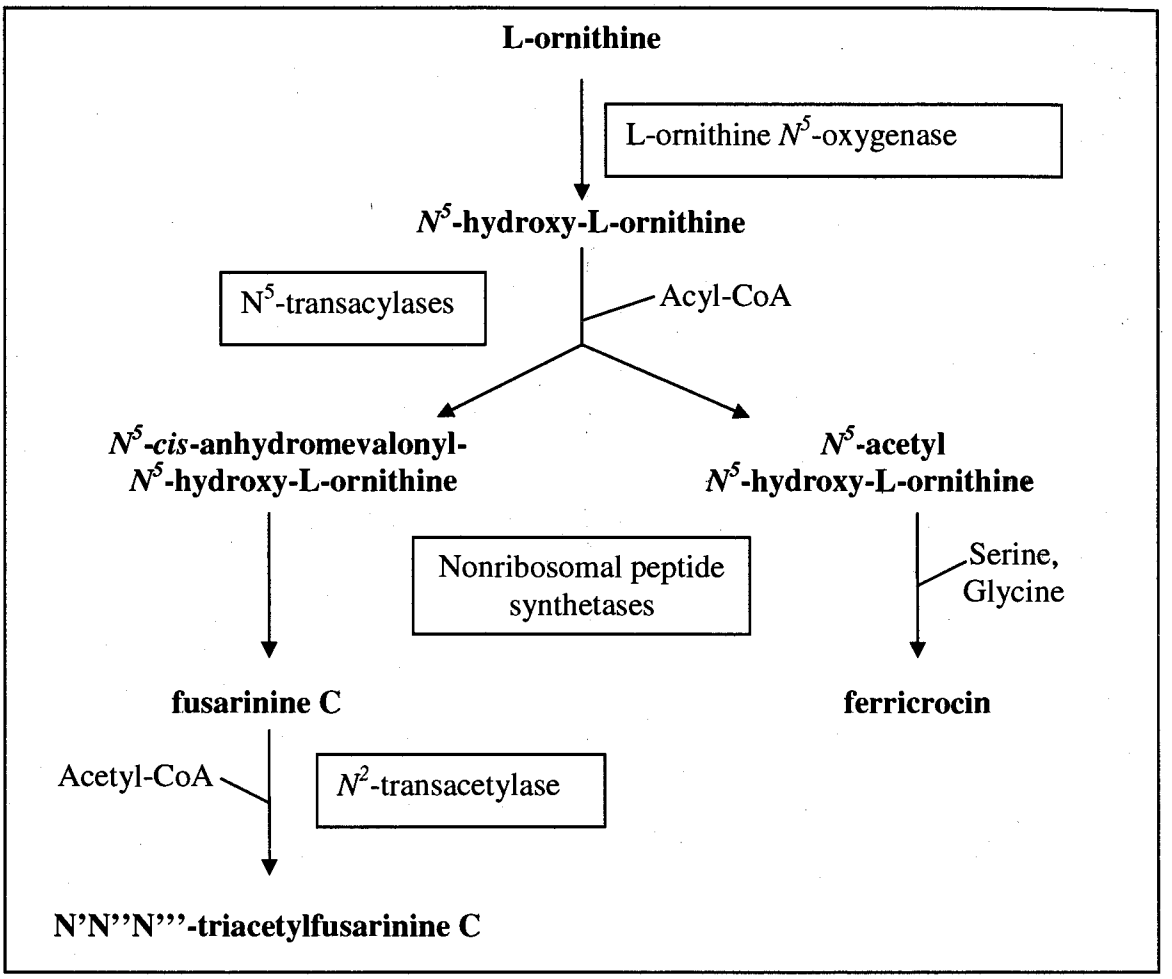


Figure 5-2: Biosynthetic pathway of hydroxamate siderophores

The biosynthesis of N'N''N'''-triacetylfusarinine C (TAF) and ferricrocin, according to Plattner and Diekmann (1994). The chemical structures of TAF and ferricrocin are shown in Figure 1-3.

Ferric reductases

Ferric reductases are important cellular enzymes. Assimilatory ferric reductase enzymes are found in almost all living creatures (Schroder *et al.*, 2003), and ferric reduction is involved in almost all iron uptake by fungi, whether it occurs extracellularly or intracellularly. These enzymes can reduce free ferric iron or ferric iron complexed to chelators. The product of the reduction is ferrous iron, which is much more weakly complexed and therefore easily released from chelators such as transferrin, heme and siderophores.

Ferric reductases have been well characterized in yeasts, where they are the primary iron uptake mechanism. In yeasts, iron uptake involves reduction of the complexed ferric iron by a plasma membrane-bound ferric reductase (Fre), followed by transport of the soluble free ferrous iron into the cell by the high affinity Fet3/Ftr1 ferroxidase/permease complex (Askwith and Kaplan, 1997). Fet3 is a multicopper oxidase which reoxidizes ferrous to ferric iron before transport by Ftr1. This strategy of reduction followed by reoxidation may be necessary because iron must first be reduced in order to free it from chelators such as siderophores. Reoxidation may occur in order to increase substrate specificity, as Ftr1 is highly specific for free ferric iron (Stearman *et al.*, 1996). Conversely, ferrous iron can also be transported by the low affinity iron transporter Fet4 (Dix *et al.*, 1997), which transports ferrous iron, in addition to numerous other divalent cations (Hassett *et al.*, 2000).

In contrast to yeast, filamentous fungi are reported to generally take up iron by transport of the ferrated siderophore into the cell (Schroder *et al.*, 2003). Ferric reductases are necessary, however, to reduce the bound iron and free it from the

siderophore. A gene, *freA*, with homology to *Saccharomyces cerevisiae* metalloreductases such as Fre2, has been characterized in *A. nidulans* (Oberegger *et al.*, 2002). Fre2 can reduce ferric citrate as well as iron bound to numerous siderophores, including desferrioxamine B, ferrichrome, TAF, and rhodotorulic acid (Schroder *et al.*, 2003). Therefore, in addition to siderophore production, there is evidence for the presence of cell-surface ferric reductases which could play a role in iron uptake by filamentous fungi.

Mouse models of invasive aspergillosis

Invasive aspergillosis can be established in many animals, including mice, rabbits, rats, guinea pigs, chicken, cows, turkeys, ducks and monkeys (Latgé, 1999). Immunosuppression treatments greatly increase the susceptibility of animals to *A. fumigatus* infection, and much like the human infection, the pattern of infection in model animals is highly dependant on the immunosuppressive routine. Immunosuppression with cortisone and/or cyclophosphamide is preferred in mice and rabbits because of its ease of use (Spreadbury *et al.*, 1989). Cortisone acetate is an ideal treatment because it generates high levels of cortisol in the bloodstream that persist for 48 hours, resulting in lengthy immunosuppression (Fauci, 1975).

Intranasal inoculation best mimics the natural route of infection, though invasive aspergillosis can also be established by intravenous (IV) injection of conidia. IV injection has the advantage of giving more reproducible response, thereby allowing the use of smaller numbers of animals; however, the affected organs (primarily kidneys and brain) are quite different from those affected in human infection (Jensen *et al.*, 1996).

Intranasal inoculation results in a highly variable response of the animals to the inoculum, but generates an infection that is much more similar to that observed in human patients.

The main drawback of animal models of invasive aspergillosis is the high dose of conidia required to reproducibly establish infection. The human disease generally results from repeated exposure to low numbers of conidia, whereas laboratory animals are given a very high dose at one time point (Latgé, 1999). The infectious dose can be reduced by increasing the severity of the immunosuppression (Smith *et al.*, 1994), but increased immunosuppression has the drawback of increasing mortality due to secondary infections or drug toxicity. A moderately high dose of immunosuppressive drugs, combined with intranasal inoculation with a reasonably high number of conidia (Dixon *et al.*, 1989) appears to offer the best available model of invasive aspergillosis.

Objectives

To investigate the importance of siderophores in the virulence of *Aspergillus fumigatus*, we have deleted *sidA*, the gene coding for L-ornithine N^5-oxygenase in *A. fumigatus*. This mutant strain should be unable to produce any hydroxamate siderophores, including TAF and ferricrocin. The ability of the Δ*sidA* strain to secrete siderophores, to grow in iron-limited media, and to remove transferrin-bound iron was examined. Because ferric reductase could present a possible alternate iron uptake pathway, ferric reductase activity of both wild type and Δ*sidA A. fumigatus* strains was measured. Finally, the virulence of Δ*sidA* and the parental strain was compared in a mouse model of invasive aspergillosis.

Materials and Methods

Strains and growth conditions

A. fumigatus (ATCC 13073), originally isolated from a human pulmonary lesion, was obtained from the American Type Culture Collection and maintained on YM slants (0.3% malt extract, 0.3% yeast extract, 0.5% peptone, 0.5% glucose) at 4° C. *A. fumigatus* 13073 (*hyg*^{S}, *sidA*$^+$) is designated in the text as wildtype or the parental strain, while 19B4 (*hyg*^{R}, *sidA*$^-$) is designated Δ*sidA*. The construction of 19B4 is described below. 13073 and 19B4 are isogenic except for the disruption of the *sidA* gene. *A. fumigatus* strains were regularly cultured on YM plates or YM plates containing 200 μg/ml hygromycin B (Roche) at 28°C for 6 days until fully conidiated. Conidia were harvested as described in Chapter 2.

Growth rate studies

A. fumigatus was inoculated into 5 ml volumes of YM medium or 5 ml of modified Grimm-Allen (GA) medium (1 g/L $KHSO_4$, 3 g/L K_2HPO_4, 3 g/L $(NH_4)_2SO_4$, 20 g/L sucrose, 1 g/L citric acid, 2 mg/L thiamine, 20 μg/L $CuSO_4$, 1 mg/L $MnSO_4$, 5.5 mg/L $ZnSO_4$, 810 mg/L $MgSO_4$, pH 6.9) (Payne, 1994) in 25 ml acid-washed flasks at a concentration of 10^6 conidia/ml. GA medium was supplemented with 5 μM $FeCl_3$ where described. Human serum (male) was obtained from Sigma, stored frozen until use and added to media at a concentration of 10% (v/v). Desferri-TAF and desferri-ferricrocin were purified from *A. fumigatus* cultures as described in Chapter 3 and treated with 8-hydroxyquinoline to remove all bound iron. Siderophore solutions were filter-sterilized prior to addition to media.

Dry weights of *A. fumigatus* cultures were measured as described in Chapter 2.

Construction of *sidA* deletion vector

Preliminary sequence data for *A. fumigatus* was obtained from The Wellcome Trust Sanger Institute (www.sanger.ac.uk). *sid1* from *U. maydis* (Mei *et al.*, 1993) was used as a probe to search the *A. fumigatus* genome for homologous genes.

Standard molecular techniques were carried out as described by Sambrook *et al.* (1989). Plasmids were propagated in *Escherichia coli* DH5α (Life Technologies). Genomic DNA was extracted from *A. fumigatus* by standard phenol chloroform extraction (Sambrook *et al.*, 1989) as described by May *et al.* (1985). pID620, composed of pBluescript SK+ (Stratagene) containing the *hph* hygromycin resistance cassette in the EcoRI site (Brown *et al.*, 1998), was kindly provided by D.W. Holden, Department of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School, Imperial College of London, UK. Custom primers were ordered through Invitrogen.

A 1.6 kb DNA fragment containing *sidA* was PCR amplified from *A. fumigatus* genomic DNA using primers 5'-AAGCTTAAGCTTTTGAACGGAAGTCAGAATCG and 5'-TCTAGATCTAGAACAGGTTCCCTCATGTCTGC which flank the *sidA* gene and contain restriction sites for *Hind*III and *Xba*I, respectively (underlined). This PCR product was digested with *Hind*III and *Xba*I, then ligated into *Hind*III- and *Xba*I-digested pID620, generating pGAW1. pGAW1 was then digested with *Sma*I and *Pst*I, excising bases 576-1078 of the *sidA* coding region. The hygromycin resistance cassette (*hph*) was PCR-amplified from pID620 using primers 5'-AACGTTAACGTTGTAAAACGACGGCCAGTG and 5'-GGAAACAGCTATGACCATG. This PCR product was digested with *Pst*I and ligated to the digested pGAW1, creating the *sidA* gene replacement vector, pGAW2. This vector

should undergo homologous recombination, resulting in deletion of the wild type *sidA* gene. The plasmids pGAW1 and pGAW2 are shown in Figure 5-4. The correct disruption of *sidA* in the transformation vector pGAW2 was confirmed by sequencing the gene deletion construct (University Core DNA and Protein Services, University of Calgary, Alberta, Canada).

Disruption of *sidA* in *A. fumigatus*

A. fumigatus was transformed by electroporation, according to the method of Weidner *et al.* (1998). Transformation reactions were plated on *Aspergillus* minimal medium (MM) containing 10g/L glucose, 0.85 g/L $NaNO_3$, 0.52 g/L KCl, 0.52 g/L $MgSO_4 \cdot 7H_2O$, 1.52 g/L KH_2PO_4, 40 µg/L $Na_2B_4O_7 \cdot 10H_2O$, 0.4 mg/L $CuSO_4 \cdot 5H_2O$, 1 mg/L $FePO_4 \cdot 4H_2O$, 0.6 mg/L $MnSO_4 \cdot H_2O$, 0.8 mg/L $Na_2MoO_4 \cdot 2H_2O$, 8 mg/L $ZnSO_4 \cdot 7H_2O$, 1 mg/L nicotinic acid, 2.5 mg/L riboflavin, 2 mg/L pantothenic acid, 0.5 mg/L pyridoxine, 10 µg/L biotin, 0.2 mg/L PABA, and 10 µM TAF, pH 6.5. The plates were incubated at room temperature overnight, then overlayed with 10 ml of MM containing 267 µg/ml hygromycin B (Roche) and 1.5% agar. Plates were then incubated at 37°C for 48 hours until colonies had conidiated. Conidia from putative transformants were screened in 2 ml modified GA medium supplemented with 10 µM TAF, incubated at 37°C for 3 days. This TAF concentration was sufficient to promote growth of siderophore secretion mutants without interfering with colourimetric detection of siderophores. Cultures were selected which did not produce any orange colour upon addition of 200 µl of 10mg/ml $FeSO_4$. Ten of 140 hygromycin-resistant colonies showed lack of siderophore secretion by this test. These transformants were further screened by

PCR to ensure deletion of *sidA* gene. One transformant, 19B4, was selected for further study.

The reversion rate of the Δ*sidA* strain was estimated by plating wild-type and Δ*sidA* strains on GA plates containing 100 μM of the iron chelator 2,2'-dipyridyl. This medium supported the growth of wild type, but completely inhibited growth of Δ*sidA* strains at concentrations up to 10^7 conidia per plate.

Gene deletion was confirmed by PCR at the *sidA* site (Figures 5-5 and 5-6) using the primers 5'-TTGAACGGAAGTCAGAATCG (oof) and 5'-ACAGGTTCCCTCATGTCTGC (oor) which flank the *sidA* gene. Gene deletion was also confirmed by PCR using primers complementary to *hph* (Figures 5-5 and 5-7): 5'-GACATATCCACGCCCTCCTA (hph1) and 5'-ACTGTCGGGCGTACACAAAT (hph2) and to a region just external to the *sidA* gene: 5'-ACGCCCTCAACTGTATGGAC (be-oor, 1.8kb upstream of *sidA* start codon) and 5'-TTTCGTGCAAAACAGTGGAG (af-oof, 1.6 kb downstream of *sidA* stop codon). PCR was carried out using 1 μg genomic DNA from either wild type or the putative Δ*sidA* strain, incubated under the following conditions: predwell at 95°C for 2 minutes, then 30 cycles of 94°C for 30 seconds, 55°C for 90 seconds, and 72°C for 30 seconds. Postdwell was for 10 minutes at 72°C.

Southern analysis was carried out on genomic DNA extracted from wild type and the putative Δ*sidA* strain. Genomic DNA was completely digested with *Eco*RV, *Pst*I and *Hind*III, fragments were separated by electrophoresis on 0.7% agarose and transferred to Hybond N+ (Amersham) using standard techniques (Sambrook *et al.*, 1989). Probes to

the *sidA* gene were constructed using the AlkPhos direct labeling kit with the CDP-star detection reagent (Amersham).

Detection of siderophores

Siderophores were purified from *A. fumigatus* wild type and Δ*sidA* cultures. Conidia (10^6/ml) were inoculated into 5 ml volumes of YM and incubated at 37°C for 3 days. Cultures were filtered to remove mycelia, and 100 mg/ml $FeCl_3$ was added to the supernatants. Ferrated siderophores were then extracted from the aqueous supernatants with 3 x 1 ml 1:1 phenol:chloroform. Combined phenol:chloroform fractions were washed with 2 ml dH_2O, and then diluted with 10 ml diethyl ether. The siderophores were extracted from the diethyl ether fraction with 2 x 0.5 ml dH_2O. Combined aqueous layers were washed with 0.5 ml diethyl ether, and then lyophilized to dryness. Extracts were redissolved in 30 µl ddH_2O and analysed on silica gel thin layer chromatography sheets using a running phase of 4:1 dichloromethane:methanol. R_f values were compared to those previously measured for TAF and ferricrocin in Chapter 3.

Ferric reductase activity

Ferric reductase assays were performed as described by Morrissey *et al.* (1996). *A. fumigatus* wild type and Δ*sidA* strains were inoculated in GA medium (10^6 conidia/ml) containing 5 µM $FeCl_3$ and incubated at 37°C and 150 rpm for 24 hours. Mycelia were filtered through Miracloth (Calbiochem), washed with distilled water, and transferred to microcentrifuge tubes. Mycelia were incubated with 0.5 ml of 0.2 mM $FeCl_3$ in PBS plus 0.5 ml 2 mM bathophenanthrolinedisulfonic acid (BPDS, Sigma-Aldrich) in PBS at 37°C and 150 rpm for 1 hour, at which time the absorbance of the supernatant was measured at 540 nm. To normalize the absorbance data to biomass, mycelia were then washed,

lyophilized and dry weights obtained. *Saccharomyces cerevisiae* is known to express ferric reductase activity (Askwith *et al.*, 1996), and was used as a positive control in the ferric reductase assay. *S. cerevisiae* CBY676 pRS426 was kindly provided by C. Beh, Department of Molecular Biology and Biochemistry, Simon Fraser University. *S. cerevisiae* was cultured at 28°C in YM or in GA supplemented with Minimal Essential Medium (MEM) amino acids solution (Invitrogen).

Iron removal from diferric-transferrin

A. fumigatus (10^6 conidia/ml) was cultured in 1 ml modified GA medium supplemented with 50 μM $FeCl_3$ at 37°C for 24 hours. Mycelia were washed 3 times with PBS and resuspended in 1 ml MEM containing no phenol red (pH 7.4, Life Technologies) supplemented with 0.2 mg/ml human diferric-transferrin (Sigma). Cultures were incubated at 37°C and 150 rpm, and 100 μl samples were removed at various intervals. The samples were lyophilized to dryness and redissolved in 10 μl water + 10 μl urea-polyacrylamide gel electrophoresis (urea-PAGE) loading buffer (1X TBE, 10% glycerol and 0.2% bromphenol blue). Urea-PAGE was used to determine the proportions of apo-, diferric-, and monoferric-transferrin in each sample. Urea-PAGE was carried out as described in Chapter 3.

Mouse aspergillosis model

Female BALB/c mice weighing from 18-22g were obtained from Charles River Breeders and given 0.5 mg/ml tetracycline in their drinking water throughout the course of the study. Mice were immunosuppressed by subcutaneous injections of 200 mg/kg cortisone acetate (Wiler-PCCA, London, Ontario, Canada) on days -3, 0, 2 and 4. Cortisone acetate was prepared as a 30 mg/ml suspension in sterile saline (Baxter

Medical). For these studies, *A. fumigatus* was cultured on YM agar at 37°C for 4 days to ensure conidia were fully mature and pigmented. Conidia were harvested as described and suspended in sterile saline. Mice were randomly assigned to one of three treatment groups: parental strain (n=10), Δ*sidA* (n=11) and saline (n=5). On day 0, mice were anaesthetised with isoflurane and $5x10^6$ conidia of either wild type or Δ*sidA* were instilled intranasally in a 20 μl volume using a micropipette and a gel loading tip. Each inoculum was plated on YM agar to determine viabilities of conidia harvested from both strains. Mice were kept anaesthetized until all the liquid was observed to be inhaled. Mice were monitored daily for 14 days to observe any clinical symptoms, and were deemed to have reached endpoint if they displayed ruffled fur and one of the following: either (a) laboured breathing, hunching and decreased movement, or (b) disorientation and loss of balance. Mice displaying either set of clinical symptoms were euthanized.

At endpoint or the end of the 14-day study, lungs were fixed by immediately opening the chest of the euthanized animal, isolating the trachea, and perfusing 10% formalin in PBS into the lung cavities. After two minutes, lungs were removed and further fixed at room temperature overnight in PBS containing 10% formalin. Lungs were subsequently paraffin-embedded, sectioned and stained with hematoxylin and eosin. Images were obtained on a Zeiss LSM10 confocal microscope equipped with a QImaging 10-bit camera.

To determine if viable fungi were present, lungs were aseptically removed from mice at endpoint or mice surviving to the end of the 14 day study. Lungs were homogenized in sterile PBS using several 5 second bursts of a polytron with cooling on

ice between bursts. Lung homogenate was plated on YM agar containing 0.1 mg/ml streptomycin and 25 units/ml penicillin G and incubated at 37°C overnight.

Statistics

The Student t-test was used for statistical analysis of data. Survival data was analysed by log rank analysis using Prism 4.0 software (GraphPad).

Results

Characterization of *sidA*

It was shown in Chapter 3 that siderophores play an important role in the growth of *A. fumigatus* in serum-containing media, and that purified *A. fumigatus* siderophores can remove iron from human diferric transferrin in vitro. To establish the role of siderophores in growth of *A. fumigatus* we created a siderophore secretion mutant by deleting *sidA*, the gene coding for L-ornithine N^5-oxygenase.

A BLAST search of the *A. fumigatus* genome revealed a sequence with high homology to published sequences for L-ornithine N^5-oxygenases (Figure 5-3). The *A. fumigatus* gene was termed *AfusidA*, according to the nomenclature recommended by the *Aspergillus* genome sequencing group (Anderson, 2004). The gene was composed of an ORF of 1564 base pairs. Using GlimmerM from the Institute for Genomic Research (TIGR) (http://www.tigr.org/software/glimmerm/), trained for *A. fumigatus*, an intron of 58 base pairs and an amino acid sequence of 501 amino acids was predicted for the *A. fumigatus* SidA protein. These sequence data have been submitted to the GenBank database under accession number AY819708.

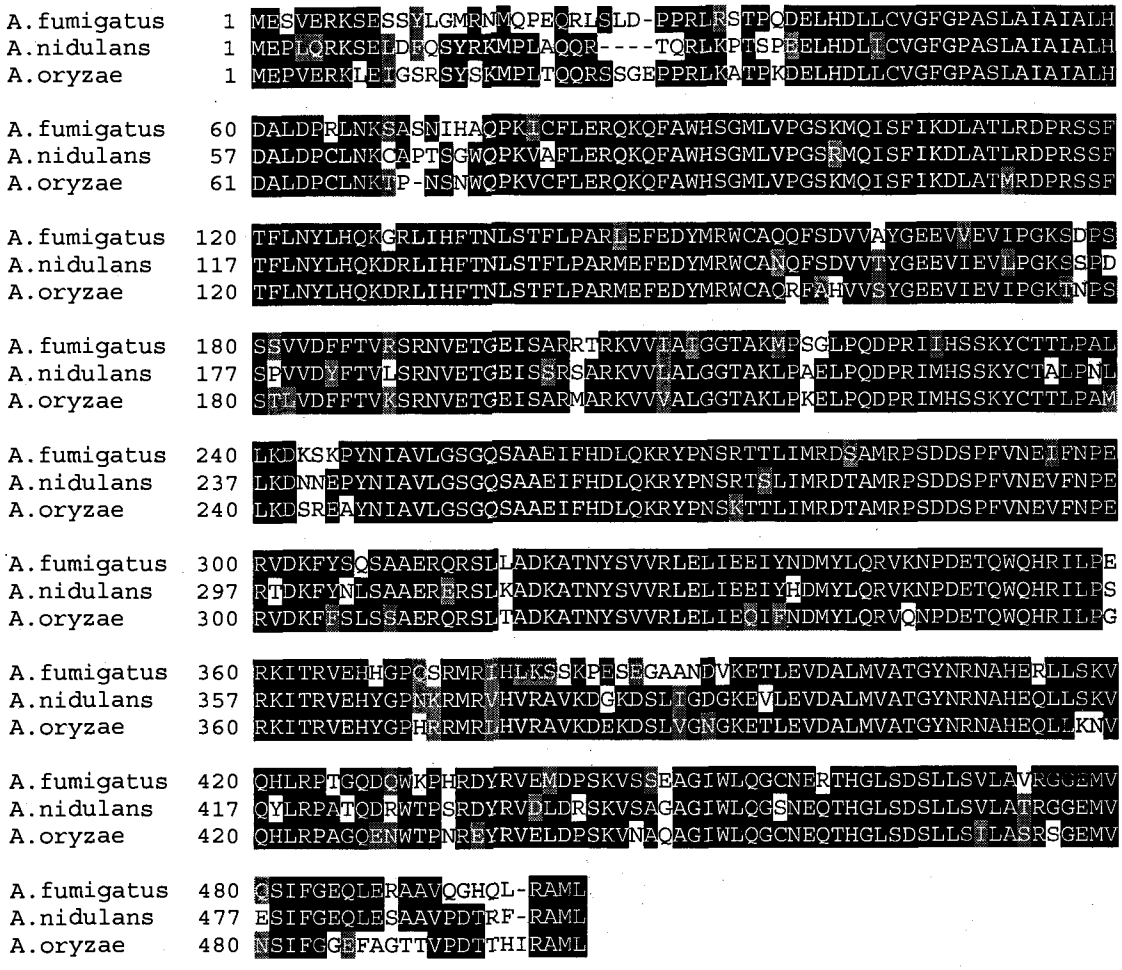


Figure 5-3: Alignment of the *A. fumigatus* SidA, *A. nidulans* SidA and *A. oryzae* Dff1 amino acid sequences

The amino acid sequence of *A. fumigatus* SidA was predicted using GlimmerM from the Institute for Genomic Research (TIGR) trained for *A. fumigatus*. Multiple pairwise alignment was performed by ClustalW (Chenna *et al.*, 2003) and the output generated by Boxshade 3.21. Black and grey boxes represent identical and similar residues, respectively.

The coding sequence of the *A. fumigatus sidA* showed very high identity to *sidA* from *A. nidulans* (75%) and *dffl* from *A. oryzae* (74%) (Figure 5-3). The *A. fumigatus* sequence contained the three signature sequences typical of amino acid hydroxylase enzymes. The first of these is the conserved putative binding sites for the substrate DXXX(L/F)ATGYXXXXP (Stehr *et al.*, 1998), located at residue 400. Typical of ornithine-binding enzymes, such as *pvdA* and *sid1*, the last P of this sequence was not conserved in *sidA* and was replaced by H. An FAD binding domain, GXGXXG, was found at residue 45, and the last glycine in this domain of *sidA* was exchanged for proline which is typical of siderophore biosynthetic enzymes (Stehr *et al.*, 1998). An NADPH binding site, GXGXXG, was found at residue 254, though again typical for siderophore biosynthetic genes, the last G in *sidA* was not conserved (Stehr *et al.*, 1998).

Deletion of *sidA*

To investigate the role of siderophores in iron uptake and virulence of *A. fumigatus*, the *sidA* gene was deleted by gene replacement. Southern analysis revealed only one copy of *sidA* in *A. fumigatus* genomic DNA (data not shown). *sidA* was PCR-amplified from genomic DNA and cloned into the multiple cloning site of pBluescript SK+ to create pGAW1. The hygromycin resistance (*hph*) cassette was inserted into the *sidA* sequence of pGAW1 to create pGAW2, a transformation vector containing a nonfunctional *sidA* gene and a selectable marker. In the pGAW2 *sidA* gene, bases 576-1078 of the *sidA* coding region were replaced by *hph* (Figure 5-4), removing required domains such as the NADPH binding site at residue 254.

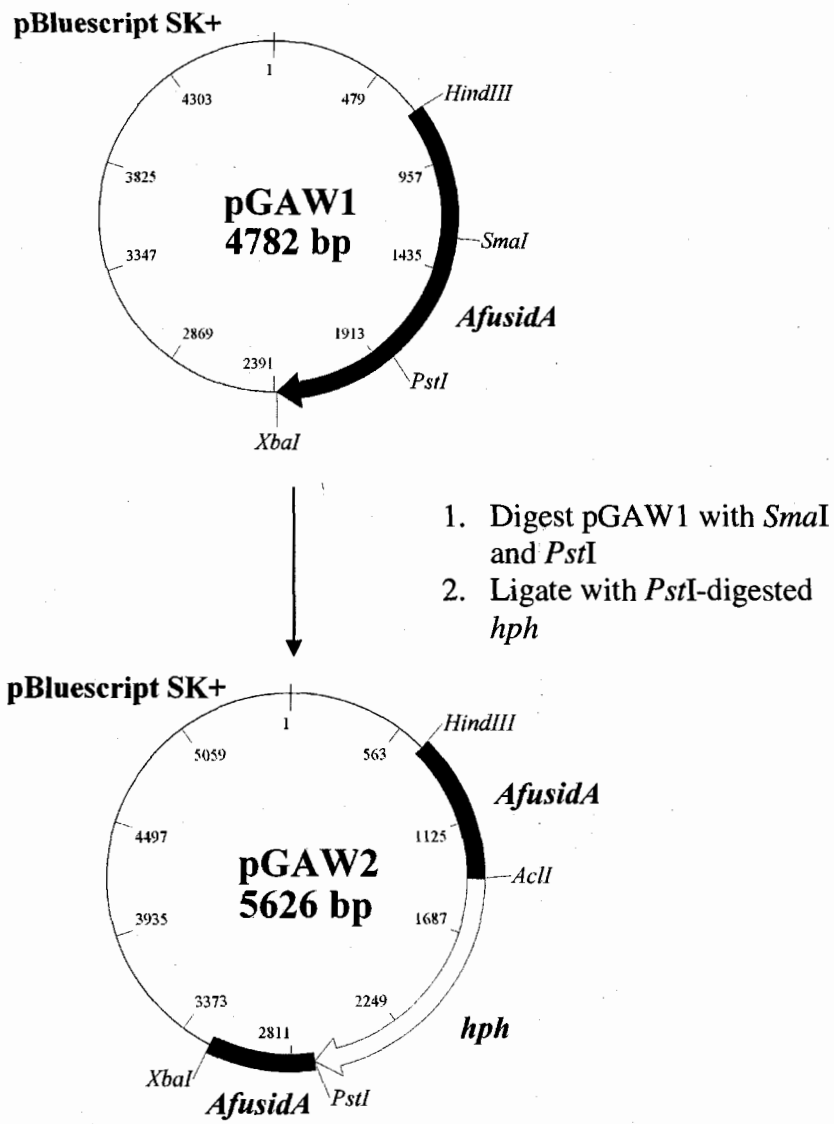


Figure 5-4: Construction of the *sidA* gene deletion vector

The 1.6 kb *sidA* gene was PCR-amplified and ligated into *Hind*III- and *Xba*I-digested pID620, generating pGAW1. pGAW1 was then digested with *Sma*I and *Pst*I, excising ~500 bases of the *sidA* coding region including the NADPH-binding domain. The *hph* hygromycin resistance cassette was PCR-amplified from pID620 and digested with *Pst*I. *hph* was ligated to the digested pGAW1, creating the *sidA* gene replacement vector, pGAW2.

A. fumigatus strain ATCC 13073 was then transformed by electroporation with pGAW2. The resulting hygromycin resistant strains were screened for absence of siderophore production in low-iron modified Grimm-Allen (GA) medium. The GA medium was supplemented with 10 μM TAF to support the growth of siderophore secretion mutants. This TAF concentration was low enough to avoid interference with the colourimetric detection of siderophores. Strains which did not produce an orange colour upon addition of $FeSO_4$ to 4-day old culture medium were examined further. The correct disruption and integration of the gene deletion construct was confirmed in one strain, 19B4, by PCR (Figure 5-5) using the *sidA* primers (Figure 5-6), as well as primers to *hph* and primers from regions upstream and downstream of the *sidA* gene (Figure 5-7). Gene deletion was also confirmed by Southern blot analysis (Table 5-1, Figure 5-8, Figure 5-9).

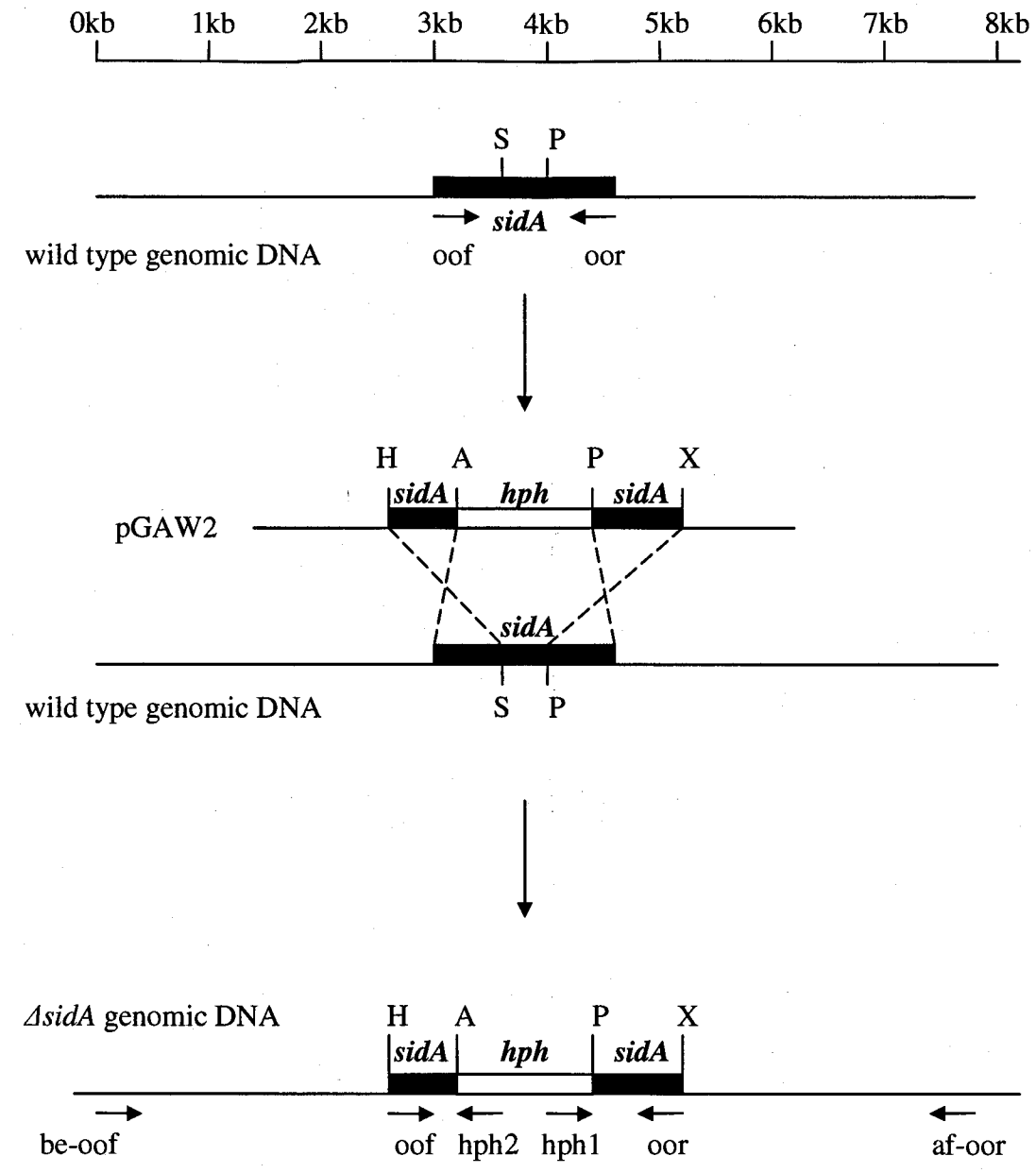


Figure 5-5: Double crossover gene deletion showing binding sites for primers used in this study

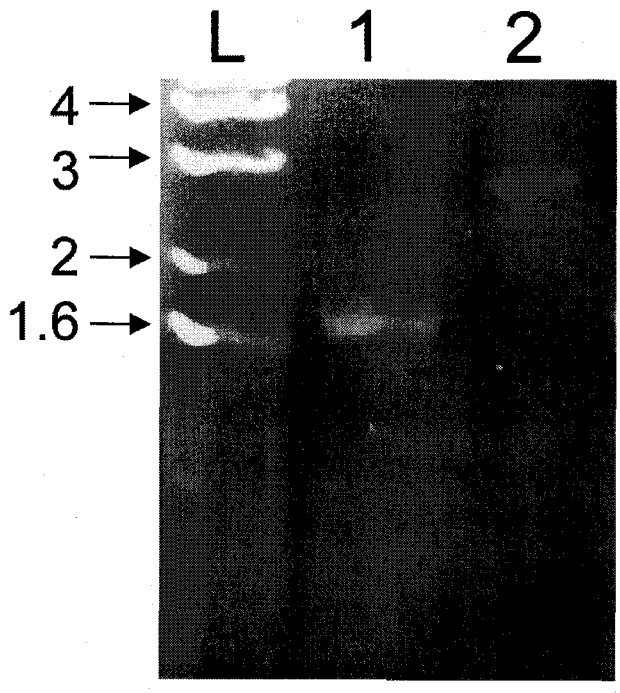


Figure 5-6: PCR evidence for deletion of *sidA*

Genomic DNA from wild type (lane 1) and Δ*sidA* (lane 2) was amplified using primers oof and oor, which flank *sidA* as shown in Figure 5-5. Wild type *sidA* gene should give a PCR product of 1.6 kb. In the gene deletion strain, Δ*sidA*, *sidA* is interrupted by *hph* and should give a PCR product of 2.8 kb. No 1.6 kb product should be present in Δ*sidA*.

L=1.6kb DNA ladder. Numbers to the left of the gel denote size of DNA ladder bands in kb.

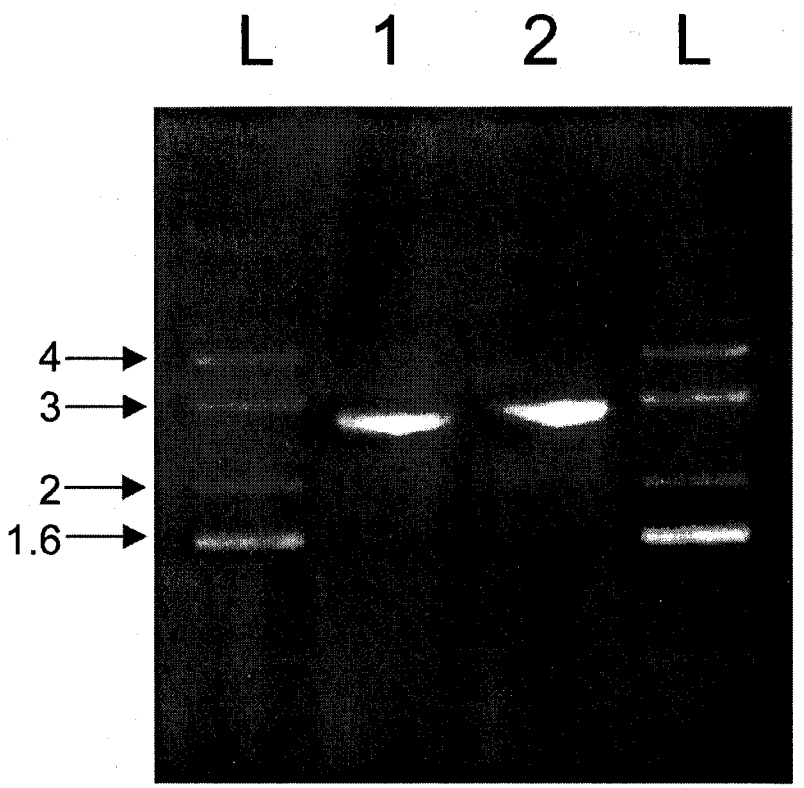


Figure 5-7: PCR evidence that pGAW2 integrated at the correct site, deleting *sidA*

Genomic DNA from Δ*sidA* was amplified using primers complementary to *hph* (hph1 and hph2) and to regions upstream and downstream of *sidA* (be-oof and af-oor) as shown in Figure 5-5. No product would be observed if the pGAW2 DNA had integrated at a location other than the *sidA* gene. A 2.7 kb product is expected for primers be-oor and hph2 (lane 1), and a 2.9 kb product is expected for primers af-oor and hph1 (lane 2) if double crossover has occurred.

L=1.6kb DNA ladder. Numbers to the left of the gel denote size of DNA ladder bands in kb.

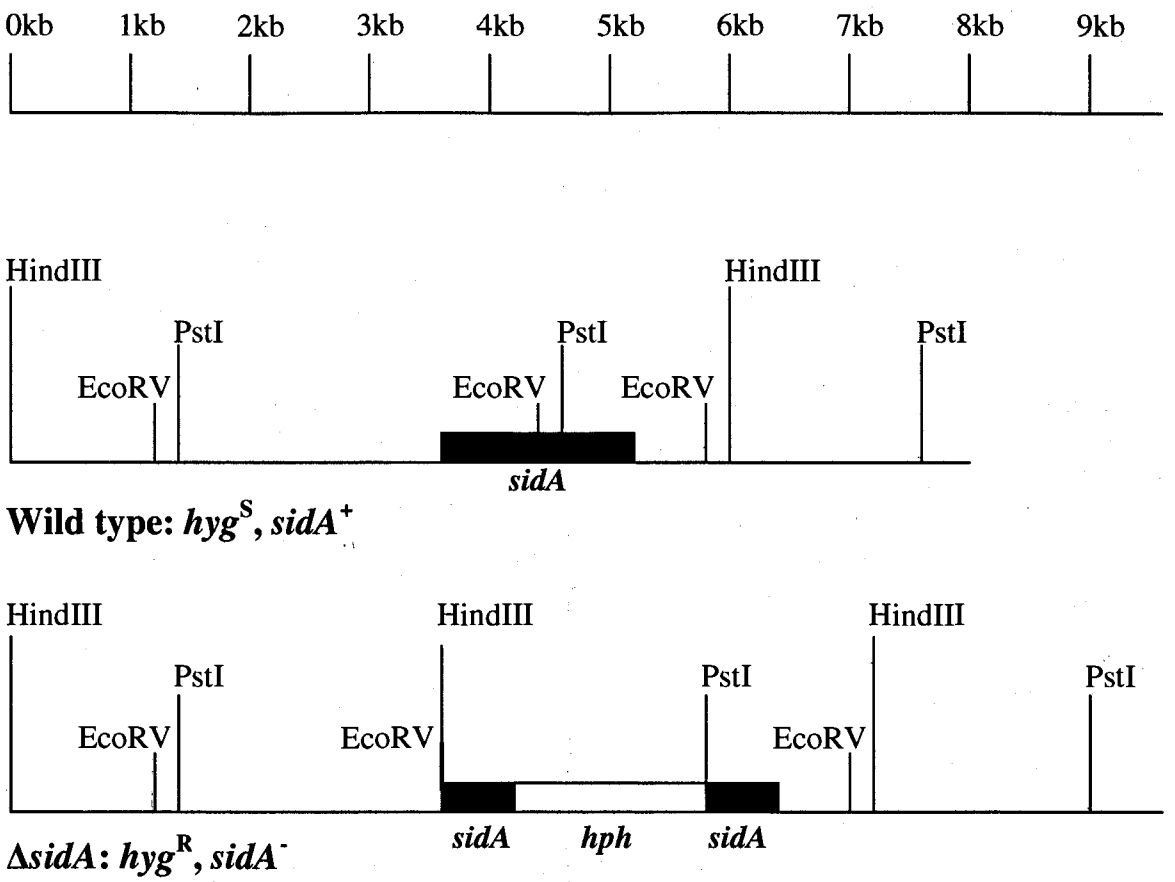


Figure 5-8: Restriction sites for *Pst*I, *Hind*III and *Eco*RV for *A. fumigatus* wild type and Δ*sidA* strains

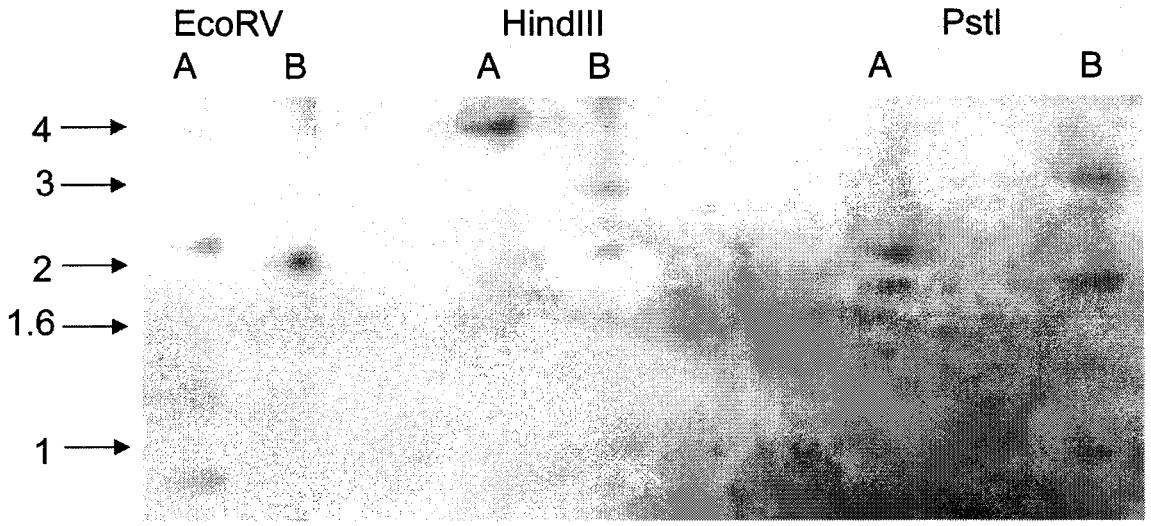


Figure 5-9: Southern blot of wild type and Δ*sidA* genomic DNA, demonstrating deletion of the *A. fumigatus sidA* gene

The Southern blot confirms the deletion of *sidA*. *A. fumigatus* genomic DNA from wild type and Δ*sidA* was completely digested with *Eco*RV, *Pst*I and *Hind*III, separated by gel electrophoresis and transferred to Hybond N+. Blots were probed using a *sidA* probe constructed using AlkPhos direct labeling kit. Detection was by the CDP-star reagent. A=wild type genomic DNA, B= Δ*sidA* genomic DNA. Numbers to the left of the blot represent the position of DNA standards in kb.

Table 5-1: Predicted and actual sizes of bands (number of bases) appearing on Southern blot

	*Eco*RV		*Hind*III		*Pst*I	
wild type predicted	2219	909	4189		2353	2038
wild type observed	2200	900	4000		2200	2000
Δ*sidA* predicted	2078	2037	2966	2217	3347	2038
Δ*sidA* observed	2000*		3000	2100	3200	2000

* bands overlap, therefore only one band was visible on Southern blot

Since we would not expect to see any revertants if the correct integration had been achieved by double crossover, reversion rates of the Δ*sidA* strain were measured. If a single crossover had occurred within the *sidA* gene, reversion could regenerate a functional gene. On Grimm-Allen plates containing 100 μM 2,2'-dipyridyl, $1.4x10^7$ Δ*sidA* conidia were screened without the appearance of a single revertant, while growth of wild type colonies was not inhibited (Figure 5-10). The lack of reversion further confirms the correct deletion of *sidA*.

***sidA* is required for growth in low iron media and serum-containing media**

The Δ*sidA* mutant and the parental strain grew at equal rates in rich media such as YG which contains yeast extract, malt and peptone (Table 5-2). The timing and extent of conidiation of the Δ*sidA* strain in complex iron-rich media were also indistinguishable from the parental strain. However, growth of Δ*sidA* was severely restricted in iron limited defined media such as GA medium. Supplementing GA with 5 μM $FeCl_3$ or 10 μM TAF restored growth of Δ*sidA* to wild-type levels (Table 5-2). In GA containing 5 μM $FeCl_3$, an increased lag period was observed for Δ*sidA*, in that germination and growth were delayed compared to the wild type for the first 12 hours of incubation.

However, after 48 hours the biomass of Δ*sidA* was identical to that of the parental strain. No delay in conidiation of Δ*sidA* in GA containing 5μM $FeCl_3$ was apparent. When GA was supplemented with 10 μM TAF, length of lag phase, rate of growth, and timing of conidiation of Δ*sidA* were identical to those of the parental strain (data not shown).

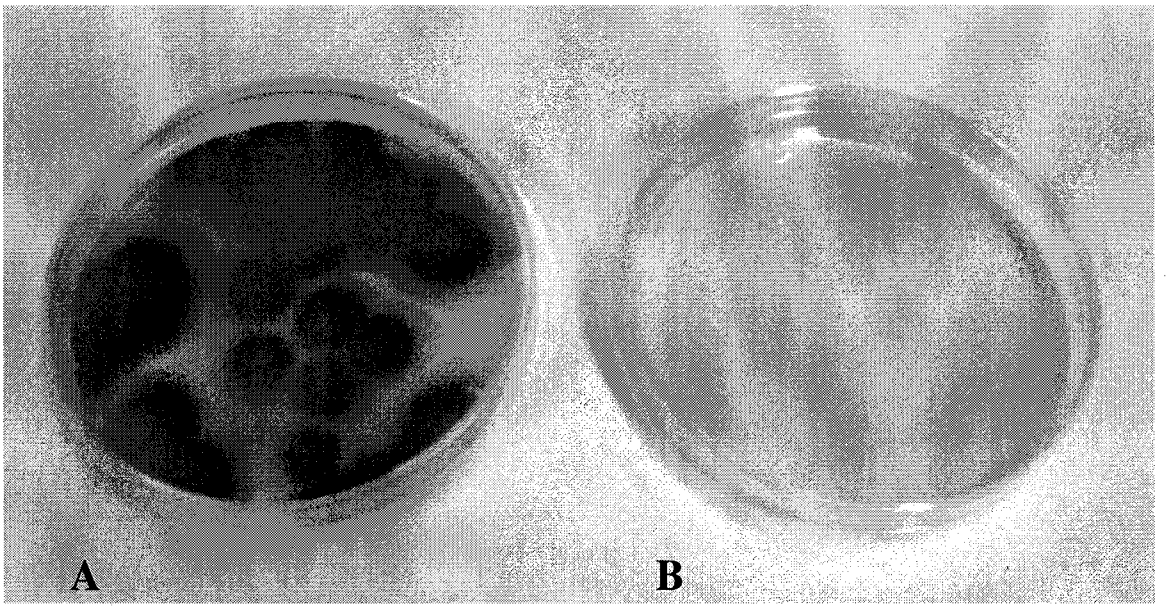


Figure 5-10: Measurement of reversion rate of Δ*sidA*

(A) Wildtype or (B) Δ*sidA* conidia (10^6/ml) were plated onto solid GA medium containing 100 μM of the iron-chelating agent 2,2'-dipyridyl.

Table 5-2: Δ*sidA* can grow in rich medium, but not iron-limited GA medium

A. fumigatus was cultured for 4 days at 37°C in 5 ml volumes of YM, GA medium, GA medium supplemented with 5 μM $FeCl_3$, or GA medium supplemented with 10 μM TAF. At the end of the incubation period, mycelia were filtered, washed with PBS, lyophilized and weighed. Data presented are averages ± standard deviations of triplicate values. This experiment was performed three times with similar results.

	dry weight (mg)	
Medium	parental	Δ*sidA*
YPD	25.7 ± 0.5	25.9 ± 0.4
GA	25 ± 2	0.1 ± 0.1 *
GA + $FeCl_3$	44.3 ± 0.1	47.4 ± 3
GA + TAF	41 ± 2	46 ± 1

* Significantly different than parental strain ($p < 0.005$)

Serum is extremely iron-limiting because it contains partially saturated transferrin, which reduces free iron concentrations to very low levels. Thus, serum is inhibitory to the growth of many microbes, including most fungi. It was demonstrated in Chapter 4 that siderophores produced by *A. fumigatus* were able to remove transferrin-bound iron. Growth of the siderophore secretion mutant, Δ*sidA*, was completely inhibited by 10% human serum (Table 5-3), but could be restored to wild-type levels by addition of 50 μM desferri-TAF or 50 μM desferri-ferricrocin. These siderophore concentrations were chosen because they are similar to the concentration of siderophore

produced by wild type *A. fumigatus* in this medium. These data indicate that siderophores are required for *A. fumigatus* growth in the presence of human serum.

Table 5-3: Growth of Δ*sidA* was inhibited by human serum and restored by addition of siderophores

A. fumigatus wild type and Δ*sidA* strains were cultured in 1 ml volumes of modified GA medium, or in GA medium supplemented with 5 μM $FeCl_3$, 10% serum, 50 μM desferri-TAF or 50 μM desferri-ferricrocin. Cultures were incubated for 4 days at 37°C and 150 rpm. At the end of the incubation period, mycelia were filtered, washed, lyophilized and weighed. Data presented are averages ± standard deviations of triplicate measurements from three independent experiments.

	dry weight (mg)	
Medium	parental	Δ*sidA*
GA	4.3 ± 1.1	1.2 ± 0.4 *
GA + $FeCl_3$	7.8 ± 0.8	7.1 ± 2.5
GA + $FeCl_3$ + serum	11.4 ± 1.2	0.3 ± 0.3 **
GA + $FeCl_3$ + serum + TAF	9.7 ± 1.0	7.2 ± 0.5
GA + $FeCl_3$ + serum + ferricrocin	11.1 ± 1.8	11.0 ± 1.2

*Significantly different than the parental strain ($p < 0.01$)
** Significantly different than the parental strain ($p < 0.001$)

sidA is required for secretion of TAF and ferricrocin

sidA catalyses the first committed step in hydroxamate siderophore biosynthesis. *A. fumigatus* is known to secrete several siderophores of the hydroxamate group, and all should be absent in the Δ*sidA* mutant. To determine whether *sidA* was required for the

production of siderophores in *A. fumigatus*, Δ*sidA* and the wild type were cultured in liquid YM medium. YM was used for this experiment because it supports identical growth rates of wild type and Δ*sidA* without inhibiting siderophore secretion by the wild type strain. Both strains were cultured at 37°C for 3 days, at which time mycelia were removed by filtration, and siderophores extracted from the culture medium as described in Materials and Methods. Solvent extracts were analyzed by thin layer chromatography using 4:1 dichloromethane:methanol as the mobile phase. TAF, ferricrocin and two unidentified siderophores were clearly visible in the extract from wild type strain; however, neither TAF, ferricrocin, nor the two additional siderophores could be observed on the TLC of the Δ*sidA* extracts (Figure 5-11). Faint yellow spots on the TLC in the Δ*sidA* lane correspond to components extracted from the medium that were also present in an uninoculated medium blank (data not shown). Thus *sidA* is required for production of TAF, ferricrocin and two other unidentified siderophores.

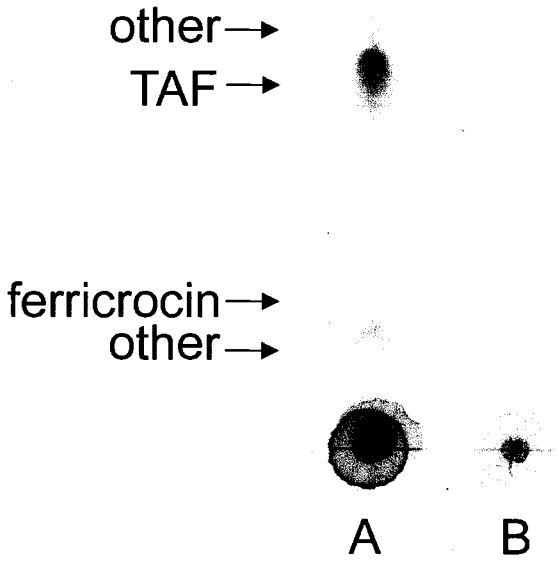


Figure 5-11: Thin layer chromatography of siderophores produced by wild type and Δ*sidA* strains of *A. fumigatus*

Siderophores were extracted from wild type and Δ*sidA* cultures grown in 5 ml volumes of modified GA medium at 37°C for 3 days as described in Materials and Methods. Extracts were analysed on silica gel thin layer chromatography sheets using a running phase of 4:1 dichloromethane:methanol. Ferricrocin, TAF, plus two unidentified siderophores (labeled 'other') are visible in the wild type extract (A), while no siderophores are visible in extract from Δ*sidA* (B). Faint yellow spots in the Δ*sidA* extracts were also present in the uninoculated control (data not shown).

A. fumigatus produces ferric reductase activity

Some pathogenic fungi do not produce siderophores and are able to grow in vivo using ferric reductase enzymes to obtain iron. The ferrous iron transport system was found to be required for virulence of *Candida albicans* (Ramanan and Wang, 2000). Extracellular ferric reductase activity has not yet been described in *A. fumigatus*. Both wild type and Δ*sidA* produced measurable ferric reductase activity when grown in GA supplemented with 5 μM $FeCl_3$ or YM medium. The yeast, *S. cerevisiae*, which is known to produce ferric reductase (Georgatsou and Alexandraki, 1994), was used as a positive control in the activity assay. Ferric reductase activity was found in both the *A. fumigatus* cells and in the culture supernatant, though the levels of cell-associated activity were much higher (Table 5-4). There was little difference observed in the levels of ferric reductase activity in GA supplemented with 5 μM $FeCl_3$ compared to YM medium (Table 5-4), nor was any significant difference observed in the levels of ferric reductase expressed by the parental or Δ*sidA* strains (Table 5-4). *S. cerevisiae* produced an absorbance at 540 nm of 0.042 ± 0.012 in the ferric reductase assay following 24 hours growth in GA supplemented with amino acids. This level of ferric reductase activity was similar to that produced by both *A. fumigatus* strains following the same incubation period in the same medium. These results indicate that for *A. fumigatus*, at least in vitro, ferric reductase activity was not upregulated in Δ*sidA* to compensate for the defect in siderophore secretion.

Table 5-4: Ferric reductase assay

A. fumigatus wild type and Δ*sidA* strains were cultured for 24 hours in 1 ml volumes of modified GA medium containing 5 μM $FeCl_3$. Mycelia were transferred to an assay buffer containing 0.5 ml 0.2 mM $FeCl_3$ in PBS and 0.5 ml 2 mM BPDS in PBS and incubated at 37°C and 150 rpm for 1 hour. The absorbances of the supernatant were read at 540 nm. Data are reported as absorbance per mg dry weight of mycelia present in the reductase assay. Data shown are average ± standard deviation from three independent experiments.

	Absorbance at 540nm / mg dry weight (x100)	
	Medium 1 (GA + 5 μM $FeCl_3$)	Medium 2 (YG)
wild type	7.6 ± 2.2	4.9 ± 0.6
Δ*sidA*	4.7 ± 1.7	4.0 ± 0.1

***A. fumigatus* requires siderophores to remove transferrin-bound iron**

The ability of the siderophore secretion mutant, Δ*sidA*, to remove iron from human diferric transferrin was assessed in vitro. Δ*sidA* and the parental strain were cultured in modified GA medium containing 10 μM $FeCl_3$ for 24 hours. Each strain produced roughly equal mycelial biomass after 24 hours, but they had not yet begun to conidiate. Mycelia were washed three times in PBS and transferred to fresh tubes containing MEM + 0.2 mg/ml human diferric transferrin. This extra incubation step was required because Δ*sidA* conidia cannot germinate and grow in MEM.

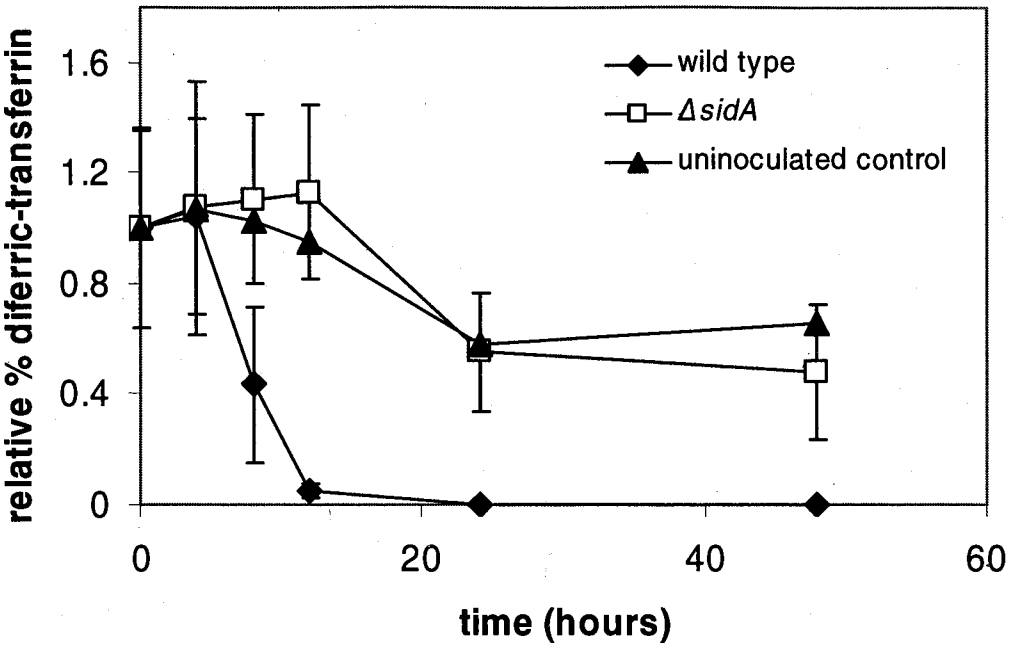


Figure 5-12: Iron saturation of human diferric-transferrin incubated with wild type or Δ*sidA* *A. fumigatus* mycelia

A. fumigatus wild type and Δ*sidA* mycelia were incubated in MEM containing 0.2 mg/ml human diferric-transferrin at 37°C and 150 rpm. Iron saturation of transferrin was assessed by urea-PAGE. Gels were stained with SYPRO orange, scanned on a Typhoon 9410 Imager and the transferrin bands quantified using ImageQuant 5.2. Data are normalized and reported as % total transferrin present in diferric form / % transferrin in diferric form at t=0. Error bars represent standard deviations of triplicate measurements.

The cultures were incubated at 37°C in MEM/diferric-transferrin and samples of supernatant were removed at various intervals. The supernatants were analysed by urea-PAGE to measure the iron saturation of transferrin. Wild type cultures consumed all of the diferric-transferrin within 12 hours (Figure 5-12), converting it to monoferric-transferrin and apotransferrin. In contrast, the levels of diferric-transferrin in the Δ*sidA* culture remained identical to those of an uninoculated control over a period of at least 48 hours (Figure 5-12). These data show that siderophores are required by *A. fumigatus* for removal of iron from human diferric transferrin.

Siderophores contribute to virulence of *A. fumigatus*.

Because Δ*sidA* was unable to grow in iron limited media, including serum, and was unable to remove iron from human diferric transferrin, we compared its virulence to that of the parental strain in a mouse model of invasive aspergillosis. Mice were immunosuppressed with cortisone acetate, then challenged intranasally with $5x10^6$ conidia of either Δ*sidA* or wild type. An aliquot of wild type and Δ*sidA* inocula were plated on YM agar, and the viabilities of conidia in both inocula were found to be identical (data not shown). One cortisone-treated animal was infected with 10^6 wild type conidia then sacrificed 24 hours post-infection. An aliquot of homogenized lung tissue was plated and 4000 colony forming units were recovered from the lungs. This indicates that 0.4% of the original inoculum was viable within the lungs after 24 hours.

Two distinct sets of clinical symptoms were observed in mice infected with the parental strain. Some developed clear signs of pulmonary infection, showing laboured breathing, hunching, decreased mobility and ruffled fur. The remaining mice displayed what appeared to be symptoms of sinusitis or central nervous system (CNS) impairment.

These mice had a characteristic head tilt, became disoriented and showed a loss of balance. They also displayed laboured breathing and eventually decreased mobility. Either one of these outcomes was classified as endpoint. Ninety percent of mice receiving the wild type strain reached endpoint by day 9 post-infection (Figure 5-13).

In contrast, Δ*sidA* was almost completely avirulent in this model of invasive pulmonary aspergillosis (Figure 5-13). All but one of the Δ*sidA*-infected mice appeared similar to the saline-inoculated mice in terms of their physical condition and behaviour. Only one of 11 mice infected with Δ*sidA* conidia reached endpoint, which was not significantly different from the mortality rate of saline-inoculated, immunocompromised mice. Endpoint was reached 10-days post-infection, which is much later than the average endpoint for wild type-infected mice. The fungal burden isolated from the endpoint Δ*sidA*-infected mouse corresponded to $2x10^3$ colony forming units within the lungs. All the fungal isolates recovered from the lungs of this mouse were morphologically identical to *A. fumigatus* and found to be hygromycin-resistant (data not shown), indicating that Δ*sidA* had survived within the lungs.

To determine the extent of fungal growth, lungs were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Lung fixation was performed when mice reached endpoint, or at 14 days post-infection for the remaining mice. Representative sections are shown in Figure 5-14. Of the four saline-treated controls whose lungs were examined, all four showed normal lung structure with open airways and no inflammation (Figure 5-14, A and B). Of the 10 mice in the group infected with wild type conidia, we examined sections from 8 of these; 7 of these mice died and one survived. Fungal hyphae were observed in sections from 6/7 symptomatic

mice that were studied. Fungal growth was accompanied by an extensive inflammatory infiltrate composed of polymorphonuclear leukocytes (PMNs) and monocytes. Extensive tissue destruction was apparent, including frank necrosis of the airway walls and large blood vessels, and complete replacement of alveolar architecture with necrosis and inflammation (Figure 5-14, C, D, and E). The mice that showed primarily CNS symptoms were also found to have fungal colonization of their lungs (see Panel C, Figure 5-14). The one mouse that survived in the group infected with wild type *A. fumigatus* showed no evidence of fungi within the lung sections (data not shown).

In the group that received the Δ*sidA* mutant strain, sections were examined from 7 of the 11 mice, including the one mouse that died. In the 6 asymptomatic mice receiving the mutant strain, the lungs of four mice showed evidence of peribronchiolar or perivascular inflammation whereas the remaining two mice had no evidence of inflammatory response. Only one of the asymptomatic mice exposed to the mutant strain had evidence of fungi within the lungs. Figure 5-14 F, G and H show representative sections in which inflammation is evident. In G, inflammatory cells appeared to be confined to the lumen of the bronchioles. In panels F and H, there are leukocytes present in the airway walls, but in contrast to mice infected with the wild type strain, little necrosis is evident. However, the mouse that died after exposure to the mutant strain had an extensive inflammatory response consisting of both PMNs and monocytes. Fungal hyphae are evident in the tissue sections (Figure 5-14, panel F, arrow); however, the fungal growth was less extensive compared to wild type and tended to remain confined to the bronchiolar lumen.

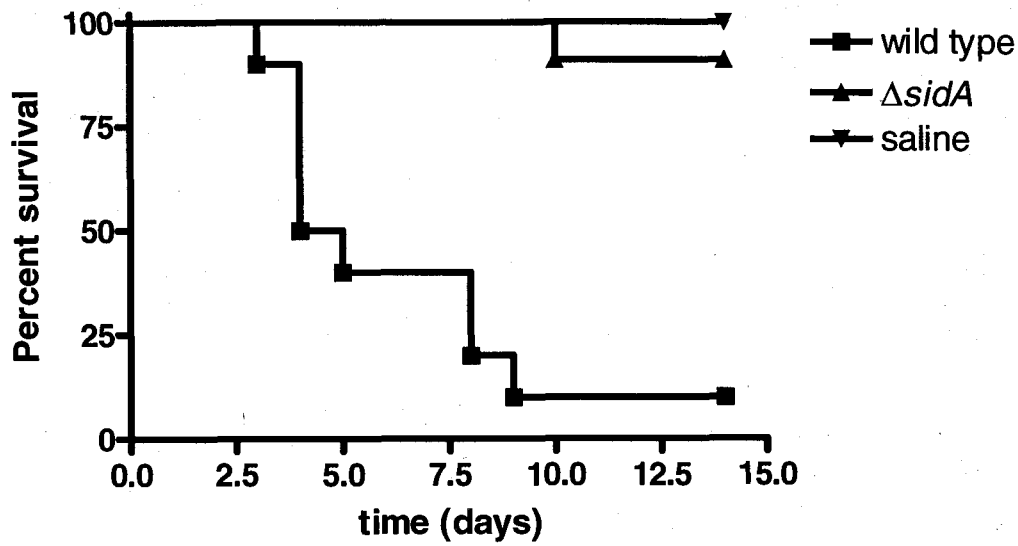
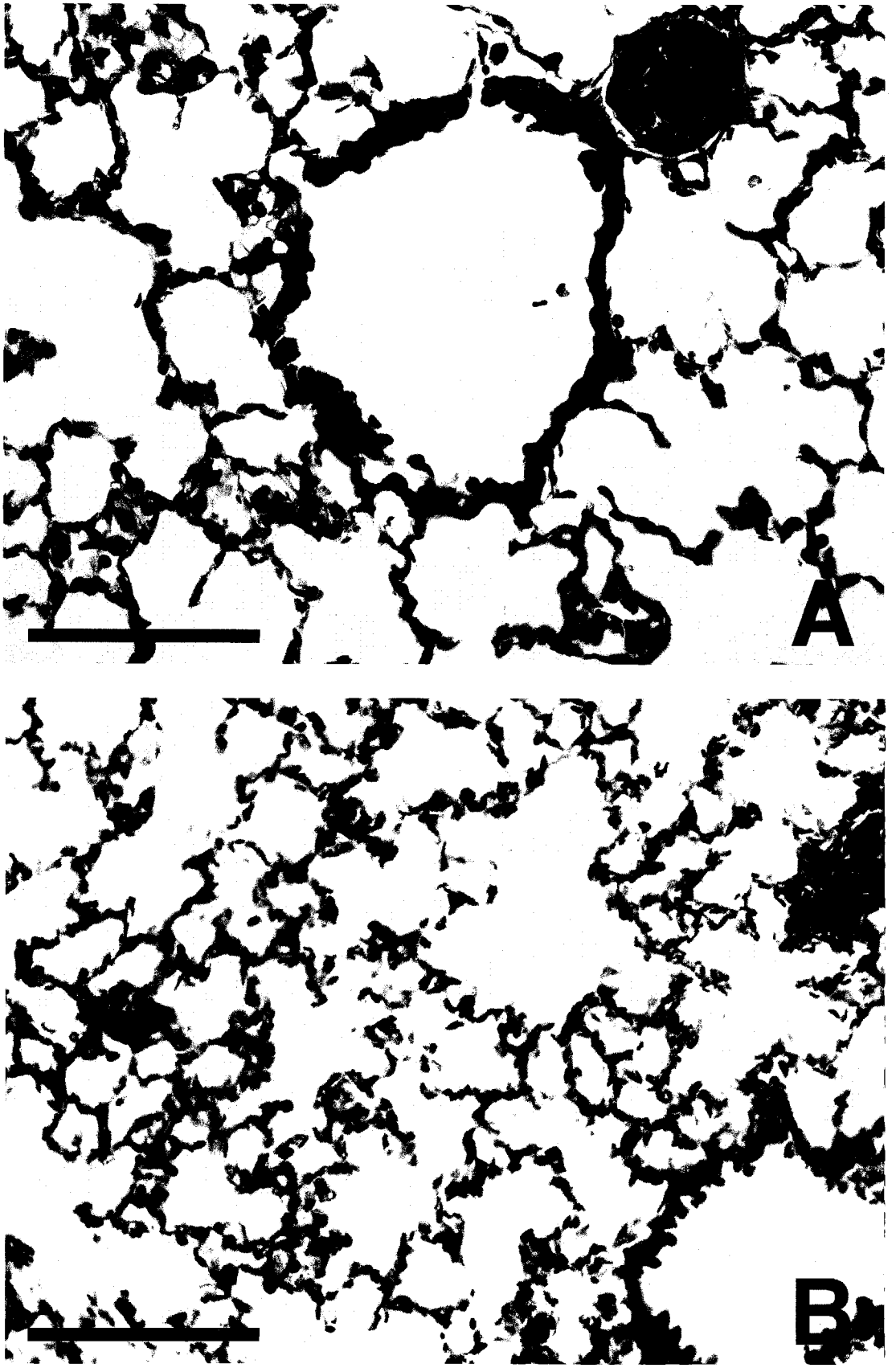
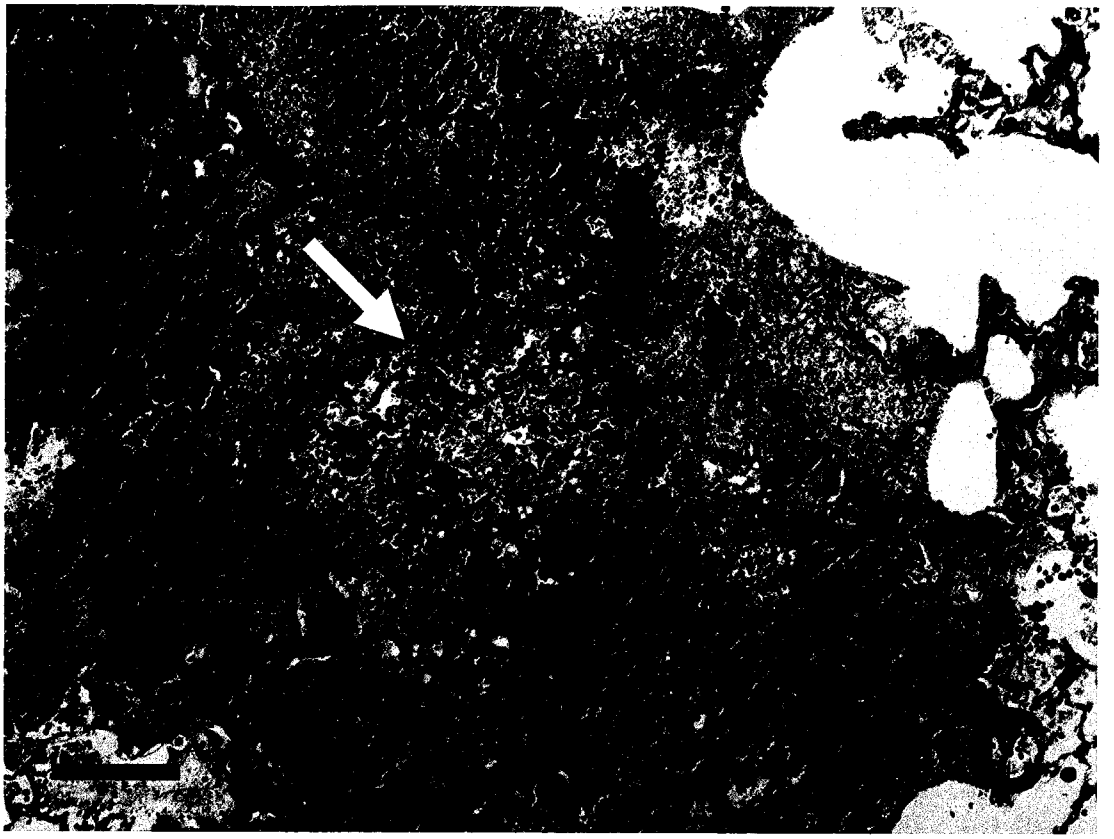
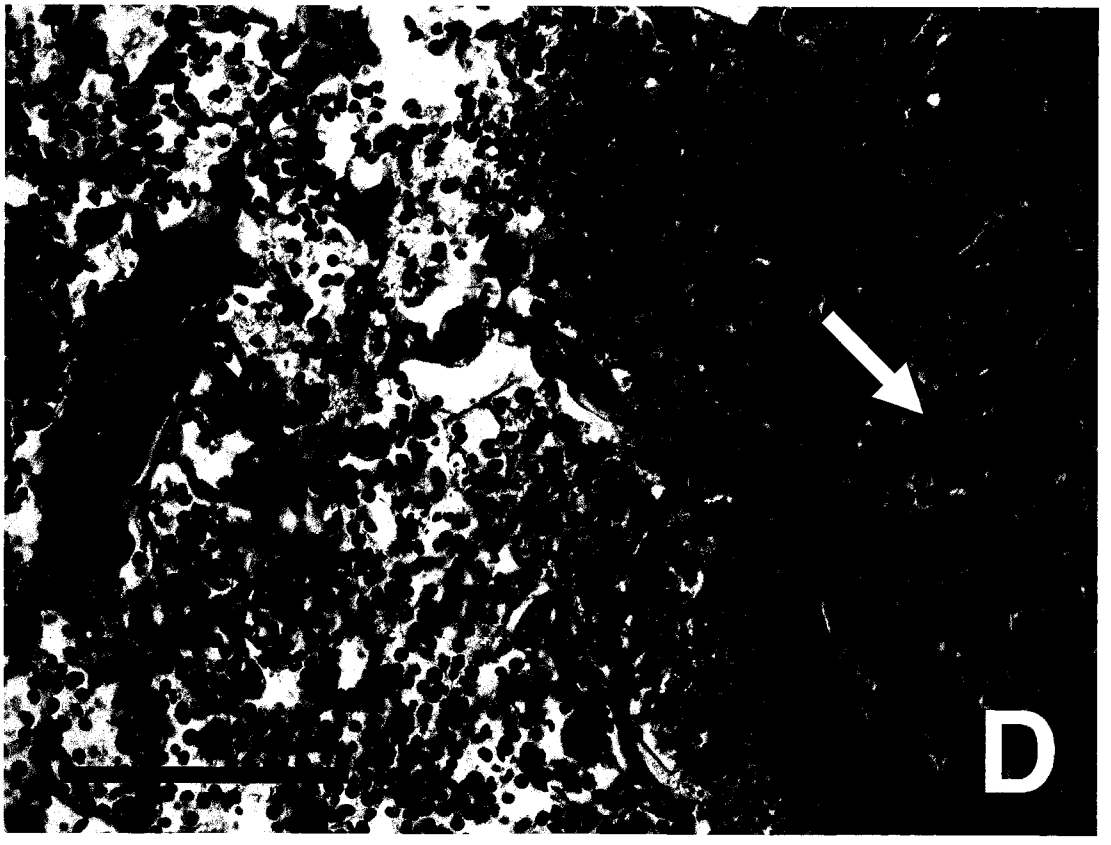


Figure 5-13: Survival curve of female BALB/c mice infected with *A. fumigatus* wild type or Δ*sidA*

Mice were immunosuppressed by subcutaneous injection of 200 mg/kg cortisone acetate on days -3, 0, 2 and 4. Mice were infected with $5x10^6$ conidia in 20 µl saline of either wild type or Δ*sidA* on day 0. Control mice were given saline alone. Mice were monitored daily and sacrificed if they displayed symptoms of infection as described in Results. Survival curves for wild type and Δ*sidA* are significantly different ($p < 0.0001$) by log rank analysis.


A
B

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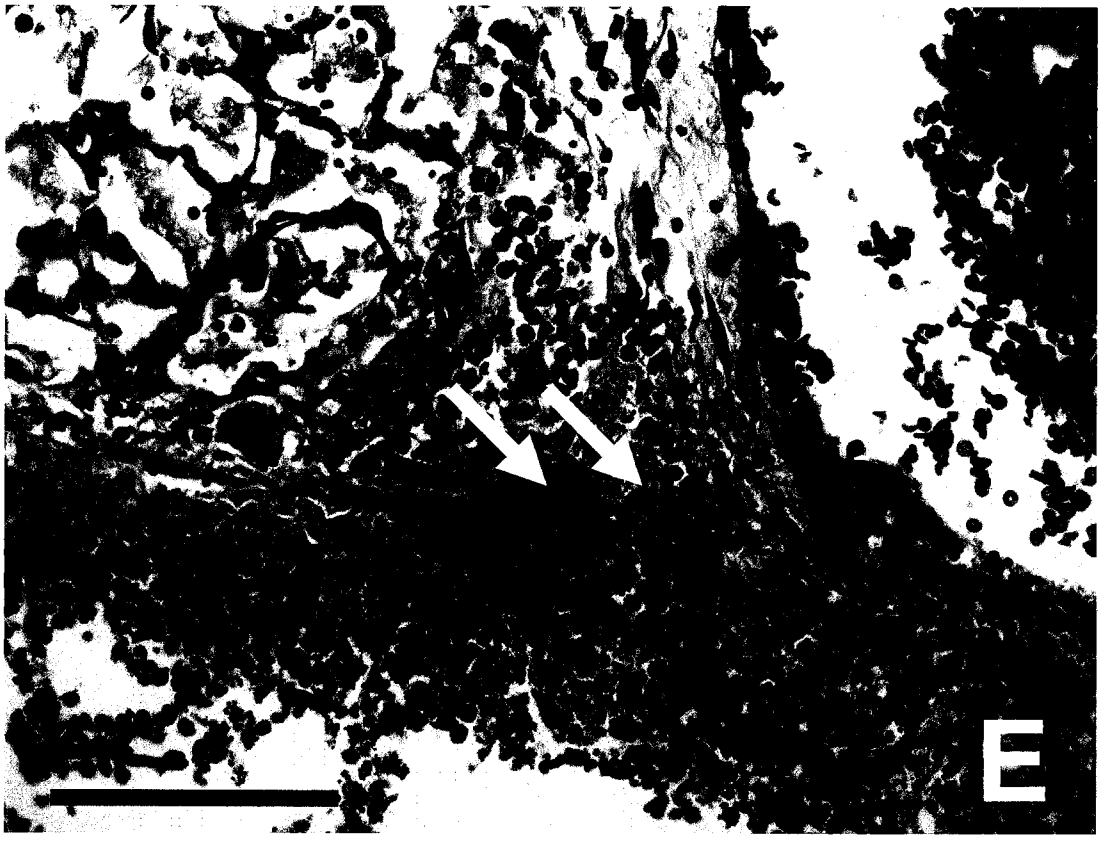
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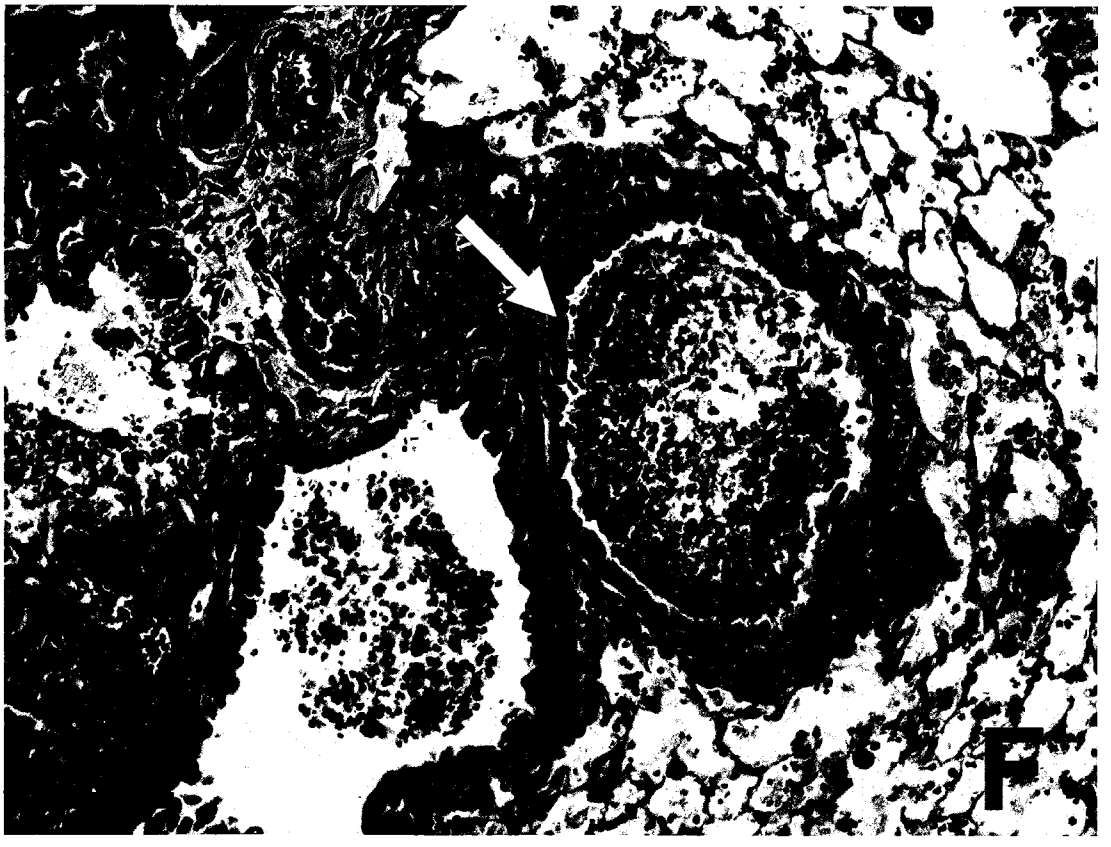
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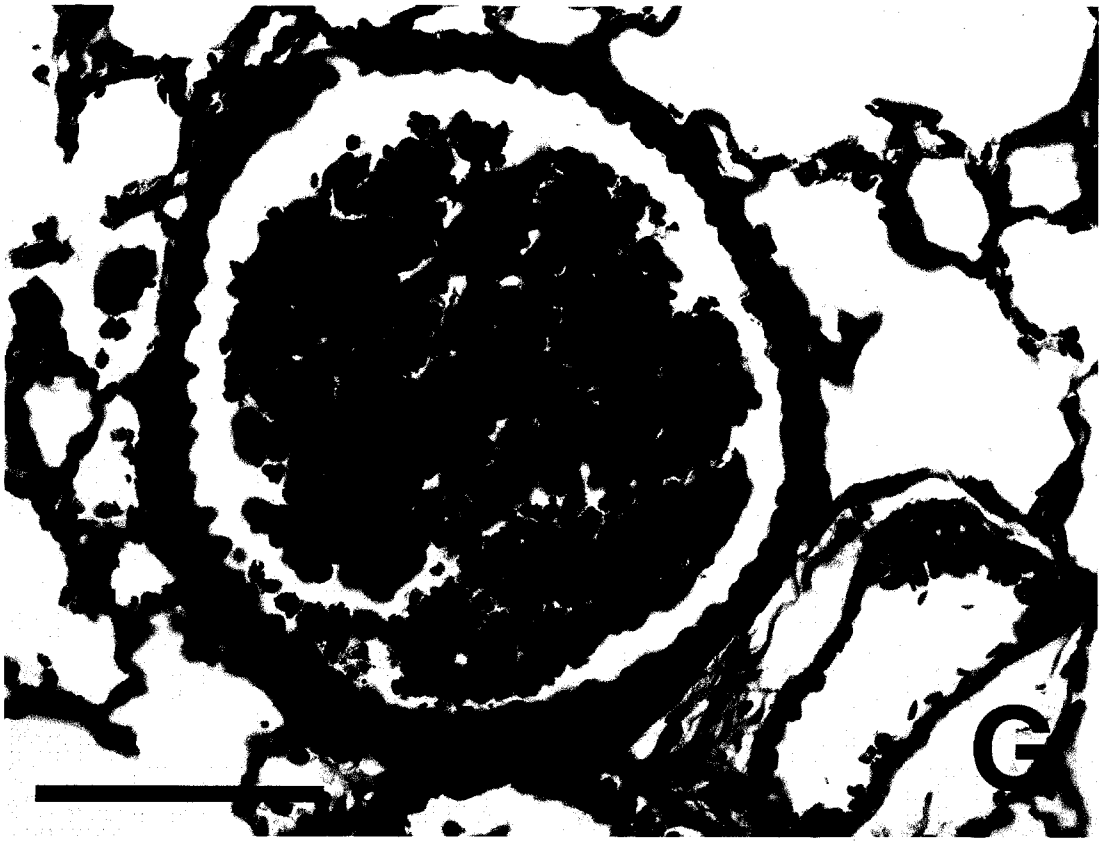
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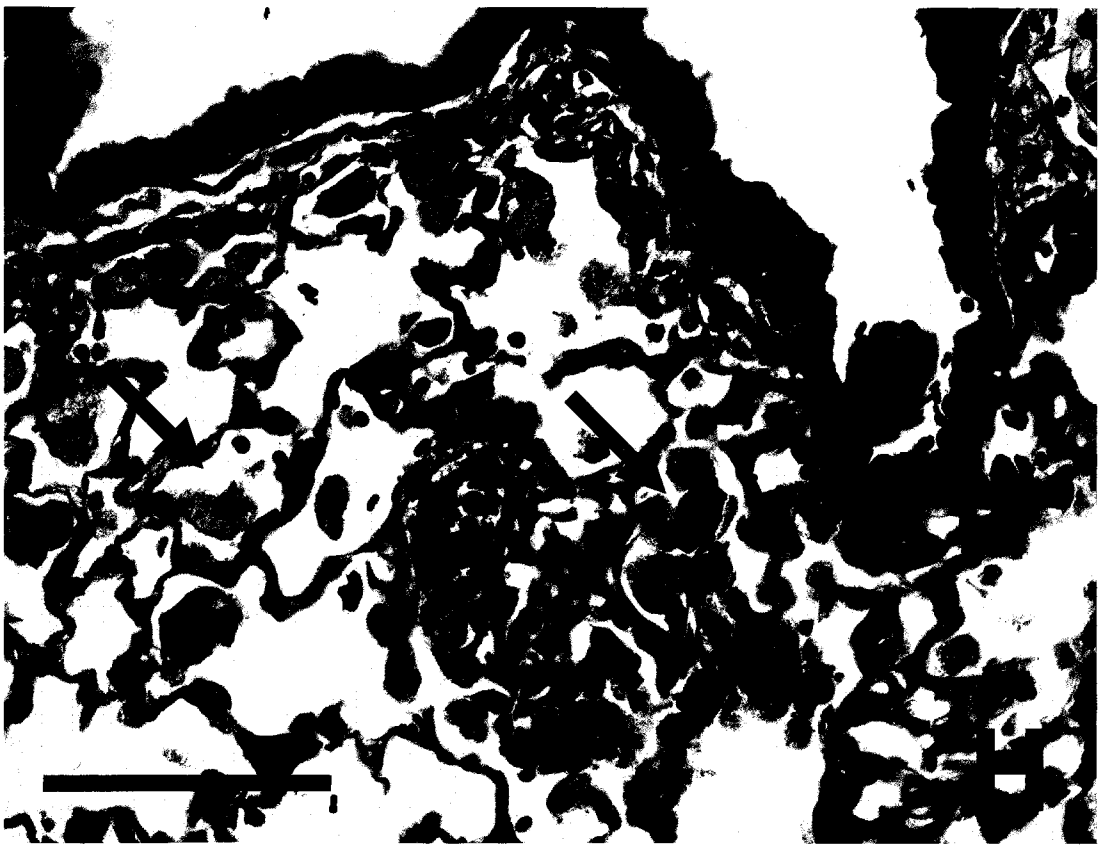

Figure 5-14: Lung tissue sections from cortisone-treated mice

Mice were exposed to one of the following treatments: saline (A and B), wild type *A. fumigatus* conidia (C, D and E), or Δ*sidA A. fumigatus* conidia (F, G and H). Each panel represents a section from a different animal. A and B (saline): lungs have normal appearance with clear airways and no inflammatory infiltrate. C (wild type): hyphal growth (white arrow) is visible within an area of extensive necrosis. Airway walls are no longer evident and a heavy inflammatory infiltrate surrounds the fungal growth. D (wild type): fungal hyphae (white arrow) within an airway are accompanied by a neutrophil and monocyte infiltration; erythrocytes are seen within the airways (black arrow). E (wild type): hyphae within a bronchiole are extending towards a blood vessel. Hyphae have also penetrated the wall of the bronchiole (arrows). Polymorphonuclear leukocytes predominate in the inflammatory cells. F (Δ*sidA*): heavy inflammation can be observed in the airway walls of the one mouse that died in the group exposed to the *sidA* mutant. Fungi are visible within the airway (arrow) but little necrosis is evident. Both PMNs and monocytes are present in the infiltrate. Normal lung is visible in this section although alveolar macrophages are numerous compared to saline-treated animals. G (Δ*sidA*): normal lung tissue surrounds a focus of inflammation confined to the lumen of a bronchiole; oedema of the wall of the adjacent arteriole is evident. H (Δ*sidA*): no fungi were observed in the lungs of this animal but foci containing large numbers of macrophages were observed (arrows). Scale bars are 20 μm in length.

Discussion

L-ornithine N^5-oxygenases

We have characterized the *A. fumigatus* L-ornithine N^5-oxygenase gene, *AfusidA*, which encodes the first committed step in hydroxamate siderophore biosynthesis. The *A. fumigatus sidA* gene showed a high degree of identity (~75%) to L-ornithine N^5-oxygenases from the closely related species, *A. nidulans* and *A. oryzae*. *AfusidA* contains the NADPH-, FAD- and substrate-binding domains (Stehr *et al.*, 1998) which are identical to those of the other characterized *Aspergillus* L-ornithine N^5-oxygenases. In addition, *sidA* had a > 50% similarity to L-ornithine N^5-oxygenases from *U. maydis* (*sid1*), as well as to the *pvdA* genes of *Pseudomonas* and *Burkholderia* species. The similarity of *sidA* to bacterial L-lysine N^6-hydroxylases (*iucD*) of *Escherichia coli* and *Yersinia* species was approximately 45%.

A Δ*sidA* strain of *A. fumigatus* was constructed by transformation with pGAW2, a *sidA* gene deletion construct. The Δ*sidA* strain did not produce detectable levels of either of the two most common *A. fumigatus* siderophores, TAF and ferricrocin, nor were any other siderophores detected. To date, all siderophores reported in *Aspergillus* species are hydroxamate siderophores (Howard, 1999), therefore it was expected that deletion of *sidA* would prevent synthesis of all *A. fumigatus* siderophores.

Alternative methods of iron uptake

Many pathogens, both bacterial and fungal, successfully colonize hosts without production of siderophores. Clearly other iron uptake mechanisms must be capable of scavenging iron within the host. Transferrin has low affinity for ferrous iron; therefore reduction of ferric iron coupled with a ferrous iron transporter presents an alternative method of iron uptake. Ferric reductases have been well characterized in fungal

pathogens such as *Candida albicans* (Knight *et al.*, 2002) and *Cryptococcus neoformans* (Jacobson *et al.*, 1998). *Histoplasma capsulatum* produces siderophores, ferric reductases and it expresses a cell surface receptor for hemin (Foster, 2002). The ferric reductases of *H. capsulatum* have been reported to remove iron from both hemin and transferrin (Timmerman and Woods, 2001), offering alternatives to siderophore production for iron uptake in vivo. A ferrous permease was found to be required for virulence of *C. albicans* (Ramanan and Wang, 2000). In addition, *C. albicans* produces the hemolytic molecule mannan, and has been demonstrated to access heme-bound iron (Santos *et al.*, 2003).

As described in Chapter 3, wild type *A. fumigatus* can efficiently remove transferrin-bound iron, even when separated by a dialysis membrane with a 10 kDa molecular weight cut-off. These data suggested that siderophores were capable of removing transferrin-bound iron, but did not eliminate the possible involvement of other iron uptake mechanisms such as low molecular weight ferric reductases. The Δ*sidA* strain, which produced no siderophores, did not remove iron from transferrin, indicating that siderophores are required for iron removal from transferrin. Thus, none of the alternative iron assimilation pathways of *A. fumigatus* were able to remove transferrin-bound iron in the absence of hydroxamate siderophores.

Growth of Δ*sidA*

Δ*sidA* was unable to grow in low iron defined medium (GA with no supplements), therefore siderophore secretion was required for growth in very low iron conditions. This growth inhibition could be overcome by addition of 5 μM $FeCl_3$; however, growth of Δ*sidA* in GA containing 5 μM $FeCl_3$ was delayed by about 24 hours.

These results suggest that uptake of $FeCl_3$ by the siderophore secretion mutant was much slower than that of the wild type, but that ferric iron could promote growth. The mechanism by which *A. fumigatus* Δ*sidA* strain can access ferric iron is not known, but could involve the reduction of ferric to ferrous iron, followed by uptake by ferrous iron transporters.

As described in Chapter 2, *A. fumigatus* ATCC 13073 grows very well in the presence of high concentrations (up to 80%) of human serum, conditions which are normally fungistatic due to the low concentration of free iron. In contrast, growth of *A. fumigatus ΔsidA* was completely inhibited by 10% human serum. Growth in GA + serum could be restored by the addition of either 50 μM desferri-TAF or 50 μM desferri-ferricrocin, indicating that siderophores are required for the growth of *A. fumigatus* in serum-containing media.

Little is known about ferric reductases in *Aspergillus* species, though *A. nidulans* has been reported to produce an iron-regulated gene, *freA*, with homology to *S. cerevisiae* metalloreductases (Oberegger *et al.*, 2002). Wild type and Δ*sidA A. fumigatus* strains both displayed cell-associated ferric reductase activity, as well as soluble ferric reductase activity. We postulated that ferric reductase activity would be upregulated in the Δ*sidA* strain to compensate for loss of siderophore-mediated iron uptake. However, no significant differences were observed between levels of ferric reductase activities of wild type and Δ*sidA* in vitro.

Despite the lack of siderophore secretion, growth of Δ*sidA* was not inhibited in rich media such as YG, which contains various organic iron sources. These organic iron sources are therefore accessible to *A. fumigatus* Δ*sidA* through alternative iron uptake

pathways. The Δ*sidA* growth defect in GA medium was completely rescued by addition of 10 μM TAF, indicating that a lack of siderophore secretion was the only cause of the growth inhibition by the GA medium. Similar results were observed when Δ*sidA* was cultured in media containing serum. These results contrast with observations for *sidA* mutants of *A. nidulans*, which were unable to grow in defined medium unless supplemented with 10 μM TAF or 1.5 mM ferrous iron (Eisendle *et al.*, 2003). Thus unlike *A. fumigatus*, *A. nidulans* is unable to access $FeCl_3$ or ferric iron from sources such as citrate without the use of siderophores. It was not stated if *A. nidulans* Δ*sidA* strain was able to grow in rich medium. These data suggest that the *A. nidulans* metalloreductase gene, *freA*, (Oberegger *et al.*, 2001) may not promote any significant degree of reductive iron assimilation. More work is necessary to elucidate ferric reductase pathways in both *A. fumigatus* and *A. nidulans*, as the two species appear to differ significantly in their non-siderophore mediated iron uptake pathways. The *A. oryzae* Δ*dff1* strain is similar to Δ*sidA* of *A. fumigatus* and can grow in rich media without addition of siderophores (Yamada *et al.*, 2003).

Role of siderophores in virulence

Siderophores have been demonstrated to play a role in pathogenesis of many different bacteria. Pyoverdin, a catecholate-hydroxamate siderophore is required for virulence of *Pseudomonas aeruginosa* in a burned mouse model of infection (Meyer *et al.*, 1996). In another study, it was found that both pyoverdin and another siderophore, pyochelin contributed to virulence of *P. aeruginosa* (Takase *et al.*, 2000). Similarly, *pvdA* mutants of the closely related *Burkholderia cepacia*, which were unable to secrete ornibactins, were less virulent than the parental strain in both chronic and acute models of

respiratory infection (Sokol *et al.*, 1999). Aerobactin, a mixed citrate-hydroxamate siderophore, is an important contributer to in vivo extracellular growth of *Escherichia coli* (de Lorenzo and Martinez, 1988), *Vibrio vulnificus* species unable to produce catechol siderophores showed reduced virulence in an infant mouse (Litwin *et al.*, 1996), and anguibactin production has been shown to contribute to virulence of *Vibrio anguillarum* in juvenile trout (Wertheimer *et al.*, 1999). In fungi, less is known about the role of siderophores in virulence because many of the common fungal pathogens do not produce siderophores. However, many fungi are able to use exogenous siderophores, and siderophore transporters have been shown to be required by the yeast *Candida albicans* for epithelial invasion and penetration (Hu *et al.*, 2002).

Corticosteroids inhibit the conidicidal activity of tissue macrophages (Ng *et al.*, 1994). In mice treated with corticosteroids, conidia are taken up by macrophages which are unable to inhibit their germination and growth. Thus, conidia are not only exposed to the airway surface fluids but also to the intracellular environment where other potential iron sources, such as ferritin, are found. Since the Δ*sidA* strain of *A. fumigatus* was unable to establish infection, none of the iron sources to which it was exposed in the host are sufficient to allow invasive growth in the absence of siderophores. In addition, siderophore mutants may be more susceptible to reactive oxygen species produced by phagocytic cells. Excess unbound iron can contribute to the formation of deleterious hydroxyl radicals, which catalyze damage to DNA and other essential molecules via Fenton chemistry. In *A. nidulans*, it has been demonstrated that lack of cellular siderophores increased sensitivity to the redox-cycling agent paraquat (Eisendle *et al.*, 2003).

Δ*sidA* strains of *A. fumigatus* were completely avirulent in a mouse model of invasive aspergillosis. To our knowledge, this is the first description of siderophores as virulence factors in fungi. In addition, *sidA* is now one of the few identified virulence factors in *A. fumigatus*. To date, the only other avirulent strains of *A. fumigatus* were a p-aminobenzoic acid (PABA) auxotroph that could not germinate in vivo unless supplied with PABA (Brown *et al.*, 2000), a *pyrG* mutant unable to germinate in the absence of uridine and uracil (D'Enfert *et al.*, 1996) and a *lysF* mutant unable to synthesize lysine (Liebmann *et al.*, 2004). These genes are not strictly virulence factors because they are also required for in vitro growth. Siderophore secretion is therefore the first true virulence factor to be discovered in *A. fumigatus*. What makes this discovery so important is the fact that siderophore biosynthesis and uptake are absent in mammalian cells. These pathways therefore represent new targets for anti-fungal drug development.

Conclusion

In conclusion, siderophore-mediated iron uptake was required by *A. fumigatus* for growth in low iron media, including serum-containing media, and is the only mechanism by which *A. fumigatus* can remove iron from transferrin in vitro. Alternative iron uptake pathways were sufficient for growth in rich media, and defined media supplemented with 5 μM $FeCl_3$. Siderophore biosynthesis was required for virulence of *A. fumigatus* in a mouse model of invasive aspergillosis. Since siderophore biosynthesis pathways are absent in humans, they represent novel targets for antifungal chemotherapy.

CHAPTER SIX: GENERAL DISCUSSION AND CONCLUSION

Background

Aspergillus fumigatus is a common soil fungus, most often found cycling nutrients within decomposing organic matter. Unfortunately, some specific traits of *A. fumigatus* allow it to colonize the lungs of immunocompromised individuals, and have caused it to be the leading cause of invasive mold infection worldwide (Denning, 1998). There are thousands of fungal species in the environment, including hundreds of *Aspergillus* species, which do not cause invasive disease. Therefore the question has been posed: what virulence factors set *A. fumigatus* apart from all the non-pathogenic similar species?

The elucidation of these virulence factors has motivated researchers to explore numerous aspects of the biology of *A. fumigatus* for the latter half of the 20th century. The first virulence factor of *A. fumigatus*, p-aminobenzoic acid biosynthesis, was identified almost 30 years ago (Sandhu *et al.*, 1976), however this species has not been one to give up its secrets easily. In the past 30 years, very few factors have been found to be essential for virulence, and all of these are mutations generating auxotrophy. The growth of these auxotrophic strains is inhibited in vitro, therefore they are not true virulence factors. This slow pace of research has lead some investigators to propose that *A. fumigatus*, as an opportunistic pathogen, might not possess virulence factors in the traditional sense (Latgé, 2001).

Rationale for study

This work was begun after the serendipitous observation that *A. fumigatus* growth was promoted, rather than inhibited, by serum (Figure 2-2). It was found that both human and fetal bovine sera promoted this effect, and that increasing the serum concentrations increased the amount of biomass produced by *A. fumigatus* cultures. This is a very unusual observation, as the growth of most fungi, including some very common pathogens, is severely inhibited by serum (Artis *et al.*, 1982; Eng *et al.*, 1981; Granger *et al.*, 1986; Minn *et al.*, 1997; Nassar *et al.*, 1995; Sutcliffe *et al.*, 1980; Taramelli *et al.*, 2000; Verdonck *et al.*, 1993). This inhibition is due to the presence of iron-binding molecules in serum, most notably transferrin. Since *A. fumigatus* was unaffected by the antifungal activity of serum, we postulated that it must possess very effective iron-uptake mechanisms.

With this background in mind, we launched our study into the role of iron in the growth of *A. fumigatus*. Iron is in limited supply in all aerobic environments. With the exception of certain bacteria that do not require iron for growth (Pandey, 1994; Posey and Gherardini, 2000), iron uptake is critically important to microbial growth in any environment. Iron is particularly unavailable within the mammalian host, where it is tightly bound by various iron-binding molecules. Host organisms tightly sequester iron for two reasons. First, they need to minimize damage resulting from Fenton chemistry (Leong and Winkelmann, 1998). Second, by withholding iron, they can limit the supply of iron available to pathogenic microorganisms. In response, pathogens have evolved several strategies for accessing this bound iron. Possible iron sources for pathogens include lactoferrin in extracellular secretions, transferrin in the bloodstream, heme within red blood cells and ferritin inside most cells (Howard, 1999).

We chose to focus our study on iron uptake from transferrin for several reasons. First, transferrin is known to be present in airway surface fluids (Govindaraju *et al.*, 1998), which is the environment encountered by inhaled *A. fumigatus* conidia. Second, transferrin is present in high concentrations, 40 μM (Low *et al.*, 1987), in serum, and it was known that *A. fumigatus* proliferated in serum-containing media. Third, there was a large body of literature demonstrating the importance of transferrin-bound iron in virulence of various bacterial species. In addition, serum iron overload is a significant risk factor for infection by several different fungal pathogens, including *Pencillium marneffei* (Taramelli *et al.*, 2000), *Mucor* spp. (Maertens *et al.*, 1999) and *Candida albicans* (Minn *et al.*, 1997).

It is worth noting that *A. fumigatus* conidia have been shown to be internalized by lung epithelial cells, within which they germinate and escape from the cell (Wasylnka and Moore, 2003). Additionally, they are internalized by macrophages (Wasylnka and Moore, 2002). Thus, conidia and mycelia are exposed to the sources of iron within these cells. Therefore, intracellular iron storage molecules such as ferritin and lactoferrin are also potential iron sources available in the early stages of *A. fumigatus* infection.

Production of siderophores is known to be a particularly effective strategy for in vivo iron uptake, and siderophore secretion is important for virulence of several bacterial species (de Lorenzo and Martinez, 1988; Litwin *et al.*, 1996; Meyer *et al.*, 1996; Sokol *et al.*, 1999; Takase *et al.*, 2000; Wertheimer *et al.*, 1999). Until this study, siderophores had never been shown to play a role in the virulence of fungal pathogens. *A. fumigatus* has long been known to secrete siderophores (Diekmann and Krezdorn, 1975; Nilius and

Farmer, 1990), however it was not known if any of these fungal hydroxamate siderophores were important for growth in vivo.

Since siderophores are often important in iron acquisition from transferrin by pathogenic bacteria, and all evidence pointed towards an important role for siderophores in removal of iron from human transferrin, we focused on siderophores as potential virulence factors in *A. fumigatus*. If siderophores were found to be important in virulence, the siderophore biosynthesis and uptake pathways could present much needed novel drug targets against *Aspergillus* infections.

Summary of results

Following the observation that *A. fumigatus* proliferated in the presence of human serum, we were able to verify that *A. fumigatus* could indeed remove iron from human serum transferrin (Figure 3-2). We next considered several possible mechanisms by which *A. fumigatus* could remove transferrin-bound iron. Proteinase secretion was one potential means of iron uptake from serum, as we had found that serum induces very high levels of proteinase secretion in *A. fumigatus* (Figure 2-2). It was also observed that proteinases produced by *A. fumigatus* were able to degrade human transferrin (Figure 3-1). The proteinases secreted by *A. fumigatus* differed under different culture conditions, and serum was required to stimulate secretion of the transferrin-degrading proteinase (Figure 3-1). However, the proteinase secretion did not occur early in the growth of *A. fumigatus*, reaching a maximum only when the cultures had reached stationary phase (Figure 2-1). In addition, no degradation of transferrin was observed in the first 24 hours of growth (Figure 3-1).

Conversely, siderophore production could be detected in the very early stages of *A. fumigatus* growth (Figure 3-3). Siderophores could be detected in the culture medium after about 8 hours incubation at 37°C, which is approximately the same time required for germination of conidia. This early production provided evidence that siderophores might be important for growth of *A. fumigatus* in serum-containing media.

Further experiments demonstrated that *A. fumigatus* was able to remove transferrin-bound iron despite physical separation from the transferrin by a dialysis membrane (Figure 3-4). This finding excluded the possibility of a role for proteinases or for transferrin receptors on the fungal cell surface. Transferrin receptors have never been reported in fungi; however, a hemin receptor has been reported in *H. capsulatum*, which would allow it to uptake heme found within macrophages (Foster, 2002). *C. albicans* also has a heme-uptake system that is independent of siderophore and ferric reductase production (Santos *et al.*, 2003).

Ferric reductases are important iron uptake strategies employed by fungi. Ferric reductases are important in *C. albicans* (Knight *et al.*, 2002) and *H. capsulatum* (Timmerman and Woods, 1999, 2001). We found that *A. fumigatus* does produce ferric reductase activity (Table 5-4); however, the ability of *A. fumigatus* to remove iron from across a dialysis membrane excludes a role for cell-associated ferric reductases or soluble ferric reductases larger than 12 kDa. Thus, although ferric reductases might contribute to iron uptake, they are not required for removal of iron from transferrin. Extracellular nonproteinaceous ferric reductant(s) have been reported in *H. capsulatum* (Timmerman and Woods, 2001), though they have yet to be identified and characterized. These small reductants are theoretically small enough to pass through a 12 kDa membrane. However,

most of the ferric reductase activity produced by *A. fumigatus* was cell-associated, only low levels were detected free in the culture medium (data not shown). Therefore, most of the evidence strongly pointed towards an important role for siderophores in iron uptake from transferrin by *A. fumigatus*.

We next wished to purify and identify the siderophores produced by *A. fumigatus* under our experimental conditions. We found at least five siderophores, or siderophore degradation products, were produced by *A. fumigatus* in low iron GA medium (Table 3-2). Two siderophores predominated in *A. fumigatus* culture medium (Table 3-2), and these were subsequently conclusively identified by NMR and mass spectrometry as N'N''N'''-triacetylfusarinine C (TAF) and ferricrocin (Tables 3-3, 3-4, 3-5, 3-6). We isolated 5mg/ml ferricrocin and over 110 mg/ml TAF. TAF and ferricrocin have very high thermodynamic iron binding constants and both bind iron more strongly than transferrin. Experiments demonstrated that TAF and ferricrocin both had the kinetic (Figure 3-5) as well as thermodynamic ability to remove transferrin-bound iron. These data further supported the hypothesis that siderophores are important in iron uptake in vivo.

More detailed kinetic studies demonstrated that both TAF and ferricrocin efficiently removed iron from human diferric-transferrin, as well as from both forms of human monoferric-transferrin (Table 4-3). It was interesting to note that both siderophores preferentially removed iron from the C-terminal lobe of human diferric-transferrin. This is unusual because most iron chelators studied preferentially remove iron from the N-terminal site of transferrin, which is thermodynamically the weaker iron-binding site (Aisen *et al.*, 1978; Evans and Williams, 1978). At least two other microbial

siderophores, aerobactin (Ford *et al.*, 1988) and amonabactin (Stintzi and Raymond, 2000), demonstrate a similar preference for iron bound to the C-terminal site of transferrin.

TAF removed iron from transferrin at a faster rate than ferricrocin; however, rates of iron removal by both siderophores were quite high and of a similar order of magnitude to those of other siderophores, such as aerobactin (Tables 4-2 and 4-3). The predominant form of transferrin in human circulation is the N-terminal monoferric-transferrin (Bali *et al.*, 1991), and it was demonstrated in this study that although iron is removed from the C-lobe preferentially, both TAF and ferricrocin can efficiently remove iron from N-terminal monoferric-transferrin (Table 4-3).

We wished to further examine the importance of siderophores in removal of iron from transferrin and in virulence by constructing a siderophore-deficient strain of *A. fumigatus*. This type of study had been previously accomplished in the fungus *Ustilago maydis* (Mei *et al.*, 1993) and the bacteria *Burkholderia cepacia* (Sokol *et al.*, 1999) and *Pseudomonas aeruginosa* (Visca *et al.*, 1994). In all cases, the siderophore secretion mutant was constructed by disruption of L-ornithine N^5-oxygenases. These enzymes are critical to the synthesis of hydroxamate siderophores because they catalyse the first step in creation of the hydroxamate functional group (Figure 5-1) . Additionally, while work was underway on this project, creation of L-ornithine N^5-oxygenase deficient strains of *Aspergillus nidulans* (Eisendle *et al.*, 2003) and *Aspergillus oryzae* (Yamada *et al.*, 2003) were reported.

We were able to take advantage of the unfinished *A. fumigatus* genome sequence to search for L-ornithine N^5-oxygenases, and found a single match with high homology to

the same protein in *U. maydis*. The genomic data were invaluable in cloning and disrupting this gene, termed *AfusidA*. The L-ornithine N^5-oxygenases cloned in *A. nidulans*, *A. oryzae* and *A. fumigatus* are highly homologous, with approximately 75% identical amino acid sequences (Figure 5-3). All appear to be necessary for production of hydroxamate siderophores (Figure 5-11)(Eisendle *et al.*, 2003; Yamada *et al.*, 2003).

In characterizing the *A. fumigatus* Δ*sidA* strain, several interesting observations were made. First, Δ*sidA* was unable to grow in low iron medium (Table 5-2), or in the presence of human serum (Table 5-3). This was strong evidence for the importance of siderophores in iron uptake by *A. fumigatus*. Secondly, without production of siderophores, *A. fumigatus* was not able to remove iron from transferrin in our in vitro experiments (Figure 5-12). Third, Δ*sidA* displayed highly attenuated virulence in a mouse model of invasive aspergillosis (Figure 5-13). This indicated that siderophores are the main method for iron uptake from in vivo sources.

In the absence of siderophores, growth of *A. fumigatus* was severely impaired within the immunocompromised mouse (Figure 5-14), resulting in a considerable reduction in its virulence. This result was interesting because several other fungal pathogens survive and multiply in vivo without production of siderophores. *C. albicans* and *C. neoformans* are examples of successful fungal pathogens that must acquire iron in vivo by alternate means, likely ferric reductase activity. Though *A. fumigatus* does produce relatively high levels of ferric reductase activity, this was not sufficient to promote growth in vivo, nor in low iron or serum-containing media.

Siderophores, including TAF and ferricrocin, are produced by several non-pathogenic fungal species, including non-pathogenic *Aspergillus* species (unpublished

observations of M. Dennis in our laboratory). However siderophore secretion appears to be necessary, if not sufficient, for virulence of this pathogen.

Siderophore-mediated iron uptake

Iron uptake systems in fungi are under control of GATA protein family of transcription factors which are regulatory proteins with consensus zinc finger motifs that bind to DNA elements containing a GATA core sequence (Kosman, 2003). In *A. nidulans*, siderophore biosynthesis and uptake are negatively regulated by a GATA-type transcription factor called SREA (Haas *et al.*, 1999). Expression of iron uptake systems in fungi is repressed by the presence of environmental iron (Kosman, 2003).

The siderophore-iron uptake pathway has been studied in fungi (De Luca and Wood, 2000). In *U. maydis*, expression of the siderophore biosynthesis genes are regulated by an iron-responsive transcriptional repressor, Urbs1 (Yuan *et al.*, 2001). Urbs1 contains the two zinc finger motifs which are characteristic of the fungal GATA-transcription factor family (Scazzocchio, 2000) and these interact with the promoter region of the siderophore biosynthetic genes. Urbs1 orthologs have been identified in many ascomycetes, including *A. fumigatus* (Haas, 2003).

There is evidence that siderophores are synthesized and stored in intracellular vesicles until required for secretion. Since siderophores are generally polar, secretion is likely to require specific transport proteins. Secretion of enterobactin in *E. coli* has recently been shown to be mediated by the EntS protein (Furrer *et al.*, 2002). After iron loading, siderophores are returned to the cell via specific siderophore transporters, or the siderophore-iron complex is utilized by the reductive iron assimilatory system. Both these systems are present in *C. albicans* (Hu *et al.*, 2002) and *S. cerevisiae* (Heymann *et*

al., 2000; Yun *et al.*, 2000). *A. nidulans* appears to lack a reductive iron assimilatory pathway (Eisendle *et al.*, 2003), however the closely related *A. fumigatus* shows evidence of iron reduction at the cell surface (Table 5-4).

Siderophore-iron receptors have very high stereo-specific affinities for their ferri-siderophore complex, indicating that binding to the transporter is not dependent on overall size or hydrophobicity but on specific binding sites (Winkelmann, 2001). These transporters are thought to be proton symport transport proteins (Pao *et al.*, 1998). It is known that *A. fumigatus* can take up ferrated ferrirubin, ferricrocin, ferrichrome, and ferrichrysin with equal efficiency (Wiebe and Winkelmann, 1975). These siderophores are all members of the ferrichrome class of siderophores and are structurally very similar. TAF-bound iron is also efficiently taken up by *A. fumigatus*, while iron bound to coprogen and ferrioxamine B, exogenous siderophores produced by *Neurospora crassa* and *Streptomyces pilosus*, is not absorbed (Wiebe and Winkelmann, 1975).

The siderophore uptake process appears to involve endocytosis of the transporter subsequent to binding of its substrate, and translocation of iron across the endosomal membrane (Kim *et al.*, 2002). Within the cell, iron is liberated from the siderophore by use of NADPH-linked ferric reductases (Ernst and Winkelmann, 1977). Siderophores of the ferrichrome class are thought to be concentrated in the deferrated state within small vesicles and shuttled back to the cell surface for exocytosis (Ardon *et al.*, 1998). In contrast, the ester bonds in TAF are hydrolysed after cellular uptake and the fusarinine moieties are excreted (Adjimani and Emery, 1987; Oberegger *et al.*, 2001). Cleavage of the siderophore or maintenance of iron-free siderophores within vesicles can be envisioned as strategies to prevent uncontrolled chelation of intracellular iron stores.

Alternatively, iron is released from the siderophore at the cell surface by reductive iron assimilation. Cell surface metalloreductases can reduce iron salts, low-stability iron chelates such as ferric citrate, and siderophores (Haas, 2003). Ferrous iron is then transported within the cell by ferrous iron transporters. This system can be used to obtain iron from siderophores which cannot be taken up as the siderophore-iron complex.

Siderophores secreted in vivo might encounter transferrin, and a model for iron release from transferrin has been described. The current accepted model has four steps: (1) conformational change of the diferric protein from a closed to an open configuration, (2) destabilization of the iron binding site by protonation of the carbonate/histidine, then protonation of lysines 209 and 296, (3) attack of the chelator and formation of a quaternary complex and (4) decay of the quaternary complex to yield the apoprotein and ferrated ligand (Grossmann *et al.*, 1992; Jeffrey *et al.*, 1998; Kilar and Simon, 1985; Pakdaman and El Hage Chahine, 1996).

Other applications for siderophore research

The study of siderophores has some other applications. Siderophores also bind metals other than iron, including Pb(II), Cr(III) and Al(III) and the actinides (Renshaw *et al.*, 2003), which are radioactive and potent carcinogens. The binding of these other metals has created potential applications for their medical and industrial use (Haas, 2003). Siderophores are used medically to treat iron and aluminum overload. The main compound used, desferroxamine B (desferral) is a siderophore produced by *Streptomyces pilosus* (Muller and Raymond, 1984), but its use is hampered by poor oral availability. Siderophores also have potential to de-incorporate actinides, as anticancer and antimicrobial agents. Additionally, it has been proposed that anti-microbial drugs could

be coupled to siderophores, allowing highly targeted chemotherapy for microbial infections (Roosenberg *et al.*, 2000). The metal chelation of siderophores could be exploited in order to reprocess nuclear fuel and treat and remediate metal-contaminated sites (Haas, 2003). These are important reasons to further the study of microbial siderophores.

Future avenues for exploration

In order to conclusively demonstrate the importance of *sidA* in virulence, a complementation study should be carried out. One of Molecular Koch's postulates states that restoration of the mutated gene should return pathogenicity to the organism (Falkow, 1988). Transformation of Δ*sidA* with a functional *sidA* gene should restore virulence to *A. fumigatus* and must be carried out in order to prove the importance of this gene in virulence.

It would be interesting to study iron uptake from alternate sources, for example lactoferrin or heme. Lactoferrin is present in bodily secretions and in neutrophils where it would be encountered by conidia. Lactoferrin is structurally very similar to transferrin, so it is likely that the kinetics of iron uptake by *A. fumigatus* siderophores would be quite similar. *A. fumigatus* also produces a hemolytic toxin (Yokota *et al.*, 1977) therefore iron uptake from heme is possible. It is not known if heme is an iron source for *A. fumigatus.*

Chapter 2 left several questions unanswered, notably the identity of the factor in serum that stimulates proteinase secretion. Also, it would be interesting to study regulation of proteinases secretion, as it appears to be quite complex. Proteinase gene expression studies might be able to answer the question of which proteinases are induced

by serum or A549 cells or basal lamina proteins. These studies might be able to determine which proteinases are important in vivo.

Little is known about the siderophore receptors in *A. fumigatus*. Siderophore receptors have been better characterized in *A. nidulans*, where three putative siderophore-transporter-encoding genes (*mirA*, *mirB* and *mirC*) have been characterized (Haas *et al.*, 2003). MirA is primarily responsible for uptake of the heterologous siderophore enterobactin, while MirB takes up TAF exclusively. It is not known whether *A. fumigatus* takes up enterobactin, or which receptors are involved in uptake of TAF and ferricrocin.

To distinguish between the importance of ferricrocin and TAF in iron uptake and virulence, mutants deficient in single siderophores could be created. *sidC*, the gene encoding a non-ribosomal peptide synthetase has been disrupted in *A. nidulans*. This strain produces TAF, but no ferricrocin (Eisendle *et al.*, 2003). Similarly, the non-ribosomal peptide synthetases responsible for a reaction in the TAF biosynthesis pathway could be disrupted. The resulting mutant strains could be used to further examine the specific roles and importance of each of the major siderophores produced by *A. fumigatus*.

Further examination of the early events in infection could uncover the reason for the attenuated virulence of the Δ*sidA* strain. For instance, it is known that Δ*sidA* can germinate in vivo, and can on rare occasion cause infection, but it is not known if germination and growth of Δ*sidA* occurs in most mice. It would be interesting to note at what stage of infection the growth of Δ*sidA* is halted, and this could be determined by histological examination of mouse lungs during the first few days post-infection.

Ferric reductases have yet to be thoroughly studied in *Aspergillus*. Ferric reductases serve two functions: intracellularly to release iron from siderophores, and extracellularly to reduce ferric iron bound to ligands in the environment to ferrous iron for uptake. It would be interesting to study these reductases for several reasons. First, there is clearly a difference between the ferric reductases of *A. fumigatus*, *A. oryzae* and *A. nidulans*. Δ*sidA* strains of *A. nidulans* are unable to grow in defined media unless supplemented with siderophores or with very high concentrations of ferrous iron. Siderophore-deficient strains of *A. fumigatus* and *A. oryzae* are more resilient and better able to use other means of iron uptake. Second, ferric reductases of the yeasts *C. albicans* and *C. neoformans* have been well studied, and are able to support in vivo growth of these species. The ferric reductases of *A. fumigatus* are unable to support in vivo growth, so it would be interesting to study the differences between the ferric reductase/ferrous iron uptake systems of these species.

There are several other important pathogens in the genus *Aspergillus*, most notably *A. flavus*. It would be interesting to closely examine siderophore secretion in this species as well.

Finally, it will be important to explore the siderophore-mediated iron uptake pathway for potential drug targets. There are several enzymes that are critical for siderophore biosynthesis, one of which is *sidA*. There are also targets such as siderophore receptors which are necessary for siderophore-mediated iron uptake. It would be possible to screen compounds for their ability to inhibit siderophore secretion in *A. fumigatus*. More study of the proteins involved in siderophore biosynthesis is necessary.

Relevance of this study

We have demonstrated that hydroxamate siderophores are required for iron uptake from transferrin and virulence of *A. fumigatus*. This has provided a greater understanding of the growth of *A. fumigatus* in vivo and is also the first demonstration that fungal siderophores can remove iron from human transferrin and play an important role in vivo. Therapies aimed at interfering with siderophore biosynthesis or uptake might also be effective against some bacterial pathogens such as *Burkholderia cepacia* and *Pseudomonas aeruginosa*, which both require siderophores for full virulence (Sokol *et al.*, 1999; Takase *et al.*, 2000). Such a therapy could be very useful for treatment of patients who often have mixed infections. New strategies for combating *Aspergillus* infections are desperately needed, and a greater understanding of the in vivo requirements of these species may someday help improve the outcome for all aspergillosis patients.

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