Heme utilization and storage by *Cryptococcus neoformans*.

by

Melanie Scofield
B.Sc. University of British Columbia, 2006

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(Microbiology and Immunology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

December 2009
© Melanie Scofield, 2009
ABSTRACT

The opportunistic fungal pathogen Cryptococcus neoformans has been previously shown to use heme as a sole iron source, but the mechanisms for heme utilization are unknown. The goal of this study was to begin a genetic analysis of heme utilization in C. neoformans by deletion of candidate genes and phenotypic characterization. The first hypothesis was that a putative heme oxygenase protein, Hmx1, was responsible for degrading heme to release iron. However, an hmx1 deletion strain was capable of growth on heme, indicating that the gene is not required for heme utilization. The expression pattern of HMX1 showed down-regulation in the presence of heme and hemoglobin indicating that HMX1 likely plays a regulatory role within the cell. Because loss of the heme-related gene HMX1 did not reveal any phenotypes related to heme as an iron source, the role of the vacuolar protein Vps41 in iron and heme utilization was also examined. The work on Vps41 was designed to test a second more general hypothesis that the vacuole is involved in heme or iron storage and utilization. It was found that vps41 mutants had heme and iron growth defects, as well as increased sensitivity to excess levels of both heme and inorganic iron. Analysis of the wild-type strain grown with heme led to the surprising discovery of dark intracellular aggregates that were visible with light microscopy. These aggregates were reminiscent of the crystallized heme (hemozoin) found in malaria parasites. In contrast, the cells of vps41 mutants became filled with diffuse heme throughout the cell, indicating that an intact vacuole was required for aggregate formation. The inability of the mutant to sequester heme in the aggregates may contribute to the observed sensitivity of the strain to heme toxicity. Overall, these results provide new insights into heme utilization and storage in C. neoformans.
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................... ii  
TABLE OF CONTENTS ............................................................................................................... iii  
LIST OF TABLES .......................................................................................................................... vi  
LIST OF FIGURES ....................................................................................................................... vii  
LIST OF ABBREVIATIONS ......................................................................................................... ix  
AKNOWLEDGEMENTS ................................................................................................................... x  
DEDICATION ..................................................................................................................................... xi  

1. INTRODUCTION ..................................................................................................................... 1  

1.1 *Cryptococcus neoformans*: Epidemiology, geographic distribution, route of infection, and virulence factors ................................................................. 1  
1.2 The importance of iron ........................................................................................................ 5  
1.3 Iron uptake in bacteria and fungi ...................................................................................... 6  
1.4 The role of iron in *C. neoformans* ................................................................................. 11  
1.5 Heme as an iron source ..................................................................................................... 14  
1.6 Thesis objectives ............................................................................................................... 21  

2. MATERIALS AND METHODS ............................................................................................. 22  

2.1 Strains, media, and growth conditions .............................................................................. 22  
2.2 Construction of mutant strains ......................................................................................... 23  
2.3 Web Resources .................................................................................................................. 26  
2.4 Genomic DNA isolation ..................................................................................................... 26  
2.5 Plate assays with different iron sources ........................................................................... 26  
2.6 Other phenotype assays .................................................................................................... 27  
2.7 Determination of culture growth and supernatant pH changes ..................................... 27  
2.8 Confirmation of the *vps41* mutant response to starvation stress ................................. 28  
2.9 Quantitative real-time PCR ............................................................................................. 28  
2.10 Growth curves and colour photography of cells ......................................................... 30  
2.11 Examination of internal heme localization by fluorescence microscopy ..................... 30  

3. RESULTS ................................................................................................................................... 31  

3.1 Characterization of the *HMX1* gene ................................................................................. 31
3.2 Construction and characterization of hmx1 mutants................................................ 36
3.3 HMX1 does not affect the expression of virulence factors ........................................ 37
3.4 HMX1 is not required for growth with heme as a sole iron source .................... 38
3.5 A deletion of HMX1 does not affect growth under other various conditions of stress and nutrition ........................................................................................................ 41
3.6 A liquid culture of hmx1 exhibits a change in supernatant pH and colour when grown in the presence of heme .................................................................................... 45
3.7 The loss of HMX1 does not affect virulence in a mouse model of infection .......... 47
3.8 HMX1 transcription is not affected by heme sources or temperature ................ 47
3.9 Loss of the high-affinity iron uptake system did not reveal additional phenotypes in the hmx1 mutant ................................................................................................. 50
3.10 Construction and characterization of VPS41 mutants ......................................... 52
3.11 The vps41 mutants showed increased susceptibility to nutrient starvation ................................................................................................................................. 55
3.12 Loss of VPS41 did not affect the expression of virulence factors .................... 56
3.13 VPS41 is required for iron acquisition from both inorganic iron and heme ................................................................................................................................. 58
3.14 A deletion of VPS41 caused sensitivity to excess iron and heme ..................... 58
3.15 A loss of VPS41 caused reduced growth in liquid media with inorganic iron and heme .................................................................................................................... 60
3.16 Heme was sequestered in wt cells but appeared in a diffuse pattern in vps41 mutants ................................................................................................................................. 63
3.17 Internal heme localization could be visualized with fluorescence microscopy using heme or the heme analog zinc mesoporphyrin IX ........................................ 67

4. DISCUSSION ..................................................................................................................... 69
4.1 Hmx1 has characteristics of a heme oxygenase but is not required for growth with heme ......................................................................................................................... 70
4.2 HMX1 regulation suggests a role in heme recycling and metabolic changes in the cell ................................................................................................................................. 73
4.3 The role of \textit{VPS41} in iron acquisition...............................................................78
4.4 The role of \textit{VPS41} in heme utilization and storage........................................80
4.5 Heme storage may involve heme crystallization................................................81
4.6 A model for heme and iron utilization in relation to the vacuole .........................84
4.7 Proposed future studies to examine the use of heme by \textit{C. neoformans} ..........85
  4.7.1 Identification of the heme uptake and degradation systems.......................85
  4.7.2 Further analysis of the role of \textit{HMX1} .........................................................86
  4.7.3 Characterization of heme-related aggregates..............................................87
  4.7.4 The role of other vacuolar proteins in heme utilization
     and storage......................................................................................................88
  4.7.5 Examination of capsule shedding in relation to possible
     excess heme excretion ..................................................................................89
4.8 Overall conclusions..............................................................................................89

REFERENCES.............................................................................................................91

APPENDIX A ...............................................................................................................102

APPENDIX B...............................................................................................................109
# LIST OF TABLES

| Table 2.1 | C. neoformans strains used in this study ............................................................. 23 |
| Table 2.2 | List of primers used to create deletion mutants in this study ......................... 25 |
| Table 2.3 | List of primers used for Real Time PCR analysis ................................................. 29 |
| Table 3.1 | Comparison of *C. neoformans* Hmx1 with homologous heme oxygenases from mammals and fungi ................................................................. 32 |
| Table 3.2 | Comparison of *C. neoformans* Hmx1 with putative heme oxygenases from basidiomycete fungi ................................................................. 35 |
| Figure 3.1 | Sequence alignment of mammalian and bacterial heme oxygenases with Hmx1 | 33 |
| Figure 3.2 | Sequence alignment of characterized fungal heme oxygenases with Hmx1 | 34 |
| Figure 3.3 | Genomic arrangement, structure of deletion constructs, and conformation of deletion of HMX1 | 36 |
| Figure 3.4 | Two hmx1 mutants are confirmed by Southern blot | 37 |
| Figure 3.5 | hmx1 mutants do not show changes in virulence-associated phenotypes compared to the wild type | 38 |
| Figure 3.6 | hmx1 mutants do not have heme or iron related growth defects | 40 |
| Figure 3.7 | The hmx1 strain does not have diminished growth in liquid media containing heme or iron | 41 |
| Figure 3.8 | hmx1 mutants do not have increased sensitivity to oxidative or nitrosative stress | 42 |
| Figure 3.9 | Wild type and hmx1 mutants grow similarly on all tested carbon sources | 44 |
| Figure 3.10 | The culture supernatant of wild type and hmx1 mutants has colour and pH differences when cells are grown with heme | 46 |
| Figure 3.11 | Influence of heme source and temperature on HMX1 transcript levels in wt C. neoformans | 48 |
| Figure 3.12 | Quantitative RT-PCR analysis of iron-related genes in wild type and hmx1 strains | 49 |
| Figure 3.13 | Two cfo1hmx1 double mutants are confirmed by PCR | 51 |
| Figure 3.14 | A deletion of both CFO1 and HMX1 did not reveal additional heme-related growth defects | 52 |
| Figure 3.15 | Genomic arrangement, structure of deletion constructs, and conformation of deletion of VPS41 | 54 |
| Figure 3.16 | Three vps41 mutants were confirmed by genomic hybridization | 55 |
Figure 3.17  The *vps41* mutants had increased susceptibility to nutrient starvation...........56
Figure 3.18  The *vps41* mutants did not show changes in virulence-associated
phenotypes compared to the wt strain.................................................................57
Figure 3.19  The *vps41* mutation caused heme and iron-related growth defects...........59
Figure 3.20  Growth of wild type and *vps41* mutants in liquid media containing
inorganic iron ......................................................................................................61
Figure 3.21  Growth of the wt strain and *vps41* mutants in liquid media
containing heme.................................................................................................62
Figure 3.22  Heme and iron uptake was altered in *vps41* mutants...............................66
Figure 3.23  Visualization of heme and zinc mesoporphyrin IX (ZnMP) localization
by fluorescence microscopy...............................................................................68
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>BPS</td>
<td>Bathophenanthroline Disulfonate</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon Monoxide</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential Interference Microscopy</td>
</tr>
<tr>
<td>DOPA</td>
<td>3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic DNA</td>
</tr>
<tr>
<td>GXM</td>
<td>Glucuronoxylmannan</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HO</td>
<td>Heme Oxygenase</td>
</tr>
<tr>
<td>HOPS</td>
<td>Homotypic Fusion and Vacuole Protein Sorting</td>
</tr>
<tr>
<td>LI</td>
<td>Low Iron</td>
</tr>
<tr>
<td>LIM</td>
<td>Low Iron Media</td>
</tr>
<tr>
<td>NAT</td>
<td>Nourseothricin Acetyltransferase</td>
</tr>
<tr>
<td>NEO</td>
<td>Neomycin</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PPIX</td>
<td>Protoporphyrin IX</td>
</tr>
<tr>
<td>SAGE</td>
<td>Serial Analysis of Gene Expression</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>YNB</td>
<td>Yeast Nitrogen Base</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast Peptone Dextrose</td>
</tr>
<tr>
<td>ZnMP</td>
<td>Zinc Mesoporphyrin IX</td>
</tr>
</tbody>
</table>
AKNOWLEDGEMENTS

I would first like to thank Dr. Jim Kronstad for his guidance, kindness, and support from start to finish of graduate school. I would also like to thank my committee, Dr. Steven Hallam and Dr. Michael Murphy for their insightful comments and feedback.

I would also like to thank the people that provided me with help and technical advice over the last several years: Dr. Wonhee Jung, for his expertise on iron as well as the donation of mutants and primers, Dr. Horacio Bach, for his valuable assistance and guidance in many things, Matthias Kretschmer, for his help with RT-PCR, and Anita Sham for her assistance in virulence assays. I would also like to thank the entire Murphy lab, for their help and generosity with supplies, and particularly Jason Grigg, who always answers all of my questions. Furthermore, I would like to thank members of the Kronstad, past and present: Emma, Jennifer, Guanggan and Joyce, and especially Brigitte, Po-Yan and Iris, for their advice, troubleshooting, and friendship.

Outside of the lab, I would like to thank my parents and Brian for all of their love, support and patience. Special thanks to the member of the Brotherhood without Banners for their endless support and encouragement, for teaching me so much about the world, and for making me laugh no matter what.
This thesis is dedicated to the members of TTTNE
1. INTRODUCTION

1.1 *Cryptococcus neoformans*: Epidemiology, geographic distribution, route of infection, and virulence factors

*Cryptococcus neoformans* is an opportunistic fungal pathogen capable of causing disease in humans and other mammals. In the environment, *C. neoformans* is found throughout the world in multiple sources including soil, trees, and bird excreta (especially that of pigeons) (Idnurm et al., 2005). The fungus is inhaled from the environment as dehydrated yeast cells or basidiospores, causing initial infection in the lungs. The pulmonary infection often does not cause symptoms in the host, and may either be cleared or become a latent infection restricted in granulomas. Cells within the granuloma can remain dormant indefinitely without causing disease. Serological studies show that the majority of adults have antibodies against cryptococcal antigens, indicating that exposure to *Cryptococcus* is common and most infections are asymptomatic (Chen et al., 1999; Deshaw and Pirofski, 1995; Goldman et al., 2001). However, if host immunity is suppressed or compromised, the dormant cells can become active and disseminate throughout the body to any organ, with an affinity for the brain (Bicanic and Harrison, 2005). Because of this predilection for the brain, *C. neoformans* proliferates in the central nervous system and causes cryptococcal meningitis, which is fatal if left untreated (Buchanan and Murphy, 1998).

Incidents of cryptococcal infection have increased greatly over the last thirty years due to the rise of immunocompromised people being treated for HIV infection, cancer, and organ transplantation (Lin, 2009). Indeed, cryptococcal meningitis is recognized as an AIDS-related infection, and is responsible for up to 30% of deaths of AIDS patients in
underdeveloped regions such as Southeast Asia and Sub-Saharan Africa (Bicanic and Harrison, 2005; Casadevall and Perfect, 1998; Lin, 2009; Perfect, 2005). Globally, over 950,000 AIDS-related cases of cryptococcal meningitis occur each year, and 624,700 of these cases are fatal by three months after infection (Park et al., 2009). Cases in the United States and other Western countries have decreased with the availability of highly active antiretroviral therapy (HAART), but cryptococcal infection is still responsible for 5% of AIDS-related death in the USA (Mirza et al., 2003).

While most *C. neoformans* infections occur in immunocompromised individuals, infection can also occur in those with seemingly normal immune systems. *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) cause the vast majority of cryptococcal disease, mainly in the immune-compromised population (Lin, 2009). *Cryptococcus gattii* (serotypes B and C) was recently raised to species level (Kwon-Chung and Varma, 2006) and is most often the cause of infection in individuals with normal immune systems. It was originally thought to be restricted to tropical and subtropical regions of the world; however an outbreak began recently on Vancouver Island in British Columbia, Canada, which has a temperate climate. The outbreak, which began in 1999, has infected over 200 people and caused 8 deaths. Large numbers of domestic and wild animals have also been infected (Fyfe et al., 2008; MacDougall et al., 2007). *C. gattii* cases have recently been reported on the mainland of British Columbia as well as in the States of Washington and Oregon, indicating that the area of the disease is spreading (Byrnes et al., 2009; MacDougall et al., 2007).
While *C. gattii* has put the spotlight on cryptococcal infections, serotype A of *C. neoformans* is still the most predominant serotype among clinical samples, and is responsible for over 95% of cryptococcosis cases worldwide (Chayakulkeeree and Perfect, 2006; Lin, 2009). The study in this thesis focuses on the biology of a representative serotype A isolate.

Several antifungal drugs are available to treat cryptococcosis, including amphotericin B, and fluconazole. However, the available antifungals are either lacking in efficacy or can cause toxicity in the host. Clinical drug resistance also occurs. The drugs are less effective in developing countries due to problems with distribution, storage and delivery to patients. Even in a hospital setting in medically advanced countries, cryptococcosis causes mortality in 10 to 25% of cases (Perfect and Casadevall, 2002). Treating fungal disease is a challenge because of the similar cellular machinery between humans and fungi, leaving fewer drug targets available than in the treatment of bacterial diseases or viruses (Idnurm *et al.*, 2005).

Cryptococcus is an environmental saprophyte with ‘ready-made’ virulence; it is capable of causing disease in mammalian hosts directly from the environment and does not require replication within a host as an essential component of its life cycle (Casadevall *et al.*, 2003). *C. neoformans* has a number of virulence factors that contribute to its ability to cause disease in the host. The three major virulence factors are the ability to grow at 37˚C, the production of a capsule, and melanin formation (Casadevall and Perfect, 1998).

The first virulence factor, the ability to grow at 37˚C, is the most direct, as the ability to survive and replicate at mammalian body temperature is an obvious requirement for invasive pathogenesis. However, very few species of fungi possess this ability and thus cannot cause
disease. In fact, *C. neoformans* and *C. gattii* are the only pathogenic members of the *Cryptococcus* genus (Perfect, 2005).

The second major virulence factor is the production of a polysaccharide capsule. This capsule is unique to *Cryptococcus* and a distinct feature of the pathogen (McFadden *et al.*, 2006). The capsule is comprised mainly of glucuronoxylomannan (GXM) and is an essential virulence factor that has multiple effects during infection (Perfect, 2005). Acapsular mutants are much less virulent than wild-type strains (Chang and Kwon-Chung, 1994). The capsule has anti-phagocytotic properties, and also promotes survival within macrophages if cells are engulfed (Janbon, 2004). The capsule also has the ability to down-regulate both the cellular and the humoral immune systems. It also interferes with immune responses when it is shed into host tissues (Bicanic and Harrison, 2005). For example, the capsule can deplete complement proteins in the host (Janbon, 2004), as well as block the recruitment of inflammatory cells, thereby increasing the ability of the pathogen to evade the host immune system. Capsule size can be influenced by the availability of iron, as well as by CO$_2$ levels, serum, and location within host tissues (Janbon, 2004).

The third major virulence factor is the production of melanin, a brown or black pigment that is produced by one or more laccase enzymes. Laccase converts diphenolic compounds such as catecholamines into melanin. During infection, melanin can interfere with ingestion by macrophages (Casadevall *et al.*, 2000), and can help prevent oxidative killing by phagocytes if the cryptococcal cells are engulfed (Bicanic and Harrison, 2005). Melanin can also play a role in interfering with antifungal susceptibility, and can provide protection against extreme temperatures (Perfect, 2005). While wild-type cells become brown on
certain agar media, such as DOPA or Niger Seed agar, laccase mutants remain white in colour and are attenuated for virulence in the mouse model, indicating an important role for melanin production \textit{in vivo} (Salas \textit{et al.}, 1996).

Two of \textit{C. neoformans'} major virulence factors, the capsule and melanin production are regulated by iron, a key nutrient for survival and pathogenesis (Lian \textit{et al.}, 2005; Jung \textit{et al.}, 2006). The next section will discuss the importance of iron for cellular metabolism and during infectious disease.

\textbf{1.2 The importance of iron}

Virtually all organisms have a nutritional requirement for iron. Iron can be found alone in the cell or incorporated into several complexes, including iron-sulphur clusters and heme. The metal has two readily available positively charged ionic forms, Fe(II) (ferrous iron) and Fe(III) (ferric iron), and the ability to readily gain or lose electrons makes iron an excellent co-factor for oxidation-reduction (redox) reactions (Howard, 1999; Wandersman and Delepelaire, 2004). It is involved in many essential processes including respiration, electron transport, deoxyribonucleotide synthesis, photosynthesis, and the synthesis of some amino acids, sterols, and lipids (Philpott, 2006; Wandersman and Delepelaire, 2004). Despite being the second most abundant metal on earth, free iron in the environment is generally biologically unavailable. In aerobic environments, iron quickly becomes oxidized to the ferric form which forms insoluble compounds. The available concentration of free iron is lower than the requirement for the survival of most microorganisms, so microbes have evolved a number of strategies to overcome the problem of iron unavailability.
Overall, there are three major strategies to increase iron solubility: acidification of the environment, reduction of Fe(III) to Fe(II), and secretion of soluble molecules that chelate iron (Philpott, 2006). These strategies are often used in combination. Pathogenic microorganisms face an additional challenge within the host. At neutral pH and aqueous conditions within a mammalian host, iron is much more soluble than in the environment (Wandersman and Delepelaire, 2004). However, mammals can tightly control their iron stores, eliminating any free iron in blood, plasma and extracellular fluids; the concentration of free iron is maintained at about $10^{-18}$ M (Bullen et al., 2006; Philpott, 2006). This low iron environment is maintained by circulating transferrin and lactoferrin, which are only saturated with iron up to 40% of their capacity, and which bind free iron with a high stability constant (Bullen et al., 2006). Pathogens have therefore developed strategies to obtain iron from mammalian proteins such as transferrin and hemoglobin. The struggle between hosts to sequester and protect their iron stores and pathogens to obtain that iron for growth and survival within the host is a key component of many bacterial and fungal infections.

1.3 Iron uptake in bacteria and fungi

Iron uptake in bacterial pathogens has been well studied, and much is known about their various uptake systems. There are two general mechanisms employed by bacteria for iron uptake, which can be classified as direct or indirect (reviewed in Ratledge and Dover, 2000; Wandersman and Delepelaire, 2004). Direct uptake involves intimate contact between the cell surface and the available iron source. This system requires active transport by cell surface receptor proteins, a key example of which is the TonB complex (Ratledge and Dover, 2000). Additional information on bacterial iron uptake is provided in section 1.5 below.
Indirect uptake occurs by way of the secretion of siderophores. Siderophores are low molecular weight compounds with high specificity and affinity for iron (Johnson, 2008). Over 500 different siderophores have been identified, mainly from bacteria. They are synthesized by the cell under conditions of iron starvation, and secreted outside of the cell. The function of siderophores is to chelate iron from extracellular compounds and proteins, such as within a host body, and then return the captured iron to the bacterial cell via specific siderophore receptors (Johnson, 2008; Wandersman and Delepelaire, 2004). The iron is released intracellularly and the iron-free siderophore is either broken down or re-released out of the cell. These high affinity iron uptake systems allow pathogens to acquire iron sequestered by the host and are often required for virulence.

While the uptake of iron is key for bacterial survival, excess free iron is toxic to cells. Free iron causes the production of free radicals via the Fenton reaction which can damage cells, so iron uptake and storage must be tightly regulated. Iron uptake systems are often repressed under iron-replete conditions (Wandersman and Delepelaire, 2004). Bacteria also contain ferritins, a family of spherical iron storage proteins. Bacterial ferritins may contain a heme molecule, and are then known as bacterioferritins. Ferritins are made up of 24 subunits that form a hollow sphere-like structure and can store thousands of Fe(III) ions (Carrondo, 2003). Ferritins store iron to prevent toxicity from radicals and for availability during periods of iron starvation.

Fungi also have a variety of methods to acquire and store iron, and the diversity for different fungal species reflects the need to ensure that iron is available while dealing with a variety of environmental challenges. The best studied fungus is the model organism
*Saccharomyces cerevisiae* (reviewed in Kosman, 2003), but other fungi such as *Candida albicans* and *Aspergillus fumigatus* have also been examined in some detail with regard to iron acquisition. *S. cerevisiae* has a high affinity reductive uptake system that consists of cell-surface reductases as well as Ftr1, an iron permease, and Fet3, a multicopper ferroxidase. This system reduces ferric iron to the ferrous form and then the Ftr1-Fet3 complex transports it across the plasma membrane with concomitant oxidation (Kosman, 2003; Philpott, 2006). *C. albicans* also has a *FTR1* ortholog, *CaFRT1*, which is required for inorganic iron and transferrin uptake and is also required for virulence (Knight *et al.*, 2005). In contrast, *A. fumigatus* also contains an Ftr1 homolog, called FtrA, but it is not required for virulence *in vivo* (Schrettl *et al.*, 2004).

Low affinity iron uptake is also used by some fungi to acquire iron, although it has not been well studied. *S. cerevisiae* has a transport protein called Fet4 that can transport Fe(II) into the cell with very low affinity, but also low specificity (Dix *et al.*, 1997). The yeast also expresses three divalent metal ion transporters, Smf1, Smf2, and Smf3, which can play a role in low-affinity uptake (Portnoy *et al.*, 2000).

Like bacteria, some species of fungi can secrete siderophores that bind iron with very high affinity. The iron-siderophore complexes are then imported into the cell via specific receptors (recently reviewed in Haas *et al.*, 2008). Some fungi, such as *A. fumigatus*, use siderophores for essential iron uptake during infection (Schrettl *et al.*, 2004, Hissen *et al.*, 2005). The siderophore synthesis gene SidA is required for survival of *A. fumigatus* in a mouse model of infection, indicating the importance of siderophores for iron uptake during disease (Schrettl *et al.*, 2004). In addition to their function in iron scavenging, siderophores
have been reported to be produced internally to act as iron storage molecules in *A. fumigatus* (Wallner *et al.*, 2009). Interestingly, several species of fungi are unable to synthesize or secrete their own siderophores, but have transporters for siderophore uptake (reviewed in Haas, 2003; Haas *et al.*, 2008). *S. cerevisiae* has four siderophore transporters, with varying degrees of specificity for different siderophores, including Arn3/Sit1, which has high affinity for the siderophore ferrioxamine B (Kosman, 2003). *C. albicans* also does not secrete siderophores, but has a similar set of receptors to *S. cerevisiae*. In *C. albicans*, Arn1/Sit1 is not required for virulence in a murine model, but is required for epithelial cell invasion (Heymann *et al.*, 2002). Presumably, these siderophore uptake receptors allow these fungi to scavenge iron from the siderophores of other microorganisms in the environment or during infections containing mixed populations, thus providing the fungi with an advantage.

One internal component of the cell that is involved in iron trafficking, storage, and degradation of iron is the endomembrane system, specifically the vacuole. The yeast vacuole is an acidic compartment that functions in metabolite storage, as well as ion and pH homeostasis. The acidic pH is required for the activity of the many hydrolases that act within the vacuole, making it the major cellular recycling center (Ostrowicz *et al.*, 2008). The vacuole plays an important role in the cell during conditions of starvation, and for the degradation of receptors on the cell surface, allowing the cell to respond and adjust to changes in extracellular signals (Ostrowicz *et al.*, 2008). Both the endocytic and secretory pathways target cargo to the vacuole, making it a key player in membrane and protein trafficking.
The role of the vacuole in metal storage and homeostasis has been shown in *S. cerevisiae* and requires genes in the vacuole protein sorting (VPS) family (Szczypta *et al.*, 1997). Mutations in *VPS* genes cause increased copper sensitivity and altered levels of Fet3, indicating that an intact vacuole is required for metal-related functions in *S. cerevisiae*. Another VPS protein, Vps41, has also been shown to play a key role in vacuolar trafficking and high affinity iron transport in yeast (Radisky *et al.*, 1997). Vps41 is a vacuolar membrane protein that is part of the homotypic fusion and vacuole protein sorting (HOPS) complex. It is required for membrane docking and fusion of the golgi-to-endosome and endosome-to-vacuole stages of protein transport (Nakamura *et al.*, 1997; Seals *et al.*, 2000). Vps41 is regulated by phosphorylation, and this phosphorylation is required for endosomal fusion with the vacuole (Cabrera *et al.*, 2009). A deletion of *vps41* causes a fragmented vacuole and abnormalities in post-golgi trafficking of vacuolar components (Nakamura *et al.*, 1997; Radisky *et al.*, 1997). In *S. cerevisiae*, a *vps41* mutant also grows poorly when inorganic iron is provided as the sole iron source (Radisky *et al.*, 1997), implicating vacuolar function in the uptake or utilization of iron.

In *S. cerevisiae*, post-transcriptional regulation of the high affinity iron uptake proteins Fet3 and Frt1 also depends on intracellular membrane trafficking and vacuolar function. When cells are starved for iron, Fet3-Frt1 is expressed constantly on the plasma membrane due to recycling by the endocytic pathway (Felice *et al.*, 2005; Strochlic *et al.*, 2007). However, when starved cells are exposed to iron, Fet3-Frt1 is rapidly internalized to the vacuole and degraded. This occurs by the proteins being internalized into vesicles that are then targeted to the vacuole for degradation. In the absence of iron, the vesicles are sorted back from the endosome to the golgi, and then returned to the plasma membrane (Strochlic *et
A defect in endocytosis causes more Fet3-Ftr1 to remain in the plasma membrane even under high-iron conditions, and a defect in a vacuolar protease causes a build-up of Fet3-Frt1 in the vacuole due to the inability to degrade the proteins (Felice et al., 2005). The iron growth defects seen in vps41 deletion strains may be related to this high affinity uptake regulation, or they may be caused by additional vacuolar defects; regardless, these studies indicate a role for trafficking and vacuolar function with relation to iron homeostasis in yeast.

*C. neoformans* also has a number of iron uptake systems, many of which have similarities to the fungal systems previously discussed. The current knowledge of iron uptake in *C. neoformans* will be examined next.

### 1.4 The role of iron in *C. neoformans*

In *C. neoformans*, serial analysis of gene expression (SAGE) was performed to examine the transcriptome under low-iron and iron-replete growth conditions to identify iron-responsive genes (Lian et al., 2005). The results revealed that metabolism (including glucose and nitrogen utilization, and respiration) appears to be substantially remodeled under conditions of iron deprivation in *C. neoformans*. The analysis of the SAGE data also revealed that the genome contains orthologues of the *FTR1* and *FET3* genes, responsible for high affinity iron uptake in *S. cerevisiae*, and that both genes are induced upon iron limitation (Lian et al., 2005). The SAGE data provided a window into the importance of iron in *C. neoformans* for a multitude of cellular functions.

The *FTR1* and *FET3* genes, called *CFT1* and *CFO1* respectively in *C. neoformans*, have been studied in detail (Jung et al., 2008; Jung et al., 2009). Cft1, the high-affinity iron permease, is required for iron uptake from inorganic iron (FeCl$_3$) as well as from transferrin
(Jung et al., 2008) A deletion of cft1 does not have any affect on growth with siderophores or heme as the sole iron sources, indicating that the pathways for these iron sources are independent of high affinity iron uptake. The cft1 mutant is also attenuated for virulence in a mouse model of infection (Jung et al., 2008). CFO1, encoding the multicopper oxidase, shows very similar iron-related defects to cft1 when deleted, and is also attenuated for virulence in the mouse model (Jung et al., 2009). However, virulence is not eliminated in either mutant, which indicates that C. neoformans is able to utilize additional iron sources or uptake systems, such as heme, or a low-affinity uptake system. Low affinity uptake of iron has been described (Jacobson et al., 1998), and cfo1 mutants grow well with high concentrations of iron (Jung et al., 2008), indicating that low affinity transport does occur, but the details and mechanism are currently unknown.

Like S. cerevisiae and C. albicans, C. neoformans does not appear to produce its own siderophores, but is able to utilize exogenous siderophores (Jacobson and Petro 1987). SAGE analysis of C. neoformans also led to the characterization of SIT1, a putative siderophore transporter (Tangen et al., 2007). This gene was required for growth on the siderophore ferrioxamine B, and a deletion of the gene reduced growth in low iron media (LIM). Sit1 mutants also had increased laccase activity, and in a serotype D background, mutants had altered melanin production and increased temperature sensitivity. Sit1 may play a role in intracellular metal homeostasis, and the loss of SIT1 may affect copper loading into laccase. However, Sit1 is not required for virulence in the mouse model of cryptococcosis (Tangen et al., 2007). Bacterial siderophores are not likely to always be found in the host during infection, and it is likely that other iron uptake systems are able to acquire sufficient iron during infection even without one or more siderophore transport genes.
As mentioned above, the role of iron in many cellular processes was indicated by SAGE analysis of the transcriptome in *C. neoformans* cells from minimal and replete iron conditions (Lian *et al.*, 2005). Iron uptake and internal iron regulation are key events that need to be tightly regulated within the cell. In *S. cerevisiae*, iron genes are regulated by the transcriptional activators Aft1 and Aft2 (Blaiseau *et al.*, 2001; Yamaguchi-Iwai *et al.*, 1995). *C. albicans* uses the transcriptional repressor Sfu1 to regulate iron-responsive genes, and other fungi have similar repressors. Some of these activators and repressors, such as Sfu1 (but not Aft1 and Aft2), have characteristics of GATA-type transcription factors. In *C. neoformans*, the GATA-type transcription factor Cir1 was identified by sequence similarity with other fungal iron regulatory proteins. A deletion of *cir1* caused a defect in all major virulence factors (growth at 37°C, melanin and capsule production) (Jung *et al.*, 2006). Microarray analysis of *cir1* mutants revealed that Cir1 is both a transcriptional activator and repressor, and that it regulates the majority of iron responsive genes. The *cir1* mutants are also completely avirulent in the mouse model of cryptococcosis, suggesting that Cir1 is a both key regulator of virulence factor expression and that iron regulation plays an important role during infection (Jung *et al.*, 2006).

The role of Vps41 and the vacuole was examined in *C. neoformans* to determine if these functions are involved in growth on inorganic iron as found in *S. cerevisiae* (Liu *et al.*, 2006). However, no growth defects were seen when cells were grown on a rich yeast extract medium that was reduced in available iron by addition of a chelator (Liu *et al.*, 2006). The *vps41* mutants showed loss of viability under conditions of nutrient starvation, and while the mutant had no reduction of virulence factor expression *in vitro*, there was dramatic attenuation of virulence in the mouse model of cryptococcosis (Liu *et al.*, 2006). While the
role of the vacuole in iron utilization in *C. neoformans* may be unclear due to the experimental conditions used for the initial analysis of Vps41 by Liu *et al.*, (2006), it is evident by the attenuation of virulence that an intact vacuole plays an important role during infection. A more detailed examination of the role of Vps41 was conducted as part of the study in this thesis.

### 1.5 Heme as an iron source

Heme is an iron-containing protoporphyrin IX molecule, which is essential for the function of many fundamental processes in the cell, particularly in aerobic growth (Li and Stocker, 2009; Ponka, 1999). Heme refers to the ferrous iron (Fe(II)) form of the molecule, while the commercially available oxidized form contains ferric iron (Fe(III)), and is called hemin. However, both of these molecules are commonly referred to as heme, and that convention will be followed here (Tong and Guo, 2009). Heme acts as a prosthetic group for a large number of proteins, known as hemoproteins, including hemoglobin, myoglobin, and cytochromes. These heme containing proteins have a wide range of functions and are involved in many cellular pathways and reactions (Li and Stocker, 2009; Ponka, 1999). Many organisms are capable of heme synthesis, creating heme molecules for use in hemoproteins, although some bacteria are unable to synthesize the porphyrin ring. For example, *Haemophilus influenza* and *Enterococcus faecalis* must rely on exogenous heme as a protoporphyrin source (Wandersman and Delepelaire, 2004). Intracellular heme levels are tightly controlled due to toxicity of excess free heme, which is toxic to cells because it can insert into membranes due to its hydrophobic nature, and the heme-iron can cause non-enzymatic redox reactions (Wandersman and Delepelaire, 2004).
The abundance of heme within host organisms also causes heme to be an important source of iron for pathogens. In fact, heme is the most abundant source of iron in the human body, with over two-thirds of iron located in heme molecules within hemoglobin (Li and Stocker, 2009). However, to be used as an iron source, heme must first be degraded (Wandersman and Delepelaire, 2004). Pathogenic organisms have evolved several strategies to acquire heme from the host to use as an iron source. Strategies for heme uptake and degradation will be reviewed below for bacteria and fungi.

Heme uptake in bacteria was recently reviewed in detail (Tong and Guo, 2009). Uptake systems in gram-negative bacteria have been more thoroughly studied than those of gram-positive bacteria. There are two major systems. The first involves the direct binding of heme or hemoproteins to specific outer membrane receptors, which then transport the heme into the cell via ABC transporters. Direct heme uptake genes are regulated by Fur, the ferric uptake regulator. This system is best studied in *Yersinia pestis*, *Y. enterocolitica*, *Pseudomonas aeruginosa*, and *Shigella dysenteriae* (Tong and Guo, 2009). The second system is similar to the siderophore system used for inorganic iron uptake. It involves the secretion of hemophores which are small, specialized proteins that bind and acquire extracellular heme from hemoproteins for subsequent uptake by specific outer membrane receptors on the cell surface (Wandersman and Delepelaire, 2004). Transport across the outer membrane dependent on the activity of TonB, and heme is transported into the cell using an ABC transporter. The most common hemophore is HasA, which is found in many Gram-negative bacteria including *Serratia marcescens*, *P. aeruginosa*, *P. fluorescenes*, *Y. pestis*, and *Y. enterocolitica* (Tong and Guo, 2009; Wandersman and Delepelaire, 2004). An additional hemophore, HxuA, was identified in *Haemophilus influenzae* and is required for
heme-hemopexin utilization (Tong and Guo, 2009; Wandersman and Delepelaire, 2004). Heme uptake systems in gram-positive bacteria are less well studied, and to date very few hemophore systems have been discovered. The first Gram-positive hemophore system was discovered recently in Bacillus anthracis and is made up of two secreted proteins that scavenge heme from hemoglobin (Maresso et al., 2008). The direct uptake strategy is similar to that in gram-negative bacteria, but due to the thick cell wall of Gram-positive bacteria, the binding proteins must be anchored in the cell wall (Ratledge and Dover, 2000). The best studied Gram-positive system is the iron-response surface determinant (Isd) locus in Staphylococcus aureus (Maresso and Schneewind, 2006; Mazmanian et al., 2003). This gene cluster encodes cell wall anchored proteins that can bind heme, hemoglobin, and hemoglobin-haptoglobin, as well as a membrane transport system and two heme oxygenases (Maresso and Schneewind, 2006). Heme is stripped from hemoproteins and relayed through the cell wall to the membrane, where it is transported across the membrane into the cytosol.

Heme uptake in fungi is less well studied than in bacteria, but several fungal systems have been examined. S. cerevisiae, the model system for yeast, is not capable of using exogenous heme as an iron source, and does not take up heme in iron limited conditions (Weissman et al., 2002). However, heme uptake is increased in mutants deficient in heme synthesis, as well as under hypoxic conditions, although the uptake system is unknown (Protchenko et al., 2008). Histoplasma capsulatum is able to grow when heme is provided as a sole iron source, but the uptake system has not yet been identified (Foster, 2002). Uptake is best understood in Candida albicans. The membrane protein Rbt5 and its close homolog Rbt51 are extracellular membrane anchored proteins that are involved in heme and hemoglobin uptake (Weissman and Kornitzer, 2004; Weissman et al., 2008). A deletion of
**RBT5** greatly reduces the ability of *C. albicans* to use heme and hemoglobin as iron sources (Weissman and Kornitzer, 2004). Rbt5 and Rbt51 are short lived in the plasma membrane, and are degraded in the vacuole (Weissman *et al.*, 2008). Rbt5 facilitates the endocytosis of hemoglobin to the vacuole. The exact mechanism is unclear, but heme/hemoglobin may be internalized as a complex with Rbt5/51 and delivered to the vacuole, where heme and/or iron are released (Weissman *et al.*, 2008). The role of endocytosis and the vacuole in hemoglobin degradation in *C. albicans* is another piece of evidence that the vacuole and trafficking systems may play a key role in iron utilization in fungi.

Regardless of the heme uptake system, once heme has been transported into the cytosol, it must be degraded to release the iron for use by the cell. The most common enzyme for heme degradation is heme oxygenase (HO), which catalyzes the oxidative cleavage of heme to biliverdin, carbon monoxide (CO) and iron, in the presence of oxygen and an electron donor (Montellano, 2000). This is a multistep process which produces hydroxyheme and verdoheme as reaction intermediates. Although heme oxygenase is present in many diverse organisms, the catalytic mechanism for heme breakdown is essentially the same for most of them. In mammals, biliverdin reductase converts the biliverdin into bilirubin which is then further modified before it is eventually secreted.

A number of HO isoforms exist across various species. Plants can express several isoforms, while bacteria and fungi usually contain one or sometimes two HO isoforms. Animals can also express one or two isoforms, with mammals generally expressing two, HO-1 and HO-2 (Kim *et al.*, 2006; Li and Stocker, 2009). HO-1 is found predominantly in the liver and spleen, and is inducible by a variety of stresses and physical factors, including heme itself. HO-1 is involved in the response to oxidative stress, and is a major regulator of
whole-body and cellular heme and iron homeostasis (Reviewed in Li and Stocker, 2009, Ryter and Choi, 2009). Excess heme is degraded, and the iron is recycled by the body. HO-2 is constitutively expressed throughout the body, and is unresponsive to induction. It is found mainly in the brain, vascular system, and testis, and is involved in the production of CO for use as a neurotransmitter.

In bacteria, HO is used to cleave heme to release iron for use in cellular functions. Most bacterial HOs have a similar mechanism of ring cleavage to the mammalian HOs. Heme oxygenases have been isolated and characterized from both gram-positive and gram-negative bacteria. The first prokaryotic HO to be characterized was HmuO from the gram-positive bacterium *Corynebacterium diphtheriae* (Schmitt, 1997). HmuO has 33% identity and 70% similarity to human HO-1. Since then, HOs have been identified and characterized in many bacteria, including *Neisseria gonorrhoeae, N. meningitides* (Zhu *et al.*, 2000a; Zhu *et al.*, 2000b), *P. aeruginosa* (Ratliff *et al.*, 2001), *Campylobacter jejuni* (Ridley *et al.*, 2006), *Leptospira interrogans* (Murray *et al.*, 2008), *E. coli* O157:H7 (Suits *et al.*, 2005), and *S. aureus* (Wu *et al.*, 2005). While many of these HOs have a high degree of sequence and/or structural homology to mammalian HO-1, the HO ChuS from *E. coli* has unique sequence and structural features (Suits *et al.*, 2005). IsdG and IsdI from *S. aureus* are small monooxygenases that degrade heme to biliverdin and iron. These three proteins lack sequence homology to mammalian HOs (Skaar *et al.*, 2004). Bacterial HOs can be clustered with genes involved in heme uptake, and are often part of an operon that is regulated by an iron-responsive factor such as Fur. Another common feature is the up-regulation of HO in response to iron and/or heme starvation (Li and Stocker, 2009). When iron or heme levels are low, HO expression is up-regulated to allow heme to be used as a sole iron source. For
example, HmuO in *C. diptheriae* is positively regulated by the presence of heme, but is repressed completely when an excess of inorganic iron is provided (Schmitt, 1997).

While heme degradation has been studied in many bacterial species, the process in fungi is not well characterized. The role of heme oxygenase has so far only been studied in *S. cerevisiae* and *C. albicans*. Both species contain one HO, called Hmx1, which were shown to have HO activity and cleave heme to produce biliverdin (Kim et al., 2006). Although, *S. cerevisiae* does not take up heme in response to iron starvation and cannot effectively use heme as a sole iron source to fulfill the cell’s nutritional requirements (Weissman et al., 2002), Hmx1 still plays an important role within the cell. ScHmx1 is mainly involved in the recycling of heme iron, similar to the HO role in mammals, and may also reduce the intracellular heme stores under conditions of iron starvation (Protchenko and Philpott, 2003). The role of CaHmx1 in *C. albicans* is more similar to bacterial HOs, and is required for the use of heme as a sole iron source (Santos et al., 2003). *CaHMX1* expression is induced by high temperature, iron starvation, and the presence of heme (Santos et al., 2003), as well as by hemoglobin (Pendrak et al., 2004). Hemoglobin induction occurs independently of the presence or absence of inorganic iron in the media, and at a more rapid rate than induction by iron starvation, indicating that the two methods of induction are separate (Pendrak et al., 2004). *C. albicans* colonies grown with heme as a sole iron source also have an unusual filamentous morphology, highlighting the role of heme in cellular processes such as cell morphology.

Another group of pathogens that can utilize host hemoglobin in a system involving the vacuole are the malaria parasites. Malaria parasites are members of the *Plasmodium* genus, and *P. falciparum* is the most serious disease-causing species. During the erythrocyte
phase of infection, malaria parasites infect the blood and live in vacuoles derived from erythrocyte membranes (Fitch and Russell, 2006). The parasites feed on the erythrocyte cytoplasm, and in the process digest up to 80% of the hemoglobin present in the red blood cell (Krugliak et al., 2002). This process takes place in the digestive vacuole of the parasite and occurs to release essential amino acids needed for protein synthesis (Krugliak et al., 2002). Heme is a byproduct of hemoglobin degradation, and is not degraded by HO (in contrast to the situation in bacteria). Free heme is toxic to cells, and malaria parasites have a unique strategy to detoxify excess heme by converting it to a crystalline form called hemozoin. Formed in the vacuole of the parasite, these dark brown or black crystals are the fate of the majority of heme released from hemoglobin (reviewed in Egan, 2008a). Hemozoin is structurally identical to β-hematin, a synthetic heme crystal, and is formed from chains of dimerized heme molecules that crystallize in the presence of lipids (Egan, 2008a, Egan, 2008b). Crystallization occurs within lipid bodies, which are made up of lipids from the membranes of endocytic vesicles that transport hemoglobin to the vacuole (Egan, 2008b). Hemozoin is important for the survival of malaria parasites, and is the target for the antimalarial drug chloroquine, which interferes with hemozoin formation. The presence of hemozoin in *P. falciparum* vacuoles has been known for many years, and hemozoin crystals have now been identified in seven *Plasmodium* species, as well as other species of blood-feeding organisms. These include several species of helminth worms, such as *Schistosoma mansoni*, and blood-sucking insects such as *Rhodnius prolixus* (Egan, 2008a). This indicates that hemozoin formation is a widespread strategy for heme detoxification in blood-feeding organisms, and also implicates the vacuole in another key role related to heme and hemoglobin in pathogenic species.
Heme uptake, degradation, and storage in *C. neoformans* have not previously been studied in detail. We have shown that *C. neoformans* grows well *in vitro* when heme is provided as a sole iron source (Jung et al., 2008). This, combined with the fact that elimination of inorganic iron and transferrin uptake attenuates virulence but does not eliminate it, points to the fact that *C. neoformans* may be able to use heme/hemoglobin as an iron source during infection.

1.6 Thesis objectives

The objective of this study was to examine and characterize key genes involved in the ability of *C. neoformans* to take up and/or utilize heme as an iron source. Our first hypothesis was that *C. neoformans* uses a heme oxygenase to degrade heme and release iron for use by the fungal cells. The *C. neoformans* serotype A genome has one HO homolog, also called Hmx1, and the first goal was to delete this gene and examine the effect on heme utilization and virulence-related phenotypes. The prediction was that deletion of *HMX1* should result in cells that were unable to grow with heme as the sole iron source. Our second hypothesis was that intact vacuolar function plays a role in iron and heme transport or storage. This was examined by deleting *VPS41*, which is known to play a role in iron utilization in *S. cerevisiae*. The effect of deleting *vps41* on heme uptake, utilization, and storage has not been examined in fungi, but we predicted that Vps41 and the vacuole were important for iron and/or heme utilization by *C. neoformans*. 
2. MATERIALS AND METHODS

2.1 Strains, media, and growth conditions

The wild type (wt) strain employed for this study was *C. neoformans* variety *grubii* strain H99 (serotype A, MATα). Mutants were derived from this strain, and all of the strains used in the study are listed in Table 2.1. Strains were maintained in yeast extract, bacto-peptone medium with 2.0% glucose (YPD, Difco) or yeast nitrogen base (YNB, Difco) with 2.0% glucose. Defined low-iron media (LIM) was prepared as described (Li et al., 2008) with some modifications. The water used for LIM and related solutions was passed over a column of Chelex 100 resin (BioRad) to chelate iron. Defined YNB media was prepared and adjusted to pH 7.0 with 3-morpholinopropanesulfonic acid (MOPS), with the addition of 200µM bathophenanthroline disulfonate (BPS) to remove iron and 25 µM CuSO₄ to ensure copper replete conditions. For solid media, agar was dissolved and incubated with Chelex 100 resin (Sigma) for 30 minutes at 65°C. The agar was then removed from the resin, autoclaved, and added to the LIM. Iron-replete conditions were achieved by adding inorganic iron (FeCl₃ or FeSO₄) or heme to the concentrations as indicated in the text. Heme was dissolved as hemin in 0.1M NaOH before being added to the media.
Table 2.1. *C. neoformans* strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H99</td>
<td>MATα</td>
<td>Joseph Heitman</td>
</tr>
<tr>
<td><em>hmx1</em>-1</td>
<td>MATα <em>hmx1Δ::NAT</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>hmx1</em>-2</td>
<td>MATα <em>hmx1Δ::NAT</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>cfo1</em></td>
<td>MATα <em>cfo1Δ::NAT</em></td>
<td>Jung <em>et al.</em>, 2009</td>
</tr>
<tr>
<td><em>cfo1hmx1</em>-23</td>
<td>MATα <em>hmx1Δ::NAT, cfo1Δ::NEO</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>cfo1hmx1</em>-94</td>
<td>MATα <em>hmx1Δ::NAT, cfo1Δ::NEO</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>vps41</em>-1</td>
<td>MATα <em>vps41Δ::NEO</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>vps41</em>-2</td>
<td>MATα <em>vps41Δ::NEO</em></td>
<td>This study</td>
</tr>
<tr>
<td><em>vps41</em>-3.1</td>
<td>MATα <em>vps41Δ::NEO</em></td>
<td>This study</td>
</tr>
</tbody>
</table>

2.2 Construction of mutant strains

The sequence of a putative heme oxygenase was obtained from the *C. neoformans* var. *grubii* serotype A genome database ([http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans](http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans)). The locus number of the gene is **CNAG_05852.2** (*HMX1*). A PCR overlap strategy was used to create the *hmx1* mutant. A deletion cassette containing the nourseothricin acetyltransferase resistance gene (NAT) and 5’ and 3’ flanking regions was used to replace the 1425 bp open reading frame of *HMX1*. The 5’ flanking region (1033 bp) was amplified from wt H99 genomic DNA using primers HOKO1 and HOKO2, and the 3’ flanking region (1007 bp) was amplified with primers HOKO3 and HOKO4. The NAT gene was amplified from the plasmid pCH233 with primers M13-F and M13-R, and the fragments were combined using overlapping PCR (Yu *et al.*, 2004). The construct was biolistically
transformed into the wt strain as described previously (Toffaletti et al., 1993). Positive transformants were identified by PCR using primers HOKO4 and Nat2TF (expected to yield a positive band in transformants with homologous integration) and HOintF and HOintR (expected to yield a negative result upon correct loss of the *HMX1* open reading frame), and confirmed by genomic hybridization. All primers used for deletion mutant creation are listed in Table 2.2.

To create the *cfo1hmx1* double mutant, the NEO-containing deletion cassette of *cfo1* was amplified from genomic DNA of the *cfo1cft1* mutant strain (created and provided by Dr. Wonhee Jung) using primers H9CFO1_KO5 and H9CFO_KO6. The cassette was then biolistically transformed into the *hmx1Δ2* strain. Positive transformants were identified by PCR using primers H9CFO1_KO4 and Nat2TF, and negative transformants were identified using primers CFO7 and CFO8.

The sequence of the *VPS41* gene was obtained from the *C. neoformans* var. *grubii* serotype A genome database ([http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans](http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans)). The locus number of the gene is **CNAG_04293.2** (*VPS41*). A PCR overlap strategy was used to create the *vps41* mutants. A deletion cassette containing the neomycin resistance gene (NEO) was used to replace the 3816 bp open reading frame of *VPS41* by homologous recombination with the 5’ and 3’ flanking regions. The 5’ flanking region (1033 bp) was amplified using primers VPS1 and VPS2, and the 3’ flanking region (1028 bp) was amplified with primers VPS3 and VPS4, from wt H99 genomic DNA. The NEO gene was amplified from the plasmid pJAF1, and the fragments were combined using overlapping PCR (Yu et al., 2004). The construct was biolistically transformed into the wt
strain as described previously (Toffaletti et al., 1993). Positive transformants were identified by PCR using primers VPS4 and Nat2TF (positive for correct integration) and VPS7 and VPS8 (negative for the wt open reading frame), and confirmed by genomic hybridization.

**Table 2.2** List of primers used to create deletion mutants in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMX1</td>
<td>HOKO1</td>
<td>GGATGGCTTGCTGCCTCAAGGATCA</td>
</tr>
<tr>
<td></td>
<td>HOKO2</td>
<td>AATTCTGCAGATATCCACTACACATGGCGCCTTGGCGGCGCA</td>
</tr>
<tr>
<td></td>
<td>HOKO3</td>
<td>AATTCCAGCAGACTGGCGGCGGTACTAGTATATGAAGCTTTAGTAGAGGTCAGGC</td>
</tr>
<tr>
<td></td>
<td>HOKO4</td>
<td>CGGCCGTTGTATCTGACTCTGACT</td>
</tr>
<tr>
<td></td>
<td>HOKO5</td>
<td>CGGTAAAGTCAGTACGAGAGAGCT</td>
</tr>
<tr>
<td></td>
<td>HOKO6</td>
<td>GTTGTACGTCAGTCATTACCAGATCCAGTCCAG</td>
</tr>
<tr>
<td></td>
<td>HOintF</td>
<td>ACATGTTCGCTACTTCCAGTT</td>
</tr>
<tr>
<td></td>
<td>HOintR</td>
<td>CGAATTTAGTCCAAAGGAGTCG</td>
</tr>
<tr>
<td>VPS41</td>
<td>VPS1</td>
<td>GATCAAGTACGTCCTCAACGAAC</td>
</tr>
<tr>
<td></td>
<td>VPS2</td>
<td>AATTCTGCAGATATCCACATTACATGGCGCATGCTGTTTAAAGGTGTATAGGATG</td>
</tr>
<tr>
<td></td>
<td>VPS3</td>
<td>AATTCCAGCAGACTGGCGGCGGTACTAGTAGTTTGTATAGTTTGTATCCGTAATACATTATA</td>
</tr>
<tr>
<td></td>
<td>VPS4</td>
<td>GCCACACGTCTGCATTTATCTGTA</td>
</tr>
<tr>
<td></td>
<td>VPS5</td>
<td>GTCGTTTACGGATGAGGAC</td>
</tr>
<tr>
<td></td>
<td>VPS6</td>
<td>TCAAGCCACATTTACCCTTCTCT</td>
</tr>
<tr>
<td></td>
<td>VPS7</td>
<td>TACAGCAGATTAAAAAGGTCGGATAC</td>
</tr>
<tr>
<td></td>
<td>VPS8</td>
<td>TCTTTGCTTTTACAGATCATGAGGCAGCC</td>
</tr>
<tr>
<td>CFO1</td>
<td>H9CFO1_KO1</td>
<td>GACGAAATCTCTCTGCGGAAAACCTTTG</td>
</tr>
<tr>
<td></td>
<td>H9CFO1_KO4</td>
<td>GGATGGTTACAGAAGCTCTTCTC</td>
</tr>
<tr>
<td></td>
<td>H9CFO1_KO5</td>
<td>CAGGGTATTTCCCACGCTCACC</td>
</tr>
<tr>
<td></td>
<td>H9CFO1_KO6</td>
<td>GGTCGACTTGAAGTACTGGGACTTTTC</td>
</tr>
<tr>
<td></td>
<td>CFO7</td>
<td>GTACCCCTGGTGATGCGCTCT</td>
</tr>
<tr>
<td></td>
<td>CFO8</td>
<td>TGGGTAAACGGAGAGTTTGAAG</td>
</tr>
<tr>
<td>Generic</td>
<td>NatT2F</td>
<td>CTTCACTGGCTCTTTGTCTCTGAAAACC</td>
</tr>
<tr>
<td></td>
<td>M13-F</td>
<td>CACGACGTTGAAAAACGACGCCAG</td>
</tr>
<tr>
<td></td>
<td>M13-R</td>
<td>CAGGAAACAGCTATGACCAGATTACG</td>
</tr>
</tbody>
</table>
2.3 Web Resources

All *C. neoformans* sequences were obtained from the *C. neoformans* var. *grubii* serotype A genome database (http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans). BLAST searches were performed using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi, Altschul *et al.*, 1997). Multiple sequence alignments were created using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html, Larkin *et al.*, 2007). Intracellular protein location was predicted using WoLFPSORT (http://wolfpsort.org/, Horton *et al.*, 2007). Transmembrane domains were predicted using the SOSUI tool (http://bp.nuap.nagoya-u.ac.jp/sosui, Hirokawa *et al.*, 1998).

2.4 Genomic DNA isolation

Genomic DNA (gDNA) isolation was performed as described previously (Pitkin *et al.*, 1996). Briefly, the method involved vigorous vortexing of cells with 0.5 mm glass beads in a phenol:chloroform:isoamyl alcohol solution followed by a series of precipitations with 100% ethanol. The precipitated DNA was re-suspended in distilled water and the concentration was measured using a Nanodrop Spectrophotometer (Thermo Scientific).

2.5 Plate assays with different iron sources

Spot plate assays were performed to examine the growth of strains on various iron sources. Plates of LIM were created as described above, with inorganic iron or heme added to the final concentrations indicated in the Results section. To deplete cells of iron, cultures were grown in LIM for two days at 30°C in a shaker. Cells were counted, washed with LIDH2O and adjusted to 10^6 cells mL^-1. Ten-fold serial dilutions of cells were spotted onto plates and incubated at 30°C and 37°C for 2-5 days before being photographed.
2.6 Other phenotype assays

For various phenotype analyses, YNB was prepared with 2% glucose and 20g Bacto-agar L⁻¹ with additional components as indicated. Cells were grown in YPD or LIM overnight at 30°C in a shaker. Cells were counted, washed with water and adjusted to 10⁶ cells mL⁻¹. Ten-fold serial dilutions of cells were spotted onto plates and incubated at 30°C and 37°C for 2-5 days before being photographed. For cell wall integrity, 300 µg of congo red mL⁻¹, 100 µg of calcofluor white, or 0.1% SDS was added to the medium. To test osmotic sensitivity, 1.5M sorbitol or 1M NaCl was added. For oxidative stress, 2 mM H₂O₂ was added to the media, and for nitrosative stress, 4 mM or 8 mM sodium nitrate was added. To test for growth on various carbon sources, YNB plates were prepared with the addition of 0.2% or 2% glucose, galactose, acetate, or 2% glycerol plus 2% ethanol. To test sensitivity to antifungal drugs, 5 µg mL⁻¹ Fluconazole, 1 µg mL⁻¹ Amphotericin B, or 1 µg mL⁻¹ Miconazole was added to YPD media with 20 g L⁻¹ BactoAgar.

To test for melanin production, minimal L-DOPA media was prepared as reported previously (Tangen et al., 2007). To test capsule production, cells were grown in liquid YPD for 1-2 days at 30°C, counted, and 10⁶ cells mL⁻¹ were transferred to capsule-inducing, synthetic low-iron media (Vartivarian et al., 1993). After incubation for 1 to 3 days, the capsule was stained with India ink and examined by differential interference contrast microscopy (DIC).

2.7 Determination of culture growth and supernatant pH changes.

To examine changes in culture supernatant pH and colour, cells were pre-starved overnight in LIM. Cells were counted, washed with LI-dH2O, adjusted to 1x10⁶ cells mL⁻¹,
and inoculated into fresh LIM or LIM plus 0.1mM or 1mM heme. At 24 hour intervals, 5 mL of culture was removed and cells were counted. Cells were then spun down by centrifugation at 3000 rpm for 10 minutes and discarded. The supernatant was collected and the pH was measured with a pH meter. Colour changes in supernatant were observed visually and photographed with a digital camera. The colour change and pH assay was repeated three times and cells were counted in two of the three trials.

2.8 Confirmation of the vps41 mutant response to starvation stress

A starvation time course assay was performed as described by Liu et al., (2006) to confirm the poor growth and survival of vps41 mutants in low nutrient conditions. The wt and mutant strains were grown overnight in liquid YPD at 30°C, collected, and washed twice with water. Cells were re-suspended to $10^8$ cells mL$^{-1}$ in 15 mL of YNB without amino acids or ammonium sulphate in a 150 mL flask. Cells were incubated at 37°C with shaking at 250 rpm min$^{-1}$ for 0 to 10 hours. At each time point, 100 mL of each culture was removed and used to determine cell viability by plating 10-fold serial dilutions on YPD agar and counting colony forming units (CFUs). The assay was repeated three times in triplicate.

2.9 Quantitative real-time PCR

Primers for RT PCR analysis were designed using Primer Express 3.0 from Applied Biosystems and are listed in Table 2.3. Cell cultures were grown overnight in LIM and then transferred to the same media containing different sources of iron as indicated; the cells were then grown for 18 hours at the temperature indicated in the text. Total RNA was extracted with the RNeasy kit (Qiagen), and treated with DNase (Qiagen). cDNA was synthesized
using the SuperScript First Strand Synthesis System (Invitrogen). Real time PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR system using Power SYBR Green PCR mix (Applied Biosystems) as described previously (Tangen et al., 2007). The cDNA of the actin gene \textit{ACT1} as well as \textit{GAPDH} were used for normalization of the data. The real-time PCR analysis was repeated three times with independent samples for each condition.

\textbf{Table 2.3} List of primers used for Real Time PCR analysis

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| \textit{HMX1} | Forward: GGCCAACCTTGCTTTTTCC  
Reverse: TGGCAGGACGTGAATGA |
| \textit{CFO1} | Forward: \textit{CFO1} primers were designed and generously donated by Dr. Wonhee Jung (personal communication)  
Reverse: CTGCGTCCCCAGCTGATC |
| \textit{CFT1} | Forward: ACGAGAACGACCTCAGTAATGAAA  
Reverse: CCCGAGTCTTGGACGAATCTC |
| \textit{LAC1} | Forward: TCGTGCGGGTCCAAATG  
Reverse: AGAACGACCTCAGTAATGAAA |
| \textit{CIG1} | Forward: GGCGTCATCGCTGGCTTA  
Reverse: TGCTTCTTCTTGCCAAAACGA |
| \textit{ACT1} | Forward: CCACACTGTCCCCATTACGA  
Reverse: CAGCAAGATCGATACGGAGGAT |
| \textit{GAPDH} | Forward: GTCTGCGGTTGTCACCTTGAT  
Reverse: CAAGAAGCGGCTGGACACGATT |
2.10 Growth curves and colour photography of cells

The growth of wt and \textit{vps41Δ} strains was measured in liquid culture with various iron and heme sources. Cultures were grown in LIM for two days at 30°C with shaking to deplete cells of iron. Cells were counted, washed with LI-dH₂O, and adjusted to 5x10⁵ cells mL⁻¹ in 3 mL fresh LIM. Various concentrations of heme and FeCl₃ were added as indicated in the text. Cultures were grown for 96 hours at 30°C with shaking. Samples (100µL) of culture were removed every 24 hours and used to determine cell viability by plating 10-fold serial dilutions on YPD agar and counting CFUs after two days of incubation. At the same time points, cells were examined for heme/iron uptake and cell morphology at 1000x magnification on a Zeiss Axioskop 2 MOT light microscope and photographed.

2.11 Examination of internal heme localization by fluorescence microscopy

To examine the internal localization of heme in wt and mutant strains, the fluorescent heme analog zinc mesoporphyrin IX (ZnMP) was used (Rajagopal \textit{et al.}, 2008). Cells were pre-starved for iron in LIM for 2 days at 30°C with shaking, and subcultured into 1 mL fresh LIM containing 1.5µM heme or ZnMP, or 1.5 µM of both compounds, as indicated. Cultures were grown overnight at 30°C with shaking, centrifuged at 8000 rpm for 1 minute, washed twice in LI-dH₂O, and resuspended in 20-100 µL LI-dH₂O. Cells were examined and photographed with both DIC and fluorescence at 1000x magnification using a Zeiss Axioplan Imaging 2 microscope.
3. RESULTS

3.1 Characterization of the HMX1 gene

Hmx1 was chosen for this study as a putative HO that may play a role in heme utilization as an iron source in *C. neoformans*. Initially, the sequence of the gene was analyzed to determine whether it was a good candidate for subsequent genetic analysis. The sequences of the *S. cerevisiae* and human heme oxygenase proteins were initially used to search the genome of the *C. neoformans* serotype A strain H99 to identify candidate heme oxygenase homologs. A single HO homolog was identified in the Broad database for strain H99 ([http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans](http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans)). The gene was located on chromosome 7 and encoded a predicted 362 amino acid protein which was designated Hmx1. Hmx1 shared 18% identity and 33% similarity with Hmx1 from *S. cerevisiae*, but was more similar to the mammalian HOs, with 23% identity and 36% similarity to human HO-1 and 24% identity and 36% similarity to HO-2 (Table 3.1). Hmx1 also shared similarity with some bacterial HOs, such as that from *C. diphtheriae*, but had very little similarity with other bacterial HOs. A BLAST search was also performed with known heme oxygenases that do not share sequence similarity to the mammalian HOs. These included ChuS from *E. coli* and IsdG and IsdI from *S. aureus*, but none of these identified any similar sequences in *C. neoformans*. A search for proteins containing the ‘heme oxygenase domain’ also did not reveal any similar sequences other than Hmx1. Overall, Hmx1 was the only identifiable heme oxygenase candidate revealed from similarity searches.
Table 3.1. Comparison of *C. neoformans* Hmx1 with homologous heme oxygenases from mammals and fungi.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Protein name</th>
<th>% identity</th>
<th>% similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. sapiens</em></td>
<td>HO-1</td>
<td>23</td>
<td>36</td>
</tr>
<tr>
<td><em>H. sapiens</em></td>
<td>HO-2</td>
<td>24</td>
<td>36</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>ScHmx1</td>
<td>18</td>
<td>33</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>CaHmx1</td>
<td>19</td>
<td>36</td>
</tr>
<tr>
<td><em>C. diphtheriae</em></td>
<td>HmuO</td>
<td>20</td>
<td>33</td>
</tr>
<tr>
<td><em>N. meningitidis</em></td>
<td>HemO</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

Hmx1 was aligned with four mammalian HOs as well as the HO from *C. diphtheriae* to further characterize the gene (Figure 3.1). This analysis revealed that Hmx1 had a 42 amino acid (a.a.) insertion at the N terminus compared to the mammals HOs, as well as an insertion in the middle of the protein at a.a. position 140. There was a high level of conservation in the center of the protein around the ‘heme oxygenase domain’ that is the signature domain of heme oxygenase proteins (black bar). The proximal histidine residue involved in heme binding was perfectly conserved (filled triangle). There was very little conservation of residues in the C-terminal region of Hmx1 compared to the mammalian HOs. However, Hmx1 was predicted to have a C terminal transmembrane domain (see Materials and Methods), which is also present in mammalian and fungal heme oxygenases (thick grey bar) (Protchenko and Philpott, 2003).
Figure 3.1. Sequence alignment of mammalian and bacterial heme oxygenases with Hmx1. The amino acid sequences were aligned with ClustalW2 (Larkin et al., 2007). The proximal histidine residue is marked with a filled triangle (▼). The heme oxygenase domain is underlined with a solid black bar, and the predicted C terminal transmembrane domain is marked with a large grey bar.

Hmx1 was also aligned with the other characterized fungal HOs from S. cerevisiae and C. albicans (Figure 3.2). The overall level of identity was lower between Hmx1 and the fungal HOs compared to the level of identity between Hmx1 and mammalian HOs as seen in...
Figure 3.1. The proximal histidine ligand was also perfectly conserved (filled triangle), but the heme oxygenase domain was less well conserved (underlined). There was a higher level of similarity in the C terminal end of the protein compared to the alignments with the mammalian proteins.

S.cer  ---MEDSNTIIPSPTDVGALANRIN 23  
C.alb  ---MQYKSSGATSKLQVEIIPAKTDVGALANRIN 32  
C.neo  MFATSQHLINSSPLDTPFRENTPFEESPAQSSAKITEPGVGLFELDLNFISSLK 60  

| S.cer | FQTRDAHNNKINTFMGIKMAIAMRH-GFIYRQGIILAYYVFDKIEIQIDRLNDFVTEEG 82  
| C.alb | LETRLSDHDAKTDTLKFALALRN--YKYVRQGIQFYHFASIEKALYQK----EKKD 86  
| C.neo | LGTTRAKHIAEHSAGAAALVGKLGEEYR1WLAALWWTIDVLEDGIQENNANPIAP 120  

Figure 3.2. Sequence alignment of characterized fungal heme oxygenases with Hmx1. The amino acid sequences were aligned with ClustalW2 (Larkin et al., 2007). The proximal histidine residue is marked with a filled triangle (▼). The heme oxygenase domain is underlined with a solid black bar, and the predicted C terminal transmembrane domain is marked with a large grey bar.
S. cerevisiae and C. albicans are ascomycete fungi and C. neoformans is a basidiomycete fungus. This may account for the variation in sequence between Hmx1 and the other characterized fungal heme oxygenases. To compare C. neoformans Hmx1 with sequences from a more similar class of fungi, the genomes of other basidiomycete fungi were examined for the presence of a heme oxygenase homolog. Four basidiomycete genomes contained putative HO orthologs that were annotated based on sequence prediction (obtained from the Broad fungal genome initiative [http://www.broadinstitute.org/science/projects/fungal-genome/initiative/current-fgisequence-projects]). C. neoformans Hmx1 had higher levels of identity and similarity to these putative heme oxygenases than to the mammalian or ascomycete fungi HOs (Table 3.2). Overall, comparing sequence data revealed that C. neoformans Hmx1 has many characteristics of a heme oxygenase protein, but is less similar to the two characterized fungal heme oxygenases. This indicates that it may function differently from CaHmx1 in C. albicans, which is required growth when heme is the sole iron source (Santos et al., 2003).

**Table 3.2.** Comparison of C. neoformans Hmx1 with putative heme oxygenases from basidiomycete fungi.

<table>
<thead>
<tr>
<th>Organism</th>
<th>% Identity</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coprinus cinereus</td>
<td>36</td>
<td>54</td>
</tr>
<tr>
<td>Ustilago maydis</td>
<td>33</td>
<td>47</td>
</tr>
<tr>
<td>Laccaria bicolour</td>
<td>37</td>
<td>54</td>
</tr>
<tr>
<td>Moniliopthora perniciosa</td>
<td>32</td>
<td>46</td>
</tr>
</tbody>
</table>
3.2 Construction and characterization of hmx1 mutants

To characterize the function of HMX1, mutants lacking the entire coding region of the gene were generated. The coding region was deleted by biolistic transformation using a transformation cassette constructed by overlap PCR and containing a NAT marker (See Materials and Methods). Two independently generated mutants were created and deletion of the HMX1 allele was confirmed by PCR (Figure 3.3) and genomic hybridization (Figure 3.4). These mutants were viable and had similar doubling times to wt cells at 30°C; both mutants were used for subsequent experiments to characterize the function of the gene.

**Figure 3.3. Genomic arrangement, structure of deletion constructs, and confirmation of deletion of HMX1.** (A) HMX1 is located on chromosome 7. The gene was disrupted by transforming the disruption cassette containing the selectable resistance marker for nourseothricin (NAT'). (B) Positive transformants were screened by PCR using primers HOKO4 and Nat2TF and negative transformants were indentified with primers HOintF and HOintR as shown. (C) Two hmx1 mutants are positive for deletion (top panel), while the wild-type strain shows presence of the HMX1 gene (bottom panel).
Figure 3.4. Two \textit{hmx1} mutants are confirmed by Southern blot. (A) Genomic DNA was digested with KpnI and NdeI and probed with a radioactively labelled DNA fragment as shown. (B) Southern blot of the digested genomic DNA hybridized with the probe as indicated in (B) confirms the \textit{HMX1} deletion in the two mutant strains.

3.3 \textit{HMX1} does not affect the expression of virulence factors.

The \textit{hmx1} mutants were tested for changes in expression of the three major virulence factors: growth at 37°C, melanin production, and production of capsule. Analysis of the virulence factor production revealed that \textit{hmx1} mutants grew well at 37°C, and had no obvious changes in capsule size in low iron medium, or melanin synthesis compared to the wt strain (Figure 3.5).
Figure 3.5. *hmx1* mutants do not show changes in virulence-associated phenotypes compared to the wild type strain. Mutants were able to grow well at 37°C (A), had normal levels of melanin production (B), and had no change in capsule size (C) compared to the wt strain.

3.4 *HMX1* is not required for growth with heme as a sole iron source.

To investigate the role of *HMX1* in heme utilization, wt and mutant strains were grown on solid low iron media containing heme as a sole iron source. The cells were first grown in liquid LIM to deplete intracellular iron stores and then plated into medium containing increasing concentrations of heme. All strains grew well on the control media YPD and YNB at 30°C and 37°C, but LIM without the addition of an iron source promoted little to no growth of any strains (Figure 3.6A). This ensured that any observed changes in growth were due to the addition of an iron source. However, the *hmx1* mutants behaved like
the wt strain at all tested concentrations of heme (Figure 3.6B). The *hmx1* mutants also showed no growth defects compared to wt when tested for their ability to utilize inorganic iron (Figure 3.6C) as well as hemoglobin as iron sources (Figure 3.6D). Growth was also measured by OD over time in liquid media containing heme or inorganic iron, and the mutant grew at the same rate as the wt strain (Figure 3.7). These results suggest that while *C. neoformans* can grow well when provided with heme, *HMX1* is not required for the utilization of heme as a sole iron source.
Figure 3.6. *hmx1* mutants do not have heme or iron related growth defects. Ten-fold serial dilutions of cells were spotted onto solid media containing various iron sources as indicated and were monitored to assess defects in growth. Plates were incubated at 30°C and 37°C for three days. (A) The wt strain and *hmx1* mutants grew well on control media YPD and YNB at 30°C and 37°C, while LIM does not support growth. The *hmx1* mutants grew at the same level as the wild type when provided with (B) heme, (C) inorganic iron, or (D) hemoglobin (Hb). The assays were repeated at least three times.
Figure 3.7. The *hmx1* strain do not have diminished growth in liquid media containing heme or iron. Cells were starved for iron in LIM and inoculated into fresh LIM containing 10µM heme, 10µM FeCl₃, or without addition, at an optical density of 0.1. The growth was measured at intervals over 68 hours by optical density (OD₆₀₀). The experiment was repeated in triplicate with error bars representing the standard deviations.

3.5 A deletion of *HMX1* does not affect growth under other various conditions of stress and nutrition.

Because mammalian HOs play a role in the prevention of oxidative stress (Li and Stocker, 2009; Ryter and Choi, 2009), the *hmx1* mutants were tested for any defects in their ability to grow on solid YNB media containing agents that cause oxidative stress (H₂O₂) or nitrosative stress (NaNO₃) (Figure 3.8). However, the *hmx1* strains did not show any change
in growth compared to the wt strain, indicating that a deletion of \textit{HMX1} does not increase susceptibility to oxidative or nitrosative stress.

\textbf{Figure 3.8.} \textit{hmx1} mutants do not have increased sensitivity to oxidative or nitrosative stress. Ten-fold serial dilutions of cells were spotted onto solid YNB media containing various stresses as indicated and were monitored to assess defects in growth. Plates were incubated at 30°C and 37°C for five days. \textit{hmx1} mutants did not have increased sensitivity to the oxidative stress agent hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) or to two concentrations of the nitrosative stress agent sodium nitrate (NaNO\textsubscript{3}). The experiment was repeated three times.

\textit{hmx1} mutants were also tested for their ability to grow on YNB media containing a variety of carbon sources. The ability to use glycerol and ethanol as carbon sources is independent of glycolysis and instead depends on the TCA cycle, gluconeogenesis and respiration. Heme is required for respiration within cytochrome proteins, and a deletion of \textit{HMX1} may cause changes in the demand for heme or changes in regulation within the cell,
which in turn could affect the growth of the mutant strain on certain carbon sources. However, there were no observed defects in the \textit{hmxl} strains compared to wt when cells were grown on YNB media containing (Figure 3.9 A) 0.2\% or 2\% glucose, (Figure 3.9 B) 0.2\% or 2\% galactose, (Figure 3.9 C) 0.2\% or 2\% acetate, or (Figure 3.9 D) 2\% glycerol + 2\% ethanol. Therefore, a deletion of \textit{HMX1} does not appear to affect carbon source utilization under the tested conditions. Additional phenotypic assays were also performed and are displayed in Appendix B.
Figure 3.9. Wild type and *hmx1* mutants grow similarly on all tested carbon sources. Ten-fold serial dilutions of cells were spotted onto solid YNB media containing various carbon sources as indicated and were monitored to assess defects in growth. Plates were incubated at 30°C and 37°C for five days. No differences in growth were seen between wt and *hmx1* mutants for any tested carbon source. The assay was repeated three times.
3.6 A liquid culture of *hmx1* exhibits a change in supernatant pH and colour when grown in the presence of heme.

Liquid LIM had a pH of 7.0, and was pale pink in colour due to the addition of the BPS chelator, while LIM containing heme was brown (Figure 3.10A). When wt cells were grown in LIM with heme and then removed by centrifugation, the culture supernatant was lighter and more pink in colour. When *hmx1* mutants were grown in the same media, the culture supernatant remained darker and more brown in colour. To investigate potential causes for the colour change, the wt strain and the *hmx1* mutants were grown with 100µM or 1mM heme, and the pH of culture supernatant was measured after the removal of the cells. The pH for both wt and *hmx1* supernatant from LIM media remained constant over time (7.0 for all strains); however the cells were not actively growing so no metabolite change was occurring. However, the light coloured supernatant from wt cells grown in the presence of 100µM heme had a pH of 4.6, and the darker *hmx1* supernatant had a higher pH of 5.7 (Figure 3.10A). A similar trend was observed for cells grown with 1mM heme (pH 5.9 and 6.5 for the wt and mutant strains, respectively). The colour difference of the supernatant appeared to be affected by pH, because the colour became lighter as the pH decreased when 1M HCl was titrated into the *hmx1* supernatant. The cells grew at the same rate for mutant and wt when provided with heme (Figure 3.10B). Therefore the colour difference is likely due to the deletion of *HMX1*, and indicates that *HMX1* does have some activity in the cell. It is possible that Hmx1 is modifying something, possibly heme itself, to cause a change in pH. Hmx1 is a cytosolic membrane bound protein and is not predicted to be secreted (See Materials and Methods). However it is possible that Hmx1 is located near the plasma membrane and a loss of the protein could change a heme-related aspect of metabolism in
some way. Overall, this result is the first indication that Hmx1 had some activity that is possibly related to heme.

Figure 3.10. The culture supernatant of wild type and hmx1 mutants has colour and pH differences when cells are grown with heme. Cells were grown for 3 days in LIM containing 0.1mM or 1mM heme. Cells were removed and the supernatant pH was measured. (A) Wt supernatant had a lower pH and was lighter in colour than the two hmx1 mutant supernatants (Δ1 and Δ2). (B) Wt and mutant strains grew to the same cell density, indicating that the pH and colour changes were not a result of difference in growth.
3.7 The loss of HMX1 does not affect virulence in a mouse model of infection.

Even without changes in virulence factor production, the deletion of a gene can sometimes cause a reduction in virulence in vivo. A small scale pilot study was used to examine the virulence of the hmx1 mutant in the mouse inhalation model of cryptococcosis. Two mice were infected with the hmx1 mutant strain, and these mice survived the same number of days as mice infected with the wt strain. Although this was a small scale experiment, the results suggests that Hmx1 does not make a contribution to virulence during infection.

3.8 HMX1 transcription is not affected by heme sources or temperature.

Even if Hmx1 only functions in a heme recycling role or other minor role in the cell, up-regulation of a heme oxygenase in response to the presence of heme would be expected. In support of this idea, the CaHMX1 gene in Candida albicans is up-regulated in the presence of heme, hemoglobin, and at 37°C (Pendrak et al., 2004; Santos et al., 2003). To examine transcript levels of HMX1 under various conditions, the wt strain of C. neoformans was grown in LIM with the addition of 10 μM heme, 10 μM hemoglobin, or no addition, at 25°C, 30°C, and 37°C. RNA was extracted from the cells after 16 hours, and the transcript level of HMX1 was measured by real-time RT-PCR. The transcript level of HMX1 decreased when heme or hemoglobin were added to the media at all temperatures (Figure 3.11). The down-regulation of HMX1 was about 2-fold at 25°C and 30°C, and about 4-fold at 37°C. These results support the idea that HMX1 in C. neoformans is regulated differently than in C. albicans. Regulation appears to be more similar to ScHMX1 in S. cerevisiae, which is up-regulated during iron starvation.
Figure 3.11. Influence of heme source and temperature on *HMX1* transcript levels in wt *C. neoformans*. Relative quantification levels of *HMX1* transcripts were measured by RT-PCR as described in Materials and Methods. *HMX1* expression in LIM conditions was used as a calibrator for each temperature. Changes less than 2-fold are not considered significant (dashed line). The experiment was repeated at least three times in triplicate with independent samples for each trial, and a representative trial is shown. Error bars represent standard error of mean expression levels.

To test the possibility that a deletion of *HMX1* may have more subtle effects in the cell related to iron homeostasis, real-time RT-PCR was next used to examine the transcription levels of known iron-related genes in the *hmx1* mutant compared to the wt strain. The four genes selected were *CFO1* and *CFT1* (Jung *et al.*, 2008; Jung *et al.*, 2009), which encode components of the high affinity reductive iron uptake system, *LAC1*, which is responsible for the production of melanin (Jung *et al.*, 2006), and *CIG1*, a mannoprotein discovered by SAGE to be highly regulated by iron levels (Lian *et al.*, 2005). The expression of these genes was examined in wt and *hmx1* strains grown in LIM with or without the addition of
heme. Expression levels of \( CFO1 \), \( CFT1 \), and \( LAC1 \) were approximately the same in wt and mutant strains, regardless of the presence or absence of heme (Figure 3.12). This implies that a deletion of \( HMX1 \) does not influence the reductive uptake system or the production of melanin, as indicated by other experiments (Figure 3.5 and Figure 3.6). However, the transcript for the mannoprotein \( CIG1 \) appears to be up-regulated slightly when \( HMX1 \) is not present. The trend of up regulation is more pronounced in the presence of heme. The fact that \( CIG1 \) levels are influenced by iron implies that \( HMX1 \) may be influencing conditions within the cell.

![Figure 3.12. Quantitative RT-PCR analysis of iron-related genes in wild type and hmx1 strains.](image)

**Figure 3.12. Quantitative RT-PCR analysis of iron-related genes in wild type and hmx1 strains.** Wild type (wt) and two hmx1 mutant strains were grown overnight in the absence of an iron source (grey bars) or the presence of heme (black bars) and gene expression changes were measured by quantitative RT-PCR. Gene expression in the wt strain was used as a calibrator for expression of each gene. Changes less than 2-fold are not considered significant (below dashed line). The experiment was repeated at least three times in triplicate with independent samples for each trial, and a representative trial is shown. Error bars represent standard error of mean expression levels.
3.9 Loss of the high-affinity iron uptake system did not reveal additional phenotypes in the \textit{hmx1} mutant.

Because \textit{C. neoformans} has several iron uptake systems that vary in affinity and specificity, it was possible that a small amount of free iron was still available to the cells and that the \textit{hmx1} mutant was acquiring this iron in another way, such as from the high-affinity reductive uptake system. To eliminate this possibility, a double mutant was created that lacked \textit{HMX1} as well as one component of the reductive uptake system, \textit{CFO1}. As mentioned previously, \textit{CFO1} encodes a multicopper ferroxidase. Furthermore, a \textit{cfo1} deletion mutant grows poorly when provided with inorganic iron as a sole iron source (Jung \textit{et al.}, 2009). The double mutant (\textit{cfo1Δ hmx1Δ2}) was constructed by amplifying the deletion cassette from the previously constructed \textit{cfo1} mutant (Jung \textit{et al.}, 2009), and transforming it into the \textit{hmx1Δ2} mutant strain (See Materials and Methods). The deletion mutations were confirmed by PCR (Figure 3.13). The double mutants grew well on control media at both 30°C and 37°C, and were tested for growth defects with inorganic iron or heme as a sole iron source. As expected, the double mutants grew poorly in the presence of 10 µM FeCl$_3$ (Figure 3.14A). However, no obvious growth defect was observed when the \textit{cfo1 hmx1} double mutants were grown in the presence of heme or even an excess concentration of heme (Figure 3.14B), indicating that redundant high affinity iron uptake is not masking the effect of the \textit{hmx1} deletion for growth on heme.
Figure 3.13. **Two cfo1hmx1 double mutants are confirmed by PCR.** The CFO1 gene was deleted in the *hmx1Δ*2 strain by transforming the disruption cassette containing the selectable resistance marker for neomycin (NEO<sup>r</sup>). (A) Positive transformants were screened by PCR using primers CFOKO4 and Nat2TF and negative transformants were identified with primers CFO7 and CFO8 as shown. (B) Two *cfo1 hmx1* mutants are positive for deletion (top panel), while the *hmx1Δ*2 strain shows presence of the CFO1 gene. The *cfo1* single mutant was included as a control.
Figure 3.14. A deletion of both COP1 and HMX1 did not reveal additional heme-related growth defects. Ten-fold serial dilutions of cells were spotted onto solid media containing various iron sources as indicated and were monitored to assess defects in growth. Plates were incubated at 30°C and 37°C for three days. (A) Wt, single mutants and cfo1hmx1 double mutants grow well on control media YPD at 30°C and 37°C, while LIM does not support growth. Both the cfo1 and cfo1hmx1 double mutants grow poorly in the presence of FeCl₃. (B) All strains grow to the same level as the wt strain when provided with heme as a sole iron source or in excess. The assays were repeated three times.

3.10 Construction and characterization of VPS41 mutants

Because the obvious heme-related gene HMX1 did not show phenotypes related to the utilization of heme as an iron source in C. neoformans, a more global examination of the genome was performed for candidate genes with possible roles in heme utilization (Table B.1 in Appendix B). Of these, the most promising target was the gene for the vacuolar protein Vps41. VPS41 has previously been implicated in high affinity iron uptake in S. cerevisiae in that mutation of vps41 caused poor growth on LIM as well as vacuolar defects (Radisky et al., 1997). Surprisingly, an iron-related defect was not observed in a previous study of
VPS41 in C. neoformans (Liu et al., 2006); however the media and growth conditions used in that study may not have been sufficient to cause iron starvation. The vacuole has also been shown to be involved in heme/hemoglobin uptake in C. albicans (Weissman and Kornitzer, 2004; Weissman et al., 2008). Taken together, these results made VPS41 an excellent target for the analysis of its role in both inorganic iron and heme utilization as iron sources in C. neoformans.

To characterize the function of VPS41, mutants lacking the entire coding region of the gene were generated. The coding region was deleted by biolistic transformation using a transformation cassette constructed by overlap PCR containing a neomycin marker (See Materials and Methods). Four independently generated mutants were created and deletion of the VPS41 allele was confirmed by PCR (Figure 3.15). Three of the putative mutants were confirmed by genomic hybridization (Figure 3.16). These mutants were viable and had similar doubling times to wt cells at 30°C, and these strains were used for subsequent experiments. An attempt was made to complement the vps41 deletion mutation by integrating a wt copy of VPS41 back into the genome, and several independent complementation strains were obtained. However, the VPS41 gene integrated into the genome at a random location, and none of the complemented strains were restored to wt phenotypes; this result may indicate that VPS41 must be present at its native location in the genome to function correctly, or that part of the promoter region was missing from the complementation construct. None of the complemented strains were used for future experiments, and multiple independent mutants were relied on instead.
Figure 3.15. Genomic arrangement, structure of deletion constructs, and confirmation of deletion of VPS41. (A) VPS41 is located on chromosome 9. The gene was disrupted by transforming the disruption cassette containing the selectable resistance marker for neomycin (NEO\(^r\)). (B) Positive transformants were screened by PCR using primers VPS4 and Nat2TF and negative transformants were indentified with primers VPS7 and VPS8 as shown. (C) Four vps41 mutants are positive for deletion by PCR (top panels), while the wt strain shows presence of the VPS41 gene (bottom panels).
Figure 3.16. Three \textit{vps41} mutants were confirmed by genomic hybridization. (A) Genomic DNA was digested with NcoI and Spe1 and probed with a radioactively labeled DNA fragment as shown. Genomic hybridization of the digested genomic DNA hybridized with the probe as indicated in (B) confirmed the \textit{VPS41} deletion in three of the four putative mutants identified by PCR. The \textit{vps41} candidate \textit{vps41\Delta4} appeared to have ectopic insertions and was not used for subsequent experiments.

3.11 The \textit{vps41} mutants showed increased susceptibility to nutrient starvation.

A deletion of \textit{vps41} has been examined previously in \textit{C. neoformans}, and the mutant strain was found to have poor survival under conditions of nutrient starvation (Liu \textit{et al.}, 2006). This starvation experiment was repeated to confirm that our mutant strains showed the same phenotype (see Materials and Methods). Cells were incubated in minimal YNB
without amino acids or ammonium sulphate at 37˚C to cause nutrient stress and the viable cells were quantified at zero and ten hours by plating on rich media and counting CFUs (Figure 3.17). The wt strain had a 25% decline in viable cells over 10 hours, while the two vps41 mutant strains decreased by 67 and 65%. It was previously reported that a vps41 mutant had an 80% decrease in viability under the same conditions (Liu et al., 2006). The similar trend of decrease in viability confirms that the vps41 mutants created in this study behaved in the same way as the previously constructed mutants.

Figure 3.17. The vps41 mutants had increased susceptibility to nutrient starvation. Strains were pre-cultured in rich media and then transferred to minimal media for 10 hours to examine the rate of death due to starvation. The vps41 mutants showed more cell death compared to the wt strain after 10 hours.

3.12 Loss of VPS41 did not affect the expression of virulence factors.

The vps41 mutants were tested for changes in expression of the three major virulence factors: growth at 37˚C, melanin production and elaboration of a polysaccharide capsule.
Analysis of the virulence factor production revealed that \textit{vps41} mutants grew well at 37°C, and had no obvious changes in capsule size in low iron medium, or melanin synthesis compared to the wt strain (Figure 3.18). However, a \textit{vps41} mutant has previously been shown to be attenuated for virulence in the mouse model of cryptococcosis (Liu \textit{et al.}, 2006), indicating that Vps41 is important during infection even without producing changes in the expression of major virulence factors.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.18}
\caption{\textbf{The} \textit{vps41} mutants did not show changes in virulence-associated phenotypes compared to the wt strain.} The mutants were able to grow well at 37°C (A), had normal levels of melanin production (B), and showed no change in capsule size (C) compared to the wt strain.
\end{figure}
3.13 VPS41 is required for iron acquisition from both inorganic iron and heme.

To examine the role of VPS41 in iron and heme utilization, growth assays were performed on solid LIM containing inorganic iron or heme as a sole iron source. While all strains grew well on the control media of YPD and YNB at both 30°C and 37°C, LIM without the addition of an iron source promoted little to no growth of any strain (Figure 3.19A). This situation ensured that any observed changes in growth were due to the addition of an iron source. The vps41 mutants showed a clear growth defect when grown on inorganic iron (Fe(II) or Fe(III)) (Figure 3.19B). This defect is more pronounced at low concentrations of inorganic iron (10 µM) and at 37°C, which suggested that the high affinity uptake system may be affected and that the low affinity iron uptake system might be allowing the mutants to grow better at high iron concentrations. The vps41 mutants also grew poorly when heme was provided as the sole iron source (Figure 3.19C). The defect was much more pronounced at 37°C, but did not appear to be concentration dependent between 1µM and 100µM heme.

3.14 A deletion of VPS41 caused sensitivity to excess iron and heme.

Excess heme and iron can both be toxic to cells, and therefore must be sequestered internally. If the vacuole is required for storage of these molecules, then a disruption of the intact vacuole may cause increased sensitivity to high levels of heme and iron. To test this idea, the wt strain and the vps41 mutants were plated on solid iron-replete media containing an additional high concentration of iron or heme. The vps41 mutants show increased sensitivity to both excess FeCl₃ and heme, and this sensitivity was more pronounced at 37°C.
(Figure 3.19D). This result supports the idea that an intact vacuole is required for protection against both heme and iron toxicity.

**Figure 3.19.** The *vps41* mutation caused heme and iron-related growth defects. Ten-fold serial dilutions of cells were spotted onto solid media containing various iron sources as indicated and the plates were monitored to assess defects in growth. Plates were incubated at 30°C and 37°C for three days. (A) The wt strain and the *vps41* mutants grew well on the control media YPD and YNB at 30°C and 37°C, while LIM did not support growth. (B) The *vps41* mutants grew poorly at low concentrations of inorganic iron at both 30°C and 37°C, and also had a slight defect with high inorganic iron at 37°C. (C) The *vps41* mutants grew poorly at all tested concentrations of heme. The effect was more pronounced at 37°C. (D) The *vps41* mutants had increased sensitivity to excess amounts of both heme and iron. The assays were repeated at least three times.
3.15 A loss of \textit{VPS41} caused reduced growth in liquid media with inorganic iron and heme.

To confirm the poor growth of \textit{vps41} mutants in the presence of excess inorganic iron and heme, wt and mutant strains were grown in liquid LIM with the addition of increasing and relatively high concentrations of FeCl$_3$ or heme at 30°C. Growth was measured by counting colony forming units to ensure that only live cells were measured. As expected, both the wt strain and the mutants grew poorly in LIM alone (Figure 3.20A). The \textit{vps41} mutants had a long lag phase in the presence of 100 µM FeCl$_3$, but then grew to levels similar to the wt strain (Figure 3.20B). Wt cells were able to grow to a low density in the presence of 1mM FeCl$_3$ but mutant cells were unable to grow. This result indicated that while this high level of iron is tolerable to some degree in the wt strain, it caused toxicity in the \textit{vps41} mutants. This was similar to the pattern of growth seen on solid media containing inorganic iron.

Mutant \textit{vps41} strains grown in the presence of heme as a sole iron source showed a similar pattern of growth as the heme concentration was increased. Mutant cells had a lag in growth for the first 24 hours, and then increased briefly in growth before dying off (Figure 3.21). At an extremely high concentration of heme (6mM), the lag phase for mutants was increased to 48 hours and the growth phase was shorter. Wt cells, on the other hand, were able to grow well even in the presence of 6mM heme, indicating that they had a storage or export system for excess heme that protected the cells from heme toxicity.
Figure 3.20. Growth of wild type and vps41 mutants in liquid media containing inorganic iron. Cells were grown in media containing increasing concentrations of inorganic iron and measured by counting colony-forming units plated on YPD media. (A) Low iron media (LIM) did not support the growth of the wt strain or the vps41 mutant. (B) The vps41 mutant grew similarly to the wt strain in 100µM FeCl₃. (C) Growth of wt cells was impaired in 1mM FeCl₃, while the vps41 mutants were unable to survive.
Figure 3.21. Growth of the wt strain and \textit{vps41} mutants in liquid media containing \textit{heme}. Cells were grown over time in media containing increasing concentrations of heme and measured by counting colony-forming units plated on YPD media. (A) Low iron media (LIM) did not support growth of the wt strain or the \textit{vps41} mutant. The \textit{vps41} mutants exhibited a 24 hour lag phase, grew to low densities and then died off at (B) 100\textmu M and (C) 1mM heme. Wt cells showed a slight decrease in growth at 72 hours but recovered in both concentrations. (D) Wt cells grew well in 6mM heme, while \textit{vps41} mutants had a 48 hour lag phase and died off quickly after 72 hours.
3.16 Heme was sequestered in wt cells but appeared in a diffuse pattern in vps41 mutants.

To further investigate the question of iron/heme storage, cells from the liquid growth curve experiment (Figures 3.20 and 3.21) were also examined by light microscopy at 24 hour intervals. Interestingly, after three days of growth, wt cells grown in 100µM had obvious dark brown or black aggregates inside that were visible without the addition of stains or labelling (Figure 3.22A). It is possible that these structures may be heme sequestered in vesicles or formed into crystals, similar to heme sequestration by malaria parasites (Egan, 2008a). Some of these aggregates were also seen in cells grown with 1mM heme, however many cells did not contain them and instead had large vacuoles that were the predominant visible intracellular feature. Cells grown in 6mM heme did not contain any dark bodies; instead all cells had a very large visible vacuole. Another interesting observation was that as the concentration of heme increased, an increasing amount of brown extracellular material was visible. C. neoformans has been previously shown to shed capsular material in vivo (reviewed in Zaragoza et al., 2009). It is possible that this extracellular matrix was capsular material, but whether it was shed as a method to remove excess internal heme or whether extracellular heme was simply precipitating onto shed material was unclear. Regardless, these results indicated that at lower concentrations of heme, the cells were able to sequester and store heme, but at higher concentrations, heme was removed or broken down in a method that appeared to involve the vacuole.

Because cells lacking VPS41 grew poorly with heme as a sole iron source and had a vacuolar defect, it was presumed that they would show different internal structures compared
to wt cells. Indeed, the vps41 mutants did not contain the black aggregates that were seen in wt cells grown with heme; instead the cells appeared to be filled with diffuse heme that was not sequestered or localized to a discrete internal compartment (Figure 3.24A). The intracellular heme became darker and more concentrated within the cell as the concentration of heme increased. At 0.1mM and 1mM heme, not every cell appeared to contain visible heme, but at 6mM heme, every cell was entirely filled with the brown heme. The brown extracellular material was seen at low levels with 0.1mM heme, and at levels similar to the wt strain with 1mM; however it was not visible at all for mutant cells grown with 6mM heme. Taken together, these results indicated that wt cells of C. neoformans have one or more systems for both heme sequestration and expulsion to protect against heme toxicity, and that these systems required an intact vacuole to function correctly. These results also imply that the brown extracellular material may indeed be part of the protection against heme toxicity, and that this system was impaired in strains lacking VPS41.

The vps41 strains also had impaired growth when provided with inorganic iron (FeCl₃), and these cells were also examined under the microscope. Wt cells show no morphology or colour changes with 0.1 or 1mM iron (Figure 3.22B). However, vps41 mutants became increasingly pink in intracellular compartments. The pink colour likely comes from the presence of the chelator BPS in the media, which becomes pink in colour when bound to iron. BPS is hydrophilic and only has limited membrane permeability; it is therefore normally not taken up by the cell (Jayasena et al., 2007; Kicic et al., 2001). However, the mutants appeared to take up iron-bound chelator, possibly because of a defect in endocytosis. It has been shown that the high affinity iron uptake proteins, Fet3 and Ftr1, are recycled to the vacuole via endocytosis in the presence of iron in S. cerevisiae (Strochlic
et al., 2007; Strohlic et al., 2008). An endocytosis defect in \textit{vps41} strains of \textit{C. neoformans} may therefore cause the endocytosis of chelator as well. Overall, the clear defects in both iron and heme storage in \textit{vps41} mutants correlated well with the poor growth of the mutant strains in the presence of increasing concentrations of inorganic iron or heme, and support the idea that an intact vacuole plays a key role in protection from toxicity.
Figure 3.22. **Heme and iron uptake was altered in vps41 mutants.** Cells were grown with increasing concentrations of heme or FeCl$_3$ and examined by light microscopy. (A) Wt cells contained dark aggregates at low concentrations of heme, but developed a large vacuole and extracellular material as the heme concentration increased (top row). The *vps41* mutants became filled with a brown compound as the concentration of heme increased (bottom row). (B) Wt cells grown with increasing concentrations of inorganic iron did not show a colour change (top row), but *vps41* strains grown with inorganic iron became increasingly pink internally (bottom row). The 6mM concentration of FeCl$_3$ was not included in these experiments due to immediate precipitation of iron, which did not support growth. All assays were repeated three times in triplicate and representative images are shown.
3.17 Internal heme localization could be visualized with fluorescence microscopy using heme or the heme analog zinc mesoporphyrin IX.

The discovery of internal heme-related aggregates was an intriguing result, and the observation of these structures was therefore pursued in a further experiment. Specifically, cells were exposed to a fluorescent heme analog, zinc mesoporphyrin IX (ZnMP), to examine heme localization in the cell, and to confirm that the dark structures in the wt strain did indeed contain heme. The auto-fluorescence of ZnMP has been used previously to identify heme localization in *C. elegans* (Rajagopal *et al.*, 2008). It was unclear if *C. neoformans* would be able to take up ZnMP, as it does not contain an iron molecule and the heme uptake system has not yet been identified. To ensure fluorescence was observed, cells were starved for iron and then grown overnight in the presence of heme alone, a combination of ZnMP and heme, and ZnMP alone, and examined by fluorescence microscopy (Figure 3.23). Wt cells showed low levels of background fluorescence when grown in YPD and LIM as controls. Surprisingly, fluorescence was visible in small punctuate dots within the cells grown solely in the presence of heme. The pattern of fluorescence was very similar to the pattern of the dark aggregates seen by light microscopy, implying that the structures did contain heme. Cells grown with a combination of heme and ZnMP also showed heme localization in bright dot-like bodies, but in addition showed a ring-like fluorescence that may be the vacuolar membrane. Wt cells grown with ZnMP alone mainly showed fluorescence only in the ring-like structure. This indicated that the ZnMP can be internalized by the cell, presumably by the heme uptake system, but it cannot be fully processed like the iron-containing heme. Iron may be required for the additional processing steps. The *hmx1* mutants were also tested and showed fluorescence patterns similar to the wt strain in all
conditions. Given the lack of heme-related phenotypes for \textit{hmxi}, the results for this strain were unsurprising. In contrast, the \textit{vps4i} mutants showed diffuse fluorescence when exposed to heme, ZnMP, or the combination of both. This was also consistent with the results from the light microscopy, and supports the idea that the brown compound seen in the cells is heme that is unable to be sequestered. These data, combined with the results of the light microscopy, support the conclusion that an intact vacuole is required for heme sequestration, which appears to occur in the form of small dark aggregates structures in \textit{C. neoformans}.

Figure 3.23. Visualization of heme and zinc mesoporphyrin IX (ZnMP) localization by fluorescence microscopy. The internal location of heme, ZnMP, or a combination of both compounds was examined after 16 hours of growth. YPD and LIM were included as controls for auto-fluorescence levels. Wt and \textit{hmxi} strains showed localized fluorescence in small punctate structures, while fluorescence was seen throughout the cell in the \textit{vps4i} strain. The assays were repeated at least three times and representative pictures are shown.
4. DISCUSSION

Iron is an essential element for the growth of virtually all organisms, and it must be acquired from the environment. For zoonotic pathogens, this entails acquiring iron from within host tissues or from host proteins. Most bacterial and fungal pathogens require a sufficient supply of iron to proliferate and cause infection in the host; however, free iron is unavailable due to sequestration by high-affinity iron binding proteins such as transferrin and lactoferrin, or because of incorporation into heme molecules found within hemoproteins (Tong and Guo, 2009). Thus, pathogens have developed various iron uptake systems to acquire sequestered iron from the host environment. Previous studies have indicated that \emph{C. neoformans} can grow well when provided with heme as a sole iron source, but to date the uptake and utilization of heme has not be examined. The overall goal of this study was to examine heme utilization and degradation by \emph{C. neoformans}.

The two candidate proteins for heme utilization that were examined in this study were Hmx1, a candidate heme oxygenase, and Vps41, a vacuolar protein. Surprisingly, Hmx1 did not play a demonstrable role in the utilization of heme as a sole iron source, but mutants defective in Hmx1 did have several subtle phenotypes, including a change in supernatant pH, that may indicate a role in heme recycling or regulation in the cell. Vps41 was required for both heme and iron uptake, and also conferred protection against toxicity from excess heme and iron. The analysis of heme uptake in the \emph{vps41} mutant led to the discovery of black aggregates in wild-type cells grown with heme. These aggregates presumably indicate the existence of a heme storage system and/or a mechanism for protection against heme toxicity. These results will be discussed in more detail in the following sections.
4.1 Hmx1 has characteristics of a heme oxygenase but is not required for growth with heme

Many bacterial pathogens can use heme or hemoglobin as an iron source during infection. While the heme uptake systems vary, once heme is transported into the cell, the most common way to release iron from heme for use by the cell is through degradation by a heme oxygenase (HO). Heme oxygenases have been characterized in many mammals and bacteria, as well as two fungi. The HO in \textit{C. albicans}, CaHmx1, is required for growth of the fungus when heme is provided as a sole iron source (Santos \textit{et al}., 2003). Thus the study of a putative HO in \textit{C. neoformans} was an excellent starting point to examine heme utilization. The genome of \textit{C. neoformans} contained one putative HO homolog, designated Hmx1. Sequence analysis of the predicted amino acid sequence revealed that Hmx1 has all of the defining characteristics of a heme oxygenase protein. It is predicted to be a cytosolic protein that is bound to an internal cellular membrane via a C terminal membrane-binding domain. For example, Hmx1 in \textit{S. cerevisiae} is localized to the ER (Protchenko and Philpott, 2003), and it is likely that Hmx1 in \textit{C. neoformans} has similar localization. Hmx1 from \textit{C. neoformans} also contains the key proximal histidine residue required for heme binding, as well as the signature ‘heme oxygenase domain’. Overall Hmx1 was a clear candidate protein for providing a heme degradation function.

The two characterized fungal HOs from \textit{S. cerevisiae} and \textit{C. albicans} are similar in sequence and structure, but have different functions within the cell. Purified, overexpressed Hmx1 proteins from \textit{S. cerevisiae} and \textit{C. albicans} were tested for heme degradation activity, and both proteins were able to degrade heme (Kim \textit{et al}., 2006). However, \textit{S. cerevisiae} is not capable of using heme as a sole exogenous iron source, and ScHmx1 likely plays a role in
heme recycling as well as lowering intracellular heme stores (Protchenko and Philpott, 2003). Similarly, Hmx1 in *C. neoformans* was not required for growth with heme as a sole iron source under any tested conditions. The results for *S. cerevisiae* and *C. neoformans* are therefore in contrast to the demonstrated role of HO in heme utilization in *C. albicans*. This was somewhat surprising because *C. neoformans* and *C. albicans* are both pathogens and it might be expected that they would degrade heme in a similar manner. The lack of phenotypes with heme implies that CnHmx1 plays a very different role than CaHmx1 in *C. albicans*, and potentially plays a role in heme recycling or regulation such as in *S. cerevisiae*. It is also possible that *C. neoformans* contains an additional HO or heme degradation system and that Hmx1 is therefore redundant. Sequence analysis did not reveal an obvious candidate protein for a second HO in the *C. neoformans* genome. However, several HOs have been discovered recently that degrade heme to release iron but that do not share sequence or structural similarity to the mammalian HOs (Skaar *et al.*, 2004; Suits *et al.*, 2005). While similarity searches with the sequences of these HOs did not reveal any additional candidate proteins for heme degradation, it is possible that *C. neoformans* contains an additional protein capable of degrading heme that has not previously been identified.

High affinity reductive iron uptake systems are very efficient at acquiring iron from the environment. Even if heme is a preferred iron source *in vivo*, fungal cells can take up any available free iron in laboratory media *in vitro*, potentially causing difficulty in studying other iron uptake systems. For example, when *C. albicans* proteins were transformed into *S. cerevisiae* to identify proteins that allowed heme uptake, a background strain deficient in the reductive iron uptake system was used (Weissman and Kornitzer, 2004). This ensured that any growth was dependent only on the ability to take up heme. A component of the
reductive uptake system in *C. neoformans*, *CFO1*, was deleted in the *hmx1* strain to confirm that the heme was truly the only available iron source and that the wt level of growth in the *hmx1* strain was not being supported by free iron. No additional defects were seen when *cfo1* was deleted, which confirms that *hmx1* can grow in the presence of heme, and also that *CFO1* is not required for heme uptake as seen previously (Jung *et al.*, 2009). Overall these results indicate that although *C. neoformans* can use heme as a sole iron source, the system for heme degradation does not require the putative HO gene *HMX1*. Therefore an unidentified protein or system to release heme from iron must exist. Screening of a mutant library for candidates that show a growth defect on heme is ongoing and may reveal candidate proteins that function in heme degradation.

A deletion of *hmx1* did not affect virulence in the mouse model of cryptococcosis, which is unsurprising given that the protein is not required for growth with heme as a sole iron source, and a deletion of the gene does not cause a reduction in virulence factor expression. However, *C. neoformans* mutants have previously been identified that do not cause a reduction in virulence factor expression but cause an attenuation of virulence in the mouse model, such as in the initial study of the *VPS41* gene (Liu *et al.*, 2006). This made a preliminary *in vivo* pilot study for virulence reasonable for the current work, but no further virulence assays were performed due to the lack of change in virulence of the *hmx1* mutant. However, it is possible that Hmx1 does play some role in virulence that was not visible in the inhalation mouse model of cryptococcosis, and other virulence models could provide insight into the effects of an *hmx1* deletion.
4.2 HMX1 regulation suggests a role in heme recycling and metabolic changes in the cell

As with their different roles in cell growth on heme, HMX1 genes from C. albicans and S. cerevisiae also differ in their regulation. In C. albicans, HMX1 is up-regulated in response to heme and hemoglobin, as well as at 37°C (Pendrak et al., 2004; Santos et al., 2003). In S. cerevisiae, HMX1 is transcribed in response to iron starvation, although the effect of temperature was not examined (Protchenko and Philpott, 2003). The differences in regulation correlate with the proposed roles of each HMX1: heme degradation in C. albicans which is likely during infection where the temperature is higher, and a response to iron starvation in S. cerevisiae leading to lower intracellular heme stores. C. neoformans HMX1 appears to be regulated more similarly to S. cerevisiae. CnHMX1 is down-regulated in response to the presence of heme or hemoglobin, regardless of temperature. This is consistent with a recycling role for HMX1, because heme would likely not need to be recycled if there was abundant heme provided to cell and thus HMX1 would be down-regulated in the presence of excess heme or hemoglobin. The lack of a pronounced temperature effect is consistent with the idea that CnHMX1 is not required during infection. In S. cerevisiae, it is hypothesized that HMX1 is up-regulated under low iron conditions to degrade heme to release iron for metabolic functions, as well as to lower intracellular heme pools (Protchenko and Philpott, 2003). C. neoformans hmx1 mutants grow as well as wt in liquid LIM, indicating that even if Hmx1 is releasing iron from heme stores, some other heme degradation or storage system can compensate for the loss of Hmx1 or that the amount of iron released is trivial and does not cause an increase in growth.
The regulation of four *C. neoformans* genes known to be regulated by iron was also examined in the wt strain and the *hmx1* mutants in the presence and absence of heme. Although *HMX1* is not required for growth with heme, it is possible that its heme recycling or unknown function within the cell could have a subtle influence by affecting the expression of other iron related genes. The expression of the components of the high affinity uptake system, *CFO1* and *CFT1*, as well as the laccase gene, *LAC1*, responsible for melanin production were unaffected by the deletion of *HMX1*, with or without heme present. This fits with the observations that there is no change in reductive iron uptake or melanin production in the *hmx1* mutant. However, the mannoprotein *CIG1* was up-regulated about 2 fold in the *hmx1* mutants in LIM compared to the wt expression under the same conditions. *CIG1* was also up-regulated slightly more (2.5-3 fold) in the *hmx1* mutants when heme was present compared to the wt expression under the same conditions. *CIG1* was chosen for this analysis because it was discovered by SAGE to be the most abundant transcript in cells grown in LIM conditions (Lian *et al.*, 2005). *CIG1* was annotated as cytokine inducing glycoprotein, and it is known to be a secreted mannoprotein that is present in the plasma membrane, cell wall, and also outside of the cell (Lian *et al.*, 2005, B. Cadieux, personal communication). It appears to have a role in cell wall integrity, and mutants deficient in *cig1* have increased sensitivity to stress. The exact function of *CIG1* is still unclear, but it is positively regulated by the iron-responsive transcription factor *CIR1* and has a clear link to iron. Elucidation of the function of *CIG1* may shed light on the relationship between *CIG1* and *HMX1*. It is possible that the lack of the heme recycling function or additional function is causing a change in the cell that is having a subtle affect either directly or indirectly on the expression
of CIG1. When further information on HMX1 or CIG1 becomes available, it will be interesting to revisit this expression relationship between the genes.

The discovery that there is a difference in supernatant colour and pH when wt and hmx1 cells are grown in LIM containing heme is intriguing. The supernatant of wt cells grown in LIM containing 0.1mM heme became lighter in colour and higher in pH than the hmx1 mutant strains under the same conditions. The colour change and pH drop also occurred in wt cells grown with 1mM heme compared to the hmx1 strains, but the neither the colour or pH differential was as drastic as with 0.1mM heme (See Figure 3.10). The change in supernatant colour appears to be related to the pH of the media. The higher pH and smaller pH change at 1mM may be due to the high concentration of heme in the media. The heme was dissolved in 0.1mM NaOH, which may be buffering the decrease in pH seen at the lower concentration of heme. As well, the medium containing 1mM heme is very dark in colour so any change in colour is less obvious than in the lighter 0.1mM heme media. This result is taken as an indication that Hmx1 has some heme-related activity within the cell. Hmx1 is probably not secreted, as it is thought to be membrane bound and was not predicted to contain any secretion signals, so it is likely causing a change from within the cell. The pH/colour change may be due to Hmx1 modifying a media component, possibly even heme itself. It is also possible that a byproduct of the heme recycling or an additional unknown function of CnHmx1 is secreted into the supernatant, and that the product is causing the pH change. As well, it could be that the function of HMX1 in the cell causes a change in regulation of a gene not tested in this study, and that the pH/colour change is a downstream affect caused by the HMX1 deletion. In S. cerevisiae, iron starved cells undergo a metabolic shift to down regulate metabolic pathways that involve heme containing proteins (Shakoury-
Elizeh et al., 2004). These pathways include the tricarboxylic acid cycle, the mitochondrial respiration and electron transport chain, and the heme and biotin biosynthetic pathways. It is quite likely that C. neoformans undergoes a similar metabolic rearrangement when starved for iron and the regulatory or additional role of *HMX1* may be involved in this shift by lowering intracellular heme pools. Metabolic changes could affect the pH of the supernatant (e.g., through the export of acidic products such as acetic acid and lactic acid), and thus the deletion of *HMX1* may influence alter related metabolic functions.

One by-product of heme degradation by heme oxygenases is CO. In mammals, this CO can act as a signalling molecule that has many molecular targets (Ryter and Choi, 2009). At low concentrations, CO modulates intracellular signalling pathways that involve hemoproteins, as well as downstream effectors. These include targets involved in vasodilation, as well as NADPH oxidase which regulates reactive oxygen species production. MAPK pathways and several transcription factors are also regulated by CO. CO can confer protective effects at low concentrations, such as an anti-apoptosis effect, likely through the pathways that it is capable of modulating (Ryter and Choi, 2009). The role of HO in mammals is clearly much more extensive than just heme recycling, and this is true of yeast as well. It was recently discovered that CO may function as a signal for changes in metabolic cycling in *S. cerevisiae* (Tu and McKnight 2009). *S. cerevisiae* cells have three phases of the metabolic cycle, oxidative phase, reductive rebuilding, and reductive charging, and the addition of CO causes cells to prematurely enter the oxidative phase (Tu and McKnight, 2009). This may be caused by the cell sensing catabolism of free heme. If free heme is available, it may indicate that the heme-containing cytochromes are loaded and the cell is
ready to undergo oxidative respiration. Heme catabolism by HO is the only known biological source of CO, and \textit{S. cerevisiae} \textit{hmx1} mutants were examined for a response to CO (Tu and McKnight, 2009). The \textit{Schmx1} strain showed a delay in entry to the oxidative phase, and was still able to respond to exogenous CO, indicating that there are signals for metabolic change other than CO. The findings in \textit{S. cerevisiae} further support the idea that Hmx1 in \textit{C. neoformans} is responsible for metabolic changes within the cell. In \textit{C. neoformans}, CO may be acting as a signal that causes a downstream effect, and this effect could be responsible for the change in supernatant colour/pH between wt and \textit{hmx1}. However the presence of an additional signal, or possibly the production of CO from the unidentified heme utilization pathway, may compensate for the loss of CO production in the \textit{hmx1} strain of \textit{C. neoformans}. It is also possible that the CO mediated signal is not required under conditions tested in this study, and that CnHmx1 may be important for growth or survival only under certain conditions.

Overall, it appears that Hmx1 does have some heme or iron related function in the cell, such as recycling iron from heme, or lowering intracellular heme pools during iron starvation. However, it is not required for growth with heme as a sole iron source and does not have an effect on the virulence of \textit{C. neoformans}. Because the goal of this study was to examine heme utilization as an iron source, other genes annotated with potential heme related functions were examined. The most promising candidate gene was \textit{VPS41}, as it is known to play a role in iron utilization in \textit{S. cerevisiae} (Radisky \textit{et al.}, 1997). It is also required for an intact and functional vacuole, which plays a role in heme/hemoglobin utilization in \textit{C. albicans} (Weissman \textit{et al.}, 2008). The findings from this study that are related to \textit{VPS41} and iron/heme are discussed in the next section.
4.3 The role of \textit{VPS41} in iron acquisition

In the first study of Vps41 in \textit{C. neoformans} by Liu \textit{et al.}, (2006), \textit{vps41} mutants were not found to have iron-related growth defects. However, the lack of phenotypes may be due to the experimental conditions employed in that study. In the present study, clear iron-related defects were seen in the \textit{vps41} mutants, indicating a discrepancy from the original study, perhaps due to careful control of the iron levels in culture media. The \textit{vps41} mutants show an interesting pattern of growth when grown with inorganic iron. There is a clear growth defect at a low concentration of iron, but the defect is less severe at an intermediate concentration, and appears to be more temperature dependent (See Figures 3.19 and 3.20). However, at a very high concentration, the mutants again show a clear defect at both temperatures. The results imply that the high affinity reductive iron uptake is affected by the deletion of \textit{VPS41}. At higher iron concentrations, another system such as low affinity iron uptake can bring in iron and rescue the iron defect. This is similar to the phenotype seen when components of the reductive uptake system, \textit{CFT1} and \textit{CFO1}, are deleted (Jung \textit{et al.}, 2006; Jung \textit{et al.}, 2008). This correlates well with what is known about endocytosis of the proteins encoded by the \textit{CFO1} and \textit{CFT1} homologs in \textit{S. cerevisiae}, \textit{FET3} and \textit{FRT1}. The Fet3-Frt1 complex is expressed constantly on the plasma membrane due to recycling by the endocytic pathway when cells are starved for iron (Felice \textit{et al.}, 2005; Strochlic \textit{et al.}, 2007). However, when starved cells are exposed to iron, the Fet3-Frt1 complex is rapidly internalized. This occurs by the proteins being internalized into vesicles that are then targeted to the vacuole for degradation. In the absence of iron, the vesicles are sorted back from the endosome to the golgi, and then returned to the plasma membrane (Strochlic \textit{et al.}, 2007; Strochlic \textit{et al.}, 2008). A similar process is likely occurring in \textit{C. neoformans}, which
explains the reductive uptake system defect in the \textit{vps41} mutant. In \textit{S. cerevisiae}, the \textit{vps41} mutant is known to have defects in endocytosis and intracellular trafficking (Radisky \textit{et al.}, 1997; Strochlic \textit{et al.}, 2007), and therefore it is likely that the reductive uptake system is being sorted and internalized incorrectly. For \textit{C. neoformans}, the toxicity effect seen at high iron levels in the \textit{vps41} mutants indicates that intracellular iron storage is affected in the mutants strain. This could be because of the lack of an intact vacuole. Intact vacuolar function and integrity is required for both copper and iron homeostasis in \textit{S. cerevisiae} (Szczypta \textit{et al.}, 1997). Mutants causing a defect in vacuolar structure also have increased sensitivity to metal toxicity. The fact that the \textit{C. neoformans vps41} strain is more susceptible to iron toxicity than the wt indicates the vacuole may be required for protection against iron toxicity in \textit{C. neoformans}, as in \textit{S. cerevisiae}. The growth of \textit{vps41} mutants at 30°C in liquid culture containing iron show the same trend as on solid media. That is, the wt strain and the \textit{vps41} mutants grow at about the same rate at an intermediate concentration of iron but the mutants are not viable at high iron. Even the wt cells are impaired by 1mM iron, indicating that while an intact vacuole is required for protection against iron toxicity, there is a limit to the effectiveness of the system.

The pink colour that is visible in \textit{vps41} cells grown with iron is likely due to the internalization of the iron chelator BPS, which turns pink upon binding to iron. BPS is included in the LIM to ensure iron depletion of the media before addition of a specific iron source. BPS is normally not membrane permeable (Jayasena \textit{et al.}, 2007; Kicic \textit{et al.}, 2001), which is supported by the wt cells, which do not have any visible colour accumulation when grown in media containing BPS and iron. However, it is possible that an endocytosis defect in the \textit{vps41} strain could be causing the cells to endocytose the iron-BPS complex. This may
be related to the proposed endocytosis of Cfo1-Cft1, or it may be a side effect of a general endocytosis defect. It is difficult to determine at this point if the internal pink colour is seen due specifically to the proposed iron storage defect because the presence of BPS inside the cell may be toxic itself, or it may be interfering with normal cellular processing of iron. Regardless of the exact reason, it is clear that Vps41, and likely an intact vacuole, is important for the utilization and storage of inorganic iron as well as protection against iron toxicity.

4.4 The role of VPS41 in heme utilization and storage

The role of Vps41 in the context of heme has not been previously examined to our knowledge. In *C. neoformans*, *VPS41* was found to be required for growth with heme as a sole iron source on solid media at 37°C, and a slight growth defect was also seen at 30°C. The more pronounced defect at 37°C indicates that *VPS41* may play a role under conditions of stress, such as within the host during infection. A *vps41* mutant is known to be drastically attenuated for virulence in the mouse model of cryptococcosis (Liu *et al.*, 2006), and the growth defect at 37°C with heme may provide an explanation. It is currently unclear whether *C. neoformans* has access to heme as an iron source *in vivo*. Most heme in the host is within hemoglobin in erythrocytes (Li and Stocker, 2009), and some pathogens have hemolytic activity to lyse erythrocytes. The genome of *C. neoformans* contains one putative homolog of hemolysin, but the results of a hemolysis test on blood agar were unclear. However it is possible that *C. neoformans* does not secrete hemolysin when other iron sources are available. More work needs to be completed on the potential hemolysin activity. However, it remains possible that heme may be an important iron source during infection, and *VPS41* appears to play a role in using heme as an iron source.
Similar to growth in excess iron, \textit{vps41} mutants are also sensitive to excess heme levels. Again, the effect is more pronounced at 37°C on solid media. In liquid culture at 30°C, \textit{vps41} mutants grown with heme have a delay at the start of growth, and then peak at a low level of growth compared to the wt and die off quickly. The growth pattern indicates that heme is internalized, but then likely becomes toxic, causing cell death. This theory is supported by the results of the light microscopy of \textit{vps41} mutants grown with heme. The cells became brown inside and this phenotype increased with higher concentrations of heme in the media. The cells appear to fill with free heme that remains diffuse throughout the whole cell. Free heme is toxic to cells because it can insert into membranes due to its hydrophobic nature, and the heme-iron cause non-enzymatic redox reactions (Wandersman and Delepelaire, 2004). Therefore, it is tempting to hypothesize that \textit{vps41} mutants can take up heme through one or more unidentified heme uptake systems, but then cannot process or store the internalized heme correctly and die due to heme toxicity. The growth defects with heme as a sole iron source, as well as sensitivity to heme toxicity, suggests that an intact vacuole is required for heme processing, utilization, and storage in \textit{C. neoformans}.

\textbf{4.5 Heme storage may involve heme crystallization}

The examination of \textit{vps41} cells grown with heme led indirectly to a surprising discovery in wt cells. Wt cells grown with heme form black aggregates inside the cell that are clearly visible without the use of dyes or labelling. The aggregates appear to consist of heme, or possibly a modified version of heme, and they are similar in appearance to hemozoin crystals formed by \textit{Plasmodium} species and other malaria parasites (Egan, 2008a). These malaria parasites do not use heme as an iron source; instead heme is a toxic by product.
of hemoglobin degradation. They also do not have a heme oxygenase to degrade heme. They deal with excess heme by forming hemozoin crystals which are chains of dimerized heme molecules formed in the presence of lipids (Egan, 2008a; Egan, 2008b). The crystallization occurs in the parasite vacuole, and the lipids are supplied by the endocytic vesicles that transport hemoglobin to the vacuole. It is reasonable to assume that a similar process is occurring in *C. neoformans*, where heme is delivered to the vacuole via intracellular vesicles and forms heme-containing crystals in the vacuole. To help confirm that the aggregates did in fact contain heme, the auto fluorescent heme analog zinc mesoporphyrin IX (ZnMP) was used to monitor heme location by fluorescence microscopy. ZnMP has been used previously to track the intracellular location of heme in other organisms (Rajagopal *et al.*, 2008). ZnMP was provided to *C. neoformans* cells alone and in combination with heme to ensure that the unidentified heme uptake system was activated. However, ZnMP alone was taken up by the cells. Interestingly, only cells with heme alone and the combination of heme and ZnMP showed fluorescence in the same punctuate pattern as the aggregates seen by light microscopy. Cells grown with ZnMP alone showed a ring-like pattern of fluorescence, which is likely to indicate localization to the vacuolar membrane. These results indicate several things. First, the unidentified heme uptake system appears to recognize the porphyrin ring and not the metal ion in its center, because ZnMP contains a similar porphyrin structure to heme but contains a zinc ion. Second, it implies that ZnMP cannot undergo the same cellular processing as heme. It appears to become concentrated at the vacuolar membrane and cannot be processed further. In *Plasmodium falciparum*, the addition of zinc protoporphyrin IX (ZnPPIX) actually inhibits hemozoin formation (Iyer *et al.*, 2003). However, *C. neoformans* cells grown with the combination of
ZnMP and heme show both the punctate and ring-like fluorescence patterns, which indicates that the heme is still able to crystallize, while the ZnMP is stuck at the vacuolar membrane. The heme dimers in hemozoin involve a bond from one porphyrin ring to the Fe(III) center of its neighbour (Slater et al., 1991), so it is probable that the zinc ion in ZnMP cannot form these same bonds, and therefore cannot form into crystals. Third, the results help to confirm that the aggregates do indeed contain heme. It was initially surprising to see autofluorescence of the aggregates when cells were grown with heme alone. However, hemozoin was recently discovered to be autofluorescent (Bellemare et al., 2009). The autofluorescence of malarial hemozoin and the synthetic hemozoin analog hematin anhydride is due to the condensed phase of heme. Therefore, it is likely that the autofluorescence seen in *C. neoformans* is also caused by condensed heme in a structure analogous to hemozoin.

The results of the light microscopy and fluorescence experiments provide strong evidence that an intact vacuole is required for the formation of the hemozoin-like aggregates to protect against heme toxicity. The *vps41* mutants do not form the aggregates, but instead fill with diffuse heme and die due to heme toxicity. However, wt cells are able to thrive even when provided with an extremely high amount of heme (6mM), indicating that they are capable of preventing death from heme toxicity. Interestingly, the strategy to prevent heme toxicity appears to change at extreme heme concentrations. The aggregates are no longer visible within the cell, and instead the cell becomes predominantly filled with a very large vacuole. As well, a large amount of brown extracellular material is visible that is not associated with cells. This material is also seen to some extent at lower concentrations of heme. It is well documented that *C. neoformans* can shed its capsule. This may occur as a defense response to affect the host immune system *in vivo*, or as a passive effect of creating new capsular
It is unclear at this point whether the extracellular material is capsular, although this is a reasonable assumption. It is also unclear if the brown colour of the material is due to secretion of excess heme by the cell, or if heme in the media is simply precipitating on the capsular material. It is possible to imagine a heme export system involving the vacuole that exports heme to the surface of the cell and expels it along with capsular material. However, the existence of this system is speculative and will need to be examined in detail to determine if it is plausible.

It is worth noting that some of the heme and iron concentrations used in this study are extremely high and would not be found in a biological situation. There may be certain in vivo conditions that could cause a local concentration of heme to be high, such as a patch of ruptured blood vessels or erythrocytes; however, overall concentrations of heme and iron would not reach the levels tested here. With this limitation in mind, these experimental conditions are still valuable because they allow visualization of cell structures or changes that are not visible with biologically relevant concentrations, such as the cellular colour changes of the vps41 mutants. As well, the fact that the wt cells can survive at very high levels of heme and iron indicates that the systems that prevent toxicity are both efficient and important in the cell. The fact that the vps41 mutants have a growth defect with 1 µM heme lends credibility to the results seen at higher concentrations of heme.

4.6 A model for heme and iron utilization in relation to the vacuole

Overall, the results presented in this study with wt and vps41 strains grown in the presence of heme provide strong evidence that the vacuole plays a key role in heme storage and utilization. Hemozoin is formed in parasite vacuoles, and the hemozoin-resembling
aggregates seen in *C. neoformans* are observed in wt cells but not *vps41* mutants. Instead, heme is diffuse throughout the mutant cells, which have a fragmented vacuole and impaired cellular trafficking. In wt cells grown with extremely high amounts of heme, the cells develop a large vacuole, indicating that the structure is key in preventing toxicity because the cells are capable of growing well. This leads to the following model of how heme utilization and storage works in *C. neoformans*.

Heme enters the cell via an unknown uptake system. Once inside, it is shuttled to the vacuole in intracellular vesicles. Heme is degraded for utilization of iron in the vacuole by an unknown protein or system. When excess heme is available, the vesicular membrane lipids facilitate the formation of hemozoin or a related crystallized heme structure, which protects the cells from heme toxicity.

In terms of inorganic iron, the model is that intracellular trafficking is required for the correct function of the reductive iron uptake system, which is likely endocytosed to the vacuole in a similar system to that described in *S. cerevisiae*. The vacuole is also involved in iron storage and the protection against iron toxicity, but the iron storage system has not yet been identified.

### 4.7 Proposed future studies to examine the use of heme by *C. neoformans*

#### 4.7.1 Identification of the heme uptake and degradation systems

Based on the results of this study, it was concluded that the putative HO Hmx1 is not required for growth with heme as a sole iron source. However, *C. neoformans* can grow well with heme, indicating that it does have a system to release iron from heme. Two missing pieces of the story are the heme uptake system and the heme degradation system or enzyme.
Recently, we discovered that heme internalized by *C. neoformans* appears to be modified, possibly by the addition of a side chain (Appendix A). This may indicate that the heme is not a substrate for a traditional heme oxygenase, and some unknown degradation system is required. As well, chitin synthases may play a role in heme uptake or modification at the cell surface. This idea is based on preliminary chemical analyses indicating that the side chain may be related to N-acetyl glucosamine, and a study suggesting that chitin synthases may bind heme (H. Bach, unpublished observations). Both of these avenues are currently being examined to determine what, if any, role they play in heme processing. In parallel, a mutant library is being screened for candidate genes that show a growth defect with heme as a sole iron source.

### 4.7.2 Further analysis of the role of *HMX1*

The synthesis of heme requires oxygen, and heme-containing cytochromes are required for respiration (Tong and Guo, 2009). In *S. cerevisiae*, heme uptake is increased when cells are grown anaerobically (Protchenko *et al.*, 2008). *C. neoformans* grows optimally at atmospheric oxygen concentrations, and an oxygen concentration less than that significantly reduces growth (Odds *et al.*, 1995). Oxygen concentrations in the human brain are reported to be drastically lower than in the atmosphere and vary significantly among anatomical sites (Erecinska and Silver, 2001). *C. neoformans* can colonize the brain, and must adapt to the low O$_2$ level. Low oxygen conditions may cause Hmx1 to have a more important role when *C. neoformans* spreads to the brain during infection. The examination of *HMX1* transcript levels by qRT-PCR under conditions of hypoxia with or without the addition of heme may indirectly shed light on the role of *HMX1* in the brain. As well, the preliminary virulence assay in this study was only a pilot study and the cells inoculated into
mice were cultured in rich media. It is possible that the role of Hmx1 in virulence cannot be visualized in the mouse model, and the interaction of the mutant strains with macrophages may be more informative (Tucker and Casadevall, 2002). Fungal cells within macrophages are assumed to face conditions of nutrient deprivation, including iron limitation; thus a macrophage assay may show a change in survival for the hmx1 strain. Although *S. cerevisiae* Hmx1 is not involved in the utilization of heme as an iron source, purified Hmx1 protein from both *S. cerevisiae* and *C. albicans* have heme degradation ability in vitro as measured by spectroscopy (Kim *et al.*, 2006). Similar analysis of expressed and purified Hmx1 protein from *C. neoformans* would confirm that the protein does have heme degradation activity and therefore support the idea that the protein functions in recycling in a manner similar to *S. cerevisiae*.

4.7.3 Characterization of heme-related aggregates

The discovery of dark aggregates in wt cells grown with heme opens the door to many questions, including what the aggregates contain and how they are formed. Isolation of the aggregates from cells would allow analysis of their structure to confirm that they are hemozoin or a related heme-crystal structure. The anti-malaria drugs chloroquine and quinacrine cause death in the malaria protozoa by inhibiting the formation of hemazoin (Harrison *et al.*, 2000). The drugs are concentrated in the protozoan food vacuole, and quinacrine is also targeted to the vacuole of *S. cerevisiae*, as it has been used as a vacuolar marker in yeast. *C. neoformans* is susceptible to both chloroquine and quinacrine, and the antifungal effects were maximal at pH 7.4 and at 37°C (Harrison *et al.*, 2000). The drugs appeared to be localized to acidic internal compartments in the cell, which likely correlated to the vacuole. This is the site of hemozoin formation in malaria pathogens and it is also the
proposed site of the hemozoin-like aggregate formation in *C. neoformans*. *C. neoformans* may have increased sensitivity to chloroquine or quinacrine when grown in the presence of heme. Analysis of the aggregate formation in cells grown in the presence of both heme and the drugs could be determined by fluorescence, and this experiment would give insight into whether the formation of the aggregates occurs by a mechanism that is similar to that of hemozoin. It would also be interesting to test the *vps41* strain for susceptibility to the drugs, as it does not appear to form the aggregates and may have a difference in survival compared to the wt strain.

### 4.7.4 The role of other vacuolar proteins in heme utilization and storage

The vacuole of *C. neoformans* has been previously implicated in virulence (Erickson et al., 2001). Acidification of the vacuole is required for the production of melanin and capsule, and a strain defective in vacuolar acidification is attenuated for virulence in the mouse model of cryptococcosis. If the vacuolar acidification mutant *vph1* also shows defects in heme utilization and storage, it would support the idea that an intact, functional vacuole is required for correct heme processing in *C. neoformans*. Examination of other genes related to proper vacuole formation or acidification may also lend credibility to the results. Many proteins involved in formation of the vacuole as well as intercellular vesicle transport and vacuolar fusion have been identified in *S. cerevisiae* (Ostrowicz et al., 2008), and many of these proteins have homologs in *C. neoformans*. Vps41 is a member of the HOPS complex, which is required for membrane docking and fusion at the Golgi-to-endosome and endosome-to-vacuole stages of protein transport (Nakamura et al., 1997; Seals et al., 2000). The HOPS complex also contains four other Vps proteins, Vps11, 16, 18, and 33, as well as Vam6 (Ostrowicz et al., 2008). Determining the involvement of each of these proteins as
well as other non-HOPS Vps proteins and SNARES in heme utilization and storage may be informative in teasing out the steps in the process.

4.7.5 Examination of capsule shedding in relation to possible excess heme excretion

Capsule shedding is a well known process by *C. neoformans* (Zaragoza et al., 2009), but the idea that the cell may use capsule shedding and excess heme excretion together to prevent heme toxicity would be interesting to test. An obvious first step in examining this theory is to use capsular antibodies or mass spectroscopy to confirm that that extracellular material seen with cells grown in the presence of excess heme is indeed capsule polysaccharide. Evaluating the formation of extracellular material and sensitivity to excess heme in mutants defective in capsule synthesis, such as *cap59* and *cap60* (Chang and Kwon-Chung, 1994, Chang and Kwon-Chung, 1998), as well as *pka1*, a strain defective in trafficking of capsule to the cell wall (Hu et al., 2007), would also provide primary insight into whether capsule shedding and protection against heme toxicity may be related processes.

4.8 Overall conclusions

In summary, this study has determined that Hmx1 may be a regulatory protein that does not play a major role in the utilization of heme as an iron source. However, it may be involved in recycling of heme as well as changes in metabolism under certain growth conditions, such as iron starvation. The analysis of *vps41* mutants indicated that the vacuole is a key feature in both heme and iron utilization and storage, and appears to be the site of heme storage in the form of aggregates that are likely crystallized heme. Further studies are ongoing to identify the heme uptake and degradation systems in *C. neoformans*. In combination, these studies will eventually lead to a mechanistic understanding of heme
uptake and utilization systems in *C. neoformans*, and an appreciation of the role of heme as an iron source during infection.
REFERENCES


Kwon-Chung, K. J., and Varma, A. (2006). Do major species concepts support one, two or more species within *Cryptococcus neoformans*? FEMS Yeast Research, 6(4), 574-587.


APPENDIX A. Isolation and structural elucidation of a putative modified heme compound

The work in the following section was performed in collaboration with Dr. Horacio Bach.

A.1 Introduction

The study described in this thesis confirmed previous findings that *C. neoformans* grows in presence of heme as a sole iron source. However, this study also revealed that a putative HO, Hmx1, is not required for growth with heme because no differences in the phenotype of *hmx1* mutants were observed when compared to the wt strain. This suggests that *C. neoformans* contains unidentified/alternative pathways for heme uptake and processing.

During the process of preparing a lysate of cells grown with heme as the sole iron source, a red cytosolic fraction was observed, in contrast to a colourless fraction obtained when cells were grown in the absence of heme. The discovery that *C. neoformans* is able to internalize the heme analog ZnMP (see section 3.17) suggests that the heme uptake system likely recognizes the porphyrin ring structure independent of the metal ion loaded in the molecule. Thus, we hypothesized that the red colour in the cytosolic fraction was caused by the internalization of heme. The goal of this section was to isolate and elucidate the structure of the candidate modified heme compound, which presumably confers the red colour to the cytosolic fraction.

A.2 Materials and Methods

To isolate the compound, a 50 mL pre-culture of *C. neoformans* was grown in LIM at 30°C with shaking for 48 hours. The pre-culture was then inoculated into 500 mL LIM with
or without the addition of 100 µM heme and grown for 48 hours at 30°C with shaking. The culture was then split into 250 mL fractions and centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed twice with Li-dH2O. The pellet was then resuspended in lysis buffer (50 mM HEPES pH 7.5, 5% glycerol, 1 mM DTT, 0.1% NP-40) followed by the addition of 100 µL of 1 M PMSF to deactivate serine proteases. Cells were then lysed with a bead basher for 2 minutes, followed by two minutes on ice, and a second treatment with the bead basher. The lysate was centrifuged at 15,000 rpm for 30 minutes and the supernatant was collected. The compound was then precipitated with 5 volumes of cold acetone at -20°C overnight, and then centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 50 mM HEPES pH 7.5, followed by the addition of 20 µg mL⁻¹ Proteinase K. The mixture was placed at 42°C overnight. The next day, the mixture was spotted onto a TLC Silica gel 60 F₂₅₄ plate (EMD Chemicals Inc.), and chromatographed in a TLC chamber using a solvent system consisting of 85% benzene, 15% methanol, and 0.1% formic acid. To further purify the compound, a more polar solvent system consisting of 50% benzene, 50% methanol, and 0.1% formic acid was used. After separation on a TLC plate, two compounds were visualized using a UV lamp; these were marked and eluted with methanol after scraping from the plate.

Cells were tested for their ability to grow using the purified compounds as a sole iron source. In the first experiment, 5 mL of wt and hmx1Δ2 strains were pre-cultured as described previously, counted, and 5x10⁵ cells mL⁻¹ were inoculated into 1 mL LIM containing 1, 5 or 10 µL of the compound mixture. Cultures of LIM supplemented with or without 10 µM heme were included as controls. Cultures were grown for 4 days at 30°C with shaking and then counted using a hematocytometer chamber. In a second experiment,
cells were tested by their ability to grow on each compound purified as described for the first experiment. Equivalent weights of each compound were measured and added to 1 mL LIM. An equal weight of heme was included as a control as well as LIM alone.

Dr. Luis Hernandez at the Department of Chemical-Biological Sciences, University of the Americas, in Puebla, Mexico, performed structure analyses using $^1$H-NMR and $^{13}$C-NMR and the compounds isolated by TLC.

A.3 Results and Discussion

When *C. neoformans* was cultured in LIM supplemented with heme as a sole iron source, a red colour was visualized in the cytosolic fraction of these cells. Interestingly, this colour was not observed in cultures without addition of heme. The presence of red colour suggests that heme was internalized into the cells by a not yet defined transport/processing system.

To identify the red compound, a cytosolic fraction of *C. neoformans* was processed as described in Section A.2. The results of TLC analysis revealed the presence of a compound more polar than heme and protoporphyrin IX (heme without iron), according to the calculated retention factors (Rf) (Figure A.1). This observation implies that heme has been modified during or after its uptake by *C. neoformans*. 
Figure A.1. TLC separation of heme, PPIX, and the purified compound. Heme (Rf: 0.57), PPIX (Rf: 0.70) and the purified compound (Rf: 0.21) run differently by TLC, indicating they are not the same compound. The background colour was changed for the compound to better visualize the spot.

To determine whether the compound isolated from the cytosolic fraction can serve as a sole iron source, both wt and hmx1 strains were cultured in its presence in LIM. The results showed that the purified compound served as a sole iron source for both strains (Figure A.2), suggesting the presence of available iron.
Figure A.2. The isolated compound supports the growth of wt and hmx1 strains in LIM. Wt (gray) and hmx1 (black) strains were starved for iron in LIM and then inoculated into fresh LIM containing the purified compound as indicated. Both strains were able to grow well in the presence of the compound as a sole iron source. Due to the limited amount of available compound, the experiment was only performed once.

To further purify the compound, a more polar solvent system was employed by decreasing the percentage of benzene in the mixture. TLC analysis revealed that the previous isolated spot was a mixture of two compounds. Both compounds were separated again as described in Section A.2., and defined as “top” and “bottom” compound according to their migration patterns on TLC plates. Both the “top” and “bottom” compounds were more polar than heme. To determine whether these compounds can serve as a sole iron source, each compound was individually supplied to a *C. neoformans* culture grown in LIM. The results showed that only the “top” compound was able to support fungal growth (Figure A.3), suggesting that it contained iron. It is possible that the “top” and “bottom” compounds are the same entity with and without iron, or that the compounds are different intermediates in the same pathway.
Figure A.3. The “top” compound supports growth of iron starved cells. Wild type cells were starved for iron and then transferred to LIM containing 0.8 or 4 mg of “top” compound, “bottom” compound, or heme. Only the “top” compound and heme could support growth of cells indicating that no iron is present in the “bottom” compound. Due to limited amount of available compound, the experiment was only performed once.

In an attempt to elucidate the structures of the compounds, NMR analyses were performed in detail on the “bottom” compound (which was produced more abundantly). $^1$H-NMR revealed that this compound contained hydrogens, which were attached to esters, and alcohols in an aromatic structure. However, this aromatic structure appeared not to be the characteristic pyrrole ring, as vinylic hydrogens were not observed, suggesting that this position has been hydroxylated. $^{13}$C-NMR revealed that the structure also contains a sugar monomer. The “top” compound was also sent for structural analysis, but it appeared to be contaminated with the “bottom” compound.

Overall, these results imply that *C. neoformans* is capable of modifying heme during or after internalization. It may be speculated that an uncharacterized heme degradation
system could be involved in modifying heme during the process of degradation. As well, it appears that both the “top” and “bottom” compounds may be intermediates. More material has been prepared for additional structure analyses such as 2D NMR, IR, UV, polarimetry, and mass spectrometry (ESI). We believe that the results of such analyses will shed light on the structure of the compounds and will give insight into their role in the heme metabolism.
APPENDIX B. Additional results

B.1. Deletion analysis of additional candidate genes for heme uptake or utilization.

Because the *hmx1* strains did not show a heme-related growth defect, other genes that were predicted to have a function related to heme were also deleted. The resulting mutants were then tested for their ability to grow with heme as a sole iron source (Table B.1).

Unfortunately, all of mutants were able to grow well with heme in a preliminary screen, and thus were not examined further.

**Table B.1.** Additional genes with potential heme-related functions that were deleted in this study.

<table>
<thead>
<tr>
<th>JEC21 ID (NCBI)</th>
<th>H99 locus (BROAD)</th>
<th>Annotation</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNJ01390</td>
<td>CNAG_04707.2</td>
<td>Conserved hypothetical protein</td>
<td>CFEM domain</td>
</tr>
<tr>
<td>CNA02900</td>
<td>CNAG_00306.2</td>
<td>Conserved hypothetical protein</td>
<td>CFEM domain</td>
</tr>
<tr>
<td>CNA04580</td>
<td>CNAG_00476.2</td>
<td>Conserved hypothetical protein</td>
<td>CFEM domain</td>
</tr>
<tr>
<td>CNA08210</td>
<td>CNAG_00844.2</td>
<td>Conserved hypothetical protein</td>
<td>Major Facilitator Superfamily</td>
</tr>
<tr>
<td>CNF01700</td>
<td>CNAG_05809.2</td>
<td>Ferrochelatase</td>
<td>Required for heme synthesis</td>
</tr>
<tr>
<td>CNH00110</td>
<td>CNAG_06976.2</td>
<td>Ferric chelate reductase</td>
<td>Heme containing membrane protein</td>
</tr>
</tbody>
</table>
B.2. Additional phenotypic assays for the *hmx1* mutants.

The *hmx1* mutant strains were tested for growth under other conditions, as described in Materials and Methods (Figure B.1). No differences were found compared with the wt strain.

![Figure B.1. Analysis of additional growth conditions for phenotypic differences between the wt and *hmx1* strains.](image)

Ten-fold serial dilutions of cells were spotted onto solid media containing various additions as indicated and were monitored to assess defects in growth. Plates were incubated at 30°C and 37°C for three to five days. The *hmx1* strains did not have a growth defect on standard control media (A), on stress inducing agents (B), or on antifungal drugs (C). All assays were repeated three times.