Identification and Characterization of *Histoplasma capsulatum* extracellular proteins and their roles in virulence.

DISSertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Graduate Program in Microbiology

The Ohio State University

2012

Dissertation Committee:

Dr. Chad Rappleye, Ph.D., Advisor

Dr. Brian Ahmer, Ph.D.

Dr. John Gunn, Ph.D.

Dr. Stephanie Seveau, Ph.D.
Abstract

*Histoplasma capsulatum* is the most common cause of endemic mycosis in the world and is endemic to the Midwestern U.S. *Histoplasma* commonly resides within soil as conidia-producing mycelia, but upon temperature shift to 37°C it undergoes a morphological shift to yeast that parasitize phagocytic cells and cause disease. The mechanisms allowing *Histoplasma* to circumvent the innate immune response and cause disease are largely unknown, however all known virulence determinants are extracellular factors that are predicted to interact with the host immune system and promote *Histoplasma* survival. The purpose of this dissertation research is to identify the extracellular proteins released by pathogenic-phase *Histoplasma* and to characterize the involvement of these proteins in *Histoplasma* pathogenesis. Utilizing shotgun proteomics we identified the 33 most abundant extracellular proteins, including factors from several diverse functional groups. The groups included proteins involved in cell wall biosynthesis, oxidative stress defense, chaperone activity and proteins of unknown function (Cfp4). We identified proteins with potential roles in virulence by determining which were expressed primarily by *Histoplasma* cells in the pathogenic-phase and that those that are expressed during residence in macrophages and during lung infections. From this, we prioritized an extracellular superoxide dismutase (Sod3), extracellular catalase (CatB) and a protein of unknown function (Cfp4) for further analysis and
Histoplasma strains depleted of these proteins were created to functionally characterize these factors.

We demonstrated that Sod3 is required for Histoplasma survival against neutrophils and activated macrophages by protecting Histoplasma against host-derived superoxide. The Sod3 protein is both secreted and attached to the yeast cell wall by a putative GPI-anchor. At the cell surface, Sod3 specifically protects Histoplasma against extracellular superoxide such as that produced by host phagocytes. Sod3 is required for the survival of Histoplasma in vivo and is required for disease causation. In contrast, the CatB protein is not required for Histoplasma survival in macrophages or in vivo, despite being required to protect yeast against hydrogen peroxide in vitro. To determine if Sod3 and CatB act synergistically protect Histoplasma against host-derived reactive oxygen we generated the first double deletion mutant in Histoplasma. Our results show that Sod3 but not CatB provide protection to Histoplasma. To determine if the non-essentiality of CatB for virulence results from redundancy with additional catalases, we investigated the contributions of an intracellular catalase, CatP. We also determined that Cfp4, is not required for Histoplasma virulence, but is recognized by the human immune system and therefore has potential as a diagnostic antigen. Our results provide a mechanistic explanation as to how Histoplasma survives the encounter with phagocytes by producing Sod3 and a dual catalase system to detoxify host-derived anti-microbial reactive oxygen.
Dedication

This document is dedicated to my family and friends for their encouragement and support throughout my life. I would especially like to thank my parents for their love and support from the day I was born, through my academic career and into the future.
Acknowledgments

I would like to thank my advisor Dr. Chad Rappleye for all of his assistance throughout my graduate career. Chad has helped me enormously in becoming a better scientist and enhancing my ability to examine all problems critically.

I also wish to thank my dissertation committee of Dr. Brian Ahmer, Dr. John Gunn and Dr. Stephanie Seveau for their advice and assistance over the years. They have helped me to become a better critical thinker and better able to consider alternative experimental techniques.

Additionally, I would like to thank the members of the Rappleye and Seveau labs for all of their help over the years. In particular I would like to thank Brian Youseff, Dr. Jessica Edwards, Olga Zemska, Katie Smolnycki, Megan Kemski, Steve Vadia and Eusondia Arnett for all of their help in the lab, for being great people and making the harder times more bearable. I truly appreciate all of the help and could not have done it without you.
Finally, I want to thank the CMIB for providing me a forum to present my work in front of so many great minds within the pathogenesis world. Additionally, I want to thank the National Institutes of Health and Public Health Preparedness for Infectious Disease for funding.
Vita

June 2006 ................................................................. B.S. Microbiology, Ohio State University

September 2006 to June 2009 ......................... Graduate Research and Teaching Associate,

Ohio State University

June 2009 to September 2011 ....................... PHPID Graduate Fellow, Ohio State

University

Present ......................................................... Graduate Research Associate, Department

of Microbiology, Ohio State University

Publications

Superoxide Dismutase Protects Histoplasma Yeast Cells from Host-Derived Oxidative
contributing authors.

extracellular proteome of pathogenic-phase Histoplasma capsulatum. J. Proteome Res.


Fields of Study

Major Field: Microbiology
# Table of Contents

Abstract .......................................................................................................................... ii
Dedication ......................................................................................................................... iv
Acknowledgments .......................................................................................................... v
Vita ..................................................................................................................................... vii
Chapter 1 : Introduction ................................................................................................. 1
  1.1 *Histoplasma capsulatum* ......................................................................................... 1
  1.2 Histoplasmosis ......................................................................................................... 1
  1.3 The mammalian immune system ............................................................................. 4
  1.4 Antimicrobial mechanisms of phagocytic cells ....................................................... 6
  1.5 Interactions between *Histoplasma* and phagocytes ............................................ 8
  1.6 *Histoplasma* virulence factors ............................................................................. 11
  1.7 Mechanisms of dimorphism ................................................................................... 17
  1.8 Secreted virulence proteins in eukaryotic pathogens ............................................. 18
  1.9 Eukaryotic secretion mechanisms ......................................................................... 21
  1.10 Research goals ...................................................................................................... 26
Chapter 2 : Definition of the extracellular proteome of pathogenic-phase *Histoplasma*
  capsulatum ....................................................................................................................... 28
  2.1 Introduction ............................................................................................................. 28
  2.2 Materials and Methods .......................................................................................... 31
    2.2.1 Fungal culture ................................................................................................. 31
    2.2.2 Culture filtrate and cellular lysate preparation .................................................. 32
    2.2.3 Electrophoretic separation and visualization of proteins .................................. 34
    2.2.4 Shotgun mass spectrometry-based identification of extracellular proteins ...... 34
    2.2.5 Identification of proteins by band excision ...................................................... 37
2.2.6 Immunoblotting ................................................................. 38
2.2.7 *Histoplasma* RNA isolation and analysis ................................ 39
2.2.8 Construction of RNAi lines .................................................. 41
2.3 Results .................................................................................... 42
  2.3.1 Characterization of the *Histoplasma* extracellular protein profile ...... 42
  2.3.2 Identification of the major constituents of the pathogenic-phase extracellular proteome ............................................................... 49
  2.3.3 Expression of extracellular proteins in the pathogenic-phase .......... 62
  2.3.4 Molecular genetic confirmation of pathogenic-phase extracellular protein identities ................................................................. 65
  2.3.5 Confirmation that pathogenic-phase extracellular factors are expressed during infection ............................................................... 70
2.4 Discussion .............................................................................. 74

Chapter 3 : Extracellular Superoxide Dismutase Protects *Histoplasma* Yeast Cells from Host-Derived Oxidative Stress ............................................................... 83
3.1 Introduction ............................................................................. 83
3.2 Material and Methods ............................................................. 86
  3.2.1 Ethics Statement ................................................................. 86
  3.2.2 Fungal strains and culture .................................................... 86
  3.2.3 Generation of the sod3Δ mutant, *SOD3*-complemented, and *SOD1*-RNAi strains ........................................................................ 88
  3.2.4 Preparation of *Histoplasma* culture filtrate and cellular lysate samples ........ 90
  3.2.4 Superoxide dismutase assay .................................................. 91
  3.2.5 Construction and analysis of strains expressing variant Sod3 proteins ........ 92
  3.2.6 Determination of susceptibility to superoxide ................................ 93
  3.2.7 PMN isolation and infection .................................................... 94
  3.2.8 Macrophage isolation and infection ........................................ 96
  3.2.9 *In vivo* virulence determination ........................................... 97
3.3 Results .................................................................................... 99
  3.3.1 The *Histoplasma SOD3* gene encodes an extracellular superoxide dismutase 99
  3.3.2 Sod3 is both secreted from and associated with yeast cells .......... 104
3.3.3 Sod3 protects specifically against extracellular reactive oxygen .............110
3.3.4 Sod3 protects *Histoplasma* yeast cells from phagocyte-derived reactive oxygen ..............................................................................................119
3.3.5 Sod3 promotes *Histoplasma* virulence *in vivo* .................................130
3.4 Discussion ............................................................................................141

Chapter 4: The extracellular catalase CatB is dispensable for *Histoplasma* virulence...
4.1 Introduction ..........................................................................................150
4.2 Materials and Methods .........................................................................153
  4.2.1 Fungal strains and culture conditions ..................................................153
  4.2.2 Generation of *catbΔ, catbΔsod3Δ, CATB-complement* and RNAi construction ...........................................................................................................154
  4.2.3 *Histoplasma* RNA isolation and qPCR .............................................156
  4.2.4 Harvesting yeast culture supernatants and catalase activity assay ........157
  4.2.5 Determining sensitivity to hydrogen peroxide *in vitro* .......................158
  4.2.6 PMN isolation and infection ...............................................................159
  4.2.7 Harvesting and differentiation of human blood monocytes ..................160
  4.2.8 Determination of virulence within the mouse model ...........................161
4.3 Results ....................................................................................................162
  4.3.1 CatB is responsible for extracellular catalase activity. .........................162
  4.3.2 CatB protects against hydrogen peroxide *in vitro* ............................167
  4.3.3 CatB protects *Histoplasma* from PMN generated hydrogen peroxide ....167
  4.3.4 *Histoplasma* does not require CatB to survive within human MDMs ....170
  4.3.5 CatB is dispensable for growth *in vivo* ..............................................177
  4.3.6 *Histoplasma* is protected by a redundant system of catalases ..........180
4.4 Discussion .............................................................................................195

Chapter 5: Cfp4 does not contribute to *Histoplasma* pathogenesis despite being an immunoreactive antigen...
5.1 Introduction ..........................................................................................203
5.2 Materials and Methods .........................................................................205
  5.2.1 Fungal strains and culture conditions ..................................................205
5.2.2 Generation of Cfp4 depletion strains ................................................................. 206
5.2.3 Macrophage killing assay ...................................................................................... 209
5.2.4 Isolation and infection of mouse peritoneal macrophages ................................. 210
5.2.5 Isolation and infection of human monocyte derived macrophages .......... 211
5.2.6 Bioinformatic analysis of Cfp4. .............................................................................. 213
5.2.7 Mouse infection assays ......................................................................................... 213
5.2.8 Cfp4 recognition by the human immune system ............................................. 215
5.3 Results ......................................................................................................................... 216
  5.3.1 Cfp4 is abundantly expressed yeast protein...................................................... 216
  5.3.2 *Histoplasma* does not require Cfp4 for growth within macrophages. ............ 220
  5.3.3 Cfp4 is dispensable for growth in the mouse ..................................................... 226
  5.3.4 Human *Histoplasma* immune serum detects Cfp4 ....................................... 231
5.4 Discussion ................................................................................................................. 236
Chapter 6: Conclusions ................................................................................................. 241
References ....................................................................................................................... 255
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Previously described virulence factors of <em>H. capsulatum</em></td>
<td>13</td>
</tr>
<tr>
<td>1.2</td>
<td>Mechanisms of nonclassical secretion in eukaryotes.</td>
<td>25</td>
</tr>
<tr>
<td>2.1</td>
<td>Protein profiles of <em>Histoplasma</em> cellular and extracellular fractions.</td>
<td>45</td>
</tr>
<tr>
<td>2.2</td>
<td>Absence of cellular factors in concentrated culture filtrate samples.</td>
<td>47</td>
</tr>
<tr>
<td>2.3</td>
<td>Concentration and recovery of yeast phase culture filtrate proteins.</td>
<td>50</td>
</tr>
<tr>
<td>2.4</td>
<td>Proteomic identification of the major constituents of the extracellular proteome of <em>Histoplasma</em> yeast.</td>
<td>54</td>
</tr>
<tr>
<td>2.5</td>
<td>Phylogenetic distribution of superoxide dismutase enzymes.</td>
<td>58</td>
</tr>
<tr>
<td>2.6</td>
<td>Confirmation of protein identities by protein excision from SDS-PAGE and LC-MS/MS.</td>
<td>61</td>
</tr>
<tr>
<td>2.7</td>
<td>Relative expression of extracellular protein-encoding genes by yeast or mycelial-phase <em>Histoplasma</em> cells.</td>
<td>64</td>
</tr>
<tr>
<td>2.8</td>
<td>RNA interference confirms mass spectrometry-derived protein identities.</td>
<td>66</td>
</tr>
<tr>
<td>2.9</td>
<td>Extracellular proteins are expressed in vivo by <em>Histoplasma</em> during infection.</td>
<td>67</td>
</tr>
<tr>
<td>3.1</td>
<td><em>Histoplasma</em> Sod3 encodes an extracellular Cu++-dependent superoxide dismutase.</td>
<td>101</td>
</tr>
<tr>
<td>3.2</td>
<td>N-terminal and C-terminal signals direct extracellular localization of Sod3.</td>
<td>105</td>
</tr>
<tr>
<td>3.3</td>
<td><em>Histoplasma</em> Sod3 does not alleviate intracellular oxidative stress.</td>
<td>112</td>
</tr>
<tr>
<td>3.4</td>
<td>Increasing amounts of xanthin oxidase increase the amount of superoxide produced.</td>
<td>115</td>
</tr>
<tr>
<td>3.5</td>
<td>Sod3 protects <em>Histoplasma</em> yeast cells from exogenous superoxide in vitro.</td>
<td>117</td>
</tr>
<tr>
<td>3.6</td>
<td>Sod3 protects <em>Histoplasma</em> yeast from PMN-derived reactive oxygen.</td>
<td>121</td>
</tr>
<tr>
<td>3.7</td>
<td>Treatment with DPI does not affect phagocyte viability.</td>
<td>123</td>
</tr>
<tr>
<td>3.8</td>
<td>Sod3 <em>Histoplasma</em> yeast from ROS produced by activated macrophages.</td>
<td>126</td>
</tr>
<tr>
<td>3.9</td>
<td><em>Histoplasma</em> virulence <em>in vivo</em> requires Sod3.</td>
<td>131</td>
</tr>
<tr>
<td>3.10</td>
<td>Lungs infected with Sod3-deficient <em>Histoplasma</em> yeast have reduced inflammation.</td>
<td>134</td>
</tr>
<tr>
<td>3.11</td>
<td>Lethal infection by <em>Histoplasma</em> requires Sod3 function.</td>
<td>136</td>
</tr>
</tbody>
</table>
Figure 3.12 Intracellular Sod1 function is dispensable for *Histoplasma* virulence. .......138
Figure 3.13 Sod3 facilitates infection through detoxification of host reactive oxygen...139
Figure 4.1 *Histoplasma* encodes a secreted and yeast associated catalase.............163
Figure 4.2 CatB protects *Histoplasma* yeast against hydrogen peroxide in vitro........168
Figure 4.3 CatB is required to protect *Histoplasma* from human PMN oxidative killing..........................................................................................................................171
Figure 4.4 CatB is dispensable for survival within resting human macrophages. ......174
Figure 4.5 CatB is not required for *Histoplasma* survival in IFNγ activated human macrophages..........................................................................................................................175
Figure 4.6 CatB is dispensable for *Histoplasma* growth in mouse lungs...............178
Figure 4.7 CatB is dispensable for *Histoplasma* dissimination to mouse spleens........181
Figure 4.8 CATB is the only yeast phase enriched *Histoplasma* catalase...............183
Figure 4.9 *Histoplasma* lacking CatB and CatP lacks intracellular and extracellular catalase activity .................................................................186
Figure 4.10 The loss of both CatB and CatP increases *Histoplasma* sensitivity to PMN killing..........................................................................................................................188
Figure 4.11 *Histoplasma* requires CatB and CatP to survive in IFNγ activated human macrophages..........................................................................................................................191
Figure 4.12 *Histoplasma* requires CatB and CatP to survive in vivo......................193
Figure 5.1 Multiple *Histoplasma* isolates express Cfp4 protein............................217
Figure 5.2 *Histoplasma* does not require Cfp4 to replicate within and kill macrophages. ..........................................................................................................................221
Figure 5.3 Cfp4 is not required for *Histoplasma* survival in human macrophages.......224
Figure 5.4 Panamerican *Histoplasma* isolates do not require Cfp4 for growth in vivo.227
Figure 5.5 North American *Histoplasma* isolates do not require Cfp4 for growth in vivo. ..........................................................................................................................229
Figure 5.6 *Histoplasma* yeast expressing Cfp4 do not have a fitness in vitro or in vivo. ..........................................................................................................................232
Figure 5.7 Cfp4 is recognized by the human immune serum during histoplasmosis infection. ..........................................................................................................................234
List of Tables

Table 2.1. *Histoplasma* strains .................................................................33
Table 2.2. Components of the *Histoplasma* Pathogenic-Phase Extracellular Proteome...56
Table 3.1. *Histoplasma capsulatum* strains ....................................................87
Table 4.1. *Histoplasma capsulatum* strains. ...................................................155
Table 5.1. *Histoplasma capsulatum* strains. ...................................................207
Chapter 1: Introduction

1.1 *Histoplasma capsulatum*

*Histoplasma capsulatum* is the causative agent of the respiratory disease histoplasmosis, and is the leading cause of endemic mycosis in the world (1). *Histoplasma* is one of the few fungi with the capability of causing disease in both immunocompetent and immunocompromised human hosts (2-4). *H. capsulatum* has a world-wide distribution, but the major endemic regions include North America, Latin America and Africa. Within the U.S. the primary endemic regions are located within the Ohio and Mississippi river valleys, and surrounding areas, where serological tests have indicated 80% of the population has been exposed (5). Within endemic regions it has been estimated that up to 200,000 individuals are infected with *Histoplasma* in the U.S. every year (6). *Histoplasma* is particularly enriched in soils containing bird or bat guano (7, 8).

1.2 Histoplasmosis
Most cases of histoplasmosis occur as isolated incidences; however, outbreaks do occur within endemic regions. These outbreaks typically involve populations of otherwise healthy individuals. One such example occurred when over 500 high school students, staff, and faculty at an Indiana high school were exposed to *Histoplasma* conidia and over 350 individuals developed acute pulmonary histoplasmosis (9). This outbreak was linked to landscaping work in a central courtyard leading to *Histoplasma* spores in the ventilation system. Even outside the endemic regions outbreaks can occur, as was the case in a Dallas, Texas medical school where a long term construction project led to 30 individuals showing signs of clinical histoplasmosis (10). Generally, such outbreaks are linked to large scale soil disturbances that lead to large numbers of conidia being released near a human population (11, 12).

Histoplasmosis is a disease with a variety of clinical manifestations that are dependent upon the extent of exposure and immunological state of the host. *Histoplasma* infections range from an asymptomatic infection to varying disease states such as acute pulmonary histoplasmosis (APH), subacute pulmonary histoplasmosis (SPH), chronic pulmonary histoplasmosis (CPH) and progressive disseminated histoplasmosis (PDH) (13). In the majority of cases the infectious dose of *Histoplasma* is low and individuals typically demonstrate no symptoms or minor symptoms that are self-resolving (4, 13). APH and SPH are clinically very similar and differ only by the length of symptom presentation. Both APH and SPH are defined as *Histoplasma* pneumonia with flu like
symptoms (fever, chills, coughing, myalgias, malaise and headaches) (14). Generally, mild cases of APH are self-resolving but may require hospitalization and ventilation if infectious dose was high. CPH occurs primarily in individuals with emphysema or chronic bronchitis and is characterized by symptoms that persist for 3 months or longer, such as weight loss, dyspnea, productive cough and hemoptysis (15). CPH disease presentation can be mistaken for tuberculosis (13). PDH is the most life-threatening form of histoplasmosis and typically results from complications involved with APH such as immunodeficiencies, i.e. AIDS, malignancy, immunosuppressive therapy, advanced age or in some cases in healthy individuals (16-22). Dissemination to the liver, spleen, lymph nodes, gastrointestinal tract, adrenal glands, bone marrow and heart often occur. PDH is often clinically indistinguishable from severe APH, and symptoms include fever, nonproductive cough, weight loss and diarrhea. Without treatment this disseminated form can reach mortality rates as high as 80%, but antifungal treatments, like amphotericin B, decrease mortality to 25% (23).

*H. capsulatum* belongs to a group of ascomycetes known as the thermally dimorphic fungi. This group consists of multiple primary pathogens including other North American endemic fungi such as *Blastomyces dermatitidis* and *Coccidioides posadasii*, and the South American *Paracoccidioides brasiliensis*. Dimorphism is defined as the ability to exist in two mutually exclusive morphological states depending on environmental conditions. Within this group, fungi exist as either a saprobic filamentous mold within the soil, or yeast, or spherule in the case of *Coccidioides*, inside the
mammalian host. The trigger between these two states is temperature, with the mold form present at ambient temperatures, 25°C, and yeast present only at mammalian body temperature of 37°C. Only the yeast form is capable of causing disease, as *Histoplasma* chemically or genetically locked into the mold form is incapable of causing disease (24, 25). The transition from mold to yeast is also characterized by large scale changes in gene expression, with an increase in transcripts for known virulence factors required for survival within the host and a decrease in transcripts potentially involved in mold-phase growth (26).

The mold-form of *H. capsulatum* produces macroconidia (8-15 um in diameter) and microconidia (2-4 um in diameter) that germinate into mold in soil and propagate the saprobic life-cycle. However, conidia can also be inhaled by a mammal where the increase in ambient temperature triggers the conversion to the parasitic life-cycle. Microconidia are small enough to reach the alveolar spaces within the lung where they encounter resident alveolar macrophages and are phagocytozed. The yeast grow and replicate within macrophages, eventually leading to the destruction of the phagocyte and releasing yeast cells to infect additional phagocytic cells. Typically, infections are self-resolving primarily through the actions of CD4+ T-cells that produce pro-inflammatory cytokines that allow macrophages to effectively control *Histoplasma* replication (27, 28).

1.3 The mammalian immune system
The mammalian immune system is characterized by an innate and adaptive response. The innate immune response is primarily characterized by phagocytic leukocytes such as neutrophils (polymorphonuclear neutrophils [PMNs]), monocytes and macrophages (29). These cells are known as professional phagocytic cells and are capable of engulfing and destroying invading organisms. In addition to destroying microbes resident tissue macrophages are also responsible for clearing damaged host cells, apoptotic bodies and other debris from tissues (30). Professional phagocytes recognize invading organisms based upon the presence of specific microbial antigens called pathogen associated molecular patterns (PAMP) interacting with pattern recognition receptors (PRR) on the phagocyte (31, 32). Recognition of antigen by PRRs triggers numerous downstream responses including signal transduction pathways, phagocytosis, an oxidative burst and cytokine/chemokine production to recruit and activate additional immune cells. These cells recognize and engulf invading organisms into specialized endosomes known as phagosomes, where microbes are exposed to numerous harsh conditions.

The adaptive immune response is the source of immune memory, allowing the immune system to respond more intensely and quickly to reinfection. The actions of the adaptive immune response are primarily mediated by B- and T- lymphocytes (33). These lymphocytes specifically recognize microbial antigens in either a soluble state, Ig receptors of B-cells, or cell-associated manner, antigen-major histocompatibility complex (MHC) recognized by T-cells. Upon recognition of antigen, B-cells mature into plasma
cells that produce antibodies specific to the microbial antigen. Antigen recognitions mediate various terminal effects such as direct pathogen killing by formation of the membrane attack complex, or by enhancing phagocytosis and killing mechanisms of innate immune cells. Upon recognition of the antigen-MHC by naïve CD4+ or CD8+ T-cells, thymocytes undergo clonal expansion, and begin to produce numerous cytokines and chemokines that act to recruit additional immune cells to sites of infection. The types of cytokines produced by CD4+ T-cells can be further broken down into Th1 or Th2 subsets. The Th1 response promotes cellular immunity (IL-12 and IFN-γ) or humoral immunity (IL-4), respectively. Additionally T-cell activation can stimulate direct killing of an infected host cell in the case of CD8+ T-cells, or activate additional antimicrobial mechanisms within phagocytes, such as macrophages, through the production of numerous cytokines with CD4+ T-cells.

1.4 Antimicrobial mechanisms of phagocytic cells

Phagocytic cells expose microbes to an array of toxic compounds and detrimental conditions that are capable of destroying the majority of microorganisms. Neutrophils and macrophages particularly are capable of producing reactive oxygen species (ROS) directly into the phagosome, or in the case of neutrophils both into the phagosome and into the extracellular environment (34). These ROS are produced by the actions of the NADPH oxidase complex that is composed of 5 proteins (gp91, p22, p40, p47, p67 phox proteins) and Rac1 or Rac2, that converts O₂ into O₂⁻. Superoxide anion is the progenitor
molecule for all ROS within the phagosome and is thought to directly damage macromolecules. Superoxide can undergo spontaneous or enzymatic dismutation into hydrogen peroxide that can more readily cross biological membranes. Hydrogen peroxide can undergo further chemical reactions to form more toxic compounds, such as reacting with iron to form extremely reactive hydroxyl radicals through the Fenton reaction. Neutrophils additionally produce myeloperoxidase that converts hydrogen peroxide and a halide cofactor into hypochlorous acid, one of the most potent antimicrobials known. Each of these compounds is intrinsically toxic and can damage numerous macromolecules including DNA, proteins and lipids (35).

Phagocytic cells utilize additional antimicrobial effects to destroy invading organisms. Phagosomes undergo acidification mediated by vacuolar-ATPases that pump $\text{H}^+$ into the phagosome (34, 36). The decrease in pH alone can be detrimental to some organisms, but additional stress conditions are also present as the phagosome is thought to be an extremely nutrient poor environment (34). These starvation conditions can prevent efficient microbial growth within the phagosome and could enhance the microbicidal functions of other effectors. Neutrophils additionally contain intracellular granules containing numerous antimicrobial compounds. The azurophilic granules contain the largest number of degradative compounds and can either undergo fusion with the phagosome or be released into the extracellular environment to mediate microbial killing (37). Macrophages also possess an organelle containing degradative enzymes called the lysosome, that can fuse to the acidified phagosome and exposes the microbe to
a variety of hydrolytic enzymes. IFN-γ activated macrophages can also generate nitric oxide through the actions of nitric oxide synthase (iNOS), which causes damage directly to a wide variety of macromolecules. Further, nitric oxide can interact with superoxide anion to produce the highly reactive and toxic peroxynitrite. All of these systems together create an extremely hostile environment within phagocytes that only specialized pathogens can overcome (34).

1.5 Interactions between Histoplasma and phagocytes

Neutrophils are known to be critical for controlling invading microbes and are one of the first immune cells recruited to a site of infection. These cells have been implicated in the control of viruses, bacteria and fungi. Neutropenia is known to exacerbates infections with numerous organisms (38-41). Neutrophils are known to be recruited to the lung during Histoplasma infection and yeast must survive this initial onslaught in order to establish an infection (42). It has been previously demonstrated that neutrophils are unable to efficiently kill Histoplasma yeast but are able to inhibit Histoplasma growth (43-45). Histoplasma yeast can induce an oxidative burst from human and murine neutrophils, but are highly resistant to oxidative killing (44-46). The specific fungistatic activity of neutrophils has been localized to the azurophilic granules (47). The molecules responsible for this activity were later determined to be the defensins HNP-1, HNP-2 and HNP-3, and to a lesser extent cathepsin G and bactericidal-permeability-increasing protein (47). Overall, Histoplasma is able to resist killing by quick responding but short-
lived neutrophils to effectively colonize the host and cause infections. The mechanisms that allow *Histoplasma* to resist these cells, however, require further investigation.

*Histoplasma* primarily resides within aveolar macrophages in the lung. The phagocytosis of *Histoplasma* by macrophages fulfills two important benefits for yeast by providing yeast a refuge from surfactant present within the aveoli, which is lethal to *Histoplasma* (48), and by providing a niche for growth within the host. *Histoplasma* primarily enters macrophages via the CR3 receptor interacting with Hsp60 on the yeast cell wall (49, 50). Within the macrophage *Histoplasma* fails to induce an oxidative burst (51), unless yeast are first opsonized in fresh or heat-killed serum (52) or macrophages are activated with proinflammatory cytokines (53, 54). In either case yeast are fully capable of surviving the oxidative burst (53-55). *Histoplasma* also prevents phagosome acidification by arresting pH at approximately 6.0, similar to the bacterial pathogen *Mycobacterium tuberculosis* (56, 57). The *Histoplasma* containing phagosome also undergoes differing levels of phagolysosomal fusion depending on the type of macrophage. Within some cell lines yeast containing phagosomes undergo fusion with lysosomes but replicate normally (58, 59). Within human monocyte derived macrophages or primary murine macrophages, yeast-containing phagosomes avoid phagolysosomal fusion and exposure to degradative enzymes (60). However, this fusion event is totally dependent on the viability of yeast cells, as phagosomes containing dead yeast fuse with lysosomes normally (60). *Histoplasma* is also able to resist killing by nitric oxide produced by activated macrophages, which only represses *Histoplasma* growth (61, 62).
Yeast replicate within macrophages and eventually lyse them and spread to other cells. It is currently unknown whether yeast lyse macrophages based on sheer fungal burden or through the activation of an apoptotic mechanism. Overall, *Histoplasma* is able to overcome the lethal macrophage effectors and utilizes them as a niche within the host to grow.

Dendritic cells (DCs) are also recruited to the sites of *Histoplasma* invasion. These cells play a key role in host clearance of *Histoplasma* by acting as a bridge between the innate and adaptive immune response, along with macrophages. DCs uptake *Histoplasma* yeast by the VLA-5 receptor (63) and are able to effectively kill yeast (64, 65). This killing is mediated by efficient phagolysosomal fusion and yeast degradation (66). Antigens are then presented to naïve CD4+ T-cells that mediate anti-*Histoplasma* activity through the production of cytokines and other effector molecules that enhance the antifungal activity of phagocytes.

The adaptive immune response is critical for the control and clearance of *Histoplasma*. Specifically, a strong cellular immune response is required through the action of pro-inflammatory CD4+ T-cells. Upon recognition of *Histoplasma* specific antigen these cells produce pro-inflammatory cytokines such as IFN-γ, TNF-α, GM-CSF and IL-1β that are all required to control *Histoplasma* growth (67). These cytokines act on macrophages and enhance anti-*Histoplasma* effects such as the production of nitric oxide, increased iron sequestration and enhanced phagolysosomal fusion (61, 68, 69). Despite
these lethal effects *Histoplasma* has been reported to become latent within the host and can be reactivated when the host immune status is altered by advanced age, AIDS or immunosuppressive therapies (70). The factors that allow *Histoplasma* latency are currently not well understood.

1.6 *Histoplasma* virulence factors

Despite *Histoplasma*’s ability to resist killing by the innate immune system there are few factors known that are attributed to yeast virulence. Even the best studied virulence factors utilize unknown mechanisms to promote *Histoplasma* pathogenesis. However, most of the known virulence factors are extracellular and thus in optimal position to interact with the host (Figure 1.1). One of the best characterized virulence determinants of *Histoplasma* is the α-glucan polysaccharide layer present on the yeast cell wall (71). This cell wall component is present only on the pathogenic yeast and is also produced by other dimorphic fungi such as *B. dermatitidis* (72–74). It has long been noted that alpha-glucan containing strains that lose the polysaccharide are attenuated (75). The first gene linked to alpha-glucan production in *Histoplasma* was alpha-glucan synthase (*AGS1*). Disruption of *AGS1* results in attenuation within the macrophage and in the intranasal mouse model of infection (76). Specifically, α-(1,3)-glucan acts to mask the extremely immunostimulatory β-(1,3)-glucan present on the fungal cell wall, which is normally recognized by the host receptor dectin-1 on macrophages (77). Normally the interaction of β-(1,3)-glucan with dectin-1 triggers the production of pro-inflammatory
cytokines such as TNF-α, which are important for yeast clearance. Two other factors have been linked to α-(1,3)-glucan synthesis including a UTP-glucose-1-phosphate uridylyltransferase (UGP1) that synthesizes UTP-glucose monomers for incorporation into polysaccharide chains and an α-amylase (AMY1) with homology to proteins possessing glycosyl hydrolases/transglycosylases specific for α-(1,4) linkages (78). Specifically, disruption or depletion of AMY1 results in attenuation within the macrophage and mouse model of infection, mirroring the phenotype observed in the ags1∆ and signifies the importance of Amy1 in the biosynthesis of α-glucan. Interestingly, most but not all Histoplasma strains produce α-glucan. The polysaccharide is produced by the Panama strain (PAN1 or G186AR) and a North American isolate (NAM1) (80). The strain not producing α-glucan is a North American isolate (NAM2 or G217B), and is fully virulent in humans and animals. Within this background the AGS1 transcript is produced at lower levels, but does not appear competent for α-glucan synthesis (81). This strain likely possesses a compensatory mechanism to avoid detection, either through cell wall modification or the production of specific factor(s) that prevents recognition.

Another well studied extracellular virulence factor is the secreted calcium binding protein (Cbp1). This protein is small (10 kDa), but extremely stable even in the presence of SDS (82). This protein is released into culture in very high quantities, and is exclusively produced Histoplasma in the pathogenic-phase (83, 84). Cbp1 is produced and accumulates within the phagosome of macrophages during Histoplasma infection (85). Deletion of this gene results in attenuation within both the macrophage and mouse
Previously described virulence factors of *H. capsulatum*. Summarized are the regulators of dimorphism and their putative interactions with one another. DRK1 and RYP1 could act independently of one another or in conjunction to control morphological transition. Additionally, RYP1 could interact with other DNA binding proteins, like MCM1, to promote transcriptional changes during transition. The primary extracellular virulence factors of the Panama, Nam1 and Nam2 *Histoplasma* strains are pictured. Adapted from (79).
models of infection (84). This protein possess the ability to bind calcium and could have a role in calcium acquisition within the host, however recent structural analysis has revealed similarity to the saposin family of proteins that are involved in lipid binding (82). Cbp1 could bind the lipids of the phagosome and act to either modify the local environment or liberate lipids to act as a carbon source for internalized yeast. Although clearly important the exact role of this protein remains to be elucidated.

Like other pathogens it is essential for Histoplasma to acquire iron within the host and specifically within the phagosome. Histoplasma possess multiple mechanisms to acquire sequestered iron, including utilizing iron reductases and siderophores. Yeast release glutathione-dependent ferric reductase complexed to a protein with \(\gamma\)-glutamyltransferase (Ggt1) activity that assists yeast in acquiring iron from multiple sources, such as holotransferrin and hemin (86-88). The silencing of GGT1 results in the inability of yeast to destroy Raw 264.7 macrophages, but the role in vivo remains unclear (86). Histoplasma also produces Fe (III)-binding hydroxymate siderophores that act in iron acquisition (89). Unusually, the genes responsible for siderophore biosynthesis are located in a localized gene cluster and the removal of Sid1, the enzyme responsible for the first committed step of siderophore biosynthesis, greatly reduces siderophore production (90). Interestingly, disruption of the SID1 gene does not result in an attenuated phenotype within the mouse, unless the \(sid1\Delta\) is grown in competition with wild-type yeast within the mouse. The fitness phenotype was only strongly observed later in co-infection, 14 days, correlating with a role during cell mediated immunity (90).
is most likely due to the iron reductase mechanism being sufficient for iron acquisition unless competing with a more fit strain expressing multiple iron savaging mechanisms. Siderophores are also important for Histoplasma’s ability to capture iron in the phagosome (91). Genes related to siderophore production and GGT1 are also transcriptionally up-regulated only under iron limiting conditions further linking them to iron homeostasis within parasitic Histoplasma (86, 90).

Another extracellular virulence factor is the secreted and cell wall associated yeast-phase specific protein (Yps3). This protein is yeast-phase specific and is expressed only by strains lacking α-glucan (92). Yps3 is very similar to the Bad1 protein of Blastomyces dermatitidis, which plays a critical role in adhering yeast to host cells and modulating the host immune response to Blastomyces infection (93, 94). In Blastomyces, Bad1 modulates the host cell response by triggering the production of anti-inflammatory cytokines such as TGF-β (93). Yps3 lacks the extensive invasion-repeats present in the center of the Bad1 protein, which account for the extreme size disparity between the two proteins (20 kDa Yps3 compared to 120 kDa Bad1) (95, 96). Yps3 does contain the epidermal growth factor domain (EGF) that has been linked to binding Bad1 to cell wall chitin. YPS3 also binds chitin within the cell wall by virtue of the EGF domain (96). Knock-down of Yps3 does not hamper the survival of yeast within Raw 264.7 macrophages, however knock-down does decrease fungal burden within mouse spleens and livers (97). Additionally, it was postulated that Yps3 plays a role in Histoplasma dissemination, as intraperitoneal infection with Yps3 knock-down strains demonstrate a
decrease in spleen and liver fungal burdens compared to the gfp-RNAi control. Yps3 appears to play a role during yeast infection of the mammalian host but whether it functions as an adhesion, immune modulating factor or dissemination factor remains to be determined.

The specific mechanism of yeast uptake also appears to be critical for *Histoplasma* survival within phagocytes, as masking β-glucans is critical for avoiding host detection. *Histoplasma*’s association and uptake by macrophages and PMN’s is mediated by the interaction between Hsp60 on the yeast cell wall and CR3 (CD18/CD11b) on the host cell (49, 50). This heat shock protein was initially uncovered by probing *Histoplasma* yeast with recombinant CR3 (50). This interaction was demonstrated to be a specific interaction based on the ability of yeast to only bind CHO cells expressing CR3, in addition to the inability of yeast to bind cells in the presence of excess recombinant Hsp60 or monoclonal-antibody blocking CR3. Interestingly, *Histoplasma* entry via CR3 would be predicted to induce minimal macrophage response, such as preventing an oxidative burst, and thus allow yeast to avoid some of the innate immune defenses (98). Hsp60 also has a role as a chaperone and appears to have some interaction with several other predicted cell wall associated proteins (99). A disruption or knock-down strain lacking Hsp60 has not been generated so any role in vivo remains speculative; however Hsp60 is a prime therapeutic target since monoclonal antibodies protect mice from a lethal *Histoplasma* infection (100, 101).
Additionally, there are two other identified extracellular factors produced by *Histoplasma*. The cell walls of Nam2 *Histoplasma* yeast have been reported to contain histone-2B-like protein (H2B). This protein is especially surprising given the fact that histones are rarely found outside of the cell and are usually associated with DNA in the nucleus. The function of this protein *in vivo* or within cells is unknown but antibodies specific to H2B are able to protect mice from *Histoplasma* infection through their ability to promote opsonophagocytosis (102). Additionally, Nam1 yeast can secrete a serine protease that degrade collagen *in vitro*. This may enhance yeast dissemination, but it is currently unknown why only the Nam1 strain appears to produce this extracellular protease and whether it has any role in *Histoplasma* pathogenesis *in vivo* (103).

1.7 Mechanisms of dimorphism

The molecular mechanisms allowing thermally dimorphic fungi to shift from mycelia to yeast morphologies have only recently begun to be uncovered (Figure 1.1). Microarray experiments have demonstrated that there are massive global shifts in gene expression from the mold morphology to the yeast morphology. Over 1600 genes undergo either induction or repression during the morphological shift (26). The first factor reported to control dimorphism was dimorphism regulatory kinase (DRK1) within *Blastomyces* and *Histoplasma* (25). This kinase is required for the expression of yeast-phase specific genes and is able to functionally complement *Saccharomyces cerevisiae* strains lacking Sln1, which controls the response to environmental stimuli. Strains of
Blastomyces lacking Drk1 remain as mold even at 37°C, and predictably are completely attenuated in mice. Depletion of Drk1 within Histoplasma also prevents transition into the yeast-phase at 37°C and leads to attenuation within the mouse model (25). A global transcriptional regulator RYP1 (regulator of yeast-phase growth) was also identified as being vitally important for phase transition. RYP1 transcript is most abundant at 37°C indicating that it is important for the formation of the pathogenic yeast form (26). The loss of RYP1 greatly alters the transcription profile upon transition from 1674 to 321 transcripts, and prevents Histoplasma from taking on the yeast morphology at 37°C demonstrating its importance to dimorphism. Additionally, Ryp1 was determined to bind at the promoters of numerous genes, including the known virulence determinant Cbp1, and appears to specifically control 98% of the genes induced at 37°C (26).

1.8 Secreted virulence proteins in eukaryotic pathogens

The role of secreted effector molecules and secretion systems are known to be critical for numerous bacterial pathogens. Bacteria utilize multiple secretion mechanisms to deliver effector molecules into the host-pathogen interface or directly into host cells. Among these specialized delivery systems are bacterial Type III and Type IV secretion systems that use needle-like structures to deliver proteins directly into mammalian cells in order to modulate their functions (104). Pathogens like Yersenia pestis and Salmonella enterica use Type III systems to inhibit the ability of phagocytes to engulf the bacteria and/or enhance survival within immune cells (105, 106). Legionella pneumophila utilize
Type IV systems to directly modulate the characteristics of bacteria-containing phagosomes and even to transport nucleic acids (107).

Secreted proteins are also critically important in eukaryotic pathogens. These proteins include adhesion proteins, oxidative defense proteins and proteolytic enzymes that can destroy immune molecules or promote survival within immune cells. The parasite *Leishmainia* spp. produces a metalloprotease, GP63, that is capable of hydrolyzing a large array of proteins, destroying complement factor C3 and is reportedly important for survival within the phagosome (108-111). Additional factors such as alkaline phosphatase, chitinase, EF-1α, and SAcP, an organic dephosphorylase, have all been implicated in virulence (112-114). In the African parasite *Trypanosoma brucei* the pseudopeptidase metacaspase 4 is required for full virulence in the mouse (115). Additionally, several other factors like *Trypanosome cruzi* oligopeptidase B, involved in cleaving peptide hormones, may also have a role in pathogenesis (116). Within fungi some of the best characterized virulence factors are adhesins or other completely soluble factors. In *Candida albicans* the agglutinin-like sequence (ALS) are GPI-linked proteins required for attachment to host cells (117) and families of serine proteases (SAPs) are required for colonization of various sites within the host (118). *Cryptococcus neoformans* has a polysaccharide capsule that prevents phagocytosis (119) and the component polysaccharide, glucuronoxylomannnan, has immunosuppressive qualities (120). Some of the few known virulence factors of dimorphic fungi are also soluble and/or cell wall associated. The cell wall associated and soluble Bad1 protein of *B. dermatitidis* is an
adhesion and immunosupresive protein (93, 94). The spherule outer wall glycoprotein (SOWgp) of *Coccidioides* spp. acts as an adhesin modulates the host response and is required for virulence (121). An extracellular metalloprotease is also required by *Coccidioides* for survival within the host and acts to assist endospores, the infectious particle of ruptured spherules, to avoid host detection by removing SOWgp (122).

Large scale proteomics analyses of extracellular proteins have been carried out for many medically important parasites and several fungi. Those studies have indicated that a diverse array of proteins are released and represent a wide range of functions. Putative functions include protease activity, adhesion, polysaccharide modification and proteins whose function cannot be predicted based on homology (123-127). Surprisingly, these extracellular proteomes are large, typically being composed of over a hundred proteins. Interestingly, many of the large (123-127) scale protein identification studies also uncover numerous unexpected proteins such as normally cytosolic proteins associated with translation, transcription, proteasome activity, internal metabolic cycles and other normally internal processes (125, 126, 128, 129). It is difficult to differentiate whether these proteins have been specifically secreted from the organism under study, or if it has been released due to cellular lysis or some other nonspecific mechanism, such as the release of degradative compartments into culture. Some studies attempt to account for this possibility by screening samples for the presence of DNA, cytoplasmic enzyme activity (i.e. GAPDH) or screening for other known internal proteins (123). However,
many studies lack such controls and could contain factors not intentionally released into culture.

1.9 Eukaryotic secretion mechanisms

Fungi utilize vesicles to transport proteins, polysaccharides and lipids to the extracellular environment. Polysaccharides transported in this manner can act as precursors to the cell wall, lipids become part of the plasma membrane and proteins can either be directly released into the surrounding environment or become attached to the cell wall (130). Proteins are transported in vesicles that follow a general path starting from the endoplasmic reticulum (ER), to the Golgi apparatus and then fuse with the plasma membrane releasing protein into the extracellular environment. Proteins targeted for secretion are translated by ribosomes on the rough endoplasmic reticulum (ER) and generally contain an N-terminal signal peptide. Within mammalian systems translation is coupled to ER entry but in S. cerevisae the process can be coupled or independent (131). The signal peptide is recognized by the multiprotein signal recognition particle (SRP) that can then interact with SRP receptors that allow the polypeptide to pass into the ER lumen. The signal peptide is removed during translocation by signal peptidases within the ER (132, 133). Proteins undergo maturation within the ER, sometimes with the assistance of chaperone proteins like calnexin and calritican, and additionally can be glycosylated with a core oligosaccharide group, which can also assist in proper protein folding (134). After maturing within the ER proteins lacking an ER retention signal are packed into
COPII coated vesicles and trafficked to the cis-Golgi network. Within the Golgi proteins can undergo additional maturation by proteolitic cleavage events and oligosaccharide chains can be further modified and expanded during movement through the Golgi (135). Movement of proteins through the Golgi apparatus is mediated by transport through COPI coated vesicles from one area of the Golgi to the next or by the dynamic movements of the Golgi itself. Within the Golgi proteins are sorted into specific vesicles that are then targeted to different parts of the cell from the trans-Golgi network. The packing of proteins into specific vesicles often involves recognition by membrane bound receptors that recognize proteins by specific modifications, such as specific oligosaccharide side chain residues. The best studied of these is the terminal mannose-6-phosphate modification on the oligosaccharide side chain of acid hydrolases, that is recognized by mannose-6-phosphate receptors within regions of the Golgi membrane (136). The acid hydrolases are concentrated at these receptors before vesicles bud off and are targeted to the lysosome. Proteins lacking such specific receptors instead follow the default transport pathway towards secretion at the plasma membrane.

The specificity of vesicle trafficking to the plasma membrane or cellular organelle is mediated by multiple factors present on the vesicle and the target membranes surface. The main determinants of specificity involve rab protein tethering mechanisms and soluble NSF attachment protein receptor (SNARE) proteins that enhance membrane fusion. The Rab proteins are a family of ras GTP-proteins that are involved in numerous stages of protein secretion as the vesicle is trafficked through the cell. One of the most
important roles for Rab proteins is to allow specific targeting to different parts of the cell (137). The rab proteins are present within the vesicle membrane. There are 11 known rab proteins utilized by yeast to mediate secretion and over 60 in mammalian systems (138). These proteins are recognized by specific Rab effector protein complexes, located in the membrane of the cellular target. The binding of Rab-GTP and Rab effector bring the vesicle in close proximity to its target membrane. After being in proximity to the target membrane vesicle SNAREs (v-SNAREs) interact with target SNARES (t-SNAREs). These α-helical proteins twist around one another and bring the membranes together and are thought to promote the fusion between membranes (139, 140). Classically, SNARE protein interactions were thought to mediate targeting specificity, however studies have indicated that the Rab protein’s play a larger role than previously thought and that SNARE proteins can be promiscuous (137, 141, 142). Within the yeast S. cerevisiae secretion occurs very near the generalized model. The rab protein Sec4-GTP is present on the secretion vesicle and is recognized by an eight protein exocyst complex (143). The Sec4-GTP membrane protein is bound by Sec15p that is associated with the complex and assists in bringing the vesicle into tight proximity to the membrane to allow SNARE protein function. Interestingly, protein secretion in yeast has been observed to be localized at sites of budding (144). Within filamentous fungi secretion is localized at the apical and subapical hyphae (145). Targeting is mediated by the protein Sec3p that is only present at bud tips or necks and is the anchoring portion of the exocyst complex.
Although the previously described mechanism is the best understood secretion pathway in eukaryotes, additional mechanisms can be utilized. Some of these mechanisms do not require nascent polypeptides to possess a canonical N-terminal secretion signal, or are transported to the plasma membrane by pathways excluding the ER, Golgi or both. These alternate systems have only recently been described mechanistically (146). The general pathways that can be undertaken are summarized in Figure 1.2. Generally, it appears that only a unique subset of proteins can traverse these alternate mechanisms. However, some organisms seem to make more preferential use of these pathways. For example, within the secreted proteomes of the parasites *Leishmania* and *Trypanosomes* only 2% and 7%, respectively, of the proteins possess a classic secretion signal and release a majority of their secreted proteins through vesicle secretion (147, 148). This process involves the release of an entire vesicle, or exosome, into culture that range in size from 40 nm- 100 nm in diameter. Overall, this system is best studied in mammalian cells where it is one of the primary mechanisms of IL-1β release (146). Some fungi, including *Cryptococcus neoformans* (149), *S. cerevisiae* (150) and two dimorphic fungi *Paracoccidioides* (151, 152) and *Histoplasma* (153) have also been reported to produce these structures in culture. This has been proposed as a mechanism that fungi and parasites utilize to deliver effectors into the host, and has been implicated in the release some *Cryptococcus* virulence factors (149, 154). Interestingly, it appears as though these vesicles primarily contain a random sampling of cytoplasmic proteins arguing against a specific role in pathogenesis (155). For example, cells expressing green
Figure 1.2 Mechanisms of nonclassical secretion in eukaryotes. Presented is a summary of the known secretion systems for proteins lacking canonical secretion sequences as adapted from (146). These pathways include secreted lysosomes (SL) and multivesicular bodies (MVB). Red arrows indicate Golgi reassembling stacking protein (GRASP) that is known to mediate several forms of nonclassical secretion pathways.
fluorescent protein (GFP) within their cytoplasm also release GFP in their exosomes (156). RNA even appears to be large portion of these vesicles (157-159). These alternate secretion mechanisms appear to be play important roles in mammalian systems where they aid in signaling (157). However, the roles of these microvesicles in pathogens remain elusive, as they contain numerous molecules that would be predicted to elicit a strong immune response. In some cases these vesicles can contain virulence determinants (149), but thus far in Histoplasma no know virulence factors have been identified (153).

### 1.10 Research goals

The purposes of my research is to (1) identify the proteins that *H. capsulatum* releases into the extracellular environment and (2) characterize the roles extracellular proteins play in promoting *Histoplasma* growth and survival within phagocytes and within animal host. These proteins are perfectly positioned to interact with the host or to protect yeast from toxic host effector molecules. Additionally, these proteins also represent the most ideal candidates for improved diagnostics for histoplasmosis due to their extracellular location. All of the known *Histoplasma* virulence factors are present extracellularly and we reasoned that other potential virulence determinants would also be present in the extracellular proteome. My work systematically identified the extracellular *Histoplasma* proteins (Chapter 2). From these, candidate virulence factors were selected and functionally characterized including the oxidative stress defense proteins superoxide dismutase (Sod3, Chapter3), catalase (CatB, Chapter 4), and an abundant protein lacking
homologies to known proteins (Cfp4, Chapter 5). Not only do these studies provide a comprehensive view of the extracellular proteins released by *H. capsulatum* pathogenic yeast, but they identify essential virulence mechanisms that protects *Histoplasma* from host produced ROS. These antioxidant defenses are critical for microorganisms that interact intimately with host phagocytes and enable their survival against such normally antimicrobial immune cells.
Chapter 2: Definition of the extracellular proteome of pathogenic-phase *Histoplasma capsulatum.*

2.1 Introduction

Invasive and systemic fungal infections continue to be an important cause of morbidity and mortality. During the past few decades, the incidence of invasive mycoses has risen and now ranks as the seventh most frequent cause of death due to infectious disease in the United States (160)(161). The majority of systemic-disease causing fungal pathogens are acquired via the respiratory tract through inhalation of spores or conidia. Lung infection by respiratory fungal pathogens results in varying degrees of pulmonary disease dependent on dose, host immunological status, and natural virulence of the pathogen. Of hospitalizations in the United States due to infection by dimorphic fungi, over half are caused by the pathogen *Histoplasma capsulatum* (1).

The dimorphism of *Histoplasma capsulatum* reflects both a change in morphology and a switch from a saprobie to a parasitic lifestyle. In the environment, *Histoplasma* exists as mycelia, extending septate hyphae into the local substrate for
absorption of nutrients. Inhalation of mycelial-produced conidia into the lung exposes them to mammalian body temperatures, which trigger their differentiation into pathogenic yeast cells (162). This temperature-controlled transition into yeast is absolutely necessary for Histoplasma pathogenesis. Although the mycelia can produce infective conidia, mycelia themselves are non-pathogenic as Histoplasma cells locked in the mycelial-phase are unable to cause disease (24, 25). Mycelia are thus designated the non-pathogenic-phase and yeast as the pathogenic-phase with conidia being the natural infective form. The extensive changes in gene expression that characterize the yeast-phase program include loci required for Histoplasma pathogenesis (26, 163).

The yeast form of Histoplasma is an adept intracellular pathogen, found almost exclusively within host phagocytic cells that normally provide the first line of protection against fungal infections. Histoplasma combats the antimicrobial effects of phagocyte-produced reactive oxygen (164) and establishes a replication-permissive niche inside the phagocyte. However, we lack many of the mechanistic details regarding how Histoplasma establishes a successful infection and thrives within host phagocytes. To date, only a handful of virulence factors have been identified for Histoplasma: mechanisms for iron acquisition (86, 90, 165); a secreted protein of unknown function (Cbp1)(84); an extracellular yeast-phase specific factor (Yps3) (97); and a cell wall polysaccharide (α-(1,3)-glucan) (76).
At the interface between host and pathogen, extracellular factors produced by pathogens are perfectly situated to influence the outcome of the interaction to the pathogen’s advantage. Microbial pathogens possess an array of molecules that facilitate adherence, invasion, inactivation of host defenses, and alteration or destruction of host cells. Among the medically relevant fungal pathogens, many of the demonstrated virulence factors are extracellular, being either secreted into the surrounding milieu or associated with the fungal cell wall. Shedding of surface components, notably the Cryptococcus capsule component glucuronoxylomannan, Blastomyces Bad1, and Coccidioides SOWgp, also affect host cell function or the host immune system (93, 94, 166-168). Underscoring the central role of secreted factors in pathogenesis, many bacterial pathogens have evolved specialized secretion systems for delivery of virulence effectors into target host cells (169). In addition to the use of the general eukaryotic secretory pathway, some fungi release protein-containing vesicles into their surroundings providing an alternate mechanism to transport proteins to the extracellular milieu (170).

Most of the factors identified to date which are necessary for Histoplasma virulence are extracellular, either present on the host-interacting pathogen surface or present in the soluble culture filtrate of yeast cells (79). These molecules are prime candidates for modifying the internal compartment in which Histoplasma yeast reside following phagocytosis. Phagocytosis of virulent Histoplasma yeast traffics them to phagosomes in which lumen acidification is impaired. In contrast, phagocytosis of killed Histoplasma yeast traffics them to acidified phagosomal compartments and the yeast are
digested by lysosomal enzymes (56, 60, 171). This suggests that *Histoplasma* yeast actively produce factors that contribute to modification of the yeast-containing phagosome, survival of host defenses, and virulence. Despite this presumed importance to *Histoplasma* pathogenesis, a clear definition of the inclusive set of soluble extracellular components produced by *Histoplasma* cells in the pathogenic yeast-phase has not been described.

In this project, we have identified the most abundant constituents of the extracellular proteome of *Histoplasma* yeast. Extracellular proteins were collected from culture filtrates of pathogenic yeast-phase *Histoplasma* cells. The proteins identities were ascertained through a shotgun mass spectrometry approach. Enriched expression of factors in the pathogenic-phase was determined by quantifying transcription of the encoding genes by yeast cells versus non-pathogenic mycelia. To validate the relevance of the secreted proteome definition to *in vivo* yeast, we confirmed secreted protein expression by yeast cells during residence in macrophages and during murine lung infection. These findings provide an important framework on which mechanistic studies of *Histoplasma* pathogenesis can be based, specifically the role of extracellular yeast-phase factors that could influence the host-pathogen interaction.

### 2.2 Materials and Methods

#### 2.2.1 Fungal culture
The clinical isolate of *Histoplasma capsulatum*, G186A (ATCC 26027) was the genetic background used for all studies. Strains constructed from WU8, a *ura5*-deletion strain derived from G186A, are presented in Table 1. Yeast- and mycelial-phase cultures were grown in *Histoplasma* macrophage medium (HMM;[172]) supplemented with 0.1 mM uracil. For maintenance of the yeast-phase, cultures were grown at 37°C under 5% CO$_2$ / 95% air with shaking at 200 rpm. Mycelial cultures were grown at 25°C -26°C in normal air. Solid media was prepared by addition of 0.6% agarose and 25 μM FeSO$_4$. For infection studies requiring uracil prototrophy, WU8 was transformed with plasmid pCR473 carrying the *URA5* gene. The growth phase of *Histoplasma* yeast cultures was determined by treating aliquots of the culture with 1 M NaOH and measuring the optical density at 600 nm.

**2.2.2 Culture filtrate and cellular lysate preparation**

Replicate 200 ml yeast-phase culture supernatants were harvested from cultures in mid- to late-exponential phase growth (OD 600 nm = 1.4 to 1.9). Cells were gently removed by allowing yeast to settle for 20 min followed by centrifugation at 1200 x g for 5 minutes to pellet residual yeast cells. Supernatants were sequentially filtered through 0.45 μm-pore and 0.21 μm-pore, membranes (Millipore). Mycelial-phase culture filtrates were prepared by filtration of mycelial cultures through Whatman #5 qualitative filter paper (approximately 2.5 μm pore size) followed by filtration through a 0.21 μm-pore membrane.
Table 2.1. *Histoplasma* strains

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Genotype</th>
<th>Other Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>G186A</td>
<td>wild type (ATCC#26027)</td>
<td></td>
</tr>
<tr>
<td>WU8</td>
<td><em>ura5-D32</em></td>
<td>WT*</td>
</tr>
<tr>
<td>WU29</td>
<td><em>ura5-D32 cbp1-D8::hph</em></td>
<td><em>cbp1Δ</em></td>
</tr>
<tr>
<td>OSU17</td>
<td><em>ura5-D32</em> / pCR473 [URA5, gfp-RNAi]</td>
<td>gfp-RNAi</td>
</tr>
<tr>
<td>OSU52</td>
<td><em>ura5-D32</em> / pEH15 [URA5, CATB-RNAi]</td>
<td>CATB-RNAi</td>
</tr>
<tr>
<td>OSU62</td>
<td><em>ura5-D32</em> / pEH11 [URA5, CFP1-RNAi]</td>
<td>CFP1-RNAi</td>
</tr>
<tr>
<td>OSU63</td>
<td><em>ura5-D32</em> / pEH09 [URA5, CFP4-RNAi]</td>
<td>CFP4-RNAi</td>
</tr>
<tr>
<td>OSU64</td>
<td><em>ura5-D32</em> / pCR438 [URA5, CFP8-RNAi]</td>
<td>CFP8-RNAi</td>
</tr>
<tr>
<td>OSU66</td>
<td><em>ura5-D32</em> / pBY08 [URA5, CATB]</td>
<td>CATB(++)</td>
</tr>
</tbody>
</table>

* WU8 is the wild-type background except that the *ura5* gene has been deleted to allow transformation with *URA5*-based plasmids
Culture filtrates were concentrated approximately 30-fold by ultrafiltration through a 10 kDa molecular weight cut off polyethersulfone membrane (Millipore) under 0.5 barr of pressure with gentle stirring. The buffer was exchanged into 10 mM ammonium acetate by dilution followed by ultrafiltration. Cytoplasmic proteins were prepared by beating yeast cells with 0.5 mm-diameter glass beads in lysis buffer (50 mM Tris-HCl pH7.5, 2 mM EDTA, 2 mM DTT, 50 mM KCl, 0.2% Triton X-100, with 1X protease inhibitor cocktail (Roche)). Protein concentrations were determined by D_2 assay (Lowry-based; BioRad) using an ovalbumin standard curve.

2.2.3 Electrophoretic separation and visualization of proteins

Culture filtrate proteins (6 μg) and yeast cellular lysates (3.5 μg) were denatured in reducing sample buffer (50 mM Tris pH 6.8, 1% SDS, 10% glycerol, and 0.1 M DTT), heated for 10 minutes at 100°C, and clarified by centrifugation for 5 minutes at 16,000 x g. Proteins were separated by electrophoresis through 10% and 12.5% NextGel polyacrylamide gels (Amresco). For visualization of separated proteins, gels were fixed for 30 minutes at room temperature in 40% ethanol / 10% acetic acid, followed by silver staining(173, 174). Silver stained images were captured with a Sony ICX267 1.4 megapixel CCD camera with Alphaimager software (Cell Biosciences).

2.2.4 Shotgun mass spectrometry-based identification of extracellular proteins
430 μg of culture filtrate sample were lyophilized by speedvac. Protein pellets were resuspended in 40 μl of Invitrosol (Invitrogen) and heated at 100°C for 5 minutes followed by 60°C for 10 minutes with intermittent vortexing. The solution was supplemented to a final concentration of 25 mM ammonium bicarbonate and 32.5 mM DTT. Reduced sulfhydryls were blocked by addition of 80 mM iodoacetamide. The protein solution was aliquoted into tubes with 8.1 μg of total protein. For proteolytic digestion, trypsin (0.81 μg/μl; Sigma) or chymotrypsin (0.91 μg/μl; Sigma) was added and reactions incubated for 5 hours at 37°C.

Total protein identification was performed by capillary-liquid chromatography-nanospray tandem mass spectrometry (Capillary-LC/MS/MS) using a Thermo Finnigan LTQ orbitrap mass spectrometer equipped with a nanospray source operated in positive ion mode on 1 μg of digested culture filtrate protein. Samples were separated on a capillary column (0.2x150 mm Magic C18AQ 3μ200A, Michrom Bioresources Inc., Auburn, CA.) using UltiMate™ 3000 HPLC system from LC-Packings A Dionex Co. (Sunnyvale, CA.). Each sample was injected into the trapping column and desalted with 50 mM acetic acid for 10 minutes before injection onto the chromatography column. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Mobile phase B was increased from 50% to 90% over 5 minutes followed by holding at 90% for an additional 5 minutes before being brought down to 2% in 1 minute. MS/MS was acquired with a nanospray source operated at a spray voltage of 2 kV and a capillary temperature of 175°C. The scan sequence of the mass spectrometer...
was based on the data-dependent TopTen™ method: the analysis was programmed for a full scan recorded between 400-2000 Da and a MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive scans of the ten most abundant peaks in the spectrum. The resolution of full scan was set at 30,000 to achieve high mass accuracy MS determination. The CID fragmentation energy was set to 35%. Dynamic exclusion was enabled with a repeat count of 30 seconds, exclusion duration of 350 seconds, and a low and high mass width of 0.50 and 1.50 daltons, respectively.

Sequence information from the MS/MS data was processed by converting the Xcalibur (version 2.0) .raw files into a merged file (.mgf) using ReAdW (version 4.3.1) and an in-house program, RAW2MZXML_n_MGF_batch (merge.pl, a Perl script). The resulting mgf files were searched using the MASCOT Daemon (Matrix Science, version 2.2.1) against an in-house generated *Histoplasma capsulatum* genome database fragmented into 10 kb contigs which contained 5196 total database entries. This database was constructed from the draft assembly of the *Histoplasma* G186A genome (December 2004 assembly, Washington University Genome Sequencing Center). The mass accuracy of the precursor ions were set to 1.2 Da and the fragment mass accuracy was set to 0.8 Da. Considered variable modifications were methionine oxidation and carbamidomethyl cysteine. Up to two missed cleavages for the enzyme were permitted and peptides with a score less than 20 were removed. *Histoplasma* contigs with a MASCOT score of 150 (for trypsin-derived peptides) or 100 (for chymotrypsin-derived peptides) were selected for further analysis (p<0.05). False discovery rates were estimated using the MASCOT
decoy feature, which matches peptides against inverted database sequences and were found to be 5%, 9% and 7% for trypsin reactions and 6%, 6% and 7% for chymotrypsin reactions. Matching peptides were mapped to the contig and were considered representative of a positive protein identification if at least two unique peptides matched within 1000 nucleotides of one another in the translated sequence and matched amino acid sequences were encoded on the same strand. Protein sequences were derived from a combination of mass spectrometry-derived peptides, protein predictions (Broad Institute), protein homologies, and manual alignment of encoding exons with consensus splicing signals. To assign potential functional descriptions, BLAST searches were performed using the predicted protein sequence against identities in the non-redundant NCBI protein database. The presence of N-terminal secretion signals were identified using the SignalP algorithm (version 3.0, (175)).

2.2.5 Identification of proteins by band excision

*Histoplasma* yeast-phase culture filtrate proteins (34 μg) were separated by one-dimensional electrophoresis through a 10% polyacrylamide gel. Gels were stained either with Coomassie blue R-250 or SYPRO Ruby (BioRad). Prominent protein bands were excised using glass Pasteur pipets and acrylamide slices transferred to a solution of 40% methanol / 5% acetic acid and stored at 4°C until peptide extraction. For peptide extraction, gel pieces were washed twice in 50% methanol / 5% acetic acid for one hour, and dehydrated in acetonitrile. Gel pieces were rehydrated and incubated with DTT
solution (5 mg/ml in 100 mM ammonium bicarbonate) for 30 minutes before addition of iodoacetamide (15 mg/ml) for 30 minutes. The gel plugs were washed again with cycles of acetonitrile and 100 mM ammonium bicarbonate in 5 minute increments. Gel plugs were dried in a speedvac, before the addition of trypsin solution (20μg/ml in 50 mM ammonium bicarbonate) and overnight incubation at room temperature. Peptides were extracted using 50% acetonitrile and 5% formic acid several times and pooling the liquid. Extracted pools were concentrated using speedvac.

Protein identification was determined as above except mobile phase A was water containing 50mM acetic acid. A 5 cm / 75μm ID ProteoPep II C18 column packed directly in the nanospray tip was used for chromatographic separations. Peptides were eluted directly off the column into the LTQ system using a gradient of 2-80% solvent B over 45 minutes, with a flow rate of 300 nl/min. The total run time was 65 minutes. The MS/MS was acquired using a nanospray source operated with a spray voltage of 3 kV and a capillary temperature of 200°C. The CID fragmentation energy was set to 35%. Dynamic exclusion was enabled with a repeat count of 30 seconds, exclusion duration of 350 seconds. and a low and high mass width of 0.50 and 1.50 daltons, respectively. Peptide information was used to search the *Histoplasma* 10 kb genome database with the MASCOT daemon as described above.

2.2.6 Immunoblotting
Five to 36 μg of protein (either from yeast-phase culture filtrate or from yeast cellular lysates) were separated by electrophoresis through 10% polyacrylamide following denaturation and proteins were transferred to PVDF in transfer buffer (16 mM Tris base, 120 mM glycine, 0.05% SDS, and 20% methanol) at 4°C. For immunoblotting, the membrane was blocked with 5% nonfat milk / 0.05% Tween-20 followed by addition of anti-tubulin antibody (Sigma B512). Detection was performed by incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody and the Immobilon Western Chemiluminescent HRP substrate (Millipore).

### 2.2.7 *Histoplasma* RNA isolation and analysis

*Histoplasma* yeast grown to exponential phase were collected by centrifugation (2000 x g) and resuspended in TRIzol (Invitrogen). Mycelia were collected from 8 day-old liquid cultures by filtration onto Whatman #5 filter paper and scraping of the retained mycelia into TRIzol. *Histoplasma* RNA from infected macrophages was obtained by infecting 6x10^6 P388D1 cells (a macrophage cell line permissive for intramacrophage *Histoplasma* yeast growth and replication) with *Histoplasma* yeast at a multiplicity of infection of 10 yeast to 1 macrophage in HMM-M (HMM buffered with 25 mM sodium bicarbonate instead of HEPES;[58]) containing 10% fetal bovine serum. Cells and yeast were incubated at 37°C with 5%CO₂ / 95% air for 40 hours. Extracellular yeast were removed by extensive washing with PBS, and P388D1 cells were lysed by a hypotonic solution of 10 mM Tris pH 8.0. The liberated yeast were collected from the macrophage
lysate by centrifugation and resuspended in TRIzol. To isolate *Histoplasma* RNA from infected mouse lungs, ten C57BL/6 mice (Harlan) were intranasally infected with $1 \times 10^7$ yeast per mouse. After 24 hours, lungs were removed and homogenized in hypotonic 10 mM Tris, 1 mM EDTA buffer to free yeast from mammalian tissue. Homogenates were pooled together and debris removed by passing through sterile gauze using a Buchner funnel. Yeast and mammalian nuclei were collected by centrifugation (3000 x g for 5 minutes) and resuspended in 6 ml of TRIzol. To reduce viscosity, the suspension was passed through a 21 gauge needle. Yeast were collected again by centrifugation and washed 4 times in TRIzol before isolating RNA.

RNA was liberated from *Histoplasma* cells in TRIzol by beating with 0.5 mm-diameter glass beads. RNA was purified by extraction with CHCl$_3$ followed by alcohol precipitation of the aqueous phase. Genomic DNA was removed by two sequential digestions with Turbofree DNase (Ambion) and lack of genomic DNA verified by PCR. Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen) using 5 μg of total RNA and random 15-mer or oligo dT 22-mer primers.

Endpoint PCR amplification of candidate genes was performed using gene specific primers with random 15-mer primed reverse-transcribed RNA templates. Amplification reactions contained 1:200 reverse-transcribed RNA templates, 0.5 μM of each primer, and 150 μM dNTPs. For amplification, 35 cycles (intramacrophage RNA samples) or 45 cycles (RNA from infected lungs) were performed with the following
parameters: 94°C for 10 seconds, 55°C for 15 seconds, 72°C for 1 minute. PCR
amplicons were detected by 1.5% agarose gel electrophoresis. Control reactions using
non-reverse transcribed RNA verified amplified products were derived from transcripts
and not genomic DNA contamination.

Quantitative PCR was performed on reverse-transcribed RNAs from three
biological replicate cultures of yeast and mycelia. Reactions were assembled using a
SYBR-green based PCR master mix (BioRad) with diluted reverse-transcribed templates
(1:200 final) and 0.5 μM each gene specific primer. Cycling was performed with an IQ5
thermocycler (BioRad) using the following conditions: 95°C for 8 minutes followed by
50 cycles of 95°C for 10 seconds, 55°C for 15 seconds, 72°C for 1 minute. Relative
transcript enrichment in yeast was determined by comparing the Ct values from yeast and
mycelia using the ΔΔCt method (176) after normalizing to levels of the RPS15 transcript.

2.2.8 Construction of RNAi lines

Plasmids for RNAi-mediated depletion of candidate factors were created by
insertion of duplicate copies of the target gene sequence in inverted orientation into a
URA5(+) Histoplasma RNAi vector as described (76). 800-1000 base pairs of target gene
sequence were amplified by PCR from Histoplasma reverse-transcribed RNA. Primers
for amplification were designed with restriction sites to facilitate cloning of the fragments
into the RNAi vector (primer sequences available on request).
RNAi constructs were introduced into WU8 *Histoplasma* yeast by electroporation of 200 ng of PacI-linearized plasmid DNA (177). Transformed yeast were plated on HMM plates to select for uracil prototrophs. Transformants were used to inoculate liquid HMM cultures, which were grown at 37°C to late exponential phase. Cell-free culture supernatants were collected from each and the proteins separated by SDS-PAGE. Protein loadings were normalized to culture density as determined by the optical density of cultures at 600 nm. When necessary, supernatants were deglycosylated first using PNGase F according to the manufacturer’s protocol (New England Biolabs). Gels were silver stained as previously described to visualize extracellular proteins.

### 2.3 Results

#### 2.3.1 Characterization of the *Histoplasma* extracellular protein profile

To identify the proteinaceous extracellular factors produced by *Histoplasma*, we collected culture filtrates from *Histoplasma* grown in the pathogenic-phase. *Histoplasma* strain G186A, a clinical strain from the Panamanian phylogenetic group, was selected for these studies because (i) it is representative of chemotype II strains which comprise five of the six phylogenetically distinguishable *Histoplasma* lineages found worldwide (178), (ii) G186A is well established as a virulent isolate, (iii) sufficient genome nucleotide sequence coverage exists to enable proteomic-based analyses. In addition, the G186A strain is the most genetically tractable of laboratory *Histoplasma* strains: it is easily
transformed, *ura5* auxotroph backgrounds exist that allow selection of *URA5*-based plasmids, and gene knock-downs and knock-outs can be generated to facilitate validation of protein identities through molecular genetics.

As our primary interest is in extracellular factors with relevance to *Histoplasma* pathogenesis, we focused on those culture filtrate proteins produced by *Histoplasma* yeast as compared to those of *Histoplasma* grown as non-pathogenic mycelia. To approximate the *in vivo* growth condition, *Histoplasma* yeast were cultured in liquid *Histoplasma*-macrophage medium (HMM), a tissue-culture based medium that supports the growth and replication of *Histoplasma* yeast cells when co-cultured with macrophages(58). Cultures were maintained as pathogenic-phase yeast by growth at 37°C under 5% CO₂/95% air. Following concentration of the culture filtrate, proteins were separated by one-dimensional SDS-PAGE and visualized by silver staining (Figure 1A). Since the already identified 8-11 kDa Cbp1 virulence factor is extremely abundant in culture filtrates and it could potentially saturate downstream protein identification procedures, we used for our proteomic analyses *Histoplasma* strain WU29, a strain derived from G186A in which the *CBP1* gene has been deleted (cbp1Δ). With the obvious exception of the Cbp1 protein, yeast-phase supernatants prepared from three replicate cbp1Δ cultures look identical to that derived from the wild-type G186A strain (Figure 2.1A). To determine the degree of uniqueness of the proteins released by pathogenic-phase *Histoplasma*, yeast-phase culture filtrate proteins were compared to the proteins secreted by mycelia as well as intracellular yeast proteins. By one-dimensional
separation, the detectable yeast-phase proteins were markedly distinct from the major proteins recovered from mycelial-phase culture filtrates as well as the majority of intracellular yeast-phase proteins (Figure 2.1A). Noticeably, yeast-phase culture filtrates are characterized by a prominent high molecular weight smear consistent with heavily glycosylated proteins. Our attempts at two-dimensional separation of proteins proved unsuccessful, possibly due to the high degree of protein glycosylation or abundant release of polysaccharides that interfere with isoelectric focusing.

Since the validity of any extracellular proteome definition depends on the fraction purity, we verified that our pathogenic-phase culture filtrate preparations were obtained from cultures with no significant cellular lysis. As indicators of cytosolic contamination (e.g., through lysis of yeast cells during culture or during harvesting of the supernatants), we tested the samples for the presence of α-tubulin by immunoblotting, for *Histoplasma* DNA using PCR, and for the cytosolic enzyme homogentisate dioxygenase by enzymatic assay. Whereas the α-tubulin protein is readily detected in cellular lysates, no α-tubulin was detectable in the culture filtrates indicating little pollution of the culture filtrate by cellular antigens (Figure 2.1B). Similarly, no *Histoplasma* DNA was detected in culture filtrates by PCR (Figure 2.2A). While cellular lysates have significant homogentisate dioxygenase activity, culture filtrate samples lack any detectable dioxygenase activity, even when 16-fold greater volume of culture filtrate volume is assayed compared to the cytosolic lysate (Figure 2.2B). Thus, the isolated culture filtrates reflect the extracellular population of factors.
Figure 2.1 Protein profiles of Histoplasma cellular and extracellular fractions. (A) *Histoplasma* culture filtrate (CF) proteins from yeast-phase (Y-phase), mycelial-phase (M-phase), and a yeast-phase cellular lysate were separated by one-dimensional SDS-PAGE (10% NextGel) and visualized by silver staining. Three independent culture filtrates from *cbp1Δ* yeast were compared for biological reproducibility and only lacked the 8-10 kDa Cbp1 protein present in wild type (WT) yeast-phase culture filtrates. (B) α-tubulin immunoblot of *Histoplasma* culture filtrates show lack of detectable cytosolic contamination. 18 μg or 36 μg of concentrated yeast-phase culture filtrate or 5 μg or 10 μg of yeast cellular lysate were probed with an antibody to the cytosolic protein α-tubulin following transfer to PVDF.
Figure 2.1

A

Y-phase CF

cbpΔ

WT

Y-phase lysate

220 kDa

100 kDa

70 kDa

50 kDa

20 kDa

B

Y-phase CF

1 2 3

20ug 10ug 20ug 10ug 20ug 10ug

Y-phase lysate

10ug 5ug
Figure 2.2 Absence of cellular factors in concentrated culture filtrate samples. The three culture filtrate samples for proteomic analysis were checked for the presence of *Histoplasma* DNA by PCR (A) or for activity of the cellular enzyme homogentisate dioxygenase (B). (A) PCR for the *Histoplasma* RPS15 gene was performed using either *Histoplasma* genomic DNA as template (6 pg to 100 ng) or culture filtrate samples (250 ng or 1000 ng of total protein). (B) Homogentisate dioxygenase activity in culture filtrate samples or in *Histoplasma* cellular lysates was determined spectrophotometrically by measuring the conversion of homogentisate to maleylacetoacetate. Phosphate-buffered saline (PBS) was included as a negative control lacking homogentisate dioxygenase. The assay was performed using 50 μg of culture filtrate (CF) total protein or cellular lysate prepared from the equivalent number of cells (1x) used to derive the culture filtrate. 1:4 or 1:16 dilutions of the lysate were also assayed to show sensitivity. Formation of maleylacetoacetate was monitored by absorbance at 330 nm.
Figure 2.2
We also verified that our culture filtrate isolation and concentration procedures did not alter the protein composition of the yeast-phase culture filtrate. To check for loss of protein due to natural extracellular protease activity, we compared culture filtrates collected in the presence or absence of protease inhibitors. No significant reduction in protein amount or change in one-dimensional electrophoretic profile was observed (Figure 2.3). To prepare samples for mass spectrometry, proteins were concentrated by ultrafiltration and lyophilization and recovered by resuspension in Invitrosol buffer. Comparison of protein samples before and after lyophilization and resuspension showed that 92% of the protein was recovered and that the final protein composition was indistinguishable from the original culture filtrate sample by one-dimensional gel electrophoresis (Figure 2.3).

2.3.2 Identification of the major constituents of the pathogenic-phase extracellular proteome

Since the major extracellular factors of pathogenic phase cells are clearly distinct from those produced by the mycelial-phase, we employed a shotgun mass spectrometry approach to identify those proteins that comprise the pathogenic-phase extracellular proteome. Culture filtrates were concentrated by ultrafiltration and subjected to proteolytic digestion. Peptide sequences were extrapolated from the peptide masses obtained through mass spectrometry. Genomic DNA sequence from strain G186A was searched using the MASCOT algorithm (179) to identify the putative secreted protein-
Figure 2.3 Concentration and recovery of yeast-phase culture filtrate proteins.

Proteins from yeast culture filtrates were collected in the presence (“+”) or absence (“-“) of protease inhibitors to check for alteration of protein composition due to natural proteolytic activity. Following concentration by ultrafiltration and lyophilization, proteins were resuspended (“resolubilized”) in an equivalent volume of resuspension buffer and checked for recovery of the original composition. Protein composition in samples is shown by one-dimensional electrophoretic separation of proteins by SDS-PAGE. Total protein was determined for each sample and the quantities normalized to the total protein from culture filtrate samples collected in the absence of protease inhibitors. The normalized protein yield for each sample is listed below the corresponding gel lane.
Figure 2.3
encoding genes. Limiting our searches to the catalog of *Histoplasma* predicted genes would be only as successful as the reliability of those predictions (which are based largely on gene homologies and hypothetical genes from other fungal genomes). To make our searches less biased towards predicted genes and their products, we instead searched the obtained peptide sequences against the translated genome sequence. Reasoning that most protein-coding genes are less than 10 kb, we fragmented the G186A genome sequence into 10 kb length contigs that overlap by 1000 base pairs and used this 10 kb contig database as the subject for our MASCOT searches. As the MASCOT algorithm interprets each 10 kb contig as a single candidate sequence, raw MASCOT scores can be artificially inflated due to multiple peptides matching the contig yet not corresponding to the same protein. Consequently we used the MASCOT scores only as a preliminary estimate of peptide matching and considered a score of 100 as an initial cut off to direct our manual inspection of peptide matches. Peptides were assigned to the same protein if (1) their sequences matched within the same 10 kb contig, (2) were encoded on the same DNA strand, and (3) were no greater than 1000 base pairs from another peptide match on a given contig.

To provide greater confidence in the protein identifications obtained through mass spectrometry, we analyzed multiple pathogenic-phase culture filtrates and employed proteases with dissimilar cleavage sites. We used three biological replicate samples as an indicator of variability in protein secretion. We obtained 5741 total peptides from the three samples (2470 total peptides for sample 1, 1345 total peptides for sample 2, and
1926 total peptides for sample 3). As shown in Figure 2.4A, the protein profiles represented by the peptides from the three replicates showed some differences. Nevertheless, the peptides corresponding to proteins in at least two of the three culture filtrate samples account for over 90% of the total peptides isolated; 547 of the 5741 peptides match proteins present in only a single sample. To guard against false positive identities, each sample was digested separately with trypsin as well as with chymotrypsin to generate a population of different proteolytic fragments representing the same extracellular proteins. We considered as positive protein identification, only those proteins defined by peptides derived from both trypsin digestion as well as from chymotrypsin. In addition, only proteins defined by at least two unique peptides and present in all three culture filtrate samples constituted the final census of extracellular proteins (Table 2.2). These criteria identified 33 proteins which were represented by over 70% of the total peptides obtained (Figure 2.4A). By consideration of consensus splicing signals and protein homologies, we mapped the obtained peptides to the conceptually translated nucleotide sequence for each of these proteins. Peptide coverage for the extracellular proteins fell between 15 and 87 percent (Table 2.2). Not all predicted peptide sequences from a given protein were recovered likely due to peptide mass-altering post-translational modifications (e.g., glycosylation) or peptides whose nucleotide coding sequence spanned exon-exon junctions.

The identified proteins were classified as to potential function using homology to other eukaryotic proteins (Figure 2.4B). Six proteins had glycosidase or glycosyl
Figure 2.4 Proteomic identification of the major constituents of the extracellular proteome of *Histoplasma* yeast. (A) Venn diagram comparing the proteins present in the three independent yeast-phase culture filtrates shows 33 factors common to all three samples. (B) Chart depicts the classification of extracellular protein identities and indicates their approximate abundance based on the number of peptides obtained. Protein identities having N-terminal signal sequences (as determined by Signal P 3.0) are indicated in bold type. Proteins in the five major classes constitute 84% of the total peptides of the extracellular proteome. Protein identities were determined by LC-MS/MS of trypsin- and chymotrypsin-digested culture filtrate samples.
Figure 2.4

A

Sample 1

Sample 2

Sample 3

B

Polysaccharide Associated / Cell Wall

Eng1  Bgl1
Exg1  Gel3
Chs1  Gel4

Unknown
Cfp1  Cfp8
Cfp4  Cfp11
Cfp7

Dehydrogenases
Gdh1  Lpd1
Mpd1  Hom1

Chaperone-like
Pdi1  Fpr2  Hsp60  Hsp82  Ssa1

Oxidative Stress Defense
CatB  Sod1  Trl1

Other (4%)
Ribosomal (3%)
Proteases (2%)
Metabolic Enzymes (7%)
Table 2.2. Components of the *Histoplasma* Pathogenic-Phase Extracellular Proteome.

<table>
<thead>
<tr>
<th>Protein</th>
<th>% Coverage</th>
<th>Total Peptides</th>
<th>Secretion Signal Score</th>
<th>Total Trypsin-Derived Peptides</th>
<th>Total Chymotrypsin-derived peptides</th>
<th>Putative Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CatB</td>
<td>73</td>
<td>439</td>
<td>0.869</td>
<td>179(25)</td>
<td>168(23)</td>
<td>Catalase B</td>
</tr>
<tr>
<td>Eng1</td>
<td>80</td>
<td>412</td>
<td>0.851</td>
<td>180(25)</td>
<td>188(32)</td>
<td>Endo-(1,3)-β-glucanase</td>
</tr>
<tr>
<td>Chs1</td>
<td>57</td>
<td>345</td>
<td>0.949</td>
<td>142(16)</td>
<td>154(18)</td>
<td>Chitinase</td>
</tr>
<tr>
<td>Pdi1</td>
<td>54</td>
<td>242</td>
<td>0.266</td>
<td>117(15)</td>
<td>114(14)</td>
<td>Protein disulfide isomerase</td>
</tr>
<tr>
<td>Cfp1</td>
<td>79</td>
<td>229</td>
<td>0.933</td>
<td>72(9)</td>
<td>119(14)</td>
<td>Aspergillus allergen Asp 4</td>
</tr>
<tr>
<td>Cfp8</td>
<td>81</td>
<td>177</td>
<td>0.872</td>
<td>49(10)</td>
<td>82(10)</td>
<td></td>
</tr>
<tr>
<td>Exg1</td>
<td>48</td>
<td>177</td>
<td>0.941</td>
<td>72(13)</td>
<td>91(16)</td>
<td>Eco-(1,3)-β-glucanase</td>
</tr>
<tr>
<td>Fpr2</td>
<td>55</td>
<td>170</td>
<td>0.197</td>
<td>117(10)</td>
<td>42(11)</td>
<td>Peptidyl-prolyl cis-trans isomerase</td>
</tr>
<tr>
<td>Cfp7</td>
<td>61</td>
<td>167</td>
<td>0.785</td>
<td>65(11)</td>
<td>82(13)</td>
<td>SUN-domain containin protein</td>
</tr>
<tr>
<td>Gor3</td>
<td>59</td>
<td>149</td>
<td>0.644</td>
<td>76(10)</td>
<td>66(12)</td>
<td>β-(1,3)-glucanoyltransferase</td>
</tr>
<tr>
<td>Mpd1</td>
<td>86</td>
<td>147</td>
<td>0.142</td>
<td>94(17)</td>
<td>53(14)</td>
<td>Mannitol phosphate dehydrogenase</td>
</tr>
<tr>
<td>Gh1</td>
<td>75</td>
<td>141</td>
<td>0.041</td>
<td>56(18)</td>
<td>29(10)</td>
<td>Glutamate dehydrogenase</td>
</tr>
<tr>
<td>Lpd1</td>
<td>62</td>
<td>140</td>
<td>0.341</td>
<td>99(19)</td>
<td>41(10)</td>
<td>Dihydrodiploamide dehydrogenase</td>
</tr>
<tr>
<td>Cfp4</td>
<td>50</td>
<td>133</td>
<td>0.901</td>
<td>21(4)</td>
<td>82(3)</td>
<td></td>
</tr>
<tr>
<td>Cip1</td>
<td>54</td>
<td>131</td>
<td>0.233</td>
<td>54(13)</td>
<td>57(13)</td>
<td>Methylcitrate synthase</td>
</tr>
<tr>
<td>Hsp82</td>
<td>48</td>
<td>126</td>
<td>0.093</td>
<td>87(19)</td>
<td>31(11)</td>
<td>Heat shock cognate molecule</td>
</tr>
<tr>
<td>Cfp11</td>
<td>44</td>
<td>124</td>
<td>0.702</td>
<td>71(12)</td>
<td>46(10)</td>
<td></td>
</tr>
<tr>
<td>Hsp80</td>
<td>53</td>
<td>112</td>
<td>0.262</td>
<td>93(19)</td>
<td>19(6)</td>
<td>Heat shock protein 60</td>
</tr>
<tr>
<td>Gnl4</td>
<td>48</td>
<td>106</td>
<td>0.726</td>
<td>29(6)</td>
<td>75(14)</td>
<td>β-(1,3)-glucanoyltransferase</td>
</tr>
<tr>
<td>Aps1</td>
<td>28</td>
<td>100</td>
<td>0.186</td>
<td>66(15)</td>
<td>28(8)</td>
<td>Amino peptidase</td>
</tr>
<tr>
<td>Rad24</td>
<td>78</td>
<td>97</td>
<td>0.106</td>
<td>64(8)</td>
<td>33(6)</td>
<td>DNA-damage checkpoint protein</td>
</tr>
<tr>
<td>Eff1b</td>
<td>66</td>
<td>75</td>
<td>0.073</td>
<td>33(7)</td>
<td>42(8)</td>
<td>Elongation factor 1β</td>
</tr>
<tr>
<td>Bgl1</td>
<td>45</td>
<td>72</td>
<td>0.84</td>
<td>26(5)</td>
<td>33(8)</td>
<td>β-glucosidase</td>
</tr>
<tr>
<td>Eno1</td>
<td>51</td>
<td>68</td>
<td>0.068</td>
<td>51(8)</td>
<td>17(7)</td>
<td>Enolase</td>
</tr>
<tr>
<td>Pfk1</td>
<td>44</td>
<td>65</td>
<td>0.034</td>
<td>57(13)</td>
<td>28(8)</td>
<td>Phosphoglycerate kinase</td>
</tr>
<tr>
<td>Ssa1</td>
<td>15</td>
<td>59</td>
<td>0.135</td>
<td>29(2)</td>
<td>30(5)</td>
<td>HSP70-like protein</td>
</tr>
<tr>
<td>Tgc1</td>
<td>42</td>
<td>55</td>
<td>0.027</td>
<td>33(5)</td>
<td>22(3)</td>
<td>Telomeric ssDNA-binding protein</td>
</tr>
<tr>
<td>Tl1</td>
<td>87</td>
<td>54</td>
<td>0.975</td>
<td>25(5)</td>
<td>29(8)</td>
<td>Thioredoxin reductase-like protein</td>
</tr>
<tr>
<td>Sba1</td>
<td>53</td>
<td>51</td>
<td>0.083</td>
<td>32(4)</td>
<td>21(5)</td>
<td>P21 cell-cycle regulator</td>
</tr>
<tr>
<td>Rpp2</td>
<td>42</td>
<td>49</td>
<td>0.134</td>
<td>22(3)</td>
<td>27(3)</td>
<td>60S ribosomal protein 2</td>
</tr>
<tr>
<td>Ipp1</td>
<td>24</td>
<td>36</td>
<td>0.375</td>
<td>19(3)</td>
<td>17(4)</td>
<td>Inorganic phosphatase</td>
</tr>
<tr>
<td>Hom1</td>
<td>38</td>
<td>35</td>
<td>0.497</td>
<td>13(5)</td>
<td>22(5)</td>
<td>Homocysteine dehydrogenase</td>
</tr>
<tr>
<td>Sod3</td>
<td>22</td>
<td>25</td>
<td>0.82</td>
<td>18(2)</td>
<td>7(2)</td>
<td>Cu/Zn superoxide dismutase</td>
</tr>
</tbody>
</table>

*A* Secretion signal scores (mean S-score) determined by the SignalP algorithm.

*B* Total peptide number with the number of unique peptides given in parentheses.
transferase domains with two predicted to function as glucanases (Exg1 and Eng1) and one as a chitinase (Chs1). Given that the yeast cell wall is comprised of glucan and chitin polymers, these proteins were designated as polysaccharide / cell wall associated. Five of the proteins showed no significant homologies to proteins with known functions and were designated as culture filtrate proteins (Cfp’s). Three proteins linked to oxidative stress (catalase B, CatB; superoxide dismutase, Sod3; and a thioredoxin reductase-like protein designated as Trl1) were found in the culture supernatant. CatB and Sod3 represent distinct enzymes from those involved in detoxification of intracellular reactive oxygen compounds (Figure 2.5). The set of extracellular proteins also included five with chaperone-like functions including protein disulfide isomerase (Pdi1), peptidyl-prolyl cis-trans isomerase D (Fpr2), and three proteins with homology to heat shock factors (Hsp60, Hsp82, and Ssa1). The extracellular proteome had four dehydrogenase enzymes with predicted specificities for glutamate (Gdh1), mannitol phosphate (Mpd1), dihydrolipoamide (Lpd1), and homoserine (Hom1). Despite the validated lack of significant cytosolic contamination of our samples, ten normally cytosolic proteins were present in the extracellular proteome and included metabolic enzymes, peptidases, and other normally cytosolic proteins. Using the number of peptides as an approximate indicator of abundance, the five major protein classes represent 84% of the peptides identified from the extracellular proteome (Figure 2.4B).

Secreted proteins typically possess a secretion signal, often a hydrophobic stretch of amino acids at the N-terminus of the protein followed by a cleavage site, which targets
Figure 2.5 Phylogenetic distribution of superoxide dismutase enzymes. Unrooted phylogenetic tree constructed from ClustalW-aligned proteins distinguishes Fe/Mn-superoxide dismutases (blue clade) from Cu/Zn-superoxide dismutases (green and red clades). The cytosolic superoxide dismutase orthologs include the *Histoplasma* Sod1 protein (HCBG_06622.2). The *Histoplasma* Sod3 protein (HCBG_03650.2) is not part of the cytosolic clade and clusters with a group of Cu/Zn-type Sod-like proteins that all have N-terminal secretion signals (red clade). Species abbreviations used are: Hc (*Histoplasma capsulatum*), Af (*Aspergillus fumigatus*), An (*Aspergillus nidulans*), Nc (*Neurospora crassa*), Ca (*Candida albicans*), Sc (*Sacccharomyces cerevisiae*), Sp (*Schizosaccharomyces pombe*), Mm (*Mus musculus*), and Hs (*Homo sapiens*). Genbank accession entries are listed except for the *Histoplasma* proteins which use the Broad Institute designations.
Figure 2.5
the protein into the eukaryotic secretory pathway. We used the Signal P algorithm (175) to determine the likelihood that each extracellular protein possessed a secretion signal. While not all secreted proteins have canonical signal peptides, 14 of the 33 *Histoplasma* extracellular proteins contained a likely N-terminal secretion signal (Mean S score > 0.7; Table 2.2). In particular, all of the proteins from the polysaccharide / cell wall class, all proteins with unknown functions, and all proteins involved in reactive oxygen stress defense had readily discernable signal sequences. Although the predicted signal sequences have not been functionally validated, they are consistent with the presence of these 14 proteins in the experimentally-derived extracellular protein fraction and lend support to their extracellular localization. Additionally, no peptides corresponding to these putative signal sequences of extracellular proteins were recovered consistent with proteolytic processing of the sequences during transit through the secretory pathway.

In addition to identification of extracellular proteins by the shotgun approach, we isolated yeast-phase protein bands from one-dimensional SDS-PAGE gels and subjected them to mass spectrometry after trypsin digestion. Peptides derived from the masses obtained were aligned to the *Histoplasma* translated genome sequence with MASCOT. The protein identities obtained through this process matched those constituents of the extracellular proteome defined by shotgun mass spectrometry, providing further validation of the ascertained protein identities. For ten of the prominent bands of electrophoretically separated culture filtrate proteins, the vast majority of tryptic peptides defined an individual extracellular antigen (Figure 2.6). Multiple extracellular proteins
Figure 2.6 Confirmation of protein identities by protein excision from SDS-PAGE
and LC-MS/MS. Thirty-four micrograms of yeast-phase culture filtrate were separated by one-dimensional SDS-PAGE and individual protein bands excised and subjected to LC-MS/MS analysis to determine protein identities. The principal protein isolated in each band is indicated by the protein designation adjacent to the respective band. Proteins present in the upper molecular weight smear are included as a group since protein bands could not be individually resolved.
were found in the slowly migrating protein smear between 100 and 200 kDa and included Chs1, Eng1, Cfp4, and Aps1.

2.3.3 Expression of extracellular proteins in the pathogenic-phase

Since the dimorphism of *Histoplasma* reflects a developmental switch to the yeast-phase regulon that is necessary for virulence, we examined the relative expression of secreted protein-encoding genes by *Histoplasma* cells growing as pathogenic yeast compared to growth as non-pathogenic mycelia. The genes that specifically characterize the yeast-phase regulon constitute probable candidate factors that promote *Histoplasma* virulence. To determine the degree of yeast-phase specificity, quantitative reverse-transcription PCR (qRT-PCR) was used to assess the levels of transcription by yeast-phase cells and by mycelia. Total RNA was isolated from wild-type G186A *Histoplasma* cells incubated at 37°C (yeast) or from cells incubated at 25°C (mycelia). Three independent RNA populations were prepared from yeast-phase and mycelial-phase cultures and reverse transcription of the RNAs was primed with oligo-dT and random 15-mer primers. To ensure that PCR targeted genuine transcript sequences, we designed quantitative PCR primers to nucleotide regions for which corresponding peptides had been identified by mass spectrometry. Individual transcript levels were normalized to expression of the small ribosomal subunit gene *RPS15* which shows equivalent expression in both phases (data not shown). The specificity for the pathogenic-phase was
determined by comparison of the relative levels of yeast-phase versus mycelial-phase expression of each secreted protein encoding gene.

Expression analysis revealed 11 extracellular-protein encoding genes with significant upregulation during pathogenic-phase growth (Figure 2.7). The yeast-phase specific gene *CBP1* showed over 4000-fold enrichment in yeast as compared to mycelia. The level of *MS8* mRNA, a transcript known to be enriched in mycelia (180), was 20 to 25 fold higher in mycelia. These results confirm the purity of the yeast and mycelia cultures from which RNAs were isolated. We classified as yeast-phase “specific” and yeast-phase “enriched” transcripts those showing at least 80-fold or at least 5-fold higher expression in the yeast-phase, respectively. Of the genes encoding secreted proteins with unknown functions, *CFP4* and *CFP17* showed strong specificity for the yeast-phase. In particular, *CFP4* transcription is essentially exclusive to the yeast-phase; *CFP4* mRNA is over 19,000-fold higher in yeast as compared to mycelia and *CFP4* mRNA was not detected in mycelia-phase RNAs by endpoint RT-PCR (data not shown).

Other yeast-phase specific transcripts include genes for the extracellular endoglucanase (*ENG1*) and chitinase (*CHS1*). Transcription of both the extracellular catalase (*CATB*) and superoxide dismutase (*SOD3*) genes were substantially higher (approximately 100-fold) in yeast-phase cells than in mycelia. The genes encoding Cf p1, Cf p7, and Cf p8, as well as the extracellular exoglucanase (Exg1), and thioredoxin
Figure 2.7 Relative expression of extracellular protein-encoding genes by yeast or mycelial-phase *Histoplasma* cells. Quantitative RT-PCR of RNA isolated from wild-type *Histoplasma* yeast and mycelial cultures shows ten proteins are enriched in yeast-phase cells. Cultures were grown at 37°C for yeast or 25°C for mycelia cultures. Gene-specific primers were used for each reaction and the fold-change in the transcript levels calculated using the ΔΔC_T method after normalizing to the ribosomal RPS15 gene. Bars represent the average of three biological replicates and error bars indicate the standard deviation. The fold change in expression (yeast relative to mycelia) for proteins with at least a 5-fold change (dotted line) is indicated above the respective bars.
reductase like factor (Trl1), while not as unique to the yeast-phase, were nonetheless expressed significantly higher by yeast-phase cells than mycelia. The genes encoding the remaining glucosidases (*BGL1*, *GEL3*, *GEL4*), and other proteins of unknown function showed no particular enrichment for the yeast-phase. While the chaperone-like proteins Pdi1, Hsp82, and Ssa1, are components of the yeast-phase extracellular proteome, their transcription is similar between pathogenic and non-pathogenic phases. Only the aminopeptidase genes *APS1* and *APS2* were more abundantly transcribed by mycelia than by yeast cells. The connection between yeast and *Histoplasma* pathogenicity focused our attention on the 11 factors whose transcripts are particularly enriched in the pathogenic-phase (i.e., Cfp1, Cfp8, Cfp4, Cfp17, Exg1, Eng1, Chs1, CatB, Sod3, and Trl1). Notably, these 11 proteins are represented by 58% of the peptides defining the extracellular proteome and each is predicted to possess a N-terminal secretion signal.

2.3.4 Molecular genetic confirmation of pathogenic-phase extracellular protein identities.

Since extracellular proteins were identified through peptide masses, we validated the deduced nucleotide coding sequence through molecular genetic-based depletion of target proteins (Figure 2.8). The inefficiency of homologous recombination in *Histoplasma* led us to use RNA interference (RNAi) to deplete target proteins. RNAi silences protein production based on nucleotide sequence identity with a targeted transcript. This provides important confirmation of novel proteins whose predicted gene structure could not be supported by homology to known proteins in public databases. We
Figure 2.8 RNA interference confirms mass spectrometry-derived protein identities.

Individual extracellular proteins were targeted for depletion by RNA interference: Cfp8 and Cfp1 (A), Cfp4 (B), and CatB (C). (A) RNAi-based targeting of Cfp8 (CFP8-RNAi) and Cfp1 (CFP1-RNAi) depletes specific protein bands in comparison to the control (WT) yeast culture filtrate. The control culture filtrate was derived from *Histoplasma* yeast transformed with a gfp-RNAi plasmid. Proteins were separated by 10% NextGel SDS-PAGE. (B) RNAi-based silencing of CFP4 expression identifies the Cfp4 band in deglycosylated culture filtrates. Deglycosylated proteins from culture filtrates from two independent CFP4-RNAi lines were compared to deglycosylated culture filtrate proteins from CFP4(+) yeast. Yeast culture filtrate proteins were oligosaccharides prior to electrophoretic separation. The 36 kDa PNGase F protein migrates just below the Cfp4 protein as shown by the lane containing only the PNGase F protein. Proteins were separated by SDS-PAGE through 12.5% NextGel. (C) RNAi-based depletion and overexpression confirms the identity of catalase B (CatB). Culture filtrates were prepared from control yeast (CATB(+)), yeast in which CatB was silenced by RNAi (CATB-RNAi) and yeast which overexpress CatB (CATB(+++)). Culture filtrate proteins were separated by electrophoresis through 8% denaturing polyacrylamide. All protein bands were visualized by silver staining following electrophoretic separation.
Figure 2.8
selected as targets, yeast-phase enriched factors for which we could monitor protein depletion by one-dimensional electrophoretic separation of culture filtrate proteins. RNAi vectors targeting the yeast-phase enriched factors Cfp1 and Cfp8 and the yeast-phase specific protein Cfp4 were constructed based on the predicted encoding gene sequences, and vectors were transformed into *Histoplasma* yeast. In the absence of available antibodies to these extracellular factors to monitor protein loss, we screened concentrated yeast-phase culture filtrates for loss of specific protein bands. *Histoplasma* transformants were grown in liquid HMM as yeast (i.e., cultured at 37°C), the cell free culture supernatants collected, and the extracellular proteins separated by one-dimensional SDS-PAGE and visualized by silver staining. Culture filtrates from yeast transformants harboring either the *CFP8*-RNAi or the *CFP1*-RNAi vector showed loss of a single 29 kDa protein band or 26 kDa protein band, respectively when compared to the extracellular protein profile of wild type yeast (Figure 2.8A). RNAi-based determination of these protein band identities corresponds to their predicted molecular weight and matches their identification as Cfp8 and Cfp1 obtained through band excision and mass spectrometry (Figure 2.6). The molecular weight based on the amino acid sequence of Cfp4 is 23 kDa, but this is inconsistent with its presence in the slower mobility smear as determined by band excision. This suggests that Cfp4 might be highly glycosylated. To test this possibility, N-linked glycans were removed from extracellular proteins by digestion of culture filtrates with PNGase F, and the resultant deglycosylated proteins were separated by one-dimensional SDS-PAGE (Figure 2.8B). After N-linked deglycosylation, the slowly migrating smear largely disappears and a prominent protein
band is detected around 38 kDa (compare Figure 2.8B to Figure 2.6). This approximate 38 kDa protein is absent in deglycosylated culture filtrates prepared from two separate CFP4-RNAi lines of *Histoplasma* yeast (Figure 2.8B) identifying the band as Cfp4. The still larger apparent molecular weight from that predicted may represent additional O-linked glycosylation which would not be removed by PNGase F and would retard its electrophoretic mobility. This result confirms the identity of Cfp4 as a member of the extracellular proteome and demonstrates the protein is substantially modified through N-linked glycosylation.

RNA interference was also used to validate the presence of catalase B in the extracellular deglycosylated by treatment with the endoglycosidase PNGaseF to remove N-linked pathogenic-phase proteome. Enzymatic analysis shows wild-type culture filtrates possess catalase activity consistent with the presence of secreted CatB (data not shown). We created an RNAi vector that specifically targets the *CATB* gene sequence and transformed it into *Histoplasma* yeast. Separation and visualization of the extracellular proteins harvested from *CATB*-RNAi yeast cultures showed a conspicuous deficiency of a 90 kDa band when compared to wild type culture filtrates (Figure 2.8C). As a secondary confirmation of this protein band as CatB, we constructed a vector in which transcription of *CATB* was initiated from a strong constitutive promoter and examined culture filtrates from *Histoplasma* yeast transformed with this overexpression vector for increased abundance of a specific protein. Consistent with the band identified as CatB
through RNAi, overexpression of CatB led to a prominent increase in this same 90 kDa band (Figure 2.8C).

We also used RNAi to deplete Gdh1 from yeast-phase culture filtrates. Although this protein was not one of the yeast-phase enriched or yeast-phase specific proteins, Gdh1 was consistently found in culture filtrates and the protein was associated with a particular band by one-dimensional gel electrophoresis of extracellular proteins. Culture filtrates from yeast carrying an RNAi vector targeting Gdh1 are specifically depleted of the Gdh1 protein band following one-dimensional electrophoresis (data not shown). We also confirmed the depletion of Gdh1 by enzymatic assay; culture filtrates from two independent GDHI-RNAi lines show at least 3-fold reduction in extracellular glutamate dehydrogenase activity compared to GDHI(+) culture filtrates (data not shown).

2.3.5 Confirmation that pathogenic-phase extracellular factors are expressed during infection.

The proteomic analyses described above identified the extracellular proteins released by Histoplasma yeast grown in liquid culture and not directly from host environments. While in vitro culture was necessary to harvest enough material for mass spectrometry, efforts were made to approximate the environment that would be encountered by yeast within the mammalian host. To confirm that the extracellular factors produced in culture are produced by Histoplasma yeast during residence within
phagocytic cells, we examined the expression of candidate proteins by yeast following infection of cultured macrophages (Figure 2.9). Macrophages were infected with *Histoplasma* yeast for 40 hours and internalization and intramacrophage residence of yeast was verified by microscopy. RNA was isolated from the co-culture and RT-PCR was performed using primers for the genes encoding each of the yeast-phase specific and yeast-phase enriched factors. All primer sets amplified specific products from *Histoplasma* genomic DNA, and with the exception of the mycelial-phase transcript MS8, all genes were expressed by *Histoplasma* yeast grown in laboratory culture. We detected transcripts corresponding to each of the pathogenic-phase extracellular factors from RNA isolated from yeast resident within macrophages confirming these factors are expressed by intramacrophage *Histoplasma* yeast. No amplicons were produced from intramacrophage yeast RNA in the absence of reverse transcription (data not shown) nor from uninfected macrophage RNA. *Histoplasma* yeast infecting murine lungs (infected lung). For intramacrophage transcription, total RNA was purified from *Histoplasma* yeast 40 h. after infection of P388D1 macrophages. Histoplasma transcription of genes during respiratory infection was determined by infection of C57BL/6 mice with *Histoplasma* yeast for 24 h. To determine if *Histoplasma* yeast express constituents of the pathogenic-phase extracellular proteome *in vivo*, we examined transcription of the candidate genes by yeast during respiratory infection of mice. Mice were infected intranasally with wild-type *Histoplasma* yeast and lungs were removed after 24 hours of infection. *Histoplasma* yeast were recovered from infected lung tissue and RNA was isolated. RT-PCR was performed using primers for genes encoding the yeast-phase specific and yeast-phase
Figure 2.9 Extracellular proteins are expressed *in vivo* by *Histoplasma* during infection. Transcription of each factor was determined by RT-PCR of template ribonucleic nucleic acids from *Histoplasma* yeast, *Histoplasma* yeast infecting macrophages (intramacrophage), and Control PCR reactions were performed with a constitutively expressed gene (RPS15), a mycelia-enriched gene (MS8), and a yeast-phase specific factor (CBP1). Genomic DNA was used as template to show the accuracy of each primer set and band shifts relative to RNA templates correspond to primer sets which span introns. Reactions lacking reverse-transcription (RT) and the absence of genomic-sized bands indicate the lack of DNA contamination of RNA templates. PCR products were specific for *Histoplasma* yeast as no products were obtained from uninfected macrophage and lung reverse-transcribed RNAs. Reverse transcription of RNAs was primed with random 15-mer and dT 22-mer oligonucleotides. PCR products were resolved by electrophoresis through 1.5% agarose and visualized by ethidium bromide staining.
Figure 2.9
enriched proteins after reverse-transcription primed by random nucleotide oligomers (Figure 2.9). In RNA harvested from yeast isolated from infected lungs, mRNA was detected for the housekeeping gene RPS15 as indicated by the faster migrating amplicon corresponding to the spliced RPS15 transcript. In the absence of reverse transcription, no amplicons were produced from yeast RNA from infected lungs (data not shown) nor were amplicons produced from reverse-transcribed RNA harvested from uninfected lungs (Figure 2.9). Similar to our results from intramacrophage yeast, yeast-phase specific and yeast-phase enriched factors were detected in RNA from yeast isolated directly from infected lungs. This data establishes the validity of the in vitro-derived extracellular proteome of pathogenic-phase Histoplasma to yeast infecting the mammalian host.

2.4 Discussion

The success of Histoplasma as an intracellular pathogen depends on avoidance or neutralization of the defense mechanisms of the host macrophage. In this study, we identified the primary constituents of the Histoplasma pathogenic-phase extracellular proteome as a foundation upon which to base studies to define the factors that influence the outcome of the host-pathogen interaction. In addition, exoantigens represent attractive molecules for antigenemia-based diagnostic tests. For example, the H- and M-antigens of Histoplasma are culture filtrate molecules β-glucosidase and catalase, respectively (181-183). Current diagnosis of disseminated histoplasmosis utilizes an ELISA-based assay for
an extracellular polysaccharide antigen (184). While the proteome assembled here identifies constituents in the cell-free supernatant, we do not distinguish between those soluble components which are not associated with the yeast cell surface and those surface-associated molecules which could be shed from the yeast cell as has been found for Yps3 (96).

Our studies focused on yeast-phase proteins since virulence is precisely linked to this state. Factors preferentially produced by *Histoplasma* yeast will thus likely be tailored to benefit the pathogenic lifestyle unique to this phase. Indeed, one-third of the major constituents of the extracellular proteome are expressed exclusively by cells in the pathogenic-phase or are significantly enriched compared to cells in the non-pathogenic phase. This pattern mirrors the phase-specific production of three of the known virulence factors identified to date: secreted Cbp1 (83), cell wall α-glucan (72, 185), and Yps3 (186). Since sufficient material for proteomics could not be isolated directly from intramacrophage *Histoplasma* yeast, we pursued an alternative approach whereby proteins were isolated from *Histoplasma* cells grown under pathogenic phase-inducing conditions followed by validation that identified factors were expressed by *Histoplasma* during infection. We attempted to mimic infection conditions during *in vitro* culture, yet *Histoplasma* may produce some extracellular factors only during residence within the phagocyte phagosome and these could have been missed. However, these potential additions to the pathogenic-phase proteome are likely few since the short time frame during which phagocytosis, the phagocyte oxidative burst, and intracellular trafficking
occur would not allow much time for de novo synthesis of molecules critical to 
*Histoplasma* survival. In support of this, we found that differentiation as yeast is 
sufficient for expression of the antioxidant defenses superoxide dismutase and catalase. 
Furthermore, all of the major yeast-phase enriched extracellular factors are expressed 
during infection. Thus, our *in vitro*-derived extracellular proteome is relevant to 
*Histoplasma* pathogenesis *in vivo*.

The different phylogenetic groups of *Histoplasma* produce different combinations 
of extracellular yeast-phase virulence factors (79). Identified extracellular factors 
produced by the North American 2 class (NAm2) of *Histoplasma* strains include 
dipeptidyl petidase and gamma-glutamyltransferase (86, 187). North America class I 
*Histoplasma* strains uniquely secrete an extracellular serine protease (103). However, 
none of these enzymes were found in the G186A extracellular proteome presented here. 
Thus the extracellular proteomes of the different *Histoplasma* strains are distinct from 
one another and our definition of the extracellular factors produced by the *Histoplasma* 
G186A background may not be fully applicable to that of other phylogenetic groups. 
These results also suggest the different strain groups may utilize different strategies for 
pathogenesis.

To provide a high degree of confidence in the protein identifications obtained, we 
used stringent criteria and multiple lines of evidence. For inclusion in our final 
extracellular proteome index, we required proteins were (1) present in three independent
culture filtrate samples, (2) represented by at least two unique peptides, and (3) defined by both trypsin-derived as well as chymotrypsin-derived peptides. For definitive confirmation, particularly of pathogenic-phase factors that lack significant homology to other defined proteins, we demonstrated that depletion of candidate factors through genetic means corresponded to loss of specific proteins from culture filtrates.

While any of the identified extracellular proteins could contribute to *Histoplasma* virulence, indicators such as (1) abundance, (2) pathogenic-phase-enriched expression, (3) depletion when secretion is blocked, and (4) *in vivo* expression focuses attention on Chs1, Exg1, Eng1, Cfp1, Cfp4, Cfp8, CatB, Sod3, and Trl1. Presumably, the exo- and endoglucanases as well as chitinase act on the yeast glucan- and chitin-rich cell wall during wall remodeling processes required for yeast growth and replication. Isotropically growing yeast cells remodel a much greater proportion of their surface than hyphae that elongate at the tips, which may account for the increased production of the cell wall degrading enzymes in yeast as compared to mycelia. Release of the glucanases and chitinase from the cell surface may account for the presence of these enzymes in yeast cell-free culture filtrates. Alternatively, extracellular glucanases and chitinase may reduce potential activation of immune defenses by degrading glycan fragments released during cell wall remodeling into smaller, less immunostimulatory oligomers; large chitin and α-glucan polymers can both stimulate the immune system through recognition by host PRRs (188)(189) whereas small soluble α-glucans do not activate the α-glucan receptor dectin-1 (188, 190-192).
The production of extracellular superoxide dismutase and catalase most likely protect *Histoplasma* yeast from phagocyte-derived reactive oxygen molecules. *Histoplasma* also expresses two other catalases, CatP and CatA (193), but their intracellular localization suggests these two catalases are involved in ameliorating fungal-derived oxygen byproducts of metabolism and respiration. Our results showing increased expression of the extracellular catalase by *Histoplasma* yeast cells contrasts with that reported by Johnson, et al. who reported CATB transcription in both phases (193). However, their analysis used a different phylogenetic strain of *Histoplasma* (NAm2; North American class 2) and was not quantitative. In the G217B NAm2 strain, CatB is primarily attached to the yeast cell wall and soluble CatB appears only after 7 days of culture, which was postulated to result from cell lysis(194). In contrast, we find abundant CatB in the cell-free yeast culture filtrate from exponentially growing (1-2 days of culture) G186A yeast cells with no indication of cellular lysis. While G186A yeast also have cell wall-associated catalase activity, this represents a minority of the total extracellular peroxidase activity (data not shown), again reflecting differences between these two phylogenetically separate strains.

The extracellular superoxide dismutase identified in this study, Sod3, is a member of a third class of extracellular fungal Cu/Zn dismutase enzymes (Figure 2.5). In contrast to the intracellular Fe/Mn-dependent superoxide dismutases associated with mitochondria, Sod3 is more similar to the Cu/Zn-type superoxide dismutases. However, Sod3 is not the cytosolic superoxide dismutase ortholog since a different *Histoplasma*
Cu/Zn Sod protein (Sod1; HCBG_06622.2) belongs to the clade of eukaryotic cytosolic superoxide dismutases (Figure 2.5). *Histoplasma* Sod3 is distinct from these cellular enzymes and shows greater similarity to a group of fungal Cu/Zn-type superoxide dismutases that all have secretion signals as do the related mammalian extracellular Sod3 enzymes. Similar to CatB, *Histoplasma* Sod3 is likely produced to specifically eliminate extracellular oxidative stress such as that generated by the phagocyte NADPH-oxidase in response to microbes. As superoxide is a charged molecule unable to cross membranes, intracellular superoxide dismutases are unable to dismutate the superoxide produced in the lumen of the phagosome and would not provide protection to the yeast. On the other hand, the extracellular superoxide dismutase Sod3 is precisely positioned to interact with and destroy the phagocyte-derived superoxide.

The functions of the remaining constituents of the pathogenic-phase extracellular proteome are more speculative at this point. The Cfp4 protein is a heavily glycosylated protein expressed exclusively by the pathogenic-phase. Sera from patients with histoplasmosis contain antibodies to this protein confirming *Histoplasma* yeast produce Cfp4 *in vivo* (195). Comparison of PNGaseF-treated and native culture filtrates indicates N-linked glycosylation is a hallmark of pathogenic-phase extracellular proteins suggesting that protein glycosylation is linked to *Histoplasma* virulence. Cfp1 has homology to an allergen from the fungal pathogen *Aspergillus fumigatus* but no function has been described (196, 197).
The *Histoplasma* extracellular proteome also contains a number of normally cytosolic factors. Three stringent tests (immunoblotting, PCR, enzymatic assay) dispute the idea that these proteins leak into the extracellular fraction through cellular lysis. Two different mechanisms may account for their extracellular localization. The extracellular localization of membrane-bound peptidyl-prolyl cis-trans isomerase (Fpr2) and the endoplasmic reticulum protein disulfide isomerase (Pdi1) may reflect insufficient retention of these chaperone proteins in organelles of the secretory pathway. Cytosolic factors can also become extracellular through vesicle secretion, a process recently demonstrated for some fungal organisms, including *Histoplasma capsulatum*, by which cytosolic constituents are packaged into vesicles and delivered across the cell membrane and wall (170). No mechanism has been described for selectivity of the proteins transported by this process and thus abundant cellular proteins (e.g., TCA cycle enzymes, chaperone molecules, and ribosomal or translation factors) could enter this secretion pathway.

Regardless of the transport mechanism, precedent exists for the unconventional extracellular localization of many of the cytosolic factors. For example, Hsp90 is found in the *Candida* cell wall proteome (198) and mammalian Hsp90 can be secreted from mammalian cells due to a C-terminal secretion motif (199). Hsp60 has been found at the yeast cell surface of NAm2 *Histoplasma* strains where it functions as an adhesin for CD18-family receptors on macrophages (200). Studies with other microbes, including fungi, have found metabolic proteins outside of the cytosol, including phosphoglycerate
kinase (Pgk1) and enolase (Eno1) (198, 199, 201-203). In addition to a role in glycolysis, enolases from *Pneumocystis* and *Leishmania* have been shown to bind host plasminogen (203, 204).

One other study has detailed a set of extracellular proteins released by *Histoplasma capsulatum* yeast through vesicular secretion and resulted in the identification of 206 factors, including ribosomal factors, metabolic enzymes, cell signaling, and cytoskeletal proteins (205). Both the study by Albuquerque, et al. and ours use proteomic approaches; Albuquerque, et al., focused on those proteins present in extracellular vesicles released from NAm2 yeast, while our study collected proteins directly from the G186A yeast culture filtrate. While there is overlap in the identities obtained, factors unique to the extracellular proteome defined by this study include Eng1, all but Cfp1 of the Cfp proteins, Lpd1, Hom1, Eno1, and all the oxidative stress defense factors (CatB, Sod3, Trl1). The catalase identified by Albuquerque, et al. is not CatB as reported but is instead CatP, a peroxisomal catalase. The superoxide dismutases reported in vesicles by Albuquerque, et al., are most similar to mitochondrial Fe/Mn-type enzymes and are distinct from the extracellular Cu/Zn-superoxide dismutase Sod3 we identified. The unique factors identified by our study comprise three of the four most abundant classes of extracellular proteins and most of these unique proteins are those predicted to have N-terminal secretion signals suggesting the transport of these relies upon the eukaryotic secretory pathway. On the other hand, the majority of the normally cellular proteins (e.g. Cit1, Gdh1, Mpd1, Rad24, Pgk1, Ssa1, Rpp2, and Ipp1), were found to be
delivered to the extracellular environment through vesicle secretion (198, 205). Whereas most of the unique extracellular proteins defined here are expressed primarily by the pathogenic-phase, the majority of proteins common between the two studies show equivalent or even higher expression in the non-pathogenic-phase.

In conclusion, we have identified the abundant constituents of the pathogenic-phase extracellular proteome. In conjunction with the previously identified secreted protein Cbp1 (83), this comprehensive catalog of extracellular factors provides a foundation for understanding those factors which manipulate the host-pathogen interface. The preferential expression of many of these extracellular proteins by pathogenic-phase cells makes them prime candidates for reverse genetic approaches to dissect Histoplasma pathogenesis. While random mutagenesis strategies are an alternative approach, they are limited to defining one factor at a time. Since pathogenesis is multi-factorial, the index of extracellular factors determined through proteomics provides a more complete framework for deciphering the multiple aspects of Histoplasma virulence.
Chapter 3: Extracellular Superoxide Dismutase Protects *Histoplasma* Yeast Cells from Host-Derived Oxidative Stress.

3.1 Introduction

Highly reactive oxygen metabolites are one of the primary effector mechanisms used by the host immune system to control or clear microbial infections. Initial host defenses against fungal invaders rely on responses of innate immune cells, particularly neutrophils (polymorphonuclear leukocytes; PMNs) and macrophages. These phagocytes produce reactive oxygen molecules through activation of the NADPH-oxidase complex that generates superoxide. Superoxide and the other reactive molecules derived from it, including peroxide and hydroxyl radicals, are collectively termed reactive oxygen species (ROS). These species can directly damage macromolecules on or in the microbe leading to death of the microbe (35). Cytokine activation of macrophages during the adaptive immune response enhances the ability of macrophages to produce ROS and this correlates with increased ability to restrict or kill microbe (206, 207). Since ROS molecules are highly toxic to microbes, effective pathogens must avoid or neutralize
phagocyte-derived ROS in order to survive within the host. This is particularly necessary for intracellular pathogens.

The yeast form of *Histoplasma capsulatum* is an intracellular pathogen that successfully infects and parasitizes phagocytes. The fungus is widespread in the Midwestern United States and throughout Latin America. It is estimated that 200,000 infections occur annually in the United States through inhalation of infectious particles (6). Macrophages efficiently ingest pathogenic *Histoplasma* yeast cells, but are unable to kill the yeasts (54, 61, 208). By itself, the innate immune system is insufficient to clear the infection. With activation of the adaptive immune response and the corresponding enhancement of phagocyte antifungal defenses, most immunocompetent individuals can restrict *Histoplasma* proliferation (27, 28).

The mechanisms *Histoplasma* employs to avoid clearance by innate immune cells are essential to its virulence. *Histoplasma* survival in macrophages may result, in part, by the lack of an oxidative burst from these phagocytes (51-54). Activation of macrophages by cytokines primes their production of ROS in response to *Histoplasma* (53, 54, 209). PMNs also participate in the initial response to respiratory *Histoplasma* infection (42, 210) and the interaction of *Histoplasma* with these phagocytes triggers a respiratory burst (44-46, 164). Nevertheless, *Histoplasma* yeast cells are not killed despite ample ROS production adequate to kill other fungi such as *Candida* (43-45, 164). How *Histoplasma*
yeasts endure this oxidative challenge and the factors enabling *Histoplasma* survival in the face of phagocyte-derived ROS are unknown.

To identify new factors facilitating *Histoplasma* pathogenesis, we recently examined the extracellular proteome of pathogenic-phase *Histoplasma* cells (211). From this, we identified three proteins potentially involved in defending *Histoplasma* from ROS. One protein is a predicted superoxide dismutase (Sod3) whose expression is greatly enriched in pathogenic-phase yeast cells suggesting its importance to virulence. This extracellular protein is distinct from other *Histoplasma* superoxide dismutases that have a higher degree of similarity to cytoplasmic enzymes (211), suggesting Sod3 functions in combating extracellular superoxide, such as that which would be produced by phagocytes.

In this paper, we define the contribution of Sod3 to *Histoplasma* pathogenesis and the role of host-derived reactive oxygen defenses. To functionally test the role of Sod3, we created a *Histoplasma* strain lacking the *SOD3* gene. Our results demonstrate that Sod3 is the major source of extracellular superoxide dismutase activity and that Sod3 prevents superoxide-dependent killing of *Histoplasma* yeast cells. In addition, we show that host control of *Histoplasma* infection requires ROS production. These results identify Sod3 as an essential virulence factor of *Histoplasma* and provide a mechanistic explanation as to how *Histoplasma* survives ROS generated by host phagocytes during infection.
3.2 Material and Methods

3.2.1 Ethics Statement

This study was carried out in accordance with The National Research Council's Guide for the Care and Use of Laboratory Animals, Public Health Service Policy on Humane Care and Use of Laboratory Animals, and AAALAC accreditation guidelines. The protocol was approved by the Ohio State University Institutional Animal Care and Use Committee (protocol number: 2007A0241). Animal procedures were performed under anesthesia as described below and all efforts were made to minimize suffering. Human cells were obtained from healthy volunteers after obtaining HIPAA research authorization and written informed consent in accordance with the Declaration of Helsinki. The human subjects protocol was reviewed and approved by the Ohio State University Biomedical Sciences Institutional Review Board (protocol number 2008H0242) under Ohio State University's Office for Human Research Protections (Federalwide Assurance number: 00006378).

3.2.2 Fungal strains and culture

*Histoplasma capsulatum* strains used in this study were derived from the wild-type strain G186A (ATCC 26027) and are listed in Table 3.1. *Histoplasma* yeasts were grown in
Table 3.1. *Histoplasma capsulatum* strains

<table>
<thead>
<tr>
<th>Strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Genotype</th>
<th>Other Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WU8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ura5-32Δ</td>
<td>sod3Δ</td>
</tr>
<tr>
<td>OSU13</td>
<td>ura5-32Δ sod3-3Δ::hph</td>
<td></td>
</tr>
<tr>
<td>OSU15</td>
<td>ura5-32Δ sod3-3Δ::hph / pCR468 [URA5, gfp:FLAG]</td>
<td>sod3Δ</td>
</tr>
<tr>
<td>OSU22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ura5-32Δ zzz::pCR482 [hph, gfp]</td>
<td></td>
</tr>
<tr>
<td>OSU45</td>
<td>ura5-32Δ / pCR468 [URA5, gfp:FLAG]</td>
<td>SOD3(+)</td>
</tr>
<tr>
<td>OSU49</td>
<td>ura5-32Δ sod3-3Δ::hph / pBY09 [URA5, SOD3]</td>
<td>sod3Δ/SOD3</td>
</tr>
<tr>
<td>OSU88</td>
<td>ura5-32Δ / pCR468 [URA5, gfp:FLAG]</td>
<td></td>
</tr>
<tr>
<td>OSU102</td>
<td>ura5-32Δ / pCR508 [URA5, SOD3&lt;sub&gt;1-78&lt;/sub&gt;:gfp:FLAG]</td>
<td>Sod3&lt;sub&gt;1-26&lt;/sub&gt;:GFP:FLAG</td>
</tr>
<tr>
<td>OSU103</td>
<td>ura5-32Δ zzz::pCR482 [hph, gfp] / pCR464 [URA5]</td>
<td>gfp(+)+</td>
</tr>
<tr>
<td>OSU104</td>
<td>ura5-32Δ zzz::pCR482 [hph, gfp] / pCR473 [URA5, gfp-RNAi]</td>
<td>gfp-RNAi</td>
</tr>
<tr>
<td>OSU116</td>
<td>ura5-32Δ sod3-3Δ::hph / pCR601 [URA5, SOD3&lt;sub&gt;1-78&lt;/sub&gt;:FLAG:SOD3&lt;sub&gt;79-693&lt;/sub&gt;]</td>
<td>sod3Δ&lt;sub&gt;1&lt;/sub&gt;FLAG:SOD3</td>
</tr>
<tr>
<td>OSU117</td>
<td>ura5-32Δ sod3-3Δ::hph / pCR602 [URA5, SOD3&lt;sub&gt;1-78&lt;/sub&gt;:FLAG:SOD3&lt;sub&gt;79-615&lt;/sub&gt;]</td>
<td>sod3Δ&lt;sub&gt;1&lt;/sub&gt;FLAG:SOD3&lt;sub&gt;1G&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> all strains were constructed in the G186A (ATCC# 26027) background

<sup>b</sup> uracil auxotroph of G186A (Marion CM, et al., 2006 [63])

<sup>c</sup> GFP sentinel RNAi background (Edwards, et al. 2011 [26])
Histoplasma-macrophage medium (HMM; (172)) or modified 3M medium (85 mM NaCl, 1 mM K₂HPO₄, 20 mM HEPES, 1.5% glucose, 15 mM (NH₄)₂SO₄, 1 mM MgSO₄, 0.2 mM CaCl₂, 15 mM glutamate, 350 μM cysteine at pH 7.0; (172)) at 37°C. Liquid cultures were continuously shaken (200 rpm) until late log / early stationary phase unless otherwise indicated. For growth of uracil auxotrophs, media was supplemented with uracil (100 μg/mL). Growth rate and growth stage of strains were determined by measurement of liquid culture turbidity at 595 nm. In some experiments media was supplemented with paraquat (856177, Sigma) to a final concentration of 5 μM and 10 μM to artificially raise the intracellular levels of superoxide. For growth on solid media, HMM was solidified with 0.6% agarose and supplemented with 25 μM FeSO₄.

Histoplasma yeasts were transformed with linear plasmids by electroporation (177) and plated on solid HMM to select Ura⁺ transformants. For experiments with defined numbers of yeasts, clumps of cells were removed by centrifugation (1 minute at 50 rcf). For precise enumeration, yeast cells were counted using a hemacytometer. The cap1Δ/cap1Δ Candida albicans strain (used to prevent filamentous growth of Candida albicans which would confuse accurate enumeration of cfu; (212)) was grown in liquid YPD medium or on YPD solidified with 2% agar. Candida cells were enumerated by hemacytometer.

3.2.3 Generation of the sod3Δ mutant, SOD3-complemented, and SOD1-RNAi strains
WU8 *Histoplasma* yeasts were transformed with the *URA5(+) plasmid pBY06* which contains a hygromycin resistance gene flanked by 2 kb of sequence upstream and 2 kb of sequence downstream of the *SOD3* coding sequence. Hygromycin-resistant transformants were grown in liquid HMM with uracil and 150 μg/mL hygromycin B, diluting the culture 20-fold when stationary phase was reached. 3 passages in liquid culture were performed after which yeast cells were plated on solid HMM containing uracil, 150 μg/mL hygromycin B, and 1 mg/mL 5-fluororotic acid (5-FOA) to counter-select retention of the knock-out allele episomal molecule. Hygromycin-resistant, 5-FOA-resistant colonies were picked and screened for the loss of the *SOD3* CDS by PCR. For complementation, the *SOD3* gene was amplified from wild-type G186A genomic DNA by PCR and cloned into pCR468 replacing the *gfp* transgene. The pCR468 vector contains the histone-2B (*H2B*) promoter for high level constitutive expression of transgenes. The *sod3Δ* mutant was transformed either with the *gfp*- (pCR468) or *SOD3*-expression vector (pBY09) and *Ura*<sup>+</sup> transformants selected to create uracil-prototrophic strains. An isogenic strain with the wild-type *SOD3* locus was also created by transformation of WU8 with the *gfp*-expression vector (pCR468).

Depletion of Sod1 was achieved using RNAi. Nucleotides -12 to 787 of the *SOD1* CDS were amplified by PCR and two copies cloned in inverse orientation into the RNAi *gfp*-sentinel vector pCR473 (26). Linearized plasmids were transformed into OSU22, a WU8-based strain that expresses a *gfp* transgene. *Ura*<sup>+</sup> transformants were screened for silencing of the GFP sentinel using a modified UV transilluminator (213, 214).
Transformants with significant loss of GFP fluorescence were selected for analysis of intracellular superoxide dismutase activity as described below.

3.2.4 Preparation of *Histoplasma* culture filtrate and cellular lysate samples

*Histoplasma* yeasts were grown to late log / early stationary phase in modified 3M medium. Yeast cells were removed by centrifugation (5 minutes at 5000 rcf) and supernatants were filtered through 0.2-μm pore polyethersulfone (PES) membranes. Culture filtrates were concentrated and proteins exchanged into PBS buffer by stir-cell based ultrafiltration using 10 kDa molecular weight cut off PES membranes (Millipore) or centrifugal devices (Sartorius Stedim Biotech). Culture filtrate protein concentrations were determined by DC Lowry assay using ovalbumin as the protein standard for comparison. Concentrated culture filtrates were treated with 50 mM diethyldithiocarbamate (DDC) for 30 minutes to remove Cu^{++} ions. DDC was removed by dialysis against Tris-buffered saline (TBS) before assaying for superoxide dismutase activity. Cu^{++} ions were replenished by incubation of Cu^{++}-depleted samples with 50 mM CuSO_{4} for 30 minutes and subsequent dialysis against TBS.

Cellular lysates were prepared by collecting yeast cells from liquid 3M cultures by centrifugation (5 minutes at 5000 rcf). Cytosolic material was liberated from yeast cells by beating the yeasts with 400 μm-diameter glass beads in PBS containing a
protease inhibitor cocktail (P2714, Sigma). Cellular lysate protein concentrations were determined by DC Lowry assay using bovine serum albumin as the protein standard.

3.2.4 Superoxide dismutase assay

Detection of superoxide dismutase activity was based on depletion of superoxide as determined by the superoxide-dependent reduction of the water soluble tetrazolium dye WST-1 (Dojindo Molecular Technologies, (213, 214)). Superoxide was generated in vitro from hypoxanthine using xanthine oxidase (X4500, Sigma). Reactions were performed in a solution consisting of 50 mM Tris pH 8.0, 100 μM hypoxanthine, 5 mU/mL xanthine oxidase, and 100 μM WST-1 to which variable amounts of *Histoplasma* samples were added. WST-1 reduction over time at 25°C was monitored by absorbance at 438 nm using a Synergy 2 plate reader (BioTek). The inhibition of WST-1 reduction was determined by subtracting the value of WST-1 reduction in each sample from that of reactions with buffer alone (corresponding to 100% WST-1 reduction). For assays not comparing cell-associated and soluble activities, culture filtrate samples were normalized to 5 μg total protein in a total volume of 200 μL and the kinetics of WST-1 reduction determined. For experiments comparing cell-associated and soluble superoxide dismutase activity, 1x10^8 cells (estimated by OD\textsubscript{600} readings without removal of clumped yeast) were collected by centrifugation and resuspended in a volume of PBS equal to the volume of culture filtrate removed. Soluble superoxide dismutase samples were obtained.
using the volume of culture filtrate equivalent to that of $1 \times 10^8$ *Histoplasma* yeast cells. Cell-free samples were concentrated and buffer-exchanged into PBS by ultrafiltration. Equivalent proportions of cells or culture filtrates were added to the superoxide generating system in a final volume of 400 µL. After 12 minutes with shaking (1000 rpm), samples were centrifuged to remove cells, 200 µL clarified sample recovered and the WST-1 reduction determined by end-point absorbance at 438 nm. The proportion of superoxide dismutase activity in culture filtrate or cell-associated samples was determined as the fraction of total inhibition of WST-1 reduction relative to the respective buffer controls.

**3.2.5 Construction and analysis of strains expressing variant Sod3 proteins**

Nucleotides 1 to 78 of the *SOD3* CDS, encoding the first 26 amino acids of the Sod3 protein, were amplified by PCR and inserted in frame in front of *gfp* with a C-terminal FLAG epitope. Constructs were cloned behind the *Histoplasma H2B* promoter in a *URA5(+)* expression vector (pCR468) to make vector pCR508. Linearized pCR508 and pCR468 plasmids were transformed into *Histoplasma* strain WU8. Culture filtrates and yeast cells were collected from liquid cultures of Ura<sup>+</sup> transformants. 5 µg of total culture filtrate and cellular lysate proteins were screened for the FLAG epitope by immunoblotting with an anti-FLAG antibody (F1804, Sigma) and for α-tubulin with the anti-tubulin antibody (B512, Sigma).
For construction of Sod3 proteins truncated from the C-terminus, a nucleotide sequence encoding the FLAG epitope was inserted before nucleotide 79 of the SOD3 CDS. Downstream SOD3 sequence ended at either nucleotide 615 or 693 of the cDNA. Fragments were amplified and spliced together by PCR. Fusion proteins were directed into the secretory pathway by insertion of sequence encoding the Sod3 signal peptide (nucleotides 1 to 78, encoding the first 26 amino acids) at the 5′ end. Constructs were cloned into the Histoplasma H2B-promoter transgene expression vector (pCR468) to generate plasmids pCR601 and pCR602. Linearized plasmids were transformed into OSU13 and Ura+ transformants assayed for cell-associated and cell-free soluble superoxide dismutase activities. Statistical significance was determined by one-tailed Student’s t-tests. Immunoblotting for the FLAG epitope in culture filtrates was performed by deglycosylating culture filtrate proteins with PNGaseF (NEB) for at least 12 hours prior to separation of proteins by SDS-PAGE and immunoblotting with the anti-FLAG antibody. Immunoblot signals were quantified using ImageJ software (http://rsbweb.nih.gov/ij/).

3.2.6 Determination of susceptibility to superoxide

For determination of superoxide susceptibility, Histoplasma or Candida yeast cells were collected from log-phase liquid cultures and 1x10^5 cells were incubated in a superoxide-generating system consisting of 50 mM Tris pH 8, 100 μM hypoxanthine, and
increasing amounts of xanthine oxidase (X4500; Sigma) in a total volume of 500 μL in wells of a 24-well tissue culture plate. Yeasts were incubated for 4 hours at 37°C with shaking (200 rpm) in a humidified chamber. After incubation, yeasts were removed and serial dilutions plated on solid media to determine viable fungal colony forming units (cfu). For determination of peroxide susceptibility, 1×10^5 yeast cells were incubated in HMM with increasing amounts of hydrogen peroxide (0 to 4 mM). Aliquots were removed at 4 hours and serial dilutions plated on solid media to determine viable cfu. The concentration of the hydrogen peroxide stock solution was determined immediately before use by spectrophotometric absorbance at 240 nm (215). Survival was statistically compared between strains using one-tailed Student’s t-tests.

3.2.7 PMN isolation and infection

PMNs were isolated from human blood samples obtained by venipuncture of healthy volunteers (216, 217). 10 mL venous blood were collected into syringes containing 250 U heparin and diluted back 2-fold in 0.9% saline solution. 10 mL of blood suspension was layered onto 4.5 mL Ficoll-Paque PLUS and PMNs were recovered by density sedimentation (40 minutes at 400 rcf at 18°C). Red pellets were harvested and suspended in 6 mL of 0.9% saline before the addition of 6 mL of 3% Dextran500. Tubes were gently mixed and incubated at 4°C for 20 minutes. The upper layer was transferred to a new tube, and cells were collected by centrifugation (15 minutes at 800 rcf at 4°C).
Residual erythrocytes were lysed by suspending pellets in cold H₂O and gently mixing for 25 seconds before restoring isotonicity by adding an equal volume of 1.8% saline solution. Recovered cells were washed and suspended in Hanks buffered saline solution (without Mg⁺⁺ or Ca⁺⁺) and cells enumerated by hemacytometer. Cell viability was determined using trypan blue. Autologous serum was prepared from separate blood samples following coagulation. PMNs were seeded into 96-well tissue culture plates at 2x10⁵ cells per well in DMEM with 10% autologous serum and cells were allowed to adhere for 20 minutes at 37°C in 5% CO₂/95% air. For infection, 2x10⁴ Histoplasma or Candida yeast cells were added to the PMNs. PMNs and fungal cells were co-cultured at 37°C in 5% CO₂ / 95% air. For inhibition of the NADPH-oxidase, 10 μM diphenylene iodonium (DPI;43088, Sigma) was added to PMNs 20 minutes before infection and maintained throughout the co-culture. At specified times following infection, the culture medium was removed and PMNs lysed by the addition of sterile water and thoroughly scraping wells with a pipette tip. Cell lysates were combined with the removed culture medium and serial dilutions plated to determine viable fungal cfu counts. Yeast survival was compared using one-tailed Student’s t-tests. Parallel cultures of PMNs, with and without DPI, were used to score PMN viability during the course of the assay. PMN viability was determined by exclusion of trypan blue. 100 cells were scored for each of seven fields by brightfield microscopy (for a total of 700 cells). For luminescence assays PMNs were collected and seeded at 2x10⁵ cells per well in 96-well plates. To measure production of ROS, the medium was replaced with luminescence buffer (115 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 2.5 mM CaCl₂, 50 μM luminol, 25 mM
NaHCO₃, 10 mM glucose at pH 7.4) containing 2x10⁵ yeasts. In some assays 10 μM DPI (diphenylene iodinium) was also added to inhibit the NADPH-oxidase. Luminescence of wells was read every 2 minutes at 37°C using a Wallac Victor plate reader.

3.2.8 Macrophage isolation and infection

Unelicited peritoneal macrophages were obtained by peritoneal lavage of C57BL/6 mice (NCI) according to standard procedures (218). Following euthanasia, 10 mL PBS were injected into the peritoneum and the peritoneum gently massaged for 1 minute. Peritoneal cells were recovered by aspiration using a 10 mL syringe with an 18-gauge needle inserted into a small incision. Peritoneal cells were collected from the lavage fluid by centrifugation (10 minutes at 700 rcf at 4°C). Cells were resuspended in DMEM with 10% fetal bovine serum and enumerated by hemacytometer. Macrophages were recovered by adherence to plastic for 12 hours and removal of non-adherent cells. 1x10⁵ macrophages were seeded into each well of a 96-well tissue culture plate. 100 U murine IFNγ and/or 10 U murine TNFα (Biolegend) were added to wells for 24 hours at 37°C in 5% CO₂ / 95% air for activation of macrophages. For infection, 2x10³ Histoplasma or Candida yeast cells were added to the macrophages. Macrophages and yeast cells were co-cultured at 37°C in 5% CO₂ / 95% air. In some wells, 10 μM DPI was added to inhibit the NADPH-oxidase. At incremental times post-infection, the culture medium was removed and macrophages lysed by addition of water. The lysate and
removed culture medium were combined and serial dilutions plated on solid media for enumeration of viable fungal cfu. Yeast survival was compared by one-tailed Student’s t-tests. Parallel cultures of macrophages, with and without DPI, were used to score macrophage viability during the course of the assay. Macrophage viability was determined by exclusion of trypan blue. 100 cells were scored for each of seven fields by brightfield microscopy (for a total of 700 cells). For luminescence experiments macrophages were harvested as described above and 1x10^5 macrophages were added to each well of a 96-well plate. ROS production was determined by replacing the medium with luminescence buffer containing 1x10^5 yeasts. Luminescence of wells was measured every 2 minutes at 37°C.

3.2.9 *In vivo* virulence determination

C57BL/6 mice (NCI or Harlan) were infected with *Histoplasma* yeasts by intranasal delivery of 1x10^4 to 2x10^4 yeast cells. *Histoplasma* yeast cells were collected from exponentially growing liquid cultures and enumerated by hemacytometer. Actual cfu in the inocula were determined by plating serial dilutions. At 4, 8, 12, 16, and 20 days post infection, mice were euthanized and lungs and spleens collected. Lung and spleen tissues were homogenized in 5 mL and 3 mL HMM, respectively, and serial dilutions of the homogenates plated on solid HMM to determine the fungal burden in each organ. For determination of virulence in mice lacking the phagocyte oxidase, p47
knock-out mice (line 4227, Taconic) or isogenic wild-type mice (line C56BL/6N, Taconic) were infected intranasally with 1x10^4 to 2x10^4 Histoplasma yeast cells and tissues harvested at 2, 4, and 8 days post-infection. To prevent bacterial infections during the assay time course, 0.2 mg/mL enrofloxacin (Bactrim) and 0.3 mg/mL tetracycline were added to the drinking water and mice were housed on non-abrasive Alpha-dri bedding (Shepherd Specialty Papers). Mean cfu counts were compared between infections by one-tailed Student’s t-test for statistical significance.

For histology analyses, mice were intranasally infected with 1x10^4 to 2x10^4 yeast cells. After 4 and 8 days, lungs were removed, perforated with a 25-gauge needle, and fixed with 5% formalin in phosphate buffered saline. Lung sections were stained with hematoxylin and eosin and examined and interpreted by a board-certified pathologist (Comparative Pathology and Mouse Phenotyping Facility, Ohio State University College of Veterinary Medicine).

For mouse survival assays, mice were intranasally infected with 7x10^6 Histoplasma yeasts. Mice were monitored daily for survival, weight loss, and symptoms of disease. Mice were determined to be moribund if they demonstrated extreme lethargy, lateral recumbence, or greater than 25% reduction in weight. Statistical analyses were performed using the log-rank test.
3.3 Results

3.3.1 The *Histoplasma SOD3* gene encodes an extracellular superoxide dismutase

Proteomic analysis of the extracellular proteins produced by pathogenic-phase *Histoplasma* cells identified a putative superoxide dismutase (211). The gene encoding this protein was designated *SOD3* to distinguish it from the intracellular Sod proteins, Sod1, Sod2, and Sod4. The *SOD3* locus contains a 771 base pair gene comprised of two exons (GI: EEH08361.1). Previous work demonstrated that the *SOD3* gene is preferentially expressed by *Histoplasma* pathogenic yeast cells compared to expression by non-pathogenic mycelia. Furthermore, *SOD3* is expressed during *in vivo* infection consistent with a role in virulence (211). *In vitro*, the Sod3 protein is produced by yeasts during growth in rich (HMM) and minimal (3M) media at 37°C with 5% CO₂/95% air (data not shown).

To validate the predicted *SOD3* gene and to enable functional analyses of Sod3, we created a mutant strain lacking the *SOD3* gene. A deletion allele was created by replacement of the *SOD3* coding region with a constitutively-expressed hygromycin resistance gene (*hph*). Two kilobases of DNA sequence corresponding to sequence immediately 5’ and 3’ of the *SOD3* gene flanked the *hph* cassette to serve as recombination substrates. The chromosomal locus was replaced with the deletion allele by allelic exchange and the rare recombinants were isolated by successive positive and
negative selections (84). We confirmed deletion of the *SOD3* gene by PCR analysis using primers specific for either the wild-type or the deletion allele (Figure 3.1A). Whereas *SOD3* coding region-specific primers detect a 770 base pair product from the parental strain (*SOD3(+)*) , no *SOD3* coding region is amplified from the *SOD3*-deletion (*sod3Δ*) strain. In contrast, a PCR product corresponding to the *hph*-marker was only amplified from the *sod3Δ* strain as expected for replacement of the wild-type locus with the deletion allele. Amplification of the *RPS15* gene indicates that the genomic DNA used was competent for PCR amplification. The *sod3Δ* strain has identical colony morphology and grows at an equal rate to the *SOD3(+)*) strain in culture, in both HMM and 3M media (data not shown).

Culture filtrate from the *sod3Δ* strain demonstrates that *SOD3* encodes the major extracellular superoxide dismutase activity produced by *Histoplasma* yeasts. To measure superoxide dismutase activity, we used an indirect assay of superoxide anion through superoxide-dependent reduction of the water soluble tetrazolium salt WST-1 (213) Culture filtrate from the *SOD3(+)*) strain substantially decreased the rate of WST-1 reduction, indicating depletion of superoxide by the Sod3-containing culture filtrate. In contrast, inhibition of WST-1 reduction by culture filtrate from the *sod3Δ* strain is nearly identical to the buffer control, indicating very little destruction of superoxide by the *sod3Δ* culture filtrate. Expression of the predicted *SOD3* genomic DNA in the *sod3Δ* mutant (*sod3Δ/SOD3*) fully complements the loss of superoxide dismutase activity in *sod3Δ* culture filtrate. These data indicate that *SOD3(+)*)
Figure 3.1 *Histoplasma* Sod3 encodes an extracellular Cu++-dependent superoxide dismutase. (A) PCR validation of deletion of the SOD3 gene. Genomic DNA from the SOD3(+) parental strain (wu8) and from the sod3Δ strain (OSU13) were tested by PCR for the ribosomal subunit gene RPS15, the wild-type SOD3 gene, and the mutant allele marked with the hygromycin resistance gene (hph). (B) Superoxide dismutase activity in culture filtrates harvested from SOD3(+) (OSU45), sod3Δ (OSU15), and the sod3Δ/SOD3 complemented (OSU49) strains. Detection of superoxide was determined through superoxide-dependent reduction of the WST-1 tetrazolium dye after generation of superoxide using hypoxanthine and xanthine oxidase. Reduction of WST-1 was monitored by absorbance at 438 nm. Buffer or culture filtrates contained 5 μg ovalbumin or total culture filtrate protein, respectively. Asterisks represent significant difference (*** p<0.001) in the inhibition of WST-1 reduction between SOD3(+) and sod3Δ culture filtrates. Data shown representative of three independent experiments each performed with triplicate samples. (C) Sod3 activity following Cu++ depletion. Culture filtrates containing 5 μg total protein from SOD3(+) (OSU45) and sod3Δ (OSU15) strains were tested for their ability to inhibit WST-1 reduction by superoxide before (no chelator), after Cu++ depletion (+DDC), and after subsequent repletion with 50 mM Cu++ (+CuSO₄). Values represent relative inhibition of WST-1 reduction by culture filtrate samples (n=3) compared to buffer controls treated in parallel. Asterisks represent significant differences from SOD3(+) culture filtrates (*p<0.05, **p<0.01).
Figure 3.1
Histoplasma yeasts produce soluble extracellular superoxide dismutase activity and that the SOD3 gene product is responsible for the majority of this superoxide dismutase activity.

Bioinformatic analyses suggested Sod3 is more similar to Cu/Zn Sod proteins than to Fe/Mn-type enzymes and we functionally confirmed this by testing Sod3-dependent superoxide dismutation following copper depletion. Culture filtrates from Sod3-producing and Sod3-deficient strains were treated with 50 mM diethyldithiocarbamate (DDC), a copper chelator, to remove copper ions from Sod3 and the resultant culture filtrates tested for superoxide dismutase activity (Figure 3.1C). Without DDC treatment, culture filtrate from SOD3(+) yeasts inhibits WST-1 reduction by nearly 60% compared to the buffer control due to superoxide dismutase activity. However, DDC treatment effectively negates the inhibition caused by culture filtrate from the SOD3(+) strain, indicating loss of superoxide dismutase activity. DDC treatment of culture filtrate from the SOD3(+) strain is similar to untreated culture filtrate from the sod3Δ mutant, consistent with loss of Sod3 activity due to Cu²⁺ chelation (Figure 3.1C). Culture filtrate from the sod3Δ mutant similarly lacks dismutase activity after treatment with DDC. Replenishing Cu²⁺ ions to DDC-treated SOD3(+) culture filtrate largely restores superoxide dismutase activity (44% inhibition of WST-1 reduction compared to 57% inhibition with untreated SOD3(+) culture filtrate). These results demonstrate that Sod3 depends on copper for enzymatic activity and classifies it as a Cu/Zn-type superoxide dismutase.
3.3.2 Sod3 is both secreted from and associated with yeast cells

Alignment of Sod3 protein sequences derived from the sequenced *Histoplasma* genomes (G186A, G217B, and NAm1) highlights regions of high conservation at the N- and C-termini (Figure 3.2A). The N-terminus of Sod3 has a predicted signal peptide (amino acids 1-21) that potentially directs the protein into the canonical eukaryotic secretion pathway consistent with Sod3’s localization to the extracellular environment (211). In contrast to the variable sequence identity found throughout the central region of the protein among *Histoplasma* Sod3 orthologs (which includes the core domain shared among enzymes with superoxide dismutase catalytic activity), the C-terminal 24 amino acids are 100% identical suggesting conservation of an important function beyond superoxide dismutase activity. Further inspection of the C-terminus identifies a potential glycophosphatidyl inositol (GPI) attachment signal with a putative omega site at residue 205. GPI signals characterize a number of *Saccharomyces cerevisiae* and *Candida albicans* cell wall proteins in which the GPI anchor is thought to be subsequently cleaved and the protein covalently attached to the cell wall (219, 220). These two motifs potentially direct secretion of Sod3 from yeast cells and may provide for its association with the yeast cell wall in addition to its observed presence in the soluble culture filtrate.

To determine if a portion of Sod3 is associated with the *Histoplasma* yeast cell wall, yeast cells and their corresponding culture filtrates were tested for superoxide dismutase activity. Addition of cells and culture filtrates to the superoxide
Figure 3.2 N-terminal and C-terminal signals direct extracellular localization of Sod3. (A) Schematic of the Sod3 protein highlighting the predicted signal peptide (SP) and the glycosylphosphatidylinositol anchor (GPI) signal motifs. Numbers represent amino acid residues in the Sod3 protein. Shading beneath the Sod3 protein indicates amino acid sequence similarity between G186A, G217B and Nam1 Sod3 proteins ranging from dark (>90% sequence identity) to light (<50% identity). (B) Relative Sod3 activity associated with the yeast cell and soluble extracellular fraction. Superoxide dismutase activities were determined by inhibition of superoxide-dependent WST-1 reduction in the presence of 1x10^8 yeast (cell-associated) or the corresponding culture filtrate (soluble) of SOD3(+) (OSU45) and sod3∆ (OSU15) strains (n=3, each). Inhibition of WST-1 reduction was normalized to reactions in the absence of yeast or culture filtrate. Asterisks represent significant differences (p<0.001) from SOD3(+) samples. (C) Determination of the localization of GFP when fused to the N-terminus of Sod3. Extracellular or intracellular GFP localization was determined by α-FLAG immunoblot of culture filtrates or cellular lysates from Histoplasma yeast strains expressing FLAG epitope-tagged GFP (GFP:FLAG; OSU88) or GFP with the first 26 amino acids of Sod3 (Sod3_26:GFP:FLAG; OSU102). Cellular lysates were also tested for α-tubulin to demonstrate equal loadings. (D) Localization of Sod3 activity after removal of the C-terminal 26 amino acids. Cell-associated and soluble superoxide dismutase activities of SOD3(+) (OSU45), sod3∆ (OSU15), and yeast expressing full length Sod3 (sod3∆/FLAG; OSU116) or Sod3 lacking the putative GPI signal (sod3∆/FLAG:SOD3∆GPI; OSU117). Results were normalized to the uninhibited reactions and plotted as the proportion of total
inhibitory activity. Asterisks represent significant difference from full length Sod3 (** p<0.01, *** p<0.001). Relative quantitation of Sod3 in culture filtrates was determined by α-FLAG immunoblot and is indicated by numbers below.
Figure 3.2

A

<table>
<thead>
<tr>
<th>1</th>
<th>21</th>
<th>205</th>
<th>231</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP</td>
<td>Sod3</td>
<td>GPI</td>
<td></td>
</tr>
</tbody>
</table>

B

![Bar graph showing inhibition of WST-1 reduction.]

D

![Bar graph showing inhibition of WST-1 reduction.]

C

![Western blot images comparing GFP::FLAG and Sod3::GFP::FLAG protein levels in culture filtrate and cellular lysate.]

- **culture filtrate**
  - GFP::FLAG
  - α-FLAG
  - α-tubulin
- **cellular lysate**
  - GFP::FLAG
  - α-FLAG
  - α-tubulin
generating/WST-1 system showed that both culture filtrates and cells prevent superoxide-dependent reduction of WST-1 (Figure 3.2B). Compared to the buffer control, cell-associated Sod activity inhibits WST-1 reduction by 61%. A comparable amount of culture filtrate inhibits WST-1 reduction by 60%. Thus, approximately 50% of the total extracellular Sod activity is associated with the cell and 50% is found in the soluble fraction. Sod activity associated with sod3Δ cells and their culture filtrates inhibit WST-1 reduction by 26% and 27%, respectively, indicating that the majority of the cell-associated and cell-free Sod activity is attributable to cell-associated and cell-free Sod3 protein.

To determine if the putative N-terminal signal peptide of Sod3 functions as a secretion signal, we tested whether the N-terminus could direct secretion of a normally cytosolic protein. Sequence encoding the first 26 amino acids of Sod3 was fused to a gfp coding sequence which had a C-terminal FLAG epitope to allow monitoring of the GFP protein localization by immunoblotting. Expression of gfp lacking the N-terminal Sod3 residues causes GFP accumulation in the cytosol of yeast cells as determined by fluorescence microscopy (data not shown) and by immunoblotting of cellular lysates (Figure 3.2C). On the other hand, GFP protein possessing the Sod3 N-terminal residues is secreted into the culture filtrate and is effectively absent from the cytosolic fraction (Figure 3.2C). These results demonstrate that the Sod3 N-terminal residues encode a signal peptide that is sufficient to direct protein secretion from yeast cells.
As yeast cells have significant Sod3 activity, we examined the role of the putative GPI signal in mediating cell-association of Sod3. To track the localization of Sod3, sequence encoding the FLAG epitope was inserted into the \textit{SOD3} cDNA at nucleotide 79 of the coding sequence thereby preserving the Sod3 signal peptide for secretion. Downstream of the FLAG epitope, the \textit{SOD3} sequence encoded either Sod3 with the putative GPI attachment signal (nucleotides 79-693 encoding amino acids 27-231) or Sod3 without the GPI signal (nucleotides 79-615 encoding amino acids 27-205). Each construct was transformed into \textit{sod3}\textit{Δ} yeasts. To determine if deletion of the putative GPI attachment signal reduced cell-association, yeast cells and their corresponding culture filtrates were assayed for their ability to inhibit WST-1 reduction corresponding to superoxide dismutase activity. Expression of FLAG-tagged Sod3 protein yields both cell-free and cell-associated superoxide dismutase activity similar to the ratio observed for \textit{SOD3}(+) yeast cells (Figure 2D). Deletion of the GPI signal from Sod3 significantly decreases cell-associated Sod activity, approaching background levels of \textit{sod3}\textit{Δ} mutant yeasts (18% and 12% inhibition of WST-1 reduction, respectively). This is consistent with diminished retention of cell-associated Sod3 when the GPI signal is removed.

From this data alone, the possibility that loss of the C-terminal GPI signal causes misfolding of Sod3, either resulting in the protein being degraded within the cell or being transported to the extracellular fraction in an inactive form, cannot be ruled out. To address this, we examined the superoxide dismutase activity in matching culture filtrates from these yeast cells. We found that the decrease in cell-associated Sod3 is accompanied
by an increase in soluble Sod3 activity in the culture filtrate (54-57% inhibition of WST-1 reduction compared to 26-27% inhibition by GPI signal-containing Sod3). As further evidence of the redirection of Sod3 from the cell surface to the soluble fraction in the absence of the GPI signal, immunoblotting of culture filtrates shows approximately 2.4-fold more Sod3 protein is released into the culture filtrate without the GPI signal than when the GPI signal is present (Figure 3.2D). Attempts to directly measure cell-associated Sod3 protein by immunoblotting failed despite treatment of the cell wall fraction with reductants (dithiothreitol), ionic detergent (SDS), or glycanases (zymolyase and chitinase) and combinations of each to release soluble Sod3 from insoluble cell wall material (data not shown). Nonetheless, depletion of cell-associated Sod3 activity and the corresponding increase in Sod3 in the culture filtrate when the GPI signal is removed indicates the GPI signal promotes cell-association of a portion of the total secreted Sod3.

3.3.3 Sod3 protects specifically against extracellular reactive oxygen

The extracellular localization of Sod3 predicts Sod3 is produced to combat exogenous but not intracellular superoxide. Indeed, *Histoplasma* yeasts express additional Sod proteins homologous to intracellular superoxide dismutase enzymes and these are more appropriately positioned to detoxify endogenous superoxide such as would be generated from aerobic respiration. The *Histoplasma SOD1* gene encodes a protein homologous to Cu/Zn superoxide dismutase enzymes commonly found in the cytosol of eukaryotes (211). To determine the specificity of Sod3 and Sod1 for
extracellular and intracellular superoxide, respectively, we compared the ability of strains
deficient for Sod3 and strains depleted of Sod1 for their ability to survive intracellular
superoxide stress. To enable these tests, we depleted Sod1 activity from *Histoplasma*
yeasts by RNA interference (RNAi). Depletion of Sod1 function in a GFP-fluorescent
RNAi sentinel strain (81) was initially determined by reduced GFP fluorescence caused
by co-targeting *gfp* and *SOD1* sequence (Figure 3.3A). Two independent RNAi lines
were created in which the fluorescence of the GFP sentinel is reduced 47%-54%, which
is indicative of knock-down of Sod1 function. To confirm depletion of Sod1 function,
yeast cellular lysates were tested for superoxide dismutase activity. While deletion of the
*SOD3* locus removes extracellular superoxide dismutase activity (Figure 3.1B), deletion
of *SOD3* does not reduce cytosolic superoxide dismutase activity compared to isogenic
*SOD3(+) yeasts (51% and 50% inhibition of WST-1 reduction, respectively; Figure
3.3A). However, depletion of Sod1 activity by RNAi diminishes cytosolic superoxide
dismutase activity compared to the isogenic *gfp*-RNAi and parental *gfp(+) strains (21%
and 13% compared to 46% and 45% inhibition of WST-1 reduction, respectively). Thus,
loss of Sod3 specifically affects extracellular but not cytosolic superoxide dismutase
activity. Conversely, depletion of Sod1 specifically reduces intracellular superoxide
dismutase activity.

Sod1-depleted and Sod3-deficient strains were tested for their sensitivities to
intracellular superoxide to ascertain their roles in protecting against intracellular
superoxide stress. Yeast cells were grown in liquid culture with increasing amounts of
Figure 3.3 *Histoplasma Sod3 does not alleviate intracellular oxidative stress* (A)

Depletion of intracellular superoxide dismutase activity by SOD1-RNAi but not by loss of Sod3. RNAi-based depletion of Sod1 was done in a GFP-expressing *Histoplasma* strain (OSU22). GFP fluorescence is shown in colony images of a strain in which gfp was not targeted (gfp(+); OSU103), gfp alone was targeted (gfp-RNAi; OSU104) or two independent isolates in which gfp and SOD1 were co-targeted (gfp:SOD1-RNAi; OSU105). Numbers below the images indicate relative GFP fluorescence. Intracellular superoxide dismutase activity was determined by inhibition of WST-1 reduction using 5 μg of cellular lysate protein from SOD3(+) (OSU45), sod3Δ (OSU15), and the RNAi strains and results plotted relative to uninhibited reactions using 5 μg BSA. Non-significant (ns) and significant (*** p<0.001) differences between SOD3(+) and sod3Δ or between the SOD1(+) strain (gfp(+)) and the gfp-RNAi or SOD1-RNAi strain are indicated above the columns. (B-D) Inhibition of yeast growth by increased intracellular reactive oxygen. Liquid growth of SOD3(+) (OSU45), sod3Δ (OSU15), SOD-proficient (gfp-RNAi; OSU104), and SOD1-RNAi (gfp:SOD1-RNAi; OSU105) strains was determined by optical density of cultures measured at 595 nm. Intracellular reactive oxygen was increased in yeast by addition of 5 μM (C) or 10 μM (D) paraquat. Growth curve points represent the mean of optical density of replicate cultures (n=3).
Figure 3.3

A

B

0 µM paraquat

C

5 µM paraquat

D

10 µM paraquat
paraquat, which interacts with intracellular redox systems and the mitochondrial respiratory chain to cause increased formation of superoxide (221, 222). Paraquat treatment of yeast cells causes a dose-dependent decrease in growth as measured by the optical density of yeast cells, indicating paraquat is detrimental to *Histoplasma* cells (Figure 3.3B-D). *Histoplasma* yeasts are susceptible to paraquat at concentrations of 5 \( \mu \text{M} \) and greater, and yeast cells lacking Sod3 show no enhanced sensitivity compared to yeasts producing Sod3 (Figure 3.3C-D). In contrast, yeast cells depleted of Sod1 show greater sensitivity to paraquat than isogenic cells expressing normal amounts of Sod1. Thus, Sod1 contributes to alleviation of intracellular superoxide stress, but Sod3 does not mitigate intracellular superoxide. These results are consistent with Sod3 specifically functioning in combating exogenous oxidative stress.

To test the role of Sod3 in protecting *Histoplasma* from exogenous superoxide, yeast cells were challenged *in vitro* with superoxide. To generate superoxide *in vitro*, graded amounts of xanthine oxidase were added to yeast suspensions in Tris buffer with excess hypoxanthine; the amount of superoxide proportionally increases with the concentration of xanthine oxidase enzyme (Figure 3.4). Parental *SOD3(+)* yeasts largely survive the *in vitro* superoxide challenge (Figure 3.5A). However, yeasts lacking Sod3 are unable to survive superoxide challenge; only 23-35\% of *sod3Δ* yeasts survive challenge with superoxide levels at which the Sod3-producing yeasts exhibit greater than 95\% survival. Challenge of yeasts with the highest level of superoxide that kills roughly 20\% of *SOD3(+)* yeasts, kills over 85\% of the *sod3Δ* mutant yeasts. Complementation of
Figure 3.4 Increasing amounts of xanthin oxidase increase the amount of superoxide produced. (A) Production of superoxide by increasing amounts of xanthine oxidase (XOD) with hypoxanthine. A two-fold dilution series of xanthine oxidase was added to 100 mM hypoxanthine and the superoxide produced was detected by reduction of WST-1. WST-1 reduction was monitored over time by absorbance at 438 nm. Data points represent the mean value of triplicate samples. (B) Linear relationship between the amount of xanthine oxidase and the superoxide produced. The amount of WST-1 reduced by superoxide in 8 minutes was determined for each concentration of xanthine oxidase tested. A linear curve was fit to the line (R-squared value=0.988).
Figure 3.4

(A) WST-1 reduction (Abs 438 nm) over time (min) for different concentrations of XOD:
- 20 mU/mL XOD
- 10 mU/mL XOD
- 5 mU/mL XOD
- 2.5 mU/mL XOD
- 0 mU/mL XOD

(B) Linear relationship between WST-1 reduction (Abs 438 nm) and [Xanthine oxidase] (mU/mL) with $R^2 = 0.988$. 
Figure 3.5 Sod3 protects *Histoplasma* yeast cells from exogenous superoxide *in vitro*. (A) Survival of yeast cells following challenge with superoxide. Yeast were incubated in increasing amounts of superoxide generated by addition of increasing amounts of xanthine oxidase to hypoxanthine. SOD3(+) (OSU45), sod3Δ (OSU15), sod3Δ/SOD3 (OSU49), and Candida albicans yeast were incubated for 4 hours at 37°C after which viable colony forming units (cfu) were determined. Results are plotted as relative yeast survival compared to viable cfu of yeast incubated in the absence of superoxide (0 mU/ml xanthine oxidase). Results represent the mean ± standard deviations from 3 replicate challenges per strain. Asterisks indicate significant differences (** p<0.002, *** p<0.001) from the SOD3(+) strain. (B) Sensitivity of *Histoplasma* yeast to hydrogen peroxide. Increasing amounts of hydrogen peroxide were added to *Histoplasma* yeast (n=3 for each strain) at 37°C and the viability of yeast after 4 hours was determined by enumeration of viable cfu. Results are plotted as relative yeast survival compared to viable cfu of yeast incubated in the absence of peroxide (0 mM hydrogen peroxide). Data is representative of 3 independent experiments.
Figure 3.5

A

![Graph showing yeast survival against xanthine oxidase concentration](image)

B

![Graph showing yeast survival against H₂O₂ concentration](image)
the sod3Δ mutant restores protection against superoxide challenge to SOD3(+) levels. Interestingly, the impaired survival of Sod3-deficient Histoplasma yeasts resembles the degree of killing of Candida albicans yeasts by superoxide (Figure 3.5A). These results demonstrate that Sod3 protects Histoplasma yeasts from exogenous superoxide.

Superoxide is only one reactive oxygen species with potentially lethal effects on Histoplasma yeasts. Spontaneous dismutation of the superoxide that is generated by the xanthine oxidase/hypoxanthine system could also expose yeasts to peroxide stress that could affect yeast cell viability. To determine if peroxide contributes to the enhanced superoxide killing of sod3Δ cells, yeasts were challenged in vitro with different concentrations of hydrogen peroxide (0 mM to 4 mM). Increasing the concentration of hydrogen peroxide kills an increasing proportion of Histoplasma yeasts (Figure 3.5B). However, Sod3-expressing and Sod3-deficient yeast have very similar susceptibilities to hydrogen peroxide. These results indicate that peroxide, a potential reactive oxygen derivative of superoxide, does not significantly contribute to the increased killing of sod3Δ mutant yeast cells during challenge with superoxide.

3.3.4 Sod3 protects Histoplasma yeast cells from phagocyte-derived reactive oxygen

The primary source of exogenous reactive oxygen encountered by Histoplasma during infection is that produced by host phagocytic cells. Of these cells, PMNs are notorious for
their strong oxidative burst in response to pathogens. To test if Sod3 defends *Histoplasma* yeasts from killing by PMNs, *SOD3(+) and sod3Δ* mutant yeasts were co-incubated with human PMNs. *SOD3(+) yeasts largely survive the encounter with PMNs exhibiting 94% and 81% viability after 2 hours and 4 hours, respectively (Figure 3.6A). Without Sod3, *Histoplasma* yeast cells are efficiently killed by PMNs; only 50% of the *sod3Δ* yeast cells remain viable after 2 hours and viability drops to 31% by 4 hours. As was observed with *in vitro* sensitivity to superoxide challenge, PMN killing of *sod3Δ* yeast cells mirrors the susceptibility of *Candida* to PMN killing (Figure 3.6A).

The fungicidal effect on Sod3-deficient yeasts depends on PMN production of reactive oxygen. Suppression of the NADPH-oxidase complex by treatment of PMNs with 10 μM diphenylene iodinium (DPI;[223]) substantially reduces PMN killing of *Histoplasma* and *Candida* yeast cells (Figure 3.6B). Survival of *sod3Δ* yeasts when co-incubated with PMNs improves from 26% to 89% when the NADPH-oxidase is inhibited, restoring yeast viability to that of Sod3-producing *Histoplasma* yeasts. Impairing NADPH-oxidase function similarly enhances *Candida* survival (Figure 3.6B). DPI itself has no detrimental effects on yeast cells (Figure 3.6B) nor does DPI impair PMN viability (Figure 3.7A,[224]).

The protective effects of DPI or Sod3 on *Histoplasma* viability derive from their ability to decrease toxic reactive oxygen molecules, either by preventing superoxide
Figure 3.6 Sod3 protects *Histoplasma* yeast from PMN-derived reactive oxygen. (A) Survival of yeast after infection of human PMNs. SOD3(+) (OSU45), sod3Δ (OSU15) and Candida albicans yeast were added to PMNs at a multiplicity of infection (MOI) of 1:10. Yeast survival was determined by enumeration of viable cfu after 2 and 4 hours of co-incubation of yeast with PMNs at 37°C. Results are plotted as relative yeast survival (mean ± standard deviation of 3 replicates) compared to viable cfu of yeast incubated in the absence of PMNs. Significantly decreased survival compared to SOD3(+) yeast is indicated by asterisks (**p<0.001). (B) Inhibition of yeast killing by PMNs upon inactivation of the NADPH-oxidase. Yeast were added to PMNs (+PMNs) and incubated for 4 hours at 37°C and viable cfu were determined. 10 μM dipheylene dinum (DPI) s added to some assays to inactivate the NADPH-oxidase. Results indicate relative yeast survival (mean ± standard deviation of 3 replicates) compared to viable cfu of yeast incubated in the absence of PMNs (no PMNs). Significant (** p<0.001) or non-significant (ns) reduction in survival compared to yeast in the absence of PMNs is indicated above the respective columns. (C) Reactive oxygen production by PMNs in response to *Histoplasma* yeast. *Histoplasma* yeast were added to PMNs at an MOI 1:1 in the presence of the lumino ROS-detection reagent and the luminol luminescence measured over time. PMNs and yeast were co-incubated in the presence (open symbols) or absence (closed symbols) of 10 μM DPI to inhibit the NADPH-oxidase. Data points represent the mean luminescence (n=3).
Figure 3.7 Treatment with DPI does not affect phagocyte viability. Human PMN (A) and murine peritoneal macrophage (B) viability following sustained treatment with 10 μM diphenylene iodonium (DPI). Relative phagocyte viability was determined by microscopy of cells plated in chambered slides as cytosolic exclusion of trypan blue (n=700 cells scored as 7 objective fields with 100 cells per field). Time points monitored correspond to the times of initial addition of DPI (-20 minutes), time of addition of yeast (0 minutes), and time points post-infection (60 minutes and 240 minutes). Error bars represent standard deviations. No significant differences between DPI-treated and untreated cell viability were found at any time point (p>0.05).
Figure 3.7

A

PMN viability

no inhibitor
10 μM DPI

0%
20%
40%
60%
80%
100%

-20 0 60 240

time (minutes)

B

Macrophage viability

no inhibitor
10 μM DPI

0%
20%
40%
60%
80%
100%

-20 0 60 240

time (minutes)
production or by dismuting PMN-produced superoxide, respectively. To demonstrate this, we measured reactive oxygen levels produced during co-incubation of yeasts and PMNs with luminol (Figure 3.6C). In the absence of yeast cells, highly reactive PMNs gradually produce reactive oxygen species as indicated by the slow rate of increasing luminol luminescence. Addition of both $SOD3(+)$ and $sod3\Delta$ *Histoplasma* cells stimulates rapid production of reactive oxygen by the PMNs, confirming PMNs react to *Histoplasma* yeasts. While reactive oxygen species persist when $sod3\Delta$ yeast cells are added, the reactive oxygen quickly disappears in the presence of yeast cells producing Sod3. Addition of DPI abrogates the luminol luminescence triggered by both $SOD3(+)$ and $sod3\Delta$ strains, consistent with its ability to inhibit the NADPH-oxidase. Together these data show that although yeast interaction with PMNs triggers production of fungicidal reactive oxygen, *Histoplasma* Sod3 eradicates the superoxide generated thereby providing for increased yeast survival.

In addition to encountering PMNs, *Histoplasma* also infects macrophages during infection of the host. To explore the role of Sod3 in this interaction, we measured the survival of *Histoplasma* yeast cells following infection of both resting and activated macrophages. 80% and 93% of $SOD3(+)\,$ yeasts remain viable after infection of resting macrophages at 2 and 4 hours (Figure 3.8A). The absence of Sod3 results in a small but significant decrease in $sod3\Delta$ yeast viability compared to $SOD3(+)\,$ yeasts consistent with minimal production of reactive oxygen by resting macrophages in response to *Histoplasma*. When macrophages are activated with IFN\(\gamma\) and TNF\(\alpha\) to
Figure 3.8 Sod3 Histoplasma yeast from ROS produced by activated macrophages.

(A-B) Survival of yeast after infection of resting (A) or cytokine-activated (B) murine macrophages. SOD3(+) (OSU45), sod3Δ (OSU15) and Candida albicans yeast were added to resident peritoneal macrophages at an MOI of 1:50. Yeast survival was determined by enumeration of viable cfu after 2 and 4 hours of co-incubation of yeast with macrophages at 37°C. In (B), 10 U TNFα and 100 U IFNγ were added to macrophages 24 hours prior to infection to enhance ROS production. Results are plotted as relative yeast survival (mean ± standard deviation of 3 replicates) compared to viable cfu of yeast incubated in the absence of macrophages. Significantly decreased survival compared to SOD3(+) yeast is indicated by asterisks (* p<0.05, ** p<0.001, *** p<0.001). (C) Prevention of yeast killing by macrophages after inhibition of the NADPH-oxidase. Yeast were added to resting and to IFNγ/TNFα-activated macrophages and incubated for 4 hours at 37°C in the absence or presence of 10 μM diphenylene iodinium (DPI) and viable cfu were determined. Results indicate relative yeast survival (mean± standard deviation of 3 replicates) compared to viable cfu of yeast incubated in the absence of macrophages. Significant (** p<0.01) or non-significant (ns) reduction in survival compared to yeast in the absence of macrophages is indicated above the respective columns. (D) Reactive oxygen production by activated macrophages in response to Histoplasma yeast. Histoplasma yeast were added to resting or activated macrophages at an MOI of 1:1 in the presence of the luminol ROS-detection reagent and the luminol luminescence measured over time. Macrophages and yeast were co-incubated
in the presence (open symbols) or absence (closed symbols) of 10 μM DPI to inhibit the NADPH-oxidase. Data points represent the mean luminescence (n=3).
Figure 3.8
enhance production of reactive oxygen species, \textit{SOD3(+)} \textit{Histoplasma} yeasts continue to survive (Figure 3.8B). However, the viability of \textit{sod3}\textsuperscript{Δ} yeasts in these activated macrophages is considerably reduced with only 62\% and 41\% surviving at 2 and 4 hours. Macrophages treated with IFN\textgreek{g}, but not TNF\textgreek{a}, similarly demonstrate anti-\textit{Histoplasma} activity, but the degree of killing is enhanced by the presence of both cytokines (data not shown). Interaction of \textit{Candida} yeasts with either resting or activated macrophages decreases \textit{Candida} viability (Figure 3.8A and 6B).

Since production of superoxide is not the only antimicrobial mechanism in the macrophage arsenal, we tested survival of yeast cells in macrophages that are unable to produce superoxide. Inhibition of the NADPH-oxidase complex with DPI prevents killing of \textit{Histoplasma} \textit{sod3}\textsuperscript{Δ} and \textit{Candida} yeasts by activated macrophages, indicating that the majority of the macrophage fungicidal activity at 4 hours post-infection requires the production of reactive oxygen compounds (Figure 3.8C). As with PMNs, treatment of phagocytes with 10 \(\mu\)M DPI does not impair host cell viability (Figure 3.7B). As independent evidence that the outcome of the interaction between yeasts and macrophages involves oxidative killing, we monitored the macrophage oxidative burst during infection with \textit{Histoplasma} yeasts using luminol (Figure 3.8D). In the absence of fungi, both resting and activated macrophages produce little reactive oxygen. When infected with \textit{Histoplasma} yeasts, resting macrophages produce negligible reactive oxygen, indicating yeasts do not stimulate an oxidative burst in these cells (data not shown). This is not the case with
activated macrophages where *Histoplasma* yeasts trigger an initial burst of reactive oxygen within 5 minutes that peaks 10-20 minutes after addition of yeasts (Figure 3.8D). Considerably less reactive oxygen is detected (65% decrease) if the infecting yeasts produce Sod3 than if the yeasts lack Sod3, consistent with Sod3 destroying superoxide. Thus, activated macrophages produce reactive oxygen species in response to *Histoplasma* yeasts, but yeast-generated Sod3 destroys these reactive compounds and this ROS diminution correlates with enhanced survival of *Histoplasma* in phagocytes.

### 3.3.5 Sod3 promotes *Histoplasma* virulence *in vivo*

To determine the contribution of Sod3 to *Histoplasma* virulence *in vivo*, we measured the ability of yeasts lacking Sod3 function to infect murine tissues. As indicators of respiratory and systemic disease, the fungal burden was determined in lung and spleen tissues, respectively, following intranasal delivery of a sublethal inoculum. *SOD3(+) yeasts infect and replicate within the lung tissue, increasing the fungal burden 40-fold through day 8. The onset of cell-mediated immunity begins to clear *SOD3(+) yeasts from the lung after day 12 (Figure 3.9A). With *SOD3(+) Histoplasma* yeasts, dissemination to the spleen occurs by day 4 and rapidly increases in this tissue (Figure 3.9B). As observed in the lung, the fungal burden begins to clear in the spleen after day 12. In contrast to *SOD3(+) yeasts, the population of sod3Δ yeasts initially decreases within the lung through day 4 without the protective function of Sod3 (Figure 3.9A).
Figure 3.9 *Histoplasma* virulence in vivo requires Sod3. (A) Kinetics of sublethal lung infection by *Histoplasma*. Wild-type C57BL/6 mice were intranasally infected with approximately $1 \times 10^4$ SOD3(+) (OSU45), sod3Δ (OSU15), or sod3Δ/SOD3 (OSU49) *Histoplasma* yeast. At 4 day intervals post-infection, the fungal burden in lungs was determined by quantitative plates for *Histoplasma* cfu. (B) Kinetics of dissemination following lung infection with *Histoplasma*. At each time point, organs were harvested and the fungal burden in spleen tissue was determine by quantitative platings for cfu. In (A) and (B), each data point represents cfu counts per organ from an individual animal (n=5 per time point) and horizontal bars represent the mean fungal burden. Asterisks indicate significant differences at each time point from animals infected with SOD3(+) organisms (* p<0.05, ** p<0.01, *** p<0.001). The actual inoculum dose is shown in graphs at day 0. The limit of detection is 100 cfu for lungs and 60 cfu for spleen tissue. (C) Inflammation and pathology of lung tissue following *Histoplasma* infection. Wild-type C57BL/6 mice were infected with SOD3(+) eosin. Arrowheads indicate detectable clusters of yeast cells. Scale bars represent 50 μm.
Figure 3.9

A

SOD3(+)

sod3Δ

sod3Δ/SOD3

cfu / lung

days post infection

B

SOD3(+)

sod3Δ

sod3Δ/SOD3

cfu / spleen

days post infection

C

SOD3(+)

sod3Δ

sod3Δ/SOD3

Histological images
Although there is some expansion of $sod3\Delta$ yeasts between day 4 and day 8, the number of mutant yeasts barely increases above the inoculum level and then shows rapid clearance starting at day 12. Dissemination of $sod3\Delta$ yeasts from the lung to the spleen is nearly undetectable, most likely as a consequence of the substantially diminished number of $sod3\Delta$ yeasts in the lung (Figure 3.9B). Complementation of the $sod3\Delta$ mutant by expression of $SOD3$ genomic DNA, restores the ability of yeasts to survive and replicate in the lung (Figure 7A), as well as dissemination to the spleen (Figure 3.9B).

Lung tissue pathology caused by *Histoplasma* infection correlates with lung colonization by Sod3-expressing and Sod3-deficient strains. At 4 days post-infection, lesions are more pronounced in lungs infected with $SOD3(+)\,$ yeasts than with $sod3\Delta\,$ yeasts (Figure 3.9C and Figure 3.10A). In $SOD3(+)\,$-infected lungs, more inflammatory foci are present (1-13 per section) with thick collars of inflammatory cells composed of PMNs with fewer alveolar macrophages and lymphocytes (Figure 3.9C). Yeasts are associated with the inflammatory foci and their numbers correlate with inflammation severity. In contrast, inflammatory foci are rare (0-2 per section) with sparse cellular infiltrates in $sod3\Delta$-infected lungs (Figure 3.9C). Sod3- deficient yeasts are rarely observed consistent with their clearance by the influx of PMNs. Those surviving yeasts that are present are presumably within macrophages. By 8 days post-infection, when $SOD3(+)\,$ fungal burdens are approaching their maximal level, thick collars of inflammatory cells (primarily macrophages and lymphocytes with fewer neutrophils) surround most blood vessels and/or bronchioles (Figure 3.10B). Interstitial myxedema
Figure 3.10 Lungs infected with Sod3-deficient *Histoplasma* yeast have reduced inflammation. Histology of murine lung sections after infection with *Histoplasma* yeast. Wild-type C57BL/6 mice were intranasally infected with approximately $1 \times 10^4$ *SOD3(+)* (OSU45), *sod3Δ* (OSU15) or *sodΔ/SOD3* (OSU49) *Histoplasma* yeasts. At 4 days (A) or 8 days (B) post-infection, lungs were removed, fixed in 5% formalin, and sections stained with hematoxylin and eosin. Representative images are shown. Scale bars represent 1 mm.
and congestion is present with inflammation extending into the parenchyma. In sod3Δ-infected lungs at 8 days, fewer and less dense inflammatory foci are present. Thus, inflammation severity and tissue pathology, as indicators of disease, closely parallel the fungal burden established by Sod3-expressing and Sod3-deficient yeasts in vivo.

To determine if establishment of lethal histoplasmosis requires Sod3, mice were inoculated with a lethal dose of Histoplasma SOD3(+) and sod3Δ yeasts. Mice infected with SOD3(+) and Sod3-complemented (sod3Δ/SOD3) strains have a median survival time of 5.5 and 5 days, respectively (Figure 3.11). In contrast, nearly all mice infected with the sod3Δ strain survive through two weeks (Figure 3.11) and appear to have fully recovered from the high inoculum as they show no adverse symptoms and have regained or surpassed their initial body weight (data not shown). Thus, the protective effects of Sod3 are required for Histoplasma to establish both lethal and sublethal infections in vivo. The differing fungal burdens, lung pathology, and host survival determined for Sod3-deficient and Sod3-producing strains demonstrate that Sod3 is essential for the full virulence of Histoplasma in vivo.

The virulence attenuation due to loss of Sod3 function contrasts with the full virulence of yeasts depleted of the intracellular superoxide dismutase, Sod1. Depletion of Sod1 by RNAi
Figure 3.11 Lethal infection by *Histoplasma* requires Sod3 function. Kinetics of mouse survival after infection with a lethal dose of *Histoplasma* yeast. Wild-type C57BL/6 mice were intranasally infected with $7 \times 10^6$ *SOD3(+) (OSU45), sod3Δ (OSU15), or sod3Δ/SOD3 (OSU49) Histoplasma* yeast (n=8 per strain). Survival time of mice infected with sod3Δ yeast differs significantly from that of infections with SOD3(+) and sod3Δ/SOD3 (p<0.0001).
Figure 3.3) from *Histoplasma* yeasts does not impair lung infection; the *gfp*-RNAi and *gfp:SOD1*-RNAi strains establish comparable fungal burdens in lungs throughout acute and clearing stages of infection (Figure 3.12). These results demonstrate that *Histoplasma* virulence specifically requires extracellular (Sod3) superoxide dismutase function.

Virulence in the animal model reflects the summation of multiple aspects of the immune response in control of *Histoplasma* yeasts. To clearly define the mechanism of the attenuation of sod3Δ yeasts *in vivo*, we tested the effect of specifically eliminating superoxide production by the host. Infections were established in mice lacking a functional phagocyte NADPH-oxidase complex (Phox) due to mutation of the p47Phox subunit (225). If the reduced virulence of sod3Δ yeasts *in vivo* results from inability to survive host-produced reactive oxygen, eliminating this host defense mechanism would restore the virulence of sod3Δ yeasts. In Phox(+/+) mice, *SOD3(+) Histoplasma* yeasts survive and establish respiratory infection as evidenced by the increasing fungal burdens over the first 8 days (Figure 3.13A). Although the magnitude of the fungal burdens in the lungs of these Phox(+/+) mice are less than that for experiments in Figure 3.9 due to different susceptibilities of wild-type mice among vendors, the upward trend in fungal burden is repeated in these mice that are isogenic with the Phox(-/-) mice. Similar to earlier results, at least half of the sod3Δ yeasts are killed in Phox(+/+) mice, in the Phox(-/-) hosts unable to produce superoxide, both strains establish similar fungal burdens reaching over $10^6$ cfu in lungs by day 8 (Figure 3.13B). In addition, Phox(-/-) mice are unable to clear the normally sublethal infection and mice become moribund at day 15.
Figure 3.12 Intracellular Sod1 function is dispensable for *Histoplasma* virulence.

Kinetics of sublethal lung infection with *Histoplasma* yeasts. Wild-type C57BL/6 mice were intranasally infected with approximately $2 \times 10^4$ *gfp*-RNAi (OSU104) or *gfp:SOD1*-RNAi (OSU105) *Histoplasma* yeasts. The fungal burden in lungs was determined by quantitative platings for *Histoplasma* cfu at the indicated times representing progressing infection. Each data point represents cfu counts per lung from an individual animal (n = 4 per time point) and horizontal bars represent the mean fungal burden. No significant differences (ns) in fungal burden are detected between infections with the SOD1-proficient strain (*gfp*-RNAi) and the SOD1-depleted strain (*gfp:SOD1*-RNAi). The actual inoculum dose is shown in graphs at day 0. The limit of detection is 100 cfu.
**Figure 3.13 Sod3 facilitates infection through detoxification of host reactive oxygen.**

Kinetics of sublethal lung infection by *Histoplasma* in animals competent for ROS production (A) or animals lacking the NADPH-oxidase function (B). Mice were intranasally infected with approximately $1 \times 10^4$ SOD3(+) (OSU45) or sod3Δ (OSU15) *Histoplasma* yeast. At 2, 4, 8 and 15 days post-infection, the fungal burden in the lungs was determined by quantitative platings for *Histoplasma* cfu. (A) Respiratory infection of Phox (+/+) mice isogenic to the p47phox knock-outs. (B) Respiratory infection of p47phox knock-out (Phox(-/-)) mice. Each data point represents cfu counts per lung from an individual animal (n=3 per time point) and horizontal bars represent the mean fungal burden. Non-significant (ns) or significant differences (* p<0.05, ** p<0.001) from animals infected with SOD3(+) organisms is indicated above the respective columns. The actual inoculum dose is shown in graphs at day 0. The limit of detection is 100 cfu.
Figure 3.13

A  Phox(+/+) mice

B  Phox(-/-) mice
with nearly $10^8$ fungal cfu per lung (Figure 3.13B). Importantly, the kinetics and fungal burdens for $SOD3(+)$ and $sod3\Delta$ yeasts in the Phox(-/-) mice are statistically indistinguishable from each other. These infection data show the overall significance of the role of host-derived reactive oxygen in limiting Histoplasma infections. Additionally, they demonstrate that the virulence attenuation of $sod3\Delta$ yeasts is due to reactive oxygen-dependent killing by the host and further confirms Sod3 promotes Histoplasma virulence in vivo by detoxifying host-derived reactive oxygen.

3.4 Discussion

We show in this study that the fungal pathogen Histoplasma capsulatum resists damage from antimicrobial ROS and demonstrate that this ability directly contributes to Histoplasma virulence. The extracellular superoxide dismutase Sod3 imparts resistance to superoxide since Histoplasma strains lacking Sod3 are susceptible to killing by superoxide anion and by macrophages and PMNs that produce ROS. Although the repertoire of phagocyte anti-Histoplasma defenses includes non-oxidative mechanisms such as hydrolytic enzymes (47), defensins (47, 226), and reactive nitrogen (61, 62), our use of an NADPH-oxidase inhibitor and Phox(-/-) mutant mice demonstrate that killing of the $sod3\Delta$ mutant is mediated by superoxide production. The protection of yeasts due to Sod3 mechanistically explains previous studies that show phagocytosis of Histoplasma
yeasts by PMNs and activated macrophages is accompanied by ROS production, yet the wild-type yeasts remain viable (44-46, 54, 164).

Extracellular superoxide dismutases are appropriately positioned to combat host-derived ROS. Unlike peroxide, superoxide is a charged molecule that does not readily cross cellular membranes. This has important consequences for intracellular pathogens. First, the lack of diffusion across membranes maintains higher concentrations of superoxide within the phagosomal lumen. Second, lack of diffusion into the fungal cell cytoplasm requires superoxide defense mechanisms to be located extracellularly. The *Histoplasma* Sod3 protein has an N-terminal signal sufficient to direct it into the secretory pathway and a C-terminal signal that promotes association with the cell surface. Cell-associated Sod3 may be covalently linked to the cell wall as has been shown for some yeast cell wall proteins with GPI signals (81, 220). Consistent with this, we have been unable to recover soluble Sod3 from cell wall preparations after treatment with reducing agents, anionic detergents, and glycanases (unpublished data). It is unknown if the portion of Sod3 protein that is not associated with yeast cells represents protein previously located at the cell surface and subsequently shed or whether it is protein secreted directly into the extracellular milieu. We suspect soluble Sod3 reflects insufficient retention on the cell since deletion of the C-terminal GPI signal shifts Sod3 from association with the cell into the soluble fraction. Deletion of the GPI signal did not completely prevent association of Sod3 with the cell, suggesting the existence of other unidentified cell-association signals. Regardless, both soluble and cell-associated Sod3
are appropriately located extracellularly in order to detoxify superoxide in the phagosomal lumen.

Our study is the first to demonstrate *Histoplasma* produces extracellular superoxide dismutase activity and identifies its source as Sod3. These results differ from earlier studies that failed to show *Histoplasma* yeasts could dismute exogenously generated superoxide *in vitro* (51, 52). A number of experimental differences likely account for this discrepancy. In the earlier *in vitro* studies, nearly 4 times more superoxide were generated and 10-fold less yeast cells were used as the potential superoxide dismutase source. In the current study, the use of a more sensitive detection reagent for superoxide (WST-1; (213, 214)) allowed us to generate less superoxide which did not overwhelm the dismutase activity of the number of yeast cells used in our assays. The deletion of *SOD3* and the corresponding loss of extracellular superoxide dismutase activity provide further evidence of the ability of Sod3-producing *Histoplasma* yeasts to destroy exogenous superoxide. While most studies have agreed that *Histoplasma* yeasts trigger ROS release from PMNs (44-46, 54, 164, 209), studies failing to detect ROS production (52) may have misinterpreted the results as lack of ROS stimulation instead of rapid destruction of ROS. Our results show that in the presence of efficient superoxide scavenging by Sod3, phagocyte ROS levels rapidly return to baseline by 10 to 15 minutes resulting in a narrow window for detection (Figure 3.6C).
The susceptibility of $sod3\Delta$ yeasts to superoxide challenge \textit{in vitro} and their attenuated virulence show superoxide is one of the major toxic oxidative compounds against which \textit{Histoplasma} must defend \textit{in vivo}. Although superoxide can be dismuted to peroxide, either through spontaneous or Sod3-catalyzed dismutation, peroxide is not the toxic form of reactive oxygen responsible for damaging the $sod3\Delta$ yeasts. As evidence of this, we show that yeasts lacking Sod3 function are killed by superoxide, but do not exhibit any increased sensitivity to peroxide (Figure 4A and 4B). Lack of peroxide sensitivity may result from efficient destruction of peroxide by \textit{Histoplasma} yeasts since they also express an extracellular catalase (181, 193, 194). As an uncharged molecule, peroxide can also diffuse into the yeast cell cytoplasm where intracellular scavenging enzymes may also protect yeasts from oxidative damage from peroxide. Alternatively, yeast viability may rely on essential target molecules that are attacked specifically by superoxide but not peroxide. The impact of exogenous peroxide and the role of \textit{Histoplasma} peroxide defenses await further genetic tests with yeasts lacking catalase antioxidant factors.

The ability to tolerate or destroy host-generated reactive oxygen is a fundamental characteristic of successful microbial pathogens. Extracellular dismutation of superoxide is a capability shared by \textit{Histoplasma} and other intracellular pathogens of macrophages including \textit{Mycobacterium tuberculosis} (227), \textit{Salmonella} spp. (228, 229), and \textit{Francisella tularensis} (230). A past study demonstrated that the resistance of fungal pathogens to killing by PMNs distinguishes primary from opportunistic pathogens; despite triggering
ROS release from PMNs, *Histoplasma*, *Blastomyces*, and *Paracoccidioides* survived whereas *Candida* and *Aspergillus* cells were efficiently killed (164). Genome analyses of the dimorphic fungal pathogens *Blastomyces*, *Paracoccidioides*, and *Coccidioides* identify proteins homologous to *Histoplasma* Sod3 and these proteins also have predicted N-terminal signal peptides (data not shown). This suggests that, like Sod3, they are extracellular superoxide dismutases that could protect these fungal cells from ROS release during interaction with host cells. The production of extracellular superoxide dismutases thus appears to be a defining characteristic of primary pathogens that are not effectively controlled by the innate immune system. Extracellular superoxide dismutation typifies intracellular pathogens in particular as the pathogenesis of these microorganisms involves engagement of and internalization into superoxide-producing phagocytes.

A number of plant and human pathogenic fungal species express multiple superoxide dismutases, which have been postulated to impart virulence potential. However, the majority of the identified Sods are intracellular enzymes (cytosolic or mitochondrial) and their loss causes sensitivity to intracellular oxidative molecules. This suggests that the *in vivo* virulence attenuation of fungi with deleted intracellular SOD genes may stem from their impaired ability to alleviate superoxide stress arising from altered metabolism imposed by growth in the host rather than insufficient detoxification of host-derived superoxide. For example, *Cryptococcus gattii* Sod1 and Sod2, *Cryptococcus neoformans* Sod1 and Sod2, and *Candida albicans* Sod1, Sod2, and Sod3 are all intracellular Sods. Deletion of their encoding genes reduces fungal virulence *in*
vivo but also increases sensitivity to pharmacologically-induced intracellular oxidative stress or growth on respiration-requiring media (231-234). Cryptococcus neoformans sod1Δ mutants survive and replicate within macrophages albeit at a slightly slower rate also consistent with Sod1 functioning in reducing metabolic rather than host-produced oxidative stress (235). Whether virulence attenuation in these mutants arises from failure to defend against host-produced oxidative stress awaits testing in hosts unable to produce superoxide. Data for the involvement of superoxide dismutases in fungal infection of plants is based primarily on correlations with SOD gene transcription during fungus-plant interaction (236-239), but genetic evidence of the role of superoxide dismutase is largely lacking or contradictory in the case of Botrytis Sod1 (240, 241). The results of our study using Histoplasma strains deficient in Sod1 and Sod3 demonstrate the spatial specificity of the Sod1 and Sod3 superoxide dismutases for internal and external (i.e., host-derived) superoxide, respectively. Only growth of the Sod1-depleted strain, but not Sod3-deficient yeasts, is impaired when intracellular superoxide levels are increased (Figure 3.3). Conversely, only Sod3-deficient but not Sod1-depleted yeasts are attenuated in virulence in vivo (Figure 3.9 and Figure 3.12). Furthermore, the restoration of Histoplasma sod3Δ mutant virulence in mice unable to produce superoxide conclusively shows that Sod3 functions in detoxification of superoxide generated by the host.

However, pathogenic potential is not solely attributable to extracellular Sods and their ability to destroy toxic ROS. For example, the opportunistic fungal pathogen Candida albicans expresses at least two extracellular superoxide dismutases (Sod4 and
Sod5) that are induced in response to oxidative stress and are required for full virulence in mice (242, 243). Conflicting results exists as to whether Sod5 affords some protection to *Candida* against superoxide-dependent killing by phagocytes *in vitro* (242-244), and one study shows Sod5 affects sensitivity to miconazole-induced intracellular ROS (245). Nonetheless, *Candida* is still effectively controlled by innate immune cells. In short term viability assays, we found PMNs kill nearly 80% of *Candida* yeasts (Figure 3.6A) suggesting *Candida* Sod4 and Sod5 proteins are insufficient to protect against the levels of superoxide generated by PMNs. Resting and activated macrophages similarly killed *Candida* to a large extent in our study (Figures 3.8A and 3.8B). Interestingly, *Histoplasma* yeasts lacking Sod3 closely resemble *Candida* yeasts in their susceptibility to *in vitro* superoxide and to phagocyte-derived ROS, showing *Histoplasma* Sod3 contributes to the greater resistance of *Histoplasma* to phagocyte killing and consequently to *Histoplasma*’s ability to cause disease in immunocompetent hosts.

In addition to detoxification of ROS, the ability to minimize stimulation of the phagocyte respiratory burst also contributes to the pathogenic potential of fungi. Similar to *Candida*, *Histoplasma* stimulates ROS production by PMNs and activated macrophages (Figure 3.6C and 6D, (53, 54, 209, 244). However, the level of ROS release triggered by sod3Δ mutant *Histoplasma* yeasts is less than that caused by other phagocyte stimuli such as phorbol myristate acetate and zymosan (data not shown). This may result from decreased phagocyte recognition of *Histoplasma* yeasts since the α-glucan polysaccharide in *Histoplasma* cell walls masks cell wall β-glucans from the phagocyte
β-glucan receptor Dectin-1 (77). On the other hand, *Candida* cell wall molecules are recognized by multiple pattern recognition receptors (246) and Dectin-1 recognition of *Candida* enhances ROS production by phagocytes (247-250).

Despite the ability of *Histoplasma* Sod3 to destroy fungicidal superoxide, host-generated ROS provides some limited control of *Histoplasma* during the innate immune response and is critically important for clearance of *Histoplasma* upon activation of immune cells. IFNγ treatment of macrophages primes host cells for ROS production in response to *Histoplasma* yeasts (55) and addition of TNFα further enhances ROS generation (53, 209). Pre-treatment of macrophages with levels of IFNγ and TNFα used in our study enhance ROS production in response to stimuli while keeping nitric oxide levels relatively low (251). *In vitro*, activation of macrophages can variably inhibit *Histoplasma* yeasts (55, 209, 252-254) while our results indicate Sod3 protects *Histoplasma* yeasts against activated macrophage ROS, at least in short term assays (up to 4 hours).

Rapid proliferation of both Sod3-producing and Sod3-deficient *Histoplasma* in the lungs of mice lacking NADPH-oxidase function highlights the critical importance of host ROS. Phox(+/+) mice, compared to Phox(-/-) mice, restrict even SOD3(+) *Histoplasma* yeasts, indicating the involvement of oxidative defense mechanisms. While this restrains the infection to some degree, the innate response is unable to clear the infection. Our results indicate that Sod3 function is a major factor in protecting the yeasts
from innate immune cells and in promoting histoplasmosis (Figure 3.9C, Figure 3.11, and Figure 3.10). Consistent with this early involvement of ROS, yeasts lacking Sod3 function are rapidly killed during the early phases of infection (Figure 3.9A and 3.13A). Nonetheless, the sod3Δ yeasts are not entirely cleared and in fact show some proliferation of the fungal burden in lungs between days 4 and 8 (Figures 3.9A and 3.13A). We suspect resting macrophages, the infection of which by *Histoplasma* does not result in significant ROS production (Figure 3.8, and (51-54)), provide a temporary refuge for Sod3-deficient yeast. However, influx of CD4+ T-cells and the corresponding increase in IFNγ in lungs after day 7 (255) promotes clearance of sod3Δ yeasts through activation of macrophages and enhancement of oxidative killing. Cytokine activation of macrophages in culture and their ability to kill sod3Δ yeasts support this model (Figure 3.8B). Thus, Sod3 is an essential virulence factor that protects *Histoplasma* yeasts from killing by ROS produced by the host, particularly during the innate immune response to infection.
Chapter 4: The extracellular catalase CatB is dispensable for *Histoplasma* virulence.

4.1 Introduction

The production of reactive oxygen species (ROS) is one of the most important antimicrobial responses generated by the innate immune system. Within mammals a specialized subset of leukocytes produces ROS, including monocytes, neutrophils, dendritic cells and macrophages. The generation of reactive oxygen by these cells is mediated by the NADPH oxidase complex that transfers electrons from NADPH to molecular oxygen, creating superoxide anions (256). Superoxide anion is the precursor molecule for all other ROS such as hydrogen peroxide, hydroxyl radical and hypochlorous acid, making the generation of superoxide anion critical for initiation of the antimicrobial innate immune response. The loss of a functional NADPH oxidase complex leads to chronic granulomatous disease, which is characterized by persistent infections with bacteria or fungi, demonstrating the importance of the oxidative burst in preventing microbial infections (257, 258). All forms of ROS are capable of damaging numerous macromolecules and killing microbes. Effective pathogens have coped with these host derived ROS by utilizing many of the same protein factors utilized to combat metabolically generated ROS.
Generally, pathogenic organisms neutralize oxidative stresses by utilizing both non-enzymatic and enzymatic strategies. Within fungi non-enzymatic strategies include the utilization of melanin and mannitol to absorb excess ROS (259, 259-261). Enzymes such as superoxide dismutase and catalase specifically destroy the ROS molecules superoxide anion or hydrogen peroxide, respectively (262). These survival strategies have been utilized by parasites (263), bacterial (264) and fungal pathogens (262) of both plants and animals. Additionally, pathogenic organisms can position these proteins to interact with the extracellular environment thus specifically placing them to defend against immune system produced ROS (181, 229, 230, 244, 265-267).

*Histoplasma capsulatum* is an intracellular pathogen capable of parasitizing phagocytic immune cells. This fungus is found worldwide, but is particularly widespread within the Ohio and Mississippi River valleys. Within these endemic areas it has been estimated that 80% of the population has been exposed to *Histoplasma* (5). In the soil *Histoplasma* grows as conidia-producing mold. Upon inhalation of conidia into the mammalian lung the temperature change to 37°C shifts *Histoplasma* into pathogenic yeast (6, 268). Yeast proliferate in the lung within the phagosomes of aveolar macrophages. The innate immune system alone is unable to control *Histoplasma* replication and yeast continue to grow in these normally fungicidal cells. However, most immunocompetent individuals are able to control yeast growth upon activation of the adaptive immune response that enhance the antifungal response of phagocytes (67).
Histoplasma’s ability to avoid or detoxify ROS is thus critical to yeast pathogenesis. Resting macrophages do not generate an oxidative burst in response to Histoplasma yeast, unless macrophages are activated or the yeast are opsonized (51, 52, 269). On the other hand polymorphonuclear neutrophils (PMNs) produce an oxidative burst to Histoplasma (44-46, 269). In either case Histoplasma yeast are able to survive oxidative challenge and proliferate. The molecular mechanisms responsible for this resistance have largely remained unknown. Recently, we demonstrated that yeast produce an extracellular superoxide dismutase that protects Histoplasma from macrophage and PMN produced ROS and is required for full virulence within a murine infection model (269). The importance of Sod3 to Histoplasma survival within the host indicates that yeast must cope with host derived ROS in order to effectively survive within the lung.

The Histoplasma genome encodes three catalase proteins CatA, CatB and CatP that could potentially assist in destroying host derived hydrogen peroxide (193). CatA is an 80 kDa cytoplasmic catalase expressed primarily in the nonpathogenic mold-phase. CatB is a 90 kDa extracellular catalase and CatP is an intracellular 60 kDa protein. CatB, also known as M-antigen, is expressed by yeast during infection and is one of primary diagnostic antigens used to identify Histoplasma infection (270, 271). It has long been speculated that CatB is a virulence factor, but evidence for this has been lacking. Recently, functional studies demonstrated that CatB breaks down hydrogen peroxide in vitro but whether this affects Histoplasma pathogenesis in vivo remains to be determined (194).
Here we examine the role of intracellular and extracellular catalases in protecting 
*Histoplasma* from host derived ROS and their contribution to *Histoplasma* virulence. To 
functionally test their roles, we constructed a *Histoplasma* strain lacking *CATB*. We 
confirmed that CatB is the primary source of extracellular peroxide-destroying activity in 
culture supernatants, and that CatB protects yeast from hydrogen peroxide challenge *in 
vitro*. Interestingly, CatB does not seem to play a major role in the mouse model of 
*Histoplasma* infection. These results indicate that *Histoplasma* CatB is not required for 
virulence as has been postulated but that CatP fulfills a redundant role in protecting 
*Histoplasma* during infection.

### 4.2 Materials and Methods

#### 4.2.1 Fungal strains and culture conditions

*Histoplasma capsulatum* strains were generated from the wild-type G186AR (ATCC 
26027) and are listed in Table 4.1. *Histoplasma* cultures were grown and maintained in 
*Histoplasma* macrophage media (HMM) (172). Liquid cultures were grown at 37°C with 
constant aeration (200 rpm) until late exponential growth phase unless otherwise noted. 
Additionally, uracil auxotrophs were grown in media supplemented with uracil (100 
µg/ml). Growth phase was determined by measuring culture turbidity at 595 nm, after 
large clumps were broken using 1M NaOH. For yeast growth on solid media HMM was
solidified with 0.6% agarose and supplemented with 25μM FeSO4. Yeast were transformed with linearized plasmids by electroporation and selected for Ura+ on HMM (177). For experiments requiring exact yeast enumeration large clumps were first removed by spinning cultures at 50 rcf and then counted on a hemacytometer.

4.2.2 Generation of catbΔ, catbΔsod3Δ, CATB-complement and RNAi construction

To generate the catbΔ strain Histoplasma (Wu8) was transformed with a Ura5 containing plasmid pBY15, that contains a hygromycin-resistance gene (HPH) flanked by 1.8 kb of sequence upstream and downstream of the CATB coding sequence (84). Ura+ transformants were picked and grown in liquid HMM containing 150 μg/ml hygromycin and uracil before diluting cultures 20-fold upon reaching stationary phase. Cultures were grown to late-exponential phase and passaged 3-times before plating on HMM containing uracil, 150 μg/ml hygromycin and 1 mg/ml 5-flourourotic acid (5-FOA) and grown at 37°C with 5% CO2/95% air. Hygromycin-resistant, 5-FOA-resistant colonies were picked and screened for the loss of CATB by PCR. The catbΔsod3Δ was created as above by flanking a neomycin resistancegene with 2 kb immediately 5’ and 3’ of the SOD3 coding region. Selections were carried out using 100 μg/ml G418, otherwise selection was identical to the standard procedure. Deletions were complemented with a wild-type genomic copy of CATB cloned into pCR468 where the gfp transgene is replaced with a genomic copy of the CATB gene. Transgene expression is driven by a strong constitutive promoter. The catbΔ was transformed with vector only (pCR468) or CATB-expressing
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WU8b</td>
<td>ura5-32</td>
<td></td>
</tr>
<tr>
<td>OSU15</td>
<td>ura5-32:: sod3-3::hph / pCR468 [URA5, gfp:FLAG] sod3Δ</td>
<td></td>
</tr>
<tr>
<td>OSU16</td>
<td>ura5-32:: catb-2::hph / pCR468 [URA5, gfp:FLAG] catbΔ</td>
<td></td>
</tr>
<tr>
<td>OSU22c</td>
<td>ura5-32:: zzz::pCR482 [hph, gfp] catbΔ</td>
<td></td>
</tr>
<tr>
<td>OSU24</td>
<td>ura5-32:: catb-2::hph catbΔ</td>
<td></td>
</tr>
<tr>
<td>OSU31</td>
<td>ura5-32:: sod1-3::hph catb-3::neo catbΔsod3Δ</td>
<td></td>
</tr>
<tr>
<td>OSU45</td>
<td>ura5-32:: / pCR468 [URA5, gfp:FLAG] CATB(+)</td>
<td></td>
</tr>
<tr>
<td>OSU46</td>
<td>ura5-32:: sod1-3::hph catb-3::neo / pCR468 [URA5, gfp:FLAG] catbΔsod3Δ</td>
<td></td>
</tr>
<tr>
<td>OSU51</td>
<td>ura5-32:: catb-2::hph / pBY08 [URA5, Pr2AP-CATB:FLAG] catbΔ/CATB</td>
<td></td>
</tr>
<tr>
<td>OSU52</td>
<td>ura5-32:: / pEH15 [URA5, CATB-RNAi]</td>
<td></td>
</tr>
<tr>
<td>OSU138</td>
<td>ura5-32:: catb-2::hph catp-1::neo catbΔcatpΔ</td>
<td></td>
</tr>
<tr>
<td>OSU139</td>
<td>ura5-32:: catb-2::hph catp-1::neo / pCR467 [URA5,] catbΔcatpΔ</td>
<td></td>
</tr>
<tr>
<td>OSU140</td>
<td>ura5-32:: catb-2::hph catp-1::neo / pCR468 [URA5, gfp:FLAG] catbΔcatpΔ</td>
<td></td>
</tr>
<tr>
<td>OSU141</td>
<td>ura5-32:: catb-2::hph catp-1::neo / pCR615 [URA5, Pr2AP-CATP:FLAG]</td>
<td></td>
</tr>
<tr>
<td>OSU142</td>
<td>ura5-32:: catb-2::hph catp-1::neo / pBY08 [URA5, PPr2AP-CATB:FLAG]</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1. *Histoplasma capsulatum* strains.

---

*a* all strains were constructed in the G186A (ATCC# 26027) background

*b* uracil auxotroph of G186A (Marion CM, et al., 2006 (78))

*c* GFP sentinel RNAi background (Edwards, et al. 2011 (81))
vector (pBY08). Ura+ transformants were selected and grown to late exponential phase in liquid HMM. Complementation was confirmed by PCR, the restoration of catalase activity to supernatants and the appearance of CatB on SDS-PAGE after silver staining.

4.2.3 *Histoplasma* RNA isolation and qPCR

*Histoplasma* yeast grown to exponential phase were collected by centrifugation (2000 x g) and resuspended in Ribozol (Bioexpress). Mycelia were collected from liquid cultures by filtration onto Whatman #5 filter paper and scraping of the retained mycelia into Ribozol. RNA was liberated from *Histoplasma* cells in Ribosol by beating with 0.5 mm-diameter glass beads. RNA was purified by extraction with CHCl₃ followed by alcohol precipitation of the aqueous phase. Genomic DNA was removed by two sequential digestions with Turbofree DNase (Ambion) and lack of genomic DNA verified by PCR. Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen) using 5 μg of total RNA and random 15-mer primers.

Quantitative PCR (qPCR) was performed on reverse-transcribed RNAs from three biological replicate cultures of yeast and mycelia. Reactions were assembled using a SYBR-green based PCR master mix (BioRad) with diluted reverse-transcribed templates (1:200 final) and 0.5 μM each gene specific primer. Cycling was performed with an IQ5 thermocycler (BioRad) using the following conditions: 95°C for 10 minutes
followed by 40 cycles of 95°C for 10 seconds, 55°C for 15 seconds, 72°C for 1 minute. Relative transcript enrichment in yeast was determined by comparing the Ct values from yeast and mycelia using the ΔΔCt method (176) after normalizing to levels of the RPS15 transcript. In other experiments the relative levels of CATP, CATB and CATA in yeast and mycelia was determined using gene specific primers after normalizing transcript levels to RPS15 and then ACT1 expression using the ΔΔCt method. Statistical analysis was performed using the student t-test.

4.2.4 Harvesting yeast culture supernatants and catalase activity assay

*Histoplasma* yeast cultures were grown in liquid HMM at 37°C with aeration until they reached late exponential/early stationary growth phases. Supernatants were clarified by pelleting yeast at 1000 xg for 5 min. and removing supernatant from cell pellet. Supernatants were additionally centrifuged at 16,000xg to remove remaining yeast. For yeast cell lysates cultures were pelleted, washed twice with PBS, resuspended in PBS and ruptured with 200 μl of 400 μm-diameter glass beads. Cytoplasmic protein was released by bead beating yeast with rests on ice. Protein concentration was determined using the Bradford method with BSA standard protein.

Catalase activity assays were performed as previously described (194). Briefly, culture supernatant volume was normalized to growth phase and the volume was brought
to 200 μl with 50 mM Na-phosphate buffer pH 7.5 before the addition of 800 μl of 10 mM H₂O₂ in 50 mM Na-phosphate buffer pH 7.5. Hydrogen peroxide destruction was measured at 240 nm with a correction at 595 nm on a spectrophotometer every 30 seconds for 5 minutes and compared to a control containing the buffer and HMM only. For catalase activity present on the yeast cells, pellets were suspended in 50 mM Na-phosphate buffer and total cell number was estimated by OD600 (1=1x10⁸ yeast/ml), using 1x10⁵ yeast per reaction. Yeast suspensions were treated like yeast supernatants above with a new reaction set up for each time point. Readings were taken every minute for 10 min. At each time suspensions were placed into Spin-X filter centrifugation columns (0.22 um) to remove yeast and clarified solutions were read in a quartz cuvette as above. To slow enzymatic destruction of hydrogen peroxide by cell associated catalases the reactions were kept at 4°C until spectrophotometer reading. CatP activity was measured by adding 12μg of cytosolic protein to the hydrogen peroxide destruction assay. Statistical analysis was performed using the student t-test.

4.2.5 Determining sensitivity to hydrogen peroxide in vitro

For determination of hydrogen peroxide sensitivity *H. capsulatum* yeast strains were collected at exponential growth phase from liquid culture and spread on solid HMM plates at 1x10⁶ yeast per plate. Lawn plates were then dried for 4 hours at 37°C to ensure even yeast distribution across the media surface. After spreading plates sterile filter disks
were treated with 20 μl of 0, 75, 150 or 300 mM hydrogen peroxide in 50 mM Na-phosphate buffer pH 7.4 and placed in each quadrant. Plates were incubated at 37°C with 5% CO2/95% air to allow for Histoplasma lawn development. After the appearance of lawns the zones of inhibition around filter disks were measured to determine sensitivity to hydrogen peroxide. Zones of inhibition diameters (mm) were measured using a light box and alpha imager software from one position through the filter disk and to the opposing point on the zone of clearance. Statistical analysis was performed using the student t-test.

4.2.6 PMN isolation and infection

PMNs were isolated from the blood of healthy human donors through venipuncture as previously described (216, 217). Briefly, 10 mls of blood was collected into syringes containing 250 U heparin and diluted into 0.9% saline solution. Blood was then overlayed onto 4.5 ml Ficoll-Paque PLUS and PMNs harvested by density sedimentation for 40 min. at 400 rcf. Erythrocytes were removed by the addition of 6 ml 0.9% saline and 6 ml of 3% Dextrose₅₀₀ and incubating at 4°C for 20 min. to allow the heavier red blood cells to settle. The upper layer was removed and pelleted at 4°C for 15 min. at 800 rcf and residual erythrocytes were lysed by the addition of 10 ml cold H₂O and mixing for 20 seconds before the addition of 10 ml of 1.8% saline solution to restore osmotic stability. PMNs were pelleted at 4°C for 10 min. at 800 rcf and washed in Hanks
balanced salt solution (lacking Ca and Mg) before pelleting and counting cells on a hemacytometer. Cell viability was determined using Trypan blue. Autologous serum was prepared from separate blood samples utilizing coagulation and centrifugation. PMNs were seeded at $2 \times 10^5$ cells per well of a 96-well plate in DMEM plus 10% autologous serum. For infections $2 \times 10^4$ yeast were added to each PMN containing well and incubated at 37°C with 5% CO$_2$/95% air. After 4 hours post infection PMNs were lysed with the addition of cold H$_2$O and scrapping the wells with a pipette tip. Lysates were diluted and plated on solid HMM to determine viable cfu numbers. Survival was calculated by comparing infected wells to wells containing yeast only. Statistical analysis was performed using the student t-test.

4.2.7 Harvesting and differentiation of human blood monocytes.

Blood was collected from healthy donors through venipuncture (272). For experiments 100 ml of blood was taken and 20 ml was diluted into 15 ml of 0.9% saline before adding the diluted blood to 14 ml of Ficoll-Paque PLUS and separating cells by density sedimentation (40 min. at 400 rcf). “Buffy” coats were collected into conical tubes and total volumes were brought to 50 ml with cold PBS. Cells were pelleted by centrifugation (15 min. 460 rcf at 4°C) followed by the addition of PBS and cell pellets being pooled in a single tube, and volumes were brought to 50 ml with cold PBS before pelleting. The cell pellet was resuspended in 10 ml RPMI 1640 and cells counted on a
hemacytometer. Total cell concentration was adjusted to $2 \times 10^6$ cells/ml in RPMI 1640 and 20% autologous serum in Teflon wells. Cells were incubated at 37°C with 5% CO$_2$/95% air for 5 days to differentiate monocytes into macrophages. After 5 days Teflon wells were put on ice for 30 min. before removing liquid and washing wells three times with 4 ml RPMI 1640. Cells were pelleted (10 min. 130 rcf at 4°C) and resuspended in 5 ml RPMI 1640 and counted on a hemacytometer. Cell suspensions were adjusted to $4 \times 10^6$ cells/ml in RPMI 1640 with 10% autologous serum and 200 μl ($8 \times 10^4$ macrophages) was added to the wells of a 96-well plate and incubated at 37°C with 5% CO$_2$/95% air. After 2 hrs. nonadherent cells were removed by washing wells three times with PBS. For infections $2 \times 10^3$ yeast were added to each monolayer and incubated at 37°C with 5% CO$_2$/95% air. After 4 hours macrophages were lysed by adding cold H2O and scraping wells with a a pipette tip. Lysates were diluted and plated on solid HMM to enumerate viable fungal cfu. Survival was determined by comparing cfu from infected wells to wells containing yeast alone. For some experiments macrophage monolayers were first treated with 1000 U/ml IFN-γ in RPMI 1640 with 10% autologous serum for 2 days at 37°C with 5% CO$_2$/95% air. Statistical analysis was performed using the student t-test.

4.2.8 Determination of virulence within the mouse model.

C57BL/6 mice (NCI) were intranasally infected with ~$2 \times 10^4$ yeast cells. *Histoplasma* yeast were grown to exponential growth phase in liquid culture and counted
by hemacytometer. The actual infectious dose was verified by plating innocula dilutions on solid media and determining cfu. At 4, 8, 12, 16 and 20 days post infection mice were sacrificed, and lungs and spleens were collected. Lungs and spleens were homogenized in 5 ml or 3 ml HMM, respectively and dilutions were plated on solid HMM to determine viable fungal cfu. Statistical analysis was performed using the student t-test.

4.3 Results

4.3.1 CatB is responsible for extracellular catalase activity.

All sequenced *H. capsulatum* genomes contain the *CATB* gene and at least two diverse strains express the *CATB* gene suggesting a conserved function. Importantly, *CATB* is expressed during macrophage infection and during mouse lung infections, consistent with a role in virulence (211). To functionally verify the role of *CATB* during infection we constructed a *Histoplasma* strain lacking the *CATB* coding region. The catb\(\Delta\) allele was created by replacing the native *CATB* gene with a hygromycin resistance gene (*hph*). The *hph* cassette was flanked by two 1.8 kb DNA fragments corresponding to the sequence 500 bp from the 5’ end and immediately adjacent to the 3’ end of *CATB*. We confirmed the deletion of *CATB* using PCR analysis with primers for the wild-type or deletion allele (Figure 4.1A). The *CATB* specific primers amplified a 500 base pair product from wild-type *Histoplasma* (*CATB(+)*) , but no product was detected in the
Figure 4.1 *Histoplasma* encodes a secreted and yeast associated catalase. (A) PCR validation of CATB deletion. Genomic DNA from CATB(+) parental strain (Wu8), catbΔ strain (OSU16) and catbΔ/CATB complement strain (OSU51) were tested by PCR for the mutant allele marked with the hygromycin resistance gene (hph), actin gene (ACT1) or the wild-type CATB gene. (B) SDS-PAGE validation of the loss of CatB protein. Culture filtrates (6μg) from wild-type CATB(+) (OSU45), CATB(-) (CATB-RNAi (OSU52) or catbΔ (OSU16)) or catbΔ/CATB (OSU51) were resolved on 8% denaturing polyacrylamide gel and visualized by silver staining. (C) Confirmation that CatB is responsible for catalase activity in *Histoplasma* supernatants. Supernatants from wild-type CATB(+) (OSU45), catbΔ (OSU16) or catbΔ/CATB (OSU51) yeast cultures were collected at late-exponential growth phase. Yeast were removed from supernatants and equal supernatant volumes were loaded into hydrogen peroxide solution (50 mM Na-phosphate pH 7.4 with 10 mM H2O2). The breakdown of hydrogen peroxide was measured at 240 nm over 5 minutes. (D) Confirmation that CatB is responsible for catalase activity on yeast cells. Yeast cells were collected from late-exponential growth phase from wild-type CATB(+) (OSU45), catbΔ (OSU16) or catbΔ/CATB (OSU51) strains and 1x10^8 yeast were incubated in hydrogen peroxide solution on ice. Yeast were removed from hydrogen peroxide solution every minute and remaining hydrogen peroxide was monitored at 240 nm for a total of 10 minutes. Data is representative of three experiments performed in triplicate. Plots in (C) and (D) represent mean values ± standard deviations of experiments performed in triplicate. Asterisks indicate significant difference from CATB(+) (* < 0.05, ** < 0.01, *** < 0.001 and ns (not significant)).
Figure 4.1

<table>
<thead>
<tr>
<th>A</th>
<th>HPH</th>
<th>ACT</th>
<th>CATB</th>
</tr>
</thead>
<tbody>
<tr>
<td>CATB(+)</td>
<td>catbΔ</td>
<td>catbΔ/CATB</td>
<td>CATB(+)</td>
</tr>
</tbody>
</table>

B

C

D

Change in Abs 240 nm

Time (min)

Change in Abs 240 nm

Time (min)
CATB deletion (catbΔ). Consistent with the replacement of the wild-type allele, we detect a 1 kb product of the hph gene in the catbΔ strain. The presence of a 500 bp ACT1 fragment indicated that all DNA was competent for PCR amplification. The catbΔ grows at a rate comparable to wild-type in both rich (HMM) and minimal (3M) media (data not shown).

The loss of CatB was verified at the protein level (Figure 4.1B). Culture supernatants from Histoplasma grown to late-exponential phase was collected and concentrated using 10 kDa MWCO viva-spin concentration columns. Denaturing polyacrylamide gels were loaded with 6 μg of total protein and proteins were visualized by silver staining. In CATB(+) supernatants we detected a distinct 90 kDa band that is lacking in catbΔ supernatants. CatB can be restored by the addition of an extrachromosomal plasmid encoding a genomic copy of the CATB coding region. Complementation molecularly confirms previous proteomics studies indicating that the 90 kDa band is CatB. Additionally, we confirmed the identity of CatB using RNAi. The CATB-RNAi strain supernatants lacked the 90 kDa CatB band (Figure 4.1B) thus independently verifying the location of the CatB on denaturing polyacrylamide gels.

To confirm that extracellular catalase activity arises from CatB, we compared the activity of the CatB-deficient strain with the wild-type. Supernatants from cultures grown to exponential growth phase were harvested from CATB(+), catbΔ or catbΔ/CATB strains and ability to destroy hydrogen peroxide was determined (Figure 4.1C). Supernatants
from \( \text{CATB}(+) \) were able to degrade \( \text{H}_2\text{O}_2 \) over time, but supernatants from \( \text{catb}\Delta \) were unable to break down \( \text{H}_2\text{O}_2 \) and behaved similarly to \( \text{H}_2\text{O}_2 \) only controls. Catalase activity was restored by the addition of a plasmid copy of \( \text{CATB} \). Catalase activity was actually higher in the complemented strain than in wild-type, which was expected since \( \text{CATB} \) expression is driven by the H2B promoter. It was previously reported that CatB is also associated with the cell wall of \( H. \text{capsulatum} \) Nam2 strains, and we sought to determine if CatB was also localized to the Panamanian strain’s cell wall. \( \text{CATB}(+) \), \( \text{catb}\Delta \) or \( \text{catb}\Delta/\text{CATB} \) strains, were isolated from cultures at late-exponential growth phase and added to reactions to monitor the destruction of peroxide. The destruction of hydrogen peroxide occurred quickly in the presence of \( \text{CATB}(+) \) cells. CatB-deficient yeast were unable to effectively destroy hydrogen peroxide, resulting in a line similar to hydrogen peroxide only controls. Activity was restored to strains containing a plasmid copy of \( \text{CATB} \). To account for how quickly yeast bound catalase destroys hydrogen peroxide we also performed hydrogen peroxide destruction reactions on ice to slow the enzymatic breakdown of hydrogen peroxide. Results were similar to those observed at room temperature with \( \text{CATB}(+) \) possessing more activity than \( \text{catb}\Delta \) containing reactions, but total activity was not alleviated in strains lacking CatB. This result was not unexpected, as hydrogen peroxide could be reacting with other proteins or freely crossing over the plasma membrane into yeast where it is being actively destroyed or removed from our assay system. Overall, CatB is clearly the source of extracellular catalase produced by \( H. \text{capsulatum} \) yeast.
4.3.2 CatB protects against hydrogen peroxide *in vitro*.

The ability of CatB to protect *Histoplasma* yeast from hydrogen peroxide stress was determined using the disk diffusion method (Figure 4.2). The CATB(+) strains are able to resist hydrogen peroxide killing as indicated by the smaller inhibition zones of 11.1, 14.4 and 18.7 mm in the presence of 75, 150 and 300 mM hydrogen peroxide, compared to the larger zones of inhibition observed for *catbΔ* at 12.6, 18.3 and 23.5 mm, respectively. The return of the CATB gene to *catbΔ* restores the ability of yeast to resist hydrogen peroxide killing as indicated by the increased diameter of clearing regions, 8.7, 12.2 and 15.9 mm. All three strains were able to grow equally well as lawns in the absence of hydrogen peroxide. This result confirms that CatB specifically protects yeast cells against hydrogen peroxide during *in vitro* growth conditions.

4.3.3 CatB protects *Histoplasma* from PMN generated hydrogen peroxide.

The primary generators of ROS in the mammalian immune system are phagocytic cells. One of the major producers of ROS among phagocytes are PMNs (Polymorphonuclear neutrophils) that are capable of producing large quantities of hydrogen peroxide in response to pathogens. To determine if CatB is involved in protecting *Histoplasma* from PMN generated hydrogen peroxide we measured the ability
Figure 4.2 CatB protects *Histoplasma* yeast against hydrogen peroxide *in vitro*.

Survival of *Histoplasma* yeast during hydrogen peroxide challenge by disk diffusion assay. Wild-type CATB(+) (OSU45), catbΔ (OSU16) or catbΔ/CATB (OSU51) strains were spread onto HMM plates at 1×10⁵ yeast/plate and sterile filter disks were added that contained 0 mM, 75 mM, 150 mM or 300 mM hydrogen peroxide. *Histoplasma* lawns were grown at 37°C and diameters of the zones of inhibition (mm) were determined using alpha imager software. Data represents three experiments performed in triplicate. Asterisks indicate significant difference from CATB(+) (* <0.05 and ** < 0.01).
Figure 4.2

![Graph showing the diameter of clearing in millimeters against hydrogen peroxide concentration in millimolar (mM). The graph compares three conditions: CatbΔ, CATB(+), and CatbΔ/CATB.]

- **CatbΔ**
- **CATB(+)**
- **CatbΔ/CATB**
of wild-type and CatB-deficient strains to survive co-incubation with PMNs. After 4 hours of co-incubation PMNs were lysed and lysates were plated to determine viable yeast cfu (Figure 4.3). Wild-type *Histoplasma* yeast are able to survive PMN exposure, 83% yeast survival, while strains lacking the known oxidative defense protein Sod3 are readily killed, 31% yeast survival. *Histoplasma* unable to produce CatB are also killed by PMNs, 63% yeast survival, but the defect is not as extreme as the loss of Sod3. This suggests that yeast are more sensitive to exogenous superoxide than peroxide stress. The survival defect observed in catbΔ can be restored to wild-type levels by returning a plasmid copy of CATB, 89% yeast survival. Additionally, the observed survival defects were attributed to oxidative killing, since the addition of the NADPH oxidase inhibitor DPI restores sod3Δ and catbΔ yeast survival to wild-type levels. To determine if CatB acted synergistically with Sod3 in response to PMN produced oxidative stress and constructed a catbΔsod3Δ, double deletion strain. This strain demonstrated wild-type growth in HMM media and lacks extracellular catalase and extracellular superoxide dismutase activity. The catbΔsod3Δ strain survived at levels equivalent to sod3Δ yeast, indicating that ROS was the primary mediator of killing in this strain.

4.3.4 *Histoplasma* does not require CatB to survive within human MDMs.

*Histoplasma* yeast primarily reside within macrophages during infection. To determine if CatB promotes *Histoplasma* survival within macrophages we co-incubated human macrophages with wild-type and catbΔ strains. After co-incubation for 4 hours the
Figure 4.3 CatB is required to protect *Histoplasma* from human PMN oxidative killing. Survival of *Histoplasma* yeast after incubation with human PMNs. Wild-type CATB(+) (OSU45), sod3Δ (OSU15), catbΔ (OSU16), catbΔ/CATB (OSU51) or catbΔsod3Δ (OSU46) were added to PMNs at a multiplicity of infection (MOI) of 1:10. Yeast survival was determined by enumeration of viable cfu after 4 hours of co-incubation with human PMNs at 37°C. 10 μM of diphenelyne iodinium (DPI) was added to some assay to inactivate NADPH oxidase. Results are plotted as relative yeast survival (means ± standard deviation of 3 replicates) compared to viable cfu of yeast incubated in the absence of PMNs. Asterisks indicate significance from wild-type (* <0.05, *** <0.001 and not significant (ns)). There is no significant difference between the % yeast survival between wild-type and DPI-treated samples. Superoxide dismutase activity (data not shown). The catbΔsod3Δ strain was co-cultured with human PMNs and plated lysates as above. We found that the double mutant behaved nearly identically to the sod3Δ alone, 32% compared to 31% yeast survival. This indicates again that superoxide not peroxide is the major anti-histoplasma compound since the additional loss of CatB did further decrease survival. To confirm that ROS were mediating yeast killing PMNs were treated with 10 μM DPI to inhibit NADPH oxidase generated ROS. After the addition of DPI the
Figure 4.3

% Yeast Survival

- CATB (+)
- Sod3Δ
- CatbΔ
- CatbΔ/CATB
- CatbΔ sod3Δ
- CATB (+)
- Sod3Δ
- CatbΔ
- CatbΔ/CATB
- catbΔ sod3Δ

10 μM DPI
number of surviving yeast was determined. We found that human monocyte derived macrophages were unable to kill *Histoplasma* yeast over the course of 4 hours, consistent with yeast avoiding a macrophage oxidative burst (Figure 4.4). *Histoplasma* strains lacking CatB, Sod3 or both proteins were also able to survive within human macrophages. This result suggests that *Histoplasma* does not induce a strong oxidative burst from human macrophages.

Human macrophages are known to produce a stronger oxidative burst upon cytokine activation (209, 269). To determine if CatB protects yeast from activated macrophages wild-type or CatB-deficient strains were co-incubated with IFN-γ activated macrophages (Figure 4.5). Wild-type *Histoplasma* yeast were able to survive within activated macrophages, 82% yeast survival, and surprisingly *catbΔ* were also able to resist activated macrophage killing, 87% yeast survival. To confirm that IFN-γ activated human MDMs were producing ROS we infected activated macrophages with *sod3Δ*, which is known to be sensitive to phagocyte-produced ROS, and found that these yeast were readily killed, 25% yeast survival. The *sod3Δ* killing could be abrogated by the the addition of 10 μM DPI, indicating that ROS are being produced by activated macrophages. The loss of both CatB and Sod3 resulted in a large survival defect, 31% yeast survival, nearly equivalent to the loss of Sod3 alone, 25% yeast survival, indicating the lack of any additive protective effects for strains containing both proteins. The survival defect could be restored to wild-type levels by the addition of DPI confirming that the survival defect is dependent on ROS. These results indicate that CatB is not
Figure 4.4 CatB is dispensable for survival within resting human macrophages.

Survival of *Histoplasma* yeast in the presence of human macrophages. Wild-type CATB(+) (OSU45), sod3Δ (OSU15), catbΔ (OSU16) or catbΔsod3Δ (OSU46) strains were coincubated at an MOI of 1:50. Yeast survival was determined by enumeration of viable cfu after 4 hours of co-incubation with human macrophages at 37°C. Results are plotted as relative yeast survival (means ± standard deviation of 3 replicates) compared to viable cfu of yeast incubated in the absence of macrophages. There is no significant difference between CATB(+) and other yeast strain survival in macrophages.
Figure 4.5 CatB is not required for *Histoplasma* survival in IFNγ activated human macrophages. Survival of *Histoplasma* yeast after infection of activated human macrophages. CATB(+) (OSU45), sodΔ (OSU15), *catbΔ* (OSU16), *catbΔ/CATB* (OSU51) or *catbΔsodΔ* (OSU46) strains were added to macrophages activated by 100 U IFNγ for 48 hours at an MOI of 1:50. Yeast survival was determined by enumeration of viable cfu after 4 hours of co-incubation with human macrophages at 37°C. Results are plotted as relative yeast survival (means ± standard deviation of 3 replicates) compared to viable cfu of yeast incubated in the absence of macrophages. Asterisks represent significant difference from wild-type (***(<0.001). The difference between wild-type and *catbΔ, catbΔ/CATB* and DPI treated wells was nonsignificant (ns).
Figure 4.5

% Yeast Survival

10 μM DPI

CATB(+)  sod3Δ  catbΔ  catbΔ/CATB  CATB(+)  sod3Δ  catbΔ  catbΔsod3Δ
essential for protecting yeast against ROS produced by IFN-γ activated human monocyte derived macrophages.

4.3.5 CatB is dispensable for growth in vivo.

We next wanted to determine if CatB was required for yeast growth within the lung. Wild-type C57BL/6 mice were intranasally infected with wild-type or mutant *Histoplasma* strains. Wild-type yeast replicate within lungs, reaching the highest fungal burden between days 8 and 12, a 500 fold increase in lungs over inoculum, before gradually declining by day 16, representing the activation of the adaptive immune response (Figure 4.6). The *catbΔ* reaches nearly identical fungal burdens in the lungs through 20 days post infection, indicating that CatB is not required for lung infection. The *catbΔ sod3Δ* demonstrated a survival defect during lung infection that is basically identical to the defect observed in strains lacking Sod3 alone. This is in good agreement with the characteristic survival defects observed during PMN and activated macrophage infection. *Histoplasma* dissemination to the spleen was detectable by day 8 post infection and fungal burden reached the highest levels between days 8 and 12 before declining in wild-type *Histoplasma* (Figure 4.7). The *catbΔ* strain reached fungal burdens identical to the wild-type yeast within the spleen, indicating that the lack of CatB does not impair dissemination. The loss of both CatB and Sod3 greatly hampered the ability of *Histoplasma* to reach the spleen in a manner identical to yeast lacking Sod3 alone.
Figure 4.6 CatB is dispensable for *Histoplasma* growth in mouse lungs. Kinetics of sublethal lung infection by *Histoplasma*. Wild-type C57BL/6 mice were intranasally infected with approximately $1 \times 10^4$ wild-type (OSU45), *catb*Δ (OSU16), *sod3*Δ (OSU15) and *catb*Δ*sod3*Δ (OSU46) strains. At 4 day intervals post infection the fungal burden in lungs was determined by quantitative plating. Each data point represents cfu count per lung from individual animals (n=4 per time point) and horizontal bars indicate the mean fungal burden. Asteriks represent significant difference from wild-type (* <0.05, ** <0.01, *** <0.001 and not significant (ns)).
Figure 4.6

CATB(+) vs. catbΔ

sod3Δ vs. catb△sod3Δ

Days post infection

CFU/Lung

Days post infection

CFU/Lung

Days post infection

CFU/Lung

Days post infection

CFU/Lung
4.3.6 *Histoplasma* is protected by a redundant system of catalases

The loss of CatB alone did not impair *Histoplasma* survival in activated human macrophages, PMNs or during mouse infection. One possible explanation for this observation is that *Histoplasma* utilizes multiple redundant defense mechanisms against hydrogen peroxide. *Histoplasma* has been previously reported to produce two additional intracellular catalase proteins CatA and CatP. Previous studies indicated that *CATA* is only transcribed at low levels in yeast (193). We examined the expression of the three *Histoplasma* catalases by yeast and mycelia using qRT-PCR (Figure 4.8A). The expression of *CATB* is 92-fold enriched in the yeast-phase, similar to our previous observations. Both *CATA* and *CATP* are equally expressed between the mycelia- and yeast- phases, 1.7-fold and 3.3-fold enriched in the yeast-phase respectively. The absolute levels of transcript, as indicated by Ct values, however indicate that *CATP* is very highly expressed whereas *CATA* expression is extremely low in both phases. *CATP* is abundantly expressed in both yeast and mycelia, with a modest increase in expression in mycelia (Figure 4.8B). This is in contrast to *CATB* expression, which is yeast-phase enriched. Additionally; *CATP* is more abundantly expressed than *CATB* in either phase, 2.6-fold higher in yeast and 44-fold higher in mycelia. The *CATA* gene is only transcribed at a very low level in either yeast or mycelia. These results suggest that only CatB and CatP would be abundant enough to protect *Histoplasma* from peroxide during infection conditions. Although CatP is not enriched in the yeast-phase it is likely that abundant
Figure 4.7 CatB is dispensable for *Histoplasma* dissemination to mouse spleens.

Kinetics of dissemination following lung infection with *Histoplasma*. Wild-type C57BL/6 mice were intranasally infected with approximately $1 \times 10^4$ wild-type (OSU45), *catbΔ* (OSU16), *sod3Δ* (OSU15) and *catbΔsod3Δ* (OSU46) strains. At 4 day intervals post infection the fungal burden in spleens was determined by quantitative plating. Each data point represents cfu count per spleen from individual animals ($n=4$ per time point) and horizontal bars indicate the mean fungal burden. Asterisks represent significant difference from wild-type (* <0.05, ** <0.01, *** <0.001 and not significant (ns)).
Figure 4.7

Wild-type

$\text{CFU/Spleen}$

$10^2$ $10^3$ $10^4$ $10^5$ $10^6$

4 8 12 16 20

$\text{Days post infection}$

$\text{catb}^\Delta$

$\text{CFU/Spleen}$

$10^2$ $10^3$ $10^4$ $10^5$ $10^6$

4 8 12 16 20

$\text{Days post infection}$

$sod3^\Delta$

$\text{CFU/Spleen}$

$10^2$ $10^3$ $10^4$ $10^5$ $10^6$

4 8 12 16 20

$\text{Days post infection}$

$\text{catb}^\Delta sod3^\Delta$

$\text{CFU/Spleen}$

$10^2$ $10^3$ $10^4$ $10^5$ $10^6$

4 8 12 16 20

$\text{Days post infection}$

ns *** ** * ns

ns *** ** * ns

ns *** ** * ns
Figure 4.8 CATB is the only yeast-phase enriched *Histoplasma* catalase. Quantitative RT-PCR of RNA isolated from wild-type *Histoplasma* yeast and mycelia cultures indicates only *CATB* is yeast-phase enriched. Cultures were grown at 37°C for yeast or 25°C for mycelia cultures. The fold-change in transcript level was calculated using the ΔΔC\(_T\) method after first normalizing transcripts to the *RPS15* gene and then comparing yeast-phase and mycelia-phase transcript levels (A) or comparing expression to the *ACT1* gene (B). Bars represent the average of three biological replicates and error bars indicate standard deviation. The fold change in expression (yeast relative to mycelia) is indicated above the respective bars.
Figure 4.8

A

Log (fold change)

Yeast-phase

Mycelia-phase

CBP
MS8
CATP
CATA
CATB

B

Fold change (Actin)

Yeast
Mold
Yeast
Mold
Yeast
Mold

***
*
***
ns
expression indicates that CatP could be important for general ROS destruction in both morphologies.

To address potential redundancies between CatB and CatP a catbΔcatpΔ strain was generated by replacing the CATP locus with a G418 resistance marker (neo). The CATP gene was knocked out and screened for as described above. The loss of CatP and CatB resulted in the loss of catalase activity in supernatants and within yeast cells (Figure 4.9). Since catbΔ possesses normal intracellular catalase activity this indicates that CatP is responsible for the majority of intracellular catalase function. Consistant with a role in protecting *Histoplasma* from metabolically-derived ROS, strains lacking CatP demonstrate a slower than wild-type growth during culture in HMM (data not shown). Intracellular catalase activity and aerobic growth rate can be restored to wild-type levels by the addition of a plasmid copy of CATP the gene confirming the decreased growth is due to CatP deficiency. Attempts to produce a catpΔ or a CATP-RNAi resulting in significant CatP knock-down have thus far been unsuccessful.

To address the contribution of CatP in *Histoplasma* resistance to phagocyte produced hydrogen peroxide catbΔcatpΔ were co-cultured with human PMN’s or human macrophages. The catbΔcatpΔ strain was susceptibility to killing by PMNs (Figure 4.10), 25% yeast survival compared to 67% for wild-type or 49% yeast survival for catbΔ yeast after co-incubation with PMNs. Double catalase knock-outs also demonstrate enhanced sensitivity to IFN-γ activated human macrophage killing (Figure 4.11). The catbΔcatpΔ
**Figure 4.9** *Histoplasma* lacking CatB and CatP lacks intracellular and extracellular catalase activity. (A) The loss of CatB and CatP results in a loss of catalase activity in *Histoplasma* culture supernatants. Wild-type (OSU45), catbΔcatpΔ (OSU140) or catbΔcatpΔ/CATB (OSU142) culture supernatants were mixed with hydrogen peroxide reaction solution at room temperature. The break down of H$_2$O$_2$ was monitored at 240 nm every 0.5 minute for 5 minutes on a spectrophotometer. Each point represents the mean of three replicates and standard deviations. (B) The loss of CatB and CatP results in a loss of catalase activity within *Histoplasma* yeast. Wild-type (OSU45), catbΔcatpΔ (OSU140) or catbΔcatpΔ/CATP (OSU141) yeast were disrupted by bead beating and 12 μg of total protein was mixed with hydrogen peroxide reaction solution at room temperature. The break down of H$_2$O$_2$ was monitored at 240 nm every 0.5 minute for 5 minutes on a spectrophotometer. Each point represents the mean of three replicates and standard deviations. Asterisks represent significant difference from wild-type (* p<0.05 and *** 0<0.001).
Figure 4.9

(A) Change in Abs240 nm

- WT
- catbΔcatpΔ
- catbΔcatpΔ/CATB

(B) Change in Abs240 nm

- WT
- catbΔcatpΔ
- CatbΔcatpΔ/CATP

Time (min.)

Change in Abs240 nm

*** P < 0.001
Figure 4.10 The loss of both CatB and CatP increases *Histoplasma* sensitivity to PMN killing. Survival of *Histoplasma* after co-incubation with human PMNs. Wild-type (OSU45), *catbΔ* (OSU16), *sod3Δ* (OSU15) or *catbΔcatpΔ* (OSU140) were added to PMN’s at a MOI of 1:10. Yeast survival was determined by enumeration of viable cfu after 4 hours of co-incubation with human PMNs at 37°C. 10 μM of diphenelyne iodinium (DPI) was added to some assays to inactivate NADPH oxidase. Results are plotted as relative yeast survival (means ± standard deviations of 3 replicates) compared to viable cfu of yeast incubated in the absence of PMNs. Asterisks represent significant difference from wild-type (* <0.05, ** <0.01). The difference between *catbΔ* and *catbΔcatpΔ* with DPI is nonsignificant (ns).
Figure 4.10

% Yeast Survival

WT  catbΔ  sod3Δ  catbΔsod3Δ  catbΔsod3Δ + 10μM DPI

*  **  **
had a strong survival defect, 40% yeast survival compared to 93% yeast survival for wild-type yeast or 94% yeast survival for catbΔ. *Histoplasma* yeast lacking CatB and CatP survived within unactivated human macrophages at levels identical to wild-type *Histoplasma* yeast (data not shown). DPI was able to restore yeast survival to wild-type levels indicating that ROS are the cause of survival defects observed in phagocytes. The restoration of CatB or CatP restores the ability of *Histoplasma* to survive within activated macrophages. Interestingly, the return of CatP resulted in greater yeast survival than the return of CatB, 97% compared to 68% yeast survival respectively.

The importance of a fully intact catalase system to *Histoplasma* survival *in vivo* was determined by performing intranasal infections and determining fungal burdens in lungs and spleens (Figure 4.12 A and B). The loss of both catalase proteins resulted in severe attenuation within the mouse 8 days post infection. Yeast were unable to survive within the lung or effectively disseminate to the spleen compared to wild-type. The total fungal burden in the lung was 100-fold higher in lungs infected with wild-type *Histoplasma* compared with the catbΔcatpΔ strain, which decreased about 3-fold compared to initial inoculum after 8 days. Additionally, the mutant was not detectable within spleens after 8-days (60 cfu limit of detection), indicating the mutant is impaired in reaching the spleen, 170-fold lower fungal burden than wild-type. This dissemination defect is most likely attributable to lower lung fungal burden.
Figure 4.11 *Histoplasma* requires CatB and CatP to survive in IFNγ activated human macrophages. Survival of *Histoplasma* yeast after infection with activated human macrophages. Wild-type (OSU45), *catb*Δ (OSU16), *sod3*Δ (OSU15), *catb*ΔcatpΔ (OSU140), *catb*ΔcatpΔ/CATB (OSU141) and *catb*ΔcatpΔ/CATP (OSU142) strains were added to macrophages activated with 100 U IFNγ for 48 hours at an MOI of 1:50. Yeast survival was determined by enumeration of viable cfu after 4 hours of co-incubation with human macrophages at 37°C. Results are plotted as relative yeast survival (means ± standard deviation of 3 replicates) compared to viable cfu of yeast incubated in the absence of macrophages. Asterisks represent significance compared to wild-type (*<0.05, **<0.01, ***<0.001 and not significant (ns)).
Figure 4.11

% Yeast survival

WT  catbΔ  sod3Δ  catbΔsod3Δ  catbΔsod3Δ + 10μM DPI  CatbΔsod3Δ /CATB  CatbΔsod3Δ /CATP

ns  **  ns  ns  **  ns
**Figure 4.12** *Histoplasma* requires CatB and CatP to survive *in vivo*. *Histoplasma* fungal burden in lungs and spleens 8 days post infection. Wild-type C57BL/6 mice were intranasally injected with approximately 1x10^4 wild-type (OSU45) or catbΔcatpΔ (OSU140) strains. At 8 days post infection the fungal burden in lungs (A) or spleens (B) was determined by quantitative plating. Each data point represents cfu count per organ from individual animals and horizontal bars indicate the mean fungal burden.
Figure 4.12

A

CFU/Lung

$10^7$ - $10^6$

$10^5$ - $10^4$

$10^3$ - $10^2$

WT $\text{catb}$$\Delta$$\text{catp}$$\Delta$

B

CFU/Spleen

$10^6$ - $10^5$

$10^4$

$10^3$ - $10^2$

WT $\text{catb}$$\Delta$$\text{catp}$$\Delta$
In this study we demonstrated that the extracellular catalase CatB is not required for the full virulence of *H. capsulatum*. The loss of CatB alone resulted in increased sensitivity to hydrogen peroxide and PMN mediated oxidative stress but not ROS from IFN-γ activated human macrophages. Additionally, yeast strains lacking CatB are able to survive and replicate normally within mouse lungs and spleens. The additional loss of CatP resulted in an increased sensitivity to hydrogen peroxide, PMN and activated macrophage mediated oxidative killing and an inability to survive within the mouse model. Our results demonstrate that *Histoplasma* uses multiple functionally redundant catalase proteins to protect itself from host-produced hydrogen peroxide stress.

We determined that CatB is an extracellular catalase that is localized to the cell wall and extracellular environment as indicated by previous studies (194). The extracellular localization of CatB is ideal for detoxifying host derived ROS before it could damage yeast, much like the extracellular Sod3 protein. The ability to detoxify hydrogen peroxide is critical for pathogen survival within phagocytes, as hydrogen peroxide is a very common and stable form of ROS produced by phagocytes. The CatB protein contains a canonical N-terminal signal peptide that targets it through the secretory pathway where the protein can then be released into the extracellular environment or attached to the cell wall, however; the mechanisms attaching CatB to the yeast cell wall are unknown (211). Interestingly, we were able to identify catalase activity in
supernatants and on the cells of yeast throughout exponential growth phase, which conflicts with a previous study reporting that CatB is released only after prolonged culture (194). We have determined that this discrepancy is attributable to strain differences between the Panamerican and Nam2 isolates, as Nam2 appears to produce lower amounts of CatB than the Panamerican strain overall, possibly indicating that Panamerican strains are more resistant to ROS or that they induce more ROS upon phagocyte entry (Data not shown).

The production of hydrogen peroxide by phagocytic cells has been well documented as a downstream product of the NADPH oxidase complex (35), and is one of the molecules that pathogens must contend with during infection. Histoplasma strains lacking CatB are more sensitive to hydrogen peroxide compared to CATB(+) strains, indicating that CatB is capable of protecting Histoplasma against hydrogen peroxide (Figure 4.2 ). Additionally, the expression of CatB, but not CatP, is increased by the presence of iron (273). This observation would also be consistent with CatB preventing the formation of hydroxyl radicals by destroying the precursor molecule hydrogen peroxide. However, this appears not to be the case, as the loss of CatB does not greatly impair Histoplasma survival against phagocytes or during lung infections.

Among phagocytic cells PMNs are known for producing large quantities of hydrogen peroxide in addition to other types of ROS (274). These phagocytes are critical for the control of numerous pathogenic organisms including bacteria like Staphylococcus
*aureus* (275) or fungi like *Aspergillus fumigatus* (276). These cells are recruited to the sites of *Histoplasma* infection (255) and have been reported to produce ROS and other toxic effector molecules, like defensins (47), in response to yeast recognition. Yeast growth is halted when exposed to these molecules but yeast are not killed (47). The loss of CatB did result in a survival defect during PMN infection, 63% survival for *catbΔ* compared to 83% survival for *CATB(+)*, indicating importance for protecting *Histoplasma* from PMN’s, however this survival defect was not as severe as the loss of Sod3, a 60% decrease in yeast survival compared to wild-type.

We postulated that multiple proteins could act together to protect *Histoplasma* from phagocyte mediated oxidative stress, accounting for the observation that only a modest survival defect of *catbΔ* in the presence of PMN’s. The Sod3 protein is predicted to act upstream of CatB and would convert superoxide anion into hydrogen peroxide, that could then be destroyed by CatB. To explore this relationship we generated the first double deletion strain in *H. capsulatum* that was depleted for both CatB and Sod3. The loss of both proteins resulted in increased sensitivity to PMN mediated killing that could be rescued by DPI (Figure 4.10). However, this defect was nearly identical to the survival defect observed in strains lacking Sod3 alone. This result confirms that Sod3 acts upstream of CatB and that superoxide anion appear to be the primary anti-*Histoplasma* oxygen molecule produced by phagocytes. It also strongly suggests that *Histoplasma* must utilize other mechanisms to resist damage due to hydrogen peroxide. *Histoplasma* expresses two additional internal catalase proteins that could also act to protect yeast.
from this stress, as hydrogen peroxide can more freely cross biological membranes (193). Our expression studies indicated that both CATB and CATP are expressed at high levels in yeast, while the CATA transcript is only expressed at low levels in yeast or mycelia (Figure 4.8). Interestingly, only CATB was highly yeast-phase enriched as CATP is highly expressed in both yeast and mycelia. This observation is consistent with CatP potentially being involved in a conserved function between the two phases, such as degrading ROS produced during metabolic activity. To determine if CatB and CatP are functionally redundant a catbΔcatpΔ double deletion was generated. The loss of both catalase proteins resulted in a significant decrease in Histoplasma survival during PMN infection, 25% survival, compared to wild-type or the loss of CatB alone, 67% or 49% (Figure 4.10) or withing IFNγ activated macrophages (Figure 4.11). This result indicates that Histoplasma can protect itself from host-derived hydrogen peroxide using extracellular and/or intracellular catalase proteins. As neutrophils are known to produce various toxic non-oxidative effector molecules such as hydrolytic enzymes and defensins, we demonstrated that all observed survival defects could be alleviated when the NADPH oxidase inhibitor DPI was added to survival assays.

The loss of CatB had no detrimental effect on Histoplasma’s ability to survive within resting human macrophages. It is likely that resting human macrophages fail to induce an oxidative burst to Histoplasma, consistant with observations from murine cells (51). Consistant with this hypothesis is that the sod3Δ is readily able to survive within resting human macrophages. Additioally the ability of human macrophages to induce an
oxidative burst (55, 277-279) is in keeping with our observation that sod3Δ are readily killed. However, the presence or absence of CatB did not affect Histoplasma survival in response to activated macrophages. The additional loss of Sod3 did not result in any additional killing of Histoplasma after co-incubation with activated human macrophages. The loss of both extracellular and intracellular catalases, CatB and CatP, decreased Histoplasma survival within activated macrophages, demonstrating the importance of catalases to combat ROS. This result demonstrates that CatP can likely functionally compensate for the loss of CatB during the infection of phagocytic cells. To conclusively explore this a catpΔ strain is required. It has been well documented that IFN-γ activation of macrophages induces other toxic effectors in macrophages, such as increased nitric oxide production or increased iron sequestration (34, 68). We ruled out the possibility of some of these mechanisms involvement since our assays were carried out over a short time frame, allowing only the more immediate effectors to become involved and because the NADPH oxidase inhibitor DPI was able to restore survival defects to wild-type levels indicating ROS involvement.

The loss of CatB alone did not attenuate Histoplasma growth within mouse lungs or spleens. We noticed a minor decrease in lung fungal burden during days 16 and 20, corresponding to a 2.2- and 5.1-fold lower fungal burden than CATB(+) strains, but overall fungal burdens at each day between CATB(+) and catbΔ were nearly identical. Fungal growth was also equivalent between strains after dissemination to spleens. This result dispels the notion that CatB is required for Histoplasma growth during infection,
despite being yeast-phase enriched and produced during infections (211). The fact that $\text{catb}\Delta$ is not attenuated within the mouse model is not without precedence in other pathogenic organisms. The loss of catalase proteins in bacteria such as Neisseria gonorrhoeae (280), S. Typhemermium (281), Haemophilus influenzae or in fungal pathogens like Candida glabrata (282) or Aspergillus fumigatus (283) does not result in attenuation during animal infections. The authors largely attribute this lack of attenuation to either redundant oxidative defense mechanism and/or lower concentrations of hydrogen peroxide produced in vivo compared to in vitro analysis. The lack of attenuation in vivo also demonstrates that hydroxyl radicals may not play a role in host defense against Histoplasma, as CatB would be the only catalase protein positioned appropriately to prevent extracellular radical formation effectively. The loss of both extracellular oxidative defense proteins, Sod3 and CatB, did not further impair the growth of Histoplasma within the mouse lung or spleen, which is consistent with our Histoplasma survival assays in PMNs or activated macrophages. This agrees with the idea that superoxide anion is one of the most detrimental molecules to Histoplasma growth within the mammalian host. The additional loss of CatP resulted in growth attenuation within the lung and spleen. The loss of CatP could have multiple affects during lung infection including impaired defense against ROS produced by the immune system or impaired growth due to an increase in metabolically produced ROS within yeast cells. The later would be predicted to be a part of this phenotype as $\text{catb}\Delta\text{catp}\Delta$ yeast demonstrated a small growth defect during growth in HMM. It will be interesting to determine which catalase is the most critical for growth in vivo either by generating a
*catpΔ* or restoring *CATP* or *CATB* to the *catbΔcatpΔ* and examining survival during murine infection.

The apparent redundancy between CatB and CatP is not surprising since hydrogen peroxide is a common stress encountered during aerobic growth and from the host during infection. It is possible that this redundancy could be incidental as CatP is likely more important for protection against metabolically derived ROS. This hypothesis is supported by the observation that *CATP* is expressed highly in pathogenic yeast and non-pathogenic mycelia. However, it appears that CatP can compensate for the loss of CatB during infections by quickly degrading hydrogen peroxide molecules that pass through the plasma membrane and into the cytoplasm. This hypothesis is strongly supported by the ability of CatP or CatB to restore the survivability of *catbΔcatpΔ* in activated macrophages. CatA seems to play no role in the observed redundancies, as the loss of CatP resulted in abolishment of nearly all intracellular catalase activity. This result differs from previous studies (193) indicating that *CATA* transcript is enriched in the mycelial phase and could represent strain differences between the North American 2 and Panamerican *Histoplasma* isolates. However, preliminary studies by our lab have indicated that *CATA* is not highly produced at high levels by either *Histoplasma* isolate in yeast or mycelia morphologies.

Taken together these results demonstrate that the abundantly secreted CatB is not absolutely required for *Histoplasma* virulence. However, *Histoplasma* does appear to
require either the presence of the CatB or CatP protein to allow full *Histoplasma* survival in phagocytes and during infections. CatB and CatP are functionally redundant and capable of protecting yeast against hydrogen peroxide mediated host-derived stress. This work mechanistically explains previous observations that have indicated that *Histoplasma* is particularly resistant to killing by oxidative stress (44-46, 269), since yeast utilize multiple defensive enzymes to detoxify ROS during infection, including Sod3, CatB and CatP.
Chapter 5: Cfp4 does not contribute to *Histoplasma* pathogenesis despite being an immunoreactive antigen.

5.1 Introduction

*Histoplasma capsulatum* is a respiratory fungal pathogen estimated to infect 200,000 individuals in the U.S. every year (6). Within endemic regions of the U.S. *Histoplasma* is enriched in soils containing bird and bat guano (7), and is estimated that over 80% of individuals have been exposed to the fungus (5). *Histoplasma* is part of a group of fungi known as the thermally dimorphic fungal pathogens. These fungi possess the ability to shift between two mutually exclusive life styles depending on environmental temperature. At ambient temperatures within the soil *Histoplasma* grows as a conidia-producing mold, but upon exposure to mammalian body temperature it undergoes a morphological shift into a yeast-like cell. Yeast are able to survive and replicate within phagocytic cells, mainly macrophages, eventually leading to the destruction of the phagocyte and dissemination to other organs.
*H. capsulatum* falls into two chemotypes that are defined based on the composition of the yeast cell wall (80, 284). Chemotype 2 strains are defined as having α-(1,3)-glucan polysaccharide layer that prevents host cell recognition by the Dectin-1 receptor (77, 81, 284). The α-(1,3)-glucan layer is required by Chemotype 2 strains for full virulence (76). Chemotype 1 strains lack the α-(1,3)-glucan polysaccharide layer but remain fully virulent within humans and animals (81). The Chemotype 1 strains transcriptionally express some of the factors required for α-(1,3)-glucan biosynthesis but the transcript is apparently not translated (81). Chemotypes roughly break down *Histoplasma* by geographical area, with Chemotype 1 strains being localized to North America, Nam2 strains, while Chemotype 2 represents *Histoplasma* throughout the world, in particular the Panamerican and North American, Nam1, isolates (178). Interestingly, despite this major difference all the aforementioned isolates are capable of causing disease. These strains share some factors in common that are required for disease causation, such as the secreted virulence factor calcium binding protein (Cbp1) (84), implying that all isolates could use conserved mechanism to establish efficient host infection.

Within the genomes of most organisms there are a large number of genes lacking sequence homology to proteins in sequenced databases. In some organisms these regions can represent up to 40% of the total protein coding sequences (285). *Histoplasma* contains a large number of such proteins, some of which appear important for host colonization. Recently, we have identified the most abundant extracellular proteins
produced by pathogenic-phase *Histoplasma*, and identified five proteins that shared no clear functional homology with other proteins (211). Three of these proteins, culture filtrate proteins (cfp) 1, 4 and 8 were highly enriched in the pathogenic-phase on both a transcript and protein level compared to the nonpathogenic mold-phase.

Here we investigated the role of the most abundant of these proteins Cfp4 for *Histoplasma* virulence. We created depletion strains in chemotype 1 and chemotype 2 strains to functionally determine the role of Cfp4 during macrophage infection, in particular potential roles in macrophage killing and yeast survival within macrophages. Additionally, we sought to determine if Cfp4 was required for yeast replication within a respiratory infection model. Surprisingly, Cfp4 does not appear to play a role during *Histoplasma*’s growth within macrophages, or during lung infection but may play a minor role during the initial stages of disseminated infection. Importantly, Cfp4 is recognized by human *Histoplasma* immune serum which provides evidence that Cfp4 could potentially be used as a diagnostic marker for histoplasmosis.

5.2 Materials and Methods

5.2.1 Fungal strains and culture conditions
*Histoplasma* capsulatum strains were derived from either the wild-type G186A (ATCC 26027) or G217B (ATCC26032) and are listed in Table 5.1. *Histoplasma* yeast or mycelia were grown and maintained in *Histoplasma*-macrophage media (HMM) at either 37°C or 25°C, respectively (172). For the growth of *Histoplasma* uracil auxotrophs, media was supplemented with uracil (100 μg/ml). Liquid cultures were aerated (200 rpm) until late exponential growth phase. Growth rate and stage were determined by measuring liquid culture turbidity at 595 nm. For growth on plates HMM was solidified with 0.6% agarose and supplemented to 25 μM FeSO₄. *Histoplasma* yeast were transformed with linearized-plasmid using electroporation, and Ura+ transformants were selected on solid HMM (177). For experiments requiring defined yeast numbers G186A strains had clumps removed by centrifugation (1 min. at 50 xg) and enumerated on a hemacytometer.

5.2.2 Generation of Cfp4 depletion strains

Insertional mutants were generated utilizing the previously described *Agarobacterium tumefaciens* method (286, 287). Briefly, *A. tumefaciens* strains LBA1100 containing plasmid pCM41, containing a T-DNA insertion region with HYG resistance marker, was grown in induction media (0.2% glucose, 200 μM acetosyringone and 100 mM MES pH 5.3) for 16 hours at 25°C. Bacteria were harvested at a OD600 of 1.5 (1.5x10⁹ bacteria/ml) by centrifugation and Wu8 *Histoplasma* yeast harvested from fresh plates seeded with 4x10⁵ yeast/cm² by flooding plates with liquid HMM. For co-
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WU8</td>
<td>G186A ura5-32</td>
<td></td>
</tr>
<tr>
<td>Wu15</td>
<td>G217B ura5-42</td>
<td></td>
</tr>
<tr>
<td>OSU6</td>
<td>G186 ura5-32Δ cfp4-1::T-DNA (RL, hph) -209 bp from start codon</td>
<td>CFP4(-)</td>
</tr>
<tr>
<td>OSU18</td>
<td>G186A ura5-32Δ ags1-4Δ::hph / pCR473 [URA5, gfp-RNAi]</td>
<td>ags1Δ</td>
</tr>
<tr>
<td>OSU22b</td>
<td>G186A ura5-32Δ zzz::pCR482 [hph, gfp]</td>
<td></td>
</tr>
<tr>
<td>OSU45</td>
<td>G186A ura5-32Δ / pCR468 [URA5, gfp:FLAG]</td>
<td>CFP4(+)</td>
</tr>
<tr>
<td>OSU75</td>
<td>G217B ura5-42Δ / pCR468 [URA5, gfp:FLAG]</td>
<td>CFP4(+)</td>
</tr>
<tr>
<td>OSU77</td>
<td>G186A ura5-32Δ / pCR468 [URA5, gfp:FLAG]</td>
<td></td>
</tr>
<tr>
<td>OSU84</td>
<td>G186A ura5-32Δ cfp4-1::T-DNA / pCR468 [URA5, gfp:FLAG]</td>
<td>CFP4(-)</td>
</tr>
<tr>
<td>OSU85</td>
<td>G186A ura5-32Δ cfp4-1::T-DNA / pCR540 [URA5, P_{h2B}-tomatoeRFP:FLAG]</td>
<td>CFP4(-)</td>
</tr>
<tr>
<td>OSU87</td>
<td>G217B ura5-42Δ / pEH09 [URA5, CFP4-RNAi]</td>
<td>CFP4(-)</td>
</tr>
<tr>
<td>OSU107</td>
<td>G186A ura5-32Δ cfp4-1::T-DNA / pCR521 [URA5, P_{h2B}-CFP4:FLAG]</td>
<td></td>
</tr>
</tbody>
</table>

* uracil auxotroph of G186A (Marion CM, et al., 2006 (78))

b GFP sentinel RNAi background (Edwards, et al. 2011 (81))
cultivation $1.5 \times 10^9$ bacteria were mixed with $5 \times 10^7$ yeast in 400 ul induction media and plated on Whatman #5 paper on solid induction media supplemented with 0.7 mM cysteine and 100 ug/ml uracil. Co-cultures were incubated at 25°C for 48 hours to promote the transfer of T-DNA into yeast. Filter paper was then transferred to selection media (HMM supplemented with 100 ug/ml uracil, 150 ug/ml hygromycin and 200 uM cefotaxim) and incubated at 37°C with 5% CO$_2$/95% air until colonies appeared. Mutants were harvested by flooding plates to create mutant pools, that were then diluted 1:200 in HMM and grown at 37°C with aeration for 48 hours. Total nucleic acids were prepared from half of the mutant pool by bead beating cell pellets, phenol:chloroform extraction of lysates and ethanol precipitation of total nucleic acid. This material was used as template for PCR reactions to determine the presence of the desired mutant in a pool. Insertions were identified using either a primer specific for the left or right T-DNA border or a primer specific for Cfp4. The individual insertion was recovered from the mutant pool using previously described methods (287).

Depletion of Cfp4 was also carried out using RNA interference. The full CFP4 coding region was amplified from cDNA using CFP4-specific primers with PCR and two copies were cloned in inverse orientation into the RNAi gfp-sentinel vector, pCR473. Linearized plasmids were transformed into OSU22 (G186A background expressing a gfp transgene) or OSU32 (G217B expressing a gfp transgene) and Ura+ transformants were screened for silencing of GFP using a UV transilluminator. With depletion or insertion mutants the loss of Cfp4 was confirmed by growing wild-type or depletion yeast in HMM
to late exponential growth phase and removing yeast from HMM by centrifugation. Supernatant volumes were normalized by growth phase and deglycosylated. Deglycosylation was performed using PNGaseF enzyme and the recommended standard deglycosylation procedure (NEB). Supernatants were treated with Denaturation buffer (0.2% SDS and 100 mM DTT) and heating at 100°C for 10 minutes before the addition of NP-40 (1%) in G7 reaction buffer (50 mM Na-phosphate pH7.2) and a 1:100 dilution of stock PNGaseF enzyme. Reactions were incubated overnight at 37°C. Samples were examined for the loss of Cfp4 using SDS-PAGE and silver staining (211). Insertion mutants were complemented by transforming OSU6 with a linearized plasmid containing a G186A genomic copy of CFP4.

5.2.3 Macrophage killing assay

To determine the role of Cfp4 in macrophage killing we utilized the previously described P338D1-lacz macrophage killing assay (288). Macrophages were maintained in Ham’s F-12 with 10% heat inactivated fetal bovine serum (FBS). For experiments macrophages were seeded at 2.5x10^4 macrophages per well of a 96-well plate and allowed to replicate for 16 hours at 37°C with 5%CO2/95% air. Wild-type, OSU6, OSU87, OSU1 or OSU75 yeast were grown to late exponential growth phase in HMM, pelleted by centrifugation and counted on a hemacytometer. Yeast pellets were suspended in HMM-M (HMM buffered to pH 7.2 with 25 mM bicarbonate) with 10% FBS and
added to macrophage containing wells at 2.5x10^4, 5x10^4 or 1x10^5 yeast per well and incubated at 37°C with 5%CO₂/95% air for 7 days. Monolayers were visually examined daily compared to wells containing uninfected macrophages or wells containing macrophages with wild-type yeast as a positive and negative control. The number of macrophages remaining was determined by measuring total β-galactosidase activity in each well. Culture media was removed and macrophages were lysed by the addition of PBS containing 0.5% Tritox X-100, 2 mM MgCl₂ and 2 mg/ml o-nitropenyl-β-D-galactopyranoside (ONPG). Yellow color was allowed to develop for 30 minutes at room temperature before reading color change at 420 nm with a correction at 595 nm on a plate reader.

5.2.4 Isolation and infection of mouse peritoneal macrophages

Resting peritoneal macrophages were isolated from five C57BL/6 mice (NCI) as previously described (218). Briefly, mice were euthanized with CO₂ and 10 ml of cold PBS was injected into the peritoneum followed by messaging the peritoneum for about 1 minute. PBS was recovered from the peritoneum using a syringe with an 18-gauge needle. Cells were collected by centrifugation (700xg for 10 min. at 4°C), suspended in DMEM with 10% FBS and enumerated with a hemacytometer. Cell viability was determined using Trypan Blue. Macrophages were seeded at 2x10^5 cells per well of a non-tissue culture treated 96-well plate and incubated at 37°C with 5% CO₂/95% air for 3
hours to allow adherence. Non-adherent cells were removed by washing wells three times with PBS leaving $1 \times 10^5$ macrophages per well. $CFP4(+)$, OSU45 and OSU75, or $CFP4(-)$, OSU84 and OSU87, Panema and North American strains were grown in HMM, pelleted and suspended in DMEM with 10% FBS before counting on a hemacytometer. Yeast were then added to macrophage containing wells at $2 \times 10^3$ yeast per well. Infected wells were incubated at 37°C with 5% CO$_2$/95% air for 4 hours before culture media was removed and macrophages were lysed with cold water and scrapping. Lysates were diluted and plated on HMM to determine viable yeast cfu. Colonies for each infection were enumerated and compared to wells containing only yeast to determine % yeast survival.

5.2.5 Isolation and infection of human monocyte derived macrophages

Blood was harvested from healthy human volunteers and cells were isolated as previously described (272). Briefly, blood was collected by venipuncture into syringes containing or lacking 1000U/ml heparin using a butterfly needle. Autologous serum was prepared by adding non-heperanized blood into glass tubes and allowing blood to coagulate for 1 hour at room temperature followed by 1 hour on ice before centrifugation (200xg for 15 minutes at 4°C). The yellow serum layer was collected and filter sterilized before storing at -20°C. About 20 ml of heparinized blood was mixed with 15 ml of PBS and 14 ml of Ficoll-Paque PLUS was underplayed into the blood suspension before
centrifugation (400 rcf for 40 minutes at 18°C). The upper plasma layer was removed and “buffy coats” and Ficoll was pooled into 50 ml conical tubes with volumes adjusted to 50 ml with PBS. Cells were pelleted (200 rcf for 15 minutes at 4°C) and resuspended in 5 ml of RPMI 1640 before counting on a hemacytometer. Total cell concentration was adjusted to 2x10⁶ cells/ml in Teflon wells with RPMI 1640 containing 20% autologous serum and incubated for 5 days at 37°C with 5% CO₂/95% air to allow differentiation. Cell suspensions were chilled on ice for 30 minutes before cell suspensions were removed and wells were washed three times with 4 ml RPMI 1640. Washes were pooled with cell suspensions and cells were pelleted (200 rcf for 15 minutes at 4°C) and suspended in 3 ml RPMI 1640 before enumerating cells on a hemacytometer. Total cell counts were determined and the cell suspension was diluted to 4x10⁶ cells/ml in RPMI 1640 with 10% autologous serum. Cells were seeded at 8x10⁴ total cells per well and incubated for two hours at 37°C with 5% CO₂/95% air. Non-adherent cells were removed by washing wells three times with PBS and cells were finally left in RPMI 1640 with 10% autologous serum. CFP4(+), OSU45 and OSU75, or CFP4(-), OSU84 and OSU87, Panama and North American strains were grown to late exponential growth phase in HMM before yeast were pelleted and enumerated with a hemacytometer. Yeast were diluted and added to macrophage containing wells at 2x10³ yeast/well and infections were allowed to proceed for 4 hours at 37°C with 5% CO₂/95%air. In some experiments macrophages were first activated with 100 U of recombinant human IFN-y (Biolegend) for 48 hours in RPMI 1640 with 10% autologous serum before infections. After 4 hours media was removed and macrophages were lysed with cold water and scraping before
diluting lysates and plating on HMM to determine viable yeast cfu. Colonies were enumerated from each infection and compared to wells containing yeast and complete media to determine % yeast survival.

5.2.6 Bioinformatic analysis of Cfp4.

The CFP4 coding region was obtained from the sequenced genomes of G186A, Nam1 and G217B from The Genome Institute at Washington University (St. Louis) and from the Broad Institute (MIT). Nucleotide sequences were translated to amino acids in silico and sequences were examined using BioEdit software. The presence of a secretion signal peptide was determined using SignalP 3.0 (175) and putative asparagine linked glycosylation sites were predicted using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/).

5.2.7 Mouse infection assays.

To determine if Cfp4 is required for *Histoplasma* growth during infection we grew OSU45, OSU75, OSU84 and OSU87 in HMM to late exponential growth phase at 37°C. Yeast were pelleted by centrifugation, suspended in HMM and enumerated with a hemacytometer. Four C57BL/6 mice (NCI) were used for each strain at each time point
and intranasally infected with $2 \times 10^4$ yeast in HMM. Initial infection innoculums were plated on solid HMM to determine the actual inoculum size. Mice were euthanized with CO$_2$ and lungs and spleens collected at 4, 8, 12, 16 and 20 days post infection. Organs were manually homogenized to liberate yeast from host tissue and lysates were diluted and plated on HMM to enumerate viable yeast cfu at 37°C with 5% CO$_2$/95% air. Statistical analysis of data was performed using the student T-test.

For competition assays the wild-type *Histoplasma* strain OSU77, Wu8 containing pCR468 (expressing GFP), and the cfp4::T-DNA OSU85, OSU6 containing pCR540 (expressing tomato RFP) were grown in HMM to late exponential growth phase at 37°C with aeration (200 rpm). Yeast were pelleted and suspended in HMM before enumeration on a hemacytometer. OSU77 and OSU85 were mixed at a 1:1 ration containing a final count of $1 \times 10^4$ yeast, $5 \times 10^3$ yeast of each strain. The mixed yeast suspension was used to infect five C57BL/6 (NCI) mice Innoculums were plated on solid HMM to determine total actual inoculum size and to confirm starting OSU77:OSU78 ratios. The infection innoculums were also used to start 3 ml liquid HMM cultures at 37°C with aeration (200 rpm) to determine if the OSU77:OSU78 ratios were significantly altered during *in vitro* growth. After 1, 3, 4 and 5 days after inoculation some of the culture was diluted and plated on solid HMM to determine viable yeast cfu and the ratio of yeast strains at each point. After 7 days post infection lungs were removed, homogenized, diluted and plated on solid HMM to determine viable cfu counts. After colonies were grown the total ratio of OSU77:OSU78 was determined on a modified UV transilluminator using filters for
GFP or RFP. GFP+ or RFP+ colonies were enumerated in addition to the total number of colonies present. Competition index was determined as previously described by using the formula \[\frac{(OSU78/OSU77)_N}{(OSU78/OSU77)_{Starting}}\](90).

5.2.8 Cfp4 recognition by the human immune system.

*Histoplasma* yeast strains Wu8, Wu15, OSU6 or OSU87 were grown in 100 ml liquid HMM cultures until late exponential growth phase. Yeast were removed by centrifugation (2000 rcf for 5 minutes) and then filtered through 0.22 um filters. Supernatants were concentrated using an Amicon Ultrafiltration unit with a 10 kDa molecular weight cut off PES membrane (Millipore). The concentrations of culture filtrates were determined using a DC protein assay with ovalbumin protein standard. Some of each sample (200 ug) was deglycosylated as described above and sent to MiraVista Diagnostic Laboratories in Indianapolis, Indiana to determine reactivity with human serum samples. About 5 ug of total native or PNGase F deglycosylated culture filtrate protein were resolved on a 10% SDS-PAGE and transferred to nitrocellulose in transfer buffer. Membranes were then blocked and probed with pooled high-titer histoplasmosis immune serum at 1:500 in blocking buffer. Protein recognition was visualized using biotinylated anti-human secondary (1:10,000) and streptavidin-horse radish peroxidase (0.2 μg/ml) with Opti 4CN Substrate color detection (Bio Rad).
Immonoblots performed with pooled high-titer Blastomyces or healthy human immune sera were performed as above.

5.3 Results.

5.3.1 Cfp4 is abundantly expressed yeast protein.

We previously identified Cfp4 during a proteomics screen of pathogenic-phase *Histoplasma* extracellular proteins (79). We found that this protein is heavily glycosylated and that transcript is extremely enriched in the pathogenic yeast-phase. This protein is encoded in the genome of multiple *H. capsulatum* clinical isolates including two North American isolates (Nam1 and Nam2) and the Panamerican strain (Figure 5.1A). The Cfp4 protein shares extremely high sequence conservation between *Histoplasma* isolates, with a minimum of 83% identity and 88% similarity, but lacks functional homology to known proteins. Using SignalP we determined that all three sequences contain strong canonical N-terminal signal peptides, targeting the protein towards the secretory pathway. The N-terminal portion of Cfp4 also contains a high percentage of proline, serine and threonine residues which is a common feature of some proteins associated with fungal cell walls.
Figure 5.1 Multiple *Histoplasma* isolates express Cfp4 protein. (A) Cfp4 protein is conserved among *Histoplasma* isolates. Amino acid sequences from Panamanian (G186AR) or North American (Nam1 or G217B) aligned using ClustalW. Darkened residues indicate amino acid identity between isolates. The signal peptide targeting Cfp4 to the secretory pathway is indicated. (B) Panama and North Americal isolates secreted Cfp4. Supernatants from wild-type G186A (OSU45), *CFP4(-) G186A* (OSU84), wild-type G217B (OSU75) or *CFP4(-) G217B* (OSU87) were deglycosylated with PNGaseF and equal volumes separated by denaturing polyacrylamide gel before visualizing total protein by silver staining.
Our previous work determined that the Panama strain Cfp4 is heavily glycosylated, especially with asparagine-linked sugars. These polysaccharide residues greatly alter protein motility on one-dimensional denaturing polyacrylamide gels, such that Cfp4 cannot be localized to any single protein band. Utilizing the NetNGlyc asparagine-linked glycosylation predictor we identified the putative N-linked glycosylation sites, NXS/T where X is any amino acid except proline, and determined that the Panama strain possesses two canonical sites while the North American isolates each contain three putative sites. Several, or all, of these sites are attached to sugar moieties since enzymatic deglycosylation by PNGase F compresses Cfp4 into a single band in both Panama and North American isolates (Figure 5.1B). PNGaseF is an enzyme that specifically cleaves polysaccharides from glycoproteins at the linkage between asparagine and the conserved N-acetylglucosamine residue. Interestingly, even after the removal of N-linked polysaccharides Cfp4 does not migrate at the predicted protein mass of 25 kDa. This strongly implies that Cfp4 also contains O-linked glycosylations since those linkages would be resistant to PNGase F cleavage. O-linked glycans are less structured, contain fewer carbohydrate residues and lack the core polysaccharide contained in N-linked glycans. Cfp4 contains numerous serine and threonine residues that could be potential sites of glycosylation. Interestingly, the molecular weight of Cfp4 varies between North American and Panamanian isolates after deglycosylation, with the Panamanian strain Cfp4 running slightly slower on the gel, 33 kDa compared to about 31 kDa. This could be indicative of differing levels of O-linked glycosylation between the two strains.
5.3.2 *Histoplasma* does not require Cfp4 for growth within macrophages.

We sought to determine if *Histoplasma* requires Cfp4 for virulence in macrophages. To determine if Cfp4 plays a functional role during macrophage parasitism we depleted Cfp4 from *Histoplasma* yeast. We depleted Cfp4 from the Panamerican strain by utilizing *Agarobacterium tumafacions* to insert T-DNA into the *Histoplasma* genome. The T-DNA insertion was 209 bp upstream of the CFP4 starting codon. To deplete Cfp4 from the Nam2 background we transformed yeast with a linearized plasmid containing *CFP4*-RNAi to knock down expression levels. We verified that both depletion strains lacked Cfp4 by treating *Histoplasma* culture supernatants with PNGaseF before separation by SDS-PAGE and visualizing protein by silver staining (Figure 5.1B). In both cases Cfp4 is no longer detected by silver staining in depletion strains but readily visible in isogenic wild-type strains. Both depletion strains grow at rates equal to wild-type (data not shown).

To determine if Cfp4 plays a role during macrophage infection we utilized P388D1 macrophage-like cells that have been transfected with a lacZ transgene. Our lab has previously utilized this model to identify *Histoplasma* yeast attenuated for growth within macrophages (288). This system quantifies the ability of yeast to destroy macrophage monolayers by measuring remaining β-galactosidase activity 7 days post infection compared to uninfected wells. Macrophages were seeded into wells of a 96-well plate and infected at MOIs of 0.5:1, 1:1 and 2:1 with Panamerican *CFP4(+) or CFP4(-)*,
Figure 5.2 *Histoplasma* does not require Cfp4 to replicate within and kill macrophages. Total macrophage survival after *Histoplasma* infection. Wild-type G186A (OSU45), CFP4(-) G186A (OSU84), a known attenuated G186A lacking α-glucan (OSU18), wild-type G217B (OSU75) or CFP4(-) G217B (OSU87) were used to infect monolayers of P388D1-lacz macrophages at an MOI of 1:1. Macrophages and yeast were co-cultured for 7 days at 37°C before supernatants were removed and the number of remaining macrophages was determined by lysing macrophages with a solution containing ONPG. Color change was monitored at 420 nm. The plotted data represents means ± standard deviations of a representative experiment performed in triplicate. Results are representative of three experiments performed in triplicate. There is no statistical difference between CFP4(+) and CFP4(-) infected macrophage monolayers.
or Nam2 containing a gfp-RNAi or CFP4-RNAi plasmid. After 7 days media was removed and macrophages were lysed with buffer containing ONPG. Yellow color development was monitored to determine the relative number of remaining macrophages (Figure 5.2). Both wild-type yeast isolates are readily able to destroy macrophage monolayers, 29% and 27% macrophages remaining, while the attenuated agsIΔ is defective in monolayer destruction, 78% macrophages remaining. However, Histoplasma lacking Cfp4 are able to destroy macrophage monolayers as efficiently as CFP4(+) yeast, indicating that Cfp4 is not involved in macrophage killing. However, P388D1 cells suffer from several limitations, including the inability to produce ROS. All strains were able to destroy macrophage monolayers at equal levels regardless of the MOI used.

Although Cfp4 was not involved in Histoplasma’s ability to replicate within and kill murine cells, it is possible that it could protect yeast from primary cells during human infection. To test this hypothesis blood was collected from healthy donors and monocytes were isolated and differentiated into macrophages. After differentiation cells were seeded into wells and the non-adherent cells were removed. Macrophages were infected with wild-type or Cfp4 depletion strains. After 4 hours macrophages were lysed with water and lysates were plated to determine viable cfu counts. We found that yeast lacking Cfp4 were able to survive at wild-type levels in human macrophages. This indicates that Cfp4 is not required for full virulence within human macrophages (Figure 5.3). Our results indicate that Histoplasma does not require Cfp4 to survive and grow within murine or human macrophages.
Figure 5.3 Cfp4 is not required for *Histoplasma* survival in human macrophages.

Survival of *Histoplasma* yeast in human monocyte derived macrophages. Wild-type G186A (OSU45), *CFP4(-)* G186A (OSU84), wild-type G217B (OSU75) or *CFP4(-)* G217B (OSU87) strains were co-incubated with macrophages at an MOI of 1:50. Yeast survival was determined by enumeration of viable cfu after 4 hours of co-incubation with human macrophages at 37°C. Results are plotted as relative yeast survival (means ± standard deviation of 3 replicates) compared to viable cfu of yeast incubated in the absence of macrophages. There is no statistical difference in yeast survival between *CFP4(+) and CFP4(-) strains.*
Figure 5.3

% Yeast Survival

CFP4(+)  CFP4(-)  CFP4(+)  CFP4(-)

G186A  G217B
5.3.3 Cfp4 is dispensable for growth in the mouse.

To address any potential role of Cfp4 in *Histoplasma* virulence we infected mice with *CFP4(+) or CFP4(-) Panamerican* or Nam2 *Histoplasma* isolates. C57BL/6 mice were intranasally infected and lungs and spleens were removed at 4, 8, 12 and 16 days post-infection. Organs were homogenized and the fungal burdens were monitored by quantitative platings (Figure 5.4 and Figure 5.5). Wild-type yeast of either strain survive and replicate within the mouse lung and increase to a fungal burden 100 times higher than inoculum 8 days post infection. The fungal burden decreases after day 12 and continues dropping through day 16 when the adaptive immune system becomes involved and infection is controlled. *Histoplasma* reaches the spleen at detectable levels by day 8 and total fungal burden peaks between days 8 and 12 post infection, and begins to decrease on later days. The Panamerican strain lacking Cfp4 grows and survives within mouse lungs and spleens identically to *CFP4(+) yeasts*. Nam2 isolates lacking Cfp4 were also able to readily replicate within mouse lungs in a manner similar to *gfp-RNAi* containing controls. Interestingly, a small defect in spleen colonization at day 8 compared to *gfp-RNAi* containing yeast was detected. This 5.2 fold decrease at day 8 was consistent through multiple mouse infections, but after day 8 post-infection *CFP4-RNAi* yeast strains reached a fungal burden identical to the *gfp-RNAi* containing wild-type, indicating the observed phenotype was only transient.
Figure 5.4 Panamerican *Histoplasma* isolates do not require Cfp4 for growth in vivo.

(A) Kinetics of sublethal lung infection by *Histoplasma*. Wild-type C57BL/6 mice were intranasally infected with approximately 1x10⁴ wild-type (OSU45) or CFP4(-) (OSU84) strains. At 4 day intervals post-infection fungal burden in lungs were determined by quantitative plating for *Histoplasma* cfu. (B) Kinetics of dissemination following lung infection with *Histoplasma*. At each time point organs were harvested and fungal burden in spleen tissue was determined by quantitative platings for cfu. In both (A) and (B) each data point represents cfu counts per organ from an individual animal (n=4 per time point) and horizontal bars represent mean fungal burden. There is no significant difference in organ fungal burden between CFP4(+) and CFP4(-).
Figure 5.4

(A) CFU/Lung

CFU/Lung

Days post infection

(CFP4(+))

(CFP4(-))

(B) CFU/Spleen

CFU/Spleen

Days post infection

228
Figure 5.5 North American *Histoplasma* isolates do not require Cfp4 for growth in *vivo*. (A) Kinetics of sublethal lung infection by *Histoplasma*. Wild-type C57BL/6 mice were intranasally infected with approximately $1 \times 10^4$ wild-type (OSU75) or CFP4(-) (OSU87) strains. At 4 day intervals post-infection fungal burden in lungs were determined by quantitative plating for *Histoplasma* cfu. (B) Kinetics of dissemination following lung infection with *Histoplasma*. At each time point organs were harvested and fungal burden in spleen tissue was determined by quantitative platings for cfu. In both (A) and (B) each data point represents cfu counts per organ from an individual animal (n=4 per time point) and horizontal bars represent mean fungal burden. Asterisks represent significant difference from wild-type (* p<0.05 and ** p<0.01), all other fungal burdens are not significantly different from CFP4(+).
Figure 5.5

A

CFU/Lung

10^7

10^6

10^5

10^4

10^3

10^2

0 4 8 12 16

Days post infection

CFP4(+)

CFP4(-)

B

CFU/Spleen

10^6

10^5

10^4

10^3

10^2

4 8 12 16

Days post infection
To determine if Cfp4 bestows an advantage to *Histoplasma* during mouse infection, the effect of Cfp4 loss on fitness compared to *CFP4(+)* yeast was determined. *Histoplasma* was transformed, the *CFP4(+) (Wu8)* or *CFP4(-) (OSU6)*, with linearized plasmids containing *RFP* or *GFP* driven by a constitutive promoter. Yeast were grown to late-exponential growth phase and mixed at a 1:1 ratio before intranasally infecting C57BL/6 mice. Liquid cultures were also grown of the mixed suspension to ensure that neither strain possessed a growth advantage *in vitro*. At 9 days post infection lungs were removed, homogenized and lung homogenates plated to determine viable yeast cfu and the ratios of OSU78 to OSU84 (Figure 5.6). The growth of both strains in liquid culture was identical resulting in a competition index (cI) of 1 for the initial mixture and 1.6 after 5 days of culture demonstrating that the loss of Cfp4 does not cause a change in fitness *in vitro*. Within the lung after 9 days the competition index reached 1.3, nearly identical to the value observed in HMM alone indicating that neither strain has a fitness advantage over the other within the lung.

5.3.4 Human *Histoplasma* immune serum detects Cfp4.

We next wanted to determine if Cfp4 was produced during human infection and if it had a potential use as a diagnostic antigen. Previous results indicated that *CFP4* is transcribed during mouse lung infection indicating possible expression during human *Histoplasma* infection (211). Yeast strains with and without Cfp4 in the North American
Figure 5.6 *Histoplasma* yeast expressing Cfp4 do not have a fitness *in vitro* or *in vivo*.

Survival of *Histoplasma* with or without Cfp4 during mouse lung infection. Wild-type C57BL/6 were intranasally infected with approximately $1 \times 10^4$ total yeast, half of these were wild-type G186A expressing GFP (OSU77) or CFP4(-) expressing RFP (OSU85). After 9 days post infection fungal burden in the lung was determined by quantitative plating for *Histoplasma* cfu. Additionally, starting inoculum was used to start a *Histoplasma* liquid culture that was maintained at 37°C, after 5 days this suspension was quantitatively plated to determine cfu. The ratio of CFP4(+) to CFP4(-) was determined by quantifying the number of GFP(+) or RFP(+) colonies using a UV transilluminator and Alpha Imager software. There is no significant difference between *Histoplasma* grown in culture or within mice.
Figure 5.6

Competition index

Starting  HMM  Lungs

Log scale for y-axis
Figure 5.7 Cfp4 is recognized by the human immune serum during histoplasmosis infection. (A) Human immune sera from patients with histoplasmosis recognize Cfp4. Culture filtrates from wild-type G186A (OSU45), G186A CFP4(-) (OSU84), sod3Δ (OSU15), wild-type G217B (OSU75) or G271B CFP4(-) (OSU87) were resolved on a polyacrylamide gel with or without prior treatment with PNGaseF. Proteins were transferred to nitrocellulose and probed with human immune sera from patients with histoplasmosis before detection. Proteins were visualized using biotin conjugated anti-human antibodies and streptavidin-HRP in the presence of developing solution. (B) Human sera from patients with blastomycosis does not strongly recognize Cfp4. As with (A) using human immune sera from patients with blastomycosis. (C) Healthy humans do not produce an antibody response to Cfp4. As with (A) and (B) using human immune sera from healthy individuals. The red arrow indicates the position of Cfp4.
Figure 5.7
and Panamerican isolates were grown to late-exponential growth phase, and culture filtrates were prepared by removing yeast by centrifugation and filtration. Culture filtrates were concentrated and deglycosylated before samples were immune blotted with pooled immune sera from patients infected with *Histoplasma* (Figure 5.7A). Interestingly, there are no visible differences between *CFP4* (+) or *CFP4* (-) strains when Cfp4 is in its native form, however after deglycosylation human immune serum readily detects the 33 kDa Cfp4 protein in both geographical isolates expressing Cfp4 but not in the Cfp4 depletion strains. This indicates that Cfp4 is produced during human infection and that it is detected by the human immune response. Additionally, performing the immunoblot with sera from individuals infected with the closely related dimorphic fungi *Blastomyces dermatitidis* only faintly recognizes Cfp4 indicating specificity to *Histoplasma* (Figure 5.7B). Serum from healthy individuals does not recognize the Cfp4 protein (Figure 5.7C).

### 5.4 Discussion

In this study we examined the potential virulence function of the highly glycosylated and abundant *Histoplasma* Cfp4 protein during yeast infection of macrophages or mice. Cfp4 plays no apparent role during *Histoplasma* growth and survival within macrophages, or during mouse infection. This result was surprising since Cfp4 is extremely yeast-phase enriched at both the transcript and protein level, which generally correlates with a role in *Histoplasma* virulence. Although Cfp4 does not play a role during infection it is readily
detected by *Histoplasma* human immune serum indicating that the abundant protein could be utilized as a potential diagnostic antigen.

The coding region of *CFP4* is found in multiple geographically distinct *Histoplasma* isolates at a high degree of sequence conservation implying that the encoded protein is generally important for *Histoplasma* yeast. The Cfp4 protein possesses an N-terminal signal peptide trafficking it to the *Histoplasma* secretory pathway and into the extracellular environment (Figure 5.1A and (211)). As is the case with most proteins secreted by eukaryotes Cfp4 contains polysaccharide structures linked to asparagine or serine/threonine residues. Depending on the *Histoplasma* isolate Cfp4 contains two or three asparagine residues that can form glycosidic bonds and that the attached polysaccharides greatly alter the mass of Cfp4 from the predicted 24 kDa to a range of molecular weights between 60 kDa or as high as 200 kDa. It is also possible that Cfp4 is associated with the yeast cell wall, as the protein contains a stretch enriched in threonine and proline that is characteristic of some fungal cell wall proteins (289). Additionally, the high sugar character associated with Cfp4 is characteristic of pathogenic fungal cell wall mannoproteins in fungi (290, 291). However, Cfp4 does not appear to play a role in cell wall stability as depletion strains are as resistant to cell wall perturbing agents, such as calcoflour white and congo red, as *CFP4(+)* strains (data not shown).

The lack of attenuation in murine and human macrophages coupled with normal growth and survival within the mouse was surprising. These results demonstrate that Cfp4 is not involved in *Histoplasma* virulence despite being highly pathogenic-phase
enriched. Cfp4 has the same general characteristics of previously identified factors required for *Histoplasma* survival in macrophages or *in vivo* like Cbp1 (83, 84), Yps3 (92, 96) and alpha-glucan biosynthesis proteins like Ags1 (72) in that all are highly enriched in the pathogenic-phase and are either highly expressed at the protein level or their end product is exclusive to the yeast-phase. Additionally, Cfp4 is similar to the secreted Cbp1 since it is expressed by strains with and without alpha-glucan on the cell wall unlike Yps3 that is only expressed by strains lacking alpha-glucan (71, 92). One possible explanation for the apparent lack of Cfp4 function in these model systems would be the presence of an extracellular factor(s) with redundant function. A search of the Panamerican isolate’s genome for proteins sharing sequence similarity to Cfp4 revealed one region encoding a putative 178 amino acid peptide that has 44% identity and 63% sequence similarity to Cfp4. This protein also contains a canonical N-terminal signal peptide marking it for the secretory pathway, however we have never identified this protein as a constituent of the yeast-phase extracellular proteome using batch Shotgun proteomics on multiple *Histoplasma* culture filtrates in either rich or minimal media.

The Cfp4 protein has been previously identified as the *Histoplasma* immunoreactive protein (195). In a previous study, Cfp4 was expressed in *E. coli* and found that it had reactive properties with *Histoplasma* immune sera from animals. Here, we provide evidence that human’s also produce antibodies to Cfp4 in response to *Histoplasma* infection. Additionally, a human immune serum from patients infected with the closely related *B. dermatitidis* does not recognize the Cfp4 protein very strongly.
indicating specificity for *Histoplasma*. Currently the most common diagnostic test for histoplasmosis involves the detection of the *Histoplasma* polysaccharide antigen within urine, sera or other bodily fluids using a sandwich ELISA method (184). This assay is a good indicator of histoplasmosis but currently most diagnostic tests for histoplasmosis suffer greatly from cross-reactivity with other endemic mycosis, and Cfp4 could potentially represent a marker that would avoid those problems. Creating an ELISA based assay to detect Cfp4 in patient samples is potentially very exciting but multiple complications remain. The most substantial problem is the high degree of protein glycosylation. So far, we have been unable to purify or visualize Cfp4 using protein staining techniques or through immunoblots for *CFP4*-FLAG. Only after removal of the N-linked glycan residues can Cfp4 be visualized by staining or immunobot. However, human immune sera does readily recognize deglycosylated Cfp4, indicating that the antibodies are produced to areas not occluded by polysaccharides and thusly accessible to potential diagnostic antibodies used in an ELISA. Recently, our lab has developed several monoclonal antibodies specific for Cfp4 that recognize deglycosylated Cfp4. These Mab also do not appear to recognize the native form of Cfp4, however it is possible that native Cfp4 detection is difficult due to the presence of numerous glycoforms, Cfp4 linked to glycoproteins of various size and/or structure that are individually to dilute to detect. Therefore, it might be possible to utilize an ELISA based technique with a monoclonal antibody that could collect all the glycoforms in a well and allow for easier detection. This possibility is exciting but requires additional experimentation to determine if Cfp4
can be detected in this manner and if Cfp4 is present in fluids from animals or humans with histoplasmosis.
Chapter 6: Conclusions

Here we have sought to identify and characterize novel extracellular proteins for roles in *Histoplasma* virulence. We identified the 33 most abundantly expressed extracellular proteins produced by *Histoplasma* during growth in HMM (Chapter 2). The proteins we identified fall into multiple putative functional categories with the largest categories being comprised of proteins involved with cell wall biosynthesis, proteins of unknown function, oxidative defense and proteins with predicted chaperone activities. Of those proteins we chose to first characterize a previously undescribed Cu/Zn superoxide dismutase (Sod3) and found that it is absolutely essential for yeast replication and disease causation in the mouse animal model (Chapter 3). Specifically this protein protects yeast from superoxide stress generated by neutrophils and activated macrophages. We examined the extracellular catalase (CatB) for a role in ROS defense but found that this protein is dispensable for yeast survival within animals (Chapter 4). We found that CatB protects yeast from hydrogen peroxide stress *in vitro* and to a minor extent in human neutrophils but plays no role during yeast survival in resting or activated human macrophages. Additionally we examined the role of an abundant protein of unknown function (Cfp4) for any potential role during infection. We determined that Cfp4 is
dispensable for survival within neutrophils, macrophages and during grown in the animal model, but Cfp4 is recognized specifically by human immune sera indicating that Cfp4 could be used as diagnostic marker.

The *Histoplasma* secreted proteome consisted of 121 total proteins that met our rigorous identification criteria. Using our criteria we hoped to avoid detection of peptide fragments representing the “dumping” of contents from within lysosome-like compartments (292). The functional diversity observed in the proteome was unsurprising, as other fungal or parasite extracellular proteomes are also extremely diverse (124, 125, 127, 147, 148, 151). When examined more closely 33 proteins represented over 70% of the total peptides identified and were present in three independent biological replicate samples. If we expand our findings to proteins identified in at least two biological replicates than we account for 76 of the total 121 proteins and over 90% of the total peptides indicating that our results were highly reproducible. Over 40% of the proteins identified in all three replicates, ranked by abundance, are predicted to contain strong canonical secretion signals by SignalP that would target them to the classical secretion pathway. This ratio is in keeping with bioinformatic studies using SignalP 3.0 indicating that 35.6% of *Histoplasma* strain G186A predicted secreted proteome contains secretion signal peptides (293). Protein identities with signal peptides represent 58% of the peptides identified in all three replicates or 46.2% for proteins found in at least two replicates. This observation indicates that *Histoplasma* makes heavy use of the classical secretion pathway for abundantly expressed proteins. The ratio of extracellular proteins
with signal peptides is high compared to parasites like Leishmania or Trypanosome where only 2% of extracellular proteins contain signal peptides (126, 127) and is similar to the ratio observed for other fungi using Signalp 3.0 prediction servers (293).

Even with a relatively high number of proteins containing signal peptides *Histoplasma* is reported to utilize additional pathways to target proteins to the extracellular environment. These non-classical mechanisms are incredibly varied and difficult to predict in any conclusive manner. In some cases we predict that proteins possessing chaperone activity such as Pdi1 and Hsp82 are not being specifically trafficked to the secretion pathway but are carried with other secretory molecules being trafficked through the Golgi. This would be consistent with previously described protein disulfide isomerase proteins that are abundant within the ER and Golgi (134) and are noted to remain attached to some proteins through the secretion pathway {{438 Benyair,R. 2011}}. Alternative methods of protein secretion have also been reported in *Histoplasma* like vesicle-secretion (198). This pathway is common in fungi and parasites and involves the release of exosome-like vesicles across the cell wall and into the supernatant (127, 149, 152). Polyclonal antibodies from *Histoplasma* infected mice are able to recognize vesicle contents which give some evidence that these structures are produced *in vivo* (198), however the study does not address the possibility that antibody recognition with vesicle proteins could be incidental. Extracellular proteins could be secreted through classical mechanisms within the host and trigger an immune response that is then capable of recognizing similar proteins with secreted vesicles *in vitro*. This
hypothesis is possible, given that known *Histoplasma* immunogenic proteins, like CatB, are not found in extracellular vesicles and two of the proteins utilized as proof of principal, H2b and Hsp60 are both known to be cell wall associated. It remains to be determined if this form of protein secretion is important for virulence. To date no known virulence determinates have been identified within *Histoplasma* secreted vesicles. Additionally, secreted vesicles are noted to contain random samplings of the cytoplasm and not a specific subset of protein cargo destined for the extracellular environment (149, 152, 198). This seems to be the case in *Histoplasma* as a large number of ribosomal proteins and proteasome components are identified within these vesicles in addition to mitochondrial superoxide dismutase proteins (198). Our study does contain some similar identifications which could represent the rupturing of these vesicles during proteomics analysis, however it is more likely that some of these proteins were captured in these secreted vesicles and released, since secreted vesicles are fairly sparse under most culture conditions and require harsh treatment, like chloroform:methanol extractions to rupture(198). Thusly, we would predict based on our observations that classical secretion appears to be the most utilized form of protein secretion in *Histoplasma*. However, this hypothesis is difficult to confirm as the *Histoplasma* genome contains three Sec4 homologs and depletion of any one of these using RNAi does not alter *Histoplasma* growth or alter protein profiles as determined by SDS-PAGE (data not shown). This could imply significant functional redundancy within the secretion system, but further work is required to functional characterize the secretion pathway in *H. capsulatum*.
One of the most interesting proteins identified during proteomics analysis was the Sod3 protein. Since this protein would be predicted to protect *Histoplasma* yeast from phagocyte derived ROS during infection. We determined that this protein was both released into the culture filtrate and is associated with yeast cells, likely by a GPI-anchor. This optimally places Sod3 to interact with ROS produced by phagocytes and would represent a spatially distinct superoxide dismutase compared to the ones required for metabolically produced superoxide. *In vitro* superoxide challenge confirmed that *sod3Δ* were far more susceptible to superoxide challenge but were resistant to hydrogen peroxide stress. This result was consistent with the role of a superoxide dismutase destroying superoxide anion, but having no role in hydrogen peroxide break down. Additionally, we demonstrated that *sod3Δ* were extremely sensitive to killing by human neutrophils and both activated mouse and human macrophages indicating that *Histoplasma* requires Sod3 to protect itself against these cells from the oxidative burst of phagocytes. The yeast strains lacking Sod3 were also unable to effectively replicate within an intranasal murine model. Based on our kinetic intranasal infection model we were able to determine that *sod3Δ* are never reach a fungal burden in excess of the starting inoculum and are cleared from the lung more quickly than wild-type *Histoplasma* yeast. The use of Phox-/- mice allowed us to conclude that yeast were being killed by host derived ROS *in vivo*. This is in very good agreement with the observations that ROS production is required for *sod3Δ* yeast killing by phagocytes. This observation sheds light on conditions *Histoplasma* likely encounters within the host during infection. The *sod3Δ* yeast encounter superoxide stress generated by neutrophils early during infection.
resulting in yeast killing, but resting macrophages could provide a safe haven for

\textit{Histoplasma} within the host as fungal burdens recover around day 8 only to be destroyed when the innate immune system is involved and superoxide levels again increase. This model would imply that Sod3 is particularly important early (≥4 days p.i) and late during infection (≤16 days p.i.), possibly promoting \textit{Histoplasma} reactivation at a later time.

Additionally, we confirmed that Sod3 is required for disease causation utilizing a lethal mouse infection system. The presence of Sod3 was absolutely required for disease causation in mice, as mice infected with yeast lacking Sod3 demonstrated few symptoms and survived through the entire experiment, 14 days p.i., while mice infected with wild-type yeast succumbed to infection by 4 days p.i.

Sod3 is the first factor identified that protects \textit{Histoplasma} specifically from host-produced ROS. This virulence factor represents a major new understanding in how \textit{Histoplasma} survives within the host and causes disease. Generally, \textit{Histoplasma} was regarded to not cause a significant oxidative burst from macrophages (51), and our results confirm this in mouse (Chapter 3) and human (Chapter 4) cells. However, neutrophils, activated macrophages and macrophages engulfing opsonized yeast were able to mediate a burst but yeast were readily able to survive this assault (44-46, 209). Sod3 appears to be responsible for the previous observations that \textit{Histoplasma} is resistant to oxidative killing (44, 45, 61, 164) and its loss causes phagocyte oxidative bursts to become extremely lethal (Chapter 3). It is especially interesting, since superoxide anion appears to be toxic to \textit{Histoplasma} yeast through interactions with a yet unknown factor. The direct toxicity
of superoxide anion has been somewhat controversial but more recently it was
determined that *E. coli* lacking all superoxide dismutases are impaired for growth (35).
The exact cellular factors superoxide interacts with are not known, but proteins with Fe-S
clusters appear especially susceptible (294). Additionally, superoxide anion is charged
and unlikely to pass through biological membranes like hydrogen peroxide and so must
be interacting with a necessary surface exposed factor, such as a protein channel.
Similarly, some bacteria like Salmonella enterica or fungi like Candida albicans also
require a periplasmic or extracellular superoxide dismutase for full virulence indicating
that superoxide defense is a trend amongst pathogens (229, 244).

Superoxide dismutases would also prevent additional downstream oxidative
stresses such as the production of peroxynitrite by activated macrophages. However,
superoxide dismutases do convert superoxide into hydrogen peroxide, which would also
be expected to damage *Histoplasma* as it can readily pass through membranes (35). Our
results indicate that this is not the case, since both wild-type and *sod3Δ* yeast are equally
resistant to hydrogen peroxide stress. *Histoplasma* contains multiple defense proteins
against hydrogen peroxide challenge, including two intracellular catalases and an
extracellular catalase (193). These proteins potentially offer redundant layers of
protection against hydrogen peroxide stress, although CatB would be predicted to be the
factor most important for challenge against ROS since it is located extracellularly,
expressed during lung infection and is the most abundant extracellular protein
*Histoplasma* produces, after Cbp1.
CatB has long been implicated in *Histoplasma* virulence and was one of the first diagnostic markers of histoplasmosis (194, 271, 295). Our study is the first to functionally characterize the role of CatB within phagocytes and in the mouse infection model. A previous study reported that CatB has catalase activity and speculated that the majority of protein released into the supernatant was shed from the cell wall or due to cell lysis (194). That study utilized the Nam2 isolate, which we have determined to produces less extracellular catalase activity than the Panameric strain (data not shown). With the Panameric strain we have seen CatB activity present in the culture filtrate and on the yeast cell during exponential growth. The creation of the catbΔ strain demonstrated that CatB is responsible for the vast majority of catalase activity on the cell and in the culture filtrate. Strains lacking CatB were more sensitive to hydrogen peroxide in vitro than CATB(+) strains indicating the importance of the protein in ROS defense. When catbΔ strains are assayed for survival against human neutrophils a modest survival defect was observed, however catbΔ strains were able to survive within resting or IFN-γ activated human macrophages at a level equal to wild-type yeast. Utilizing an intranasal mouse model it was determined that catbΔ yeast replicate in mouse lungs at a rate equal to wild-type through 12 days post infection. After 16 post infection the catbΔ strains reach a slightly lower fungal burden in the lungs than wild-type and this difference is largest at 20 days post infection, although still not statistically significant. These results taken together indicate that CatB does not appear to play a significant role in *Histoplasma* virulence, except potentially at later infection times.
CatB lacking a role in virulence was surprising, since the protein is best situated to deal with phagocyte produced hydrogen peroxide. The conflicting results observed during *in vitro* hydrogen peroxide and culture with phagocytes was surprising as both neutrophils and macrophages produce hydrogen peroxide as part of their oxidative burst. A small survival defect was observed when the *catbΔ* strain is co-cultured with human neutrophils, consistent with the *in vitro* observations, but no such defect is observed in resting or activated human macrophages. The most likely explanation for the discrepancy is the level of hydrogen peroxide present in each system, as catalase proteins can be critical for protection against high levels of hydrogen peroxide but not for protection against levels encountered *in vivo* (282). This could be the result of a more intensive oxidative burst by neutrophils compared to macrophages. It is possible that CatP or CatA compensate for the loss of CatB in yeast, although CatA expression is low in yeast cells (193). CatP is transcribed at a high level and is responsible for nearly all intracellular catalase activity in yeast.

Although the loss of CatB alone had no detrimental affect on *Histoplasma* survival within the lung, the additional loss of Catp resulted in yeast destruction by human PMNs or activated human macrophages. It is likely that CatP is involved primarily in removing metabolically produced hydrogen peroxide stress from aerobic respiration, as expression levels are equivalent in pathogenic and non-pathogenic *Histoplasma*. The loss of CatP in the CatB background additionally caused somewhat slower growth during in HMM supporting the idea that CatP is primarily involved in
detoxifying metabolically derived hydrogen peroxide. Interestingly, the restoration of CatB or CatP to the catb∆catp∆ provides insight into the relative importance of these proteins relative to one another. The return of either enhances the survival of Histoplasma against macrophages, however the restoration of CatP grants a greater protective effect than CatB. This could be due to the ability of hydrogen peroxide to pass through membranes, which would allow peroxide escaping destruction by CatB to enter cells and cause severe damage. Conversely the presence of CatP would allow for the protection of intracellular targets, indicating that CatP could be more important for defense against hydrogen peroxide stress.

Although the lack of attenuation in catb∆ was surprising it is not unprecedented in other pathogenic organisms (280-283, 296). However, the placement of CatB and Sod3 on Histoplasma suggest potential cooperation as Sod3 would quickly dismute superoxide anion into hydrogen peroxide that could then be converted into oxygen and water by CatB. Interestingly, yeast lacking Sod3 and CatB behave in a manner identical to the sod3 alone in human neutrophils, activated mouse peritoneal or human macrophages and within the mouse infection model. Thus the loss of both proteins does not have a synergistic effect on Histoplasma survival during infection conditions, indicating that Sod3 is more important as it acts upstream of CatB and other factors can compensate for the loss of CatB in vitro and in vivo.
The protein Cfp4 was also a protein of high interest for a role in virulence. It is abundantly expressed by yeast and is one of the most yeast-phase enriched proteins produced by *Histoplasma*, on a scale near Cbp1 (211). This protein lacks sequence homology to other proteins, so no putative functionality could be assigned. Cfp4 was depleted in two *Histoplasma* medical isolates, the Pan-American and Nam2 strains to determine if the protein had a similar function in both isolates. As Cfp4 is transcriptionally enriched in both strains to a similar level we hypothesized that it might have a similar role in both strains. However, Cfp4 depletion strains demonstrated the ability to replicate and kill macrophages, survive within resting and activated macrophages and survive neutrophil antimicrobial activity at wild-type levels. Cfp4 is also dispensable for *Histoplasma* replication within the lung, as strains lacking Cfp4 reached identical fungal burdens within the lung as wild-type throughout the time course (20 days p.i). However, within the spleen the Nam2 strain containing CFP4-RNAi demonstrated a minor defect in spleen colonization at day 8 post infection, but at no other time point. This result was interesting as it was not observed in the Panamerican strain, and was isolated to one specific time in Nam2 isolates. What this result means is unclear as yeast disseminate through the movements of immune cells and not through their own motility (297). One potential role could be that Cfp4 is specifically recognized by some phagocytic cells and leads to more efficient cell migration to other organs. If this is the case it is apparent that other mechanisms are also involved as the loss of Cfp4 is not detrimental to yeast during growth within the host.
Although Cfp4 lacks a discernible virulence defect in phagocytic cells or in mice we wanted to determine if this protein could be used as an exoantigen marker of histoplasmosis. Currently the diagnosis of histoplasmosis is based on multiple clinical tests including immunodiffusion assays, complement fixation assays, direct fungal culture, serological testing or directly testing for *Histoplasma* antigen using ELISA (298). The later test is the most modern and screens for the presence of the *Histoplasma* polysaccharide antigen (184), containing >90% sugar by weight and likely only a very minor peptide component. This test involves the testing of urine, serum or bronchoalveolar lavage fluid for the presence of PA. The test is very sensitive but lacks specificity as assays cross-react with other endemic mycosal infections. Cfp4 potentially circumvents this problem, as no other endemic fungi encode a similar protein. Cfp4 is expressed during mouse infection and the protein is very abundantly produced during growth *in vitro*, potentially indicating a high level of protein expression *in vivo*. Our studies have also revealed that the expression of CFP4 is similar *in vitro* to that in the lung during infection giving evidence that protein levels are likely similar. Thus, Cfp4 appears to be a prime candidate as a potential exoantigen diagnostic marker. With collaborators from MiraVista Diagnostics we verified that human immune sera from histoplasmosis patients recognizes Cfp4 in both Panamerican and Nam2 wild-type strains but not in the depletion strains. Additionally, human serum from blastomycosis patients does not significantly recognize Cfp4 indicating that this protein is extremely *Histoplasma* specific.
The role of Cfp4 as a potential histoplasmosis diagnostic marker is exciting but some complicating factors and questions remains. We are currently unsure if Cfp4 is found at detectable levels within bodily fluids such as urine, blood, serum or lavage fluid. Recently the lab has developed a monoclonal antibody to Cfp4, and want to determine if an ELISA assay for Cfp4 is feasible at the sensitivity and specificity required for clinical tests. The biggest complicating factor is the level of polysaccharide present on Cfp4. The protein in its native form cannot be visualized by protein staining, immunobloting for CFP4-FLAG or with monoclonal antibodies or purified to a high degree in its native form. This greatly complicates the potential usefulness of Cfp4 as a potential diagnostic antigen unless the protein is first deglycosylated. However, human histoplasmosis serum contains antibodies recognizing Cfp4 in its native form and deglycosylated forms indicating that antibody recognition occurs at sites accessible to proteins regardless of glycosylation state. Therefore, it is likely possible to develop diagnostic assays for Cfp4, but specific antibodies will have to be generated to very specific epitopes.

Overall, my work has clearly demonstrated that novel proteins in the Histoplasma secreted proteome are required for the full virulence of H. capsulatum. We identified numerous extracellular proteins that potentially are required for yeast replication and survival within phagocytes and the host. We demonstrated that CatB and Cfp4 appear to lack a significant role during yeast replication within phagocytes or the lung despite being highly yeast-phase enriched and very abundant proteins. However, Cfp4 has potential as a diagnostic marker for histoplasmosis. Sod3 is a novel virulence factor that
is required for *Histoplasma* growth in phagocytes, the lungs and spleen, and for disease causation. The identification and characterization of Sod3 sheds light on how *Histoplasma* defends itself against host-derived oxidative stress and identifies superoxide anion as the toxic effector molecule *Histoplasma* must defend against during an effective infection.
References


274. Winterbourn CC & Kettle AJ (2012) Redox reactions and microbial killing in the neutrophil phagosome. *Antioxid Redox Signal*


