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Identification and characterization of virulence factors in the fungal pathogen Histoplasma capsulatum

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Identification and characterization of virulence factors in the fungal pathogen

Histoplasma capsulatum

by

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DISSERTATION

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Approved:

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For my family
Thanks for everything!!

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Identification and characterization of virulence factors in the fungal pathogen *Histoplasma capsulatum*

Dervla Tamara Isaac

ABSTRACT

Histoplasma capsulatum, a fungal pathogen, colonizes macrophages during infection of mammalian hosts. *In vitro* studies showed that the fungus survives and replicates within the macrophage phagosome, ultimately lysing host cells. The fungal factors that promote these processes are largely unknown. Similarly, it is unknown whether a high intracellular fungal burden is sufficient to cause host cell lysis, or whether *Histoplasma* expresses factors that actively induce host cell death. To identify genes required for virulence in the macrophage, I designed a genetic screen to identify *H. capsulatum* mutants that failed to lyse macrophages. I tested 14,000 insertion mutants and identified twenty-six *l*ysis-*d*e*f*ective (LDF) mutants with a range of lysis defects and growth kinetics within macrophages. Several mutants appeared to be defective in genes that encode predicted secreted factors and metabolic proteins. Additionally, a number of the LDF genes had no orthologs in other fungal genomes, suggesting they play a role in *Histoplasma*-specific virulence mechanisms. One such gene, *CBP1*, had previously been identified in another lab as being required for lysis of macrophages and virulence in the mouse model of histoplasmosis. Surprisingly, the growth characteristics of the *cbp1* mutant within macrophages had not previously been determined. I observed that Cbp1 is largely dispensable for intracellular growth within macrophages, but clearly required for

macrophage lysis. These data provide the first evidence that a high intracellular fungal burden is not sufficient to trigger macrophage lysis.

Since Cbp1 is a secreted factor, I reasoned that it might interact directly with host factors and modulate the host response. We used MEEBO microarrays to compare the transcriptional profile of macrophages infected with wild-type *H. capsulatum* and the *cbp1* mutant. Cbp1 was required for the induction of a set of macrophage genes that was normally induced during infection with live *H. capsulatum* but not during infection with other intracellular pathogens. Since Cbp1 is also required for host-cell lysis, these data suggest that induction of the Cbp1-dependent transcriptional signature in macrophages is a molecular marker that correlates with host-cell death. Future work will address the molecular mechanism of Cbp1-dependent macrophage lysis.

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CHAPTER ONE

Introduction

The success of microbial pathogens lies in their ability to subvert or bypass host immune defenses. These microbes have evolved factors and mechanisms to achieve this goal, converting an otherwise hostile host environment into one in which they not only survive, but thrive. Detailed knowledge and understanding of these virulence determinants is the key to disease prevention and eradication.

The goal of my thesis work is to identify and characterize virulence factors in the intracellular fungal pathogen *Histoplasma capsulatum.* Previous studies of *H. capsulatum* infection characterized the intracellular host environment of the fungus, establishing that it can subvert host microbicidal defenses such as the oxidative burst, phagosome acidification, and phagosome-lysosome fusion. The effect of this host manipulation on the growth and survival of the fungus, however, remains unclear and few studies have adequately explored the molecular mechanisms driving this host subversion by *H. capsulatum*. At the time I initiated my studies, three virulence factors had been identified, but their molecular function was unknown. I have taken an unbiased genetic approach to identify *H. capsulatum* factors required for normal interaction with host cells. In a screen of approximately 14,000 random insertion mutants, I have identified 26 *H. capsulatum* mutants that fail to lyse macrophages. These mutants have allowed me to explore the molecular underpinnings of *H. capsulatum* pathogenesis.

***Histoplasma capsulatum*: Disease and interactions with the host**

Histoplasmosis is the respiratory disease that results from infection with the fungus *H. capsulatum*. Disease severity depends on the immune status of the host and the level of exposure to fungal particles. Immunocompromised individuals, such as those

with AIDS, undergoing chemotherapy, or taking immunosuppresive drugs, are at greatest risk for fatal, systemic disease. Healthy hosts exposed to a low dose of *H. capsulatum* are usually asymptomatic. Exposure to a large number of fungal particles will result in a self limiting infection that can be controlled by the cell-mediated immune response. It is important to note that a healthy host controls, but does not clear, the fungus. A small number of fungal cells remain present in granulomas, restricted in their growth and virulence. If the immune status of a previously exposed individual changes and the fungus can no longer be contained within granulomas, the host can experience a life-threatening reactivation of histoplasmosis.

H. capsulatum is a dimorphic intracellular mammalian pathogen. In the United States, it is endemic to the Ohio and Mississippi Valley. In the soil, or at 25^{o}C in the laboratory, the fungus exists as a multicellular mycelium. When the soil is disrupted, mycelial fragments and the vegetative spores they produce are easily aerosolized. These airborne fungal particles are then inhaled by mammalian hosts. Upon entering the lungs, or being shifted from 25^{o}C to 37^{o}C in the laboratory, these fragments and spores undergo a morphological transition. The vegetative spores germinate to form budding yeast and the mycelia switch growth patterns from polar filamentous growth to bipolar budding yeast growth. This conversion to the yeast form is required for successful *H. capsulatum* infection (Medoff *et al.*, 1986). These pathogenic yeast cells are subsequently phagocytosed by alveolar macrophages. Within these host immune cells, *H. capsulatum* is able to survive and replicate.

The ability of *H. capsulatum* to grow within the macrophage phagosome is intriguing. Macrophages are sentinels of the immune system whose primary function is to ingest and digest invading microbes. These microbes are typically exposed to extremely

harsh phagosomal conditions – oxidative stress, due to the release of reactive oxygen intermediates, acidic pH, due to phagosome maturation, and degradative stress due to the activity of hydrolytic enzymes present in the lysosome. *H capsulatum*, however, evades this harsh intracellular fate.

Upon phagocytosis, invading microbes are exposed to a number of reactive oxygen species (ROSs) in a process known as the respiratory burst. This involves the activation of the membrane bound NADPH oxidase to generate highly reactive superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. Exposure to these compounds in the phagosome can cause damage to microbial membranes, enzymes, and DNA. *H. capsulatum* actively prevents the generation of these ROSs in murine macrophage models of infection, thereby escaping their detrimental effects (Wolf *et al.*, 1987).

Proper maturation of the phagosome is another key aspect of macrophage immune function. This maturation involves phagosome acidification and fusion with the lysosome. The resulting phagolysosome has a lumenal pH of 4.5, which is required for the activation of lysosomal hydrolytic enzymes. Eissenberg *et al.* showed that the *H. capsulatum* phagosome fuses with lysosomes (Eissenberg *et al.*, 1988). This data suggests that *H. capsulatum* would be exposed to hydrolytic enzymes present in the phagolysosome. However, *H. capsulatum* is able to prevent phagosome acidification, and instead maintains a near-neutral phagolysosomal pH of 6.5 (Eisenberg *et al.*, 1993, Strasser *et al.*, 1999). This inhibition of phagosome acidification would prevent the activation of lysosomal pH dependent enzymes and remove their threat to the survival of the fungus. The pH of phagosomes containing heat-killed and methanol-killed *H. capsulatum* is 4.7, indicating that pH modulation by *H. capsulatum* is an active process that requires fungal viability. Interestingly, *H. capsulatum* can modulate the pH of its environment in culture as well as in macrophages. *H. capsulatum* neutralizes culture media, regardless of the starting pH (Berliner, 1973). While inhibition of

phagosome acidification appears to be important for *H. capsulatum* pathogenesis, the *H. capsulatum* factors that are involved have yet to be determined.

H. capsulatum virulence factors

Only three *H. capsulatum* virulence factors had been identified at the time I initiated this research - α-1,3-glucan, Yps3, and Cbp1. These three factors are active in the G186AR strain of *H. capsulatum*, but only Yps3 and Cbp1 are active in the G217B strain. Interestingly, these virulence determinants are only expressed during *H. capsulatum* growth in the yeast form, the pathogenic form of the fungus. Additionally, α-1,3-glucan is expressed on the fungal cell surface and Cbp1 and Yps3 are secreted. The extracellular localization of these factors during infection suggests that they might play a role in manipulating the host during infection.

α 1,3 glucan

α-1,3-glucan is a carbohydrate that is found in the cell wall of many fungi, including *H. capsulatum* and the closely related fungi *Blastomyces dermatitidis* and *Paracoccidiodes brasiliensis*. The presence of this polysaccharide in the cell wall contributes to rough colony morphology, to autoaggregation during growth in culture, and to virulence during infection. The role of α-(1,3)-glucan in *H. capsulatum* pathogenesis was established when spontaneous smooth colony variants of the rough colony strains, G184AR and G186AR, were identified. These smooth colony variants, G184AS and G186AS respectively, were avirulent in the mouse model of infection. It was later determined that the smooth strains lacked the α-(1,3)-glucan in their cell walls (Klimpel *et al.*, 1987; Klimpel *et al.*, 1988).

Rappleye *et al.*. later took a more targeted approach to determining the role of α-(1,3)-glucan in virulence by knocking down *AGS1*, the α-1,3 glucan synthase, using RNA interference (RNAi) in the G186AR strain (Rappleye *et al.*, 2004). The targeted knockdown recapitulated the phenotype of the spontaneous isogenic variants. The *ags1* mutant had a smooth colony morphology and was avirulent. Consequently, α-1,3-glucan is an *H. capsulatum* virulence factor. Interestingly, not all virulent strains of *H. capsulatum* express *AGS1*. This is the case for one of the most virulent *H. capsulatum* strains, G217B. It has a smooth colony morphology, but is even more virulent that G186AR. This indicates that α-1,3-glucan is not necessary for virulence in all strains of *H. capsulatum*.

Studies exploring the molecular function for α-1,3-glucan during infection have only recently been published. α-1,3-glucan is present on the outermost layer of the *H. capsulatum* yeast cell wall, where it is thought to shield an inner layer of β-glucans (Rappleye *et al.*, 2007). The shielding is thought to be important because it prevents the immune system from recognizing and responding to the β-glucans via Dectin-1. As a result TNFα, a proinflammatory, anti-*H. capsulatum* cytokine, is not induced. In summary, α-1,3-glucan serves as a shield to hide *H. capsulatum* from the immune system. G217B, which has no α-1,3-glucan on its surface must have an alternate strategy for evading the immune response to β-glucan on its cell surface.

Yps3

Another surface associated virulence factor, yeast phase specific protein 3 or *YPS3*, was first identified in a differential hybridization screen for *H. capsulatum* genes whose expression was specific to the pathogenic yeast form of the fungus (Keath *et al.*, 1989). *YPS3* shares homology to the *B. dermatitidis* adhesion and virulence factor *BAD1*.

Like *BAD1*, *YPS3* is both secreted and cell wall associated. Secreted *YPS3* binds the cell surface via interactions with chitin (Bohse *et al.*, 2005). *YPS3* expression in different strains of *H. capsulatum* correlates with their virulence. The highly virulent G217B strain expresses *YPS3*, while the avirulent Downs strain does not (Keath *et al.*, 1989).

Studies exploring the role of *YPS3* during infection showed that it was not required for virulence in the macrophage cell line RAW264.7, but was required for full virulence in the mouse model of infection. When the authors knocked down *YPS3* using RNAi, they showed that expression of this gene was required for proper organ colonization in the intranasal and intraperitoneal mouse models of infection. In fact, the colonization defect was more dramatic in the livers and spleens of animals infected intranasally (Bohse *et al.*, 2007). While there was a $\log_{10}$ difference of 1.28 in CFU between the control and the *yps3* knockdown strain in the lungs 7 days post infection, in the liver and spleen this difference was 2.48 and 2.66 respectively. Taken together, this data indicates that Yps3 is an *H. capsulatum* virulence factor that is required for dissemination in the host. How *YPS3* performs this function, however, remains unclear.

Cbp1

In vitro, *H. capsulatum* yeast cells are able to grow under calcium limiting conditions while mycelia can not. Given that conversion to the yeast from is required for virulence and that the ability to acquire calcium is thought to be important for microbial survival in the host, Batanghari *et al.* sought to identify the *H. capsulatum* gene was responsible for this phenotype (Batanghari *et al.*, 1997). They identified the abundantly secreted factor *CBP1*, calcium binding protein 1.

The role of *CBP1* during infection was examined in later studies. RT-PCR of infected P388D1 macrophage lysates and microscopic analysis of Cbp1-gfp fusion protein expression demonstrated that *CBP1* was expressed during macrophage infection (Batanghari *et al.*, 1998, Kugler *et al.*, 2000). The Δ*cbp1* mutant was avirulent during macrophage infection and was defective in lung colonization in the intranasal mouse model of infection, indicating that *CBP1* is required for virulence (Sebghati *et al.*, 2000). The molecular function of this protein is still unknown. It is worth noting, however, that the avirulent G184AS and G186AS strains still express *CBP1* (Batanghari *et al.*, 1997; Batanghari *et al.*, 1998). Consequently, expression of *CBP1* is necessary but not sufficient for virulence.

Identifying virulence factors in other intracellular pathogens

Identifying *H. capsulatum* virulence factors is key to understanding how this fungus interacts with and manipulates the host. Previous research in this field has relied on *in vitro*, host-free systems in their quest to uncover these factors. For instance, the role of α-1,3-glucan was discovered because rough colony morphology in other pathogens correlated with virulence (Klimpel *et al.*, 1987). *YPS3* was identified because it was expressed only in the parasitic yeast form of the fungus. Finally, Cbp1 was identified because the ability to acquire calcium is important for survival (Batanghari *et al.*, 1997). While these host free approaches have been valuable in identifying *H. capsulatum* virulence factors, they have inherent biases.

A more direct and comprehensive approach to identifying virulence factors is to ask which genes are required for a microbes ability to survive within and kill its host. *In vitro* cell culture models of infection provide an excellent system for doing these types of

experiments in a high-throughput manner. This approach has been successful in identifying key virulence factors in other clinically relevant intracellular pathogens. These include *S. typhimurium* and *L. pneumophila*.

Salmonella typhimurium

Salmonella typhimurium, an intracellular bacterial pathogen, causes gastroenteritis in infected hosts. It is able to replicate in the harsh environment of the macrophage phagolysosome (Carroll *et al.*, 1979). To identify *S. typhimurium* factors that promoted intracellular replication, Fields *et al.* generated a library of transposon insertion mutants in a virulent strain of *S. typhimurium* and directly screened 9,516 mutants for their ability to grow in thiogycollate-ellicted peritoneal macrophages (Fields *et al.*, 1986). Avirulence was measured by the decrease in bacterial CFUs recovered from infected macrophages 24 hours post infection. 115 macrophage survival (MS) mutants were identified. Of these, 83 showed decreased or no virulence in the mouse model in infection. Thirty-four mutants were found to be defective in one of several virulence associated traits. They were either sensitive to oxidative stress, hypersensitive to serum induced cell death, non-motile, or had aberrant colony morphologies. The authors also tested the MS mutants for sensitivity to antimicrobial defensins and identified a class of mutants that was more sensitive that wild-type *S. typhimurium* (Fields *et al.*, 1989).

Subsequent mapping of 23 MS mutants provided further insights into *S. typhimurium* virulence (Baulmer *et al.*, 1994). Only 7 mutants could be mapped to known genes. Two mutants had disruptions in genes homologous to the *E coli* proteases *HTRA* and *PRC*. Disruption of these genes in *E coli* results in a periplasmic leakage phenotype.

Three mutants mapped to a small uncharacterized region of the *S. typhimurium* genome, potentially uncovering a new virulence cluster. Finally, 16 of the 23 mapped mutant insertions were in putative *S. typhimurium* genes. These studies generated an extremely powerful library of MS mutants that fueled the field for the next 20 years.

Legionella pneumophila

L. pneumophila is an intracellular bacterial pathogen. It is the causative agent of Legionnaires' pneumonia. It replicates within macrophages, creating a specialized phagosome that is re-routed from normal traffic though the endocytic pathway. This phagosome does not acidify and does not fuse with lysosomes. It does, however, acquire mitochondria and rough endoplasmic reticulum. To identify virulence determinates responsible for *L. pneumophila's* hijacked phagosome, Andrews *et al.* carried out a screen to identify *L. pneumophila* mutants that were defective for intracellular growth in macrophages (Andrews *et al.*, 1998). EMS mutagenesis was used to create a library of mutants. Of the 4,960 mutants screen, 17 had an intracellular growth defect. Secondary analysis of the intracellular trafficking of these mutants showed that 6 trafficked to the lysosome. This analysis resulted in the identification of 3 genes that were required for the formation of the *L. pneumophila* replicative vacuole and intracellular survival in macrophages.

Identifying virulence genes in *H. capsulatum*

Genetic screens monitoring microbial survival and growth during *in vitro* macrophage infection have successfully identified virulence determinants in *S.*

typhimurium and *L. pneumophila*. I have employed a similar strategy to the identification of virulence factors in *H. capsulatum*. I designed a genetic screen to identify *H. capsulatum* genes required for lysis of macrophages during infection. I will now discuss the main considerations involved in the design of the screen, including the choice of the host cell model system and the choice of virulence phenotype to monitor.

I used the macrophage as the model host system in this screen. Macrophages are one of the primary host cells for *H. capsulatum* during infection of mammalian hosts. Additionally, macrophages are readily parasitized by *H. capsulatum in vitro* (Figure 1-1). Yeast cells bind to and are phagocytosed by these host immune cells. Inside the macrophage phagosome, these yeast cells replicate to very high numbers. This fungal colonization results in the lysis of the host macrophage. Two types of macrophages were used for infections in this screen, the macrophage cell line, J774.A1, and primary bone marrow-derived macrophages (BMDMs). This was done to ensure that virulence factors identified in the screen were not specifically required for infection of particular macrophage cell types.

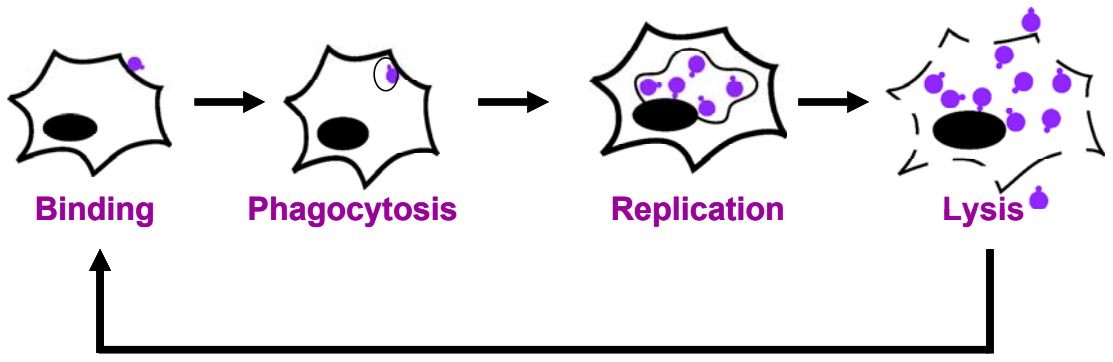

Figure 1-1. *H. capsulatum* infection of macrophages

I designed the screen to monitor macrophage lysis since it is the terminal result of a successful *H. capsulatum* infection. There are several advantages to using macrophage lysis as a screening phenotype. First, mutants that fail to lyse macrophages could identify genes that are required for all stages of infection. This includes genes that are required for binding and entry into the macrophage, genes required for acquiring nutrients and subverting host defenses, and genes required for killing the macrophage. Second, macrophage lysis is a phenotype that can easily be monitored in a high-throughput manner. There are both quantitative and qualitative assays available to do this. For the screen, I chose to use the visual crystal violet assay to monitor macrophage lysis. The crystal violet dye is used to stain the macrophage monolayers after infection to detect any remaining, intact cells. Macrophage monolayers infected with wild-type *H. capsulatum* will not stain with crystal violet several days after infection due to the death of all macrophages in the monolayer. Macrophage monolayers infected with an lysis defective (LDF) mutant, on the other hand, will remain intact and stain positive for crystal violet (Figure 1-2). A quick visual scan for the purple/blue monolayers will permit the easy detection of avirulent mutants. This assay was used to screen 14,000 *H. capsulatum* insertion mutants. The results of these experiments will be described in the following chapters.

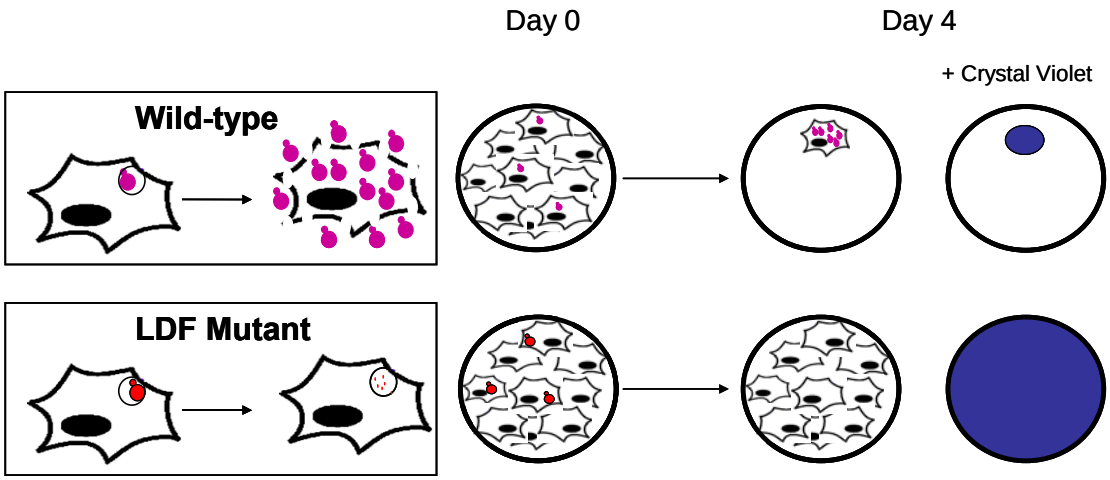

Figure 1-2. Crystal Violet Assay

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CHAPTER TWO

A genetic screen identifies *Histoplasma capsulatum* virulence factors

Introduction

H. capsulatum is an intracellular fungal pathogen. Mammalian hosts are infected after inhaling infectious mycelial fragments and vegetative spores. Within the lungs, mycelia and vegetative spores change growth patterns, converting to budding yeast cells. These yeast cells are the parasitic form of the fungus that causes disease. They are phagocytosed by a number of host immune cells within the lungs, including alveolar macrophages.

Macrophages are one of the primary host cells for *H. capsulatum*. Consequently, studying the molecular interactions between *H. capsulatum* and these immune cells can provide insights into how this microbe causes disease. *Ex vivo* macrophage cell culture systems have been developed to overcome the complexities inherent in studying host-microbe interactions in the infected animal. *In vitro, H. capsulatum* infection of macrophages has shown that the fungus is able to colonize and kill macrophages.

Macrophages employ an arsenal of antifungal defenses to defeat microbes. *H. capsulatum* virulence during macrophage infection is due to the ability of the fungus to subvert the immune defense mechanisms of this host cell. For example, *H. capsulatum* inhibits the production of reactive oxygen species (ROS), which are normally produced in the phagosome soon after phagocytosis occurs. These toxic products, which include hydrogen peroxide and superoxide anions, readily react with lipids, proteins, and nucleic acids. The resulting damage to microbial membranes and disruption of protein function is often lethal to an invading microbe. *H. capsulatum,* however, inhibits the production of ROS in murine macrophages and is able to survive exposure to these reactive molecules during infection of human macrophages (Wolf *et al.*, 1987).

Another key host defense mechanism that is subverted during *H. capsulatum* infection of macrophages is the process of phagosome maturation. Phagosome maturation is the process by which the microbial phagosome matures into a phagolysosome. The resulting phagolysosome is extremely acidic, usually at a pH of 4.5, and is highly degradative, due to the presence of hydrolytic enzymes that are activated at acidic pH. During a macrophage infection, the *H. capsulatum* phagosome fuses with the lysosome, but fails to acidify (Eisenberg *et al.*, 1993, Strasser *et al.*, 1999). Instead, the pH within the phagosome lumen is 6.5. The ability of *H. capsulatum* to inhibit phagosome acidification is thought to protect the fungus from the activity of the lysosomal hydrolases that are not active at this neutral pH. This hypothesis, however, has not been tested experimentally. The role of inhibition of phagosome acidification in *H. capsulatum* pathogenesis remains unclear.

Previous experiments have demonstrated that *H. capsulatum* is able to manipulate the normal biological functions of the macrophage. These manipulations are followed by the growth and accumulation of the fungus in the macrophage prior to host cell lysis. There is, however, very little known about the *H. capsulatum* factors and mechanisms involved in these subversive interactions with host cells. At the time this study was initiated, only 2 virulence factors had been identified in the *H. capsulatum* strain G217B. These factors included Cbp1, a secreted factor that is required for virulence in macrophages and colonization of the lung in the murine pulmonary infection model, and Yps3, another secreted factor that is required for virulence, but only during mouse infection (Sebghati *et al.*, 2000; Bohse *et al.*, 2007). While these factors have been shown to be required for virulence, their molecular functions remain a mystery.

Our understanding of *H. capsulatum* pathogenesis is hindered by our limited knowledge of the fungal factors required to promote survival within host macrophages. In this work, I describe an unbiased and comprehensive genetic strategy to identify *H. capsulatum* virulence factors. I designed and implemented a qualitative, high-throughput screen to test *H. capsulatum* insertion mutants for their ability to lyse macrophages during infection. I screened a library of 14,000 random insertion mutants and identified 26 lysis defective (LDF) mutants. Genomic and phenotypic analyses of these insertion mutants, as well as bioinformatic analysis of the fungal genes identified, are described in this chapter.

Materials and Methods

Strains and culture conditions

H. caspsulatum strain G217B (ATCC 26032) and G217B *ura5*Δ (WU15) were obtained from William Goldman (Washington University, St. Louis, MO). Yeast cells were grown in liquid *Histoplasma* macrophage media (HMM) or on HMM agarose plates (Warsham and Goldman, 1988). Where indicated, HMM liquid media was supplemented with 200 ug/ml uracil (HMM/ura), 200 ug/ml hygromycin B (Roche) (HMM/hyg), or both (HMM/hyg/ura). In pH modulation experiments, HMM, pH 4.5, was made without HEPES and $FeSO_4$, and 40 mg/ml bromcresol purple was added (pH-HMM). Where indicated, HMM-agarose plates were supplemented with 400 ug/ml uracil (HMM/ura), 200 ug/ml hygromycin B (HMM/hyg), or both (HMM/hyg/ura). Plates were supplemented with 200 uM cefotaxime (Sigma) where indicated. Liquid cultures were grown in an orbital shaker at 37^{o}C with 5% CO_2. Stock cultures were maintained by passaging every 2-3 days at a 1:25 dilution. Plates were incubated in a humidified chamber at 37^{o}C with 5% CO_2.

For macrophage infections, an overnight, mid-log culture of yeast cells (OD_{600} = 5-7) was prepared. Approximately 18 hours prior to infection, a two-day late log/stationary phase culture (OD_{600} = 10-12) was diluted 1:5 into the appropriate HMM media. The diluted cells were then incubated at 37^{o}C with 5% CO_2 overnight to obtain mid-log cultures at the time of infection. Culture ODs were measured using an Eppendorf BioPhotometer.

Generation of *H. capsulatum* insertion mutants

H. capsulatum insertions mutants were generated using *Agrobacterium*-mediated transformation of G217B and WU15 as previously described (Nguyen and Sil, 2008). We selected for hygromycin resistant (Hyg^R) insertion mutants on HMM/hyg/cefo and HMM/hyg/cefo/uracil agarose plates.

Macrophage Culture Conditions

Bone marrow derived macrophages (BMDMs) were isolated as described previously (Hwang *et al.*, 2008). They were cultured in Bone Marrow Macrophage Media (BMM) containing DMEM (Dulbecco's Modified Eagle Medium, High Glucose) (UCSF Cell Culture Facility), 20% Fetal Bovine Serum (Hyclone), 10% v/v CMG supernatant (source of CSF-1), 2 mM glutamine (UCSF Cell Culture Facility), 110 mg/mL sodium pyruvate (UCSF Cell Culture Facility), and penicillin/streptomycin (UCSF Cell Culture Facility). For LDH release assays, BMM without phenol red (BMM-pr), made using DMEM w/o phenol red, was used.

J774 macrophage-like cells (ATCC-TIB67) were cultured in J774 macrophage media (JMM) containing DMEM, 10% Fetal Bovine Serum, and penicillin/streptomycin. For LDH release assays, JMM without phenol red (JMM-pr), made using DMEM w/o phenol red, was used.

When indicated, macrophage media was supplemented with 400 ug/ml uracil (BMM/ura or JMM/ura).

LDF Screen

Growth of insertion mutants in 96-well plates. Individual insertion mutants and WU15 were inoculated into the wells of a 1ml-96-well plate (Corning 3598) containing 600 ul HMM/hyg/ura and HMM/ura respectively (Figure 2-1). A schematic of the mutant plate layout is shown in Figure 2-2. These plates were then sealed with Neptune Bioseal Breathable Tape (Continental Life Products 2424.S) and incubated at 37°C with 5% CO_2 in a *Multitron* incubator shaker, *AJ140* (ATR, Laurel, MD) at 800 rpm. Four days later the cultures were passaged into a fresh 1ml-96-well plate containing 600 ul HMM/hyg/ura and HMM/ura in the appropriate wells at a 1:25 dilution. After two days growth, these cultures were passaged in preparation of infection.

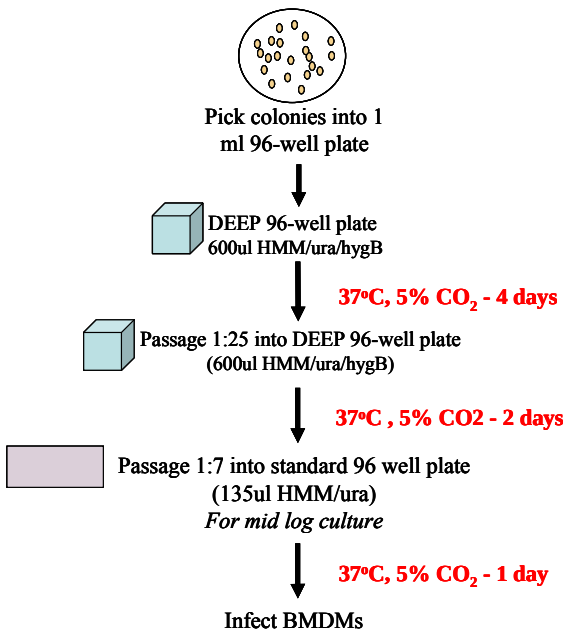

Figure 2-1. Protocol for preparing *H. capsulatum* insertion mutants for infection in the LDF screen

Preparation of mutants for infection in the LDF screen. Approximately 18-20 hours before infection, 20 ul of the two- day culture was passaged into 115 ul HMM/ura in a standard 96-well plate (BD Falcon 353072) for a final volume of 135 ul. This plate was then incubated at 37^{o}C with 5% CO_2 overnight. This dilution ensured that the mutants were in mig-log growth when they were used to infect macrophages. At the time of the infection, the OD_{600} of each well was measured using the Spetramax Plus 384 plate reader (Molecular Devices). As the OD_{600} varied well-to-well, we determined the median OD_{600} of the entire plate and calculated a median *H. capsulatum* concentration for the plate using a conversion factor of 1.1 x 10^5 Hc/ul per OD_{600} unit. Macrophages were then infected at an MOI of 10 based on this median concentration. The same volume of yeast cells was used for each insertion mutant in the plate to infect macrophages.

Macrophage infections. 5.5 x 10^4 J774 macrophages, seeded approximately 16 hours before infection, were infected, in duplicate, at an MOI of 10. A diagram of the infection plate setup is shown in Figure 2.2. Each row of the *H. capsulatum* insertion mutant 96-well plate was used to infect two consecutive rows of macrophages in the infection plate. Ultimately, there were two macrophage infection plates for every plate of *H. capsulatum* insertion mutants. After a 1-hour period of incubation, the infected macrophages were washed once with DMEM and 150 ul JMM/ura was added to each well. In the wells designated *ura5*Δ-uracil, 150 ul JMM was added. Two days post-infection, 50 ul JMM±ura was added to the appropriate wells. At 4 days post-infection, the media in each well was replaced with pre-warmed, fresh media. At 5 days post-infection, the media was removed from each well and the cells were fixed with 150 ul of 10% formaldehyde in PBS. After 5 minutes of incubation, the formaldehyde solution was removed and 150 ul

PBS was added. The samples were stored at room temperature prior to crystal violet staining.

BMDMs were infected similarly, with several modifications. $8x10^4$ BMDMs were seeded in 96-well plates and were cultured in BMM±ura when appropriate. BMDM monolayers were fixed and stained 3-4 days post infection.

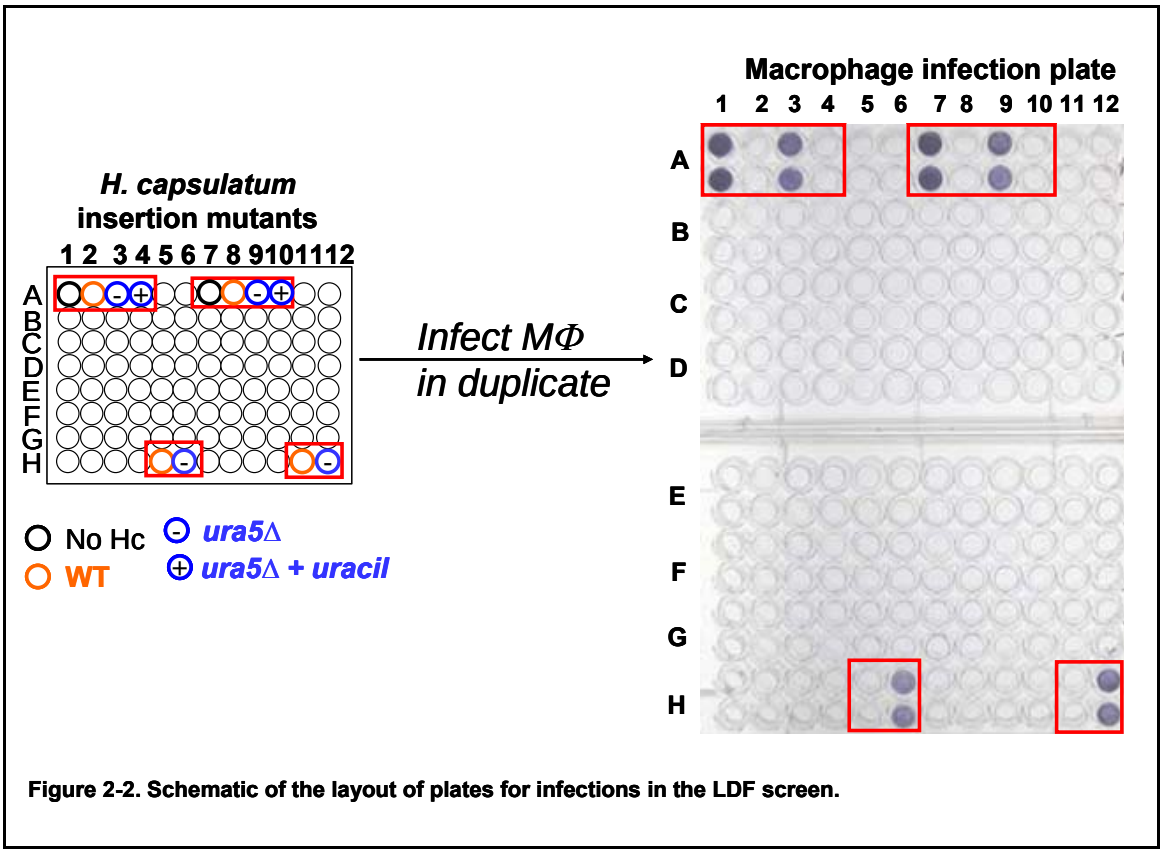

Figure 2-2. Schematic of the layout of plates for infections in the LDF screen.

Crystal violet assay. PBS was removed from each well and replaced with 75 ul crystal violet staining solution (CVSS) (0.2% w/v crystal violet, 2% v/v EtOH in ddH_2O). After 5 minutes of incubation, the CVSS was replaced with PBS and samples were allowed to incubate for 5 minutes before the PBS was removed and the wells were allowed to air dry. Plates were photographed with an Olympus digital camera.

The LDH release assay

In 24-well tissue-culture treated dishes, 2 x 10^5 BMDMs were infected, in duplicate, with *H. capsulatum* strains at various MOIs. In preparation for the infections, logarithmically growing *H. capsulatum* cultures were pelleted, resuspended in DMEM w/o phenol red, sonicated, counted by hemacytometer, and diluted to the appropriate concentration prior to being added to BMDMs. After a 2-hour incubation period, the media was removed from the infected BMDMs, the monolayers were washed twice with DMEM w/o phenol red and 750 ul BMM/ura-pr was added to each well. The infected macrophages were then incubated at 37^{o}C with 5% CO_2. Approximately 48 hours post infection, 250 ul fresh BMM/ura-pr was added to each well.

At various timepoints post infection, LDH levels in the infected macrophage supernatants were measured. At each time-point, the volume in each well was brought up to 1 ml with BMM/ura-pr. 200 ul of the culture supernatant from the infected macrophages were transferred to two wells of a 96-well plate. To measure the total LDH, at 2 hours post infection two mock-infected BMDM monolayers were lysed with 1 ml of lysis solution (1% Triton in DMEM w/o phenol red). 200 ul of this mock-infected macrophage lysate was transferred to two wells of the 96-well plate. This 96-well plate was then centrifuged to pellet any cells or debris that might be present in the supernatants or lysate. 20 ul of the clarified culture supernatant and lysate was transferred to a fresh 96-well plate containing 30 ul DMEM-phenol red in each well. The LDH released into the culture supernatant was assessed using the Promega Cytotox96 non-radioactive cytotoxicity (Roche) assay according to the manufacturer's protocol. The percent BMDM lysis at each time-point is

calculated as the percentage of the total LDH from uninfected cells at 2 hours post infection.

Southern Blot Analysis

Genomic DNA from *H. capsulatum* strains was prepared using Puregene Genomic DNA Purification Kit (Gentra Systems). This DNA was the digested with SpeI. The digested DNA was then separated by gel electrophoresis, transferred to a nylon membrane and probed with ^{32}P-radiolabeled *hph* probe.

Mapping the insertion site in the mutants - Inverse PCR

3 ug of genomic DNA was digested with HindIII in a 50 ul reaction. The digested DNA was then used in an intramolecular ligation reaction with a final volume of 600 ul using T4 DNA ligase (New England Biolabs). The DNA was then precipitated and divergent primer pairs were used to amplify the genomic sequence flanking the left and right borders of the insertion (Figure 2-3). Primers OAS908 (5'-GTTGCGCAGCCTGAATGGCG-3') and OAS907 (3'-GTAAGCGCCCACTCCACATC-5') were used to amplify the left border genomic DNA sequence. Primers OAS750 (5'-GGCTCCTTCAACGTTGCGGT -3') and OAS1322 (3'-GGGCGACACGGAAATGTTGAATACTC-5') were used to amplify the right border genomic DNA sequence. PCR products were gel purified and sequenced.
BLAT (BLAST-Like Alignment Tool), was used to determine the location of the amplified sequence in the *H. capsulatum* G217B genome (Kent, 2002).

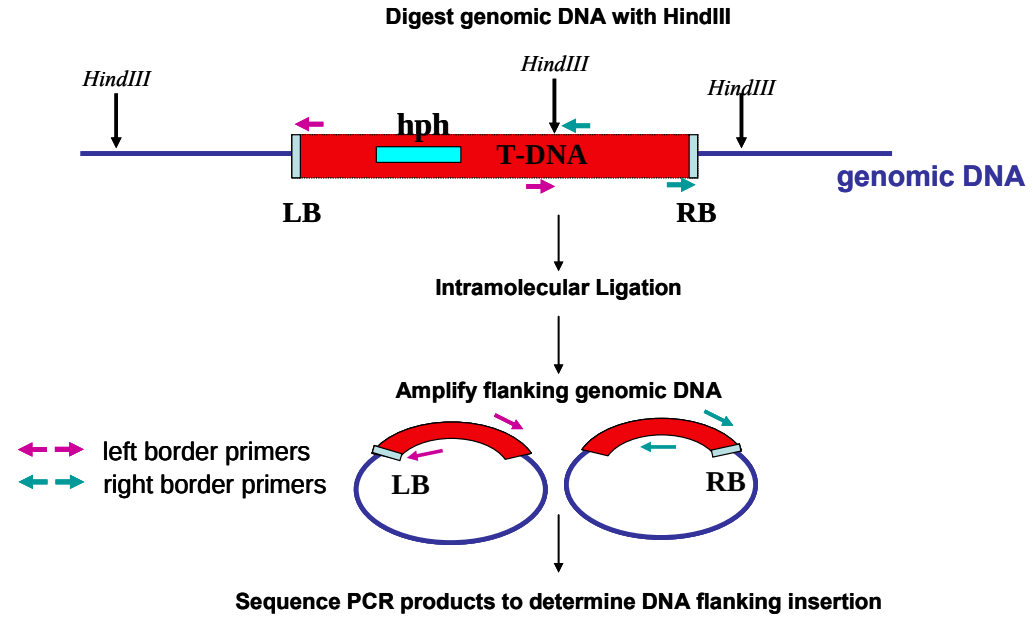

Figure 2-3. Schematic of the inverse PCR protocol

Northern Blot Analysis

Two-day *H. capsulatum* cultures were pelleted and RNA was harvested from the yeast cells as previously described (Hwang *et al.*, 2003). 4 ug of total RNA from each strain was separated on a 1.5% denaturing, formaldehyde agarose gel and transferred to a positively charged nylon membrane (Roche 11209299001). The membrane was then subject to Northern Blot analysis according to the Digoxigenin (DIG) Northern Blot protocol (Roche Applied Biosciences). DIG labeled probes were generated from WU15 genomic DNA using the PCR DIG probe synthesis kit (Roche 11636090910). The genes of interest and the primers used to generate their DIG labeled probes are listed in Table 1. The membrane was then exposed to film and developed.

Mutant	Predicted gene	Primer	Sequence
138-G1	*BUB2*	OAS1979	GCTTTCACCAAGGAGCCATGAT
		OAS1980	TTGAGGAAGTTCATTGAGCATGAGT
	CBP2	OAS1981	GCACCAATTGCAGCGTTCGG
		OAS1982	CCAGCACTAGTCACGGTGGATGT
157-C1	*CKH1*	OAS1983	ATGCAGGACGAGCCTCCTTG
		OAS1984	GAACACAGTGGGGGAGACGG
155-B1	*ATP1*	OAS1985	ATGCCACGGAAAGCAATCGA
		OAS1986	CCAATTGATGAGCTCTTGCAGC
172-C5	*CBP1*	OAS1987	GAAAACCCAGCGAAAATCACCTCC
		OAS1988	GATGATGATGATGCTGGTGAGAGG
UA6-C8	*TBF1*	OAS1532	GGTATCGATGCTTTGCAGCA
		OAS1611	CGGTCTTCATTGTTGGGGTT
FE6-C3	*HCL1*	OAS1592	GGGGACAAGTTTGTACAAAAAAGCAGGCTC
			CTTGTTTGCCGCTGCTACAGAAGC
		OAS1593	GGGGACCACTTTGTACAAGAAAGCTGGGT
			CCGCATTCCGAGACTGTGGAGAGTG

Table 2-1. Primers used to generate probes for Northern blot analysis

Intracellular growth assay

In 24-well tissue culture treated dishes, 2 x 10^5 BMDMs were infected, in duplicate, with *H. capsulatum* strains at various MOIs. After a 2-hour incubation period, the media was removed from the infected BMDMs, the monolayers were washed twice with DMEM and 1ml BMM/ura was added to each well. The infected macrophages were then incubated at 37^{o}C with 5% CO_2. The media was changed at 48 hours post infection and every day thereafter.

At various timepoints post infection, the media was removed from each well and 500 ul ddH_2O was added. After 5 minutes of incubation, the macrophages were mechanically lysed by vigorous pipetting. The lysate was collected, sonicated, diluted in HMM and plated for *H. capsulatum* colony forming units (CFUs) on HMM/ura-agarose plates. Colonies were counted 14 days later and the doubling times were calculated.

Growth on minimal media

Serial dilutions of late-log/stationary phase cultures of various LDF mutants were spotted onto HMM/ura-agarose plate and 3M-agarose plates. The components of 3M have been described previously (Warsham and Goldman, 1988).

pH modulation assay

25 mls *H. capsulatum* liquid cultures (OD_{600}=4-5) were pelleted and resuspended in pH-HMM containing 40 ug/ml bromcressol purple, pH 4.5. Cultures were incubated at 37^{o}C with 5% CO_2 in an orbital shaker. At various timepoints, a 1 ml sample was removed from each culture. The pH was measured using a digital pH meter and the OD_{600} was measured using the Eppendorf Biophotometer.

Results

A genetic screen identifies *H capsulatum* lysis defective (LDF) mutants

I designed a screen to identify *H. capsulatum* insertion mutants that fail to lyse macrophages. These mutants will be referred to as lysis-defective (LDF) mutants (Figure 2-4). A library of 14,000 random *H. capsulatum* mutants was generated using *Agrobacterium tumefaciens*-mediated transformation. The resulting transformants contained a fragment of *A. tumefaciens* DNA, referred to as T-DNA, randomly inserted into their genome. This library was then used to infect two types of macrophages, J774 macrophage-like cells and primary BMDMs. The mutants that exhibited a phenotype in both cell types were the most robust LDF mutants and were the only mutants that were assessed. J774 macrophage-like cells were infected, in duplicate, at an MOI of 10. Five days later, lysis of the macrophage monolayer was assessed using the crystal violet assay. Primary BMDMs were also infected, but at an MOI of 5, and lysis was monitored 3 days post-infection. Forty-seven insertion mutants reproducibly exhibited a lysis defect in both J774 macrophages and BMDMs as measured by the crystal violet assay.

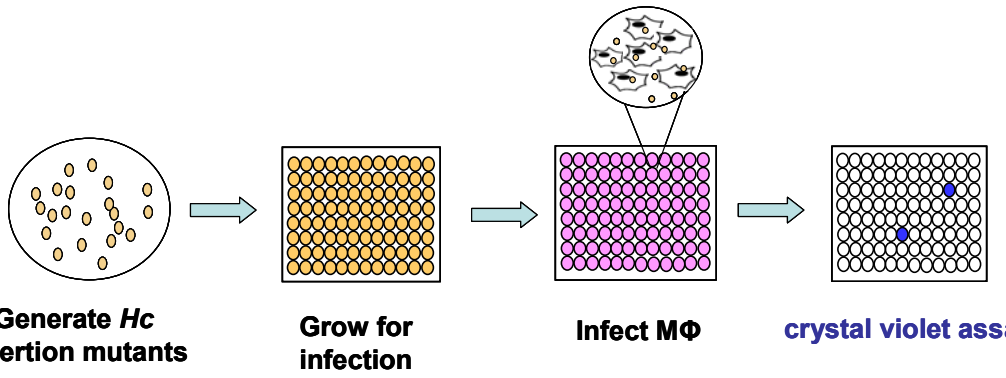

Figure 2-4. Schematic of LDF screen

The lysis defect of the LDF mutants was verified and quantified using the lactate dehydrogenase (LDH) release assay. This assay measures the release of the stable cytosolic enzyme lactate dehydrogenase into the culture supernatant when macrophages lyse. BMDMs were infected with putative LDF mutants at an MOI of 5 and BMDM lysis was monitored over the course of a 5-6 day infection using the LDH release assay. Two putative LDF mutants no longer exhibited a lysis defect (data not shown) in this assay. These mutants were not analyzed further. The lysis defect of the remaining LDF mutants ranged in severity from moderate to severe. While wild-type *H. capsulatum* started to lyse BMDMs within 24 hours post-infection, macrophages infected with severe LDF mutants did not exhibit significant lysis during the course of the infection. This group of severe LDF mutants included *cbp1*, *bub2*, and *hcl1* (Figure 2-5). The majority of the LDF mutants had a moderate lysis defect as exemplified by *atp1* and *ldf4*. These mutants exhibited a delay in kinetics of macrophage lysis, where the onset of lysis did not occur until 3 days post-infection.

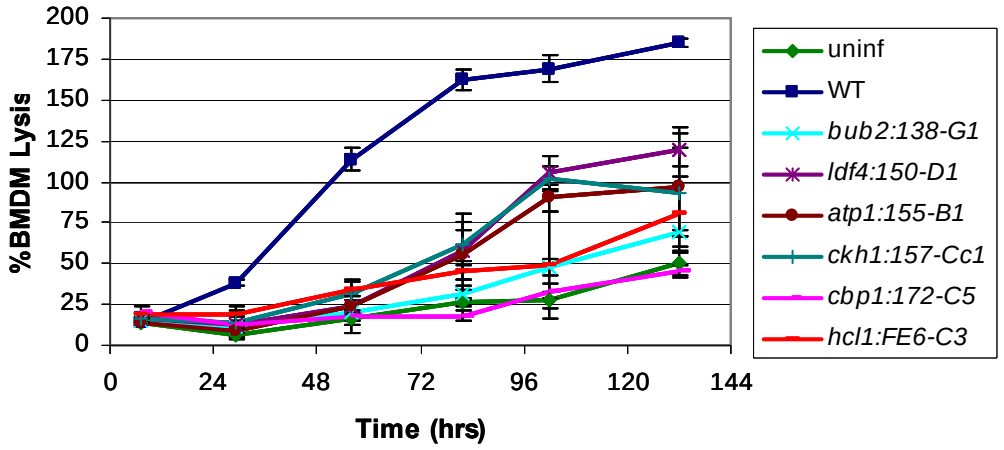

Figure 2-5. LDF mutants exhibit a range of lysis defects.
BMDMs were either mock infected or infected with various LDF mutants at MOI = 5. At 7, 30, 56, 82, 102, and 132 hrs post-infection, the supernatant was removed from the infected monolayers and lactate dehydrogenase activity was measured to monitor the percentage of BMDM lysis that occurred. The mean ± standard deviation is shown.

Genotypic analysis of the LDF mutants

Determining the number of insertion sites in each LDF mutant

A. tumefaciens-mediated transformation typically yields single-site genomic insertions in 80% of transformants. To determine the number of insertion sites in the LDF mutants, I performed Southern Blot analysis. Genomic DNA from each mutant was digested with SpeI, a restriction site that is not present in the inserting T-DNA. This digested DNA was then probed for the presence of the *hph* gene which is located within the T-DNA. Single-site insertion mutants would contain only one SpeI fragment that hybridized to the *hph* probe. This analysis indicated that three putative LDF mutants had insertions at multiple sites in the genome (Figure 2-6). An example of one such mutant, 197-D9, is shown. These mutants were not analyzed further. I was unable to detect the *hph* gene by Southern Blot analysis in two LDF mutants, despite the ability of these

mutants to grow in media supplemented with hygromycin. Mutant 114-G2 is an example of one such LDF mutant. These mutants were not analyzed further.

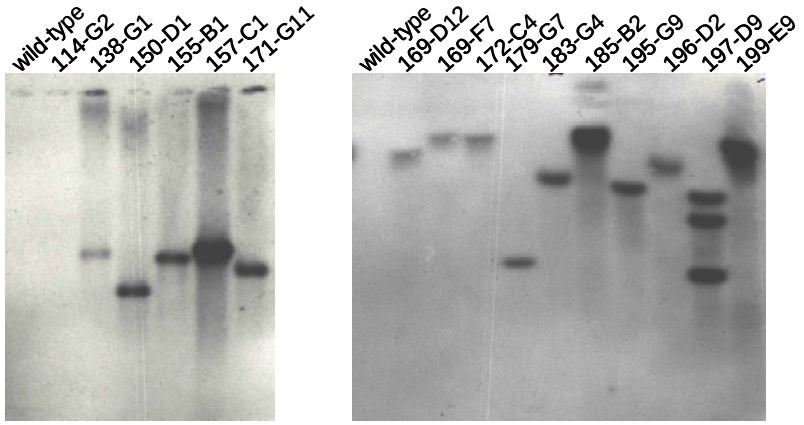

Figure 2-6. Southern blot analysis identifies single-site insertion mutants
Genomic DNA was harvested from each mutant and digested with SpeI. The digested DNA was then separated by gel electrophoresis, transferred to a nylon membrane and probed with ^{32}P-radiolabeled *hph* probe.

Mapping the site of insertion

To identify the genes that were disrupted in LDF mutants, I mapped the location of the T-DNA insertion in the G217B genome. The *H. capsulatum* G217B genome was sequenced to 13.2X coverage and assembled into 261 contigs (Genome Sequencing Center (GSC), Washington University). I used inverse PCR to map the T-DNA insertions sites for 38 LDF mutants (Figure 2-3). Genomic DNA was isolated from each mutant and digested with HindIII. This restriction enzyme cuts once within the T-DNA insertion, in addition to cutting within the genome. This digest liberates the left and right borders of the insertion with the corresponding flanking genomic DNA. After performing an intra-molecular ligation, I amplified the flanking genomic DNA using inverse PCR with

divergent primers that annealed to the T-DNA sequence. The PCR products were sequenced and the resulting sequences were aligned to the G217B genome using the BLAST-like alignment tool (BLAT) (Kent, 2000).

I was able to map both the left and the right borders for 21 LDF mutants. In 80% of the mutants, the genomic DNA flanking the left and the right borders of the T-DNA insertion were adjacent to each other (Figure 2-7A). This indicates that the T-DNA inserted without causing any genomic rearrangement at the locus. For five LDF mutants, however, the borders did not map to adjacent regions in the genome. In two instances, the sequences for the left and the right border were located on the same contig, but several kb apart – 11kb and 140kb, suggesting an intervening deletion had occurred. In three LDF mutants, the left and the right border were located on different contigs. It is likely that the T-DNA insertion in these five mutants resulted in a genomic rearrangement that affects the expression of multiple genes. These five mutants were withdrawn from further analysis.

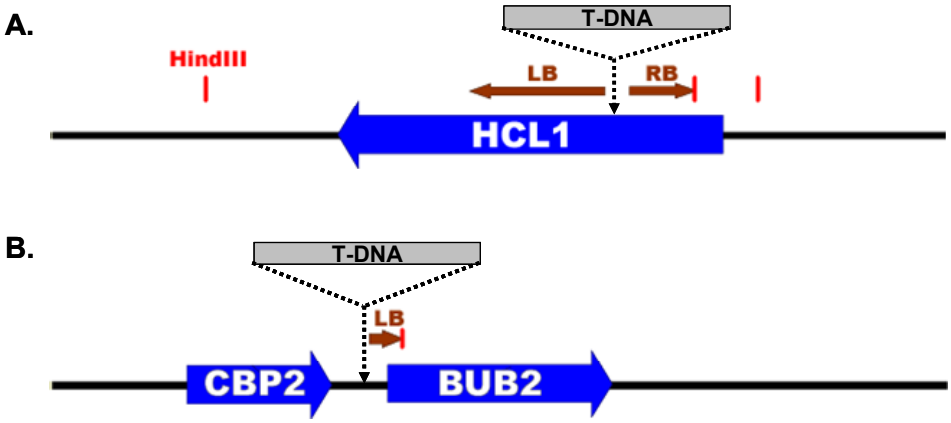

Figure 2-7. Inverse PCR identifies the site of insertion in the LDF mutants.
Inverse PCR was used to amplify the genomic DNA flanking the left border (LB) and the right border (RB) of the T-DNA insertion. These PCR products were then sequence and aligned to the G217B genome.

A. The genomic locus containing the T-DNA insertion in mutant FE6-C3 (*hcl1*) is diagrammed. The left and right border genomic sequences are located adjacent to each other in the *HCL1* ORF.

B. The genomic locus containing the T-DNA insertion in mutant 138-G1 (*bub2*) is diagrammed. Only the left border genomic DNA sequence could be identified. It mapped to an intergenic region between *CBP2* and *BUB2*.

Several mutants could not be mapped completely using inverse PCR. Fourteen LDF mutants yielded PCR products and sequence data for only one border of the T-DNA insertion, usually the left border. For ease of analysis, I presumed that these insertions occurred with no resulting genomic rearrangements. Four LDF mutants had insertions in repeat regions and transposon-associated regions of the genome. This made it difficult to map the specific location of the insertion. No PCR products were obtained for 2 LDF mutants. Consequently I was unable to map their sites of insertion in the genome. These 6 mutants were not analyzed further. At the conclusion of this genomic analysis of the LDF mutants, I had a final set of 26 LDF mutants that were mapped to single sites in the genome.

Based on the genomic location of the T-DNA insertion, I was able to predict which genes would be disrupted in the LDF mutants. This analysis was most straightforward when the T-DNA inserted into the ORF or the promoter of a gene.

Thirteen mutants contained insertions in predicted ORFs. Another thirteen mutants contained intergenic insertions, five of which appeared to be in gene promoters. I was unable to predict the disrupted gene in mutants that contained intergenic insertions located either between divergently transcribed genes or between the 3' ends of two genes. There were 5 such ambiguous LDF mutants. Complementation with either gene to rescue the lysis defect in these mutants or targeted deletion of these genes to determine which deletion recapitulates the LDF phenotype will provide definitive evidence regarding which gene plays a role in virulence.

One of the LDF mutants identified in this screen contains an insertion in the promoter of the known virulence factor *CBP1*. This LDF mutant has a severe lysis defect, which is consistent with the published data (Sebghati *et al.*, 2000). Our ability to identify the one virulence factor known to play a role in the virulence of the G217B strain validates our screening methodology.

Annotation of the LDF genes

I mapped the genomic disruption site in twenty-six single-insertion LDF mutants. For 18 of these LDF mutants, I could predict which gene was most likely to be disrupted based on the location of the insertion. These mutants had insertions in the ORF of a gene, in the promoter of a gene, or downstream and proximal to a gene. This analysis, however, is complicated by the fact that there is little experimental validation of the *H. capsulatum* G217B gene set. There is only limited experimental data available to verify that these annotated genes are real and expressed.

Recent work from our lab has addressed this issue by characterizing the yeast transcriptome of *H. capsulatum* G217B using whole genome tiling arrays (WGTA) (Voorhies *et al.*, in preparation). PolyA-selected RNA was harvested from yeast cells grown under various conditions - early, middle, and late logarithmic growth, stationary phase, heat shock, oxidative stress and sulfhydryl reducing stress. RNA was also prepared from yeast cells cultured in different media, including HMM, 3M (minimal media) and YPD. This RNA was then hybridized to WGTAs and analyzed. A GSC predicted gene was determined to be expressed if at least 50% of the predicted base pairs overlapped with the transcript detected by the WGTA. Together with Mark Voorhies, I analyzed the set of 18 LDF genes to determine if their expression was detected on the G217B WGTA and found that 10 of the 18 genes were detected (data not shown).

Because the WGTA data do not comprehensively represent all transcriptional states of *H. capsulatum*, I also interrogated spotted oligonucleotide microarray data that included expression profiling from morphological forms of *H. capsulatum* that are distinct form the yeast form. Spotted microarrays with oligonucleotides representing the G217B predicted genes had been used to characterize the gene expression profile of G217B yeast, mycelia, and conidia (Nyugen *et al.*, 2008, Inglis *et al.*, in preparation). I examined the set of 18 LDF genes to determine which had corresponding 70-mer oligonucleotides probes that hybridized to RNA in any of the *H. capsulatum* growth phases. 70-mer oligonucleotides representing 16 genes hybridized to RNA, providing experimental validation for the expression of these genes. Consequently, 89% of the LDF genes were validated experimentally. There was no evidence for expression of the disrupted gene in the LDF mutants UA37-C11 and 176-C10. This could be due to the fact

that these predicted genes are not real genes or that we have not examined the conditions under which these genes are expressed.

To better understand the function of the LDF genes, Mark Voorhies and I examined orthologs in more well characterized fungal systems. Twelve non-*Histoplasma* genomes were analyzed, including *Coccidioides immitis, Aspergillus nidulans, Aspergillus fumigatus, Aspergillus oryzae, Neurospora crassa, Magnaporthe grisea, Fusarium graminearum, Candida albicans, Saccharomyces cerevisiae, Saccharomyces pombe, Cryptococcus neoformans,* and *Ustilago maydis*. Five *Histoplasma* genomes were also examined; including two independently sequenced G186AR genomes, WU24, H88, and Hl43. Seven LDF genes had orthologs in at least 12 fungal genomes (Figure 2-8 and Table 2-2). The remaining 11 genes were found almost exclusively in *H. capsulatum* species and will be referred to as "Histoplasma-specific" genes . (Figure 2-8). *CBP1*, the known virulence factor that was isolated in the LDF screen, is a member of this group. Interestingly, 61% of the LDF gene set represents *Histoplasma*-specific genes, an enrichment when compared to the 20% of the G217B predicted gene set they represent. The prevalence of these genes in *H. capsulatum* genomes and their identification in the LDF screen suggests that they may play roles in *H. capsulatum*-specific virulence mechanisms.

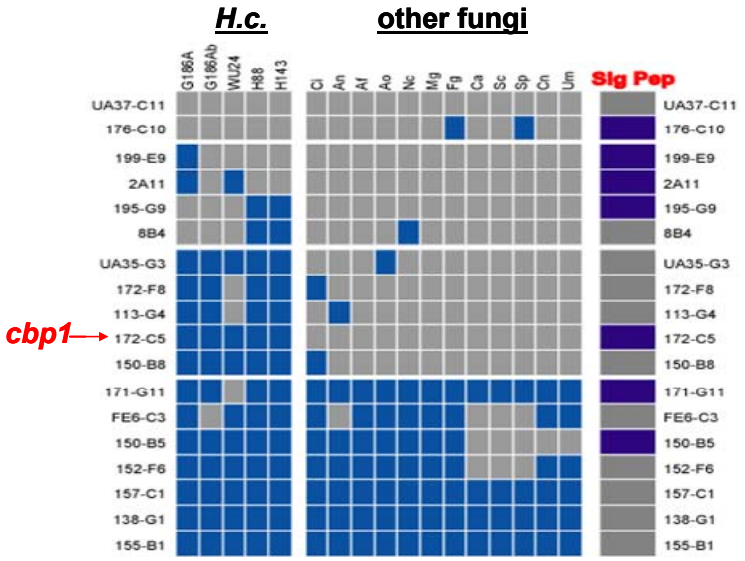

Figure 2-8. The majority of LDF genes are *Histoplasma*-specific genes.
Orthologs for LDF genes were identified in 16 fungal genomes (4 *Histoplasma* and 12 non-*Histoplasma*). The presence of an ortholog is indicated with a blue box, while the absence of an ortholog is indicated by a grey box. The presence of an N-terminal signal peptide motif was determined using InterProScan. The results are shown in the Sig Pep column. Blue indicates the presence of a predicted signal peptide.

To gain further insights into the potential function of the LDF genes, I used InterProScan to identify amino acid motifs. One motif that was significantly represented among the LDF genes was the signal peptide motif. This motif is of particular interest in virulence-associated genes because it suggests that the corresponding proteins are either secreted or present on the surface of the fungus, where they can potentially interact with host factors. Seven genes had a predicted signal peptide motif present at the N-terminus of their protein sequence (Figure 2-8). Interestingly, five of these genes are *Histoplama*-specific genes.

Mutant	Predicted gene	Putative Function
171-G11	*MVP1*	Sorting Nexin
FE6-C3	*HCL1*	HMG CoA lyase
150-B5	*GSH1*	Glycoside hydrolase
152-F6	*ABC1*	ABC transporter
157-C1	*CKH1*	Casein kinase homolog
138-G1	*BUB2*	RAB GTPase
155-B1	*ATP1*	N-acetyl transferase/ATPase

Table 2-2. Genes conserved in non-*Histoplasma* fungal genomes
The seven LDF genes that are conserved among diverse fungi are shown (refer to Figure 2-8). Putative functions are based on motif analysis.

Monitoring the expression of disrupted genes

The location of the T-DNA insertions allowed me to predict which genes were disrupted in the LDF mutants. I then used Northern blot analysis to experimentally verify that the expression of these genes was affected in the mutants. I hypothesized that the disrupted gene would either no longer be expressed in the mutant or would be aberrantly expressed. Such aberrant expression would result if the upstream regulatory region of the genes was affected by the T-DNA insertion. Initial experiments examined the expression of the disrupted genes in the most severe LDF mutants. RNA was extracted from wild-type *H. capsulatum* and several LDF mutants and was then probed for expression of the predicted disrupted gene.

This analysis was performed on six LDF mutants. Four mutants (*atp1*, *hcl1*, *ckh1*, and *tbf1*) contained insertions in their respective ORFs. Of the ORF insertion mutants

analyzed, the *atp1* mutant (155-B1) did not express *ATP1*, and the *hcl1* (FE6-C3) and the *ckh1* (157-C1) mutants expressed altered *HCL1* and *CKH1* transcripts that were smaller than their respective transcripts in wild-type *H. capsulatum* (Figure 2-9 and data not shown). The expression of a smaller transcript may be the result of transcription initiation in the T-DNA, which has been observed previously. Consistent with this hypothesis, the insertions in these mutants are located in the 5' end of the ORFs. The *tbf1* mutant (UA6-C3), on the other had, expressed wild-type levels of *TBF1*, suggesting that the LDF phenotype was not linked to the T-DNA insertion at the *TBF1* locus (data not shown).

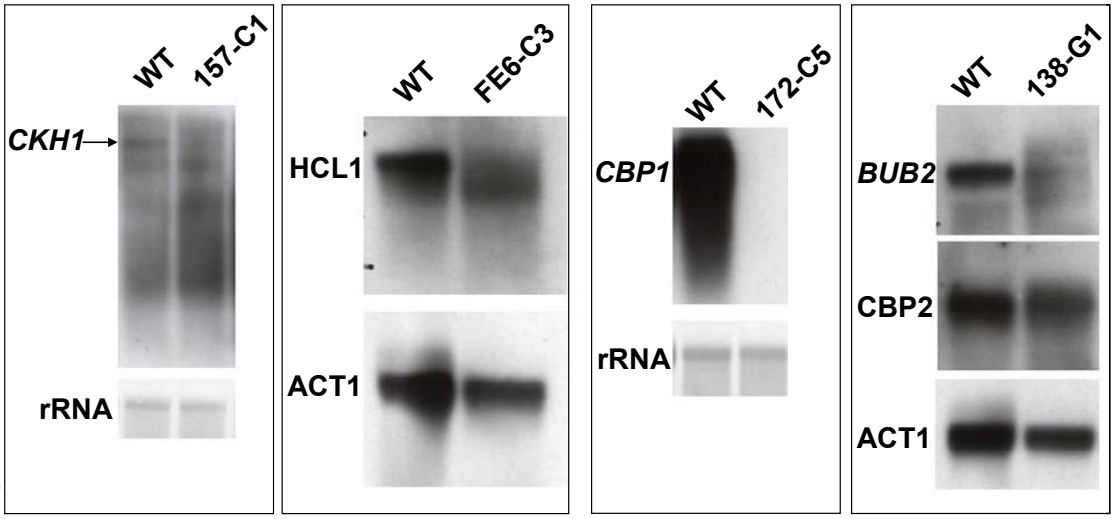

Figure 2-9. LDF mutants are defective in their expression of the disrupted gene
RNA was harvested from the LDF mutants during growth in culture was separated by gel electrophoresis, transferred to a nylon membrane and probed for expression of the indicated gene. Methylene blue was used to stain rRNA.

Two mutants *cbp1* (172-C5) and *bub2* (138-G1)) contained promoter insertions. The *cbp1* mutant contained an insertion that was located in a 5kb intergenic region. The insertion was located approximately 200 bp upstream of the *CBP1* ORF and approximately 4kb downstream of the proximal gene. The location of this insertion suggested *CBP1* was the gene most likely to be disrupted. Northern blots analysis confirmed this was indeed the case and showed that *CBP1* was not expressed in the LDF mutant 172-C5. The *bub2* mutant contained an insertion in an intergenic region, 138bp upstream of the *BUB2* gene (Figure 2-9b). Due to the small size of this intergenic region, 415bp, we examined the expression of both *BUB2* and the other gene flanking the insertion, *CBP2* (no relationship to *CBP1*), using Northern blot analysis. *CBP2* expression in the mutant was similar to its expression in wild-type *H. capsulatum*. The *BUB2* transcript, however, was no longer expressed. This result indicated that disruption of *BUB2* was likely responsible for the LDF phenotype. Verifying that the genomic insertion affects the expression of the disrupted gene is important for ascribing the LDF phenotype to a *H. capsulatum* factor. Here, I have shown that this is the case for 5 of 6 LDF mutants.

Verifying the disrupted gene in responsible for LDF phenotype

Once the disrupted gene has been identified and the expression of this gene is shown to be altered in the LDF mutant, one step remains in definitively linking the LDF phenotype to the disrupted gene. This involves either rescuing the lysis defect of the LDF insertion mutant by expressing a wild-type copy of the disrupted gene, or recapitulating the LDF phenotype by targeting this gene for disruption in wild-type *H. capsulatum*.

These experiments are ongoing in the lab. To date, we have complemented two LDF mutants, *cbp1* (172-C5) (see chapter 3) and *hcl1* (FE6-C3) (data not shown; Alison Coady, personal communication).

Phenotypic analysis of the LDF mutants

Genomic analysis of the LDF mutants allowed me to identify *H. capsulatum* genes associated with virulence during macrophage infection. While sequence homology to genes in other organisms can provide clues to the function of a gene, this bioinformatic analysis is of limited utility because only a small fraction of the LDF genes have well-characterized homologs. To better understand the role these LDF genes play during infection, I performed a number of assays to monitor virulence-associated phenotypes. These phenotypes include ability of these mutants to grow within BMDMs, to express *CBP1*, to grow on minimal media and to modulate pH *in vitro*. I also examine the relative expression of each of the LDF genes in the various growth phases of *H. capsulatum* – yeast, mycelia, and conidia – as differentially higher expression in the yeast phase is a characteristic of the previously identified G217B virulence factors *CBP1* and *YPS3*.

Intracellular growth

To monitor the intracellular viability and replication of the LDF mutants, I performed intracellular CFU assays. BMDM monolayers were infected with wild-type *H. capsulatum* or the LDF mutants at MOIs ranging from 0.25 to 2. At various timepoints post-infection, the infected BMDMs were lysed and the lysate was plated to measure fungal CFUs. Timepoints were taken for each strain until the onset of lysis to avoid

quantifying the extracellular replication of yeast cells released from lysed BMDMs. CFUs from each timepoint were counted and intracellular doubling times were calculated.

To date, this analysis has been performed for 17 of the 26 single-site insertion LDF mutants. Surprisingly, none of the LDF mutants showed a loss of overall viability within the macrophage. An increase in CFUs over time was observed for all of the LDF mutants tested. However, the rate of increase differed among the LDF mutants. This was determined based on the intracellular doubling times. Wild-type *H. capsulatum* had an intracellular doubling time of 13.87±2.13 hours. LDF mutant 179-G7 replicated as well as wild-type *H. capsulatum* in BMDMs (Figure 2-10). The majority of the LDF mutants had a mild to moderate decrease in their rate of intracellular replication that was characterized by a doubling time less than or equal to 1.5 times that of wild-type *H. capsulatum*. Two LDF mutants exhibited severely impaired growth kinetics. *tbf* (UA6-C8) and *hcl1* (FE6-C3) had intracellular doubling times that were greater that twice the doubling time of wild-type *H. capsulatum*, 30 and 36 hours respectively.

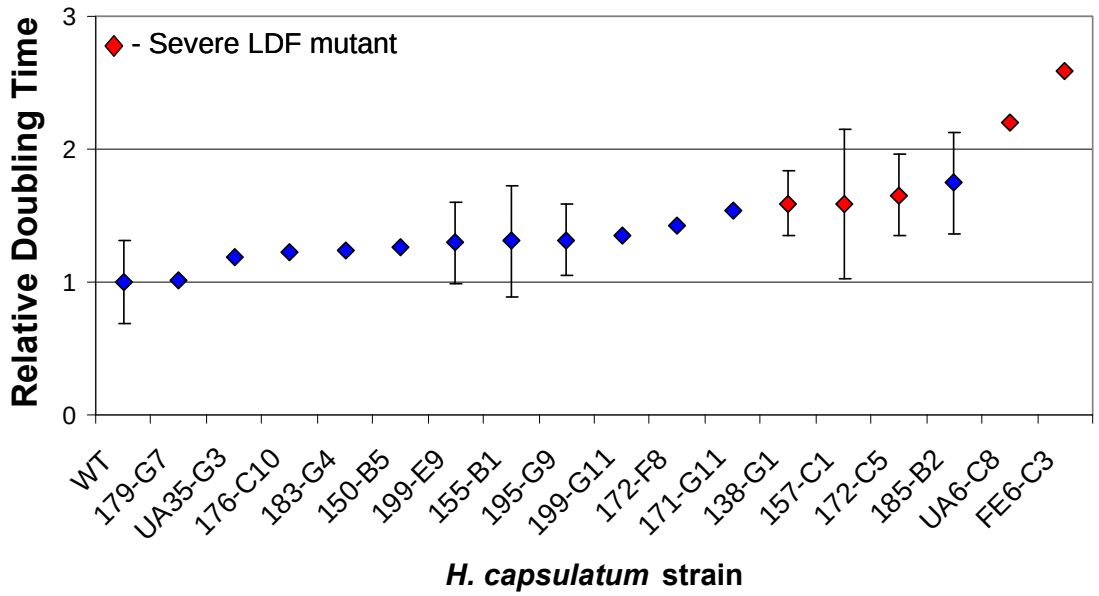

Figure 2-10. The LDF phenotype does not always correlate with an intracellular growth defect. BMDMs were infected at various MOIs with the indicated LDF mutants. At each timepoint, infected BMDMs were lysed and lysates were plated for *H. capsulatum* CFUs. Doubling times were calculated. Average doubling time relative to wild-type is shown ± standard deviation when applicable.

The relationship between the intracellular replication kinetics defect and the lysis defect was not straightforward. On one hand, the *tbf1* (US6-C8) and *hcl1* (FE6-C3) mutants had a severe macrophage lysis defect as well as a slower growth rate in the macrophage. On the other hand, the severe LDF mutants, 138-G1 (*bub2*), 157-C1 (*ckh1*), and 172-C5 (*cbp1*), had intracellular doubling times that were similar to those of more moderate LDF mutants. Additionally, the LDF mutants 138-G1 (*bub2*), 172-C5 (*cbp1*), and UA6-C8 (*tbf1*) achieved high fungal burdens within BMDMs without causing lysis (Figure 2-11 and data not shown). These results indicate that the ability to achieve a high intracellular fungal burden is not sufficient to cause macrophage lysis.

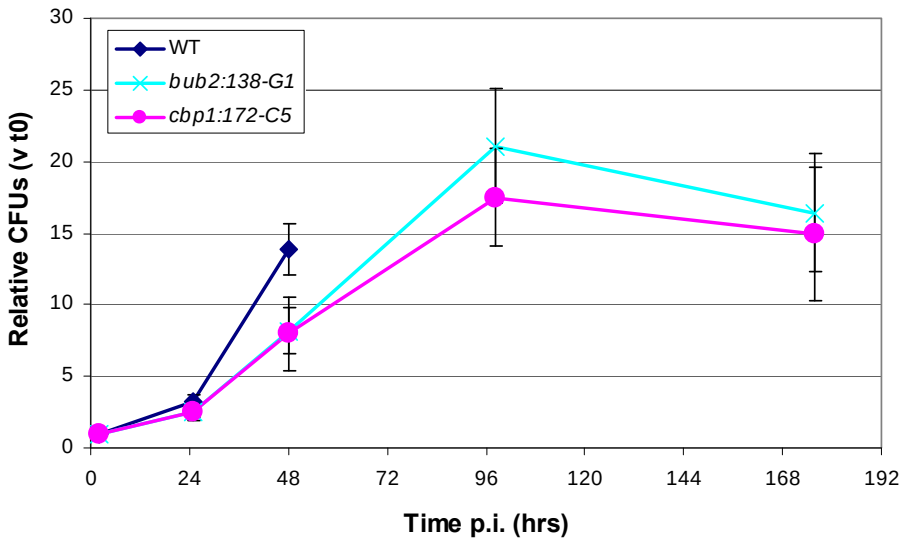

Figure 2-11. LDF mutants 138-G1 (*bub2*) and 172-C5 (*cbp1*) achieve a high fungal burden in BMDMs. BMDMs were infected at an MOI of 2. At each timepoint, infected BMDMs were lysed and lysates were plated for *H. capsulatum* CFUs. Relative CFUs, as a fraction of CFUs at the 2 hour timepoint are graphed above. Average relative CFUs are shown ± standard deviation.

CBP1 and *YPS3* expression

Two LDF mutants, 172-C5 (*cbp1)* and 138-G1 (*bub2)*, were able to grow to wild-type numbers in BMDMs, despite exhibiting a severe lysis defect. I hypothesized that these mutants might be defective in similar virulence mechanisms. Since *cbp1* was one of the mutants in this group, I decided to test a subset of the LDF mutants for expression of *CBP1*. With the exception of *CBP1* transcript – UA6-C8 (*tbf1),* 172-C5 (*cbp1)* and 138-G1 (*bub2),* all the other LDF mutants expressed a normal *CBP1* transcript (Figure 2-12).

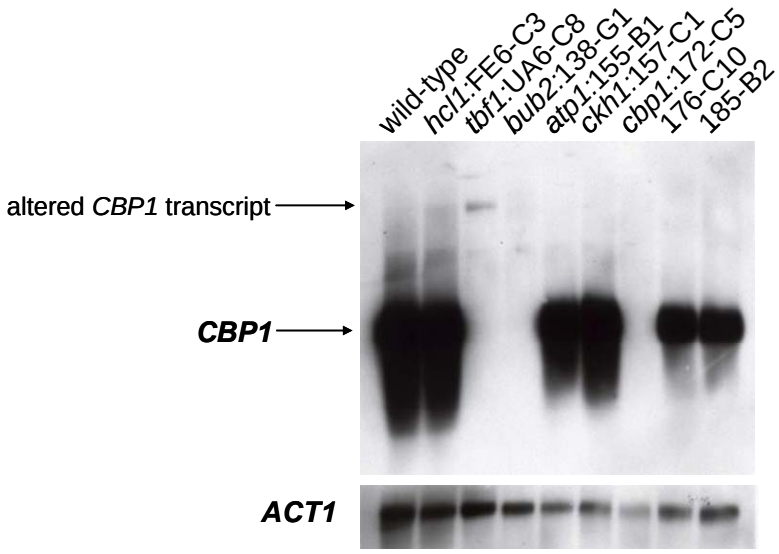

Figure 2-12. Severe LDF mutants 138-G1 *(bub2)*, 172-C5 *(cbp1)*, and UA6-C8 *(tbf1)* do not express *CBP1*
4 ug of total RNA from all strains were separated on a 1.5% denaturing, formaldehyde agarose gel and transferred to a positively charged nylon membrane The membrane was then subject to Northern blot analysis to determine levels of *CBP1*. *ACT1* expression is monitored as a loading control.

In the *tbf1* mutant, the *CBP1* probe hybridized to a transcript that was significantly larger in size and decreased in abundance when compared to the *CBP1* transcript in wild-type *H. capsulatum*. When I amplified a 4kb region of the *CBP1* genomic locus in the *tbf1* mutant, I observed a product that was larger than expected, indicating that there was a genomic rearrangement at the *CBP1* locus in the *tbf1* mutant (Figure 2-13c). The LDF phenotype could be a result of this genomic rearrangement and not a consequence of the insertion in the *TBF1* gene. The wild-type level of *TBF1* gene expression observed in the *tbf1* mutant was consistent with this hypothesis.

The *cbp1* and *bub2* mutants did not express detectable levels of *CBP1* transcript by Northern blot analysis. This was expected for the *cbp1* insertion mutant and accordingly amplification of the *CBP1* genomic region did not yield a product (Figure 2-13b). This was likely due to the large T-DNA insertion in that region. PCR amplification of the

CBP1 genomic locus in the *bub2* mutant showed no signs of gross genomic rearrangement. It is still possible that there is a single-site mutation at the *CBP1* locus in this mutant that abrogates *CBP1* expression. Such a change would be undetectable by PCR. Alternatively, the lack of *CBP1* expression in the *bub2* mutant could occur because *BUB2* is required for *CBP1* expression.

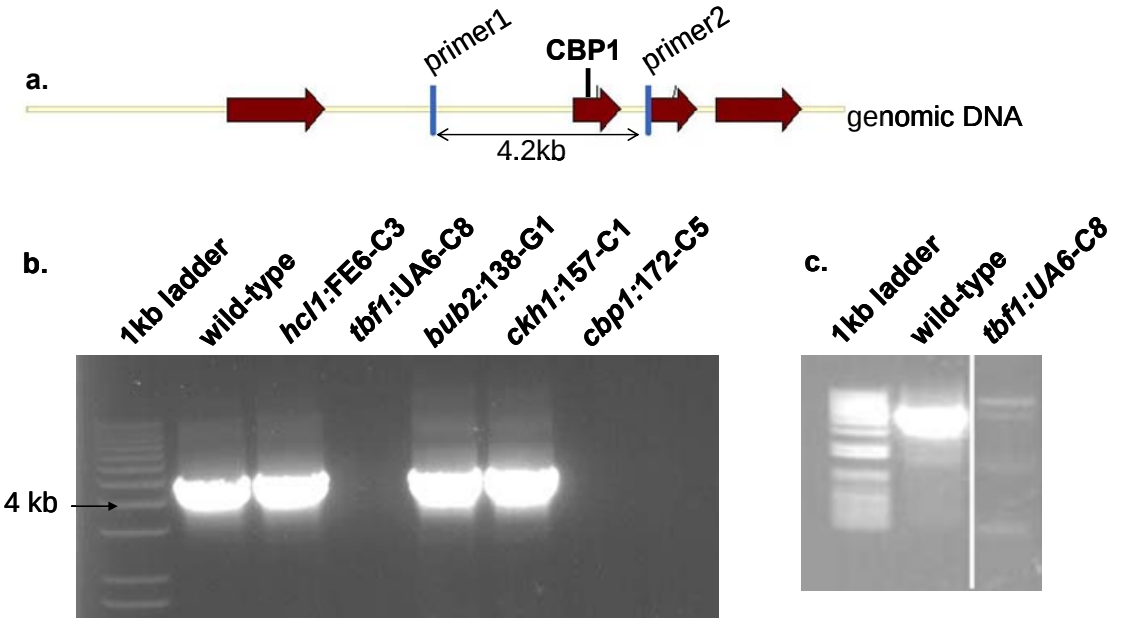

Figure 12-13. Examination of *CBP1* locus in LDF mutants.
a. Schematic depicting the *CBP1* locus and the location of primers used in PCR.
b. PCR amplificaton of *CBP1* locus from genomic DNA of indicated *H. capsulatum* strains. Products are separated on 0.8% agarose gel.
c. Amplification of the *CBP1* locus in *tbf1* mutant (UA6-C8) with longer extension time.

The screen effectively identified 3 "*cbp1"* mutants. Each mutant achieves wild-type levels of intracellular growth without causing macrophage lysis. This data strongly indicates that a high fungal burden is not sufficient to cause macrophage lysis and suggests that *CBP1* may play a critical role in promoting lysis during infection.

Yeast phase expression of the LDF genes.

H. capsulatum must convert to the yeast form in order to parasitize the host and cause disease. It is therefore not surprising that many of the virulence factors that were identified prior to this study were predominantly expressed during yeast phase growth. This includes α-1,3-glucan, *CBP1*, and *YPS3*. This data suggested that there might be a correlation between the yeast phase expression of a gene and the role of that gene in virulence. To determine if there was a correlation, Mark Voorhies and I examined the relative expression of the 18 LDF genes in *H. capsulatum* yeast, mycelia, and conidia in data from transcriptional profiling experiments previously performed in the lab (Inglis *et al.*, in preparation) (Figure 2-14). Few genes were significantly differentially expressed, using a four-fold cutoff, in one state versus the others. Genes that were enriched greater than 4-fold in yeast cells compared to either mycelia or conidia included *LDF1* (mutant 2A11) and *CBP1* (mutant 172-C5). In fact, *LDF1* is the most highly expressed gene in the yeast form of the fungus. Genes disrupted in LDF mutants 8B4 and UA35-G3 were more highly expressed in G217B conidia than in yeast or mycelia. The disrupted genes in 152-F6 was more highly expressed in mycelia and conidia, compared to yeast cells, and the disrupted genes in both 150-B5 and 157-C1 were more highly expressed in yeast and mycelia compared to conidia. It may be the case that some of these mycelial- or conidial-induced transcripts are induced in yeast form cells during infection of macrophages. Nevertheless, there is very little enrichment for yeast-phase specific expression *in vitro* for the LDF gene set.

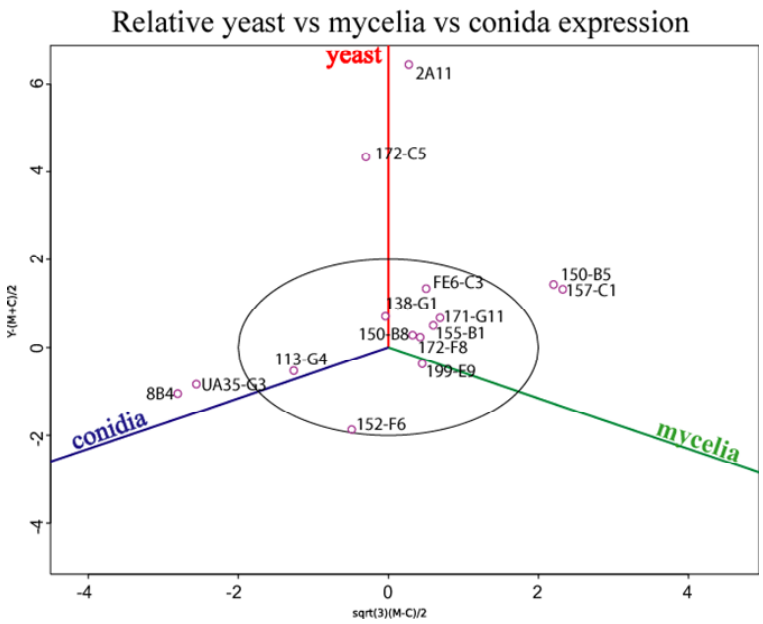

Figure 2-14. Relative expression of LDF genes during *H. capsulatum* growth as yeast, mycelia, and conidia.
The relative expression of the LDF genes in the different phases of *H. capsulatum* growth are represented in $\log_2$. The circle is drawn at the 4-fold differential expression cut-off.

Growth on minimal media

A nutritional auxotroph would exhibit an LDF phenotype during macrophage infection. Such a mutant would be unable to acquire the nutrients necessary for survival within the host macrophage. To determine if any LDF mutants were auxotrophs for essential nutrients, I patched and plated serial dilutions of stationary cultures on HMM/ura and minimal 3M/ura plates. UA37-C11 was the only LDF mutant that displayed a growth defect on minimal media (Figure 2-15).

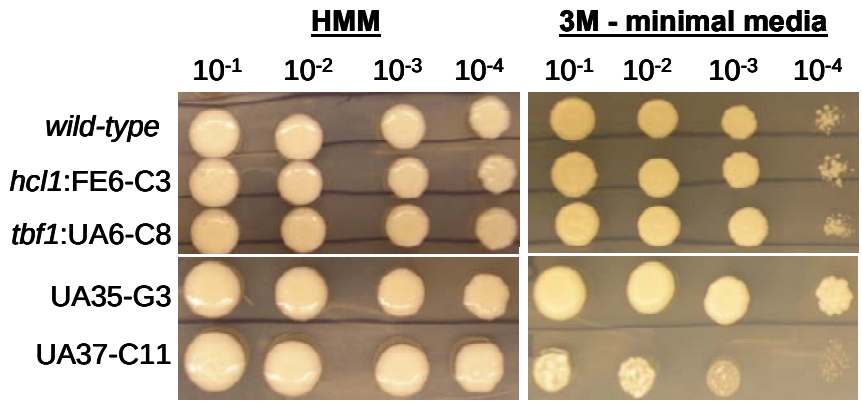

Figure 2-15. LDF mutant UA37-C11 has a growth defect on minimal media.
Serial dilutions of mid/late log cultures were spotted onto HMM or 3M plates that were supplemented with uracil. These images were taken 13 days later.

pH modulation *in vitro*

H. capsulatum inhibits the acidification of its phagolysosome and is able to maintain a near-neutral pH of 6.5 (Eisenberg *et al.*, 1993; Strasser *et al.*, 1999). While this inhibition of phagosome acidification is a well-documented feature of *H. capsulatum* infection of macrophages, its role in pathogenesis has yet to be determined. The identification of an LDF mutant that fails to inhibit phagolysosome acidification would provide the genetic evidence that is necessary to demonstrate that the ability to modulate phagosome pH is a key component of *H. capsulatum* virulence.

In addition to modulating the pH of the phagolysosome, *H. capsulatum* can modulate the pH of its culture media to a neutral pH (Berliner *et al.*, 1973). This occurs irrespective of the starting pH. This ability to modulate pH *in vitro* could serve as a proxy for the ability to modulate the pH of the phagosome. To determine if the LDF mutants retain their ability to modulate culture pH, I performed a pH modulation assay. In this

assay, yeast cells were grown to mid log phase, pelleted and resuspended in unbuffered acidic HMM. The pH of the culture was then monitored over the course of two days using a digital pH meter. OD_{600} was also measured to determine if exposure to the initial acidic pH affected growth. Several LDF mutants were monitored for their ability to modulate pH *in vitro*. All mutants were able to neutralize the pH of the media within 8 hours, similar to wild-type *H. capsulatum* (Figure 2-16). Interestingly, one mutant, the *hcl1* mutant, was not able to maintain a neutral pH. After 24 hours, the pH of the media started to re-acidify. The *hcl1* mutant has a pH modulation defect *in vitro* and this might explain the LDF phenotype of the mutant. If the *hcl1* mutant phagosome does not remain neutral, the fungus might be more susceptible to host antimicrobial defense mechanisms, including the activity of lysosomal hydrolytic enzymes.

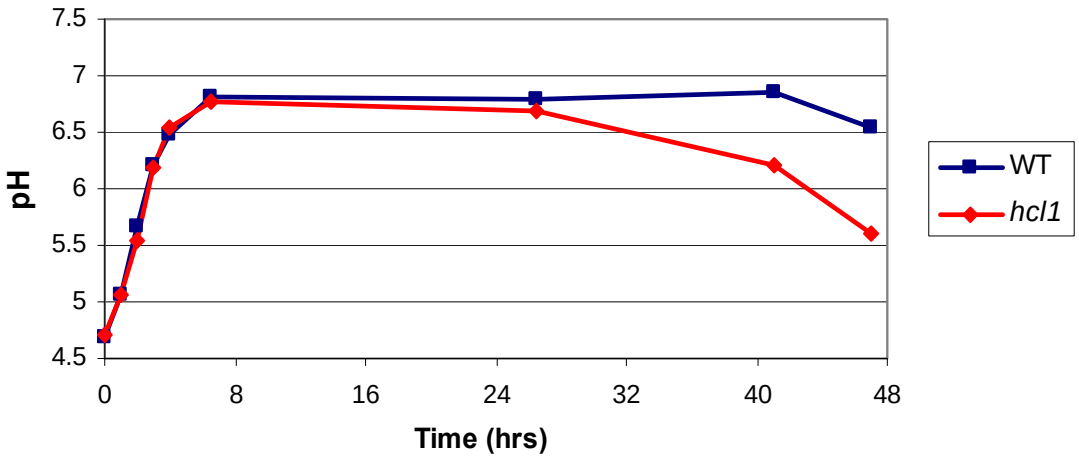

Figure 2-16. The *hcl1* (FE6-C3) mutant fails to maintain a neutral pH in culture
Mid-log cultures of wild-type *H. capsulatum* and the *hcl1* mutant were pelleted, re-suspended in HMM/ura (no buffer) at pH 4.5, and incubated at 37ºC, 5%CO_2. pH measurements were taken with a digital pH meter at the indicated timepoints.

Discussion

The goal of this work was to identify *H. capsulatum* virulence factors. To do this, I designed a forward genetic screen to test *H. capsulatum* insertion mutants for their ability to lyse macrophages. I screened 14,000 insertion mutants for their ability to lyse J774 macrophage-like cells and primary BMDMs during infection. Forty-seven LDF mutants displayed a lysis defect in the qualitative crystal violet assay. The quantitative LDH release assay demonstrated that the mutants had a range of lysis defects. Macrophage lysis was never observed upon infection with 5 severe LDF mutants. The remaining mutants displayed a more moderate lysis defect in which the kinetics of macrophage lysis were slower than they were for macrophages infected with wild-type *H. capsulatum*. Genomic analysis revealed that 26 mutants contained insertions at single sites in their genomes. These 26 mutants identified putative virulence determinants.

A key finding that comes my of our analysis of the LDF mutants is that intracellular replication is not sufficient to cause macrophage lysis. Prior to this study, the prevailing dogma in the literature was that *H. capsulatum* infected macrophages died in response to the high fungal burden at the later stages of infection. This hypothesis would predict that any *H. capsulatum* strain that could achieve a high intracellular fungal burden would be able to lyse macrophages. In this work, I monitored intracellular replication of the LDF mutants using a standard CFU assay. I showed that all LDF mutants replicated within macrophages, although with different kinetics than wild-type *H. capsulatum*. Interestingly, several severe LDF mutants can replicate to high numbers within the macrophage without causing lysis. Additionally, there is no strict correlation between the rate of intracellular replication and the severity of the macrophage lysis defect. Mutants

with similar doubling times, such as 171-G11 and 138-G1, have a moderate and severe lysis defect respectively. My results indicate that a high fungal burden is not sufficient to cause macrophage lysis and suggests that *H. capsulatum* is actively lysing macrophages via an *H. capsulatum*-mediated mechanism that has yet to be elucidated.

A better understanding of macrophage lysis during infection may come from studies of the *H. capsulatum* factor Cbp1. Cbp1 was previously identified as a secreted virulence factor required for macrophage lysis (Sebghati *et al.*, 2000). Its molecular function, however, is unknown. I isolated a *cbp1* insertion mutant in the LDF screen and demonstrated it achieve a high fungal burden, similar to that of wild-type *H. capsulatum*, inside BMDMs. It did this despite having a severe lysis defect. Interestingly, there were two additional severe LDF mutants, *bub2* and *tbf1,* that grew to wild-type levels within the macrophage. The *bub2* mutant did not express *CBP1*. The *tbf1* mutant had a genomic rearrangement at the *CBP1* locus and failed to express a normal *CBP1* transcript. These data indicate that I have effectively isolated three insertion mutants that are defective for expression of *CBP1*, and they are characterized by similar phenotypes – a severe lysis defect despite their ability to achieve a high fungal burden macrophages. These data point to a role for *CBP1* in mediating macrophage lysis during infection. It also suggests that the *BUB2* gene may play a role in regulating the expression of *CBP1*. The role of *CBP1* during macrophage lysis will be discussed in more depth in Chapter 3.

In addition to providing insights into factors required for host cell lysis, the LDF mutants allow us to investigate the intra-phagosomal environment encountered by *H. capsulatum* during infection. A well-characterized aspect of this environment is its near-neutral pH. This pH of 6.5 is observed despite the fact that the *H. capsulatum* phagosome

fuses with lysosomes, which typically have a very acidic pH. While it has been hypothesized that this inhibition of acidification is important for *H. capsulatum* pathogenesis, its importance has not been experimentally demonstrated. The role, if any, of pH modulation during *H. capsulatum* infection remains unclear. The identification of the *hcl1* mutant in the LDF screen may provide the first evidence that inhibition of acidification is important for *H. capsulatum* growth and virulence. HMG-CoA lyase 1 (*HCL1*) is a metabolic gene that catalyzes the last step in leucine catabolism. An *HCL1* deficiency in humans results in metabolic acidosis and the build-up of acidic intermediates in the urine. I hypothesize that the growth of the *hcl1* mutant in the phagosome results in a similar accumulation of acidic intermediates, which in turn lowers the pH of the phagosome and affects virulence during macrophage infection. Direct measurements of the phagosomal pH would allow us to determine if the pH of the *hcl1* phagosome is more acidic than that of the wild-type *H. capsulatum* phagosome. Further evidence for the importance of inhibition of phagosome acidification would be provided if the lysis defect of the *hcl1* mutant could be rescued by chemically neutralizing the macrophage phagosome. I have performed preliminary experiments exploring the ability of the *hcl1* mutant to modulate the pH of culture media *in vitro*. I observed that the *hcl1* mutant is able to neutralize culture pH as quickly as wild-type *H. capsulatum*. However, the *hcl1* mutant culture starts to re-acidify after 24 hours. This is likely due to the accumulation of acidic intermediates. Whereas *HCL1* is not a direct *H. capsulatum* virulence factor, it can be a powerful tool that allows us to explore the intracellular environment of the *H. capsulatum* phagosome.

To further our understanding of the LDF genes, Mark Voorhies and I examined their orthologs across fungal genomes. We hoped to gain insights into the function of these genes by identifying orthologs in more well-characterized fungi. We looked for orthologs for the 18 LDF genes that could be predicted based on the site of insertion. Only 7 LDF genes were conserved among the fungal species examined. These included *HCL1* (FE6-C3), a putative ABC transporter (152-F6), a putative casein kinase homolog (157-C1), a putative RAB GTPase (138-G1), a putative N-acetyl transferase (155-B1), a putative glycoside hydrolase (150-B5) and a putative sorting nexin (171-G11). The remaining 61% of the LDF genes were found almost exclusively in *Histoplasma* genomes. The LDF genes are enriched for these '*Histoplasma*-specific' genes, as only 20% of the *H. capsulatum* G217B predicted genes are only found in *H. capsulatum* genomes. *CBP1* is among this group of LDF genes. These genes are of particular interest because they may play roles in *H. capsulatum* specific virulence mechanisms. I then used InteproScan to identify motifs in these mutants and observed that 5 of the 11 *H. capsulatum*-specific genes contained a predicted N-terminal signal peptide sequence. The presence of signal peptide motifs in these genes is of particular interest because it suggests that the genes encode proteins that are either localized to the cell surface of the fungus or secreted into the macrophage environment, where they can manipulate the host and cause death. Determining where these factors localize during infection, as well as identifying their target in host cells, would greatly expand our knowledge of fungal and host pathways involved in infection. It is exciting to speculate that these genes may be part of an arsenal of secreted effectors that designed by *H. capsulatum* to manipulate and kill host cells.

Though the conversion to yeast phase growth is critical for virulence in the host, there is no enrichment for yeast expressed genes among the LDF genes during growth *in vitro*. When we examined the expression of the LDF genes in all morphological states of *H. capsulatum*, including yeast, mycelia, and conidia, we found that only 2 genes were significantly enriched in yeast cells versus mycelia and conidia. They included *CBP1* (mutant 172-C5) and *LDF1* (mutant 2A11), where *LDF1* was the most highly expressed gene in the yeast phase. While there is a subset of yeast enriched genes that are important for virulence during macrophage infection, enriched yeast phase expression *in vitro* is neither a necessary nor sufficient condition for identifying virulence determinants.

The LDF screen was an excellent first step in identifying *H. capsulatum* virulence factors. For other intracellular pathogens, virulence in macrophages has reliably served as a proxy for virulence in the animal and was thus a good model system in which to perform a high-throughput screen. One of the next steps in this analysis is to demonstrate that the LDF mutants have a virulence defect in the mouse model of infection. This can be done by monitoring disease progression and survival of animals after intranasal challenge with the LDF mutants. Organ colonization and dissemination are other criteria that can be examined. Due to the fact that the screen was performed in macrophage monolayers, outside of the complex cellular milieu of the host, it would not be surprising if a few LDF mutants did not exhibit a virulence defect in the mouse pulmonary infection model. In an *S. typhimurim* screen for virulence factors, only 72% of the mutants that exhibited a virulence defect in macrophages displayed a defect during animal infection (Fields *et al.*, 1986).

Once virulence in the animal is determined, there are a number of secondary virulence-associated assays that can be performed to elucidate the function of the LDF genes. For example, the ability of the mutants to grow under nutrient limiting conditions, such as iron starvation, can be monitored to gain insights into the conditions *H. capsulatum* encounter in the phagosome and the fungal factors that play a role in overcoming them. The ability of the LDF mutants to manipulate the maturation of the phagosome, including pH modulation and fusion with lysosomes, can also be monitored it gain insights into the molecular pathway that govern this interaction. Finally, if the LDF mutants are defective in the response to reactive oxygen and nitrogen species, the mutants would provide insight into *H. capsulatum* genes that play a role in subverting or neutralizing these toxic species during infection.

The LDF screen has yielded a number of interesting mutants and genes to pursue. However, there are a number to technical limitations involved in performing a screen in this manner. The *H. capsulatum* genome was not screened to saturation. There are still many virulence factors that have yet to be identified. Additionally, this screen monitored virulence in macrophages as a proxy for virulence in the animal. It was very satisfying to identify the one G217B virulence factor, *CBP1,* known at the time to be required for virulence in macrophages. *YPS3* and similar genes that are dispensable during macrophage infection, but are required during an infection of an animal, would not be identified in this screen. Other screening strategies, such as signature tagged mutagenesis, would identify genes that are required for colonization and virulence of the host *in vivo.*

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CHAPTER THREE

Histoplasma capsulatum actively triggers macrophage lysis during infection utilizing the secreted factor Cbp1.

Introduction

Endemic to the Ohio and Mississippi Valley regions, *Histoplasma capsulatum* is one of the most common causes of fungal respiratory infection in the United States. *H. capsulatum* is a dimorphic intracellular pathogen, existing in one form in the environment and another in the host. Mycelia and associated spores are the infectious form of the fungus that is found in the environment, in the soil or in the refuse of bats and chickens. Upon inhalation by a mammalian host, the mycelia and spores convert to the parasitic form of the fungus, the budding yeast.

Within the lungs, the yeast cells are quickly phagocytosed by alveolar macrophages (AVMs). These macrophages are one of the primary host cells for *H. capsulatum*. *In vitro* studies of macrophage infection with *H. capsulatum* show that the fungus replicates to high levels intracellularly and ultimately lyses this host cell. Knowledge of the fungal factors that mediate this macrophage colonization and death is critical to our understanding of *H. capsulatum* pathogenesis and our ability to treat disease.

Cbp1, calcium binding protein 1, was one of the first *H. capsulatum* virulence factors to be identified. It is a small secreted protein, approximately 7 kDa in size. It was initially identified because it conferred yeast cells with the ability to grow under calcium limiting conditions *in vitro*. *CBP1* is only expressed during *H. capsulatum* growth in the parasitic yeast form and is one of the most highly expressed yeast-phase genes. Consistent with the high levels of expression, Cbp1 is the most abundantly secreted protein during growth in culture.

Cbp1 is also expressed and secreted during macrophage infection (Batanghari *et al.*, 1998; Kugler *et al.*, 2000). *CBP1* was shown to be required for H. *capsulatum* virulence during macrophage infection when the Δ*cbp1* mutant was unable to lyse P388D.1 macrophage-like cells (Sebghati *et al.*, 2000). The authors assumed this failure was due to an inability of the Δ*cbp1* mutant to survive within macrophages. This Δ*cbp1* mutant is also unable to colonize the lungs of intranasally-infected mice, indicating that *CBP1* is required for growth in the animal.

It is clear that Cbp1 plays an important role in *H. capsulatum* pathogenesis. The molecular function of this virulence factor, however, is still unknown. No insights can be gained from primary sequence homology to functionally characterized genes in other organisms because *CBP1* is only present in the genomes of *H. capsulatum* and the closely related fungus *Paracoccidiodes brasiliensis*. Studies of the Cbp1 protein have been more informative. The recent solution of the NMR structure of Cbp1 revealed a structural similarity to mammalian Saposin B, a small lysosomal protein that acts as a cofactor for lipid-degrading enzymes (Beck *et al.*, 2009).

In this study, I uncover a novel phenotype for a *cbp1* insertion mutant. This mutant was isolated in a screen to identify *H. capsulatum* factors that were required for macrophage lysis. When the intracellular fate of the *cbp1* mutant was analyzed, I discovered that the lysis defect was not due to an intracellular survival defect and that the mutant cells grew and accumulated within macrophages to similar levels as wild-type cells. These results suggested that Cbp1 may play a role in mediating macrophage lysis. Since *CBP1* is a secreted factor, we hypothesized that it would play a role in manipulating the macrophage response to *H. capsulatum* infection. Together with

Charlotte Berkes, I analyzed the macrophage transcriptional response to *H. capsulatum* infection and identified a cluster of genes whose induction was *CBP1* dependent and correlated with lysis.

Materials and Methods

Strains and Culture Condition

H. caspsulatum strain G217B *ura5*Δ (WU15) was obtained from William Goldman (Washington University, St. Louis, MO). Yeast cells were grown in liquid *Histoplasma* macrophage media (HMM) or on HMM agarose plates (Warsham and Goldman, 1988). Liquid cultures were grown in an orbital shaker at 37^{o}C with 5% CO_2. Stock cultures were maintained by passaging every 2-3 days at a 1:25 dilution. Plates were incubated in a humidified chamber at 37^{o}C with 5% CO_2.

For macrophage infections, an overnight, mid-log culture of yeast cells (OD_{600} = 5-7) was prepared. Approximately 18 hours prior to the infection, a two-day late log/stationary phase culture (OD_{600} = 10-12) was diluted 1:5 into HMM media. The diluted cells were then incubated at 37^{o}C with 5% CO_2 overnight to obtain mid-log cultures at the time of infection. Culture ODs were measured using an Eppendorf BioPhotometer.

Insertion mutagenesis of *H. capsulatum*

H. capsulatum insertions mutants were generated using *Agrobacterium*-mediated transformation of WU15 as previously described (Nguyen and Sil, 2008). We selected for transformants (hygromycin resistant (Hyg^{R})) on HMM/uracil plates containing 400 ug/ml uracil, 200 μg/ml hygromycin B and 200 μM cefotaxime. 14,000 insertion mutants were screened for their ability to lyse BMDMs and J774.1 macrophages. Forty-seven

mutants were defective for macrophage lysis and one mutant contained an insertion upstream of the ORF for *CBP1*. This mutant was designated as the *cbp1* mutant.

Complementation of the *cbp1* mutant

Transformation. An episomal complementation plasmid, pDTI22, containing the *CBP1* ORF, 2764 bp of 5' flanking sequence and 554 bp of 3' sequence was generated. 100 ng of PacI digested pDTI22 was transformed into the *cbp1* mutant as previously described (Woods *et al.*, 1998), creating the *cbp1+CBP1* strain. The *cbp1* mutant was also transformed with pLH211 as a vector control, creating the *cbp1+URA5* strain. The *ura5*Δ parental strain, WU15, was also transformed with pLH211, generating the *ura5*Δ*+URA5* strain. This strain served as our wild-type control. Transformants were selected on HMM-agarose plates.

Northern Blot. Two-day cultures of *ura5*Δ*+URA5*, *cbp1+URA5*, *cbp1+CBP1* were pelleted and RNA was harvested from the yeast cells as previously described (Hwang *et al.*, 2003). 4 ug of total RNA from all strains were separated on a 1.5% denaturing, formaldehyde agarose gel and transferred to a positively charged nylon membrane (Roche 11209299001). The membrane was then subject to Northern Blot analysis according to the Digoxigenin (DIG) Northern Blot protocol (Roche Applied Sciences). DIG-labeled probes were generated from WU15 genomic DNA using the PCR DIG probe synthesis kit (Roche 11636090910). Primers OAS1987 (5'-GAAAACCCAGCGAAAATCACCTCC-3') and OAS1988 (3'-GATGATGATGATGCTGGTGAGAGG-5') were used to generate the *CBP1* probe and primers OAS1055 (5'- AAGTCGCTGCCCTCGTTAT -3') and OAS1056 (3'-

TAGAAGCACTTGCGGTGGAC -5') were used to generate the *ACT1* probe. The membrane was then exposed to film and developed.

In vitro H. capsulatum growth

Two-day, late log cultures of the *ura5*Δ+*URA5*, the *cbp1*+*URA5*, and the *cbp1*+*CBP1* strains were used to inoculate 30 ml HMM media to a starting OD_{600}=0.1. At 20, 26, 47, 69, 97, 124, and 169 hours post innoculation, 3 x 1 ml samples were removed from each culture, vortexed for 30 seconds to break up clumps, and their OD_{600} was measured.

Macrophage culture

Bone marrow derived macrophages (BMDMs) were isolated as described previously (Hwang *et al.*, 2008).

Alveolar macrophages (AVMs) were obtained by broncho-alveolar lavage (BAL) from the lungs of 12- to 15- week-old female C57Bl/6 mice. 20 mls of BAL fluid (5 mM EDTA in PBS without Ca^{2+} and Mg^{2+}) was collected from each mouse. The cells were pelleted and resuspended in AKT lysis solution (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in ddH_2O, pH 7.2-7.4). The remaining cells were pelleted and resuspended in AVM media, which consists of Dulbecco's Modified Eagle Medium (DMEM) High Glucose (UCSF Cell Culture Facility), 10% Fetal Bovine Serum (Hyclone, Thermo Fisher, www.hyclone.com), penicillin and streptomycin (UCSF Cell Culture Facility).

Cytotoxicity Assay

Bone marrow derived macrophages (BMDMs). In 24-well tissue-culture treated dishes, 2 x 10^5 BMDMs were infected, in duplicate, with *H. capsulatum* strains at an MOI of 2. In preparation for the infections, logarithmically growing *H. capsulatum* cultures were pelleted, resuspended in DMEM without phenol red, sonicated, and counted by hemacytometer. After a 2-hour incubation period, the media was removed from the infected BMDMs, the monolayers were washed twice with DMEM without phenol red and 750 ul BMM without phenol red (DMEM High Glucose without phenol red (UCSF Cell Culture Facility), 20% Fetal Bovine Serum, 10% v/v CMG supernatant, 2 mM glutamine, 110 mg/mL sodium pyruvate, penicillin and streptomycin) was added to each well. The infected macrophages were then incubated at 37^{o}C with 5% CO_2.
Approximately 48 hours post-infection, 250 ul fresh BMM was added to each well.
At various timepoints post-infection, LDH levels in the infected-macrophage supernatants were measured to monitor BMDM lysis. At each time-point, the volume in each well was brought up to 1 ml with BMM. 200 ul of the culture supernatant from the infected macrophages were transferred to two wells of a 96-well plate. To measure the total LDH, at 2 hours post-infection two mock-infected BMDM monolayers were lysed with 1 ml of lysis solution (1% Triton X-100 in DMEM without phenol red). 200 ul of this mock-infected macrophage lysate was transferred to two wells of the 96-well plate. This 96-well plate was then centrifuged to pellet any cells or debris that might be present in the supernatants or lysate. 20 ul of the clarified culture supernatant and lysate was transferred to a fresh 96-well plate containing 30 ul DMEM-phenol red in each well. 60

ul of complete LDH solution (equal parts 2 mg/ml INT (IodoNitroTetrazolium chloride) in DMSO, 36 mg/ml Lithium L-lactate in 10 mM Tris, pH 8.5, and 1x NAD+/diaphorase in 1% PBS/BSA; 10x NAD+/diaphorase is composed as follows: 13.5 u/mL Diaphorase, 3 mg/mL NAD+, 0.03% BSA, and 1.2% sucrose in PBS) was then added to each well. The plate was then incubated for 30 minutes in the dark. 40 ul of 1 M acetic acid was added to stop the reaction. The OD_{490} was then measured using the Molecular Devices Spectramax Plus 384 plate reader. The % BMDM lysis at each time-point is calculated as the percentage of the total LDH from uninfected cells at 2 hours post-infection.

Alveolar macrophage (AVMs). In 96-well tissue-culture treated dishes, 1.5 x 10^5 BMDMs were infected, in duplicate, with *H. capsulatum* strains at an MOI of 5. After a 6-hour incubation period, the media was removed from the infected AVMs, the monolayers were washed twice with DMEM without phenol red and 200 ul AVM media without phenol red (DMEM without phenol red, 10% Fetal Bovine, penicillin and streptomycin) was added to each well. The infected macrophages were then incubated at 37^{o}C with 5% CO_2. At various timepoints post-infection, LDH levels in the infected macrophage supernatants were measured to monitor AVM lysis. At each time-point, 150 ul of the culture supernatant from each infected macrophage monolayer was transferred to one well of a fresh 96-well plate. To measure the total LDH, at 6 hours post-infection, one mock-infected macrophage monolayer was lysed with 200 ul of lysis solution (1% Triton X-100 in DMEM-phenol red). 150 ul of this mock-infected macrophage lysate was transferred to one well of the 96-well plate. This 96-well plate was then centrifuged to pellet any cells or debris that might be present in the supernatants or lysate. 50 ul of the clarified

culture supernatant and lysate is transferred to a fresh 96-well plate and LDH release was assessed as previously described for BMDMs.

Intracellular Replication Assay

In 24-well tissue culture treated dishes, 2 x 10^5 BMDMs were infected, in duplicate, with *H. capsulatum* strains at an MOI of 2. After a 2-hour incubation period, the media was removed from the infected BMDMs, the monolayers were washed twice with DMEM and 1 ml BMM was added to each well. The infected macrophages were then incubated at 37^{o}C with 5% CO_2. The media was changed at 48 hours post-infection and every day thereafter.

At various timepoints post-infection, the media was removed from each well and 500 ul ddH_2O was added. After 5 minute of incubation, the macrophages were mechanically lysed by vigorous pipetting. The lysate was collected, sonicated, diluted in HMM and plated for *H. capsulatum* colony forming units (CFUs) on HMM-agarose plates. Colonies were counted 14 days later. The relative CFU at time x is calculated as $(CFU_{tx})/(CFU_{t0})$.

Microscopic analysis of intracellular growth and dissemination

BMDMs. 3.25 x 10^5 BMDMs per well were seeded in 12-well tissue-culture treated dishes containing 15 mm glass coverslips. Approximately 18 hours later, these BMDMs were infected with 8.1 x 10^4 yeast cells per well, centrifuged for 5 minutes at 800 x g, and incubated for 2 hours. The media was then removed from the infected BMDMs, the monolayers were washed twice with DMEM and 2 ml BMM was added to each well. The

infected macrophages were then incubated at 37°C with 5% CO_2. The media was changed at 48 hours post-infection and every day thereafter.

At various timepoints post-infection the media was removed from each well and the monolayers were fixed with 1 ml PAS fixative (3.7% formaldehyde in 100% ethanol) for 1 minute. The fixative was removed and cells were washed twice with PBS before being stored in 2 ml PBS at 4°C. Coverslips were then stained with Periodic acid-Schiff reagent and methyl green as previously described (Webster and Sil, 2008).

Microscopic images were taken with the Leica DM 1000 microscope.

To quantify dissemination, the number of infected macrophages in 3 x 25 consecutive fields were counted for each strain at each timepoint. The average of the 3 sets of 25 fields for each is graphed ± standard deviation.

AVMs. 3 x 10^5 AVMs per well were seeded in 8-well glass LabTek Chambers (Nunc 177402). Approximately 18 hours later, these AVMs were infected with 3 x 10^4 yeast cells per well and incubated for 5.5 hours. The media was then removed from the infected BMDMs, the monolayers were washed once with DMEM and 2 ml BMM was added to each well. The infected macrophages were then incubated at 37°C with 5% CO_2. The media was changed at 48 hours post-infection and every day thereafter.

At various timepoints post-infection, the media was removed from each well and the monolayers were fixed with 500 ul PAS fixative (3.7% formaldehyde in 100% ethanol) for 1 minute. The fixative was removed and cells were washed twice with PBS before being stored in 500 ul PBS at 4°C. Slides were then stained with Periodic acid-Schiff reagent and methyl green as previously described (Webster and Sil, 2008). Microscopic images were taken using the Leica DM 1000 microscope.

BMDM transcriptional analysis

BMDM infections. In 6-well tissue culture treated dishes, 7 x 10^5 BMDMs were infected, in duplicate, with various *H. capsulatum* strains at an MOI of 5. After a 1-hour incubation period, the media was removed from the infected BMDMs, the monolayers were washed twice with BMM and 2 ml BMM was added to each well. The infected BMDMs were then incubated at 37^{o}C with 5% CO_2. At 3, 6, and 12 hours post-infection, the media was removed from each well and replaced with 350 ul Buffer RLT+βME (Qiagen). Lysates from duplicate wells were pooled, flash frozen in liquid nitrogen, and store at -80^{o}C.

BMDM RNA Isolation. The QIAGEN RNeasy mini kit was used to isolate total RNA from infected macrophages. Briefly, lysates were thawed, *H. capsulatum* yeast cells were pelleted by high speed centrifugation, and the supernatants were homogenized using the QIAshredder homogenizer (Qiagen). RNA was precipitated for the homogenized lysate according to the manufacturer's instructions for RNeasy Mini purification of total RNA from animal cells using spin technology. The purified total RNA was eluted off the RNeasy column in 30-50 ul RNAse free water and RNA quality was analyzed using the Agilent Bioanalyzer.

Microarrays. aRNA, amplified RNA, was generated from the total RNA using the Ambion Amino-Allyl Message Amp II aRNA Amplification Kit (Applied Biosystems) according to the manufacturer's protocol. This amplified RNA was hybridzed to mouse MEEBO arrays and scanned as described previously (Leber *et al.*, 2008).

Microarray analysis. The microarray results were gridded and the data was normalized as described (Leber *et al.*, 2008). Statistical Analysis of Microarrays (SAM), was used to

determine which genes were significantly differentially expressed between BMDMs infected with *ura5*Δ*+URA5*, *cbp1+URA5*, and *cbp1+CBP1*.

Results

Cbp1 is required for BMDM lysis, but is largely dispensable for intracellular growth.

A *cbp1* insertion mutant was isolated in a genetic screen to identify *H. capsulatum* virulence factors (see Chapter 2). The insertion was located approximately 200 bp upstream of the *CBP1* ORF. Northern blot analysis showed that this disruption completely abrogated *CBP1* expression in the mutant (Figure 3-1)

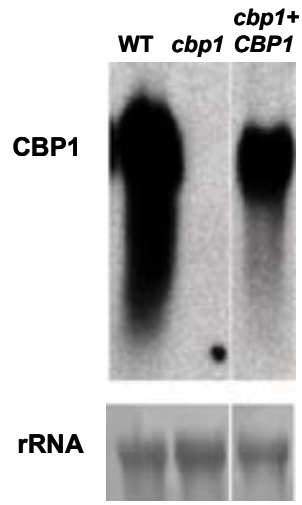

Figure 3-1. The *cbp1* insertion mutant does not express *CBP1*
Total RNA was isolated from mid-log cultures of the WT, *cbp1*, and *cbp1+CBP1* strains growing at 37°C, 5% CO_2, separated by gel electrophoresis and transferred to a nylon membrane. A 4.2 kb probe containing the *CBP1* ORF was hybridized to the membrane. Methylene blue was used to stain rRNA.

To quantify the lysis defect of the *cbp1* mutant I performed a lactate dehydrogenase (LDH) release assay. I infected bone marrow-derived macrophages (BMDMs) with either wild-type *H. capsulatum,* the *cbp1* mutant, or the complemented strain, *cbp1+CBP1*, and measured the release of cytosolic LDH from the macrophages into the culture supernatants at 2, 24, 32, 40, 48, 72, 96, 120, and 144 hours post-infection. Lysis of BMDMs infected with wild-type *H. capsulatum* began after 24 hours of infection (Figure 3-2). The BMDM monolayer was completely destroyed by six days post-infection. No significant lysis was observed in macrophages infected with the *cbp1* mutant. In fact, the lysis profile of the *cbp1* mutant-infected macrophages was indistinguishable from the lysis profile of uninfected macrophages. To verify that this severe lysis defect was due to the disruption of *CBP1*, I complemented the *cbp1* mutant with an epsisomal copy of the wild-type *CBP1* gene. Reintroduction of *CBP1* into the *cbp1* mutant rescued the BMDM lysis defect (Fig 3-2).

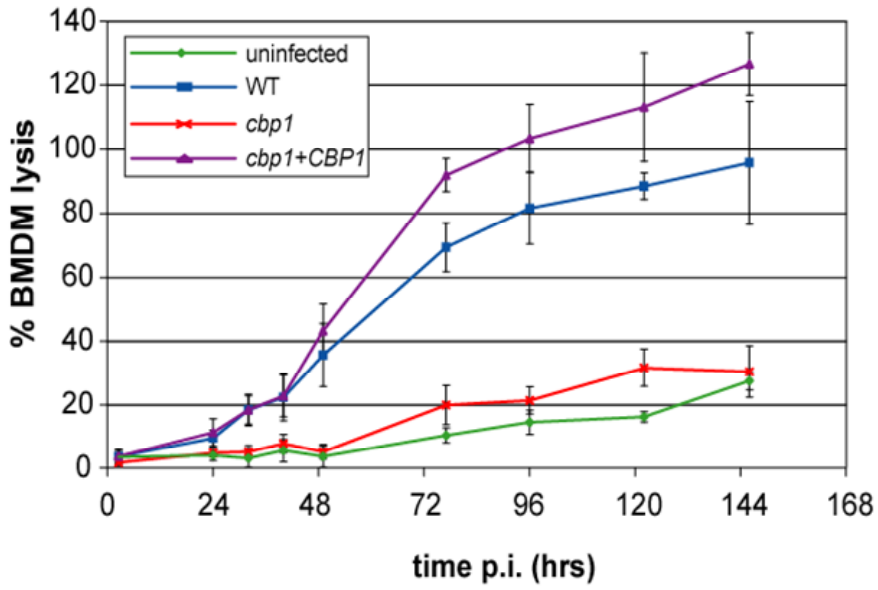

Figure 3-2. CBP1 is required for BMDM lysis.
BMDMs were either mock infected or infected with WT, *cbp1,* and *cbp1+CBP1* yeast cells at MOI = 2. At 2, 24, 32, 40, 48, 72, 96, 120, and 144 hrs post infection, the supernatants was removed from the infected monolayers and lactate dehydrogenase activity was measured to monitor the % BMDM lysis. The average of four measurements ± standard deviation is shown.

One explanation for the *cbp1* mutant lysis defect could be that *CBP1* is required for the survival of *H. capsulatum* in BMDMs. To address this possibility, I examined the intracellular viability and replication of the *cbp1* mutant. BMDMs were infected with either wild-type *H. capsulatum*, the *cbp1* mutant or the *CBP1* complemented strain, *cbp1+CBP1*. At 2, 12, 24, 36, 48, 72, 96, 120, 144, and 168 hours post-infection, the infected BMDMs were lysed and the lysates were plated for *H. capsulatum* colony forming units (CFUs). Measurements were terminated for each strain at the onset of macrophage lysis to avoid measuring the extracellular replication of yeast cells that had been released from lysed macrophages. Wild-type *H. capsulatum* experienced approximately 4 rounds of replication in the BMDMs before the macrophages started to lyse (Figure 3-3). The doubling time was 13.87 ± 2.13 hours. The *cbp1* mutant also

replicated within BMDMs. The intracellular fungal burden increased 16-fold during the first 4 days and this level of colonization was maintained throughout the final 3 days of the infection. Although the *cbp1* mutant grew to similar intracellular levels as wild-type *H. capsulatum*, it did so with an almost two-fold slower doubling time of 22.93 ± 0.71 hours. Complementation with *CBP1* rescued this growth delay as well as BMDM lysis (Figure 3-3). No differences in growth were observed when these strains were grown in culture (Figure 3-4). These results indicated that *CBP1* was not required for *H. capsulatum* to accumulate to wild-type levels in BMDMs. Importantly, this data provides the first evidence that a high intracellular fungal burden is not sufficient to cause macrophage lysis during *H. capsulatum* infection.

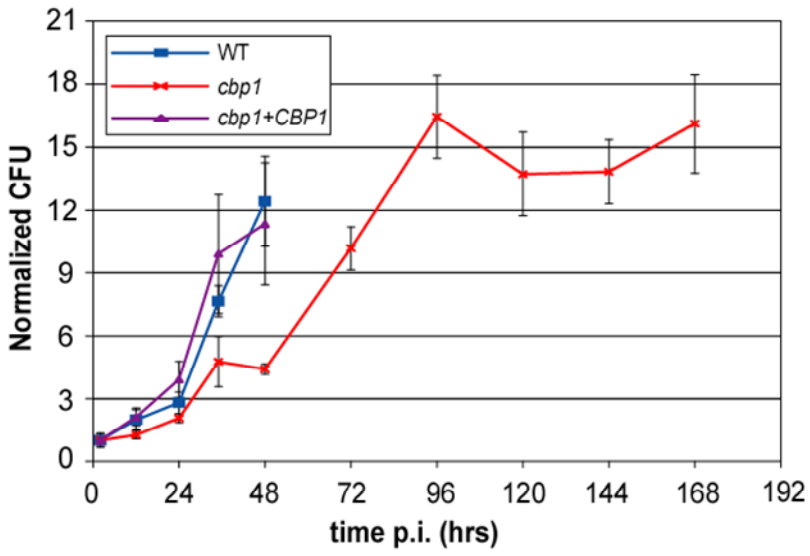

Figure 3-3. *CBP1* is dispensable for *H. capsulatum* replication in BMDMs.
BMDMs were infected with WT, *cbp1,* and *cbp1+CBP1* yeast cells at MOI = 2. At 2, 12, 24, 36, 48, 72, 96, 120, 144, and 168 hrs post infection, the BMDMs were lysed with ddH_2O and the lysates were plated for *H. capsulatum* CFUs on HMM agarose plates. Measurements were terminated at the onset of macrophage lysis. Consequently, no measurements were made for BMDMs infected with WT and *cbp1+CBP1* after 48 hours of infection. Each measurement is the average of 4 platings (duplicate infections/ duplicate platings) ± standard deviation.

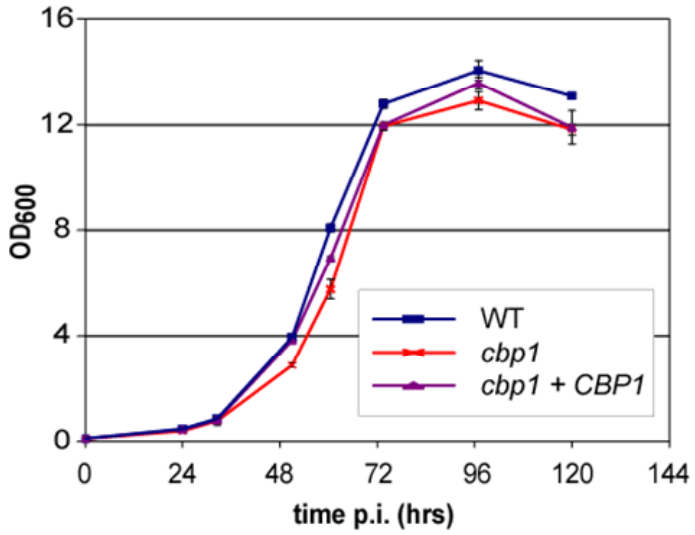

Figure 3-4. The *cbp1* mutant has wild-type growth kinetics *in vitro*.
The WT, *cbp1*, and *cbp1+CBP1* strains were innoculated into HMM at a starting OD_{600} = 0.1. Triplicate 1ml samples were taken from each culture at the indicated timepoints. The samples were vortexed and diluted when necessary to measure the optical density. The average of three measurements ± standard deviation is shown.

The results of the LDH release and the intracellular growth assays suggest that the *cbp1* mutant would be defective in its ability to disseminate throughout the BMDM monolayer. To test this hypothesis I infected BMDMs with wild-type *H. capsulatum*, the *cbp1* mutant and the complemented strain at a very low MOI of 0.25. The infected monolayers were fixed and stained 3, 24, 48, 72, 96, and 120 hours post-infection. Microscopic analysis of the infected monolayers confirmed that the *cbp1* mutant was able to grow to high levels within individual BMDMs as quantified in the CFU experiment (Figure 3-5). At 5 days post-infection, most infected BMDMs in the *cbp1* mutant infected monolayer contained greater than 20 fungal cells, compared to the 1-5 that were present at the beginning of the infection.

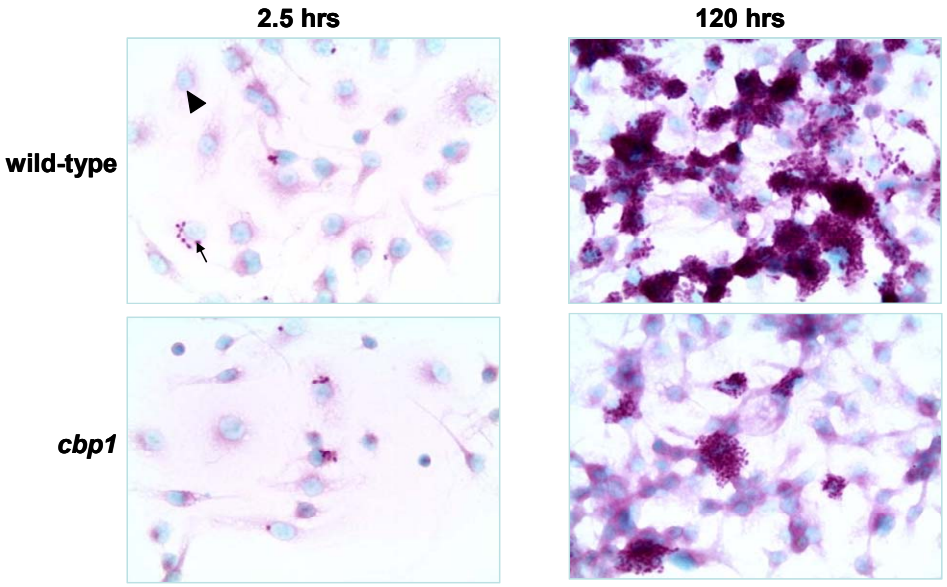

Figure 3-5. The *cbp1* mutant fails to disseminate during *in vitro* BMDM infection
BMDMs were infected with WT, *cbp1,* and *cbp1+CBP1* yeast cells at MOI = 0.25. At 2, 24, 48, 72, 96, and 120 hours post infection, the infected monolayers were fixed. The *H. capsulatum* yeast cells were stained with periodic acid/Schiff (PAS) reagent (arrows) and the macrophage nuclei were counter-stained with methyl green (arrowhead). Representative images at 40x magnification are shown.

Further analysis of these stained monolayers showed that the *cbp1* mutant was defective in dissemination. I counted the number of infected macrophages in a unit area and saw a 2-3 fold increase in the number of infected macrophages when I infected with the *cbp1* mutant, compared to a 6-7 fold increase observed when I infected BMDMs with either wild-type *H. capsulatum* or the complemented strain (Figure 3-6). This difference in dissemination is illustrated in Figure 3-5. After 5 days of infection, wild-type *H. capsulatum* had spread rampantly, leaving few macrophages uninfected. Despite its ability to replicate intracellularly, the *cbp1* mutant does not disseminate, and most macrophages within the monolayer remain uninfected.

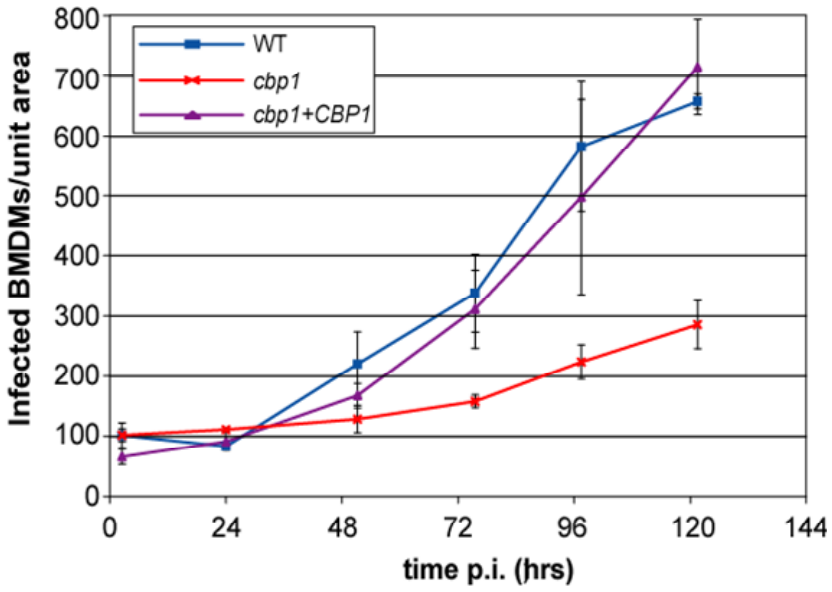

Figure 3-6. The *cbp1* mutant fails to disseminate during *in vitro* BMDM infection
Quantitation of stained macrophage monolayers described in Figure 3-5. The number of infected macrophages in 25 consecutive microscopic fields were counted. The data shown represents the average of three distinct areas of each coverslip ± standard deviation.

CBP1 is required for the induction of the *Histoplasma* response cluster.

As a first step to understanding the effect of Cbp1 on the biology of the host cell, Charlotte Berkes and I compared the transcriptional response of BMDMs infected with wild-type *H. capsulatum* to BMDMs infected with the *cbp1* mutant strain. Previous work in the laboratory had defined a transcriptional host signature that was induced only when BMDMs were infected with live, replicating yeast cells (Figure 3-7). Infection with UV-treated yeast cells, which do not lyse macrophages, did not induce the expression of these genes. Consequently, the induction of these genes correlated with either *H. capsulatum* growth in the macrophage or with the lysis to the macrophage. Additionally, the induction of these genes was not part of a general macrophage response to intracellular

pathogens, as they were not induced upon infection with *Listeria monocytogenes, Francisella tularensis,* or *Mycobacterium tuberculosis* (Sajeev Batra, personal communication). This BMDM transcriptional signature is unique to *H. capsulatum* infection and shall be referred to as the *Histoplasma* response cluster (HRC).

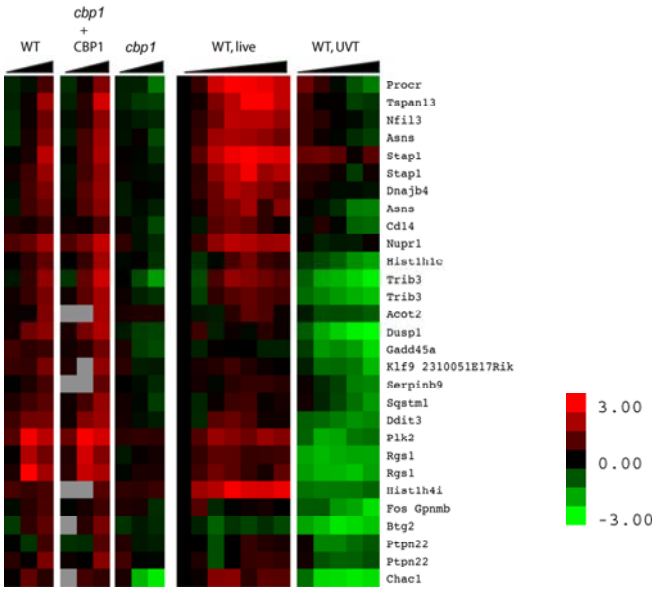

Figure 3-7. *CBP1* is required for the induction of the *Histoplasma* response cluster
Cluster analysis of gene that are significantly differentially expressed between the indicated strain by SAM analysis. Gene expression ratios are relative to the mock-infected sample at the first timepoint.

To examine the role of Cbp1 in the macrophage response to *H. capsulatum* infection we infected BMDMs with wild-type *H. capsulatum*, the *cbp1* mutant and the complemented strain at an MOI of 5. At 3, 6 and 12 hours post-infection, macrophage RNA was collected, amplified, and hybridized to MEEBO microarrays. Although the *cbp1* mutant replicated inside BMDMs, it did not induce the HRC (Figure 3-7). The transcriptional profile of BMDMs infected with the *cbp1* mutant looked very similar to

the transcriptional profile of BMDMs infected with UV-treated *H. capsulatum*. This ruled out the possibility that the induction of the HRC was a response to *H. capsulatum* growth in the macrophage. The induction of the HRC correlated with macrophage lysis resulting from *H. capsulatum* infection. It is worth noting that the failure of the *cbp1* mutant to induce the HRC was not due to an inability of the macrophage to detect the fungus, as the *cbp1* mutant induces general inflammatory response genes similarly to wild-type *H. capsulatum* (Figure 3-8*).* This data indicated that Cbp1 was necessary for the induction of the HRC, a cluster of genes whose expression in BMDMs during *H. capsulatum* infection correlates with lysis.

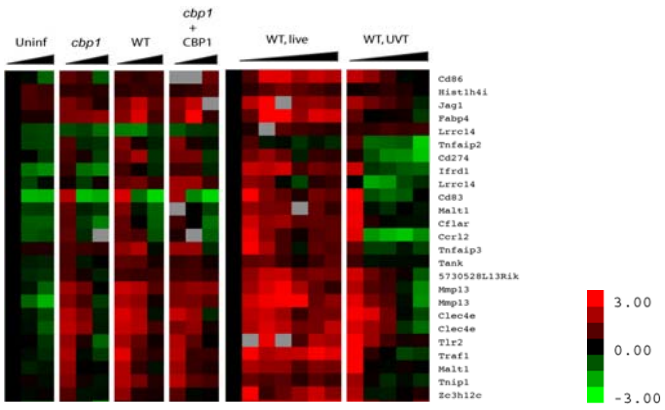

Figure 3-8. *CBP1* is dispensable for the induction of inflammatory genes
Cluster analysis of gene that are significantly differentially expressed between the indicated strain by SAM analysis. Gene expression ratios are relative to the mock-infected sample at the first timepoint.

CBP1 is required for lysis of alveolar macrophages during infection

Alveolar macrophages (AVMs) are one of the primary host cells for *H. capsulatum* during pulmonary infection. To determine if *CBP1* plays a similar role in these macrophages, I isolated AVMs from the lungs of C57Bl/6 mice by bronchoalveolar lavage and infected them with wild-type *H. capsulatum*, the *cbp1* mutant and the complemented strain at an MOI of 5. At 6, 26, 49, 73, 97, and 122 hours post-infection, the LDH release assay was performed on culture supernatants to monitor AVM lysis. Lysis was initially observed in AVMs infected with wild-type *H.capsulatum* 24 hours post-infection. (Figure 3-9). No lysis was observed during infection with the *cbp1* mutant, and this lysis defect was rescued by complementation with a wild-type copy of the *CBP1* gene. As is the case in BMDM infection with *H. capsulatum*, *CBP1* is required for lysis of AVMs.

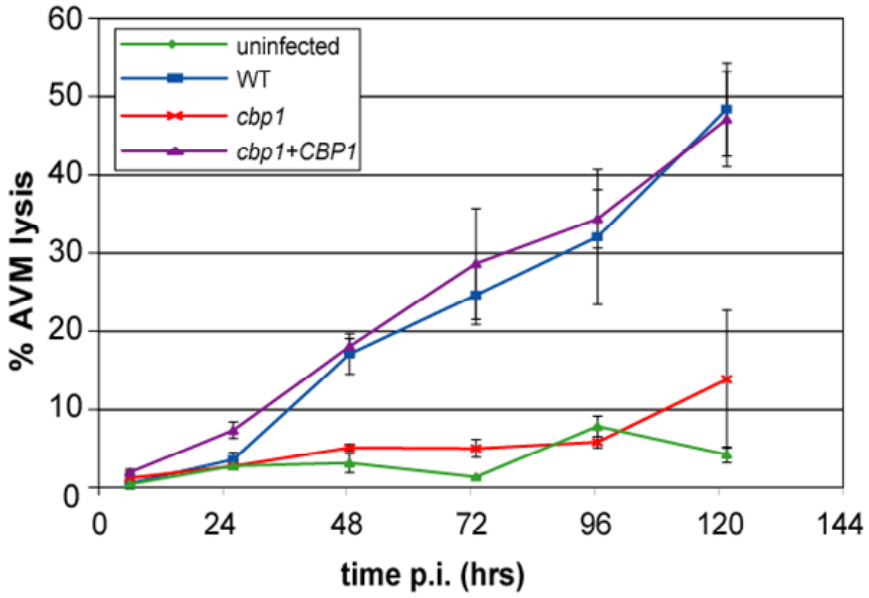

Figure 3-9. CBP1 is required for AVM lysis.
AVMs were either mock-infected or infected with WT, *cbp1,* and *cbp1+CBP1* yeast cells at MOI = 5. At 6, 26, 49, 73, 97, and 122 hrs post infection, the supernatants were removed from the infected monolayers and lactate dehydrogenase activity was measured to monitor the %AVM lysis. The average %AVM lysis for 4 measurements of 2 infected wells ± standard deviation is shown.

I next sought to determine if the *cbp1* mutant was able to replicate within AVMs. I infected AVMs with wild-type *H. capsulatum*, the *cbp1* mutant and the complemented strain at an MOI of 0.1 and microscopically monitored intracellular growth. I observed that the *cbp1* mutant, like wild-type *H. capsulatum* and the complemented strains, was able to replicate in AVMs (Figure 3-10). *CBP1* was dispensable for *H. capsulatum* replication during AVM infection. Thus, the phenotypes observed in the BMDMs were reproduced in AVMs.

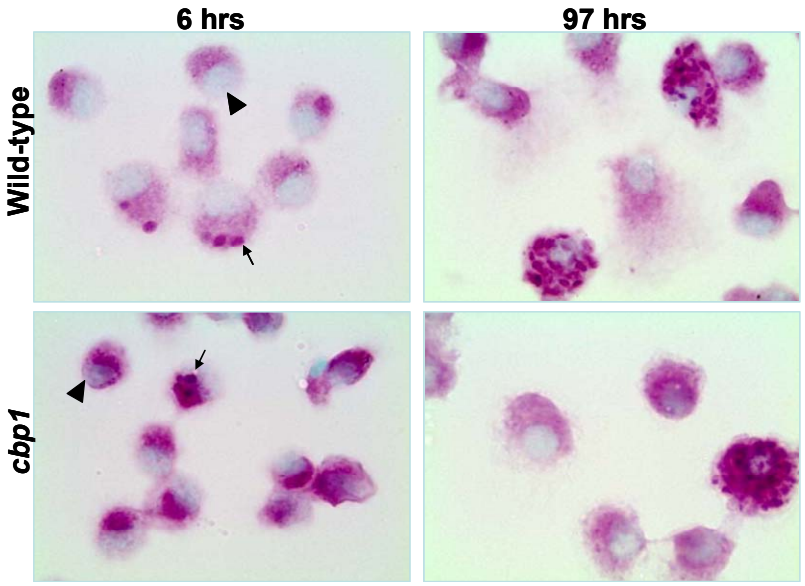

Figure 3-10. The *cbp1* mutant replicates in alveolar macrophages
AVMs were infected with *WT* and *cbp1* yeast cells at MOI = 0.1. At 6 and 97 hours post infection, the infected monolayers were fixed and stained. The *H. capsulatum* yeast cells were stained with periodic acid/Schiff (PAS) reagent (arrows) and the macrophage nuclei were counter-stained with methyl green (arrowhead). Representative images at 100x magnification are shown

Discussion

CBP1 was first implicated as an *H. capsulatum* virulence factor because it was required for growth in culture under calcium-limiting conditions (Batanghari *et al.*, 1997; Sebghati *et al.*, 2000). *CBP1* was also observed to be required for fungal proliferation *in vivo*. The Δ*cbp1* mutant failed to colonize the lungs of mice when examined 8 days after intranasal infection (Sebghati *et al.*, 2000). This mutant was also unable to lyse P388D.1 macrophage-like cells during infection. These findings led the authors to conclude that *CBP1* was indispensable for survival in both the macrophage and the mouse model of pulmonary infection, even though survival of the mutant in macrophages had not been directly monitored.

I isolated a *cbp1* insertion mutant in a screen to identify *H. capsulatum* factors required for macrophage lysis. This insertion mutant did not express *CBP1*, nor was it able to lyse primary BMDMs or J774 macrophage-like cells. The lysis defect was rescued by restoring a wild-type copy of the *CBP1* gene in the mutant. Consistent with the published data for the Δ*cbp1* mutant, our *cbp1* insertion mutant exhibited wild-type growth kinetics during *in vitro* culture.

When I analyzed the intracellular fate of the *cbp1* mutant in primary BMDMs, I reached a strikingly different conclusion than that reached by Sebghati *et al.*. The *cbp1* mutant survived and replicated in BMDMs. Wild-type *H. capsulatum* and the *cbp1* mutant both underwent approximately four rounds of doubling within these macrophages, though with different kinetics. Lysis was initially observed in wild-type *H. capsulatum*-infected macrophages at 2 days post-infection. The *cbp1* mutant did not reach similar

intracellular levels until 4 days post-infection and did so without causing BMDM lysis for up to 6 days post-infection. This data indicated that *CBP1*, while required for BMDM lysis, was largely dispensable for intracellular growth. This observation leads us hypothesize that Cbp1 is an *H. capsulatum* lysis factor.

I allowed the *cbp1*-mutant infections to progress to 168 hours without observing BMDM lysis, despite the high fungal burden. Interestingly, the *cbp1* mutant did not replicate beyond the initial 4 doublings, although the additional 72 hours would have allowed time for 3 more rounds of replication. This plateau in intracellular fungal load is reminiscent of the stationary phase of the fungal growth curve, when the fungus dramatically slows its rate of replication to compensate for a lack of available nutrients. This would suggest that lysis of the host macrophages is critical for *H. capsulatum* survival as it would allow the fungus to gain access to the nutrient rich intracellular environment of neighboring uninfected macrophages.

While these results suggest that Cbp1 plays a role in macrophage lysis, the molecular mechanism of its function remains elusive. On intriguing hypothesis is that Cbp1 is a factor that can directly mediate host cell lysis. Experiments exploring the effects of ectopic expression of *CBP1* in host cells would begin to address this issue. If it were the case that *CBP1* expression was sufficient to cause host cell lysis, it would be interesting to determine if this effect was host cell dependent. Was ectopic expression of *CBP1* sufficient for lysis of macrophages, but not other cell types?

Recently, Beck *et al.* solved the NMR structure of Cbp1 (Beck *et al.*, 2008). The authors reported that Cbp1 had structural similarity to mammalian saposins. Saposins and saposin-like proteins (SAP-LIPs) make up a large family of structurally homologous, but

functionally diverse, proteins that bind lipids and interact with membranes. Saposin B, the closest structural homolog to Cbp1, is a sphingolipid activator that localizes to lysosomes, where *H. capsulatum* resides during infection. Interestingly, there is a class of saposins that have cytolytic activity against mammalian cells. These include the amoebapores from *Entamoeba histolytica*, the Naegleriapores for *Naegleria fowleri*, and clornorin from *Clonorchis sinensis*. They mediate their cytotoxic effects by forming pores in the membranes of the infected cells. This structural similarity supports the idea that *CBP1* may be acting as an *H. capsulatum* lysis factor and suggests experiments to examine the ability of purified Cbp1 to interact with lipids and/or form pores in membranes.

Though my findings contradict published statements claiming that Cbp1 is essential for growth in macrophages, they are not inconsistent with published data regarding growth in the animal during infection. My results would predict that the *cbp1* mutant would grow within primary infected alveolar macrophages during the early stages of infection. The inability to lyse these host cells would prevent the spread of the fungus, making it easier for infiltrating neutrophils and other immune cells to control and clear the infection. When Sebghati *et al.* examined the growth of the Δ*cbp1* mutant in mouse lungs, they did not look at the fungal burden during the early stages of the infection. In these experiments, mice were intranasally infected with wild-type *H. capsulatum* and the Δ*cbp1* mutant and the fungal burden in the lungs was only measured after 8 days of infection. They observed a log increase in the number of yeast cells recovered from the lungs of wild-type-infected mice compared to the infecting inoculum. No CFUs could be recovered from the lungs of mice infected with the same inoculum of the Δ*cbp1* mutant.

The failure to see intracellular replication could simply be due to the limited time resolution in this experiment. A finer timecourse must be performed to really understand the role of Cbp1 in the intracellular replication of *H. capsulatum in vivo*.

In our endeavor to understand the function of Cbp1 during macrophage infection, Charlotte Berkes and I examined how the presence and absence of this secreted factor affected the macrophage transcriptional response to *H. capsulatum*. . Transcriptional analysis of BMDMs infected with either live or UV-treated *H. capsulatum* yeast cells identified a cluster of macrophage genes that was induced specifically upon infection with live, virulent yeast. We refer to this set of induced genes as the *Histoplasma* response cluster (HRC) because it was not induced upon infection with other intracellular pathogens, including *M. tuberculosis, F. tularensis*, and *L. monocytogenes*. *CBP1* is required for the induction of these genes, as the *cbp1* mutant did not induce the HRC.

This result raises the question of how Cbp1, an *H. capsulatum* factor secreted into the phagosome, promotes a transcriptional response in the macrophage nucleus. Determining where Cbp1 localizes after it is secreted into the phagosome is one of the first steps in answering this question. Identification of the macrophage factors that interact with Cbp1, if any, would also be valuable. The knowledge of Cbp1 cellular localization and interactions could provide insights into the host pathways involved in Cbp1 manipulation of the host and the mechanisms by which nuclear transcription is activated.

There are two models that explain how the induction of the HRC relates to macrophage lysis during *H. capsulatum* infection. In one model, these genes are induced

as part of the lytic program that is initiated during infection with virulent *H. capsulatum*. In an alternate model, the genes of the HRC promote macrophage lysis. This is our preferred model.

Once induced, we hypothesize that the induction of the HRC triggers macrophage cell death. Consistent with this hypothesis, several genes in this cluster have been implicated in the regulation of cell death in other systems. Ddit3 (aka CHOP) and Trib3 are induced during ER-stress induced apoptosis (Ohoka *et al.*, 2005). Ddit3, Trib3, Nurp1 (aka p8), and Asns are transcriptionally induced after tumor cells are treated with Δ^9-tetrahydrocannabinol (THC) and their induction promotes apoptosis (Carracedo *et al.*, 2006). These four genes are induced in BMDMs infected with *H. capsulatum* in a *CBP1*-dependent manner. This data suggests a causative role for the genes of the HRC in macrophage lysis during *H. capsulatum* infection. Abrogating the function of these genes in the context of *H. capsulatum* macrophage infection will provide evidence for the role of this transcriptional response in *H. capsulatum* pathogenesis. Another key aspect of this analysis will involve examining whether these genes are induced in the animal during infection. Determining if animals deficient for HRC genes have altered susceptibility to *H. capsulatum* infection would be extremely informative.

This transcriptional analysis highlights another aspect of the *H. capsulatum*-macrophage interaction that has yet to be explained. What is the mechanism of host cell death? The genes induced in the HRC suggest that *H. capsulatum* induced cell death is apoptotic. This, however, has not been demonstrated. Determining the mode of host cell death would provide key insights into and context for the role of Cbp1 and the HRC in *H. capsulatum* virulence.

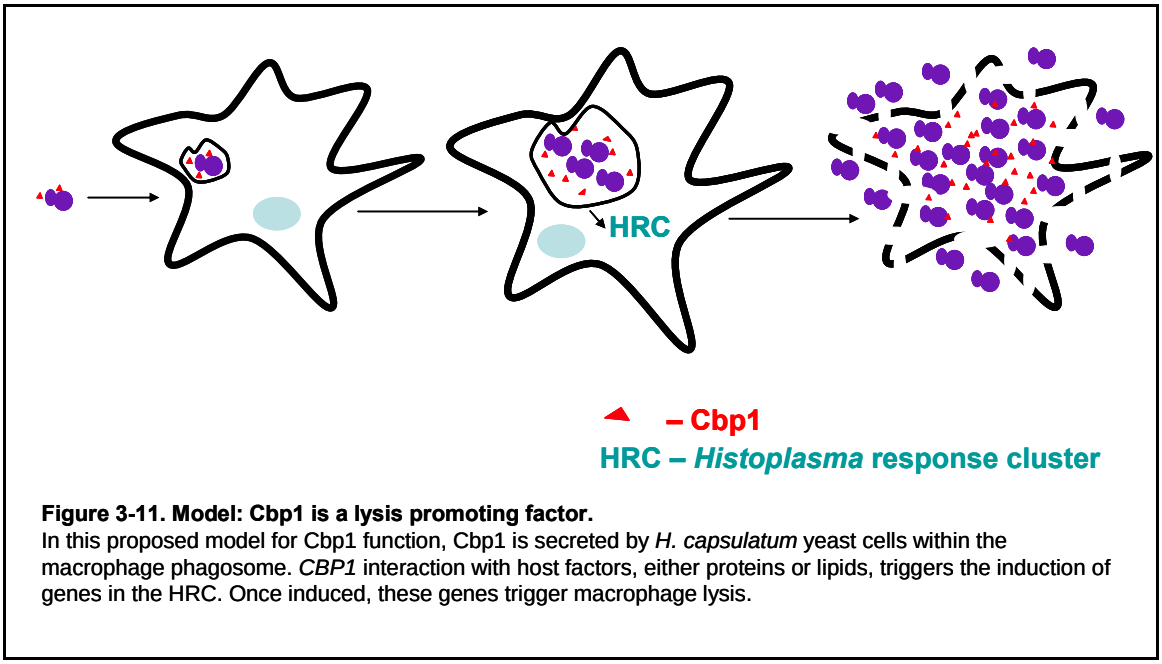

Figure 3-11. Model: Cbp1 is a lysis promoting factor.
In this proposed model for Cbp1 function, Cbp1 is secreted by *H. capsulatum* yeast cells within the macrophage phagosome. *CBP1* interaction with host factors, either proteins or lipids, triggers the induction of genes in the HRC. Once induced, these genes trigger macrophage lysis.

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CHAPTER FOUR

Conclusions and Future Directions

Conclusions

Successful microbial pathogens must subvert or evade host immune defenses in order to promote their survival and cause disease. *H. capsulatum,* a dimorphic fungus, is one such pathogen. This fungus survives and replicates within macrophages, ultimately killing the phagocytic immune cells whose primary function is to kill invading microbes. The creation of this hospitable, replicative niche inside the macrophage requires that *H. capsulatum* evade the antimicrobial effects of toxic reactive oxygen and nitrogen species, as well as the effects of hydrolytic lysosomal enzymes.

Understanding the fungal mechanisms involved in meeting these antimicrobial challenges is the key to understanding *H. capsulatum* pathogenesis. Unfortunately, our ability to understand the virulence strategies employed by *H. capsulatum* has been hampered by a limited knowledge of the fungal factors that promote survival in the macrophage. Additionally, the molecular functions of the fungal factors that have been identified are still unknown.

The experiments described in this work address these limitations and further our understanding of *H. capsulatum* pathogenesis. I performed a genetic screen to identify *H. capsulatum* virulence factors and isolated 26 lysis defective (LDF) mutants that failed to lyse macrophages. Characterization of these mutants is still ongoing, but their identification in this screen suggests that the disrupted genes play a role in *H. capsulatum* pathogenesis. One mutant identified in the screen contained an insertion in the promoter of the previously identified virulence factor Cbp1. Analysis of the interactions between

the *cbp1* mutant and host macrophages has given us insights into the molecular function of this secreted factor. I will now discuss these findings in greater detail.

H. capsulatum actively induces macrophage lysis during infection

When I initiated these studies, the mode of host cell death in response to *H. capsulatum* infection was not well understood. Despite a lack of experimental evidence, the prevailing presumption in the field was that macrophages died as a result of the high fungal burden at the later stages of infection (Sebghati *et al.*, 2000). An examination of the intracellular replication of the LDF mutants revealed that this was not the case. I monitored the intracellular replication of 17 LDF mutants inside BMDMs and found that they were all able to grow. Most mutants replicated with slightly delayed kinetics, exhibiting a doubling time that was approximately 1.5 times that of wild-type *H. capsulatum*. Two severe lysis defective mutants, *cbp1* (172-C5) and *bub2* (138-G1), achieved a high fungal burden within macrophages. These results indicated that intracellular replication was not sufficient to cause macrophage lysis and demonstrated that macrophage lysis was actively triggered by *H. capsulatum* during infection.

CBP1 is required for the induction of a unique macrophage transcriptional response that correlates with lysis

Prior to these studies, other labs assumed the macrophage lysis defect of the Δ*cbp1* mutant was due to an intracellular survival defect. This conclusion was made in the absence of data examining intracellular growth. In this work, we monitored the intracellular replication of the *cbp1* mutant and showed that it grew to high levels within

the macrophage without causing lysis. This data suggested that the *H. capsulatum* secreted factor Cbp1 was dispensable for accumulation of *H. capsulatum* cells, and thus may play a more direct role in mediating macrophage lysis than was previously thought. Since Cbp1 is a secreted protein, it may be ideally positioned to interact with host factors to manipulate the macrophage response to infection and promote host cell lysis. Together with Charlotte Berkes, I examined the effect of *CBP1* on the transcriptional response of macrophages during infection and found that this factor was required for the induction of the genes in the *Histoplasma* Response Cluster (HRC) (see Chapter 3). This cluster of genes is induced when macrophages are infected with virulent *H. capsulatum* strains that ultimately lyse the macrophage. Consistent with the hypothesis that the induction of these genes promotes lysis, several genes in this cluster have been shown to play a role in regulating and promoting cell death in other experimental systems (Ohoka *et al.*, 2005; Carracedo *et al.*, 2006).

Future directions

Our analysis of virulence factors in *H. capsulatum* has given rise to further questions and lines of experimental inquiry. I will outline some of some these questions below.

Do the *H. capsulatum* specific LDF genes point to *H. capsulatum* specific virulence mechanisms?

The LDF gene set is enriched for genes that are only predominantly found in *Histoplasma* species. Sixty-one percent of these genes have no orthologs in other fungi. One trivial explanation for this finding is that these genes were falsely annotated by the various *H. capsulatum* sequencing projects. Our ability to detect these genes using whole genome oligonucleotide expression arrays argues against this explanation. Another, more intriguing, hypothesis is that these genes define novel virulence mechanisms that are specific to *H. capsulatum* infection. The fact that *CBP1* is one of these genes is consistent with this hypothesis. In addition to being required for virulence in macrophages and mice, we show that *CBP1* is required for the induction of a macrophage transcriptional response that is not observed upon infection with other intracellular pathogens. Similar experiments monitoring the role of these novel virulence factors in the manipulation of the macrophage response during infection will begin to answer this question.

Is *CBP1* expression sufficient to cause macrophage lysis?

The *cbp1* mutant grows to high numbers within macrophages without causing lysis. This data suggests that the role of Cbp1 during infection is to promote host cell

lysis, possibly through a direct role as a lysis factor. This hypothesis can be tested experimentally by ectopically expressing *CBP1* in host cells. Ectopic expression can be achieve by directly expressing *CBP1* in macrophages under a macrophage promoter or by infecting macrophages with a fungus, *Candida glabrata*, that has been engineered to express and secrete *CBP1*. If ectopic expression is sufficient to cause host cell lysis, then Cbp1 can be classified as a lysis factor. We can also determine if ectopic expression of *CBP1* is sufficient to induce the HRC.

What is the fate of the LDF mutant phagosome?

H. capsulatum must create a hospitable, replicative niche inside the macrophage phagosme. A subset of the LDF mutants might fail to generate such a haven, an thus will identify fungal genes that are required for macrophage colonization. Characterizing the LDF mutant phagosome, with respect to pH, membrane markers, and oxidative character, will provide insights into the role of the corresponding LDF genes in pathogenesis.

How do LDF genes affect the host response to *H. capsulatum*?

A number of LDF genes are predicted to have N-terminal signal peptides. This suggests that these genes encode proteins that may be secreted or found on the fungal cell surface. Exposed to the macrophage environment, these factors may function to manipulate the host during infection. Comparing the transcriptional profile of the macrophage response to infection with these mutants versus infection with wild-type *H. capsulatum* could provide insights into the host pathways that are being manipulated by the LDF genes.

What is the mode of macrophage cell death in response to infection with *H. capsulatum*?

The mode of macrophage cell death during *H. capsulatum* infection is unknown. Interestingly, a number of the genes in the HRC are involved in promoting apoptotic cell death in other systems. Future experiments will address whether macrophage lysis in response to *H. capsulatum* is accompanied by Cbp1-dependent DNA fragmentation, phosphotidylserine exposure on the plasma membrane, and Caspase-3 activation, which would suggest the activation of apoptotic pathways.

Does Cbp1 interact with host factors? Which ones?

Cbp1 is a secreted factor that is required for the induction of a macrophage transcriptional signature that correlates with host cell lysis during *H. capsulatum* infection. We hypothesize that Cbp1 is interacting with host factors to mediate its function. Identification of these host factors would provide insights into the molecular mechanisms of Cbp1 function. Immunoprecipitation of Cbp1 from infected macrophages or 2-hybrid screens with a host library could be used to identify macrophage binding factors.

Does expression of the genes in the HRC cause macrophage lysis?

Several genes in the HRC have been implicated in the regulation of cell death in other systems. These include Ddit3, Trib3, Nurp1 (aka p8), and Asns. These four genes are induced in BMDMs infected with *H. capsulatum* in a *CBP1*-dependent manner. This

data suggests a causative role for the genes of the HRC in macrophage lysis during *H. capsulatum* infection. The role of these genes in causing host cell lysis during infection can be dissected by infecting macrophages that are deficient in individual HRC genes. If wild-type *H. capsulatum* is unable to lyse these mutant macrophages, it will be clear that the HRC gene in question is required for host cell lysis.

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