Analysis of Hypoxia Response Mechanisms in *Histoplasma capsulatum*

Honors Research Thesis

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by

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Abstract

Histoplasma capsulatum is a dimorphic fungal pathogen endemic to the Ohio River valley. This pathogen is particularly harmful because of its ability to cause disease in both immunocompromised and immunocompetent hosts. *Histoplasma* primarily affects the respiratory system, leading to colonization of the lungs, but can disseminate to other organs such as the spleen and bone marrow. The mechanisms of *Histoplasma* virulence and how it adapts to growth within host cells and the host environment are relatively unknown. We have isolated a strain of *H. capsulatum* with a mutation in the *DSC2* gene, which is postulated to regulate the fungal response to hypoxia. The Dsc complex responds to decreased sterol synthesis, which is coupled to decreased oxygen levels. We hypothesize that the Dsc complex may allow *Histoplasma* to survive in the potential hypoxic environments of different organs. To test this, we developed a complemented strain and analyzed the ability of the wild-type, the *dsc2* mutant, and complemented strains to grow under low oxygen, conditions that mimic hypoxia in vitro (e.g., growth with $CoCl_2$), and infection of cultured macrophages. The data obtained showed some indication of decreased survival of the *dsc2* mutant in comparison with the wild-type and complemented strains. We also examined the progression of disease of the *dsc2* mutant strain in vivo using a murine model of respiratory and disseminated histoplasmosis. A better understanding of *Histoplasma* virulence mechanisms such as the response to hypoxia could help to indicate new targets for potential therapeutics for histoplasmosis.

Introduction

Histoplasma capsulatum is a fungal pathogen that is particularly prevalent in the Ohio and Mississippi river valleys. It is a dimorphic fungus, existing as filamentous mycelia in lower temperature environments and infectious yeasts at 37˚ (1). *Histoplasma* initially infects the lungs,

which means the conidia are inhaled and then convert to yeasts due to the increased temperature of the body. Within the lungs, *Histoplasma* proliferates in macrophages and can disseminate to other areas of the body. *Histoplasma* causes the disease histoplasmosis which ranges from subclinical symptoms to life-threatening disseminated disease (2). It is particularly unique among fungal pathogens because of its ability to cause disease in both immunocompromised and immunocompetent hosts. In hosts with a healthy immune system, *Histoplasma* may only cause a mild or asymptomatic infection. In patients who do not possess a fully functioning immune system, however, it may manifest in more severe forms, including possible dissemination (2).

During infection of the lungs, *Histoplasma* must combat many host factors in order to survive and replicate to become a persistent infection. *Histoplasma* faces obstacles from the immune system including phagocytosis and antimicrobial reactive oxygen species produced by immune cells. In addition, it may experience conditions of hypoxia in the lung. While the lung is normally a highly oxygenated tissue, formations of large masses of cells such as infiltrating neutorophils and other inflammatory cells can decrease the oxygen availability (3, 6).

One potential way that *Histoplasma* may survive in these hypoxic environments is through adaptation to hypoxia which is controlled by a protein complex called Dsc. In *Aspergillus fumigatus*, another fungal pathogen, it has been shown that Dsc is necessary for survival in hypoxia (4). As *Histoplasma* is also a pathogen of mammalian lungs, Dsc may also facilitate *Histoplasma* infection. To test this hypothesis, I am investigating the gene *DSC2*, which forms part of the Dsc complex. In the proposed mechanism of action, Dsc recognizes low oxygen levels and then cleaves the N-terminal helix-loop-helix motif of the protein Srb1. This Srb1 cleavage product is then transported to the nucleus where it acts as a transcription factor for genes that regulate *Histoplasma*'s response to hypoxia. The loss of Dsc2 would thus result in

decreased cleavage of Srb1 in response to hypoxia which would lead to an inability to produce the active transcription factor needed to initiate the hypoxia response.

Materials and Methods

Strains and growth conditions

The *dsc2* mutant strain OSU152 was created previously using transposon mutagenesis in a uracil auxotroph background. To generate uracil prototrophs for use in infections, this strain was then transformed with plasmid pCR628 which expresses the *URA5* gene. The *dsc2* complemented strain, OSU213, was created by transforming the *dsc2* mutant strain with a plasmid containing the wild-type *DSC2* gene.

Strains were grown at 37° on *Histoplasma*-macrophage media (HMM, 5). Liquid cultures were grown up to late exponential/early stationary phase with continuous shaking of the cultures at 200 rpm.

$CoCl_2$ assay

$CoCl_2$ was used to mimic hypoxia conditions. Wild-type, mutant, and complemented strains were grown in 96-well plates with variable concentrations of $CoCl_2$. Growth of *Histoplasma* yeasts over time was monitored by OD_{595} on a Synergy 2 plate reader (BioTek).

Growth at normoxic, hypoxic, and anaerobic conditions

Liquid cultures of the wild type, mutant, and complemented strains were grown to late exponential/early stationary phase and the yeasts suspension enumerated by hemacytometer. Equivalent concentrations of yeasts were spotted on HMM plates in triplicate. Plates were stored in a normal incubator (approx. 20% O_2), a hypoxic incubator (0.5% O_2, 5% CO_2, 94.5% N_2), and

in an anaerobic chamber flushed with N_2. Yeasts were incubated at 37° until growth was observed.

Macrophage assay

P388D1 macrophages were seeded in a 96-well plate at 2 x 10^4 cells/well in complete F12 media (Ham's F12, 10% FBS, L-glutamine, 10 µg/mL tetracycline, 20 µg/mL gentamycin), and left to adhere at 37° for 24 hours prior to infection. During infection, 2 x 10^4 *Histoplasma* yeasts were added to the wells in HMM-M complete (HMM-M, 10% FBS, L-glutamine, 10 µg/mL tetracycline, 20 µg/mL gentamycin). After 4 hours, the media was removed and replaced with fresh media. Intracellular survival and replication of yeasts was determined by plating of macrophage lysates. For plating, every 24 hours macrophages were lysed with sterile TE buffer (10 mMTris, pH 8.0, 1 mM EDTA, pH 8.0) and dilutions of the lysate plated on HMM to determine viable colony forming units. Plates were grown at 37°, 5% CO_2/95% air.

In vivo competition assay

For competition assays, wild type was marked with red-fluorescent protein, the *dsc2* mutant with green-fluorescent protein, and the *dsc2/DSC2* complemented strain as non-fluorescent. Yeasts for inoculation of mice were counted by hemacytometer. Each C57BL/6 mouse was infected intranasally with 3 x 10^4 yeasts in a volume of 45 microliters. At four day intervals post infection (4, 8, 12, 16, and 20 days), mice were euthanized and the lungs and spleens were removed and homogenized in 5 mL and 3 mL HMM, respectively. Dilutions of homogenates were plated on HMM to determine viable colony-forming units (cfu) in each tissue. Strain genotypes were inferred using red, green, or non-fluorescence of colonies as measured with a UV transilluminator with appropriate filters for GFP and RFP fluorescence emission.

Construction of SRB1-RNAi strain

An *SRB1*-RNA interference (RNAi) strain was created to knockdown expression of *SRB1*. A fragment of the *SRB1* coding sequence was cloned into an RNAi vector with a GFP sentinel to provide a visual indicator of target gene knock-down. In the RNAI vector, two copies of the gene fragment are inserted in reverse orientation to create an RNA hairpin after transcription. The hairpin is cleaved by *Histoplasma* proteins and then the RNA fragments induce the cleavage of the corresponding gene in the cell. The *GFP* gene can be included in the hairpin, which will then act in trans to knock-down GFP fluorescence in the cell. The *SRB1*-RNAi plasmid was transformed into *Agrobacterium tumefaciens* by electroporation to enable *Agrobacterium*-mediated transformation of *Histoplasma*. *Agrobacterium* was cocultured with *Histoplasma* strain OSU194 (a GFP fluorescent wild type background) to induce uptake and integration of the plasmid DNA, and knock-down of GFP fluorescence of transformant colonies tested. *Histoplasma* strain OSU194 with *gfp*-RNAi plasmid pED02 (lacking sequences for SRB1 knock-down) was used as the control.

Results

dsc2 mutant is more susceptible to effects of $CoCl_2$

As an in vitro test of sensitivity to hypoxia, yeasts were treated with $CoCl_2$ which mimics many of the aspects of growth in low oxygen. A graded series of $CoCl_2$ concentrations were tested to establish a dose-response curve (Figure 1). Whereas the wild-type had an IC50 of 0.45 mM, the *dsc2* mutant showed increased sensitivity to $CoCl_2$ with an IC50 of 0.034 mM. Complementation of the *dsc2* mutation restored the resistance of *Histoplasma* yeast to $CoCl_2$ with an IC50 of 0.38 mM. This shows that (1) the *dsc2* mutation is more sensitive to $CoCl_2$ treatment and (2) complementation of the *dsc2* mutation reveals the sensitivity is directly linked

to the loss of Dsc function. Since $CoCl_2$ mimics many of the aspects of hypoxia, we infer that Dsc functions to facilitate growth of *Histoplasma* yeasts in low oxygen environments.

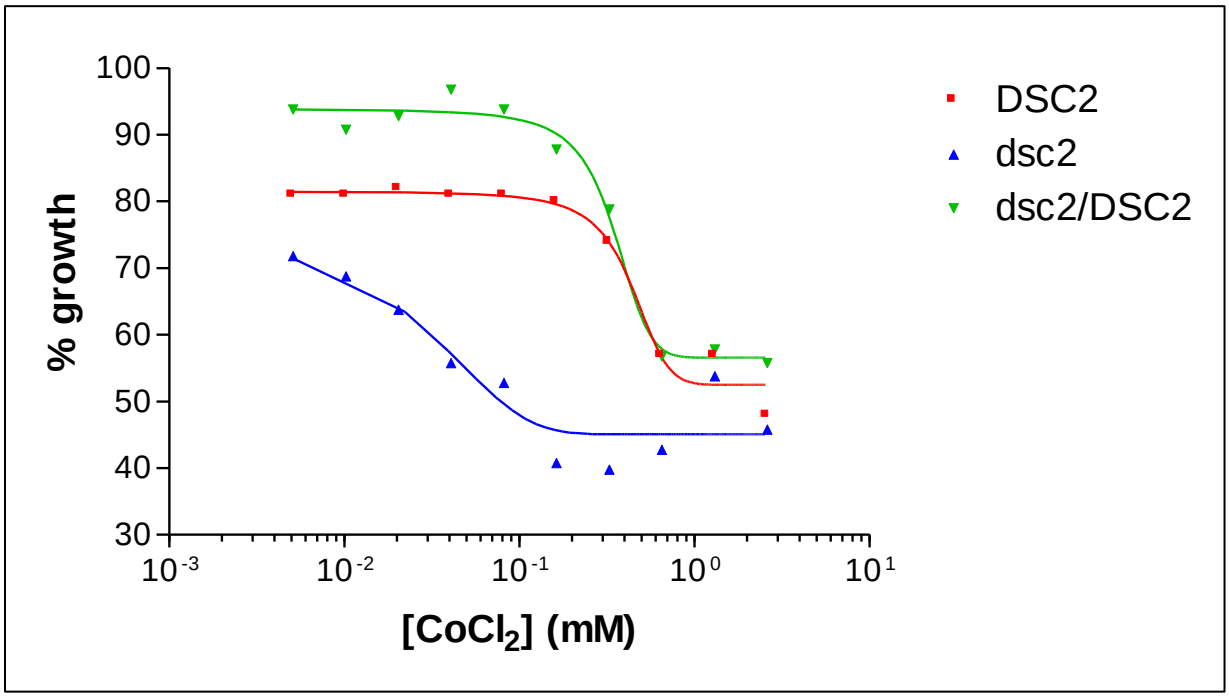

Figure 1: Effects of [$CoCl_2$] on *Histoplasma capsulatum DSC2* (WU15), *dsc2* (OSU152), and *dsc2/DSC2* (OSU213) strains.

Growth of dsc2 mutant is attenuated under lower oxygen conditions

As a more direct test of the sensitivity of *Histoplasma* yeast to hypoxia, wild-type and *dsc2*-mutant yeasts were grown under hypoxic and anaerobic conditions. In normoxia, the wild-type and the *dsc2* mutant produced similarly sized colonies after 7 days of incubation (Figure 2A). In hypoxia (0.5% oxygen), growth rate was significantly reduced (data not shown) but wild-type and *dsc2*-mutant strains grew similarly (Figure 2B). In more severe reduction of oxygen, the *dsc2* mutant showed greater impairment in growth than the wild-type and the *dsc2/DSC2* complemented strain (Figure 2C). The *dsc2* mutant was not impaired in the number

of colonies formed, but instead in the size of the colonies, indicating viability was not impaired but growth was significantly reduced. These data confirm the $CoCl_2$ tests and indicate that *DSC2* is involved in maintaining normal growth in very low oxygen conditions.

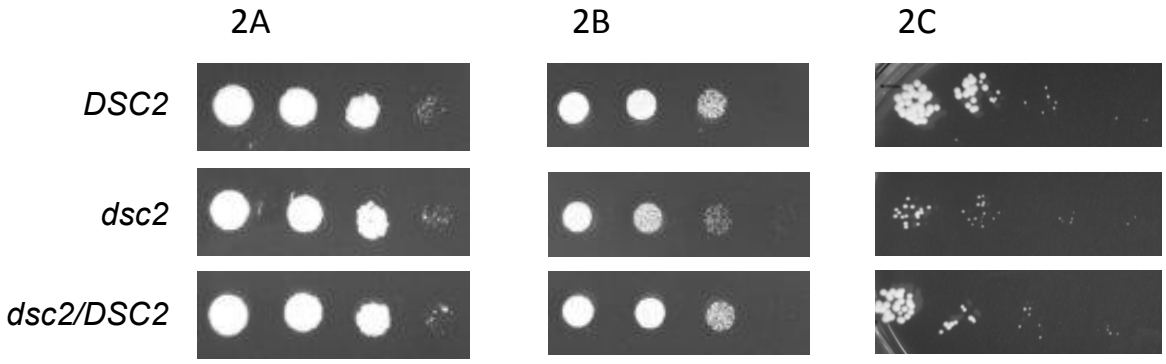

Figure 2A: Growth under normoxic conditions (approximately 20% O_2) **2B-C:** Growth under hypoxic (0.5% O_2) and anaerobic (0% O_2) conditions.

Dsc2 is necessary for intramacrophage growth

Since the host cell for *Histoplasma* is the alveolar macrophage, we tested whether *dsc2* (and thus adaptation to hypoxia) was required for growth in macrophages using the P388D1 macrophage cell line. Macrophage cells were infected with wild-type, *dsc2*-mutant, and *dsc2/DSC2* yeasts at a multiplicity of infection of 0.5 yeasts/macrophage. At incremental time points, the number of yeasts was quantified by lysing the macrophages and plating of the lysate for viable colony forming units. The wild-type and *dsc2/DSC2* strain both survived and replicated in macrophages similarly, increasing 30-fold by 48 hours. In contrast, the *dsc2* mutant showed only 15-fold increase in cell number indicating the ability to replicate in macrophages is attenuated with Dsc2 function (Figure 3).

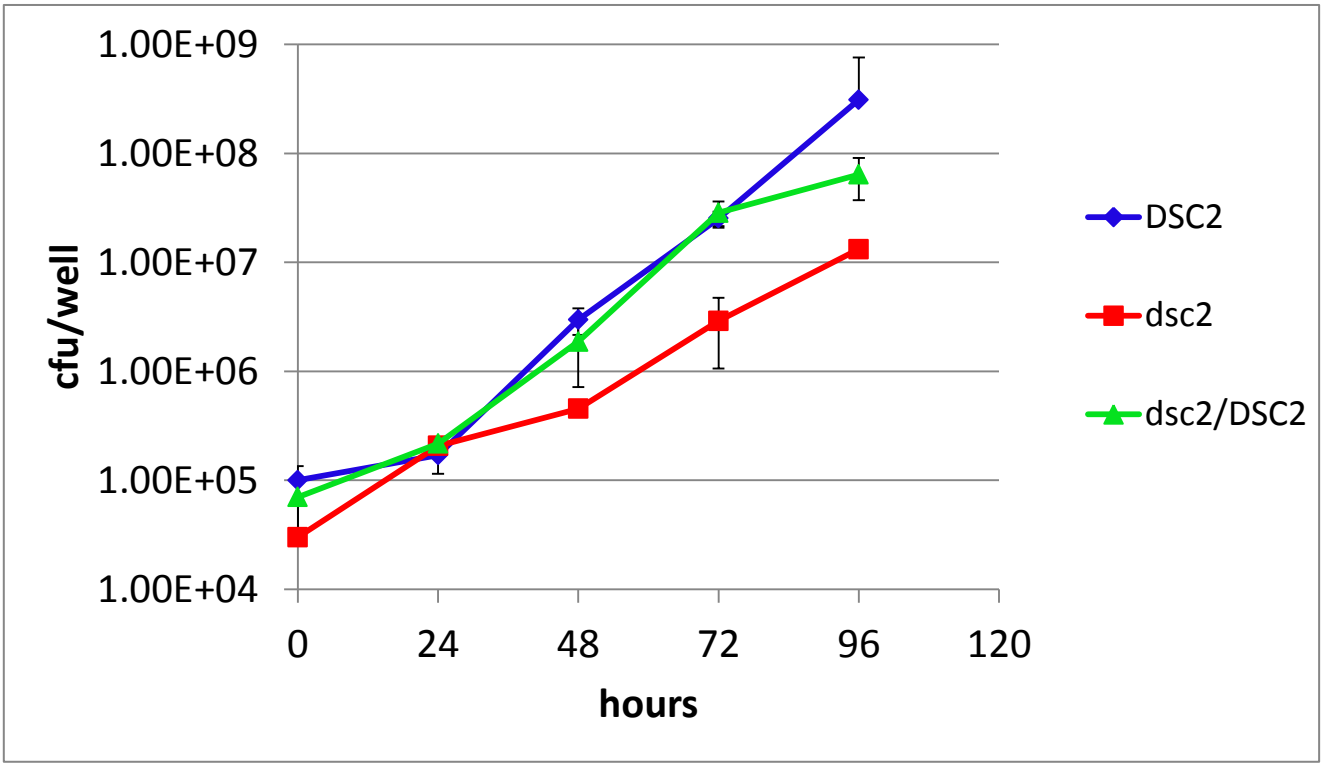

Figure 3: Survival of *Histoplasma* wild-type, *dsc2* mutant, and *dsc2/DSC2* complemented strains in P388D1 macrophage cell line.

Dsc2 is not required for infection in vivo

To determine whether *Histoplasma* yeasts must adapt to hypoxia in vivo, mice were co-infected with equal amounts of the wild-type, *dsc2* mutant, and complemented strains. This competition assay is a sensitive measure of whether Dsc2 confers a fitness advantage in vivo. After 8 days, the fungal burden in lungs and in spleens was determined as indicators of acute respiratory infection and dissemination capability, respectively. Colony counts of the strains indicated that there was no significant difference in infection of the lungs nor in spleens of the mice at day 8 (Figure 4). This trend also continued throughout the course of the infection (data not shown). This indicates that Dsc2 may not be required for infection in mice.

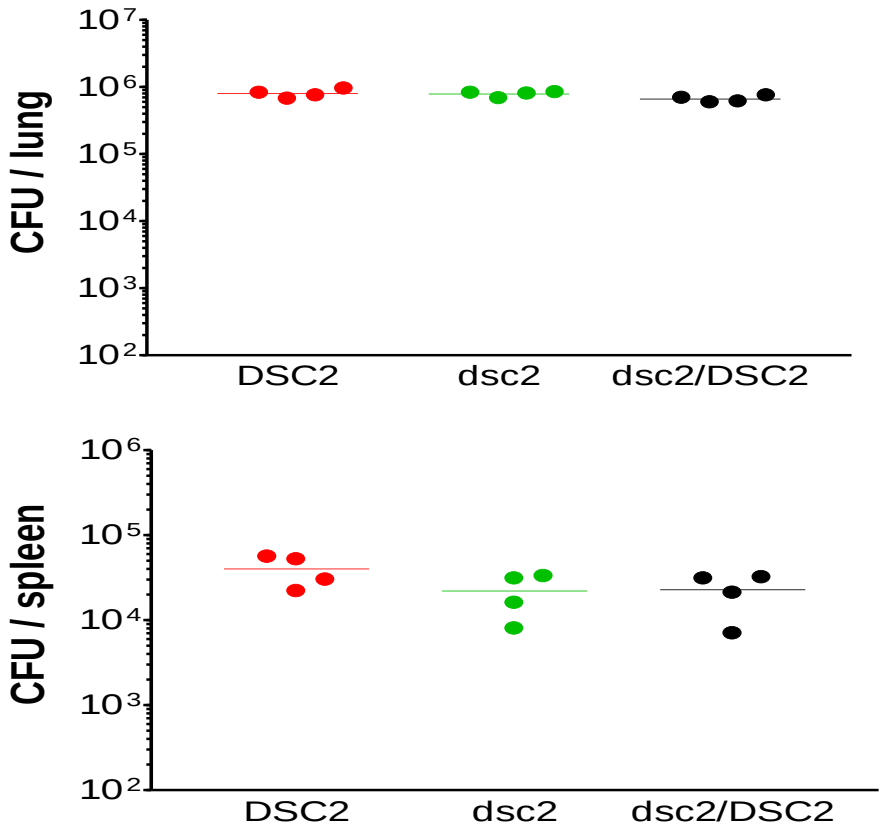

Figure 4: Competition assay to examine the relative fitness of the wild-type, *dsc2* mutant, and complemented strains in vivo.

Srb1 is required in Histoplasma

We sought to determine if the downstream effector of Dsc activation was involved and so *SRB1* expression was targeted for knocked down by RNAi (Figure 5). Unfortunately, no transformants were recovered that had knock-down of the gfp sentinel. Conversely, this indicated that there was no knock-down of the *SRB1* gene. This was in contrast to transformation with an RNAi plasmid lacking the SRB1 sequences. This data indicates that no viable transformants with *SRB1*-knock down were obtained which strongly suggests that knock-down of *SRB1* is lethal to the yeasts.

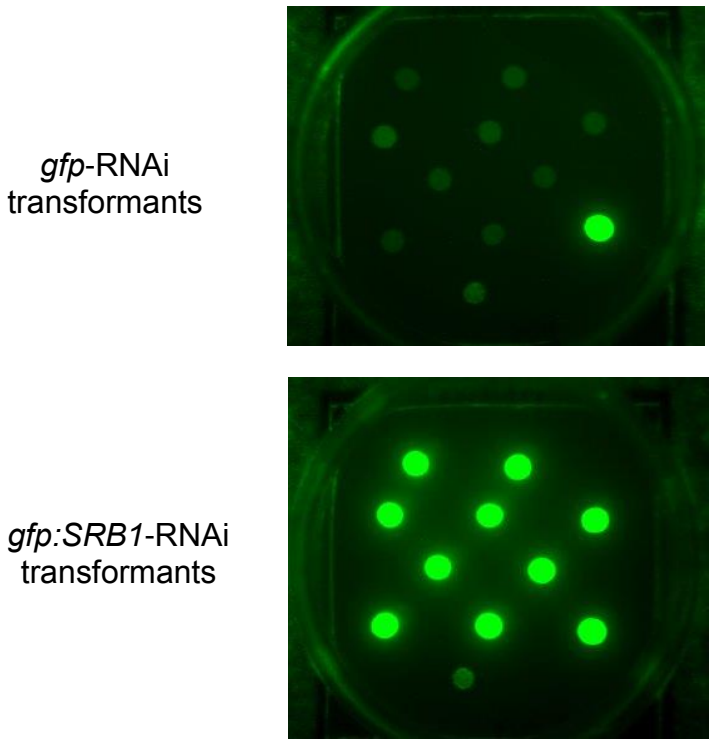

Figure 5: Use of RNA interference (RNAi) to observe knock-down of the *Histoplasma SRB1* gene.

Discussion

This study shows that *DSC2* is required by *Histoplasma* for growth under low oxygen in vitro. These findings are similar to those of the fungal pathogen *Aspergillus fumigatus* (4), which indicates that the mode of action of Dsc may be similar in both organisms. In the $CoCl_2$ assay, there was approximately a 15-fold decrease in IC50 of the *dsc2* mutant compared to wild type. This is reflected when the strains were grown on under very low oxygen conditions. There was attenuation in colony size rather than colony number which means it may be possible for *Histoplasma* to establish growth in hypoxic conditions, but not to maintain it. The plates with *Histoplasma* spots were not prepared under hypoxic conditions, so any remaining oxygen in the media may have been enough to begin growth of the *dsc2* mutant because the strains were not

immediately under hypoxic conditions. This is unlikely, however, due to the greater amounts of time it takes *Histoplasma* to begin to grow.

When measuring survival and proliferation of the *Histoplasma* yeasts in macrophages, intramacrophage survival was decreased 20-fold in the *dsc2* mutant compared to wild type. This indicates that intracellular proliferation requires Dsc2 in *Histoplasma*. All three *Histoplasma* strains were phagocytized equally by the macrophages, but *dsc2* mutant yeasts were unable to replicate as efficiently as wild type. This indicates that the deficiency is due to loss of Dsc2-dependent adaptation to the intracellular environment. Given these results, it was surprising that in vivo infection showed no requirement for Dsc2. This fact that Dsc2 is dispensable suggests that in vivo, *Histoplasma* does not encounter significant hypoxic conditions. This could be due to the possibility that the mice lungs are not as hypoxic as other animals, although work with pulmonary *Aspergillus* infections indicates some hypoxia is created within the lung (4). However, *Aspergillus* evokes a profound inflammatory response whereas *Histoplasma* yeasts reside within immune cells and do not stimulate as much activation. In longer term infections, *Histoplasma* yeasts trigger granuloma formation (an organized immune response designed to "wall-off" the infection) and these granulomas can become calcified. This granuloma environment can become significantly hypoxic (6) so it would be interesting to see if Dsc2 is required for long-term infections in mammals.

Although we were able to successfully mutate *DSC2*, we were unable to interfere with the function of the downstream effector Srb1. Our results using a gfp-sentinel strain suggests that Srb1 is essential. This indicates that Dsc2-dependent Srb1 activation is likely only part of the upstream inputs into Srb1. Srb1 has also been proposed to function in the production of responses to iron limitation which would be essential. Additional testing to determine the

presence of an Srb1 cleavage product and localizing Srb1 in the cells would be beneficial in further characterization of Dsc2 and its interactions with Srb1 in *Histoplasma*.

Our results with Dsc2 suggest that *Histoplasma* does not encounter significant hypoxia in vivo, at least in acute disease. Future experiments addressing the role of Dsc2, and thus adaptation to hypoxia, in establishment of latent infections will be needed to determine if targeting hypoxia response pathways could be a viable therapeutic option.

Superoxide dismutase (*SOD3*)

As a supplement to my work on *DSC2*, I have also been working on comparing the roles of superoxide dismutase (*SOD3*) in two divergent *Histoplasma* strains, G186A and G217B. *Histoplasma* secrets the superoxide dismutase Sod3 in order to help protect itself from extracellular reactive oxygen species (ROS) produced by the host cells that combat the fungal infection. Superoxide is a type of ROS produced by the host which is broken down by superoxide dismutase. The mechanisms of Sod3 virulence have been characterized in the G186A strain previously (7), but the comparison has not been made between G186A and G217B. G217B has greater virulence than G186A in mice and one potential mechanism is greater resistance to host-produced superoxide. To test this idea, we have created mutant strains in both backgrounds G186A and G217B, respectively. I constructed complemented strains for each background by cloning the wild-type *SOD3* gene into *Agrobacterium* vector pCR639 and transforming the resulting plasmids (pED07 and pED08 for G186A and G217B, respectively) into *Histoplasma* via *Agrobacterium*. These plasmids are then integrated into the recipient cell DNA. Cell cultures were grown in HMM liquid media and supernatants were isolated from the cultures by spinning the cells down at 2000 rcf for 2 minutes and then filter sterilizing the supernatant with 0.2 µm

pore polyethersulfone (PES) membranes. A portion of the culture filtrates was concentrated using acetone precipitation and then run on a polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane for immunoblotting with α-Sod3 antibody in order to confirm the construction of the mutant and complemented of the mutation. As expected, Sod3 protein was not present in the mutant strains (Figure 6) but was produced by the wild-type parent and the *sod3/SOD3* complemented lines. With confirmed strains, we can now test the relative contribution of Sod3 to the virulence of G186A and G217B.

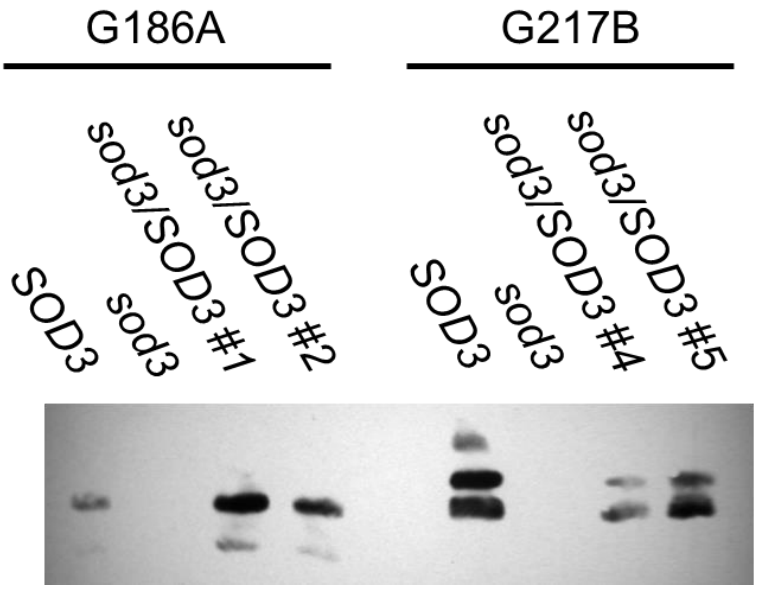

Figure 6: Immunoblot with α-Sod3 antibody of wild-type, *sod3* mutant, and two complemented strain isolates for each *Histoplasma* background.

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