December 2019

Role of *Cryptococcus neoformans* Pyruvate Decarboxylase and Aldehyde Dehydrogenase Enzymes in Acetate Production and Virulence

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A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biochemistry and Molecular Biology

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by
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December 2019

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ABSTRACT

The basidiomycete *Cryptococcus neoformans* is is an invasive opportunistic pathogen of the central nervous system and the most frequent cause of fungal meningitis. *C. neoformans* enters the host by inhalation and protects itself from immune assault in the lungs by producing hydrolytic enzymes, immunosuppressants, and other virulence factors. *C. neoformans* also adapts to the environment inside the host, including producing metabolites that may confer survival advantages. One of these, acetate, can be kept in reserve as a carbon source or can be used to weaken the immune response by lowering local pH or as a key part of immunomodulatory molecules. Thus, cryptococcosis could be treated by targeting acetate production. The Smith laboratory has identified two potential pathways for acetate production. The xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (Xfp) - acetate kinase (Ack) pathway, previously thought to be present only in bacteria, converts phosphoketose sugars to acetate through acetyl-phosphate. The pyruvate decarboxylase (Pdc) and acetaldehyde dehydrogenase (Ald) pathway, found in other fungi, converts pyruvate to acetate through acetaldehyde. The genes encoding enzymes from these pathways have been shown to be upregulated during infection, suggesting that acetate production may be a required part of cryptococcal infection. In *Saccharomyces cerevisiae*, Pdc works with one or more Alds to produce acetate. Eight of the nine *C. neoformans* Alds and the sole Pdc all contributed to the cellular acetate pool, and loss of some of these enzymes reduced cell survival during growth on various carbon sources,

under oxidative or nitrosative stress, under pseudo-hypoxia conditions, and when the cell wall integrity was disrupted. In addition, deletion mutants of of some of these enzymes affected capsule formation and melanization, two primary determinants of *Cryptococcus*, and led to decreased virulence in macrophages and *Galleria mellonella*, an invertebrate model of infection.

Metabolic adaptability is an important attribute for fungal pathogens to successfully infect and cause disease. Carbon metabolism is critical for virulence in *C. neoformans*, but little is known about which carbon sources are utilized during infection. Lung alveolar macrophages, the first line of host defense against *C. neoformans* infection, provide a glucose- and amino acid-poor environment, and nonpreferred carbon sources such as lactate and acetate are likely important early in establishment of a pulmonary infection. A global screening was undertaken to identify *C. neoformans* proteins necessary in acetate utilization, as possible drug targets. From two libraries, together comprising 3936 gene knockouts, 41 mutants failed to grow on media with either glucose or acetate as the carbon source, or on both media. Of the known proteins lacking in these mutants, most function in gluconeogenesis, arginine biosynthesis, or mitochondrial transmembrane transport. Overall, this work elucidated the roles of *C. neoformans* acetate production and utilization pathways in virulence.

DEDICATION

It is with my genuine gratefulness and warmest regard that I dedicate this dissertation to my family: my beloved mom, who has always been there for me when I needed emotional support, my dad, whose dream, before he passed away, was for me to obtain my Ph.D. in the United States, and whose encouraging voice I still hear whenever I am struggling, my husband, Akram, who always puts me first, my daughter Mariam, my bright star, and my brothers Mohamed, Adel, Salah, and Nuri. Without these people in my life, this dissertation would not have been completed. I love you all!

ACKNOWLEDGMENTS

I would like to thank my advisor and committee chairman, Dr. Kerry Smith, for his continuous support of my studies and research over the years, as well as my dissertation committee members, Dr. Julia Frugoli, Dr. Cheryl Ingram-Smith, and Dr. Lukasz Kozubowski for their patience, motivation, enthusiasm, and guidance throughout my time in graduate school.

The completion of my project would not have been possible without the support of everyone, including the professors, staff, and graduate students, of the Eukaryotic Pathogens Innovation Center and of the Genetics and Biochemistry Department at large. I would like to especially express my gratitude to Dr. Meredith Morris, Dr. Lesly Temesvari, and Dr. Kimberly Paul for making my day and life more cheerful and happier in hard times. I am also extremely grateful to all my fellow lab members, past and present, who helped me with my experiments and assisted me in myriad ways throughout my time at Clemson.

I would like to thank my Libyan family: my husband Akram and my sweet daughter Mariam for their constant love and support, my beloved mother Salma, and my brothers, who have been my source of inspiration and who have given me strength and their continuous moral, spiritual, and emotional support when I thought of giving up.

Finally, last but by no means least, I am also grateful to my American family and friends: Cheryl Jones, Nicole Clark-Mount, Hollie H. Smith, Sylvia Cole,

Sophia Altamirano, and Archana Nalin Mankad for their love and for supporting me along the way. Julia Frugoli and Cheryl Ingram Smith, who, in addition to agreeing to be on my committee and giving me technical advice, also acted as a personal friend and extended family member.

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CHAPTER ONE

LITERATURE REVIEW

***Cryptococcus neoformans*: Epidemiology and sources of exposure**

Cryptococcus neoformans, an opportunistic pathogen that belongs to the phylum *Basidiomycota* (Loftus *et al*., 2005; Sorrell, 2001), has two species that are most often found to infect humans: *Cryptococcus neoformans*, consisting of cryptococcal serotypes A and D, as well as a hybrid serotype AD, and *Cryptococcus gattii*, consisting of serotypes B and C (Litvintseva *et al*., 2011; Chayakulkeeree and Perfect, 2008). The vast majority of patients with cryptococcosis have defects in cell-mediated immunity, specifically in $CD4^+$ lymphocytes. AIDS is a major risk factor with mortality rates in AIDS patients ranging from 15-20% in the US and 55-70% in Latin America and sub-Saharan Africa (Park *et al.*, 2009). Immunosuppressive therapy for solid organ transplant patients is another major risk factor (Neofytos *et al*., 2010).

The current incidence of cryptococcal meningitis is estimated to be approximately 220,000 cases per year among HIV infected patients (Rajasingham *et al*., 2017) and possibly higher overall, leading to almost 150,000-200,000 deaths annually (Limper *et al.*, 2017). The incidence rate is down from the ~1 million cases of fungal meningitis and over 600,000 deaths from cryptococcal meningitis estimated to have occurred in 2006 (Park *et al.*, 2009). This recent decrease may seem contrary to the disproportionately high number of HIV-associated *Cryptococcus* infections, and deaths, in regions like sub-Saharan Africa;

nevertheless, the reasons for both of these effects are related. The general fate of HIV infected patients in developed, industrialized nations dramatically improved during the 1990's with the introduction of Highly Active Anti-Retroviral Therapy (HAART); the mortality rate was significantly reduced and the incidence of various HIV-associated opportunistic infections like cryptococcosis has been decreasing since in wealthier countries (Kaplan *et al*., 2000; Mirza *et al*., 2003; Jahnke *et al*., 1999). However, in parts of the world (and even in parts of the U.S.) where this therapy is still not affordable, HIV-associated opportunistic infection still poses an immense threat (McQuiston and Williamson, 2012; Kaplan *et al*., 2000; Mirza *et al*., 2003). AIDS-related cryptococcosis cases and deaths due to cryptococcal meningitis are much more prevalent in developing nations because the HIV pandemic rate is significantly higher, and/or access to proper medical treatment and therapeutic measures, including antifungal medications, is limited or entirely lacking, in these countries (Warnock, 2006; Park *et al*., 2009; Pyrgos *et al*., 2013). The majority of cryptococcal meningitis cases are found in low-income and middle-income countries; almost 73% of the cases up until 2014 have been reported as occurring in sub-Saharan Africa (Rajasingham *et al.*, 2017). Similarly, the highest rates of death due to cryptococcal meningitis were and still are observed in sub-Saharan Africa (Figure 1.1), to a great extent because of the relatively high number of untreated HIV/AIDS patients there (Mitchell and Perfect, 1995; Park *et al.*, 2009; Rajasingham *et al.*, 2017). Hence, in the United States, AIDS patients face a mortality rate of 15-20%, whereas the rate is 55-70% in Latin America and Sub-

Saharan Africa (Brown *et al*., 2012). This indicates that greater patient access to HAART in developing nations may reduce the prevalence of cryptococcosis, fungal meningitis, and associated death, just as it has in developed ones. However, even in countries where HAART has reduced the mortality in HIV-infected patients, cryptococcosis remains a major cause of invasive fungal infection in non-HIV patients who are immunocompromised or immunosuppressed due to therapies they are receiving in the form of monoclonal antibodies, corticosteroids, or other immunosuppressive agents, and cryptococcosis can even occur in immunocompetent non-HIV patients (Snydman *et al*., 2008; Wu *et al.*, 2009; McQuiston and Williamson, 2012).

Cryptococcosis is the third most common invasive fungal infection in sold organ transplant recipients after infections by *Candida* and *Aspergillus* species (Neofytos *et al*., 2010), and, in the U.S. alone, 20-60% of invasive fungal infections in non-HIV-infected patients have been reported to be cryptococcosis (Vilchez *et al*., 2002). With the increasing application of immunosuppressive therapeutic regimens, cryptococcosis and other fungal infections are suspected to rise proportionally as the leading symptomatic diseases in non-HIV-infected patients (Aratani *et al*., 2006). Furthermore, amongst the most common diseases prevalent in sub-Saharan Africa excluding HIV/AIDS, cryptococcal infection is the fourth leading cause of death (Figure 1.2; Park *et al.*, 2009), and it is estimated that the mortality rate of infections caused by pathogenic fungi such as *C. neoformans*, *C. albicans*, and *A. fumigatus* is actually much higher than that of malaria and

tuberculosis (Denning and Bromley., 2015; Meyer *et al*., 2016). Therefore, it is of great importance to better understand the metabolism and virulence-related pathways of *C. neoformans* in order to develop drugs that can kill this pathogen.

Ecological niche of *Cryptococcus*

Outside of the human host, *C. neoformans* is found in very diverse ecological niches, depending on the local environment. The first strain of *C. neoformans* was isolated in 1894 by Sanfelice from fruit juice (Emmons, 1951). Moreover, the presence or absence of such ecological habitats can affect the distribution and virulence of *C. neoformans* in any given geographic region. *C. neoformans* is commonly found in decaying material in the hollows of at least eighteen different tree species around the world, including eucalyptus, as well as in avian excreta, particularly the waste of feral pigeons (Chowdhary *et al.*, 2012; Randhawa *et al*., 2003; Reimão *et al*., 2007). Pigeons only develop a latent infection, not an active one, as their higher body temperature is not suitable for fungal growth, but they act as good vectors, since their excrement provides the required nutrients for *C. neoformans var. grubii* cells to survive until the cells can enter the human host as basidiospores through inhalation (Littman and Borok, 1968; Hiremath *et al*., 2008). Furthermore, since *C. neoformans* can survive in its saprophyte form in excreta, it can be found on any surface that comes into contact with bird droppings, including contaminated soil, fruits, and vegetables, as well as inside homes in regions of the world where birds can enter through open windows; this is particularly dangerous for HIV seropositive patients in such regions, since

they can be regularly re-infected in their own homes (Jarvis and Harrison, 2007; Chowdhary *et al.*, 2012; Swinne *et al*., 1989, 1994). *Cryptococcus* species can reside inside soil nematodes and free-living amoebae and use them as replicative hosts; both *C. neoformans* and *C. gattii* are capable of doing this in the laboratory (May *et al*., 2016). However, prevalence of *C. neoformans* is more widespread than *C. gatti* in terms of host tree species that it was isolated from (17 host tree species for *C. neoformans* in contrast to 12 for *C. gatti*) (Chowdhary *et al.*, 2012), which results in wide arboreal distribution for *C. neoformans* and therefore increased chance of host pathogen interaction

Mechanism of infection and host response

Within the human host, *Cryptococcus neoformans* can gain access to the respiratory system by inhalation of spores or desiccated airborne yeast cells from the environment (Taylor-Smith, 2017). Encapsulated fungal cells often measure around 5-10 μm, and are therefore susceptible to removal from lung epithelia by mucociliary clearance (Okagaki and Nielsen, 2012). However, basidiospores or desiccated cells of *C. neoformans* isolated from soil or from bird droppings measure around 0.6-3 μm, which is a sufficiently small size for alveolar deposition following inhalation (Levitz, 1991; Lin and Heitman, 2006), possibly via the mucociliary movement itself (Sabiiti and May, 2012). Within the lungs, there are four possible outcomes to the infection (Sabiiti and May, 2012). The two less harmful outcomes are that the fungi can be controlled and cleared by the host immune system or that the infection can become latent and remain in the lungs

symptom-free (Sabiiti and May, 2012; Jarvis and Harrison, 2007; Goldman *et al*., 1994). The third outcome is that the latent infection can revive in the lungs upon weakening of the immune system, such as due to HIV infection or pharmacological immune suppression, leading to more serious outcomes (Goldman *et al*., 1994; Garcia-Hermoso *et al*., 1999; Lindell *et al*., 2006; Jarvis and Harrison, 2007) and cause lung inflammation and pulmonary disease. The fourth outcome and the most dangerous is dissemination of *Cryptococcus* from pulmonary site to other organs such as the urinary tract, the prostate gland, the skin, bones, the liver, the spleen, lymph nodes, and, especially, the brain (Liu *et al*., 2009; Jarvis and Harrison, 2007; Hernandez, 1989; Sabesin *et al*., 1963). In fact, the organs most commonly affected when *C. neoformans* infects a host are the lungs and the brain, and, in immunocompromised patients, not only can lung infection lead to pneumonia, but infection of the blood (fungemia) which often leads to the fatal meningoencephalitis (Goldman *et al*., 1994; Chrétien *et al*., 2002; Sabiiti and May, 2012).

In immunocompetent individuals, there are a number of stages of the immune response to infection. When *C. neoformans* cells enter the alveoli of the lungs, alveolar macrophages attempt to phagocytose the fungal cells and either kill them or sequester them in granulomas (Rohatgi and Pirofski, 2015; Vecchiarelli *et al*., 1994a; Mitchell and Friedman, 1972; McQuiston and Williamson, 2012; Sabiiti and May, 2012). If a macrophage is successful in engulfment, the fungal cell is sequestered inside the phagolysosome, an organelle formed by the fusion of a phagosome and a lysosome to produce a local environment of low pH,

hydrolytic enzymes, antimicrobial peptides, and toxic free radicals that the macrophage utilizes to try to destroy the pathogen (Hampton *et al*., 1998; Vieira *et al*., 2002; Cox *et al*., 2003; Tohyama *et al.,* 1996; Ma and May, 2009). Free radicals, specifically reactive oxygen species (ROS) and reactive nitrogen species (RNS), are capable of damaging the fungal cell wall and cell membrane and may attack the DNA and cellular proteins as well (Bergamini *et al*., 2004; Dedon and Tannenbaum, 2004; Upadhya *et al*., 2013), while the hydrolytic enzymes break apart pathogen proteins into peptides. Optimally, the pathogen is destroyed and its peptides are presented by the macrophage via the Major Histocompatibility Complex (MHC) cell surface receptors to activate T-cells, and the macrophage is also activated to release cytokines that attract neutrophils and other immune cells (Dong *et al*., 1997; Del Poeta, 2004). In particular, $CD4^+$ helper T cells are activated upon binding of their T cell receptors to the cryptococcal peptides, and these helper T cells, in turn, release cytokines that regulate the attack upon the fungal pathogen (Campbell *et al*., 2008). One subset of these helper T cells are Th1 cells, and overexpression of Th1 cytokines like tumor necrosis factor α (TNFα) and interferon γ (IFNγ) has been shown to effectively control the fungal burden by increasing the inflammatory and immune response (Kawakami *et al*., 1995; Wormley *et al*., 2007). Once macrophages and helper T cells recruit the rest of the immune system to the site of infection, the cell mediated immune response against *C. neoformans* is mostly driven by interleukin 2 (IL-2) activated $CD8^+$ cytotoxic T cells and Natural Killer (NK) cells, which can detect and destroy the pathogen in an MHC dependent

or independent manner (Levitz *et al*., 1994). Natural killer cells and cytotoxic T cells secrete various proteins, including granulysin, perforin, and granzymes, which induce cryptococcal lysis and permeabilization and also force infected host cells to undergo apoptosis (Ernst *et al*., 2000; Voskoboinik *et al*., 2006).

Cryptococcal response to immune attack and dissemination

C. neoformans is able to survive in the lungs and disseminate to other organs because of its ability to evade, counteract, or circumvent the immune response. For example, the pathogen exploits the fact that, after activating T helper cells, macrophages have essentially opposite responses to feedback regulation imposed on them by the cytokines secreted by Th1 versus Th2 T helper subsets (Voelz *et al.,* 2009). This opposing feedback regulation is the reason for the almost direct correlation, in HIV infected patients, between cryptococcal virulence and the degree to which the release of Th1 "pro-inflammatory" cytokines is compromised, and for the similar correlation observed between virulence and the increased release of "anti-inflammatory" cytokines by Th2 cells in transplant recipients given immunosuppressive drugs to counter transplant rejection (Voelz and May, 2010). Th1 cytokines recruit and activate macrophages and neutrophils and make them release microbicidal oxidative and nitrosative bursts (Shoham and Levitz, 2005; Voelz *et al*., 2009). In contrast, macrophages activated by Th2 cytokines have a lower expulsion probability, and allow a higher intracellular proliferation of the fungal pathogen, compared with those activated by Th1 cytokines (Voelz *et al*., 2009). *C. neoformans* inhibits the Th1 response by expressing eicosanoids

(Noverr *et al*., 2003), uses components of its polysaccharide-based capsule to decrease the release of Th1 pro-inflammatory cytokines while increasing the release of anti-inflammatory IL-10 from Th2 cells (Shoham and Levitz, 2005), and shifts the Th1-Th2 balance more towards Th2 cells (Voelz and May, 2010), thereby manipulating the host immune system to its own advantage.

C. neoformans utilizes a number of mechanisms to ensure that it can remain and survive in the host. Immediately after entry into the alveoli of the lungs, *C. neoformans* can secrete Phospholipase B, an enzyme that breaks down dipalmitoyl phosphatidylcholine (DPPC), the major lipid present in the natural, tension-reducing surfactant coating the epithelial lining, thus allowing the fungal cells to attach directly to lung epithelial cells (Ganendren *et al.*, 2006). *C. neoformans* can grow into extremely large, polyploid cells, called titan cells, that have ten-fold the diameter of normal *C. neoformans in vivo* via pheromone signaling following mating between opposite mating types (a or α) and therefore resist phagocytosis, as well as block the phagocytosis of nearby normal-sized fungal cells (Okagaki *et al*., 2010; Zaragoza *et al*., 2010; Okagaki and Nielsen, 2012; May *et al.*, 2016). Just like these titan cells, cryptococci that enlarge their capsules after they reach the lungs resist being engulfed by macrophages (Levitz and Tabuni, 1991; Xie *et al*., 2012; May *et al.*, 2016). This permits the cryptococcal cells to pass into the bloodstream and thereby enter the systemic circulation, causing fungemia, via direct internalization by the lung epithelial cells to which they had attached (Sabiiti and May, 2012; May *et al.*, 2016). The polysaccharide

capsule also prevents phagocytosis by macrophages in other ways, including by interaction with the surfactant in the host lung. Inside the lungs of immunocompetent individuals, opsonization of pathogenic microorganisms by surfactant proteins A and D (SPA and SPD) normally promote engulfment by alveolar macrophages, but SPA has no effect on phagocytosis of *C. neoformans* (Geunes-Boyer *et al.*, 2012; Schelenz *et al*., 1995; Giles *et al*., 2007). Similarly, *C. neoformans* cells either use the capsule components glucuronoxylomannan (GXM) and mannoprotein 1 (MP1) to prevent opsonization by SPD, or, if they lack an effective capsule, can actually use the SPD coating to protect themselves from the ROS and RNS mediated chemical attack after engulfment by macrophages (Geunes-Boyer *et al.*, 2012; Schelenz *et al*., 1995; Levitz and Tabuni, 1991; Van de Wetering *et al*., 2004).

If phagocytosed, *C. neoformans* can use multiple virulence factors, including but not limited to the capsule and its components, to survive inside and to parasitize macrophages, thereby giving rise to a dormant infection (Feldmesser *et al*., 2001). Upon subsequent immunosuppression of the host, latent cryptococci in the granulomas initially produced by macrophages to protect the host, or the cryptococci surviving inside parasitized macrophages, are reactivated and can enter the systemic circulation by leaving the macrophages through vomocytosis, a nonlytic exocytosis (Nicola *et al*., 2011; Alvarez and Casadevall, 2006). Worse, *C. neoformans* cells that have parasitized macrophages can use the macrophages

as "Trojan Horse" vehicles to travel to other parts of the body, effectively hidden from the immune system (Charlier *et al*., 2009; Santiago-Tirado *et al*., 2017).

As mentioned above, among the various tissues and organs to which *C. neoformans* can disseminate, the most notable are those of the central nervous system (CNS), especially the brain. The CNS is both an immune-privileged site, allowing *C. neoformans* cells that have traveled there to evade immune surveillance, and is a highly sterile environment, eliminating competition from other pathogens (May *et al*., 2016). Normally, the blood-brain barrier (BBB) prevents pathogens from reaching the brain, but *Cryptococcus neoformans* has evolved methods to traverse the BBB and reside in the meningeal tissues surrounding the brain or in the neurons of the brain itself (Charlier *et al*., 2005). There are three different proposed mechanisms for dissemination of *C. neoformans* to the CNS (Figure 1.3). First, the fungal cells can cross the BBB by forcing their way between the tight junctions of the vascular endothelial cells in a process known as paracytosis (Chen *et al*., 2003; Vu *et al*., 2013; May *et al*., 2016). In the second method, called transcytosis, *C. neoformans* uses hyaluronic acid on its surface to bind to CD44 on the luminal endothelium, leading to a protein kinase C-dependent actin remodeling activity in the endothelial cells that drives these cells to engulf the cryptococcal cells. The fungal cells then exit out the other side, thereby crossing the BBB by going through the endothelial cells (Chang *et al*., 2004; Jong *et al*., 2008; May *et al*., 2016). The third mechanism is known as the ‘Trojan Horse’ hypothesis, in which cryptococcal cells cross the BBB by hitchhiking within host

phagocytes (such as macrophages), which are then able to breach the BBB using enzymes the same way that they normally cut through extracellular matrix or tight junctions to reach a site of infection (Sorrell *et al*., 2016; May *et al.*, 2016; Santiago-Tirado *et al*., 2017).

The physiological changes that *C. neoformans* goes through after it has traversed the BBB into the CNS are not very well understood, but it is known that fungal cells found in the cerebrospinal fluid (CSF) are metabolically very active and upregulate various genes involved in core metabolic processes and stress responses (Chen *et al.*, 2014). The transcriptional profile approximately matches that of cryptococci grown in nutrient-rich media (Chen *et al.*, 2014), which suggests that CSF provides many of the essential nutrients that are required for the growth and activity of *C. neoformans*. In fact, the pathogen can, depending on the location to which it has disseminated, upregulate a wide variety of metabolic pathways at the site of the infection, including those involved in energy production, protein synthesis and degradation, as well as upregulate genes involved in stress responses, transport of small molecules, and signaling pathways, even under low nutrient and hypoxic conditions (Steen *et al*., 2003; Kronstad *et al*., 2012). This metabolically active state of *C. neoformans* and its responsiveness to the environmental conditions is likely one of the causes of its survival and virulence.

This responsiveness to local environmental conditions may be just as important as the ability to counteract the immune response in the survival and virulence of the pathogen. *C. neoformans* faces a number of environmental

challenges inside the human host, including a temperature that is higher than the optimal growth temperature of *Cryptococcus* species and low oxygen conditions found in many cells and tissues of the human body. *C. neoformans* grows optimally at atmospheric oxygen concentration (~21%), and its growth is significantly reduced at lower oxygen concentrations (Odds *et al*., 1995; Ingavale *et al*., 2008). Oxygen concentrations in the human body can range widely between 1% and 15%, with a normal oxygen concentration of ~14% in the lungs, ~5.3% in the venous blood, and only 1-5% in the brain (Erecinska and Silver, 2001; Carlsson *et al.*, 2001; Studer *et al.,* 2000). In order for *C. neoformans* to invade the CNS after inhalation into the lungs, reach the brain, and cause meningitis, it must be able to cope with the significantly lower concentrations of oxygen in the brain (Ingavale *et al*., 2008). Furthermore, oxygen levels in host tissues can be further reduced by thrombosis, inflammation and necrosis associated with the infection (Chun *et al*., 2007; Nau and Brück, 2002; Sawyer *et al.,* 1991), thus posing a challenge for the survival of the pathogen.

Virulence factors

Given the number of hurdles that cryptococci must overcome to infect and spread in a body, it is not surprising that many virulence factors help with the pathogenesis of *C. neoformans* by allowing survival and proliferation inside the host, evasion or escape from the host immune system, and exploitation of damage to the host to provide nutrients and survival factors to the pathogen. The virulence of *C. neoformans* is due to a concert of many factors and cannot be attributed to

any single one. However, some of the main virulence factors that contribute to the pathogenicity of *C. neoformans* (and *C. gattii)* are polysaccharide capsule formation, melanin production and deposition along the cell wall, and the ability to grow at 37°C (Coelho *et al.*, 2014; Casadevall *et al*., 2000; Kronstad *et al*., 2011; Ma and May, 2009). The cryptococcal capsule and melanin, together, are protective against phagocytosis and the ROS secreted by immune cells (Wang *et al*., 1995; Mednick *et al*., 2005; Shoham and Levitz, 2005; Rohatgi and Pirofski, 2015).

Several proteases, lipases, and other enzymes, as well as many metabolites produced by *C. neoformans* after infection, also play a role in its virulence. For example, *C. neoformans* Phospholipase B breaks down host cell lipids to allow fungal cell exit from macrophages or for use as fungal nutrients (Cox *et al*., 2001; Chrisman *et al*., 2011). As mentioned above, this enzyme is also used by the fungus to cleave one of the lung surfactant lipids, thereby helping with adherence to alveolar epithelial cells (Ganendren *et al.*, 2006; Sabiiti and May, 2012), and to produce eicosanoids out of macrophage lipids, which are then used to suppress the Th1 immune response (Noverr *et al*., 2003; Sabiiti and May, 2012).

Metabolites produced by the fungus that aid in virulence include the carbohydrate trehalose, which assists in the ability of the fungus to survive at the host body temperature (Chen and Haddad, 2004; Gancedo and Flores, 2004), and the lipid prostaglandin E2, which downregulates host antifungal activity (Valdez *et al*., 2012).

Ion acquisition by the pathogen is assisted by the upregulation of expression of specific fungal genes and their encoded proteins during infection (e.g. copper or dual copper/iron acquisition is increased via expression of Ctr4 or Cft1/Cfo proteins, respectively), to facilitate survival (Jung *et al*., 2009; Ding *et al*., 2013). Homeostasis of iron and copper is critical for virulence, including survival in the high-copper environment of the lungs and low copper environment of the brain, or for dissemination to the brain, and mutation or deletion of many of the genes involved in iron or copper transport, redox state, and homeostasis, including CFT1, CFT2, FRE2, FRE4, and CUF1 reduces virulence (Jung *et al*., 2008, 2009; Saikia *et al*., 2014; Waterman *et al*., 2012). Some proteins, like Cft1, Cft2, Cfo1, Ctr1, Ctr4, and Cu-detoxifying metallothionein (CMT) are expressed differently in response to, or used to counteract, temperature, oxidative, and/or osmotic stress or variability in iron and copper levels (Jung *et al*., 2008, 2009; Lee *et al*., 2014; Saikia *et al*., 2014; Zhang *et al*., 2016; Sun *et al*., 2014; Ding *et al*., 2013; Waterman *et al*., 2012).

Polysaccharide capsule

One of the most complex and versatile virulence factors is the fungal capsule. It provides protection from antimicrobial compounds, toxic free radicals, and various stress conditions (Zaragoza *et al*., 2008; Zaragoza, 2011). It has been shown that acapsular mutants of *C. neoformans* are avirulent (Chang and Kwon-Chung, 1994). During inhalation, the fungal spores are generally unencapsulated (Velagapudi *et al.*, 2009) as smaller size helps in passage through the airway, but

capsule size increases dramatically during infection as the spore reaches the alveoli (Bergman, 1965; Cruickshank *et al*., 1973). Furthermore, the location of infection plays an important role in the regulation of capsule biosynthesis. It has been shown that the environment in the lung is a potent inducer of capsule formation and enlargement (Zaragoza *et al*., 2003), while, in the brain, the capsule is smaller than in the lungs, but larger than that of cells outside the host (Rivera *et al*., 1998; Zaragoza *et al*., 2003). A larger capsule, especially combined with a large (titan) cell, blocks phagocytosis of the fungal cells by macrophages in the lungs (Okagaki *et al*., 2010; Zaragoza *et al*., 2010; Okagaki and Nielsen, 2012; May *et al.*, 2016). The ability of the capsule to interfere with phagocytosis has been demonstrated *in vitro*, where there is essentially no phagocytosis of encapsulated *C. neoformans* in the absence of opsonins (Shoham and Levitz, 2005). If phagocytosis does occur, capsule polysaccharides are released into vesicles around the phagosome (or phagolysosome) inside the engulfing macrophages, and accumulation of these vesicles in the cytoplasm of the host cell leads to macrophage dysfunction and lysis (Tucker and Casadevall, 2002). Furthermore, the capsule is also used to counteract attempts by the macrophage to destroy the fungal cell by neutralizing the reactive oxygen and nitrogen species produced by the macrophage to attack the cryptococci (Zaragoza *et al*., 2008). Additionally, capsule polysaccharides act as a acid-base buffer to maintain a pH that is optimal for fungal cell growth but non-optimal for the acid hydrolases used by macrophages to break down pathogen proteins (De Leon-Rodriguez *et al*., 2018).

Cryptococcal capsules and their components have very strong immunomodulatory properties, (Chang *et al.*, 2006; Chiapello *et al.*, 2008) which *C. neoformans* can use to evade or counteract the host immune response, secondary to preventing phagocytosis. The capsule, or polysaccharides shed from it, interfere with the ability of macrophages to present cryptococcal peptides via MHC receptors, including by downregulating the expression of the MHC receptors; they also make the macrophages express Fas ligand (FasL) that binds to Fas receptor on the surface of nearby T cells, thereby inducing the T cells to undergo apoptosis as well as drive the macrophages to undergo apoptosis (Vecchiarelli *et al*., 2003; Monari *et al*., 2005; Villena *et al*., 2008; Siegemund and Alber, 2008; Ben-Abdallah *et al*., 2012). In addition, capsular material is also reported to delay maturation and activation of, and antigen presentation by, human dendritic cells (Vecchiarelli *et al*., 2003). The capsule or its components also function to inhibit the secretion of pro-inflammatory cytokines by the host immune cells, reduce complement activation, inhibit antibody production, and reduce leukocyte migration, thereby conferring a considerable survival advantage to *C. neoformans* within the host (Zaragoza, 2011; Siegemund and Alber, 2008; Villena *et al.*, 2008; Vecchiarelli, 2007; Ellerbroek *et al.*, 2004b, 2004c; Bose *et al*., 2003; Buchanan and Murphy, 1998; Vecchiarelli *et al*., 1994b). Leukocyte migration to the site of infection is impeded in a number of ways. These include interfering with chemokine receptor function and reducing the attraction of leukocytes to chemokines, forcing the leukocytes to shed from their membranes the L-selectins they use to migrate

along the blood vessel endothelial cell lining, increasing IL-10 production (by anti-inflammatory immune cells), and repressing neutrophil expression of C5a and TNF-α receptors (Ellerbroek *et al*., 2004b, 2004c). These processes are all mediated by the abundant polysaccharides shed from the capsule into the host bloodstream (Ellerbroek *et al*., 2004b, 2004c) or via the fully formed capsule.

To form a capsule, *C. neoformans* produces glucuronoxylomannan (GXM), which comprises about 90-95% of the capsule polysaccharides, and galactoxylomannan (GalXM), which makes up another ~5% of the capsule polysaccharides (Rakesh *et al.*, 2008; Vecchiarelli *et al*., 2011). GXM is a large polymer made of repeating units of α-1,3-mannose, with β-D-xylopyranosyl, β-D-glucopyranosyluronic acid, and 6-O-acetyl groups branching off of the mannose sugars (Cherniak *et al*., 1998; Rakesh *et al.*, 2008). These three different mannose modifications vary in number and order within the GXM polymer, leading to the formation of different serotypes with sharp antigenic heterogeneity (McFadden *et al*., 2007). GXM has been found to be associated with lipids of intracellular and extracellular vesicles (Oliveira *et al.*, 2009). The presence of GXM in *C. neoformans* extracellular vesicles suggests that the fungus synthesizes its capsule from the outside of the cell to allow for the cell to grow (Rodrigues *et al*., 2007). The abovementioned induction of apoptosis of nearby T cells via forced upregulation of FasL in macrophages have been attributed to GXM that is either released from inside macrophages or secreted into the blood, from where it binds to Toll-like receptors (e.g. TLR4) on macrophages or other peripheral blood

mononuclear cells to induce the production of FasL (Shoham *et al*., 2001; Monari *et al.*, 2005; Vecchiarelli, 2007). This FasL induction occurs without stimulating the activation of NF-κB or the production of TNFα (Shoham *et al*., 2001; Monari *et al.*, 2005; Vecchiarelli, 2007).

GalXM is an α-1,6-galactan that contains branches of β-1,3-galactose, α-1,4-mannose and α-1,3 mannose chains, and therefore is technically a glucuronoxylomannogalactan (Vaishnav *et al.*, 1998; Heiss *et al*., 2009; Vecchiarelli *et al*., 2011). GalXM is a critically important factor in the suppression of T cell activation and in the induction of the subsequent FasL-mediated apoptosis of those T cells, as well as in the indirect induction of B cell and macrophage apoptosis (Pericolini 2006, 2010; Vecchiarelli *et al*., 2011). Together, these polysaccharides interfere with the activation and proliferation of T cells, thereby reducing the effect of the cell-mediated immune response (Yauch *et al.*, 2006). These capsule polysaccharides are produced under low iron conditions in the presence of CO_2 (Granger *et al*., 1985; Vartivarian *et al*., 1993; Vecchiarelli *et al.*, 2011). Along with GXM and GalXM, β1,4 N-acetylglucosamine molecules similar to those polymerized into the chitin layer between the cell membrane and the cell wall, and several mannoproteins (the latter of which constitute <1% of the capsule components) are also found to be associated with the capsule and help in anchoring the GXM polymers to the fungal cell wall (Ramos *et al.*, 2012; Vecchiarelli *et al.*, 2011; Fonseca *et al.*, 2009; Rodrigues *et al.*, 2008a; Huang *et al.*, 2002; Levitz, 2001; Van Dyke and Wormley, 2018; Erwig and Gow, 2016).

Addition of chitinase to *C. neoformans* cells causes detachment of the capsule from the cell wall (Rodrigues *et al.*, 2008a). Acetate and its metabolism are important in capsule formation, stability, and function (Hu *et al*., 2008), and, once converted into acetyl CoA, it can be used in the formation of the β1,4 N-acetylglucosamine that links the cell wall to the capsule (Hu *et al*., 2008). Additionally, acetate is the source for the acetylation of GXM, and, despite the surprising hypervirulence of *C. neoformans* mutants lacking acetyl groups on their capsule (Janbon *et al*., 2001), this acetylation is required for the GXM-mediated reduction of neutrophil migration to the site of infection (Ellerbroek *et al*., 2004a).

There are several genes that are involved in capsule formation and synthesis. *CAP59* was the first capsule-associated gene to be isolated, and it encodes a transmembrane protein (Chang and Kwon-Chung, 1994, 1998, 1999; Chang *et al.*, 1996) that is required in the process of export of GXM out of the cell and into the capsule (Garcia-Rivera *et al.*, 2004). *CAP64* was the second gene identified in the capsule formation pathway, and deletion of this gene results in an acapsular phenotype (Chang *et al.*, 1996, 1997). *CAP60* and *CAP10* must also be involved in capsule formation, since deletion of these genes results in an acapsular phenotype, although the biochemical function of their products is still elusive (Chang and Kwon-Chung, 1998, 1999). UDP glucuronic acid (UDP-GlcA) is a critical precursor in the biosynthesis of cryptococcal polysaccharides and mutations in the *UGD1* gene, encoding the UDP-glucose dehydrogenase that is necessary to synthesize UDP-GlcA, or in *CAP10*, *CAP59*, *CAP60*, or *CAP64*, lead

to an acapsular phenotype (Moyrand and Janbon, 2004; Griffith *et al.*, 2004). A deficiency in UDP-glucose dehydrogenase leads to temperature sensitivity and loss of thermotolerance, preventing growth at the 37°C host temperature, as well as morphological defects and an overall decreased virulence (Moyrand and Janbon, 2004; Griffith *et al.*, 2004). The production of UDP-GlcA is also important because it can be converted into UDP-xylose; about 20% of GalXM molecules contain xylose, while 40-60% of GXM molecules contain xylose and GlcA (Griffith *et al.*, 2004).

Proteins required for transporting the products of enzymes encoded by CAP genes are as important as the synthesizing enzymes themselves. For example, the UDP-GlcA transporter, Uut1, has a vital role in capsule formation. *C. neoformans* with a reduced ability to transport UDP-GlcA exhibit growth defects and metabolic abnormalities, resulting in greater phagocytosis by macrophages *in vitro* and faster clearance of the infection *in vivo* (Li *et al.*, 2018).

Several other genes and their corresponding proteins play an important but non-essential role in capsule synthesis. For instance, *CAS1* and *CAS3* are required for acetylation of GXM (Janbon *et al.*, 2001; Moyrand and Janbon, 2004). Other genes, including *UXS1*, *UGD1*, *CAS31*, *CAS32*, *CAS33*, *CAS34* and *CAS35,* are important for xylosylation of GXM (Bar-Peled *et al.*, 2001; Moyrand and Janbon, 2004). Computational analysis of the approximately 20 megabase genome of *C. neoformans* revealed 30 new genes that have potential roles in capsule synthesis (Loftus *et al.*, 2005). These include seven members of the

CAP64 capsule associated gene family and a second *CAP10* family of six capsule associated genes (Loftus *et al*., 2005). Capsule formation is controlled largely by the Gα protein-cAMP-PKA signaling cascade (Alspaugh *et al*., 2002; Hicks *et al*., 2004), but is also negatively regulated by the high osmolarity glycerol (HOG) pathway. (Bahn *et al*., 2005). The HOG pathway, also referred to as the *HOG1* pathway based on the major gene of the pathway, has been shown to be involved in the response to external environmental stress, such as high temperature, oxidative stress, or osmotic shock (Bahn *et al*., 2005). Hog1, an upstream MAP kinase in the HOG pathway, may have distinct functional roles depending on the cryptococcal serotype. For example, deletion of *HOG*1 induces the synthesis of capsule and melanin in *C. neoformans var. grubii*, which is serotype A, but not in *C. neoformans var. neoformans,* which is serotype D (Bahn *et al*., 2005).

Melanin production and laccase activity

C. neoformans produces melanin for use as a protective molecule. The exact molecular structure of the cryptococcal melanin pigment is unknown and it has often been said to be 'melanin-like' pigment; regardless, it is a negatively charged, hydrophobic molecule of high molecular weight, which is synthesized from phenolic and/or indolic components via oxidative polymerization (Frases *et al*., 2007; Steenbergen and Casadevall, 2003; Nosanchuk and Casadevall, 2003; Hamilton and Gomez, 2002; Jacobson, 2000). Although melanized cryptococcal cells have been shown to be responsible for the initial human infection, the environmental sources of melanin production prior to infection of a host are still

unclear (Williamson, 1997; McFadden and Casadevall, 2001; Steenbergen and Casadevall 2003; Frases *et al*., 2007; Eisenman *et al*., 2007). During infection, cryptococcal melanin is synthesized from exogenous precursors such as catecholamines, including epinephrine, norepinephrine, L-DOPA, dopamine, and aldehydes, obtained from host cells, but not from endogenous chemicals like amino acids (Williamson, 1997; McFadden and Casadevall, 2001; Steenbergen and Casadevall 2003; Frases *et al*., 2007; Eisenman *et al*., 2007).

Melanin (or the cryptococcal melanin-like pigment) has the properties of a stable free-radical that allow it to function as an efficient antioxidant (Jacobson and Emery, 1991a; Jacobson and Tinnell, 1993; Steenbergen and Casadevall 2003) and also provides protection against both cold and heat, helping *C. neoformans* to survive at the host body temperature (Nosanchuk and Casadevall, 2003). Melanin can also induce complex immunomodulatory effects that increase the virulence of *C. neoformans* by eliciting changes in the host cytokine/chemokine response to infection (Mednick *et al.*, 2005; Huffnagle *et al.*, 1995). Additionally, melanized cryptococcal cells are less susceptible to the action of amphotericin B (Van Duin *et al.*, 2002), an anti-fungal agent.

C. neoformans melanin or melanin-like pigments are produced by enzymes known as laccases, members of the multicopper oxidase family of proteins, using copper as the co-factor (Walton *et al*., 2005). These laccase enzymes are important, since laccase-deficient mutants of *C. neoformans* with a corresponding melanin deficiency have reduced virulence (Kwon-Chung *et al.*, 1982; Rhodes *et*

al., 1982). In *C. neoformans*, melanin is produced using two laccase enzymes, Lac1 and Lac2, which are spatially separated. Lac1 is abundantly associated with the cell wall, whereas Lac2 is present in the cytoplasm (Zhu *et al*., 2001; Waterman *et al*., 2007). Although it has been suggested that Lac1 plays the predominant role in infection, the genes for the two enzymes share 75% identity, and both isoforms are required for full virulence (Missall *et al*., 2005; Pukkila-Worley *et al.*, 2005).

Once produced, melanin is deposited in the cell wall, creating an electron dense layer (Solano, 2014) from where the melanin fulfils its antioxidant function, offering protection to the cryptococcal cells from free oxygen and nitrogen radicals and other toxic molecules generated within macrophages to attack the fungal cell wall, membrane and other internal components (Williamson, 1997; Eisenman and Casadevall, 2012; Wang and Casadevall, 1994a, 1994b, 1994c). It is possible that more than one melanin-like pigment is produced, and that insoluble melanin is deposited in granules in the cell wall, while soluble melanin-like pigments are also localized to the cell wall but can diffuse (Nosanchuk and Casadevall, 2003). Regardless, this cell wall deposition is probably achieved by vesicular secretion of melanin outside the cell membrane and even of the laccase enzymes themselves (Rodrigues *et al*., 2008b, Eisenman *et al*., 2009). Both are commonly found in cryptococcal extracellular vesicles (Rodrigues *et al*., 2008b). Such vesicle mediated melanin deposition has also been reported in other fungi like *Candida albicans* (Walker *et al*., 2010).

The laccases have additional roles besides melanin production. For example, by catalyzing the production of prostaglandin E2 from extracellular, macrophage-derived arachidonic acid (Erb-Downward *et al*., 2008), laccases can modulate the immune response and interfere with the control of infection by the host. Prostaglandins have been shown to reduce phagocytosis, lymphocyte proliferation, chemokine production, and Th1 response. (Erb-Downward and Huffnagle, 2007; Noverr *et al* 2002). Moreover, by functioning as an iron oxidase, laccase enzymes can convert Fe(II) to Fe(III), thereby neutralizing the antimicrobial oxidative effect of hydroxyl radicals generated inside the macrophages, and protecting the fungal cells (Liu *et al*., 1999; Casadevall *et al*., 2000). Laccase activity in *Cryptococcus* is induced by metals or metal ions like copper and calcium, and is repressed by the presence of nutrients such as nitrogen and glucose and nitrogen (Zhu and Williamson, 2004; Wang *et al*., 2001; Nyhus and Jacobson, 1999; Jacobson and Compton., 1996; Zhu *et al* 2003). Copper regulates laccase activity not only as a co-factor, but also through the transcription factor encoded by *CUF1*, which drives the expression of *LAC1* (Jiang *et al*., 2009). The presence of iron within the phagolysosome of an engulfing macrophage should be toxic to the fungal cell; instead, it induces the abovementioned iron oxidase function of the laccase, which acts to reduce potentially toxic Fenton reactants and hydroxyl radicals (Liu *et al.*, 1999). Likewise, infected brain tissue has decreased levels of glucose, which should decrease survival by *C. neoformans* inside the neurons, but, instead, the low glucose levels induce laccase expression (*i.e.*, expression of both

LAC1 and *LAC2*) through the protein encoded by *TSP2-1*, facilitating further infection of brain cells by the pathogen (Zhu and Williamson, 2004; Li *et al*., 2012; Pukkila-Worley *et al*., 2005). Fortunately for the host, other metals and biomolecules, as well as elevated host temperatures, can repress laccase activity (Jacobson and Emery, 1991b; Zhu and Williamson, 2004). Thus, while inflammation of the brain is harmful, it may also be the way that the body tries to raise the brain temperature to repress laccase activity, as well as the activity of other enzymes, in meningoencephalitis.

Laccase gene expression is also regulated by signaling pathways. It has been observed that the Gα-cAMP-PKA signaling pathway, initiated by the activation of the Gα protein Gpa1 upon binding of extracellular stimuli like hormones and nutrients to receptors, might be involved in the modulation of laccase expression in *C. neoformans* (Pukkila-Worley *et al*., 2005; Zhu and Williamson, 2004; Alspaugh *et al*., 1997, 2002). Likewise, the *HOG1* pathway negatively regulates melanin production on a serotype-specific basis; serotype A (H99 strain) but not serotype D (strain JEC21) *hog1* mutants exhibit significantly increased melanin production (Bahn *et al*., 2005). Other studies have shown a role for MAP kinase cascade targets Ste12a and Ste12alpha in regulating laccase expression (Chang *et al*., 2000, 2001). Wang *et al*. (2001) have demonstrated a role for two homologues of cyclophilin A, a protein involved in the inflammatory response in humans, in laccase activity, and *C. neoformans* lacking both isoforms of cyclophilin A have reduced melanin levels.

Thermotolerance

C. neoformans is capable of growing at the mammalian host body temperature of 37°C, which gives *C. neoformans* an advantage over other fungi when it comes to infecting mammalian hosts (Steenbergen and Casadevall, 2003; Perfect, 2005). Less than 0.01% of outdoor fungi possess this virulence factor; most soil fungi and even most cryptococcal species lack this ability and are therefore non-pathogenic (Steenbergen and Casadevall, 2003; Perfect, 2005). The importance of thermotolerance as a virulence factor is evidenced by the inability of temperature-sensitive mutants of *C. neoformans* to infect a mammalian host, despite being capable of producing a capsule and melanin (Kwon-Chung *et al.*, 1982; Odom *et al.*, 1997). However, at temperatures of 39°C-40°C, even *C. neoformans* grows poorly, and intracellular vacuolization, aberrant budding, and the formation of pseudohyphal structures indicate that the cells are not healthy at this temperature (Mitchell and Perfect, 1995; Steenbergen and Casadevall, 2003).

Cryptococcal thermotolerance involves a number of cellular pathways. A genome-wide transcriptome analysis of *C. neoformans* cells subjected to two different temperatures (25°C and 37°C) revealed the upregulation of several important genes at the higher temperature (Steen *et al.*, 2002). In particular, differences were observed in the levels of *C. neoformans* mRNAs encoding histones, heat shock proteins, components of the translational machinery, mitochondrial proteins, and stress-response proteins such as superoxide dismutase (Steen *et al.*, 2002). The upregulation of the expression of (the mRNAs

for) heat shock proteins (and chaperones) Hsp60 and Hsp70 was especially significance, as these proteins have been found to be prominent antigens in cryptococcal infection of animals and humans (Steen *et al.*, 2002). Additionally, higher levels of transcripts encoding phenolic metabolism enzymes at 37°C suggest a direct correlation between increased temperature and a greater need for melanin synthesis inside the mammalian host (Steen *et al.*, 2002).

In addition to the observed upregulation of certain genes in response to higher temperature, which may or may not be necessary for virulence, several genes have been demonstrated to be important for the basic survival of *Cryptococcus* at the human physiological temperature (Perfect, 2005). For example, the vacuolar ATPase VPH1, CCN1 (a protein used in DNA replication, transcription and splicing), basic amino acid metabolism genes such as *ILV2*, and signaling pathways genes such as *RAS1*, *CNA1*, *CNB1*, *MPK1* and *CTS1*, are all important in high temperature growth (Perfect, 2005). Disruption of *RAS1* or *CNA1* causes severe deleterious effects on fungal virulence at 37°C, as does disruption of *TPS1* or *SOD2* (Perfect, 2005; Alspaugh *et al*., 2000). It makes sense that *SOD2*, which encodes the *C. neoformans* mitochondrial manganese superoxide dismutase involved in neutralizing ROS (Giles *et al*., 2005a), is necessary to deal with the stress caused by higher temperature. Likewise, the Ras proteins regulate cellular morphogenesis and *RAS1* encodes a *C. neoformans* Ras protein responsible for attachment, mobility, and cytoskeletal integrity at high temperatures (Alspaugh *et al*., 2000). Ras1 fulfills these functions via Cdc42,

expression of which is increased in, and necessary for survival of, cryptococcal cells at 37°C (Ballou *et al*., 2010). *TPS1* and *TPS2* encode trehalose-6-phosphate synthase and trehalose-6 phosphate phosphatase, respectively, and both are necessary to provide the thermotolerance effect of the trehalose sugar metabolite (Perfect, 2005). Trehalose has been reported to play an important role in the survival of several bacteria and fungi under stress conditions (Chen and Haddad, 2004; Gancedo and Flores, 2004), and likely does so for *C. neoformans*. Other genes, such as *STE20α* and *CPA1* (which encodes cyclophilin) are required for survival at very high temperature (39°C to 40°C), even if they are not strictly necessary for growth at 37°C (Perfect, 2005).

Resistance to hypoxia

Since the brain uses a vast amount of ATP energy and is therefore a highly oxidative organ, it consumes a disproportionately large portion of the body's oxygen pool (Erecinska and Silver, 2001). Despite this, there is a highly variable but generally low oxygen concentration and partial pressure in the brain (Erecinska and Silver, 2001), posing a challenge to the survival of *C. neoformans* cells that have disseminated into brain tissue. However, the pathogenic fungus adapts to these conditions and causes infection in both immunocompromised and normal patients (Ingavale *et al*., 2008). In order to adapt to the variable oxygen concentrations in the brain and other tissues that it infects, *C. neoformans* must first be able to sense to the concentration of oxygen. In *Schizosaccharomyces pombe,* cells respond to environmental oxygen concentrations by sensing changes

in sterol levels in cell membranes, using the sterol response element binding protein (SREBP) pathway in which the proteins Sre1 and Scp1 monitor oxygen dependent sterol synthesis (Hughes *et al.*, 2005; Todd *et al*., 2006). Under low sterol conditions, SREBP (Sre1) is cleaved by SREBP cleavage activating protein (SCAP/Scp1), and is thus activated (Hughes *et al.*, 2005). The activated Sre1 facilitates the transcription of genes involved in sterol biosynthesis and uptake (Hughes *et al.*, 2005; Todd *et al*., 2006). In *S. pombe*, the Sre1 protein is also activated in response to low oxygen conditions, and stimulates the expression of genes required for survival under hypoxia (Hughes *et al*., 2005). These genes have orthologues in *C. neoformans* that have been shown to be important for cryptococcal survival under hypoxic conditions; for example, deletion of *SRE1* in *C. neoformans* decreases the growth of the pathogen under low oxygen conditions, both *in vitro* and in an animal model (Chang *et al.*, 2007; Bien *et al.*, 2007). The *in vitro* experiments also established that, like in *S. pombe*, *C. neoformans* Sre1p is activated by hypoxia via cleavage of the precursor protein (Chang *et al.*, 2007; Chun *et al*., 2007). Hypoxia can be mimicked *in vitro* by the use of cobalt chloride, and both Sre1p and Scp1p have been shown to be essential during growth under a cobalt chloride induced pseudo-hypoxic environment (Lee *et al*., 2007). In addition to the Sre1 pathway, Tco1, a member of a highly conserved family of fungal-specific histidine kinases, also plays a role in the resistance to low oxygen conditions (Chun *et al.*, 2007). The *C. neoformans tco1* mutant is sensitive to hypoxia, and a *tco1-sre1* double knock-out strain shows even

more sensitivity to hypoxia than the individual single mutants, verifying that Tco1 functions in a pathway parallel to Sre1 (Chun *et al.*, 2007). Tco1 positively regulates the aforementioned HOG/MAPK pathway, and thereby negatively regulates melanin synthesis (Chun *et al.*, 2007). Besides these pathways, several genes in *C. neoformans* related to sterol, heme, and fatty acid metabolism are upregulated, and pathways involved in translation, vesicle trafficking, and cell wall and capsule synthesis are downregulated, in response to low oxygen conditions (Chun *et al*., 2007).

Response to oxidative and nitrosative stress

Upon entry into the host pulmonary system, *C. neoformans* encounters alveolar macrophages, the first line of host defense. Once the pathogen is phagocytosed by the macrophage, it faces the toxic effects of the ROS and RNS molecules that break down the fungal cell's membrane lipids and proteins, as well as other cellular molecules, including the DNA (Bergamini *et al*., 2004; Dedon and Tannenbaum, 2004; Upadhya *et al*., 2013; Missall *et al.*, 2004a; Hampton *et al.,* 1998; Vieira *et al.,* 2002; Cox *et al.,* 2003; Liu *et al.,* 1999; Tohyama *et al.*, 1996). It has been shown that macrophages produce nitric oxide (NO), a key type of RNS, in response to infection (Tripathi *et al*., 2007; Maffei *et al*., 2004) and that the anti-cryptococcal activity of macrophages is mostly dependent on RNS, and, to a lesser degree, ROS (Cox *et al.,* 2003; Tohyama *et al.*, 1996; Maffei *et al*., 2004). Therefore, it is important for *C. neoformans* to protect itself from nitrosative (and oxidative) damage in order to survive inside the host system. The pathogen also

alters gene expression in response to ROS (Upadhya *et al*., 2013), and genomic and proteomic analyses of *C. neoformans* cells subjected to nitrosative stress revealed an upregulation of proteins involved in the oxidative stress response and downregulation of proteins involved in the osmotic and starvation stress response pathways (Missall *et al.*, 2006),. Additionally, RNS specifically attacks and inactivates proteins that contain iron, iron-sulfur clusters, and copper, or thiol groups, including aconitase and ribonucleotide reductase, as well as cytochrome c oxidase and proteins of complexe I and II of the electron transport chain, which requires the pathogen to mount a specific response to nitrosative stress (Missall *et al*., 2004a). Therefore, it is notable that the enzymes transaldolase (Tal1), aconitase (Aco1) and thioredoxin-dependent thiol peroxidase (Tsa1) were repeatedly found to be altered in their expression levels as well as modified post-translationally in response to NO (Missall *et al.*, 2006). Likewise, *TSA1* and another thiol peroxidase gene, *TSA3*, are transcriptionally induced in *C. neoformans* treated with hydrogen peroxide, a form of ROS (Missall *et al*., 2004b). *C. neoformans tsa1* mutants have reduced growth and virulence (Missall *et al*., 2004b). Similarly, cryptococcal mutants of glutathione reductase (*GLR1*), which is upregulated following nitrosative stress, were found to be avirulent in a mouse infection model and are also specifically sensitive to nitrosative burst (Missall *et al*., 2006). *C. neoformans* also relies on cytochrome C peroxidase (Ccp1), superoxide dismutase (Sod1), and catalase enzymes to defend itself from oxidative stress (Cox *et al*., 2003; Giles *et al*., 2005b, 2006) and the absence of

any one of these enzymes adversely affects the virulence of the pathogen (Narasipura *et al*., 2003). *PKC1*, another important gene involved in resistance to environmental stress that is employed in maintaining cell wall integrity, is also upregulated in wild type *C. neoformans* following oxidative and nitrosative burst (Gerik *et al.*, 2008), while *pkc1* mutants have defects in the production of melanin and capsular polysaccharides important for protecting the pathogen against free radicals and are unable to withstand nitrosative and oxidative stress (Gerik *et al*., 2008).

Degradative enzymes involved in *Cryptococcus* virulence

Phospholipases B and C

Phospholipases are members of a heterogeneous group of hydrolases that break down the ester linkages in glycerophospholipids, which are the principal components of cell membranes and of lung surfactants (Djordjevic, 2010). Thus, fungal phospholipases play a significant role in the process of pulmonary invasion (Djordjevic, 2010). Phagocytosed *C. neoformans* cells secrete phospholipases that can degrade membrane phospholipids in order to lyse the host cell and escape into the bloodstream or brain (Santangelo *et al*., 2004; Maruvada *et al*., 2012). Furthermore, even inside phagocytic cells, phospholipid degradation facilitates damage to phagolysosomal membranes, which allows the engulfed fungus to access the host cytoplasmic components and intracellular nutrients, thereby ensuring fungal survival inside the host cell (Feldmesser *et al*., 2000).

C. neoformans expresses two classes of phospholipases to promote its own virulence, phospholipase B and phospholipase C (Chen *et al*., 1997a; Chrisman *et al.*, 2011). A secreted phospholipase, encoded by *PLB1*, can function as phospholipase B, lysophospholipase, or lysophospholipase transacylase, depending on the substrate (Chen *et al*., 1997a,b; Chrisman *et al.*, 2011). *C. neoformans* is sensitive to the presence of phospholipids, and addition of phospholipids to cryptococcal culture causes significant induction of Plb1 activity (Coelho *et al.*, 2014). Plb1 has been shown to act primarily on dipalmitoyl phosphatidylcholine (DPPC) and phosphatidylglycerol (PG), which are abundant in lung surfactant (Merkel *et al*., 1999; Chen *et al*., 2000). Thus, *C. neoformans* utilizes Plb1 to drive the adhesion of cryptococci to human lung epithelial cells (Ganendren *et al.*, 2006), the initiation of interstitial pulmonary infection, and dissemination from the lung via lymphatic and blood vessels (Santangelo *et al.*, 2004). Plb1 is also essential in the production of eicosanoids, such as prostaglandins and leukotrienes, which are bioactive anti-inflammatory molecules that suppress the host immune system, thereby promoting survival and dissemination of the pathogen within the host (Noverr *et al*., 2002, 2003; Ganendren *et al.,* 2006). The precursor of these eicosanoids, arachidonic acid, is not present within the fungal cell itself; rather, it is suspected that the pathogen appropriates the host cell arachidonic acid and derives the necessary eicosanoids from it (Wright *et al*., 2007). Plb1 also plays a major role in the capsule enlargement of *C. neoformans* in response to macrophage interaction (Chrisman *et al*., 2011)

and in the formation of titan cells (Evans *et al*., 2015). Plb1 is also employed by the fungus to activate the Rac1 GTPase of endothelial cells and thereby alter the actin cytoskeleton of these cells to allow transcytosis and penetration of the blood brain barrier by the pathogen; lack of Plb1 significantly reduces the transcytosis efficiency (Maruvada *et al*., 2012). The importance of this enzyme to the virulence of the pathogen is confirmed by the fact that mice infected with Plb1-deficient mutants of *C. neoformans* survive better than mice infected with the wild type strain, which suggests that Plb1-deficient cryptococci have reduced pathogenicity, including having greater difficulty in invading brain tissue (Cox *et al.*, 2001). Intracellular growth and survival within macrophages is also reduced in *plb1* mutants (Evans *et al*., 2015).

C. neoformans has two phospholipase C enzymes, Plc1 and Plc2 (Chayakulkeeree *et al*., 2008). PLCs act on phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PIP), and phosphatidyl inositol-4,5-bisphosphate (PIP_2), as the substrate (Heinz *et al*., 1998). In fact, in *C. neoformans*, Plc1 acts on PIP_2 to produce inositol-3,4,5-triphosphate (IP_3) for use by enzymes such as the inositol polyphosphate kinase Arg1; a deficiency in either Plc1 or Arg1 impairs thermotolerance, as well as capsule formation and melanin production, and compromises the integrity of the cell wall (Lev *et al*., 2013). The secretion of fungal Plb1 depends on the removal of a glycosylphosphatidylinositol (GPI) anchor (Djordjevic *et al*., 2005; Chayakulkeeree *et al.,*2008), which might be a function of one of these phospholipase C enzymes, especially Plc1. Regardless,

deletion of *plc*1 also produces avirulent cryptococcal cells that are unable to produce melanin or induce nonlytic exocytosis (Chayakulkeeree *et al.,* 2008). In addition to Plc1 and Plc2, a *C. neoformans* inositol phosphorylsphingolipid-phospholipase C (encoded by *ISC1*) protects the pathogen from excessively low pH and from oxidative and nitrosative stresses, conditions found in the phagolysosome (Shea *et al*., 2006).

Urease

Urease is a nickel-dependent enzyme that hydrolyzes urea into ammonia and carbamate, and the degradation of the urea causes alkalization of the surrounding environment, thereby facilitating acquisition of nitrogen (Coelho *et al*., 2014). Urease has been found to be an important virulence factor for bacterial pathogenesis (Eaton *et al*., 1991) and has been postulated to have a major role in cryptococcal pathogenesis. One feature that distinguishes *C. neoformans* from other yeast species in clinical specimens is the detection of urease activity (Canteros *et al*., 1996). In *C. neoformans*, urease is initially an apoenzyme, encoded by *URE1* (Cox *et al*., 2000). Similar to other organisms that use the urease system, a nickel transporter, encoded by *NIC1*, and accessory proteins encoded by the *URE4*, *URE6*, and *URE7* genes, are required for the apoenzyme to become functional (Singh *et al*., 2013). Another gene, *URE2*, is also expressed in *C. neoformans* and appears to be necessary for utilization of urea as a nitrogen source (Varma *et al*., 2006). Urease deficient strains of *C. neoformans* have been isolated from clinical samples only rarely, suggesting the importance of the urease

activity in virulence, and a *ure1* mutant strain was less virulent in both inhalation and intravenous injection mouse infection models (Cox *et al*., 2000); however, the effect of *URE2* deficiency on virulence may depend on the route of infection, since some *ure2* mutants exhibit delayed dissemination from the lung, while others are no different from wild type controls in virulence (Varma *et al.*, 2006). The urease system and its components enhance either passage across the lung epithelium into the bloodstream or passage through the endothelial layer of the blood vessel wall into the brain (Singh *et al*., 2013; Shi *et al*., 2010; Olszewski *et al*., 2004). Furthermore, the pattern of dissemination of urease-deficient *C. neoformans* strains in the brain, spleen, and other organs after intravenous inoculation is different from that of the wild type strain, suggesting that urease enhances the sequestration of the pathogen within microcapillary beds, thereby facilitating invasion into the CNS, but may not actually be required for dissemination to the brain (Olszewski *et al.*, 2004).

Proteinases

Once *C. neoformans* enters the human body via the respiratory pathway, the fungus utilizes enzymes that can degrade proteins and lipids (*i.e.*, proteinases/proteases and lipases, respectively), present in the lung membrane, to penetrate lung parenchyma within a few hours after its entry into the alveolar space (Goldman *et al.*, 1994). Both environmental and clinical isolates of *C. neoformans* have been shown to have proteinase activity (Casadevall and Perfect, 1998), and the first report of the detection of extracellular proteinase activity from

C. neoformans dates back to 1972 (Müller and Sethi, 1972). The action of cryptococcal proteases on host membranes during infection was evident in histopathological sections of *Cryptococcus*-infected tissues from beige mice, in which collagen fibrils were found to be degraded (Salkowski and Balish, 1991). Similarly, Chen *et al*., (1996) confirmed the presence of protease activity capable of degrading collagen fibers, and possibly elastin and fibrinogen proteins, of the extracellular matrix around host cells (Ma and May, 2009). Cryptococcal supernatant containing secreted serine proteinases is also capable of degrading human fibronectin, as well as laminin and type IV collagen (Rodrigues *et al*., 2003). Besides degrading host membranes and extracellular matrix proteins, fungal proteinase activity may also be utilized by the pathogen to compromise host immunity by degrading immunologically important proteins, such as antibodies and complement factors (Chen *et al.*, 1996).

Cytoplasmic and extracellular cryptococcal proteases include at least one membrane-bound aspartyl protease and an elastinolytic-like metalloprotease, as well as multiple serine proteases/proteinases, some of which are embedded in the membrane by GPI anchors (Eigenheer *et al*., 2007; Il Yoo *et al*., 2004; Pinti *et al*., 2007; Rodrigues *et al*., 2003). Metalloprotease deficient strains of *C. neoformans* are incapable of crossing the endothelium of the blood-brain barrier and the Mpr1 metalloprotease is required to invade the CNS (Vu *et al*., 2014), verifying the importance of proteases in cryptococcal pathogenesis. In addition to the

metalloproteases, Cryptococcal serine protease activity also augments the permeability of the blood-brain barrier (Xu *et al.,* 2014).

Metabolites important for *C. neoformans* infection

Besides the virulence factors discussed above, *C. neoformans* utilizes small metabolites to inhibit the immune system of the mammalian host and for other survival purposes. Heat inactivated supernatant of *C. neoformans*, in which proteins are presumably denatured, can induce apoptosis of neutrophils, and characterization of these supernatants from cryptococcal cultures revealed the presence of thirty different metabolites, including amino acids, alditols, nucleosides, acetate, and ethanol (Wright *et al.*, 2002). These findings confirmed the results from a second, earlier study, in which *C. neoformans* grown in culture also secreted a number of these metabolites, including mannitol, glucitol, erythritol, glycerol, derivatives of choline and ethanolamine, γ-aminobutyric acid (GABA, an inhibitory neurotransmitter), nucleosides, and amino acids, and, most of all, ethanol and acetic acid (Bubb *et al*., 1999).

Mannitol

Mannitol is one of the alditol metabolites released in the culture media by *C. neoformans*, and the presence of mannitol correlates with cryptococcal virulence (Wong *et al.*, 1990). As an intracellular product of the pathogen, it is believed to protect *C. neoformans* from heat and osmotic stress and likely helps during infection by scavenging and neutralizing extracellular hydroxyl radicals produced by phagocytes (Chaturvedi *et al.*, 1996a). Mutants of *C. neoformans* that

produce less mannitol are more susceptible to *in vitro* killing by normal neutrophils and ROS, suggesting that mannitol is important for virulence (Chaturvedi *et al.*, 1996b). Addition of mannitol in the medium as a carbon source induces capsular polysaccharide production in *C. neoformans in vitro*, and, *in vivo*, GXM production was found to be regulated by mannitol supplementation in a mice model (Guimarães *et al*., 2010). Moreover, the large amounts of mannitol released by *C. neoformans* following brain infection may increase the osmolarity of the tissue, thereby causing cerebral edema (Chaturvedi *et al.*, 1996a; Wong *et al*., 1990), which benefits the pathogen. Increased mannitol production is thought to have contributed to the raised intracranial pressure that resulted in mortality of cryptococcal meningitis patients (Hoang *et al*., 2004). The presence of mannitol in the CSF of HIV infected patients with cryptococcal meningitis was previously reported to increase intracranial pressure (Megson *et al*., 1996), but this finding has also been disputed (Liappis *et al.,*2008).

Ethanol

Ethanol is another metabolite that has been found to be secreted *in vitro* by *C. neoformans* cultures, at a concentration as high as 0.3% (w/v), into the culture supernatant (Bubb *et al*., 1999; Wright *et al*., 2002). However, this concentration is much higher than the highest amount of ethanol produced (0.064%) by one strain of *C. neoformans* in a previous study (Pappagianis *et al*., 1966), and ethanol levels as low as 0.0004% have been reported in spinal fluid inoculated with cryptococci (Dawson and Taghavy, 1963). Likewise, although an ethanol concentration above

0.79% is detrimental to leukotriene generation and to the subsequent responses of human neutrophils, such as oxidative metabolism, aggregation, elastase release, migration, and chemotaxis (Nilsson *et al.*, 1995), this concentration is higher than the maximum concentration secreted from the *in vitro* culture. Thus, the effect of the ethanol produced and secreted by *C. neoformans* during infection and its significance as a virulence factor is questionable.

Trehalose

Alpha-glucopyranosyl-alpha-D-glucopyranoside, more commonly called trehalose, is a disaccharide produced by several organisms, including plants, fungi, and invertebrates. Trehalose is rapidly produced in these organisms in response to cellular stress to act as a stress protectant (Elbein, 1974; Gancedo and Flores, 2004), and its production in *S. cerevisiae* has been shown to increase resistance to heat (Lewis *et al.*, 1995; De Virgilio *et al.*, 1993; Attfield, 1987) and to dehydration and desiccation (Gadd *et al.*, 1987; Hottiger *et al.*, 1987), mostly by preventing the denaturation of certain proteins (Hottiger *et al.*, 1987). Besides preventing denaturation of protein, trehalose also suppresses the aggregation of already denatured proteins (Singer and Lindquist, 1998). It has also been shown that trehalose can protect the cell membrane from stressful conditions (Crowe *et al.*, 1984). Like other sugars, trehalose can also be utilized by cells as a source of carbon and energy (Francois and Parrou, 2001; Kane and Roth, 1974; Lillie and Pringle; 1980; Thevelein and Hohmann, 1995).

The phosphorylated trehalose sugar (trehalose-6-phosphate, T6P) has been shown to regulate glycolytic flux by regulating hexokinase II in some fungi (Blázquez *et al.*, 1993). Studies conducted in *S. cerevisiae* have revealed that trehalose synthesis is dependent on two enzymes, T6P synthase (Tps1) and T6P phosphatase (Tps2) (Bell *et al*., 1992; Vuorio *et al*., 1993), while unutilized trehalose is hydrolyzed by a neutral trehalase encoded by NTH1 (Kopp *et al.*, 1993).

Expression of the homologous genes involved in the trehalose synthesis pathway has been detected in *C. neoformans* by transcriptional analysis of the CSF in an experimental model of cryptococcal meningitis utilizing infected rabbits (Steen *et al.*, 2003), indicating that, like *S. cerevisiae*, *C. neoformans* produces trehalose *in vivo.* Similarly, nuclear magnetic resonance (NMR) studies of tissues surrounding cryptococcomas, large-mass lesions resulting from infection, in infected rat brain and lungs, revealed a high abundance of trehalose (Himmelreich *et al*., 2001, 2003).

Trehalose has been shown to be important for *C. neoformans* virulence. In a murine infection model, a trehalose synthesis-deficient *C. neoformans tps1* mutant was far less lethal, allowing the mice to survive for over 60 days after infection, than the H99 wild type strain that killed infected mice by day 16 (Petzold *et al.*, 2006). In contrast, mutations to trehalose degrading enzyme (Nth1) did not have much of an effect on the virulence of the pathogen in this model (Petzold *et al.*, 2006). However, a recent study by Botts *et al*. (2014) demonstrated that *NTH1*

deletion results in defective germination and sexual development of cryptococcal spores. Interestingly, deletion of a previously unrecognized and only recently identified gene for trehalase, *NTH2*, results in hypervirulent fungal strains (Botts *et al*., 2014), possibly indicating that accumulation of trehalose (as a result of a degradation defect) provides for greater survival and infectivity of *C. neoformans*.

Acetate

Acetate is one of the major metabolites secreted by cryptococci cultured *in vitro* (Bubb *et al.*, 1999). Using NMR, substantial amounts of acetate have been detected from pulmonary cryptococcomas (Himmelreich *et al.*, 2001). Acetate is believed to provide a survival advantage to the pathogen by two mechanisms, the first of which is through its effect on pH. An excess of acetate in the infected tissue presumably decreases the pH in cryptococcomas (Wright *et al.*, 2002), thereby facilitating the activity of phospholipase B at the sites of cryptococcal infection (Evans *et al*., 2015; Sharon *et al*., 2000). Reduced pH in the environment around *C. neoformans* can also shield the pathogen from an immune attack, by either reducing the migration of neutrophils or increasing their necrosis (Hu *et al.*, 2008; Wright *et al*., 2002). Lowering of pH also increases neutralization of free radicals and specifically decreases the formation of superoxide (Wright *et al*., 2002), thus limiting the ability of immune cells to use such chemicals to kill infecting cryptococci.

Four eukaryotic acetate production pathways in protists

Acetate metabolism has been studied for many years in bacteria, but has received less attention in eukaryotic microbes even though acetate is a major metabolic end product (Wright *et al*., 2002; Bubb *et al.*, 1999). Four different pathways for the production of acetate from acetyl-CoA have been identified in eukaryotic microbes (Tielens *et al*., 2010). In the first pathway, ADP-forming acetyl-CoA synthetase is used in acetate production by amitochondriate protists (like *Entamoeba histolytica* and *Giardia* sp.) and some species of Archaea (Reeves *et al*., 1977; Mazet *et al*., 2013). Kinetoplastids and *Trichomonas* use the second pathway, in which acetate:succinate-CoA transferase is employed as the primary enzyme for acetate production (Mazet *et al*., 2013). The third pathway is the phosphotransacetylase-acetate kinase (Pta-Ack) pathway, which is required for interconversion of acetate and acetyl-CoA, and is found in green algae (such as *Chlamydomonas*) and *Phytophthora* (Atteia *et al*., 2006; Ingram-Smith *et al*., 2006). In *Chlamydomonas*, two parallel Pta-Ack pathways have been identified, and upregulation of the mRNAs encoding the enzymes of these pathways (Ack1, Ack2, Pat1, and Pat2, with "Pat" used instead of "Pta" in *Chlamydomonas*) under dark, anaerobic conditions correlates with the production and excretion of acetate under these conditions (Yang *et al.,* 2014; Mus *et al.,* 2007). Based on both proteomics analyses (Atteia *et al.,* 2006; Terashima *et al.,* 2010) and localization studies, the enzymes of one of the two *Chlamydomonas* pathways, Pat1 and Ack2, are localized to the mitochondria, while those of the other pathway, Pat2 and Ack1,

are localized to the chloroplasts (Yang *et al*., 2014). Null *ack1* and *pat2* mutants are highly sensitive to anoxia (Yang *et al*., 2014), possibly indicating the importance of the Pta-Ack pathway to the production of ATP under fermentation conditions. In fact, the Ack-Pta pathway has been shown to be critical for *Chlamydomonas* survival under anoxia, although some acetate was still produced under anoxia in an *ack1-ack2* double mutant, suggesting that other pathway(s) may also be used to produce acetate and ATP in *Chlamydomonas* (Yang *et al*., 2014). In the fourth pathway, acetyl-CoA hydrolase is employed in peroxisomal acetate production, including in animals and in fungi like *S. cerevisiae* (Lee *et al*., 1990; Tielens *et al*., 2010).

Acetate production pathways in fungi

In addition to the above peroxisomal acetate production pathway present in yeast, three cytoplasmic or mitochondrial acetate production pathways have been identified in fungi. The pathways employ 1) acetyl-CoA synthetase, 2) xylulose 5-phosphate/fructose 6-phosphate phosphoketolase and acetate kinase, or 3) pyruvate decarboxylase and acetaldehyde dehydrogenase.

Acetyl-CoA synthetase

Acetyl-CoA synthetase (Acs) has generally been considered to operate solely in the direction of acetyl-CoA formation, so enzymatic studies have largely ignored the enzymatic reaction in the acetate-forming direction. Acetylation of a conserved lysine in Acs enzymes from bacteria to mammals blocks acetyl-CoA formation during the first step of the reaction (Starai *et al*., 2002, Starai and

Escalante-Semerena, 2004; Gardner *et al*., 2006), and this acetylation is presumed to be a deliberate regulatory mechanism by which the enzymatic activity is directed. In *Aspergillus nidulans*, acetylation of the enzyme influences the directionality of the enzymatic reaction, such that unacetylated Acs works to synthesize acetyl-CoA, whereas acetylation of the enzyme favors synthesis of acetate (Takasaki *et al.*, 2004). This acetylation of Acs occurs under anaerobic growth conditions in which the fungus both ferments ammonia and substantially increases acetate production (Takasaki *et al.*, 2004). The site of acetylation has not yet been not identified, so whether this acetylation is at the conserved lysine residue discussed above or not is unknown.

Xylulose 5-phosphate/fructose 6-phosphate phosphoketolase-acetate kinase pathway

However, similar to other fungal species, and some bacterial species, *C. neoformans* may utilize alternate sources of acetate, apart from acetyl-CoA, and therefore alternative pathways. In lactic acid bacteria and in species of the genus *Bifidobacterium*, acetate kinase can make acetate from the acetyl phosphate produced by xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (Xfp1/Xfp2) out of either xylulose-5-phosphate or fructose-6-phosphate (Meile *et al.*, 2001), and homologous enzymes are present in *C. neoformans* (Ingram-Smith *et al*., 2006). Although it is missing in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, an open reading frame (ORF) encoding Ack is present in euascomycete and basidiomycete fungi, (Ingram-Smith *et al*., 2006;

Papini *et al*., 2012). However, these fungi lack an ORF encoding Pta and instead express one or two *XFP* genes (*XPF1/2*), encoding xylulose 5-phosphate phosphoketolase to convert xylulose 5-phosphate to acetyl phosphate and glyceraldehyde 3-phosphate and/or fructose 6-phosphate phosphoketolase to convert fructose 6-phosphate to acetyl phosphate and erythrose 4-phosphate (Jeffries, 1983; Ingram-Smith *et al*., 2006). This pathway appears to function in a manner similar to the modified pentose phosphate pathway (called the pentose phosphoketolase pathway) used by heterofermentative bacteria, as well as lactic acid bacteria and bifidobacteria, to produce ethanol, acetate, and lactate (Jeffries, 1983; Meile *et al*., 2001; Kleijn *et al*., 2005; Papini *et al*., 2012). Therefore, in euascomycete and basidiomycete fungi, instead of partnering with Pta, Ack processes the acetyl-phosphate product of Xfp1/2 to acetate (Meile *et al*., 2001; Ingram-Smith *et al*., 2006), making ATP in the process. The existence of this pathway is supported by the fact that all fungi with an open reading frame encoding an acetate kinase also have at least one open reading frame encoding an Xfp enzyme, and in some cases, such as in *C. neoformans*, there are two, phylogenetically distinct Xfps, designated as Xfp1 and Xfp2 (Ingram-Smith *et al*., 2006). In fact, in *Aspergillus nidulans*, which has a single gene encoding an Xfp, the Xfp-Ack pathway is known to function in central carbon metabolism (Panagiotou *et al.,*2008), although Xfp is designated Phk in *A. nidulans*. Over-expression of *PHK* increased growth on xylose, glycerol, and ethanol as carbon sources (Panagiotou *et al.,*2008). Similarly, *Aspergillus niger* has genes encoding

both Xfp1 and Xfp2, as well as encoding Ack (Poulsen *et al*., 2012). Likewise, the fungus *Metarhizium anisopliae*, an insect pathogen, has two *XFP* genes, encoding Xfp1 and Xfp2, and *xfp2* mutants have reduced virulence (Duan *et al*., 2009).

The *S. pombe* Xfp has not been specifically characterized, but the phosphoketolase has been recognized in proteomics analyses (Beltrao et al., 2009; Gunaratne *et al*., 2013; Meyer *et al*., 2014). Analysis of the *S. pombe* response to environmental stresses, such as oxidative and osmotic stresses, heat, alkylation, and heavy metal toxicity, found that the expression of mRNA encoding fructose-6-phosphate phosphoketolase (Xfp1) is increased as part of the core stress response (Chen *et al*., 2003α), and the level of this mRNA doubles during cell entry into the G0 quiescent state in response to limited nitrogen availability, relative to during normal growth (Marguerat *et al.,*2012). This indicates that the *S. pombe XFP1* may serve an important role in the stress response; however, an *S. pombe XFP1* deletion mutant is able to grow at temperatures between 25-32°C in YES medium enriched with glucose (Kim *et al*., 2010; Hayles *et al*., 2013), so Xfp1 may not be necessary for growth under ideal circumstances. When fused via its C-terminus to yellow fluorescent protein (YFP), *S. pombe* Xfp1 was found in both the nucleus and cytosol (Matsuyama *et al.,*2006). Additionally, it is phosphorylated at Ser^{454} (Beltrao *et al*., 2009), an amino acid that is conserved in other fungal Xfp1 proteins but that is altered to glycine in both bacterial and fungal Xfp2 proteins, indicating that Xpf1 proteins may need to be phosphorylated to function, but Xfp2 proteins may not (Kerry Smith, Ph.D., personal communication).

As mentioned above, there are two ORFs for Xfp enzymes, *XFP1* and *XFP2* in *C. neoformans* (Ingram-Smith *et al*., 2006). However, only Xfp2 has been biochemically characterized, and it has been found that the enzyme prefers xylulose-5-phosphate over fructose-6-phosphate as a substrate (Glenn *et al*., 2014). *C. neoformans* Xfp2 utilizes divalent cation in its function, preferably Mg^{2+}, but potentially Ca^{2+}, Co^{2+}, Mn^{2+}, and Ni^{2+} instead (Glenn *et al*., 2014). It functions optimally in the 37-40°C temperature range (Glenn *et al*., 2014), indicating that it might be used after infection of a mammalian host. It requires a pH range of 4.5 to 6.0 for optimal function, with a maximal activity at pH 5.5, and does not function well at a pH of 7.0 or above (Glenn *et al*., 2014). *C. neoformans* Xfp2 is subject to allosteric inhibition by ATP, phosphoenolpyruvate (PEP), and oxaloacetate (OAA), as well as positive allosteric regulation by AMP, positive cooperativity when bound at the active site by phosphoketose substrates, and negative cooperativity when bound at the active site by inorganic phosphate (Glenn *et al*., 2014). *C. neoformans* also expresses an Ack enzyme, for which the crystal structure has been determined (Thaker *et al*., 2013).

Pyruvate decarboxylase – acetaldehyde dehydrogenase pathway

In *S. cerevisiae*, the primary biosynthetic pathway for cytosolic acetate production during growth on glucose is the pyruvate dehydrogenase bypass, which utilizes pyruvate decarboxylase (Saint-Prix *et al*., 2004; Remize *et al.*, 2000; Pronk *et al*., 1996). Through this pathway, pyruvate is decarboxylated to form acetaldehyde, which is oxidized to acetate by acetaldehyde dehydrogenase in the

presence of $NADP^+$ (Saint-Prix *et al*., 2004; Remize *et al.*, 2000; Pronk *et al*., 1996). Acetate produced by this pathway is then used for producing acetyl-CoA (Saint-Prix *et al*., 2004; Remize *et al.*, 2000). Again, the homologues for these enzymes are present in *C. neoformans* (Hu *et al*., 2008). However, there are a number of *C. neoformans* aldehyde dehydrogenases, and which one participates in acetate production is not known. Therefore, a better understand of the function of each of the *C. neoformans* aldehyde dehydrogenases would advance the overall understanding of *C. neoformans* metabolism. Analysis of the *C. neoformans* pyruvate decarboxylase, including by comparison to the *S. cerevisiae* homologue, and determination of whether the *C. neoformans* pyruvate decarboxylase and acetaldehyde dehydrogenase function in a single pathway, would also inform possible treatment of cryptococcosis.

Pyruvate decarboxylase (PDC)

Pyruvate decarboxylase, a tetrameric enzyme that requires Mg^{2+} and thiamine pyrophosphate as cofactors, catalyzes the decarboxylation of pyruvate to acetaldehyde and CO_2 (Lohmann and Schuster, 1937; Schellenberger, 1967; Pronk *et al*., 1996). In *S. cerevisiae*, Pdc is a key player in the acetate production pathway (Saint-Prix *et al*., 2004; Remize *et al*., 2000) as the Xfp-Ack pathway is absent and is likely to be important for acetate production in *C. neoformans* (Hu *et al*., 2008). It might therefore be an important target for therapeutic reduction of fungal virulence in the treatment of cryptococcal meningitis.

Pyruvate decarboxylase is a branch point in the choice of energy derivation pathway used in *Saccharomyces* (Agarwal *et al.*, 2013; Møller *et al.*, 2004), so an overview of Pdc deficient mutants and of structural and regulatory *PDC* genes in *S. cerevisiae* is called for and yields important information. Schmitt and Zimmermann (1982) isolated several *S. cerevisiae* point mutants with reduced Pdc activity and categorized them into two complementation groups, PDC1 and PDC2. The *pdc-1* mutants have growth defects and are semi-dominant, *i.e.* the diploid organism produced by mating with the wild type strain has reduced pyruvate decarboxylase activity, but not to the extent of the haploid mutant (Schmitt and Zimmermann, 1982). One of these mutants, *pdc1-8,* completely fails to grow on glucose-containing medium (Schmitt and Zimmermann, 1982), indicating that *S. cerevisiae* requires pyruvate decarboxylase activity for normal growth. In contrast, the *pdc-2* mutants have reduced enzyme activity as a recessive phenotype; diploid strains produced by mating with the wild type strain have normal growth (Schmitt and Zimmermann, 1982). The *pdc-1, pdc-2* double mutants do not show any pyruvate decarboxylase activity and are unable to ferment glucose (Schmitt and Zimmermann, 1982).

Since the work of Schmitt and Zimmermann (1982), it has been discovered that there are actually six Pdc-encoding genes, *PDC1* through *PDC6*, in *Saccharomyces* species (Seeboth *et al.*, 1990; Kaiser *et al.*, 1999; Agarwal *et al.*, 2013). However, under normal growth conditions, the enzyme encoded by the *PDC1* gene is the main contributor of pyruvate decarboxylase activity in wild type

strains of fermenting yeast cells (Schmitt and Zimmermann, 1982; Schmitt *et al.*, 1983; Kellermann *et al.*, 1986; Hohmann and Cederberg, 1990; Hohmann 1991a; Hohmann, 1993). Mutation of the *PDC1* gene causes decreased growth because of over accumulation of pyruvate, which can slow down the production of energy by glucose breakdown in *S. cerevisiae* (Schmitt and Zimmermann, 1982). For example, the *pdc1-8* point mutant exhibits excessive secretion of pyruvate and a redox imbalance in the cytosol (Van Maris *et al.*, 2004) and, even though pyruvate decarboxylase is normally connected with a fermentation pathway, this *S. cerevisiae* mutant is unable to use glucose to grow under either respiratory or fermentative conditions (Schmitt *et al.*, 1983).

Surprisingly, unlike with the abovementioned point mutants, a complete deletion mutant of the *PDC1* gene (*pdc1*^{0}) generated by Seeboth *et al*. (1990) has no effect on the growth of *S. cerevisiae* on glucose-containing medium, since 60-70% of pyruvate decarboxylase activity is still present. This remaining activity is the result of upregulation of expression of the *PDC5* gene, which is 88% identical to *PDC1*, in response to the complete absence of *PDC1* in these *S. cerevisiae* mutants (Seeboth *et al.*, 1990; Agarwal *et al.*, 2013; De Assis *et al.*, 2013). It appears that Pdc5 is expressed at low levels when functional Pdc1 is present in wild type cells, and is expressed at a much higher level in null or deletion mutants than in point mutants of the *PDC1* gene, suggesting that expression of PDC genes is autoregulated in *S. cerevisiae* based on the level of pyruvate decarboxylase activity already present in the cell (De Assis *et al.*, 2013; Muller *et al.*, 1999;

Eberhardt *et al.*, 1999; Hohmann and Cederberg, 1990; Schaaff *et al.*, 1989; Kaiser *et al.*, 1999). However, even if Pdc5 and/or the other pyruvate decarboxylases are expressed in *pdc1* null mutants, the rate of the reaction they catalyze appears to be one-fourth that of Pdc1, therefore allowing the level of pyruvate in the *pdc1* null mutant to accumulate to over twice that of the wild type strain; yet, the rate of reaction appears to be high enough to produce enough acetaldehyde (and ethanol) to match the relatively low levels present in the wild type strain (Remize *et al.,* 2000). Because of this (or due to the production of acetate via another pathway) the acetate concentration in the *pdc1* null mutant is about the same as that of the wild type strain (Remize *et al.,* 2000).

Insertional mutagenesis of both *PDC1* and *PDC5* genes results in double-mutant *S. cerevisiae* strains with essentially no detectable pyruvate decarboxylase activity, and a triple mutant that is also knocked out for *PDC6* cannot grow for very long using glucose as the energy source (Flikweert *et al.*, 1996). The *S. cerevisiae PDC6* gene is weakly expressed and the function of the corresponding protein is not clear (Hohmann, 1991b; Kaiser *et al.*, 1999), but sulfur limiting conditions and high sugar stress have been shown to induce ScPdc6 expression (Erasmus *et al.*, 2003; Fauchon *et al.,*2002). In summary, under normal growth conditions, Pdc1p appears to be the major pyruvate decarboxylase in *S. cerevisiae* and, when it cannot be expressed, the yeast prefers to use Pdc5p instead of Pdc6p unless the cells are under certain types of stress (De Assis *et al.*, 2013; Muller *et al.*, 1999;

Eberhardt *et al.*, 1999; Hohmann and Cederberg, 1990; Schaaff *et al.*, 1989; Kaiser *et al.*, 1999).

Both PDC1 and PDC5 mRNA levels and the activity levels of the corresponding enzymes are dependent on the carbon source; low levels of PDC1 and PDC5 mRNA are observed when *S. cerevisiae* is provided with ethanol as the carbon source, while the addition of glucose leads to rapid increase in the level of PDC1 mRNA (Hohmann and Cederberg, 1990; Schmitt *et al.*, 1983). This is because of ethanol-repressed elements in the promoters of the *PDC1* and *PDC5* genes that suppress the expression of the genes in the presence of ethanol (Liesen *et al.*, 1996). Thus, while Pdc1 activity is regulated by glucose concentrations (Schmitt and Zimmermann, 1982), the transcription of *PDC1* is regulated based on the presence or absence of ethanol (Liesen *et al.*, 1996).

The regulation of transcription of *PDC1* and *PDC5* and the level of Pdc1p and/or Pdc5p enzyme activity also depends on the product of the *PDC2* gene, which has an important but indirect role in pyruvate metabolism as a regulatory protein in *S. cerevisiae* (Hohmann, 1993; Kaiser *et al.*, 1999). Transcription of the *PDC5* gene, and high-level transcription of the *PDC1* gene, requires the presence of Pdc2p, a transcriptional activator that is strongly expressed in fermenting yeast, and Pdc2p may be involved in the *PDC1*/*PDC5* autoregulation in *S. cerevisiae* (Hohmann, 1993; Kaiser *et al.*, 1999). A *PDC2* deletion mutant and, to a lesser extent, a point mutant of the gene, both demonstrate significantly reduced pyruvate decarboxylase activity, and exhibit slow, oxygen-dependent growth on glucose

and accumulation of pyruvate, as expected of *S. cerevisiae* mutants that are unable to use fermentation and the pyruvate decarboxylase-dependent pyruvate dehydrogenase by-pass pathway (Hohmann, 1993). In short, although *PDC2* does not encode an enzyme, Pdc2p is still important for pyruvate decarboxylase enzyme activity, and mutants with reduced Pdc2p levels are unable to grow on glucose because of their inability to properly express the *PDC1* and *PDC5* genes (Velmurugan *et al.*, 1997).

PDC3 was identified by Wright *et al.*, (1989) as the affected gene in the *pdc1-30* mutant that complemented both, and was therefore not allelic to either, the *pdc1* and *pdc2* mutant groups identified by Schmitt and Zimmermann (1982). Like *PDC2*, *PDC3* also encodes a regulatory subunit, based on the finding that, unlike PDC1 mRNA, the level of PDC3 mRNA does not change between glycolytic and gluconeogenic growth, indicating that *PDC3* is not a structural gene (Wright *et al.*, 1989). Similarly, *PDC4* also appears to encode a regulatory protein (Seeboth *et al.*, 1990). Thus, while *S. cerevisiae* has six *PDC* genes, Pdc2p, Pdc3p, and Pdc4p regulate the expression and/or activity of Pdc1p, Pdc5p, and Pdc6p, the three pyruvate decarboxylase enzymes.

Unlike *S. cerevisiae*, there is only one known *PDC* gene in *C. neoformans*, which was found to be expressed abundantly at both 25°C and 37°C, *i.e.* room and mammalian body temperatures (Steen *et al*., 2002). This implies that there may be a role for this enzyme in the thermotolerance. A transcriptomic analysis of *C. neoformans* isolated from the lungs of infected mice showed an elevated

expression of the *PDC* transcript, along with the transcripts of other genes putatively involved in acetate formation (Hu *et al*., 2008). Pdc was identified among the proteins present in the extracellular vesicles produced by *C. neoformans* (Rodrigues *et al*., 2008b).

Aldehyde dehydrogenases (ALDs)

Organic compounds like aldehydes are present ubiquitously in nature; even low level accumulation of many of these aldehydes causes toxicity and thus, it is important to regulate their levels in the cells (Perozich *et al.*, 1999). The aldehyde dehydrogenase (Aldh) superfamily constitutes a class of enzymes that catalyzes the oxidation of aldehydes to their corresponding carboxylic acids, which are less toxic to the cells (Perozich *et al.*, 1999). This superfamily is highly conserved throughout Eubacteria, Archaea, and Eukarya, which suggests an important role of this enzyme throughout evolutionary history (Perozich *et al.*, 1999). In eukaryotes, there are close to twenty families of aldehyde dehydrogenase (Aldh or Ald) enzymes (Sophos and Vasiliou, 2003). In recent years, a great deal more information regarding gene and protein sequences of a large number *ALDH* genes (also referred to as *ALD* genes) has been made available than has been previously analyzed. For example, in 2002 alone, 555 *ALD* gene sequences were reported, including 32 sequences from Archaea, 351 from Eubacteria, and 172 from Eukarya (Sophos and Vasiliou, 2003).

The large variety of Aldhs take part in a wide range of different physiological processes and exhibit specificity for various substrates. Some Aldhs act on only a

few specific substrates, while others have a broader specificity, yet most Aldhs require the presence of NAD^+ or $NADP^+$ as a cofactor in order to function (Lindahl, 1992; Yoshida *et al.*, 1998). Although most Aldh family members protect the cell by keeping in check the levels of harmful aldehydes, they also demonstrate enzymatic functionality beyond detoxification. For example, Aldhs are important for the synthesis of important biomolecules like retinoic acid, betaine, and folate (Marchitti *et al.*, 2008; Vasiliou *et al.*, 2000; Sobreira *et al.*, 2011). Aldhs also modulate cell proliferation, differentiation, and cell survival, mainly by participating in retinoic acid synthesis (Marchitti *et al.*, 2008). Some of the members of the superfamily also exhibit roles independent of their enzymatic activity, including acting as crystallins and absorbing ultraviolet irradiation in the cornea and binding to hormones and other small molecules including androgens, cholesterol, thyroid hormone, and acetaminophen (Pereira *et al.*, 1991; Marchitti *et al.*, 2008; Estey *et al.*, 2007). ALDH activity is important for cellular function, and enzyme deficiency in humans leads to diseases and medical conditions such as cataracts (*ALDH1A1*, *ALDH3A1*, *ALDH18A1*), seizures (*ALDH7A1*), hyperprolinaemia (*ALDH4A1*), heart disease (*ALDH2*), alcohol sensitivity (*ALDH1A1, ALDH1B1, ALDH2*) and some cancers (*ALDH2*) (Marchitti *et al.*, 2008).

Aldehyde dehydrogenase in fungi

Fungi are continually exposed to different stresses during their growth; in particular, the metabolic products ethanol and acetaldehyde can accumulate to toxic levels (Ingram and Buttke, 1985; Jones, 1990). In order to convert these and

other aldehyde toxic by-products to less harmful chemicals, yeast employ dedicated enzymatic pathways that involve one or more of the aldehyde dehydrogenases (Marchitti *et al*., 2008; Vasiliou and Nebert, 2005; Black *et al*., 2009; Sládek, 2003). For example, via pathways utilizing acetaldehyde dehydrogenase, acetaldehyde derived from pyruvate, ethanol, or other sources is oxidized to form acetate (Pritchard and Kell, 2002; Saint-Prix *et al*., 2004). Just like *C. neoformans*, *S. cerevisiae* also has multiple aldehyde dehydrogenases, but, in the latter species, the role of each of the enzymes in a number of both distinct and overlapping metabolic pathways on a number of aldehyde substrates, including acetaldehyde, has been thoroughly investigated (Aranda and del Olmo, 2003; Saint-Prix, *et al.,* 2004). In *S. cerevisiae*, Ald6p, Ald2p, and Ald3p are cytosolic aldehyde dehydrogenases, while Ald4p and Ald5p are mitochondrial (Aranda and del Olmo, 2003; Meaden *et al.,* 1997; Saint-Prix *et al.,* 2004). The cytosolic enzymes are activated by Mg^{2+}, use $NADP^{+}$ as a coenzyme, and are expressed independent of the level of glucose (Aranda and del Olmo, 2003; Meaden *et al*., 1997). In contrast, the mitochondrial enzymes are activated by K^{+} and thiols and use NAD^{+} or $NADP^{+}$ as coenzymes, and their expression is suppressed in the presence of glucose (Aranda and del Olmo, 2003). Functionally, the cytosolic enzymes form acetate (from acetaldehyde) as a final product of anaerobic glucose metabolism, whereas the mitochondrial enzymes are employed when *S. cerevisiae* needs to utilize ethanol as the carbon source (Aranda and del Olmo, 2003). Nevertheless, the mitochondrial enzymes can play an important role in

acetate production in *S. cerevisiae*; studies on aldehyde dehydrogenase mutants suggest that cytosolic and mitochondrial enzymes can compensate for each other (Aranda and del Olmo, 2003).

Similarly, the *S. cerevisiae* enzyme encoded by *ALD6*, a homologue of at least one Ald in *C. neoformans*, plays an important role in the formation of acetate, as demonstrated by Meaden *et al*. (1997), and Ald6p may function as the major cytosolic acetaldehyde dehydrogenase. This is supported by Remize *et al.* (2000), who found that *S. cerevisiae* cells lacking Ald6p display abnormal growth and reduced acetate production when undergoing fermentation. In agreement with these other two groups, Saint-Prix *et al.* (2004) analyzed the growth and acetate production of several single-deletion and multiple-deletion *ald* mutants in two *S. cerevisiae* strain backgrounds, and found that both Ald6p and Ald5p play an important role in acetate production during fermentation. Deletion of either *ALD5* or *ALD6* reduces acetate production significantly during anaerobic growth (Saint-Prix *et al*., 2004). Although this result was in contrast to a study by Wang *et al*., (1998), Saint-Prix *et al*., (2004) concluded that Ald5p is required for full acetate production, based on a decrease in acetate production by 22-26% in yeast deleted for *ALD5*, and based on the observation that the levels of ALD5 mRNA are upregulated in the exponential growth phase and during fermentation (Saint-Prix *et al*., 2004).

Another *S. cerevisiae* aldehyde dehydrogenase, Ald4p, localized in the mitochondria, is not considered to be primarily involved in acetate formation

(Aranda and del Olmo, 2003; Meaden *et al.,* 1997; Saint-Prix *et al.,* 2004; Remize *et al.,* 2000). *ALD4* expression starts only in the late exponential phase of fermentation, and deletion of *ALD4* does not affect the acetate levels in at least two *S. cerevisiae* strain backgrounds (Saint-Prix *et al*., 2004). However, it is possible that the activity of Ald4p is dependent on the level of glucose present and on genetic background (Saint-Prix *et al*., 2004). Furthermore, it appears that Ald4p plays a minor role in acetaldehyde metabolism and is partly able to compensate for Ald6p, since the *ald6*, *ald4* double mutant has a reduced growth rate, and exhibits reduced acetate production, relative to either single mutant (Saint-Prix *et al*., 2004; Remize *et al.,* 2000). Furthermore, the *ald6* mutant yeast appears to use the partial redundancy between Ald4p and Ald6p to its advantage; Ald4p levels, and even ALD4 mRNA levels, are increased in this mutant, and *ALD4* expression begins earlier than in wild type yeast, to allow the mutant to survive by compensating for the loss of Ald6p with increased levels and possible accumulation of Ald4p (Saint-Prix *et al*., 2004).

Similar to the limited acetaldehyde dehydrogenase function of the mitochondrial Ald4p, and unlike the confirmed role of cytosolic Ald6p, whether the other two cytosolic aldehyde dehydrogenases, Ald2p and Ald3p, are actually acetaldehyde dehydrogenases is uncertain, and the evidence indicates that they are not involved in acetate production in *S. cerevisiae* (Navarro-Aviño *et al*., 1999; Saint-Prix *et al*., 2004). Double mutants of the two genes have lower growth rates on ethanol (Navarro-Aviño *et al*., 1999), but Ald2p and Ald3p may function primarily

in redox metabolism under stress, particularly under osmotic stress but also in glucose depletion, heat shock, and oxidative stress (Navarro-Aviño *et al*., 1999; Blomberg and Adler, 1989). In fact, Saint-Prix *et al*. (2004) suggests that Ald2p and Ald3p are not involved in the acetate production pathway at all, since the *ald2*, *ald3*, *ald4*, *ald6* quadruple mutant phenotype is no different than that of the *ald4*, *ald6* double mutant, and a mutant that is null for *ALD2*, *ALD3*, *ALD4*, *ALD5*, and *ALD6* has a similar phenotype to that of the *ald4*, *ald5*, *ald6* triple mutant.

Given the number and possible functional overlap of aldehyde dehydrogenases in *S. cerevisiae*, the regulation of expression of each of the genes is also important. Although which of the specific *S. cerevisiae ALD* genes is expressed under various growth conditions, and which of the corresponding enzymes are active, can depend on both environmental conditions and genetic background, one environmental condition of interest is the ethanol and acetaldehyde stress faced by *S. cerevisiae* specifically during the fermentation and biological ageing of wine (Aranda and del Olmo, 2003). In order to determine the effect of these stress conditions on aldehyde dehydrogenase activity, Aranda and del Olmo (2003) studied the differential *ALD* gene expression and the corresponding enzymatic activities in fermentative and flor yeast strains, the latter of which are industrial strains responsible for the biological aging of sherry wines, when these two types of yeast were grown in media with and without acetaldehyde. Previously, Aranda *et al*. (2002) had demonstrated that aldehyde stress can modulate transcription of heat shock protein genes, which may, in turn, regulate

the stability of other proteins. Aranda and del Olmo (2003) determined that acetaldehyde stress can also affect the expression of aldehyde dehydrogenase genes directly, although the effect of aldehyde stress on the transcription of *ALD* genes is also determined by the genetic background of the yeast strain, the carbon source available for yeast growth, and the gene itself. In particular, when acetaldehyde is added to glucose-rich media, yeast generally decrease expression of *ALD6*. However, if yeast are grown in ethanol-rich media, acetaldehyde stress increases ALD6 mRNA levels well above that of the normally low *ALD6* expression in ethanol without acetaldehyde (Aranda and del Olmo, 2003). Conversely, in flor yeast grown in glucose media, supplementation with acetaldehyde increases ALD2 and ALD3 mRNA levels from the normally low level of expression in glucose media only, while the mRNA levels decrease in ethanol upon addition of acetaldehyde (Aranda and del Olmo, 2003). Likewise, *ALD2* and *ALD3* expression is increased with growth in ethanol versus with growth in glucose, while *ALD6* expression is the opposite (Aranda and del Olmo, 2003). Thus, the expression patterns of *ALD2* and *ALD3* are essentially inverted to those of *ALD6*, even though all three genes encode cytosolic aldehyde dehydrogenases, possibly indicative of the relative roles of the corresponding enzymes in the acetate production (or acetaldehyde degradation) pathway. Similar functional compensation between aldehyde dehydrogenases, such as between Ald6p, Ald5p, and, under certain conditions, Ald4p, in *S. cerevisiae*, as well as expression regulation of the

corresponding genes, may also apply to the *C. neoformans* aldehyde dehydrogenases.

Aldehyde dehydrogenase pathways are of significance in *C. neoformans* since acetate production is tightly correlated with the virulence of this pathogen (Hu *et al*., 2008). *ALD* transcripts were found to be upregulated in the fungus during the onset of pulmonary infection in mice and cryptococcal meningitis in rabbits (Hu *et al*., 2008; Steen et al., 2003). *ALD* gene expression, in particular the expression of *ALD5*, was also increased in *C. neoformans* (*var grubii*) following engulfment by macrophages (Derengowski *et al*., 2013), even though expression of Pdc, which produces acetaldehyde, was decreased (Derengowski *et al*., 2013). Thus, elucidation of the function of the various aldehyde dehydrogenases, and of which ones, if any, work with *C. neoformans* pyruvate dehydrogenase to produce acetate, may reveal whether or not these enzymes can be targeted in order to treat potentially lethal cryptococcal meningitis.

Summary and Study Objective

Cryptococcus neoformans, the most frequent cause of fungal meningitis, is a huge threat to immunocompromised patients (Loftus *et al*., 2005; Sorrell, 2001; Litvintseva *et al*., 2011; Chayakulkeeree and Perfect, 2008; Park *et al*., 2009; Rajasingham *et al*., 2017; Limper *et al*., 2017; Brown *et al*., 2012). The pathogen enters the respiratory system upon inhalation and the spores can pass through the lungs to the alveoli to establish a successful pulmonary infection (Taylor-Smith, 2017; Sabiiti and May, 2012). From there it can disseminate to other organs like

the brain, and can cause meningoencephalitis or other lethal conditions, especially if combined with immunosuppression such as occurs in HIV-infected patients (Liu *et al*., 2009; Jarvis and Harrison, 2007; Hernandez, 1989; Sabesin *et al*., 1963; Goldman *et al*., 1994; Chrétien *et al*., 2002; Sabiiti and May, 2012; Park *et al.*, 2009; Rajasingham *et al*., 2017; Limper *et al.*, 2017). The death toll is larger in parts of the world where efficient medical treatment is not available or where the HIV pandemic rate is higher (Mitchell and Perfect, 1995; Park *et al.*, 2009; Rajasingham *al.*, 2017; Brown *et al*., 2012; McQuiston and Williamson, 2012; Kaplan *et al*., 2000; Mirza *et al*., 2003; Warnock, 2006; Pyrgos *et al*., 2013). Therefore, *C. neoformans* infection is a serious issue regarding the health of immunocompromised individuals in a large part of the world.

Once within the host, *C. neoformans* can counter attack by multiple components the cell mediated immune system; it can even survive within engulfing macrophages after being phagocytosed and manipulate the macrophages or the other aspects of cellular immunity to spread throughout the host (Sabiiti and May, 2012; Noverr *et al*., 2003; Shoham and Levitz, 2005; Voelz and May, 2010; Okagaki *et al*., 2010; Zaragoza *et al*., 2010; Okagaki and Nielsen, 2012; May *et al.*, 2016; Levitz and Tabuni, 1991; Xie *et al*., 2012; Geunes-Boyer *et al.*, 2012; Schelenz *et al*., 1995; Van de Wetering *et al*., 2004; Feldmesser *et al*., 2001; Nicola *et al*., 2011; Alvarez and Casadevall, 2006; Charlier *et al*., 2005, 2009). Such robust survival is mostly attributed to various virulence factors, including the ability to grow at human physiological temperature, the production of capsule and

melanin, resistance to hypoxia and to oxidative and nitrosative stress, and the use of many degradative enzymes (Coelho *et al.*, 2014; Casadevall *et al*., 2000; Kronstad *et al*., 2011; Ma and May, 2009; Cox *et al*., 2001, 2003; Chrisman *et al*., 2011; Ganendren *et al.*, 2006; Sabiiti and May, 2012; Noverr *et al*., 2002, 2003; Ingavale *et al*., 2008; Missall *et al.*, 2004a, 2004b, 2006; Giles *et al*., 2005b, 2006; Santangelo *et al*., 2004; Maruvada *et al*., 2012; Feldmesser *et al*., 2000; Chen *et al*., 1997a,b; Chayakulkeeree *et al*., 2008; Goldman *et al.*, 1994). The pathogen also produces a variety of metabolites that confer survival advantages, as well as create a suitable microenvironment for the fungi, such as mannitol, trehalose, ethanol, and acetate (Bubb *et al.*, 1999; Wright *et al*., 2002; Chen and Haddad, 2004; Gancedo and Flores, 2004; Perfect, 2005; Wong *et al.*, 1990; Chaturvedi *et al.*, 1996a; Guimarães *et al*., 2010; Steen *et al*., 2003; Himmelreich *et al*., 2001, 2003; Hu *et al.*, 2008).

As a metabolite, acetate could play a crucial role in the survival of *C. neoformans* under anaerobic conditions, including the potential use as a source of carbon and/or energy in a glucose deficient environment (Price *et al*., 2011; Hu *et al.*, 2008; Kronstad *et al*., 2012). Acetate can also be used by *Cryptococcus* to change the local environmental pH to increase the efficiency of fungal enzymes that act as virulence factors while decreasing the effectiveness of the immune response to infection, as well as to neutralize reactive oxygen and nitrogen species used by the immune system to destroy the pathogen (Hu *et al.*, 2008; Wright *et al.*, 2002). This might explain the findings that acetate is one of the major metabolites

secreted by *C. neoformans*, both when this pathogen is grown as an *in vitro* culture (Bubb *et al.*, 1999) and when the cryptococci are localized in pulmonary cryptococcomas following infection (Himmelreich *et al.*, 2001). Two possible pathways have been identified for the production of acetate in *Cryptococcus*: the Xfp1/2 – Ack pathway that produces acetate from D-xylulose 5-phosphate or D-fructose 6-phosphate and the Pdc-Ald pathway to produce acetate from pyruvate. Surprisingly, relatively little attention has been given to uncovering the precise metabolic pathway for the production of acetate and to revealing its role in the virulence of this pathogen. Given that it might be possible to treat cryptococcal meningitis or general cryptococcal infection by inhibiting the corresponding enzymes in order to save lives, it would be especially unfortunate this avenue remains unexplored. Therefore, even gaining a better understanding of the enzymes in one or more of the putative acetate production pathways could be highly useful.

In *S. cerevisiae* which lacks the Xfp-Ack pathway (Ingram-Smith *et al.*, 2006), the pathway for acetate synthesis is the pyruvate dehydrogenase bypass (Saint-Prix *et al.*, 2004; Remize *et al.*, 2000), which appears to depend on either cytosolic or mitochondrial acetaldehyde dehydrogenases. The enzyme encoded by *ALD6* is the primary cytosolic aldehyde dehydrogenase in *S. cerevisiae*, and mutants of this gene produce reduced levels of acetate and exhibit growth defects (Remize *et al.*, 2000; Saint-Prix *et al.*, 2004). The mitochondrial aldehyde dehydrogenase encoded by *ALD5* is upregulated in *S. cerevisiae* during

fermentation, and *ald5* mutants produce reduced acetate levels (Saint-Prix *et al.*, 2004). Similarly, *C. neoformans PDC* and *ALD* genes, especially *ALD5*, are upregulated in the fungal cells recovered from the lungs of infected mice (Hu *et al.*, 2008). Together, these findings support a pathway involving the use of Ald and Pdc enzymes in the production of acetate by *C. neoformans* that is highly advantageous, if not absolutely necessary, for the growth of the pathogenic fungus inside a human host. The possible contribution of Ald and Pdc enzymes in the survival, growth, and virulence of *C. neoformans* under physiological conditions was investigated, and the functional family of *C. neoformans* aldehyde dehydrogenases was studied, as described herein.

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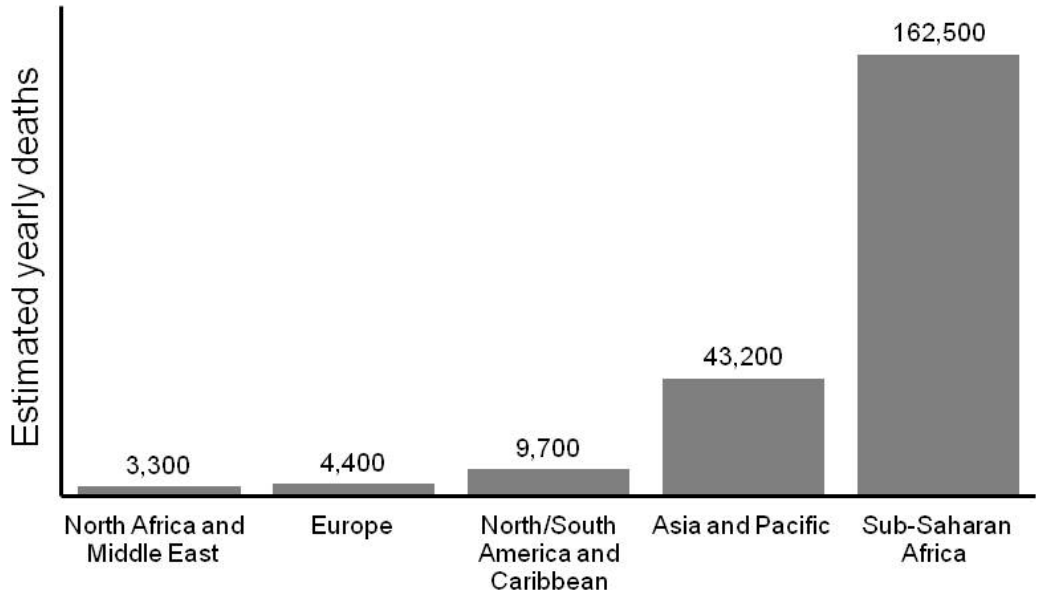

Figure 1.1: Global burden of HIV-related cryptococcal meningitis in 2017. The estimated yearly cases of meningitis caused by cryptococcal infection in HIV patients in various geographical regions across the world are shown. Sub-Saharan Africa has the highest number of occurrences per year. Data adapted from Rajasingham *et al*., Lancet Infectious Diseases 2017.

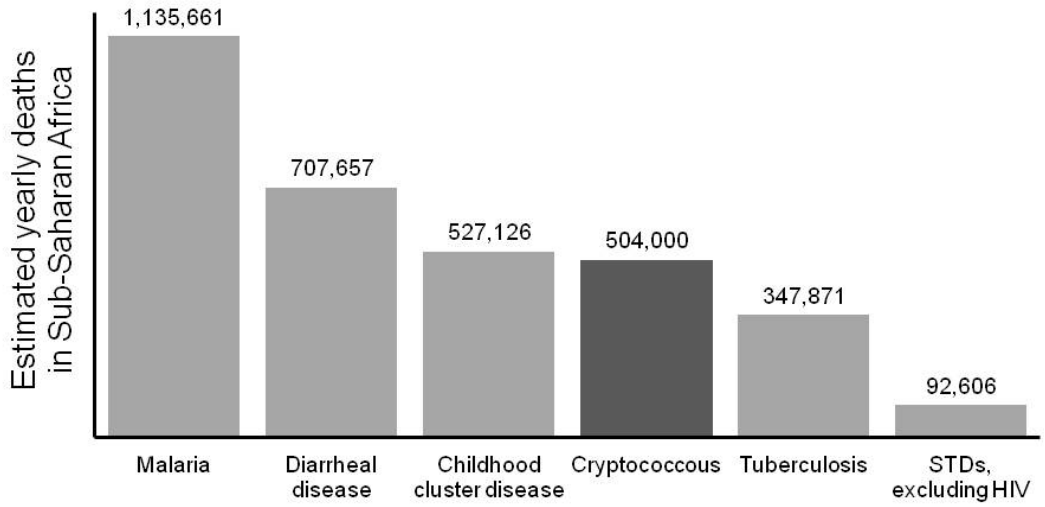

Figure 1.2: Cryptococcal infection is the fourth leading cause of death, excluding HIV, in Sub-Saharan Africa. Deaths due to cryptococcal infection are more prevalent than those due to tuberculosis, but less prevalent than those due to malaria. Image adapted from Park *et al.*, AIDS 2009.

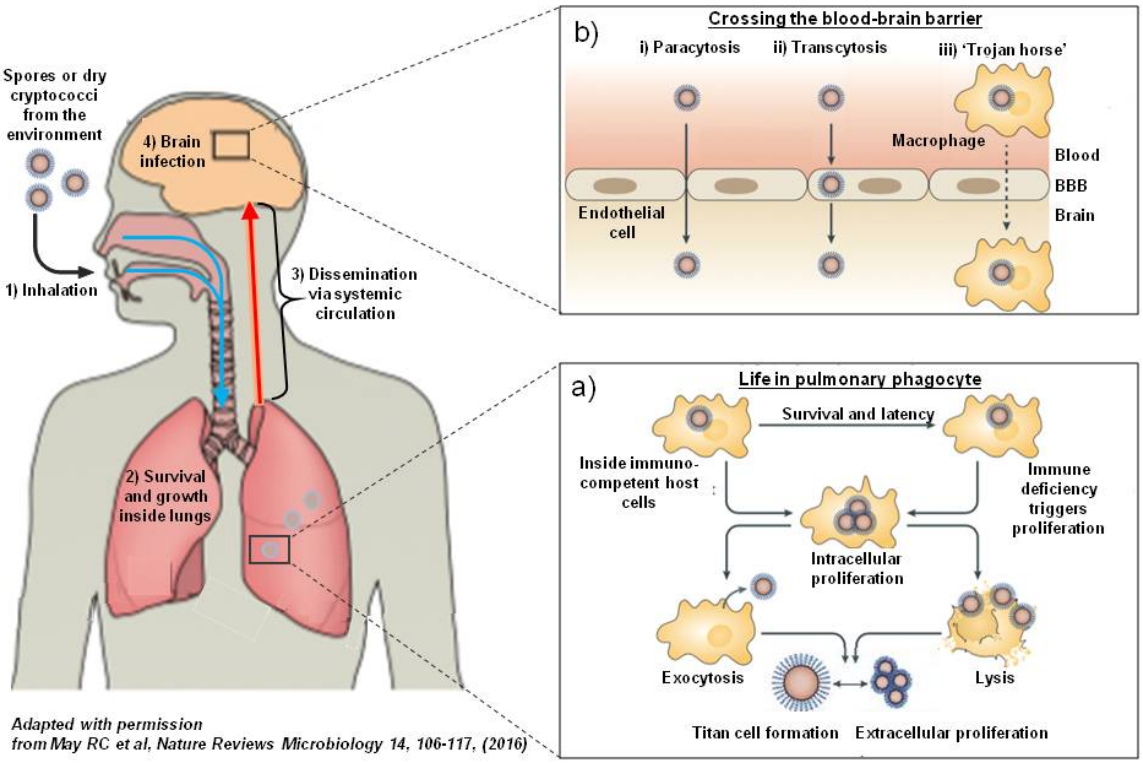

Figure 1.3: *Cryptococcus neoformans* pathogenesis cycle. After spores or dry cryptococcal cells are inhaled, they reach the alveoli in the lungs via mucocilliary movement. Activated alveolar macrophages attempt to phagocytose and kill the fungal cells, but *C. neoformans* can survive due to antiphagocytic functions and other protective properties of the fungal capsule (Sabiiti and May, 2012;). **a)** Upon immuno-suppression of the host, latent cryptococci in granulomas or inside parasitized macrophages are reactivated and can enter the systemic circulation by lytically or non-lytically exiting the macrophages, resulting in fungemia, or reside, replicate, and ride inside the macrophages (Sabiiti and May, 2012). Alternatively, *C. neoformans* can form large "titan cells" that resist phagocytosis and thereby enter the systemic

circulation directly (Sabiiti and May, 2012). **b)** Once it moves through the circulation to the capillaries of the blood brain barrier (BBB), the fungal cell gets through the BBB via any one of three mechanisms: **i) Paracytosis:** entry between endothelial cells, enhanced by damage to tight junctions (facilitated by activation of host plasminogen by fungal mannoproteins); **ii) Transcytosis:** adherence to brain microvascular endothelial cells, followed by endocytosis and then exocytosis on the other side of the cell; or **iii) Trojan horse:** riding in parasitized immune cells such as macrophages (Sabiiti and May, 2012) that can force their way through tight junctions. Image adapted from May *et al.*, 2016, with permission.

CHAPTER TWO

THE ROLE OF *C. NEOFORMANS* ALDEHYDE DEHYDROGENASE FAMILY MEMBERS IN STRESS AND VIRULENCE

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Abstract

Cryptococcus neoformans is a fungal pathogen that quickly senses changes in the host environment and adapts to it by producing a range of metabolites important for optimizing the pH of the host environment, evading the host immune system, use as carbon sources, or other purposes. One of the major metabolites found in biopsies of infected mouse brain and lung tissues, and secreted *in vitro*, is acetate. Herein, aldehyde dehydrogenases, previously identified in *S. cerevisiae* as important in acetate production, are characterized in terms of their role in the pathogenesis and stress survival response of *C. neoformans*. Comparison of the nine different aldehyde dehydrogenases (Alds) of *C. neoformans* with putative homologues suggested that they may have diverse functions. Subsequent analysis of knockouts of eight of the nine *ALD*, revealed interesting deficiency-associated phenotypes. All of the mutants displayed a statistically significant but incomplete reduction in acetate production, but only three mutants, CNAG_06628 (*ald5*Δ), CNAG_05113 (designated *ald*13Δ), and CNAG_03269 (designated

*ald*69Δ), displayed growth defects when cultured on media containing different carbon sources. The *ald5*Δ mutant failed to grow on ethanol-containing media suggesting that it might encode an acetaldehyde dehydrogenase. The *ald*13Δ mutant produced small colonies on acetate and ethanol but grew normally on glucose. Conversely, the *ald*69Δ mutant failed to grow on glucose but grew normally on acetate or ethanol, likely reflecting distinct roles for the corresponding enzymes in the use of different carbon sources. With regard to virulence factors, none of the mutants produced a capsule of normal thickness. Only the *ald*69Δ mutant showed a reduced level of melanin, whereas the *ald5*Δ mutant exhibited increased melanization. The deficiency of certain aldehyde dehydrogenases negatively impacted various stress responses, with the exception of a normal response to osmotic stress. The *ald*29Δ mutant had reduced growth under hypoxia-mimicking conditions, but, notably, was rescued with acetate. The *ald*69Δ mutant could not maintain cell wall integrity nor survive under oxidative stress, and demonstrated partial inhibition of the adenine biosynthesis pathway under nitrosative stress. The observed abnormal phenotypes apparently affected *in vitro* virulence, since the *ald5*Δ, *ald*29Δ, and *ald*69Δ mutants had a statistically significant reduction, as did the *ald*78Δ and *ald*13Δ mutants, but to a lesser extent, in survival after engulfment by murine macrophages. Similarly, *Galleria mellonela* larvae, an *in vivo* infection model, injected with *ald5*Δ or *ald*78Δ mutant *C. neoformans* (but not those injected with the *ald*13Δ strain) survived longer than those injected with the wild type strain, indicating that the *ald5*Δ and *ald*78Δ

mutants were significantly less virulent. Overall, these genetic and biochemical assays establish the roles of different Ald enzymes in modulating the stress response and virulence of *C. neoformans*.

Introduction

Cryptococcus neoformans is an invasive opportunistic pathogen of the central nervous system (CNS) and the most frequent cause of fungal meningitis in humans (Lin and Heitman, 2006), leading to significant annual global mortality, especially in sub-Saharan Africa (Park *et al.*, 2009, Pappas, 2001; Lui *et al.*, 2006; Franzot *et al.*, 1999; Dromer *et al.*, 1996; Tortorano *et al.*, 1997; Powderly, 1993; Jarvis and Harrison, 2007; Kisenge *et al.*, 2007). This fungus mainly affects immunosuppressed individuals (Hull and Heitman, 2002), especially HIV/AIDS patients and solid-organ transplant recipients, with a reported mortality of up to 82% (Abhilash *et al.*, 2015; Park *et al.*, 2009). The source of *C. neoformans* infections are desiccated yeast cells or spores, which are found in the environment after deposition or aerosolization of bird guano as well as in decomposing organic material and in the soil (Steenbergen and Casadevall, 2003; Velagapudi *et al.*, 2009). These desiccated cells or fungal spores are inhaled by mammalian hosts and pass into the lungs, leading to an initial pulmonary infection which often disseminates to the brain, causing a potentially lethal meningoencephalitis (Sabiiti and May, 2012; Velagapudi *et al.*, 2009). There are a number of virulence factors that allow the pathogen to survive and spread in the host body, including production of a polysaccharide capsule and melanin (or a melanin-like pigment) and the ability to grow at the 37°C host body temperature (Coelho *et al.*, 2014; Casadevall *et al.*, 2000; Kronstad *et al.*, 2011; Ma and May, 2009). One of the other mechanisms *C. neoformans* utilizes to propagate and disseminate inside the

host body, in which the availability of glucose can vary, is to adapt to utilization of different carbon sources to obtain energy (Price *et al.*, 2011). *C. neoformans* can utilize glucose or ethanol inside the host to produce energy, and it can also use acetate for a similar purpose (Hu *et al.*, 2008; Price *et al.*, 2011). *C. neoformans* also adapts metabolically to the environment inside the mammalian host to infect, survive, spread, and cause disease. In particular, the pathogen produces a range of metabolites including acetate (Figure 2.1) that confer survival advantages by creating an optimal microenvironment for itself (Bubb *et al.*, 1999; Wright *et al.*, 2002; Hu *et al.*, 2008; Price *et al.*, 2011; Himmelreich *et al.*, 2001; Kronstad *et al.*, 2012).

Acetate is one of the major metabolites secreted by cryptococci cultured *in vitro* (Bubb *et al.*, 1999). Acetate has also been found to be a major metabolite associated with infection, based on analyses of brain and lung tissue biopsies from infected rats (Wright *et al.*, 2002; Himmelreich *et al.*, 2003). Furthermore, substantial amounts of acetate have been detected from pulmonary cryptococcomas, using nuclear magnetic resonance (NMR) (Himmelreich *et al.*, 2001). Although the role of acetate in virulence has not yet been fully elucidated, there is much evidence that it augments fungal survival, perhaps via immunomodulatory mechanisms (Rakesh *et al.,* 2008; Vecchiarelli *et al.,* 2011; Hu *et al.,* 2008; Ellerbroek *et al.,* 2004a, b, c; Li *et al.,* 2018; Siegemund and Alber, 2008; Villena *et al.,* 2008; Yauch *et al.,* 2006).

Two putative pathways for the production of acetate in *C. neoformans* have been identified: the xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (Xfp) – acetate kinase (Ack) pathway and the pyruvate decarboxylase (Pdc) – acetaldehyde dehydrogenase (Ald) pathway. In fungi that lack the Xfp – Ack pathway such as *Saccharomyces cerevisiae*, the primary pathway makes use of pyruvate decarboxylase (Pdc) and an acetaldehyde dehydrogenase function of one or more of the aldehyde dehydrogenase (Ald) enzymes (Saint-Prix *et al*., 2004; Remize *et al*., 2000; Pronk *et al*., 1996).

We have identified nine *C. neoformans* aldehyde dehydrogenases, but which, if any, participate in acetate production is not known. The expression of the transcript encoding one of these aldehyde dehydrogenases (*ALD5*) is increased in *C. neoformans* obtained from the lungs of infected mice, as is the expression of mRNAs encoding other enzymes (*i.e.* pyruvate decarboxlyase and acetyl-CoA synthetase) believed to function in acetate metabolism in this pathogen (Hu *et al.*, 2008). An mRNA encoding a *C. neoformans* aldehyde dehydrogenase was also found to be upregulated in a rabbit infection model (Steen *et al*., 2003). Similarly, various other studies using nuclear magnetic resonance (NMR) analysis have reported high levels of acetate in the media from *C. neoformans* culture and from infected-tissues (Bubb *et al.*, 1999; Himmelreich *et al.*, 2003). Thus, it is likely that at least one *C. neoformans* Ald is an acetaldehyde dehydrogenase that may plays a role in cryptococcal virulence. A thorough characterization of these nine putative aldehyde dehydrogenases has not yet been reported, and the exact role of each

aldehyde dehydrogenase in cryptococcal virulence needs to be fully elucidated. A better understanding of the function of each of the *C. neoformans* aldehyde dehydrogenases would advance the overall understanding of *C. neoformans* metabolism.

For eight out of the nine Ald genes, knockout mutants were available from the Madhani laboratory (UCSF) through the Fungal Genetics Stock Center (Chun and Madhani, 2010). In order to determine which, if any, of these genes are important in *C. neoformans* pathogenesis, the mutants were assayed for phenotypes associated with virulence, including growth on different carbon/energy sources, survival and growth under various stress conditions (*i.e.* osmotic stress, oxidative and nitrosative stresses, and hypoxia), and maintenance of cell wall integrity. The effect of the loss of each Ald on the avoidance of, or survival after, phagocytosis by immune cells was also assayed, as was *in vivo* virulence using an invertebrate infection model. The effect of deficiency of each of the aldehyde dehydrogenases on these phenotypes was correlated with the effect on acetate production by the corresponding mutant, which was also assayed. Overall, this study demonstrated that deficiency of at least some of the aldehyde dehydrogenases negatively impacts the production of acetate and the survival and growth of *C. neoformans in vitro* and *in vivo.*

Materials and methods

Fungal Strains:

A wild type strain in the KN99α background was used as the control in all experiments unless specified otherwise. All eight *ald* mutant strains of *Cryptococcus neoformans var grubii* (in a KN99α strain background) were obtained from the Madhani lab University of California San Francisco (UCSF) gene deletion collection (via the Fungal Genetics Stock Center). These single knockout mutants were maintained under selection for nourseothricin (NTC) resistance, since a nourseothricin acetyltransferase cassette (*NAT*, which encodes an enzyme that neutralizes NTC) was used to replace each gene by insertional mutagenesis mediated by homologous recombination (Chun and Madhani, 2010). Table 2.1 shows the gene identification numbers of all of the strains (and corresponding gene and protein identification numbers) used in this study, the predicted mitochondrial or cytoplasmic cellular location of each protein (determined using the MitoFates website; Fukasawa *et al.*, 2015), as well as the chromosomal location of each corresponding gene (obtained from the Fungi Database, https://fungidb.org/fungidb/).

Phylogenetic tree:

The amino acid sequences of 43 proteins including of the *C. neoformans* aldehyde dehydrogenases and putative homologs in other species were downloaded from the FungiDB website (http://fungidb.org/fungidb/srt.jsp). Alignment and phylogeny reconstruction were performed using Molecular

Evolutionary Genetics Analysis (MEGA X) software (Kumar *et al.*, 2001). First, alignment was carried out with Clustal Ω software (Thompson *et al.*, 1994). Then, aligned sequences were used to construct a Maximum Likelihood (ML) tree, under an assumption of a uniform rate of substitution among all amino acid sites, using the Jones-Taylor-Thornton model (Jones *et al.*, 1992), with 1000 bootstrap replicates (Felsenstein, 1985) to verify the reliability of the tree. The initial tree was constructed using both Neighbor Joining (NJ) and BioNJ algorithms and optimized via the Nearest Neighbor Interchange (NNI) version of the ML heuristic method with a "moderate" branch swap filter setting.

Confirmation of deletion of the corresponding gene in each aldehyde dehydrogenase mutant by PCR analysis:

Deletion of the aldehyde dehydrogenase genes was confirmed by PCR analysis using primers specific to each gene (Table 2.2) and assessing the absence of PCR product. Since all of the *ald* mutant strains at UCSF were generated in the Madhani lab by replacing the corresponding gene with the *NAT* gene conferring resistance to the nourseothricin (NTC) antibiotic (Chun and Madhani, 2010), the presence of *NAT* was also verified using primers synthesized based on sequences obtained from the Madhani lab, as follows:

NAT Forward primer (107): CCTAGCAGCGGATCCAAC

NAT Reverse primer (108): CGCATCCCTGCATCCAAC

DNA was isolated from all the strains using the cetyl/hexadecyl-trimethylammonium bromide (CTAB) DNA isolation protocol (Pitkin *et at*, 1996).

Gene amplification was performed using the KOD Hot Start DNA Polymerase kit (Novagen/Millipore-Sigma, St. Louis, MO) with a thermocycler program as follows for the *ALD* genes: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec., annealing at 60°C for 30 sec., and extension at 72°C for 2 min., followed by final extension at 72°C for 5 minutes. The thermocycler program utilized to amplify *NAT* differed only in that 30 cycles were used and the annealing temperature was set to 55°C.

Quantitative and qualitative growth analysis in various carbon sources:

C. neoformans strains mentioned above were cultured overnight in Yeast Extract Peptone Dextrose (YPD) media (1% Difco Yeast extract, 2% Bacto Peptone, and 2% glucose) at 30°C, in a rotating shaker at 200 rpm (Barnstead MaxQ 4000 Orbital Incubator Shaker). The next morning, ~25 µl from each overnight culture was used to inoculate a corresponding 2 ml volume of YPD, to produce a refreshed culture with an optical density at 600 nm wavelength (OD_{600}) of ~0.075, which was then incubated to OD_{600} of ~0.2 as measured using an Evolution 60 spectrophotometer (ThermoScientific, Waltham, MA). The refreshed cells were pelleted at 8,000×g, washed twice in Corning Dulbecco's Phosphate Buffered Saline (D-PBS; LifeSciences, Oneonta, NY), and counted using a hemocytometer.

For qualitative analysis, spot assays were performed. Refreshed cells were initially diluted to 5×10^3 cells/µl, and then 10-fold serially diluted three times. From each dilution, 2µl were spotted onto Yeast Nitrogen Base (YNB; HiMedia) plus

agar (YNB-agar) supplemented with 2% of either glucose, acetate, or ethanol. Thus, 1×10^4 cells, 1×10^3 cells, 100 cells, and 10 cells of the wild type strain and each of the mutant strains were plated and were allowed to grow at 30°C for approximately 3 days. Images of colonies were captured using the Canon EOS Rebel T1i camera.

For quantitative growth analysis 1×10^4 cells/µl were used to inoculate YNB media supplemented with 2% of either glucose, acetate, or ethanol as the carbon source. Cultures were set up in each medium in triplicate in microtiter plates. Cells were allowed to grow in a shaking incubator at 30°C for 48 hours, and their growth was monitored over time using the Epoch Multi-Volume Microplate Reader (BioTek Instruments, Inc., Winooski, VT) to measure the OD_{600}.

Measurement of acetate production:

To analyze acetate production by *C. neoformans*, the hydroxamate assay was performed as described previously (Fowler *et al.*, 2012; Aceti and Ferry, 1988; Rose *et al.*, 1954). As detailed above, *C. neoformans* strains were grown overnight in YPD media at 30°C. To refresh cells, 25 µl from each of these overnight cultures was used to inoculate a corresponding 5 ml volume of YNB supplemented with 2% glucose, which was incubated to an OD_{600} of ~0.2. Cells were counted using a hemocytometer and each of the 5 ml cultures were adjusted to a concentration of 1×10^4 cells/µl. The 5 ml cultures were vortexed briefly to fully resuspend the cells and 1 ml of each culture was transferred to a corresponding eppendorf tube, which was centrifuged at 10,000×g. The recovered supernatants were transferred to

fresh tubes and frozen down at –80°C. The remaining culture volumes were incubated at 30°C, and, every 12 hours, up to and including 48 hrs, the process was repeated. Once all supernatants were collected, the hydroxamate assay was performed on triplicate 225 µl samples from each 1 ml of supernatant media from each strain as described by Aceti and Ferry (1988). Briefly, 75 µl of hydroxamate reaction mix (2.4 M hydroxylamine-HCl at pH 7.0, 0.4 M Tris at pH 7.5, 80 mM $MgCl_2$, and 90 µM ATP disodium salt hydrate) was added to each 225 µl of supernatant and the mixture was incubated at 37°C for 5 minutes. Then, 5 µl of *Methanosarcina thermophila* acetate kinase (purified by Dr. Cheryl Ingram-Smith) was added to a final concentration of 0.023 ng/µl, and the reaction was mixed to homogeneity by pipetting and incubated at 37°C for 15 minutes. Finally, the reaction was quenched using an equal volume of a stop solution (10% trichloroacetic acid and 2.5% $FeCl_3$ in 2 N HCl) and the absorbance was read at 540 nm using the Epoch Multi-Volume Microplate Reader spectrophotometer. A standard curve was prepared by performing exactly the same protocol using known concentrations of acetate solution ranging from 0.1 mM - 2 mM instead of supernatant media.

Capsule formation assay:

Capsule production by *C. neoformans* was induced using previously published conditions (Zaragoza and Casadevall, 2004). Briefly, wild type and mutant strains were grown overnight in 2 ml of Sabouraud medium (4% glucose and 1% bacto peptone, pH 5.6) at 30°C. The following day, cells were collected by

centrifugation (8,000-10,000×g) for 2 minutes at room temperature, washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.5 mM Na_2HPO_4), and resuspended in 0.1x Sabouraud medium (pH of 7.3). Cells were counted, diluted to 5×10^6 cells/ml in 2 ml of the above 0.1x Sabouraud medium, and incubated for 24 hours at 37°C to induce capsule formation.

Capsules of the wild type and mutant strains were observed under the Axiovert Inverted Microscope (Carl Zeiss, Inc., Thornwood, NY) after 10 µl of each cell suspension were mixed with 10 µl of India Ink (Becton Dickinson, NJ) to provide contrast. Images were taken at 40x magnification and processed with Zeiss software and Image J. At least five different fields were randomly chosen and photographed, and 25 to 30 cells were observed.

Melanin production assay:

C. neoformans wild type and *ald* mutant strains were grown as described above and then harvested by centrifugation for 2 min at room temperature, washed twice in D-PBS, and counted using the hemocytometer. Each strain was diluted to 1×10^5 cells/ml and a 5µl volume of each strain was plated on agar-containing melanin-induction medium (8 mg/ml KH_2PO_4, 2 mg/ml glucose, 2 mg/ml L-glycine, 1 µg/ml D-biotin, 1 µg/ml thiamine, 0.92 mg/ml $MgSO_4{\cdot}7H_2O$, and 0.4 mg/ml L-3,4-dihydroxyphenylalanine [L-DOPA]) in order to induce melanin production (Li *et al*., 2018). Plates were incubated at 30°C for 2-3 days, and then imaged using the Canon EOS Rebel T1i camera. The experiment was performed thrice.

Growth under hypoxia and under oxidative and nitrosative stresses:

C. neoformans wild type and *ald* mutant strains were grown overnight and refreshed the next morning as described above, then harvested by centrifugation for 2 min at room temperature, washed twice in D-PBS, counted using the hemocytometer, and diluted to 5×10^3 cells/µl. Next, three 10-fold serial dilutions of cells were prepared, and 2µl of each of these four dilutions were spotted at 1×10^4, 1×10^3, 100 and 10 cells per spot onto media that mimicked different stress conditions that the fungus encounters during infection. To mimic hypoxia, cells were spotted onto YES-agar (0.5% yeast extract, 2% glucose, 1.5% agar and 225 µg/mL of each of uracil, adenine, leucine, histidine, and lysine) supplemented with 0.7 mM $CoCl_2$ (Lee *et al.*, 2007). To test the effects of oxidative and nitrosative stresses, cells were spotted onto sodium succinate-adjusted YNB-agar medium (1.34 g/liter yeast nitrogen base without amino acids, 4 g/L glucose, and 5 mM sodium succinate to adjust the pH to 4.0, filter sterilized and mixed 1:1 with 4% agar to a final concentration of 2% agar) supplemented with either 1 mM hydrogen peroxide (H_2O_2) or 1.5 mM sodium nitrite ($NaNO_2$), respectively, or not supplemented as control (Gerik *et al.,* 2008). Plates were incubated at 30°C for 2-3 days and then imaged using the Canon EOS Rebel T1i camera. Each experiment was repeated thrice.

Growth under osmotic stress:

Wild type and *ald* mutant fungal cells were cultured overnight, refreshed and counted the next day, diluted, and spotted as described above, except that osmotic

stress generating medium (1% yeast extract, 2% Bacto Peptone, 2% glucose, and 2% agar, supplemented with either 1.5 M NaCl or 1.2 M KCl) was used. Plates were incubated at 30°C for 3 days and then imaged using the Canon EOS Rebel T1i camera. Each experiment (with NaCl or KCl) was repeated thrice.

SDS and Congo red assays for cell wall integrity:

Wild type and *ald* mutant fungal cells were cultured overnight, refreshed and counted the next day, diluted, and spotted onto plates as above, except that the various strains were spotted onto media with 1% yeast extract, 2% Bacto Peptone, 2% agar, and 1% glucose (all from Difco) supplemented with either 0.5% Congo red (Sigma-Aldrich/Millipore Sigma, St. Louis, MO) or 0.03% sodium dodecyl sulfate (SDS; Calbiochem/Millipore Sigma, St. Louis, MO). Plates were incubated at 30°C for ~72 hours and then images were captured using the Canon EOS Rebel T1i camera. Each experiment was repeated three times.

Macrophage culture:

A murine macrophage-like cell line J774A.1 (gift From Dr. Jeffrey Anker, Clemson University), originally derived from a BALB/c mouse reticulum cell sarcoma (Fan *et al.*, 2005), was maintained in macrophage medium (Dulbecco's Modified Eagle Medium [DMEM] supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1% penicillin-streptomycin, and 10% NCTC-109 medium, all from Gibco Life Technologies). Cells were passaged at a 1:10 split approximately every 5-7 days when they reached 80% confluence.

Macrophage survival assay:

At ~80% confluence, cultured macrophages were washed twice with D-PBS, harvested by scraping after addition of fresh macrophage medium, and then diluted to 1×10^3 cells/µl. Into two 96-well flat-bottom tissue culture treated plates (Corning Inc., Corning NY), one for one-hour co-incubation and the other for 24-hour co-incubation with fungal cells, 100 µl of the cell suspension (1×10^5 cells) in macrophage media were transferred into three wells per *C. neoformans* strain. Both plates were incubated overnight at 37˚C in the 5% CO_2 incubator.

Concurrently, wild type (KN99α) and mutant *C. neoformans* strains were grown overnight in liquid YPD media at 30°C. The next day, the strains were pelleted by centrifugation at 8,000×g for 1 min, washed twice with D-PBS, and resuspended in macrophage medium. The fungal cells were then diluted to 125 cells/µl in 1 ml of macrophage media (*i.e.* 1.25×10^4 *C. neoformans* cells per 100 µl). To enhance macrophage phagocytosis by opsonization, each strain of *C. neoformans* was incubated for one hour at 37˚C with 1µg/ml (final concentration) of the MAb18B7 monoclonal antibody (gift from Dr. Arturo Casadevall, Johns Hopkins University School of Medicine) that binds to the glucuronoxylomannan (GXM) capsule component (Casadevall *et al.*, 1998). As soon as the *C. neoformans* strains with antibody were placed in the incubator, two plates containing macrophages were processed as follows. The media was gently removed from each well and replaced with 100 µl macrophage media containing

10 nM phorbol myristate acetate (PMA), and then both plates were incubated at 37˚C in 5% CO_2 for one hour to activate the macrophages (Forman and Torres, 2001).

After separate one hour incubations of the macrophages and fungal cells, the medium in each well from both 96-well macrophage plates was replaced, without disrupting the macrophage monolayer, with a 100 µl volume of cell suspension (*i.e.* 1.25×10^4 cells) of one of the wild type or *ald* mutant *C. neoformans* strains, in triplicate, at an 8:1 macrophage to fungal cell ratio. In a third, 96-well tissue culture treated plate, the cell suspensions of *C. neoformans* wild type or mutant strains were added, in triplicate, at 100 µl (*i.e.* 1.25×10^4 cells) per well, in the absence of macrophages as a control. All three plates were left at 37˚C in 5% CO_2 for one hour, after which, each well in the one-hour and 24-hour plates with macrophages, but not in the control plate without macrophages, were gently washed thrice with 200 µl D-PBS to remove non-phagocytosed *C. neoformans*. To the 24-hour plate, 100 µl of fresh macrophage media were added to each well, and the plate was placed back into the 37˚C, 5% CO_2 incubator for 24 hours.

To the one-hour plate, 200 µl of sterile distilled, deionized water (ddH_2O) were added to each well, and the plate was incubated at room temperature for 5 minutes to lyse the macrophages. A pipette tip was then scraped against the bottom of the wells to lift up the adherent macrophages, and the cells suspended in the water were pipetted up and down to disrupt and further lyse the macrophages. The 200 µl lysate was then transferred to a microfuge tube. Again,

200 µl of sterile ddH_2O were added to each well, the wells were scraped, and the suspensions were pipetted up and down. The additional lysates generated were combined with the corresponding original lysates. These steps were repeated twice more, but with 300 µl of sterile ddH_2O, so that, by the end of the procedure, each microfuge tube held 1 ml final volume of lysate. For the plate of *C. neoformans* strains without macrophages, since the wells were not washed prior to addition of sterile ddH_2O, the volume of original media plus sterile ddH_2O transferred to each microfuge tube was 300 µl; then 200 µl, 200 µl, and 300 µl volumes were used and transferred to the corresponding microfuge tube. The same procedure that was used on the one-hour plates with macrophages was used, without modification, to obtain macrophage lysates from the 24-hour plate the next day. For each 96-well plate, macrophage lysis (or recovery of fungal cells from wells without macrophages) was conducted in sets of one well per strain, in triplicate sets, to obtain the fungal cells from the three wells per strain.

Immediately after *C. neoformans* cells were obtained from the macrophage lysates or from the fungal cell suspensions (*i.e.* from the no-macrophage control plate), the cells were diluted 1:10, then again 1:2.5 for a final dilution of 1:25, or a (theoretical) maximum concentration of 500 cells/ml in a total volume of 0.5 ml. For each *C. neoformans* strain, from this final dilution, 100 µl were plated on YPD-agar plates, one plate per corresponding well from each 96-well plate. This resulted in triplicate YPD-agar plates for each wild type and mutant strain, with a maximum of 50 cells per plate from each corresponding well of the 96-well plates (at least from

the 1-hour plate, although due to replication of the fungal cells, more than this number was possible from the 24-hour plate). YPD-agar plates were incubated at 30°C for ~48 hours and then colony forming units (CFU) were counted for each plate and the percentage survival was calculated for each strain using the following formula, in which MΦ refers to macrophages and the CFU are the average of the individual CFU from the triplicate YPD-agar plates:

% Phagocytosis = ($CFU_{C.\ neoformans\ \text{with MΦ @ 1hr}}$ ÷ $CFU_{C.\ neoformans\ \text{without MΦ}}$) × 100

% Survival = ($CFU_{C.\ neoformans\ \text{with MΦ @ 24 hrs}}$ ÷ $CFU_{C.\ neoformans\ \text{with MΦ @ 1 hr}}$) × 100

***In vivo C. neoformans* virulence assay using an invertebrate model:**

To examine the virulence of mutant *C. neoformans* strains, either the wild type *C. neoformans* strain or one of *ald5*, *ald*13, or *ald*78 mutant strains, all in the H99 strain background, were injected into the larvae of the greater wax moth, *Galleria mellonella*, and larval survival was assayed. *C. neoformans* strains to be injected were grown in YPD media at 30°C overnight and refreshed the next morning by inoculation of new 2 ml cultures and incubation to an OD_{600} of ~0.2. The cells were then pelleted and washed once with sterile water and twice with D-PBS. Each strain was then resuspended in D-PBS to a final concentration of 1×10^5 cells/µl. Subsequently, milky-white *G. mellonella* larvae without any dark spots and weighing between 0.27 g and 0.30 g were chosen. Ten larvae per *C. neoformans* strain were selected and disinfected using alcohol wipes just prior to inoculation. Using Hamilton syringes, each of the ten larvae were injected in the bottom-left proleg with 10 µl of one of the *C. neoformans* strain resuspensions. To determine

the level of death due solely to injection, 10 µl of D-PBS were injected into ten more larvae (as a control) and survival was monitored over time. Similarly, as a negative control, the *C. neoformans* wild type strain (in the H99 background) was heat-killed via incubation at 65°C for one hour and 1×10^6 of these dead cells were injected into each of ten larvae. After all strains and controls were injected into *G. mellonella*, the larvae were incubated at 37°C and monitored daily. Death was scored based on blackened appearance (indicative of either necrosis or complete melanization) and a lack of normal rolling-over behavior. The numbers of dead larvae in each injection group were tallied and graphed in a Kaplan-Meier survival curve.

Statistical analysis of results:

All of the graphs were prepared using GraphPad Prism; the significance of the results were determined using the analysis of variance (ANOVA) software within Prism, with Dunnet, Sidak, or other post-test multiple comparison, as recommended by the software. One-way ANOVA was used when triplicate values generated at the end of the experiment were compared, but when multiple time points were compared as well as the various strains, two-way ANOVA was utilized. For the macrophage survival assay, data was first analyzed using Microsoft Excel to calculate the relative percent survival (or recovery, versus the wild type strain) after phagocytosis of each strain prior to graphing the data and conducting statistical analyses via Prism. For Kaplan-Meier survival curves, the Gehan-

Breslow-Wilcoxon test and/or Log-Rank test was used to compare the virulence of mutant strains to that of the wild type strain.

Results

Phylogeny of the *C. neoformans* Ald family:

In *C. neoformans*, nine genes have been predicted to encode proteins that could have aldehyde dehydrogenase (Ald) activity. In order to further understand the function of these genes, sequence similarity analysis was performed on the corresponding protein sequences using Molecular Evolutionary Genetics Analysis (MEGA X) (Kumar *et al.*, 2001) phylogenetic software (Figure 2.2).

A mutant strain deficient for one aldehyde dehydrogenase in *C. neoformans*, CNAG_02377, also known as CKF44_002132 (in the H99 and H99-derived KN99 strains, respectively), was not available for experimental analysis. However, by the phylogenetic analysis, the protein sequence for this Ald was found to diverge from a common ancestor with, and was evolutionarily most related to, CNBG_4084, an Ald from *Cryptococcus deuterogattii*. CNAG_02377 is next most closely related to AGR57_15224, the NAD-Ald of *Phanerochaete chrysosporium,* a basidiomycota, and then to FOC4_Q10007570, the lactaldehyde dehydrogenase of *Fusarium oxysporam*. These four Ald sequences form GROUP 1 in the phylogenetic tree (Figure 2.2).

CNAG_05029 (Ald29) in the H99 strain of *C. neoformans* (*var grubii*), known as CKF44-004510 in the KN99 strain, is closest to CC1G_03873, the Ald of *Coprinopsis cinerea,* a basidiomycota fungi. According to the fungi database, both proteins are referred to as meiotic Sister-Chromatid recombination aldehyde dehydrogenases (https://fungidb.org/fungidb/app/record/gene/CNAG_05029, and

https://fungidb.org/fungidb/app/record/gene/CC1G_03873). The next most closely related protein to these is PH9BL_176604, a betaine aldehyde dehydrogenase from *Phycomyces blakesleeanus,* a filamentous fungi in the phylum Zygomycota. These sequences form GROUP 2 of the phylogenetic tree.

CNAG_06018 (Ald18) and CNAG_06010 (Ald10) in the H99 strain have corresponding putative enzymes in the KN99 strain, CKF44_005401 and CKF44_005393, respectively. CNAG_06010 is most closely related to CGBM_0210C, an Ald in *Cryptococcus gattii*, but CNAG_06018 is the next most closely related aldehyde dehydrogenase from among all of those considered. These sequences, along with the next most closely related sequence, AGR57_7717, the NAD-aldehyde dehydrogenase from *Phanerochaete chrysosporium,* a basidiomycota fungi, form GROUP 3 of the phylogenetic tree.

CNAG_03269 (Ald69) in the H99 strain, known as CKF44_002909 in the KN99 strain, is closest to CGB_G2310C, an Ald in *C. gattii*. Together with the next closest protein, ACLA_062670, an Ald in *Aspergillus clavatus*, these sequences form GROUP 4 of the phylogenetic tree.

Phylogenetic GROUP 5 consists of CNAG_00735 (Ald35) in the H99 strain or CKF44_000642 in the KN99 strain, along with AGR57_4875, an NAD-Ald in *P. chrysosporium*, and BDEG_01128, the Ald family 7 member A1 of *Batrachochytrium dendrobatidis,* a fungus known to cause chytridiomycosis in amphibians (Rebollar *et al.,* 2014; Zumbado-Ulate *et al.,* 2019).

CNAG_01078 (Ald78) and CNAG_05113 (Ald13) in the H99 strain have corresponding proteins CKF44_000949 and CKF44_004585, respectively, in the KN99 strain. CNAG_05113 is most closely related to the CNBG_4577 NAD-Ald from *C. deuterogatti,* but is next most closely related to CNAG_01078. CNAG_06628 (Ald5) in the H99 strain, or CKF44_005930 in the KN99 strain, is the next most closely related Ald to both CNAG_01078 and CNAG_05113 and completes the phylogenetic GROUP 6. These three *C. neoformans* Alds are the most closely related, among all *C. neoformans* Alds, to *S. cerevisiae* Ald4p, Ald5p, and Ald6p, which can act as acetaldehyde dehydrogenases to produce acetate (Remize *et al.,* 2000; Saint-Prix *et al.,* 2004). However, as *C. neoformans* Ald5, Ald13, and Ald78 are more closely related to each other than any is to *S. cerevisiae* Ald4p, Ald5p, and Ald6p, and, conversely, since these *S. cerevisiae* enzymes are more closely related to each other than to any *C. neoformans* Alds, it is difficult to predict, based on the function of the *S. cerevisiae* enzymes, which one of Ald5, Ald13, or Ald78 may function in acetate production.

Verification of *ALD* deletions:

The *ald* mutants obtained from the Madhani deletion collection (Chun and Madhani, 2010) were checked for the deletion of the appropriate *ALD* and for the presence of the nourseothricin acetyltransferase (*NAT*) cassette, that was used to replace the *ALD* gene. Primers specific for each *ALD* were designed and used in PCRs to amplify a portion of the corresponding gene from wild type and mutant strain genomic DNAs. Similarly, primers specific for the *NAT* cassette were used

to amplify this gene from genomic DNA from the mutant strains. The results from each PCR confirmed the deletion of the respective *ALD* genes in the mutants used in this study (Figure 2.3a). Amplification of *NAT* (~1600 bp) from all of the *ald* mutants, but not from the wild type KN99α strain, was also observed (Figure 2.3b).

Impact of *ALD* deletions on the growth of *C. neoformans* on different carbon sources:

Under normal conditions, *C. neoformans* favors glucose over other carbon sources for growth (Hu *et al*., 2008; Sabiiti and May, 2012). However, during early stages of infection, it is challenged by low glucose and low amino acid conditions and must utilize alternative carbon sources like acetate and lactate to survive (Hu *et al.,* 2008; Price *et al.,* 2011). To investigate whether each of the *ald* mutants could grow in media supplemented with alternative carbon sources like acetate and ethanol as well as they could in glucose-rich media, spot assays and growth curve analyses were performed by spotting the yeast on YNB-agar, or by culturing them in YNB liquid medium, respectively, supplemented with 2% glucose, 2% acetate, or 2% ethanol.

All of the *ALD* mutants exhibited normal growth when cultured on YNB-agar supplemented with 2% glucose except the *ald*69Δ (CNAG_03269) mutant, which demonstrated a major growth defect on this media (Figure 2.4a). Under this growth condition, the *ald*69Δ mutant produced fewer colonies of very small size, a phenotype which was clearly visible at the lower dilutions of cells (Figure 2.4a). In contrast, the *ald*69Δ mutant strain did not have a significant defect in growth on

YNB-agar supplemented with either 2% acetate or 2% ethanol (Figure 2.4b and 2.4c).

Conversely, in the *ald*13Δ (CNAG_05113) mutant, the absence of the Ald13 protein, which is predicted to reside in the mitochondria (MitoFates website, http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi; Fukasawa *et al.*, 2015), resulted in smaller sized colonies on YNB-agar plates containing 2% acetate or 2% ethanol relative to colonies on YNB-agar plates containing 2% glucose, and versus the average colony size of the wild type strain on 2% acetate or 2% ethanol supplemented YNB-agar (Figure 2.4). However, no difference in the number of colonies, as compared with the wild type strain (or other mutant strains), was observed.

In addition to the phenotypes of the *ald*69Δ and *ald*13Δ strains, the *ald5*Δ mutant strain exhibited a severe growth defect (*i.e.* no growth) specifically when plated on YNB-agar with 2% ethanol, but not on YNB media supplemented with 2% glucose or 2% acetate (Figure 2.4c).

In order to quantify the previous decline in growth of *ald*69Δ and *ald5*Δ mutants, but not of other mutants, all aldehyde dehydrogenase mutants were cultured in liquid medium containing 2% glucose, 2% acetate, or 2% ethanol as the carbon source. A quantitative growth analysis was performed over a 48-hour time course. The *ald*69Δ mutant displayed a growth defect reminiscent of the smaller number of colonies observed in the spot assay. Specifically, this mutant exhibited a slower growth rate at later time points in medium with 2% glucose

(Figure 2.5a; P-value = 0.0210, 0.0429, 0.0315, and 0.0320 at 24, 31, 38, and 48 hours, respectively, all less than the 0.05 cutoff value for significance), but not in media with 2% acetate or 2% ethanol. Similarly, the *ald5*Δ mutant grew poorly in medium supplemented with 2% ethanol (Figure 2.5c; P-value = 0.0052 for the overall growth curve versus the wild-type growth curve), just as it did on the equivalent plates in the spot assay, but grew normally in media with 2% glucose (Figure 2.5a) or 2% acetate (Figure 2.5b), just as it did on the 2% glucose and 2% acetate supplemented YNB-agar. The quantitative growth curve analysis conducted over 48 hours correlated with the qualitative spot assay results, with clear growth defects in *ald5*Δ and *ald*69Δ. All strains, including the wild type strain, exhibited slower growth in media supplemented with 2% acetate, so no significant differences were observed between the wild type and any of the mutant strains (Figure 2.5b).

Acetate production is reduced in *ald* mutants:

Acetaldehyde dehydrogenase has been shown to be involved in the production of acetate in *S. cerevisiae* (Pronk *et al.*, 1996). In order to determine whether each Ald enzyme in *C. neoformans* functions in the production of acetate, the levels of acetate produced by the various *ald* mutants cultured in YNB media supplemented with 2% glucose were measured. The concentration of acetate produced by the wild type strain and each of the various strains deficient in different aldehyde dehydrogenases was determined every 12 hours. All of the mutants could utilize glucose in the media and produce acetate. However, although the

acetate production by the *ald* mutants was not eliminated, it was significantly reduced relative to the wild type KN99α strain at all time points after 12 hours (Figure 2.6; P-value < 0.0001 overall). In particular, after 12 hours, *ald5*Δ, *ald*10Δ, *ald*18Δ, *ald*35Δ, and *ald*78Δ exhibited very significantly (P-value < 0.01) reduced acetate production versus the wild type strain, (with P-values of 0.0095, 0.005, 0.0033, 0.0092, and 0.0053, respectively), while *ald*13Δ and *ald*29Δ exhibited highly significantly (P-value < 0.001) reduced acetate production versus the wild type strain (with P-values of 0.0008 and 0.0002, respectively), and *ald*69Δ exhibited extremely significantly (P-value < 0.0001) reduced acetate production versus the wild type strain.

Capsule formation:

C. neoformans forms a polysaccharide capsule that protects the fungal cell from the immune response and increases fungal virulence (Reese and Doering, 2003; Bose *et al.,* 2003). To characterize capsule formation in wild type and *ald* mutants, strains were incubated in capsule-inducing medium and then stained with India Ink to visualize capsule thickness under the microscope. It was found that the capsules of all *ald* mutants were smaller than that of the wild type KN99α strain, although the *ald*10 mutant had a larger capsule than the other mutants (Figure 2.7). This suggests that production and completion of the capsule is highly compromised in the absence of any one of the Alds.

Melanin production:

Melanin neutralizes reactive oxygen species and prevents oxidative killing by phagocytes, thus acting as another important virulence factor during infection (Ma and May, 2009). Spot assays were performed in L-DOPA supplemented plates to determine whether a deficiency of any of the aldehyde dehydrogenases has an effect on melanin production. Most of the mutants, namely *ald*13Δ, *ald*78Δ, *ald*10Δ, *ald*18Δ, *ald*29Δ, and *ald*35Δ, had no melanin production defect and produced melanin at levels comparable to the wild type strain (Figure 2.8). However, there was a significant reduction in the production of melanin by the *ald*69Δ mutant, suggesting a role for the corresponding enzyme in the production of melanin (Figure 2.8). Surprisingly, the *ald5*Δ mutant displayed increased melanin production, relative to the wild type strain (Figure 2.8), suggesting that the normal function of the Ald5 enzyme has, either directly or indirectly, a negative effect on the production of melanin. This data suggests that Ald69 is required for optimal production of melanin, while functional Ald5 apparently reduces the need for melanin.

Response to hypoxia, and oxidative and nitrosative stresses:

In the host brain, *C. neoformans* faces low oxygen conditions, which are detrimental to its survival (Ingavale *et al.*, 2008; Erecińska and Silver, 2001). In order to understand whether any of the Alds are important for the survival of the pathogen under hypoxic conditions, each mutant was tested for the ability to grow in YES medium supplemented with $CoCl_2$, which induces the Sre1p ergosterol

production pathway used by the cells as an oxygen sensing pathway, and thereby drives the cells into a metabolic state similar to that induced by hypoxia (Lee *et al.*, 2007). All of the mutants survived normally when subjected to hypoxia conditions except *ald*29Δ (Figure 2.9b). The *ald*29Δ mutant, which grew normally in YES media without $CoCl_2$, *i.e.* under normoxia (Figure 2.9a), exhibited defective growth when subjected to the low oxygen simulating conditions (Figure 2.9b). Surprisingly, when this mutant was provided with acetate in the $CoCl_2$ containing plates, it recovered normal growth (Figure 2.9c).

While surviving hypoxia matters after dissemination to the brain, at the initiation of infection, upon inhalation of cryptococcal cells or spores, the fungal cells encounter alveolar macrophages in the lungs that attempt to engulf them and to destroy them using ROS and RNS (Hampton *et al.,* 1998; Vieira *et al.,* 2002; Cox *et al.,* 2003; Tohyama *et al.,* 1996). To determine if any of the Alds play a role in the survival of the pathogen under oxidative conditions, wild type and *ald* mutant strains were grown on YNB-agar (buffered to pH 4.0 using sodium succinate), either alone as control or supplemented with 1 mM H_2O_2. When subjected to this oxidative stress condition, all of the mutants except *ald*69Δ had growth comparable to the wild type KN99α strain (Figure 2.10). The *ald*69Δ mutant displayed reduced growth under oxidative stress (Figure 2.10). This result suggests a specific requirement for the Ald69 enzyme in *C. neoformans* survival under the oxidative stress conditions encountered within macrophages, indicating that this Ald may play a role in *C. neoformans* virulence.

Similarly, when the wild type and mutant strains were grown under nitrosative stress by 1.5 mM sodium nitrite, only the *ald*69Δ mutant displayed a phenotype different from that of the wild type strain (Figure 2.11b). However, instead of showing a defect in growth, the *ald*69Δ mutant formed pink/red colonies (Figure 2.11b, bottom row). It has long been established that *S. cerevisiae* mutants with deficiencies in either adenine or biotin production grow as pink/red colonies (Smirnov *et al.*, 1967). Therefore, to test whether the *ald*69Δ mutant turned pink under nitrosative stress due to adenine deficiency, 225 µg/ml adenine was added to the media along with 1.5 mM sodium nitrite and the growth of all wild type and mutant strains was reanalyzed. The red pigmentation was not observed, even in the *ald*69Δ mutant, when the cells were grown in adenine-supplemented nitrosative stress media (Figure 2.11c). This suggests that the *ALD* genes are not required for growth under nitrosative stress conditions; however, the function of the Ald69 enzyme plays a role in the production of adenine in the pathogen under nitrosative stress.

All *ald* mutants exhibited a normal osmotic stress response:

C. neoformans has evolved specialized pathways, including the production and use of mannitol (Chaturvedi *et al.*, 1996) and the *HOG1* pathway (Bahn *et al.*, 2005), to survive high osmolarity environments inside the host body and the corresponding osmotic stress. To investigate whether the *C. neoformans* aldehyde dehydrogenases function in the osmotic stress response, the wild type strain and each of the *ald* mutants were grown on YPD-agar supplemented with either 1.5 M

NaCl or 1.2 M KCl to osmotic stress. It was found that all of the *ald* mutants grew normally under these high salt conditions, that is, to a degree similar to that of the growth of the wild type strain (Figure 2.12). These results suggest that the *ALD* genes are not required for growth under osmotic stress.

The *ald* mutants, especially *ald*69Δ, have partially compromised cell wall integrity:

The *C. neoformans* cell wall protects the fungal cell from environmental challenges. in order to survive attack by ros and rns produced by macrophages when they phagocytose a pathogen, the cryptococcal cells upregulate pathways used to build the cell wall (Gerik *et al.*, 2005). The capsule is attached to the cell membrane via the cell wall, and melanin is deposited in the cell wall; therefore, these virulence factors that protect against reactive oxygen species require an intact cell wall for their functioning (Baker *et al.*, 2007; Van Dyke and Wormley, 2018). The integrity of the cell wall of the wild type and aldehyde dehydrogenase mutant strains was tested by spotting serial ten-fold dilutions of cells on YPD-agar treated with sodium dodecyl sulfate (SDS) or Congo red. Congo red binds to components of chitin and of the cell wall and interferes with cell wall construction, while SDS dissolves and thereby disrupts the plasma membrane, lysing the cells (García *et al.*, 2015; Banks *et al.*, 2005; Ram and Klis., 2006; Baker *et al.*, 2007; Gerik *et al.*, 2005; Wood *et al.*, 1983). Strains with cell wall integrity defects will not survive in the presence of either Congo red or SDS, depending which layer of capsule, cell wall, or chitin is deficient. The growth of cryptococcal cells on media

with SDS was not affected in the *ald* mutant strains (Figure 2.13b), indicating that the SDS could not pass through the chitin layer to dissolve the cell membrane in these mutants. While media with Congo red inhibited the growth of both wild type and mutant strains (Figure 2.13c) in comparison to normal media (Figure 2.13a), the Congo red affected the growth of *ald* mutants more. Even when spotted at higher cell concentrations, mutant cells did not grow as robustly as the wild type cells on the Congo red supplemented media, and the mutant strains displayed smaller colonies overall (Figure 2.13c), possibly reflecting the effect of their smaller capsules. The most affected mutant was *ald*69Δ, which did not grow at all when lower concentrations of cells were spotted, and grew poorly when the highest concentration of cells was used (Figure 2.13c, bottom row). This indicates that the corresponding enzyme may have a special role in the maintenance of cell wall integrity.

The majority of *ald* mutants exhibited reduced survival and/or recovery after phagocytosis:

To investigate the contribution of the various aldehyde dehydrogenases to the survival of *C. neoformans* against attack by host immune cells, the percentage of wild type or *ald* mutant fungal cells surviving engulfment by murine macrophages was determined. First, fungal cells were co-incubated, in triplicate, with macrophages for 1 hour, after which the non-phagocytosed fungal cells were removed, the macrophages were lyzed, and the fungal cells from within those macrophages were plated onto YPD. The average percent phagocytosis of each

triplicate set was determined by comparing the number of colonies to the colonies from identically cultured fungal cells without macrophages, and then normalized to the wild type result. The relative percentages of input cells phagocytosed (*ald5Δ*: 111.32%; *ald*10Δ: 98.44; *ald*13Δ: 133.74; *ald*18Δ: 98.8; *ald*29Δ: 144.32; *ald*35Δ: 107.08; *ald*69Δ: 165.17; *ald*78Δ: 136.76) were not significantly different from that of the wild type strain (100%). In a parallel plate, after removal of non-internalized fungal cells, the macrophages were given 24 hours to destroy the phagocytosed *C. neoformans* cells and were then lysed to release surviving, presumably growing, *C. neoformans* cells, which were plated on YPD. The number of colonies of each strain were divided by the average percent phagocytosis from the previous step to determine the percent survival (and recovery) after phagocytosis. The percent survival was reduced, relative to the wild type strain, for most, but not all, mutant strains (Figure 2.14). The mutant strains designated *ald10*, *ald18*, and *ald35* did not exhibit a significant difference in survival/recovery after phagocytosis compared with the wild type strain. In contrast, the *C. neoformans* cells from the *ald5*, *ald*13, *ald*29*, ald*69*,* and *ald*78 mutants, showed significantly reduced relative survival (and recovery) inside the macrophages (40.45%, 65.47%, 28.08%, 22.95% and 30.09% survival, respectively, with P-value = 0.0006, P-value = 0.0417, P-value < 0.0001, P-value = 0.0004, P-value = 0.0028, respectively). In short, the lack of the corresponding enzymes in the *ald5*Δ, *ald*29Δ, and *ald*69Δ mutant strains, significantly diminished survival after engulfment by macrophages.

The *ald5* and *ald*78 mutants had significantly reduced virulence in the invertebrate *Galleria mellonella* infection model:

Virulence of mutant strains of *C. neoformans in vivo* is often assayed using an invertebrate model, the larvae of the greater wax moth *Galleria mellonella*. To determine whether each of the corresponding Ald enzymes has a role in pathogenesis, in three independent experiments, 1×10^6 of the wild type or *ald5*Δ, *ald*13Δ, or *ald*78Δ mutant *C. neoformans* H99 strains were injected into ten healthy larvae in parallel.

The results of a representative experiment demonstrated increased survival of the *G. mellonella* larvae that were infected with any of the three *ald* mutant *C. neoformans* strains, when compared with those infected with the wild type H99 strain (Figure 2.15). Specifically, larvae infected with wild type H99 cells started dying on day 6 post-injection and all such larvae were dead by day 9, while at least some larvae infected with the *ald5*Δ, *ald*13Δ, or *ald*78Δ strain survived until day 12, 13, or 17 respectively, with the *ald*78Δ strain-infected larvae starting to die only on day 9. For comparison, the two negative controls, larvae injected with PBS or with heat-killed wild type H99 cells, only started to die on day 8, and at least some larvae survived until day 18 at minimum. Larvae injected with the *ald*78Δ strain did not display any significant difference from the negative control larvae. The median survival time of wild type H99-injected larvae was 8 days, whereas the median survival time of *ald5*Δ strain-injected larvae was 8.5 days, and that of *ald*13Δ strain-injected larvae was 8.5 days. The *ald*78Δ strain-injected

larvae had a median survival time of 12 days. The survival curve of the *ald5*Δ strain-injected larvae was statistically different from that of the wild type H99-injected larvae (P-value = 0.0243, as determined using a Gehan-Breslow-Wilcoxon test, or, according to the Log-Rank test, P-value = 0.0188), but the survival curve of the *ald*13Δ strain-injected larvae was not statistically different from that of the wild type injected larvae (P-value = 0.1386 using the Gehan-Breslow-Wilcoxon test, or, using the Log-Rank test, P-value = 0.0734). The *ald*78Δ strain-injected larval survival curve was extremely statistically significantly different from the survival curve of larvae injected with wild type *C. neoformans* (P-value < 0.0001 from both statistical tests). These results indicate that the absence of Ald5 has a greater effect on the virulence of *C. neoformans* than that of the aldehyde dehydrogenase deficient in the *ald*13Δ mutant, but still allows for pathogenicity, unlike a deficiency of the aldehyde dehydrogenase deficient in the *ald*78Δ mutant, which appears to abolish virulence.

Discussion

C. neoformans has evolved several mechanisms to survive inside the host system by producing virulence factors like the capsule and melanin, enzymes like phospholipase B, urease, and proteases, and metabolites like trehalose, ethanol and acetate (Ma and May., 2009; Coelho *et al*., 2014; Bubb *et al*., 1999; Wright *et al*., 2002). This pathogen also shows the ability to switch to different carbon sources and use them as sources of energy whenever necessary, and therefore can survive in host tissues with low glucose (Hu *et al.*, 2008; Price *et al.*, 2011). One of the carbon sources that *C. neoformans* can depend on is acetate, which the pathogen can convert into acetyl-CoA to use in the tricarboxylic acid cycle, gluconeogenesis, or the glyoxalate cycle (Hu *et al.*, 2008).

It has also been seen that high amounts of acetate are produced by *C. neoformans*, both *in vitro* and after infection of lung tissue in a mouse model (Bubb *et al*., 1999; Wright *et al*., 2002; Hu *et al*., 2008). Acetate is believed to provide a survival advantage to the pathogen, among other ways, through its effect on pH. Unlike other fungi, such as *Aspergillus fumigatus* or *Candida albicans*, *C. neoformans* grows only in a specific pH range; however, this range includes a pH of 7.4, which is the pH of human blood and cerebrospinal fluid, as well as in the more acidic environment inside the macrophage phagolysosome, with a pH of 4.0-5.0 (O'Meara et al., 2010; Nyberg et al., 1992), which it actually prefers. When growing in a location in the host body that has a pH outside this preferred acidic range, such as in cerebral cryptococcomas, the pathogen secretes excess acetate

into the infected tissue in order to decrease the pH of the local environment (Wright *et al.*, 2002), thereby optimizing the activity of phospholipase B, and potentially other enzymes, at the site of cryptococcal infection (Evans *et al*., 2015; Sharon *et al.*, 2000). Reduced pH in the environment around *C. neoformans* can shield the pathogen from an immune attack by either reducing the migration of neutrophils or increasing their necrosis, as well as by permitting the neutralization of free radicals and decreasing the formation of superoxide, thereby limiting the ability of immune cells to use such chemicals to kill infecting cryptococci (Hu *et al.*, 2008; Wright *et al.*, 2002).

The exact pathway(s) by which *C. neoformans* produces acetate is unknown. In *S. cerevisiae*, the primary biosynthetic pathway for cytosolic acetate production during growth on glucose is the pyruvate dehydrogenase by-pass, which utilizes pyruvate decarboxylase; in this pathway, pyruvate, originally produced via glycolysis, is decarboxylated to form acetaldehyde, which is oxidized to acetate by one or more of the aldehyde dehydrogenase (Ald) enzymes functioning as an acetaldehyde dehydrogenase (Saint-Prix *et al*., 2004; Remize *et al*., 2000; Pronk *et al*., 1996). Not only does *C. neoformans* express similar enzymes, but mRNAs encoding both pyruvate decarboxylase and an aldehyde dehydrogenase are increased in *C. neoformans* cells recovered from the lung tissue of infected mice (Hu *et al*., 2008) or from the cerebrospinal fluid of a rabbit cryptococcal meningitis model (Steen *et al*., 2003). An aldehyde dehydrogenase has also been shown to be upregulated following engulfment by macrophages

(Derengowski *et al*., 2013). This suggests that the Pdc-Ald pathway may be used by *C. neoformans* to generate acetate, which may augment the virulence of the pathogen. However, *C. neoformans* may also use Pdc and Ald to enhance virulence in other ways. To better understand the functions of *C. neoformans* aldehyde dehydrogenases in growth and pathogenicity, mutants for the genes encoding aldehyde dehydrogenase enzymes were analyzed via various assays. This approach was complicated by the fact that nine different genes coding for different aldehyde dehydrogenases exist in the *C. neoformans* genome. These aldehyde dehydrogenases may have a wide array of functions, including reducing the toxicity of chemicals that the fungus may encounter, or producing various metabolites.

Like *C. neoformans*, *S. cerevisiae* has multiple aldehyde dehydrogenases, so a review of the functions of the different *S. cerevisiae* enzymes and of their roles in survival under stress can inform the analysis of the results obtained in *C. neoformans* in the assays described above. To cope with the continuous stress they are subjected to during growth, most yeast species have devised stress detection and response mechanisms, including the high osmolarity glycerol (HOG) response pathway and pathways that respond to oxidative stress and DNA damage, heat shock, and nutritional starvation (Brewster *et al*., 1993; Hohmann, 1997; Siderius *et al*., 1997; Estruch, 2000). Most relevant here, the accumulation of ethanol and acetaldehyde in growing cells can eventually lead to toxic cell death (Ingram and Buttke, 1985; Jones, 1990), so yeast utilize alcohol dehydrogenase

and one or more of the aldehyde dehydrogenases, respectively, to metabolize them into less toxic molecules (Aranda and del Olmo, 2003). For example, more than one *S. cerevisiae* aldehyde dehydrogenase can function as an acetaldehyde dehydrogenase to oxidize acetaldehyde into acetate (Pritchard and Kell, 2002; Saint-Prix *et al*., 2004).

In *S. cerevisiae*, although the cytosolic aldehyde dehydrogenases Ald2p and Ald3p may not function as acetaldehyde dehydrogenases (Navarro-Aviño *et al*., 1999; Saint-Prix *et al*., 2004), the cytosolic Ald6p and the mitochondrial Ald5p, as well as the mitochondrial Ald4p specifically in *ALD6* deletion strains, all appear to convert acetaldehyde to acetate, such that these three can partly, but not completely, compensate for each other (Aranda and del Olmo, 2003; Meaden *et al.,* 1997; Saint-Prix *et al.,* 2004; Remize *et al.* 2000). For example, both Ald6p and Ald5p play an important role in acetate production, and deletion of either corresponding gene reduces acetate production and growth under anaerobic conditions; additionally, levels of ALD5 mRNA are upregulated during both exponential growth and fermentation (Remize *et al.* 2000; Saint-Prix *et al.,* 2004). Similarly, in the *ald6*Δ mutant, ALD4 mRNA is expressed earlier, and the levels of this mRNA and of Ald4p protein are increased to compensate for the loss of Ald6p with increased levels and possible accumulation of Ald4p (Saint-Prix *et al*., 2004). Thus, in an organism in which more than one aldehyde dehydrogenase exists, the enzymes might compensate for each other (Remize *et al.,* 2000). This would explain the results from the various *C. neoformans ald* mutants, which had

reduced, but not abolished, acetate production, indicating a similar partial redundancy of the various Ald enzymes.

Functions and evolutionary relatedness of aldehyde dehydrogenases in various species

Aldehyde dehydrogenases are not only present in fungi, but also in a vast array of species, since their substrates, organic compounds known as aldehydes, are present ubiquitously in nature (Perozich *et al.*, 1999). Accumulation of these aldehydes causes toxicity, so it is important to regulate their levels in the cells (Lindahl, 1992; Perozich *et al.*, 1999; Kozak *et al.*, 2014). The aldehyde dehydrogenase (ALDH) superfamily consists of a class of enzymes that catalyzes the oxidation of aldehydes to their corresponding carboxylic acids, which are less toxic to the cells (Perozich *et al.*, 1999). The various activities of these enzymes, including but not limited to this detoxification, have likely been critical throughout evolutionary history, since this superfamily is highly conserved in Eubacteria, Archaea, and Eukarya (Perozich *et al.*, 1999). As seen in *S. cerevisiae*, some ALDHs are localized to the mitochondria, while others are localized to the cytosol, depending on the specific enzyme (Marchitti *et al.*, 2008; Vasiliou and Nebert, 2005; Black *et al.*, 2009; Sládek *et al.*, 2003).

There are a large variety of ALDHs in nature, and they take part in different physiological processes and exhibit specificity for various substrates. Some ALDHs will act on only a few specific substrates, while others have a broader specificity, yet most ALDHs require the presence of NAD^+ or $NADP^+$ as a cofactor

in order to function (Lindahl, 1992; Yoshida *et al.*, 1998). Although most ALDH family members protect the cell by keeping in check the levels of harmful aldehydes, they also demonstrate functionality beyond detoxification; for example, aldehyde dehydrogenases are important for the synthesis of vital biomolecules like retinoic acid, folate, and betaine (Marchitti *et al.*, 2008; Vasiliou *et al.*, 2000; Sobreira *et al.*, 2011). ALDHs also modulate processes like cell proliferation, differentiation, and cell survival, mainly through participation in retinoic acid synthesis (Marchitti *et al.*, 2008). Some of the members of the ALDH superfamily also exhibit functions independent of their enzymatic activity. These alternate activities include acting as crystallins in the cornea, absorbing ultraviolet radiation there, and binding to hormones or other small molecules, including androgens, cholesterol, thyroid hormone, and acetaminophen (Pereira *et al.*, 1991; Marchitti *et al.*, 2008; Estey *et al.*, 2007), as well as roles in abiotic stress tolerance, male fertility/sterility, embryo development, and seed viability and maturation in plants (Kotchoni *et al.*, 2010). ALDH activity is also required for catalysis of the phytohormone indole-3 acetic acid (IAA) from indole-3-acetaldehyde (Cooney and Nonhebel, 1989; Basse *et al.*, 1996; Tam and Normanly, 1998; Fedorova *et al.*, 2005; Spaepen *et al.*, 2007; Reineke *et al.*, 2008). Moreover, ALDH proteins can participate in osmoregulation and can operate as antioxidants (Marchitti *et al.*, 2008; Vasiliou and Nebert, 2005).

The protein sequences of human and horse mitochondrial and cytosolic ALDHs, as well as the sequence of rat dioxin-inducible ALDH, have been known

since as early as 1988 (Von Bahr-Lindström *et al.*, 1984; Hempel *et al.*, 1985, 1989; Johansson *et al.*, 1988). In 1999, Perozich and coworkers investigated the evolutionary relationships between 145 ALDH family members, spanning multiple phyla. This led to the identification of several conserved residues and motifs in the ALDH superfamily (Perozich *et al.*, 1999). There are ten highly conserved motifs, all clustered around the active site, suggesting the importance of these motifs to the function of all ALDH enzymes, regardless of specific substrate (Perozich *et al.*, 1999). Perozich *et al.,* (1999) reported that there were 13 ALDH families, but this number has been increased to 20 (Sophos and Vasiliou, 2003), following the availability of improved and more efficient sequencing and alignment technologies and access to sequences of more aldehyde dehydrogenases. In fact, a great deal of information is now available for gene and protein sequences of far more ALDH-encoding genes (usually referred to as *ALD* genes) than previously analyzed. As of 2002, 555 *ALD* gene sequences were reported, including 351 from Eubacteria, 32 from Archaea, and 172 from Eukarya (Sophos and Vasiliou, 2003).

The evolutionary relatedness of the nine *C. neoformans* aldehyde dehydrogenases to each other and to enzymes from other fungal species was analyzed (Figure 2.2), to attempt to discern specific roles for each *C. neoformans* aldehyde dehydrogenase. Three important observations were made from the phylogenetic tree. First, the nine proteins could be divided principally into two major groups based on their predicted common ancestor. CNAG_01078 (Ald78), CNAG_05113 (Ald13), and CNAG_06628 (referred to as Ald5 because that is the

official designation of the protein) formed the first monophyletic group, whereas CNAG_06010 (Ald10), CNAG_06018 (Ald18), CNAG_05029 (Ald29), CNAG_03269 (Ald69), CNAG_00735 (Ald35), and CNAG_02377 formed a second monophyletic group. Within this second group, Ald10 and Ald18 are most closely related, and then most closely related to Ald29, then to CNAG_02377, next to Ald69, and finally to Ald35. All of the enzymes in this second group are evolutionarily very distant from the aldehyde dehydrogenases of *S. cerevisiae* and *A. nidulans*, which was the second major observation.

The above two major groups could be further divided into six groups, in which one or more of the proteins showed evolutionary relatedness to specific aldehyde dehydrogenases from other species. For example, CNAG_05029 (Ald29), the predicted meiotic sister-chromatid recombination aldehyde dehydrogenase in *C. neoformans var grubii*, is closely related to the meiotic sister-chromatid recombination aldehyde dehydrogenase of *Coprinopsis cinerea*, supporting the prediction of a role for Ald29 in sister-chromatid exchange, and also to the betaine aldehyde dehydrogenase of *Phycomyces blakesleanus*. These three enzymes are predicted to have oxidoreductase activity, utilizing NAD^+ as an electron acceptor.

Ald18 (CNAG_06018) and Ald10 (CNAG_06010) were found to be closely related, not only to each other, but also to aldehyde dehydrogenases in *Cryptococcus gattii* and *Phanerochaete chrysosporium*, suggesting that they could function in a similar role in *C. neoformans* as these enzymes. Unfortunately, the

specific functions of those enzymes in *C. gattii* and *P. chrysosporium* are not known. Nevertheless, the phylogenetic relatedness of CNAG_06018 and CNAG_06010 is based on their similarity in amino acid sequence, indicating these proteins are sufficiently alike that they may have redundant or overlapping functions.

*ALD*10 and *ALD*18 (CNAG_06010 and CNAG_06018, respectively) are not only evolutionarily closely related to each other, but also physically close to each other in the same chromosome, which might lead one to speculate that they may be similar to the *S. cerevisiae* genes *ALD2* and *ALD3*, which are also located in tandem (Navarro-Aviño *et al*., 1999). However, *S. cerevisiae* ald2p and ald3p are outgroups in the MEGA phylogeny tree and therefore are not closely related to any of the *C. neoformans ALD* genes assayed herein. According to the phylogenetic tree, they are not even closely related to each other, with Ald3p acting as the outlier group to all other aldehyde dehydrogenases.

Ald69 (CNAG_03269) was found to be most closely related to a *C. gattii* aldehyde dehydrogenase (CGB_G2310C), which, according to the Fungi Database (https://fungidb.org/fungidb/app/record/gene/CGB_G2310C), is in turn homologous to vanillin dehydrogenases from other fungal species, indicating that Ald69 may function as a vanillin dehydrogenase or other oxidoreductase. Ald35 (CNAG_00735) was found to be most closely related to an NAD-aldehyde dehydrogenase in *P. chrysosporium* (AGR57_4875), which the Fungi Database

indicated is homologous to succinate-semialdehyde dehydrogenase enzymes from other species, suggesting that Ald35 may have this function in *C. neoformans*.

Ald78 (CNAG_01078) and Ald13 (CNAG_05113) were found to be most closely related to an aldehyde dehydrogenase from *Cryptococcus deuterogattii*; unfortunately, a search in the Fungi Database for this protein did not reveal a more specific function. However, these aldehyde dehydrogenases, along with Ald5 (CNAG_06628) and the equivalent aldehyde dehydrogenase in the KN99 strain, were determined to be similar to each other and evolutionarily related to *S. cerevisiae* aldehyde dehydrogenases Ald4p (YOR374W), Ald5p (YER073W), and Ald6p (YPL061W) that all act on acetaldehyde as a substrate and/or produce acetate under different conditions (Aranda and del Olmo., 2003; Saint-Prix *et al*., 2004). This was the third major observation based on the phylogenetic analysis, and it was an important one, since it strongly suggests that one or more of CNAG_01078, CNAG_05113, and CNAG_06628 (Ald5) are most likely to be the acetaldehyde dehydrogenase(s) in *C. neoformans* that function in acetate production.

All *ald* mutants have reduced acetate production

In order to determine whether any of the eight out of nine *C. neoformans* Alds for which mutants were obtained from the Madhani library, especially Ald5, Ald13, or Ald78, can function as an acetaldehyde dehydrogenase (leaving CNAG_02377 as the last option if not), all of the mutants were confirmed to be deleted for the corresponding gene and assayed for production of acetate. It was

observed that acetate production was decreased in each of the mutant strains relative to the wild type strain (Figure 2.6), but, interestingly, was not completely eliminated. In other words, a complete loss of acetate production did not occur in cells deficient for any given Ald, suggesting that when a single *ALD* gene is deleted, either one or more of the aldehyde dehydrogenases encoded by the other genes may partially compensate and produce acetate. As mentioned above, this has been previously shown for the *ald6* mutant in *S. cerevisiae*, in which the yeast compensates for the deletion of *ALD6* by upregulating *ALD4* (Saint-Prix *et al*., 2004). Alternatively, other pathways not utilizing aldehyde dehydrogenases, such as one employing Xfp1/2 and Ack, may be used for the production of acetate when one of the *ALD* genes is lost, or may contribute to acetate production in general (Figure 2.1). The retention of some degree of acetate production disproves the hypothesis that only one of Ald78, Ald13, or Ald5 is the sole *C. neoformans* acetaldehyde dehydrogenase, but it strengthens the hypothesis that one of these may be the major acetaldehyde dehydrogenase, with the other Alds acting as "backup." Therefore, it is informative to review the results of the other assays to determine the relative contribution of the various aldehyde dehydrogenases to the degradation of acetaldehyde (to prevent toxicity) and to the production of acetate, as well as to identify clues as to the alternative, specific functions of the eight aldehyde dehydrogenases for which mutants were available.

Growth assays reveal abnormalities in three *C. neoformans ald* mutants

The first of these other assays measured growth using glucose or alternative primary carbon sources. In order to propagate and disseminate inside the host body despite possible location-to-location differences in the availability of glucose, *C. neoformans* utilizes different carbon sources to obtain energy, including glucose, ethanol, and acetate (Hu *et al.*, 2008; Price *et al.*, 2011). Therefore, the growth of the mutants on glucose, ethanol, or acetate supplemented minimal media was compared, both qualitatively and quantitatively, with the growth of the wild type strain, to determine whether each of the aldehyde dehydrogenases was necessary for the growth on alternative carbon sources, and therefore in virulence.

The *ald5*Δ mutant had a growth defect when it was provided with ethanol instead of glucose as the primary carbon source (Figures 2.4c and 2.5c). Under anaerobic conditions, pyruvate, produced from glucose, or ethanol, via alcohol dehydrogenase, can be converted to acetaldehyde and then to acetate in order to store energy (within NADPH) in *S. cerevisiae* (Pronk *et al*., 1996; Cheung *et al*., 2017). At the same time, acetaldehyde is toxic to *S. cerevisiae* at or above certain levels (Aranda and del Olmo, 2003; Aranda and del Olmo, 2004), so an inability to convert it to acetate could kill the cells. In *C. neoformans,* when ethanol is the sole or primary carbon source, as it was when the *ald5*Δ mutant was unable to grow normally, the ethanol is likely converted to acetaldehyde by the fungus, even under the aerobic conditions of the experiment. It is therefore possible that the growth

defect of the *ald5*Δ mutant may be due to accumulation of acetaldehyde to toxic levels if deletion of *ALD5* results in deficiency of the acetaldehyde dehydrogenase that converts the acetaldehyde to acetate. In other words, the growth defect on ethanol suggests that *ALD5* encodes the principal acetaldehyde dehydrogenase and that, to the extent that any mutant cells are able to grow on ethanol, the aldehyde dehydrogenases encoded by the other *ALD* genes may partially compensate by processing the acetaldehyde at lower rates of conversion.

In contrast, the growth assays did not support a role for the enzyme deficient in the *ald*13Δ strain as an acetaldehyde dehydrogenase. The *ald*13Δ mutant displayed small colonies in the spot assay when grown using acetate or ethanol (Figure 2.4b or c, respectively), but otherwise grew normally, including producing normal size colonies when grown using glucose as the carbon source (Figure 2.4a). It also grew normally in the quantitative growth curve assay in liquid media (Figure 2.5), in which colonies do not form, indicating that deficiency of the Ald13 enzyme has a relatively minor, non-cell autonomous effect on the growth of *C. neoformans*, since it did not affect the cells individually, only *en masse*. Thus, other than concluding that Ald13 facilitates the use of alternative carbon sources besides glucose, and that accumulation of its aldehyde substrate by multiple cells eventually hinders colony growth, it is difficult to discern the specific role for this enzyme based on the growth assays alone.

The *ald*69Δ mutant exhibited diminished growth (relative to the wild type strain) in media supplemented with glucose (Figure 2.4a, 2.5a), whereas it grew

normally on media containing acetate or ethanol as the primary carbon source (Figure 2.4 b and c; Figure 2.5 b and c), suggesting that the Ald69 enzyme has a role in a glucose metabolism pathway. One possible role for this aldehyde dehydrogenase is as a glyceraldehyde-3-phosphate dehydrogenase in glycolysis. However, two other *C. neoformans var. grubii* glyceraldehyde-3-phosphate dehydrogenases, CKF44_06699 and CKF44_004057 (FungiDB; http://fungidb.org/fungidb/), have been identified, and their protein sequences are not similar enough (*i.e.* sharing only 17.67% and 16.67% identity, respectively) with that of Ald69 to support this hypothesis. Nevertheless, the fact that the *ald*69Δ mutant can grow using acetate or ethanol, but not glucose, supports a role for the enzyme in an upstream part of a glucose metabolism pathway, and not a role as an acetaldehyde dehydrogenase.

Capsule defects and loss of cell wall integrity was observed in the *ald* mutants

In addition to the use of organic molecules as carbon sources to obtain energy from the environment, *C. neoformans* also uses them to form, among more complex molecules, carbohydrates employed as virulence factors. For example, acetate, via acetyl-CoA, is important in acetylation of the GXM capsule polysaccharide that constitutes 90-95% of the capsule carbohydrates, and this acetylation is necessary for the GXM that is shed from the capsule to be able to reduce neutrophil migration (Rakesh *et al.*, 2008; Vecchiarelli *et al.*, 2011; Hu *et al.*, 2008; Ellerbroek *et al.*, 2004a, b, c). Additionally, GXM or other capsule

polysaccharides, when shed into the extracellular space, can inhibit the ability of host immune cells to generate and secrete pro-inflammatory cytokines (Li *et al*., 2018; Siegemund and Alber, 2008; Villena *et al*., 2008), deplete complement proteins, and reduce the migration of immune cells, including leukocytes, to sites of infection (Li *et al*., 2018; Ellerbroek *et al*., 2004a,b,c). These capsule polysaccharides, when shed near or within macrophages, can also directly interfere with phagocytosis and with the ability of macrophages to present peptides from phagocytosed fungal cells via their surface MHC molecules (Li *et al*., 2018; Siegemund and Alber, 2008; Villena *et al*., 2008), and even cause macrophage death, as well as induce macrophages to express Fas ligand and thereby trigger apoptosis of nearby Fas receptor-expressing T cells (Monari *et al*., 2005; Ben-Abdallah *et al*., 2012; Lupo *et al*., 2008; Vecchiarelli *et al*., 1994b). The shed polysaccharides can also drive down the T-cell response in other ways and interfere with the activation and proliferation of T cells (Vecchiarelli *et al*., 2011; Yauch *et al*., 2006). Hence, capsule polysaccharides are used for a number of virulence-enhancing immunomodulatory functions and the fungus may use acetate to enhance these functions.

The intact capsule is also important for *C. neoformans* virulence. Upon entering the lungs, *C. neoformans* attempts to evade phagocytosis by macrophages by enlarging its polysaccharide capsule (Okagaki *et al.*, 2010; Zaragoza *et al.*, 2010; Okagaki and Nielsen, 2012; Levitz and Tabuni, 1991; Xie *et al.*, 2012; Sabiiti and May, 2012; May *et al.*, 2016). A larger capsule prevents

macrophages from phagocytosing the fungal cells and allows any phagocytosed *C. neoformans* to neutralize the destructive reactive oxygen species produced by the macrophage (Giles *et al*., 2009, Velagapudi *et al*., 2009). Capsule attachment to the cell wall depends on the chitin monomer, β-1,4 N-acetyl glucosamine, the production of which requires acetyl groups that can be generated from acetate (Ramos *et al.*, 2012; Vecchiarelli *et al.*, 2011; Fonseca *et al.*, 2009; Rodrigues *et al.*, 2008a; Huang *et al.*, 2002; Levitz, 2001; Van Dyke and. Wormley, 2018; Erwig and Gow, 2016; Hu *et al*., 2008). Although mutants that are defective in capsule acetylation are actually hypervirulent (Janbon *et al*., 2001), acetate production is likely very important in capsule formation, stability, and function.

It is possible that, through a negative effect on acetate production, or via other mechanisms, the lack of one or more of the aldehyde dehydrogenases could negatively affect capsule formation. Therefore, capsule formation/size in the *ald* mutants was compared to that of the wild type strain. All of the mutants had much thinner capsules than the wild type strain, except for the *ald*10Δ mutant, which also displayed a smaller capsule, but which was affected to a lesser degree (Figure 2.7). As a further step, the capsule and cell wall integrity of the mutant and wild type strains were assayed on media containing Congo red or SDS. By binding to (1,4) β-glucans (or to the chitin monomer, β-1,4 N-acetyl glucosamine), Congo red interferes with cell wall construction and with the attachment of the chitin and capsule to the cell wall, while SDS that traverses gaps in the capsule, chitin, or cell wall lyses the cells by dissolving the plasma membrane (García *et al*., 2015; Banks

et al., 2005; Ram and Klis, 2006; Baker *et al*., 2007; Gerik *et al*., 2005; Wood *et al*., 1983). Thus, in the assays, cells with incompletely formed capsules or cell walls will have growth defects on these media. While the SDS had no visible effect on colony growth of either the wild type or mutant strains, all of the mutants were more seriously affected than the wild type strain by the Congo red, and developed fewer, smaller colonies (Figure 2.13c), indicating a partial loss of capsule and/or cell wall integrity. In other words, the lack of ability to grow in media containing Congo red may reflect a greater permeability for the dye due to the thinner capsules of the *ald* mutants. The *ald5*Δ mutant (Figure 2.13c, second row from the top) while unable to grow as well as the wild type strain, was least affected by the Congo red, which may given the potential importance of acetate in capsule formation, weaken the argument for Ald5 being the principle acetaldehyde dehydrogenase. In contrast, the *ald*69Δ mutant exhibited the most severe growth defect. It did not grow at all if plated at low cell concentrations and grew poorly at higher concentrations of plated cells (Figure 2.13c, bottom row). This indicated that the enzyme lacking in the *ald*69Δ mutant is necessary for capsule and cell wall integrity, likely via a role in cell wall construction or attachment of the cell wall to the capsule; this may involve a function for the enzyme in the production or utilization of acetate. Alternatively or additionally, the failure to process a particular aldehyde due to a lack of the aldehyde dehydrogenase in each mutant may lead to accumulation of the respective aldehyde to levels that impede capsule formation. The aldehyde processed by the Ald69 enzyme might be the most toxic, potentially most strongly

inhibiting an enzyme, or inhibiting the most enzymes, involved in cell wall production or in maintaining cell wall and capsule integrity, while the aldehyde processed by Ald5 appears to be least inhibitory to the maintenance of capsule/cell wall integrity. This interpretation of the results also matches with the acetate production results, in which all of the mutants exhibited a similarly reduced ability to make acetate. If acetate production was the limiting factor in capsule and cell wall integrity, all of the mutants should have been equally affected by Congo red.

The *ald*69Δ mutant was sensitive to oxidative and nitrosative stress.

One of the reasons why capsule and cell wall integrity matter to *C. neoformans* virulence is that, upon engulfment by an alveolar (or other) macrophage, *C. neoformans* is chemically attacked by high concentrations of reactive oxygen and nitrogen species inside the phagolysosome, leading to its destruction if it cannot neutralize these free radicals (Missall *et al.,* 2004a). Therefore, to be pathogenic, *C. neoformans* that is unable to avoid phagocytosis must be able to survive oxidative and nitrosative stress, and, to do this, it must be able to maintain cell wall and capsule integrity (Gerik *et al.*, 2005). As mentioned above, the capsule protects against reactive oxygen and nitrogen species produced by phagocytes by neutralizing them and generally keeping them away from the cell wall (which acts as a second protective layer) and from the cell membrane (Zaragoza *et al.*, 2008; Zaragoza, 2011). Furthermore, if one or more of the aldehyde dehydrogenases that are variously deficient in the mutants produces acetate, since acetate itself or the change in pH it can induce in the

environment protects the pathogen from the ROS and RNS produced by macrophages (Giles *et al*., 2009, Velagapudi *et al*., 2009), the mutant lacking the appropriate Ald enzyme should be more sensitive to oxidative or nitrosative stress. Thus, it was important to determine whether the various *ald* mutants were more sensitive to ROS or RNS than the wild type strain. All strains were grown on plates with hydrogen peroxide as an ROS source or sodium nitrite as an RNS source (Figures 2.10 and 2.11, respectively). Surprisingly, in both cases only the *ald*69Δ mutant was affected. Under oxidative stress, the *ald*69Δ mutant grew poorly (Figure 2.10b, bottom row), while under nitrosative stress it turned pink (Figure 2.11b, bottom row), a phenotype reversed when adenine was added to the media with the sodium nitrite (Figure 2.11c, bottom row). This indicated that the *ald*69Δ mutant was inhibited in the production of adenine, such that the accumulation of either of two red intermediates in the adenine biosynthesis pathway caused the color change, whereas providing adenine to the cells eliminated the need to use the pathway and produce the intermediate. The same phenotype has been observed in *S. cerevisiae* mutants with deficiencies in adenine production (Smirnov *et al*., 1967). Whether the lack of the enzyme deficient in the *ald*69Δ mutant caused inhibition of the adenine production pathway via the lack of the enzyme product or by accumulation of the aldehyde substrate of the enzyme, is unknown. Likewise, whether an already existing suppression of the adenine biosynthesis pathway due to lack of Ald69 was enhanced by nitrosative stress or the nitrosative stress activated the adenine synthesis pathway, which was then inhibited downstream by

the lack of Ald69, is also unknown. Together with the growth defect on glucose observed for the same mutant, it does seem that the *ald*69Δ mutant may have a more general growth defect than that expected from a role as an acetaldehyde dehydrogenase or from a lack of acetate production alone. Regardless, it is interesting that only the *ald*69Δ mutant, and not the *ald5*Δ, *ald*13Δ, or *ald*78Δ mutant, or, for that matter, any other aldehyde dehydrogenase deficient mutant, exhibited sensitivity to both ROS and RNS.

Melanin production is altered in the *ald*69Δ as well as in the *ald5*Δ mutant

The correlation between resistance to ROS/RNS and virulence is well established, indicating that more than one mechanism must be used by virulent cryptococci to neutralize free radicals. Indeed, secondary to the capsule, melanin production and deposition in the cell wall promotes resistance to ROS and RNS (Wang *et al.*, 1995; Xie *et al.*, 1997; Wang and Casadevall, 1994) because melanin is itself a stable free radical that can react with and directly neutralize ROS/RNS (Jacobson and Emery, 1991a; Jacobson and Tinnell, 1993; Steenbergen and Casadevall, 2003). Melanin is therefore used by fungal cells to provide protection from oxygen and nitrogen derived radicals produced by macrophages, thus protecting *C. neoformans* from immune attack, by scavenging these free radicals (Samarasinghe *et al*., 2018; Jacobson and Tinnell, 1993; Polacheck and Kwon-Chung, 1988). In fact, melanin deficient mutants of *C. neoformans* that produce 100-fold less melanin are less virulent than melanin producing strains (Wang *et al*., 1995).

C. neoformans synthesizes melanin (or at least a black, melanin-like pigment) from chemicals obtained from host cells or from the environment, namely, catecholamines like L-DOPA, and potentially, aldehydes (Williamson, 1997; Casadevall *et al*., 2000; McFadden and Casadevall, 2001; Steenbergen and Casadevall, 2003; Frases *et al.*, 2007; Eisenman *et al.*, 2007). Conversely, lack of melanin production may make a mutant more vulnerable to aldehyde accumulation resulting from ROS/RNS-mediated membrane damage. A failure to counteract oxidative stress leads to oxidative degradation of cell membrane lipids (*i.e.* lipid peroxidation), which gives rise to hundreds of different reactive and toxic aldehydes (Singh *et al.*, 2013). Thus, *C. neoformans* aldehyde dehydrogenases may be necessary to protect against the effects of ROS and RNS by reducing the levels of those aldehyde by-products (Singh *et al.*, 2013). The *ald*69Δ mutant produced abnormally low levels of melanin, which may be directly related to the growth defect under oxidative and nitrosative stress also exhibited by this mutant. In contrast, the *ald5*Δ mutant appeared more melanized than the wild type strain, which should have made it more resistant to ROS/RNS than the wild type strain, but it was not. Thus, the mechanisms for the melanin phenotypes of the two mutants appear to be divergent.

Melanin, and the laccase enzyme that produces it, are located in the cryptococcal cell wall (Perez-Dulzaides *et al*., 2018; Walton *et al*., 2005; Zhu *et al*., 2001; McFadden and Casadevall, 2001), which allows the melanin to neutralize any free oxygen and nitrogen radicals before they can reach the cell membrane

(Wang *et al*., 1995; Wang *et al*., 1996). However, this localization also means that disruption of cell wall integrity leads to leaking out and loss of the melanin (Perez-Dulzaides *et al*., 2018). Along with low melanin levels, the *ald*69Δ mutant also exhibited significant defects in capsule formation and thickness, as well as in cell wall integrity in the Congo red assay. So, it is reasonable to hypothesize that the loss of cell wall integrity in the *ald*69Δ mutant is directly responsible for the lack of melanin, and that, in combination with a deficiency in the ability to eliminate a potentially toxic aldehyde from the cell, this results in increased cell death under oxidative stress in the *ald*69Δ mutant. The reduced capsule thickness observed in the *ald*69Δ mutant supports this melanin leakage hypothesis. However, since melanization of the cell wall increases cell wall thickness (McFadden and Casadevall, 2001; Feldmesser *et al*., 2001), it is also possible that the lack of melanin contributes to the lack of cell wall integrity, either causatively or in a negative feedback cycle. The block in the adenine synthesis pathway observed in the *ald*69Δ mutant under nitrosative stress may be an indicator of a constitutive defect (that is simply enhanced in the presence of RNS) resulting in generally low cyclic AMP (cAMP) levels. Since laccase function is regulated by a signal transduction pathway that is dependent on cAMP (Pukkila-Worley *et al*., 2005), low levels of cAMP in the *ald*69Δ mutant could result in low laccase activity, low melanin production, and low cell wall integrity, in that order.

As mentioned above, in contrast with the *ald*69Δ mutant, the *ald5*Δ mutant produced more melanin. The capsule of the *ald5*Δ mutant is no larger than the

capsule of the *ald*69Δ mutant, precluding the possibility that more melanin is simply being retained. Therefore, the simplest explanation for the increased pigmentation of the *ald5*Δ mutant is that deficiency of the Ald5 enzyme lead either to a lack of a metabolic product that normally inhibits laccase activity or to an excess of the aldehyde substrate of Ald5, which then accumulated and was used as a substrate by one or both laccases to produce more melanin (Williamson, 1997). Another possibility is that the *ALD5* gene might enhance or play a role in the high osmolarity glycerol/*HOG1* pathway that inhibits melanin production specifically in serotype A strains of *C. neoformans*, so a deficiency of *ALD5* lead to increased melanin in the KN99α-derived, and thus serotype A (Nielsen *et al*., 2003), *ald5*Δ mutant strain assayed herein, similar to that observed in the *hog1* mutant of this serotype (Bahn *et al*., 2005). However, if the *ALD5* deficiency does interfere with the high osmolarity glycerol pathway, then the *ald5*Δ mutant should have had an abnormal response to hyperosmolarity in the NaCl/KCl osmotic stress assay, which neither it nor any other *ald* mutant exhibited. Yet, it is still possible that Ald5 directly or indirectly inhibits melanin production downstream of the *HOG1* pathway, or that the aldehyde accumulated in the *ald5*Δ mutant inhibits the *HOG1* pathway downstream of the response to hyperosmolarity. The substrate or product of Ald5 may also regulate melanin production by influencing some other signaling pathway.

Since melanin pigmentation was found to be unaltered in the other *ald* mutants, either the corresponding genes play no role in the regulation of melanin

production or the corresponding enzymes are somewhat redundant in function, so loss of one aldehyde dehydrogenase is compensated for by the other enzymes and thus has no effect on melanin production. In either case, it is clear that at least Ald5 plays either a metabolic or regulatory role in melanin production, regardless of whether or not it functions in acetate production, while the enzyme deficient in the *ald*69Δ strain plays a role either in melanin production or in cell wall or capsule integrity, or both.

Only the *ald*29Δ mutant has a defective hypoxia response, which is rescued by the presence of acetate

After dissemination, *C. neoformans* encounters and must cope with a hypoxic state in infected tissues (Erecińska and Silver, 2001; Kronstad *et al.*, 2012; Chang *et al.*, 2007). For example, oxygen concentrations in the human brain are significantly lower, at 1-5%, than atmospheric levels and levels present in the lungs (*i.e.* ~14-21% oxygen), requiring *C. neoformans* to adapt to this hypoxia to infect the brain (Erecińska and Silver, 2001; Kronstad *et al.*, 2012; Chang *et al.*, 2007). Growth under hypoxia forces the use of fermentation, in which acetaldehyde dehydrogenase activity may be utilized by wild type *C. neoformans* to produce acetate and NADPH. So, a growth defect of an *ald* mutant under hypoxia might signify a deficiency of the acetaldehyde dehydrogenase function of the corresponding enzyme. Therefore, the survival/growth of the wild type and mutant strains under conditions mimicking hypoxia was assayed.

Many mammalian studies have used cobalt chloride ($CoCl_2$) to induce hypoxia-mimicking conditions (Goldberg *et al.*, 1987; Wang and Semenza, 1993a,b; Wang *et al.*, 2000; Huang *et al.*, 2003; Grasselli *et al.*, 2005). Likewise, it has been shown that growth of fungi on media containing $CoCl_2$ can be used to mimic hypoxia in fungal assays, although the mechanism of action is different from that in animals (Goldberg *et al.*, 1987; Wang and Semenza, 1993a, b; Lee *et al.*, 2007; Chun *et al.*, 2007; Chang *et al.*, 2007). In fungi, including in *C. neoformans*, $CoCl_2$ induces the Tco1/HOG pathway, as well as expression and activation of Sre1p and Scp1p, which drive the oxygen dependent, and therefore oxygen sensing, sterol synthesis pathway; $CoCl_2$ does this in the same way as hypoxia itself, making the fungal cells behave as if they are in low oxygen (Lee *et al.*, 2007; Chun *et al.*, 2007; Chang *et al.*, 2007; Bahn *et al*., 2005). Hence, the growth of the *ald* mutant and wild type strains on cobalt chloride containing media was assayed. Of note, cobalt chloride treatment also increases the production of ROS (Guzy *et al.*, 2007), since the cobalt ion in the cobalt chloride can drive the production of ROS via the Fenton reaction just like iron (Ingavale *et al*., 2008), so results from the hypoxia and oxidative stress assays were analyzed together to determine if any mutant phenotype was due to hypoxia or ROS.

Only the *ald*29Δ mutant (and not the *ald5*Δ nor the *ald*69Δ strain) was sensitive to the use of cobalt chloride to mimic hypoxia. Moreover, this sensitivity was due to the lack of ability of the mutant to respond properly to hypoxia, and not due to a sensitivity to ROS or RNS, since this mutant grew normally on media with

hydrogen peroxide or sodium nitrite. This, together with the finding that supplementing $CoCl_2$ treated plates with acetate restored normal growth of the *ald*29Δ mutant, strongly suggests that the corresponding aldehyde dehydrogenase functions in fermentation and acetate production. Therefore, it is possible that Ald29 functions as a major acetaldehyde dehydrogenase and produces acetate, despite the lack of homology to the major acetaldehyde dehydrogenases in *S. cerevisiae*. The specific requirement for the Ald29 enzyme for cryptococcal survival under low oxygen conditions indicates that this Ald is a virulence factor in *C. neoformans* and that either it plays a role in the production of acetate by an anaerobic, fermentation-based pathway such that supplementing with acetate eliminates the need for the enzyme, or it functions in a hypoxia response pathway that can be replaced by a secondary, acetate-dependent pathway.

In vitro and _in vivo_ virulence assays reveal a role for several Alds in pathogenicity

Once the effects of a deficiency of the various aldehyde dehydrogenases on survival under the assortment of stresses associated with infection of a host were determined, the next logical step was to test the effects on virulence itself, using *in vitro* and *in vivo* assays. Part of the virulence for *C. neoformans* involves evading or surviving phagocytosis. *C. neoformans var neoformans* isolated from the lungs of previously infected mice express high levels of an ALD transcript (Hu *et al.*, 2008) encoding a protein with 96.25% sequence identity to Ald5 from *C. neoformans var. grubii*. Moreover, after ingestion by macrophages, *C. neoformans*

var grubii upregulates expression of *ALD5* (Derengowski *et al.*, 2013). This indicates that expression of this aldehyde dehydrogenase is important to protect the fungal cell from phagocytosis or from destruction by the macrophage after engulfment. Expression of other *ALD* genes may also be required to survive phagocytosis. Furthermore, the smaller capsules observed for the aldehyde dehydrogenase mutants in the capsule formation assay should make these mutants more prone to phagocytosis and destruction by macrophages. Therefore, the effect of deficiency of each Ald, in the corresponding knockout mutant, on phagocytosis and survival after engulfment was quantified in an *in vitro* macrophage assay (Figure 2.14). The *C. neoformans ald5*Δ, *ald*13Δ, *ald*29Δ, *ald*69Δ, and *ald*78Δ mutants exhibited statistically significantly reduced survival inside the macrophages and recovery afterwards, versus the wild type strain (40.45%, 65.47%, 28.08%, 22.95% and 30.09% survival after phagocytosis, respectively, with P-value = 0.0006, P-value = 0.0417, P-value < 0.0001, P-value = 0.0004, and P-value = 0.0028, respectively). This confirms a role for each of the corresponding Ald enzymes in virulence. However, it does not reveal their specific roles in pathogenesis. Whether the inability of the *ald5*Δ mutant to grow on ethanol plays any role in its low survival inside macrophages is uncertain, and the *ald5*Δ and *ald*69Δ mutants have opposite defects in melanization, eliminating a common melanin-production defect as the cause for low survival. While the growth defect of the *ald*29Δ mutant under hypoxia-mimicking conditions, and the dependence of this defect on the absence or presence of acetate, as well as the lack of cell wall

integrity and sensitivity to ROS/RNS of the *ald*69Δ mutant, are most likely relevant to the decreased survival of these mutants inside the macrophages, these phenotypes are not shared between *ald5*Δ, *ald*29Δ, and *ald*69Δ mutants, so no one phenotype is associated with reduced recovery from phagocytosis. Likewise, all of the mutants, including those that had normal survival after phagocytosis, exhibited smaller capsules and produced lower than normal levels of acetate, so these phenotypes, while potentially relevant, are not the cause of the reduced post-engulfment survival of the *ald5*Δ, *ald*13Δ, *ald*29Δ, *ald*69Δ, and *ald*78Δ strains. Thus, it appears that each of these mutants was more vulnerable to phagocytosis than the wild type strain due to the loss of individual pathways resulting from the deletion of the corresponding gene. Nevertheless, Ald5 and the enzymes deficient in the *ald*29Δ and *ald*69Δ mutants are clearly required for virulence of *C. neoformans*, at least *in vitro*.

For the *in vivo* virulence assay, the larvae of *Galleria mellonella*, commonly known as the greater wax moth, were used. *G. mellonella* is an ideal model to study the processes of *C. neoformans* infection and virulence. The larvae are quite easy to obtain, are inexpensive to grow and maintain in the lab since they can be kept in Petri dishes with wood chips, and can be infected or treated with various reagents via any one of multiple delivery systems, including topical application, oral delivery, or injection, without significantly traumatizing the insect (Fuchs *et al.*, 2010; Kavanagh and Sheehan, 2018). The latter property permits relatively quick infection of multiple larvae with specific fungal pathogens. *G. mellonella*

encounters various microbes in its natural environment and has therefore evolved the capacity to produce specific immune responses that are similar to those of potential mammalian hosts of *C. neoformans* (Fuchs *et al.*, 2010; Kavanagh and Sheehan, 2018). Specifically, the early phagocyte response can be studied using this model organism, as it possess 6 different types of phagocytic cells: prohemocytes, coagulocytes, spherulocytes, oenocytoids, plasmatocytes, and granulocytes; additionally, a change in the concentration of hemocytes subsequent to infection reflects the overall immune response (Arvanitis *et al.*, 2013; Tsai *et al.*, 2016). Moreover, the structural, cellular, humoral, and molecular level responses of these phagocytic cells are similar to those of the mammalian innate immune response (London, *et al.*, 2006). Furthermore, the ability of *G. mellonella* to grow at 37°C, which is human body temperature, as opposed to 25°C, the preferred temperature for many other non-mammalian model organisms, makes it an ideal system to study factors required for mammalian infection by fungi like *C. neoformans*, without the expense associated with a mammalian model organism (Fuchs, *et al.*, 2010; Kavanagh and Sheehan, 2018). To study the virulence of *C. neoformans*, the mortality rate of the larvae can be assessed over a short span of time after infection, due to a relatively short time course from infection to lethality, and thus, the importance of numerous *C. neoformans* genes in virulence can be investigated with high efficiency (Arvanitis *et al.*, 2013). Here, the wild type and certain *ald* mutant strains were injected into the larvae and their virulence was assayed *in vivo*. At the time that the *G. mellonella* larvae were available in the lab,

only a version of the *ald5*Δ, *ald*13Δ, and *ald*78Δ mutant strains in the H99 background, had been obtained. When these mutants were tested for their ability to kill the *G. mellonella* larvae, the *ald5*Δ strain exhibited statistically significantly reduced virulence relative to the wild type H99 strain, in that the larvae infected with *ald5*Δ mutant cells survived longer than the larvae infected with the same number of wild type cells (Figure 2.15). Larvae injected with the *ald*13Δ strain also survived longer than those injected with the wild type strain, but the overall difference in the larval survival curves was not significant, likely because the first larvae of each group died on the same day post-injection. The *ald*78 mutant exhibited severely reduced virulence, and larvae injected with this mutant had close to the same survival as larvae injected with PBS alone or with heat killed wild type cells, the two negative controls. These results confirm that Ald5 is required for full *C. neoformans* virulence *in vivo* just as it is *in vitro*, and strongly supports the *in vitro* assay result that the enzyme deficient in the *ald*78Δ strain is critical for the virulence of *C. neoformans*. It is possible that, if the virulence of the other mutant strains were assayed in the *G. mellonella* model, the *ald*29Δ and *ald*69Δ strains would also take longer to kill the larvae, and this should be tested in the future.

In summarizing the evidence for a role in overall pathogenicity across multiple assays, several Ald enzymes rise to prominence. Ald5 seems to be an important virulence factor, based on the results of the melanin production assay, the macrophage survival assay, and the *Galleria* infection/survival assay (Figures

2.8, 2.14, and 2.15, respectively). The enzyme deficient in the *ald*78Δ mutant is also important for pathogenicity, since the mutant has reduced survival after macrophage engulfment and severely attenuated virulence in *Galleria* larvae (Figures 2.14 and 2.15, respectively). The fact that the *ald*29Δ mutant is unable to grow under hypoxia-mimicking conditions unless acetate is provided (Figure 2.9b and 2.9c, respectively) and exhibits reduced survival/recovery after engulfment by macrophages relative to the wild type strain (Figure 2.14), strongly suggests that the corresponding enzyme is also required for *C. neoformans* virulence. The *ald*69Δ mutant not only lacked the ability to utilize glucose (Figure 2.4a and Figure 2.5a), but also exhibited a growth defect under oxidative stress (Figure 2.10), a mutant phenotype, albeit an unexpected one, under nitrosative stress (Figure 2.11), a severe growth defect when its cell wall integrity was challenged (Figure 2.13c), reduced levels of melanin compared with the wild type strain (Figure 2.8), and, finally, a statistically significant reduced survival after engulfment by macrophages (Figure 2.14), all indicative of a role for the corresponding enzyme in virulence.

Ald5 and Ald29 are the leading candidates for the *C. neoformans* acetaldehyde dehydrogenase

Following infection, *C. neoformans* may depend for survival inside the host on alternate carbon sources like acetate or ethanol (Ries *et al*., 2018), the latter of which is first converted to acetaldehyde and then, via acetaldehyde dehydrogenase, to acetate for use. The evolutionary relatedness of *C. neoformans*

Ald5 (CNAG_06628), Ald78 (CNAG_01078), and Ald13 (CNAG_05113) to the *S. cerevisiae* acetaldehyde dehydrogenases Ald4p, Ald5p, and Ald6p (Saint-Prix *et al.,* 2004), suggests that may be used by *C. neoformans* to produce acetate. The finding that the *ald5*Δ mutant was unable to grow on ethanol, both in the spot assay (Figure 2.4c) and in the liquid media quantitative assay (Figure 2.5c), is in line with a role for Ald5 as an acetaldehyde dehydrogenase. In contrast, although the *ald*13Δ mutant also exhibited a growth defect on acetate or ethanol supplemented minimal media, the lack of a cell autonomous mutant phenotype in the equivalent growth curve assays indicates that Ald13 is not likely to function as an acetaldehyde dehydrogenase. The *ald*78Δ mutant grew normally on ethanol, implying that Ald78 also is not an acetaldehyde dehydrogenase.

Ald29 is not as closely related to the *S. cerevisiae* acetaldehyde dehydrogenases, yet it is a candidate for the *C. neoformans* acetaldehyde dehydrogenase. The *ald*29Δ mutant is unable to grow under hypoxia mimicking conditions (Figure 2.9b), but recovers when provided acetate (Figure 2.9c), which suggests that the corresponding enzyme may be an acetaldehyde dehydrogenase, but, given the lack of a mutant phenotype under other stresses, may be used only under hypoxia. Under this hypothesis, deficiency of this enzyme prevents anaerobic production of sufficient levels of acetate for survival. One can speculate that the pathogen primarily uses Ald5 to produce acetate to alter the pH in an environment in which oxygen is present, for an immunomodulatory or other protective purpose, while using Ald29 to generate acetate and NADPH under

anaerobic/hypoxic conditions for use in fermentation and growth in ethanol. If so, Ald29 might have evolved an acetaldehyde dehydrogenase function independently, and not from any evolutionary relationship with Ald5, Ald78, or *S. cerevisiae* aldehyde dehydrogenases.

Potential future experiments

While the assay results described herein provide clues as to which *C. neoformans* aldehyde dehydrogenases may function as acetaldehyde dehydrogenases, greater certainty of this role might be possible with more evidence.

As mentioned above, the reduced (but not eliminated) acetate production in each *C. neoformans ald* mutant is similar to that observed in *S. cerevisiae* mutants deficient in Ald6p or deficient in Ald5p. In *S. cerevisiae* this was considered evidence for partial compensation for deletion of one *ALD* gene by the remaining enzymes via upregulation of the remaining aldehyde dehydrogenase genes (Aranda and del Olmo, 2003; Meaden *et al.,* 1997; Saint-Prix *et al.,* 2004; Remize *et al.* 2000). The hypothesis that the *C. neoformans* enzymes also partly compensate for each other via the same upregulation mechanism can be tested by quantifying the expression of the remaining *ALD* genes in each *C. neoformans ald* deletion mutant.

Additionally, quantifying the level of the mRNAs encoding each aldehyde dehydrogenase in wild type (or pyruvate decarboxylase deficient) *C. neoformans* grown on media supplemented with glucose, ethanol, acetate, or acetaldehyde might also reveal which Ald functions in acetaldehyde/acetate metabolism. When Aranda and del Olmo (2003) quantified the mRNA levels of the three *S. cerevisiae* cytoplasmic aldehyde dehydrogenases in fermentative and flor (industrial wine aging) yeast strains grown in glucose or ethanol containing media, they found that

ALD2 and *ALD3* expression is increased with growth in ethanol versus with growth in glucose, while *ALD6* expression is the opposite. Furthermore, yeast grown in glucose generally decrease expression of *ALD6,* while yeast grown in ethanol increase expression of ALD6, after acetaldehyde is added (Aranda and del Olmo, 2003). Conversely, flor yeast grown in glucose increase, while those grown in ethanol decrease, ALD2 and ALD3 mRNA levels when acetaldehyde is added (Aranda and del Olmo, 2003). These inverse expression patterns of the *S. cerevisiae* cytosolic aldehyde dehydrogenase mRNAs may be imitated by the *C. neoformans* aldehyde dehydrogenases. The major *C. neoformans* acetaldehyde dehydrogenase(s) should have an *ALD6*-like expression pattern in glucose or ethanol with and without acetaldehyde when measured by quantitative reverse transcription PCR (qPCR).

Additionally, based on the findings of Aranda and del Olmo (2003), as well as the SAGE analyses of Hu *et al*. (2008) and Derengowski *et al*. (2013) after cryptococcal infection of model organisms, qPCR or RNA-Seq experiments can be used to determine which genes involved in various metabolic and virulence pathways are upregulated or downregulated in the mutants grown on various carbon sources (*i.e.* glucose, ethanol, or acetate) with or without acetaldehyde or under various stresses. Changes in the gene expression profile of the mutants versus the wild type strain under these various assay conditions combined with a gene ontology analysis of the transcriptome could provide clues about the function

of the enzyme deficient in each mutant, including whether or not the enzyme is necessary to process acetaldehyde.

The results of these assays could also be correlated with measurement of acetate production by the strains grown with and without non-toxic levels of acetaldehyde, since one would expect that the mutant lacking acetaldehyde dehydrogenase function would not have increased acetate production through the conversion of the acetaldehyde, but all other mutants would increase the expression of the corresponding gene missing in this mutant.

It would be informative to conduct the assays involving growth under oxidative and nitrosative stress, growth on Congo red containing medium, and induction of capsule and melanin formation, all using media supplemented with acetate to see if the mutants that exhibit phenotypes in those assays are rescued by supplementation with acetate that they are perhaps unable to make on their own, just as acetate supplementation rescued the *ald*29Δ mutant in the hypoxia assay. If so, the deficient enzyme is likely to have a significant role in the production of acetate, likely as one of the acetaldehyde dehydrogenases.

It might also be informative to produce *ald* double and triple mutants in *C. neoformans*. Such mutants are informative in *S. cerevisiae*. For example, the reduced growth of the *ald4*, *ald6* double mutant compared with either single mutant indicated a previously overlooked role for Ald4p. The role of Ald5p in acetaldehyde and acetate metabolism is emphasized by the fact that an *ald4*, *ald5*, *ald6* triple knockout has a more severe phenotype, both in terms of a growth defect and in

terms of acetate production, than the *ald4*, *ald6* double mutant (Saint-Prix *et al*., 2004). In contrast, Ald2p and Ald3p were revealed to be irrelevant to acetate production based on the finding that the *ald2*, *ald3*, *ald4*, *ald6* quadruple mutant phenotype is no different than that of the *ald4*, *ald6* double mutant, and a mutant that is null for *ALD2*, *ALD3*, *ALD4*, *ALD5*, and *ALD6* has a similar phenotype to that of the *ald4*, *ald5*, *ald6* triple mutant (Saint-Prix *et al*., 2004). Thus, generation of double, triple, or quadruple *ald* mutant strains of *C. neoformans*, followed by measurement of their acetate production, might reveal the relative contributions of each *C. neoformans* Ald in the production of acetate, and assaying the growth and other phenotypes of these multiple mutants might uncover the relative importance of each Ald in virulence.

While the assays already conducted strongly suggest which aldehyde dehydrogenase enzymes are most important for virulence of *C. neoformans*, it might be informative to repeat the *Galleria* larval survival assay using the KN99α strain mutants (as opposed to the H99α-based mutants), in particular the *ald5*Δ, *ald*13Δ, *ald*29Δ, *ald*69Δ, and *ald*78Δ strains that exhibited significant differences from the wild type strain in one or more of the other assays described herein. On the other hand, while *G. mellonella* is an optimal invertebrate model to study the processes of *C. neoformans* infection and virulence, the study of dissemination of *C. neoformans* to various organs cannot be assessed using this model organism, due to vastly different visceral organs between insects and vertebrates and the lack of lungs and a true brain in *G. mellonella*. The macrophage survival assay can

address some of these concerns, since it uses mouse macrophages, making at least some analysis of post-infection host cell genome expression changes possible. However, in order to truly understand the progress of infection, vertebrate organisms, generally mice or zebrafish, must be used as infection models, especially to study the effect of deficiency of a particular *C. neoformans* aldehyde dehydrogenase on dissemination to the brain as a critical step in virulence. Moreover, the results already described from the *Galleria* survival assay may reflect a disparity between insect immune systems and mammalian immune systems (Tojo, S. *et al*., 2000), and the use of a mammalian model organism is required in order to verify these results. Therefore, it would be advisable to test the *ald5*Δ, *ald*13Δ, *ald*29Δ, *ald*69Δ, and *ald*78Δ strains, as well as the wild type strain, all in a KN99α background, for their virulence (along with PBS and heat killed wild type strain as negative controls) in a mouse model of pulmonary infection or cryptococcal meningitis.

Based on the results already obtained in this work, a complete analysis of these mutants, including the experiments proposed above, will likely reveal one or more of the corresponding aldehyde dehydrogenases to be a suitable target for pharmaceutical intervention to treat cryptococcal meningitis in humans and reduce its lethality.

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Table 2.1: List of genes/enzymes deficient in the mutants used in this study.

Name used in this study	Official Enzyme name†	Identification Number†	Chromosome†	Location‡
ald5	Aldehyde dehydrogenase (NAD)	CNAG_06628	7	Cytoplasmic
*ald*10	Aldehyde dehydrogenase (NAD), variant	CNAG_06010	12	Cytoplasmic
*ald*13	Aldehyde dehydrogenase (NAD)	CNAG_05113	4	Mitochondria
*ald*18	Aldehyde dehydrogenase (NAD), variant	CNAG_06018	12	Cytoplasmic
*ald*29	Meiotic Sister-Chromatid recombination aldehyde dehydrogenase	CNAG_05029	4	Cytoplasmic
*ald*35	Aldehyde dehydrogenase family 7 member A1	CNAG_00735	1	Mitochondria
*ald*69	Aldehyde dehydrogenase	CNAG_03269	8	Mitochondria
*ald*78	Aldehyde dehydrogenase (NAD)	CNAG_01078	5	Cytoplasmic

†The official enzyme name and identification number, as well as the chromosomal location of each corresponding gene, were obtained from the Fungi Database, https://fungidb.org/fungidb/app/record/gene/CNAG_06628.

‡The predicted mitochondrial or cytoplasmic cellular location of each protein was determined using the MitoFates website http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi; (Fukasawa *et al.*, 2015).

Table 2.2: List of primers used to verify deletion of *ALD* genes in mutant strains.

Mutant strain	ID #	Forward primer	Reverse primer	bp product
ald5	CNAG_06628	cgcttgttgaggatgcattg	tatcaaggtcccgctccatc	1496
*ald*10	CNAG_06010	caatctcctcgttcgccaag	ggtactgaagaagcacctcg	1381
*ald*13	CNAG_05113	gcaactggtgaatgagtccc	caagcttatcactgtcccgc	1473
*ald*18	CNAG_06018	tgaacccctcatgtcccttc	tgcttgatactcatacagcgc	1317
*ald*29	CNAG_05029	agtcgtcgagttctgcatcc	gcacccaaccacgaacattac	1641
*ald*35	CNAG_00735	tcgattgcttgtttccctcg	gatttggcgaacgacctctc	1630
*ald*69	CNAG_03269	ccgaaaagctcagtcactcg	ctcacgtttccagttcaccg	1559
*ald*78	CNAG_01078	acgtcacggaatttcttgcc	tctttgctggcggtttgaac	1718

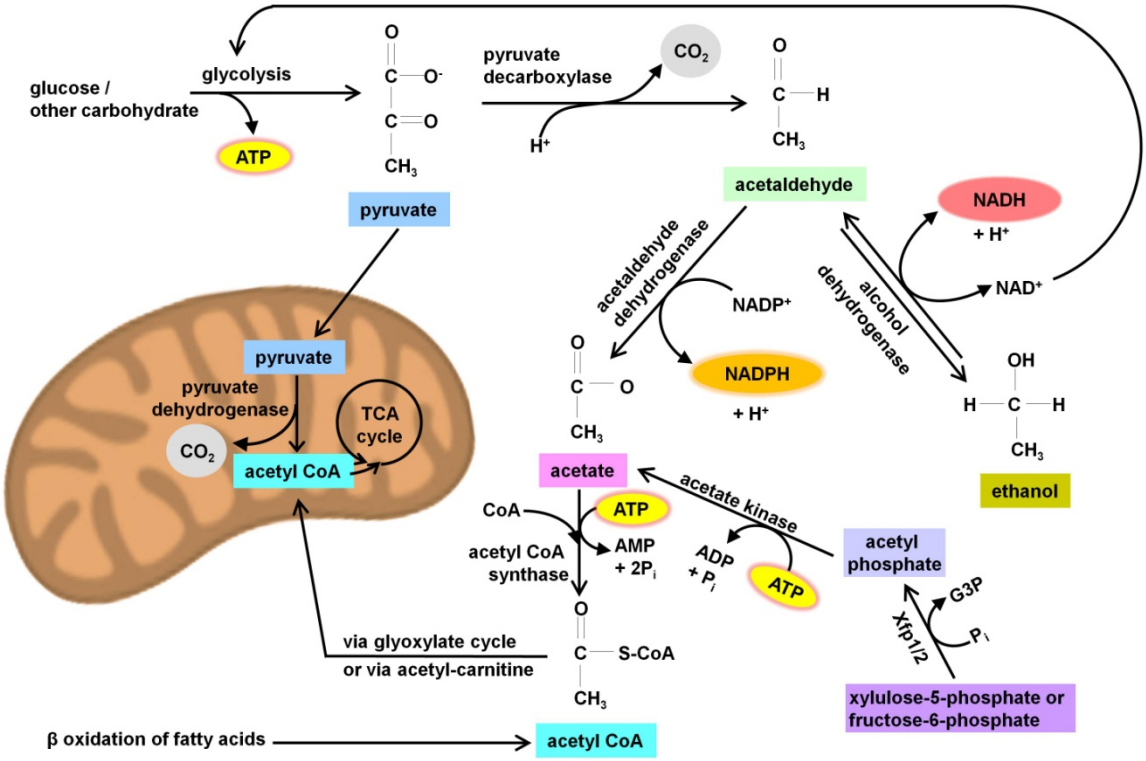

Figure 2.1: Various *C. neoformans* metabolic pathways that utilize pyruvate. Ethanol, glucose and acetate can be used as sources of carbon by *C. neoformans*. Under anaerobic conditions glucose is converted to acetate by the action of pyruvate decarboxylase (Pdc) and acetaldehyde dehydrogenase enzymes. Ethanol in the cells is converted to acetaldehyde via alcohol dehydrogenase, which is then converted to acetate via acetaldehyde dehydrogenase.

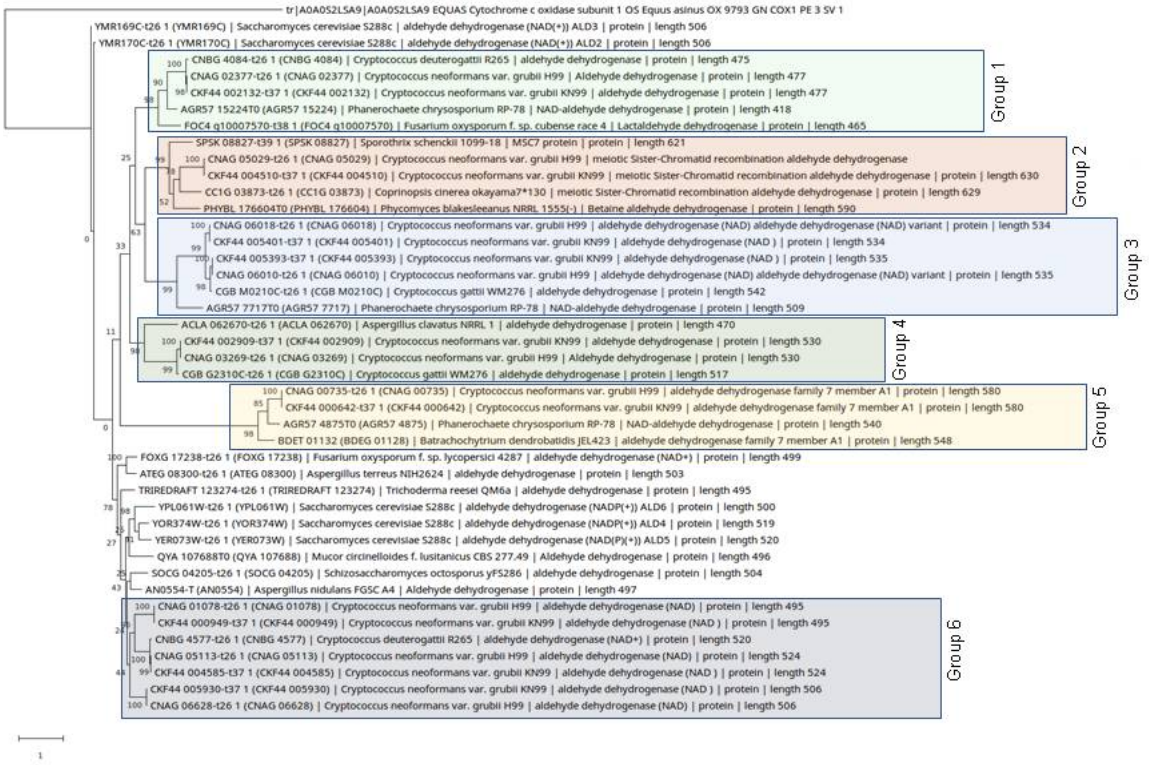

Figure 2.2: Phylogenetic tree analysis showing evolutionary relatedness between specific aldehyde dehydrogenases (Alds) of *C. neoformans* and Ald6p, Ald4p and Ald5p of *S. cerevisiae*. The sequences of putative cryptococcal aldehyde dehydrogenases and potential homologues from other fungal species were analyzed using Molecular Evolutionary Genetics Analysis (MEGA X) software to build a phylogenetic tree. Depending on the sequence similarity, each of the Alds could be subdivided into 6 groups (shown in different colors). From the analysis, it was deduced that CNAG_01078 (Ald78), CNAG_05113 (Ald13), and CNAG_06628 (Ald5) of *C. neoformans* were closer to aldehyde dehydrogenases Ald4p, Ald5p, and Ald6p of *S. cerevisiae* than CNAG_06010 (Ald10), CNAG_06018 (Ald18), CNAG_05029 (Ald29),

CNAG_00735 (Ald35), CNAG_03269 (Ald69), or CNAG_02377. *C. neoformans* Ald78, Ald13 and Ald5 were also closely related to each other, while Ald10 and Ald18 were closely related to each other, and to lesser extent, to Ald29.

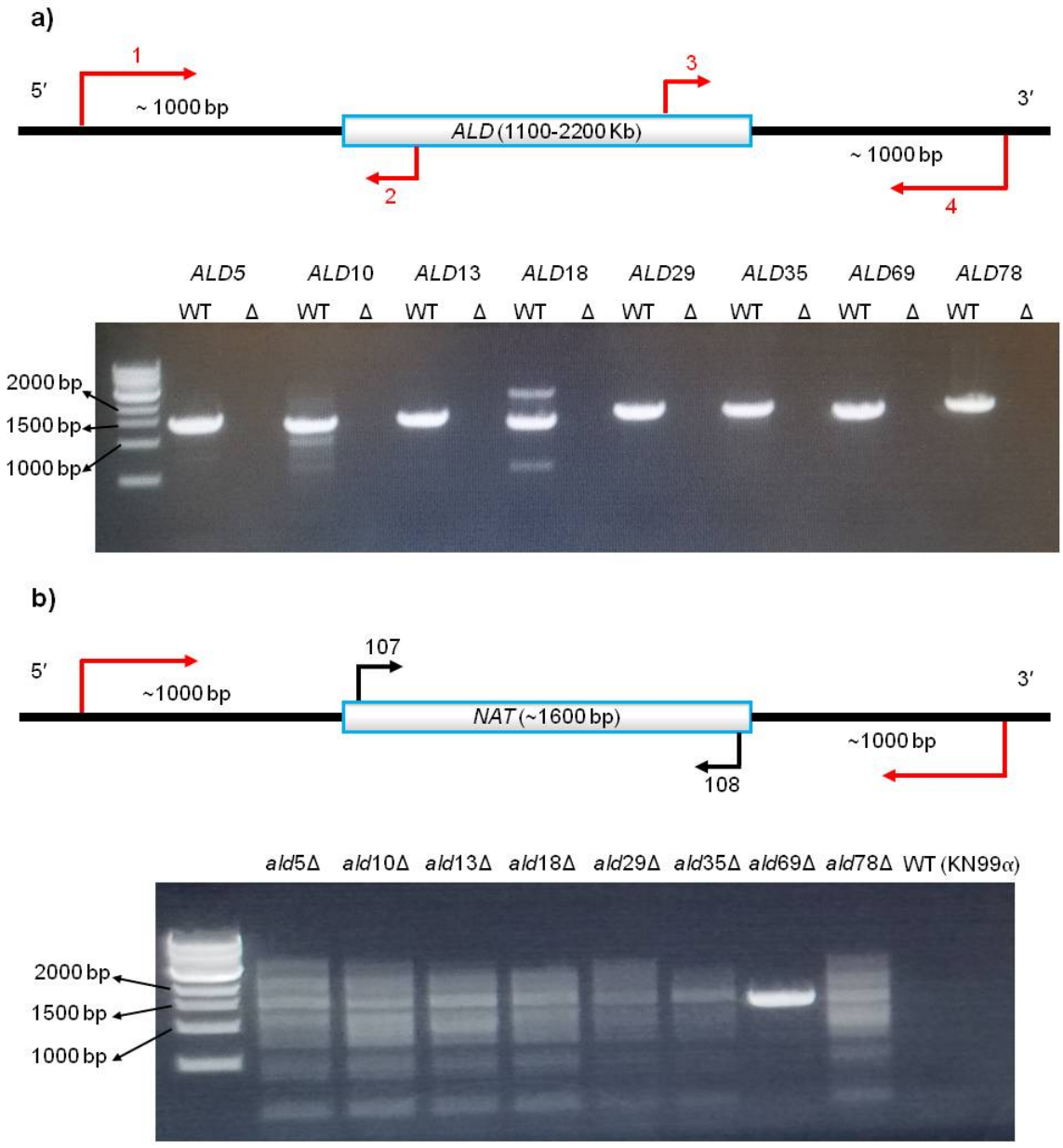

Figure 2.3: Verification of the deletion of each *ALD* gene in the corresponding mutant. Primers specific for each aldehyde dehydrogenase gene (either 1 and 2, or 3 and 4, as shown above) and for the *NAT* gene conferring resistance to the nourseothricin antibiotic (107 and 108 above) were used in PCR to confirm the insertional mutagenesis and deletion of the aldehyde

dehydrogenase gene in each of the corresponding *ald* mutants obtained from the Madhani lab at UCSF. Specifically, these mutants were checked for the replacement of the *ALD* genes with the nourseothricin resistance cassette (*NAT*). **(a)** The presence of a band representing each gene in the lanes corresponding to the reactions with wild type (WT) strain DNA, but not in the lanes corresponding to the respective mutants (Δ), corroborated the deletion of the respective *ALD* genes in the mutants used in this study. **(b)** The presence of the *NAT* gene (~1600 bp) in all of the *ald* mutants, but not in the wild type strain (KN99α) was confirmed, verifying that resistance to nourseothricin in the mutants is due to the presence of the *NAT* cassette and not due to metabolic changes in the cells.

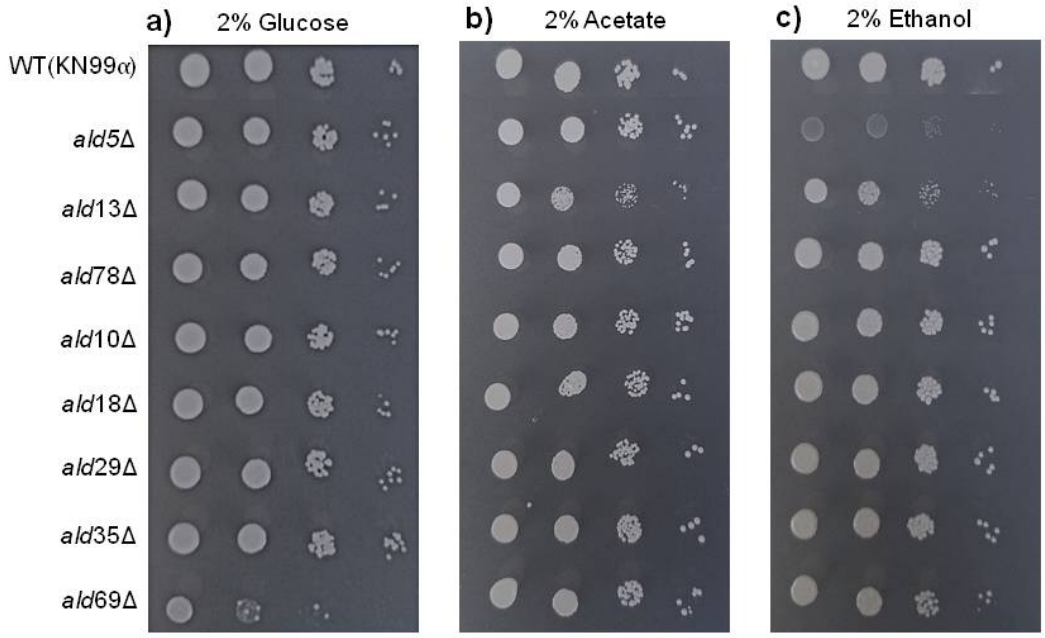

Figure 2.4: Qualitative growth analysis revealed growth defects in *ald5*Δ, *ald*13Δ, and *ald*69Δ mutants. Four 10-fold serial dilutions (2 µl of 5000, 500, 50, and 5 cells/µl) of each mutant strain, and of the wild type (WT) KN99α strain, of *C. neoformans* were spotted from left to right on YNB-agar media supplemented with **(a)** 2% glucose, **(b)** 2% acetate, or **(c)** 2% ethanol. The *ald*69Δ mutant strain exhibited reduced growth (relative to wild type) on **a)** 2% glucose, while the *ald*13Δ mutant strain had a smaller colony size (relative to wild type) on **b)** 2% acetate, and **c)** 2% ethanol. The *ald5*Δ mutant grew extremely poorly on **c)** 2% ethanol, but grew normally on **a)** 2% glucose and on **b)** 2% acetate.

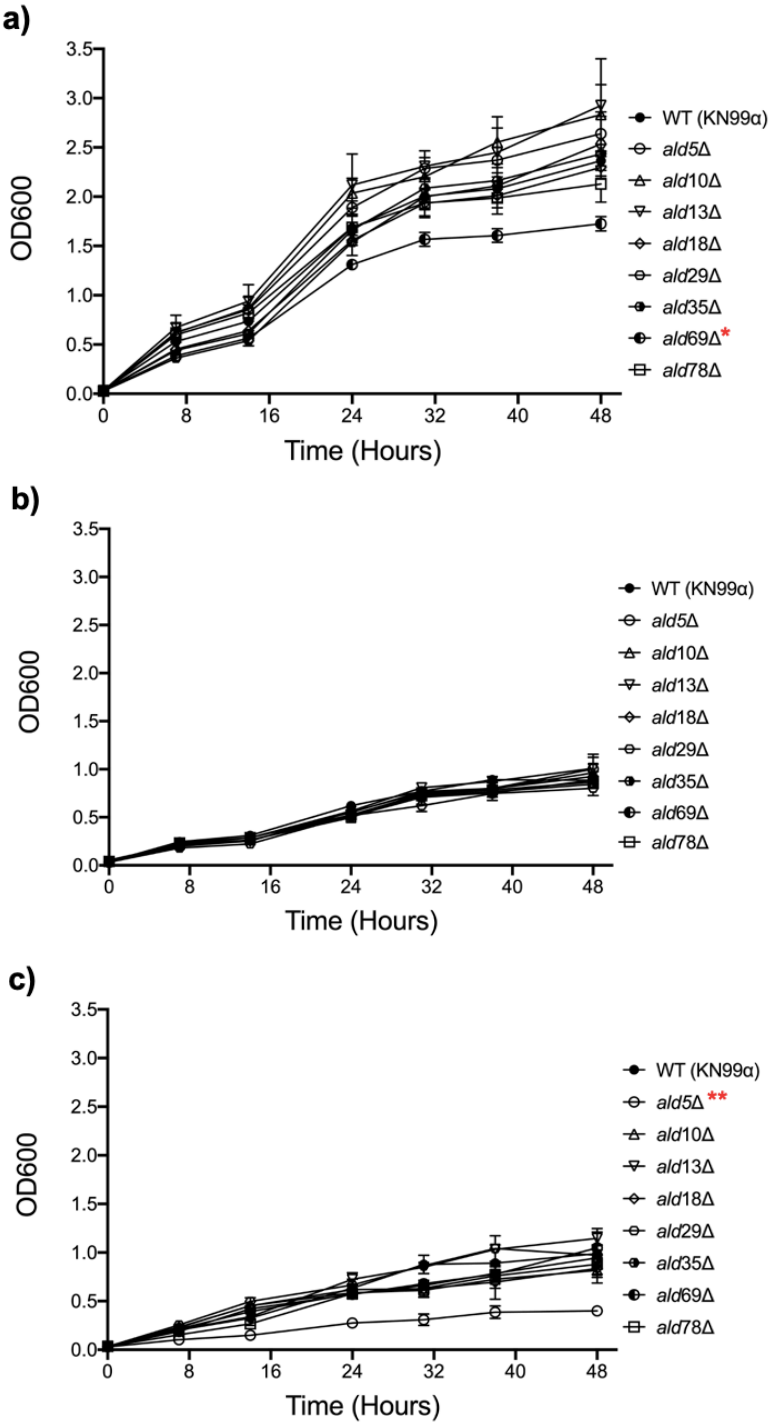

Figure 2.5: Quantification of the impact of deletion of *ALD* genes on growth in liquid media with various primary carbon sources. Growth curves for the

wild type (KN99α) strain and for each *ald* mutant strain of *C. neoformans* were generated over a 48-hour time course in YNB media supplemented with **a)** 2% glucose, **b)** 2% acetate, or **c)** 2% ethanol as the primary carbon source. The *ald*69Δ mutant displayed a reduced growth rate in **a)** glucose (* = significant, P< 0.05; P-value = 0.0210, 0.0429, 0.0315, and 0.0320 at 24, 31, 38, and 48 hours, respectively), but not in **b)** acetate or **c)** ethanol, while the *ald5*Δ mutant displayed a reduced growth rate in **c)** ethanol (** = very significant, P<0.01; P-value = 0.0052 overall), but not in **a)** glucose or **b)** acetate.

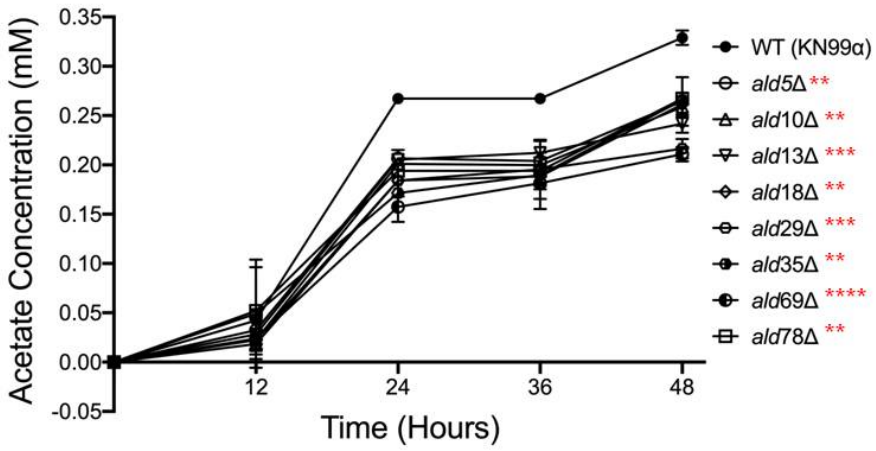

Figure 2.6: All *ald* mutants produced significantly less acetate than the wild type strain after 12 hours. A 48-hour time course analysis revealed significantly reduced acetate production by all of the *ald* mutant strains after 12 hours (P-value < 0.0001 overall), compared with the KN99α wild type (WT) strain. The degree of significance of the difference between the acetate production by each mutant and the wild type strain is indicated as follows: ** P-value < 0.01 (very significant), *** P-value < 0.001 (highly significant), **** P-value < 0.0001 (extremely significant).

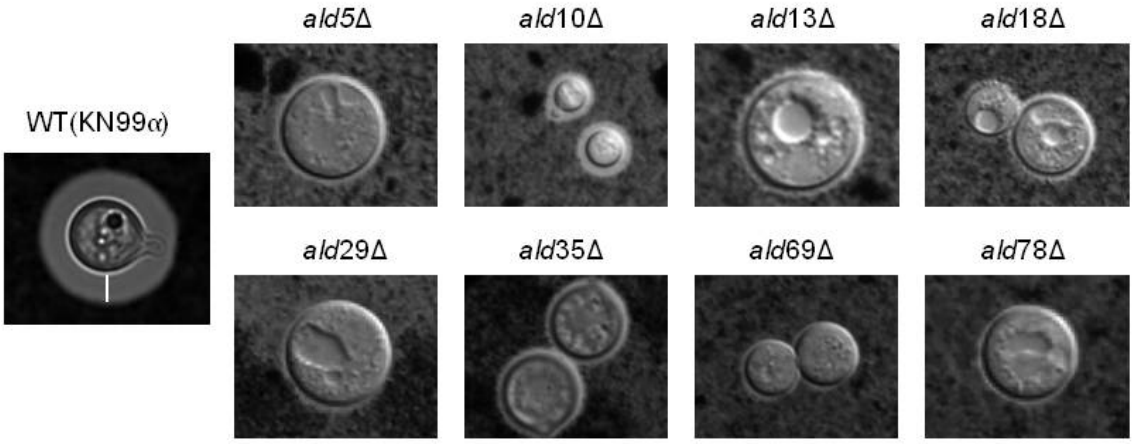

Figure 2.7: *ALD* genes played an important role in capsule formation. Cells from each of the various mutant strains and from the wild type KN99α strain were cultured in capsule-inducing medium and stained with India Ink. A white line shows the width of the capsule in the wild type (WT) KN99α strain (left-most image). The width of the capsule in all of the mutant strains was reduced relative to that of wild type, as observed visually at 40x magnification, although the capsule of the *ald*10Δ mutant was slightly larger than that of the other mutants.

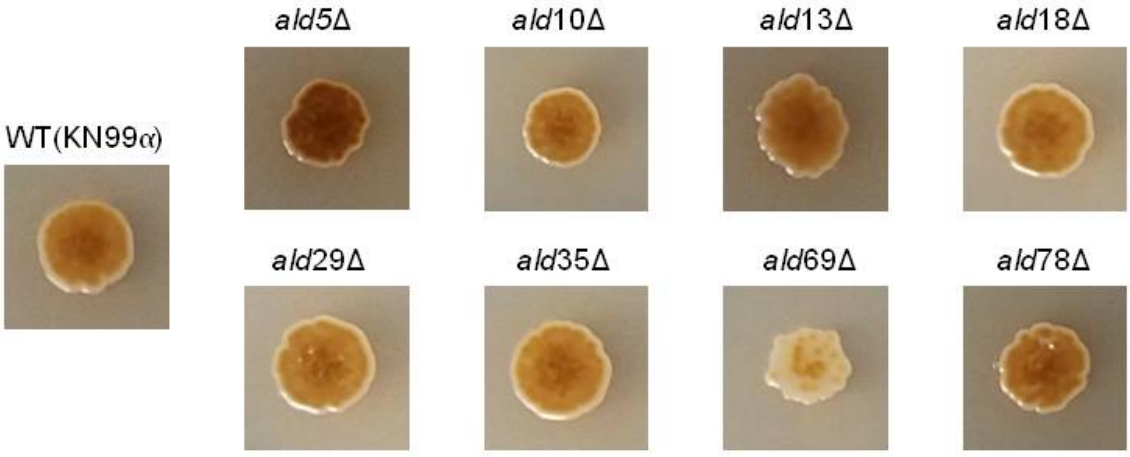

Figure 2.8: The *ald*69Δ mutant produced less melanin and the *ald5*Δ mutant produced more melanin than the wild type strain. A spot assay was conducted to visually compare the level of melanin production in *ald* mutants relative to the wild type (WT) KN99α strain. Cells from each strain were allowed to grow on plates supplemented with L-DOPA, which is converted to the brown, melanin-like pigment by laccase enzymes (Williamson, 1997; Li *et al*., 2018). The *ald*69Δ mutant displayed a lighter coloration, whereas the *ald5*Δ mutant exhibited a darker coloration, compared with the wild type strain.

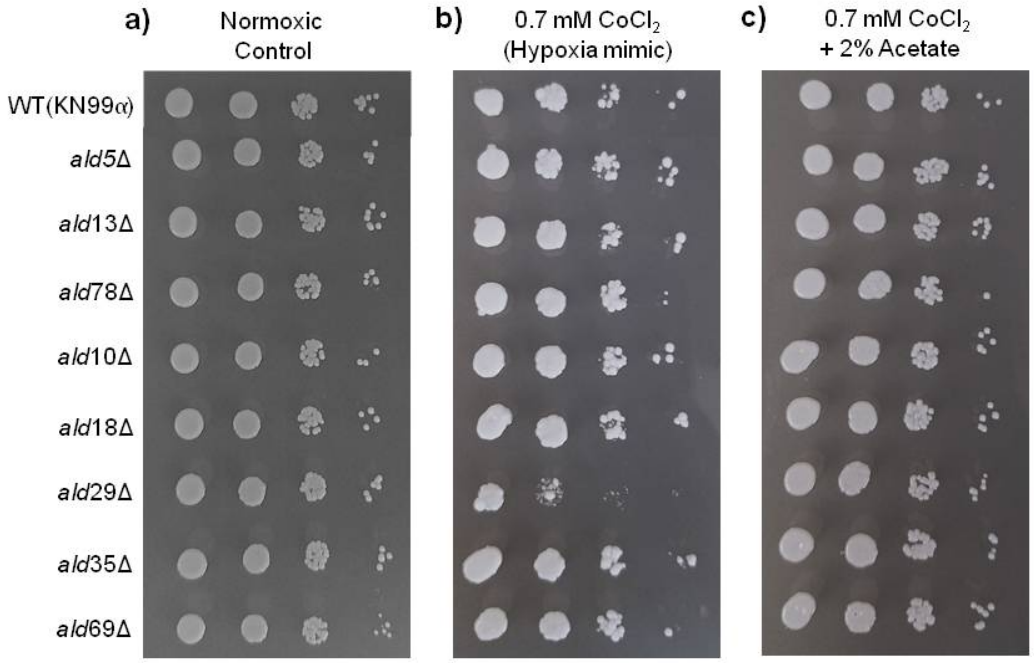

Figure 2.9: The *ald*29Δ mutant grew poorly under hypoxia-mimicking conditions. Spot assays were conducted in which the wild type (WT) KN99α strain and *ald* mutants (in the same strain background) were grown on **a)** nutrient rich YES media without $CoCl_2$ as normoxic control (Normoxic Control), or **b)** YES media supplemented with 0.7 mM $CoCl_2$, which induces cells to behave physiologically as if they were growing in hypoxia (0.7 mM $CoCl_2$). The *ald*29Δ mutant grew normally on the control plate but exhibited a growth defect when conditions mimicked hypoxia. **c**) This growth defect was reversed when plates with $CoCl_2$ were supplemented with acetate (0.7 mM $CoCl_2$ + 2% Acetate).

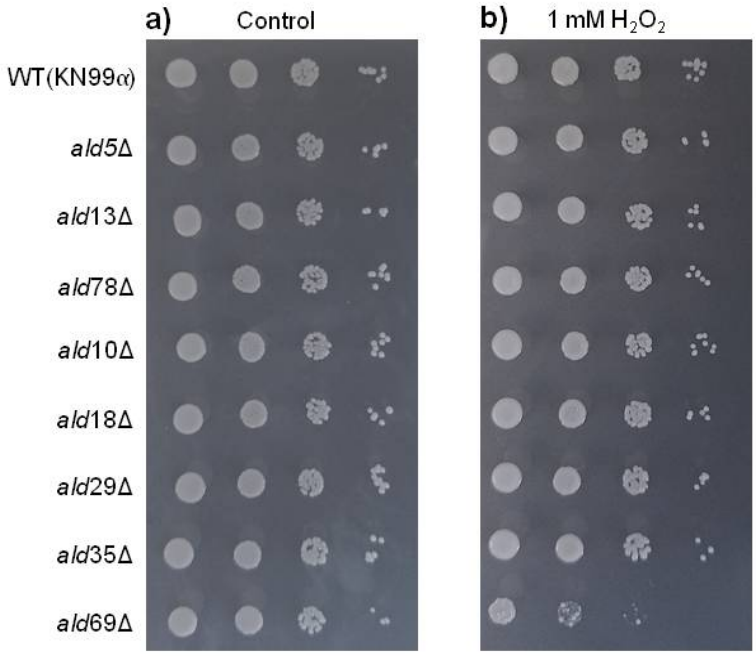

Figure 2.10: Ald69 was important for the survival of *C. neoformans* under oxidative stress. Wild type (WT) and *ald* mutant strains (in the KN99α strain) were grown on YNB-agar media with sodium succinate pH 4.0 buffer, either **a)** alone as control or **b)** supplemented with 1 mM H_2O_2 to generate reactive oxygen species. All mutants grew normally relative to the wild type strain, except the *ald*69Δ mutant (bottom row), which exhibited a growth defect when subjected to oxidative stress.

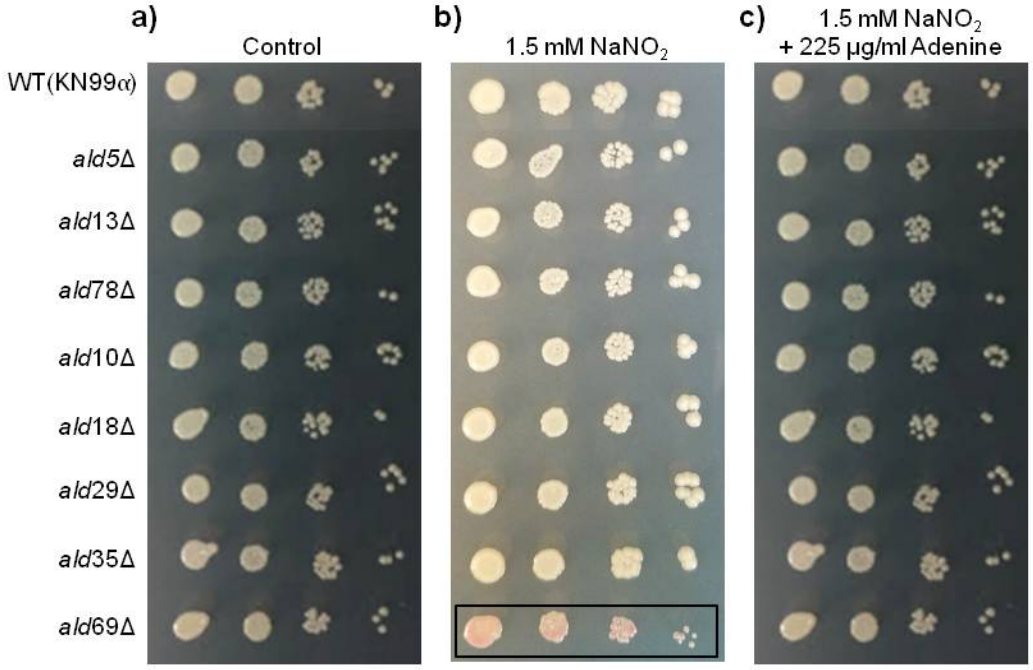

Figure 2.11: The *ald*69 mutant exhibited an adenine production defect under nitrosative stress. Wild type and *ald* mutant strains (in a KN99α background) were grown on YNB-agar media with sodium succinate pH 4.0 buffer, either **a)** alone as control or **b)** supplemented with 1.5 mM $NaNO_2$. All mutants grew normally relative to the wild type strain, except that the *ald*69Δ mutant grew as pink colonies under nitrosative stress (**b**, bottom row), possibly indicative of accumulation of a red substrate, P-ribosylamino imidazole (AIR) or P-ribosylamino imidazolecarboxylate (CAIR), (Sharma *et al.*, 2003; Smirnov *et al.*, 1967) in the adenine synthesis pathway due to a blockage in the pathway. **c)** Nitrosative stress from the $NaNO_2$ was confirmed to cause this adenine production defect, and this defect was confirmed to be the reason for the pink colonies, when all strains were grown on media supplemented with both 1.5 mM $NaNO_2$ and 225 µg/ml adenine;

the addition of adenine negated the need for the manufacture of adenine by the cells and thereby eliminated the pink color of the *ald6*9Δ mutant colonies. The global contrast of this image was increased by 20% to better reveal the pink color.

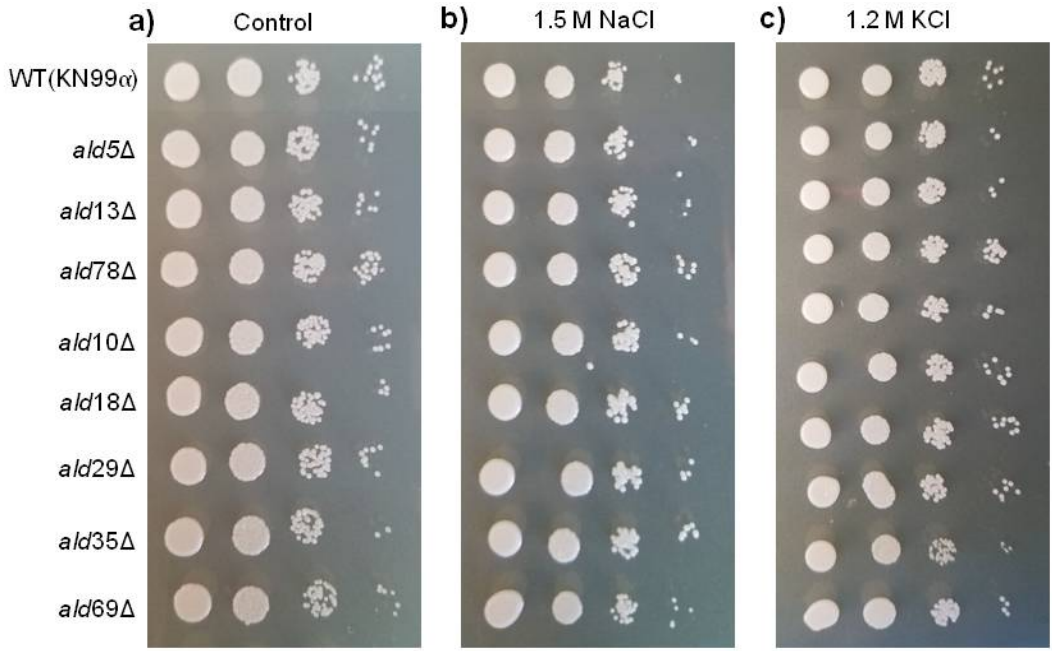

Figure 2.12: *ald* mutants grew normally under high salt conditions. Wild type and *ald* mutant strains (in the KN99α background) were grown on YPD-agar plates **a)** without further salt added (as control) or supplemented with **b)** 1.5 M NaCl or **c)** 1.2 M KCl in order to induce osmotic stress. All mutants grew normally relative to the wild type strain.

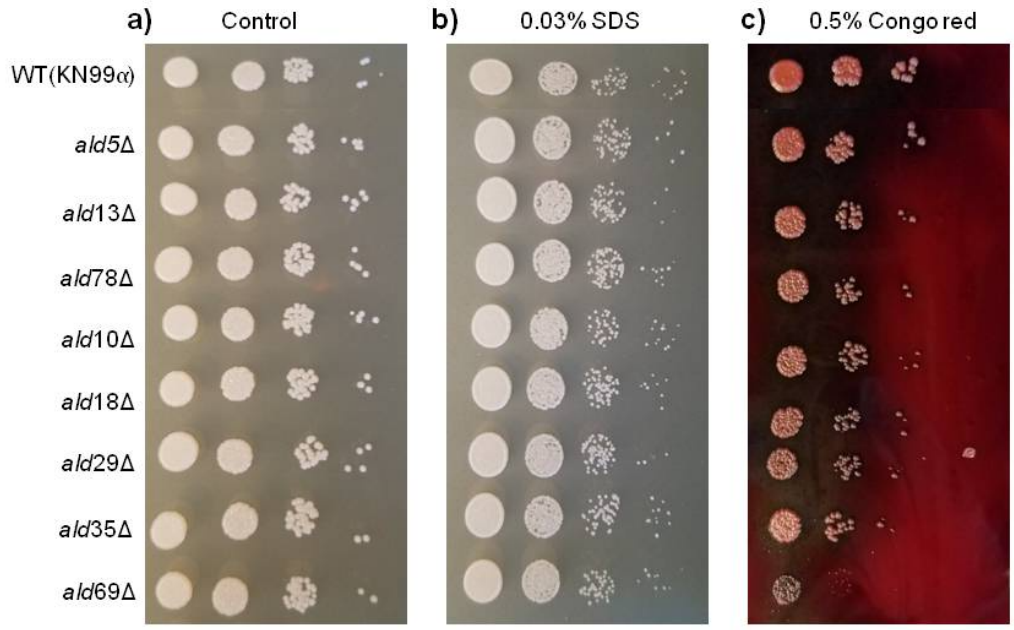

Figure 2.13: The *ald*69Δ mutant had defects in cell wall and/or capsule integrity. Wild type (WT) and *ald* mutant strains (in a KN99α background) were grown on YPD-agar plates, either **a)** alone or supplemented **b)** with 0.03% SDS for cell membrane disruption or **c)** with 0.5% Congo red, which disrupts cell wall production by passing through the capsule and binding to components of the cell wall and chitin layers. No growth defect was observed on SDS containing media **(b)**, whereas, in Congo red containing media **(c)**, all mutants exhibited a slight growth defect and smaller colonies, either reflecting the greater permeability of their thinner capsules or indicating a loss of cell wall integrity and resulting reduction in fitness. The *ald*69Δ mutant displayed the most obvious growth defect in the presence of Congo red (**c**, bottom row, left spot).

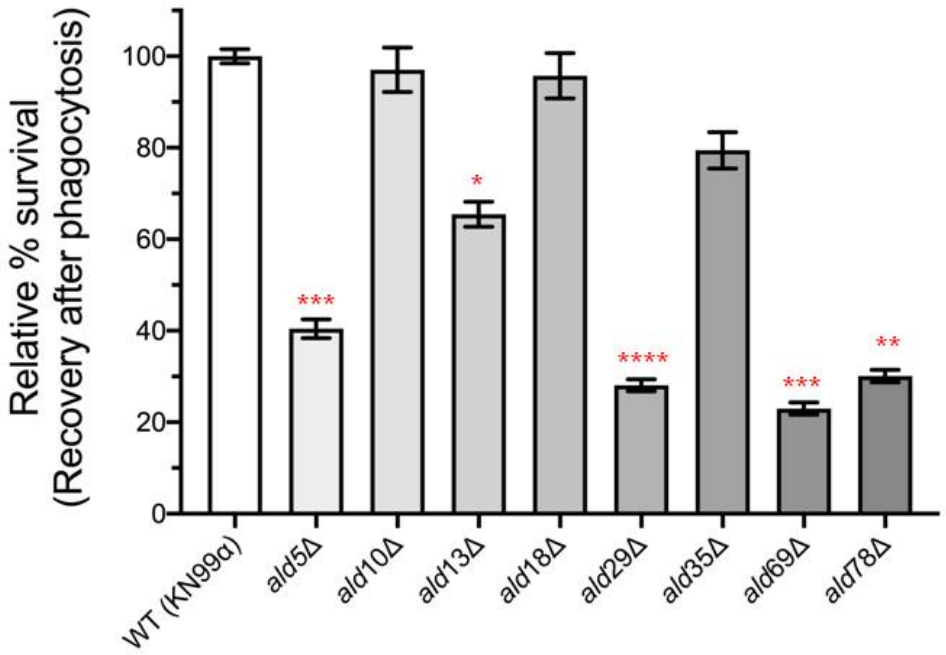

Figure 2.14: Percent survival of wild type and *ald* mutant *C. neoformans* strains after engulfment by macrophages. The *ald5*Δ, *ald*13Δ, *ald*29Δ, *ald*69Δ, and *ald*78Δ mutants demonstrated statistically decreased survival and recovery, relative to the wild type KN99α strain, after phagocytosis and attempted killing by macrophages (* = significant, P-value < 0.05; ** = very significant, P-value < 0.01; *** = highly significant, P-value < 0.001; **** = extremely significant, P-value < 0.0001), but not *ald*10Δ, *ald*18Δ, or *ald*35Δ.

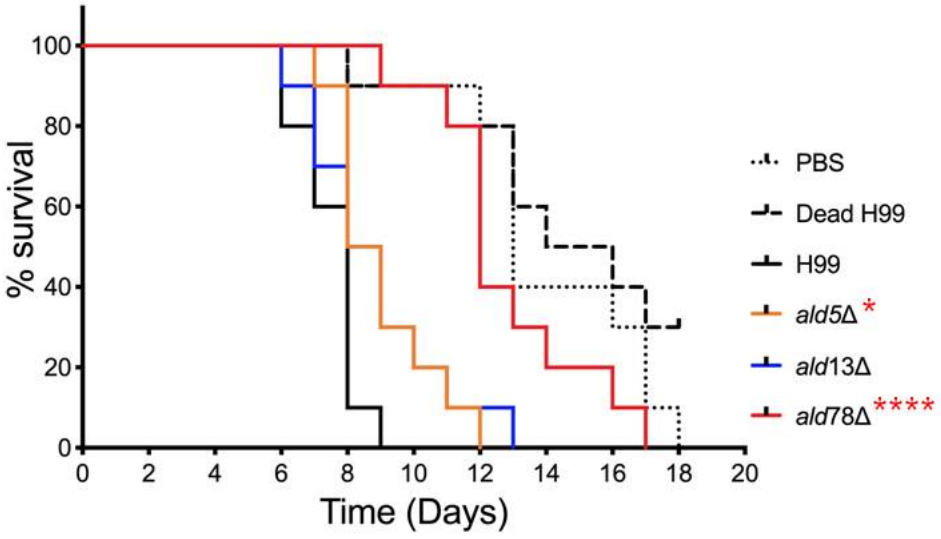

Figure 2.15: *Galleria mellonella* survival assay. The survival of *Galleria mellonella* larvae was analyzed after infection with 1×10^6 fungal cells of various strains of *C. neoformans,* ten larvae per strain. The percent survival of these larvae over time, after injection with the wild type H99 (black), *ald5*Δ (orange), *ald*13Δ (blue), or *ald*78Δ (red) strains of *C. neoformans*, or with the negative controls of phosphate buffered saline (PBS; dotted black) or heat-killed wild type strain (Dead H99; dashed black), are graphed as Kaplan-Meier survival curves. Statistical analysis of the overall survival curves, using either a Gehan-Breslow-Wilcoxon test or Log-Rank test, indicated that the *ald5*Δ strain was less pathogenic (* P-value < 0.05) than the wild type strain, while the *ald*13Δ strain was either only slightly (non-significantly) less pathogenic, or no less pathogenic, than the wild type strain. Deletion of the corresponding gene in the *ald*78Δ strain effectively eliminated pathogenicity of this mutant strain (**** P-value < 0.0001).

CHAPTER THREE

THE ROLE OF PYRUVATE DECARBOXYLASE IN THE STRESS RESPONSE AND PATHOGENESIS OF *C. NEOFORMANS*

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Abstract

Acetate is one of the major metabolites secreted by cryptococci cultured *in vitro* and found in biopsies of infected rat brain and lung tissues. In this study, the role of *Cryptococcus neoformans* pyruvate decarboxylase, the first enzyme in a putative acetate-production pathway, in the stress response and pathogenesis of *C. neoformans* was investigated. A *C. neoformans pdc*Δ mutant was ineffective in producing acetate and exhibited substantially reduced growth in an environment where glucose was the only carbon source. Additionally, melanization and capsule formation, which are important for pathogen virulence, negatively affected in the mutant. Surprisingly, despite the significant capsule formation defect, the ability of the pathogen to neutralize reactive oxygen and nitrogen species and to grow under high osmolarity conditions was not affected in the *pdc*Δ strain. However, the *pdc*Δ mutant was unable to cope in an assay mimicking the low oxygen conditions found in the host brain and the survival of the pathogen inside murine macrophages was

compromised. Nevertheless, no changes were observed in the ability of the *pdc*Δ mutant versus wild type *C. neoformans* to kill injected larvae of the greater wax moth *Galleria mellonela* in an *in vivo* infection model. Overall, by using genetic and biochemical techniques, this work provides evidence for the role of Pdc in acetate production by *C. neoformans*, and in the hypoxia stress response and virulence of this pathogen.

Introduction

Cryptococcus neoformans is an invasive fungal pathogen that infects lung and brain tissue and is the most frequent cause of fungal meningitis in humans, leading to significant annual global mortality (Lin and Heitman, 2006; Park *et al.*, 2009, Pappas PG., 2001; Lui *et al.*, 2006; Franzot *et al.*, 1999; Dromer *et al.*, 1996; Tortorano *et al.*, 1997; Powderly, 1993). When desiccated yeast cells or spores, found in bird droppings, decomposing organic material, or soil (Steenbergen and Casadevall., 2003; Velagapudi *et al.*, 2009) are inhaled by mammalian hosts, they enter the lungs and then may disseminate to the brain, causing a potentially lethal meningoencephalitis (Sabiiti and May, 2012; Velagapudi *et al.*, 2009). In order to survive and disseminate, the pathogen requires a number of virulence factors, including a polysaccharide capsule, melanin, and the ability to grow at the host body temperature of 37°C (Coelho *et al.*, 2014; Casadevall *et al.*, 2000; Kronstad *et al.*, 2011; Ma and May, 2009). *C. neoformans* also undergoes metabolic adaptation to conditions inside the mammalian host. For example, the pathogen produces many metabolites, including acetate, that it then may use to create an optimal microenvironment (Bubb *et al.*, 1999; Wright *et al.*, 2002; Hu *et al.*, 2008; Price *et al.*, 2011; Himmelreich *et al.*, 2001; Kronstad *et al.*, 2012).

Acetate is one of the major metabolites secreted by cryptococci cultured *in vitro* and found in biopsies of infected rat brain and lung tissues (Bubb *et al.*, 1999; Wright *et al.*, 2002; Himmelreich *et al.*, 2001, 2003). It is believed to provide a

survival advantage to the pathogen by two mechanisms: altering pH and providing a source of carbon or energy.

The fairly narrow pH range in which *C. neoformans* can grow (relative to that of some other fungal species) includes the pH 4.0-5.5 environment inside the macrophage phagolysosome, as well as the pH 7.4 of human blood and cerebrospinal fluid (O'Meara *et al.*, 2010; Nyberg *et al.*, 1992), although the pathogen actually prefers the former, more acidic environment as an optmal pH range. If the pathogen infects or disseminates to a location in the body that is more basic than this optimal range, it can secrete excess acetate into the local environment to reduce the pH (Wright *et al.*, 2002). This lower pH enhances the activity of the fungal phospholipase B and potentially other enzymes at the site of cryptococcal infection (Evans *et al.*, 2015; Sharon *et al.*, 2000) and can shield the pathogen from an immune attack by neutralizing free radicals and decreasing the formation of superoxides produced by phagocytes, or by reducing the migration or increasing the necrosis of neutrophils (Hu *et al.*, 2008; Wright *et al.*, 2002).

Two putative pathways for the production of acetate in *C. neoformans* have been identified: the xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (Xfp) – acetate kinase (Ack) pathway and the pyruvate decarboxylase (Pdc) – acetaldehyde dehydrogenase (Ald) pathway. In fungi that lack the Xfp-Ack pathway, such as *Saccharomyces cerevisiae*, the primary acetate production pathway utilizes pyruvate decarboxylase (Pdc) and aldehyde dehydrogenase (Ald) (Saint-Prix *et al*., 2004; Remize *et al*., 2000; Pronk *et al*., 1996).

In *S. cerevisiae*, pyruvate is an important metabolic intermediate that is acted upon by different enzymes at the branch point of oxidative respiration and non-oxidative fermentation, depending on growth conditions (Agarwal *et al.*, 2013; Møller *et al.*, 2004). During aerobic respiration, pyruvate can be converted via pyruvate dehydrogenase to acetyl-CoA, which can then be fed into the TCA/Krebs cycle to produce energy. This pathway works inside mitochondria (Møller *et al.*, 2004; Remize *et al.,* 2000), and the mitochondrial acetyl-CoA cannot diffuse into the cytoplasm (Remize *et al.,* 2000). During non-aerobic fermentation, acetyl-CoA for use in the cytosol can be produced from pyruvate via the pyruvate dehydrogenase by-pass pathway. In this cytosolic pathway pyruvate decarboxylase converts pyruvate to acetaldehyde, which is oxidized via acetaldehyde dehydrogenase to acetate and subsequently to acetyl-CoA via acetyl-CoA synthetase (Pronk *et al.*, 1994; Remize *et al.*, 2000). In this process, the oxidation of acetaldehyde is used to reduce NAD^+/$NADP^+$ to NADH/NADPH to store energy and to maintain a redox balance in the cell (Saint-Prix *et al.*, 2004; Remize *et al.,* 2000). Alternatively, the acetaldehyde can serve as an electron acceptor and be reduced to ethanol, thereby allowing the oxidation of NADH to NAD^+ for use in glycolysis (Pronk *et al.*, 1994). At the branch point between the respiratory and fermentative pyruvate catabolic pathways, the efficiency of pyruvate decarboxylase determines whether pyruvate will be diverted either towards the TCA cycle or towards fermentation (Agarwal *et al.*, 2013). A similar set of pathways is believed to exist in *C. neoformans* (Figure 3.1).

S. cerevisiae requires pyruvate decarboxylase activity for anaerobic growth, as is evident from early studies of *pdc* null mutants which were either unable to grow or grew more slowly on media supplemented with glucose when forced to utilize fermentation (Lam and Marmur, 1977; Schmitt and Zimmermann, 1982). There are actually multiple Pdc-encoding genes in *Saccharomyces* species; however, under normal growth conditions, Pdc1p is the main contributor of pyruvate decarboxylase activity in wild type strains undergoing fermentation (Schmitt and Zimmermann, 1982; Schmitt *et al.*, 1983; Kellermann *et al.*, 1986; Hohmann, 1991a). Pdc1 deficiency affects the enzymatic activity more severely than deficiency of either of the other two major Pdc enzymes, Pdc5 and Pdc6 (Flikweert *et al*., 1996). Nevertheless, a mutant of the *PDC1* gene (*pdc1*^{0}) generated by Seeboth *et al*. (1990) could grow on glucose even when cellular respiration was blocked, and 60-70% of pyruvate decarboxylase activity was retained in the mutant, probably due to the upregulation of expression of the *PDC5* gene in these mutants (Seeboth *et al.*, 1990; Hohmann and Cederberg, 1990). Similarly, deletion of the *PDC1* gene in *S. cerevisiae* results in no significant change in the acetate yield or in the amount of secondary metabolites, possibly because Pdc5 can be used instead of Pdc1 (Remize *et al*., 2000). The *PDC6* gene is weakly expressed and the function of the corresponding protein is not clear (Hohmann, 1991b). The *PDC2* gene, on the other hand, has an important but indirect role in pyruvate metabolism, in that it is involved in regulating the expression of *PDC1* and *PDC5* or the corresponding enzyme activities (Hohmann,

1993). Pdc activity in *S. cerevisiae* is low under ethanol-only growth conditions, whereas it increases by 10 to 20 fold over time under high glucose conditions (Schmitt and Zimmermann, 1982). The expression of the *PDC1* gene also increases in glucose supplemented media versus in media with ethanol as the only major carbon source due to an ethanol-dependent repression (Schmitt and Zimmermann, 1982; Schmitt *et al.*, 1983). Thus, Pdc activity may play a role in the utilization of alternative carbon sources in *S. cerevisiae*.

In *C. neoformans,* unlike in other fungal species, there is only one putative *PDC* gene, which may play a role in virulence. The levels of mRNAs encoding acetyl-CoA synthetase (Acs1), aldehyde dehydrogenase, and pyruvate decarboxylase, enzymes that may function in the production of acetyl-CoA from pyruvate via acetaldehyde and acetate, are increased in *C. neoformans* cells recovered from pulmonary tissue in a mouse infection model (Hu *et al.*, 2008). This suggests that pyruvate decarboxylase plays an important role during pulmonary infection.

Additionally, Rodrigues *et al.* (2008) identified Pdc as one of the components of extracellular vesicles (EVs) utilized by the fungus, further evidence for a role of *C. neoformans* Pdc in the virulence of the pathogen. EVs are produced in all domains of life (Raposo and Stoorvogel, 2013) and in fungi were first identified in *C. neoformans* (Rodrigues *et al.*, 2007). Vesicular secretion is a common mechanism used by *C. neoformans* for transporting many virulence-related components, including capsular GXM polysaccharide (Rodrigues *et al.*,

2007), phospholipase B (Cox *et al.*, 2001), urease (Cox *et al.*, 2000; Perfect and Casadevall., 2002), melanin (Eisenman *et al.*, 2009), superoxide dismutase (Cox *et al.*, 2003), and laccase (Rodrigues *et al.*, 2008; Salas *et al.*, 1996) through the cell wall for use outside the cell. The presence of Pdc in these EVs is highly unusual, since, unlike the other components and enzymes listed, Pdc is a metabolic enzyme involved in energy production. The fact that *C. neoformans* exports it out of the cell may indicate a role for the enzyme in virulence, such as in production of acetate that is used in the acetylation of cell wall and chitin carbohydrates as part of cell wall and capsule formation and attachment of the capsule to the cell wall (Rakesh *et al.*, 2008; Vecchiarelli *et al.*, 2011; Hu *et al.*, 2008; Ellerbroek *et al.*, 2004).

Thus, the role of the *C. neoformans* Pdc in growth on glucose, ethanol, and acetate, during hypoxia, oxidative and nitrosative stresses, and osmotic stress, in the production of the capsule and melanin, which are important virulence factors, in cell wall and membrane integrity, and in actual virulence *in vitro* and *in vivo*. was assayed herein using a knockout mutant of the *C. neoformans PDC* gene, and was compared with the effect of deficiency of the pyruvate decarboxylase enzyme on acetate production.

Materials and methods

Fungal Strains:

Wild type and pyruvate decarboxylase (*pdc*Δ) mutant strains of *Cryptococcus neoformans var grubii*, in the KN99α background, were obtained from the Madhani lab, University of California San Francisco (UCSF) gene deletion collection (via the Fungal Genetics Stock Center). These were used for all of the experiments, except for the *in vivo Galleria mellonella* virulence assay, in which similar strains in an H99α background (Fungal Genetics Stock Center) were used. The *pdc*Δ knockout mutants in each background were maintained under selection for nourseothricin (NTC) resistance, since a nourseothricin acetyltransferase (*NAT*) cassette was used to disrupt the *PDC* gene by insertional mutagenesis (Chun and Madhani, 2010). Information on the *C. neoformans* pyruvate decarboxylase gene/enzyme, including the gene and protein identification numbers and the predicted mitochondrial or cytoplasmic cellular location of the protein (determined using the MitoFates website, http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi; Fukasawa *et al.*, 2015), as well as the chromosomal location of the gene (obtained from the Fungi Database, https://fungidb.org/fungidb/) is provided in Table 3.1.

Phylogenetic tree:

Fungal pyruvate decarboxylase protein sequences were downloaded from the FungiDB (http://fungidb.org/fungidb/srt.jsp). Alignment and phylogeny reconstruction were performed using Molecular Evolutionary Genetics Analysis

(MEGA X) software (Kumar *et al.*, 2001) as follows. Alignment was carried out with Clustal Ω (Thompson *et al.*, 1994), and the aligned sequences were used to construct a Maximum Likelihood (ML) tree, under an assumption of a uniform rate of substitution among all amino acid sites, using the Jones-Taylor-Thornton model (Jones *et al.*, 1992), with 500 bootstrap replicates (Felsenstein J., 1985) to verify the reliability of the tree. The initial tree was constructed using both Neighbor joining (NJ) and BioNJ algorithms and optimized via the Nearest Neighbor Interchange (NNI) version of the ML heuristic method with a "moderate" branch swap filter setting.

Confirmation of deletion of *PDC* in the mutant by PCR analysis:

The deletion of *PDC* in the pyruvate decarboxylase (*pdc*Δ) mutant from the Madhani lab was confirmed by PCR using the following gene-specific primers and assaying for a lack of PCR product.

PDC forward primer: TGACGGTTCTTTGCAGTTGG

PDC reverse primer: TTGAAGGCGAAGGTTGTGTG

Additionally, the presence of the *NAT* gene (in replacement of *PDC*) was verified using primers synthesized based on sequences obtained from the Madhani lab:

NAT forward primer (107): CCTAGCAGCGGATCCAAC

NAT reverse primer (108): CGCATCCCTGCATCCAAC

DNA was isolated from wild type and mutant strains using the cetyl/hexadecyl-trimethylammonium bromide (CTAB) DNA isolation protocol (Pitkin *et at*, 1996). Gene amplification was performed using the KOD Hot Start DNA Polymerase kit (ThermoScientific) with a thermocycler program as follows for the *NAT* gene: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 2 min, followed by final extension at 72°C for 5 min. The thermocycler program utilized to amplify *PDC* differed only in that 35 cycles were used and the annealing temperature was set to 60°C.

Quantitative and qualitative growth analysis in various carbon sources:

C. neoformans wild type and *pdc*Δ strains were cultured overnight in Yeast Extract Peptone Dextrose (YPD) media (1% Difco Yeast extract, 2% Bacto Peptone, and 2% glucose) at 30°C, in a rotating shaker at 200 rpm (Barnstead MaxQ 4000 Orbital Incubator Shaker). The next morning, the cultures were refreshed: 25 µl from each of the overnight cultures were used to inoculate a corresponding 2 ml volume of YPD, which was incubated to an optical density at 600 nm wavelength (OD_{600}) of ~0.2 as measured using an Evolution 60 Spectrophotometer (ThermoScientific, Waltham, MA). These cells were pelleted at 8,000×g and washed twice in Dulbecco's Phosphate Buffered Saline (D-PBS; LifeSciences, Oneonta, NY), then counted using a hemocytometer.

For qualitative analysis, spot assays were performed. Refreshed cells were initially diluted to 5×10^3 cells/µl, and then 10-fold serially diluted three times. From each

dilution, 2µl were spotted onto Yeast Nitrogen Base (YNB; HiMedia) plus agar (YNB-agar) supplemented with 2% of either glucose, acetate, or ethanol. Thus, 1×10^4 cells, 1×10^3 cells, 100 cells, and 10 cells of the wild type and *pdc*Δ mutant strains were each plated and were allowed to grow in an incubator at 30°C for approximately 3 days. Images of colonies were captured using the Canon EOS Rebel T1i camera.

For quantitative growth analysis (growth curve assay) 1×10^4 cells/µl were used to inoculate YNB media supplemented with 2% of either glucose, acetate, or ethanol, as the carbon source. Cultures were set up in each medium in triplicate in microtiter plates. Cells were allowed to grow in a shaking incubator at 30°C for 48 hours, and their growth was monitored over time using the Epoch Multi-Volume Microplate Reader (BioTek Instruments, Inc., Winooski, VT) to measure the OD_{600} at each time point.

Measurement of acetate production:

To analyze acetate production by *C. neoformans*, the hydroxamate assay was performed as described previously (Fowler *et al.*, 2012; Aceti and Ferry, 1988; Rose *et al.*, 1954). As detailed above, *C. neoformans* strains were grown overnight in YPD media at 30°C. To refresh cells, 25 µl from each of these overnight cultures was used to inoculate a corresponding 5 ml volume of YNB supplemented with 2% glucose, which was incubated to an OD_{600} of ~0.2. Cells were counted using a hemocytometer and the 5 ml cultures were adjusted to a concentration of 1×10^4 cells/µl. The 5 ml cultures were vortexed briefly to fully resuspend the cells and 1

ml of each culture was transferred to a corresponding eppendorf tube, which was centrifuged at 10,000×g; the recovered supernatants were transferred to fresh tubes and frozen down at –80°C. The remaining culture volumes were incubated at 30°C, and every 12 hours, up to and including 48 hrs, the process was repeated. Once all supernatants were collected, the hydroxamate assay was performed on triplicate 225 µl samples from each 1 ml of supernatant media from each strain as described by Aceti and Ferry (1988). Briefly, 75 µl of hydroxamate reaction mix (2.4 M hydroxylamine-HCl, pH 7.0; 0.4 M Tris pH 7.5; 80 mM $MgCl_2$; 90 µM ATP disodium salt hydrate) was added to each 225 µl of supernatant and the mixture was incubated at 37°C for 5 min. Then, 5 µl of *Methanosarcina thermophila* acetate kinase (purified by Dr. Cheryl Ingram-Smith) was added to a final concentration of 0.023 ng/µl, mixed to homogeneity by pipetting and incubated at 37°C for 15 minutes. Finally, the reaction was quenched using an equal volume of a stop solution (10% trichloroacetic acid and 2.5% $FeCl_3$ in 2 N HCl) and the absorbance was read at 540 nm using the Epoch Multi-Volume Microplate Reader spectrophotometer. A standard curve was prepared by performing exactly the same protocol using known concentrations of acetate solution ranging from 0.1 mM - 2 mM instead of supernatant media.

Capsule formation assay:

Capsule production by *C. neoformans* was induced as previously described (Zaragoza and Casadevall, 2004). Briefly, wild type and *pdc*Δ strains were grown overnight in 2 ml Sabouraud medium (4% glucose and 1% bacto peptone, at pH

5.6) at 30°C. The following day, cells were collected by centrifugation (8,000-10,000×g) for 2 min at room temperature, washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.5 mM Na_2HPO_4), and resuspended in 0.1x Sabouraud medium (pH7.3). Cells were counted, diluted to 5×10^6 cells/ml in 2 ml of the above 0.1x Sabouraud medium, and incubated for 24 hours at 37°C to induce capsule formation. Capsules were observed under the Axiovert Inverted Microscope (Carl Zeiss, Inc., Thornwood, NY) after 10 µl of each cell suspension were mixed with 10 µl of India Ink (Becton Dickinson, NJ) to provide contrast. Images were taken at 40x magnification and processed with Zeiss software and Image J. At least five different fields were randomly chosen and photographed, and 25 to 30 cells were observed.

Melanin production assay:

C. neoformans wild type and *pdc*Δ mutant strains were grown overnight and refreshed the next morning in the same way as described above and then harvested by centrifugation for 2 min at room temperature, washed twice in D-PBS, counted using the hemocytometer. Each strain was diluted to 1×10^6 cells/ml and a 5µl volume of each strain was plated on agar-containing melanin-induction medium (8 mg/ml KH_2PO_4, 2 mg/ml glucose, 2 mg/ml L-glycine, 1 µg/ml D-biotin, 1 µg/ml thiamine, 0.92 mg/ml $MgSO_4{\cdot}7H_2O$, and 0.4 mg/ml L-3,4-dihydroxyphenylalanine [L-DOPA]) in order to induce melanin production (Li *et al.,* 2018). Plates were incubated at 30°C for 2-3 days and the imaged using the Canon EOS Rebel T1i camera. The experiment was repeated thrice.

Growth under hypoxia and under oxidative and nitrosative stresses:

Wild type and *pdc*Δ strains were grown overnight and refreshed the next morning as described above, then harvested by centrifugation for 2 min at room temperature, washed twice in D-PBS, counted using the hemocytometer, and diluted to 5×10^3 cells/µl. Next, three 10-fold serial dilutions of cells were prepared, and 2µl of each of these four dilutions were spotted at 1×10^4, 1×10^3, 100 and 10 cells per spot onto media that mimicked different stress conditions that the fungus encounters during infection. To mimic hypoxia, cells were spotted onto YES-agar (0.5% yeast extract, 2% glucose, 1.5% agar and 225 µg/mL of each of uracil, adenine, leucine, histidine, and lysine) supplemented with 0.7 mM $CoCl_2$ (Lee *et al.*, 2007). To test the effects of oxidative and nitrosative stress, cells were spotted onto sodium succinate-adjusted YNB-agar medium (1.34 g/liter yeast nitrogen base without amino acids, 4 g/L glucose, and 5 mM sodium succinate to adjust the pH to 4.0, filter sterilized and mixed 1:1 with 4% agar to a final concentration of 2% agar) supplemented with either 1 mM hydrogen peroxide (H_2O_2) or 1.5 mM sodium nitrite ($NaNO_2$), respectively, or not supplemented as control (Gerik *et al.,* 2008). Plates were incubated at 30°C for 2-3 days and then imaged using the Canon EOS Rebel T1i camera. Each experiment was repeated thrice.

Growth under osmotic stress:

Fungal cells were cultured overnight, refreshed and counted the next day, diluted, and spotted the same way as above, except that the wild type and *pdc*Δ strains were grown on osmotic stress generating medium (1% yeast extract, 2%

Bacto Peptone, 2% glucose, and 2% agar, supplemented with either 1.5 M NaCl or 1.2 M KCl). Plates were incubated at 30°C for 3 days and then imaged using the Canon EOS Rebel T1i camera. Each experiment (with NaCl or KCl) was repeated three times.

SDS and Congo red assays for cell wall integrity:

Fungal cells were cultured overnight, refreshed and counted the next day, diluted, and spotted onto plates as above, except that the wild type and *pdc*Δ strains were spotted onto media with 1% yeast extract, 2% Bacto Peptone, 2% agar, and 1% glucose supplemented with either 0.5% Congo red (Sigma-Aldrich/Millipore Sigma, St. Louis, MO) or 0.03% sodium dodecyl sulfate (SDS; Calbiochem/Millipore Sigma, St. Louis, MO). Plates were incubated at 30°C for ~72 hours and then images were captured using the Canon EOS Rebel T1i camera. Each experiment was repeated three times.

Macrophage culture:

A murine macrophage-like cell line J774A.1 (gift from Dr. Jeffrey Anker, Clemson University), originally derived from BALB/c mouse reticulum cell sarcoma (Fan et al.,2005), was maintained in macrophage medium (Dulbecco's Modified Eagle Medium [DMEM] supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, 1% penicillin-streptomycin, and 10% NCTC-109 medium, Gibco Life Technologies). Cells were passaged at a 1:10 split approximately every 5-7 days when reached 80% confluence.

Macrophage survival assay:

At ~80% confluence, cultured macrophages were washed twice with D-PBS, harvested by scraping after addition of fresh macrophage medium, and then diluted to 1×10^3 cells/µl. These macrophages were transferred into three wells each for the wild type and *pdcΔ C. neoformans* strains into two 96-well flat-bottom tissue culture treated plates (Costar; Corning Inc., Corning NY), one for one-hour co-incubation and the other for 24-hour co-incubation with fungal cells, with 100 µl of the macrophage suspension (1×10^5 cells in macrophage media) added to each well. Both plates were incubated overnight at 37˚C in the 5% CO_2 incubator.

Concurrently, wild type (KN99α) and *pdcΔ C. neoformans* strains were grown overnight in liquid YPD media at 30°C. The next day, the strains were pelleted by centrifugation at 8,000×g for 1 min, washed twice with D-PBS, and resuspended in macrophage medium. The fungal cells were then diluted to 125 cells/µl in 1 ml of macrophage media (*i.e.* 1.25×10^4 *C. neoformans* cells per 100 µl). To enhance macrophage phagocytosis by opsonization, the two *C. neoformans* strains were each incubated for one hour at 37˚C with 1µg/ml (final concentration) of the MAb18B7 monoclonal antibody (gift from Dr. Arturo Casadevall, Johns Hopkins University School of Medicine) that binds to the glucuronoxylomannan (GXM) capsule component (Casadevall *et al.*, 1998). As soon as the *C. neoformans* strains mixed with antibody were placed in the incubator, the two plates containing macrophages were processed as follows. The media was gently removed from each well and replaced with 100 µl macrophage

media containing 10 nM phorbol myristate acetate (PMA), and then both plates were incubated at 37˚C in 5% CO_2 for one hour to activate the macrophages (Forman and Torres, 2001).

After separate one hour incubations of the macrophages and fungal cells, the medium in each well from both 96-well macrophage plates was replaced, without disrupting the macrophage monolayer, with a 100 µl volume of either the wild type or *pdcΔ* mutant cell suspension (*i.e.* 1.25×10^4 cells), in triplicate per *C. neoformans* strain, at an 8:1 macrophage to fungal cell ratio. In a third 96-well tissue culture treated plate, as a control, 100 µl (*i.e.* 1.25×10^4 cells) per well of the cell suspensions of *C. neoformans* wild type or *pdcΔ* strains were added, in triplicate, in the absence of macrophages. All three plates were left at 37°C in 5% CO_2 for one hour, after which each well in the one-hour and 24-hour plates with macrophages, but not in the control plate without macrophages, were washed gently three times with 200 µl D-PBS to remove non-phagocytosed *C. neoformans*. To the 24-hour plate, 100 µl of fresh macrophage media were added to each well, and the plate was placed back into the 37˚C, 5% CO_2 incubator for 24 hours.

To the one-hour plate, 200 µl of sterile distilled, deionized water (ddH_2O) were added to each well, and the plate was incubated at room temperature for 5 minutes to lyse the macrophages. A pipette tip was then scraped against the bottom of the wells to lift up the adherent macrophages, and the cells suspended in the water were pipetted up and down to disrupt and further lyse the macrophages. The 200 µl lysate was then transferred to a microfuge tube. Again,

200 µl of sterile ddH_2O were added to each well, the wells were scraped, and the suspensions were pipetted up and down. The additional lysates generated were combined with the corresponding original lysates. These steps were repeated twice more, but with 300 µl of sterile ddH_2O, so that, by the end of the procedure, each microfuge tube held 1 ml final volume of lysate. For the plate of *C. neoformans* strains without macrophages, since the wells were not washed prior to addition of sterile ddH_2O, the volume of original media plus sterile ddH_2O transferred to each microfuge tube was 300 µl; then 200 µl, 200 µl, and 300 µl volumes were used and transferred to the corresponding microfuge tube. The same procedure that was used on the one-hour plates with macrophages was used, without modification, to obtain macrophage lysates from the 24-hour plate the next day. For each 96-well plate, macrophage lysis (or recovery of fungal cells from wells without macrophages) was conducted in sets of one well per strain, in triplicate sets, to obtain the fungal cells from the three wells per strain.

Immediately after wild type or *pdcΔ* mutant *C. neoformans* cells were obtained from the macrophage lysates or from the fungal cell suspensions (*i.e.* from the no-macrophage control plate), the cells were diluted 1:10, then again 1:2.5 for a final dilution of 1:25, or a (theoretical) maximum concentration of 500 cells/ml in a total volume of 0.5 ml. From this final dilution, 100 µl were plated on YPD-agar plates, one plate per corresponding well from each 96-well plate, resulting in triplicate YPD-agar plates of wild type and *pdcΔ* mutant cells. From the 1-hour plate, it was expected that a maximum of 50 cells from each corresponding

well of the 96-well plates would form colonies, but, due to replication of the fungal cells, more than this number of colonies was possible from the 24-hour plate. YPD-agar plates were incubated at 30°C for ~48 hours and then colony forming units (CFU) were counted for each plate and the percentage survival was calculated for each strain using the following formula, in which MΦ refers to macrophages and the CFU are the average of the individual CFU from the triplicate YPD-agar plates:

% Phagocytosis = ($CFU_{C.\ neoformans\ \text{with MΦ @ 1hr}} \div CFU_{C.\ neoformans\ \text{without MΦ}}$) × 100

% Survival = ($CFU_{C.\ neoformans\ \text{with MΦ @ 24 hrs}} \div CFU_{C.\ neoformans\ \text{with MΦ @ 1 hr}}$) × 100

***C. neoformans* virulence assay with invertebrate model:**

To examine the virulence of mutant *C. neoformans* strains, the survival of larvae of the greater wax moth, *Galleria mellonella*, was assayed after injection, in parallel, with either the wild type or *pdc*Δ mutant *C. neoformans* strain; for this assay, both strains were in an H99 strain background. For use in this assay, the two *C. neoformans* strains were grown in YPD media at 30°C overnight and refreshed the next morning by inoculation of new 2 ml cultures and incubation to an OD_{600} of ~0.2. The cells from each strain were then pelleted and washed once with sterile water and twice with D-PBS, before being resuspended in D-PBS to a final concentration of 1×10^5 cells/μL. Subsequently, milky-white *G. mellonella* larvae without any dark spots and weighing between 0.27 g and 0.30 g were chosen. Ten larvae per *C. neoformans* strain were selected and disinfected using alcohol wipes just prior to inoculation. Using Hamilton syringes, the ten larvae were each injected in the bottom, left proleg with 10 μl (1×10^6 cells) of either the wild

type or *pdc*Δ strain. To determine the level of death due solely to injection, 10 µl of D-PBS were injected as a control into another ten larvae and death was monitored over time. Similarly, as a negative control, the wild type *C. neoformans* strain (in the H99 background) was heat-killed via incubation at 65°C for 1 hour and 1×10^6 of these dead cells were injected into each of another ten larvae. After all strains and controls were injected into *G. mellonella*, the larvae were incubated at 37°C and monitored daily. Death was scored based on blackened appearance (indicative of either necrosis or complete melanization) and a lack of normal rolling-over behavior. The numbers of dead larvae in each injection group were tallied and graphed in a Kaplan-Meier survival curve.

Statistical analysis of results:

All of the graphs were prepared using GraphPad Prism. When multiple time points were being analyzed in the experiment, the significance of the results were determined using the analysis of variance (ANOVA) software within Prism, with Dunnet, Sidak, or other post-test multiple comparison, as recommended by the software, generally using one-way ANOVA to compared the wild type to the *pdc*Δ strain. When only triplicate values at one time point (such as in the macrophage survival assay, in which the relative percent survival values were first calculated using Microsoft Excel) were analyzed, a parametric, paired, two-tailed t-test was conducted. For Kaplan-Meier survival curves, the Gehan-Breslow-Wilcoxon test and/or Log-Rank test was used to compare the virulence of the mutant strain to that of the wild type strain.

Results

Phylogenetic tree analysis:

The phylogeny of the pyruvate decarboxylase (Pdc) family was generated with protein sequences from *C. neoformans var. grubii* and *var. neoformans* and putative homologs from various other fungi (Figure 3.2) using MEGA X (Kumar *et al.*, 2001) software. The *C. neoformans var. grubii* Pdc was, as expected, most closely related to that of *C. neoformans var. neoformans* and of *Cryptococcus gattii*, and, together with a hypothetical protein from *Tremella mesenterica*, form a monophyletic group (GROUP 1 in Figure 3.2) in the tree. These Pdc sequences are next most closely related to the putative Pdc1 from *Sporisorium reilianum*, a putative indolepyruvate decarboxylase from *Ustilago maydis* (UMAG_03994), the Pdc sequence from *Coprinopsis cinerea* (CC1G_03453), and the Pdc sequence from *Phanerochaete chrysosporium* (AGR57_8688), which, together, form the monophyletic GROUP 2 in Figure 3.2. Surprisingly, the *C. neoformans var. grubii* Pdc is only distantly related to Pdc proteins from a third monophyletic group (GROUP 3 in Figure 3.2) that includes Pdc1p, Pdc5p, and Pdc6p from *S. cerevisiae* and Pdc1p and Pdc4p from *Schizosaccharomyces pombe*, but is more closely related to those than to the regulatory *S. cerevisiae* protein Pdc2p (Hohmann, 1993; Kaiser *et al.*, 1999) or to the related regulatory protein Pdc2p from *Candida albicans* (Kaiser *et al.*, 1999). It is therefore likely that *C. neoformans var. grubii* and *var. neoformans* pyruvate decarboxylases have similar enzymatic function as Pdc1p, Pdc5p, and Pdc6p from *S. cerevisiae*.

Verification of *PDC* deletion:

The *pdc*Δ mutant (in the KN99α strain background) obtained from the Madhani deletion collection (Chun and Madhani, 2010) was checked for the replacement of *PDC* with a nourseothricin acetyltransferase (*NAT*) cassette by PCR. The results confirmed the presence of *PDC* in the wild type strain (also in the KN99α strain background) and the deletion of the gene in the *pdc*Δ strain (Figure 3.3, lanes 2 and 3, respectively). No PCR product for *NAT* was observed for the wild type strain, while a band of the correct size (~1600bp) was observed for the *pdc*Δ strain (Figure 3.3, lanes 4 and 5, respectively).

Absence of Pdc impacts glucose-dependent growth of *C. neoformans*:

C. neoformans prefers glucose over other carbon sources for growth (Hu *et al*., 2008; Sabiiti and May, 2012), but inside the host it encounters conditions such as low glucose and low amino acid concentrations, which forces it to utilize acetate, lactate, or other alternative carbon sources to survive (Hu *et al.,* 2008; Price *et al.,* 2011). Therefore, the growth of the *pdc*Δ mutant on YNB medium supplemented with glucose as the conventional carbon source, or with acetate or ethanol as alternative carbon sources, was investigated. Specifically, spot assays and growth curve analyses were performed on solid or liquid media supplemented with 2% glucose, 2% acetate, or 2% ethanol. In the qualitative spot assay, the *pdc*Δ mutant grew similarly to the wild type KN99α strain on all three media, suggesting that the function of the Pdc enzyme is not necessary for utilization by *C. neoformans* of different carbon sources (Figure 3.4). In the quantitative growth analysis, the *pdc*Δ

mutant did not grow as well as the wild type strain in medium supplemented with glucose (Figure 3.5a; P-value = 0.0011 overall), especially after 14 hours (P-value = 0.0053, 0.0084, 0.0047, and 0.0063 at 24, 31, 38, and 48 hours, respectively). Unlike in the colonies formed in the spot assay, in the quantitative growth assay, the constant shaking of fungal cells grown in liquid medium tended to separated individual cells from each other, preventing them from contributing to each other's growth and survival via some type of *en masse* metabolic compensation mechanism; this might explain the discrepancy between the results of the two assays. Thus, the overall results of the growth assays demonstrated that the pyruvate decarboxylase-deficient mutant exhibited inefficient cell autonomous utilization of glucose, but could make use of acetate or ethanol as effectively as the wild type strain, since the two strains exhibited equivalent growth in the corresponding media (Figure 3.5b, c).

Acetate production is reduced in the *pdc*Δ mutant:

Pyruvate decarboxylase is used by *S. cerevisiae* to convert pyruvate to acetaldehyde, which is then be converted to acetate via an acetaldehyde dehydrogenase (Pronk *et al.*, 1996). In order to determine whether the *C. neoformans PDC* gene is similarly involved in the production of acetate, the amount of acetate secreted into the extracellular media by wild type and *pdc*Δ strains grown on YNB supplemented with 2% glucose was measured every 12 hours. It was observed that the *pdc*Δ mutant could produce acetate, but at a concentration very significantly (P-value < 0.01) lower concentration than that

produced by the wild type KN99α strain, (Figure 3.6; P-value = 0.0035 overall, with P-value = 0.0053 and P-value = 0.0206 at 36 and 48 hours, respectively), implying that *C. neoformans* Pdc is utilized in acetate production.

Capsule formation is compromised in the *pdc*Δ mutant:

The *C. neoformans* polysaccharide capsule protects against attack by the host immune system by downregulating inflammatory cytokines, decreasing the levels of complement proteins, and reducing the antigen presenting capacity of monocytes (Retini *et al.*, 1998; Vecchiarelli *et al.*, 2003; Vecchiarelli *et al.*, 1995), as well as by protecting against phagocytosis by macrophages (Panepinto *et al.*, 2007; Cross and Bancroft, 1995) and quenching phagocyte-produced reactive oxygen species if the pathogen is engulfed (Zaragoza *et al.*, 2008). To determine whether Pdc activity is important for production of the polysaccharide capsule, cells from wild type and *pdc*Δ mutant *C. neoformans* strains in the KN99α background were incubated in capsule inducing medium and then stained with India Ink to visualize capsule thickness under the microscope. Capsule thickness was reduced in the *pdc*Δ mutant relative to the wild type strain (Figure 3.7), indicating that pyruvate decarboxylase function is relevant to capsule production.

Melanization is reduced in the *pdc*Δ mutant:

Melanin is produced by *C. neoformans*, and protects the pathogen from attack by reactive oxygen and nitrogen species produced by macrophages after phagocytosis (Ma and May, 2009), so melanin deficient mutants of *C. neoformans* are less virulent (Wang *et al.*, 1995). Wild type and *pdc*Δ mutant

cells were spotted onto plates supplemented with L-DOPA, which *C. neoformans* converts to melanin using the laccase enzymes (Williamson, 1997; Li *et al*., 2018). The *pdc*Δ mutant colonies had visibly reduced coloration as compared with the wild type colonies, indicating that this pyruvate decarboxylase deficient mutant had defective melanin production (Figure 3.8).

Deficiency of pyruvate decarboxlyase affects response to hypoxia:

C. neoformans causes fungal meningitis by infecting the host nervous system and then surviving and growing under the low oxygen conditions found in the brain (Ingavale *et al.*, 2008; Erecińska and Silver, 2001). Thus, identifying the enzymes used by *C. neoformans* to withstand hypoxia could generate therapeutic targets. Cobalt chloride induces both the Sre1p ergosterol production pathway and transcription of the fatty acid desaturase gene *OLE1*, which function in coordinating the cellular low-oxygen response (Guzy *et al* 2007; Lee *et al.*, 2007). Cells react to $CoCl_2$ as if they were under hypoxia (Guzy *et al* 2007; Lee *et al.*, 2007). To determine whether Pdc plays a role in the survival of the pathogen under hypoxia, the *pdc*Δ mutant strain was grown in YES medium supplemented with $CoCl_2$. The *pdc*Δ mutant grew normally in YES medium under normoxic conditions (Figure 3.9a), but exhibited defective growth when subjected to the artificial hypoxia (Figure 3.9b). This suggests that Pdc is important for survival of the pathogen under low oxygen. However, when the *pdc*Δ mutant was cultured on $CoCl_2$-containing medium to which 2% acetate was added, it grew normally (Figure 3.9c). Therefore, the observed growth defect is likely due to insufficient production of

acetate by this pyruvate decarboxylase deficient mutant. Alternatively, Pdc functions in a hypoxia response pathway that can be replaced by a secondary, acetate-dependent pathway, although how this would work is uncertain.

Pyruvate decarboxylase is not required for survival under oxidative, nitrosative, or osmotic stress

While the pathogen needs to be able to survive hypoxia after dissemination to the brain, at a much earlier stage of infection immediately after inhalation into the host lungs, the cryptococcal cells (or spores) must be able to survive engulfment by alveolar macrophages, which generate and use reactive oxygen and nitrogen species to destroy the engulfed pathogen (Hampton *et al.,* 1998; Vieira *et al.,* 2002; Cox *et al.,* 2003; Tohyama *et al.,* 1996). To determine whether *C. neoformans* Pdc activity assists in the survival of the pathogen under oxidative or nitrosative conditions, the wild type and *pdc*Δ strains were grown on YNB-agar (buffered to pH 4.0 using sodium succinate) to which 1 mM H_2O_2 or 1.5 mM $NaNO_2$ was added, respectively. There was no significant difference in the growth of the *pdc*Δ mutant under either oxidative (Figure 3.10a) or nitrosative (Figure 3.10b) conditions relative to the wild type strain, indicating that Pdc plays no role in the neutralization of oxygen or nitrogen free radicals produced by the host immune system or in survival under oxidative or nitrosative stress.

C. neoformans is also challenged with osmotic stress inside the human body, and produces metabolites like mannitol (Chaturvedi *et al.*, 1996a,b) or utilizes the *HOG1* pathway (Bahn *et al.*, 2005) to survive in high osmolarity

environments. To assay whether Pdc has any function in the osmotic stress response, the wild type strain and the *pdc*Δ mutant were grown on YPD plates supplemented with 1.5 M NaCl and 1.2 M KCl to generate high salt conditions. It was found that the *pdc*Δ mutant grew as well as the wild type strain under these high salt conditions (Figure 3.11). This observation suggests that there is no involvement of the Pdc enzyme in the growth of *C. neoformans* under osmotic stress.

Cell wall integrity is not affected in the *pdc*Δ mutant:

The cell wall is an essential component that provides structure to cryptococcal cells and that protects *C. neoformans* from the oxidative environment inside the macrophages generated and used by these phagocytic cells to destroy the pathogen (Gerik *et al.*, 2005). The cell wall plays a role in the attachment of the capsule to the cell membrane and harbors the laccase enzymes that produce melanin, which is deposited in the cell wall and protects against ROS and RNS (Baker *et al.*, 2007; Van Dyke and Wormley, 2018). Therefore, an intact cell wall is required for full virulence. The cell wall integrity of the *pdc*Δ mutant strain was assayed by spotting decreasing concentrations of cells on media containing sodium dodecyl sulfate (SDS) or Congo red. Congo red binds to (1,4) β-glucans (or to the monomer, β-1,4 N-acetyl glucosamine), and thereby interferes with cell wall construction and with attachment to the cell wall of the chitin and capsule, while SDS that passes through gaps in the capsule, chitin, and cell wall dissolves the plasma membrane and lyses the cells (García *et al.*, 2015; Banks *et al.*, 2005;

Ram and Klis, 2006; Baker *et al*., 2007; Gerik *et al*., 2005; Wood *et al*., 1983). Hence, strains with cell wall integrity defects will not survive in the presence of either Congo red or SDS, depending which layer of capsule, cell wall, or chitin is deficient. The growth of the *pdc*Δ strain was not affected by SDS (Figure 3.12b) but was severely reduced at low cell concentrations or Congo Red (Figure 3.12c), indicating that the Pdc enzyme does play a role in building the cell wall or maintaining cell wall integrity, but the lack of this enzyme does not affect the integrity to such an extent that SDS can traverse the cell wall.

Deficiency of Pdc greatly impairs survival after phagocytosis :

To investigate the contribution of Pdc in the survival of *C. neoformans* against attack by host immune cells, the percentage of fungal cells surviving engulfment by murine macrophages was assayed. Wild type and *pdc*Δ mutant fungal cells were each co-incubated, in triplicate, with macrophages for 1 hour in a 96-well plate, and the fungal cells from lysed macrophages were plated on YPD. The relative percent phagocytosis of the mutant was determined by comparing colony forming ability of phagocytosed fungal cells to that of non-phagocytosed control cells and then to this ratio for wild type cells. In a parallel plate, the macrophages were given 24 hours to destroy internalized *C. neoformans* cells (after non-phagocytosed fungal cells were removed), and were then lysed. Surviving *C. neoformans* cells were plated on YPD, and the number of colonies of each strain were divided by the average number of colonies from phagocytosed cells of that strain from the percent phagocytosis analysis. The percent

phagocytosis of the mutant was marginally significantly increased relative to the wild type strain (161.39% vs. 100%; P-value = 0.0293). In contrast, the percent survival and recovery of growth capability inside the macrophages was highly significantly reduced for the *pdc*Δ strain, at only 39.11% (P-value = 0.0003) relative to the 100% normalized value for the wild type strain (Figure 3.13). These results indicate that deficiency of the pyruvate decarboxylase enzyme does negatively affect survival of *C. neoformans* inside macrophages *in vitro*.

The *pdc*Δ mutant exhibits normal virulence in the invertebrate *Galleria mellonella in vivo* infection model:

Since Pdc was found to be important for the production of the capsule and melanin, both of which are important virulence factors, and for survival in macrophages, an *in vitro* assay for virulence, it was reasonable to expect a role for Pdc in pathogenesis *in vivo*. Therefore, the virulence of the *pdc*Δ mutant strain was assessed in the invertebrate model, the larvae of the greater wax moth, *Galleria mellonella*. Fungal virulence was measured based on the number of days required for the first larva to die and then for all of the larvae to die, represented as a Kaplan-Meier survival curve (Figure 3.14). The two negative control groups of larvae only started to die off on day 8, and at least some larvae survived until day 18 at minimum. In contrast, larvae infected with wild type *C. neoformans* cells started dying on day 6 post-injection and all such larvae were dead by day 9. Similarly, larvae infected with *pdc*Δ mutant cells started dying on day 6 post-injection and all larvae were dead by day 10. In fact, the survival curve

of the *pdc*Δ strain-injected larvae was not statistically different from that of the wild type H99-injected larvae, as determined using a Chi-square-based Gehan-Breslow-Wilcoxon test (P-value = 0.3395) or using a Log-Rank test (P-value = 0.2700), whereas the two control survival curves were statistically different from the survival curve of the wild type injected larvae by either test (P-value < 0.0001 for both controls). The median survival of the larvae injected with *pdc*Δ mutant cells was 8 days, the same as that of larvae injected with wild type *C. neoformans* cells; in contrast, the median survival of larvae injected with PBS or heat-killed *C. neoformans* cells were 13 and 15 days, respectively. These observations suggest that deficiency of pyruvate decarboxylase does not affect the virulence of *C. neoformans* when utilizing *G. mellonella* as a model, despite the effect of this deficiency on reducing capsule size, melanin content, and even survival in macrophages.

Discussion

This is the first investigation of the role of *Cryptococcus neoformans* pyruvate decarboxylase in the virulence of the pathogen. *S. cerevisiae* requires Pdc activity for normal growth (Schmitt and Zimmermann, 1982) and has three structural *PDC* genes, *PDC1, PDC5,* and *PDC6* (Seeboth *et al*., 1990; Kaiser *et al*., 1999; Agarwal *et al*., 2013). The enzyme encoded by *PDC1* is the major pyruvate decarboxylase used during fermentation (Schmitt and Zimmermann, 1982; Schmitt *et al*., 1983; Kellermann *et al*., 1986; Hohmann and Cederberg, 1990; Hohmann 1991a; Hohmann, 1993). Mutation of *PDC1* decreases growth because of excessive accumulation of pyruvate, which can slow down glycolysis in *S. cerevisiae* (Schmitt and Zimmermann, 1982). Some point mutants of *PDC1* are completely unable to grow on glucose-containing media under either respiratory or fermentative conditions (Schmitt and Zimmermann, 1982; Schmitt *et al*., 1983), and exhibit a cytosolic redox imbalance, as well as secrete excess cytosolic pyruvate into the growth medium (Van Maris *et al*., 2004). Complete deletion of the *PDC1* gene apparently rescues growth on glucose, and the mutant retains 60-70% of pyruvate decarboxylase activity. The total lack of Pdc1 expression causes the upregulation of expression of the *PDC5* gene, which is 88% identical to *PDC1* (Seeboth *et al*., 1990; Agarwal *et al*., 2013; De Assis *et al*., 2013). In short, Pdc1p appears to be the major pyruvate decarboxylase in *S. cerevisiae* and the yeast uses Pdc5p instead when it cannot express Pdc1p (De Assis *et al*., 2013; Muller *et al*., 1999; Eberhardt *et al*., 1999; Hohmann and Cederberg, 1990;

Schaaff *et al*., 1989; Kaiser *et al*., 1999). A mutant in both *PDC1* and *PDC5* genes has no detectable pyruvate decarboxylase activity, and cannot grow for very long on glucose as the sole energy source, and the same is true of a triple mutant also lacking the weakly expressed *PDC6* (Flikweert *et al*., 1996; Hohmann, 1991b; Kaiser *et al*., 1999). During fermentation, *S. cerevisiae* can use the Pdc activity in the pyruvate dehydrogenase by-pass pathway to convert pyruvate to acetyl-CoA, via acetaldehyde and acetate (Pronk *et al*., 1994; Remize *et al*., 2000), so loss of Pdc function is expected to decrease the level of acetate, but *PDC1* gene mutation did not change the cytoplasmic acetate levels, even though it approximately doubled the level of pyruvate (Remize *et al*., 2000). The normal level of acetate may or may not result from low level expression and activity of Pdc5p or Pdc6p.

A distant relative of *S. cerevisiae*, *Saccharomyces kluyveri*, also has three *PDC* genes that code for functional Pdc enzymes. (Møller *et al.*, 2004). Another taxonomic relative of *S. cerevisiae*, *Kluyveromyces marxianus*, has two genes coding for Pdc enzymes, *KmPDC1* and *KmPDC5*; deletion of *KmPDC1* results in decreased or no growth in aerobic or anaerobic conditions, respectively (Choo *et al.*, 2018), indicating that it encodes the major Pdc. *PDC* is also reported to have homologues in several other yeast species, including in *Pichia pastoris* (Agarwal *et al.*, 2013), *Scheffersomyces stipitis* (Lu *et al.*, 1998), *Wickerhamomyces anomalus* (Fredlund *et al.*, 2006) and *Candida utilis* (Franzblau and Sinclair, 1983), as well as in *Ogataea polymorpha* (Ishchuk *et al.*, 2008), which, along with

Ogataea parapolymorpha, is actually a strain of the species *Hansenula polymorpha* (Suh and Zhou., 2010; Ravin *et al.*, 2013).

The fungal pathogen *Candida albicans* has 3 *PDC* genes, *PDC2*, *PDC11* and *PDC12* (Bahn *et al.*, 2007; Kaiser *et al.*, 1999). *PDC12* likely encodes the actual pyruvate decarboxylase. *PDC11* seems to encode a functionally related enzyme that localizes to the hyphal cell surface and that is upregulated under hyphae-inducing conditions in a cAMP dependent manner, but which is not found in the yeast cells themselves (Bahn *et al.*, 2007). *PDC12* is also upregulated by the cAMP signaling pathway, but is expressed in the yeast cells (Bahn *et al.*, 2007). In contrast to the proteins encoded by *PDC11* and *PDC12,* the protein encoded by *PDC2* has a regulatory role (Kaiser *et al.*, 1999). *C. albicans* requires Pdc activity to grow under hypoxic/anaerobic conditions. Pyruvate decarboxylase expression is highly responsive to oxygen levels in *C. albicans*, with expression of the Pdc apoform down-regulated in oxygen-rich environments, and upregulated in anaerobic conditions (Tylicki *et al.*, 2008). The maximal rate of Pdc activity in *C. albicans* is reached at a much lower concentration of pyruvate than in *S. cerevisiae* (Tylicki *et al.*, 2008), indicating that the Pdc enzymes in different fungal species, including in *C. neoformans*, may have different substrate affinities and enzyme kinetics.

When the protein sequence for the single *C. neoformans* Pdc was analyzed using phylogenetic software, it was found to be more closely related to the probable Pdc1 from *Sporisorium reilianum*, a putative indolepyruvate

decarboxylase from *Ustilago maydis* (UMAG_03994), the pyruvate decarboxylase from *Coprinopsis cinerea* (CC1G_03453), and the pyruvate decarboxylase from *Phanerochaete chrysosporium* (AGR57_8688) than to Pdc1p, Pdc5p, and Pdc6p from *S. cerevisiae* or to the *C. albicans* proteins, making it difficult to determine the function of the *C. neoformans* Pdc based on the *S. cerevisiae* or *C. albicans* proteins. Nevertheless, the review of Pdc enzymes from the latter two model organisms provided insight that allowed the interpretation of the results of biochemical and cellular assays of the *C. neoformans pdc*Δ mutant.

As *C. neoformans* disseminates from the lungs, it encounters different environments, such as those inside of macrophages, in the blood, or in the brain, and it must adapt to utilize different available nutrients; the fungus even changes the expression of genes important in the utilization of acetate, lactate, ethanol, or glucose as part of adjusting to the use of alternative carbon sources (Hu *et al*., 2008). Therefore, the growth of the *C. neoformans pdc*Δ mutant on glucose, ethanol, or acetate as the sole carbon source was assayed. As mentioned above, *S. cerevisiae pdc1* point mutants have reduced growth on glucose while deletion mutants grow normally by upregulating Pdc5p (Schmitt and Zimmermann, 1982; Schmitt *et al*., 1983; Seeboth *et al*., 1990; De Assis *et al*., 2013; Muller *et al*., 1999; Eberhardt *et al*., 1999; Hohmann and Cederberg, 1990; Schaaff *et al*., 1989; Kaiser *et al*., 1999). It was therefore surprising, despite the lack of evolutionary relatedness between the *C. neoformans* and *S. cerevisiae* enzymes, that the *C. neoformans pdc*Δ mutant grew normally on solid media with glucose as the sole

carbon source (Figure 3.4a) but displayed a growth defect in the equivalent media in liquid form (Figure 3.5a). This indicates that, despite having only one Pdc enzyme, *C. neoformans* cells deleted for the corresponding gene somehow work together to circumvent the function of the pyruvate metabolism pathway via some kind of compensation mechanism when grown at high density as colonies. This mechanism appears to function only when the cells are *en masse*, so that, when isolated from each other in liquid culture with constant agitation, the mutant cells do not grow as well as wild type cells. The *C. neoformans pdc*Δ mutant did not exhibit a growth defect on ethanol. Just as *S. cerevisiae* represses the expression of both *PDC1* and *PDC5* in the presence of ethanol (Liesen *et al.*, 1996) since it can make acetaldehyde, and therefore acetate, from ethanol instead of from pyruvate, *C. neoformans* also should not need pyruvate decarboxylase when grown on ethanol. Likewise, if Pdc plays a role in acetate production, it should not be necessary for growth on acetate. In support of this potential function, the *pdc*Δ mutant grew normally on media containing acetate as the primary carbon source.

In addition to serving as a possible carbon source for *C. neoformans*, acetate is one of the major metabolites in infected tissues (Himmelreich *et al.,*2003) as well as in culture (Bubb *et al*., 1999; Wright *et al*., 2002). Based on studies in *S. cerevisiae*, pyruvate produced in the cryptococcal cell by glycolysis either could be shuttled to mitochondria to generate acetyl-CoA (via the TCA cycle) or could be channeled into a anaerobic pathway to make acetaldehyde, which would then be converted to acetate and acetyl-CoA via the functions of

acetaldehyde dehydrogenase and acetyl-CoA synthetase enzymes, respectively (Saint-Prix *et al*., 2004; Remize *et al*., 2000; Pronk *et al*., 1994, 1996). The latter pathway, again, based on *S. cerevisiae*, is also likely to be the major source of cytoplasmic acetate (Saint-Prix *et al*., 2004; Remize *et al*., 2000; Pronk *et al*., 1994, 1996). Furthermore, Hu *et al*. (2008) reported that *C. neoformans* enzymes involved in metabolism or transport of acetate, including two acetate transporters, acetyl-CoA synthetase, an aldehyde dehydrogenase, and pyruvate decarboxylase, and are all upregulated during pulmonary infection. In order to assess the effect of the loss of the Pdc enzyme in *C. neoformans* on acetate production, the levels of acetate secreted by the wild type strain and by the *pdc*Δ mutant at different time points during growth in liquid media were quantified. A statistically significant decrease in the levels of acetate was observed in the *pdc*Δ mutant relative to the wild type strain (Figure 3.6), indicating that deficiency of pyruvate decarboxylase impaired acetate production and suggesting that Pdc plays an important role in the generation of acetate by *C. neoformans*. This is contradictory to the observation in *S. cerevisiae*, in which mutation of the *PDC1* gene did not affect the levels of acetate (Remize *et al*., 2000). The reason for this difference is likely that *S. cerevisiae* has at least two functioning Pdc enzymes that can compensate for each other (De Assis *et al*., 2013; Muller *et al*., 1999; Eberhardt *et al*., 1999; Hohmann and Cederberg, 1990; Schaaff *et al*., 1989; Kaiser *et al*., 1999), while *C. neoformans* has only one *PDC* gene, loss of which severely decreases the amount of acetate produced by *C. neoformans*. In combination with

the reduced acetate secretion observed in all of the *C. neoformans* aldehyde dehydrogenase mutants in the prevous chapter, the reduced levels of acetate secreted by the *pdc*Δ mutant supports the notion that Pdc and the Ald enzymes form a metabolic pathway by which a significant portion of the acetate generated by *C. neoformans* is made, although each could, instead, be part of different, parallel pathways.

Given the fact that acetate is produced by *C. neoformans* during *in vivo* infection (Himmelreich *et al.,*2003), it was obviously important to investigate whether reduced acetate levels, or reduced Pdc activity overall, could affect the virulence of the pathogen. Therefore, the status of various virulence factors, including capsule formation, levels of melanin, and cell wall integrity were assayed in the *C. neoformans pdc*Δ mutant. Indeed, the mutant exhibited a smaller and thinner capsule than the wild type strain (Figure 3.7). However, whether this reduction in capsule size is deleterious for fungal infection and survival in the host cell is actually questionable. *C. neoformans* spores are generally unencapsulated as smaller size helps in passage through the airway during inhalation (Velagapudi *et al.*, 2009). Infection with hypocapsular strains leads to worse clinical outcomes because of the higher possibility of passage into the lungs (Sabiiti *et al*., 2014). Furthermore, at least one study showed that hypercapsular mutants of *C. neoformans* were less virulent, while hypocapsular strains were more virulent, and disseminated more effectively to the brain, in a mouse model (Pool *et al*., 2013). On the other hand, completely acapsular

mutants of *C. neoformans* have been shown to be avirulent (Chang and Kwon-Chung, 1994), and cryptococci enlarge their capsules after reaching the lungs to prevent engulfment by macrophages (Levitz and Tabuni, 1991; Xie *et al*., 2012; May *et al*., 2016). Therefore, the lack of a proper capsule alone was not sufficient to predict the virulence of the *pdc*Δ mutant.

Besides the polysaccharide capsule, the survival of *C. neoformans* inside the host cells also depends on melanin. Melanin not only contributes to the maintenance of cell wall integrity, but also accumulates at the cell wall and neutralizes the free oxygen and nitrogen radicals produced by phagocytes before they can reach the fungal cell membrane, thereby protecting the pathogen from the chemical attack that is part of the immune response mounted by host cells (Wang *et al*., 1995; Wang *et al*., 1996; McClelland *et al*., 2006; Steenbergen and Casadevall, 2003). Analysis of the *C. neoformans* strain deficient for the Pdc enzyme showed a reduction in the level of melanin in the cells (Figure 3.8), which may have an impact on virulence. This decrease could be due to perturbation of the regulation of the melanin synthesis pathway, but a limited defect in cell wall integrity observed in the Congo red assay (Figure 3.12) could also contribute to the similarly limited reduction in melanization, since both melanin and the laccase enzymes that produce it are located in the *C. neoformans* cell wall (Perez-Dulzaides *et al*., 2018; Walton *et al*., 2005; Zhu *et al*., 2001; McFadden and Casadevall, 2001), and disruption of cell wall integrity leads to leaking out of melanin (Perez-Dulzaides *et al*., 2018). It is also possible that the smaller

capsule of the *pdc*Δ mutant (Figure 3.7) allowed the laccase enzyme and/or the melanin to escape, preventing the accumulation of the latter.

Since the *pdc*Δ mutant had both a smaller capsule and low melanin levels, the effect on growth of reactive oxygen and nitrogen species and of hyperosmolarity, which should affect these "unprotected" cells more, was assayed, but the mutants were unaffected by these stresses (Figures 3-10 and 3-11). This was an intriguing result, since it indicated that, either the degree of reduction in capsule size, cell wall integrity, and melanization was insufficient to increase sensitivity to these stresses, or other factors besides the capsule and melanin were protecting the cells from ROS/RNS-mediated cell damage or water loss. It is possible that the accumulation of pyruvate in these *pdc*Δ mutant cells might have been put to use by the cells to somehow neutralize the ROS/RNS and that the increasing concentrations of pyruvate increased the osmolarity within the cell, counteracting the hyperosmolarity in the media.

Just as *C. neoformans* cells have to be able to cope with oxidative (and nitrosative) stress, they also have to be able to cope with the near opposite condition, too little oxygen. *C. albicans* requires Pdc activity to grow under hypoxic/anaerobic conditions (Tylicki *et al.*, 2008). Therefore, it is possible, that pyruvate decarboxlyase activity may also provide resistance to hypoxia stress in *C. neoformans*, just as it appears to do in *C. albicans*. Indeed, when tested for growth on media with cobalt chloride, which induces cells to behave as if they are in hypoxic conditions, the *pdc*Δ mutant was more sensitive than the wild type strain

(Figure 3.9b). The cobalt ion in the cobalt chloride can drive the production of ROS via the Fenton reaction just like reduced iron (Ingavale *et al*., 2008); however, the sensitivity of the *pdc*Δ mutant to cobalt chloride was due to its lack of ability to respond properly to hypoxia, and not due to a sensitivity to ROS or RNS, since the mutant grew normally on media supplemented with hydrogen peroxide or sodium nitrite. The *pdc*Δ mutant also grew normally on media supplemented with $CoCl_2$ when 2% acetate was also added to the media. This indicates that the partial dependence of *C. neoformans* on Pdc for survival under hypoxia is based on the role of pyruvate decarboxylase in acetate production, or at least, that the pathogen needs acetate to contend with hypoxia-like conditions, and the *pdc*Δ mutant is unable to make wild type levels of acetate, as demonstrated by the hydroxamate assay.

Once it was determined that important virulence factors like melanin production, capsule formation, and cell wall integrity were negatively affected in the *pdc*Δ mutant, and that this correlated with at least one phenotype potentially associated with reduced virulence (reduced growth under hypoxia), the next logical step was to test whether the lack of the Pdc enzyme reduced the actual virulence of the mutant *in vitro* and/or *in vivo*. In fact, the *pdc*Δ mutant did demonstrate significantly reduced survival (and recovery) after phagocytosis relative to the wild type strain in an assay utilizing mouse macrophages (Figure 3.13). This indicates that, while the smaller capsule, reduced cell wall integrity, and decreased melanization observed in the mutant did not affect the response to ROS/RNS, they

did affect the ability to survive the other types of attacks mounted by the macrophages after engulfment. Thus, deficiency of pyruvate decarboxylase activity did reduce *C. neoformans* virulence *in vitro*.

Nevertheless, the effects on virulence of deletion of a gene are best determined using an *in vivo* assay. Other *C. neoformans* mutants with defective melanin production have reduced virulence in the *in vivo Galleria mellonela* infection model (Firacative *et al*., 2014). Therefore, since the *pdc*Δ mutant strain had reduced melanization in addition to reduced survival after macrophage engulfment, it was expected to have reduced virulence when injected into *G. mellonela* larvae. However, *pdc*Δ mutant cells killed the larvae just as effectively as *C. neoformans* wild type cells. This discrepancy may be due to differences in genetic background between the strains used in the larval assay and those used in the other assays; unlike in the other assays, in which the mutant and wild type strain were from the KN99α background, in the *G. mellonella* survival assay, the strains used were in the H99α background. The H99α background strains were used in the *in vivo* assay because the KN99α strains had not yet been obtained when the larvae were available. On the other hand, this strain difference should have minimal effect on the assay results, since the KN99α background strain is derived from the H99 background strain. Even if differences in genetic background played no role in the dissimilarity between the virulence of the *pdc*Δ mutant as measured by the *in vitro* macrophage assay and that measured by the *in vivo Galleria* survival curve assay, the apparently contradictory results are not that

surprising. Work by Bouklas *et al*. (2015) shows that phagocytosis and killing by murine macrophages does not necessarily correlate with virulence in *Galleria;* instead, the results of assays in which killing by macrophages was used to assess virulence are moderately similar to the results obtained from a murine infection model. This suggests that the *Galleria* model works well for testing some virulence factors but it does not consistently show a correlation with a murine model for many other virulence factors. Since this is the case, and since a murine model of infection is much more likely to demonstrate the effects on virulence in humans of mutations in *C. neoformans* genes, it is recommended that the *pdc*Δ mutant be tested for virulence in a mouse pulmonary infection or cryptococcal meningitis model.

Overall, this study shows that Pdc is an important metabolic enzyme in *C. neoformans* that can regulate synthesis of the capsule and the level of melanin in fungal cells, as well as the ability to grow in hypoxic conditions in an acetate-dependent manner, but that might or might not influence the virulence of the pathogen.

Potential future experiments

The current results point to a role for the *PDC* gene in acetate production, since it is now clear that the lack of the Pdc enzyme caused reduced growth under hypoxia-mimicking conditions in an acetate-dependent manner, based on the rescue of normal growth via supplementation with acetate. However, the importance of this putative role and of normal acetate levels to phenotypes associated with virulence needs to be investigated further by the addition of acetate to capsule induction and melanin induction media, and to the Congo red containing media in order to uncover whether the capsule formation, melanization, and cell wall integrity phenotypes are due to insufficient acetate production by the mutant. Likewise, culturing the wild type and *pdc*Δ mutant strains in media containing acetate prior to using them in the macrophage survival assay will reveal whether the mutant is less able to counteract the destruction of the fungal cells in the phagolysosome because it cannot produce enough acetate.

While the *pdc*Δ mutant had reduced survival when engulfed by murine macrophages, it exhibited normal virulence in the *Galleria mellonella in vivo* assay. However, the mutant (and wild type) strain used in the *G. mellonella* assay was in the H99α background, and was the KN99α background strain used in other assays, so the *G. mellonella* assay result should be confirmed with wild type and *pdc*Δ mutant strains in the KN99α background. If the mutant still does not exhibit reduced virulence in the larvae, and therefore contradicts the macrophage survival assay result, then this might indicate an issue with the use of an invertebrate

infection model. In other words, the use of mammalian organisms as an *in vivo* infection model would better match the use of murine macrophages, which are part of the vertebrate immune system, than the use of insect larvae, which have macrophage-like cells, but not actual macrophages (Arvanitis *et al.*, 2013; Tsai *et al.*, 2016). So, further testing of the virulence of the *pdc*Δ mutant should be undertaken using murine or zebrafish infection models involving injection or inhalation of *C. neoformans*. In particular, it is recommended that the *pdc*Δ mutant be tested for virulence in a mouse pulmonary infection or cryptococcal meningitis model. Since oxygen levels in the brain are lower than in many parts of the body after infection (Erecińska and Silver, 2001; Kronstad *et al*., 2012; Chang *et al*., 2007), cryptococcal dissemination and the development of cryptococcal meningitis requires *C. neoformans* to be able to grow under low oxygen. Therefore, the *pdc*Δ mutant, which exhibited a growth defect under hypoxia-mimicking conditions, is expected to be less virulent than the wild type pathogen in a mouse infection model.

Furthermore, the expression of various virulence-related and metabolic genes in the *pdc*Δ mutant could be analyzed after the *C. neoformans* are used in the infection of the mice. Hu *et al*. (2008) showed that levels of the mRNA encoding Pdc are upregulated in *C. neoformans* recovered from the lungs of mice from a pulmonary infection model. Therefore, the levels of the *C. neoformans* PDC mRNA could be determined by quantitative PCR after the fungal cells are grown under physiological conditions, under various stress conditions, or in or on different

carbon sources (*i.e.* glucose, acetate, or ethanol) to shed light onto the regulation of Pdc (mRNA) expression in *Cryptococcus*, and the level of expression of *PDC* in wild type cells, and of stress-response and metabolic genes in the wild type and *pdc*Δ mutant strains, could be determined after the fungal cells infect mouse lungs or brain tissue.

Finally, growth analysis of two double mutants should be undertaken. The first, a *pdc*Δ, *ack*Δ double mutant has been generated to determine whether the two corresponding enzymes function in a single pathway or in parallel pathways in acetate production; this double mutant is in the process of characterization. Likewise, since both the *ald*29Δ and *pdc*Δ mutants exhibited a growth defect under hypoxia-mimicking conditions, and both recovered normal growth when provided acetate, it is possible that the corresponding enzymes work in a single acetate production pathway. Generation of a *pdc*Δ, *ald*29Δ double mutant and analysis of the growth of this second double mutant, in this case versus that of each single mutant and of the wild type strain on media with $CoCl_2$ or with $CoCl_2$ and acetate, would determine whether the two enzymes work in series or in parallel pathways. If they function in the same pathway, the double mutant phenotype should be no worse than that of either single mutant, while, if they operate in parallel, the double mutant should be completely unable to grow on cobalt chloride supplemented media, unless acetate is provided. Analysis of the phenotype of the double mutant versus each single mutant (and wild type) strain in other assays, such as the

hydroxamate assay for acetate production, should confirm the findings of the hypoxic growth assay.

All of these experiments would further the understanding of the role of pyruvate decarboxylase in *C. neoformans* growth and virulence, completing the work begun here.

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Table 3.1: Information about the gene/enzyme deficient in the *pdc*Δ mutant strains used in this study.

Gene	Enzyme name†	Identification Number†	Chromosome†	Predicted Location‡
PDC	Pyruvate decarboxylase	CNAG_04659	10	Cytoplasmic

† The official enzyme name and identification number, as well as the chromosomal location of the corresponding *PDC* gene was obtained from the Fungi Database, https://fungidb.org/fungidb/app/record/gene/CNAG_04659.

‡ The predicted mitochondrial or cytoplasmic cellular location of the protein was determined using the MitoFates website http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi; (Fukasawa *et al.*, 2015).

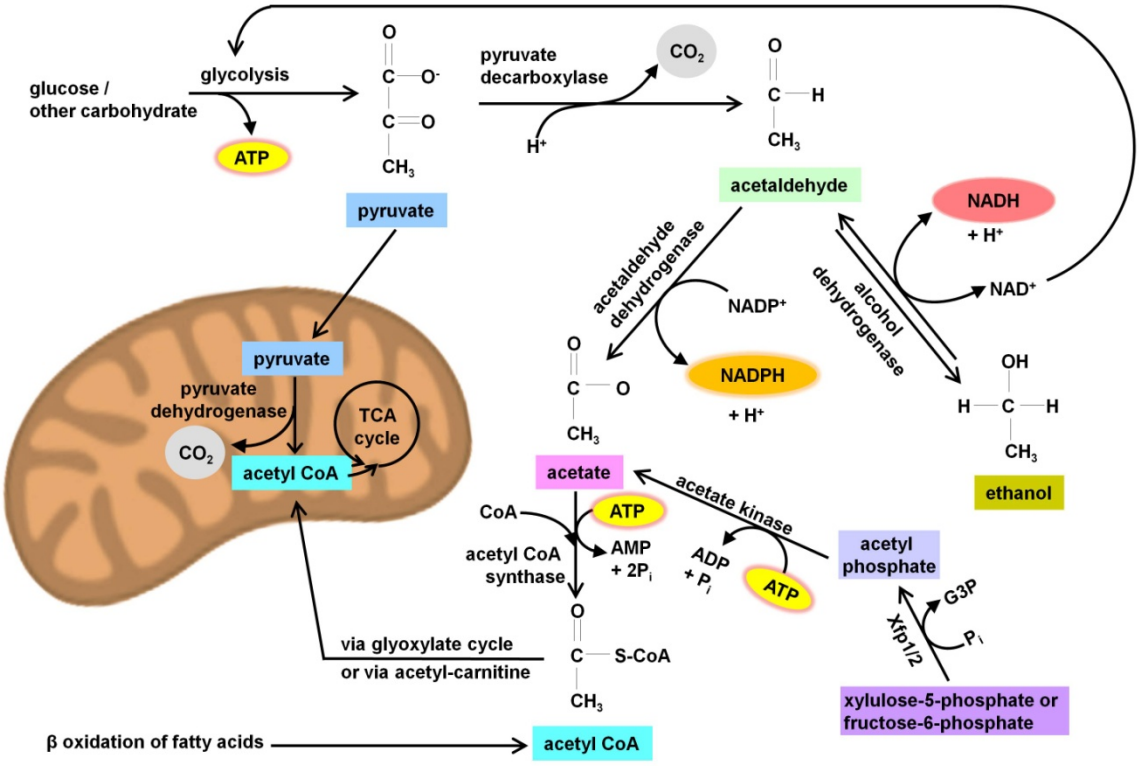

Figure 3.1: Various metabolic pathways utilized by *C. neoformans* to harness energy and invade the host immune system. Glucose, ethanol, and acetate can be used as sources of carbon by *C. neoformans*. Under anaerobic conditions, glucose is likely converted to acetate by the action of pyruvate decarboxylase (Pdc) and acetaldehyde dehydrogenase enzymes. Ethanol in the cells is converted via alcohol dehydrogenase to acetaldehyde, which is then likely converted to acetate via acetaldehyde dehydrogenase.

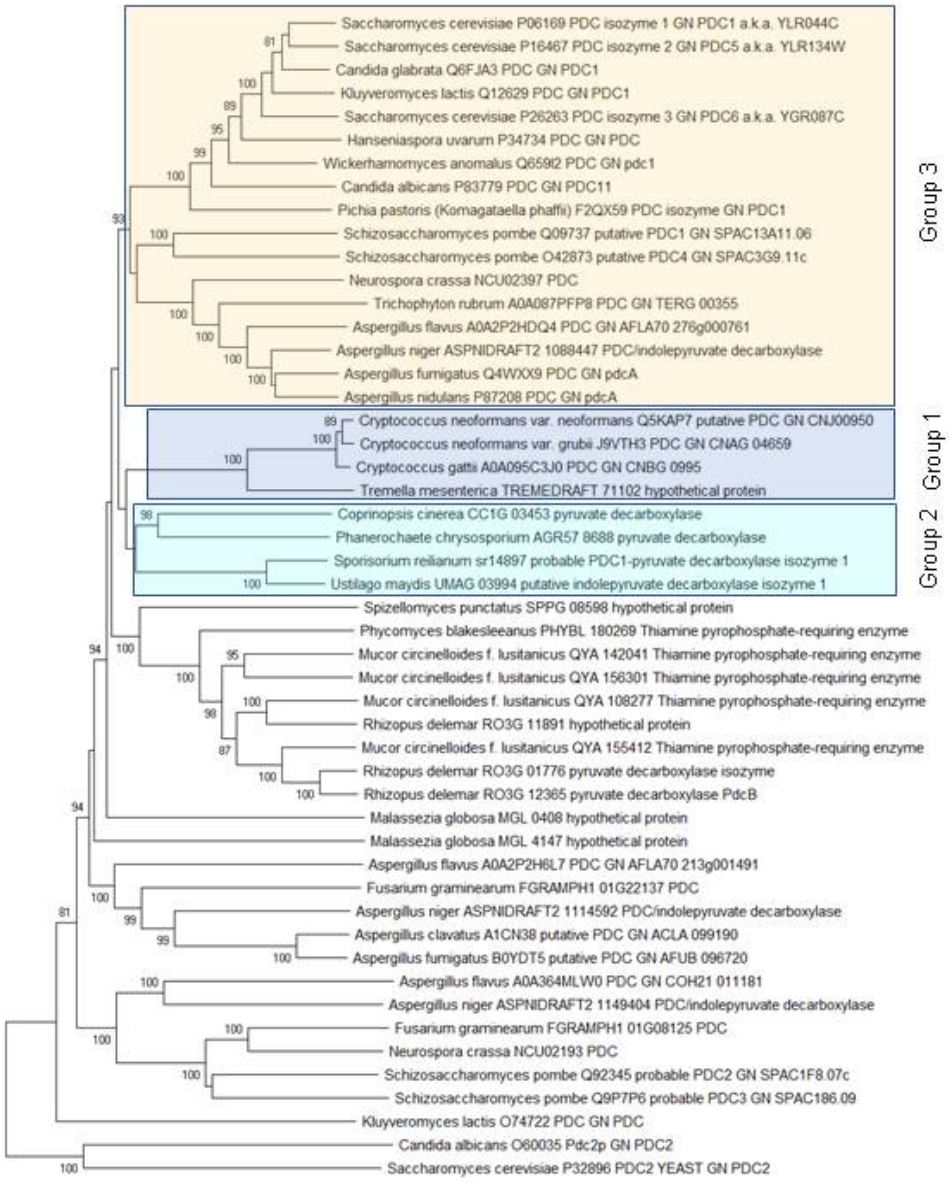

Figure 3.2: Phylogenetic tree analysis shows evolutionary relatedness of *Cryptococcus neoformans* pyruvate decarboxylase enzymes to those of other fungal species. The protein sequences of cryptococcal pyruvate decarboxylase (Pdc) enzymes and potential homologues from other fungal species were analyzed using Molecular Evolutionary Genetics Analysis (MEGAX) software to build a phylogenetic tree. The Pdc enzymes from *C. neoformans var*

grubii and *C. neoformans var neoformans* were most similar to each other and then to the homologue from *C. gattii*, and, as a group, were next most closely related to enzymes from *Sporisorium reilianum*, *Ustilago maydis*, *Coprinopsis cinerea*, and *Phanerochaete chrysosporium*. The cryptococcal proteins were only distantly related to the enzymatic proteins Pdc1p, Pdc5p, and Pdc6p from *Saccharomyces cerevisiae*, but these were more closely related than were regulatory proteins like Pdc2p from *S. cerevisiae* or from *Candida albicans*.

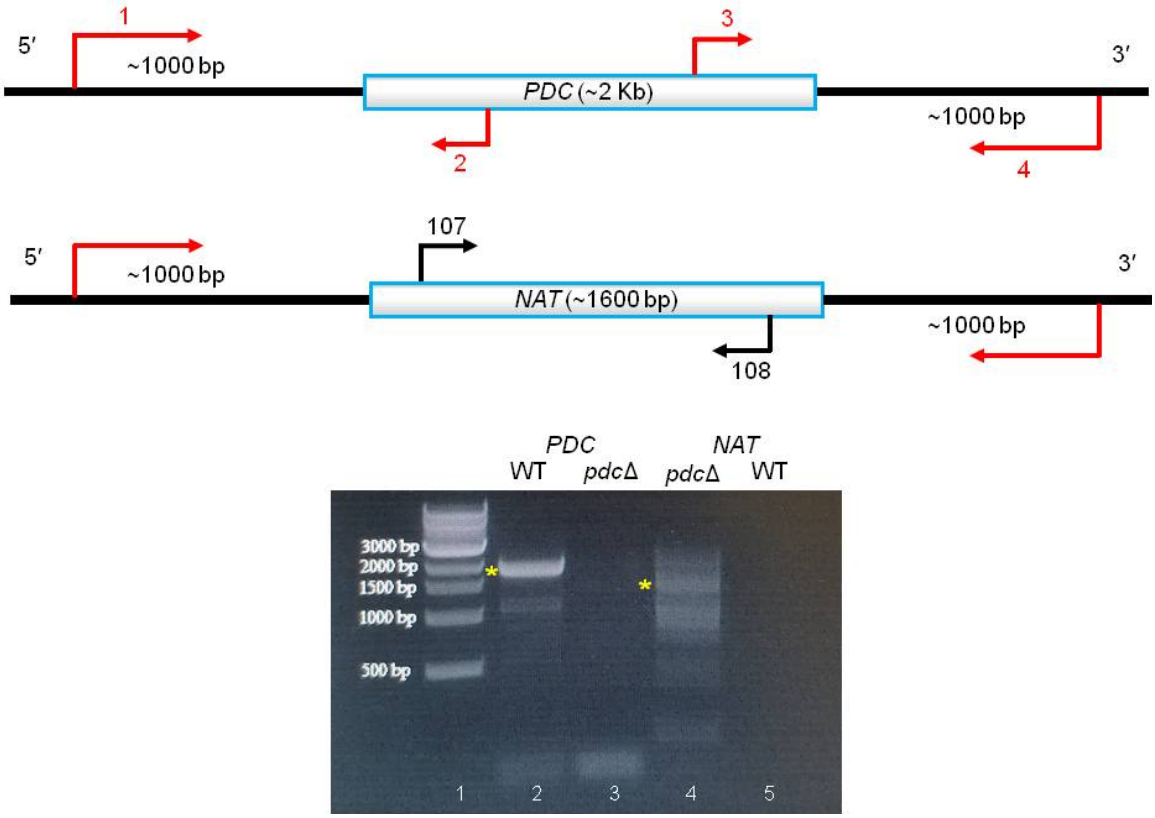

Figure 3.3: Verification of the deletion of *PDC* in the corresponding mutant.

PCR primers specific for the pyruvate decarboxylase gene (3 and 4 above) and for the *NAT* gene conferring resistance to the nourseothricin (NTC) antibiotic (107 and 108) were used to confirm the replacement of *PDC* with the *NAT* cassette in the *pdc* mutant (*pdc*Δ) obtained from the Madhani lab at UCSF. The presence of a band representing *PDC* from the wild type DNA (WT; lane 2, yellow asterisk), but not from the mutant DNA (*pdc*Δ; lane 3), corroborated the deletion of *PDC* in the mutant. The presence of the *NAT* gene in the *pdc*Δ mutant (lane 4, yellow asterisk, ~1600 bp), but not in the wild type strain (lane 5), also confirmed that insertional mutagenesis had occurred.

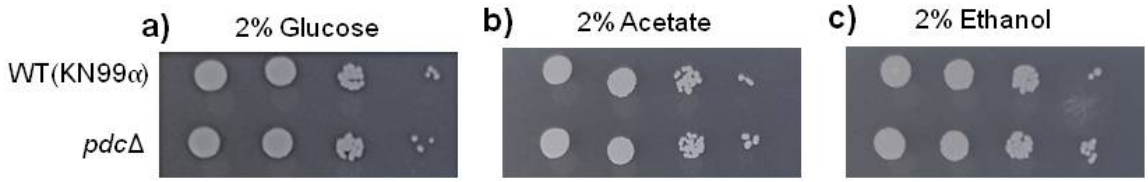

Figure 3.4: No qualitative difference in growth using glucose, acetate, or ethanol as the carbon source was observed between wild type and *pdc*Δ mutant strains. Four 10-fold serial dilutions (2 µl of 5000, 500, 50, and 5 cells/µl) of the *pdc* mutant strain (*pdc*Δ), and of the wild type (WT) strain of *C. neoformans* (both in a KN99α strain background) were spotted from left to right on YNB-agar plates supplemented with (a) 2% glucose, (b) 2% acetate, or (c) 2% ethanol. The *pdc*Δ mutant strain exhibited normal growth (relative to the wild type strain) on all three media.

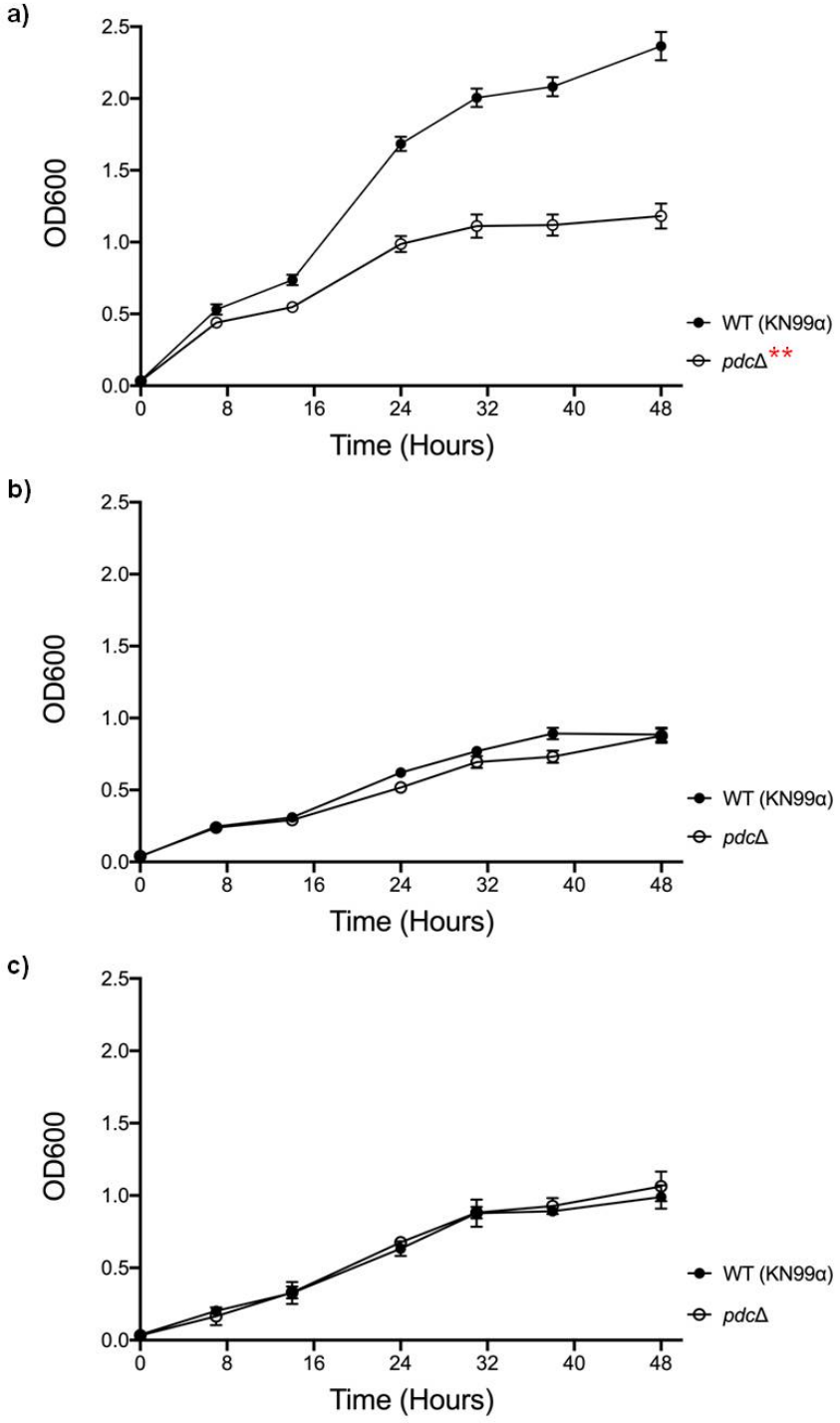

Figure 3.5: Quantification of the impact of deletion of *PDC* on growth in liquid media with various carbon sources. Growth curves for the wild type strain and for the pyruvate decarboxylase deficient strain (*pdc*Δ) of *C. neoformans* (both in a

KN99α strain background) were generated over a 48-hour time course in media supplemented with (a) 2% glucose, (b) 2% acetate, or (c) 2% ethanol as the primary carbon source. The *pdc*Δ mutant displayed a very statistically significant (** P-value < 0.01) reduced growth rate in glucose, but not in acetate or ethanol.

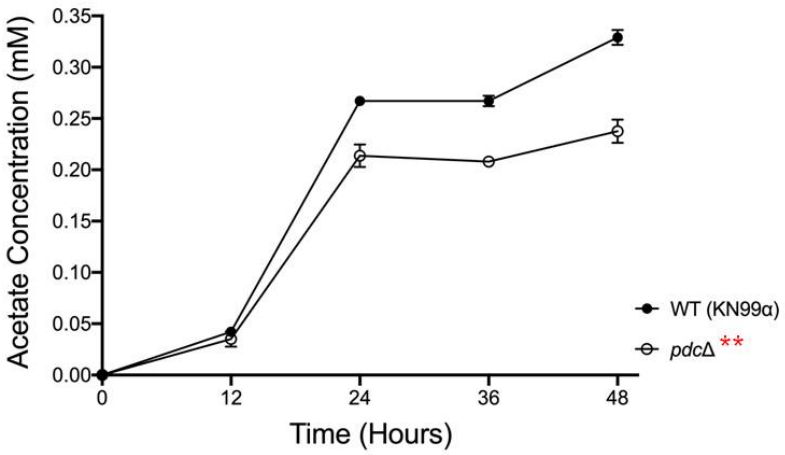

Figure 3.6: The *pdc*Δ mutant produced significantly less acetate than the wild type strain. A time course analysis over 48 hours revealed that the *pdc* mutant strain (*pdc*Δ) produced less acetate, than the wild type (WT) strain (both in the KN99α background). This difference was very statistically significant (** P-value < 0.01).

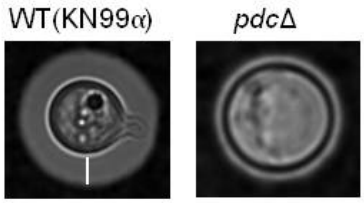

Figure 3.7: The *PDC* gene plays an important role in capsule formation. Cells from the *pdc* mutant (*pdc*Δ) and wild type strain (in the KN99α background) were cultured in capsule-inducing medium, stained with India Ink, and observed under a Zeiss microscope at 40x magnification. A white line indicates the width of the capsule (the layer of secreted polysaccharides outside the cell wall, appearing as a grey zone),in the wild type strain. The capsule of the pyruvate decarboxylase deficient mutant is clearly reduced in width.

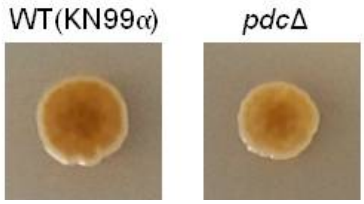

Figure 3.8: The *pdc*Δ mutant produces less melanin than the wild type strain. Wild type and *pdc* mutant strains (*pdc*Δ) in a KN99α strain background were grown in a spot assay on plates supplemented with L-DOPA, the substrate converted to a brown melanin-like pigment by laccase enzymes (Williamson, 1997; Li *et al*., 2018). The *pdc*Δ mutant accumulate less of the pigment and was lighter in color.

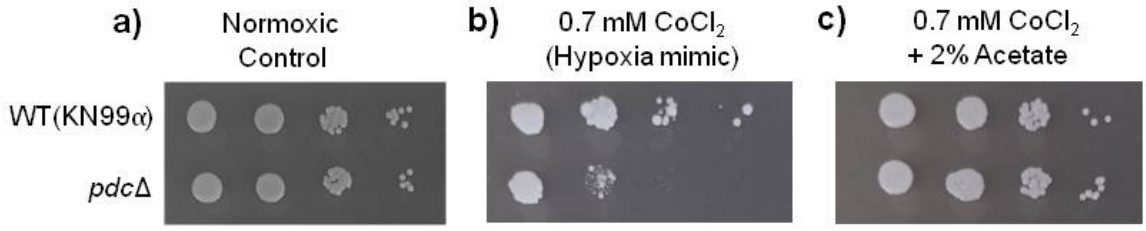

Figure 3.9: The *pdc*Δ mutant grew poorly under hypoxic conditions. A spot assay mimicking growth under low oxygen, in which cobalt chloride induces cells to act metabolically as if they were growing in hypoxia, was conducted. The wild type strain (WT, in the KN99α background) and the pyruvate decarboxylase mutant (*pdc*Δ, also in the KN99α background) were grown on **a)** YES-agar media lacking $CoCl_2$ as the normoxic control, in which the *pdc*Δ mutant grew normally, or **b)** YES-agar media supplemented with 0.7 mM $CoCl_2$, (0.7 mM $CoCl_2$; Hypoxia mimic), in which the *pdc*Δ mutant exhibited a growth defect, indicative of an abnormal response to hypoxia. **c)** This growth defect was reversed when media with $CoCl_2$ were supplemented with acetate (0.7 mM $CoCl_2$ + 2% Acetate).

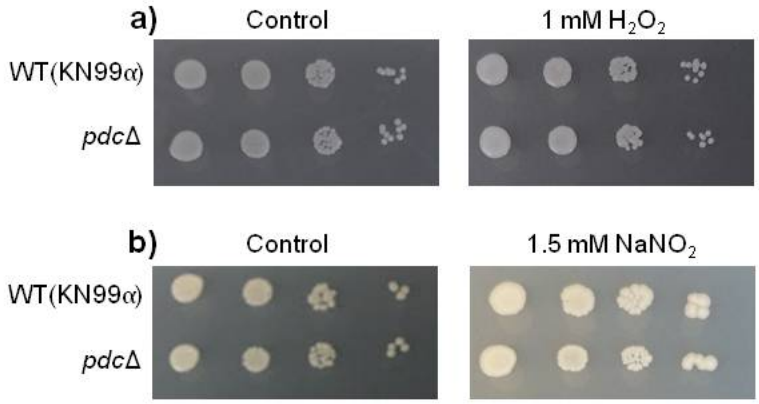

Figure 3.10: The *pdc*Δ mutant grew normally under oxidative and nitrosative stresses. The wild type and *pdc* mutant strains (*pdc*Δ), both in the KN99α background, were grown on **a)** YNB-agar media (buffered to pH 4.0 with sodium succinate) either not supplemented (Control) or supplemented with 1mM H_2O_2 to assess sensitivity of the mutant to oxidative stress, or **b)** on sodium succinate buffered YNB-agar media not supplemented (Control) or supplemented with 1.5mM $NaNO_2$ to assess sensitivity of the mutant to nitrosative stress. Growth of the *pdc*Δ mutant was normal (comparable to wild type) under both stress conditions.

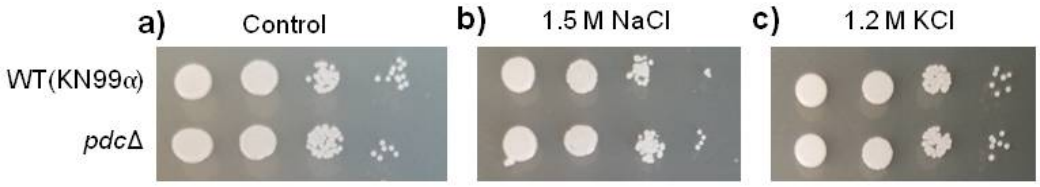

Figure 3.11: The *pdc*Δ mutant grew normally under high salt conditions. Wild type and pyruvate decarboxylase mutant (*pdc*Δ) strains (both in a KN99α background) were grown on **a)** YPD-agar alone as control, **b)** YPD-agar supplemented with 1.5 M NaCl, or **c)** YPD-agar supplemented with 1.2 M KCl in order to induce osmotic stress via the excess sodium or potassium chloride. The *pdc*Δ mutant grew normally relative to the wild type strain under all of these conditions.

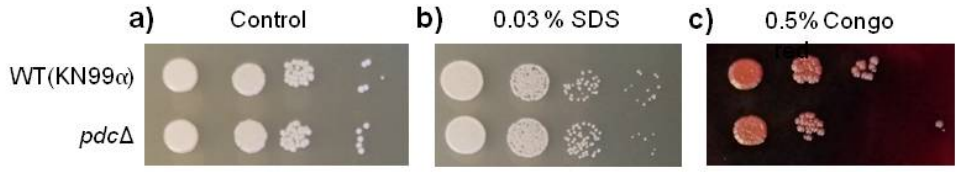

Figure 3.12: The *pdc*Δ mutant had abnormal cell wall and capsule integrity. Wild type and pyruvate decarboxylase mutant (*pdc*Δ) strains (in a KN99α background) were grown on **a)** YPD-agar alone as control, **b)** YPD-agar supplemented with 0.03% sodium dodecyl sulfate (SDS), which can disrupt the cell membrane, or **c)** YPD-agar supplemented with 0.5% Congo red, which can disrupt the cell wall production and integrity by binding to components of the cell wall and chitin layers. The *pdc*Δ mutant was not sensitive to SDS, but the deficiency of the pyruvate decarboxylase enzyme did affect survival of cells spotted at low density on Congo red media, indicating that Pdc is required for full cell wall integrity.

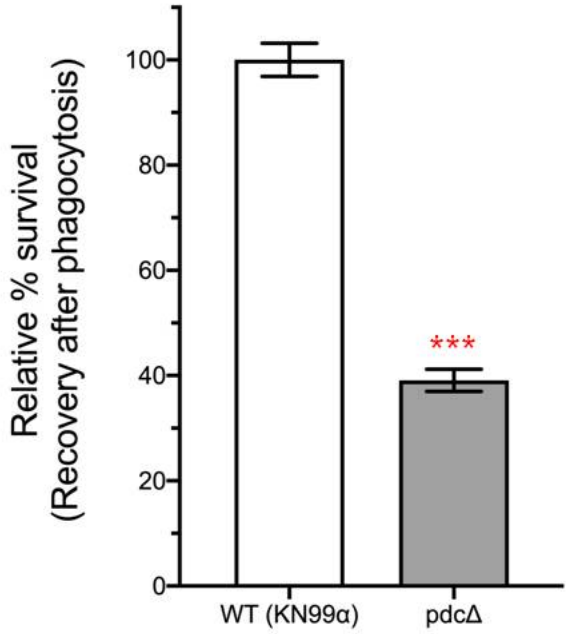

Figure 3.13: Percent survival of wild type and *pdc*Δ mutant *C. neoformans* cells after engulfment by macrophages in an *in vitro* assay. The *pdc*Δ mutant exhibits reduced survival and recovery (*** P-value < 0.001) after phagocytosis by macrophages than the wild type KN99α strain.

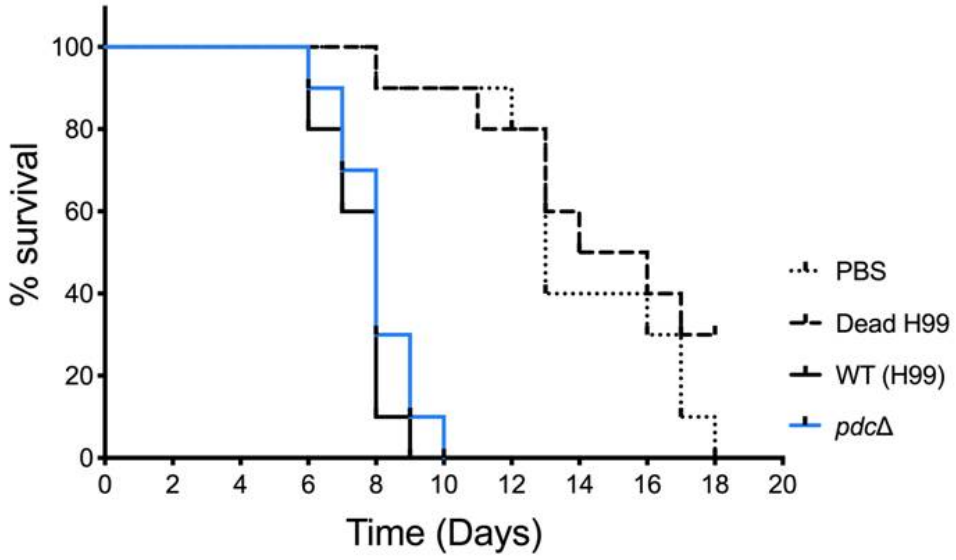

Figure 3.14: *Galleria mellonella* survival assay. The survival of *Galleria mellonella* larvae was analyzed after injection with the *pdc* mutant (*pdc*Δ; blue line) or wild type (WT; black line) strains of *C. neoformans*, in the H99 background, or, as control for death of the larvae due to injection-related damage or excessive immune response to fungal infection, with phosphate buffered saline (PBS; dotted black line) or heat-killed H99 wild type strain (Dead H99; dashed black line), respectively. Here, the survival percentages, over time, of the larvae after injection with one million cells of the appropriate type are graphed as Kaplan-Meier survival curves. The survival curves of the *pdc*Δ and wild type strain-injected larvae were not statistically different, as determined via either a Gehan-Breslow-Wilcoxon test (P-value = 0.3395) or a Log-Rank test (P-value = 0.2700), while the survival curves for the two negative controls were statistically different (from the wild type survival curves) by either test (P-value < 0.0001 for both controls).

CHAPTER FOUR

USE OF A GENETIC SCREEN TO IDENTIFY ACETATE UTILIZATION GENES IN *C. NEOFORMANS*

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Abstract

Cryptococcus neoformans is an opportunistic fungal pathogen that can adapt to use different carbon sources as needed. Additionally, it can produce and utilize acetate, ethanol, or other alternate carbon sources whenever there is insufficient glucose in the environment. The ability to use alternative carbon sources can be studied to develop drugs against the relevant pathways. In this study, a global screening approach was used to identify genes required for growth of the pathogen when provided with acetate or glucose as the only carbon source. A total of 3,936 distinct strains from two gene-knockout libraries were screened for their ability to grow on acetate-containing medium. In total, 41 individual mutants failed to grow on either glucose- or acetate- containing medium. Of these, 12 failed to grow on medium containing glucose as the only carbon source, 15 mutants failed to grow on medium with acetate as the only carbon source, and 14 failed to grow on both

of these media. The corresponding genes were categorized, as far as possible, based on the pathways to which they belong. Many of these genes are involved in gluconeogenesis, arginine biosynthesis, or mitochondrial transport. Some of these genes have known homologues in other fungal species, like *Saccharomyces cerevisiae, Aspergillus nidulans*, *Candida albicans*, and *Neurospora crassa,* and the roles of these homologues have been studied with respect to acetate utilization. However, some of the genes identified in this screening are novel and have not been previously studied in the context of acetate utilization pathways. Herein, the *C. neoformans* genes identified by this screen for growth on acetate as an alternate carbon source, and, taking insights from other fungal species, the possible roles of some of these genes, are presented.

Introduction

Cryptococcus neoformans is an opportunistic, invasive pathogen that often disseminates to the central nervous system (CNS) and is the most frequent cause of human fungal meningitis (Lin and Heitman, 2006). It causes significant annual global mortality (Park *et al*., 2009, Pappas, 2001; Lui *et al*., 2006; Franzot *et al*., 1999; Dromer *et al*., 1996; Tortorano *et al*., 1997; Powderly, 1993). This fungus mainly affects HIV/AIDS patients, transplant recipients who are immunosuppressed to prevent immune-mediated graft rejection, and other immunocompromised individuals (Hull and Heitman, 2002). When *C. neoformans* cells enter the lungs, alveolar macrophages attempt to phagocytose the fungal cells and kill them, or at least sequester them into granulomas (Rohatgi and Pirofski, 2015; Vecchiarelli *et al*., 1994a,b; Mitchell and Friedman, 1972; McQuiston and Williamson, 2012; Sabiiti and May, 2012). If phagocytosis occurs and the pathogen is trafficked into the macrophage phagolysosome, a low pH environment, hydrolytic enzymes, antimicrobial peptides, and toxic free radicals are used to try to destroy the pathogen (Hampton *et al*., 1998; Vieira *et al.*, 2002; Cox *et al.,* 2003; Tohyama *et al.,* 1996; Ma and May, 2009) and to break apart pathogen proteins into peptides for presentation to T cells in order to activate the adaptive immune response (Vecchiarelli *et al*., 1994a). In preparation for immune attack, *C. neoformans* increases the production and use of a capsule and of melanin to prevent phagocytosis, or if phagocytosed, to counteract the reactive oxygen species (*i.e.* free radicals) and modulate pH, thereby blocking the initial

steps of immune activation by macrophages (Wang *et al*., 1995; Mednick *et al*., 2005; Shoham and Levitz, 2005; Tucker and Casadevall, 2002; Zaragoza *et al*., 2008; De Leon-Rodriguez *et al*., 2018; Rohatgi and Pirofski, 2015). This, along with other virulence factors, including the ability to grow at the host body temperature of 37°C and the use of degradative enzymes, allows the pathogen to survive and spread inside the host (Coelho *et al*., 2014; Casadevall *et al*., 2000; Kronstad *et al*., 2011; Ma and May, 2009).

Metabolic adaptability and flexibility are important attributes for fungal pathogens to successfully infect and cause disease. Although carbon metabolism is critical for virulence in *C. neoformans* (Price *et al*., 2011), very little is known about which carbon sources are utilized during infection. Lung alveolar macrophages, which present a first line of host defense against *C. neoformans* infection (Feldmesser *et al*., 2000; Feldmesser *et al*., 2001), provide a glucose- and amino acid-poor environment {Barelle *et al*., 2006; Lorenz *et al*., 2004), and nonpreferred carbon sources such as lactate and acetate are likely important early in establishment of a pulmonary infection (Price *et al*., 2011).

To identify *C. neoformans* genes not previously known to be important in acetate utilization, strains from two gene knockout libraries were screened for the ability to utilize acetate as the sole carbon source. From this screen, 26 genes were identified that encode proteins necessary for growth on acetate as a sole carbon source. Some of these genes, like those encoding acetyl-CoA synthetase and phosphoenolpyruvate carboxy kinase, were previously identified in other

fungal species like *S. cerevisiae*, *Aspergillus nidulans*, and *Neurospora crassa,* (Apirion, 1965; Sealy-Lewis, 1994; Flavell and Fincham, 1968a; Schüller, 2003) or even in *C. neoformans* itself (Hu *et al.*, 2008), as important acetate utilization enzymes, thereby verifying the sensitivity of the assay, while others have been identified as important in acetate metabolism in *C. neoformans* for the first time.

Materials and methods

Strains

Two libraries of *C. neoformans* strains produced in 2015 and 2017 by the Madhani laboratory at the University of California San Francisco (UCSF) (Chun and Madhani, 2010; NIH R01AI100272), were obtained from the Fungal Genetic Stock Center.

Screening of single-deletion strains from the libraries via spot assays

Thawed 96-well plates with strains from the original knockout libraries were used to produce duplicate 96-well plates by inoculating corresponding wells containing 100 µl YPD with 2 µl from each of the wells of the original plates. The yeast in these duplicate plates were allowed to grow overnight at 30°C with constant shaking. The following day, cells from each well were refreshed by inoculating a corresponding well of 100 µl fresh YPD media in new 96-well plates (second duplicate plates) with 2 µl of the overnight culture. After a 3 hr incubation, 2.5 µl of cells from each well were spotted onto corresponding locations on each of two YNB-agar plates, one supplemented with 2% glucose and the other supplemented with 2% acetate. After a ~48-hour incubation at 30°C, the glucose or acetate supplemented plates were imaged using a Canon EOS Rebel T1i camera. The identity of the genes mutated in strains that showed growth inhibition in either test condition were recorded.

For the strains that did not grow on either glucose or acetate supplemented YNB-agar, the corresponding well in the duplicate plate #2 was assessed under a

light microscope under 20x magnification to ensure that there were cells in the wells. If no cells were observed, the same well in duplicate plate #1 was also checked. If neither duplicate plate had cells, the original stock was re-thawed, a small volume of cells from the corresponding mutant strain was plated onto YPD-agar media, and a glycerol stock was generated to add to the original library stock. Once cells from the strains that did not grow on glucose or acetate supplemented media were obtained from either of the two duplicate plates or, if necessary, from the original stock, they were grown on YPD media and then plated once again on glucose or acetate supplemented YNB-agar to attempt to replicate the original result.

Results

Previous reports suggest that acetate is a key carbon source for the survival of *C. neoformans* and other fungal pathogens at early stages of infection (Hu *et al*., 2008; Bubb *et al*., 1999; Wright *et al*., 2002; Himmelreich *et al*., 2001, 2003; De Sousa Lima *et al*., 2014; Baeza *et al*., 2017). Thus, obtaining a global overview of which fungal genes are involved in the synthesis, utilization, or transport of acetate in cryptococcal cells is potentially of both scientific and medical importance. Therefore, a library 3,936 distinct *C. neoformans* knockout strains, each produced by deletion within the open reading frame of the corresponding genes via insertional mutagenesis, was screened by spotting each individual strain on YNB-agar plates supplemented with either glucose or acetate as the sole carbon source. The growth of each of the mutant strains was assayed after incubating the plates for 48 hours at 30°C. The majority of the deletion mutants grew normally in the presence of either carbon source. However, some mutants were found to be compromised in their ability to utilize glucose and/or acetate as the sole carbon source, and grew poorly or not at all in the corresponding medium. Representative glucose and acetate supplemented plates are shown in Figures 4-1a and 4-1b, respectively. Some strains, like the deletion mutant for SWR1-Complex protein 4 (CNAG_02020), had an unusual phenotype of growth into seemingly more dense, slightly larger, creamy-white colonies on media with either glucose or acetate as the sole carbon source, or on both media (Figure 4.1 c).

Further analysis of these mutants is beyond the scope of this screen, but may be undertaken at a later date.

Mutants that failed to grow were plated from the original library stock onto YPD-agar media to provide nutrient-rich growth conditions, and then the strains on these plates were transferred to YNB-agar plates supplemented with either glucose or acetate to confirm that the growth defect observed was both verifiable and dependent on the requirement to use a specific carbon source, not a general growth defect. In total, 12 deletion mutants failed to grow on media supplemented with glucose (Table 4.1), another 15 deletion mutants failed to grow on media containing acetate as the only carbon source (Table 4.2), and 14 deletion mutants failed to grow on glucose or acetate as the only carbon source (Table 4.3). The strains that failed to grow on glucose-containing medium included those mutant for genes encoding ste20 protein kinase (CNAG_00405), the V-type H^+-transporting ATPase subunit AC39 (CNAG_00448), chitin deacetylase 2 (CNAG_01230), and the calcium sensor and enzyme regulator calmodulin (CNAG_01557), the latter of which plays a role in many cell functions, including thermotolerance (Kraus *et al*., 2005).

The strains that failed to grow on medium containing acetate as the sole carbon source included mutants with defects in genes encoding glucose-6-phosphate isomerase (CNAG_03916), which is important for melanin and capsule production, as well as in cell wall integrity and stress resistance (Zhang *et al*., 2015), fructose-1,6-bisphosphatase I (CNAG_00057), and phosphoenolpyruvate

carboxykinase (CNAG_04217), all of which function in gluconeogenesis (Hynes *et al*., 2002; Zhang *et al*., 2015). A mutant for the gene encoding acetyl-CoA synthetase (CNAG_00797), which converts acetate to acetyl-CoA that can be used for multiple purposes, including gluconeogenesis (Hu *et al.*, 2008), was also unable to grow on the acetate supplemented, glucose-free medium. Two members of the solute carrier family 25, a mitochondrial carnitine/acylcarnitine transporter (CNAG_00499) and a mitochondrial citrate transporter (CNAG_02288), as well as two carnitine acetyltransferases (CNAG_00537 and CNAG_06551), also failed to grow on acetate-containing medium. Mutant strains that grew on neither acetate-supplemented nor glucose supplemented media included those deleted for the mitochondrial amino-acid acetyltransferase (CNAG_02826), carbamoyl-phosphate synthase arginine-specific large chain (CNAG_06112), ornithine carbamoyltransferase (CNAG_02812), and argininosuccinate lyase (CNAG_02825), all of which function in arginine biosynthesis (Rhodes and Howard, 1980), as well as the large subunit of a carbamoyl-phosphate synthase (CNAG_07373) that functions in other biosynthesis pathways.

Discussion

Acetate is a two-carbon metabolite that plays a significant role in energy production in fungal pathogens after conversion to acetyl-CoA, especially after infection. The acetyl-CoA can be channeled into the tricarboxylic acid cycle, the glyoxylate cycle, or the gluconeogenesis pathway (Hu *et al*., 2008). A genetic screen for growth on two different carbon sources, glucose and acetate, was performed on an array of *C. neoformans* deletion mutants to uncover enzymes (or other proteins) not previously characterized as contributing to the growth of the pathogen when acetate is the only available carbon source. In total, 41 mutants were identified that failed to grow on media with either glucose or acetate as the only carbon source. Of these, 12 failed to grow on medium with glucose as the only source of carbon, another 15 did not grow on medium containing acetate as the sole carbon source, and 14 did not grow on either medium.

Analysis of the mutants identified several genes that play a central or secondary role in the metabolism of acetate in the fungus. These mutants can be divided into three categories based on the types of pathways affected by the corresponding protein deficiencies. Those that could not grow with glucose as the only carbon source but could grow on acetate probably have a more general glucose utilization defect and will not be discussed further. Those deletion mutants that could not grow with acetate as the sole carbon source but could grow on glucose are likely to be deficient in either the conversion of acetate to acetyl group-containing molecules used in various critical cellular functions or in the import of

these molecules (but not of glucose-derived alternatives) into the mitochondria for use in cellular respiration to produce ATP. Finally, those mutants that were able to grow on neither glucose nor acetate as the sole carbon source likely have a defect in either a metabolic pathway that combines acetyl group-containing molecules with biological derivatives of glucose or in (the downstream part of) a pathway by which acetate is utilized after it is derived from glucose or obtained from the environment.

Of the mutants that failed to grow on acetate (but that grew on glucose), some have not been previously identified, demonstrating the value of this screen. Among the proteins identified with predicted function, most were either mitochondrial transporters or enzymes in acetate or acetyl-CoA metabolic pathways, namely acetyl-CoA synthesis and gluconeogenesis, as determined based on the reported functions of homologues in other fungal species like *S. cerevisiae*, *N. crassa*, and *A. nidulans* (Apirion, 1965; Sealy-Lewis, 1994; Flavell and Fincham, 1968a; Schüller, 2003). For these, possible explanations for the inability of the corresponding *C. neoformans* mutant to grow on acetate as the sole carbon source are discussed below.

Detection of an *acs1* mutant validates the sensitivity of the screen

A growth defect on acetate supplemented, but not glucose supplemented, media was observed for the *C. neoformans acs1* mutant. *ACS1* (CNAG_00797) encodes acetyl-CoA synthetase, which generates acetyl-CoA from acetate (Schüller, 2003; Hu *et al*., 2008). Acetyl-CoA synthetase mutants of *A. nidulans*

(*facA*) (Apirion, 1965; Sealy-Lewis, 1994), in *N. crassa* (*acu-5*)(Flavell and Fincham, 1968a), and *S.* cerevisiae (acs1) (De Virgilio *et al*., 1992) also fail to grow on media containing acetate as the only carbon source. More importantly, a *C. neoformans acs1* deletion mutant has been previously described as being unable to grow on acetate (Hu *et al*., 2008), so the identification of the *acs1* mutant from the Madhani library verified the sensitivity of the screen used in the present study. Furthermore, acetyl-CoA synthetase is upregulated in *C. neoformans* isolated from pulmonary tissue obtained from a mouse infection model and *acs1* loss of function mutants have reduced growth and virulence in this model (Hu *et al*., 2008), indicating that the library screening is capable of uncovering other genes that may connect acetate metabolism with *C. neoformans* virulence. Supporting these findings, *acs1* has been shown to be a possible drug target (Koselny *et al.,* 2016a, 2016b).

Mitochondrial transporters are necessary for growth of *C. neoformans* on acetate

The screening also demonstrated that a mutant that is deficient in solute carrier family 25 member 20/29 (AcuH; CNAG_00499), also known as mitochondrial carnitine/acylcarnitine transporter (or mitochondrial carnitine/acylcarnitine carrier) was unable to grow on media containing acetate as the only carbon source. The equivalent *S. cerevisiae* carnitine/acylcarnitine carrier Crc1p, is located in the mitochondria, presumably in the inner mitochondrial membrane like the mammalian homologue, and exchanges carnitine present in

the mitochondrial matrix with acylcarnitine present in the cytosol (Indiveri *et al*., 1990, 1994; Swiegers *et al*., 2001; Palmieri *et al*., 1999a; Van Roermund *et al*., 1995). In *S. cerevisiae* and other fungi, acetyl-CoA first produced by β-oxidation in peroxisomes is converted by peroxisomal carnitine acetyltransferase to acetylcarnitine, which is exchanged with cytosolic carnitine, possibly by a predicted peroxisomal membrane-bound transporter similar to the mitochondrial carnitine/acylcarnitine transporter (Van Roermund *et al*., 1995; Elgersma *et al*., 1995; Swiegers *et al*., 2001; Palmieri *et al*., 2006). In *S. cerevisiae*, the cytosolic acetylcarnitine is then imported into the mitochondria in exchange for the mitochondrial carnitine (Van Roermund *et al*., 1995; Elgersma *et al*., 1995; Palmieri *et al*., 1999a). In multiple fungi, including *S. cerevisiae,* a mitochondrial carnitine acetyltransferase then reverses the conversion, producing acetyl-CoA and carnitine from acetylcarnitine, and the acetyl-CoA is released into the mitochondrial matrix for use in the TCA cycle (Strijbis *et al*., 2010; Swiegers *et al*., 2001; Stemple *et al*., 1998; Kohlhaw and Tan-Wilson, 1977). The modification of carnitine to acylcarnitines (especially to acetylcarnitine) is one of the few ways, if not the only way, by which acetyl-CoA from the peroxisomes or produced from acetate in the cytoplasm can enter the mitochondria, at least in *S. cerevisiae*, since mitochondrial and peroxisomal membranes have very low permeability or are impermeable to acetyl-CoA (Swiegers *et al*., 2001; Kohlhaw and Tan-Wilson, 1977; Van Roermund *et al*., 1995; Elgersma *et al*., 1995; Todd *et al*., 1997; Palmieri *et al*., 1999a). This *S. cerevisiae* peroxisome-cytosol-mitochondria carnitine/acylcarnitine exchange

process is expected to be similar in *C. neoformans*, in which the mitochondrial import of acetylcarnitine most likely occurs via solute carrier family 25 member 20/29.

This similarity extends to the use of carnitine O-acetyltransferases, and *C. neoformans* mutants lacking either of two carnitine O-acetyltransferases, CNAG_00537 or CNAG_06551, were unable to grow on media supplemented with acetate as the sole carbon source in the current screen. Both *S. cerevisiae* and *C. albicans* have three carnitine acetyltransferases, two in the cytoplasm and one that can localize to either the mitochondria or peroxisome (Strijbis and Distel, 2010; Strijbis *et al*., 2010). A third carnitine O-acetyltransferase, CNAG_05042, does exist in *C. neoformans*, but the Madhani laboratory was unable to produce a deletion mutant of the corresponding gene. Just like loss of the Cat2 peroxisomal/mitochondrial carnitine acetyltransferase in *C. albicans* precludes growth on acetate or ethanol (Strijbis *et al*., 2008), single *C. neoformans* mutants of either CNAG_00537 or CNAG_06551 were unable to grow on acetate, indicating that each of these enzymes plays a unique, non-redundant, and critical role in acetate utilization in *C. neoformans*. In *A. nidulans*, there are two carnitine acetyltransferases, FacC and AcuJ, the first of which is in the cytoplasm, while the second contains both peroxisomal and mitochondrial targeting sequences (Hynes *et al*., 2011; Stemple *et al.,* 1998), much like the Cat2 enzyme in *C. albicans* or *S. cerevisiae* (Elgersma *et al*., 1995; Strijbis and Distel, 2010). While the location of the three carnitine acetyltransferases in *C. neoformans* is uncertain, CNAG_06551

is the most similar in amino acid sequence to *A. nidulans* FacC, indicating that it may be the cytoplasmic carnitine acetyltransferase.

In addition to having at least one similar carnitine acetyltransferase to *C. neoformans*, *A. nidulans* also has the overall acetyl-CoA/carnitine/acylcarnitine shuttling pathway (Hynes *et al*., 2011; Stemple *et al*., 1998). A carnitine transporter, encoded by *acuH*, has been identified in *A. nidulans* and lack of a functional version of this transporter results in loss of growth on media containing acetate as the only carbon source (De Lucas *et al*., 1999; Armitt *et al*., 1976). Toxic accumulation of acetyl-CoA in the cytosol has been observed in the *A. nidulans acuH* mutant cultured on acetate and is the probable cause of the growth defect; it could also be the reason for the defect in growth of the *C. neoformans* CNAG_00499 (*acuH*) mutant (Armitt *et al*., 1976; De Lucas *et al*., 1997, 1999, 2008; Martínez *et al*., 2007). Another possible reason for the inability of the *C. neoformans* mutant to grow on acetate could be insufficient levels of acetyl-CoA in the mitochondria for continuous use in the TCA cycle (Strijbis *et al*., 2010; Stemple *et al*., 1998; Kohlhaw and Tan-Wilson, 1977).

A *C. neoformans* mutant deficient for another solute carrier family 25 member, solute carrier family 25 member 1 protein (AcuL; CNAG_02288), exhibited complete growth failure on medium with acetate as the sole carbon source. The *S. cerevisiae* homologue to AcuL is referred to as the succinate-fumarate carrier or succinate-fumarate antiporter (Sfc1p/Sfa), and it functions in a pathway that allows yeast to use acetate in the generation of ATP or to undergo

gluconeogenesis when acetate is the sole carbon source (Palmieri *et al*., 1997, 2000). While succinate can be formed by succinyl-CoA synthetase in the mitochondrial TCA cycle, it is also made by isocitrate lyase in the glyoxylate cycle in the cytoplasm (Przybyla-Zawislak, *et al*., 1998; Schüller, 2003). In fungi such as *S. cerevisiae* and in plants, the glyoxylate cycle utilizes a combination of enzymes in the cytosol, peroxisomes, and mitochondria, so the products of theses enzymes need to be transported between these compartments (Kunze *et al*., 2006; Flipphi *et al*., 2014). In particular, *S. cerevisiae* uses Sfc1p (or the dicarboxylate carrier, Dic1p, both of which are localized in the mitochondrial inner membrane) to transfer succinate produced in the cytoplasm into the mitochondria (Flipphi *et al*., 2014; Palmieri *et al*., 1999b, 2000; Lançar-Benba *et al*., 1996). Since Sfc1p (also called Acr1p) is an antiporter, it couples mitochondrial succinate import with export of fumarate into the cytoplasm by a strict counter-exchange mechanism (Palmieri *et al*., 1997) that makes the fumarate available for use in the cytoplasm. Fumarate is converted to malate and then to oxaloacetate which is used as a substrate for gluconeogenesis or is joined with acetyl-CoA in the TCA cycle after import into the mitochondria (Palmieri *et al*., 1997, 1999b; Flipphi *et al*., 2014). Reduction in the levels of succinate and oxaloacetate in mitochondria (the latter due to lack of fumarate in the cytoplasm) due to absence of the Sfc1p antiporter can hinder the TCA cycle and therefore usage of acetyl-CoA, which may accumulate in the mitochondria and in the cytoplasm. Acetyl-CoA toxicity has been described in *A. nidulans acuL* mutants grown on acetate (Armitt *et al*., 1976; De Lucas *et al*.,

1997,1999, 2008; Martínez *et al*., 2007). This toxicity may explain the findings that *S. cerevisiae acr1* or *dic1* mutants, as well as the *A. nidulans acuL* mutant cannot grow on either ethanol or acetate as the sole carbon source (Palmieri *et al*., 1997, 1999a; Fernández *et al*., 1994; Flipphi *et al*., 2014), corroborating the equivalent severe phenotype of the *C. neoformans sfc1* mutant observed in the present screen. Given that the lack of export of fumarate to the cytoplasm can lead to toxic acetyl-CoA accumulation, it is noteworthy that Sfc1 actually plays multiple roles during growth on ethanol or acetate; not only is it required for the mitochondrial succinate-fumarate antiport, but Sfc1/Acr1 also regulates the expression of Acs1 (Palmieri 1997, 2000). *SFC1* expression is itself upregulated in *C. neoformans* during infection in a mouse model (Hu *et al*., 2008).

Interestingly, the induction of *SFC1*/*ACR1* expression is co-regulated with that of the genes encoding isocitrate lyase and malate synthase of the glyoxylate cycle and phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase of the gluconeogenesis pathway (Bojunga *et al*., 1998; Redruello *et al*., 1999). Mutations in phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase have been shown here and in the published literature to prevent or reduce growth on acetate of *C. neoformans* or other fungal species, including *A. nidulans*, *C. albicans*, *N. crassa* and *S. cerevisiae* (Rude *et al*., 2002, Flavell and Fincham, 1968a; Armitt *et al*., 1976; Lorenz and Fink, 2001; Hynes *et al*., 2002).

The gluconeogenesis pathway is important for *C. neoformans* growth on acetate

Two mutants from the Madhani libraries, one deficient for phosphoenolpyruvate carboxykinase (Pck1; CNAG_04217), and the other for fructose-1,6-bisphosphatase I (Fbp1; CNAG_00057), were unable to grow on acetate as the sole carbon source. In many fungal species, including *A. nidulans* and *C. neoformans*, Pck1 converts oxaloacetate produced in the TCA cycle to phosphoenolpyruvate in the cytoplasm. This allows the fungi to undergo gluconeogenesis and thereby grow on carbon sources that are processed into TCA cycle intermediates used to produce the oxaloacetate (Hynes *et al*., 2002). Similarly, Fbp1 converts fructose-1,6-bisphosphate to fructose-6-phosphate and is important for growth of fungi on all carbon sources that can only (or optimally) be utilized via gluconeogenesis (Hynes *et al*., 2002). *A. nidulans acuF* and *acuG* mutants lacking Pck1 and Fbp1 activity, respectively, fail to grow on media containing acetate as the sole carbon source (Armitt *et al*., 1976), just like the *C. neoformans* mutants in this study. Of importance, Pck1 activity was highly induced, and expression of the *PCK1* gene was increased, in *C. neoformans* cells recovered from infected mouse brain or lung (Price *et al*., 2011; Hu *et al*., 2008; Fan *et al*., 2005). Furthermore, lack of *PCK1* in *C. neoformans* leads to impaired virulence in a murine inhalation model, even though it does not affect the persistence of the pathogen or its ability to disseminate to the brain in a rabbit cryptococcosis model (Panepinto *et al*., 2005; Price *et al*., 2011). Both *PCK1* and *FBP1* are upregulated

in *C. neoformans* after phagocytosis by macrophages (Derengowski *et al*., 2013). Together, these findings suggests that enzyme of gluconeogenesis, especially Pck1 and Fbp1, play an important role in the utilization of acetate as a carbon source and therefore, potentially, growth of the pathogen during infection, by converting two-carbon compounds (such as acetate) and intermediates of the TCA cycle to glucose, to allow continuous energy production even when glucose (or other hexoses) are not available. It is uncertain why gluconeogenesis is required for growth on acetate when TCA cycle intermediates such as oxaloacetate, or even acetyl-CoA, could be used to generate ATP via the TCA cycle itself, but it is clear that this is, in fact, the case.

In further support of this idea, a mutant that was deficient in glucose-6-phosphate isomerase (CNAG_03916), which converts glucose-6-phosphate to fructose-6-phosphate and vice-versa in glycolysis and gluconeogenesis, respectively, also exhibited a growth defect in this screen on acetate as the sole carbon source. A similar result was observed in another study (Zhang *et al*., 2015), in which a mutant of glucose-6-phosphate isomerase (also known as phosphoglucoisomerase) that retains only 1.9% of the original enzyme activity due to insertional disruption of the promoter has reduced growth on media where acetate is the only carbon source. Surprisingly, just like the leaky mutant in Zhang *et al*. (2015), the mutant tested in the current screen, which is expected to entirely lack glucose-6-phosphate isomerase, was able to grow when provided with glucose as the sole carbon source, even though, without this enzyme, the

glycolysis pathway should not function. It is possible that *C. neoformans* is able to bypass glycolysis using the pentose phosphate pathway when deficient in glucose-6-phosphate isomerase, much like *Trichoderma reesei* (Limón *et al*., 2011), even though this does not occur in *S. cerevisiae* (Sierkstra *et al*., 1993; Heux *et al*., 2008). Just like the glucose-6-phosphate isomerase mutant, a mutant deficient for transketolase (CNAG_06172) in the pentose phosphate pathway, is able to grow on the glucose-only medium in this screen, but this is not surprising, since it can utilize the glycolysis pathway. To test the hypothesis that the mutant lacking glucose-6-phosphate isomerase grew on glucose because it used the pentose phosphate pathway, one can generate a double mutant deficient in both CNAG_03916 and CNAG_06172 and test its ability to utilize glucose as the sole carbon source.

While glucose-6-phosphate isomerase is employed in both gluconeogenesis and glycolysis, Pck1 and Fbp1 are used only in gluconeogenesis, and, in fact, are regulated such that they cannot be used when glucose is present. In *S. cerevisiae*, Pck1 activity is repressed and degradation of the enzyme is induced in the presence of glucose (Burlini *et al*., 1989; Haarasilta and Oura, 1975). Similarly, *FBP1* is induced in the presence of non-glucose carbon sources, and its expression is repressed, while the corresponding enzyme is degraded via a ubiquitination-mediated pathway, in the presence of glucose (Giardina *et al*., 2012; Gancedo, 1971; Holzer, 1989; Schork *et al*., 1995; Schüle *et al*., 2000). Thus, overall, these results reflect the importance of the gluconeogenesis pathway

in the growth of *C. neoformans* when provided with acetate as the sole carbon source.

The essential amino acid arginine is necessary for growth of *C. neoformans in vitro*

Several of the mutants that were detected in this screen as being unable to grow on both medium supplemented with glucose-only and that supplemented with acetate-only were knocked out for genes encoding enzymes in the arginine biosynthetic pathway. It is not surprising that these mutants were unable to survive, since as an amino acid, arginine is necessary for the production of many proteins with physiological and biochemical functions in the cell.

The arginine biosynthesis pathway in *S. cerevisiae* and other fungi combines several enzymatic reactions to form L-citrulline from ornithine and carbamoyl phosphate, and then converts the citrulline to arginine via a series of steps (Jauniaux, *et al*., 1978). The process could be considered to begin in the mitochondria, where amino acid acetyltransferase, also known as N-acetylglutamate synthase, reacts L-glutamic acid with acetyl-CoA to form N-acetyl L-glutamic acid, which is then converted via three intermediates into L-ornithine (Jauniaux, *et al*., 1978). In parallel and possibly in the cytoplasm, depending on the species of fungus, carbamoyl phosphate synthetase, in particular the large subunit of an arginine-pathway-specific carbamoyl phosphate synthetase, catalyses the formation of carbamoyl phosphate from ammonia, bicarbonate, and ATP (Price *et al*., 1978; Jauniaux, *et al*., 1978). The carbamoyl from the carbamoyl

phosphate is then transferred to the L-ornithine, in a reaction catalyzed by ornithine carbamoyltransferase, to form L-citrulline (Price *et al*., 1978; Jauniaux, *et al*., 1978). Argininosuccinate synthase converts the L-citrulline into argininosuccinate, which is turned into arginine by argininosuccinate lyase (Jauniaux, *et al*., 1978).

Thus, *C. neoformans* mutants that were deficient for amino acid acetyltransferase (CNAG_02826), the arginine-specific carbamoyl phosphate synthetase large subunit (CNAG_06112), or ornithine carbamoyltransferase (CNAG_02812) failed to grow on media containing either glucose or acetate as the carbon source because these mutants were unable to make L-ornithine, carbamoyl phosphate, or, citrulline, respectively. Without the ability to produce citrulline, these mutants were unable to make arginine, and were therefore unable to grow at all, regardless of the carbon source provided. These mutants, when grown on media with acetate or glucose as the carbon source but supplemented with arginine, grew normally (H. Al Mousa personal comunication), indicating that the lack of arginine was, in fact, the reason for the original lack of growth. In contrast, a mutant lacking the non-specific carbamoyl-phosphatase synthase (CNAG_07373), which primarily plays a role in synthesis of pyrimidines, was unable to grow even when provided with arginine (H. Al mousa personal comunication), since it was unable to make the pyrimidines needed for DNA and RNA synthesis. This also means that the (vast majority of) carbamoyl phosphate made by CNAG_06112 is used to produce citrulline, and is therefore unavailable (possibly due to compartmentalization of the enzymes and products of the arginine pathway) to

make sufficient pyrimidines for survival of the CNAG_07373-deficient mutant. The reverse is also apparently true, since the mutant deficient in CNAG_06112 is unable to make (sufficient) arginine from the carbamoyl phosphate produced by CNAG_07373 to survive without arginine supplementation. The inability to produce arginine does reduce virulence in other pathogenic fungal species. For example, it reduces or eliminates the penetration by *Colletotrichum higginsianum* of its (plant) host and the pathogenicity of *Fusarium oxysporum*, a melon fungus (Huser *et al*., 2009), as well as the pathogenicity of *Magnaporthe oryzae*, a fungus that infects rice plants (Liu *et al*., 2016). Similarly, inability to produce arginine reduces the virulence of *C. neoformans* in a mouse infection model, although an arginosuccinate lyase mutant is able to regain virulence, presumably by obtaining arginine from the host (Rhodes and Howard, 1980).

Other acetate utilization enzymes function in diverse pathways

Other *C. neoformans* mutants that failed to grow on acetate as the only carbon source included those deficient in 8-Amino-7-oxononanoate synthase (CNAG_00236), cytosine deaminase (CNAG_00613), a 53 kDa brg1-associated factor (CNAG_04048), a HAL protein kinase (*HRK1*; CNAG_00745), and a AGC/AKT protein kinase (*YPK1*; CNAG_04678). The identification of two kinases is of interest. Hrk1 functions downstream, as well as independently, of the high osmolarity glycerol response (HOG) pathway, which controls the stress response to a hypertonic environment through regulation of ergosterol biosynthesis and of expression of virulence factors in *C. neoformans* (Kim *et al.,* 2011; Bahn and Jung,

2013). There are homologous proteins in *S. cerevisiae* (Rck1/2) and in *Schizosaccharomyces pombe* (Srk1) to *C. neoformans* Hrk1 (Kim *et al.,* 2011), and inhibition of ergosterol biosynthesis enzymes with drugs results in toxic stress and disrupts membrane integrity in *S. cerevisiae* (Lee *et al.,* 2012). The expression of *HRK1* depends on the central protein in the HOG pathway, a mitogen activated protein kinase (MAPK) called Hog1, but an *hrk1*, *hog1*Δ double mutant is even more sensitive to hyperosmolarity than the *hog1*Δ mutant alone (Kim *et al.,* 2011; Bahn and Jung, 2013) and than an *hrk1*Δ only *C. neoformans* mutant, which was not sensitive to osmolar stress (Lee *et al.,* 2016). Hrk1 is not required for capsule biosynthesis, and is dispensable for *C. neoformans* virulence, but the protein does appear to be involved in melanin biosynthesis (Kim *et al.,* 2011; Lee *et al.,* 2016), although its role may be minor (Liu *et al.,* 2008). Hrk1 also appears to be involved in metabolite transport (Liu *et al.,* 2008). Furthermore, deficiency of this kinase confers sensitivity to sodium dodecylsulfate (SDS) in one *hrk1*Δ mutant, indicative of a cell wall or cell membrane integrity defect (Liu *et al.,* 2008); however, this defect is not observed in another *C. neoformans hrk1*Δ mutant (Lee *et al.,* 2016).

A recent study in *Komagataella phaffii* (previously *Pichia pastoris*) found that an *hrk1* mutant is sensitive to the cytoplasmic acidity generated by entry of acetate into the cell (Xu *et al.,* 2019). Moreover, in *S. cerevisiae,* expression of an Hrk1 protein, which regulates a membrane bound ATPase-associated proton pump in response to glucose metabolism changes, protected the yeast from this acetate-induced acidity (Mira *et al.,* 2010). The identification, in the present screen,

of a *C. neoformans hrk1*Δ mutant among those unable to grow on acetate is in line with these findings in other species, and indicates that this screen was able to successfully detect genes involved in acetate-related processes beyond those functioning only in acetate utilization.

The other protein kinase detected in this screen, *Ypk1,* is a member of the AGC kinase (protein kinases A, G or C) family and therefore likely plays a role in cell survival, growth, and proliferation (Lee *et al.,* 2012). *C. neoformans* Ypk1 shares 55% identity with *S. cerevisiae* Ypk1 and 69% identity with *S. pombe* Gad8, suggesting that these proteins are homologues (Lee *et al.,* 2012). Just like the *C. neoformans hrk1*Δ mutant, the *C. neoformans ypk1*Δ mutant also exhibits sensitivity to inhibition of ergosterol (or other lipid) biosynthesis, which correlates with a similar phenotype in *S. cerevisiae ypk1* mutants (Kim *et al.,* 2011; Lee *et al.,* 2012; Lee *et al.,* 2016). In *C. neoformans*, a kinase-dead mutant exhibits the same sensitivity to inhibition of ergosterol biosynthesis as the deletion mutant (Lee *et al.,* 2012). The *C. neoformans ypk1*Δ mutant also demonstrates similarity to the *hrk1*Δ mutant in that it has a melanin production defect (Kim *et al.,* 2011; Lee *et al.,* 2012; Lee *et al.,* 2016). However, the *C. neoformans ypk1* mutant has been reported to have several other phenotypes as well (Lee *et al.,* 2012; Lee *et al.,* 2016). It has a mild general growth defect and a mild temperature sensitivity, sensitivity to osmolar and oxidative stresses, and sensitivity to Congo red, Calcofluor white, and SDS, indicative of loss of cell wall and/or cell membrane integrity (Lee *et al.,* 2016). In

contrast to Hrk1, Ypk1 plays a role in *C. neoformans* virulence, since the *ypk1*Δ mutant exhibits reduced virulence in mice (Lee *et al.,* 2012).

Comparison to previously published screening results

This screen identified some of the proteins important for acetate utilization, including acetyl-CoA synthetase (Acs1; CNAG_00797), Phosphoenolpyruvate carboxykinase (Pck1; CNAG_04217) and fructose-1,6-bisphophatase (Fbp1; CNAG_00057), and acetate transporters like the acetylcarnitine transporter (AcuH; CNAG_00499) and the succinate-fumarate antiporter (AcuL; CNAG_02288). However, previously published, alternate screening assays in other fungal species and in *C. neoformans* identified some acetate utilization mutants that were not detected in this screen. Thorough analysis of the literature indicated five possible reasons for the failure to identify previously recognized acetate utilization mutants and their corresponding genes in this screen. First, some published screens detected mutants only when the fungus was grown for screening at a different temperature than that used in this screening. For example, Hu *et al*. (2008) identified a mutant for Snf1 (CNAG_06552; a serine/threonine protein kinase also known as CAMK/CAMKL/AMPK protein kinase) that grows normally on glucose, acetate, and sucrose media at 30°C but at 37°C has reduced growth on media with sucrose and ethanol and exhibits a severe growth defect on acetate as the sole carbon source. This Snf1 protein kinase regulates the growth of fungi by modulating gluconeogenesis and alternative carbon source utilization pathways (Celenza and Carlson, 1986) and functions, in part, as a regulator of Acs1, which

converts acetate to acetyl-CoA in yeast (Young *et al*., 2003). *C. neoformans* cells grown on low glucose or acetate media have high levels of *Snf1* mRNA, although cells recovered from infected mouse lung do not (Hu *et al*., 2008). This suggests that although Snf1 is not necessary for virulence, it is required for cryptococcal growth under stress, such as when glucose is not available and acetate is the sole carbon source, or, possibly, under higher temperatures, explaining the finding that the Madhani library mutant did not exhibit growth defects in the current screen because all mutants were grown at 30°C.

The second potential reason is the difference in the carbon source concentrations used in the screening. For instance, an acetate transporter mutant, *ady2*Δ, was determined in the Smith lab to have an extreme growth defect in media supplemented with 0.2% acetate but was able to grow normally in 2% acetate (Kisirkoi, 2017). The equivalent mutant from the Madhani (combined) library, deleted for the gene designated CNAG_05678, was also able to grow at the 2% acetate concentration used in the current screen, meaning that the growth defect was suppressed at the higher acetate concentration. This presumably because acetate at higher concentrations is able to diffuse adequately across the membrane to support growth in the absence of Ady2 (Kisirkoi, 2017). The current screening result supports this hypothesis.

The third potential reason for differences between the current screen in *C. neoformans* and screens of other fungal species is that there may be multiple protein isoforms encoded by paralogous genes or other functionally redundant

proteins in *C. neoformans*. In the present screen, a *C. neoformans* mutant lacking a NAD^+ specific malate dehydrogenase (CNAG_03266) grew normally on both acetate and glucose supplemented media, but this enzyme has previously been shown to be important for acetate utilization in other fungal species. For instance, an acetate-inducible NAD^+-specific malate dehydrogenase mutant in *N. crassa* (*acu-13*) failed to utilize acetate as the sole carbon source and demonstrated a growth defect on 40 mM acetate (Owen *et al*., 1992). However, a search of the *C. neoformans* genome via the FungiDB website (https://fungidb.org/fungidb/) revealed the presence of a second malate dehydrogenase gene (CNAG_03225) located elsewhere in the same chromosome but coding for a similar protein (with 56.51% identity by Clustal Omega analysis) that likely compensates for the absence of the first enzyme and thereby masks the effect of the loss of the first protein. Unfortunately, a double mutant of CNAG_03266 and CNAG_03225 was not available for analysis.

The fourth reason is that the two libraries used in this screen do not cover the entire genome, so knockout mutants for many of the genes identified as critical for acetate utilization in other screens were unavailable for testing. There are ~6500 intron-rich genes in total in the *C. neoformans* genome (Loftus *et al*., 2005), but using the Madhani libraries it was only possible to screen 3936 of these genes. One of the important proteins encoded by a gene missing from the libraries is isocitrate lyase (CNAG_05303), a key enzyme of the glyoxylate cycle, which, in *S. cerevisiae*, is encoded by *ICL1* (Fernández *et al*., 1992; Fernandez *et al*., 1993;

Schüller, 2003). In *S. cerevisiae*, the glyoxylate cycle permits and is essentially required for yeast to grow on two-carbon molecules like ethanol or acetate as the sole carbon source. Acetyl-CoA produced from acetate that is not used in the tricarboxylic acid (TCA) cycle is fed into the glyoxylate cycle (Schüller, 2003; Lorenz and Fink, 2001; Fernández *et al*., 1992). Mutations in *S. cerevisiae ICL1* and in homologues of this gene in *A. nidulans* (*acuD*), *N. crassa* (*acu-3*), and *C. albicans (ICL1),* as well as *C. neoformans,* caused defects in acetate utilization and in the growth of the fungi in acetate-containing media (Armitt *et al*., 1976; Flavell and Fincham, 1968a, Idnurm *et al*., 2007). *C. neoformans* cells grown on acetate as the sole carbon source and those recovered from a rabbit infection model both upregulate *ICL1* transcription (Rude *et al*., 2002). However, while *C. albicans icl1* deletion mutants have clearly diminished virulence in mice (Lorenz and Fink, 2001), *S. cerevisiae icl1* deletion mutants have only slightly reduced virulence in a mouse infection model (Goldstein and McCusker, 2001; Rude *et al*., 2002), and *C. neoformans icl1* mutants exhibited no difference from wild type cells in growth inside macrophages, nor in virulence in mouse inhalation-based and rabbit meningeal infection models (Rude *et al*., 2002). Hence, isocitrate lyase seems to be critical for acetate utilization and growth on acetate-containing media, and perhaps enhances virulence, but is not required for pathogenesis, especially in *C. neoformans* (Idnurm *et al*., 2007; Hu *et al*., 2008). Thus, while it is extremely important to be able screen mutants for genes such as *ICL1* that are not available

in the Madhani libraries, some of the acetate utilization defective mutants that were missed in the current screen may not themselves be of critical importance.

The fifth and final reason for the failure to identify genes in the current screen that in other species are important for growth on acetate is the absence of homologues of these genes in *C. neoformans*. Proteins like acetyl-CoA hydrolase, which is required for growth of *N. crassa* on acetate (Owen *et al*., 1992), and *A. nidulans* facB, a master transcriptional regulator of genes involved in acetate utilization, including genes encoding acetyl-CoA synthetase, isocitrate lyase, malate synthase, and phosphoenolpyruvate carboxykinase, (Todd *et al.,*. 1997; Apirion, 1965; Armitt *et al*., 1976; Hynes, 1977; Kelly and Hynes, 1982), do not have corresponding homologues in the *C. neoformans* genome. *A. nidulans* and *N. crassa* are from the phylum Ascomycota and evolved separately from *C. neoformans* of the phylum Basidiomycota, so it is not surprising that they harbor several genetic differences from *C. neoformans*.

Although some acetate utilization mutants identified in other screens were not detected in this screen, several *C. neoformans* enzymes previously known to function in pathways related to the use or transport of acetate as well as some proteins that have not previously been linked to acetate utilization were identified in this screen. *C. neoformans* may employ these enzymes to enhance growth and virulence after infection, such as in the host central nervous system. This study opens up new avenues for both fundamental research and drug discovery to understand and treat cryptococcal meningitis. Larger scale screenings, as

described by Motaung (2018), may provide yet more target proteins for the development of therapeutic pharmaceuticals to treat and reduce the lethality of *C. neoformans*-driven cryptococcal meningitis.

Summary and Conclusions

The ability to utilize alternate carbon sources provides *C. neoformans* with survival advantages inside the host system, especially in organs like the brain where glucose levels are low (Polacheck *et al*., 1982; Zhu and Williamson, 2004**)**. Targeting this ability to switch between carbon sources for drug development can open up new avenues for treatment of meningitis caused by cryptococcal infection. This study a global screen to discover genes that are important for acetate utilization and presumably would be important for targeting cryptococcal infections in the human brain. Fourteen genes that were important for acetate utilization by *C. neoformans* were identified that have homologues in other fungal species. Most of these genes belong to the gluconeogenesis pathway, mitochondrial membrane transport pathways, or the arginine biosynthesis pathway. Further characterization of the remaining genes, deleted in mutants not previously characterized as having a growth defect on acetate, will be required to determine their exact role in acetate utilization.

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Table 4.1: Mutants from the Madhani laboratory *C. neoformans* strain libraries that were unable to grow on media with glucose as the only carbon source.

Identifier	Protein description
CNAG_00405	Ste/ste20/ysk protein kinase
CNAG_00448	V-type H^+-transporting ATPase subunit AC39
CNAG_00458	hypothetical protein
CNAG_00461	Protein kinase C substrate 80K-H
CNAG_01230	Chitin deacetylase 2
CNAG_01487	hypothetical protein
CNAG_01557	Calmodulin
CNAG_02066	hypothetical protein
CNAG_02588	Avenacinase
CNAG_03168	Sulfite reductase (NADPH) flavoprotein alpha-component
CNAG_05668	hypothetical protein
CNAG_06230	hypothetical protein

Table 4.2: Mutants from the Madhani laboratory *C. neoformans* strain libraries that were unable to grow on media with acetate as the only carbon source.

Identifier	Protein description
CNAG_00057	Fructose-1,6-bisphosphatase I
CNAG_00236	8-Amino-7-oxononanoate synthase
CNAG_00403	mitochondrial protein
CNAG_00499	Solute carrier family 25 (Mitochondrial carnitine/acylcarnitine transporter), member 20/29; Carnitine/acyl-carnitine carrier; AcuH
CNAG_00537	Carnitine O-acetyltransferase
CNAG_00613	Cytosine deaminase
CNAG_00745	HAL protein kinase
CNAG_00797	Acetyl-CoA synthetase
CNAG_02288	Solute carrier family 25 (Mitochondrial citrate transporter), member 1; Mitochondrial succinate-fumarate anti-porter; sfa; acuL
CNAG_03916	Glucose-6-phosphate isomerase
CNAG_04048	53 kDa brg1-associated factor b
CNAG_04217	Phosphoenolpyruvate carboxykinase (ATP)
CNAG_04290	hypothetical protein
CNAG_04678	AGC/AKT protein kinase
CNAG_06551	Carnitine O-acetyltransferase

Table 4.3: Mutants from the Madhani laboratory *C. neoformans* strain libraries that failed to grow on glucose and on acetate, each as the only carbon source.

Identifier	Protein description
CNAG_00734	Dihydroorotase, homodimeric type, dihydroorotase, homodimeric type, variant
CNAG_02795	Phosphoribosyl glycinamide formyl transferase
CNAG_02812	Ornithine carbamoyltransferase
CNAG_02825	Argininosuccinate lyase
CNAG_02826	Amino-acid acetyltransferase, mitochondrial
CNAG_02853	Amido phosphoribosyl transferase
CNAG_03174	4'-phosphopantetheinyl transferase
CNAG_04196	Actin binding protein
CNAG_04647	Glutathione synthetase
CNAG_05074	hypothetical protein
CNAG_05445	Smooth muscle cell associated protein-1 isoform 2
CNAG_06112	Carbamoyl-phosphate synthase arginine-specific large chain
CNAG_06314	Phosphoribosylamine-glycine ligase
CNAG_07373	Carbamoyl-phosphate synthase, large subunit

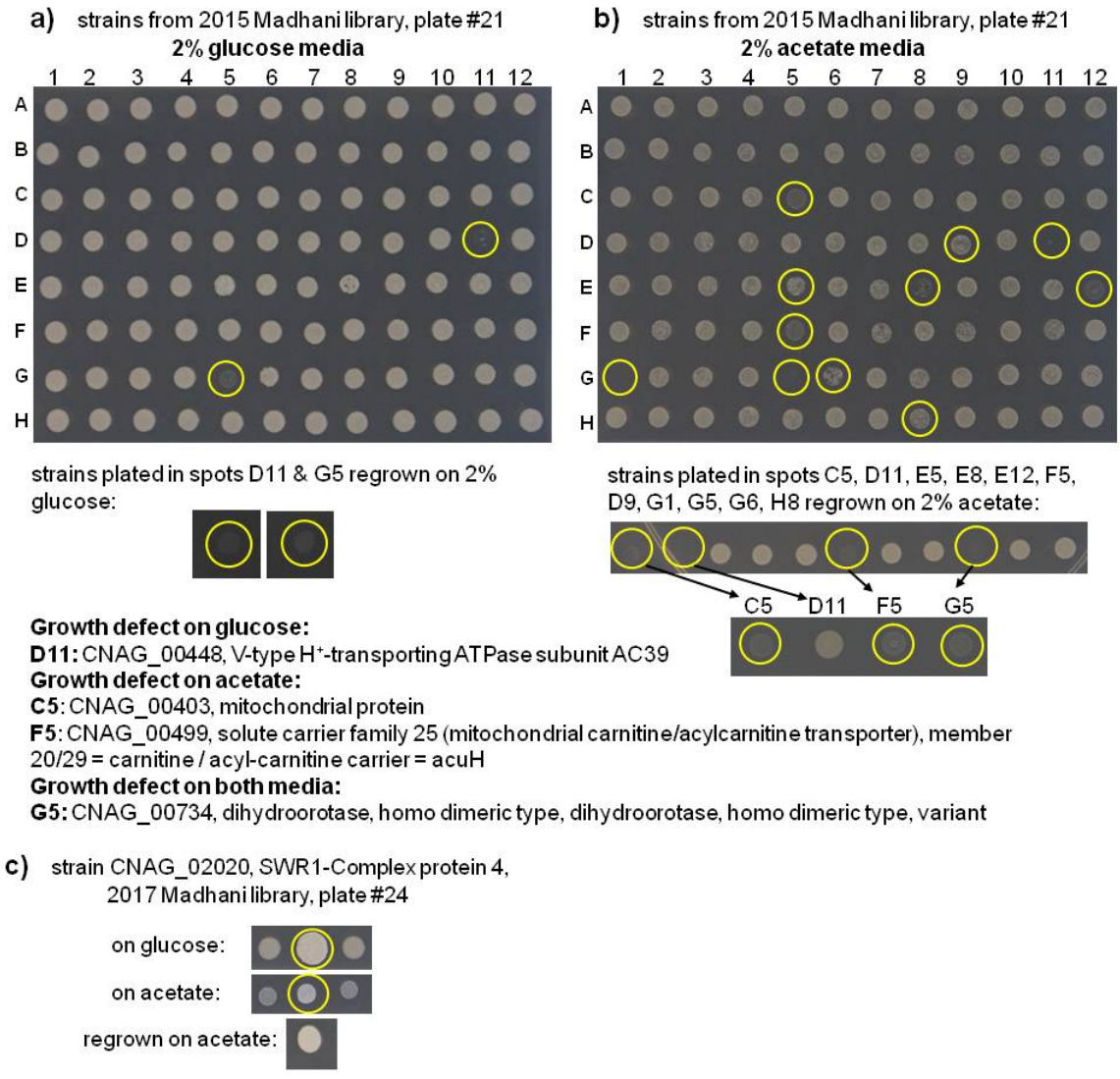


Figure 4.1: Representative plates of Madhani library strains screened for growth on a) glucose or b) acetate as the only carbon source. Strains that did not grow on original plates (circled) and that were re-tested are shown below each plate. **c)** Some strains, like the strain mutant for SWR1-Complex protein 4 (CNAG_02020), had an odd phenotype; they grew into seemingly more dense,

slightly larger, creamy-white colonies on media with either glucose (top, circled) or acetate (middle, circled) as the sole carbon source, or on both media. This phenotype was also visible when the CNAG_02020-deficient mutant was re-grown on acetate as the sole carbon source (bottom).

APPENDIX

This section contains supplementary material for Chapters Two and Three

Given the small capsules of *ald*Δ mutants, and, in particular, the loss of cell wall integrity in the Congo red assay, extremely low melanization, and sensitivity to reactive oxygen species (ROS) and reactive nitrogen species (RNS) of the *ald*69Δ mutant, quantification of the extent of membrane damage due to uptake of ROS seemed likely to be informative. Similarly, since the *pdc*Δ mutant exhibited a smaller than normal capsule, reduced melanization, and loss of cell wall integrity in the Congo red assay, even though it did not demonstrate sensitivity to ROS or RNS, determining the level of ROS-mediated membrane damage also was likely to be informative.

Method: Flow cytometry analysis of oxidative stress and cellular integrity

The concentration of ROS generated by treatment of cells with hydrogen peroxide as well as the level of ROS-induced cell wall/membrane damage in wild type and mutant *C. neoformans* strains were measured using a procedure modified from Peng *et al.*, 2018. Colonies of *C. neoformans* wild type and mutant strains grown on YPD-agar media were transferred to 2ml YPD liquid medium and incubated overnight at 30°C with constant shaking. Cells were centrifuged at 5000×g for 5 min and resuspended in 850 µl YPD medium. For each strain, the cell suspension was diluted in YNB medium (adjusted to pH 4.0 with sodium succinate) to a final OD_{600} of ~0.2 before cells were counted using a hemocytometer and the concentration was corrected to ~1×10^4 cells/µl. The cell

suspension of each strain was aliquoted into six tubes at 2 ml per tube, and either H_2O_2 to a final concentration of 5 mM, or sterile water as control, was added to three pairs of tubes each. The cultures were incubated at 30°C for 3 hours with constant shaking. Next, cells were harvested by centrifugation at 5000×g for 5 min, washed with 1X Dulbecco's Phosphate-Buffered Saline without calcium and magnesium (D-PBS w/o Ca^{2+}, Mg^{2+};Corning Inc., Corning, NY), resuspended in D-PBS w/o Ca^{2+}, Mg^{2+} supplemented with 20 μM 2′,7′ dichlorofluorescin diacetate (H_2DCFDA; Sigma), and then transferred to fresh microfuge tubes, which were immediately covered with aluminum foil. The tubes were incubated for 20 min at 30°C with constant shaking, after which cells were centrifuged again at 5000×g for 5 min., washed with D-PBS w/o Ca^{2+}, Mg^{2+}, and then resuspended in 1 ml D-PBS w/o Ca^{2+}, Mg^{2+} supplemented with 5 μg/ml propidium iodide (PI; Sigma). The tubes were covered again with aluminum foil and incubated for 40 min at 30°C with constant shaking. After this final incubation, cells were centrifuged one more time at 5000×g for 5 min, washed and then resuspended in 1 ml D-PBS w/o Ca^{2+}, Mg^{2+}, and sonicated (Q500 Sonicator, Qsonica) with 20% output strength for 5 seconds to ensure a single-cell suspension. Fluorescence was detected by flow cytometry with the Accuri C6 flow cytometer (BD Biosciences, San Jose, CA). The H_2DCFDA was expected to react with ROS to form 2',7'-dichlorofluorescein (DCF), which was detected and quantified via the FITC-A channel (533/30 nm), while the PI that entered cells through gaps in the cell wall and membrane was detected and quantified via the PerCP-A channel (670 nm).

Results

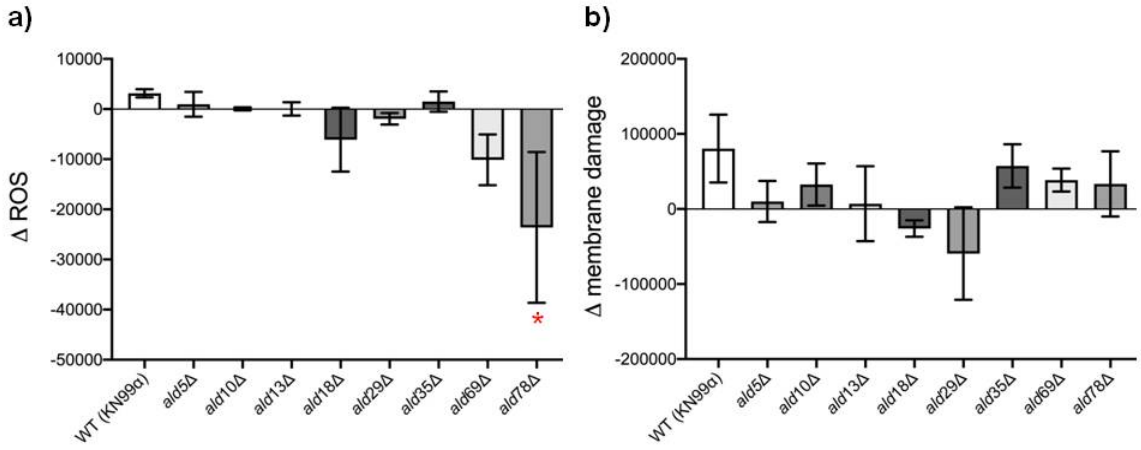
It was considered informative to determine, quantitatively, whether oxidative stress and the introduction of ROS caused increased cell wall and membrane damage in aldehyde dehydrogenase mutants, or whether they were able to compensate by neutralizing the ROS or repairing their membranes. To establish which was true for each mutant, biological triplicates of the wild type and *ald* mutant strains, as well as of the *pdc*Δ mutant, were treated with hydrogen peroxide, or not, as control, and then stained with a compound that converts to a green fluorescent dye upon reacting with ROS, as well as with propidium iodide (PI), a red fluorescent molecule to which the cell membrane is impermeable. The intensity of fluorescence of the two dyes in the triplicate samples of wild type or mutant cells was quantified by flow cytometry. The intensity of the green fluorescence was assumed to be roughly indicative of the amount of ROS inside the cell. Cells with a high intensity of PI fluorescence were considered to have lost capsule, cell wall, and/or cell membrane integrity (referred to as "membrane damage" hereafter), allowing entry of the PI. Only the PI intensity of cells with high ROS was quantified. Of the *ald* mutants, only the *ald*78Δ mutant exhibited a statistically significant difference in the amount of ROS taken into the cell compared with the wild type strain (Supplementary Figure 2.1a; P-value = 0.0269). Strangely, the *ald*78Δ mutant has less ROS than the wild type strain. Similarly, none of the *ald* mutants exhibited increased membrane damage in cells with high ROS, in the sense that the cells of the wild type strain with high ROS had the highest membrane damage

of all strains, but not significantly so (Supplementary Figure 2.1b). The *pdc*Δ mutant appeared to be resistant to ROS uptake and membrane damage relative to the wild type cells (Supplementary Figure 3.1a and 3-1b, respectively), but the results were not statistically significant, indicating that lack of Pdc did not affect response to ROS in this assay. It is worth noting that there was a moderate amount of sample-to-sample variation in ROS and PI intensity measurements of several strains, making these results susceptible to error. Thus, it is difficult to conclude with certainty that the mutants were resistant to ROS and/or able to repair their membrane damage, but, tentatively, this appears to be the case.

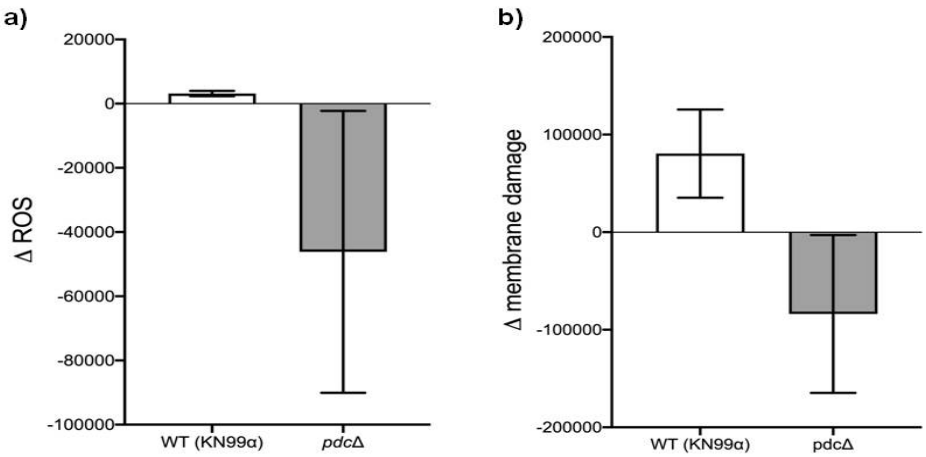
Overall conclusions and future directions

The quantification of uptake of reactive oxygen species (produced from hydrogen peroxide) and of the unrepaired membrane damage resulting from the associated chemical attack on membrane lipids is a useful endeavor. It suggests how the wild type and mutant strains might respond within the phagolysosome of macrophages after engulfment. However, the current results are mostly not significant because of high sample-to-sample variation in signal intensity within each wild type or mutant strain. Thus, the experiment needs to be repeated after the technique is further optimized. Additionally, conducting the experiment with sodium nitrite to generate reactive nitrogen species may also be informative, especially since sodium nitrite is more stable than hydrogen peroxide, and since the chemical attack on pathogens that occurs in phagolysosomes of macrophages primarily utilizes reactive nitrogen species (Tohyama *et al.*, 1996; Maffei *et al*., 2004).

Quantifying the extent of unrepaired ROS- or RNS-induced membrane damage of the pyruvate decarboxylase deficient mutant of *C. neoformans* or of mutants deficient in each of the aldehyde dehydrogenases should illuminate the role of these enzymes, and, potentially, of acetate production, in the neutralization of reactive oxygen and nitrogen species and/or in repair of membrane, cell wall, and capsule damage.



Appendix Figure 2.1: Effect of hydrogen peroxide treatment on reactive oxygen species (ROS) content and membrane damage. a) Change in ROS with H_2O_2. * significant, P-value < 0.05, **b)** Change in membrane damage (and, presumably, loss of cell wall and capsule integrity), based on propidium iodide (PI) entry into cells, with H_2O_2.



Appendix Figure 3.1: Effect of hydrogen peroxide treatment on reactive oxygen species content and membrane damage on wild type and *pdc*Δ mutant strains of *C. neoformans*. a) Change in reactive oxygen species (ROS) content with H_2O_2. **b)** Change in membrane damage, based on propidium iodide (PI) entry into cells, with H_2O_2. While it appears that the mutant had decreased ROS uptake and membrane damage relative to the wild type cells, the results were not statistically significant.

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