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Histoplasma mechanisms of pathogenesis – one portfolio doesn't fit all

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Abstract

Histoplasma capsulatum is the leading cause of endemic mycosis in the world. Analyses of clinical isolates from different endemic regions show important diversity within the species. Recent molecular studies of two isolates, the Chemotype I NAm2 strain G217B and the Chemotype II Panamanian strain G186A, reveal significant genetic, structural, and molecular differences between these representative *Histoplasma* strains. Some of these variations have functional consequences, representing distinct molecular mechanisms that facilitate *Histoplasma* pathogenesis. The realization of *Histoplasma* strain diversity highlights the importance of characterizing *Histoplasma* virulence factors in the context of specific clinical strain isolates.

Keywords

virulence determinants; dimorphic fungi; α-glucan; siderophore; Cbp1

INTRODUCTION

Histoplasma capsulatum is the etiologic agent of histoplasmosis, a fungal disease that can affect both immunocompromised and immunocompetent individuals. Cases of histoplasmosis occur worldwide with endemic regions present in North America, Latin America, and parts of Africa. Within the Ohio and Mississippi River valley areas, more than 80% of individuals exhibit serological evidence of infection (Edwards, *et al.*, 1969). The site of initial infection is the lung and pulmonary disease presents with a range of non-specific respiratory symptoms, the severity of which is determined by the immune status of the host and the number of infectious conidia inhaled (Rippon, 1988). From the lung, *Histoplasma* disseminates throughout the body, most commonly infecting organs populated with reticuloendothelial cells (i.e., liver, spleen, lymph nodes, and bone marrow). Progressive disseminated histoplasmosis is the most lethal form of the disease.

Within the lung, *Histoplasma* cells infect host macrophages. *Histoplasma* survives within these innate immune cells suggesting the operation of specific virulence factors designed to avert or neutralize immune defenses. In immunocompetent individuals, immune control of *Histoplasma* infection requires that sensitized T cells activate macrophages to kill the fungal invader (Newman, 2001). If cell-mediated immunity is inadequate, such as in AIDS patients (McKinsey, *et al.*, 1997), organ transplant patients (Freifeld, *et al.*, 2005), or individuals receiving cytokine-blocking therapies, the risk of progressive disseminated disease increases (Lee, *et al.*, 2002, Wood, *et al.*, 2003). Even following activation of cell-mediated immunity,

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infections may not be completely cleared and latent *Histoplasma* cells may persist constituting a reservoir of organisms that can seed reactivation disease upon diminished immune function (Wheat, 1992, Allen & Deepe, 2006).

Histoplasma belongs to a group of ascomycetes termed the dimorphic fungal pathogens, which includes *Blastomyces dermatitidis, Coccidioides immitis, Paracoccidioides brasiliensis*, *Sporothrix schenkii*, and *Penicillium marneffei*. These dimorphic fungi exhibit two distinct morphologies dependent upon environmental conditions: a filamentous mold within the soil, and a yeast or spherule (*Coccidioides spp.*) within the mammalian host. This thermal dimorphism is not restricted to cellular morphology but also reflects the adoption of saprophytic (mold) or parasitic (yeast) growth. The mold form is avirulent, as preventing the switch of mycelia to yeast during growth at 37°C renders the organism unable to cause disease (Medoff, *et al.*, 1986). Regulation of the transition to the yeast phase requires expression of regulatory factors including the dimorphism regulating kinase Drk1 (Nemecek, *et al.*, 2006); the Wor1 homologue, Ryp1 (Nguyen & Sil, 2008); and two velvet-family regulators, Ryp2 and Ryp3 (Webster & Sil, 2008). As this transition to the yeast form is essential for pathogenesis, and highly homologous proteins are encoded in multiple sequenced isolates, these signaling mechanisms are likely conserved among *Histoplasma* strains.

The *H. capsulatum* species is not monophyletic and has been subdivided into geographically distinct phylogenetic lineages. Based on concordance of multiple gene sequence geneologies, *Histoplasma* strains separate into at least six major clades: North American class 1 (NAm1), North American class 2 (NAm2), a Panamanian clade, Latin American group A (LAmA), Latin American group B (LAmB), and an African clade (which includes the *Histoplasma capsulatum* variety *dubosii*) (Kasuga, *et al.*, 1999, Kasuga, *et al.*, 2003). Interestingly, clinical differences in histoplasmosis disease manifestation exist among the groups. For example, some African clade strains cause primarily cutaneous and subcutaneous lesions rather than pulmonary involvement, and these have historically been classified as *Histoplasma capsulatum* var *dubosii*. Whether this manifestation is determined by genetic differences in *Histoplasma* strains is unclear since pulmonary disease-causing strains are also part of the African clade (Kasuga, *et al.*, 2003). In North America, a correlation between NAm1 infections and hosts with AIDS has been suggested, whereas NAm2 strains are isolated from histoplasmosis patients regardless of HIV-status (Medoff, *et al.*, 1986, Spitzer, *et al.*, 1990). However, another study identified a NAm1-class strain from an HIV-negative individual (Jiang, *et al.*, 2000). As all these findings are based on relatively small sample sizes, better epidemiological data is necessary to establish the link between NAm1 *Histoplasma* strain infection potential and the immune status of the host. In mouse studies, Latin American and NAm2 isolates differ in acute and chronic disease potential (Durkin, *et al.*, 2004) as well as the extent of cutaneous disease presentation (Karimi, *et al.*, 2002). Differences in surfactant-sensitivity have also been reported between NAm2 and Panamanian strains (McCormack, *et al.*, 2003). Together these findings suggest important diversity in virulence, infectivity, and pathogenesis among strains and indicate that sequence variations between phylogenetic groups are not inconsequential.

In this review, we discuss important genetic and functional differences in virulence determinants of *Histoplasma*. As establishment of functional roles relies on molecular genetic manipulation, we focus on two *Histoplasma* clinical isolates with sequenced genomes and in which genes have been disrupted or gene products depleted: a NAm2 strain, G217B, and an isolate from Panama, G186A.

GENETIC DIFFERENCES

To date, genomes from the NAm1 strain WU24, a NAm2 strain (G217B), a Panamanian strain (G186A), and two strains from the African phylogenetic clade (H143 and H88), have been sequenced and are maintained through the Broad Institute and the Genome Sequencing Center at Washington University (http://www.broadinstitute.org, http://www.genome.wustl.edu). G186A has four chromosomes whereas G217B has only three (Steele, *et al.*, 1989). However, the total genome size of G217B is roughly 30% larger than G186A (41 megabases versus 30.4 megabases, respectively) primarily due to repetitive DNA, which includes mobile DNA insertions, retrotransposons and multiple copies of a crypton (Goodwin, *et al.*, 2003). This suggests that the non-repetitive "core" *Histoplasma* genome is roughly 26–28 megabases. Bioinformatics analyses of the sequence predicts that the *Histoplasma* genome encodes between 9,000 and 10,000 genes (http://www.broadinstitute.org). Large regions of synteny exist between G186A and G217B and much of the "extra" DNA is located intergenically as clusters of repetitive sequence. Nucleotide sequence identity for homologous genes is roughly 97% ± 2% between G186A and G217B (Edwards J.A. and Rappleye C.A., unpublished results) suggesting differential gene regulation, rather than amino acid change, is an important contributor to phenotypic differences between strains.

Histoplasma capsulatum is a haploid organism and has a heterothallic mating system (Kwon-Chung, 1973). A mating type locus (*MAT* locus) is present in the genome and two *MAT* alleles are correlated with opposite mating types in clinical strains; G217B has the *MAT1-1* allele whereas G186A has the *MAT1-2* allele (Bubnick & Smulian, 2007). Some correlation exists between mating type and virulence. Considerable variation exists in the proportions of mating types (designated as + or −) in environmental sources of *Histoplasma* (Kwon-Chung, *et al.*, 1974, Gaur & Lichtwardt, 1980), however in clinical samples, - mating types predominate (Kwon-Chung, *et al.*, 1974, Kwon-Chung, *et al.*, 1984). The significance of this correlation is presently unknown.

Attempts to manipulate G186A and G217B in the lab have indicated differences in the efficiency of homologous recombination between the two strains. Whereas several gene deletion strains have been created through allelic replacement in the Panamanian background (G186A or G184A strains) (Woods, *et al.*, 1998, Sebghati, *et al.*, 2000, Tian & Shearer, 2002, Rappleye, *et al.*, 2004, Marion, *et al.*, 2006, Hwang, *et al.*, 2008, Hilty, *et al.*, 2011), only a limited number of gene knockout alleles exist in the NAm2 isolate G217B (Marion, *et al.*, 2006, Cooper & Woods, 2009). As a consequence, RNAi has been adopted as a more practical means to deplete gene functions in *Histoplasma* (Rappleye, *et al.*, 2004) when efforts to delete genes through homologous recombination fail.

ESTABLISHED VIRULENCE FACTORS

In the mouse model of histoplasmosis, G186A and G217B cause similar respiratory and systemic disease, however, G217B intranasal infections led to higher organ fungal burdens and increased lethality compared to G186A (Tewari & Berkhout, 1972, Mayfield & Rine, 2007, Edwards, *et al.*, 2011). In *Histoplasma*, only a handful of factors have been demonstrated to contribute to virulence in vitro or in vivo, and even fewer have been tested for virulence roles in both strain backgrounds. In the following sections, we will discuss studies in G186A and G217B as representative for the Panamanian and NAm2 phylogenetic clades, respectively.

Cbp1

The secreted protein Cbp1 was the first *Histoplasma* virulence factor to be established through genetics. Both G217B and G186A yeast cells produce abundant Cbp1 during liquid culture (Kugler, *et al.*, 2000, Youseff, *et al.*, 2009), and the *CBP1* gene is expressed by both strains during intramacrophage growth and during in vivo infection (Batanghari, *et al.*, 1998, Edwards, *et al.*, 2011). Cbp1 is required for the full virulence of G186A and G217B. Genetic mutations for proof of this were provided through the creation of a *cbp1*-deletion allele in the G186A background (Sebghati, *et al.*, 2000) and isolation of a T-DNA insertion mutant in the *CBP1* gene in the G217B background that prevents Cbp1 production (Youseff, *et al.*, 2009). In the absence of Cbp1, *Histoplasma* yeast grow at a similar rate in culture; however, the yeast are attenuated in both macrophage and mouse assays of virulence (Sebghati, *et al.*, 2000, Edwards, *et al.*, 2011). While the exact mechanism of Cbp1 contribution to virulence remains unknown, the Cbp1 homodimer has structural similarity to mammalian saposin B (Beck, *et al.*, 2009) suggesting a role in transforming the phagocytic compartment into a permissive environment for yeast survival and replication. The Cbp1 requirement for both G186A and G217B virulence indicates conservation of at least one mechanism for pathogenesis.

Cell wall α-glucan

G186A and G217B yeast cells have similar size and morphology when viewed by light microscopy, however structural and chemical differences exist between their respective cell walls. Electron microscopy shows that the cell wall of G186A is more than twice as thick as the cell wall of G217B (Edwards, *et al.*, 2011). Biochemical analysis of the cell walls following sodium hydroxide or glucanase treatment classifies strains as one of two chemotypes based on the polysaccharide composition of the yeast cell wall (Domer, 1971, Kanetsuna, *et al.*, 1974, Reiss, 1977, Reiss, *et al.*, 1977). Chemotype II comprise those strains for which the yeast cell wall contains α-glucan whereas Chemotype I strains lack α-glucan in the yeast cell wall. Follow-up studies using immunogold labeling confirmed the presence of α-glucan in the yeast cell walls of Chemotype II strains G186A (Panamanian class) and UCLA531 (a North American isolate with the same RFLP pattern and fatty acid profile as the Downs NAm1 strain) (Eissenberg, *et al.*, 1997, Zarnowski, *et al.*, 2007). In contrast, the NAm2 strain G217B lacks α-glucan defining it as Chemotype I (Eissenberg, *et al.*, 1991). Thus, the yeast cell wall of NAm2 differs significantly from the cell walls of other North and Latin American strains.

The production of α-glucan is critical to the virulence of Chemotype II *Histoplasma* yeast. The importance of α-glucan was first suggested by the isolation of “smooth” variants of Chemotype I strains (NAm1, Panamanian, and African strains) that spontaneously lost α-glucan, and the demonstration that, in contrast to the parent yeast, these variants have significantly attenuated virulence (Klimpel & Goldman, 1987, Klimpel & Goldman, 1988, Eissenberg, *et al.*, 1997). Creation of a G186A strain in which the α-glucan synthase (*AGS1*) gene is deleted provided the genetic proof of the importance of α-glucan to Chemotype II strain virulence; *ags1*-mutant yeast have cell walls that lack α-glucan and, although they grow normally in laboratory culture, these cells lacking α-glucan are substantially decreased in virulence (Rappleye, *et al.*, 2004). Through mutagenesis screens, two additional genes important for α-glucan biosynthesis in G186A have been identified: *AMY1* that encodes a protein with homology to α-(1,4)-amylase and *UGP1* that encodes UTP-glucose-1-phosphate uridyltransferase (Marion, *et al.*, 2006). As with deletion of *AGS1*, the loss of either *AMY1* or *UGP1* results in loss of α-glucan from the cell wall and decreased virulence. Functionally, α-glucan promotes *Histoplasma* virulence by preventing recognition of yeast by host immune cells. The α-glucan polysaccharide forms the outermost surface of the yeast cell wall, effectively concealing cell wall β-glucans that would normally be detected by

Dectin-1 receptors on host macrophages (Rappleye, *et al.*, 2007). While α-glucan masks G186A from immune detection, it also prevents entry of chemotype II yeast into epithelial cells whereas G217B can readily enter this cell type (Eissenberg, *et al.*, 1991).

Although the genome of chemotype I strains (i.e., G217B) encodes the *AGS1*, *AMY1*, and *UGP1* genes required for α-glucan synthesis, these NAm2 strains do not produceα-glucan, at least during laboratory culture of yeast. This difference from G186A yeast results, at least in part, from transcriptional changes in the NAm2 lineage. While G186A and G217B both transcribe *AMY1* and *UGP1* at similar levels, *AGS1* expression levels are significantly reduced in G217B (Edwards, *et al.*, 2011). Molecular analysis of the G217B *AGS1* promoter identified an insertion of repetitive DNA sequence that disrupts *AGS1* transcription efficiency in this strain (Edwards, *et al.*, 2011). No substantial change in *AMY1* and *UGP1* expression exist between the strains. Thus, impaired transcription of *AGS1* in NAm2 appears to be responsible for the lack of α-glucan.

How does G217B remain virulent if it doesn't produce α-glucan that is essential for chemotype II yeast virulence? One possibility is that G217B actually produces α-glucan, but does so only in vivo and not during laboratory culture. To test this possibility, Edwards et al. analyzed a mutant G217B strain in which any production of α-glucan was prevented by inactivation of the *AGS1* gene by a T-DNA insertion. Infection of mice with this mutant strain demonstrated blocking α-glucan synthesis has no effect on G217B virulence (Edwards, *et al.*, 2011). Analysis of a G217B strain in which α-glucan synthesis was independently blocked by RNAi showed a similar lack of requirement for α-glucan in G217B intramacrophage replication and in lung infection. Interestingly, although G217B yeast cells lack α-glucan, they can still prevent Dectin-1 recognition of cell wall β-glucan (Edwards, *et al.*, 2011). The growth stage-dependent mechanism by which G217B yeast accomplish this is unknown. Thus, G217B (representing chemotype I) and G186A (representing the chemotype II lineages) significantly differ in their mechanisms of pathogenesis with regards to yeast cell wall glucans and avoidance of detection by host immune cells.

Yps3

Yps3 is a secreted and cell wall factor with sequence homology to the *Blastomyces dermatitidis* adhesin BAD1. Similar to BAD1, the Yps3 protein interacts with chitin on the G217B yeast cell wall (Bohse & Woods, 2005). G217B yeast in which Yps3 production is blocked by RNAi grow similar to the wild-type strain in vitro and exhibit similar virulence in macrophages. However, the *Yps3*-deficient strain is defective in dissemination to the spleen and liver, implicating *Yps3* in progression towards disseminated disease (Bohse & Woods, 2007). Although the *YPS3* gene is transcribed transiently by G186A strains upon shift from 25°C to 37°C, expression is not maintained in the yeast phase (Keath, *et al.*, 1989). Sustained expression of the gene and production of the Yps3 protein is restricted to NAm2 strains such as G217B, in vitro (Bohse & Woods, 2007). Yps3 production in vivo remains to be tested for all *Histoplasma* strains. In addition, the *YPS3* genes of different strains encode proteins with variable numbers of tandem repeats (2 in NAm2, 11–12 in Panamanian strains, and 18–20 in NAm1). Thus, both structural and regulatory differences exist among the strains with regards to Yps3. No genetic tests have been performed to test whether G186A virulence requires Yps3, but the lack of Yps3 production by G186A suggests that Yps3 represents a distinct pathogenic mechanism for NAm2 strains.

Iron homeostasis

Histoplasma yeast are sensitive to the availability of iron and express factors to acquire sufficient iron from the environment. Iron restriction by the host is an important mechanism

for restriction of *Histoplasma* yeast growth similar to control of other intracellular pathogens (Newman, *et al.*, 1994). *Histoplasma* yeast require iron for both in vitro growth (Timmerman & Woods, 1999, Timmerman & Woods, 2001) and growth in macrophages (Lane, *et al.*, 1991, Newman, *et al.*, 1994, Newman, *et al.*, 1995). Genetic studies have identified the several gene products as important mechanisms for *Histoplasma* iron acquisition (Hwang, *et al.*, 2003, Hilty, *et al.*, 2008, Zarnowski, *et al.*, 2008, Hilty, *et al.*, 2011). Of these genes, only *SID1* has been depleted in both G217B and G186A strains (Hwang, *et al.*, 2003, Hilty, *et al.*, 2011). *SID1* encodes the enzyme whose function represents the committed step in siderophore biosynthesis and strains deficient in Sid1 are unable to produce siderophores and unable to grow on iron-depleted media. In both the G186A and G217B backgrounds loss of siderophore production impairs intramacrophage growth and modestly decreases virulence in vivo. While siderophore production is conserved in both strains, G217B has a greater reliance on this virulence mechanism since siderophore deficiency reduces lung infection to a greater degree in this background than its loss in G186A (Hilty, *et al.*, 2011).

G217B also utilizes iron acquisition mechanisms that depend on the vacuolar ATPase and an extracellular glutathione-dependent iron reductase. The *VMA1* gene encodes the V-ATPase catalytic subunit A required for vacuolar acidification. Mutation of this gene severely reduces *Histoplasma* virulence in macrophages and in mice (Hilty, *et al.*, 2008). Supplementation with iron restores intramacrophage growth of the *vma1* mutant linking the vacuolar ATPase to iron homeostasis. G217B yeast secrete a gamma-glutamyltransferase (*Ggt1*) which catalyzes a two-step glutathione-dependent reaction to reduce iron to its ferrous state (Zarnowski, *et al.*, 2008). Loss of this iron reductase activity reduces the virulence of *Histoplasma* yeast in cultured macrophages although the importance of this function in vivo has yet to be determined.

The relative contributions of each of these iron acquisition mechanisms to *Histoplasma* pathogenesis are becoming clear for G217B with the creation of mutants and RNAi lines that lack these factors. However, parallel studies of *Vma1*- and *Ggt1*-deficient G186A yeast are lacking. The finding that siderophore production is more important for G217B than G186A virulence suggests different, and perhaps compensatory, mechanisms for iron acquisition and storage may be in operation among the different clades. In support of this, the G186A genome, but not that of G217B, contains the *FET3* and *FTR1* genes that encode for components of a high-affinity iron transport system (Hilty, *et al.*, 2011). Thus, while iron acquisition is an essential virulence requirement shared by *Histoplasma* strains, the molecular mechanisms to achieve this are specific to the different *Histoplasma* phylogenetic groups.

Adhesins

The adhesins used by *Histoplasma* to gain entry into host macrophages have only been determined for G217B to date. It has been assumed that G217B and G186A use common factors for binding to host cells. For G217B yeast, cell-surface localized Hsp60 acts as the adhesin that mediates attachment of yeast cells to CD18-family complement receptors on macrophages (Long, *et al.*, 2003, Habich, *et al.*, 2006). For binding to dendritic cells, a different adhesin-receptor pair is used; G217B yeast cells utilize cell surface-localized cyclophilin A to bind to host VLA-5 (Gomez, *et al.*, 2008). As the G217B cell wall does not contain α-glucan, the yeast cell surface that interacts with host phagocytes is potentially quite different for NAm2 yeast cells compared to G186A and thus different adhesins/host receptors may be used by different *Histoplasma* strains. Because of the importance of the different yeast ligands and host receptors on the intracellular fate of phagocytosed yeast, the repertoire of surface molecules that engage host phagocytes might contribute to phenotypic

differences between *Histoplasma* strains. Future experiments that examine blockage of the candidate adhesins in G186A yeast will be needed to resolve this question.

POTENTIAL VIRULENCE MECHANISMS

CatB

Catalases are hydrogen peroxide metabolizing enzymes often utilized by pathogens to ameliorate the effects of anti-microbial reactive oxygen. The immunoreactive M-antigen found in *Histoplasma* culture filtrates corresponds to the CatB catalase protein (Hamilton, *et al.*, 1990, Zancope-Oliveira, *et al.*, 1999). Although originally prepared from mycelial-phase cultures, CatB is also an exoantigen of both G186A and G217B yeast cells. Patient antibodies to CatB confirm that the yeast produce this protein during infection. However, CatB regulation differs between strains. In G186A, the *CATB* gene shows approximately 100-fold higher expression in yeast than in mycelia, and this protein is expressed by G186A yeast in vitro, in macrophages, and in the mouse lung (Holbrook, *et al.*, 2011). In contrast, there is equivalent transcription of *CATB* in both yeast and mycelial phases of G217B (Johnson, *et al.*, 2002). In addition, differences have been found in the extracellular localization of CatB between the strains. In G186A, cell wall-associated catalase is a minor contributor to the total extracellular peroxidase activity with the majority present in the soluble extracellular fraction (Holbrook, *et al.*, 2011). For G217B, CatB is found primarily associated with the yeast cell wall, being released only after 7 days of culture (Guimaraes, *et al.*, 2008). The functional consequences of the differing regulation and localization of CatB remain to be determined but these findings continue to highlight the variability between strains that may contribute to differences in virulence phenotypes.

Other inter-strain variations

Additional variability in cellular composition and secreted factors correlate with the deeply-branching *Histoplasma* phylogenetic groups. In a survey of cellular lipids, distinct fatty acid compositions of yeast cells were found to exist among the *Histoplasma* strains (Zarnowski, *et al.*, 2007). The *Histoplasma* H-antigen (Hag1; β-glucosidase) is produced by all strains, but G217B yeast release over ten times as much β-glucosidase activity (Fisher, *et al.*, 1999). Additionally, the H-antigen produced by each strain varies in size with Panamanian strains producing a smaller protein than NAm1 and NAm2 strains. Both NAm2 and Latin American strains express surface-localized Histone-2B and melanin on yeast cells (Nosanchuk, *et al.*, 2002, Nosanchuk, *et al.*, 2003). A recent study of extracellular proteins from G186A identified ten proteins with enriched expression in the yeast phase which included four novel culture filtrate proteins (Cfps), three polysaccharide metabolism factors, and three related to reactive oxygen neutralization (Holbrook, *et al.*, 2011). Some of these factors are also produced by G217B (Holbrook E.D., Youseff B.H., and Rappleye C.A., personal communication). Finally, only NAm1 strains produce an extracellular serine-protease activity (Zarnowski, *et al.*, 2007). No studies have been done to determine if any of these variations contribute to *Histoplasma* pathogenesis.

CONCLUSIONS AND FUTURE OUTLOOK

The completion of genome sequences from multiple phylogenetic groups and the continued development and application of molecular genetic techniques are furthering our understanding of the pathogenic mechanisms that underlie *Histoplasma* virulence. For two of the most studied strains, G186A and G217B, both conserved components (e.g., Cbp1, Sid1) and distinct factors (e.g., α-glucan, Yps3) shape the resultant pathogenesis (Table 1). The examples of *AGS1* and *YPS3* highlight the influence of dissimilar transcriptional regulation on variation between strains with highly similar genome sequences. Surprisingly

few mechanistic studies have been performed with multiple *Histoplasma* strains, making it difficult to extrapolate experimental results from one strain to the others. Based on the variation in the few virulence factors examined to date, additional aspects distinguishing *Histoplasma* strains are expected. Establishment of the relevance of such mechanistic differences to *Histoplasma* pathogenesis will require recognition of the dissimilarities between strains and performance of comparative studies using the molecular genetic tools now available.

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Table 1

Histoplasma virulence factor requirements[1]

	Factor	G186A	G217B	References
Transition to the yeast phase	Drk1		Y[3]	(Nemecek, *et al.*, 2006)
	Ryp1		Y[3,4]	(Nguyen & Sil, 2008)
	Ryp2/Ryp3		Y[4]	(Webster & Sil, 2008)
Surface characteristics	α-glucan	Y[2,3,4]	N[3,4]	(Rappleye, *et al.*, 2004), (Marion, *et al.*, 2006), (Edwards, *et al.*, 2011)
	Yps3	N[6]	Y[3]	(Bohse & Woods, 2007)
	Hsp60		Y[5]	(Long, *et al.*, 2003)
	Histone-2B			(Nosanchuk, *et al.*, 2003)
	Melanin			(Nosanchuk, *et al.*, 2002)
Iron acquisition	Siderophores (Sid1)	Y[2]	Y[3]	(Hwang, *et al.*, 2008), (Hilty, *et al.*, 2011)
	Fet3/Ftr1		N[6]	(Hilty, *et al.*, 2011)
	Ggt1		Y[3]	(Zarnowski, *et al.*, 2008)
	Vma1		Y[4]	(Hilty, *et al.*, 2008)
Secreted factors	Cbp1	Y[2]	Y[4]	(Sebghati, *et al.*, 2000), (Edwards, *et al.*, 2011)
	Hag1			(Fisher, *et al.*, 1999)
	CatB			(Johnson, *et al.*, 2002), (Guimaraes, *et al.*, 2008), (Holbrook, *et al.*, 2011)
	Cfps[7]			(Holbrook, *et al.*, 2011)

[1] "Y" = required for full virulence, "N" = not required for virulence, blank = untested

[2] evidence = gene deletion

[3] evidence = RNA interference

[4] evidence = T-DNA insertion mutant

[5] evidence = competitive inhibitor

[6] gene not expressed or not present in genome

[7] Cfps = Culture filtrate proteins