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Dimorphism and virulence in fungi

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Abstract

The signature feature of systemic dimorphic fungi – a family of six primary fungal pathogens of humans – is a temperature-induced phase transition. These fungi grow as a mold in soil at ambient temperature and convert to yeast after infectious spores are inhaled into the lungs of a mammalian host. Seminal work 20 years ago established that a temperature-induced phase transition from mold to yeast is required for virulence. Several yeast-phase specific genes, identified one-by-one and studied by reverse genetics, have revealed mechanisms by which the phase transition promotes disease pathogenesis. Transcriptional profiling of microarrays built with genomic elements of *Histoplasma capsulatum* and ESTs of *Paracoccidioides brasiliensis* that represent partial genomes has identified 500 genes and 328 genes, respectively, that are differentially expressed upon the phase transition. The genomes of most of the dimorphic fungi are now in varying stages of being sequenced. The creation of additional microarrays and the application of new reverse genetic tools promise fresh insight into genes and mechanisms that regulate pathogenesis and morphogenesis. The use of insertional mutagenesis by *Agrobacterium* has uncovered a hybrid histidine kinase that regulates dimorphism and pathogenicity in *B. dermatitidis* and *H. capsulatum*. Two-component signaling appears to be a common strategy for model and pathogenic fungi to sense and respond to environmental stresses.

Introduction

The systemic dimorphic fungi represent a family of six phylogenetically related ascomycetes: *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Sporothrix schenkii, and Penecillium marnefiii.* These primary pathogens are capable of converting from a nonpathogenic mold in the soil to a pathogenic yeast (or spherule in *C. immitis*) after infectious spores are inhaled into the lungs of human or other mammalian hosts. These fungi collectively cause over a million new infections a

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year in the United States alone, and remain latent after prior infection in tens of millions of people worldwide, in whom they may reactivate if the host becomes immune-deficient [1–5].

The biological importance of phase transition in dimorphic fungi

The morphologic conversion of the dimorphic fungi from mold to yeast is required for virulence. In experimental studies of *H. capsulatum,* the transition to the yeast form is required for the establishment of disease. Treatment of mycelia with the sulfhydryl inhibitor p-chloromercuriphenylsulfonic acid (PCMS) permanently and irreversibly prevents the transition to yeast at 37°C. PCMS-treated *H. capsulatum* failed to cause illness in a mouse model of lethal experimental infection, and no fungal colonies were recoverable from spleens of the infected mice [6]. This evidence suggests that the conversion to yeast is necessary for virulence in *H. capsulatum*. Conversion to the yeast form may offer protection against killing by neutrophils, monocytes, and macrophages. Drutz and colleagues [7] showed that *B. dermatitidis* yeast are too large to be ingested by polymorphonuclear neutrophils (PMNs), unlike the much smaller conidia. In addition, both PMNs and peripheral blood monocytes are more efficient at killing conidia than yeast.

Yeast phase specific factors and their role in virulence

The phenotypic switch from an environmental mold morphotype to a pathogenic yeast morphotype results in a change not only in cell shape, but also in the composition of the cell wall, the presence of antigenic molecules, and the expression of virulence traits. In *B. dermatitidis*, the conversion from mold to yeast results in an increased cell wall content of α-(1,3)-glucan and a decreased β-(1,3)-glucan content [8]. In the pathogenic yeast forms of several of the dimorphic fungi, including *B. dermatitidis*, *H. capsulatum* and *P. brasiliensis,* the level of α-(1,3)-glucan in the cell wall correlates with the level of virulence [9]. Additionally, as cells adapt to changes in temperature, multiple changes occur in the lipid composition of the plasma membrane, which leads to remodeling and reorganization of the membrane [10].

The role of α-(1,3)-glucan in virulence of *H. capsulatum* was formally investigated in recent work. α-(1,3)-glucan synthase (*AGS1*), the gene that encodes this cell wall polymer, was demonstrated to be essential in pathogenicity of the fungus [11]. Either targeted gene disruption or silencing of its expression by RNA interference significantly impaired the growth of the yeast in macrophages *in vitro*, and its ability to colonize the lungs of mice. In additional studies [12], the regulation of α-(1,3)-glucan production was found to require the function of the *AMY1* gene product, a novel protein with homology to the alpha-amylase family of glycosyl hydrolases, and UGP1, a UTP-glucose-1-phosphate uridylyltransferase that synthesizes UDP-glucose monomers. Loss of *AMY1* function in turn attenuated the ability of *H. capsulatum* to grow in and kill macrophages and to colonize murine lungs.

The exact mode by which α-(1,3)-glucan influences the pathogenesis of infections with dimorphic fungi remains poorly understood. However, recent work has shed new light on how it may influence host-pathogen interactions. Successful infection by fungal pathogens depends on the subversion of host immune mechanisms that detect conserved cell-wall components such as β-glucans, which are recognized by dectin-1 receptors on host phagocytes [13]. Dectin-1 mediates the inflammatory response to fungi and facilitates pathogen clearance [14]. α-(1,3)-glucan in the outermost layer of the *H. capsulatum* yeast cell wall may contribute to pathogenesis by concealing immunostimulatory β-glucans from detection by host phagocytic cells. In a recent study, the production of the proinflammatory cytokine TNF-α by phagocytes was suppressed either by the presence of the α-(1,3)-glucan layer on yeast cells or by RNA interference based depletion of the host β-glucan receptor

dectin-1. These findings may reveal an important mechanism by which *H. capsulatum* thwarts the host immune system.

During conversion to the pathogenic form, the dimorphic fungi express other phase-specific products that have been shown to be essential in virulence (Table). In *B. dermatitidis*, only the yeast phase expresses the immunoreactive 120 kDa protein antigen BAD1 (formerly named WI-1) [15,16]. BAD1 binds to chitin on the yeast cell wall, and about 4.7×10^6 molecules are estimated to be present on each individual yeast cell [17]. This surface molecule functions as an adhesin and essential virulence factor that binds the fungus to complement type 3 receptors and CD14 on macrophages and lung tissue [18,19]. BAD1 also alters the host's immune response by down-regulating production of the pro-inflammatory cytokine TNF-α in phagocytes and up-regulating production of the anti-inflammatory cytokine TGF-β, aiding in the progression of a pulmonary infection [20]. Additionally, BAD1 contains 35 copies of a 25 amino acid tandem repeat, each harboring an EF-hand that binds calcium, which enables growth of yeast in a calcium poor environment [21].

Likewise, in *H. capsulatum*, only the pathogenic yeast phase expresses a released calcium-binding protein (CBP1), which is essential for growth in macrophages *in vitro*, and survival in the host and pathogenicity *in vivo* [22,23]. *H. capsulatum* yeast also produce Yps3, a cell wall-localized protein that is not produced by the mycelial phase [24]. Yps3 localizes to the cell surface via an epidermal growth factor (EGF)-like domain that fixes chitin fibrils [25]. RNA interference of Yps3 expression significantly impaired the growth of the fungus in lungs and spleen in a murine model of pulmonary infection, indicating that expression is required for virulence (personal communication; Jon P. Woods).

The search for virulence determinants in other dimorphic fungi has lead to the identification of a parasitic phase-specific adhesin SOWgp in the spherule form of *C. immitis* that binds to host extracellular matrix proteins that is important for both pathogenesis and survival in the host [26]. In *P. marneffei*, a catalase peroxidase has been identified as a phase specific product that is expressed during transition to the yeast form at 37°C [27]; its role in virulence has not been investigated.

Expression analysis of phase-specific genes in dimorphic fungi

The studies above have identified, characterized and analyzed, one-by-one, a handful of individual phase specific factors in the systemic dimorphic fungi. Some of these are indispensable for pathogenicity. Undoubtedly there are many more that remain to be identified and analyzed. The genomes of several of the dimorphic fungi are in varying stages of being sequenced and completed and this information along with microarrays should provide new insight into the identity and function of phase regulated genes that contribute to pathogenicity. Pending these studies, initial transcriptional profiling has been done in *H. capsulaum* and *P. brasiliensis* that sheds new light on phase regulated genes.

Hwang and colleagues [28] created a 10, 000 element genomic shotgun microarray with genomic fragments that are estimated to represent one-third of the genome. On probing the array with cDNA from mold and yeast, they identified approximately 500 genes that are expressed at a significantly higher level (>5-fold) in one phase versus the other. Of these, 217 genes were up-regulated in the yeast form, and 271 in the mold form. Among the yeast-specific genes, one was up-regulated from 50- to 100-fold, and 20 from 20- to 50-fold. Conversely, 3 genes were up-regulated in the mold from 50- to 100-fold. In the latter group, *TYR1* was upregulated in mold vs. yeast by nearly 120-fold. *TYR1* is an ortholog of a tyrosinase gene (*MelC2*) from the bacterium *Streptomyces griseus. MelC2* is needed to produce melanin. Under normal culture conditions, *H. capsulatum* mycelia produce melanin,

whereas yeast do not. *TYR1* is believed to be a candidate for the regulation of melanin production and establishment or maintenance of *H. capsulatum* in the mycelial form.

In addition to confirming the yeast-phase specific expression of *CBP1* and *yps-3* with the shotgun microarray analysis, Hwang and colleagues [28] identified several genes involved in sulfur metabolism that were up-regulated in the yeast phase. This is of interest because classical studies have established that sulfur metabolism influences the morphological state of *H. capsulatum* and other dimorphic fungi [29]. For example, the addition of exogenous sulfhydryl reducing agents (dithiothreitol) to the media locks cells in the yeast form independent of temperature, whereas the addition of sulfhydryl oxidizing agents (PCMS) locks cells in the mycelial form independent of temperature [29]. In *H. capsulatum*, some strains exhibit a requirement for the presence of cystine or cysteine in the culture medium to grow in the yeast phase. Microarray analysis identified a yeast-specific cysteine dioxygenase gene that was expressed 11-fold higher in yeast than in mycelia. In addition, several yeast-expressed genes share sequence similarity to genes that are involved in sulfur metabolism in other organisms: choline sulfatase; ATP sulfurylase (the first enzyme in the sulfate-assimilation arm of the methionine/cysteine biosynthetic pathway); glutamate-cysteine ligase, which affects glutathione and glutamate metabolism); and methionine permease, which can mediate both methionine and cysteine uptake in *Saccharomyces cerevisiae* [30].

Using a different approach, Felipe et al. [31] analyzed the transcriptional profiles of *P. brasiliensis* to identify mycelial and yeast specific transcripts. They took advantage of 6,022 ESTs (an estimated 80% of the fungal genome) from *P. brasiliensis* and used cDNAs from each phase to probe micro-array membranes. They identified 328 genes that were differentially expressed during the phase transition; 58 in the mycelium and 270 in the yeast. These up-regulated genes fell into multiple categories relating to the cell cycle, stress response, drug resistance, and signal transduction pathways to name a few. The role of yeast- and mold-phase specific genes in establishing and maintaining these distinct morphological forms in *P. brasiliensis* and *H. capsulatum*, and in disease pathogenesis remains to be investigated.

Regulation of dimorphism and virulence in fungi

From the foregoing, it is well recognized that transition from the mold to yeast form is a signature feature of this class of agents, and is required for the expression of virulence genes and pathogenicity. A major question is the field has been: How do these fungi sense a change in temperature and regulate the phase transition.

Nemecek et al. [32] recently uncovered a long-sought regulator that controls the switch from a non-pathogenic mold form to a pathogenic yeast form in dimorphic fungi. They found that a hybrid histidine kinase (*DRK1*) functions as a global regulator of dimorphism and virulence in *B. dermatitidis* and *H. capsulatum. DRK1* is required for phase transition from mold to yeast, expression of virulence genes, and pathogenicity *in vivo*. Disruption of *DRK1* locks *B. dermatitidis* in the mold form at temperatures (37°C) that normally trigger phase transition to yeast. RNA silencing of *DRK1* expression in *B. dermatitidis* results in impaired *BAD1* expression, severe alterations in the cell wall, and reduction in transcription of α-(1,3)-glucan synthase and the yeast-phase specific gene *BYS1*. In *H. capsulatum*, *DRK1* also regulates expression of the yeast-phase specific genes *CBP1*, *AGS1* and *yps-3*. A *DRK1* homolog is present and highly conserved in *C. immitis*, but has not yet been studied functionally. Hence, the hybrid histidine kinase *DRK1* functions as a sensor of environmental change in the dimorphic fungi; it dictates their adaptation to environment stress inside mammalian hosts and their ability to cause disease.

Two-component signaling systems are widespread in prokaryotes where signal transduction occurs via phosphorelay reactions [33]. When stimulated, a sensor histidine kinase autophosphorylates a histidine residue and transfers this phosphate to an aspartate residue on a response regulator resulting in activation. The active response regulator controls transcription to elicit a cellular response from the histidine kinase. Eukaryote signal transduction has been thought to rely mainly on serine, theonine and tyrosine kinases, but histidine kinase two-component systems have been identified in lower eukaryotes and plants [34]. These pathways have recently been implicated in environmental sensing and cell development in eukaryotes [35], including in the opportunistic fungal pathogen *Candida albicans*, where they regulate filamentation and virulence [36–38].

The most well understood fungal two-component signaling system is the *SLN1* pathway of *S. cervisiae*. Sln1, a membrane bound hybrid-histidine kinase, is regulated by hyperosmotic stress [35]. Under normal osmotic conditions, Sln1 is autophosphorylated and transfers this phosphate to the phosphotransferase Ypd1 [39]. Ypd1 shuttles the phosphate from Sln1 to a response regulator, Ssk1, repressing its activity. When yeast are exposed to hyperosmotic stress, Sln1 is inactive resulting in an unphosphorylated, active Ssk1. The response regulator turns on the Hog1 mitogen-activated protein kinase (MAPK) pathway, which increases production of the osmolyte glycerol [40]. Homologues of the Sln1 pathway components have been identified in a variety of pathogenic fungi including *C. albicans*, *Aspergillus fumigatus, Cryptococcus neoformans, B. dermatitidis*, *H. capsulatum*, and *C. immitis* [32,36,38,41,42].

Two-component signaling systems in the pathogenic fungi have diverse regulons including cell wall biosynthesis, virulence factor expression, drug resistance, and morphogenesis [32,42]. Though signaling pathway components are often similar in fungi, system structures and mechanisms of activation may differ from species to species. The HOG pathway of *C. neoformans* is an example. Unlike the canonical HOG pathway of *S. cerevisiae*, *C. neoformans* Hog1 is phosphorylated under normal conditions and functions as a repressor. When *C. neoformans* is exposed to stress, Hog1 is unphosphorylated, resulting melanin and capsule synthesis [43].

Pathogenic fungi contain multiple two-component signaling pathways. *S. cerevisiae* contains only one sensor histidine kinase (Sln1) as compared to three in *C. albicans* and 11 in *N. crassa* [36,44]. Signaling pathways of multiple sensor kinases often overlap resulting in redundant responses to stimuli, which complicates their characterization. For example, the three sensor histidine kinases of *C. albicans* (Sln1, Chk1, and Nik1) have distinct and redundant responsibilities in morphogenesis, cell-wall biosynthesis, and virulence [36].

Though challenging to delineate, two-component signaling systems regulate dimorphism and virulence in multiple fungal pathogens. As described above, Drk1 in *B. dermatitidis* and *H. capsulatum* regulates the mold to yeast phase transition [32]. Deletion of *CHK1*, *SLN1*, or *NIK1* in *C. albicans* impairs the formation of hyphae [36]. OS-1 of *N. crassa* is required for normal hyphal development as well as conidiation [45]. Importantly, inactivation of *SLN1* homologues in fungal pathogens also attenuates their virulence. The involvement of two-component signaling systems in fungal sensing, morphogenesis and virulence, and their absence in humans, makes inhibitors of Sln1 homologues or its pathway components attractive drug targets [46].

Conclusions

It has long been known that morphogenesis from an environmental mold form to a pathogenic yeast form is essential for virulence in a family of ascomyetes that comprise the

systemic dimorphic fungi. This event is triggered by exposure to host conditions, particularly temperature, and leads to programs needed for adaptation to the host environment, including genes for survival and virulence. Over the years, some of these genes have been identified and studied, one-by-one, providing a glimpse of how they promote disease pathogenesis. New tools for genetically manipulating these fungi, the sequencing of their genomes, and microarray analyses have provided a much deeper understanding of the many genes and some of the regulators involved during this extreme makeover inside the host. The future promises exciting new developments and further understanding of the regulatory networks, the downstream targets, and the role of these host-specific genetic programs essential for survival and virulence in mammalian hosts.

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Table 1

Partial list of phase-specific factors that have been investigated in dimorphic fungal pathogens

Fungus	Gene	Expression Phase	Function	Reference
Blastomyces dermatitidis	*BAD1*	yeast	adhesion, phagocytosis, calcium binding, modulation of host response	[20,21,47,48]
	bys1	yeast	unknown	[49]
Histoplasma capsulatum	*CBP1*	yeast	calcium binding	[23]
	yps-3	yeast	unknown	[24]
	yps 21:E-9	yeast	unknown	[50]
	MS8	mold	unknown	[51]
Coccidioides Immitis	*SOWgp*	spherule	binds laminin and fibronectin	[26]
Sporothrix schenkii	**	mold	dextranase	[52]
Penicillium Marneffei	*cpeA*	yeast	catalase-peroxidase	[27]

** genetic locus not identified