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Thermally Dimorphic Human Fungal Pathogens—Polyphyletic Pathogens with a Convergent Pathogenicity Trait

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Fungi are adept at changing their cell shape and developmental program in response to signals in their surroundings. Here we focus on a group of evolutionarily related fungal pathogens of humans known as the thermally dimorphic fungi. These organisms grow in a hyphal form in the environment but shift their morphology drastically within a mammalian host. Temperature is one of the main host signals that initiates their conversion to the "host" form and is sufficient in the laboratory to trigger establishment of this host-adapted developmental program. Here we discuss the major human pathogens in this group, which are *Blastomyces dermatiditis*, *Coccidioides immitis*/*posadasii*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*/*lutzii*, *Sporothrix schenckii*, and *Talaromyces marneffei* (formerly known as *Penicillium marneffei*). The majority of these organisms are primary pathogens, with the ability to cause disease in healthy humans who encounter them in endemic areas.

Dimorphism is defined as the ability of a fungus to generate free-living vegetative cell types that are either yeast or hyphal (Fig. 1), although we discuss some exceptions to this precise definition below. The most parsimonious explanation for the origin of fungi begins with a unicellular eukaryotic cell from which multicellular filamentous forms evolved. Presumably, the first filamentous forms would have been locked into that mode of growth by simple mutations in the cell division machinery, and a regulated transition between the unicellular and multicellular vegetative forms (dimorphism) evolved over time. For the filamentous growth form, the ability to generate independent, unicellular forms has reemerged several times in the guise of modern developmental programs such as asexual and sexual reproduction, which produce differentiated, dormant spores.

Dimorphism in fungi is likely to have arisen independently a number of times. The polyphyletic nature of dimorphism is evident in the distribution of organisms with this capability across the fungal kingdom (see Fig. 2). Dimorphic fungi exist in the three major phyla of fungi: the Ascomycota, Basidiomycota, and Zygomycota. In the Ascomycota, they are distributed across several orders and are closely related to many nondimorphic fungi. The largest cluster of thermally dimorphic fungi includes *Histo-*

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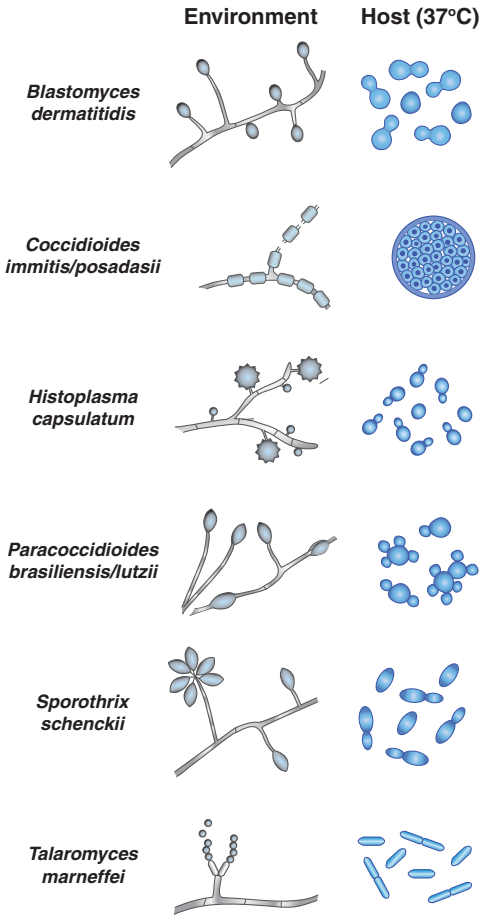

Figure 1. Simplified schematic of temperature-regulated forms of thermally dimorphic fungal pathogens. Simple cartoons of the environmental and host forms of each fungal species are shown. In the environmental hyphal form of these organisms, oval swellings depict vegetative conidia. In the case of *H. capsulatum*, tuberculate macroconidia and microconidia are shown. In the case of *Coccidioides* spp., disarticulating arthroconidia are shown. Some of the characteristic differences between yeast-phase growth are depicted: *Blastomyces dermatitidis* yeast cells have a broad bud neck, *Paracoccidioides* yeast can be multibudded, and *T. marneffei* yeast divides by fission rather than budding. Note that the relative scale of different cell types (within and between species) is not meant to be accurate. (Illustration provided by Davina Hocking Murray.)

plasma capsulatum, *Blastomyces dermatiditis*, *Coccidioides*, and *Paracoccidioides* species of the order Onygenales. Similarly, there are a number of dimorphic species in the Ophiostomatales order including the various *Sporothrix* species. In contrast, *Talaromyces marneffei* is the only known dimorphic species in the large order of Eurotiales and is, in fact, the only dimorphic fungus in which yeast cells divide by fission rather than budding. There are far fewer known dimorphic fungi in the other phyla with the best known basidiomycete being the maize smut *Ustilago maydis* and zygomycete animal pathogens represented by various *Mucor* species. Indeed, many of the dimorphic fungi are pathogens of animals or plants.

As mentioned above, dimorphism in its strictest sense involves the ability of a fungus to generate two types of vegetative cells—those that are either yeast or hyphal in morphology. *Coccidioides* species do not precisely fit this definition because they do not produce free-living

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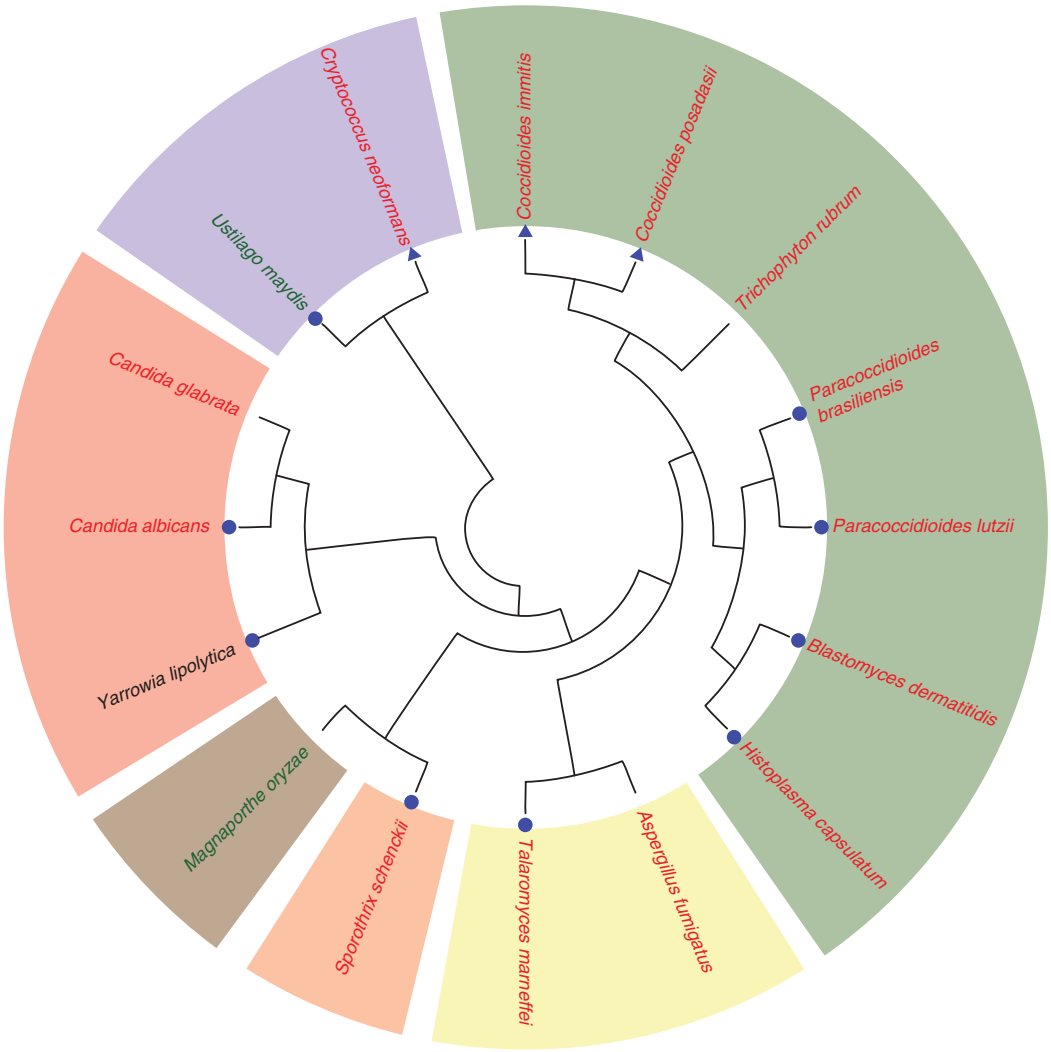

Figure 2. Molecular phylogenetic analysis of dimorphic fungal pathogens. The evolutionary tree is based on a protein comparison from the major dimorphic human pathogens and includes a number of other fungal pathogens for reference. Human pathogens (red typeface), plant pathogens (green typeface), and nonpathogens (black typeface) are shown and marked as true dimorphic species with free-living vegetative cell types (blue circle) and species with morphological transitions that are not free-living vegetative cell types (blue triangle). With the exception of *C. neoformans* and *U. maydis*, which are in the phylum *Basidiomycota*, all of the other species are in the phylum *Ascomycota*. These species cover a diverse range of orders (*Onygenales*, green; *Eurotiales*, yellow; *Ophiostomales*, orange; Magnaporthales, brown; and *Saccharomycetales*, red). To generate the phylogenetic relationships between organisms, the Pfam domain Gcd10p was used to identify Gcd10 sequences from the 16 species of interest. CLUSTALX 2.1 (Larkin et al. 2007) was used to align the full-length protein sequences and generate a bootstrapped neighbor-joining tree (1000 bootstraps; all internal nodes had a bootstrap value of at least 700). (Mark Voorhies contributed to this analysis.)

vegetative yeast cells but rather spherules that encompass numerous single cells known as endospores (Fig. 1). A number of other fungi exhibit aspects of dimorphism in their growth and morphogenesis patterns. A well-characterized example is pseudohyphal growth in the ascomycete yeast *Saccharomyces cerevisiae*; when diploid *S. cerevisiae* cells experience nitrogen limitation, they undergo a morphological transition to an elongated cell shape, with unipolar budding and incomplete separation of mother and daughter cells. This leads to hyphal-like filaments and is a program exhibited by a number of yeasts including the human pathogen *Candida albicans*, which can form pseudohyphae as well as true hyphae. The basidiomycete yeast *Cryptococcus neoformans* is also capable of forming hyphal filaments as part of a mating program.

As described below, thermally dimorphic fungal pathogens are endemic worldwide. Nonetheless, our understanding of the environmental niche and epidemiology of these dimorphic pathogens is far from complete. Because these organisms replicate in the absence of a mammalian host, the environmental pressures that have selected for host-specific traits is unclear. All of these fungi show robust, albeit sometimes slow, prototrophic growth in the hyphal form in the environment. These hyphae produce asexual spores (conidia) that often constitute the most common infectious propagule for a given organism. Conidiating hyphae are thought to exist in a mix of elusive nonhost environments and growth on dead hosts. Interestingly, it has been proposed that eukaryotic predators such as amoebae and slime molds could provide a selective pressure for virulence traits that are required to survive the antimicrobial onslaught from mammalian phagocytic cells (Steenbergen et al. 2004).

Blastomyces dermatitidis

The first known case report of infection with *B. dermatitidis* occurred in 1894 when T.C. Gilchrist reported a patient with a skin disease supposedly caused by a protozoan infection. However, Gilchrist and colleagues soon noticed a budding yeast form in tissues and cultured a hyphal organism from patient samples that they named *B. dermatitidis* (Gilchrist 1894; Gilchrist and Stokes 1896, 1898). Following Gilchrist's discovery, it took at least an additional 50 years to understand that the cutaneous manifestation of disease was associated with pulmonary involvement, suggesting that all cases of skin involvement resulted from dissemination from the lungs. It is now clear that blastomycosis is primarily a lung infection that can disseminate to the skin, osteoarticular structures, genitourinary tract, and other organs (Smith and Kauffman 2010).

B. dermatitidis is challenging to isolate from the environment, making the epidemiology of blastomycosis less well defined than histoplasmosis or coccidiomycosis. Based mainly on case reports, *B. dermatitidis* is known to be endemic in the Mississippi and Ohio River Valleys, the Midwestern states, the Canadian provinces that border the Great Lakes, and the area of New York and Canada adjacent to the St. Lawrence Seaway (Klein et al. 1986, 1987; Crampton et al. 2002; Cano et al. 2003; Dworkin et al. 2005). Additionally, sporadic cases of blastomycosis have been reported from around the world (De Groote et al. 2000; Arnett et al. 2008; Smith and Kauffman 2010). The majority of cases are asymptomatic, but individuals who are either exposed to a large dose of infectious particles or have a defect in cell-mediated immunity develop a more severe, progressive disease (Maresca and Kobayashi 2000; Smith and Kauffman 2010). Acute respiratory distress syndrome can develop in rare cases (Smith and Kauffman 2010), but chronic skin and bone lesions are the most common extrapulmonary complications. The incidence of infection in males is higher than females. Additionally, dogs in endemic regions are susceptible to blastomycosis, making it an important veterinary problem (Baumgardner et al. 1995).

B. dermatitidis undergoes temperature-dependent morphogenesis, growing in a hyphal form in the laboratory at temperatures below 30°C and in a yeast form at 37°C (Fig. 1) (Maresca and Kobayashi 2000; Nemecek et al. 2006). Like the other thermally dimorphic fungi, its morphology is reversible by changing the tem-

perature of its environment. Infection occurs when conidia or hyphal fragments are inhaled, and conversion to the yeast form occurs within the host. In contrast to *H. capsulatum*, which is found intracellularly within phagocytes during infection, *B. dermatitidis* grows as extracellular yeasts in microabcesses (Maresca and Kobayashi 2000; Smith and Kauffman 2010). *B. dermatitidis* yeasts tend to have a broad neck between the mother and bud (Fig. 1), and the yeast cells are usually, but not always, larger than those of *H. capsulatum*.

A number of fundamental tools have made it possible to identify virulence factors and explore basic processes in *B. dermatitidis*, including identification of the immunomodulatory adhesin Bad1 as well as factors that regulate morphogenesis and iron homeostasis (Brandhorst et al. 2002; Sullivan et al. 2002; Krajaejun et al. 2007; Gauthier et al. 2010). A huge advance to understanding temperature-regulated morphogenesis was the identification of a hybrid histidine kinase that is required for yeast-phase growth and virulence gene induction in response to temperature in both *B. dermatitidis* and *H. capsulatum* (Nemecek et al. 2006). Additionally, seminal studies of the host response to *Blastomyces* and other thermally dimorphic fungi have yielded rich insight into the immunobiology of fungal infections (Wuthrich et al. 2011, 2012; Wang et al. 2014).

Coccidioides immitis/posadasii

In 1891, a medical student in Argentina named Alejandro Posadas saw a patient with an unusual skin lesion. Posadas and Robert Wernicke noted a coccidia-like "parasite" in the lesion (Posadas 1892; Wernicke 1892). The first North American case was reported in 1894 (Rixford 1894). Over the next 20 years, both the dimorphism of the organism (Ophuls and Moffitt 1900) and its prevalence in California (Dickson 1915) became obvious.

Coccidioides is a New World pathogen. The genus *Coccidioides* was recently resolved into two species (Fisher et al. 2002): *immitis*, endemic in central and southern California and Northern Mexico; and *posadasii*, found throughout Arizona, Texas, Mexico, and parts of South America (Chiller et al. 2003; Galgiani et al. 2005; Pfaller and Diekema 2010; Marsden-Haug et al. 2013). *Coccidioides* is associated with small mammals in the environment (Ashburn and Emmons 1942; Emmons 1962), although the precise nature of that association and its role in the ecology of the organism is somewhat controversial.

The disease caused by *Coccidioides* species, coccidioidomycosis, is also known as San Joaquin Valley fever, or simply Valley fever, because of its prevalence in the Central Valley of California. Notably, *Coccidioides* infections are on the rise in endemic areas, with >90% increase in incidence in Arizona and California between 2001 and 2006. The resultant public health impact is enormous (Hector et al. 2011).

Coccidioides spores (arthroconidia) are produced from alternating cells of hyphae (Fig. 1). As the hypha differentiates, individual cells separate from each other, and alternating cells become 3–5 μm barrel-shaped arthroconidia while the remaining cells undergo autolysis. Once inhaled into a mammalian host, arthroconidia undergo a dramatic transformation, ultimately producing a structure that is unique to the genus *Coccidioides* (Fig. 1). Arthroconidia undergo isotropic growth, expanding in size and modifying the cell wall to produce a spherule, a cellular structure up to 100 μm in diameter. The spherule contains 100 to 300 single-celled units known as endospores. Rupture of the spherule in host tissues releases these endospores, each of which is capable of developing into a new spherule (Nguyen et al. 2013). The spherule itself is refractory to phagocytosis. Spherule formation can be induced in vitro by culturing the fungus in liquid modified Converse medium at 37°C–40°C (Converse 1955; Sun et al. 1976).

As is the case for the majority of fungi discussed in this article, the predominant route of infection with *Coccidioides* spp. is inhalation. The majority of cases are asymptomatic, but some patients develop a complicated pneumonia. Disseminated coccidioidomycosis refers to disease outside of the chest, most commonly to sites such as the skin, joints, bones, and menin-

ges. Less common is dissemination to the larynx, abdomen, adnexa, and pericardium. African American individuals have a greater risk of developing disseminated coccidioidomycosis than other ethnic groups, suggesting that a genetic component of the host influences the severity of disease outcome (Pappagianis et al. 1979; Pappagianis 1988; Kirkland and Fierer 1996; Nguyen et al. 2013). Similar to the other thermally dimorphic fungal pathogens, individuals with defective cell-mediated immunity are more susceptible to severe disease.

A robust molecular toolbox exists for *Coccidioides* spp. gene disruption, *Agrobacterium*-mediated transformation, and transcriptional profiling have defined a number of factors critical for pathogenesis, such as the immunomodulatory spherule outer wall glycoprotein (SOWgp) and a urease (URE) that is thought to contribute to host tissue damage (Abuodeh et al. 2000; Hung et al. 2007, 2012). Gene expression studies of *Coccidioides* hyphae and spherules are defining the transcriptional program of the distinct morphological states of this organism (Johannesson et al. 2006; Whiston et al. 2012; Viriyakosol et al. 2013), which will provide critical clues as to the molecular program that is initiated in response to host signals such as temperature. Additionally, population genomics studies are being used to great effect to define genes that may underlie the virulence of individual *Coccidioides* isolates (Sharpton et al. 2009; Neafsey et al. 2010).

Histoplasma capsulatum

Histoplasmosis was first described in 1906 by Samuel Darling, an American physician working at the Ancon Canal Zone Hospital in Panama (Darling 1906). During an autopsy of a patient from Martinique who died from a devastating infection, Darling found intracellular organisms that appeared to have capsules. He misidentified this organism as a new protozoan parasite and named it *H. capsulatum* owing to its residence within "histiocytes," or macrophages. Over the next few decades, it became obvious that *H. capsulatum* is actually an environmental fungus with no capsule. The organism was first isolated from soil by Chester Emmons in 1948 (Emmons 1949).

Histoplasmosis in humans is caused by two distinct varieties of *H. capsulatum* that manifest in very different clinical outcomes: *H. capsulatum* variety *capsulatum* and *H. capsulatum* variety *duboisii* (Kauffman 2007). *H. capsulatum* (*Hc*) var. *capsulatum* is found globally (mainly North, South, and Central America, Southeast Asia, and Africa) and is associated with pulmonary and systemic (i.e., classical) histoplasmosis. In contrast, *Hc* var. *duboisii* is predominantly found in Western and Central Africa. Because *Hc* var. *duboisii* causes skin and bone lesions, it was initially distinguished from variety *capsulatum* on the basis of disease symptoms (Cockshott and Lucas 1964). The disease triggered by variety *Hc* var. *duboisii* is given the name African histoplasmosis, and little is known about this variety on a molecular level. Both variety *capsulatum* and *duboisii* strains have been sequenced by the Broad Institute and the Washington University St. Louis Genome Sequencing center, which will shed light on fundamental molecular differences between *H. capsulatum* isolates. Additionally, phylogenetic analyses based on genome sequence variation of four protein-coding genes were performed on 137 *Histoplasma* isolates from six continents (Kasuga et al. 2003). These isolates fell into eight clades, seven of which represent distinct phylogenetic species. Although all *Hc* var. *duboisii* isolates were represented in the African clade, this clade also included variety *Hc* var. *capsulatum* individuals. In addition to these two varieties, a third variety, *Hc* var. *farciminosum*, which is a pathogen of horses, was found distributed within three clades. More molecular work is necessary to understand the biological differences between these varieties and their relationship to disease.

From here on, we focus on classical histoplasmosis caused by *Hc* var. *capsulatum*, referred to as *H. capsulatum* for simplicity. Although the organism is found all over the world, it tends to cause the highest disease burden in Central and North America. Along with *B. dermatitidis* and *Coccidioides* spp., *H. capsulatum* is thought to be the most common cause

of fungal pulmonary infections in immunocompetent individuals. A study of U.S. hospitalizations for endemic mycoses reveals that these fungi cause significant morbidity and mortality in healthy hosts in endemic regions (Chu et al. 2006), with approximately 25,000 estimated life-threatening infections per year in the Midwestern United States caused by *H. capsulatum* infection (Brown et al. 2012). In the United States, *H. capsulatum* is endemic in the Ohio and Mississippi River Valleys. The organism is found in soil containing large amounts of bird or bat guano, and bats have been proposed as a vector of spread, both because the fungus grows well in soil contaminated with bat guano, and because bats themselves can be colonized with *H. capsulatum* (Hoff and Bigler 1981). The vast majority of human infections are asymptomatic, but acute severe pulmonary infection occurs in either an immunosuppressed host or an immunocompetent host who inhales a large inoculum of *H. capsulatum* cells. Outbreaks of anywhere from a few to tens of thousands of people have occurred (Brodsky et al. 1973; Wheat et al. 1981; Wheat 1997).

The hyphal form of *H. capsulatum* undergoes asexual sporulation to give rise to at least two types of conidia, macro- and microconidia (Fig. 1), which are distinguished mainly on the basis of size (Pine 1960). Microconidia range in size from 2 to 6 μm, whereas macroconidia have been reported to range in size from 8 to 14 μm to 10–25 μm, depending on the strain and growth conditions. It is thought that the microconidia are the appropriate size to lodge in the alveoli of the lungs and thus represent the most prevalent infectious propagule. Conidia and/or hyphal fragments are inhaled by the host and then taken up by macrophages and other phagocytic cells (Eissenberg and Goldman 1991; Bullock 1993; Woods 2003). Once inside the host, both spores and filaments give rise to yeast cells, which evade phagocytic killing and multiply within alveolar macrophages. Subsequently, yeast cells use phagocytic cells as vehicles to spread to multiple organs of the reticuloendothelial system such as the spleen, liver, lymph nodes, and bone marrow. Hematogenous dissemination from the lungs via infected macrophages is thought to occur even in asymptomatic infections (Kauffman 2007). In patients with disseminated disease, other organs such as the skin, heart, brain, adrenal glands, and gastrointestinal tract can be colonized. In the majority of hosts, cell-mediated immunity and the corresponding activation of macrophages serve to curtail the infection (Eissenberg and Goldman 1991; Newman 1999; Deepe 2000; Huffnagle and Deepe 2003). Nonetheless, *H. capsulatum* can remain latent and reactivate years after the original infection (Kauffman 2007). Patients at particular risk for disseminated histoplasmosis include those individuals with deficient cell-mediated immunity, including infants, patients with AIDS, transplant recipients, those with hematologic malignancies, and patients undergoing treatment with corticosteroids or tumor necrosis factor antagonists (Kauffman 2007; Smith and Kauffman 2009).

The transformation of *H. capsulatum* hyphal cells to yeast cells, or vice versa, can be recapitulated in culture simply by shifting the growth temperature (Maresca and Kobayashi 1989; Maresca et al. 1994). When *H. capsulatum* cells are grown at room temperature, they grow in the hyphal form. When these cells are shifted to 37°C, they shift to the budding yeast form. A number of biological assays have been applied to yeast and hyphal cells grown in the laboratory. The first *H. capsulatum* virulence factors (Cbp1 [calcium-binding protein 1] and Yps3 [yeast-phase specific gene 3]) were identified by searching for high-abundance secreted factors that were produced only by yeast cells (Keath and Abidi 1994; Batanghari and Goldman 1997; Batanghari et al. 1998; Patel et al. 1998; Kugler et al. 2000a,b; Sebghati et al. 2000; Bohse and Woods 2007). Building on a robust extrachromosomal plasmid system (Woods and Goldman 1992, 1993) and electroporation technologies (Woods et al. 1998a), a combination of gene disruption (which is inefficient in *H. capsulatum* due, at least in part, to the high frequency of illegitimate recombination) (Woods et al. 1998b; Sebghati et al. 2000), *Agrobacterium*-mediated insertional mutagenesis (Sullivan et al. 2002; Youseff et al. 2009) and RNA interference (Rappleye et al. 2004) have been used to identify *H. capsulatum*

factors involved in iron acquisition and homeostasis (Hilty et al. 2008, 2011; Hwang et al. 2008, 2012), intracellular parasitism (Sebghati et al. 2000; Edwards et al. 2011b; Isaac et al. 2013), cell wall α-glucan synthesis (Rappleye et al. 2004; Marion et al. 2006; Edwards et al. 2011a), vitamin acquisition (Garfoot et al. 2014), and superoxide detoxification (Youseff et al. 2012; Holbrook et al. 2013). In addition to these molecular genetic approaches, a number of genomics and proteomics approaches have been used to determine genes whose expression is enriched in yeast or hyphae (Hwang et al. 2003; Nguyen and Sil 2008; Beyhan et al. 2013; Edwards et al. 2013), and the *H. capsulatum* secreted proteome (Albuquerque et al. 2008; Holbrook et al. 2011), which is presumed to include novel virulence factors. Finally, key regulatory molecules required for the temperature-dependent switch from the hyphal to yeast forms have been identified (Nemecek et al. 2006; Nguyen and Sil 2008; Webster and Sil 2008; Beyhan et al. 2013). These factors will shed light on the temperature-sensing mechanism that is key to the dimorphic switch in these organisms.

Paracoccidioides brasiliensis

P. brasiliensis is the causative agent of paracoccidioidomycosis, a disease first described in 1908 (see Goldani and Sugar 1995). It is the most common systemic mycosis in Latin America with an estimated number of infected individuals close to 10 million (Bethlem et al. 1991; Brummer et al. 1993). *P. brasiliensis* as a species was shown to consist of a number of distinct phylogenetic lineages that have recently been resolved into two species, *P. brasiliensis* and *P. lutzii* (Matute et al. 2006a,b). *Paracoccidioides* is considered a primary pathogen, infecting individuals deemed to be otherwise healthy. However, infections are most often asymptomatic, and it is believed that the fungus remains dormant in many of these instances. Disease progression is likely to be associated with some form of immunodeficiency in the host (Franco et al. 1987). A perplexing aspect of paracoccidioidomycosis is the apparent low prevalence with AIDS despite the high incidence of coexistence with HIV infection in some areas. Part of the explanation for this may relate to common prophylactic use of trimethprim-sulfamethoxazole against *Pneumocystis carinii*; this drug is also effective against *P. brasiliensis*. The other possibility, common to many fungal infections, is poor detection or misdiagnosis coupled with the fact that autopsies are not routinely conducted on AIDS fatalities (Goldani and Sugar 1995). Paracoccidioidomycosis is generally described as a chronic granulomatous inflammation but individual patients can exhibit a range of clinical manifestations ranging from a localized, benign disease to a disseminated, systemic mycosis. Disseminated disease is often fatal unless treated (Franco et al. 1987).

P. brasiliensis is endemic and restricted to South and Central America. The main environmental reservoir identified to date is in armadillos endemic to the region, but it has also been detected in a number of other mammals (Richini-Pereira et al. 2008). The high association between *Paracoccidioides* and armadillos (*Dasypus septemcinctus*), whose body temperature is 32°C–35°C, have a purported weak immune system and are burrowing animals, may underlie the evolution of pathogenicity in *Paracoccidioides*. The fungus has also been sporadically isolated from a number of other sources including bat guano and penguins (reviewed in Bagagli et al. 2008). Growth tests under various nonhost conditions show a clear preference for certain soils with high water content.

The dimorphic switch in *P. brasiliensis* is also triggered by temperature. At 25°C, elongated hyphal cells are produced that grow apically and divide by septation. Subapical branches produce a mycelial network. Within this network of hyphal cells, asexual conidia are produced without the elaboration of a complex conidiophore structure (see Goldani and Sugar 1995). In addition, arthroconidiation has also been observed (Fig. 1) (Bagagli et al. 2008). At 37°C, the yeast cells divide by budding but complete separation of mother and daughter cells is slow. This leads to a typical multibudded arrangement of cells (Goldani and Sugar 1995). It has been suggested that this large clump of cells is refractory to phagocytosis, and this is

consistent with the observation that *P. brasiliensis* cells in the host are not routinely intracellular. However, it is also clear that inhaled conidia are likely to be phagocytosed by alveolar macrophages before they are able to germinate and produce these multibudded yeast forms. The transition from hyphal to yeast cells in vitro is slow, taking 10–20 d to fully manifest. Although a teleomorphic stage has not been described, there is suggestive evidence of a sexual cycle in some isolates (based on recombination), whereas others appear asexual (Silva et al. 2008).

Paracoccidioides infections are presumed to be initiated by inhalation of either conidia or arthroconidia and primarily occur in the lung. Some observations of *Paracoccidioides* inside host cells (epithelial and alveolar) have been noted, and it is thought that initial survival of the fungus within macrophages may be important for virulence (Tavares et al. 2007). Nonetheless, *Paracoccidioides* is not predominantly an intracellular pathogen; presumably, as mentioned above, the multibudded yeast form is too large to be readily phagocytosed by innate immune cells (Mendes-Giannini et al. 2008). As an extracellular pathogen, adherence to host cells is important. Infections can be asymptomatic or symptomatic, and in the former case the disease can become evident many years after the initial exposure, indicative a period of latency. Beyond the primary pulmonary disease, disseminated disease can also manifest, affecting the reticuloendothelial system, lymph nodes, skin, and mucosa (Goldani and Sugar 1995).

Molecular genetic tools for the manipulation of *P. brasiliensis* have been developed. The DNA-mediated transformation system is based on the *Agrobacterium* T-DNA transformation system developed for *H. capsulatum* using a dominant selectable marker for resistance to hygromycin (Almeida et al. 2007). The system has been coupled with antisense RNA constructs to knock down gene expression. Gene targeting has not been developed yet. Fluorescent proteins genes have been show to work in *P. brasiliensis* (Almeida et al. 2009). There are robust cell-based and mouse models for assessing virulence (Defaveri et al. 1982; Kerr et al. 1982; Robledo et al. 1982). Transcriptional profiling using microarrays has been used extensively (Nunes et al. 2005; Andrade et al. 2006; Ferreira et al. 2006; Tavares et al. 2007; Monteiro et al. 2009).

Sporothrix schenckii

S. schenckii is a fungus with worldwide distribution, unlike many other dimorphic pathogens and more akin to the better known but nondimorphic *Aspergillus fumigatus. S. schenckii* is associated with soil and plants. Unlike the other dimorphic pathogens, *S. schenckii* predominantly causes cutaneous infections that initiate by minor skin trauma such as cuts and scrapes that come from handling infected material. It is often known as rose-grower's disease. Sporotrichosis was first reported in 1896 when the fungus was identified in subcutaneous abscesses (Schenck 1898). A particularly notorious outbreak of sporotrichosis occurred in South Africa where 3000 gold miners were infected by contaminated timber (cited in Lopez-Romero et al. 2011). The disease is often manifested as a chronic granulomatous infection. Like *Paracoccidioides*, *S. schenckii* is now considered a species complex that is comprised of six species, all of which are of medical interest, and, unless otherwise stated, a reference to *S. schenckii* is to the species complex (Lopez-Romero et al. 2011).

Despite its global distribution, certain regions like Peru appear to show much higher incidences of infection by *S. schenckii* (sometimes termed "hyperendemic"), probably as a consequence of higher frequencies of activities that contribute to infection (Pappas et al. 2000). Contact is also thought to be the reason why men show a much higher frequency of infection by *S. schenckii*. It is present in decaying (or not) plant material. A number of factors contribute to the development of sporotrichosis including the inoculum size and location, the host's immune state, and the virulence of the strain (Dixon et al. 1991). Generally infections are restricted to acute or chronic subcutaneous lesions but serious disseminated disease is evident with increasing numbers of immunocompromised individuals, particularly AIDS (Lopez-Romero

et al. 2011). Infection via animals such as cats has also been documented (Barros et al. 2004).

Sporothrix differs from the other dimorphic fungi in that hyphal and yeast cells can coexist, and temperature does not appear to be a strict cue for transition between the two cell types. The hyphal cells grow by apical extension, divide by septation, and produce a number of morphologically different asexual conidia. Some conidia are oval or elongate in shape that can be produced individually or in small groups in a sympoidal manner from specialized conidiogenous cells. The other conidial cell type has a thick wall, is darkly pigmented, and is generally produced individually. This conidial type differs in shape among the *Sporothrix* species. *Sporothrix* yeast cells are oval in shape and divide by budding. Cell separation can occur with each division or it can be delayed to produce multibudded cells. These cells can be produced over a range of temperatures (25°C–37°C) and generally emerge from hyphal cells at their apical tips or septation sites. There is no known sexual stage (Lopez-Romero et al. 2011).

For *S. schenckii*, unlike all the other dimorphic pathogens discussed here, the primary route of infection is not by inhalation of spores (although there is evidence of this possibility) but instead by the hyphal form on inoculation through superficial wounding. Infections are usually localized to the skin resulting in an ulcerated nodule and sometimes spread to the lymphatic system. In immunocompromised individuals such as those with AIDS, serious disseminated disease is common and can affect many parts of the body including the bones, joints, and the central nervous system (Lopez-Romero et al. 2011).

There is a paucity of molecular genetic tools available for *Sporothrix*. A DNA-mediated transformation system utilizing *Agrobacterium* T-DNA-mediated insertion was developed based on that described for *T. marneffei* and *Aspergillus awamori* and was used for an insertional mutagenesis screen (Zhang et al. 2011). Gene targeting has not been developed yet. Pulse-field gel electrophoresis has been used to examine chromosomal polymorphism in isolates (Sasaki et al. 2014). There are robust cell-based and mouse models for assessing virulence (Hachisuka and Sasai 1981; Kennedy et al. 1982; Dickerson et al. 1983).

Talaromyces marneffei

Penicillium marneffei, recently renamed *T. marneffei* on the basis of new sequence data that has seen a division of the classically defined Penicillium group of fungi (Samson et al. 2011), was originally identified in 1956 from a bamboo rat in Vietnam (Segretain 1959). Despite its coexistence with rats in the endemic region, it has rarely been isolated from the environment, suggesting that the reservoir may in fact be the rat. The first documented human infection was by an accidental needle stick of a researcher (DiSalvo et al. 1973). It has risen to prominence as a significant human pathogen across Southeast Asia with the global AIDS pandemic (Vanittanakom et al. 2006). The disease penicilliosis marneffei occurs predominantly in immunocompromised individuals and initiates as a pulmonary infection followed by hematogenous dissemination to a systemic mycosis (Cheng et al. 1998; Rimek et al. 1999; Garbino et al. 2001). As such, it is considered an opportunistic pathogen; however, systemic disease is fatal if untreated. A small number of cases of infection in "immunocompetent" individuals have been described, but it should be noted that in none of these cases has immune status been adequately tested, and "immunocompetency" is often used interchangeably (and incorrectly) with HIV-negative status. The British novelist and travel writer Bruce Chatwin (1963–1989) was diagnosed with penicilliosis marneffei (Shakespeare and Neely 2011).

T. marneffei is endemic to Southeast Asia and as a consequence of its escalating incidence represents an "AIDS-defining pathogen" in this region (Supparatpinyo et al. 1994; Ustianowski et al. 2008). The increasing frequency of travel to Asia and the capacity of *T. marneffei* to exist without a host has seen a spread of incidents across Asia and confirmed cases in Europe, North America, Africa, and Australia have been documented (Vanittanakom et al. 2006). Despite its prototrophy and ability to grow on a

wide range of nutrient sources, suggestive of a saprophytic lifestyle in the hyphal form, *T. marneffei* is almost always associated with bamboo rats in the environment (for review, see Vanittanakom et al. 2006). Part of the reason for this may lie in the fact that it is relatively slow growing and may not compete well with other microbes in the soil (Vanittanakom et al. 1995; Joshi et al. 2003). The bamboo rat as an environmental niche may have contributed to the evolution of its pathogenic potential and it is unclear whether the yeast growth form is found outside mammalian hosts. Population genetic studies of isolates from bamboo rats and humans across Asia suggest a highly clonal structure but with clear signs of recombination. These populations appear spatially restricted and overlap with characterized bamboo rat distributions. Coupled with data from experiments in mice, it appears that recombination may be occurring within hosts, and, if this is a requirement, it may represent population barriers for *T. marneffei* (Henk et al. 2012). There is no evidence that *T. marneffei* infections in humans are derived zoonotically and it is generally accepted that, like many other fungi, infection is initiated by inhalation of dormant conidia. In support of this are data linking infections with agricultural occupation and increased incidence during the rainy season, at least in Northern Thailand (Chariyalertsak et al. 1996).

Like the other important dimorphic fungal pathogens, *T. marneffei* exhibits dimorphic growth producing two distinct cellular forms, unicellular yeast and multicellular hyphae, under specific environmental conditions (Andrianopoulos 2002). *T. marneffei* is the only known *Talaromyces* (*Penicillium*) species that is dimorphic or a human pathogen, suggesting that these are linked emergent traits. The switch between growth forms is regulated by temperature (Andrianopoulos 2002). At 25°C, *T. marneffei* grows as multinucleate, septate, branched hyphae. These hyphae produce conidia, the infectious agent, from specialized multicellular structures termed conidiophores. When switched to 37°C, *T. marneffei* undergoes a morphogenetic process termed arthroconidiation. Cellular and nuclear division become coupled, double septa are laid down, and hyphae fragment at these septation sites to liberate uninucleate yeast cells that divide by fission (Andrianopoulos 2002). The yeast cells are the pathogenic form and are found in the pulmonary alveolar macrophages and peripheral blood mononuclear cells of infected individuals (Vanittanakom et al. 2006). In contrast to the hyphal-yeast dimorphic transition in vitro, conidia that are phagocytosed by macrophages germinate directly into yeast cells, bypassing the arthroconidiation process. Intracellular yeast cells are morphologically similar to those of other intracellular fungal pathogens such as *H. capsulatum* but can be distinguished at division because *T. marneffei* yeast cells divide by fission (Ignatov and Keath 2002).

T. marneffei infection is likely to occur through inhalation of the conidia, which are phagocytosed by host pulmonary alveolar macrophages (Vanittanakom et al. 2006). How *T. marneffei* is able to survive in the stressful intracellular environment of the phagocytic macrophage is not completely clear. Conidia phagocytosed by alveolar macrophages germinate inside these host cells into the pathogenic yeast form and proliferate. This leads to a pulmonary infection that disseminates haemotogenously to the lymphatic system, liver, spleen, and bones (Kudeken et al. 1996). Skin lesions are often evident occurring mostly on the face and neck. Untreated disseminated disease is often fatal. Experiments in mice have shown that, in the absence of immunosuppression, infections with high numbers of conidia (4×10^5) are eventually cleared, whereas athymic mice die from the infection (Kudeken et al. 1997). This shows that immune status of the host is a key factor in the outcome of infection.

An extensive set of molecular genetic tools has been developed for *T. marneffei* using the type strain (FRR2161/ATCC18224). These tools have been used to probe the molecular mechanisms that control the dimorphic switch and allow the fungus to survive within the host. These include (1) a very high frequency DNA-mediated transformation procedure using PEG-based protoplast fusions that results in integration of exogenous DNA (Borneman et al. 2001), (2) a collection of nutritional and

dominant selectable markers with matching recipient strains for transformation (Boyce et al. 2012), (3) enhanced strains in which DNA integration is strictly by homologous recombination using mutants in the nonhomologous end-joining (NHEJ) system (Bugeja et al. 2012), (4) strains expressing fluorescent proteins for live-cell tracking (Boyce et al. 2012), (5) antisense RNA knockdown systems (Canovas et al. 2011), (6) gateway systems for rapid construct generation (Boyce et al. 2012; Bugeja et al. 2012), (7) promoters for controlled expression of genes (Borneman et al. 2000; Boyce et al. 2001), (8) genomic and transcriptomic technologies (Pasricha et al. 2013), and (9) cell culture, zebrafish, and mouse-based systems for virulence testing (Boyce and Andrianopoulos 2007; Ellett et al. 2011; Henk et al. 2012). A procedure for DNA-mediated transformation using the *Agrobacterium* transfer-DNA system has also been described (Kummasook et al. 2010).

CONCLUSION

The thermally dimorphic fungi are a captivating group of organisms that use a myriad of strategies to manipulate the progression of disease in the host. The evolutionary link between dimorphism and virulence is a subject of great interest for microbiologists, and the advent of robust molecular approaches and next-generation sequencing make it a propitious time to explore the pathogenesis and basic biology of these fascinating organisms.

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