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Cryptococcus neoformans and *Cryptococcus gattii*, the Etiologic Agents of Cryptococcosis

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Cryptococcus neoformans and *Cryptococcus gattii* are the two etiologic agents of cryptococcosis. They belong to the phylum Basidiomycota and can be readily distinguished from other pathogenic yeasts such as *Candida* by the presence of a polysaccharide capsule, formation of melanin, and urease activity, which all function as virulence determinants. Infection proceeds via inhalation and subsequent dissemination to the central nervous system to cause meningoencephalitis. The most common risk for cryptococcosis caused by *C. neoformans* is AIDS, whereas infections caused by *C. gattii* are more often reported in immunocompetent patients with undefined risk than in the immunocompromised. There have been many chapters, reviews, and books written on *C. neoformans*. The topics we focus on in this article include species description, pathogenesis, life cycle, capsule, and stress response, which serve to highlight the specializations in virulence that have occurred in this unique encapsulated melanin-forming yeast that causes global deaths estimated at more than 600,000 annually.

HISTORY OF THE ETIOLOGIC AGENTS AND CRYPTOCOCCOSIS

Cryptococcus neoformans was first isolated from peach juice by Sanfelice in 1894 in Italy and was named *Saccharomyces neoformans* (Sanfelice 1894). In the same year, Busse provided the first description of a case of cryptococcosis (Busse 1894) and isolated a yeast culture from a sarcoma-like lesion in the infected young woman's tibia. Busse called the fungus *Saccharomyces*, while naming the disease *Sac-*

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charomycosis hominis (Busse 1895). Around the same time, Curtis studied a yeast-like fungus isolated from a tumor in a patient's hip, and, noting its difference from the cultures of both Busse and Sanfelice, described the fungus as *Saccharomyces subcutaneous tumefaciens* n. sp. (Curtis 1986). Curtis' strain was later determined to be the first clinical isolate of *C. gattii* (Kwon-Chung et al. 2002). Because the Busse and Sanfelice's strains lacked the sugar fermentation and ascospore formation that are the hallmarks of the genus *Saccharomyces*, Vuillemin reclassified the yeasts as *Cryptococcus hominis* and *C. neoformans*, respectively, in 1901 (Vuillemin 1901). The characteristic neurotropism of *C. neoformans* was first recognized in 1914 by Versé (1914), and 2 years later by Stoddard and Cutler (1916). However, Stoddard and Cutler called the etiologic agent *Torula histolytica* and the disease "torulosis" by misinterpreting the fungal capsule as evidence of fungal histolytic action in the host tissue (Kwon-Chung and Bennett 1992). Confusion about the identity of the cryptococcosis agent persisted until Benham performed comprehensive studies with clinical *Cryptococcus* strains and concluded that all of the strains from human infections belonged to one species with two varieties based on serological differences (Benham 1935, 1950). She proposed to replace "torulosis/torula meningitis" with cryptococcosis and to conserve the fungal name *C. neoformans* (Benham 1950). Cryptococcal antigenic heterogeneity was confirmed in 1950 by Evans who identified three serotypes: A, B, and C (Evans 1950). A fourth serotype, D, was discovered in 1968 (Wilson et al. 1968).

The environmental source of *C. neoformans* was unknown until Emmons isolated *C. neoformans* from soil collected in Virginia in 1951, reporting that the pathogen was abundant in pigeon nests and droppings (Emmons 1951, 1955). It took nearly 40 more years to discover the environmental source of *C. gattii* as trees when Ellis reported isolation of serotype B strains from *Eucalyptus camaldulensis* in Australia in 1990 (Ellis and Pfeiffer 1990).

The laboratory diagnosis of *C. neoformans* was drastically simplified by the early 1960s when Seeliger and Staib discovered that *C. neoformans* could be distinguished from other white clinical yeasts by their urease activity (Seeliger 1956) and melanin formation (Staib 1962b) besides the presence of capsule. By the mid-1970s, the complete life cycles of *C. neoformans* and *C. gattii* became known when heterothallic sexuality was discovered in both species (Kwon-Chung 1975, 1976b). The discovery of a heterothallic life cycle ushered *Cryptococcus* into the modern era, facilitating the development of the tools for classical genetic analysis and providing evidence that virulence factors such as capsule and melanin formation followed Mendelian inheritance (Kwon-Chung et al. 1982c). These discoveries were timely, as cryptococcosis had been considered a rare disease until immunosuppressive therapy came into wide use starting in the 1970s. The importance of understanding the pathogen became even more pressing as an unprecedented rate of increase in cryptococcosis began in the early 1980s, with AIDS becoming the leading risk factor of the disease. Building on these critical early advances and the need to develop a deeper understanding to combat this emergent threat, the *Cryptococcus* community has rapidly expanded into a robust and highly collaborative field of international researchers who have established a broad array of molecular techniques (Edman and Kwon-Chung 1990; Toffaletti et al. 1993; Lodge et al. 1994) and genomic sequences (Loftus et al. 2005; Kronstad et al. 2011) with which to develop a deeper understanding of these deadly pathogens.

TAXONOMY AND NOMENCLATURE OF *C. neoformans* AND *C. gattii*

For several decades, the agents of cryptococcosis were grouped into two varieties that included five serotypes: *C. neoformans* var. *neoformans* for serotypes A, D, and AD (now known to be a hybrid between A and D strains), and *C. neoformans* var. *gattii* for serotypes B and C (Kwon-Chung et al. 1982a). When teleomorphs of the two species were discovered, they were classified in the genus *Filobasidiella* as *Filobasidiella neoformans* (serotypes A and D) and *F. bacillispora*

(serotype B and C) under the family Filobasidieceae of the order Filobasidiales in Basidiomycota (Kwon-Chung 1975, 1976b). Discoveries of the two distinct teleomorphs were the first steps toward defining what are now recognized as two distinct species, *C. neoformans* and *C. gattii*. Molecular phylogenetic studies have since shown that *C. neoformans* and *C. gattii* cluster with the members of the order Tremellales instead of Filobasidiales (Fonseca et al. 2011). Furthermore, although the teleomorph names have been used to denote that both pathogenic species of *Cryptococcus* belong to a genus within Basidiomycota, its use has been abandoned as of 2013 and only *Cryptococcus* is used in accordance with the new fungal nomenclatural rule of one name for one fungus (Hawksworth et al. 2011).

After PCR and DNA sequencing became readily accessible, classification of the etiologic agents of cryptococcosis was greatly facilitated by molecular approaches. First, strains of serotype A were recognized as a separate variety, *C. neoformans* var. *grubii*, from strains of *C. neoformans* var. *neoformans* (serotype D), based on the divergence in *URA5* gene sequence (Franzot et al. 1999). In 2000, *C. neoformans* var. *gattii* was elevated to a species, *C. gattii* (Kwon-Chung et al. 2002) and the taxon *C. neoformans* was reserved for the varieties *neoformans* and *grubii*. Subsequent molecular clock analysis has estimated that these species diverged approximately 50 million years ago (Ngamskulrungroj et al. 2009a). Genotyping of numerous *C. neoformans* and *C. gattii* global strains revealed that each species is composed of at least four major genetically diverse subgroups (Meyer et al. 2011). *C. neoformans* contains molecular types VNI-VNIV and VNB with var. *grubii* VNI, VNII, and VNB strains, estimated to have diverged from the single var. *neoformans* molecular type VNIV (serotype D) approximately 25 million years ago, and serotype AD (VNIII) strains representing hybrids of the two varieties. *C. gattii* molecular types VGI and VGII usually correspond with serotype B, and molecular types VGIII and VGIV with serotype C; these molecular types have been estimated to have diverged over 8.5–12.5 million years. Furthermore, some molecular types may contain subtypes that can be identified by multilocus sequence typing (MLST). For example, *C. gattii* VGII and VGIII include at least two or three subtypes each, VGIIa/VGIIb/VGIIc and VGIIIa/VGIIIb, respectively (Kidd et al. 2004; Byrnes et al. 2010, 2011). Although serotype AD hybrids are common, these are sterile, and hybrids between other molecular types are rare, raising the possibility that each haploid type may in fact represent different species. However, elevating the eight major haploid molecular types to separate species status is controversial at present and a consensus on how to treat the *C. neoformans*/*C. gattii* species complex taxonomically has yet to be achieved among medical mycologists (de Hoog et al. 2013).

ECOLOGY

Although *C. neoformans* was first isolated from peach juice, the most important saprophytic sources globally are weathered droppings from pigeon (*Columba livia*) and soil (Emmons 1955). The fungus is not the normal flora of soil; samples positive for *C. neoformans* have mostly been from areas frequented by pigeons, chickens, turkeys, or occasionally other avian species (Emmons 1955; Ajello 1958). The species has also been recovered from the guano of a range of bird species including canaries, parrots, munia birds, and budgerigars (Staib 1962a; Swinne-Desgain 1975; Bauwens et al. 1986). Although the ecological relationship between *C. neoformans* and avian species has been globally consistent, the precise link between birds and the cryptococcal natural habitat has yet to be defined. Pigeons are rarely infected because of their high body temperature (41°C–42°C) exceeding the growth temperature ranges of *C. neoformans*, but isolation from crops, beaks, or feet suggests that the feed they ingest could be contaminated with the fungus (Littman and Borok 1968; Khan et al. 1977). Recently, arboreal sources of *C. neoformans* have been increasingly reported in various parts of the world. Isolation of *C. neoformans* from the bark, tree-trunk hollows, and decaying wood of more than 36 arboreal genera have been reported (Mitchell

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et al. 2011). The vast majority of environmental strains, regardless of the geographical source, belong to serotype A molecular type VNI. Serotype D molecular type VNIV strains from the environment are mostly from central Europe (Mitchell et al. 2011).

C. gattii, on the other hand, has not been isolated from avian guanos (Kwon-Chung and Bennett 1992; Xu et al. 2000) and subsequent to the first report on the isolation of *C. gattii* serotype B molecular type VGI strains from *E. camaldulensis* in Australia (Ellis and Pfeiffer 1990), a growing number of trees have been identified as environmental reservoirs of the species (Stephen et al. 2002; Mitchell et al. 2011), with trees that harbor both cryptococcal pathogens overlapping in various parts of the world (Mitchell et al. 2011). Although both species can infect *Arabidopsis thaliana* under laboratory conditions (Xue et al. 2007, 2011; Warpeha et al. 2013), cryptococcosis agents are not yet known to cause plant disease in nature. Another important ecologic factor of *C. neoformans* may be its interaction with other organisms in soil such as various bacteria, amoebas, mites, sow bugs, and worms (Nielson et al. 1978; Ruiz et al. 1981; Steenbergen et al. 2001). In fact, Castellani (1931) first reported isolation of *Acanthamoeba castellani* as a contaminant of *Cryptococcus* cultures. The interaction between *C. neoformans* and soil amoeba is viewed as an important factor for the evolution of *C. neoformans* as a successful facultative intracellular pathogen. *C. neoformans* can survive in amoebae and the fungus can utilize the same pathogenic strategy in human macrophages, which in some respect provides a similar environment. It has been proposed that such predation in the environmental niche has selected for the cryptococcal virulence traits that contribute to pathogenesis in human hosts (Steenbergen et al. 2001; Casadevall and Pirofski 2007).

EPIDEMIOLOGY

Cryptococcosis caused by *C. neoformans* is worldwide in distribution and the vast majority of patients with symptomatic disseminated infection have an identified underlying immunocompromised condition (Perfect 2010; Sorrell et al. 2011). As the most common predisposition to cryptococcal meningoencephalitis globally is HIV infection, the rate of cryptococcosis mirrors the spread of the AIDS pandemic; by extension, the highest number of cases occur in sub-Saharan Africa where over 25 million people are living with AIDS (Park et al. 2009, 2011). The global burden of the disease is estimated to be close to 1 million cases with 700,000 deaths annually (Park et al. 2009). Other important known underlying conditions are all associated with a compromised immune status, and include prolonged treatment with corticosteroids, organ transplantation, advanced malignancy, diabetes, sarcoidosis, and idiopathic CD4 lymphocytopenia (Casadevall and Perfect 1998; Perfect 2010). Although the vast majority of patients with disseminated cryptococcosis are immunocompromised, *C. neoformans* also causes disease in apparently normal hosts. The proportion of "apparently normal" versus immunocompromised patients is significantly higher with *C. gattii* infection (Chen et al. 2000, 2008; Sorrell et al. 2011). These apparently normal patients could have some unknown immunological defects that are not detected by routine tests employed in clinical laboratories. Recently, GM-CSF-neutralizing autoantibodies were detected in the plasma of otherwise immunocompetent patients with *C. gattii* infection (Saijo et al. 2014), a finding that suggests that expanded immunological tests may uncover hitherto unknown risks in otherwise healthy cryptococcosis patients.

Strains of *C. neoformans* molecular type VNI are the predominant etiologic agents of cryptococcosis isolated worldwide (63%) followed by VNII/VNIII (6% each) and VNIV (5%) type (Meyer et al. 2011). Regardless of molecular type, global clinical isolates are almost always mating type α (*MAT*α) except for certain areas in Africa such as Botswana, where *MAT*a can be as common as 10% of strains (Litvintseva et al. 2003). Consistent with the presence of both mating types in the region, population analysis of clinical isolates has shown evidence of sexual recombination between genetically isolated subgroups of *C. neoformans*

serotype A strains from sub-Saharan Africa (Litvintseva et al. 2003).

Cryptococcosis caused by *C. gattii* is significantly less frequent globally (<20%) than *C. neoformans* (80%), and the major risk factors for *C. gattii* infection remain unclear. Distribution of the four major molecular types of *C. gattii* strains among 2046 cryptococcal isolates from clinical and veterinary sources indicat that the VGI type is most common (9%) followed by VGII (7%), VGIII (3%), and VGIV (1%) in decreasing order (Meyer et al. 2011). In early studies, *C. gattii* infection was noted as prevalent only in subtropical and tropical regions and was infrequently found in temperate zones of the world (Kwon-Chung and Bennett 1984, 1992). Although the prevalence of *C. gattii* infection remains more pronounced in the tropical and subtropical parts of the world, recent studies have indicated that the species has expanded to temperate zones. The recent outbreaks of *C. gattii* infection on Vancouver Island in Canada (Hoang et al. 2004; MacDougall et al. 2007) and the Pacific Northwestern United States (MacDougall et al. 2007; Byrnes and Heitman 2009; Byrnes et al. 2010) are good examples. The Vancouver Island major outbreak strain, VGIIa, and the strains that emerged in the Pacific Northwest, VGIIc, were found to be significantly more virulent in mice than other molecular types of *C. gattii* (Kidd et al. 2004; Fraser et al. 2005a; Byrnes et al. 2010; D'Souza et al. 2011). Importation of *Eucalyptus* trees from Australia, the accelerated rate of human international travel, and climate changes may all have contributed to the spread of *C. gattii* to temperate zones.

HOST RESPONSE AND PATHOGENESIS

Cryptococcosis begins with inhalation of desiccated airborne yeast cells, or possibly sexually produced basidiospores, into the lungs (Fig. 1). Because the propagules are small (1.5–3.5 μm), they reach the distal airways and come into contact with alveolar macrophages. Serologic evidence indicates that cryptococcal infection in humans is prevalent (Goldman et al. 2001) but disease is rare. Activated alveolar macrophages recruit other immune cells through cytokines and chemokines and elicit a proper Th1 response and granulomatous inflammation. Our understanding of the host response to cryptococci is mostly based on animal data (Olszewski et al. 2010) and readers are referred to a recent review (Wozniak et al. 2012) for detailed information on host response in the lung.

In a normal host, an effective immune response eliminates most inhaled cryptococci. In contrast, in an immunocompromised host, the cryptococcal cells proliferate, hematogenously disseminate to the brain by crossing the blood–brain barrier (Chang et al. 2004; Shi et al. 2010), and adapt to the suboptimal levels of oxygen and nutritional conditions of the brain to multiply and cause meningoencephalitis (Chang et al. 2007; Chun et al. 2007). Although virtually every organ in the body can be involved, infection of the central nervous system (CNS) is the most common clinical manifestation of cryptococcosis and the most common cause of death. Untreated CNS infection is uniformly fatal (Kwon-Chung and Bennett 1992; Casadevall and Perfect 1998; Perfect 2010). Another commonly occurring cryptococcal infection is the formation of a small lung–lymph complex where yeasts remain viable but dormant and these patients remain clinically asymptomatic (Salyer et al. 1974; Baker 1976) until loss of local immunity resulting from various causes such as corticosteroid treatment, progression of an HIV infection, or other immunosuppressive conditions (Perfect 2010). Upon this loss of immunity, the dormant yeast cells are activated and begin to multiply in the pulmonary–lymph node complex and disseminate into extrapulmonary sites. This reactivation of latent infection has been seen mainly with *C. neoformans* infection as the most common incidence in AIDS patients, contributing to the definition of cryptococcosis as an AIDS-defining illness (Dromer 2011). In contrast, infection caused by *C. gattii* occurs more often in immunocompetent patients with or without any known underlying conditions (Sorrell et al. 2011). Although *C. neoformans* primarily presents as meningoencephalitis, pulmonary infection is considerably more common with

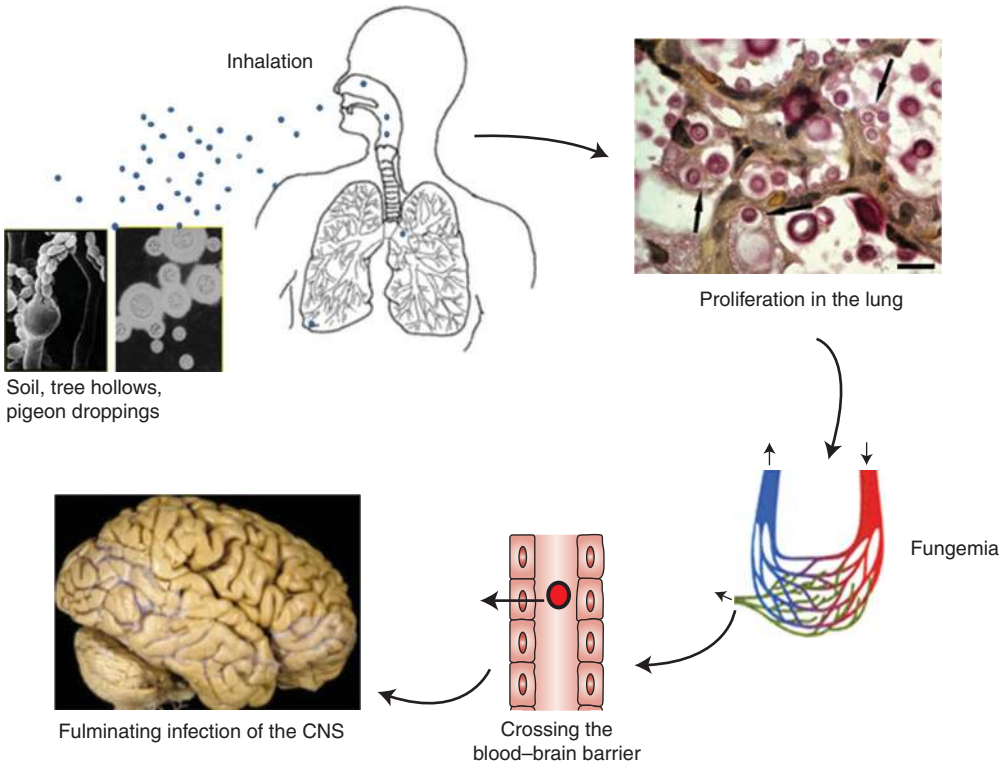

Figure 1. Route of cryptococcal meningoencephalitis. Airborne cryptococcal cells are inhaled by the host and proliferate in the lung before they hematogenously disseminate to the brain.

C. gattii infection (Chen et al. 2000; Galanis et al. 2010). Animal studies supported these differences in the primary target organs between the two species; mice infected with *C. neoformans* succumbed to infection by CNS infection, whereas mice infected with *C. gattii* died by pulmonary infection (Ngamskulrungroj et al. 2012c).

TREATMENT AND RESISTANCE TO DRUGS

The Infectious Diseases Society of America (IDSA) published the "Practice Guidelines for Management of Cryptococcal Disease" in 2000 and an updated version in 2010 (Perfect et al. 2010). Amphotericin B, a secondary metabolite of *Streptomyces nodosus*, has been the major therapeutic agent for cryptococcal meningoencephalitis since the late 1960s (Sarosi et al. 1969). Although sometimes used alone, it became common in the early 1980s to use this polyene in combination with flucytosine, a fluorinated pyrimidine analog (Bennett et al. 1979). Following the release of fluconazole in 1990, the existing regimen of amphotericin B/flucytosine combination for induction therapy was expanded to be followed by maintenance regimens using fluconazole (Perfect et al. 2010). More important, the treatment strategy for cryptococcosis may vary depending on the different categories of risk groups: HIV-infected patients, organ transplant recipients, non-HIV-infected, and nontransplant patients. Specific recommendations also exist for other risk groups such as pregnant women, patients in resource-limited environments, and those with a *C. gattii* infection (Perfect et al. 2010). Detailed information on the strategies of treatment and management of cryptococcosis patients are discussed in Whitney and Bicanic (2014).

There have been a number of reports on cryptococcal resistance to antifungal drugs. Fluconazole resistant strains have been the most common, arising from AIDS patients undergoing long-term azole maintenance therapy (Paugam et al. 1994; Rodero et al. 2003; Pfaller et al. 2011; Sionov et al. 2012). So far, only two fluconazole resistant mutant strains isolated from such patients have been molecularly characterized and both were found to contain a missense mutation in the lanosterol 14α demethylase encoding-*ERG11* gene (Rodero et al. 2003; Sionov et al. 2012). Recently, both *C. neofomans* and *C. gattii* (Sionov et al. 2009; Varma and Kwon-Chung 2010) have been reported to be innately heteroresistant to azoles. Characterized by the emergence of minor subpopulations in each clone that can adapt to higher concentrations of azoles, this acquired resistance is reversed upon release from drug stress (Sionov et al. 2009, 2010). The lowest level of fluconazole at which the heteroresistant minor subpopulation emerges (LHF) and the frequency of resistant subpopulation varies depending on the strain. A majority of the clinical isolates obtained before the birth of azoles had an LHF of 16 μg/mL or lower (Sionov et al. 2009), whereas the LHF of *C. gattii* was generally higher (Varma and Kwon-Chung 2010).

Molecular analyses have shown that the emergence of heteroresistant clones is caused by the formation of disomy (two copies) of chromosomes that contain key genes associated with azole resistance. Heteroresistant clones, therefore, are temporary aneuploids in one or more chromosomes (Sionov et al. 2010). Chromosome 1, which contains the genes encoding the azole target Erg11 and the azole efflux pump Afr1 (Posteraro et al. 2003), always duplicates at the lowest azole concentration at which the heteroresistant clones emerge. Consistent with this, emergence of disomic chromosome 1 in yeast cells was observed in vivo in mouse brains during prolonged treatment with fluconazole (Sionov et al. 2013). As drug concentration was increased, chromosome 4 and other chromosomes subsequently duplicated in a stepwise manner to resist the higher drug concentrations (Sionov et al. 2010; Ngamskulrungroj et al. 2012a). Heteroresistance has now been suggested to be the most common cause of azole treatment failure that occurs during suppression therapy in AIDS patients (Sionov et al. 2013).

MYCOLOGY

Life Cycle

C. neoformans (Fig. 2) and *C. gattii* (Fig. 3) are both heterothallic haploid yeasts that exist in two mating types, *MAT*α and *MAT*a. However, *MAT*α strains are predominant in nature, and, as a consequence, the vast majority of clinical isolates are *MAT*α strains (Kwon-Chung and Bennett 1978, 1984; Kidd et al. 2004; Litvintseva et al. 2005; Chen et al. 2008) and the reasons for paucity of *MAT*a strains are unclear. The haploid cells propagate asexually by budding until the strains of opposite mating type come into contact on substrates conducive for mating. Cells of the *MAT*a strains secrete MFa pheromone and *MAT*α cells secrete MFα pheromone; in response to the mating partner's pheromone, the *MAT*a cells undergo isotropic growth, whereas the *MAT*α cells form conjugation tubes (McClelland et al. 2004; Stanton et al. 2010; Gyawali and Lin 2013) to enable fusion and produce dikaryotic hyphae with fused clamp connections (Fig. 2E). During the early mating process, mitochondria from the *MAT*α partner are eliminated by the functions of the transcription factor Mat2 and the homeodomain protein complex Sxi1α/Sxi2a (Yan et al. 2004; Gyawali and Lin 2013) and only *MAT*a mitochondria are subsequently inherited by the progeny produced from the mating (Xu et al. 2000). In the terminal swollen part of the basidium, the two nuclei from opposite mating types fuse to form a single transient diploid nucleus that undergoes one cycle of meiotic division producing four haploid nuclei (Kwon-Chung 1976a, 1980). These meiotic products then repeatedly undergo mitotic division to basipetally bud four long chains of basidiospores (sexual spores) consisting of *MAT*α and *MAT*a type spores in an equal ratio (Kwon-Chung 1980). These basidiospores can cause infection

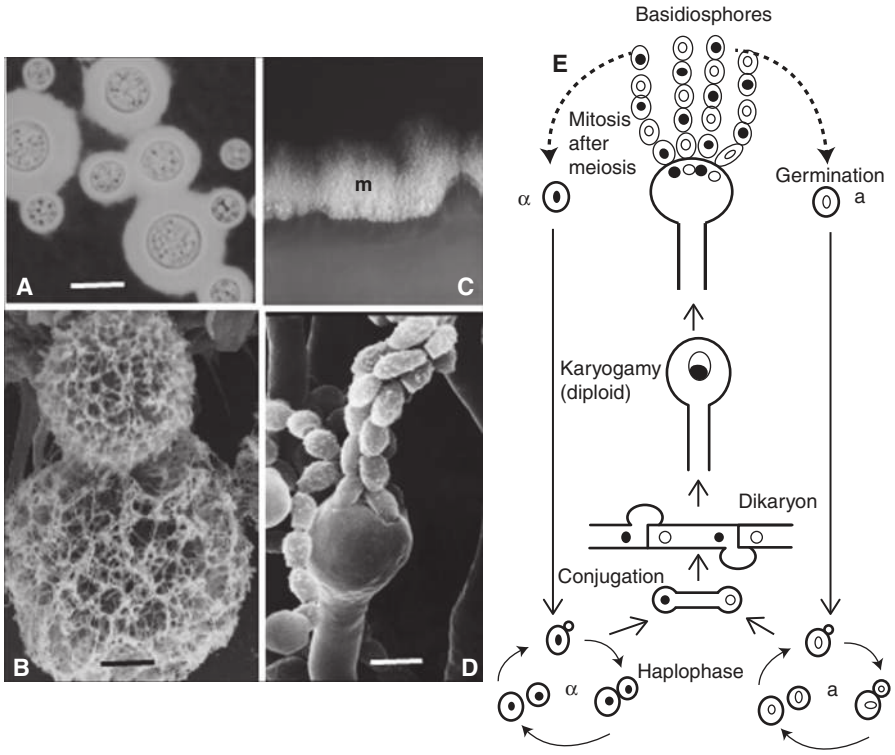

Figure 2. Sexual and asexual states of *Cryptococcus neoformans*. (*A*) Yeast cells stained by India ink showing encapsulated globose cells. Scale bar, 5 μm. (*B*) Scanning electron micrograph of budding yeast cells (image courtesy of Sabriya Stukes). Scale bar, 1 μm. (*C*) Dikaryotic hyphal production (m) at the edge of mated strains between JEC20 (*MAT*a) X JEC21(*MAT*α). (*D*) Scanning electron micrograph of basidia-bearing chains of oval to elliptical basidiospores (Samson et al. 1983). Scale bar, 5 μm. (*E*) Life cycle of *Cryptococcus neoformans*. α, cells of *MAT*α type; a, cells of *MAT*a type.

in a mouse model just as efficiently as vegetative yeast cells, and may play the role of infectious particles (Giles et al. 2009).

Traditional bipolar mating of cells of opposite mating types is not the only sexual cycle available to *C. neoformans*. In this species, unisexual reproduction has also been described in *MAT*α strains that results in the four chains of basidiospores morphologically indistinguishable from basidiospores formed by partners of two opposite mating types instead of formed when partners are of opposite mating types (Lin et al. 2005; Ni et al. 2013). Unlike traditional mating, during this process the clamp connections remain unfused (Hsueh et al. 2011b), and by necessity the mitochondria are inherited from a *MAT*a parent (Xu et al. 2000; Hsueh et al. 2011a; Ni et al. 2013). More intriguing, the developmental cascades for both modes of reproduction have been found to be controlled by the same genetic circuits, demonstrating that the spores produced in unisexual reproduction are also the result of meiosis (Lin et al. 2005; Feretzaki and Heitman 2013). Furthermore, a recent report indicates that monokaryotic hyphal production unrelated to unisexual reproduction can be induced by high-temperature arrest of yeast cell cycle in the G_2 stage (Fu et al. 2013). Whether the genetic circuits that control monokaryotic hyphal formation induced by high-temperature cell-cycle arrest at G_2 overlap with those of sexual reproduction is a compelling question that remains to be determined.

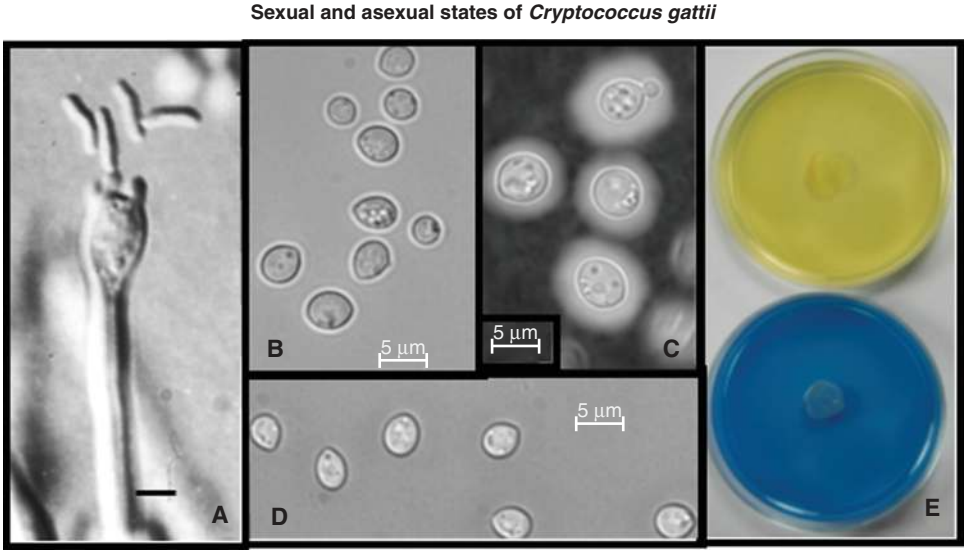

Figure 3. Sexual and asexual states of *Cryptococcus gattii*. (*A*) Basisia and basidiospores. Scale bar, 5 µm. (*B*) Globose to oval-shaped yeast cells of R265 (VGII type). (*C*) India ink–stained yeast cells of NIH319 (VGIII type). (*D*) Oval- to tear-shaped yeast cells of R-1407 (VGIV type). (*E*) CGB agar for differentiation of *C. neoformans* (no reaction) from *C. gattii* (cobalt blue).

Phenotypic Differences between *C. neoformans* and *C. gattii*

The two etiologic agents of cryptococcosis can be differentiated by their teleomorph, serotype, and biochemical characteristics in addition to their epidemiological and ecological differences. Although the morphology of *C. neoformans* yeast cells are almost uniformly globose, those of *C. gattii* are a mixture of globose and oblong to elliptical cells (Kwon-Chung et al. 1982a); this proportion of globose to oblong cells in *C. gattii* is strain dependent (Fig. 3B–D). The morphology of the two teleomorphs are even more different: rough-walled oblong to eliptical basidiospores are produced in *C. neoformans*, whereas the basidiospores produced by *C. gattii*, as suggested by the original teleomorph designation of *F. bacillispora*, are in bacillary form with smooth walls (Fig. 3A) (Kwon-Chung 1975, 1976b). The species are also distinguishable by their considerable differences in their ability to utilize nitrogen (Lee et al. 2011; Ngamskulrungroj et al. 2012b) and carbon sources (Bennett et al. 1978). Taking advantage of their differences in nitrogen assimilation, the widely used one-step diagnostic media canavanine-glycine-bromothymol blue (CGB) agar was formulated to distinguish the two species (Kwon-Chung et al. 1982b). The resistance of *C. gattii* to L-canavanine and the ability to use glycine as a nitrogen and carbon source (and hence alter the medium pH) provides two levels of selection, enabling robust identification of *C. gattii* strains on CGB medium with growth changing the media color to cobalt blue, whereas CGB agar inoculated with strains of *C. neoformans* remain unchanged (Fig. 3E). CGB agar medium was instrumental in discovering *Eucalyptus camaldulensis* as the first environmental source of *C. gattii* (Ellis and Pfeiffer 1990).

Genotypic Differences between *C. neoformans* and *C. gattii*

The differences between *C. neoformans* and *C. gattii*, and between their molecular types, are even more obvious at the genomic level. The first basidiomycete to have its genome sequenced to completion, the *C. neoformans* genome heralded the beginning of the postgenomic era for *Cryptococcus* (Loftus et al. 2005).

This data from the closely related VNIV (serotype D) laboratory strains JEC21 and B-3501A revealed the 20 Mb haploid genome to consist of 14 chromosomes ranging in size from 762 kb to 2.3 Mb (Fraser et al. 2005b; Loftus et al. 2005). With >6500 genes with an average size of 1.9 kb, the chromosomes are extremely gene-rich with the exception of a single large cluster on each composed of degenerating transposons predicted to represent regional centromeres.

The availability of the draft *C. neoformans* var. *grubii* VNI type strain H99 (Janbon et al. 2014) genome enabled comparisons to soon follow. Dietrich and colleagues revealed via whole genome comparison that the genomes exhibited 85%–90% identity with few exceptions (Kavanaugh et al. 2006). Among these were the predicted regional centromeres, conserved in location but highly divergent in sequence consistent with their makeup of degenerating rearranged transposable elements, the highly rearranged subtelomeric regions, and a region where identity between the genomes was 98.5%, evidence of recent introgression of var. *neoformans* (molecular type, VNIV) genetic material into var. *grubii* (molecular type VNI). It has subsequently been shown that since the divergence of var. *grubii* and var. *neoformans*, few genomic rearrangements have occurred in the var. *grubii* lineage and even more in var. *neoformans* (Sun and Xu 2009; Morrow et al. 2012). Given the known propensity of var. *grubii* to undergo karyotypic change during the infection process (Perfect et al. 1993; Fries et al. 1996), the small number of changes fixed within the var. *grubii* population in particular over the past ~25 million years is remarkable. This suggests that the pathogen undergoes gross chromosomal rearrangements at a massively accelerated pace during infection of the human host.

Consistent with their phylogenetic relationship, comparison of the var. *neoformans* and var. *grubii* genome sequences with the genomes of *C. gattii* VGI strain WM276 and VGIIa strain R265 revealed ~85%–87% identity (D'Souza et al. 2011). Like *C. neoformans*, *C. gattii* has 14 chromosomes, and although the predicted centromere locations are conserved, the chromosomes have been rearranged by inversions and balanced translocations. Once again, the regions of lowest conservation are the centromeres and subtelomeres. However, intriguing differences between *C. neoformans* and *C. gattii* still remain to be discovered. For example, recent work has revealed that in contrast to *C. neoformans*, *C. gattii* VGII strains have lost the RNAi mechanism (Billmyre et al. 2013).

Virulence Factors

Early classical genetic studies on virulence determinants focused on the distinctive phenotypic characteristics of these species that were also used for diagnostic purposes (i.e., polysaccharide capsule formation [Jacobson et al. 1982; Rhodes et al. 1982; Kwon-Chung and Rhodes 1986] and melanin formation [Kwon-Chung et al. 1982c; Rhodes et al. 1982]). The third trait essential for virulence is the ability to grow at mammalian body temperature (Petter et al. 2001; Perfect 2006). Molecular genetic studies using gene-deletion strains have confirmed the role of these three traits in cryptococcal virulence (Chang and Kwon-Chung 1994; Salas et al. 1996; Odom et al. 1997; Janbon et al. 2001). In fact, the molecular study into the role of capsule as the fungal virulence factor in *C. neoformans* was the first fulfillment of the molecular Koch's postulates (Chang and Kwon-Chung 1994).

Capsule

The cryptococcal capsule is anchored by the cell wall, which is composed of glucans, chitin, chitosan, and glycoprotein (Doering 2009; Gilbert et al. 2011). The capsule is composed primarily of two large virulence-implicated repeating polysaccharides (Kumar et al. 2011), glucuronoxylomannan (GXM, ~1–7 million Da) and glucuronoxylomannogalactan (GXMGal, ~100,000 Da), whose structures are depicted in Figure 4. The capsule may also include mannoproteins, hyaluronic acid, and sialic acid. The capsule is displayed on the cryptococcal surface and component polysaccharides are also shed into the environment. Although it is likely that both forms play roles in pathogenesis,

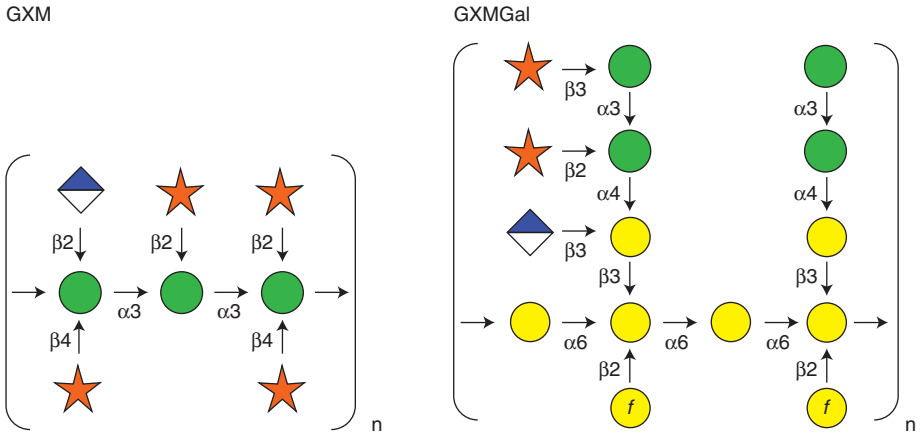

Figure 4. Structures of the cryptococcal capsular polysaccharide. The polymer repeat units of glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal) are shown. (*Left*) The maximally substituted structural reporter group of GXM. Other structural reporter groups vary in the extent and position of xylose addition (Cherniak et al. 1998). Mannose, green spheres; xylose, red stars; glucuronic acid, half-filled diamonds. Mannose acetylation is not shown. (*Right*) The repeat unit of glucuronoxylomannogalactan (GXMGal) (Vaishnav et al. 1998; Heiss et al. 2009). Trisaccharide side branches on alternate galactose residues may be modified with xylose and glucuronic acid; the two extremes of substitution are shown. Symbols are as above, with galactose as yellow spheres. A fraction of the branched backbone galactoses residues is also modified with β-1,2-linked galactofuranose, indicated by *f*; all other residues are in the pyranose form.

most structural work has been done on the shed material, which may differ from that associated with the cell (Frases et al. 2008; Kumar et al. 2011).

Capsule polysaccharides are likely made in the Golgi (Yoneda and Doering 2006; T Doering, unpubl.), requiring synthesis of activated sugar donors, transport of these compounds into the Golgi, polymerization of capsule components, export of product polysaccharides, and assembly at the cell surface (Doering 2009). Based on the capsule structures (Fig. 4), the sugar donors are expected to include GDP-mannose, UDP-glucuronate, UDP-xylose, UDP-galactofuranose, and UDP-galactopyranose. Enzymes required for synthesis of the first four have been biochemically characterized, and genes encoding synthetic machinery for all five have been identified and deleted to assess the impact on cells (Bar-Peled et al. 2001, 2004; Wills et al. 2001; Moyrand et al. 2002, 2007, 2008; Griffith et al. 2004; Moyrand and Janbon 2004; Beverley et al. 2005; Doering 2009). The lack of these proteins reduces capsule production and virulence in animal models, except for the enzyme responsible for UDP-galactofuranose synthesis; this may be because galactofuranose is a minor component of capsule polysaccharides (Heiss et al. 2013). Two nucleotide sugar transporters for GDP-mannose and one for UDP-galactose have also been investigated (Cottrell et al. 2007; Moyrand et al. 2007; Wang et al. 2014). Although the genome encodes other putative nucleotide sugar transporters, they have not been matched to specific functions required for capsule synthesis. The glycosyltransferases that directly mediate capsule synthesis are largely uncharacterized, with only one demonstrated to date (Klutts et al. 2007; Castle et al. 2008; Klutts and Doering 2008). A second enzyme, Cas1, has been suggested to function in capsule polysaccharide acetylation, but its activity has yet to be directly demonstrated (Janbon et al. 2001).

How the capsule is associated with the cell wall and how it grows are outstanding questions in the field. It is clear that an intact cell wall (Gilbert et al. 2010), and, more specifically,

cell wall α-glucan (Reese and Doering 2003; Reese et al. 2007), is required for capsule association, although whether this is a direct or indirect interaction has not been determined. Chitin has also been implicated in capsule association with the cell wall (Rodrigues et al. 2008; Fonseca et al. 2009), and cations likely play a significant role in intermolecular interactions of capsule polysaccharides that contribute to capsule formation (Nimrichter et al. 2007). Defining the machinery of capsule biosynthesis poses a considerable challenge, because of both the complexity of the process and the difficulty of assigning function to glycoactive enzymes based on sequence alone.

Further biochemical studies and new approaches to gene identification should elucidate this fascinating area.

Genetic Control and Regulation of Capsule Formation and Secretion

The size and antigenicity of the *C. neoformans* capsule is dynamic and regulated by the fungus in response to different environmental cues. More important, the in vivo capsule variability suggests an important role for this regulation in the pathophysiology of infection (Dykstra et al. 1977; Granger et al. 1985; Rivera et al. 1998; Chretien et al. 2002; Garcia-Hermoso et al. 2004). In vitro, diverse signals like iron concentration, CO_2 level, pH, and nitrogen or glucose concentrations are important modulators of capsule size (Staib et al. 1976; Dykstra et al. 1977; Granger et al. 1985; Vartivarian et al. 1993). Numerous genes functioning in different transduction pathways are necessary for *C. neoformans* to sense any alteration in its environment. Activation of these pathways induces or alters expression of specific transcription factors necessary for *C. neoformans* to adapt to its new environment. For example, the genes that encode the transcription factors Cir1 and HapX are necessary for iron homeostasis and are specifically induced under low iron conditions (Kronstad et al. 2013). Similarly, Rim101 also plays a central role in pH response and capsule regulation (O'Meara et al. 2010). These studies have also shown that the capsule-regulating transduction pathways do not function independently. Rather, significant cross talk exists between them. Activation of the cAMP/PKA pathway, which can be triggered by altered CO_2 levels, low glucose, low nitrogen, or low iron resulting in direct activation of the transcription factor Nrg1, can also result in the regulation of Hog1 and/or Rim101 in response to pH alteration and osmotic stress, respectively (Bahn et al. 2005b; Cramer et al. 2006; O'Meara et al. 2010). Thus, multiple transduction pathways respond to each environmental signal and a single transduction cascade can respond to multiple signals.

Despite numerous studies on the transcriptional response associated with the activation of these transduction pathways, the genetic basis of capsule size regulation has not been elucidated (Haynes et al. 2011; O'Meara and Alspaugh 2012). This might be a result of the fact that numerous elements of the capsule biosynthetic pathways have still not been identified (Doering 2009). It is also possible that the regulation of capsule size is affected more by secretion than the capsule biosynthetic pathways per se. In fact, the transduction pathways that regulate capsule size also seem to regulate capsule secretion (Choi et al. 2012). However, secretion of the capsule and the genetics of its regulation have not been studied extensively. In the last decade, two *C. neoformans* Sec proteins, homologous to *Saccharomyces cerevisiae* proteins necessary for vesicle-mediated exocytic secretion, have been shown to play a major role in GXM secretion (Yoneda and Doering 2006; Panepinto et al. 2009). Although these data support a classical post-Golgi model, a nonconventional capsule secretion pathway has also been identified (Kmetzsch et al. 2011). These data together with the existence of extracellular vesicles containing capsule polysaccharides in *C. neoformans* suggests an elaborate regulatory mechanism for capsule secretion (Rodrigues et al. 2008; Rodrigues and Djordjevic 2012). Each capsule secretion pathway can presumably result in a different outcome for the capsule polysaccharides. One part might be cell wall linkage (Reese and Doering 2003), and the other is secretion, which is a model supported by some mutations

that decrease the quantity of the capsule polysaccharide secreted in the environment without affecting capsule size (Kmetzsch et al. 2010), whereas some hypocapsular strains are able to secrete normal levels of polysaccharides (O'Meara et al. 2010). Studies of these two putative pathways might represent a major challenge to the field of capsule genetics in future years.

Melanin Formation

Melanin is produced by a wide variety of fungal species and the pigment deposited in the cell wall is known to play an important protective role against environmental stress (Nosanchuk and Casadevall 2006). Unlike the black fungi commonly found in soil or on plants, which synthesize dihydroxynaphthalene (DHN) melanin constitutively, *C. neoformans* produces eumelanin only in the presence of substrates such as 3,4-dihydroxyphenylalanine (DOPA) and other di/polyphenolic compounds (Chasakes 1975; Polacheck et al. 1982). The early observations, using melanin-lacking mutants isolated by UV irradiation, suggesting the importance of melanin as a virulence factor (Kwon-Chung et al. 1982c; Rhodes et al. 1982) was confirmed by the use of *LAC1* gene-deletion mutants (Salas et al. 1996). *C. neoformans* contains two laccase genes, *LAC1* and *LAC2*, in the genome but only *LAC1* is expressed significantly under most conditions and virulence is reduced only when the *LAC1* gene is deleted (Zhu and Williamson 2004; Pukkila-Worley et al. 2005).The cryptococcal laccase, a member of the multicopper oxidases is localized in the cell walls (Zhu et al. 2001; Waterman et al. 2007b) and its transport to the cell wall is Sec6 dependent (Panepinto et al. 2009). Melanization of cryptococci require numerous additional genes such as the copper transporter Ccc2, the copper chaperone Atx1, the chitin synthase Chs3, the transcriptional coactivator Mbf1, the chromatin remodeling enzyme Snf5 (Walton et al. 2005), the transcription factor Rim101, and its regulatory gene Rim20 (Liu et al. 2008). As in the case of capsule formation, melanization is regulated by number of different pathways (Liu et al. 2008).

How does melanin provide a survival advantage in the host? There have been numerous studies regarding the role of cryptococcal melanin in protection from phagocytosis (Wang et al. 1995; Liu et al. 1999), killing by host cells (Blasi et al. 1995; Wang et al. 1995), oxidants (Wang and Casadevall 1994; Blasi et al. 1995; Jacobson and Hong 1997), and microbicidal peptides (Doering et al. 1999). In addition, melanin is reported to protect cryptococci from antifungal agents including amphotericin B (Ikeda et al. 2003; Martinez and Casadevall 2006), caspofungin (van Duin et al. 2002; Martinez and Casadevall 2006), and azoles (van Duin et al. 2002, 2004; Ikeda et al. 2003).

Growth at Mammalian Body Temperature

Although the ability to grow at 37°C is not sufficient by itself to be a mammalian pathogen, it is essential for any microbial pathogen to be able to cause invasive disease. The primary reason that both *C. neoformans* and *C. gattii* are the only successful pathogens among the more than 70 *Cryptococcus* species is their ability to grow robustly at physiological temperatures. All the remaining cryptococcal species produce a polysaccharide capsule with or without melanin but fail to grow or grow poorly at 37°C (Petter et al. 2001; Fonseca et al. 2011). The first molecular study related to the cryptococcal growth at mammalian body temperature has identified calcineurin, the highly conserved Ca^{2+}/calmodulin-activated serine/threonine-specific phosphatase encoded by *CNA1*, to be essential for growth at 37°C but not at 24°C. As a consequence, *CNA1* disrupted mutant strains are avirulent (Odom et al. 1997; Fox et al. 2001) and it was proposed that the signaling cascade involving calcineurin is required for cryptococcal pathogenesis (Odom et al. 1997). The importance of calcineurin for cryptococcal growth at 37°C but not at 24°C also explained the reason why mice treated with cyclosporin A (CsA), which inhibits signal transduction at 37°C but not at 24°C, are protected from cryptococcosis (Mody et al. 1988) and that CsA is toxic to *C. neoformans* only at 37°C but not at 24°C (Odom et al. 1997). Subsequently, many genes

required for cryptococcal growth at physiological temperatures and the genes that are significantly up-regulated during growth at elevated temperatures have been identified (Perfect 2006) using various molecular methods such as complementation cloning (Chung et al. 2003), insertional library (Idnurm et al. 2004), genomic-DNA microarrays (Kraus et al. 2004), serial analysis of gene expression (SAGE) (Steen et al. 2002), signature-tagged mutagenesis (Liu et al. 2008), and representational difference analysis (Rosa e Silva et al. 2008). The functions of these genes varied ranging from cell-wall assembly, stress signaling, membrane integrity, basic metabolism, pre-mRNA splicing, chromatin remodeling, and others (Steen et al. 2002; Chung et al. 2003; Kraus et al. 2004; Liu et al. 2008; Rosa e Silva et al. 2008). Functional studies of the genes identified as important for growth at 37°C, using targeted disruption, showed correlation with low to no infectivity (Kraus et al. 2004; Liu et al. 2008). However, temperature-regulated genes did not necessarily correlate with a temperature-sensitive phenotype (Akhter et al. 2003; Cox et al. 2003).

Degradation Enzymes

C. neoformans produces many degradation enzymes; some of them have been confirmed as virulence determinants. Urease (Cox et al. 2000, 2001; Osterholzer et al. 2009; Shi et al. 2010; Bahn and Jung 2013; Singh et al. 2013) and phospholipase B (Cox et al. 2001; Ganendren et al. 2006; Wright et al. 2007; Chayakulkeeree et al. 2011) are the two most studied degradation enzymes that have a role in cryptococcal pathogenicity. The functions of these enzymes promote intracellular survival of the yeasts (Wright et al. 2007), hydrolysis of host cell membranes to penetrate into tissue (Chen et al. 1997), immunomodulation (Noverr et al. 2003; Osterholzer et al. 2009), and the enhancement of fungal dissemination from the lung to the brain (Cox et al. 2000; Noverr et al. 2003; Wright et al. 2007; Shi et al. 2010; Singh et al. 2013). Unlike the polysaccharide capsule, however, a lack of these enzymes results in reduced rather than a complete loss of virulence. The correlation between in vitro phospholipase B activity of cryptococcal strains and virulence in mice was first demonstrated in 1997 (Chen et al. 1997) and subsequent work with phospholipase B gene (*PLB1*)-deletion strains confirmed the importance of the enzyme as a virulence determinant (Cox et al. 2001; Noverr et al. 2003). Phospholipase B is transported to the cell surface in vesicles (Eisenman et al. 2009) and its secretion is dependent upon Sec14, a phosphatidylinositol transfer protein (Chayakulkeeree et al. 2011). Transport of the enzyme to the cell surface enhances adhesion of the cryptococcal cells to human lung epithelial cells, the first step toward initiation of interstitial pulmonary cryptococcosis (Ganendren et al. 2006). It also disrupts the host cell membranes by hydrolysis of the ester linkages on membrane phospholipids, which enables penetration into the host tissue (Chen et al. 2000). Plb1 also supports the intracellular survival of cryptococci within macrophages in connection with lipid metabolism (Wright et al. 2007), which is key for the eventual dissemination of cryptococci to the brain.

Cryptococcal urease activity is important for fungal propagation in the lung as the enzyme promotes accumulation of immature dendritic cells as well as the nonprotective T2 immune response (Osterholzer et al. 2009). Urease activity is also important in the fungus' ability to cross the blood–brain barrier (Olszewski et al. 2004; Shi et al. 2010) by enhancing sequestration of the yeast cells within microcapillary beds (Olszewski et al. 2004). The mechanism of urease activation has been studied extensively in bacteria and plants but rarely in fungi. The cryptococcal urease activation system has recently been deciphered, which showed that the factors required for activation of the urease apoenzyme encoded by *URE1* resemble plants more than bacteria (Singh et al. 2013). As with other ureases, the cryptococcal urease is a nickel enzyme and requires the accessory proteins, Ure4, Ure6, and Ure7, which are homologs of the bacterial accessory proteins UreD, UreF, and UreG, respectively. The cryptococcal genome lacks a homolog of bacterial UreE, a nickel chaperone. However, Ure7 (the homolog of bacterial UreG)

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appears to combine the functions of bacterial UreE and UreG. Strains harboring an intact *URE1* but disrupted accessory proteins disseminated to the brain at rates similar to the *ure1* mutant, which indicates that it is the urease activity and not the Ure1 protein that is a virulence factor in *C. neoformans* (Singh et al. 2013). The enzyme appears to require *SEC6* for secretion to the surface of the yeast cells (Panepinto et al. 2009).

SENSING AND RESPONDING TO ENVIRONMENTAL VARIABLES

More recently, other virulence factors have emerged that are being investigated from the perspective of sensing and signaling, often affecting the classical virulence traits. Three recent developments are described below.

The first is metal homeostasis. A role of calcium in signaling in *C. neoformans* has been well studied in the context of calcineurin signaling (Steinbach et al. 2007), so is not covered further. Iron and copper are the next best-studied metals. Both are relevant to pathogenesis because of their availability to the pathogen, the interconnection between oxygen availability, metal uptake, heme (with iron as a cofactor), and heme-dependent sterol biosynthesis. Thus, altered Fe or Cu homeostasis affects multiple aspects of *C. neoformans* biology that are clinically relevant, including antifungal drug efficacy. Iron can be either limiting or in excess in a host depending on health status. Effects of iron limitation on *C. neoformans* have been known since the early 1990s, because one consequence is the enhancement of capsule formation (Vartivarian et al. 1993). *C. neoformans* senses iron levels, and regulates a suite of genes using the Cir1 transcription factor (Jung et al. 2006). Those genes include an adjacent pair of genes for iron oxidation and high-affinity uptake, which are also required for full virulence (Jung et al. 2008, 2009). Other factors, such as the siderophore transporter Sit1, are not required for pathogenesis although this may reflect gene redundancy (Tangen et al. 2007). Another source of iron for *C. neoformans* is heme, yet use of this iron source is currently less well established (Jung et al. 2010; Cadieux et al. 2013).

The link between copper and melanization has also been known for two decades. For example, mutants impaired in melanin synthesis can be rescued by the addition of copper to the medium (Torres-Guererro and Edman 1994; Walton et al. 2005). Copper is a cofactor for multicopper oxidases, which include the ferroxidase Cfo1 for iron uptake and laccase for melanin synthesis. Altered copper homeostasis influences these two properties. The *MAC1* gene was identified as naturally variable in populations, affecting mating efficiency and melanization (Lin et al. 2006). *MAC1* (*CUF1*) encodes a copper-response transcription factor that regulates target genes (Ding et al. 2011). A fascinating discovery is that the effects of mutation that impair two totally opposite responses to copper (i.e., to low or high Cu concentrations) are the same. That is, loss of the Ctr4 transporter for uptake under low Cu concentrations (Waterman et al. 2007a, 2012) or loss of the metallothioneins used in sequestration of Cu under toxic high concentrations (Ding et al. 2013) both reduced virulence.

Two more recently studied metals are nickel and zinc. Nickel is a cofactor for urease. Like urease deficient strains, those impaired in nickel homeostasis have defects in dissemination after pulmonary infection (Olszewski et al. 2004; Singh et al. 2013). Disruption in *C. gattii* of the *ZAP1* gene, which encodes a zinc sensor and transcription factor, reduces virulence (Schneider et al. 2012). Zinc is a cofactor in many proteins, including zinc finger transcription factors, so loss of virulence may reflect an impact on a number of key processes.

The second example is the impact of gases on pathogenesis. O_2 and CO_2 vary in concentration in different parts of the body, lower or higher, respectively, than the natural environment in which *Cryptococcus* species reside. CO_2 sensing uses carbonic anhydrase and adenylyl cyclase. Carbonic anhydrases are zinc proteins that convert CO_2 to bicarbonate ($HCO3^-$). Two homologs are expressed in *C. neoformans*, with Can2 having the major function. Deletion of *CAN2* prevents growth under low CO_2 concen-

trations; however, virulence is unaffected presumably because of the high CO_2 concentrations within the host (Bahn et al. 2005a). The other CO_2 sensor, adenylyl cyclase (Klengel et al. 2005; Mogensen et al. 2006), synthesizes the signaling molecule cyclic AMP, and is also part of the Gα signaling pathway, and the *cac1* mutants are nonpathogenic (Alspaugh et al. 2002).

Concentrations of O_2 are sensed by Scp1, a candidate endoplasmic reticulum peptidase. Scp1 cleaves the inactive Sre1 into an active transcription factor that is shuttled into the nucleus. Deletion of either gene reduces the ability of *C. neoformans* to grow at $<3\%$ oxygen levels, and reduces virulence (Chang et al. 2007; Chun et al. 2007). Genes regulated by hypoxia include those for sterol biosynthesis and metal homeostasis. The deleterious effects of low O_2 levels are likely in part through altered membrane fluidity. For instance, overexpression of the *ERG25* gene for sterol biosynthesis can alleviate the effects of deleting *SRE1* (Lee et al. 2007).

The third example is the potential role of light sensing in pathogenesis. Deletion of genes that encode a blue-light-sensing complex reduces virulence (Idnurm and Heitman 2005; Zhu et al. 2013). It is unknown whether darkness represents a specific signal and the relevant genes are also unknown. Although genes for heme biosynthesis and iron uptake are induced by light, the light-sensing mutants do not have phenotypes that indicate any change in these properties (Idnurm and Heitman 2010). Hence, the three best-established virulence traits and growth or stress phenotypes are unaffected, and further analysis may enable discoveries about virulence factors operating in *Cryptococcus* species.

The three examples above highlight more recent experiments about sensing and signaling to alter pathogenesis. Interacting factors include pH and nutrient sensing (O'Meara et al. 2010, 2013; Yang et al. 2010). One theme that is emerging is how signals or pathways influence a common set of virulence traits, like the three classical properties of *C. neoformans*, and the potential for cross regulation among them.

SIGNALING PATHWAYS INVOLVED IN STRESS RESPONSE

In the host environment, cryptococci respond and adapt to the drastic change in conditions by activating stress defense systems such as the high osmolarity glycerol response (HOG), protein kinase C (Pkc1)/MPK1 MAPK, protein kinase A (Ras, cAMP/PKA), calcineurin, and unfolded protein response (UPR) pathways (Fig. 5). Several studies comparing the stress management between *C. neoformans* and *C. gattii* have revealed both redundant and distinct roles of the diverse signaling pathways.

For the initial establishment of infection and cellular proliferation within the host, cryptococci undergo adaptation to host body temperatures. In *C. neoformans*, in addition to the Ras, calcineurin, and UPR pathways, which play major roles, the HOG and Pkc1/Mpk1 pathways also play a significant role in thermotolerance (Bahn and Jung 2013). Interestingly, inhibition of the calcineurin pathway is fungistatic against the highly virulent *C. gattii* VGIIa strain R265 at 37°C, whereas it is fungicidal against the less virulent *C. gattii* VGIIb strain R272 and *C. neoformans* (Chen et al. 2013). This indicates functional divergence of this pathway among different genotypes of *Cryptococcus*. Comparative microarray analysis revealed *MPK1* to be more up-regulated in R265 than in R272 and its deletion caused temperature sensitivity, defects in cell wall integrity, and a reduced production of melanin and capsule (Ngamskulrungroj et al. 2011). Induction of the nonreducing disaccharide trehalose and transcription of its biosynthetic genes is required for high-temperature growth, which affects the virulence of both *C. gattii* and *C. neoformans* (Ngamskulrungroj et al. 2009b). Interestingly, the trehalose pathway modulates production of melanin and capsule as well as cell wall integrity in *C. gattii*, but not in *C. neoformans*.

Oxidative and nitrosative damage are critical stresses for *C. neoformans* and *C. gattii*. During the initial stages of infection and dissemination through the bloodstream, the pathogen needs to survive reactive oxygen species (ROS)

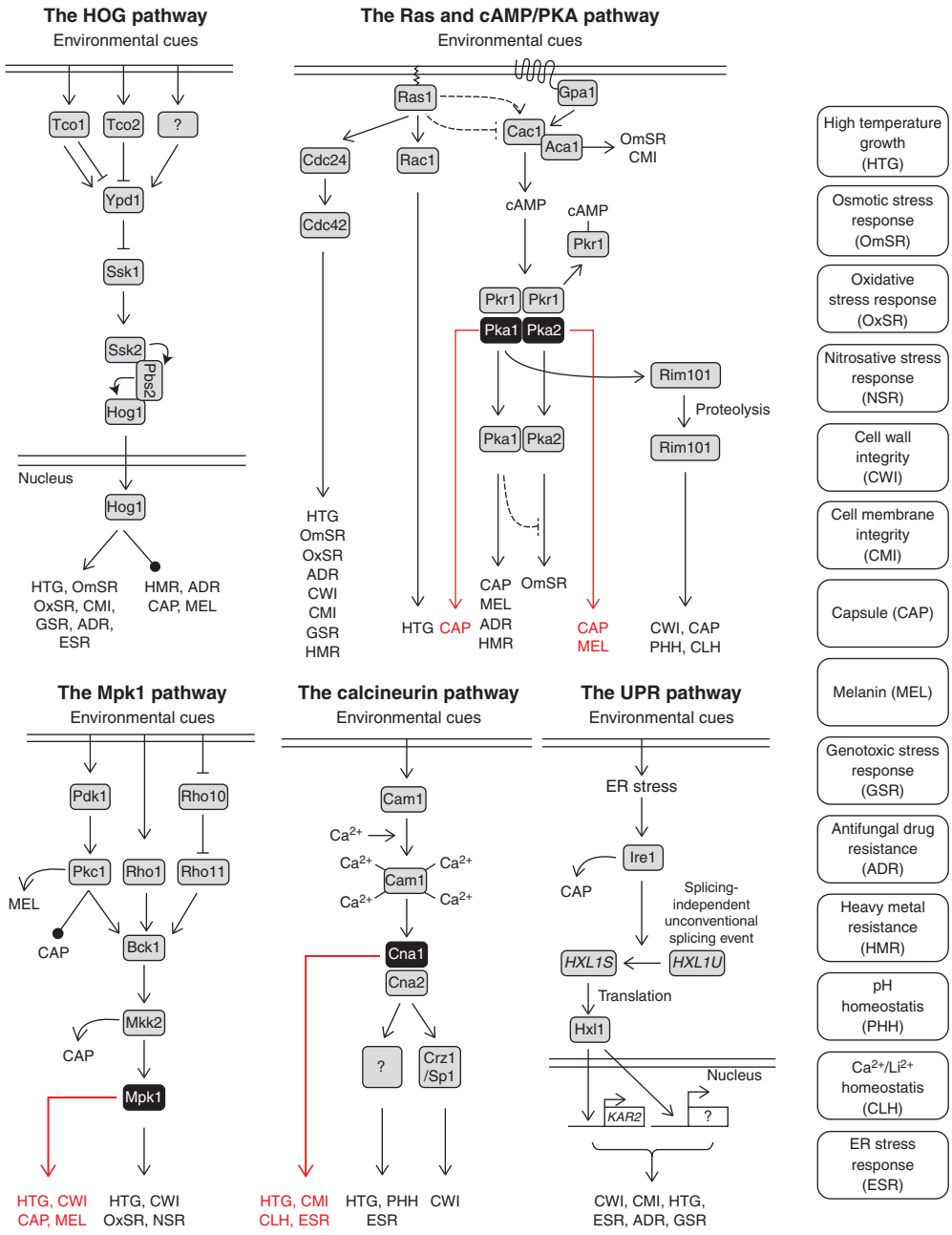

Figure 5. The stress-signaling pathways in *C. neoformans* and *C. gattii*. The HOG pathway consists of a phosphorelay system (Tco, hybrid sensor histidine kinase [HHKs]; Ypd1, a His-containing phosphotransfer protein [HPt]; Ssk1, a response regulator [RR]) and a MAPK module; (Ssk2, MAPKKK; Pbs2, MAPKK; Hog1K, MAPK). The Mpk1 MAPK pathway, which is composed of Bck1 MAPKKK, Mkk2 MAPKK, and Mpk1 MAPK, appears to be activated by multiple upstream signaling pathways, including Pkc1 and Rho-type GTPases (Rho1, Rho11, and Rho10). Key components of the cAMP/PKA pathway include Cac1 and Aca1 for cAMP production and PKA, which consist of Pkr1 (a regulatory subunit) and Pka1/2 (catalytic subunits). Cac1 is activated by Gpa1 (a Gα subunit). (*Legend continues on following page.*)

or reactive nitrogen species (RNS)-rich conditions in macrophages and neutrophils. Similar to other eukaryotes, *Cryptococcus* contains a series of conserved ROS/RNS defense proteins, including superoxide dismutases (SODs), catalases (Cat1-4), glutathione/thioredoxins, and their peroxidase systems (Brown et al. 2007). In *C. gattii*, Sod1 not only counteracts superoxide anions, but also promotes production of virulence factors such as melanin, urease, and phospholipase (Narasipura et al. 2003). To control these ROS and RNS defense systems, *C. neoformans* primarily utilizes two stress-activated MAPKS: Hog1 and Mpk1 (Bahn et al. 2005b; Gerik et al. 2008). In *C. gattii*, ROS and RNS defense systems are also important for antifungal drug resistance because itraconazole (but not fluconazole) and amphotericin B induce ROS and RNS production, which causes lipid peroxidation and oxidative degradation of lipid membranes (Ferreira et al. 2013). It is not known whether such events occur in *C. neoformans*.

The ability to maintain osmotic balance across the cell membrane is related to cation homeostasis and pH homeostasis within the cell. In fact, a key cation transporter, Ena1 (Na^+/ATPase), which plays an essential role in osmotic stress response, survival under alkaline pH conditions and plasma membrane potential, is required for virulence of *C. neoformans* (Idnurm et al. 2009; Jung et al. 2012). Following osmotic shock intracellular glycerol synthesis rapidly increases by inducing transcription of glycerol-3-phosphate dehydrogenases genes to counteract external osmotic pressures. Major controllers of these defense systems in *C. neoformans* include the HOG and Rim101 pathways (Ko et al. 2009; O'Meara et al. 2010). The corresponding pathways in *C. gattii* have not been characterized.

Finally, upon entering the host, *Cryptococcus* is subjected to drastic changes in CO_2 levels from 0.0036% (ambient air) to 5%. High CO_2 levels also induce the capsule. To survive in low CO_2 conditions, *Cryptococcus* employs carbonic anhydrases, which catalyze the reversible interconversion between CO_2/H_2O and HCO_3^-/H^+ that occurs spontaneously under high CO_2 conditions (Bahn et al. 2005a). Therefore, carbonic anhydrases do not affect virulence of *Cryptococcus* within the host, but may influence its survival on the outer surface (e.g., the skin) of the host and in the natural environment. Similar carbonic anhydrases are observed in *C. gattii*, but their functions are not known.

CONCLUDING REMARKS

The *Cryptococcus* field has made significant progress on all fronts, which includes basic biology, laboratory identification of different molecular types, mechanisms involved in pathogenesis, fungal–host interactions, and challenges in adaptive resistance incurred during long-term therapy for improved clinical outcome. A recent report on the detection of cytokine neutralizing autoantibodies in otherwise immunocompetent patients with *C. gattii* infection suggests the need of expanded immunological tests to uncover unknown risk factors in otherwise healthy cryptococcosis patients. There are a number of genetically diverged subspecies within each agent of cryptococcosis. Accurate identification of the subspecies is not only of taxonomic interest but may be clinically relevant because patients infected by these subspecies could have varying risk factors.

Figure 5. (*Continued*) Rim101 is a PKA-dependent transcription factor, which undergoes proteolysis for activation. Ras1 signaling bifurcates into Rac1, a G protein of the Rho family, and Cdc24, a guanine nucleotide exchange (GEF) factor. The calmodulin protein Cam1 binds Ca^{2+} via four EF-hand motifs and activates the Ser/Thr-specific phosphatase, calcineurin, which consists of Cna1 (a catalytic A subunit) and Cnb1 (a regulatory B subunit). The UPR pathway is composed of the Ire1 kinase, the bZIP transcription factor Hxl1, and their target genes, including *KAR2*. The described component functions of the signaling pathways are mainly derived from *C. neoformans* var. *grubii*. The components characterized in both *C. gattii* as well as *C. neoformans* are indicated in black boxes. The functions of these components in *C. gattii* are marked by a red line. Dotted arrows or line indicate potential but unclear regulation.

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