Dimorphism and haploid fruiting in *Cryptococcus neoformans*: Association with the α -mating type

(monokaryotic/dikaryotic/clamp connection/hyphae)

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ABSTRACT *Cryptococcus neoformans* is a major opportunistic fungal pathogen in AIDS and other immunosuppressed patients. We have shown that wild-type haploid *C. neoformans* can develop an extensive hyphal phase under appropriate conditions. Hyphae produced under these conditions are monokaryotic, possess unfused clamp connections, and develop basidia with viable basidiospores. The ability to undergo this transition is determined by the presence of the α -mating type locus and is independent of serotype. The association of the hyphal phase with the α -mating type may explain the preponderance of this mating type in the environment and the nature of the infectious propagule of *C. neoformans*.

Since the discovery of the sexual or perfect state of the human fungal pathogen *Cryptococcus neoformans* by Kwon-Chung (1, 2), it has been considered a bipolar heterothallic basidiomycete with two mating types, *a* and α (*MATa* and *MAT α*). The imperfect state grows as a budding yeast. Under suitable environmental conditions, fusion between the two mating types occurs and generates the perfect state, *Filobasidiella neoformans*. The perfect state is characterized by transient dikaryotic hyphae with typical basidiomycetous clamp connections. Basidia are formed at the hyphal termini wherein karyogamy and meiosis occur. Continuous post-meiotic replication and basidiosporogenesis result in four long chains of basidiospores on the basidial surface (2). Upon germination, the haploid spores regenerate the imperfect yeast state.

The various components of the life cycle of *C. neoformans* have been well characterized by both genetic and ultrastructural studies (2, 3). Genetic analysis of the individual products of meiosis (basidiospores) has demonstrated 1:1 segregation of mating types in laboratory crosses. However, clinical and environmental isolates are predominantly of the α -mating type, ranging from 40:1 ($\alpha:a$) for environmental isolates to 30:1 for clinical isolates (4–7). These results suggest that clinical and environmental isolates do not represent sexually-reproducing populations of *C. neoformans*. It has also been shown that α cells are more virulent than *a* cells (8). This increased virulence could explain the preponderance of the α -mating type in clinical samples. There is, however, no adequate explanation for the overrepresentation of α in environmental samples.

Infection with *C. neoformans*, like many other systemic fungal pathogens, begins with inhalation and is first established in the lungs. Effective penetration to the lung parenchyma requires particles $<2.0\ \mu\text{m}$ in diameter (9). However, yeast cells are 4–8.0 μm in diameter or larger, if one includes the polysaccharide capsule. It has been demonstrated that aerosolized particles $<2.0\ \mu\text{m}$ can cause cryptococcosis in experimental animals (10). This observation was attributed to yeast cells that had become desiccated and, as a result, were small enough to penetrate into the alveoli. However, in the labora-

tory, desiccated yeast cells display very poor viability, an observation confirmed for environmental isolates as well (11). Therefore, the desiccated yeast cell is an unlikely cause of cryptococcosis. On the other hand, *C. neoformans* basidiospores are $1.8 \times 2.0\ \mu\text{m}$ in size, present on aerial hyphae, easily aerosolized, infectious, and much more resistant to desiccation than yeast cells (12). These characteristics would appear to favor the basidiospore over the yeast cell as the infectious form.

The ability of hyphae to form fruiting body-like structures in the vegetative phase is called monokaryotic, homokaryotic, or haploid fruiting (13). Although haploid fruiting is common in the higher basidiomycetes (13–15), it has not been characterized in *C. neoformans*, and association with mating type has never been described. Recent observations made in this laboratory and a reassessment of past studies (5, 10, 16) allow us to propose a new hypothesis for the biased mating type ratios and the nature of the infectious propagule of *C. neoformans*. We show here that α strains of *C. neoformans* can undergo a true dimorphic transition from a haploid yeast phase to a hyphal phase from which vegetative growth can continue indefinitely. The hyphal phase is induced by nitrogen starvation on a solid surface. Basidia bearing viable basidiospores are also produced, all of which are α in mating type. The ability of α but not *a* cells to form basidiospores under these conditions provides an attractive explanation for both the mating type bias and the nature of the infectious propagule of *C. neoformans*.

MATERIALS AND METHODS

Strains. The strains used in this study are listed in Table 1. Auxotrophic strains were derived from JEC20 (*MATa*) and JEC21 (*MAT α*). JEC20 and JEC21 are a well-characterized, haploid, congenic pair of prototrophic laboratory strains that presumably differ only at the mating type locus (17).

Media. Media components obtained from Difco were Bacto-agar, Noble agar, yeast nitrogen base (YNB) without amino acids, YNB without amino acids and without ammonium sulfate (YNB-AS), yeast extract, and peptone. Other components including amino acids, agarose, carbohydrates, nucleosides, and salts were obtained from Sigma. Yeast extract/peptone/glucose (YPG) contained 1% yeast extract, 2% peptone, and 2% glucose. Synthetic dextrose (SD) contained 6.7 g/liter YNB, 20 g/liter glucose, and appropriate amino acid or nucleoside supplements. V8 juice medium contained 5% V8 juice, 0.5 g/liter KH_2PO_4 , and 4% agar adjusted to pH 7.0 with KOH. Filament agar contained 6.7 g/liter YNB-AS, 0.5% glucose, and 4% Bacto-agar at pH 5.0. Unless otherwise specified, solid media contained 2% Bacto-agar. Manipulations of media conditions were carried out by preparing concentrated stocks of the individual components and diluting to the appropriate final concentrations.

Table 1. Strains used in this study

Strain	Serotype	Genotype
JEC20	D	<i>MATα</i>
JEC21	D	<i>MATα</i>
JEC30	D	<i>MATα lys1</i>
JEC50	D	<i>MATα ade2</i>
JEC229	D	<i>MATα</i>
JEC230	D	<i>MATα</i>
NIH332	D	<i>MATα</i>
NIH424	D	<i>MATα</i>
NIH530	D	<i>MATα</i>
NIH264	D	<i>MATα</i>
NIH430	D	<i>MATα</i>
NIH291	A	<i>MATα</i>
NIH371	A	<i>MATα</i>
NIH201	A	<i>MATα</i>
NIH288	A	<i>MATα</i>
H99	A	<i>MATα</i>
NIH112	B	<i>MATα</i>
NIH189	B	<i>MATα</i>
NIH444	B	<i>MATα</i>
NIH34	C	<i>MATα</i>
NIH401	C	<i>MATα</i>

NIH strains are courtesy of K. J. Kwon-Chung (Laboratory of Clinical Investigation/National Institute of Allergy and Infectious Diseases Bethesda, MD). JEC strains are from the collection of J.C.E. H99 is courtesy of J. Perfect (Duke University Medical Center, Durham, NC)

Optimization of Hyphal Induction. A number of different variables, both physical and nutritional, were tested for their effect on hyphal induction. The physical parameters included agar concentration, incubation temperature, moisture content, solid or liquid growth substrate, pH, and to a limited extent, oxygen tension. Nutritional factors included agar type, nitrogen concentration and source, carbohydrate concentration and source, and seed culture conditions.

Microscopy. Hyphal development and basidiospore production from haploid yeast cells were followed by patching approximately 1×10^7 cells of JEC21 onto filament agar plates and incubating at 25°C. Micrographs were taken directly from the plate with an Olympus BX-40 microscope. Strains JEC21 and NIH430, which produced the most extensive hyphae and basidiospores in a standard cross, were used for microscopy of dikaryotic hyphae. An agar block was removed from the hyphal region of this cross and lightly touched to a microscope slide containing a thin layer of 2% filament agar. The slide was placed over glass rods inside a Petri dish containing water and incubated at 25°C for 4–5 days. Monokaryotic hyphae were prepared from strains JEC229, JEC230, or JEC21 by patching these isolates onto filament agar plates and incubating for 4–5 days at 25°C.

Nuclei and septa were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI; Sigma) and Calcofluor White M2R (Calcofluor; Sigma) without prior fixation using a modification of the procedure by Fischer and Timberlake (18). Microscope slides containing growing cultures of dikaryotic hyphae were submerged in staining solution (2 μ g/ml DAPI/1 μ g/ml Calcofluor in 3.7% formaldehyde) for 5–10 min at room temperature prior to microscopic examination. Nuclei and septa of monokaryotic hyphae were examined by scraping the edge of a patch and suspending the material in a drop of staining solution on a microscope slide. The slide was incubated 5 min at room temperature prior to microscopic examination. Stained preparations were visualized and photographed with a Zeiss IM35 inverted microscope.

Genetic Manipulations. Crosses were performed on V8 juice agar (19). After 5–7 days, individual basidiospores were dissected by micromanipulation onto YPG plates. Strains

JEC20, JEC21, JEC50, and JEC30 were used to assess linkage of haploid fruiting to mating type. Strain JEC21 was used for mating type determination of basidiospores produced by haploid fruiting.

RESULTS

Discovery of the Haploid Hyphal Phase of *C. neoformans*.

The recognition of hyphae production from haploid yeast cells was made during the course of a series of transformations intended to optimize conditions for gene disruption in *C. neoformans*. A number of transformants from a disruption experiment were patched onto YNB-AS medium. After 72–96 hr it was found that the majority of the patches had visibly rough edges that were unlike the typical smooth margins of yeast colonies. Repatching to fresh YNB-AS again yielded this morphology. Microscopic investigation revealed that the rough appearance was due to the presence of abundant hyphae. The isolates were confirmed to be *C. neoformans* by melanin production on bird seed agar, lack of growth on lactose, and the presence of urease. When the hyphae-forming isolates were patched onto YNB, hyphae were not observed, only typical yeast-like colonies. YNB-AS was then tested to see if it would support hyphal growth in untransformed strains. Hyphae were indeed produced by these strains demonstrating that this characteristic was not a transformation-mediated phenomenon. These observations suggested that hyphae production in haploid strains of *C. neoformans* was an innate characteristic that could be influenced by growth conditions.

The Hyphal Phase in *C. neoformans*. Fig. 1 shows *C. neoformans* growing vegetatively as hyphae. The hyphal phase is induced on filament agar and is characterized by extensive branched hyphae that grow on and into the agar. Occasional aerial hyphae can also be observed. Numerous blastospores can be seen that bud directly off the sides of each hypha. Basidia with four chains of viable basidiospores are produced, although the frequency is much lower than in a standard *MAT α -MAT α* cross and, on average, only 5–10 spores per chain were observed.

FIG. 1. Dimorphism and haploid fruiting in *C. neoformans*. (A) Colony morphology of strain JEC21 (*MAT α*) grown on filament agar for 8 weeks. (Bar = 3.0 mm.) (B) JEC20, a *MAT α* strain grown under the same conditions as A. (Bar = 3.5 mm.) (C) Strain JEC229 (*MAT α*) grown on filament agar for 11 days showing hyphae and blastospores. (Bar = 180 μ m.) (D) JEC229 grown for 11 days on filament agar. A single basidium bearing four chains of basidiospores (arrow) is clearly visible. (Bar = 20 μ m.) (E) Same as D. Arrow designates a single chain of basidiospores. (Bar = 20 μ m.)

Optimum Conditions for Hyphal Induction. Two laboratory strains of *C. neoformans* (JEC21 and JEC20) were selected for optimization of hyphal induction. A number of parameters were found to influence the production of hyphae, but hyphae were only seen in JEC21, an α strain. The medium that induced the most extensive hyphae was called filament agar and consisted of 4% Bacto-agar, 0.5% glucose, and 6.7 g/liter YNB-AS with the pH adjusted to 5.0. The optimum concentration of YNB-AS was approximately four times the manufacturer's recommended amount. This concentration was subsequently found to be identical to the concentration first reported by Gimeno and Fink in their study of pseudohyphal growth in *Saccharomyces cerevisiae* (20). Plates needed to be dry with no visible condensation on the lid (dried 3–4 days on the bench or 1–2 hr in a hood). The seed culture was best if grown for 48 hr on YPG agar and inoculated onto filament agar by streaking. Incubation at 25–30°C was found to be optimal. In some cases, induction of hyphae could be enhanced by transferring 2–3 day old yeast cells growing on filament agar to new filament agar. Heat shock and cold shock did not induce hyphae, although the absence of hyphae may have been more a function of using a water suspension than the specific insult, since plating water suspensions of unshocked yeast cells severely depressed hyphal induction. In fact, liquid medium consisting of various dilutions of supernatants from washed agar did not support hyphal growth. This observation suggests that available water, or lack thereof, may be an important regulator of the hyphal phenotype. Covering inocula with coverslips, stabbing cells into the agar, or embedding cells in molten medium generally resulted in decreased or absent hyphae. The nature of the carbon source had no effect. All carbon sources that were tested (glucose, sucrose, maltose, galactose, mannitol, and inositol) supported hyphal growth, although concentrations higher than 0.5% decreased the amount of hyphae produced. Other factors such as charcoal or EDTA, which have been effective in inducing hyphae in *Ustilago* (21, 22), did not have any effect on *C. neoformans*.

The type and concentration of agar were also found to be critical. Four percent Bacto-agar was optimal. Noble agar

supported hyphae production, but to a lesser degree. Agarose did not yield any hyphae, suggesting that one or more inducing factors are present in Bacto-agar. In fact, 4% water agar was sufficient to induce hyphae. The addition of ammonium sulfate as a nitrogen source completely repressed hyphae, even when the concentration was reduced to 0.1 g/liter, which is 2.0% of normal YNB concentration. Incorporation of proline into filament agar at concentrations ranging from 5–100 mM neither induced nor repressed hyphae, although hyphae that were produced were completely overgrown after 48 hr by yeast cells.

Comparison of Dikaryotic and Monokaryotic Hyphae. Monokaryotic and dikaryotic hyphae were compared by fluorescence microscopy after simultaneously staining with DAPI and Calcofluor. Fig. 2 *A* and *B* shows hyphae produced by crossing JEC21 and NIH430. Typical basidiomycetous clamp connections are observed, and two nuclei per hyphal cell are clearly evident. Fig. 2 *C* and *D* shows hyphae produced by JEC229. Clamp connections are not fused (false clamps), and only a single nucleus per cell is visible. However, the possibility that the monokaryons formed during haploid fruiting are diploid cannot be ruled out at this time.

Another difference between monokaryotic and dikaryotic hyphae was the abundance of blastospores. Dikaryotic hyphae produce few blastospores. In contrast, blastospores produced by monokaryotic hyphae appear very early in development and are produced continuously. Eventually, enough are produced to form microcolonies, which appear along each hypha at regular intervals (Fig. 3) and often cover the whole surface of the hypha. When these cells are transferred to YPG or SD agar, they give rise to budding yeasts. If they are transferred to new filament agar, they give rise to both yeast and hyphae.

Dimorphism and Monokaryotic Fruiting Are Linked to *MAT α* . Multiple strains representing all four serotypes of *C. neoformans* were analyzed for the ability to produce hyphae. The majority of the *MAT α* isolates that were tested were able to produce hyphae on filament agar. No hyphae were detected in any *MAT α* isolates. There were different degrees of hyphal production with some strains producing more extensive hy-

FIG. 2. Dikaryotic versus monokaryotic hyphae. (*A* and *B*) Dikaryotic hyphae produced by crossing JEC21 (*MAT α*) and NIH430 (*MAT α*). Clamp connections (black arrows, *A*), calcofluor-stained septa (thick white arrows, *B*) and pairs of DAPI-stained nuclei (thin white arrow, *B*) are evident. (*C* and *D*) Monokaryotic hyphae produced by strain JEC230 (α). Unfused clamp connection (black arrow, *C*), calcofluor-stained septa (thin white arrow, *D*) and single DAPI-stained nuclei per hyphal compartment (thick white arrow, *D*) are evident. (Bars = 20 μ m.)

FIG. 3. Blastospore development in *C. neoformans*. (A–C) Blastospore development in JEC229 (*MATα*) at 2, 3, and 7 days, respectively. Arrowheads designate single hyphae and arrows designate emerging blastospores. (D) JEC20 (*MATα*) after 7 days. (Bars = 30 μ m.)

phae than others. Strains NIH424(α), NIH530(α), JEC229(α), JEC230(α), and JEC21(α) produced extensive branched hyphae, basidia with basidiospores, and abundant blastospores. Strains H99(α), NIH444(α), NIH34(α), NIH112(α), NIH189(α), and NIH401(α) produced less extensive hyphae with some branching. Blastospores were produced but basidiospores were not detected. Strains NIH288(α), NIH332(α), NIH291(α), NIH371(α), NIH201(α), JEC20(α), NIH264(α), and NIH430(α) produced either a single hypha or none at all. Blastospores and basidiospores were not observed in these isolates.

To confirm the association of haploid fruiting and mating type, crosses between JEC20 and JEC21 and between JEC30 and JEC50 were performed on V8 agar. The mating type of each meiotic segregant was determined by backcrossing. Table 2 shows the results of these crosses. None of the *MATα* progeny and all of the *MATa* progeny were able to undergo haploid fruiting, showing that the ability to undergo haploid fruiting lies within, or is very tightly linked to, the *MATα* locus.

One isolate, JEC21, was selected for analysis of basidiospore progeny produced by haploid fruiting. Nineteen of 47 single basidiospores germinated on YPG agar. These progeny were tested for mating type by crossing to both JEC20 and JEC21. All were found to be *MATα*, ruling out the possibility of a mating type switch as an explanation for basidiospore production. These data, in addition to the extensive characterization of this isolate in earlier studies (17, 23), also rule out the possibility that JEC21 is naturally diploid or aneuploid.

DISCUSSION

Dimorphism in *C. neoformans*. Dimorphism is the ability of fungi to grow vegetatively as either yeast or hyphae (24) and is common among the pathogenic fungi. Many human fungal

pathogens including *Candida albicans*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*, and *Sporothrix schenckii* exhibit some form of dimorphism with most undergoing a hyphal to yeast transition in the host. Such transitions are thought to be triggered by a combination of environmental cues including temperature, oxidative stress, and nitrogen source (25, 26). *C. neoformans*, although capable of producing true hyphae, was not generally considered to be dimorphic because the hyphal phase is usually transient and is observed exclusively during mating. The few reports of hyphal production in *C. neoformans* occurring under nonmating conditions (5, 16, 27, 28) are the result of diploids or aneuploids which are self-fertile (5, 29).

Prior to the current report, hyphae production in haploid *C. neoformans* was noted under two conditions. In *MATα* cells transformed with the *MFα* pheromone gene, hyphae are produced in starvation medium (19). These hyphae are aseptate and largely devoid of nuclei. It is likely that these hyphae probably represent exaggerated conjugation tubes similar to those seen in *Tremella* spp. (30), *Rhodospiridium toruloides* (31), and *Ustilago maydis* (32). Wild-type *MATα* cells also produce short hyphae under stress conditions (nitrogen deprivation, toxins), but these hyphae are rare, only 20–50 μ m long, and do not progress (J.C.E. and K. J. Kwon-Chung, unpublished observations). In both of these instances, the hyphae show no evidence of basidia formation. These observations, and the data presented in this paper, strongly suggest that the ability to produce hyphae and basidiospores in the absence of mating is associated with the α -mating type. A more complex issue is how haploid fruiting and hyphae production effect pathogenesis in *C. neoformans*.

The Nature of the Infectious Particle in Cryptococcosis. An evaluation of earlier studies in light of the present data provides new insight as to how *C. neoformans* infections may be initiated. These early studies of *C. neoformans* pathogenesis suggested that desiccation of yeast cells results in enough shrinkage so that they are small enough to penetrate into the alveoli when inhaled (6, 10, 33, 34). In a study by Neilson *et al.* (10), which was conducted by incubating *C. neoformans* in sterilized soil and assaying for viability and particle size by air sampling, it was found that during the first week of incubation, a substantial reduction in viable cell number occurred. As incubation time increased, the number of viable particles <2.0 μ m in size increased, whereas the number of viable particles >2.0 μ m decreased. These authors suggested that *C. neoformans* cells either shrink and/or lose their capsule thereby becoming small enough for alveolar deposition. Additional evidence in support of the yeast cell being the infectious particle was proposed by Jong *et al.* (6) who analyzed mating type ratios in a tower that was highly contaminated with pigeon dung. In spite of the fact that *C. neoformans* was present at very high densities (3×10^6 organisms per g), all 193 isolates tested were α in mating type. These authors concluded that in the absence of *a* cells, mating could not occur and, as a result, basidiospores would not be produced. They reasoned, therefore, that the yeast cell must be the infectious particle because basidiospores would not be present in a population composed of a single mating type. Ruiz *et al.* (34) studied the relationship of moisture to-viability and identified two subpopulations that showed size-dependent susceptibilities to drying. One population, 3.3–11.0 μ m in size, experienced a 63–79% decrease in viability over the study period. The second population, 0.65–

Table 2. Segregation of haploid fruiting phenotype with mating type

Cross	No. basidiospores	% Germinated	<i>MATα</i> : haploid fruiting	<i>MATa</i> : haploid fruiting
JEC21 $\times$ JEC20	59	50.8	19 (0/19)	11 (11/11)
JEC50 $\times$ JEC30	47	82.9	20 (0/20)	19 (19/19)

3.3 μM in size, showed no decrease in viability over the same period. The conclusion reached by these authors was that drying has a severe effect on *C. neoformans* viability, although this effect is reduced for cells < 3.3 μM in size.

Our findings indicate that the small particle responsible for infection is not a desiccated yeast cell, but instead is an asexual basidiospore produced during haploid fruiting. These conclusions are based on a number of observations made in this study that are consistent with the data cited above. For example, microscopic analysis of *C. neoformans* grown on filament agar reveals that hyphae generally are present after 1–3 days, blastospores and basidia after 2–4 days, and basidiospores after 3–6 days. The increase in colony forming units and particles < 2.0 μM in size observed by Neilson *et al.* (10) coincides with our observation in which microcolonies (which would contribute to colony forming units) and basidiospores (which would contribute to < 2.0 μM particles) become abundant. The decrease in viability of larger cells (> 3.3 μM) and resistance of smaller cells (< 3.3 μM) to drying reported by Ruiz *et al.* (34) are easily explained if the smaller particles are basidiospores which, like most spores, are more resistant than yeast cells to extreme environmental conditions. In fact, we have observed drastic decreases in the viability of yeast cells on filament agar. After 2 weeks of incubation, > 99% of cells from the initial patch were dead. Finally, the conclusion by Jong *et al.* (6) that the yeast cell must be the infectious particle because α cells were not recovered in their study does not contradict the theory of the basidiospore as the infectious particle if basidiospores arise by haploid fruiting. Haploid fruiting may also explain the mating type bias. There is no physiological difference (i.e., biochemical profile, growth rate, etc.) between α and α cells. Therefore, it is difficult to reconcile the biased mating type ratios seen in nature. If α cells were less fit, this genotype would have been selected against long ago. A simpler explanation is that under certain conditions, α cells are produced in much greater numbers than α cells. These conditions may occur during the course of haploid fruiting. In addition to the ability to produce hyphae and basidiospores, the production of large numbers of blastospores may provide an additional unknown survival advantage while inadvertently skewing the mating type ratio in favor of α .

The Role of Hyphae and Haploid Fruiting in *C. neoformans* Biology. Most systemic fungal infections rely on accidental hosts. As is often the case with microbes that possess complex life cycles or morphologies, infection may result when increased host susceptibility intersects with a specific stage of microbial development. Although very little is known regarding what form *C. neoformans* assumes in nature, it now seems apparent that a hyphal phase is an intimate aspect of *C. neoformans* biology. In *C. neoformans*, haploid fruiting may function in a manner similar to other basidiomycetes. Stahl and Esser (14) suggested that haploid fruiting ensures spore production if the ability to fuse with the opposite mating type is lost, thereby increasing the chances of survival. This characteristic would be important in organisms such as *Ustilago maydis*, which requires a dikaryon to complete the life cycle (22). Circumstances that are consistent with this theory were demonstrated by Jong *et al.* (6), who could not recover a single α isolate out of almost 200 environmental isolates of *C. neoformans*.

It is also quite possible that haploid fruiting in *C. neoformans* may be a consequence of, and not a reason for, the ability to produce hyphae. In culture at least, hyphae are induced under conditions similar to pseudohyphae formation in *S. cerevisiae* (20). Gimeno and Fink suggest that the production of pseudohyphae in *S. cerevisiae* enhances foraging capacity under low nutrient conditions (35). We have seen, in addition to

extensive lateral hyphae, hyphae that penetrate through the agar to the bottom of the Petri dish and then proceed along the dish–agar interface. In *S. cerevisiae*, which normally does not produce pseudohyphae, extreme conditions are required to induce this morphology. The same seems to be true for *C. neoformans*. Filament agar provides a severe stress to the organism, possibly due to nitrogen starvation and/or water deprivation. It is likely that one or both of these stimuli are sufficient to induce hyphae formation. However, these are laboratory conditions and although they are undoubtedly stressful, they cannot begin to approach the severity of the environment. It is likely, therefore, that the hyphal phase is far more prominent in *C. neoformans* biology than we can presently measure.

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