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Variability of phenotypic traits in *Cryptococcus* varieties and species and the resulting implications for pathogenesis

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Abstract

Variability of phenotypic characteristics in *Cryptococcus neoformans var. grubii* and var. *neoformans* as well as *Cryptococcus gattii* can have diverse effects on the virulence of these fungi and are thus important for pathogenesis. This article summarizes the diverse phenotypic changes that these fungi can manifest. We divide changes into those that affect the entire fungal population and are predominantly induced by environmental signals, and those that involve subpopulations of the fungal population and have to be selected. Last, the article summarizes the experimental evidence that epitopes on the polysaccharide capsule also vary, which may have implications for the pathogenesis as these findings would further diversify the fungal population.

Keywords

pathogenesis; phenotypic switching; phenotypic variability

Cryptococcus neoformans var. *neoformans*, var. *grubii* and *Cryptococcus gattii* are ubiquitous encapsulated yeasts that cause chronic meningoencephalitis, pneumonia and disseminated disease in both immunocompetent as well as immunocompromised individuals. More than 600,000 people worldwide die of cryptococcosis per year [1] and prospective studies have suggested that 10–20% of all deaths in HIV-infected patients in Africa are attributable to cryptococcal infection [2,3]. The majority of clinical isolates are *C. grubii* although both *C. neoformans* [4,5] and *C. gattii* infections also occur [6].

Cryptococci are environmental microbes that accidentally invade the host. Virulence traits of these fungi constitute standard survival mechanisms that are advantageous in the host [7]. Phenotypic variation allows rapid adaptation to a constantly changing environment. Variegated expression of genes can be the result of many different mechanisms and contributes to heterogeneity within populations of genetically identical fungal cells. Such variation affects the host–pathogen interaction and may facilitate evasion of host defenses. Phenotypic changes are common in fungi and are induced by different mechanisms. In this article we will discuss different forms of phenotypic changes in *C. neoformans, C. grubii* and *C. gattii*. We divide them into changes that involve the whole fungal population, those that involve only a subset and those showing variability among individual cells. Global changes of phenotypic traits and classic cellular morphology involve the entire fungal population. They are induced by

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environmental signals such as iron concentration, starvation, temperature, pH change and mating-associated factors. Examples of global changes in phenotypic traits include induction of the polysaccharide capsule and melanization. Examples that involve morphological transitions are hyphal formation and sporulation. Biofilm formation also results from a global phenotypic change to an adherent population, although individual cells within the biofilm may manifest different changes. Phenotypic switching (PS) only occurs in a small percentage of cells. PS of colony morphology is a random, reversible process and is usually not induced by external signals. Last, we discuss the evidence for antigenic variation in the polysaccharide capsule of *C. neoformans*.

Phenotypic traits & morphological transitions of *Cryptococcus* species

Cellular changes in fungi can be quite striking and are relevant for pathogenesis in most fungi, including *Cryptococcus* species and varieties. Examples in other fungi include 'phase variation', which allows dimorphic fungi such as *Histoplasma capsulatum* and *Coccidioides immitis* to grow as filamentous saprophobic molds at ambient temperatures and as yeasts within the mammalian host [8,9]. Another example is the yeast–hyphal transition in *Candida* species, whereby the yeast form is associated with dissemination, and the hyphal form with tissue invasion [10–12].

As part of its lifecycle, *C. neoformans* (both var *grubii* and var *neoformans*) and *C. gattii* manifest both unique phenotypic traits and also morphological transitions common to many fungal species. These processes involve remodeling of the outer surface. *C. neoformans, C. grubii* and *C. gattii* have a somewhat unique morphology because, unlike other fungi, they are encapsulated. The induction and remodeling of the cryptococcal polysaccharide capsule is a clinically highly relevant change analogous to capsule induction in encapsulated bacteria [13]. In addition, melanization and biofilm formation are phenotypic traits that can be modified. Change involves the entire fungal population and affects pathogenesis however, it is not unique to these fungal species. Hyphae, pseudohyphae and spore formation occur during mating and monokaryotic fruiting but are not commonly found *in vivo*. Spore formation is essential to the pathogenesis of cryptococcosis, as spores are the infectious propagule that is inhaled from the environment [14–16]. Expression of morphology-specific genes, many of which are glycosylphosphatidylinositol-anchored, is controlled by diverse signal transduction pathways in *C. neoformans* and *C. grubii*, which is reviewed in more detail elsewhere [17–19]. The mitogen activated protein kinase [20–22], high osmolarity glycerol [23,24], calcineurin [25, 26], cyclic AMP [27–29], regulation of Ace2p activity and cellular morphogenesis [30] and sterol regulatory element binding proteins [31,32] pathways respond to environmental signals and are key regulators of cellular morphogenesis and proliferation. They are generally conserved among fungi; however, significant differences in these regulatory networks occur even in the closely related *C. grubii* and *C. neoformans* [23,33]. In this section we will only briefly review morphological transitions as they have been reviewed in detail elsewhere [34, 35], and will instead focus on reviewing phenotypic traits in *C. neoformans* varieties and species that can be modified during chronic cryptococcosis.

Capsule induction

The cryptococcal polysaccharide capsule is composed primarily of two polysaccharides, glucuronoxylomannan (GXM) and galactox-ylomannan (Figure 1A). The size of capsule is highly variable and dependent on environmental conditions [36]. Within a fungal population, size variability is observed and for some strains the capsule size is more variable and can change during chronic infection [37]. Recent investigations have demonstrated that spores are also coated with GXM on the surface [14]. Most investigations on the polysaccharide capsule are performed with *C. neoformans* or *C. grubii* strains. *In vivo* infection studies demonstrated that the lung environment is a powerful inducer of capsule growth [38]. Capsule enlargement is a

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controlled reproducible event and does not occur over a certain capsule size in serially passaged yeast cells [39]. Capsule changes during infection are associated with differences in the binding pattern of capsule-specific antibodies [40]. The *in vivo C. grubii* strain H99 has a larger capsule than cells of *C. gattii* strain R265, which may explain why these strains elicit different immune responses [41].

Serum [42], high CO_2 concentration [43], iron deprivation [44,45] and slight alkaline conditions can facilitate capsule enlargement [46]. Many of these inducing conditions are present in the human host. It is thought that capsule growth requires the acidic group of glucuronic acid residues to be ionized, possibly so that they can react with divalent cations for capsule assembly [47]. The ability of cryptococcal strains to respond to serum is affected by species and variety of the strain [48]. Both antibody (Ab) as well as complement-mediated *in vitro* phagocytosis assays demonstrate that increased capsule volume of both *C. neoformans* and *C. gattii* strains negatively affects phagocytosis, which is a major defensive mechanism in the host [48]. In addition, if *C. neoformans* is carried across the blood–brain barrier in phagocytic cells as suggested, then we would expect that capsule size would affect dissemination [49].

Several genes that are either directly or indirectly involved in capsule biosynthesis have been identified. Most of the null mutants of these genes either lack a capsule altogether or are hypocapsular (for a review see [50]). Accordingly, the majority of these mutants exhibit attenuated virulence when tested in animal models. Of particular interest is the *ALL1* gene, which is regulated by PS and thus during chronic infection modifies capsule size in a complex manner. The capsule of the *all1*Δ mutant in *C. neoformans* is slightly larger at baseline, but it only induces to approximately two-thirds of the wild-type capsule volume both *in vivo* and *in vitro*. Despite the slightly impaired induction, this mutant exhibits enhanced virulence both in the pulmonary as well as in the intracisternal infection model. The mutant grows at the same rate *in vivo* but affects the host–pathogen interaction, thereby impairing clearance [51]. Capsule formation is a complex and coordinated biosynthetic pathway. Well-studied signaling pathways are involved and include the protein kinase/cAMP and protein kinase C/mitogen activated protein kinase pathways [52]. The most rigorously studied transduction pathway is the cAMP pathway, for which elements have been cloned and disrupted [53]. The protein kinase C pathway does not play a role in capsule induction. Its influence on capsule formation is likely to be indirect, through impaired cell wall organization, as suggested by work in *pkc1*Δ null mutants [54,55].

The process of capsule assembly has been the subject of debate and the focus of many different studies. The first study used a monoclonal antibody (mAb) and radioactive xylose [56] and concluded that capsule grows by accumulation in the inner part of the capsule, displacing the old capsule to the edge. A different approach using a marker that covalently bonded to the capsule [57,58], suggested that during capsule induction the old polysaccharide fibers remained in a position close to the cell wall. Results from light scatter analysis of capsule-associated polysaccharide implied that capsule growth was achieved by the addition of molecules with a larger effective diameter. It showed that some polysaccharide molecules span the entire diameter of the capsule [59]. It is noteworthy that biophysical investigations have revealed differences in the molecular weight of GXM among strains and in switch variants [60] and also changes of GXM viscosity, which could potentially promote the development of high intracerebral pressure *in vivo* [61,62].

Hyphal formation

Cryptococcus neoformans, *C. grubii* and *C. gattii* typically grow as a haploid yeast, but undergo hyphal differentiation in response to environmental stresses or when confronted with an appropriate mating partner (Figure 1B) [15,63,64]. Diploid cryptococcal strains have been

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described [65] as well as same-sex mating [66]. Furthermore, different species and strains differ in their ability to mate [67,68]. Hyphal transitions are required for spore formation, which are proposed to be the infectious propagules. They can produce lethal infection in mice at very low doses, are resistant to desiccation and nutrient deprivation, easily aerosolized, and are of an ideal size to lodge in the alveoli of the lung [16]. Spores from the three species differ in size [14]. In addition, *C. neoformans* produce both intercalary and terminal chlamydospores, which are capable of generating new branches and yeast cells. Their relevance for pathogenesis is unknown [69] and it is not known if they occur in all cryptococcal species.

Factors that enhance hyphal growth include ambient temperatures, nitrogen starvation, dehydrated substrates, darkness and the presence of mating pheromone [70,71]. Dikaryotic hyphae contain two parental nuclei per hyphal compartment, fused clamp connections and are produced during sexual reproduction between a and α cells [63,72]. Monokaryotic hyphae contain one nucleus per hyphal compartment, unfused clamp connections and are produced during fruiting of α and some a cells, mostly in the setting of high ammonium sulfate levels [15,73]. Thus, both pathways lead to hyphal growth and basidiospore production and promote environmental distribution of *C. neoformans* and ultimately the incidence of cryptococcosis. In addition, cryptic same-sex reproduction can contribute to the production of infectious spores [66,74]. The biological importance of pseudohyphae is not well understood and its definition is unprecise. The observation that cryptoccocal cells in the pseudohyphal form resist phagocytosis by soil ameba [75], the natural predators of cryptococci, suggests that pseudohyphae formation might constitute a survival mechanism [75,76]. Although *C. neoformans* encounters low glucose conditions in the CNS, India ink preparations of spinal fluid from infected patients usually show mostly yeast forms and no hyphal forms. However, occasional reports of abnormal forms can be found and may be under-reported [77–79]. This suggests that suppression of filamentous growth may be required for growth in the host niche similar to dimorphic fungi [80].

Melanization

Melanization leads to a change in a phenotypic trait that is not necessarily macroscopically evident. It occurs in several fungal species including both *C. neoformans* and *C. gattii*. Melanin production requires *o*-diphenolic or *p*-diphenolic compounds such as L-3,4-dihydroxyphenylalanine as a substrate. The key enzyme laccase (CNLAC1) has been characterized [81–84] and promotes virulence by inhibiting the oxidative burst in the phagosomal space of macrophages [85]. Deletion mutants of *CNLAC1* manifest decreased virulence [86] and melanin-deficient mutant strains are avirulent in murine models of cryptococcal infection [50–51]. Several complex pathways that control the biosynthesis of melanin have been identified [18,87]. Disruption of a gene encoding a cAMP-dependent protein kinase results in both amelanotic and hypocapsular mutants [88], which highlights that other virulence traits are also regulated but makes it difficult to determine if melanization is important but not essential for virulence. All cryptococcal varities and species melanize, although they may differ in the degree of melanization [89,90]. Only rarely have melanin deficient strains been isolated from human specimens [91]. In addition, staining with melanin-specific reagents revealed melanin in the cell walls of cryptococci in human brain tissue and melanin ‘ghosts’ can be recovered from infected mouse tissue [92]. Interestingly, heterogeneity in melanization is described [92]. These results indicate that *C. neoformans* universally melanizes during human infection and supports the concept that this change in a phenotypic trait may be relevant for pathogenesis.

Biofilm formation

Biofilms are communities of microbes that are attached to surfaces and held together by an extra-cellular matrix, often predominantly consisting of polysaccharides. Biofilm formation is

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a common phenotypic trait in pathogenic yeasts and makes the yeast cells less susceptible to host defense mechanisms [93]. Biofilm-like structures have also been reported in human cases of cryptococcosis on ventriculoperitoneal shunts [94]. *In vitro* experiments have demonstrated that biofilm-associated *C. neoformans* are significantly less susceptible than planktonic cells to azoles, amphotericin B and various microbial oxidants and peptides [95,96]. *C. neoformans* biofilm development is dependent on the release of capsular polysaccharide to create an exopolysaccharide matrix. Biofilm formation can be inhibited by protective antibodies and not by nonprotective antibodies [97]. Antibodies interfere with capsular polysaccharide release from the fungal cell. Interestingly, lactoferrin – an effector molecule of the innate immune system – inhibits bacterial but not fungal biofilm formation [95]. Furthermore, biofilm-like microcolonies are released by macrophages after antibody-mediated phagocytosis of *C. neoformans* (var *grubii* and *neoformans*) and *C. gattii*, which would reduce fungal cell dispersion *in vivo* but would promote cryptococcoma formation [98]. To what extent *in vivo* biofilm formation contributes to treatment failure is still not clear; however, experimental evidence suggests that they impair the host's ability to eradicate *C. neoformans* in collaboration with antifungal treatment

Phenotypic switching

Fungal populations manifest distinct epigenetic states at a low frequency in order to maintain adaptability to a changing environment. Colony switching is defined as the spontaneous emergence of colony variants. This phenomenon is mainly described in yeasts because single colonies are hard to distinguish in molds.

Phenotypic switching in *Cryptococcus* species

Phenotypic switching is described in strains of three species, namely *C. grubii*, (serotype A SB4, J32) [99], *C. neoformans* (serotype D 24067A, RC2) [61] and *C. gattii* (serotype B NP1) [100]. Colony variants arise at a frequency of approximately 1 in 10^4–10^5. Smooth colonies (S and SM) of SB4, 24067 and NP1 have a smooth dome, and mucoid (M and MC) colonies have a shiny and mucoid colony surface. Wrinkled (WR), serrated (C) and pseudohyphal colonies exhibit an irregular dome surface with or without serrated margins and are rarely observed in clinical isolates. Most *C. neoformans* and *C. grubii* strains manifest a smooth most *C. gattii* strains exhibit a mucoid colony morphology.

Cryptococcus neoformans strain (serotype D) RC2 switches consistently between the parent SM to the MC variant and another variant strain of ATCC strain 24067 (24067a) switches from an avirulent SM parent to virulent WR and pseudohyphal variants [61,101]. SB4 and J32 are clinical *C. grubii* strains that also switch but at lower frequencies from S to WR and C (SB4) and M to S (J32) [99]. In *C. gattii* strain NP1 two colony morphologies, one mucoid (NP1-MC) and one smooth (NP1-SM), were isolated from an immunocompetent patient with meningitis. Switching experiments confirmed that they were the result of PS [100]. PS from NP1-MC to NP1-SM occurred at a frequency of 1 in 2×10^5 colonies whereas reversion occurred 1 in 7×10^5. By contrast, the two rates of switching and reversion in the *C. neoformans* strain RC2 are comparable and occur at approximately 1 in 10^4 when 5×10^4 colonies are plated. RC2 is the dominant strain used for research.

Phenotypic switching alters the polysaccharide capsule & other cellular characteristics

All cryptococcal switching strains exhibit changes in the polysaccharide capsule, which is important because changes in this phenotypic trait may affect phagocytosis and rapid destruction by macrophages [102,103]. For RC2 the MC variant has a larger capsule than the SM variant and produces a viscous exopolysaccharide. Hence, in RC2, PS alters biophysical properties of GXM [60,61]. Although not proven, this may result from changes in the spacing

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of charged β-D-glucupyranosyluronic acid (*GlcpA*) along the mannose backbone, which are not apparent by nuclear magnetic resonance and explains why the GXMs of the two switch variants manifest the same nuclear magnetic resonance pattern.

In the switching strains SB4 and 24067a, the biochemical composition of the GXM changes by PS. GXM is composed of linear α-D-manno-pyranan chain with β-D-xylopyranosyl (Xylp) and GlcpA side residues. Six (M1–6) structural reporter groups (SRGs) are defined by amount and position of linked Xylp and GlcpA residues. In *C. grubii* strain SB4 and *C. neoformans* strain 24067A PS results in changes of SRGs [101]. GXMs derived from SB4-C are composed of M2 and M3 SRGs whereas GXM shed by SB4-SM is composed of only M2. It is noteworthy that the addition of Xylp at the 4–0 position in M3 most likely requires activation of different enzymes, that are traditionally thought to be used only by *C. gattii*.

The doubling time of *C. neoformans* strain RC2 is shorter compared with *C. gattii* strain NP1 but in both switching systems the switch variant (RC2-MC and NP1-SM) grows slower when compared with the parent strain (RC2-SM and NP1-MC). MICs of amphotericin B and fluconazole for switch variants are comparable. Both mucoid switch variants (RC2-MC and NP1-MC) have altered cell walls and exhibit increased sensitivity to lysing enzyme. Cell charge and melanization are not affected by PS in the two switch systems; however, biofilm formation can differ in the switch variants [104].

Phenotypic switching occurs *in vivo* & directly affects outcome

One of the strengths of *C. neoformans* as a model organism is that the effects of PS on the pathogenesis of chronic cryptococcosis can be studied *in vivo*. It was demonstrated that PS of RC2 from a SM parent to a MC variant occurs *in vivo* during chronic infection in mice [61]. To avoid contamination of the original inoculum with switch variants, the mice were infected with very low inocula and statistical methods were applied that proved that PS occurred *in vivo* opposed to selection of variants in a heterogenous inoculum. Furthermore, emergence of MC variants was observed in rats that were infected with doses as low as 100 colony-forming units (CFU) [62]. PS occurs at a low rate and in order for switch variants to dominate the pathogen population selection is required. Selection pressure favors the MC switch variant and therefore emergence of this switch variant is only observed in infected mice. Mice infected with MC do not manifest switching to the SM variant, although the *in vitro* frequency of SM to MC is comparable to that for PS from SM to MC in RC2. Highly relevant for the clinical scenario is the finding that treatment with amphotericin or anticapsular mAb can promote the selection of MC variants in mice [105].

Selection pressures can differ among switching strains and depends on the human niche. In murine infection with *C. gattii* strain NP1, both phenotypes NP1-SM and NP1-MC were recovered in the lungs, similar to the cerebrospinal fluid of the patient from which the strain was originally grown. By contrast, from the brains of mice infected intravenously or intratrachealy, only the smooth phenotype was recovered regardless of whether the mouse was infected with the NP1-SM or NP1-MC. This supports the notion that in the patient, the PS occurs after dissemination to the CNS [100]. Thus, in contrast to RC2, both phenotypic switch variants in NP1 appear to have selection pressure and in this strain, PS may be necessary for dissemination to the CNS.

Phenotypic switching alters host pathogen interaction & inflammatory response

Phenotypic switching of *C. neoformans* strains affects virulence by altering the host–pathogen interaction and pathogenesis of the disease. RC2-MC variant is significantly more virulent in all murine and rat animal models. Even in interaction with human monocytes, differences in inflammatory response were noted [106]. In a murine pulmonary infection model, histological

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analysis demonstrated significant differences in the inflammatory tissue response elicited by RC2-SM and RC2-MC. Specifically, at day 14 postinfection, lungs of RC2-SM mice exhibited moderate inflammatory changes with cellular infiltrates composed primarily of lymphocytes and only a few macrophages [61]. These cellular infiltrates progressed to orderly granuloma formation with little concomitant lung damage by day 28 postinfection. This host immune response is distinct from that elicited by RC2-MC at day 14 and involves changes in Th1 and Th2 cytokine production [107]. Here, infected lungs exhibited extensive cellular infiltrates that extend beyond the peribronchial regions, and were predominantly composed of macrophages and neutrophils with only a few lymphocytes. Near the time of death the inflammatory response increased and resulted in extensive destruction of alveolar membranes. Of high clinical relevance is the finding that the RC2-MC variant was able to promote increased intracranial pressure in a rat model of cryptococcal meningitis [62]. In human infection, increased intracranial pressure is the leading cause of high morbidity and mortality [108]. Investigations of lung-associated macrophages derived from RC2 SM- and MC-infected mice demonstrated that macrophages were alternatively and not classically activated. However, they differed in their level of activation. MC-infected macrophages exhibited high arginine production, IL-6 and MCP production and elicited more Th17-excreting T cells [109]. These findings are consistent with findings in other fungal infection models. Although inflammation is an essential component of the protective response to fungi, its dysregulation may significantly worsen fungal diseases and limit protective, antifungal immune responses. As such, the Th17 pathway may play a damage-promoting inflammatory role previously attributed to uncontrolled Th1 cell responses [110–112].

Virulence is also affected by PS of NP1 [100]. Experiments in mice demonstrated that NP1-SM-infected mice survived significantly longer than NP1-MC infected mice in intratracheal ($p = 0.021$) as well as in intravenous ($p = 0.008$) infection models. Consistent with this the CFU in the lung of NP1-SM-infected mice after 14 days was significantly lower ($p \leq 0.03$) than NP1-MC-infected mice. The inflammatory response also differed for the NP1-SM and NP1-MC; however, in this strain a damage-promoting, overstimulated inflammatory response was not documented. Histological analysis of the lung sections demonstrated an appropriate and effective inflammatory response in lung tissue infected with NP1-SM. The mononuclear inflammation was composed of lymphocytes and macrophages. By contrast, the lungs of NP1-MC-infected Balb/c mice exhibited minimal inflammatory response and, consistent with the failure to elicit an inflammatory response, a large accumulation of yeast cells in lakes of polysaccharide consistent with cryptococcomas was observed on lung tissue sections. Histological analysis of the brain of NP1-SM- and NP1-MC-infected mice demonstrated multiple cryptococcomas but the cryptococcomas of NP1-SM-infected mice were smaller and elicited more inflammation in brain tissue. In summary, PS mainly affects virulence by altering the host immune response.

Molecular mechanism of PS

The molecular mechanisms mediating PS in *C. neoformans* are currently not understood. Although karyotype instability was observed in strain 24067A and SB4 [99,113], similar to the switching *Candida albicans* strain 3153A, it could not consistently be correlated with phenotypic variability and was not reversible. PS was associated with the downregulation of genes. These genes were not located in clusters or in telomeric regions but rather distributed across all chromosomes. Most of the regulated genes are not characterized with respect to function. One gene that is among the most prominently downregulated genes in RC2-MC relative to RC2-SM is *ALL1*. Interestingly, null mutants *of ALL1* exhibit similar phenotypic traits to the MC variant. Specifically, *all1*Δ exhibits a slightly enlarged capsule at baseline that sheds a more viscous GXM than the wild-type SM parent. Most importantly, in infection models with *all1*Δ it mimics the hypervirulence of the MC variant including the overstimulated

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host response [51] and the increased intracranial pressure. Also of note was the finding that epigenetic control of the 'phenotypic switch state' appeared to loosen with senescence. Accordingly, it was recently shown that *C. neoformans* cells (RC2) of advanced generational age exhibit up to 11-fold higher switch rates [114]. In addition, some experimental data suggested that older cells may be more resistant to antifungals and phagocytosis and thus have a selective advantage *in vivo* and accumulate. This concept is novel and could have broad implications for the pathogenesis of chronic diseases.

Comparison of PS in *Cryptococcus* with PS in other pathogenic fungi

High-frequency PS has also been studied in diverse *Candida* species, mainly *C. albicans* and *Candida glabrata* [115–118]. The model *C. albicans* strain WO-1 switches between two colony morphologies, namely white and opaque, at a frequency of 1 in 10^4–10^5. Opaque-phase cells are mating competent whereas white-phase cells survive better within the mammalian host, yet can switch to mating-competent cells when required [119]. This phenotypic switch is relevant because clinical *C. albicans* strains undergo white to opaque switching (WOS) if they are homozygous (*a/a* or α/α) whereas heterozygous (*a*/α) strains cannot switch [120,121]. White-phase cells are more virulent in intravenous infection [122] and opaque-phase cells colonize skin more effectively [123]. WOS also affects other virulence traits, including the bud–hyphal transition [124], sensitivity to neutrophils and oxidants [125], antigenicity [126], adhesion [127], secretion of proteinase [128,129], drug susceptibility and phagocytosis by macrophages [130]. All of these altered traits can potentially affect survival in the mammalian host. *Wor1* has been identified as a master regulator of WOS, as its deletion blocks opaque cell formation [131]. Interlocking feedback loop networks maintain the epigenetic state of switch variants through cell divisions. This circuit is not present in closely related fungi and could be a recent adaptation in the mammalian host [132,133].

Candida glabrata, another pathogenic yeast, undergoes 'core switching' [118] on agar containing $CuSO_4$. Core switching occurs in the majority of clinical strains and results in white, light brown, dark brown, very dark brown and irregular wrinkle colonies. PS may play a fundamental role in virulence of this fungus because dark brown predominates among natural isolates and in mice has a colonization advantage over other colony types [134]. In *Candida lusitaniae*, PS is associated with emergence of amphotericin resistance (amphotericin B). PS happens at high frequency (1 in 10^2–10^4) and may confer a selective advantage in a host that is treated with amphotericin B [135].

Phenotypic switching is a mechanism that facilitates change in important phenotypic traits, as has been demonstrated in *C. neoformans*, *C. grubii* and *C. gattii*. These changes affect host–pathogen interactions and thus contribute to virulence. Although PS shares some basic similarities with PS described in other fungi, the effect of this process in *Cryptococcus* species and varieties is different from that described in *Candida* species.

Antigenic variation of the polysaccharide capsule

Traditionally, antigenic variation is achieved by varying expression of surface proteins. Such antigens are often recognized by the host immune response and, therefore, antigenic variation may constitute a mechanism to evade the immune response. Unlike most other fungi, *C. neoformans* is encapsulated and thus exhibits a different surface. The polysaccharide capsule contains many epitopes, and there is experimental evidence that the capsular surface varies its antigen epitopes.

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Antigenic variation of *C. neoformans*

Antibody staining with capsule-specific antibodies has demonstrated that *C. neoformans* cells manifest antigenic variation in the polysaccharide capsule during murine infection and transmigration of the blood–brain barrier [37,40,49]. Owing to the complexity of the polysaccharide capsule it is difficult to precisely characterize this antigenic variation. It was proposed that this variation is generated by an infinite combination of polysaccharide triads [60]. Evidence that selection occurs during *in vitro* passage exists. Specifically, when passaged isolates were analyzed by agglutination assay, flow cytometry and indirect immunofluorescence, it was demonstrated that epitope expression of mAb 18B7 varied (they could be gained or lost) [136]. Analysis of the capsular antigenic properties by mAb binding and Scatchard analysis revealed fluctuations in the binding affinity within the capsule but not in the number of antibody binding sites, suggesting that the spatial organization of high- and low-affinity epitopes within the capsule change according to radial position. It is noteworthy that the structure of the capsule also changes with capsule age, since the capsule of older cells becomes more resistant to γ radiation-induced ablation [58]. In summary, the epitopes of a large polysaccharide such as GXM are manifold and this explains why it is inherently challenging to examine the variability of these epitopes. However, experimental data mainly comparing binding of mAbs to epitopes suggests that antigenic epitopes vary. Except for one gene, which encodes for Cas3p and affects the acetylation of the polysaccharide, and therefore also mAb binding, very little is known about the genetic mechanisms that would control such antigenic variation [137,138].

Comparison with antigenic variation in other pathogenic fungi

In *Pneumocystis* spp. a gene family called ‘major surface glycoprotein’ (*MSG*) encodes for surface proteins that undergo antigenic variation [139–145]. In *P. carinii* 85 distinct *MSG* genes are organized in clusters [146] and the current model proposes the existence of only one fixed expression. *P. carinii* populations are dominated by a single *MSG* gene at the expression site [147,148]. Patients with multiple independent infections are infected by distinct strains [149]. Hence, antigenic variation may be a survival strategy in this host-dependent fungus to avoid eradication by the host. Another example are the *EPA* genes in *C. glabrata*. *EPA1* is a glycosylphosphatidylinositol-anchored cell-wall protein with 23 paralogues. Most of these *EPA* genes are located in subtelomeric positions, where they are transcriptionally silenced [150]. Transcription of some subtelomeric *EPA* genes can be derepressed by limitation of NAD^+ precursors [151]. Null mutations in *SIR3*, *SIR4* and *RIF1* lead to expression of many *EPA* genes, resulting in a hyperadherent phenotype [150,152,153].

In summary, antigenic variation in other fungi involves proteins whereas the outer surface of *Cryptococcus* species is dominated by a polysaccharide capsule that can also be varied however this process is more complex.

Future perspective

Over the next 10 years, *in vitro* studies are needed that focus on research investigating the molecular mechanisms that control PS and phenotypic variation since the mechanisms are virtually unknown. Such studies could be significantly aided by novel epigenomic techniques such as Chip. So far, the majority of studies are performed with a few laboratory model strains, many of which have been passaged *in vitro* for years. These adapted strains may not adequately represent the breadth of phenotypic variations that can be found in clinical *C. gatti* and *C. neoformans* strains. It is not know if *in vivo* strain evolution contributes to outcome. Given the availability of spinal fluids with up to 10^5 *C. neoformans* cells per ml, the variability of phenotypic traits should be studied in cells derived directly from the host. Understanding antigenic variation of complex polysaccharides remains a major challenge that will require

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new methods to be explored. Novel *in vivo* imaging techniques may allow the design of studies that are concentrated on elucidating mechanism of lung to CNS dissemination in rat and mouse animal models. The emergence of a new *C. gattii* strain that causes infection in immunocompetent individuals in the northwestern states of the USA [154] demand more research on this species. With rising antifungal resistance [155] and the devastating prognosis of chronic cryptococcosis [1,156–159], vaccine development to prevent reactivation of *C. neoformans* would be highly desirable. This will, however, require better understanding of the relevance, the selection and the molecular regulation of phenotypic changes *in vivo* as vaccination could promote selection of variants.

Executive summary

- This article discusses how phenotypic traits in *Cryptococcus* species can vary and how the variability affects the pathogenesis of chronic cryptococcosis.
- Global changes of phenotypic traits occur in the whole fungal population under certain conditions. These changes are capsule induction, melanization, biofilm formation and hyphal/spore formation. Capsule induction is unique to *Cryptococcus* species. The other phenotypic traits are also encountered in other fungi.
- Phenotypic switching involves only a small subpopulation but usually these variants are selected and thus can become dominant because the switched phenotypes are stable epigenetic states. This process is also found in several *Candida* species; however, the phenotypic traits affected are very different. In *Candida* species, this process commonly changes cellular morphologies but can also change antifungal resistance and copper metabolism. By contrast, in *Cryptococcus* the main phenotypic trait affected is the polysaccharide capsule.
- Antigenic variation in *Cryptococcus* affects the antigens within the polysaccharide capsule and is very complex, highly variable and affects antibody binding. This process is very different from other fungi where antigenic variation affects the modification of surface proteins.

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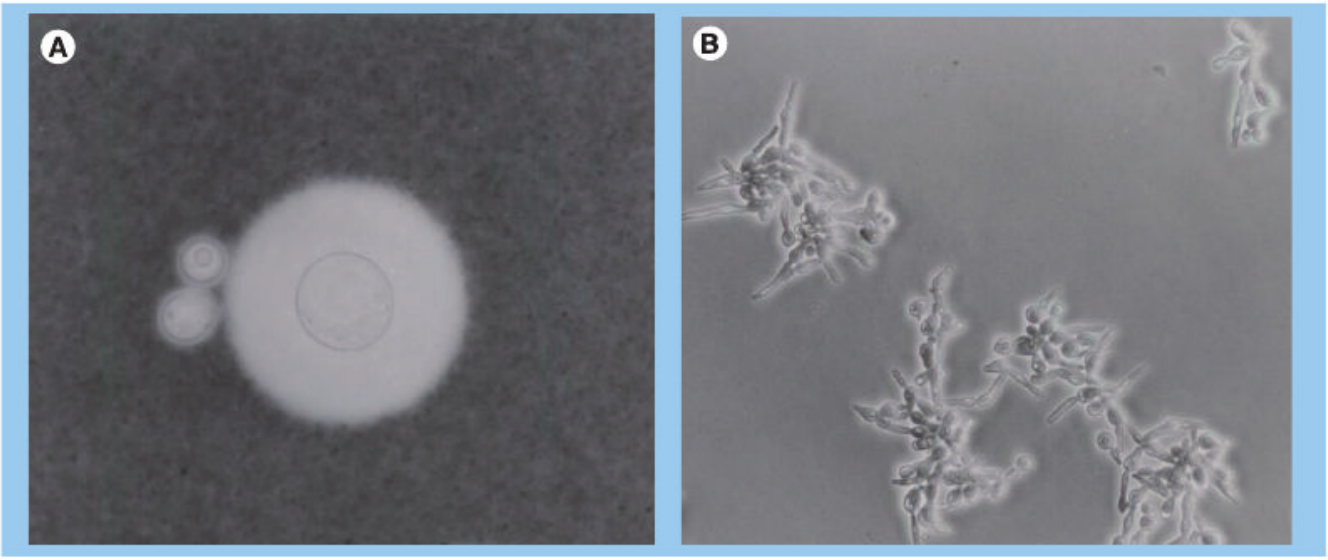

Figure 1. Phenotypic variations of *Cryptococcus neoformans*
(A) Uninduced capsule and induced capsule of *C. neoformans* cell. **(B)** Hyphal formation of *C. neoformans*.