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Antigenic and phenotypic variations in fungi

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Summary

Mechanisms to vary the phenotypic characteristics of fungi are diverse and can be important for their life cycle. This review summarizes phenotypic variability in fungi and divides this phenomenon into three topics: (i) morphological transitions, which are environmentally induced and involve the entire fungal population, (ii) reversible phenotypic switching between different colony morphologies, which is restricted to a small fraction of the population, and (iii) antigenic variation of surface antigens, which can be immuno-dominant epitopes happens in individual fungal cells.

Introduction

Phenotypic variation in microorganisms allows rapid adaptation to a constantly changing environment. Most pathogenic fungi are environmental microbes that accidentally invade the host. The exceptions are *Candida albicans* and *Pneumocystis* spp., which reside predominantly in their hosts. Variation of the microbial cell surface can impact host–pathogen interaction, and facilitate evasion of an evolving host immune response. Virulence traits often constitute important survival mechanisms in the environment and are selected in the host.

Phenotypic changes are common in fungi and induced by many different mechanisms. For clarity we will differentiate three kinds of phenotypic changes, albeit this division has many overlaps. First, morphological transitions (MT) are described in most fungi and induced by environmental signals like starvation, temperature, pH change and mating associated factors. Examples include sporulation-associated MTs, bud-hyphal MTs, capsule induction and phase variation in dimorphic fungi. These MTs are easily distinguishable, highly controlled and involve the entire population. Second, complex phenotypic changes can be achieved through reversible phenotypic switching (PS). PS occurs in a small fraction of the population, is random, reversible and represents an epigenetic state that is not necessarily induced by external signals. Third, antigenic variation (AV) involves alternating the expression of surface proteins (or carbohydrates), which happens on cellular level in the entire population but can underlie strong selection pressure. Overlaps are common, e.g. MTs can involve AVs and PS can affect the percentage of MTs in a colony.

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MT in fungi

As part of their life cycle most fungi manifest many phenotypic morphologies that involve remodelling of the outer surface. In particular hyphae, biofilm and spherule formation as well as capsule induction contribute to virulence and evasion of the host response. Expression of morphology-specific genes, many of which are GPI-anchored, are controlled by diverse signal transduction pathways, including MAP kinase, HOG, calcineurin, cyclic AMP pathway, that respond to environmental signals. Although these pathways are generally conserved, significant differences in these regulatory networks occur even in closely related species.

Phase variation in dimorphic fungi

The dimorphic fungi can cause diverse diseases after inhalation. At ambient temperatures, they grow as filamentous saprophobic molds in the environment. Elongating hyphae asexually produce propagules in the form of conidia or arthroconidia, which can be inhaled. Spores from *Coccidioides* spp. undergo isotropic growth and convert into multinucleate round cells (spherules) in the lungs. The mycelial forms are adapted to climate and soils, which determines the geographical restriction. The yeast morphology is exclusively associated with the pathogenic life phase in the mammalian host. Thus genes that are differentially regulated in the yeast versus the hyphal forms are often important for virulence. Examples include CBP, α-(1,3)-glucan in *Histoplasma capsulatum* (Kugler *et al.*, 2000), BAD1 in *Blastomyces dermatitidis*, and SOWgp and Mep1 in *Coccidioides immitis* and *C. posadasii* (Rappleye and Goldman, 2006).

Hyphal formation in pathogenic yeasts and molds

Candida albicans is both a commensal and a pathogen, which can exhibit a yeast, hyphal or pseudohyphal morphology. The yeast form is associated with dissemination, and the hyphal form with adhesion, tissue invasion and proteolytic activity (Whiteway and Bachewich, 2007). Accordingly genes involved in these functions (*ALS3*, *SAP4-6*, *HWP1*, *HYR1*, *ECE1*) are differentially expressed (Birse *et al.*, 1993; Bailey *et al.*, 1996; Sanglard *et al.*, 1997; Hoyer *et al.*, 1998; Staab and Sundstrom, 1998; Martchenko *et al.*, 2004). The MAP kinase, cAMP and the pH-sensing Rim101 signal transduction pathways (Whiteway and Bachewich, 2007) regulate cellular morphology and expression of hyphal associated genes. These MTs may enhance colonization at different anatomical sites, or invasion and is also seen in other *Candida* species.

Aspergillus fumigatus also undergoes MT to cause invasive aspergillosis. Inhaled conidia must complete germination, which involves isotropic growth, and emergence of the initial germ tube. The germling then elongates by apical extension to tube-like invasive hyphae. Genes involved in MT include *pkaR*, the regulatory subunit of the cAMP-dependent protein kinase, and *rasB*, a *ras* family subhomologue (Fortwendel *et al.*, 2005; Zhao *et al.*, 2006). Mutants of the calcineurin pathway also exhibit defects in conidial germination and polarized hyphal growth (Cramer *et al.*, 2008).

Capsule induction

Cryptococcus neoformans var. *neoformans* (*C. neoformans*) and var. *grubii* (*C. grubii*), and *Cryptococcus* var. *gattii* (*C. gattii*) are ubiquitous encapsulated yeasts that cause chronic meningoencephalitis, pneumonia and disseminated disease in susceptible individuals. *In vivo*, they induce their polysaccharide capsule in response to low iron, higher pH and CO_2 levels, and starvation (reviewed in Zaragoza *et al.*, 2009). Capsule induction is required for virulence as it affects phagocytosis and migration across the blood brain barrier (Garcia-Hermoso *et al.*, 2004; Charlier *et al.*, 2005).

Biofilm formation

Surface-associated fungi can grow embedded in extracellular matrix (ECM) that is composed of carbohydrates and proteins. Other *Candida* spp. (Iraqui *et al.*, 2005) and *Cryptococcus* spp. (Martinez and Casadevall, 2005) and even *A. fumigatus* (Mowat *et al.*, 2009) form biofilms with ECM. Biofilm-associated gene regulation is predominantly studied in *C. albicans* (for review, Blankenship and Mitchell, 2006), where hyphal formation is important for robust biofilm formation (BF). Two transcription factors, Tec1 and Bcr1, are important and regulate hypha-specific genes, and genes downstream of hyphal differentiation (Schweizer *et al.*, 2000; Nobile *et al.*, 2006). *Candida* genes that control adherence, attachment hyphal formation and quorum sensing molecules also regulate BF. Biofilms are resistant to antifungal drugs and thus greatly contribute to fungal pathogenesis.

Phenotypic switching

Colony switching is mainly described in yeasts because it is defined as the spontaneous emergence of colony variants, a phenomenon harder to distinguish in molds.

Candida albicans

High frequency PS was first described in *Candida* strains 3153A and WO-1 (Slutsky *et al.*, 1985; 1987; also Soll, 1992). The model strain WO-1 switches between two colony morphologies, whereas 3153A generates many unstable colony phenotypes (Slutsky *et al.*, 1985; 1987) White-to-opaque switching (WOS) occurs reversibly at a frequency of 1 in 10^4–10^5 (Fig. 1, upper panel). White-phase cells are round whereas large opaque-phase cells are elongated (Anderson and Soll, 1987; Slutsky *et al.*, 1987; Rikkerink *et al.*, 1988). Opaque-phase cells are mating-competent whereas white-phase cells survive better within the mammalian host, yet can switch to mating-competent cells when needed (Miller and Johnson, 2002). Physiological CO_2 levels induce WOS at 37°C (Huang *et al.*, 2009). Clinical isolates can undergo WOS if they are homozygous (*a*/*a* or α/α) whereas heterozygous (*a*/α) strains cannot switch (Lockhart *et al.*, 2002; Legrand *et al.*, 2004).

Phase-specific genes are regulated by phase-specific *trans*-acting factors (Srikantha *et al.*, 1995; Lockhart *et al.*, 1998; Sonneborn *et al.*, 1999) and implicated in virulence (Morrow *et al.*, 1992; Hube *et al.*, 1994; Srikantha *et al.*, 1995; Balan *et al.*, 1997). White-phase cells are more virulent in intravenous infection (Kvaal *et al.*, 1999) and opaque-phase cells colonize skin better (Lachke *et al.*, 2003). WOS also affects other virulence traits, including the bud-hyphal transition (Anderson *et al.*, 1990), sensitivity to neutrophils and oxidants (Kolotila

and Diamond, 1990), antigenicity (Anderson *et al.*, 1990), adhesion (Kennedy *et al.*, 1988), secretion of proteinase (Morrow *et al.*, 1992; Vargas *et al.*, 2000), drug susceptibility and phagocytosis by macrophages (Lohse and Johnson, 2008). All of these altered traits can potentially affect survival in the mammalian host.

More than 373 genes are differentially expressed in WO variants (Lan *et al.*, 2002). These genes represent diverse functions including metabolism, adhesion, cell surface composition, stress response, signalling, mating type and virulence. Metabolic specialization of switch phenotypes may enhance selection at different anatomical sites. Consistent with that hypothesis, opaque cells expressed genes associated with oxidative metabolism whereas genes in white cells were associated with fermentative metabolism. Wor1 has been identified as a master regulator of WOS, as its deletion blocks opaque cell formation (Huang *et al.*, 2006). Interlocking feedback loop networks maintain the epigenetic state of switch variants through cell divisions. *MTLa1*, *MTLα2*, *WOR1*, *CZF1*, *WOR2* and *EFG1* constitute a circuit that regulates WOS. This circuit is not present in closely related fungi and could be a recent adaptation in the mammalian host (Zordan *et al.*, 2006; 2007).

Candida glabrata

Candida glabrata, another pathogenic yeast, undergoes ‘core switching’ (Lachke *et al.*, 2002) on agar containing $CuSO_4$. Core switching occurs in the majority of clinical strains and results in white (W), light brown (LB), dark brown (DB), very dark brown (vDB) and irregular wrinkle (IWr) colonies, which results from graded conversions of Cu^{2+} to Cu^{1+} and reduction of SO_4^{2-} to S^{1-}. Transcriptional profiling showed that most upregulated genes in DB were involved in $CuSO_4$ assimilation and stress responses (Srikantha *et al.*, 2005). PS may play a fundamental role in virulence because DB predominates among natural isolates and in mice has a colonization advantage over other colony types (Srikantha *et al.*, 2008).

Candida lusitaniae

Candida lusitaniae, a rare human pathogen (Merz, 1984), frequently develops resistance to amphotericin B (AMB) during infection (Young *et al.*, 2003). *C. lusitaniae* can switch between LB, DB and W colonies on $CuSO_4$-supplemented agar. High AMB resistance is associated with W, whereas low AMB resistance and filamentation is associated with LB and DB colonies. PS happens at high frequency (1 in 10^2–10^4) and may confer a selective advantage in a host that is treated with AMB.

Cryptococcus *spp*

Phenotypic switching occurs in *C. grubii* (SB4, J32) (Goldman *et al.*, 1998), *C. neoformans* (24067A, RC-2) (Fries *et al.*, 2001) and *C. gattii* (NP-1) (Jain *et al.*, 2006). Colony variants arise at a frequency of about 1 in 10^4–10^5. Smooth colonies (S and SM) of SB4, 24067 and NP-1 have a smooth dome, and mucoid (M and MC) colonies have a shiny and mucoid appearing colony surface. Wrinkled (WR), serrated (C) and pseudohyphal (PH) colonies exhibit an irregular dome surface with or without serrated margins and are rarely observed in clinical isolates (Fig. 1, upper panel). Serotype D strain RC-2 has stable switching rates and is used for research.

RC-2-MC has a larger capsule than RC2-SM and produces a viscous exo-polysaccharide. The predominant capsular polysaccharide Glucuronoxylomannan (GXM) is composed of linear α-D-mannopyranan chain with β-D-xylopyranosyl (Xyl*p*) and β-D-glucupyranosyluronic acid (Glc*p*A) side-residues. Six (M1–M6) structural reporter groups (SRG) are defined by amount and position of linked Xyl*p*, and Glc*p*A residues. In strains SB4 and 24067A PS results in changes of SRGs (Fries *et al.*, 1999). GXMs of SB4-C are composed of M2 and M3 SRGs whereas SB4-SM exhibits only M2. The addition of Xyl*p* at the 4-0 position in M3 most likely required activation of different enzymes, which are traditionally thought to be used only by *C. gattii*. In RC-2, PS alters biophysical properties of GXM (McFadden *et al.*, 2007). Although not proven this may result from changes in the spacing of charged *GlcpA* along the Mannose backbone, which are not apparent by NMR.

Phenotypic switching to RC-2-MC results in enhanced virulence in mice or rats. Similar effects are seen for 24067A and SB4. For *C. gattii* strain NP1, NP1-MC was more virulent in the pulmonary model; however, only NP1-SM could elicit CNS infection. PS to NP1-SM promoted crossing of the blood brain barrier. Infection with RC-2-MC but not RC-2 SM caused elevated intracranial pressure in rats (Fries *et al.*, 2005a). RC-2-MC was selected *in vivo* in the setting of antifungal drug therapy and antibody (Ab) administration (Fries *et al.*, 2005b). Therefore it is conceivable that PS may contribute to treatment failures.

The molecular mechanisms mediating PS in *C. neoformans* are not understood. PS was associated with down-regulation of genes in RC2-MC relative to RC2-SM among them *ALL1* whose function is unclear but associated with capsule formation. Loss of *ALL1* function mimics the hypervirulence of the MC variant including the over-stimulated host response (Jain *et al.*, 2009).

Antigenic variation

Antigenic variation can be achieved by varying expression of surface proteins that are controlled by subtelomeric silencing. In addition intragenic tandem repeats can play an important role because they can be varied and affect the expression and possibly the function of important surface molecules.

Pneumocystis *spp*

Pneumocystis is a genus containing many species, which infect different mammalian hosts. The human pathogen *Pneumocystis jiroveci* causes a severe pneumonia (PCP) in immunocompromised patients (Stringer *et al.*, 2002). *Pneumocystis carinii* infects rats and is the model species used to study AV because *Pneumocystis* spp. do not grow *in vitro*. Expression cloning from infected rat tissue identified a gene family called ‘Major Surface Glycoprotein’ (*MSG*) encoding a surface antigen that is also called gpA. This gene family has been identified in several *Pneumocystis* spp. (Haidaris *et al.*, 1992; Kovacs *et al.*, 1993; Wada *et al.*, 1993; Garbe and Stringer, 1994; Kitada *et al.*, 1994; Linke *et al.*, 1994; Wright *et al.*, 1995). Less is known about *MSR*1 and *PRT1*, two other families not present in all species. In *P. carinii* 85 distinct *MSG* genes are organized in clusters (Sunkin *et al.*, 1994). They vary in structure, and are composed of one or more *PRT–MSR–MSG* clusters. The number of *MSG* genes between the last *MSR* gene and the subtelomere varies from 1 to 3

(Keely *et al.*, 2005). The current model proposes the existence of only one fixed expression site that includes an Upstream Conserved Sequence (UCS) that lies upstream of a constitutively active transcriptional promoter. Gene conversion appears to be the primary mechanism of *MSG* sequence change. Multiple *PRT* genes and *MSR* genes are expressed in populations of *P. carinii* dominated by a single *MSG* gene at the expression site (Keely *et al.*, 2003; Ambrose *et al.*, 2004). The *MSR* gene family may also contribute to surface variation. Three different types of *MSR* genes (Keely *et al.*, 2005) that differ in size are described. One *MSR* type contains a long tract of G:C base-pairs in the middle. Variation of that G:C tract can occur by several mechanisms and cause frameshifts during translation that produce altered Msr proteins similar to other microorganisms.

The current hypothesis is that AV allows *Pneumocystis* to persist long enough in a healthy host to assure transmission to the next host. Consistent with these data, drug resistance alleles are found in patients never treated with sulfonamides (Iliades *et al.*, 2004; Meneau *et al.*, 2004). Patients with multiple-independent episodes of PCP are infected by distinct genetic strains (Keely *et al.*, 1996). Hence, AV may be a survival strategy in this host-dependent fungus to avoid eradication by the host.

C. glabrata

Epa1 mediates adherence of *C. glabrata* to mammalian epithelial cells. *EPA1* is a GPI-anchored cell wall protein. In *C. glabrata* strain BG2 23 paralogues of *EPA1* are characterized, all encoding proteins highly related to Epa1. Most of these *EPA* genes are located in subtelomeric position, where they are transcriptionally silenced (Castano *et al.*, 2005). Transcription of some subtelomeric *EPA* genes can be de-repressed by limitation of NAD+ precursors (Domergue *et al.*, 2005). Null mutations in *SIR3*, *SIR4* and *RIF1* and deletion of the C-terminal 28 amino acids of Rap1 lead to expression of many *EPA* genes, resulting in a hyper-adherent phenotype when cells are grown under standard laboratory conditions (De Las Penas *et al.*, 2003; Castano *et al.*, 2005; Iraqui *et al.*, 2005). While SIR proteins are required for silencing of *EPA* genes, the Rif1 and the Ku proteins regulate silencing only in a subset of telomeres (Rosas-Hernandez *et al.*, 2008).

C. posadasii *and* C. immitis

Coccidioides spp. can cause pneumonia, meningitis and disseminated disease in immuno-suppressed and -competent individuals. SOWgp is an immuno-dominant antigen of the spherule outer wall, which elicits both antibody- and cell-mediated responses in patients. This glycoprotein consists of a signal peptide and propeptide, with a tandem repeat motif, and a GPI anchor signal consensus sequence. The repeat domain contains three to six copies of proline- and aspartic acid-rich sequences (strain-dependent), which is recognized by Abs of infected individuals. A metalloproteinase (Mep1) produced during the endosporulation event digests SOWgp. Thus, Mep1 contributes to *Coccidioides* virulence by ensuring endospores are devoid of SOWgp, thereby allowing them to evade host detection. It is conceivable that this mechanism could contribute to AV *in vivo* (Hung *et al.*, 2000; 2002; 2005; Johannesson *et al.*, 2005).

C. neoformans

Antibody staining with capsule specific Abs has demonstrated that *C. neoformans* cells manifest AV in the polysaccharide capsule during murine infection (Garcia-Hermoso *et al.*, 2004; Charlier *et al.*, 2005). This variation is generated by infinite combination of polysaccharide triads. Evidence that selection occurs during cloning passage exists (McFadden *et al.*, 2007).

Summary and future perspective

Understanding the dynamic qualities of genomes that allow pathogens to survive, adapt and escape immune responses is vital to our knowledge about host–pathogen interactions. Future studies should focus on studying phenotypic variation *in vivo* with a combination of proteomic and genomic approaches. Determining variation resulting from post-translation modifications of proteins remains a challenge. Understanding the relevance and the selection of phenotypic changes *in vivo* will be of considerable importance for vaccine development.

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Fig. 1.

Upper panel shows sectored and wt WO-1 *C. albicans* strain phenotype on Phloxine B containing agar. Sectors denote the different switch types. Lower panel shows a wrinkled melanized and a mucoid switch variant of 24067 *C. neoformans* strain; both variants exhibit enhanced virulence *in vivo*.