Phenotypic switching and filamentation in *Candida glabrata*

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***Candida glabrata* switches spontaneously, reversibly and at high frequency among the following four phenotypes distinguishable by graded colony colouration on CuSO₄-containing agar: white (Wh), light brown (LB), dark brown (DB) and very dark brown (vDB). These phenotypes also differ in a graded fashion in the level of expression of the metallothionein gene *MTII* (Wh < LB < DB ≤ vDB), the frequency of switching (Wh > LB > DB > vDB) and colouration on phloxine B-containing agar (Wh > LB > DB > vDB). Switching among the four graded phenotypes is referred to as 'the core switching system'. An additional switch phenotype, 'irregular wrinkle' (IWr), has been identified, which exhibits a highly wrinkled colony morphology. The characteristics of IWr suggest that switching to and from this phenotype represents a second high-frequency switching system. A microscopic analysis revealed that during the first 3 days of colony development, cells in the centres of Wh, LB, DB and vDB colonies expressed almost exclusively the budding yeast phenotype. After 3 days, however, pseudohyphae and cells extending tubes accumulated, so that by 7 days the proportions of these two cellular phenotypes reached 40–50% and 10–20%, respectively. In contrast, IWr colonies were composed almost exclusively of pseudohyphae through the first 6 days of colony development. After 6 days, IWr colonies began to accumulate both budding yeast cells and tubes. The tubes formed by *C. glabrata* reached lengths of up to six cell diameters, but the tubes did not represent traditional compartmentalized hyphae. Tube growth ended when the tube tip expanded to form a bud. Tubes then functioned as corridors for daughter nucleus migration to the apical bud, and were ultimately left uncompartimentalized and nucleus free. Core switching, pseudohypha formation and tube formation occurred in a majority of 62 tested clinical isolates, demonstrating that these developmental programmes are general characteristics of most strains of *C. glabrata*.**

Keywords: *Torulopsis glabrata*, high-frequency switching, irregular wrinkle switching, pseudohypha formation, tube formation

INTRODUCTION

Candida glabrata now represents the second most pervasive fungal pathogen behind *Candida albicans* in most categories of commensalism and infection (Fidel *et al.*, 1999; Hazen, 1995; Lockhart *et al.*, 1999; Odds, 1988; Pfaller *et al.*, 1998). Like *C. albicans*, it can be carried in a variety of body locations, and can cause superficial mucosal infections as well as bloodstream

infections in compromised hosts (Fidel *et al.*, 1999; Pfaller *et al.*, 1998). Its success is particularly worrisome because it is naturally drug resistant (Berrouane *et al.*, 1999; Fidel *et al.*, 1999; Hazen, 1995; Pfaller *et al.*, 1999; Sanglard *et al.*, 1999, 2001). Epidemiologically, the success of *C. glabrata* mimics that of *C. albicans* (Odds, 1988) and for that reason one might expect it to possess similar virulence traits. However, until quite recently it had been generally assumed that *C. glabrata* lacked two important developmental programmes that contribute to phenotypic variability and, hence, to rapid adaptation of *C. albicans*, namely high-frequency phenotypic

Abbreviations: DB, dark brown; vDB, very dark brown; IWr, irregular wrinkled; LB, light brown; Wh, white.

switching and the bud–hypha transition. Indeed, the absence of hyphae has been used as a discriminating characteristic in typing *C. glabrata* (Bale *et al.*, 1997). Believing that both developmental programmes may have been overlooked, we first took advantage of observations that *C. glabrata* could rapidly alter its resistance to copper by increasing the rate of transcription of metallothionein genes and by amplifying one of these genes, *MTII* (Mehra *et al.*, 1989, 1990; Zhou & Thiele, 1991), and developed an indicator agar containing CuSO_4 , which, when reduced to CuS , causes cells and colonies to turn brown. Plating *C. glabrata* cells on this agar revealed a spontaneous, reversible, high-frequency switching system that included the following three colony phenotypes: white (Wh), light brown (LB) and dark brown (DB) (Lachke *et al.*, 2000). In an analysis of gene expression, it was further demonstrated that switching in *C. glabrata* selectively regulated the transcription level of the metallothionein gene *MTII* in a graded hierarchy similar to that of colouration on CuSO_4 -containing medium ($\text{DB} > \text{LB} > \text{Wh}$) and the opposite of the hierarchy of switching frequency ($\text{Wh} > \text{LB} > \text{DB}$) (Lachke *et al.*, 2000; Soll, 2002a). These results demonstrated for the first time that *C. glabrata*, like several other infectious fungi (Enger *et al.*, 2001; Fries *et al.*, 1999; Gil *et al.*, 1990; Goldman *et al.*, 1998; Joly *et al.*, 1996; Odds & Merson-Davies, 1989; Slutsky *et al.*, 1985, 1987; Soll *et al.*, 1988), undergoes phenotypic switching, and that like switching in *C. albicans* (Soll, 2002a, b), switching in *C. glabrata* regulates the expression of selected genes (Lachke *et al.*, 2000). However, in contrast to *C. albicans*, switching in *C. glabrata* represents a graded system.

In regard to the bud–hypha transition, Brandao *et al.* (1995) described the formation of ‘germ-like tubes’ in three strains of *C. glabrata*, suggesting that they may be capable of forming hyphae. Odds *et al.* (1997) identified several apparent *Torulopsis* species (the previous genus name of *C. glabrata*) that formed pseudohyphae, and Csank & Haynes (2000) observed pseudohypha-like projections at the periphery of *C. glabrata* colonies grown on nitrogen-starvation medium. Together, these results suggested that like *C. albicans*, *C. glabrata* is capable of expressing different cellular phenotypes in colonizing populations through both high-frequency phenotypic switching and a dimorphic transition. To understand further the developmental capabilities of *C. glabrata*, and the relationship between switching and dimorphism in particular, we analysed in more detail the switching repertoire and the relationship of switching and cellular phenotype. From these analyses, we have (1) expanded the core switching system to include four phenotypes distinguishable on CuSO_4 -containing agar, Wh, LB, DB and very dark brown (vDB), (2) demonstrated that colonies of all four core switch phenotypes produce similar proportions of budding cells, pseudohyphae and tubes during colony development, (3) tentatively identified a second reversible switching system between core phenotypes and an irregular wrinkle

(IW_r) colony phenotype that is composed almost exclusively of pseudohyphae, (4) demonstrated that *C. glabrata* does not form true compartmentalized hyphae, but rather unique, nucleus-free tubes which swell into buds at their apices and then act as corridors for daughter nuclei to access the apical bud, and (5) demonstrated that switching, pseudohypha formation and tube formation represent general developmental characteristics of a majority of 62 tested clinical isolates.

METHODS

Strain origin and maintenance. The *C. glabrata* strain used for the basic characterization of switching and filamentation was 35B11, which was isolated from the buccal epithelium of a healthy 65-year-old individual (Lachke *et al.*, 2000; Lockhart *et al.*, 1999). Additional strains used to test the universality of switching and pseudohypha and tube formation were as follows: 32TL10, 29TL1, 32BLWR, 86FL2, 32PL1, 28TL1, 32TL1, 64PL4, 25Pp1, 7549, 39FL5, 65FLOP, 64BL1, 64TL6, 25TL1, 21BLOP, 26BL2, 26TL1, 26B10, 28BL1, 698, 26PL1, 25TL1, 24BL1, 1480.41, 1480.42, 1480.43, 1480.44, 1480.46, 1480.47, 1480.48, 1480.49, 1480.50, 86BL1, 79FLOP1, 79TL1, 79FL1, 65TL1, 75PL1, 86TL1, 24TL1, 75BL1, J932288, B63155, J932597, J932340, J932285, J931019, J932318, J932273, J932405, J932387, J941814, J932474, J932277, J932436, J942007, J932258/1, J932476, J942283, J932278, J931012. They were obtained from a collection used by Lockhart *et al.* (1999) in the development of complex DNA fingerprinting probes for *C. glabrata* (Lockhart *et al.*, 1997) and in an analysis of *Candida* species colonizing the oral cavities of the very aged (Lockhart *et al.*, 1999). All isolates were initially verified as *C. glabrata* by sugar assimilation patterns, and then by DNA fingerprinting with the species-specific complex DNA fingerprinting probe Cg6 (Lockhart *et al.*, 1997). All clones were stored at room temperature on YPD agar slants (2% glucose, 2% Bacto peptone, 1% yeast extract, 2% agar) in capped tubes. Switch phenotypes were grown and propagated on YPD agar plates containing 1 mM CuSO_4 at 25 °C (Lachke *et al.*, 2000). Cells expressing the different switch phenotypes were stored in glycerol at –80 °C.

Measurements of phenotypic switching. The methods for measuring switching were similar to those previously described in detail (Lachke *et al.*, 2000). In brief, cells from an individual 3-day-old colony exhibiting a homogeneous colony morphology were diluted into YPD liquid medium containing 1 mM CuSO_4 and grown for 6–8 h at 25 °C to a density of approximately 8×10^6 cells ml^{-1} . Cells were then diluted and plated at a density of approximately 50 c.f.u. per plate. In the case of the irregular wrinkle (IW_r) phenotype, cells from a fresh 3-day-old colony were removed from a sectorless region of a colony, diluted and plated at a density of 50 c.f.u. per plate. Plates were incubated at 25 °C for 5 days, and the numbers of colonies exhibiting the original and variant phenotypes were counted and converted to percentage of population. It should be noted that such frequencies represent the proportions of variant c.f.u. in the original colony, not the rate of switching per cell (Bergen *et al.*, 1990; Rikkerink *et al.*, 1988; Soll *et al.*, 1991). When analysing switching in the absence of CuSO_4 , 5 mg phloxine B l^{-1} was substituted for CuSO_4 .

Northern analysis of gene expression. Cells from 3–4-day-old colonies were pooled, washed in RNase-free sterile water and homogenized with acid-washed beads in a bead beater (Bio-spec Products). RNA was then extracted using the RNeasy kit (Qiagen) according to the manufacturer’s direc-

tions, and separated on a 1.2% agarose/formaldehyde gel. RNA was transferred to Zetabind nylon membrane (Amersham Life Sciences) and probed for transcripts of the genes *MTII* and *PDH1* according to methods previously described (Lachke *et al.*, 2000).

Fluorescence microscopy and scanning electron microscopy.

Cells were stained for chitin by washing them twice with water, then incubating them in a solution of 0.1 mg Calcofluor ml^{-1} (Cabib & Bowers, 1975; Hayashibe & Katohda, 1973) (Fluorescent Brightener 28, Sigma Diagnostics) for 2 h. Cells were stained for nuclei by first washing them twice in HEPES balanced salt solution (HBSS: 10 mM HEPES, 150 mM NaCl). The final pellet was resuspended and cells incubated in 200 μl HBSS plus 200 μl 80% ethanol for 10 min. Hoechst stain (Sigma) was then added to a final concentration of 10 μM and the cells incubated in this solution for 20 min. Twenty microlitres of either preparation was pipetted onto a 12 mm glass coverslip coated with poly-L-lysine and incubated for 10 min. Stained cells were viewed and photographed, first by differential interference contrast (DIC) microscopy and then by epifluorescence microscopy using a UV 365 nm excitation filter in a Zeiss Axioplan microscope. For presentation of nuclear-stained cells, the DIC and fluorescent images were merged using Adobe Photoshop software. For scanning electron microscopy, cells were rinsed with double-distilled water and distributed on a 12 mm glass coverslip coated with poly-L-lysine (Sigma Diagnostics). The cells were then fixed for 50 min in a 2.5% solution of glutaraldehyde in 0.1 M cacodylate buffer, washed three times in 0.1 M sodium cacodylate buffer, postfixed in 10% OsO_4 in 0.1 M cacodylate buffer, rinsed in 0.1 M cacodylate buffer, and treated with 6% thiocarbonylhydrazide to enhance surface architecture. Following a second fixation in 1% OsO_4 to enhance detail, the cells were finally washed in double-distilled water, dehydrated through a graded series of ethanol solutions, dried, mounted on aluminium stubs, and sputter-coated with gold/palladium. Samples were scanned with a Hitachi S-4000 scanning electron microscope.

RESULTS

Expanding the 'core' switching system to include vDB

By plating cells of strain 35B11 on agar containing 1 mM CuSO_4 at low density, we previously demonstrated that *C. glabrata* switched spontaneously and reversibly between discrete white (Wh), light brown (LB) and dark brown (DB) colony phenotypes at frequencies ranging from 4×10^{-1} to 3×10^{-3} (Lachke *et al.*, 2000). The frequencies of switching of the three phenotypes were graded according to the hierarchy Wh > LB > DB. This hierarchy was most obvious in the frequencies of sectoring in aged colonies. Two additional colony phenotypes were isolated in the original study (Lachke *et al.*, 2000), very dark brown (vDB) and very white (vWh). Because of their infrequent appearance, neither of these phenotypes was included in the core switching system in the original report (Lachke *et al.*, 2000). In subsequent plating experiments that included larger numbers ($\sim 10^4$) of vWh cells, we found no colonies exhibiting the colony phenotypes Wh, LB and DB, and with further incubation, no sectors of the latter three phenotypes. Hence, we conclude that vWh does not switch reversibly to Wh, LB or DB, and, therefore, is not

a member of the core switching system. In contrast, in plating experiments of vDB cells (5×10^4), Wh, LB and DB colonies formed at frequencies of 5×10^{-4} , 8×10^{-3} and 5×10^{-4} , respectively. We therefore conclude that vDB represents a fourth member of the core switching system (Fig. 1A).

Core switching occurs in the absence of CuSO_4

In addition to graded colouration on agar containing 1 mM CuSO_4 , the phenotypes of the core switching system can be distinguished by graded colouration on agar containing phloxine B (Fig. 2), a vital dye used previously to discriminate between the white and opaque phases of *C. albicans* (Anderson & Soll, 1987). When cells from Wh, LB, DB and vDB colonies on CuSO_4 -containing plates were replated at low density on agar lacking CuSO_4 and containing phloxine B, the majority of colonies stained dark pink, medium pink, light pink and white, respectively, a hierarchy of pink intensities opposite to the hierarchy of brown intensities on agar containing CuSO_4 (Fig. 2A). In Fig. 2(B–D), examples are presented of cells from Wh, LB and DB colonies picked from CuSO_4 -containing agar plates that were then plated at low density on phloxine B-containing plates. The majority of colonies were dark pink, medium pink and light pink, respectively. When cells from dark pink, medium pink, light pink and white colonies on phloxine B-containing agar were replated on agar containing CuSO_4 , they in turn generated predominantly Wh, LB, DB and vDB colonies, respectively (data not shown), demonstrating that the switch phenotypes originating on CuSO_4 -containing plates maintained their phenotype in the absence of CuSO_4 . When cells from a light pink sector and a light pink colony that appeared through switching in a dark pink population were replated on agar containing CuSO_4 , they formed predominantly DB colonies (Fig. 2B); when cells from light pink to white sectors that appeared through switching in a medium pink population were replated on agar containing CuSO_4 , they formed predominantly DB colonies (Fig. 2C); and when cells from a medium pink colony that appeared through switching in a light pink population were replated on agar containing CuSO_4 , they formed predominantly LB colonies (Fig. 2D). Finally, the frequency of switching of the core phenotypes on agar containing phloxine B was graded dark pink > medium pink > light pink > white. This hierarchy is evident in the frequencies of variant colonies and sectors in the dark pink, medium pink and light pink populations in Figs 2(B), (C) and (D), respectively. This hierarchy was the same as that for switching frequencies observed on CuSO_4 (i.e. Wh > LB > DB > vDB). Together, these results demonstrate that neither core switching nor maintenance of core phenotypes depends on the presence of CuSO_4 .

The 'irregular wrinkle' switching system

In expanded plating experiments of LB and DB cells, a new colony phenotype was identified in which the colony dome was irregularly wrinkled, hence the name

A. Core Switching System

B. Irregular Wrinkle Switching System

Fig. 1. *C. glabrata* possesses two switching systems: (A) the 'core switching system', which includes white (Wh), light brown (LB), dark brown (DB) and very dark brown (vDB) phenotypes; and (B) the 'irregular wrinkle (IW_r) switching system'. In the latter system, cells switch from a core phenotype to IW_r. IW_r switches back to a core phenotype, but prefers switching back to the core phenotype of origin. Hence, IW_r formed from LB is labelled IW_r_{LB}, and IW_r formed from DB is labelled IW_r_{DB}. The frequencies above the arrows in the core switching system refer to the proportions of cells in the phenotype at the base of the arrow that exhibit the phenotype at the head of the arrow.

'irregular wrinkle' (IW_r) (Fig. 3A–F). Two independent IW_r clones were isolated in LB platings, and five independent IW_r clones in DB platings. The frequency at which the IW_r phenotype appeared in both the LB and DB populations was approximately 10^{-4} . The colour of the IW_r colonies on CuSO₄-containing agar was white to very light brown (Fig. 3A, B) and on phloxine B-containing agar dark pink (data not shown). Colonies of the seven independent IW_r clones were phenotypically indistinguishable, suggesting that regardless of core phenotype origin, IW_r colonies expressed the same colony phenotype.

When cells from IW_r colonies were in turn plated on agar containing either CuSO₄ or phloxine B, the

majority formed IW_r colonies, but a sizeable minority (between 2 and 37%) switched to core phenotypes, demonstrating a high frequency of reversibility (Fig. 3C, D). When IW_r colonies were incubated for more than 7 days, all colonies formed smooth sectors of the core phenotypes (Fig. 3E, F). An analysis of sector phenotypes suggested that IW_r cells originating from the LB core phenotype switched preferentially to LB, and that IW_r cells originating from the DB core phenotype switched preferentially to DB (Table 1). To test this possibility, cells from two independent IW_r colonies emanating from LB and two independent IW_r colonies emanating from DB were plated, incubated for 10 days, and the phenotypes of sectors scored (Table 1). Because of wrinkling at the colony periphery, it was difficult to

Fig. 2. (A) Whereas CuSO₄ stains the core phenotypes white (Wh), light brown (LB), dark brown (DB) and very dark brown (vDB), phloxine B stains the same four core phenotypes dark pink, medium pink, light pink and white. (B–D) Switching occurs on phloxine B-containing agar. When cells from a light pink colony, or a light pink sector formed by dark pink cells (B) or medium pink cells (C) on phloxine B plates were replated on CuSO₄-containing agar, they expressed the dark brown (DB) phenotype. When cells from a medium pink colony formed by light pink cells (D) on a phloxine B plate were plated on CuSO₄-containing agar, they expressed the light brown (LB) phenotype.

distinguish between Wh and LB sectors, and between DB and vDB sectors, so sectors were first scored in the categories Wh/LB and DB/vDB. For both IWr clones obtained from LB, the great majority of sectors (90 % and 89 %, respectively) were Wh/LB, and for both IWr clones obtained from DB, the great majority of sectors (100 % in both cases) were DB/vDB (Table 1). When cells from 12 randomly selected Wh/LB sectors from IWr clones originating from LB were in turn plated at low density, 75 % formed LB colonies, and when cells from 12 randomly selected DB/vDB sectors from IWr clones originating from DB were in turn plated at low density, 100 % formed DB colonies. These results indicate that when cells from different core phenotypes switch to IWr, they retain their original core phenotype identity even though they are morphologically indistinguishable.

Several characteristics suggest that switching to and from IWr represents a phenotypic transition distinct from core switching. First, while IWr cells retain their original core phenotype identity, cells exhibiting core phenotypes do not. For instance, LB cells originating from Wh and DB switch in a similar fashion, showing no preferential switching to Wh and DB, respectively. Second, IWr colonies, regardless of core phenotype origin, exhibit many characteristics of the Wh core phenotype, including colouration on CuSO₄-containing

agar, colouration on phloxine B-containing agar, high switching frequencies and, as will be demonstrated, gene expression. Third, IWr does not exhibit a smooth colony dome, a characteristic of all core phenotypes. Fourth, cells in IWr colonies are restricted to the pseudohyphal growth form during the first 6 days of colony development, which is in marked contrast to cells in all core colony phenotypes (see below). Finally, cells in an IWr colony frequently and unpredictably exhibit unusually high reversion rates to their original core phenotype. The unpredictable nature of this reversion suggests that switching from IWr to core phenotype may be far more sensitive to environmental perturbations than core switching phenotypes (data not shown). For these reasons, switching to and from IWr has been tentatively separated into a system distinct from core switching, as described in Fig. 1(B).

Gene expression in vDB and IWr

In our original description of switching in *C. glabrata* (Lachke *et al.*, 2000), we demonstrated by slot-blot analysis that the metallothionein gene *MTII* was expressed in a graded fashion that correlated with the colouration hierarchy on CuSO₄-containing agar (Wh < LB < DB). We also demonstrated that for a majority of tested genes, including *PDH1*, expression was constitutive and similar in the three core phenotypes

Fig. 3. Dark brown (DB) cells plated on CuSO_4 -containing agar form irregular wrinkle (IWr) sectors (A, arrow) and colonies (B). When IWr cells are in turn plated, they form predominantly IWr colonies (C, D). When IWr colonies are incubated for extended periods (>7 days), they form sectors expressing core phenotypes (E, F).

(Lachke *et al.*, 2000). If vDB is at the high end of the hierarchy of graded phenotypes in the core switching system, it should also be at the high end of *MTII* expression. Northern analysis demonstrated that this was indeed the case. In repeat experiments, the level of *MTII* expression in vDB cells was equal to or slightly higher than that in DB cells (Fig. 4). In contrast, the level of *PDH1* expression in vDB cells was similar to that in Wh, LB and DB cells (Fig. 4). These results support the inclusion of vDB in the core switching system (Fig. 1A).

In the case of IWr cells, colouration on CuSO_4 -containing agar, colouration on phloxine B-containing agar and the high frequency of switching together suggest that regardless of core phenotype origin, IWr cells exhibit several of the characteristics of the Wh core phenotype. If this is correct, then IWr cells should express *MTII* at the low end of the expression hierarchy. Northern analysis demonstrated that this was indeed the case. In repeat experiments the level of *MTII* expression in IWr cells was similar to that in Wh cells (Fig. 4). In addition, if IWr represents a reversible switch phenotype, then a switch from IWr derived from DB to DB should result in the re-establishment of *MTII* expression to the same level as that of DB cells. Northern analysis demonstrated that this was indeed the case (Fig. 4). These results support the conclusions that IWr cells exhibit many of the phenotypic characteristics of the Wh core phenotype, and that IWr switches back to bona fide core phenotypes.

Cell morphologies in colonies of the core phenotypes

In *C. albicans*, switching between the white and opaque phase involves a dramatic change in cellular morphology (Anderson & Soll, 1987; Slutsky *et al.*, 1987; Soll, 1992;

Table 1. IWr cells switch preferentially to the core phenotype from which they originally arose

Two IWr clones from LB and two IWr clones from DB were analysed for switching back to core phenotypes. Cells from a 3-day-old colony of each clone were plated and assessed first for the frequency of colonies exhibiting a core phenotype after 7 days. The predominant IWr colonies were then assessed after 7 additional days for the proportion forming sectors and the phenotypes of those sectors.

Origin of IWr clone	Clone no.	No. of colonies analysed	Frequency of core phenotype colonies*	Percentage of IWr colonies that form sectors	Percentage of core phenotype sectors in IWr colonies†	
					Wh/LB	DB/vDB
LB	1	2046	10^{-1} (LB)	100	90	10
	2	2171	2×10^{-2} (LB, DB)	100	89	11
DB	1	1256	3×10^{-1} (DB)	100	0	100
	2	1273	4×10^{-1} (DB)	100	0	100

* The frequency of all colonies exhibiting a predominant core phenotype is presented. The major core phenotype is given in parentheses. For LB clone 2, the proportions of LB and DB phenotypes were both 1%.

† When cells from 12 Wh/LB sectors emanating from IWr clones originating from LB were in turn plated, 9 out of 12 formed LB colonies, 1 formed Wh colonies and 2 formed DB colonies. When cells from 12 DB/vDB sectors emanating from IWr clones originating from LB were in turn plated, all 12 formed DB colonies.

Fig. 4. Northern blot analysis of the expression of the metallothionein gene (*MTII*) in Wh, LB, DB, vDB, IWr, and IWr that switched to DB. As a control, the same Northern blot was rehybridized with the constitutively expressed gene, *PDHI*. To assess loading, the ethidium-bromide-stained 26S rDNA band is presented below.

Fig. 5. Chitin distribution and cell morphologies of yeast budding cells (A–D), pseudohyphae (E–H), tubes (I–L) and tubes that have formed or are forming apical buds (M–P). Cells were stained with Calcofluor to visualize chitin-containing septa; fluorescent images are shown in the left panel of each pair. In (A–D), septa between mother and daughter yeast cells are indicated with arrowheads. In (I–L), tubes lacking septa are indicated with arrows. In (M) and (N), the septum separating tube and apical bud is indicated by arrowheads. In (O) and (P), erratic light chitin rings are indicated along expanding tubes by unfilled blunt arrows. Bars, 10 μm.

Soll *et al.*, 1991). To test whether switching in *C. glabrata* also affects cellular morphology, samples were removed from the centres of colony domes after 5 days

of growth at 25 °C, and examined microscopically. Samples from all of the core phenotypes (Wh, LB, DB, vDB) consisted of approximately 50% budding yeast

Fig. 6. Nuclear staining of budding cells (A–C), pseudohyphae (D–F), tubes that have not undergone apical expansion (G–K), and tubes that have undergone apical expansion and apical bud formation (L–R). Cells were stained with Hoechst reagent. The phase-contrast images of the cell and fluorescent images of the nuclei were then merged. Note that tubes (T) that have not undergone apical expansion regardless of length are nucleus free. Tubes undergoing apical expansion can contain elongated nuclei in the process of nuclear division (L, arrowed), or one or both daughter nuclei (N, O, arrowed). Cells with developing or complete apical buds contain nuclei in the mother cell and daughter bud, but not in the intervening tube (M, P, Q, R). T, tube; R, apical bud. Bar, 10 μ m.

Fig. 7. Scanning electron microscopy (A, B, H, I, J) and phase-contrast (C–G) images of long tubes that have not undergone apical expansion (A, B), tubes undergoing apical expansion (C–E), tubes that have formed distinct apical buds (F–H), and tubes that have formed apical buds that then grew in the pseudohyphal form (I, J). Arrows indicate apical buds in different phases of growth. Bars, 10 μ m.

cells, 40 % pseudohyphae and 10 % unique tubes. These cellular morphologies are described below.

Budding yeast cells. The budding yeast cells in all four core phenotypes were round to ellipsoidal, and Calcofluor staining revealed chitin-containing septa at mother cell–daughter cell junctions and less intense staining in the general wall (Fig. 5A–D). Budding was primarily axial. The budding yeast cells of the four core phenotypes were morphologically indistinguishable.

Pseudohyphae. Pseudohyphae consisted of ellipsoidal to elongated cells forming chains that were frequently branched (Fig. 5E–H). They conformed to the growth characteristics of pseudohyphae formed by *C. albicans* (Merson-Davies & Odds, 1989; Odds, 1988). Calcofluor staining revealed chitin-containing septa at the junctions between compartments and less intense staining in the general cell wall (Fig. 5E, G). All internal cellular compartments in pseudohyphae and large distal compartments contained nuclei (Fig. 6D–F). The pseudohyphae of the four core switch phenotypes were morphologically indistinguishable.

Tubes. Tubes consisted of elongated projections emanating from round or ellipsoidal mother cells (Fig. 5I–P). In the majority of cases, the length of the tube was between one and two mother cell diameters, and the diameter approximately 20 % of the mother cell diameter (Fig. 5I–J). In some cases, tubes grew to lengths up to six times the diameter of the mother cell, with tube diameter roughly one-third the mother cell diameter, as is evident in the scanning electron micrographs (SEMs) in Fig. 7(A, B). In many cases, the apices of tubes expanded into buds, as is evident in both the phase-contrast micrographs in Fig. 7(C–G) and the SEMs in

Fig. 7(H–J). In some cases, the apical bud multiplied in the pseudohyphal growth form (Fig. 7I, J). Narrow tubes that had still not expanded at their apices were completely devoid of chitin septa along their lengths (e.g. Fig. 5I, K). In the case of tubes that had undergone apical expansion, a chitin septum was present at the junction between tube and apical bud (Fig. 5M). In cases in which the tube began to expand erratically prior to apical bud formation, lightly stained chitin rings were evident along the distal portion of the tube preceding the apical bud (Fig. 5O).

Tubes that had not undergone apical expansion were in most cases devoid of a nucleus (Fig. 6G–K). However, tubes that had begun to undergo apical expansion frequently contained two nuclei, one in the mother cell and one in the apical bud, but not along the tube (Fig. 6M, P, Q, R). Occasionally during apical expansion, an elongated nucleus could be found stretched through a portion of the tube, presumably in the process of nuclear division (Fig. 6L), or two daughter nuclei could be found in the tube, presumably just after nuclear division and prior to migration of one back to the mother cell and the other to the apical bud (Fig. 6O). Tube development appeared to be similar in the colony centres of the four core switch phenotypes.

To test whether the proportions of the three cell types differed between the core switch phenotypes during colony development, samples were removed at time intervals from the centres of developing colonies beginning at 3 days, and the proportions of the three cell types computed by microscopic analysis. At 3 days of development, 90–95 % of cells in colonies of the four core phenotypes were in the budding yeast form. The proportion of budding yeast cells then decreased from roughly 90 % at 3 days to 40–50 % at 6–7 days, followed

Fig. 8. Proportions of budding cells, pseudohyphae and tubes in the centres of colonies of the different core phenotypes and IWr as a function of colony development time.

by an increase in all four core phenotypes (Fig. 8A–D). The proportion of cells in the pseudohyphal growth form increased from 10% or less at 3 days to 40–50% between 7 and 9 days of colony development, and then decreased in all four core phenotypes (Fig. 8A–D). The proportion of tubes increased from less than 2% at 3 days to 10–20% between 5 and 6 days of colony development, then decreased in all four core phenotypes (Fig. 8A–D). Pseudohyphae and tubes were also observed in samples taken from colony edges of all four core phenotypes, but the proportions were far lower than in parallel samples taken from colony centres through 11–12 days of development (data not shown). Therefore, the changes in the proportions of budding yeast cells, pseudohyphae and tubes accompanying colony development were similar in the four core phenotypes.

IWr colonies are composed exclusively of pseudohyphae during early development

During the first 5 days of growth, IWr colonies were composed almost exclusively of pseudohyphae (Fig. 9A–D; Fig. 8E). This was true for samples from the

centres as well as the edges of colonies. The morphologies of IWr pseudohyphae were indistinguishable from those formed in the colonies of the core phenotypes. After 6 days of colony development, however, the pseudohyphae in IWr colonies began to form tubes (Fig. 9E, F) and release budding yeast cells. The proportion of budding yeast cells and tubes reached 10 and 30%, respectively, after 11 days of development (Fig. 8E). Therefore, the regulation of cellular phenotype during IWr colony development differs markedly from that during colony development of the core phenotypes, supporting the separation of IWr switching from core switching (Fig. 1).

Switching and filamentation are general characteristics of *C. glabrata*

To test whether switching, pseudohypha formation and tube formation represent general characteristics of all strains of *C. glabrata*, we plated at low density each of 62 independent clinical isolates of *C. glabrata* on three agar plates (~50 colonies per plate) containing 1 mM CuSO₄, and counted the proportions of variant colony phenotypes (i.e. phenotypes other than the dominant phenotype) after 6 days. At the end of 6 days, cells were removed from the centres of representative colonies exhibiting the dominant phenotype and variant phenotypes, and the cell populations (200 cells) scored microscopically for budding yeast cells, pseudohyphae, tubes and tubes with apical expansion. Switching was observed in 25 of the 62 isolates (40%). The dominant phenotypes most often expressed by the isolates were DB (50%) and LB (39%). Of the 25 isolates exhibiting multiple phenotypes in the 200 analysed colonies, 65% exhibited a mixture of LB and DB, 23% LB, DB and Wh, 4% Wh and vDB and 4% LB and Wh. Since only 150 colonies were scored on average for each strain, it was highly likely that switching was missed in many strains with switching frequencies lower than 7×10^{-3} (i.e. less than one variant in 150 colonies). We therefore tested whether selected strains forming no variant colonies in the initial plating did so in more expanded analyses (20 plates, providing a sensitivity of 1 in 10^3 colonies). Three strains originally forming only LB colonies and three originally forming only DB were tested. In all cases, switching was demonstrated in the expanded analyses, leading us to conclude that most, if not all, strains of *C. glabrata* undergo core switching.

Of 93 samples (dominant and variant phenotypes) analysed for cell type, 100% contained budding yeast cells, 96% contained pseudohyphae and 85% contained tubes. Of the samples with tubes, 61% exhibited expansion at the tube apices. In the cases in which apical expansion was not observed, the frequency of tube formation was usually low. When more cells were examined in two isolates that had a low tube number and no expanded apices in the original analysis, apical expansion of tubes was observed. These results, therefore, indicate that most if not all strains of *C. glabrata* form both pseudohyphae and tubes, and undergo apical tube expansion.

Fig. 9. Phase-contrast images of cells from IWr colonies after 5 days (A–D) and after 12 days (E, F). Arrows indicate examples of the pseudohyphal growth form in the 5-day cultures, and examples of tubes in the 12-day cultures. Bar, 10 µm.

DISCUSSION

An updated description of switching in *C. glabrata*

By developing an indicator agar containing CuSO_4 , we initially discovered a spontaneous, reversible, high-frequency switching system in *C. glabrata* that included the three core phenotypes Wh, LB and DB (Lachke *et al.*, 2000). We also identified in that study two additional phenotypes, vWh and vDB, which we suggested may be additional members of the same switching system, each at an opposite end of the switching hierarchy (i.e. vWh, Wh, LB, DB, vDB). Here, we have first demonstrated that vWh, which was isolated only once from a DB population, is not reversible and, therefore, does not appear to be part of the core switching system. In contrast, vDB is reversible, emanating from all other core switch phenotypes, and capable of switching back to all other core switch phenotypes. vDB has, therefore, been incorporated into the ‘core’ switching system (Fig. 1A) and has been positioned at the upper end of the CuSO_4 -based colour hierarchy. In support of that

decision, we have demonstrated that the expression of the switching-regulated gene *MTII* in vDB cells is at the high end of the expression hierarchy.

We have also presented proof here that the switch phenotypes discriminated on CuSO_4 -containing agar maintain their identities in the absence of CuSO_4 and that switching between core phenotypes is spontaneous and occurs in the absence of CuSO_4 . When cells from Wh, LB, DB and vDB colonies on CuSO_4 -containing medium were grown on phloxine B-containing agar, they exhibited a hierarchy of opposite intensities (dark pink, medium pink, light pink, white, respectively), and when they were returned to CuSO_4 -containing agar, they again expressed the phenotypes originally expressed on CuSO_4 . In addition, when variant colonies and sectors formed on phloxine B were plated on CuSO_4 -containing agar, they generated colonies with correlate colours (e.g. white colonies or sectors on phloxine B formed DB colonies on CuSO_4 ; medium pink colonies or sectors on phloxine B formed LB colonies on CuSO_4).

Identification of a second switching system in *C. glabrata*

In large plating experiments of LB and DB cells performed in the present study, we identified a new switch phenotype, IWr, that exhibits several characteristics of the core Wh cell phenotype, including a white to very light brown colour on CuSO_4 -containing agar, a medium to dark pink colour on phloxine B-containing agar, a low to negligible level of *MTII* expression, and a very high switching frequency. However, several unique characteristics suggest that switching to and from IWr represents a second switching system. First, unlike all of the core switch phenotypes (Wh, LB, DB, vDB), which form smooth round colony domes regardless of colour, the colony dome of IWr is highly wrinkled, and the perimeter irregular. Second, unlike all of the core switch phenotypes, which are composed of similar proportions of budding yeast, pseudohyphae and tubes, the colony domes of IWr are composed almost exclusively of pseudohyphae through the first 6 days of colony development. Finally, while core phenotypes emanating from different origins (e.g. from LB or DB) then switch to other core phenotypes with similar frequencies, IWr colonies exhibit a distinct propensity to switch back to the core phenotype of origin, suggesting that they maintain their original core phenotype identity. Together these characteristics are more consistent with a model in which switching to and from IWr represents a second spontaneous and reversible switching system.

Although multiple switching systems have been demonstrated previously in *C. albicans*, the different switching systems have been demonstrated to function in different strains. The switching systems of *C. albicans* include the ‘3153A switching system’, characterized in strain 3153A (Slutsky *et al.*, 1985) and strain CA14 (Perez-Martin *et al.*, 1999; Srikantha *et al.*, 1998), the ‘white–opaque transition’, characterized in strain WO-1 (Slutsky *et al.*,

1987) and the 'mycelated–unmycelated transition', demonstrated in select isolates from vaginitis patients (Soll *et al.*, 1987). The core and IWr switching systems of *C. glabrata* are the first that have been demonstrated in the same strain and the first that have been shown to interact. To corroborate this model, it must still be demonstrated that Wh and vDB can generate IWr clones that exhibit the same characteristics as IWr cells emanating from LB and DB, and that switching to and from IWr involves developmental programmes and the expression of phase-specific genes distinct from the programmes and genes involved in and regulated by core switching.

Pseudohypha and tube formation in Wh, LB, DB and vDB colonies

We have also confirmed previous observations that *C. glabrata* forms pseudohyphae (Csank & Haynes, 2000) and germ-tube-like structures (Brandao *et al.*, 1995). We have demonstrated here that the centres of 3-day-old colonies of all four core phenotypes contain primarily budding yeast cells, and that during subsequent colony development, colonies accumulate significant levels of pseudohyphae and tubes. We have demonstrated that the formation of pseudohyphae and tubes does not require nitrogen-starvation conditions. The pseudohyphae formed by *C. glabrata* resemble those formed by *C. albicans* (Merson-Davies & Odds, 1989; Odds, 1988). In no case, however, were true hyphae identified. Instead, we observed the frequent formation of tubes, which grew to lengths up to six cell diameters (9 µm). These tubes did not compartmentalize like true hyphae (Odds, 1988). Instead, they swelled at their distal ends to form a budding cell or the first in a sequence of pseudohyphal compartments. In cells that formed these tubes, the nucleus divided along the tube, and one daughter nucleus migrated back to the mother cell, while the other entered the apical bud. The *C. glabrata* tube, therefore, appears to function as a corridor during mitosis through which one daughter nucleus migrates to the new apical bud. Unlike true hyphae such as those found in *C. albicans*, the tubes of *C. glabrata* are not compartmentalized and end up nucleus free. It is not unreasonable to suggest that these tubes, like true hyphae, may serve as a vehicle for the dissemination of *C. glabrata* through tissue. Such a role would be supported by their identification in infected tissue.

Switching and the regulation of cellular phenotype

Although core switching has no apparent effect on the proportions and morphologies of pseudohyphae and tubes formed in a developing colony, switching to IWr has dramatic effects. During the first 6 days of development, IWr colonies are made up almost exclusively of pseudohyphae. This contrasts markedly with the colonies of all core phenotypes, which are made up predominantly of budding cells at 3 days of development, and slowly accumulate pseudohyphae, which reach a maximum of 40–50% after 7 days of development. After 6 days, IWr colonies begin to ac-

cumulate budding yeast cells and tubes. Switching in *C. albicans* also affects cellular phenotype. In the white–opaque transition in *C. albicans* strain WO-1, cells switch between a round budding cell and an elongated budding cell, phase-specific phenotypes with very different environmental restrictions on the bud-to-hyphae transition (Anderson *et al.*, 1989; Slutsky *et al.*, 1987; Soll, 1992; Soll *et al.*, 1991). In the more complex 3153A-type switching system in *C. albicans* (Perez-Martin *et al.*, 1999; Slutsky *et al.*, 1985; Soll, 1992; Srikantha, 1998), the differences in colony morphologies reflect dramatic spatial and temporal differences in pseudo-hypha and hypha formation in the colony dome. Finally, in the unmycelated–mycelated transition (Soll *et al.*, 1987), the difference in colony formation reflects differences in hypha formation at the colony periphery. We may, therefore, suggest that switching in these different *Candida* species may target similar pathways that regulate filamentation.

Switching and pseudohypha/tube formation are global characteristics of *C. glabrata* strains

We originally demonstrated that a single strain of *C. glabrata*, 35B11, and three additional clinical isolates, 65FLOP, 65TL1 and 75PL1, switched between Wh, LB and DB (Lachke *et al.*, 2000). Here we have demonstrated that in limited plating experiments of 62 additional *C. glabrata* isolates, 40% exhibited multiple core colony phenotypes, and in expanded plating experiments of isolates that did not exhibit multiple phenotypes in the initial plating, all formed multiple colony phenotypes; these results lead us to conclude that the great majority of *C. glabrata* strains undergo core switching. Because of the lower frequency of switching to IWr, an assessment of this switching system in the 62 isolates was untenable. Microscopic analysis of cells from colonies of these 62 isolates also revealed that 96% formed pseudohyphae and 85% tubes, and that again these were very likely underestimates, leading us to conclude that in addition to switching, the great majority of *C. glabrata* strains form pseudohyphae and tubes. Because *C. glabrata* is related to *Saccharomyces cerevisiae* (Barns *et al.*, 1991; Santos *et al.*, 1997), and because it is haploid (Doi *et al.*, 1992), the developmental programmes newly established in this pernicious opportunistic yeast pathogen may ultimately be more amenable to analysis than the comparable programmes in diploid *C. albicans* (Soll, 2000b).

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