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Candida glabrata displays pseudohyphal growth

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Abstract

The ability to undergo morphological change has been reported as an advantageous trait in fungal pathogenesis. Here we demonstrate that *Candida glabrata* ATCC2001, like diploid *Saccharomyces cerevisiae* strains, forms elongated chains of pseudohyphal cells on solid nitrogen starvation media (SLAD). Constrictions were apparent between adjoining cells; no parallel-sided hyphae were seen and pseudohyphae invaded the agar. When SLAD was supplemented with ammonium sulfate both *C. glabrata* and diploid *S. cerevisiae* strains lost their ability to undergo pseudohyphal growth. However, on this media *C. glabrata* yeast cells invaded the agar in a similar fashion to the invasive growth mode exhibited by haploid strains of *S. cerevisiae* cultured on rich media such as YPD. *C. glabrata* was not capable of invading YPD demonstrating that the process of filamentation is distinct in these two fungi. To our knowledge this is the first report to demonstrate that *C. glabrata* can undergo morphological change and grow as an invasive filamentous organism.

Keywords: Filamentous growth; Pseudohypha; Nitrogen starvation; Hypha; *Candida torulopsis*

1. Introduction

The incidence of nosocomial fungal infection has increased dramatically over the last few years to the extent that 10% of such infections are now caused by fungi and *Candida* species are now the fourth leading cause of bloodstream infections in the US with an attributable mortality of 38% [1,2]. While *Candida albicans* remains the most commonly isolated species, *Candida glabrata* is now encountered regularly and is responsible for up to 1:5 cases of candidosis [3,4].

C. albicans is a diploid organism with no known sexual cycle. Blastoconidia of this organism readily undergo the morphological change from yeast to hyphal or pseudohyphal growth in response to a wide variety of conditions including nitrogen starvation [5]. Diploid *Saccharomyces cerevisiae* also switch to pseudohyphal growth when starved for nitrogen [6] and in response to a limited range of other stimuli [7]. Haploid *S. cerevisiae* cells, on the other hand, form short, invasive pseudohyphal like cells on rich media but not on nitrogen starvation media. This is referred to as haploid invasive growth.

The signalling pathway and transcription factor genes that are known to govern pseudohyphal growth in *S. cerevisiae* are at least partially conserved in *C. albicans*. Mutants lacking these genes in *C. albicans* are reduced in their ability to filament in vitro and exhibit attenuated virulence in systemic mouse models of candidosis [5,8–11]. A double *STE12*(*CaCPH1*)/*PHD1*(*CaEFG1*) mutant is locked in the yeast form and is avirulent [12]. The ability to filament has therefore become accepted as being advantageous for virulence.

Unlike these two organisms, *C. glabrata* has never been observed in a filamentous form. This inability to adopt a filamentous growth mode has made *C. glabrata* relatively easy to distinguish from other *Candida* species observed in medical practice [13,14]. However, isolates of asexual budding yeast with no known filamentous form such as *C. glabrata* were originally assigned to the genus *Torulopsis* and the organism was originally designated *Torulopsis glabrata*. Despite the merger of *Torulopsis* into the genus *Candida* this designation has persisted in the medical liter-

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ature. The merger of the two genera was debated for many years because of the budding only morphology of *C. glabrata* and the historical definition of the genus *Candida* as 'pseudomycelial'. However, it was finally agreed upon, because the genus *Candida* includes species for which pseudohyphae are absent or rudimentary and many isolates of *Torulopsis* form pseudohyphae [14]. Recent DNA sequence data have confirmed this relationship and resulted in the reassignment of the genus *Candida*; family Candidaceae (formerly of the form-order Cryptococcales; form-class *Deuteromycetes*) to the order *Saccharomycetales*; phylum *Ascomycota*. This also includes the fully sequenced model organism *S. cerevisiae* which is very closely related to *C. glabrata* (http://www3.ncbi.nlm.nih.gov/Taxonomy/tax.html).

The ability of these organisms to undergo morphological change in response to nitrogen starvation plus the close taxonomic relationship between these organisms, *S. cerevisiae* and *C. glabrata*, in particular, prompted us to investigate the response of *C. glabrata* to growth on solid nitrogen starvation media. Here we report that the *C. glabrata* does indeed produce invasive pseudohyphae under nitrogen limiting conditions on solid media and in addition forms invasive yeast cells on the same media supplemented with ammonium sulfate. To our knowledge this is the first report of morphological transition in this increasingly important pathogenic fungus.

2. Materials and methods

2.1. Strains

C. glabrata ATCC2001 was obtained from the American Type Culture Collection (Rockville, MD, USA). The diploid *S. cerevisiae* strains used were L5366 *ura*3–52/*ura*3–52 (Σ1278b background) [15] which has been transformed to prototrophy with the vector pRS426 containing the UR*A3* gene and the prototrophic pseudohyphal strain L6294. The haploid prototrophic strain was 10560-5A. All *S. cerevisiae* strains were gifts from the laboratory of G.R. Fink (Whitehead Institute, Cambridge, MA, USA). *C. albicans* SC5314, a wild-type clinical isolate, was used throughout.

2.2. Media and culture conditions

Solid synthetic low ammonia dextrose nitrogen starvation medium (SLAD), was prepared as described by Gimeno et al. [6,16]. SLAD contains 2% (w/v) D-glucose (BDH Inc., Toronto, Canada), 1.7 g l^{-1} yeast nitrogen base without amino acids or ammonium sulfate (Difco Laboratories, Detroit, MI, USA), 10–50 μM ammonium sulfate (BDH Inc., Toronto, Canada) as a sole nitrogen source, and 2% (w/v) washed Bacto-Difco agar (granulated agar from BBL, Becton Dickinson and Co., Cockeysville, MD, USA was also used). To prepare the medium a 4× stock of the yeast nitrogen base (6.7 g l^{-1}), a 10× (0.5 mM) stock of ammonium sulfate, and a 40% (w/v) glucose stock were filter sterilized through a 0.45 μm Millipore filter, diluted to 2× the appropriate concentrations, warmed to 50°C and added to an equal volume of 50°C 4% (w/v) agar which had been washed four times with distilled deionized water prior to autoclaving. SLAD supplemented with 7.6 mM ammonium sulfate (SHAD) was also prepared.

Lee's solid medium (SM), developed for hyphal growth of *C. albicans*, was prepared by mixing autoclaved agar (4% w/v) and filter sterilized 2× media described in Lee et al. [17].

For examination of filamentous growth, cultures were grown overnight at 30°C in 10 ml YPD broth (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose), washed once with sterile distilled water and inoculated to obtain approximately 50 cells per plate.

To examine haploid invasive growth on rich medium (YPD) [18] cells were streaked onto the agar in quadrants and incubated at 30°C for 3 days followed by 1 day at room temperature prior to washing of agar surfaces. Agar invasion was investigated by gently scraping plates with a plastic inoculating needle and washing thoroughly under a stream of distilled water.

Cells were incubated at either 30°C (*S. cerevisiae*) or at 30°C and/or 37°C (*C. glabrata* and *C. albicans*). Experiments were duplicated in two independent laboratories.

2.3. Calcofluor staining

To determine the nature of budding in *C. glabrata*, cells were cultured overnight at 37°C on solid YPD plates containing 2% (w/v) agar. Cells were removed into 4% (v/v) formaldehyde, spun down immediately, washed 3× in PBS and resuspended in 100 μl sterile distilled water. Aliquots of 10 μl were mixed with 10 μl 1 mg ml^{-1} Calcofluor (Sigma) and left at room temperature for 5 min. The cells were washed 3× with sterile distilled water and resuspended in 100 μl volumes of PBS.

2.4. Microscopy

Colonies and cells were photographed using either a Nikon TMS inverted microscope and Kodak TMAX film and scanned into the computer, or a Zeiss Axiophot microscope and captured using a Hamamatsu CCD camera, and Improvision software. Plates for invasive growth on YPD were scanned directly into Adobe Photoshop (Adobe Systems Inc.) and all images were assembled using this program.

Budding patterns were visualized using a Nikon Eclipse E600 microscope with a 100× fluorescence objective and an excitation wavelength of 330–380 nm. Images were captured using a Nikon CoolPix 950 digital camera.

3. Results and discussion

On YPD and Lee's solid medium, which promotes radial hyphal growth of *C. albicans* colonies [19,20], *C. glabrata* formed smooth and circular colonies (Fig. 1A (a)) composed of yeast form cells. This smooth circular morphology is characteristic of unicellular yeast growing in the budding form [5,6,20,21].

On the other hand, on nitrogen starvation SLAD medium, *C. glabrata* developed colonies with regions of lateral growth which extended beyond colony borders (Fig. 1A (b, c)). This distinctive morphology is typical of *S. cerevisiae* and *C. albicans* filamenting colonies and is the result of growth proceeding away from the oldest cell combined with the ability of filamentous cells to penetrate agar [5,6,19,20]. Regions of *C. glabrata* radial growth occurring on SLAD were composed of chains of cells morphologically comparable to that of diploid *S. cerevisiae* pseudohyphae (Fig. 1B). Both *S. cerevisiae* and *C. glabrata* cells had an elongated cell shape and constrictions separated adjoining cells (Fig. 1B), consistent with the distinct pseudohyphal cell type of *S. cerevisiae* [6].

To quantify our observations we calculated a 'morphology index' $(2+1.78 \log sl/d^2$ where s is the diameter of the septal junction, l is the length of the cell, and d is the maximum diameter of the cell) for the three species growing under various conditions. This index was devised as an objective indicator of cell shape in *C. albicans* [22]. *C. albicans* cells growing exclusively in the spherical yeast form have an M_i between 1 and 1.5. We calculated an M_i of 1.34 for *C. glabrata* yeast cells. Elongated yeast cells have an M_i of about 2, and long pseudohyphal cells have an M_i of 2.5–3.4. True hyphae have M_i greater than 3.4 [22]. The morphology indices for *C. glabrata* and diploid *S. cerevisiae* grown on SLAD were 2.4 (range 1.9–3.2) and 2.5 (range 1.6–3.4) respectively, and thus the cells were similar in cell shape and had M_i greater than that of spherical yeast cells. Thus our findings support the existence of a filamentous, pseudohyphal growth mode for *C. glabrata*.

In *C. albicans* and diploid *S. cerevisiae* filamentous growth of SLAD is accompanied by invasion of the media [8,25,28]. In addition haploid *S. cerevisiae* strains invade rich solid media below the colonies as very short chains of cells, distinct from diploid pseudohyphae. Haploid invasive growth occurs on solid rich media, but not in response to nitrogen starvation and is accompanied by a switch from axial to bipolar, but not unipolar, bud site choice [18]. Haploid *S. cerevisiae* are capable, however, of undergoing pseudohyphal growth in response to butanol [7]. Each of these developmental alterations involves components of the same MAP kinase cascade [7,18].

We investigated the ability of *C. glabrata* to undergo invasive growth on both rich YPD media and on nitrogen starvation SLAD medium. On SLAD, both *C. glabrata* and *S. cerevisiae* diploids formed pseudohyphal filaments

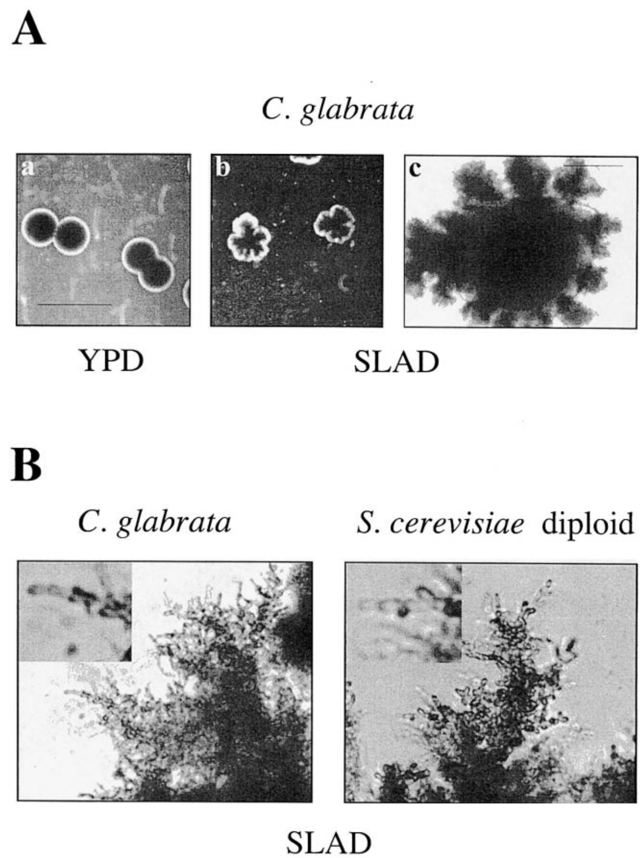

Fig. 1. *C. glabrata* ATCC2001 exhibits pseudohyphal growth in response to nitrogen starvation. A: *C. glabrata* ATCC2001 cells were incubated at 37°C for 2 weeks on Lee's solid medium (a), where they grew as round colonies made up of spherical yeast form cells or on nitrogen starvation solid medium (SLAD) (b and c), where they developed polarized colonies. The colony in (c) is a blow up of one of the colonies shown in (b). (a, b) bar = 3.1 mm; 2× objective; (c) bar = 0.62 mm; 10× objective. B: Pseudohyphal chains of *C. glabrata* cells observed from the perimeter of the colony shown in A (b, c), compared to pseudohyphal diploid *S. cerevisiae* cells (pRS426 transformed L5366) also cultured on SLAD for 2 weeks at 30°C (observed with a 40× objective; bar = 0.15 mm).

that invaded the agar (Fig. 2). On YPD invasive growth was observed only for *S. cerevisiae* haploids, neither *S. cerevisiae* diploids nor *C. glabrata* haploids invaded the agar (Fig. 3). However, when SLAD was supplemented with 7.6 mM ammonium sulfate *C. glabrata* invaded the agar as clumps of yeast cells beneath the colony (Fig. 2) in a similar manner to the haploid invasive growth of *S. cerevisiae* (Fig. 3). Therefore the morphogenetic and invasion phenotypes of *C. glabrata* are distinct and separable from both the pseudohyphal and invasive growth phenotypes observed in *S. cerevisiae* [6,18]. The molecular mechanisms underlying these phenotypes in *C. glabrata* are unexplored. However, recent work has demonstrated that it may be different to that seen in *S. cerevisiae*. The *C. glabrata STE12* homologue lacks a consensus site for binding of Dig1p and Dig2p (K. Haynes, F. Muehlschlegel, T. Rogers and M. Jones, unpublished data)

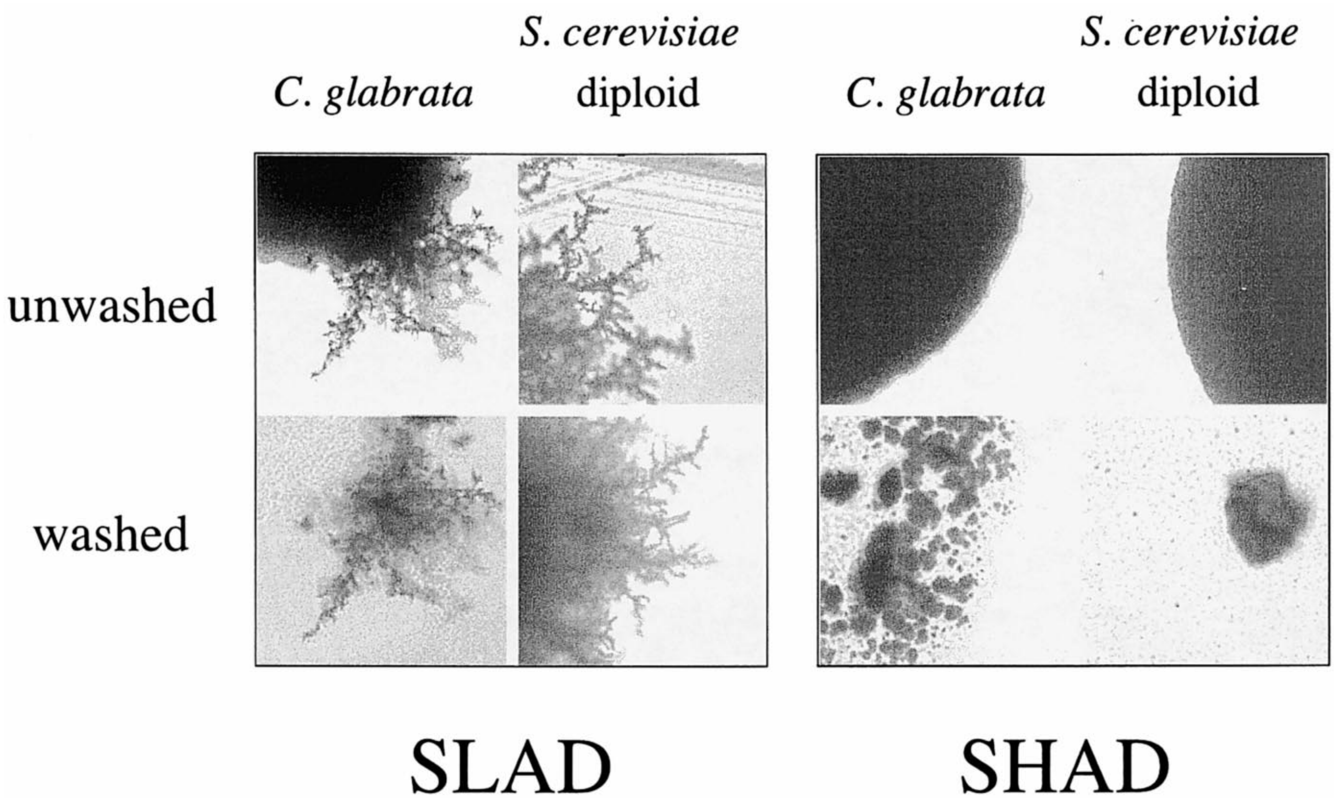

Fig. 2. *C. glabrata* ATCC2001 exhibits invasive growth as pseudohyphae on nitrogen starvation media and as yeast cells on defined media containing 7.6 mM ammonium sulfate. Cells were cultured on low ammonia nitrogen starvation media (SLAD) or SLAD supplemented with 7.6 mM ammonium sulfate (SHAD) for 15 days at 30°C (diploid *S. cerevisiae* L5366 (transformed with pRS426)) or 37°C (*C. glabrata* ATCC2001). Colonies growing on agar were photographed with a 10× objective and then plates were washed thoroughly with distilled water and photographed again. As expected the diploid *S. cerevisiae* strain produced pseudohyphae on SLAD which were capable of invading the agar as they were not removed by washing. In contrast, when cultured on SHAD *S. cerevisiae* L5366 (transformed with pRS426) grew as non-invasive spherical yeast cells which could be removed from the agar by washing. Similarly *C. glabrata* ATCC2001 produced invasive pseudohyphae on SLAD. However, when cultured on SHAD, this *C. glabrata* strain invaded the agar directly below the colony as yeast cells which could not be removed by washing.

which in *S. cerevisiae* help to inhibit the transcription factor until the MAP kinase cascade is activated [23,24].

When growing pseudohyphally *S. cerevisiae* and *C. albicans* cells do not undergo cytokinesis but form chains of cells separated by constrictions [6,21,22]. Daughter cells in these chains grow away from the oldest cell in the chain because new buds emerge at the pole opposite the previous mother/daughter junction (unipolar) [6]. Unlike *S. cerevisiae*, *C. albicans* has a true hyphal form which undergoes apical growth and is composed of elongated cellular compartments that are separated by perpendicular septa [22,25]. In order to undertake pseudohyphal growth cells must be able to bud in a unipolar fashion [6,21,26]. Budding growth of *S. cerevisiae*, *C. albicans*, and *C. glabrata* results in the formation of a small, spherical, outgrowth which eventually breaks away from the mother cell to form a new individual [4,21]. On rich liquid media diploid *S. cerevisiae* and *C. albicans* have a polar budding pattern, whereas haploid *S. cerevisiae* strains exhibit axial budding which results in the formation of tight clusters of cells (Fig. 4A) [18]. *C. glabrata* also demonstrated a polar budding pattern (Fig. 4A and B) and the pattern of cell growth appeared consistent with asymmetric and unipolar bud site selection. Daughter cells tended to emerge from the pole distal to the site of emergence of the mother cell resulting in the formation of branched chains (Fig. 4A). The degree of this asymmetrical growth pattern was most evident when cells were stained with Calcofluor White to visualize bud scars (Fig. 4B). *C. glabrata* therefore demonstrates a budding pattern consistent with the ability to undergo pseudohyphal growth.

It has been suggested that filament formation in *S. cerevisiae* may have been used to seek out new nutrient supplies [6]. Indeed, recent evidence has demonstrated that one of the genes induced by the heteromeric filamentation transcription factor (Tec1p/Ste12p) encodes a secreted endopolygalacturonase (Pgu1p) that is capable of degrading the plant specific polysaccharide pectin [27]. Nitrogen starvation induces a MAPK cascade in *S. cerevisiae* which results in the 'activation' of this heteromeric filamentation transcription factor (Tec1p/Ste12p). In the human pathogen *C. albicans*, filamentous growth has been linked to virulence [12]. Strains lacking functional copies of *CPH1* (the homologue of *STE12*) and *EFG1* (the homologue of *PHD1*) are unable to form hyphae in vitro and are attenuated in animal models of candidosis. However, recent work suggests that effectors involved in processes other than filamentation may play a part in this attenuation as filamentous forms of this mutant were seen in infected gnotobiotic pigs [28]. The role of filamentation in *C. glabrata* disease remains unexplored, however to our knowledge no description of *C. glabrata* growing in vivo as anything other than a budding yeast exists.

The morphological similarities between *C. glabrata* and *S. cerevisiae* pseudohyphae, and the similar environmental conditions required for inducing the yeast to pseudohyphal switch, suggest that the molecular mechanisms underlying these alterations may be similar. In both *S. cerevisiae* and *C. albicans*, at least two independently regulated biochemical cascades induce the yeast to hyphal transition and are required for the virulence of *C. albicans* [12]. Whether invasion of tissues by pseudohyphae plays a role in the virulence of *C. glabrata* is not yet known, although only budding growth has been observed in animal tissues [4]. The answer to this and many other interesting aspects of *C. glabrata* pathobiology will require the cloning and disruption of *C. glabrata* homologues of *S. cerevisiae* and *C. albicans* filamentation genes. In addition the haploid nature of *C. glabrata* renders certain genetic screens practical [29]. It is anticipated that further analysis will reveal novel genes involved in filamentation and pathogenicity. The demonstration, here, of dimorphism and invasive behaviors for this genetically tractable haploid fungal pathogen, enhances the reputation of *C. glabrata* as a model organism for the analysis of fungal virulence.

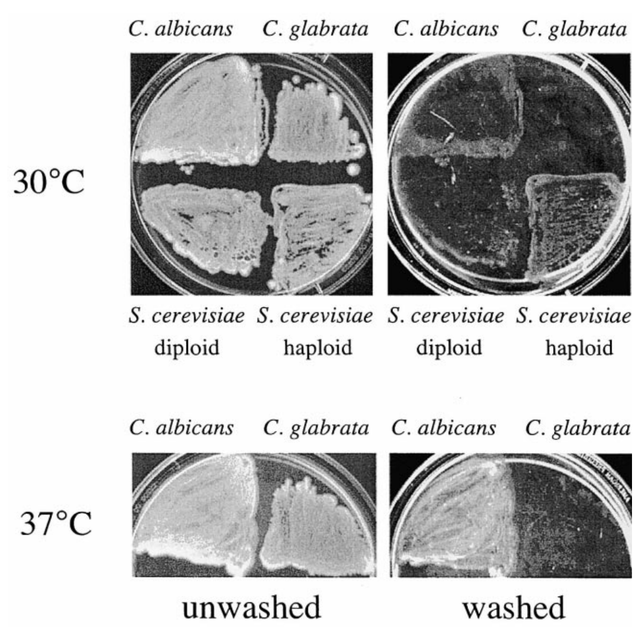

Fig. 3. *C. glabrata* ATCC2001 does not exhibit invasive growth on YPD. Cells were incubated at either 30°C (*S. cerevisiae* 10560-5A, *S. cerevisiae* L6294) or 37°C (*C. glabrata* ATCC2001 and *C. albicans* SC5314) for 2 days on YPD. Colonies growing on agar were photographed and then plates were washed thoroughly with distilled water and photographed again. As expected haploid *S. cerevisiae* and *C. albicans* were able to invade YPD, while diploid *S. cerevisiae* did not invade the agar. *C. glabrata* ATCC2001 grew as yeast cells on YPD but did not invade the agar at either 30°C or 37°C.

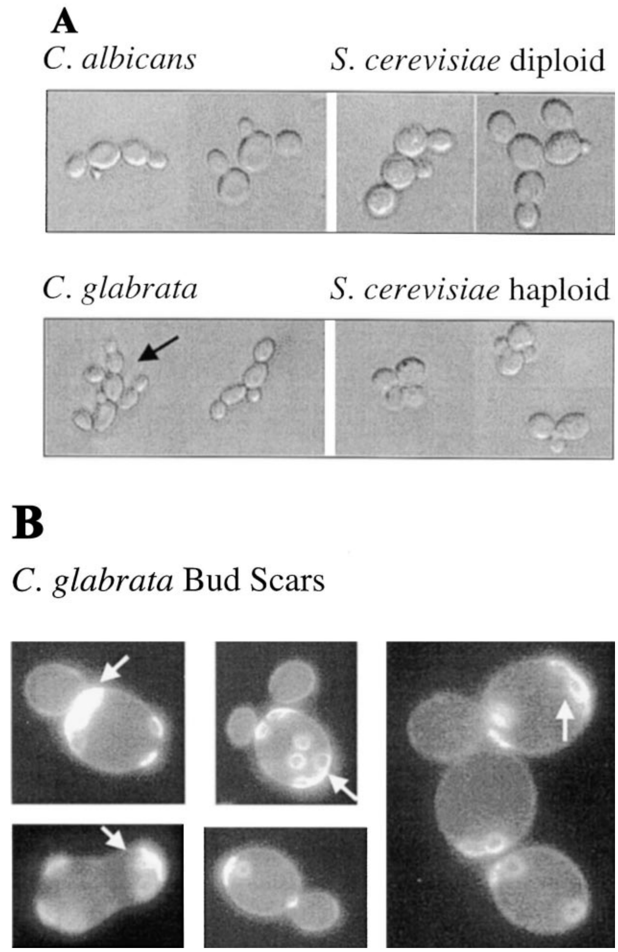

Fig. 4. *C. glabrata* ATCC2001 exhibits a polar budding pattern. A: Cells were incubated at either 30°C (*S. cerevisiae* 10560-5A, *S. cerevisiae* L6294) or 37°C (*C. glabrata* ATCC2001, *C. albicans* SC5314) overnight in liquid YPD. They were then inoculated on to solid YPD and incubated for 2 days at 30°C or 37°C, prior to 1 day at room temperature. Growing cells were then viewed using Nomarski optics (63× objective). *C. glabrata* ATCC2001 had a distinct polar budding pattern (arrow), similar to that seen in *C. albicans* and diploid *S. cerevisiae* but distinct from the axial budding pattern seen in haploid *S. cerevisiae*. B: *C. glabrata* cells prepared as in A were stained with Calcofluor White and observed with a fluorescent microscope (excitation wavelength 330–380 nm, 100× objective). The presence of bud scars at opposite poles of the yeast cells (arrows) indicates that *C. glabrata* ATCC2001 undergoes polar budding.

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