



Colony variation in *Candida glabrata* isolates from patients with vaginitis

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Summary

The ability of *Candida glabrata* to switch between different colony phenotypes on a Sabouraud's-chloramphenicol agar supplemented with phloxine B was assessed in 14 *C. glabrata* isolates. Three phenotypes (pale pink smooth, dark pink smooth and fuchsia petite) were observed *in vitro* and two of them (pale pink smooth and dark pink smooth) in fresh clinical isolates plated directly from vaginal specimens from patients with *C. glabrata* vaginitis and patients colonized with *C. glabrata*. The pale pink smooth phenotype was the predominant and the remaining phenotypes can be derived from it. No changes in susceptibility to different antifungals were observed in the pale pink smooth, dark pink smooth, fuchsia petite phenotypes but they showed differences in cell wall antigens.

Key words

Candida glabrata, Phenotypic switching, Vulvovaginal candidiasis, Antigenic variation

Variación en las colonias de *Candida glabrata* procedentes de pacientes con vaginitis

Resumen

Hemos estudiado en 14 aislamientos clínicos de *Candida glabrata* la capacidad de transformación entre diferentes morfotipos coloniales en agar de Sabouraud con cloranfenicol suplementado con floxina B. Se observaron tres fenotipos *in vitro* (rosa pálido liso, rosa oscuro liso y pequeño fucsia) y dos de estos fenotipos (rosa pálido liso y rosa oscuro liso) en aislamientos sembrados directamente a partir de muestras de pacientes con infección o colonización vaginal por *C. glabrata*. El fenotipo rosa pálido liso era el predominante y los otros dos fenotipos son probablemente derivados de éste. No se observaron diferencias de sensibilidad *in vitro* a los antifúngicos entre los fenotipos obtenidos pero se encontraron diferencias en los antígenos de la pared celular de los mismos.

Palabras clave

Candida glabrata, Transformación fenotípica, Vulvovaginitis candidiásica, Variación antigénica

Vulvovaginal candidiasis is a highly prevalent condition affecting millions of women [1]. The predominant causative agent is *Candida albicans* but other *Candida* species may also be involved [2]. Among them, *Candida glabrata* is involved in 16% of cases [3], being the second causative agent of these infections [3,4]. *C. glabrata* can colonize the oral cavity, respiratory, gastro-intestinal and genito-urinary tracts of humans [5] and it has increasingly been reported as an opportunistic pathogen in immunocompromised patients [6,7].

The pathogenesis of *C. glabrata* vulvovaginitis is to a large extent unknown but it is supposed to be similar to that of *C. albicans*. Certain states of the host, like an immunodeficient status, diabetes, the ingestion of the contraceptive pill, antibiotic and steroid therapy reputedly favor *Candida* infection [8,9]. However, also some fungal factors may play a role in the development of the full-blown clinical syndrome [10]. Among them, the switching at high frequency between an apparently limited number of phenotypes distinguishable by colony morphology described in *C. albicans*, *Candida tropicalis* and *C. glabrata* [11-13] seems to provide the fungal cells with the plasticity to evade the immune system and to develop resistance to antifungal drugs [12]. In fact, it has been reported that a single *C. albicans* strain, which was responsible for suc-

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cessive episodes of recurrent vaginitis, switched colony phenotypes between episodes [14]. In this report, we have studied the switching capacity of *C. glabrata* isolates from patients colonised or infected by this fungus and the influence of the switching on the antigenic expression and the susceptibility to different antifungals.

Materials and methods

Isolation of *C. glabrata* strains and culture conditions. Samples were removed from the wall of the vaginal tract of patients with *C. glabrata* vulvovaginal infection or colonised with *C. glabrata* with either a sterile cotton swab or a vaginal wash as previously described [15]. The swabs or the sediments from the vaginal washes were inoculated on Sabouraud's chloramphenicol agar (bioMérieux, France), and incubated at 37°C for 48h. All the yeasts isolated were identified as *C. glabrata* by the ID 32C yeast identification system (bioMérieux). *C. glabrata* NCPF 3203, obtained from the National Collection of Pathogenic Fungi (Bristol) was used as control. To study the phenotypic switching and assess the switching frequencies of primary and subsequent colonies, a modification of the method described by Soll *et al.* [12] was used. Briefly, the cells were removed from individual clonal colonies, suspended in sterile water, counted and plated at 150-200 cells per plate on Sabouraud's chloramphenicol agar to which phloxine B (5 µg/ml, Sigma Chemical Co. USA) (SCF agar) was added (SCF agar). The plates were then incubated for five days at 37 °C before analysed for colony morphology, size and color. The frequency of switching was determined by analyses of colony morphologies in clonal plating experiments. To avoid variability in colony colors or colony morphology, all the plates were poured with 25 ml of SCF agar per plate. In some experiments, the clinical specimens were plated directly on SCF agar and incubated as described above. Three colors were observed: pale pink = Pantone 176C, dark pink = Pantone 1785C and fuchsia = Pantone 1955. Pantone (Pantone Inc., USA) is a registered trade mark for color standardization of publishing and graphic design work.

Antifungal susceptibility. The susceptibility of the colonial phenotypes to 5-fluorocytosine, nystatin, amphotericin B, miconazole, econazole and ketoconazole was evaluated by the automated API-ATB-Fungus® (bioMérieux), as described previously [16].

Antibodies. The rabbit polyclonal Candida Check factor 6 antiserum was purchased from Iatron Laboratories Inc. (Japan). An anti-*C. glabrata* NCPF 3203 antiserum was prepared by monthly intravenous inoculations of a New Zealand White rabbit with 5 x 10⁸ yeasts.

Antigenic extraction, SDS-PAGE and Western blotting. The cell wall of the different colonial phenotypes was extracted with dithiothreitol according to a previously described method [18]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [19] in a minigel system (Bio-Rad Laboratories). The total amount of protein loaded per lane was 5 µg for each extract. Electrophoresis was carried out in 10% (w/v) gels at 200 V for 1 h. Subsequently, the gels were either stained with Coomassie blue or were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad) for 30 min at 60 V, 10 W and 5 mA/cm² according to Towbin *et al.* [20]. After the transfer, the nitrocellulose membranes were blocked in 10% (w/v) nonfat dry milk in Tris-buffered saline, washed in Tris-buffered saline and incubated with a 1:1, 1:20 and

1:20 dilutions of the anti-factor 6 and anti-*C. glabrata* antiserum respectively, washed, and incubated with peroxidase-labeled, affinity-purified goat anti-mouse IgM or goat anti-rabbit IgG (Sigma). Immunoreactive bands were visualized after staining for 30 min with a substrate solution (0.05% (w/v) 4-chloro-1-naphtol [Sigma] and 0.015% (v/v) H₂O₂ in Tris-buffered saline).

Results

The ability of *C. glabrata* NCPF 3203 to switch between different colonial phenotypic variants was initially studied on SCF agar. The vast majority of colonies obtained (6,460) showed a dark pink smooth phenotype and only 10 colonies showed a fuchsia petite phenotype (frequency 0.0015). To assess if the switching occurred also in fresh clinical isolates, we plated cells directly from the vaginal specimens taken from 4 patients with *C. glabrata* vaginitis and nine patients colonised with *C. glabrata* (Table 1). We found that seven of the 13 vaginal specimens had only one type of colonies, which was characterized as pale pink smooth. However, the remaining six specimens presented two different colonial phenotypic variants both identified as *C. glabrata*. Both colonial types presented the same size but they differed in their color, since one type was pale pink smooth and the other dark pink smooth (Figure 1a). No apparent relationship was found between the isolation of multiple phenotypes and the presence or absence of *C. glabrata* infection.

Table 1. Colony description of the strains isolated from vaginal swabs.

Specimen	Patient status	Type	Colony description (colour, texture, size)
S-1	Colonisation	1	pale pink, smooth, large
		2	dark pink, smooth, large
S-2	Infection	1	pale pink, smooth, large
		2	dark pink, smooth, large
S-3	Infection	1	pale pink, smooth, large
		2	dark pink, smooth, large
S-4	Colonisation	1	pale pink, smooth, large
		2	dark pink, smooth, large
S-5	Colonisation	1	pale pink, smooth, large
		2	dark pink, smooth, large
S-6	Colonisation	1	pale pink, smooth, large
		2	dark pink, smooth, large
S-7	Infection	1	pale pink, smooth, large
S-8	Infection	1	pale pink, smooth, large
S-9	Colonisation	1	pale pink, smooth, large
S-10	Colonisation	1	pale pink, smooth, large
S-11	Colonisation	1	dark pink, smooth, large
S-12	Colonisation	1	dark pink, smooth, large
S-13	Colonisation	1	pale pink, smooth, large

In an attempt to test whether the two *C. glabrata* isolates present in the same specimen represented switch phenotypes of the same strain or were separate strains, we clonally expanded cells from both phenotypes. When we plated the pale pink smooth phenotype we obtained three different phenotypes, the parental, which was the predominant, and the dark pink smooth and fuchsia petite phenotypes (Table 2, Figure 1). Plating of the dark pink smooth phenotype resulted in two types of phenotypes, the parental, which was the predominant, and the fuchsia petite phenotype. However, all the secondary colonies

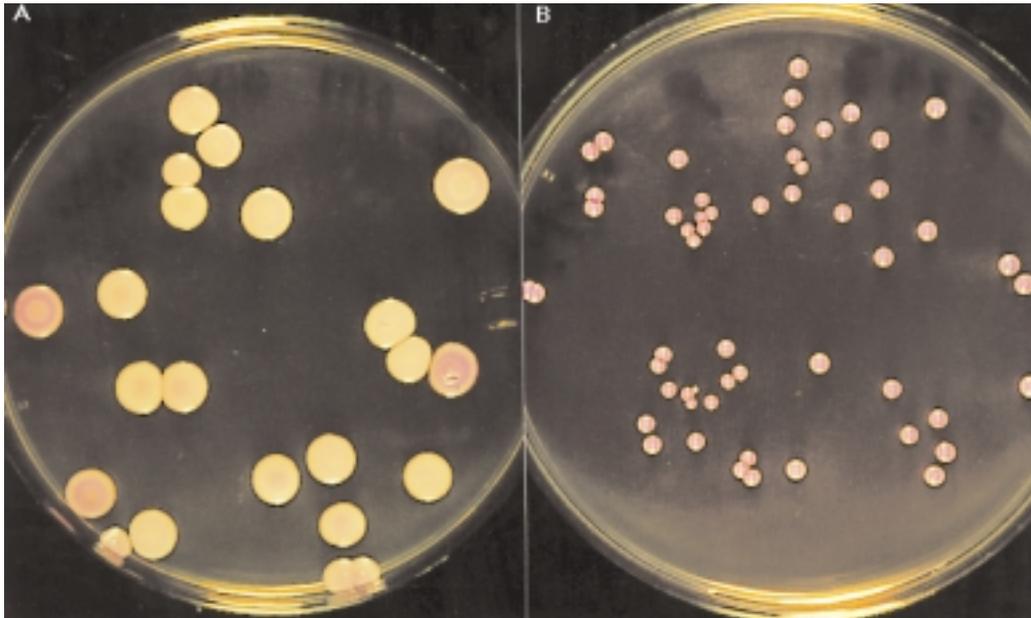


Figure 1 Colonial phenotypes of *Candida glabrata* in Sabouraud's-chloramphenicol agar supplemented with phloxine B. A: Pale pink smooth and dark pink smooth; B: Fuchsia petite.

obtained after plating the fuchsia petite phenotype exhibited the parental phenotype.

No differences in susceptibility to six antifungals were observed in the three phenotypes studied (data not shown). However, the three phenotypes could be distinguished by their protein patterns and antigenic reactivity of cell wall extracts. The three phenotypes showed identical protein patterns for cell wall proteins of molecular masses <50 kDa (Figure 2a). The pale pink smooth and dark pink smooth phenotypes showed also similar proteins of high molecular mass but those proteins were not observed in the fuchsia petite extract. Similar results were observed with the phenotypes derived from another isolate (data not shown).

The sera from a rabbit immunized with *C. glabrata* was initially used to assess the differences in reactivity of cell wall components from the three phenotypes. The pattern of reactivity in the pale pink smooth and dark pink smooth phenotypes cell wall extracts was very similar, showing well-defined bands for proteins of molecular masses >30 kDa (Figure 2b). However, the extract from the fuchsia petite phenotype showed a lower reactivity and a band of 48 kDa not observed in the other phenotypes. Similar results were observed with cell wall extracts obtained from colonial phenotypic variants derived from another isolate (data not shown). Interestingly, when the antigenic extracts were probed with an antiserum against the factor 6, only the pale pink smooth extract showed reactivity in a component of 50 kDa (Figure 2c).

Discussion

The capacity to switch between different colonial morphologies has been reported in a group of clinically relevant *Candida* species including *C. albicans*, *C. tropicalis*, *Candida parapsilosis* and *Candida krusei* [12,21]. Lachke *et al.* [13] have recently demonstrated that *C. glabrata* undergoes reversible, high-frequency phenotypic switching between a white, light brown, and dark brown colony phenotype discriminated on an indicator agar con-

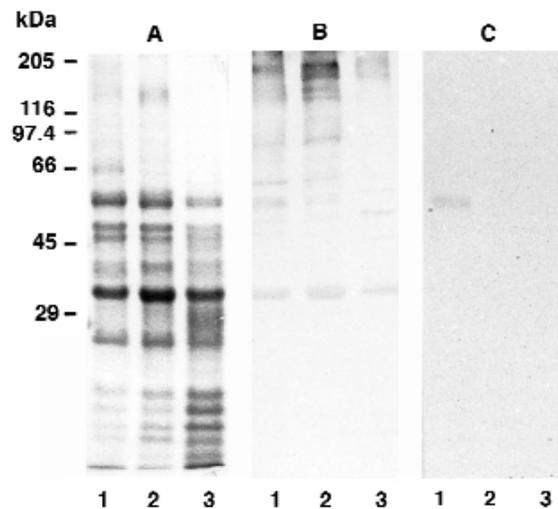


Figure 2. A. Coomassie blue staining of cell wall extracts from the pale pink smooth (lane 1), dark pink smooth (lane 2) and fuchsia petite (lane 3) colonial phenotypic variants of *C. glabrata*, separated by SDS-PAGE using a 10% acrylamide slab gel. Western blots of the same antigenic extracts showed in A stained with the serum from a rabbit immunized with *C. glabrata* (B) and with an antiserum against factor 6 (C).

taining 1 mM CuSO_4 . In this study, we have observed a similar phenomenon in *C. glabrata* isolates and we have described three phenotypes using Sabouraud's dextrose agar supplemented with the red dye phloxine B. Our first observation was made on Sabouraud's dextrose agar, where we distinguished two types of colonies on the basis of their size (large and petite). In order to distinguish the colonies by characteristics other than size, we tested media used by other authors for switching studies with *C. albicans* but we confirmed that *C. glabrata* did not grow on those media [21]. However, when using the SCF agar, we observed that the two colonial types of

C. glabrata stained different, the petit colonies staining fuchsia and the large staining pink. Additional experiments showed that the large colonies could be divided into two types (light and dark) according to the intensity of the color. Therefore, SCF agar seems to be a suitable culture medium to study the colony variation in *C. glabrata*.

As in *C. albicans*, the phenotypic switching in *C. glabrata* can occur both *in vitro* and *in vivo*. Five phenotypes were observed *in vitro* but only two phenotypes were observed, in some patients, in fresh clinical isolates plated directly from vaginal specimens from patients with *C. glabrata* vaginitis and patients colonised with *C. glabrata*. The pale pink smooth phenotype seems to be the predominant, both in patients in whom no spontaneous switching was observed and in those in whom high frequency switching was observed. *In vitro* cloning experiments demonstrated that the pale pink smooth phenotype was the predominant and that the remaining phenotypes can be derived from it.

The colony switching described in *C. albicans* and *C. tropicalis* seems to develop resistance to antifungal drugs by the fungal cells and to induce antigenic changes [12]. We have not been able to demonstrate differences in antifungal susceptibility in the three phenotypes studied in *C. glabrata*, but Bouchara *et al.* have described a petite mutant of *C. glabrata* isolated from a stool specimen from a bone marrow transplant patient treated with fluconazole that was resistant *in vitro* to azole antifungals [22]. However, we have observed antigenic differences in the three phenotypes that may facilitate the evasion of the

immune system by some cells. When compared to the other phenotypes, the fuchsia petite phenotype showed important differences in the protein composition and antigenicity of cell wall extracts. Conversely, the pale pink smooth and dark pink smooth phenotypes showed a similar cell wall protein pattern and antigenic reactivity with a rabbit anti-*C. glabrata* antiserum but they differed in the expression of antigen 6, an antigen which confers the serotype specificity in *C. albicans*. Interestingly, this antigen has been shown to be expressed in *C. glabrata* [17], and although the influence of switching on the expression of this antigen was not studied, that observation is in agreement with the expression of the antigen 6 by the pale pink smooth phenotype, which is usually the predominant in the primary cultures of *C. glabrata*. Antigenic changes have been described during white-opaque transition in *C. albicans* [23] and between colonial phenotypic variants of *C. albicans* [24].

In conclusion, we have demonstrated the capacity of *C. glabrata* to produce colonial variants *in vitro* showing antigenic differences but not changes in susceptibility to different antifungals. The phenotypic switching was also observed in fresh clinical isolates plated directly from vaginal specimens, and this capacity is showed not only by isolates from vaginal infections but also by isolates from other origins, such as catheters, liver and peritoneum (data not shown).

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