Biological consequences of petite mutations in *Candida glabrata*

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**Objectives:** To define the pathogenicity of respiration-deficient mutants of *Candida glabrata*, which present a reduced susceptibility to azoles, that are easily induced *in vitro* by exposure to these drugs or to ethidium bromide and that may be selected *in vivo* in patients receiving fluconazole.

**Methods:** Two wild-type isolates of *C. glabrata* were compared with their respective fluconazole- or ethidium bromide-induced petite mutants, regarding the carbohydrate and protein composition of the cell wall, as well as their surface physical properties, and also their adherence abilities and virulence in mice.

**Results:** Flow cytometric analysis of cell wall carbohydrates using several fluorescent lectins showed an increased binding of mutant cells to concanavalin A compared with their parent isolates, suggesting a greater availability or an increased amount of glucose–mannose residues at the cell surface in petite mutants. Likewise, some quantitative differences between parent and mutant isolates were shown by SDS–PAGE in protein extracts from blastoconidia. Regarding the surface physical properties, no significative differences were seen in the electrophoretic mobility determined by microelectrophoresis, but the two-phase partitioning method revealed a lower cell surface hydrophobicity for petite mutants. Moreover, mutant cells exhibited significant overexpression of *CgEPA1* as revealed by real-time reverse transcription-PCR, but the adherence capacities to Caco-2 cells, a human enterocyte line, were not significantly different. Finally, in agreement with their slower growth, petite mutants were less virulent than parent isolates in a murine model of systemic infection.

**Conclusion:** This low virulence in mice suggests that petite mutants could be disregarded clinically although they may arise during fluconazole therapy.

Keywords: petite mutants, cell surface hydrophobicity, adherence, virulence

**Introduction**

*Candida glabrata* is usually a commensal of the digestive tract and of the skin in humans. However, this opportunistic yeast can also be responsible for oral or deep infections in AIDS and other immunocompromised patients, and it is increasingly reported as a causative agent of candidiasis. To fight these infections, two classes of antifungal drugs are commonly used: the fungicidal polyene drugs such as amphotericin B and the fungistatic azoles, mainly the triazoles fluconazole and voriconazole, which have become the primary treatment of many fungal infections. However, their widespread use has led to the development of azole resistance and to the emergence of intrinsically resistant or less-susceptible *Candida* species such as *Candida krusei* and *C. glabrata*.

Investigations of the mechanisms of azole resistance have been focused mainly on *Candida albicans*, and different mechanisms have been described including overexpression of the *ERG11* gene encoding the azole target, point mutations in the nucleotide sequence of this gene, and reduced intracellular accumulation of the drug due to overexpression of genes encoding efflux pumps.

In *C. glabrata*, similar mechanisms have been reported, and we have recently identified the molecular mechanisms of azole resistance in some respiration-deficient petite mutants, thus confirming the previous results of the group of Sanglard.
Petite mutations, which are due to a partial or total loss of mitochondrial DNA (mtDNA), have been induced in several yeasts, categorized as petite-positive yeasts. In contrast, respiratory deficiency is usually lethal in almost all species of the Candida genus including C. albicans, which are therefore classified among petite-negative yeasts. However, C. glabrata, which is very closely related to Saccharomyces cerevisiae, is a petite-positive yeast. Indeed, petite mutants can be easily induced in vitro in C. glabrata by exposure to fluconazole or to the intercalating agent ethidium bromide (ETB), leading to a decrease in the susceptibility to azoles with a concomitant increased susceptibility to polyenes. In addition, we demonstrated a close relationship between respiration and susceptibility to azoles. Indeed, blockage of respiration by cultivation of the fungus in the presence of sodium azide induced a decreased susceptibility to azoles, culminating in azole resistance due to the deletion of mtDNA. Furthermore, we showed that the selection of azole-resistant petite mutants of C. glabrata may occur in vivo after oral administration of fluconazole.

However, very little information is available regarding the pathogenicity of these respiration-deficient mutants. Here, we compared two wild-type isolates of C. glabrata and their respective fluconazole- or ETB-induced petite mutants, in terms of carbohydrate and protein composition of the cell wall, as well as also their adherence abilities and virulence in mice.

Materials and methods

Yeast strains and culture conditions

This study was carried out using two clinical isolates of Candida glabrata, designated 90.1085 and 94.5579, obtained in our hospital laboratory in 1990 from a urine sample and in 1994 from a broncho-alveolar lavage, respectively. For each clinical isolate, a fluconazole-resistant mutant was induced for each clinical isolate by exposure to fluconazole for 2 min in 500 µL of SDS–PAGE sample buffer (Tris/HCl 62.5 mM pH 6.8, glycerol 10%, SDS 1%, β-mercaptoethanol 5%, bromophenol blue 5%), followed by centrifugation for 5 min at 10000 g. Proteins in the supernatants (20 µL samples), which corresponded to cell wall components, were separated on a 12% polyacrylamide gel. Electrophoresis was performed as described by Laemmli, and the gel was stained with Coomassie Brilliant Blue. Molecular mass of the proteins (in kDa) was interpolated from the migration of the protein standards (Amersham Biosciences Europe GmbH, Saclay, France): phosphorylase b, 94; albumin, 68; ovalbumin, 43; carbonic anhydrase, 30; trypsin inhibitor, 20 and α-lactalbumin, 14.4.

Surface physical properties of blastoconidia

The relative cell surface hydrophobicity (CSH) was measured using a modification of the method described by Rosenberg et al., which relies on the partitioning of a yeast cell suspension between an aqueous phase and a hydrocarbon phase due to differences in CSH. To do this, cells grown to stationary phase on YEPD plates were washed and then resuspended in PBS to obtain an absorbance of 0.6–1.0 at 490 nm. Then 2.5 mL aliquots of each yeast suspension were distributed in four glass test tubes and overlaid with 0.5 mL of hexadecane, except for one tube which was used as a control. After equilibration for 10 min at 37°C, the samples were vortexed for 30 s and then settled at 37°C for 30 min to allow the two phases to separate. The absorbance of the aqueous phase was then measured spectrophotometrically at 490 nm. The percentage of exclusion of the cells from the aqueous phase was calculated by comparison with the control and was recorded as the relative value of CSH. Experiments were repeated three times and mean values (±SD) were calculated.

Likewise, zeta potentials were determined by microelectrophoresis according to Busscher and Weerkamp as a measure for the net charge of blastoconidia. To this end, blastoconidia were isolated from 36 h YEPD cultures and suspended in sterile distilled water at a concentration of 10⁹ cells/mL. The zeta potentials were calculated from the electrophoretic mobility of the cells, measured with a Zetasizer 2000 (Malvern Instruments Ltd., Malvern, UK). Each sample was analysed three times and the measurements were carried out in fully automatic mode at 25°C and 300 V.

Quantification of CgEPA1 expression by RT–PCR

In order to quantify the expression of CgEPA1 (GenBank accession number AY344226) encoding an adhesin identified by Cormack et al., total RNA from each isolate was prepared as previously described. The cDNAs were synthesized from 2 µg of RNA at 37°C for 1 h in a thermocycler in a total volume of 50 µL with 2.65 µM random hexamers (Amersham Biosciences), 2 µL 10 mM dNTP, 40 U

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rNAsin ribonuclease inhibitor and 200 U M-MLV reverse transcriptase (Promega, Charbonnières, France). For the no-RT control, 1 μL diethylpyrocarbonate-treated water was added instead of the M-MLV reverse transcriptase. After purification of cDNA with QIAquick PCR Purification kit (Qiagen S.A., Courtaboeuf, France), quantitative PCR was performed and analysed on a capillary real-time thermocycler (LightCycler; Roche Diagnostics, Mannheim, Germany). Experiments were performed in duplicate and results were normalized to β-actin mRNA. Amplification was done in a 10 μL final volume using the LightCycler-Faststart DNA Master SYBR Green I kit (Roche Diagnostics): Five microlitres of cDNA (diluted 1:10) was mixed with 5 nM forward primer (GTGATTAGTACACCTCC for CgEPA1, or TATTGACAACGGTTCGG for CgACT1) and 5 nM reverse primer (GGAAGTATGCATTGAAGTC for CgEPA1, or TAGAAAGTGTGATGCCAG for CgACT1), and 4 mM MgCl2. The following run protocol was used: denaturation at 95°C for 10 min, followed by 40 cycles consisting of 15 s at 95°C for denaturation, 11 s at 54°C for annealing and 22 s at 72°C for elongation.

Adherence assay to human enterocytes in vitro

Adherence assay was performed on Caco-2 cells grown on 11 mm diameter glass coverslips in 24-well plastic dishes, and differentiated as previously described. After 15–21 days at 37°C, the medium in each well was replaced by 300 μL of PBS pre-incubated at 37°C containing 10⁵ stationary growth-phase blastoconidia. Dishes were then incubated for 30 min at 37°C, the monolayers were washed three times with PBS at 37°C to remove non-adherent blastoconidia, fixed in 2% glutaraldehyde for 10 min, and washed twice.

For detection of adherent blastoconidia, the monolayers were incubated sequentially for 1 h at 37°C with monoclonal antibody 5B2 (30 μg/mL), a mouse-rat chimeric IgM that reacts with β-linked mannose residues, and Alexa Fluor 488 goat anti-mouse IgM (1/200 dilution in PBS; Molecular Probes, Leiden, The Netherlands). After two washes with PBS at 37°C, attached yeasts were counted using a Zeiss Axioscope 2 microscope with an excitation filter at 360 nm and a suppression filter at 460 nm. The percentage of adherence in each culture was determined as the ratio of the number of adherent yeasts on the entire surface of the coverslip to the inoculum evaluated by quantitative culture. Each isolate was tested in triplicate, and three separate experiments were performed.

Experimental animal model

Eight- to nine-week-old female Swiss mice (Charles River Laboratories, Wilmington, DE, USA) were used to compare the relative pathogenicity of parent isolates and of their respective mutants. Animals (10 per isolate) were maintained in accordance with standard guidelines. Mice were inoculated with 2 x 10⁸ stationary growth-phase cells in 250 μL of PBS via the lateral tail vein. At day 10 after inoculation, mice were sacrificed for determination of fungal burden in organs. Kidneys, brain and liver were aseptically removed and homogenized in sterile PBS containing penicillin 100 IU/mL and streptomycin 100 μg/mL. Tissue homogenates from individual mice were serially diluted in sterile PBS and plated on YEPD agar plates. Colonies were counted after incubation at 37°C for 48 h. Recovery of the wild-type isolates or respiration-deficient mutants from the organs was confirmed by testing one colony from each plate for growth on yeast extract-peptone agar containing 2% glycerol. All mouse experiments were carried out according to French legal requirements for animal studies.

Growth kinetics

Approximately 10⁶ blastoconidia of each parent or mutant isolate were inoculated into 50 mL of YEPD broth and cultured at 37°C. Growth was monitored by measuring the absorbance at 490 nm of 200 μL aliquots at regular time intervals. Experiments were performed in triplicate and mean values were calculated.

Results

Carbohydrate content of the cell wall

Different FITC-labelled lectins were used to quantify the carbohydrates at the surface of parent or mutant blastoconidia. Very weak and non-specific binding was observed with WGA and PNA for all the cells (data not shown). Conversely, the parent and mutant cells incubated with FITC-ConA were intensely labelled, and the fluorescence intensity was even higher for the mutants (Figure 1). Moreover, incubation of parent or mutant cells with α-D-mannopyranoside resulted in a marked decrease in fluorescence intensity (more than 90% reduction), demonstrating the specificity of the binding to ConA. Finally, the absence of cell

Figure 1. Flow cytometric detection of glucose–mannose residues at the surface of blastoconidia of C. glabrata parent isolates and their derived petite mutants. Blastoconidia were incubated for 30 min with FITC-labelled ConA alone (thick black line) or in the presence of α-D-mannopyranoside (pale grey area). The data presented are representative of two independent experiments. The dark grey areas correspond to the controls performed by incubation of the cells without any ligand.
autofluorescence was attested by the very low fluorescence intensity of the controls.

Strikingly, a double peak of fluorescence intensity was seen for all the cell populations incubated with FITC-Con A (Figure 1). However, analysis of histograms corresponding to the number of cells according to their forward or light scatter properties showed that the addition of Con A (alone or in combination with its specific sugar) did not modify the distribution of the cells compared with the control, suggesting the absence of agglutination of the cells induced by the lectin.

Protein analysis

Comparison of the electrophoretic patterns did not reveal qualitative differences between the parent isolates and their respiration-deficient mutants (Figure 2). About 30 polypeptide bands with molecular mass ranging from 90 to 16 kDa were detected for each isolate, eight major bands of 55, 48, 41, 37, 35, 27, 25 and 23 kDa being detected for both parent isolates (lanes 1 and 4). However, a few quantitative changes were seen, with a marked decrease in the expression of the 37 and 35 kDa polypeptide bands in petite mutants, whereas the expression of other polypeptides (of 55 and 33 kDa) was slightly enhanced (lanes 2, 3, 5 and 6).

Surface physical properties

The surface physical properties of parent isolates and of their derived petite mutants were compared by measurement of their relative CSH and of their electrophoretic mobility (Table 1).

Table 1. Cell surface hydrophobicity (CSH) and zeta potential measurements for C. glabrata parent isolates and their derived petite mutants

<table>
<thead>
<tr>
<th>Yeast isolate</th>
<th>CSH (%)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate 90.1085</td>
<td></td>
<td></td>
</tr>
<tr>
<td>parent</td>
<td>34.5 ± 3.0</td>
<td>−40.8 ± 0.9</td>
</tr>
<tr>
<td>fluconazole-resistant mutant</td>
<td>12.5 ± 2.1*</td>
<td>−44.4 ± 2.8</td>
</tr>
<tr>
<td>ETB-induced mutant</td>
<td>8.4 ± 2.4*</td>
<td>−38.5 ± 1.2</td>
</tr>
<tr>
<td>Isolate 94.5579</td>
<td></td>
<td></td>
</tr>
<tr>
<td>parent</td>
<td>23.6 ± 2.8</td>
<td>−44.8 ± 0.9</td>
</tr>
<tr>
<td>fluconazole-resistant mutant</td>
<td>10.9 ± 0.6*</td>
<td>−40.3 ± 0.9</td>
</tr>
<tr>
<td>ETB-induced mutant</td>
<td>5.9 ± 0.6*</td>
<td>−44.4 ± 0.9</td>
</tr>
</tbody>
</table>

Table 2. Analysis of CgEPA1 expression by real-time RT–PCR for C. glabrata parent isolates and their derived petite mutants

<table>
<thead>
<tr>
<th>Yeast isolate</th>
<th>CgACT1 Mean Ct</th>
<th>CgEPA1 Mean Ct</th>
<th>Induction factor (2−∆∆Ct)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate 90.1085</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parent</td>
<td>15.14</td>
<td>23.95</td>
<td></td>
</tr>
<tr>
<td>fluconazole-resistant mutant</td>
<td>18.54</td>
<td>22.98</td>
<td>20.7</td>
</tr>
<tr>
<td>ETB-induced mutant</td>
<td>17.92</td>
<td>24.22</td>
<td>5.7</td>
</tr>
<tr>
<td>Isolate 94.5579</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>parent</td>
<td>16.25</td>
<td>26.22</td>
<td>13.1</td>
</tr>
<tr>
<td>fluconazole-resistant mutant</td>
<td>17.33</td>
<td>23.58</td>
<td></td>
</tr>
<tr>
<td>ETB-induced mutant</td>
<td>14.66</td>
<td>18.96</td>
<td>50.9</td>
</tr>
</tbody>
</table>

Analysis of relative CSH by two-phase partitioning with hexadecane as the organic phase revealed a lower hydrophobicity for all the petite mutants compared with their respective parent isolates. Indeed, the mean percentages of cells excluded from the aqueous phase for parent isolates were significantly higher than for their respective petite mutants. In contrast, no significant differences were seen in the electrophoretic mobility of the cells between parent and mutant isolates.

Expression of the CgEPA1 gene

The expression of CgEPA1 coding for an adhesin in the parent isolates and their respective petite mutants was determined by quantitative real-time RT–PCR. The CgEPA1 mRNA values were normalized to β-actin mRNA. Table 2 shows the mean cycle threshold (Ct) values for each parent isolate and for their mutants tested in duplicate, and the induction factors calculated for the mutants. Compared with parent isolates, mRNA levels of
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*CgEPAl* in the mutants were ~5- to 50-fold higher depending on the mutant studied.

**Adherence to human enterocytes**

Adherence assays showed weak adherence to Caco-2 cells for all the isolates without significant differences between parent and mutant blastoconidia (Figure 3). Indeed, the mean percentages of attachment to Caco-2 cell monolayers for parent isolates were 90.1085 and 94.5579, respectively, 11.4 ± 2.6 and 10.3 ± 1.2, versus 11.7 ± 4.4 and 12.8 ± 4.2 for their respective fluconazole-resistant mutants, or 10.6 ± 1.2 and 11.7 ± 2.2 for the ETB-induced mutants.

**Pathogenicity in immunocompetent mice**

None of the mice intravenously inoculated with $2 \times 10^7$ cells of the parent isolates or of their derived petite mutants died during the 10 days of observation. Thus, tissue distribution of blastoconidia was determined at day 10 by culturing brain, kidneys and liver (Table 3). Cultures were always positive for yeasts in organs collected from all mice inoculated with parent isolates, whereas yeasts were recovered in only 46 out of the 120 organs tested from the 40 mice inoculated with petite mutants. In addition, controls revealed the expected results. Thus, respiration-competent cells (as attested by their growth on both YEPD agar and glycerol-containing agar) were subcultured from organs of mice inoculated with the parent isolates. In contrast, respiration-deficient cells (as attested by the small colonies produced on YEPD agar and the lack of growth on glycerol-containing agar) were identified from all positive cultures from organs of mice inoculated with petite mutants.

Moreover, the fungal burden in the organs varied greatly depending on the organs, but also on whether the inoculum was composed of parent or mutant blastoconidia. In particular, fungal burdens were higher in kidneys than in brain or liver whatever the isolate tested. Likewise, while the fungal burden ranged from 500 to $5 \times 10^6$ colony forming units (cfu) per organ for the parent isolates, it was between 0 (in 74 out of the 120 organs) and $2.5 \times 10^3$ cfu per organ for the mutants. These results show that inoculation of mice with a relatively high number of *C. glabrata* blastoconidia results in a chronic non-fatal infection. In addition, they demonstrate that petite mutants are significantly less virulent than their parent isolates since low numbers of viable organisms were detected in only one-third of the organs tested for petite mutants whereas cultures from the other organs were negative.

### Table 3. Number of viable yeast cells recovered after 10 days from organs of immunocompetent mice intravenously inoculated with *C. glabrata* parent or mutant isolates

<table>
<thead>
<tr>
<th>Organ</th>
<th>Isolate 90.1085</th>
<th>Isolate 94.5579</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>parent</td>
<td>resistant mutant</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>$5 \times 10^3$</td>
<td>0</td>
</tr>
<tr>
<td>max</td>
<td>$6.5 \times 10^4$</td>
<td>$3 \times 10^2$</td>
</tr>
<tr>
<td>mean</td>
<td>$2.2 \times 10^4$</td>
<td>$9 \times 10^1$</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>$4.5 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>max</td>
<td>$1.6 \times 10^6$</td>
<td>0</td>
</tr>
<tr>
<td>mean</td>
<td>$4.7 \times 10^5$</td>
<td>0</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>min</td>
<td>$1 \times 10^3$</td>
<td>0</td>
</tr>
<tr>
<td>max</td>
<td>$5 \times 10^5$</td>
<td>$1.5 \times 10^3$</td>
</tr>
<tr>
<td>mean</td>
<td>$1 \times 10^4$</td>
<td>$4.2 \times 10^2$</td>
</tr>
</tbody>
</table>

Ten Swiss mice were inoculated with $2 \times 10^7$ cells for each isolate by tail vein injection. Results correspond to the minimum, maximum and mean number of cfu recovered from the organs.

![Figure 3. Adherence of *C. glabrata* parent isolates and their derived petite mutants to Caco-2 cells. Monolayers of Caco-2 cells containing adherent blastoconidia were incubated sequentially with a mouse-rat chimeric IgM that reacts with β-linked mannose residues and a fluorescent goat anti-mouse IgM. The percentage of attachment in each culture was determined as the ratio of the number of adherent yeasts on the entire surface of the coverslip to the inoculum evaluated by quantitative culture. The values are the means (and SD) of three independent experiments.](image-url)
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Figure 4. Growth curves of C. glabrata parent isolates (filled circles) and of their derived fluconazole-resistant (open circles) or ETB-induced (filled triangles) petite mutants. Growth of each isolate in YEPD broth was monitored by measuring the absorbance at 490 nm. Results correspond to mean values of triplicate experiments. Less than 10% variation was observed within triplicates.

Growth rates

Growth of petite mutants and of their parent isolates was compared in YEPD broth at 37°C. As shown in Figure 4, growth was slower for the four mutants with periods of latency of 5, 12, 15 or 21 h versus 4 h for the parent isolates, and a generation time of 2 or 4 h for the petite mutants whereas it was only 1 h for the parent isolates. Moreover, absorbance at the stationary phase was always lower for the mutants than for their parent isolates.

Discussion

Despite the high frequency of petite mutations in C. glabrata and their induction byazole drugs, particularly fluconazole, isolation of such mutants from patients undergoing antifungal therapy or prophylaxis remains very rare.10 Because of the extensive cross-talk between nucleus and mitochondria, the total or partial deletion of mtDNA observed in petite mutants leads to the deregulation of the expression of several nuclear genes.17 Thus, the acquired resistance to azoles which is seen in all petite mutants of C. glabrata seems to be linked to the overexpression of nuclear genes encoding the efflux pumps CgCdr1p and, to a lesser extent, CgCdr2p.5,6 Likewise, petite mutants present an increased susceptibility to polyene drugs, due to an increased cellular content in free ergosterol because of a defect in sterol esterification, which is mediated in S. cerevisiae by two nuclear genes, ARE1 and ARE2. One may therefore speculate that other nuclear genes, encoding molecules involved in pathogenicity of C. glabrata, are also affected by petite mutations, leading to a diminished virulence.

Little is known about the virulence factors allowing this yeast to colonize the human host and to cause disease. However, it is usually considered that adherence of microorganisms to the host tissues is one of the most important determinants of pathogenesis. Adherence may result from non-specific interactions involving the entire surface of the interacting cells and due to the electrostatic charge or to the surface hydrophobicity of the cells, or from specific interactions mediated by lectins or integrins located at the surface of the host or fungal cells. In C. glabrata, Cormack et al.14 demonstrated that adherence of blastoconidia to cultured human epithelial cells is mediated mostly by a single adhesin, Epa1p, belonging to a large class of glycosylphosphatidylinositol (GPI)-anchored cell wall proteins, which specifically recognizes asialo-lactosyl-containing carbohydrates. However, although resulting in a drastic reduction in the adherence abilities of blastoconidia, deletion of CgEPA1 does not lead to significantly decreased virulence in murine models of systemic or vaginal candidiasis. In fact, CgEPA1 is part of a large family of highly related genes that are transcriptionally repressed in vitro and that may compensate in vivo for the absence of CgEPA1.18 In contrast, CgSTE12 and CgSTE20, which have been recently cloned and characterized by Calcagno and coworkers,19,20 are required to maintain wild-type levels of virulence in a murine model of systemic candidiasis.

The fungal cell wall plays a major role in host–pathogen interactions. It is composed primarily of polysaccharides and glycoproteins which contribute, by their free hydroxyl, amine, or carboxylic groups, to the net surface charge and hydrophobicity of blastoconidia. Comparison of our petite mutants with their parent isolates showed that petite mutations lead to an increased availability or to an increased amount of glucose–mannose residues at the cell surface. Likewise, some quantitative differences in the protein composition of the blastoconidial extracts (which correspond mainly to cell wall components), were seen by SDS–PAGE between mutants and parent isolates. These biochemical changes were associated with a lower hydrophobicity of mutant cells compared with their parent isolates, but the electrostatic charge almost remained unchanged. These results are in agreement with previous work on C. albicans in which a direct correlation between CSH and the level of cell surface protein mannosylation was established.21,22 That is, increased hydrophobicity has been linked to reduced glycosylation of surface proteins. Moreover, changes in the biochemical composition of the cell wall have also been demonstrated in S. cerevisiae petite mutants by Wilkie and Evans23 who reported a higher aggregation of the cells with conA. However, we did not find any modifications of the distribution of the cells induced by the addition of Con A for our petite mutants.

Additionally, increased expression of CgEPA1 which is a GPI-anchored protein, was observed for all our petite mutants. Interestingly, Ferreira et al.24 demonstrated in S. cerevisiae that deletion of the HEM1 gene involved in haem synthesis, which like petite mutations, mimics conditions of anaerobic growth, also leads to an increased cellular content in phosphatidylinositol. Thus one
may speculate that all conditions required for an increased expression of CgEpa1p at the cell surface (and therefore for an increased adherence capability of blastoconidia) are gathered in petite mutants.

For numerous microorganisms including the pathogenic yeasts C. albicans and C. glabrata, CSH plays a key role in adherence. However, adherence of C. glabrata blastoconidia to host tissues seems to be mediated mainly by specific interactions involving the lectin Epa1p. Nevertheless, the lower hydrophobicity nor the increased expression of CgEPA1 observed in petite mutants were associated with changes in the adherence of these mutants to Caco-2 cells. This discrepancy with the results of Cormack et al. may be related to the use of a different epithelial cell line, but also to differences in the growth phase of blastococonidia. Our experiments were performed using Caco-2 cells, a human enterocyte cell line, and it seems likely that distinct adhesins mediate the adherence of this yeast to different host tissues. In addition, CgEPA1 is expressed at low levels in the stationary phase. Alternatively, changes that we previously reported in the ergosterol pathway for these mutants could be responsible for a defect in trafficking of membrane proteins to their lipid rafts, and for instance for reduced availability of CgEpa1p at the surface of blastoconidia despite increased synthesis of the protein. Indeed, lipid rafts in yeast are composed of sphingolipids and ergosterol but also of proteins (some of them presenting a GPI-anchor), and it has been demonstrated in S. cerevisiae that conditions impairing the synthesis of sphingolipids and ergosterol disrupt raft association of Gas1p, a GPI-anchored protein.

Petite mutants were also compared to their parent isolates with regard to their virulence in a model of systemic candidiasis in immunocompetent mice. In accordance with previous studies, this model showed that C. glabrata, despite a relatively large inoculum, only produces a chronic non-fatal infection. By measuring fungal burden in the kidneys, brain and liver of mice sacrificed at day 10 following inoculation, we demonstrated that adherence of C. glabrata petite mutants seemed to be related to their slower growth, although a 20% to 30% reduction in adherence was slower growth, although a 20% to 30% reduction in adherence was also noted using a human keratinocyte cell line or exfoliated human buccal epithelial cells, respectively. The markedly reduced virulence of the C. glabrata mutants described in the present work also seemed to be related to the slower growth of petite mutants compared with their parent isolates, with longer periods of latency and longer generation times. This may be caused by the defect in energy production related to respiratory deficiency. However, the absence of sterol esterification previously demonstrated in these mutants could also be responsible for growth perturbations as occurs in S. cerevisiae.

Together, these results indicate that C. glabrata petite mutants may be selected in vivo could be disregarded clinically, since these mutations lead to a drastic reduction in virulence. However, in patients infected with C. glabrata and treated with fluconazole, resistance to azoles may confer to petite mutants a selective advantage, yet allowing them to be virulent in such hosts. Thus, a follow-up of patients undergoing fluconazole therapy is needed to determine the prevalence of petite mutations and to confirm the absence of clinical relevance. Moreover, although petite mutations result in a lower hydrophobicity of the cells and an overexpression of CgEPA1 gene, no changes were seen in adherence to human enterocytes, and other adherence mechanisms must be investigated. Additionally, due to their markedly decreased virulence, these mutants may constitute valuable tools for the characterization of virulence factors in C. glabrata, and proteomic studies of the changes associated with petite mutations are now in progress.


