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MYCOLOGY

# In-vivo selection of an azole-resistant petite mutant of *Candida glabrata*

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**Two isolates of *Candida glabrata* from the same stool sample from a bone marrow transplant recipient treated with fluconazole, and designated 1084-L for large colonies on yeast extract-peptone-dextrose-agar and 1084-S for small colonies, were analysed. In-vitro susceptibility tests with a commercially available disk diffusion procedure showed that isolate 1084-L had a susceptibility pattern typical of wild-type strains of *C. glabrata* with sensitivity to polyenes and the presence of resistant colonies randomly distributed within the inhibition zones for all azole compounds except tioconazole. In contrast, isolate 1084-S, which was found by pulsed-field gel electrophoresis and random amplification of polymorphic DNA to be genetically closely related to isolate 1084-L, exhibited cross-resistance to the azole compounds except tioconazole. Determination of MICs by the E-test method confirmed these results, showing that isolate 1084-S had greater sensitivity to amphotericin B and complete resistance to ketoconazole and fluconazole. Growth on agar plates containing glucose or glycerol as the sole carbon source suggested that the resistant isolate had a respiratory deficiency, which was further demonstrated by flow cytometric analysis of the fluorescence of rhodamine 123-stained blastoconidia. Restriction endonuclease analysis of mitochondrial DNA (mtDNA) established the mitochondrial origin of the respiratory deficiency. However, PCR amplification of the mtDNA with primers ML1 and ML6, as well as transmission electron microscopy, suggested a partial deletion of the mtDNA analogous to that described for *rho*$^-$ petite mutants of *Saccharomyces cerevisiae*. Together, these results provided evidence that the selection of azole-resistant petite mutants of *C. glabrata* may occur *in vivo* after fluconazole administration, which might explain, therefore, clinical failure of antifungal therapy.**

## Introduction

Along with the widespread use of antibiotics, the development of organ or bone marrow transplantation and the increasing number of immunocompromised patients, the prevalence of candidosis has increased markedly during the past two decades. Therefore, several antifungal agents have been developed, particularly imidazoles and triazoles. Among them, the water-soluble agent fluconazole demonstrated rapid absorption following oral administration, a remarkably long half-life and high efficacy against clinically relevant yeasts with low toxicity and few adverse effects [1]. Accordingly, fluconazole has become the most widely used agent in prophylaxis and therapy of candidosis in AIDS patients and organ or bone marrow transplant recipients, as well as in patients in intensive care units or undergoing chemotherapy or radiotherapy.

However, the extensive use of fluconazole has also resulted in a shift in the causative agents of candidosis, with an increased incidence of non-*albicans Candida* species [2, 3]. Thus, *C. glabrata* – which was for a long time the second most frequent yeast in candiduria and vulvovaginal candidosis – has also become the second most frequently isolated yeast species in systemic mycoses [4]. For instance, its frequency

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in candidaemia increased from 2% in 1987 to 26% in 1992 in the USA [5] and from 6% in 1987 to 17% in 1995 in The Netherlands [6].

The cytochrome P-450 14$\alpha$-sterol demethylase (P-$450_{14DM}$, also called Erg11, Erg16 or CYP51A1), which plays a key role in the synthesis of egrosterol, is the target of azole antifungal agents. Several resistance mechanisms have been described for pathogenic yeasts, including point mutations in the *CYP51A1* gene leading to reduced affinity of the target enzyme for azole derivatives [7–11], overexpression or amplification of the *CYP51A1* gene [12–15], or mutations in genes encoding other enzymes involved in the ergosterol biosynthesis pathway, particularly sterol $\Delta$(5-6)-desaturase [16]. More frequently, an enhanced efflux of the drug is reported, involving membrane proteins responsible for multi-drug resistance which belong to the ABC (ATP-binding cassettes) or to the major facilitator transporter superfamilies [13, 14, 17–21]. A previous report proposed an alternative mechanism for the acquired resistance to azoles in *C. glabrata* [22]. Indeed, it showed that mutations in the mitochondrial DNA (mtDNA) frequently occur *in vitro* in this yeast species, leading to a respiratory deficiency associated with resistance to azoles. However, the clinical significance of this resistance mechanism remained uncertain, as such azole-resistant mutants had not been isolated from clinical samples. This study sought evidence for the selection of azole-resistant petite mutants of *C. glabrata in vivo* after fluconazole administration.

## Materials and methods

### *Yeast isolates and culture conditions*

This study was performed with two clinical isolates of *C. glabrata* designated 1084-L (for large colonies) and 1084-S (for small colonies) isolated from the same stool sample from a bone marrow transplant recipient. Two weeks after transplantation, therapy was started with netilmycine and cefepime from 13 Oct. 1998 to 23 Oct. 1998, followed by phenoxymethylpenicillin alone for 1 week and then with sulphamethoxazole and trimethoprim until the end of December, and finally with vancomycin, ceftriaxone and tobramycin from 4 Jan. 1999. One week later, mycological examination of a stool sample revealed the presence of $10^2$–$10^3$ cfu of *C. albicans*/g. Therefore, the patient was treated with fluconazole orally 100 mg/day from 17 Jan. 1999 to 29 Jan. 1999, and then 400 mg/day. However, cultures on CHROMAgar Candida (Becton Dickinson, Le-Pont-de-Claix, France) from a stool sample taken on 5 Feb. 1999 revealed a massive digestive tract colonisation (>$10^6$cfu/g) of the patient with an association of four different colonies. The green colonies were identified directly as *C. albicans*. On the basis of their carbohydrate assimilation pattern on Auxacolor test strips (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) and their morphology on rice cream-agar-Tween, the other yeasts were identified as *C. inconspicua* for the white colonies and *C. glabrata* for the large (1084-L) and the small (1084-S) purple colonies.

These two isolates of *C. glabrata* were maintained by bi-weekly passages on yeast extract-peptone-glucose (YEPD) agar containing (g/L) yeast extract (5), peptone (10), glucose (20), chloramphenicol (1) and agar (20) with incubation at 37°C. Growth of the two isolates was also compared on yeast extract-peptone-agar plates containing glycerol 2% as the sole carbon source.

### *Antifungal susceptibility testing*

Antifungal susceptibility of the isolates was determined by the disk diffusion method on Casitone (Difco, Detroit, MI, USA) agar with antifungal NeoSensitabs tablets (Rosco Diagnostica, Taastrup, Denmark) as described previously [22]. After incubation for 48 h at 37°C, the diameter of the inhibition zones was measured. The presence of large colonies randomly distributed within the inhibition zones was also noted, and isolates were classified as sensitive, intermediate or resistant according to the manufacturer's recommendations.

Susceptibility of the two isolates to amphotericin B, ketoconazole and fluconazole was also determined by measuring MICs of the antifungal agents by the E-test procedure performed as recommended by AB Biodisk (Solna, Sweden) on Casitone agar plates buffered at pH 7.2 (Axcell Biotechnologies, St Genis l'Agentière, France). Plates were incubated at 37°C and MICs were read after 48 h as the drug concentration at which the inhibition ellipse intercepted the scale on the antifungal strips.

### *Molecular typing methods*

*Pulsed-field gel electrophoresis (PFGE).* The preparation of yeast chromosomal DNA and PFGE were performed by SigmO (Vertou, France) as described previously [23]. Briefly, cells from cultures grown in YEPD broth were harvested by centrifugation, washed with 50 mM $Na_2EDTA$, pH 8, and resuspended at a concentration of $1.5 \times 10^9$ cells/ml in 0.9 M sorbitol, 0.1 M $Na_2EDTA$, pH 7.5, containing Zymolase 20T (Seikagaku, Tokyo, Japan) 0.2 mg/ml and 2-mercaptoethanol (2-ME) 2%. A sample of this suspension was immediately mixed with an equal volume of Fast lane FMC agarose (TEBU, Le Perray-en-Yvelines, France) 1% in TAFE buffer (10 mM Tris, 0.5 mM acid-free EDTA, 4.35 mM acetic acid, pH 8.2) and distributed in plastic moulds. The resulting agarose blocks were then sliced and incubated in 3 ml of a 4 mM citric acid, 120 mM $Na_2HPO4$, 20 mM $Na_2EDTA$ buffer, pH 6.1, for 4 h at 37°C. Plugs were finally incubated overnight in 0.45 M $Na_2EDTA$, sarkosyl 1%, 10 mM Tris buffer, pH 8.1, containing pronase E (Eurogentec, Seraing, Belgium) 0.3 mg; afterwards they were washed four

times for 15 min in 10 mM Tris buffer, pH 8, containing 1 mM $Na_2EDTA$ (TE buffer). PFGE was performed on a TAFE apparatus (Beckman Geneline 1; Beckman, Gagny, France) in a Fast lane FMC agarose 1% gel in TAFE buffer at 12°C with a constant electric current of 150 mA and with pulse times of 30 s for 2 h, 60 s for
Cells corresponding to 2-ml cultures were harvested by centrifugation for 2 min at 500 *g* at room temperature. Total DNA was prepared according to the method of Ness *et al*. [24]. Amplification was performed in a Perkin Elmer thermocycler 480 programmed as described previously [23]: 4 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. The primers used in this study, 5′-GGGT AACGCC-3′ for A9, 5′-CAATCGCCGT-3′ for A11, 5′-TTATCGCCCC-3′ for E12 and 5′-GGACTGCAGA-3′ for E18, were from Operon Technologies (Alameda, CA, USA). Reaction mixtures (25 $\mu$l final volume) consisted of 50 mM KCl, Triton X100 0.1%, gelatin 0.2 mg/ml, 10 mM Tris-HCl buffer, pH 9, containing 1.5 mM $MgCl_2$, 200 $\mu$M each desoxyribonucleotide triphosphate (dNTP), 5 pmoles of primer, 25 ng of genomic DNA and 1 U of Taq DNA polymerase (Appligène-Oncor, Illkirch, France). All experiments were repeated twice. The amplified fragments were separated by agarose 2% gel electrophoresis in TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM $Na_2EDTA$) with mol. wt standards (100-bp ladder from Pharmacia-Biotech, St Quentin, France). After electrophoresis, the gels were stained with ethidium bromide and examined under UV illumination.

### *Flow cytometry*

The respiratory status of the two isolates was investigated by flow cytometry after staining the cell populations with rhodamine 123 as described previously [25]. Blastoconidia grown to stationary phase in YEPD liquid medium were harvested by centrifugation and washed in 0.15 M phosphate-buffered saline (PBS), pH 7.2. Cells ($2 \times 10^6$) were incubated for 30 min at 37°C with constant shaking and with 250 $\mu$l of a rhodamine 123 (Sigma, St Quentin-Fallavier, France) 10 $\mu$g/ml solution. To inhibit the electron flow of the respiratory chain, samples of the cell suspensions were first incubated for 2 h at 37°C with 1 mM sodium azide before the addition of rhodamine 123.

After washing in PBS, the cell fluorescence was quantified with a FACScan flow cytometer (BDIS Europe, Erembodegem, Belgium) equipped with an air-cooled 15-mW argon-ion laser operating at 488 nm. Cell debris were excluded by gating the blastoconidia on the basis of their rectilinear forward and side light scatter properties. The fluorescence intensity was quantified at 535 nm. Each sample was collected in a list mode file of 10 000 events and data were analysed with the CELLQuest software from BDIS. The data presented correspond to fluorescence frequency distribution histograms (rela-

6 h and 90 s for 14 h. Gels were stained for 20 min in ethidium bromide 0.5 mg/L, de-stained for 10 min and photographed under UV illumination.

*Random amplification of polymorhpic DNA (RAPD).*
tive number of blastoconidia *versus* relative fluorescence intensity expressed in arbitrary units on a logarithmic scale).

### *mtDNA analysis*

mtDNA was extracted as previously described [23] by lysis of the cell envelope of blastoconidia by incubation for 2 h at 37°C in 0.5 M sorbitol, 10 mM $Na_2EDTA$, 50 mM Tris buffer, pH 7.6, containing Zymolase 20T 2 mg/ml and 2-ME 0.2%, followed by two successive centrifugations of 10 min at 1000 *g* to remove nuclei and intact cells, and at 15 000 *g* to pellet the mitochondria. After lysis of the resulting pellet with sarkosyl 1%, nucleic acids were purified according to standard procedure, and RNAs were eliminated by RNAase. Thereafter, mtDNA was digested by *Eco*RV or *Hin*fI (Eurogentec) at 37°C for 2 h in a 10-$\mu$l volume with 1 unit of the enzyme. Finally, the digestion products were analysed by agarose 0.8% gel electrophoresis in TBE buffer together with mol. wt markers ($\lambda$ DNA digested by *Hin*dIII and *Eco*RI), stained with ethidium bromide and visualised under UV illumination.

mtDNA of the two isolates was also analysed in two distinct generations of the cell populations by PCR with primers ML1 (5′GTACTTTTGCATAATGGGT-CAGC-3′) and ML6 (5′-CAGTAGAAGCTGCA-TAGGGTC-3′) [26]. The reaction mixtures (25 $\mu$l final volume) which contained the mtDNA and 5 pmoles of each primer, were as described for RAPD experiments. PCR conditions were as follows: 35 cycles of 30 s at 94°C for denaturing, 50 s at 50°C for annealing and 1 min at 72°C for elongation. The amplified products were detected by agarose 2% gel electrophoresis in TBE buffer and visualised under UV illumination after ethidium bromide staining of the gel.

### *Transmission electron microscopy (TEM)*

Samples for electron microscopy were prepared according to the method of Aoki *et al*. [27] with minor modifications. Blastoconidia grown on YPDA plates were washed with PBS and fixed for 1 h at room temperature with glutaraldehyde (Merck, Darmstadt, Germany) 2.5% buffered at pH 7.2 with 0.1 M sodium cacodylate (Sigma). Cells were then washed with cacodylate buffer, and fixed with $KMnO_4$ (Sigma) 1.5% for 18 h at 4°C. After washing, the cells were post-fixed with osmium tetroxide (SPI Supplies, West Chester, PA, USA) 1% for 2 h at room temperature. Samples were dehydrated with gradient series of ethanol and embedded in Epon (SPI). Ultra-thin

sections were stained with uranyl acetate and examined with a 100 CX JEOL microscope (Jeol, Paris, France).

## Results

### *Antifungal susceptibility patterns*

In-vitro antifungal susceptibility testing performed by an agar disk diffusion method with Neosensitabs tablets revealed distinct susceptibility patterns for the two isolates (Table 1). Isolate 1084-L, which showed well-defined clear-cut inhibition zones, was sensitive to polyenes and tioconazole. However, resistant colonies randomly distributed within the inhibition zones were seen for the other azoles tested, some growing in direct contact with the antifungal tablet. Conversely, isolate 1084-S was completely resistant to isoconazole, clotrimazole, ketoconazole and fluconazole, and of intermediate resistance to econazole and miconazole. Strikingly, it remained sensitive to polyenes and tioconazole with larger inhibition zones.

Determination of MICs by the E-test method confirmed these results, showing that isolate 1084-L was sensitive to amphotericin B and ketoconazole, and had a relatively high fluconazole MIC. Conversely, isolate 1084-S showed greater sensitivity to amphotericin B and complete resistance to the azole compounds ketoconazole and fluconazole with MICs >32 and 256 mg/L, respectively.

### *Molecular typing methods*

Two distinct typing methods were used to analyse these isolates. PFGE suggested that the two isolates were genetically closely related. The chromosomal DNA of isolate 1084-L was separated into 10 bands ranging in size from 0.5 to *c*. 2 Mb (Fig. 1a, lane 1) and isolate 1084-S gave an identical banding pattern (Fig. 1a, lane 2). RAPD, which was repeated twice with different subcultures of the isolates, confirmed these results showing similar electrophoretic patterns for the two isolates whatever the primer used (Fig. 1b). For example, primer A9 generated four major bands of *c*. 0.65, 0.85, 1.6 and >2 kb and nine identical minor bands for the two cell populations (Fig. 1b, lanes 4 and 5). Likewise, no differences were observed between the two isolates in amplicons obtained with primers A11 (Fig. 1b, lanes 6 and 7), E12 (Fig. 1b, lanes 9 and 10) and E18 (Fig. 1b, lanes 11 and 12).

**Table 1.** Antifungal susceptibility patterns of the two *C. glabrata* isolates

| Antifungal agent | Diameter of inhibition zones (mm) | | MIC (mg/L) | |
|---|---|---|---|---|
| | 1084-L | 1084-S | 1084-L | 1084-S |
| Amphotericin B | 27 | 36 | 0.125 | 0.064 |
| Nystatin | 31 | 35 | ND | ND |
| Tioconazole | 35 | 40 | ND | ND |
| Econazole | 22* | 15 | ND | ND |
| Miconazole | 25* | 16 | ND | ND |
| Isoconazole | 20* | CR | ND | ND |
| Clotrimazole | 26* | CR | ND | ND |
| Ketoconazole | 27* | CR | 1 | >32 |
| Fluconazole | 22* | CR | 32 | >256 |

CR, complete resistance with homogeneous growth directly in contact with the antifungal tablet; ND, not determined.
*Presence of large colonies randomly distributed within the inhibition zone and, therefore, considered to be resistant mutants.

### *Growth studies and flow cytometry evidence for the respiratory deficiency*

Comparative growth studies on agar media containing glucose or glycerol 2% suggested that there was a respiratory deficiency in isolate 1084-S. When cultivated on glucose-agar plates, this isolate produced small colonies and did not grow with glycerol 2% as the sole carbon source. Conversely, isolate 1084-L produced large colonies on glucose-containing agar and its growth was only slightly reduced when cultivated with glycerol.

Flow cytometric analysis of cells stained with rhodamine 123, a lipophilic cationic fluorochrome which is incorporated into mitochondria through a transmembrane potential-dependent mechanism, confirmed the respiratory deficiency of isolate 1084-S. Blastoconidia of isolate 1084-L exhibited a uniform staining after incubation with the fluorochrome, as illustrated in Fig. 2a, and a significant reduction of the fluorescence intensity was observed when cells were treated with the respiratory chain inhibitor sodium azide before staining (Fig. 2b). Conversely, cells of isolate 1084-S were stained poorly by rhodamine 123. Their fluorescence intensity was similar to the residual fluorescence exhibited by cells of the respiratory-competent isolate pretreated with sodium azide and it was not affected by this pretreatment (Fig. 2c and d).

### *mtDNA analysis*

Analysis of the mtDNA showed great differences between the two isolates. Restriction endonuclease analysis of mtDNA performed with *Eco*RV, which has been used successfully to discriminate strains of *C. glabrata* [24], resulted in three restriction fragments of *c*. 20, 5 and 1.7 kb for the respiratory-competent isolate (Fig. 3a, lane 2), while no mtDNA fragments were detected for the respiratory-deficient isolate (Fig. 3a, lane 3). Confirmatory results were obtained with *Hin*fI digests as shown in Fig. 3a, lanes 5 and 6.

To analyse the extent to which the mtDNA was deleted, PCR experiments were performed on the mitochondrial fraction of two distinct generations of the isolates with primers ML1 and ML6, which amplify a segment of ribosomal DNA genes of fungal mitochondria [26]. The results are presented in Fig. 3b. The respiratory-competent isolate 1084-L exhibited an intense band of *c*. 1500 bp close to the expected size for the target

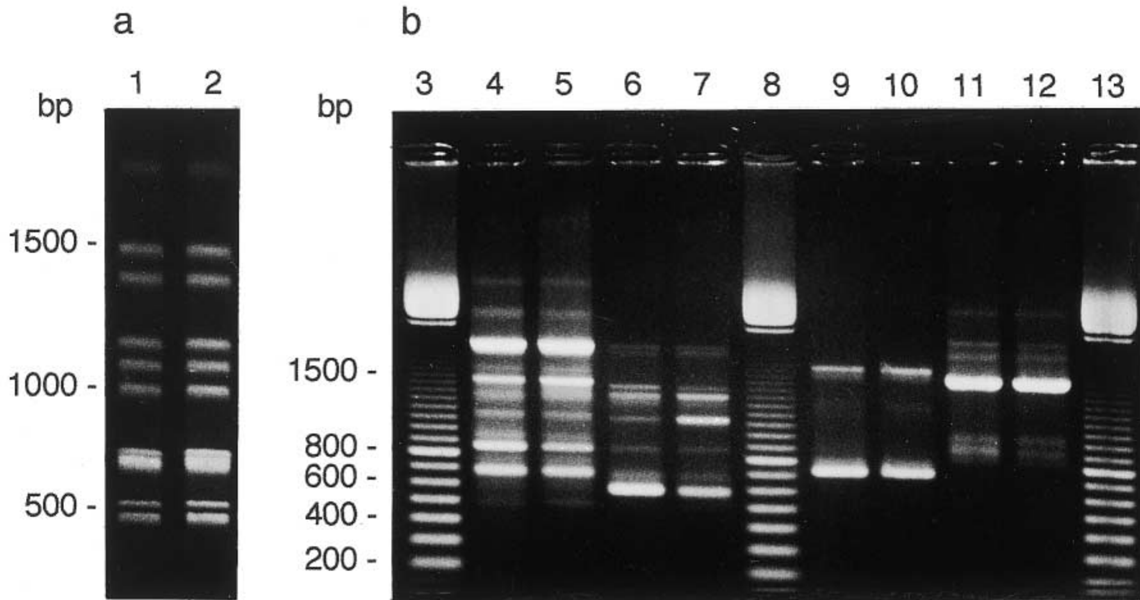


**Fig. 1.** PFGE(**a**) and RAPD(**b**) patterns of the two *C. glabrata* isolates 1084-L (lanes **1**, **4**, **6**, **9** and **11**) and 1084-S (**2**, **5**, **7**, **10** and **12**). Amplified products obtained with Operon decamer primers A9 (lanes **4** and **5**), A11 (**6** and **7**), E12 (**9** and **10**) and E18 (**11** and **12**) were analysed by agarose 2% gel electrophoresis. Mol. wt markers (100 bp ladder from Pharmacia-Biotech) are presented in lanes **3**, **8** and **13**.

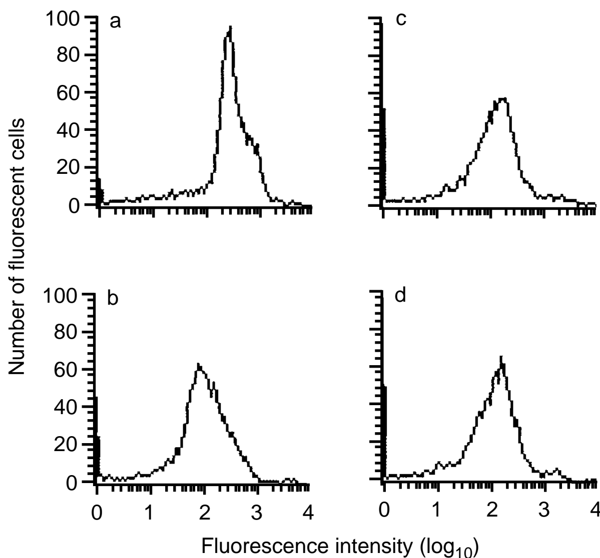


**Fig. 2.** Flow cytometric analysis of rhodamine 123-stained cells of *C. glabrata* isolates 1084-L (**a**, **b**) and 1084-S (**c**, **d**). Untreated blastoconidia (a, c) or cells pre-incubated with sodium azide (b, d) were incubated with rhodamine 123 for 30 min, then the intensity of fluorescence was measured with a FACScan cytometer. *x* axis, log of the fluorescence intensity; *y* axis, number of fluorescent cells.

of the primers used, and also non-specific bands (Fig. 3b, lanes 9 and 10). With the respiratory-deficient counterpart 1084-S, a weaker band of similar size was observed (Fig. 3b, lanes 11 and 12), indicating that the mtDNA of this isolate was not completely deleted.

### *Electron microscopy*

EM confirmed the mitochondrial origin of the respiratory deficiency. Whereas numerous mitochondrial sections were present in blastoconidia of the wild-type isolate 1084-L (Fig. 4), only very few mitochondrial sections looking ‘thread-like’ without any cristae were observed in most of the cells for the mtDNA-deficient mutant (Fig. 4b).

## Discussion

With the widespread use of azole antifungal agents in the prophylaxis or therapy of candidiasis, acquired resistance of yeast isolates to azoles has been reported increasingly, and it is most often seen in *C. glabrata* for fluconazole. However, little information is available concerning the resistance mechanisms in this emerging yeast pathogen. Studying two isolates obtained from the same patient before and after fluconazole treatment, Hitchcock *et al.* [28] demonstrated that the post-treatment resistant isolate failed to accumulate the drug, and further studies demonstrated an enhanced energy-dependent efflux in this organism, probably in relation to the hyperexpression of a multi-drug resistance-type transporter [29]. More recently, a homologue of the ABC transporter *PDR5* gene of *S. cerevisiae* has been identified in resistant isolates of *C. glabrata* [21]. However, the two isolates studied by Hitchcock *et al.* [28] were found to be genetically unrelated, suggesting the in-vivo selection of a resistant isolate within a double population of yeasts, one susceptible to fluconazole and the other resistant. Conversely, the two isolates pre- and post-treatment studied by Vanden Bossche *et al.* [12, 15], which were obtained from another patient undergoing fluconazole therapy, were clonally related as demonstrated by restriction fragment length polymorphism analysis of genomic DNA. The acquired resistance developed by

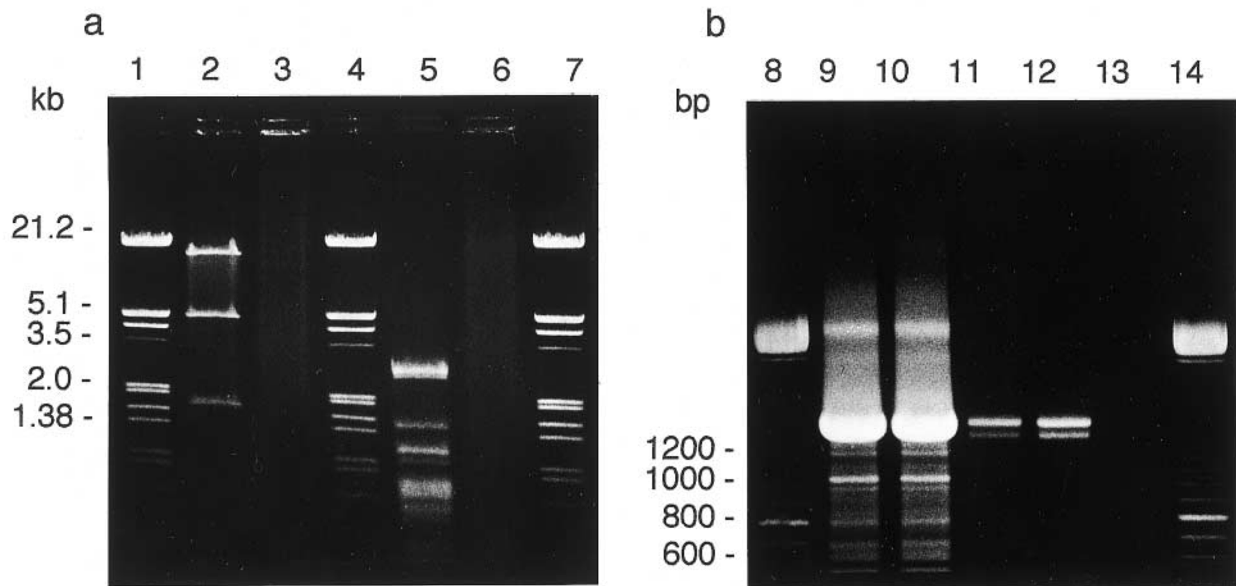


**Fig. 3.** Electrophoretic patterns of the mtDNA of the two *C. glabrata* isolates 1084-L (lanes **2**, **5**, **9** and **10**) and 1084-S (**3**, **6**, **11** and **12**). mtDNA was analysed by agarose gel electrophoresis after digestion with *Eco*RV (**a**, lanes **2** and **3**) or *Hin*fI (**a**, lanes **5** and **6**), or by PCR with primers ML1 and ML6 (**b**, lanes **9–12**). Mol. wt markers ($\lambda$ DNA digested by *Hin*dIII and *Eco*RI in **a** and 100 bp ladder from Pharmacia-Biotech in **b**) are shown in lanes **1**, **4**, **7**, **8** and **14**. Lane **13**, control without DNA.

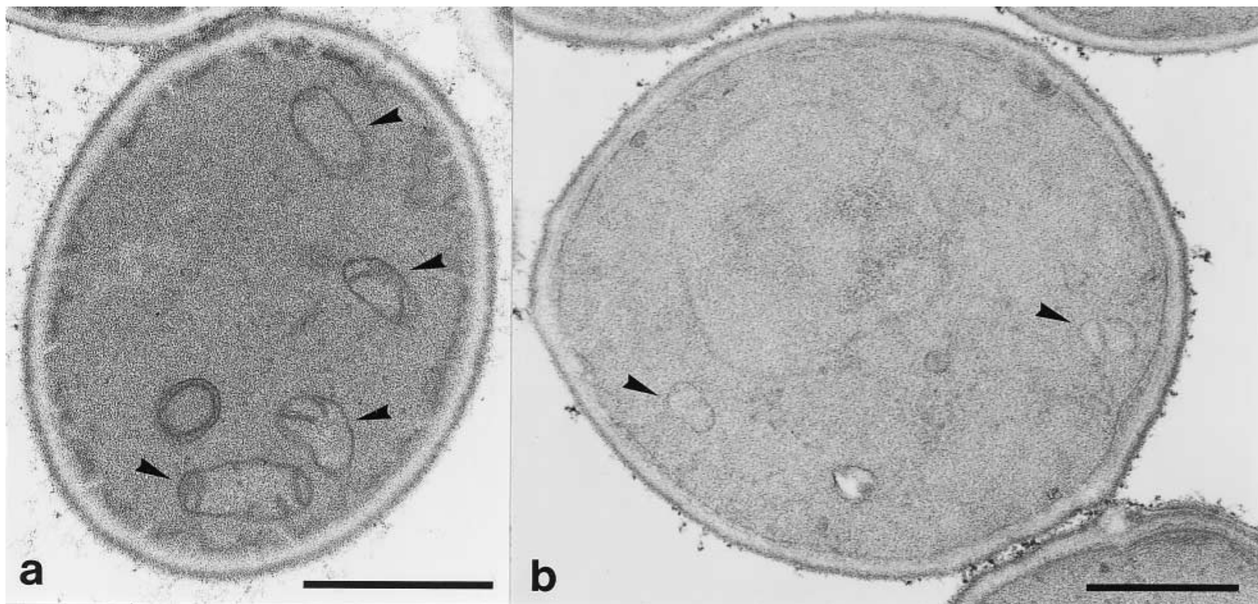


**Fig. 4.** Transmission electron micrographs of the two *C. glabrata* isolates 1084-L (**a**) and 1084-S (**b**). Bars = 1 $\mu$m. Note the very few mitochondrial sections (arrowheads) in the respiratory-deficient isolate compared with its parent strain.

the post-treatment isolate seemed to be related to an enhanced efflux of the drug associated with an increased synthesis of CYP51A1 due to gene amplification. The two isolates described here were from the same stool sample from a bone marrow transplant recipient treated with fluconazole for digestive tract candidosis due to *C. albicans*, and PFGE and RAPD clearly established that the two isolates were genetically related. In a previous study performed on 21 epidemiologically unrelated isolates of *C. glabrata*, PFGE had a high discriminatory power (calculated as described by Hunter [30], $D = 0.99$), and although less discriminant, RAPD was shown to be useful as a complementary technique [23]. Twenty distinct genotypes were obtained by PFGE, and combining the results of both genotyping methods allowed the differentiation of all the isolates.

Isolate 1084-L was characterised by the production of large colonies on YPDA and of small colonies on glycerol-agar plates. In-vitro susceptibility testing revealed an antifungal susceptibility pattern typical of

wild-type strains of *C. glabrata* [22] with sensitivity to polyenes and tioconazole and, for the other azoles tested, the presence of resistant colonies randomly distributed within the inhibition zones. The E-test method, which has been shown to give good agreement with the macrobroth dilution reference method [31, 32], confirmed these results. The isolate appeared sensitive to amphotericin B and presented a relatively high fluconazole MIC – consistent with previous reports which showed the low susceptibility of this yeast species to fluconazole [22, 33, 34]. Conversely, the second isolate (1084-S), characterised by the presence of small colonies on YPDA and the lack of growth on glycerol-containing agar which suggested a respiratory deficiency, presented a susceptibility pattern similar to that reported for in-vitro selected fluconazole-resistant and ethidium bromide-induced petite mutants of *C. glabrata* [22]. Indeed, it was completely resistant to isoconazole, clotrimazole, ketoconazole and fluconazole, and weakly inhibited by econazole and miconazole. Strikingly, it remained sensitive to polyenes and tioconazole with diameter of the inhibition zones and amphotericin B MIC greater than those obtained for isolate 1084-L, as previously reported for in-vitro selected fluconazole-resistant mutants [22].

The respiratory deficiency which resulted in a slower and reduced growth due to less efficiency in the utilisation of carbon substrates and which led to cross-resistance to the azole compounds except tioconazole, indicated a mitochondrial origin, as evidenced by flow cytometry with rhodamine 123 and mtDNA analysis. Rhodamine 123 has been shown to selectively stain mitochondria when either isolated [35] or in living cells [36]. Cells of isolate 1084-S were poorly stained by rhodamine 123. Their fluorescence intensity was similar to that of the susceptible isolate when pretreated with sodium azide, and it was not affected by this respiratory chain inhibitor, as previously described for petite mutants of *S. cerevisiae* [25].

Restriction endonuclease analysis of mtDNA confirmed the mitochondrial origin of the respiratory deficiency. No mtDNA was detectable by electrophoresis after *Eco*RV or *Hin*fI digestion, thus suggesting a total deletion of the mtDNA as it occurs for $rho^0$ petite mutants of *S. cerevisiae* [37]. However, PCR amplification of the mtDNA with primers ML1 and ML6 showed the presence of mtDNA in the resistant isolate, suggesting that it was analogous to petite $rho^-$ of *S. cerevisiae* [37]. TEM confirmed the resemblance of isolate 1084-S to petite mutants of *S. cerevisiae* and *C. albicans* [27, 38].

In conclusion, these results demonstrated that the selection of azole-resistant petite mutants of *C. glabrata* may occur *in vivo* in patients undergoing fluconazole prophylaxis or therapy. As suggested previously [22], this acquired resistance is thought to be related to a secondary increased synthesis of CYP51A1, as it is well established that any condition leading to the inhibition of respiration – such as an excess of glucose in the culture medium, growth under anaerobic conditions or petite mutations – also induces an overexpression of *CYP51A1* [39]. The clinical relevance of these petite mutants remains questionable. In contrast to petite negative yeasts such as *C. albicans*, which do not usually survive mutations in mtDNA, petite mutants are easily obtained *in vitro* in *C. glabrata* [40]. However, as yet such azole-resistant mutants have not been isolated from clinical samples in patients treated with fluconazole. Moreover, it seems likely that petite mutations lead to a reduced virulence. In *C. albicans*, altered hyphal growth [41] and loss of virulence in different animal models of candidosis [42] associated with acquired resistance to azoles have been described. Likewise, a recently characterised petite mutant of *C. albicans* demonstrated a reduced adherence ability to various mammalian cells [38]. A prospective study including mycological examination of systematic oral, stool and urine samples before, during and after azole treatment and comparison of the results with the clinical evolution would make it possible to determine the real incidence of petite mutation *in vivo* and its clinical impact.

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