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Mechanisms of Azole Resistance in Petite Mutants of *Candida glabrata*

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We previously showed that resistant colonies of *Candida glabrata* inside the azole inhibition zones had respiratory deficiency due to mutations in mitochondrial DNA. Here, we analyzed the mechanisms of azole resistance in petite mutants of *C. glabrata* obtained by exposure to fluconazole or induced by ethidium bromide. The respiratory deficiency of these mutants was confirmed by oxygraphy and flow cytometric analysis with rhodamine 123, and its mitochondrial origin was demonstrated by transmission electron microscopy and restriction endonuclease analysis of the mitochondrial DNA. Flow cytometry with rhodamine 6G suggested an increased drug efflux in mutant cells, which was further supported by Northern blot analysis of the expression of the *C. glabrata CDR1* (*CgCDR1*) and *CgCDR2* genes, encoding efflux pumps. Conversely, the expression of *CgERG11*, which encodes the azole target, was not affected by petite mutations, and no differences were seen in the sequence of this gene between parent isolates and mutants. Moreover, sterol analysis showed similar overall amount of sterols in parent and mutant cells, but quantitative modifications were observed in the mutants, with almost undetectable biosynthesis intermediates. Further analysis performed after separation of free sterols from steryl esters revealed a defect in sterol esterification in mutant cells, with free ergosterol representing 92% of the overall sterol content. Thus, resistance or decreased susceptibility to azoles in petite mutants of *C. glabrata* is associated with increased expression of *CgCDR1* and, to a lesser extent, of *CgCDR2*. In addition, the marked increase in free ergosterol content would explain their increased susceptibility to polyenes.

The incidence of life-threatening fungal infections has been increasing in recent years, particularly among immunocompromised patients. To treat these infections, fluconazole, a water-soluble triazole with greater than 90% bioavailability after oral administration, is by far the most commonly used antifungal. This practice is most likely responsible for the emergence of intrinsically resistant or less-susceptible *Candida* species such as *C. krusei* and *C. glabrata*. In particular, *C. glabrata* has emerged as an important nosocomial pathogen during the past two decades (5, 24).

Azole antifungals selectively inhibit lanosterol 14α-demethylase, a cytochrome P-450 enzyme which is an essential participant in the ergosterol biosynthesis pathway. The mechanisms of azole resistance have been studied primarily in *Candida albicans* (23). Several findings indicate that increased azole efflux due to the overexpression of genes coding for membrane transport proteins belonging to the ATP-binding cassette (ABC) transporter family (*CDR1* and *CDR2*) or to the major facilitator superfamily (*MDR1* and *FLU1*) is the major mechanism of resistance. Different mechanisms are frequently combined, resulting in the development of fluconazole resistance.

In *C. glabrata*, studies of azole-resistant isolates have shown decreased intracellular azole concentrations (10, 21, 33), increased energy-dependent azole efflux (10, 19, 21, 28, 33), and increased expression of the ABC transporter genes *C. glabrata CDR1* (*CgCDR1*) and *CgCDR2* (21, 32, 33). However, there is also evidence that azole resistance may arise from increased expression of the gene coding for the azole target (*CgERG11*) due to gene duplications (21, 42). Likewise, point mutations in the *ERG11* gene may lead to a decreased affinity of azoles for their target and therefore to acquired azole resistance, as has been demonstrated in *C. albicans* (23). Another postulated mechanism of azole resistance is mutation in the *ERG3* gene, encoding the $\Delta^{5,6}$-sterol desaturase, leading to the accumulation of 14α-methylfecosterol, which can partially overcome the lack of ergosterol in the plasma membrane. This type of mutation induces cross-resistance to azoles and amphotericin B in the Darlington strain (22) as well as in some clinical isolates (13, 25) of *C. albicans*. However, inactivation of this enzyme in *C. glabrata* results in an altered sterol composition of the membrane but not in fluconazole resistance (7).

For this species, we previously noticed the presence of resistant colonies inside the inhibition zones for azoles during in vitro susceptibility testing by a disk diffusion method. These mutants, which showed increased susceptibility to polyenes and cross-resistance or susceptibility to all the azoles tested except tioconazole, represented a respiratory deficiency due to mutations in mitochondrial DNA (mtDNA). Moreover, petite mutants obtained from a wild-type isolate by exposure to ethidium bromide (ETB) were shown to be resistant or poorly susceptible to azole antifungals, except tioconazole, with a concomitant increased susceptibility to polyenes (4). More recently, we demonstrated a close relationship between respiration and susceptibility to azoles in *C. glabrata* (3). Indeed, blockage of respiration induces decreased susceptibility to azoles, culmi-

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nating in azole resistance due to the deletion of mtDNA. Here, we analyzed the mechanisms of azole resistance of these petite mutants.

MATERIALS AND METHODS

Yeast strains and culture conditions. This study was carried out with two clinical isolates of *C. glabrata*, designated 90.1085 and 94.5579, isolated in our hospital laboratory in 1990 from a urine sample and in 1994 from a bronchoalveolar lavage sample, respectively. For each clinical isolate, a fluconazole-resistant mutant was selected in the course of antifungal susceptibility testing performed by a disk diffusion method on Casitone agar plates (Bacto-Casitone, 9 g/liter; glucose, 20 g/liter; yeast extract, 5 g/liter; chloramphenicol, 0.5 g/liter; agar, 18 g/liter; pH 7.2) as previously described (4). Likewise, a respiration-deficient mutant was induced for each clinical isolate by exposure to the intercalating agent ETB (Sigma Chemical Co., St Louis, Mo.).

The parent isolates and their fluconazole-resistant or ETB-induced mutants were maintained by biweekly passages on yeast extract-peptone-glucose (YEPD) agar containing yeast extract, 5 g/liter; peptone, 10 g/liter; glucose, 20 g/liter; chloramphenicol, 0.5 g/liter; and agar, 20 g/liter. Mutants were also subcultured on yeast extract-peptone agar containing glycerol 2% (wt/vol) as the sole carbon source to ascertain their respiratory deficiency. All isolates were preserved in glycerol 20% (wt/vol) at −80°C.

Antifungal susceptibility testing. Antifungal susceptibility was determined by a disk diffusion method on Casitone agar plates with azole or polyene Neosensitab tablets from Rosco Diagnostica (Taastrup, Denmark) as described previously (4). After incubation for 48 h at 37°C, the diameters of the inhibition zones were measured.

The MICs of amphotericin B, ketoconazole, and fluconazole were determined on Casitone agar plates by the Etest procedure as recommended by the manufacturer (AB Biodisk, Solna, Sweden). MICs were read after 48 h at 37°C as the drug concentration at which the inhibition ellipse intercepted the scale on the antifungal strips.

For azole drugs not available as Etest strips, MICs were determined with a microdilution assay in Casitone broth inoculated with 10^3 blastoconidia/ml (200 μl per well of the microtiter plates). Azoles were dissolved in dimethyl sulfoxide to reach final concentrations ranging from 0.125 to 128 μg/ml. The microtiter plates were incubated for 48 h at 37°C, and the absorbance was read at 595 nm. The MIC_{90} was defined as the lowest concentration of antifungal that inhibited growth by at least 90% compared with a drug-free control. For the Etest procedure, two independent experiments were performed in triplicate, and the results were analyzed with the Kruskall-Wallis test.

Characterization of petite mutants. The respiratory status of the two parent isolates and their fluconazole-resistant or ETB-induced mutants was investigated by flow cytometry with rhodamine 123 and by oxygraphy as previously described (3). The mtDNA of parent and mutant cells was analyzed after digestion by EcoRV (Roche Diagnostics GmbH, Mannheim, Germany), and the presence of mitochondria was investigated by transmission electron microscopy as previously described (3).

Induction of petite mutation by azoles. Mutation rates in the presence of azoles were evaluated for both clinical isolates on Casitone agar plates containing an inhibitory concentration of drug. Fungal suspensions in sterile distilled water (from 10^9 to 10^7 blastoconidia per ml) were prepared from fresh YEPD cultures and inoculated on five plates (100 μl per plate) for each inoculum and each azole. For each plate, five randomly selected colonies were subcultured on YEPD and glycerol-containing agar plates to test respiratory deficiency. Mutation rates were calculated from the average colony number obtained for each azole after a 48-h incubation at 37°C.

***CgERG11* gene sequencing.** Five pairs of oligonucleotide primers were synthesized by Genset SA (Paris, France) from the *CgERG11* GenBank sequence (accession number L40389) in order to cover the whole gene (Table 1). The genomic DNA of parent and mutant isolates was extracted with the DNeasy plant minikit (Qiagen Inc., Valencia, Calif.) and used as a template for PCR amplification. PCR conditions were as follows: 5 min of denaturation at 94°C, followed by 30 cycles consisting of 30 s at 94°C for denaturation, 40 s at 52°C for annealing, and 50 s at 72°C for elongation, and finally 10 min more of elongation at 72°C. After purification of the PCR products with the High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany), sequencing was performed with a Quick Start kit on a CEQ 2000 DNA analysis system (Beckman Coulter Inc., Fullerton, Calif.) with the forward and reverse primers previously used to synthesize the PCR products.

Flow cytometric analysis of the efflux of rhodamine 6G. The efflux of rhodamine 6G, which uses the same membrane transporter as fluconazole in yeasts (18), was evaluated by flow cytometry with stationary-phase blastoconidia. Yeast cells of parent and mutant isolates (10^7) grown in YEPD were incubated for 30 min at 30°C in 1 ml of the same medium containing rhodamine 6G (Sigma Aldrich Ltd.) at a final concentration of 100 μM. Uptake of rhodamine 6G was stopped by cooling the tubes on ice (33). The reaction mixture was then diluted 40-fold in cold sterile phosphate-buffered saline (pH 7.2), and the fluorescence of the cells was immediately quantified at 535 nm with a FACScan flow cytometer (BDIS Europe, Erembodegem, Belgium). The cells were then washed three times with cold YEPD medium to remove excess rhodamine 6G, and efflux of the dye was finally evaluated after an additional 15-min incubation at 30°C in the same medium by measuring the fluorescence of the cells after 1:40 dilution in phosphate-buffered saline. Ten thousand events were collected for each sample, and the data were analyzed with CellQuest software from BDIS. The data presented correspond to fluorescence frequency distribution histograms (relative number of blastoconidia versus relative fluorescence intensity, expressed in arbitrary units on a logarithmic scale).

TABLE 1. Oligonucleotides used for *CgERG11* sequencing

Primer	Nucleotide sequence (5′-3′)	Nucleotide coordinates[a]
1 sense	CTACAATCGAGTGAGCTTG	+17 to +35
1 antisense	GTAGAACACAAGTGGTGG	+729 to +746
2 sense	CCATCACATGGCAATTGC	+688 to +705
2 antisense	GGTCATCTTAGTACCATCC	+1445 to +1463
3 sense	GGTCGTTGAACTATTGGAG	+584 to +602
3 antisense	GGACCCAAGTAGACAGTC	+863 to +880
4 sense	CGTGAGAAGAACGATATCC	+1380 to +1398
4 antisense	CACCTTCAGTTGGGTAAC	+2047 to +2064
5 sense	CGCTTACTGTCAATTGGG	+1991 to +2008
5 antisense	GTCATATGCTTGCACTGC	+2397 to +2414

[a] Nucleotide coordinates refer to the *CgERG11* GenBank sequence (accession no. L40389).

mRNA extraction and Northern blotting. Total RNA from the parent isolates and their mutants was obtained from logarithmic-phase cultures in YEPD medium. Cells were collected by centrifugation for 5 min at 3,000 × *g* and resuspended in 2 ml of 50 mM sodium acetate (pH 5.3)–10 mM EDTA–10% (wt/vol) sodium dodecyl sulfate. The suspensions were shaken vigorously, and 2 ml of phenol at pH 5.3 was added. The mixtures were incubated for 4 min at 65°C and then frozen in liquid nitrogen. After thawing, the samples were centrifuged, and the aqueous phase was collected and subjected to phenol-chloroform extraction. Total RNAs were precipitated with 2.5 volumes of pure ethanol and resuspended in 100 μl of diethylpyrocarbonate-treated water.

Northern blot hybridization was performed as described by Ziadi et al. (46). [α-^{32}P]dUTP-labeled antisense RNA probes were prepared with the Lig'nScribe and the Strip-EZ RNA kits according to the manufacturer's instructions (Ambion Inc., Austin, Tex.). Probes were generated from genomic DNA of parent isolate 90.1085 by PCR amplification (PCR conditions were as given above except that the annealing temperature was 49°C). The amplified fragments were as follows (numbers represent positions in the GenBank sequences; GenBank accession numbers are given in parentheses): *CgCDR1*, positions 1084 to 1400 (AF109723); *CgCDR2*, positions 362 to 712 (AF251023); *CgERG11*, positions 781 to 1571 (L40389); and *CgACT1*, positions 949 to 1194 (AF069746). For densitometric analysis, signals were quantified with the Bio 1D software (Voilab, Saint-Herblain, France). Relative values were adjusted for differences in sample loading based on quantification of the control gene *CgACT1*. A twofold increase in the densitometric values of the mutants compared to the level of expression in clinical isolates was considered significant.

Sterol analysis. Sterols were extracted from lyophilized cells of the parent isolates or their mutants grown to stationary phase in YEPD broth. Dried cells (50 mg) were saponified by methanolic 40% (wt/vol) KOH in the presence of pyrogallol at 90°C for 1 h. After cooling and addition of water, the unsaponified fractions were removed with 3 volumes of heptane. The UV spectrum of the desiccated heptane solutions was determined, and the amount of ergosterol was calculated from the maximum absorbance at 281.5 nm (34). The different sterol species were then separated by gas chromatography (GC) with an AT-1 capillary column (25 m by 0.32 mm; Alltech Canada Biotechnology Centre Inc., Guelph, Canada) and identified by their retention times relative to a cholesterol standard.

Further analysis of the sterol content was performed on lyophilized cells of the parent isolate 94.5579 or its fluconazole-resistant mutant by GC after separation of free sterols from steryl esters (31). To do this, cells rehydrated with 2 ml of

TABLE 2. Susceptibility to polyenes and azoles of *C. glabrata* parent isolates and their derived petite mutants[a]

Antifungal agent	Diam (mm) of growth inhibition zone					
	Isolate 90.1085			Isolate 94.5579		
	Parent	Resistant mutant	ETB-induced mutant	Parent	Resistant mutant	ETB-induced mutant
Amphotericin B	28	40	40	26	30	30
Nystatin	28	40	40	26	35	32
Tioconazole	40	32	32	40	40	33
Miconazole	24(M)	TR	11	25(M)	17	15
Isoconazole	24(M)	TR	TR	24(M)	11	11
Ketoconazole	28(M)	11	TR	30(M)	12	12
Fluconazole	26(M)	TR	TR	28(M)	TR	TR

[a] In vitro susceptibility testing was performed by the disk diffusion method on Casitone agar plates with Neosensitab tablets (containing 10 μg of drug for amphotericin B, tioconazole, miconazole, and isoconazole; 15 μg for ketoconazole and fluconazole; and 50 μg for nystatin). Results, which are representative of two independent experiments, correspond to the diameter of growth inhibition zones. M, presence of resistant mutants within the inhibition zone. TR, total resistance.

distilled water were broken by vigorous shaking with a Mini-Beadbeater (Biospec Products Inc., Bartlesville, Okla.) in the presence of 500 μl of glass beads (diameter, 0.3 to 0.4 mm; Sigma Aldrich Ltd.). Cellular lipids were extracted with chloroform-methanol (2:1, vol/vol) as described by Folch et al. (6). The organic phase was taken to dryness, and the lipids were dissolved in 200 μl of hexane (neutral lipids). Free sterols were separated from steryl esters by thin-layer chromatography in dichloromethane on coated Silica gel 60 F_{254} plates (VWR International, Fontenay-sous-Bois, France). Sterols were then visualized under UV after spraying of a primuline solution (Sigma Aldrich Ltd.) at 0.05 mg/ml in acetone-water (80:20). Free sterol and steryl ester spots were scraped from the plate, and steryl esters were finally subjected to saponification.

All experiments were performed with two independent cultures to ensure the reproducibility of the results.

RESULTS

Antifungal susceptibility. In vitro antifungal susceptibility testing of the mutants by the agar disk diffusion method revealed resistance or markedly decreased susceptibility to all the azoles tested except tioconazole, whereas the parent isolates were susceptible to azoles (Table 2). However, resistant colonies randomly distributed within the inhibition zones were seen for parent isolates around the azole tablets, some even growing in contact with the antifungal tablet. Despite relatively poor correlation with the disk diffusion method, determination of azole MICs by the Etest procedure or by a microdilution method in Casitone broth confirmed the resistance or decreased susceptibility of the mutants to azoles (Table 3). Higher MICs were obtained with the mutants than with their parent isolates. Conversely, mutant cells exhibited increased susceptibility to polyenes, as attested by the larger growth inhibition zones around the amphotericin B or nystatin tablet and by slightly but significantly lower MICs of amphotericin B determined with the Etest strips ($P < 0.02$, Kruskall-Wallis test).

Characterization of petite mutants. Flow cytometric analysis of parent and mutant cells stained with rhodamine 123, a lipophilic cationic fluorochrome that is incorporated into mitochondria through a transmembrane potential-dependent mechanism, suggested respiratory deficiency for both fluconazole-resistant and ETB-induced mutants. Indeed, mutant cells stained with rhodamine 123 exhibited lower fluorescence intensity than parent cells, and incubation with sodium azide did not reduce their fluorescence intensity, whereas about a 50% reduction was observed for parent cells in the presence of azide (Fig. 1). Comparison of oxygen consumption in parent and mutant cells by oxygraphy confirmed the respiratory deficiency of the mutants (Fig. 2), and no mitochondria were observed for mutant cells by transmission electron microscopy, attesting to the mitochondrial origin of this respiratory deficiency. The results obtained for parent isolate 90.1085 and its mutants are illustrated in Fig. 3, and similar results were obtained for parent isolate 94.5579 and its mutants. Finally, restriction endonuclease analysis of the mtDNA from the two parent isolates and their mutants revealed two fragments of about 20 and 5 kbp for parent cells, whereas only one fragment of about 18 kbp was detected for mutant cells, as illustrated in Fig. 4 for parent isolate 90.1085 and its mutants. These results demonstrated a partial mtDNA deletion in the respiration-deficient mutants.

TABLE 3. MICs of polyenes and azoles for *C. glabrata* parent isolates and their derived petite mutants[a]

Antifungal agent	MIC (μgml)					
	Isolate 90.1085			Isolate 94.5579		
	Parent	Resistant mutant	ETB-induced mutant	Parent	Resistant mutant	ETB-induced mutant
Amphotericin B	0.032	0.012*	0.016*	0.047	0.012*	0.023*
Tioconazole	<0.125	<0.125†	<0.125†	<0.125	0.125†	<0.125†
Miconazole	<0.125	2	2	<0.125	2	1
Econazole	<0.125	1	1	<0.125	2	0.5
Ketoconazole	0.38	6	8	0.125	16	12
Fluconazole	32	>256	>256	64	>256	>256

[a] Results are representative of two independent experiments performed in triplicate. Data were obtained by the Etest procedure for amphotericin B, ketoconazole, and fluconazole. For tioconazole, miconazole, and econazole, Etest strips were not available, and for these drugs, sensitivity was tested by a microbroth dilution method. Data were analyzed with the Kruskall-Wallis test. For all values, $P < 0.001$ except *, $P < 0.02$, and †, no differences observed.

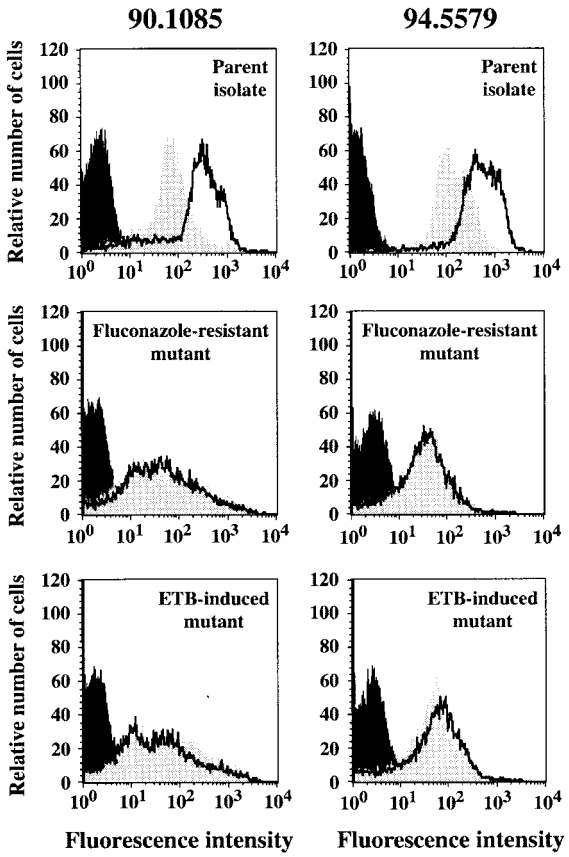

FIG. 1. Flow cytometric analysis of rhodamine 123-stained cells of *C. glabrata* parent isolates and their fluconazole-resistant or ETB-induced mutants. Yeasts were incubated (grey area) or not (black thick line) with 1 mM sodium azide before rhodamine 123 staining. The fluorescence of cells incubated without the fluorochrome (black area) is presented as a control.

Induction of petite mutation by azoles. The effect of azole antifungals on the frequency of the petite mutation was also investigated. As shown in Table 4, the addition of an azole drug to the culture medium resulted in progressively increasing mutation rates from miconazole to fluconazole resistance. Mutation rates higher than 10^{-5} were calculated for fluconazole and ketoconazole for the two clinical isolates and for econazole for isolate 94.5579.

Analysis of *ERG11* gene sequences. Comparison of the *ERG11* gene sequences for the parent isolates and their respiration-deficient mutants and of the *CgERG11* gene sequence available in the GenBank database showed six point mutations located at the same positions (A649G, T754C, C1331T, C1397T, G1586A, and A2120T) in each cell population, including the parent isolates. These point mutations did not affect the amino acid sequence, suggesting that this genetic polymorphism was functionally silent.

Accumulation and efflux of rhodamine 6G. The accumulation and efflux of rhodamine 6G in parent and mutant cells were evaluated by flow cytometry. The respiration-deficient mutants accumulated less rhodamine 6G than their parent isolates (Fig. 5), and accumulation of the dye was not modified by first treating the blastoconidia with 1 mM sodium azide for 2 h (data not shown). Indeed, the mean fluorescence intensity of the cells was 2.5- or 3.1-fold lower for 90.1085 mutants than for their parent isolate and 2.7- or 3-fold lower for the mutants generated from isolate 94.5579. After removal of free rhodamine 6G and an additional 15-min incubation in YEPD, the fluorescence intensity of mutant cells was almost equivalent to that of unloaded control cells, corresponding to a fluorescence intensity diminution of 85 and 89% for the 90.1085 mutants and 93 and 95% for the 94.5579 mutants. Conversely, a slight decrease in fluorescence intensity was observed for the parent blastoconidia (40 and 20% for isolates 90.1085 and 94.5579, respectively). These results demonstrated an increased efflux of rhodamine 6G in the respiration-deficient mutants compared to their parents.

Expression of azole resistance genes. Northern blot analysis of total RNA extracted from the four respiration-deficient mutants and their parent isolates was performed with antisense RNA probes for genes encoding the ABC transporters Cdr1p and Cdr2p and lanosterol 14α-demethylase. The *CgACT1* gene was used as an internal control for the standardization of signals. Figure 6A shows that signals for *CgCDR1* expression were intense in the four mutants, whereas no signal was detectable in the parent isolates. The relative increases in *CgCDR1* expression obtained in mutant cells were 10- to 31-fold those in the parent isolates, whereas the expression of *CgCDR2* was slightly enhanced in the petite mutants (Fig. 6B). Conversely, the expression of *CgERG11* was lower or 1.6-fold higher in two mutants than in the parent isolates.

Sterol composition. Comparison of the sterol content of the parent isolates and their respiration-deficient mutants showed no qualitative differences after separation of the different molecular species by GC (Fig. 7). The overall amount of sterols

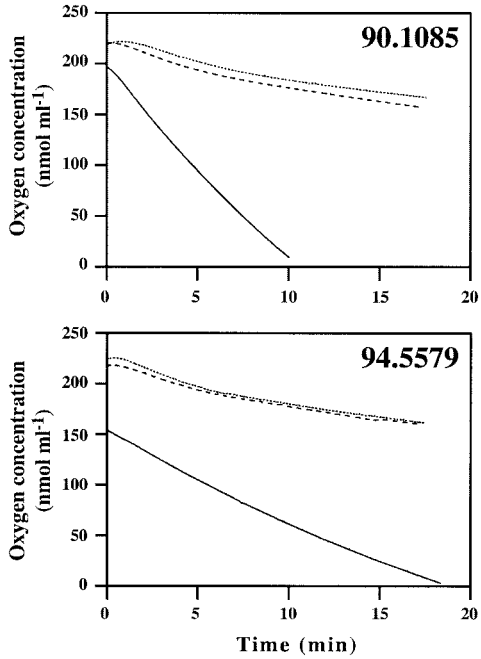

FIG. 2. Oxygen consumption by the *C. glabrata* parent (solid line) and fluconazole-resistant (dashed line) or ETB-induced (dotted line) mutant strains. A marked decrease in oxygen consumption was found for the mutant cells.

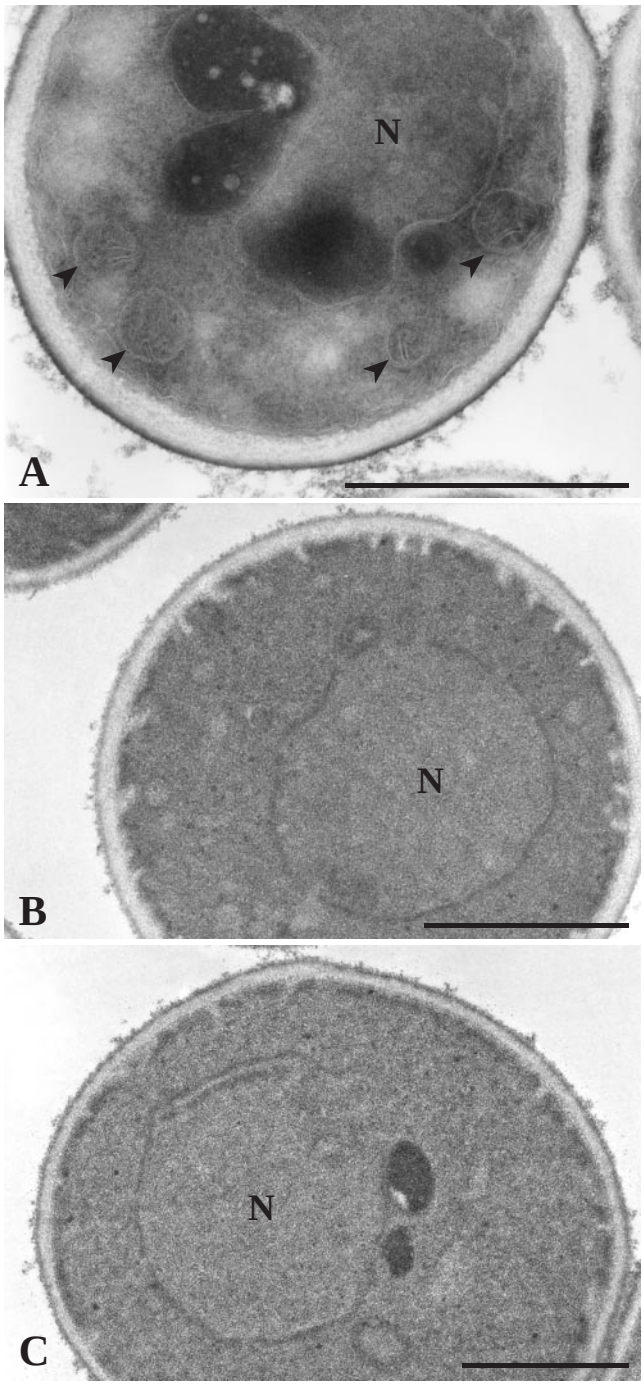

FIG. 3. Transmission electron micrographs of *C. glabrata* parent isolate 90.1085 (A) and its fluconazole-resistant (B) and ETB-induced (C) mutants. Note the absence of mitochondria in the respiration-deficient cells compared to the numerous mitochondrial sections (arrowheads) with obvious cristae in the parent cell. N, nucleus. Bars, 1 μm.

was very similar between the parent (3.98 ± 0.2 μg/g dry weight) and the mutant cells (3.84 ± 0.19 μg/g dry weight), and the same biosynthesis intermediates, characterized by their retention times, were found in all cell populations. However, the sterol repartition was quite different, since ergosterol represented about 50% of total sterols in the parent isolates and

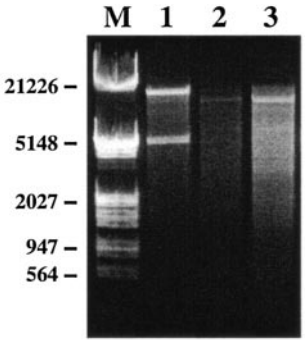

FIG. 4. Electrophoretic patterns of mtDNA of *C. glabrata* parent isolate 90.1085 (lane 1) and its fluconazole-resistant (lane 2) and ETB-induced (lane 3) mutants. mtDNA was analyzed by agarose gel electrophoresis after digestion with EcoRV. Lane M, molecular size markers (Marker III; Roche Molecular Biochemicals, Meylan, France). Sizes are shown in base pairs.

more than 90% of total sterols in the mutants. Furthermore, squalene plus lanosterol constituted more than 30% of the sterols in the parent isolates, whereas sterol intermediates were almost totally lacking in the mutants.

Considering these results, the sterol content was further analyzed after separation of free sterols from steryl esters by thin-layer chromatography. Due to the high reproducibility of the results for the two parent isolates as well as the four mutants, this analysis was performed with only one parent isolate (94.5579) and its fluconazole-resistant mutant. Major differences were seen between the two cell populations, with a defect in esterification in the mutant. Indeed, 17% of the ergosterol and 54% of the biosynthesis intermediates were esterified in the parent isolate, whereas in the mutant free ergosterol represented 92% of the overall sterol content and biosynthesis intermediates were almost undetectable.

DISCUSSION

It is generally considered that antifungal treatment may lead to the selection of resistant isolates. Previous in vitro studies from our laboratory detected resistant mutants upon exposure to fluconazole for most of the *C. glabrata* isolates and demonstrated a relationship between this acquired resistance and the petite mutation (3, 4). In addition, resistant petite mutants may also emerge in patients undergoing fluconazole prophylaxis or therapy (2). However, the results reported here demonstrated

TABLE 4. Mutation rates of *C. glabrata* parent isolates in the presence of azole antifungals

Azole antifungal (μg/ml)	Mutation rate	
	Parent isolate 90.1085	Parent isolate 94.5579
Tioconazole (20)	ND[a]	ND
Miconazole (1)	7×10^{-6}	5×10^{-5}
Econazole (1)	1×10^{-5}	2×10^{-4}
Ketoconazole (1)	1.4×10^{-4}	2×10^{-3}
Fluconazole (100)	4×10^{-4}	1×10^{-3}

[a] ND, azole-resistant petite mutants were not detected (mutation rate, $<10^{-6}$).

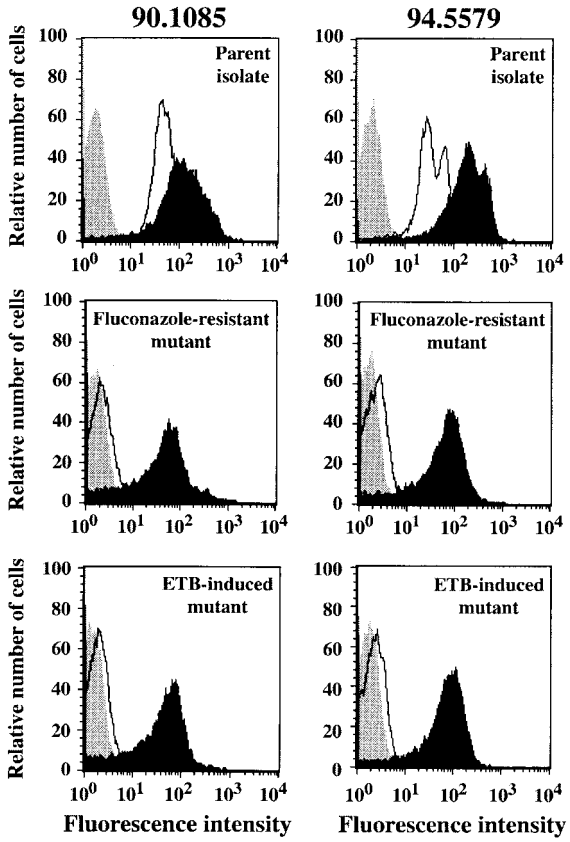

FIG. 5. Flow cytometric analysis of rhodamine 6G uptake and efflux for *C. glabrata* parent isolates and their derived petite mutants. Histograms are given for cells incubated with rhodamine 6G (black area) and after removal of the free dye and an additional 15-min incubation (black line). Unlabeled controls are represented in grey.

that some azoles may act as direct inductors of the petite mutation in *C. glabrata*.

Indeed, in our experiments, petite mutation rates in the presence of fluconazole or ketoconazole for the two isolates of *C. glabrata* studied (or of econazole for one of them) were higher than the spontaneous mutation rate reported by O'Connor et al. (26), which was 10^{-5}. Our results are in agreement with those of Sanglard et al. (32), who reported a high frequency (2×10^{-4} to 4×10^{-4}) of azole resistance in *C. glabrata* upon exposure to fluconazole and linked this high-frequency azole resistance to mitochondrial loss. Ketoconazole and fluconazole were also reported to be potent inducers of petite mutagenesis in *Saccharomyces cerevisiae* by Kenna et al. (14) but without changes in azole susceptibility. Nevertheless, petite mutants of *S. cerevisiae* were shown more recently to be resistant to fluconazole (16). This inductor effect of azoles on petite mutation may be related to the blockage of mitochondrial functions, resulting in the accumulation of mutagenic reactive oxygen species in mitochondria (15). Furthermore, a direct action of miconazole and ketoconazole on respiration has already been demonstrated in *C. albicans* by using intact blastoconidia or isolated mitochondria (35, 36, 39). Moreover, we recently showed a close relationship between respiration and azole susceptibility in *C. glabrata*, since blockage of the respiratory chain by sodium azide is sufficient to reduce susceptibility to azoles, culminating in azole resistance due to the deletion of mtDNA (3).

According to Sanglard et al. (32), azole resistance in petite mutants of *C. glabrata* is linked to the upregulation of nuclear genes encoding efflux proteins. However, other mechanisms of resistance were not investigated in that study. Due to the extensive cross talk between the nucleus and mitochondria, partial or total loss of mitochondrial functions in petite mutants also affects the expression of other nuclear genes (38). Thus, we compared two susceptible isolates of *C. glabrata* and their petite mutants obtained by exposure to fluconazole or to ETB. Our experiments demonstrated that, whatever the mechanism by which they were formed, these mutants presented the same behavior. Comparison of the sterol content between parent and mutant cells showed very close overall amounts of sterols but different proportions of various molecular species. Indeed, petite mutant cells exhibited a marked increase in ergosterol

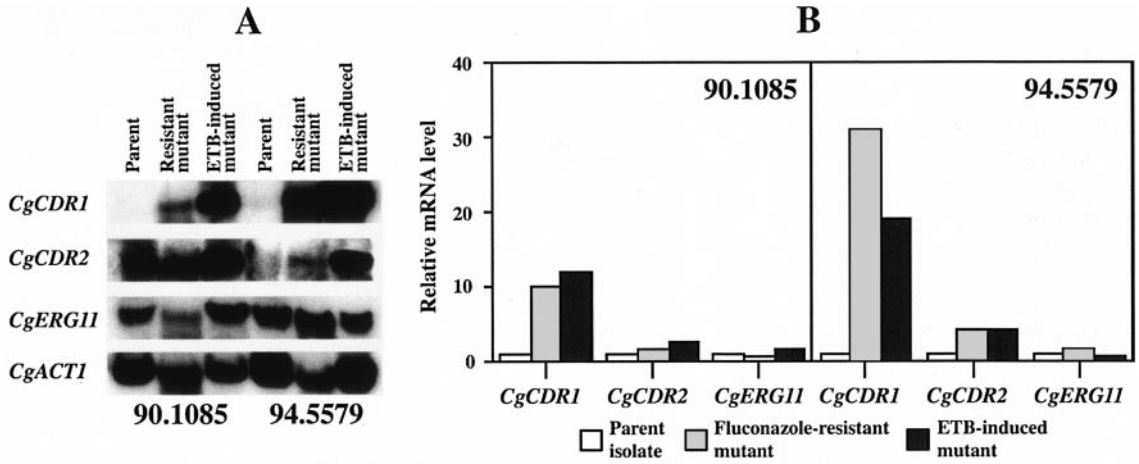

FIG. 6. Expression of azole resistance genes in *C. glabrata* parent isolates and their derived petite mutants. (A) Northern blot analysis. Total RNAs were extracted, separated, and blotted as indicated in Materials and Methods. Hybridizations were carried out with ^{32}P-labeled antisense RNA probes specific for either *CgCDR1*, *CgCDR2*, *CgERG11*, or *CgACT1*. (B) Relative mRNA levels in parent isolates and their derived fluconazole-resistant and ETB-induced mutants. The intensities of the autoradiographic signals were quantified and are shown as the ratio of *CgCDR1*, *CgCDR2*, or *CgERG11* to *CgACT1*.

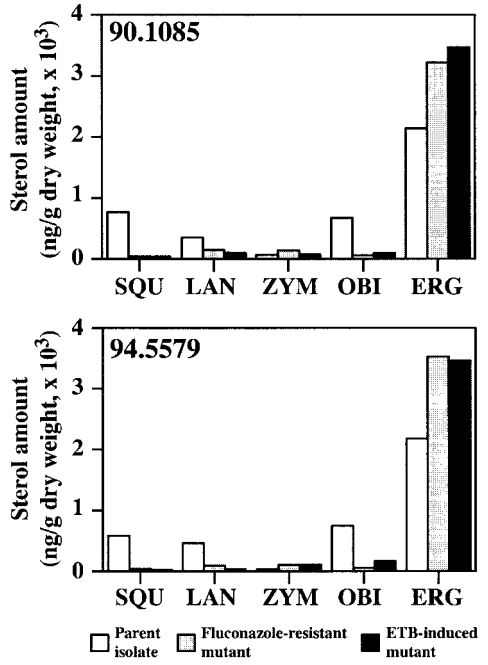

FIG. 7. Analysis of the sterol composition of *C. glabrata* parent isolates and their fluconazole-resistant and ETB-induced mutants. Sterols were extracted from approximately 50 mg of lyophilized cells. The different sterol species were separated by GC and identified by their retention times relative to cholesterol. Data are representative of the results obtained from two independent cultures. SQU, squalene; LAN, lanosterol; ZYM, zymosterol; OBI, other biosynthesis intermediates; ERG, ergosterol.

content, with an almost complete absence of biosynthesis intermediates. However, all the intermediates that were detected by GC were strictly identical to those of the parent isolates and their mutants, demonstrating the absence of an alternative metabolic pathway in petite mutants which would have led to the synthesis of nontoxic 14α-methylfecosterol in place of ergosterol. Conversely, the Darlington strain of *C. albicans* is resistant to fluconazole because of a mutation in the *ERG3* gene, encoding $\Delta^{5,6}$-sterol desaturase, which results in the accumulation of 14α-methylfecosterol in the presence of fluconazole (11, 22). Deletion of this gene in *S. cerevisiae* also leads to azole resistance (43), whereas its inactivation in *C. glabrata* results in the accumulation of toxic C-14-methylated sterols in the presence of fluconazole (7).

In addition, analysis of the different sterol species after separation of free sterols from steryl esters revealed a defect in esterification in the petite mutant studied. Indeed, free ergosterol represented almost all the sterols in the mutant, whereas 25% of sterols were esterified in the parent cells. Sterol esterification in *S. cerevisiae* is catalyzed by two acyl-coenzyme A:sterol acyltransferases encoded by the nuclear genes *ARE1* and *ARE2* (45). Are2p is the major yeast steryl ester synthase and has a significant preference for the end product ergosterol as a substrate, whereas Are1p esterifies sterol intermediates, mainly lanosterol, as well as ergosterol (12). Disruption of both *ARE1* and *ARE2* results in a viable phenotype with a significant increase in the ratio of ergosterol to biosynthesis intermediates (1). Indeed, the absence of sterol esterification renders all intermediates accessible to the enzymes of the ergosterol pathway, and thus complete conversion to ergosterol is not prevented.

Changes in sterol esterification are mediated at least partially by differential transcriptional regulation of *ARE1* and *ARE2* genes by heme. Indeed, heme deficiency in *S. cerevisiae* as well as anaerobiosis leads to increased activity of Are1p, with a concomitant decrease in Are2p activity, and therefore to a diminished level of ergosterol esters (12, 40, 41). Our experiments with *C. glabrata* petite mutants also suggested a decrease in expression of the homologue of *ARE2*, responsible for the presence of mainly free ergosterol. As free sterols are incorporated into the plasma membrane, this would explain the increased susceptibility of our petite mutants to amphotericin B. Surprisingly, a petite mutant of *C. albicans* induced by ETB possessed a lower level of ergosterol than the parent isolate and demonstrated a small but statistically significant increase in tolerance to amphotericin B (8). Likewise, the exposure of *C. albicans* to the macrolide antibiotic erythromycin, which inhibits protein synthesis in mitoribosomes and therefore respiration, results in severely decreased ergosterol levels correlated with elevated tolerance to amphotericin B (9). However, biosynthesis intermediates were not quantified, and esterification was not evaluated in these studies.

In addition, a wide range of petite mutations have been described, from partial to total deletions of the mtDNA, and their repercussions on sterol composition could vary. Additionally, it is more difficult to induce petite mutants in *C. albicans* than in *S. cerevisiae* and *C. glabrata*. Geraghty and Kavanagh (8) screened 2,500 cells of *C. albicans* grown in the presence of ETB and found only one respiration-deficient mutant, suggesting that deletion of the mtDNA in this species is lethal. The available genomic data suggest that *C. glabrata* is much more closely related to *S. cerevisiae* than to the other *Candida* species (44). Moreover, the high degree of homology with the mitochondrial genome of *S. cerevisiae* would explain why it is so much easier to induce petite mutants in *C. glabrata* than in *C. albicans* (17).

Sequencing of *CgERG11* and Northern blot analysis of its expression showed that it was not mutated or overexpressed in petite mutants. In contrast, flow cytometry showed an increased efflux of rhodamine 6G, suggesting the overexpression of nuclear genes encoding some efflux pumps, which was confirmed by Northern blotting for *CgCDR1* and, to a lesser extent, for *CgCDR2*. These results, which are consistent with the findings of Sanglard et al. (32), establish that azole resistance in petite mutants is associated with increased expression of efflux transporter genes. It is well known that the functional state of mitochondria influences nuclear gene expression in yeasts (27); for instance, petite mutations induce the overexpression of genes encoding membrane transporters in *S. cerevisiae* (38). Moreover, increased expression of efflux pump genes is the most frequent mechanism of acquired azole resistance reported in clinical isolates of *Candida* spp., usually alone, but sometimes associated with upregulation of or with point mutations in the *ERG11* gene.

Recently, a number of resistant clinical isolates of *C. albicans* showed overexpression of genes encoding efflux pumps, associated with the G464S amino acid substitution in lanosterol 14α-demethylase, affecting its affinity for fluconazole

(20). Likewise, one of the resistant isolates of *C. glabrata* studied by Redding et al. (30) showed overexpression not only of *CgCDR1* and *CgCDR2* but also of *CgERG11*. In *C. dubliniensis*, resistant isolates from a human immunodeficiency virus-infected patient were found to have combined overexpression of the *CdMDR1*, *CdCDR1*, and *CdERG11* genes, whereas isolates from another patient demonstrated overexpression of only *CdMDR1* (29). In addition, 14 point mutations were found in the *CdERG11* gene of these isolates, and two of these mutations have been described to decrease the affinity of azoles for the enzyme in *C. albicans* isolates.

In summary, we show here that resistance or decreased susceptibility to azoles in *C. glabrata* petite mutants is associated with increased expression of *CgCDR1* and, to a lesser extent, of *CgCDR2*, two genes coding for ABC transporters. In addition, the marked increase in free ergosterol content would explain their increased susceptibility to amphotericin B. The pathogenicity of these petite mutants, which can be selected in vivo, remains to be defined. Because of the deficit in energy production linked to impairment or absence of the mitochondrial respiratory chain, petite mutants yield small colonies and present a growth defect compared to their parent isolates. Moreover, the absence of sterol esterification could also result in perturbations of growth (37). Thus, one may speculate that petite mutants of *C. glabrata* present attenuated virulence compared to parent isolates, and work is in progress to evaluate their pathogenicity in animal models.

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