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Molecular Biological Characterization of an Azole-Resistant *Candida glabrata* Isolate

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Two isolates of *Candida glabrata*, one susceptible and one resistant to azole antifungals, were previously shown to differ in quantity and activity of the cytochrome P-450 14α-lanosterol demethylase which is the target for azole antifungals. The resistant isolate also had a lower intracellular level of fluconazole, but not of ketoconazole or itraconazole, than the susceptible isolate. In the present study a 3.7-fold increase in the copy number of the *CYP51* gene, encoding the 14α-lanosterol demethylase, was found. The amount of *CYP51* mRNA transcript in the resistant isolate was eight times greater than it was in the susceptible isolate. Hybridization experiments on chromosomal blots indicated that this increase in copy number was due to duplication of the entire chromosome containing the *CYP51* gene. The phenotypic instability of the resistant isolate was demonstrated genotypically: a gradual loss of the duplicated chromosome was seen in successive subcultures of the isolate in fluconazole-free medium and correlated with reversion to susceptibility. The greater abundance of the amplified chromosome induced pronounced differences in the protein patterns of the susceptible and revertant isolates versus that of the resistant isolate, as demonstrated by two-dimensional gel electrophoresis (2D-GE). Densitometry of the 2D-GE product indicated upregulation of at least 25 proteins and downregulation of at least 76 proteins in the resistant isolate.

The incidence of life-threatening fungal infections has increased dramatically over the last decade. The antifungal agents available to treat these infections can be divided into four groups according to their modes of action. They impair membrane integrity (polyenes), interact with microtubules (griseofulvin), or inhibit macromolecule synthesis (flucytosine) or ergosterol biosynthesis (azoles, morpholines, and allylamines). For treatment of systemic *Candida* infections only polyenes, flucytosine, and azoles are used.

Resistance to flucytosine is common and usually arises from the loss or mutation of cytosine permeases (the enzymes involved in its uptake), of enzymes involved in the conversion of flucytosine to phosphorylated derivatives of 5-fluorouracil, the antifungally active metabolite, or of enzymes involved in its incorporation into RNA (37). Resistance to amphotericin B, a polyene, has been seldom observed in the clinic during its use for three decades. Most amphotericin B-resistant isolates contain abnormally low numbers of ergosterol molecules in their plasma membranes, thereby limiting the numbers of available binding sites for the polyene and thus preventing the membrane damage. Up to the late eighties, azole resistance was also a rare phenomenon and limited to chronic mucocutaneous candidiasis patients. The AIDS epidemic, caused by the human immunodeficiency virus type 1, drastically changed the situation. Large-scale prophylactic and therapeutic use of fluconazole led to both the occurrence of fluconazole-resistant *Candida albicans* isolates and to increased colonization of patients with other *Candida* species, such as *C. krusei* and *C. glabrata*, that are less sensitive to fluconazole than *C. albicans* (25).

Despite the numerous reports on resistance only a few mechanistic studies were published until recently, when some multidrug resistance proteins were identified in *C. albicans*. The identification of the *CaMDR1* gene (8) (initially designated *BEN*^{r}) as a member of the major facilitators and that of *CDR1* (24) as a member of the ATP-binding cassette (ABC) family have boosted the number of studies concerning these transport molecules. Indeed, it has been shown that *CDR1* expression confers resistance not only to all types of the antifungal azoles but also to other ergosterol biosynthesis inhibitors such as the squalene epoxidase inhibitor terbinafine (26) and the Δ14-reductase and/or Δ8-7-isomerase inhibitor (23) amorolfine (2, 4, 29, 30). By contrast, expression of *CaMDR1* results in resistance only to fluconazole and not to other azoles, possibly because of this agent's hydrophilic properties (29). Recently, it has been demonstrated by Sanglard et al. that overexpression of *CaMDR1* in *Saccharomyces cerevisiae* confers resistance to terbinafine, implying that terbinafine is a substrate for this transporter (31).

Several homologs to these two multidrug resistance genes have now been identified, indicating that in *C. albicans*, as in *S. cerevisiae*, a plethora of transport systems which could contribute to lower intracellular levels of antifungals exists. Studies dealing with other possible underlying resistance mechanisms, such as alteration or overproduction of a target enzyme, changes in membrane composition, and mutation of other ergosterol biosynthesis genes are limited in number (9, 12, 16, 32, 35, 36). We previously studied two *C. glabrata* isolates, one sensitive (prefluconazole treatment) and one resistant (post-treatment). It was concluded that resistance to fluconazole was

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at least partly due to both a lower intracellular fluconazole content and an increased P-450-dependent ergosterol synthesis, whereas only the latter contributed to itraconazole resistance (36). The increased ergosterol synthesis, measured in intact cells and in subcellular fractions, was attributed to an increased cellular content and activity of cytochrome P-450 14α-lanosterol demethylase (CYP_{51} product of the *CYP51* gene; also called *ERG11* in *S. cerevisiae* and *ERG16* in *C. albicans*) and to an increased squalene epoxidase or oxidosqualene cyclase activity (36). This increase of squalene epoxidase activity was thought to be the origin of cross-resistance to terbinafine. We also demonstrated the phenotypic instability of azole resistance in the posttreatment isolate by showing a gradual increase in susceptibility to azoles when the isolate was repeatedly subcultured in fluconazole-free medium (36). After 115 subcultures the itraconazole sensitivity of the posttreatment isolate was similar to that of the pretreatment isolate, whereas fluconazole sensitivity was only partly restored even after 159 subcultures (36).

In the present study we have directly examined the content and expression of gene *CYP51* in pretreatment (susceptible), posttreatment (resistant), and revertant (mostly susceptible) isolates of *C. glabrata* from the previous publication (36) and have used two-dimensional electrophoresis of cellular proteins in the three isolates to allow direct visualization of the complex changes in protein synthesis that accompany the azole-resistant phenotype.

MATERIALS AND METHODS

Strains. *C. glabrata* B57148 (originally isolated as 173/8) was isolated from the mid-stream urine of a 57-year-old female patient before treatment with intravenous ciprofloxacin and 400 mg of fluconazole once daily. Satisfactory levels of fluconazole were achieved in both the patient's blood (up to 44 mg liter^{-1}) bile (up to 58 mg liter^{-1}) and urine (up to 141 mg liter^{-1}). Strain B57149 (originally isolated as 2895) was isolated from the mid-stream urine of the same patient after 9 days of treatment (38). To study its phenotypic and genotypic stability, strain B57149 was repeatedly subcultured up to 159 times in CYG medium (casein hydrolysate [Merck, Darmstadt, Germany], yeast extract [Difco, Detroit, Mich.], glucose, each at a concentration of 5 g liter^{-1}). This isolate, with a revertant phenotype to azole susceptibility (36), was designated B57149-159. To ensure experimental reproducibility with strains demonstrating unstable phenotypes, stock suspensions in 10% glycerol were prepared from these cultures and stored at −70°C to provide sufficient starting material for each individual experiment to begin with the same inoculum. The isolates used in this study were genotypically typed and compared with at least five unrelated clinical *C. glabrata* isolates by restriction fragment length polymorphism (RFLP) analyses of genomic DNA with *Hin*dIII, *Hin*fI, and *Bgl*II as the restriction enzymes as described by Smith et al. (33). Arif et al. (1) reported that RFLP analysis with *Hin*fI was the most suitable typing technique for differentiation of *C. glabrata* isolates. To increase the probability of clonal relatedness, hybridization of *Eco*RI digests with a highly discriminatory oligonucleotide probe (a gift from D. Soll, University of Iowa) was performed and revealed again identical patterns for the B57148, B57149, and B57149-159 isolates used in this study. This pattern differed substantially from those found with 20 unrelated clinical *C. glabrata* isolates.

Media. *C. glabrata* strains were grown in either CYG medium or in PYG_2 medium (10 g of Polypeptone [Becton Dickinson, Sparks, Md.], 10 g of yeast extract [Difco], and 40 g of glucose liter^{-1}).

MIC determination. MICs were determined spectrophotometrically by a broth microdilution method (22) based on the National Committee for Clinical Laboratory Standards (NCCLS) reference method (19). The MIC was the lowest concentration that inhibited growth by more than 50%; this end point showed the best correlation with results from the NCCLS broth macrodilution method. Quality control yeasts *C. krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were tested in parallel and were inhibited at MICs in the correct ranges for the antifungals tested (19).

Drug uptake. Accumulation experiments with [^{3}H]itraconazole (specific activity, 75 mCi mmol^{-1}; Janssen Research Foundation, Beerse, Belgium) and [^{14}C]fluconazole (specific activity, 6.8 mCi mmol^{-1}; gift from Pfizer Central Research, Sandwich, United Kingdom) were performed as described previously (36).

Isolation of DNA. (i) Isolation of DNA for restriction analysis and Southern blotting. Stationary-phase cells (6×10^9) grown in CYG medium were collected by centrifugation at 1,500 × *g*, washed twice in TE_1 buffer (10 mM Tris, 1 mM EDTA [pH 7.5]), and resuspended in 6 ml of 0.9 M sorbitol–0.1 M EDTA–14 mM β-mercaptoethanol (pH 7.5). Zymolyase (180U; 5,000 U g^{-1}; *Arthrobacter lutens*; Seikagaku Kogyo, Tokyo, Japan) was added for spheroplast formation, and the mixture was incubated for 60 min at 30°C with mild agitation. For quantification of spheroplast formation, 50 μl of cell suspension and 950 μl of water (MilliQ-plus; Millipore, Bedford, Mass.) were combined and the absorbance at 600 nm was measured 20 s after the dilution. Typically, absorbance decreased by 80 to 90% after 60 min of incubation with Zymolyase. Spheroplasts were collected by centrifugation, suspended in 3 ml of TE_2 buffer (50 mM Tris, 50 mM EDTA [pH 8.0]), and lysed with the addition of 0.3 ml of 10% sodium dodecyl sulfate (SDS). After a 30-min incubation at 65°C proteins were precipitated by the addition of 1 ml of 5 M potassium acetate. After a 2-h incubation in ice, precipitated proteins were removed by 10 min of centrifugation at 15,000 × *g* at 4°C. The supernatant thus obtained was phenol extracted according to the protocols of Sambrook et al. (28). Nucleic acids were precipitated from the aqueous phase by the addition of 0.1 volumes of 3 M sodium acetate and 2 volumes of 100% ethanol (−20°C). The pellet was collected after centrifugation for 10 min at 10,000 × *g* at 4°C. The pellet was rinsed with 50% ethanol, air dried, and resuspended in 3.5 ml of TE_1 buffer, and 150 μl of DNase-free RNase (10 mg ml^{-1} in 10 mM sodium acetate [pH 7.0]) was added. After 30 min at 37°C insoluble material was precipitated by 10 min of centrifugation at 8,000 × *g* and the DNA in the supernatant was precipitated with 1 volume of isopropanol. The pellet was rinsed with 50% isopropanol and suspended in 500 μl of TE_1 buffer. The DNA concentration was determined in a spectrofluorimeter (excitation, 345 nm; emission, 460 nm) after addition of 2.5 ml of bisbenzimide (0.1 μg ml^{-1}). The signal thus obtained was linear for final DNA concentrations between 0.04 and 5 μg ml^{-1}.

(ii) Isolation of chromosomal-grade DNA. Intact chromosomes were isolated according to the method of Den Dunnen et al. (6). *C. glabrata* strains were grown to a density of up to 5×10^7 cells ml^{-1} in CYG medium. Fifty-milliliter aliquots of these cultures were centrifuged at 1,500 × *g* and resuspended at a concentration of 1.5×10^9 cells ml^{-1} in CPE buffer (40 mM citric acid, 120 mM Na_2HPO_4 [pH 6.0], with 2 ml of 0.8 M EDTA [pH 8.0] added per 50 ml). An equal volume of 2% low-melting-point agarose (Seaplaque GTG; FMC, Rockland, Maine) dissolved in CPE buffer at 60°C was added to the cell suspension, and the suspension was mixed gently and distributed immediately in small lots into the precooled DNA plug kit (Bio-Rad, Hercules, Calif.). The plugs were placed in the refrigerator for 1 h. The solidified plugs were incubated for 2 h at 30°C in 2 ml of CPE buffer containing NOVOZYM 234 (Novo Biolabs, Bagsvaerd, Denmark) (2 mg ml^{-1}). The plugs were washed twice in TE_1 buffer and incubated for 48 h at 50°C in 2 ml of 0.8 M EDTA containing 100 μg of pronase (20 mg ml^{-1}) and 100 μl of 20% Sarkosyl. Plugs were thoroughly washed four times with TE_1 buffer and were treated twice for 30 min at 50°C with 10 ml of TE_1 buffer containing 10 μl of 14 mM phenylmethylsulfonyl fluoride stock solution in (dimethyl sulfoxide). Plugs were stored at 4°C in 0.8 M EDTA, pH 8.0.

Isolation of RNA. To minimize degradation by RNases, all glassware used was baked for 8 h at 180°C and all solutions, if possible, were treated with 0.1% diethylpyrocarbonate and subsequently autoclaved. Samples of 10^{10} cells from 16-h CYG medium cultures of *C. glabrata* strains were used to inoculate 500-ml volumes of PYG_2 medium in 500-ml Erlenmeyer flasks equipped with water traps. Cultures were agitated for 6 h on a magnetic stirrer at 30°C. Cells (4×10^{10}) were collected by centrifugation and resuspended in 2 ml of GTC buffer (6 M guanidine thiocyanate, 5 mM sodium citrate · $2H_2O$, 0.1 M β-mercaptoethanol, 0.5% sodium lauryl-sarcosinate [pH 7.0]) in a 25-ml glass tube. Glass beads (0.45 mm in diameter) were added up to the meniscus. Cells were homogenized by vortexing 15 times at high speed for 20 s each with intermittent cooling on ice. The glass beads were washed four times with GTC buffer. The pooled homogenates were layered onto 28 ml of 5.7 M CsCl containing 10 mM EDTA in sterile polyallomer centrifuge tubes (Beckman, Palo Alto, Calif.). After 16 h of centrifugation at 135,000 × *g* at 20°C in a Beckman Optima L70 ultracentrifuge with a Beckman SW28 swing-out rotor, the RNA pellet was collected, washed with 70% ethanol, air-dried, and suspended in 100 μl of TE_1 buffer containing 0.1% SDS. RNA was dissolved by incubation at 65°C and intermittent vortexing. The RNA was stored at −70°C.

Electrophoresis. (i) DNA restriction digests. Isolated DNA was digested enzymatically according to the instructions of Boehringer. Denatured DNA digests were separated in 0.8% agarose NA (Pharmacia, Uppsala, Sweden) in 1× TBE buffer (10× TBE buffer is 0.45 M Tris, 0.44 M boric acid, and 10 mM EDTA [pH 8.0]) containing ethidium bromide (0.5 μg ml^{-1}) by applying an electric field of 1 V cm^{-1} overnight in a Bethesda Research Laboratories Horizon 11.14 submarine electrophoresis apparatus.

(ii) Pulsed-field electrophoresis. Chromosomes of *C. glabrata* were separated in 1% pulsed-field certified agarose in 0.5× TBE at 14°C with Bio-Rad contour-clamped homogeneous electric field (CHEF) mapper DRII. To separate the whole set of chromosomes at low resolution (100 to 1,800 kb), the separation variables were as follows: run time, 27 h 52 min at 6 V cm^{-1}; 120°; initial switch time, 12.55 s; and final switch time, 3 min 0.01 s. For increased resolution in the 300- to 700-kb range, the settings used were as follows: run-time, 35 h 14 min at 6 V cm^{-1}; 120°; initial switch time, 35.4 s; and final switch time, 1 min 3.8 s. Chromosomes were visualized by staining the gel for 30 min with 1 μg of ethidium bromide ml^{-1} in 0.5× TBE and subsequent destaining in MilliQ water. *S. cerevisiae* chromosomal markers (Bio-Rad) were used for size calibration.

(iii) RNA. RNA samples (6 μl) were denatured by the addition of 18 μl of denaturation solution containing, per milliliter, 640 μl of formamide, 230 μl of formaldehyde, and 130 μl of 10× running buffer (10× running buffer was 0.4 M MOPS [morpholine propanesulfonic acid], 0.1 M sodium acetate · $3H_2O$, 0.01 M EDTA [pH 7.2]) and 15 min of incubation at 60°C. Denatured RNA samples were immediately chilled on ice, 6 μl of dye solution (15% Ficoll 70, 0.25% bromophenol blue, 0.25% xylene cyanol) was added, and the mixture was separated overnight at 1 V cm^{-1} in a 1% agarose gel dissolved in 1× running buffer supplemented with 18% formaldehyde.

Southern blotting. Alkaline vacuum transfer was used to blot agarose-separated DNA restriction digests to nylon Zeta-probe membranes (Bio-Rad) according to the instructions of the manufacturer of the vacuum blot apparatus (Pharmacia). Pulsed-field-separated gels were capillary blotted for 72 h with 10× SSC (20× SSC is 3 M NaCl plus 300 mM sodium citrate) after 15 min of depurination in 0.2 M HCl, two 15-min periods of denaturation (0.5 M NaOH, 1.5 M NaCl), and two 15-min periods of neutralization (1.5 M NaCl, 0.5 M Tris-HCl, 1 mM EDTA [pH 7.2]). Membranes were baked at 80°C for 2 h to immobilize the DNA on the membranes.

Northern blotting. RNA formaldehyde agarose gels were blotted under 40 mbar of vacuum with 20× SSC buffer for 3 h and baked between filter paper sheets for 2 h at 80°C. RNA was visualized by staining with 0.02% methylene blue–0.3 M sodium acetate, pH 5.5, for 15 min, followed by a 10-min wash in diethylpyrocarbonate-treated MilliQ water.

Probes. (i) *CYP51* (*ERG11*; encoding cytochrome P-450 14α-lanosterol demethylase. A 24-mer antisense oligonucleotide, TAG CCA CCA GTT GTA TCA AGA CGA, was used to detect the cytochrome P-450 gene or its mRNA transcript. This sequence was conserved in *S. cerevisiae*, *Candida tropicalis*, and *C. albicans.*

(ii) *ACT1*. Actin, a 988-bp *Acc*I-*Ava*II fragment of pYactI (20) (obtained from J. L. Looper) containing the *S. cerevisiae ACT1* gene, was used as a probe.

(iii) *ERG1*. Squalene epoxidase, a 618-bp PCR-amplified *C. glabrata* fragment was used as a probe. PCR primers were designed from conserved regions from the published *ERG1* sequences for the following species: *S. cerevisiae* (11) *Rattus norvegicus* (27), and *Mus musculus* (13). The PCR forward primer was GTTG CTTTTGTTCATGGTAGATTC; the PCR reverse primer was CGACAGTCA TACCACCACCAGT. PCR conditions were as follows: 30 cycles; annealing at 52°C for 1 min, elongation at 72°C for 2 min, and denaturation at 92°C for 1 min. The 618-bp amplification product was sequenced by the dideoxy-dye terminator method with an ABI 373A sequencer from Applied Biosystems (14). The sequence is given in Fig. 7.

Probe labeling. The oligonucleotide was end labeled with [γ-^{32}P]ATP (ICN-Flow, Costa Mesa, Calif.) by using the Ready-To-Go T4 polynucleotide kinase kit (Pharmacia) according to the instructions of the manufacturer. Other probes were randomly labeled with [α-^{32}P]dCTP (ICN-Flow), also by using the Ready-To-Go random labeling kit (Pharmacia).

Hybridization. Membranes were prehybridized for 1 h in 50 ml of Denhardt's solution (3× SSC, 1% SDS, 5× Denhardt [50× Denhardt is 1% {wt/vol} Ficoll 400, 1% {wt/vol} polyvinylpyrrolidone, 1% {wt/vol} bovine serum albumin], 0.1 mg of salmon testis DNA ml^{-1}) and hybridized overnight at 45°C for oligonucleotide labeling or at 65°C for the random-labeled probes (28). After hybridization, membranes were rinsed once with 2× SSC and washed once for 15 min with 2× SSC–1% SDS at room temperature and three times for 10 min with 0.2× SSC–1% SDS at the hybridization temperature. The damp membrane was sealed in a plastic bag, and radioactive fractions were visualized on Reflection X-ray films (Dupont NEN, Bad Hamburg, Germany) with an intensifying screen exposed at −70°C. The radioactive markings on the film were digitized with a video camera and quantified with Image 1.41 software (36).

Labeled probes were stripped from the membranes by boiling for 15 to 30 min in 0.1× SSC–1% SDS. The process was validated by exposing the stripped membranes again to an autoradiography film.

Protein labeling and two-dimensional protein separation. Exponentially growing *C. glabrata* cells (3× 10^6 cells) were labeled for 2 h in 333 μl of methionine-deficient Dulbecco's modified essential growth medium supplemented with 333 μCi of [^{35}S]methionine (Dupont NEN; specific activity, 1,175 Ci $mmol^{-1}$). Labeled cells were collected by centrifugation, washed in MilliQ water, recentrifuged, decanted, and immediately frozen in liquid nitrogen. Samples were stored at −70°C until further processing.

Frozen pellets were thawed in 120 μl of lysis buffer (9.5 M urea, 2% [wt/vol] Nonidet P-40 [NP-40], 2% [wt/vol] ampholines [pH range 7 to 9; Pharmacia], 5% [wt/vol] 2-mercaptoethanol) and sonicated in an ice-ethanol cooling bath for three 5-s intervals with 30-s cooling intervals between. Samples were then shaken at room temperature for a minimum of 4 h. The degree of [^{35}S]methionine incorporation was then determined.

Polyacrylamide gels (3.5%, 185 by 1.55 mm, 8 M urea, 2% NP-40) containing for the IEF (isoelectric focusing) gels (2% ampholines at a pH range of 3.5 to 10, 2% ampholines at a pH range of 5 to 7, and 2% servalytes at a pH range of 5 to 7 [Serva, Heidelberg, Germany]). Because considerable variation was found among different batches of ampholytes, the actual mixture of ampholytes was calibrated. Phosphoric acid (10 mM) was used as the anode buffer and degassed NaOH (20 mM) was used as the cathode. IEF gels were prefocused at 1,200 V and 133 μA (limiting values) per tube until the limiting values were reached. Then, 5 × 10^5 trichloroacetic acid-precipitable dpm was applied to the IEF gels, and samples were overlaid with overlay buffer (8 M urea, 0.8% ampholines at a pH range of 5 to 7, and 0.2% ampholines at a pH range of 3.5 to 10). Electrophoresis was carried out at 1,200 V and 133 μA (limiting per gel) for 18 h. The gels were then extruded with compressed air and equilibrated for 6 min before being frozen at −70°C until needed. For the second-dimension gel electrophoresis, gels were thawed quickly in a water bath (80°C), incubated for 2 min at room temperature, and loaded onto 12.5% polyacrylamide gels (200 by 200 by 1 mm) at 20°C. The gels were run in slowly (5 mA per gel for 1 h), followed by 15 mA per gel until the bromophenol blue line reached the bottom of the slab gel. The gels were fixed for 45 min in 45% methanol–7.5% acetic acid, and then they were treated for 45 min in Amplify (Amersham, Bucks, England) to enhance the fluorographic detection, dried, and exposed to X-ray film (type XB-200; X-Omat, United Kingdom) at −70 C for 5 days. The developed images were then compared either visually or with an image-processing computer by using the Bio Image 2D program, version 6.03, on a Sun workstation. Molecular weight and isoelectric point values were assigned with reference to known *S. cerevisiae* proteins.

Nucleotide sequence accession number. The GenBank entry for the *C. glabrata* squalene epoxidase fragment is AF006033.

TABLE 1. Effects of azole antifungal agents, 5-flucytosine, terbinafine, and amorolfine on growth of *C. glabrata* isolates[a]

Drug	IC_{50}[b] (μg · ml^{-1}) for:				
	Pre-treatment B57148	Post-treatment B57149	B57149-73	B57149-111	Revertant B57149-159
5-Flucytosine	≤0.13	≤0.13	<0.13	<0.13	≤0.13
Fluconazole	16–32	256–512	32–256	64	32–64
Ketoconazole	0.5–1	8–16	1–4	1–2	0.5–2.0
Itraconazole	1.0	4–16	1.0	1.0	0.5–1.0
Terbinafine	>16	>16	>16	>16	>16
Amorolfine	2–4	>16	8	8	4–8

[a] B57149-73, B57149-111, and B57149-159 are, respectively, the 73th, 111th, and 159th subcultures of B57149 in fluconazole-free medium.
[b] Data are the ranges of quadruplicate tests with the isolates.

RESULTS

Effects of antifungal agents on growth. Fluconazole-resistant *C. glabrata* isolate B57149 was cross-resistant to other azole antifungals that are inhibitors of the cytochrome P-450 14α-lanosterol demethylase reaction in ergosterol biosynthesis and to amorolfine, an inhibitor of the Δ14-reductase and/or Δ8-7-isomerase (23), but not to 5-flucytosine, a compound inhibiting cellular DNA and RNA synthesis. Repetitive subculturing of the resistant *C. glabrata* isolate in fluconazole-free medium was shown to be sufficient to fully restore susceptibility to itraconazole both in CYG medium, as described previously (36), and in RPMI medium (Table 1). However, despite 159 subcultures, the revertant still exhibited resistances to fluconazole, ketoconazole, and amorolfine that were higher than those of the pretreatment isolate by one or two dilution levels. The maintenance of some resistance is more pronounced in the shaken-CYG culture conditions used in the previous paper (36) than in those reported in this paper, which involved RPMI medium. By the broth microdilution method, all *C. glabrata* isolates were found to be insensitive to terbinafine, which is in accordance with the reported MIC values (>128 μg · ml^{-1}) for this inhibitor for this species (18).

Intracellular drug level. Under aerobic conditions eight times more itraconazole (576 pmol/10^9 cells) than fluconazole (72 pmol/10^9 cells) was found in B57148 cells (Table 2). In the resistant isolate, this ratio was even higher (14-fold; itraconazole: 490 pmol/10^9 cells; fluconazole: 36 pmol/10^9 cells). In the 159th subculture, a similar high value (13-fold; itraconazole: 480 pmol/10^9 cells; fluconazole: 38 pmol/10^9 cells) was found. Both the intracellular contents of fluconazole and itraconazole exhibited a statistically significant decrease in the

TABLE 2. Cellular contents[a] of fluconazole and itraconazole in *C. glabrata* isolates B57148, B57149, and B57149-159[b]

Drug	Cellular content (pmol/10^9 cells) in:		
	Pretreatment B57148	Posttreatment B57149	Revertant B57149-159
Fluconazole	72 ± 2[c]	36 ± 1	38 ± 2
Itraconazole	570 ± 8[c]	490 ± 11	480 ± 4

[a] Cells were first grown for 4 h under aerobic conditions (CYG medium) and were then incubated for 1 h in the presence of 0.3 μM [^{3}H]itraconazole or [^{14}C]fluconazole. Results are averages ± standard deviations for at least three experiments.

[b] B57149-159 is the 159th subculture of B57149 in fluconazole-free medium.

[c] Statistically significant difference ($P < 0.05$; Mann-Whitney U test) relative to the posttreatment values.

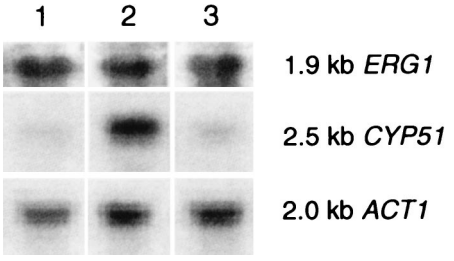

FIG. 2. Northern blots of total RNA isolated from *C. glabrata* isolates B57148 (1), B57149 (2), and B57149-159 (3). RNA was purified by means of CsCl gradient centrifugation as described in Materials and Methods. Five micrograms of RNA was loaded for electrophoresis. After vacuum transfer to a Zeta-probe membrane, the blot was stained with methylene blue to verify the quality of the RNA preparation and equal loading. Hybridization, stripping, visualization, and quantification were as described in the legend for Fig. 1.

resistant isolate. However, the fluconazole content dropped to 50% of that of the sensitive isolate, whereas for itraconazole only a 15% decrease was found. The intracellular contents for both itraconazole and fluconazole did not change significantly with repeated subcultivation.

Molecular biological approach. The increased cytochrome P-450 and squalene epoxidase and/or oxidosqualene cyclase activities in B57149 described in a previous paper (36) could be due to increased copy numbers of the genes coding for these enzymes. To compare the copy numbers of the different *C. glabrata* isolates, genomic DNA was restricted with *Hin*dIII, separated by electrophoresis, transferred to nylon membranes, and hybridized with appropriate probes. As a reference probe the *ACT1* gene, encoding actin, was chosen. This probe revealed two bands, at 6.3 and 3.5 kb, in the Southern blot (Fig. 1) because the probe contained an internal *Hin*dIII restriction site, which is conserved in the unknown *C. glabrata* sequence. No differences were seen in intensities of the hybridization signals for the three isolates of *C. glabrata*, B57148, B57149, and B57149-159, with the *ACT1* probe relative to that of the ethidium bromide signal, suggesting that the copy number of the *ACT1* gene was constant. However, substantial differences were seen among DNA samples from the three isolates with respect to the hybridization signals obtained with the P-450 oligonucleotide probe. Indeed, for the B57148 isolate a *CYP51*/*ACT1* band intensity ratio of 0.07 was found. This ratio increased to 0.26 in the resistant B57149 strain, an increase of 3.7-fold. For the revertant isolate, B57149-159, the ratio was 0.06, similar to that found for the B57148 isolate. Similar ratios were found when different amounts of DNA were loaded on the gel (results not shown). The *CYP51* probe hybridized to a single 6.7-kb fragment in all three isolates. Southern blots of DNA digested with restriction enzymes other than *Hin*dIII also showed single hybridization bands, indicative of amplification of a fairly large fragment. With the *ERG1* probe, a completely different result was obtained. The band intensities, and thus, presumably, the relative copy numbers of the gene encoding the squalene epoxidase, were almost identical for all three isolates. Due to uneven loading of the lanes in the gel, lack of overexpression of the *ERG1* gene was substantiated by the *ERG1*/*ACT1* ratio data. For the B57148, B57149, and B57149-159 isolates *ERG1*/*ACT1* intensity ratios found were, respectively, 0.07, 0.07, and 0.05. To rule out the possibility that the B57148 or B57149 isolates were polyclonal, the *CYP51* copy numbers of single colonies (six for each isolate) were determined. Variation in the copy number was less than 10%.

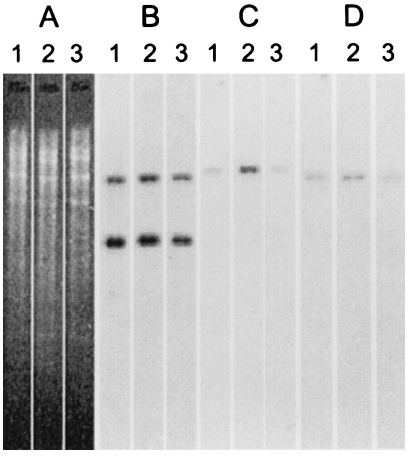

FIG. 1. (A) Ethidium bromide-stained *Hin*dIII restriction patterns of genomic DNA isolated from *C. glabrata* isolates B57148 (lane 1), B57149 (lane 2), and B57149-159 (lane 3). Two micrograms of DNA was digested and separated in 0.8% agarose. (B to D) Southern blots of vacuum-transferred digests, sequentially probed with [^{32}P]-labeled *ACT* (B), *CYP51* oligonucleotide probe (C), and *ERG1* probe (D). Between the different hybridizations, the membrane was stripped in boiling 0.1× SSC–1% SDS. Radioactive fractions were visualized by autoradiography at −70°C and quantitated by densitometry.

Greater differences were observed at the transcription level (Fig. 2). Five micrograms of total RNA was sufficient to obtain a clear hybridization signal with all probes used. A constant *ACT1* hybridization signal was seen for all three isolates tested, which allowed us to use it as a reference. The *CYP51*/*ACT1* hybridization intensity ratio for the *C. glabrata* B57148 isolate (0.04) was eight times lower than that found in the B57149 isolate (0.3) and almost two times lower than the value for the B57149-159 (0.08) isolate. Again, similar values were found when other quantities of RNA were loaded on the gel (results not shown). With the *ERG1* probe, a constant level of transcription in all three isolates was observed. For the B57148, B57149, and B57149-159 isolates *ERG1*/*ACT1* intensity ratios found were, respectively, 0.04, 0.04, and 0.03.

The increase in copy number of *CYP51* in the resistant B57149 isolate could be due to an amplification of a large fragment. This should increase the size of a chromosome sufficiently to induce a visible shift in the mobility of the chromosome involved after a chromosomal separation. In Fig. 3 the results for the CHEF-separated chromosomes of the three *C. glabrata* isolates are given. By comparison with size markers, it was found that the sizes of the different chromosomes correlated very well with those published by Doi et al. (7). In their publication they hypothesized that *C. glabrata* contained 12 chromosomes with lengths of 2,100, 1,720, 1,420, 1,380, 1,150,

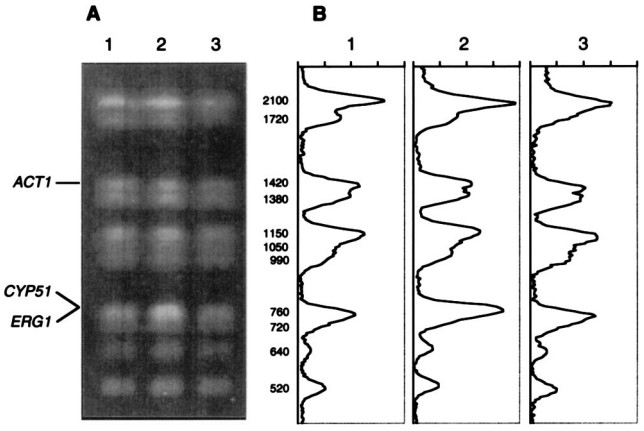

FIG. 3. (A) Ethidium bromide staining of CHEF-separated chromosomal DNA of *C. glabrata* isolates B57148 (lane 1), B57149 (lane 2), and B57149-159 (lane 3). CHEF electrophoresis was run under the following conditions: run time, 27 h 52 min; 6 V cm^{-1}; 120°; initial switch time, 12.55 s; and final switch time, 3 min 0.01 s. At the left side the locations where the [^{32}P]-labeled *CYP51* oligonucleotide probe, the *ACT1* probe, and the *ERG1* probe hybridized to a Southern blot of capillary-transferred chromosomes are given. Hybridization, stripping, visualization, and quantification were as described in the legend for Fig. 1. At the right side the estimated sizes of the chromosomes are given. Estimates were calculated from *S. cerevisiae* chromosomal size markers. (B) Intensity profiles of the ethidium bromide-stained chromosomes shown in panel A. Intensities were normalized on the 1,420-kb chromosome. The profile numbers correspond to the lane numbers in panel A.

1,050, 990, 760, 720, 640, 520, and 510 kb. In the present study the chromosomal patterns for all three *C. glabrata* isolates were found to be identical. No major shift in mobility was observed, but the 760- to 720-kb region of the B57149 isolate showed increased ethidium bromide staining. It is generally accepted that the ethidium bromide staining intensity of a band is proportional to its DNA content and the size of the band. In Fig. 3B the ethidium bromide staining intensities, normalized on the 1,420-kb chromosome, are given for the three isolates. A small increase in intensity is seen for the largest chromosome, which contains the RNA coding sequences. A more substantial increase is seen in the 760- to 720-kb region, indicating an increase in the copy number of at least one chromosome in that region. Further confirmation of this hypothesis was found with hybridization experiments. The *ACT1* probe hybridized to the 1,420-kb band, and both the *CYP51* and the *ERG1* probes hybridized with the 760- to 720-kb region. When the signal ratios for each isolate relative to *ACT1* were determined, again only that of *CYP51* was increased for the resistant B57149 isolate. No such increase was seen with the *ERG1* probe, a finding in complete agreement with that concerning the hybridization signals of the restriction digests. The fact that both probes hybridized to the same region is consistent with the chromosomal amplification theory only if this region contained more than one chromosome and the *ERG1* and *CYP51* genes are located on different chromosomes. To verify this hypothesis, a higher-resolution separation in the region was performed. As shown in Fig. 4, the 760- to 720-kb region contained not two but three chromosomes, and from the hybridization experiments with *ERG1* and *CYP51* probes, it could be concluded that *ERG1* is located on the 720-kb chromosome, whereas *CYP51* is located on the 740-kb chromosome, the chromosome that showed increased ethidium bromide staining. In Fig. 5 the normalized intensity ratios relative to the *ACT1* signal obtained in chromosomal blots are given for the B57148 and B57149 isolates and also for several subcultures of the B57149 isolate. Again, B57149 contained almost four times more *CYP51* than the B57148 isolate, a value which correlates with the amplification ratio found in the restriction digests and Southern blots. A gradual loss of amplification was

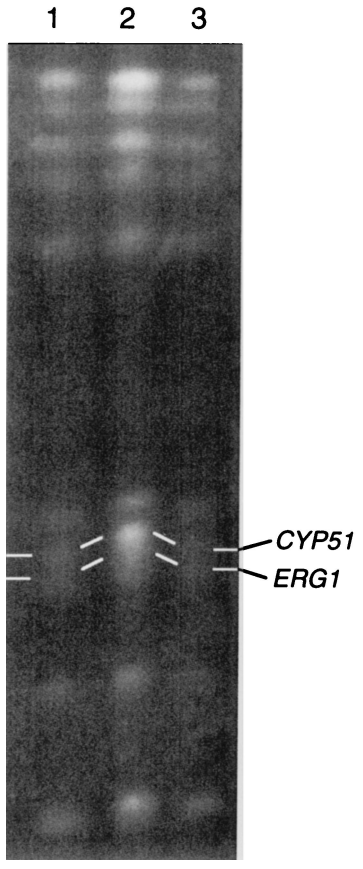

FIG. 4. Ethidium bromide staining of CHEF-separated chromosomal DNA of *C. glabrata* isolates B57148 (lane 1), B57149 (lane 2), and B57149-159 (lane 3). Higher-resolution CHEF electrophoresis was run under the following conditions: run time, 35 h 14 min; 6 V cm^{-1}; 120°; initial switch time, 35.4 s; and final switch time, 1 min 3.8 s. At the right side the location where the [^{32}P]-labeled *CYP51* oligonucleotide probe and *ERG1* probe hybridized to a Southern blot of capillary-transferred chromosomes is given. Hybridization, stripping, visualization, and quantification were as described in the legend for Fig. 1.

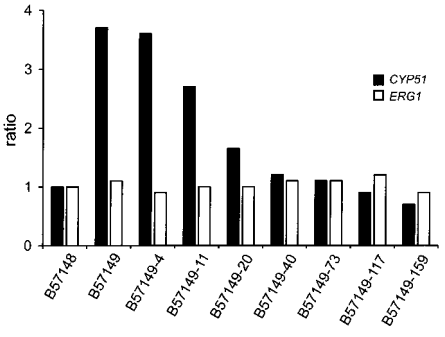

FIG. 5. Ratios of the intensities of the hybridization signals obtained with the *CYP51* probe and the *ERG1* probe relative to the signal of the *ACT1* probe obtained with the CHEF-separated chromosomal DNA isolated from *C. glabrata* isolates B57148, B57149, and B57149 after 4, 11, 20, 40, 73, 117, and 159 subcultures. To visualize the amplification of the hybridization signals relative to the *ACT1* signal, normalization was performed such that all ratio data for isolate B57148 equals one; all other ratios are reported relative to this standard.

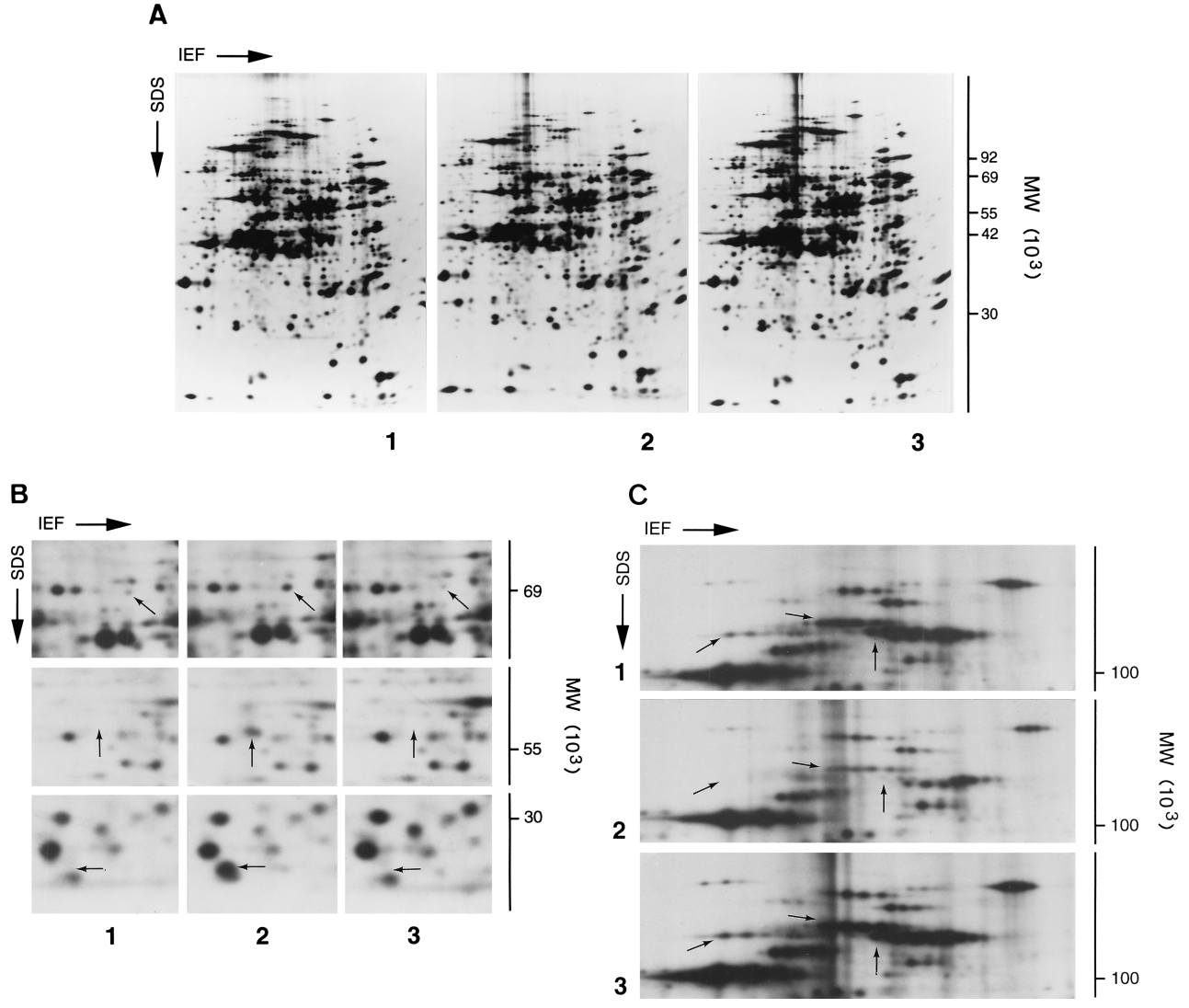

FIG. 6. (A) Two-dimensional IEF–SDS-polyacrylamide gel electrophoresis gels of proteins extracted from *C. glabrata* isolates B57148 (1), B57149 (2), and B57149-159 (3). (B to C) Higher magnification of selected regions of pictures shown in panel A. Arrows indicate proteins that were up- or downregulated in the B57149 isolate, as indicated by their intensities relative to their intensities in both the B57148 and B57149-159 isolates. MW, molecular weight.

seen upon repeated subculture of B57149 in fluconazole-free medium. After 40 subcultures almost the same value was found as for the B57148 isolate. This correlates with the gradual decrease of microsomal cytochrome P-450 content and the regained susceptibility of these isolates as described in Table 1 and in the previous paper (36). The signal of *ERG1* remained constant for all isolates tested.

The amplification of an entire chromosome would be expected to have tremendous effects on the protein expression in the cells. Indeed, when [^{35}S]methionine-labeled proteins were extracted from these *C. glabrata* strains and separated by two-dimensional gel electrophoresis, out of 1,377 proteins identified, 25 were upregulated by more than a factor of three and 76 were downregulated by more than a factor of three (Fig. 6). Figures 6B and 6C show selected regions of the pictures shown in Fig. 6A at a higher magnification to demonstrate the up- (Fig. 6B) or downregulation (Fig. 6C) of selected proteins in the resistant isolate. These figures demonstrate that the observed chromosome duplication resulted in pleotropic effects on other genes in addition to the CYP51 gene and that the disappearance of the differences in proteins expressed correlated with reversion to the nonduplicated state, as would be expected for mutations involving chromosome duplication. Experiments are ongoing to identify the upregulated products to verify if their coding genes also map to this amplified chromosome.

DISCUSSION

Clonal relatedness of clinical isolates is an important topic in both epidemiologic and resistance studies. Although it is almost impossible to obtain 100% proof of clonal relatedness, one can increase the probability that a statement of clonal relatedness is true by applying different available highly discriminatory DNA typing techniques such as Southern blot hybridization with probes, RFLP, random amplified polymorphic DNA analysis, or karyotyping. The *C. glabrata* isolates used in this study, B57148, B57149 and B57149-159, were classified into a single group by both RFLP and Southern blot hybridization. No single other *C. glabrata* isolate tested fell into the

same category. In the chromosomal CHEF separations shown in Fig. 3, no differences in size or number of resolved chromosomes were seen among the three isolates, although increased ethidium bromide staining for some of the chromosomes was observed. Apart from this increased ethidium bromide staining, which is hypothesized to be at least partly the cause of the resistance, no evidence was found to support the hypothesis that these strains are not clonally related.

A number of reviews have been written on the mechanism of resistance to azoles (10, 15, 21, 37). Probably the most frequent cause of resistance to azoles is a decreased intracellular level of the azole. Recent reports suggest that this phenomenon results from the presence or induction of proteins that transport the azoles out of the cell. Active export could be driven by ATP hydrolysis, as in the case of the ATP-binding cassette class of proteins, or by other energy sources such as those for the major facilitators. Such an energy-dependent efflux system for fluconazole has indeed been described for a fluconazole-resistant *C. glabrata* strain (5). In the *C. glabrata* isolates used in this study, similar impaired accumulation of the azole could contribute significantly to fluconazole resistance and only marginally to the cross-resistance observed with ketoconazole and itraconazole, because only small differences in intracellular levels of these drugs between the sensitive and resistant isolates were found (reference 36 and table II). It could be hypothesized that a transporter with high affinity for fluconazole and low affinity for the other two azoles is expressed in both the B57149 and the B57149-159 isolates and that this limits the intracellular fluconazole level. Another explanation would be the expression of a nonselective transporter with a limited capacity that will only be of significance to those azoles with limited intracellular contents.

Another mechanism by which yeasts can develop resistance is a mutation in the target enzyme, which in the case of the azoles is CYP_{51} (14α-lanosterol demethylase). Because in a previous study it was shown that the azole sensitivities of the isolated enzymes of the sensitive and the resistant isolates were the same, no attempts were made to make sequence comparisons between the *CYP51* genes from the two yeasts. Overproduction of P-450 is a third possibility for induction of resistance. Until now only evidence from recombinant studies was available to demonstrate this (3). The up to threefold-higher *CYP51* mRNA levels found in clinical azole-resistant *C. albicans* isolates support this hypothesis (29).

In the present study we have demonstrated that duplication of the chromosome containing the *CYP51* gene is at least a partial cause of the resistance of the posttreatment isolate. This rare phenomenon could explain the unusually fast development of resistance after only 9 days of combination therapy with 400 mg of fluconazole and intravenous ciprofloxacin. Indeed, until now most cases of resistance developed after administration of suboptimal doses of azole over a long period of time. The chromosomal duplication itself could be due to a defect in the separation process of the chromosomes during cellular division. Whether the clinical treatment or other environmental parameters interfered with the separation process itself or provided the ideal selection environment for the mutated cell is still unknown. It is known that *C. glabrata* accomplishes amplification of genes as a response to environmental stress conditions; e.g., the metallothionein genes are amplified after exposure to copper (34, 39). In *S. cerevisiae* the *CUP1* gene, coding for a metallothionein, is located on the same chromosome as *CYP51* (*ERG11*) (17). In this way the *CYP51*-containing chromosome could be prone to amplification upon exposure of cells to environmental copper stress conditions. However only small differences in copper sensitivity were

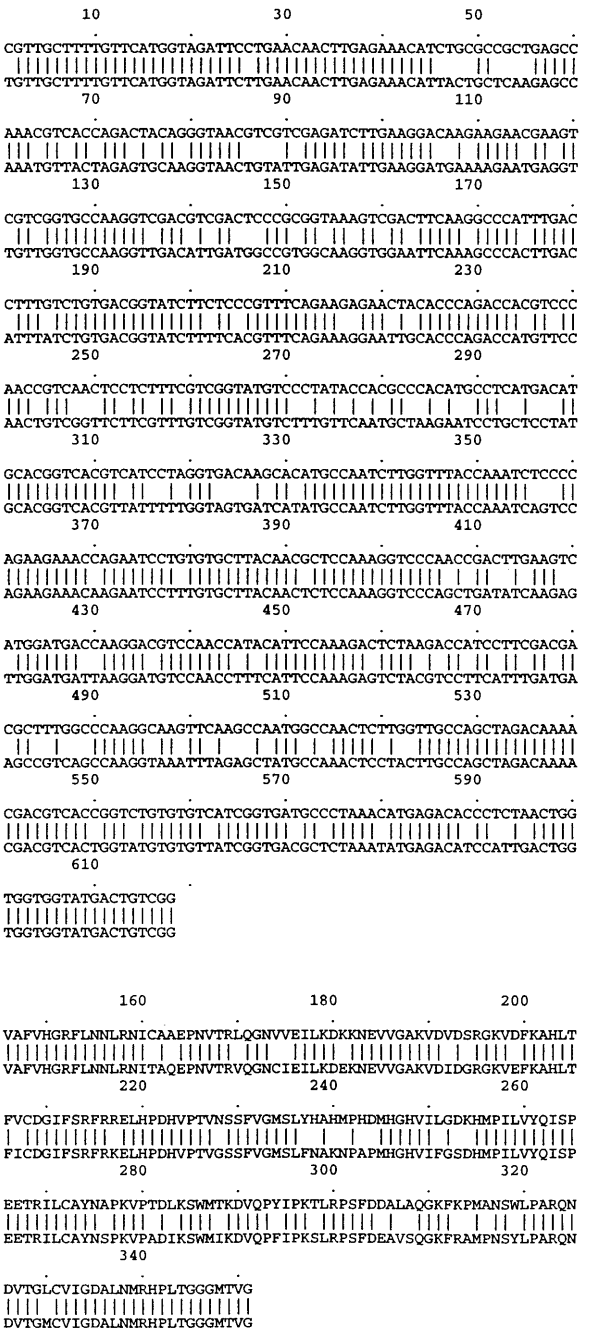

FIG. 7. Nucleotide and amino acid sequence alignment of the 618-bp PCR product amplified from *C. glabrata* DNA with the sequence of the *S. cerevisiae ERG1* gene (10). The sequence of the probe was determined by using Dideoxy-Dye Terminator chemistry on the ABI 373A sequencer from Applied Biosystems. Upper sequence, squalene epoxidase gene from *C. glabrata*; lower sequence, squalene epoxidase gene from *S. cerevisiae.*

found among the azole-sensitive (50% inhibitory concentration [IC_{50}], 0.3 mg · ml^{-1}), azole-resistant (IC_{50}, 0.5 mg · ml^{-1}), and revertant (IC_{50}, 0.5 mg · ml^{-1}) *C. glabrata* isolates.

Other still-unanswered questions are why the posttreatment isolate is cross-resistant to other nonazole ergosterol biosynthesis inhibitors such as amorolfine and why *C. glabrata* lacks susceptibility to terbinafine. In a previous study it was shown that in a subcellular ergosterol biosynthesis system using *C. gla-*

brata B57148 protein, terbinafine inhibited ergosterol formation by 50% at 5×10^{-8} M (36). This is twice the concentration needed by itraconazole to induce the same effect. In that study it was also shown that 3.3 times more fluconazole than itraconazole was needed to inhibit the cytochrome P-450 in a microsomal fraction (IC_{50}s were 112 nM for fluconazole versus 34 nM for itraconazole). However in intact cells much higher concentrations of fluconazole are needed to inhibit growth. Terbinafine has no effect, even up to 16 $\mu g \cdot ml^{-1}$. It is possible that a lower intracellular content contributes to this phenomenon. It has to be examined whether constitutive expression of a major facilitator such as *CaMDR1*, which takes both fluconazole and terbinafine, but not itraconazole, as a substrate (31), contributes. Four-times-higher concentrations of terbinafine (IC_{50}, 2×10^{-7} M) were needed to inhibit ergosterol synthesis in the B57149 isolate, and in this strain 2.2 times more lanosterol was formed from squalene, indicating doubled squalene epoxidase and/or oxidosqualene cyclase activity (36). No *ERG1* gene amplification in the resistant isolate was found. Furthermore, it was found that this gene is located on a different chromosome. The likelihood that the *C. glabrata ERG1* probe, which was PCR amplified with conserved-region primers, was not coding for the squalene epoxidase is small, because of the high sequence homology with the published *S. cerevisiae ERG1* sequence (11). Indeed, in Fig. 7 the best-fit alignment is given between the *C. glabrata* PCR amplification product and *ERG1sc*. The numbering used is that of the *Saccharomyces* sequence. Over the 618-bp segment an identity of 77% at the nucleotide level and of 83% at the amino acid level was found. It is possible that higher levels of NADPH-cytochrome P-450 reductase, a coenzyme in the squalene epoxidase reaction, contributed to the terbinafine resistance, but no increased activity of this enzyme was measured in the resistant isolate (36).

Another likely explanation for the shift in the subcellular sensitivity of ergosterol synthesis to terbinafine is an affinity alteration of the squalene epoxidase enzyme by specific mutations in the resistant isolate. More underlying mechanisms of resistance could perhaps be elucidated from the identification of the 25 strongly upregulated and 76 downregulated proteins in the two-dimensional gel analysis. Knowing the identities of other expressed genes located on the amplified chromosome could also contribute to the unravelling of the resistance enigma.

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