

## *Candida albicans* Mutations in the Ergosterol Biosynthetic Pathway and Resistance to Several Antifungal Agents

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The role of sterol mutations in the resistance of *Candida albicans* to antifungal agents has not been thoroughly investigated. Previous work reported that clinical *C. albicans* strains resistant to both azole antifungals and amphotericin B were defective in *ERG3*, a gene encoding sterol  $\Delta^{5,6}$ -desaturase. It is also believed that a deletion of the lanosterol 14 $\alpha$ -demethylase gene, *ERG11*, is possible only under aerobic conditions when *ERG3* is not functional. We tested these hypotheses by creating mutants by targeted deletion of the *ERG3* and *ERG11* genes and subjecting those mutants to antifungal susceptibility testing and sterol analysis. The homozygous *erg3/erg3* mutant created, DSY1751, was resistant to azole derivatives, as expected. This mutant was, however, slightly more susceptible to amphotericin B than the parent wild type. It was possible to generate *erg11/erg11* mutants in the DSY1751 background but also, surprisingly, in the background of a wild-type isolate with functional *ERG3* alleles under aerobic conditions. This mutant (DSY1769) was obtained by exposure of an *ERG11/erg11* heterozygous strain in a medium containing 10  $\mu$ g of amphotericin B per ml. Amphotericin B-resistant strains were obtained only from *ERG11/erg11* heterozygotes at a frequency of approximately  $5 \times 10^{-5}$  to  $7 \times 10^{-5}$ , which was consistent with mitotic recombination between the first disrupted *erg11* allele and the other remaining functional *ERG11* allele. DSY1769 was also resistant to azole derivatives. The main sterol fraction in DSY1769 contained lanosterol and eburicol. These studies showed that *erg11/erg11* mutants of a *C. albicans* strain harboring a defective *erg11* allele can be obtained in vitro in the presence of amphotericin B. Amphotericin B-resistant strains could therefore be selected by similar mechanisms during antifungal therapy.

*Candida albicans* can cause fungal diseases in immunocompromised patients, including cancer patients, transplant patients, and those with human immunodeficiency virus infections (6). The antifungal agents that are available for the treatment of *C. albicans* infections can be categorized into several chemical classes with different cellular targets. Enzymes of the ergosterol biosynthetic pathway are important targets of several classes of antifungals used to treat *C. albicans* infections, and among those, the polyenes and the azoles have a dominant position. Polyenes such as amphotericin B act at the level of ergosterol by binding tightly to this molecule. This effect damages the cell plasma membrane, thus resulting in leakage of intracellular ions. Azoles such as fluconazole, itraconazole, or voriconazole inhibit a cytochrome P450 (Erg11p) responsible for the 14 $\alpha$  demethylation of lanosterol and thus block ergosterol biosynthesis. In the late steps of this ergosterol biosynthesis pathway, azoles inhibit also the  $\Delta^{22}$  desaturation of the sterol moiety (Fig. 1). Other antifungal classes less relevant for the treatment of *C. albicans* infections, i.e., allylamines (terbinafine) and morpholines (amorolfine), inhibit ergosterol biosynthesis by blocking the activity of squalene epoxidase and sterol  $\Delta^{14}$ -reductase or  $\Delta^{7-8}$ -isomerase, respectively. Several mechanisms have been documented to be involved in the resistance to the azole and polyene antifungal

classes in *C. albicans*. They include active efflux of the antifungals, target enzyme alterations, and the absence of the target enzyme (for amphotericin B resistance) (27, 28, 31, 34). The development of compensatory pathways that circumvent the inhibitory effects of antifungals may be another mechanism for resistance to antifungals (19). Compensatory pathways have been documented for the mechanisms of resistance to the azole and polyene classes and involve alterations of specific steps in ergosterol biosynthesis. For example, analysis of the sterol compositions of two separate azole-resistant *C. albicans* clinical isolates revealed the accumulation of ergosta-7,22-dienol, which is a feature consistent with the absence of sterol  $\Delta^{5,6}$ -desaturase activity, which is encoded by *ERG3* (17, 18, 25). Azole resistance in these two cases was coupled with resistance to amphotericin B because of the absence of ergosterol in these cells. In *Saccharomyces cerevisiae*, mutants with mutations in *ERG4* (35), *ERG6* (7), and *ERG3* (1) are also devoid of ergosterol and are resistant to polyene agents. The role of *ERG3* in azole resistance originates from the observation that treatment of yeasts with azoles results in the accumulation of 14 $\alpha$ -methylated sterols and 14 $\alpha$ -methylergosta-8,24(28)-dien-3,6-diol. Formation of the latter sterol metabolite is thought to be catalyzed by sterol  $\Delta^{5,6}$ -desaturase; thus, inactivation of *ERG3* can suppress toxicity and therefore causes azole resistance (19). In *S. cerevisiae* mutations of the *ERG3* gene can result in azole resistance (33). However, inactivation of *ERG3* does not always result in azole resistance: in *Candida glabrata*, a null mutation in *ERG3* does not result in azole resistance (8). Another mutation potentially linked to azole resistance is the

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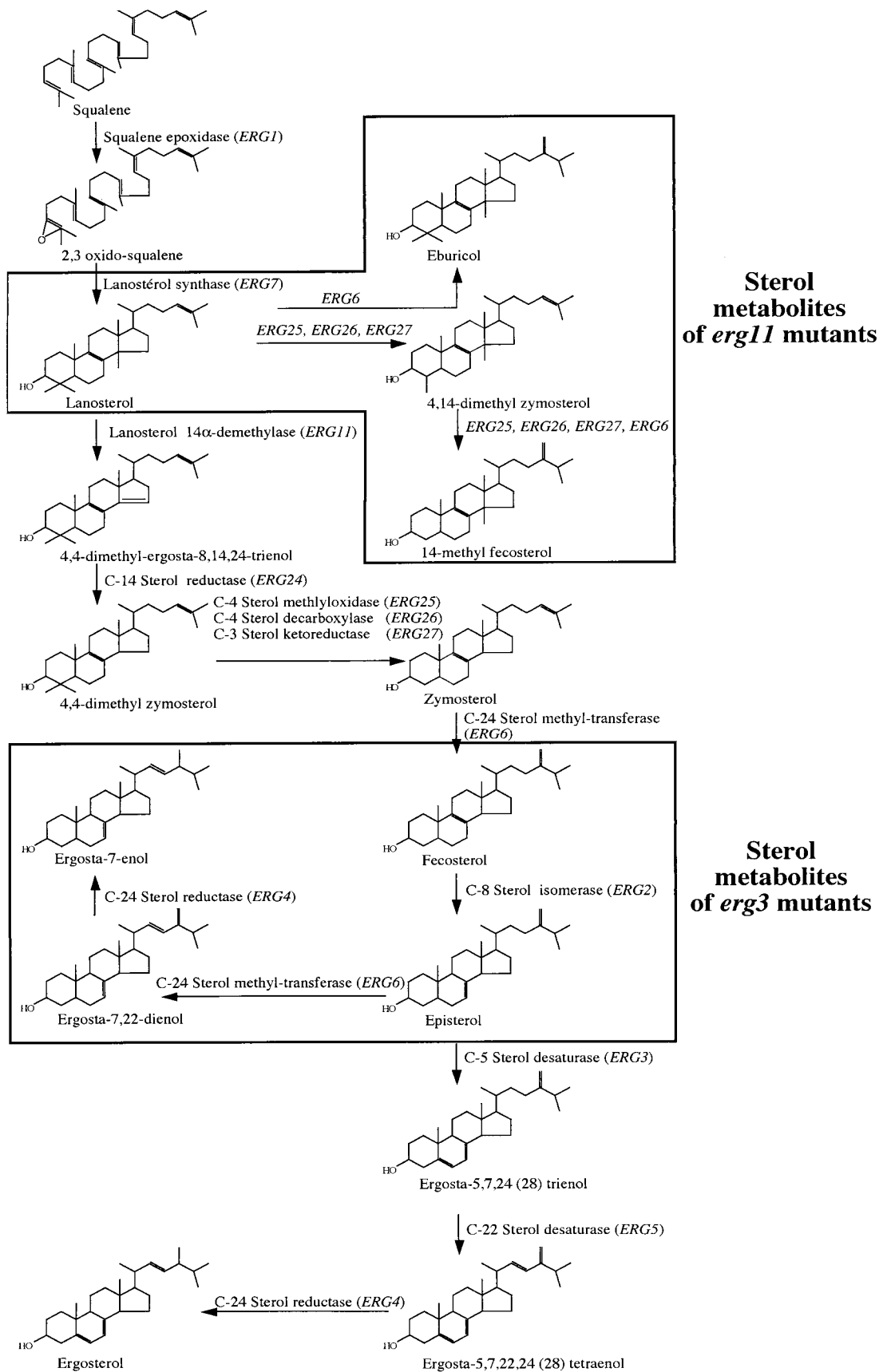


FIG. 1. Schematic representation of the ergosterol biosynthetic pathway in *C. albicans*. Blocking of the functions of *ERG11* and *ERG3* results in the accumulation of the boxed metabolites. A detailed description of this pathway is also given by Parks et al. (26).

TABLE 1. Genotypes of *C. albicans* mutants used in this study

Strain	Parent strain	Genotype	Reference
CAF4-2	CAF2-1	<i>ura3Δ::imm434/ura3Δ::imm434</i>	5
DSY1216	CAF4-2	<i>erg11Δ::hisG-URA3-hisG/ERG11</i>	This study
DSY1217	DSY1216	<i>erg11Δ::hisG/ERG11</i>	This study
DSY1336	DSY1217	<i>erg3AΔ::hisG-URA3-hisG/ERG3 erg11Δ::hisG/ERG11</i>	This study
DSY1338	DSY1336	<i>erg3AΔ::hisG/ERG3 erg11Δ::hisG/ERG11</i>	This study
DSY1751	DSY1338	<i>erg3AΔ::hisG/erg3BΔ::hisG-URA3-hisG erg11Δ::hisG/ERG11</i>	This study
DSY1769	DSY1216	<i>erg11Δ::hisG-URA3-hisG erg11Δ::hisG-URA3-hisG</i>	This study
DSY1758	DSY1751	<i>erg3AΔ::hisG/erg3BΔ::hisG erg11Δ::hisG/ERG11</i>	This study
DSY1764	DSY1758	<i>erg3AΔ::hisG/erg3BΔ::hisG erg11Δ::hisG/erg11Δ::hisG-URA3-hisG</i>	This study
DSY1431	DSY1338	<i>erg3AΔ::hisG/ERG3 erg11Δ::hisG/erg11Δ::hisG</i>	This study

loss of function of *ERG11*, since it eliminates the target site of these agents. Unfortunately, yeasts with the *ERG11* mutation are not viable under aerobic conditions. Viability with the *ERG11* mutation is possible only when it is accompanied by the inactivation of *ERG3*, as reported for *S. cerevisiae* and *C. glabrata* (3, 15). Little is known about the effects of these mutations on azole or polyene resistance in *C. albicans*. A study has reported on the characterization of *ERG3* from an azole-resistant strain known as the Darlington strain. This strain carries defective mutated *ERG3* alleles, which thus contribute to azole resistance. Unfortunately, the effects of the mutated *ERG3* alleles were masked in this strain by other azole resistance mechanisms (21). In this study, we therefore constructed *erg3/erg3* and *erg11/erg11* mutants and tested their susceptibilities to antifungal agents. Surprisingly, we were able to create *erg11/erg11* mutants by positive selection on a polyene-containing medium under aerobic conditions without the need for *ERG3* inactivation.

#### MATERIALS AND METHODS

**Strains and media.** The *C. albicans* strains used in this study are listed in Table 1. The *C. albicans* strains were grown either in complete medium consisting of YEPD liquid medium (1% Bacto Peptone [Difco], 0.5% yeast extract [Difco], 2% glucose [Fluka]) or in minimal medium consisting of yeast nitrogen base (YNB medium; Difco) and 2% glucose (Fluka). When the strains were grown on solid media, 2% agar (Difco) was added to either medium. *Escherichia coli* DH5 $\alpha$  (10) was used as a host for plasmid construction and propagation. DH5 $\alpha$  cells were grown in Luria-Bertani (LB) broth or on LB plates, which were supplemented with ampicillin (0.1 mg/ml) when required.

**Construction of mutants with *ERG3* and *ERG11* mutations.** The deletion of *ERG3* from *C. albicans* was performed by using two different constructs based on comparisons with the *S. cerevisiae* *ERG3* gene and available *C. albicans* genome data. For the first construct, a fragment amplifying the first *ERG3* allele (*ERG3A*) was generated by PCR with *C. albicans* genomic DNA, Pwo polymerase (Roche), and primers ERG3-A (5'-AAATTCATTCTTTTCACCGAT-3') and ERG3-B (5'-ATCTGGTCTTCTGTAAGATT-3'), whose sequences matched the sequence between positions +637 and +1037 with respect to the first ATG codon of *ERG3*. The PCR fragment generated was subcloned into the compatible *HincII* and *SmaI* sites of pBluescript KS to obtain pDS545. A deletion between nucleotides +833 and +850 was created by PCR with primers ERG3-SALI (5'-GCGCAAAGTCGACGCAATGGGAATAATAATGGGTA AAGATGTT-3') and ERG3-BGLII (5'-GCGCAAAGATCTTTTGTTCACCT TTTGTTAACTTTTGGACTG-3') and pDS545 as the template. The product of this PCR was digested with *SalI* and *BglII* and ligated to a 3.7-kb *SalI-BglII* fragment from pMB7 containing the *hisG-URA3-hisG* "Ura blaster" cassette (5) to yield pDS546. The first *ERG3* disruption cassette was liberated by *ApaI* and *SacI* digestion, and this linear fragment was used for disruption of the first *ERG3* allele. For the second *ERG3* disruption cassette, a fragment corresponding to the second *ERG3* allele (*ERG3B*) was amplified from *C. albicans* genomic DNA with primers ERG3-XBA (5'-TACAATCTAGATATCTTTGGACATTC-3') and ERG3-XHO (5'-GCGCAAAGTCGAGAAGATTATTTTCAA TTATCAACA CCAAATTG-3') and cloned into a compatible restriction site of pBluescript

KS(+) to yield pDS749. A deletion between nucleotides +390 and +1028 with respect to the first ATG codon was created by PCR with primers ERG3-PST (5'-GCGCAAAGACGTCCCAACCAATATAGTAGTGATAATG-3') and ERG3-BGL (5'-GCGCAAAGATCTCAGATGATTCATTGTTGT-3') and with pDS749 as a template. The product of this PCR was digested with *PstI* and *BglII* and ligated to a 3.7-kb *PstI-BglII* fragment from pMB7 to yield pDS765. The second *ERG3* disruption cassette was liberated by *ApaI* and *SacI* digestion, and this linear fragment was used for disruption of the remaining *ERG3B* allele after marker regeneration, as described by Fonzi and Irwin (5). Schematic restriction maps are also given in Fig. 2.

For the disruption of *ERG11*, this gene was first cloned from pDS271, a plasmid that has been reported by Sanglard et al. (29) and that contains *ERG11*. A 3.5-kb *EcoRV-ClaI* fragment containing *ERG11* was cloned into compatible sites of pMTL21, yielding pDS501. After the removal of an internal 1-kb *XbaI-AccI* fragment in pDS501 and blunt ending of the *XbaI* site, the Ura blaster cassette was inserted as a *BglII-AccI* fragment to create pDS506. A linear *ApaI-SacI* fragment was obtained from pDS506 for transformation into CAF4-2. Schematic restriction maps are also given in Fig. 3.

**Northern and Southern blotting.** Northern blotting and Southern blotting were carried out as described previously (31).

***C. albicans* transformations.** The different *C. albicans* strains were transformed by the lithium acetate procedure adapted by Sanglard et al. (30). After transformation with a linear DNA fragment, the cells were plated on YNB selective medium and incubated for 2 to 3 days at 30°C.

**PCR.** Pwo polymerase (Roche) was used in the PCR to generate DNA fragments for use in cloning steps or as probes. The PCR buffers and conditions were those suggested by the manufacturer. PCR was carried out in a Thermal Cycler 480 instrument (Perkin-Elmer), with a first cycle at 94°C for 2 min, followed by 30 cycles of annealing at 54°C for 2 min. Elongation was performed at 72°C for 2 min. Yeast DNA templates for PCR were prepared from overnight cultures by mechanical breakage with glass beads, as described previously (30).

**Susceptibility assays.** The *C. albicans* strains were cultivated overnight in YEPD liquid medium at 30°C with constant shaking. Saturated cultures were diluted to an inoculum size of 10<sup>4</sup> cells per ml. Susceptibility assays were performed in YEPD liquid medium in microtiter plates and were inspired by the standard protocol approved by the National Committee of Clinical Laboratory Standards (24). Antifungal agents were diluted by stepwise twofold dilutions in a total volume of 50  $\mu$ l. One hundred fifty microliters of the diluted culture was next added to each antifungal agent-containing well. A drug-free culture and a sterile control were included in each microtiter plate. The microtiter plate was then sealed with Parafilm, and incubation was carried out at 35°C for 24 h. After this incubation period, the optical density of each well of the microtiter plate was read with a microplate reader (Bio-Rad) at 540 nm. The drug MIC was defined as the drug concentration needed to decrease the optical density by at least 50% compared with the optical density of the drug-free culture.

**Sterol analysis.** *C. albicans* strains were cultured to saturation in YEPD medium, and the cells were harvested by centrifugation. The frozen cell pellets were thawed on ice and resuspended in ice-cold H<sub>2</sub>O at 200 mg/ml. One milliliter of each suspension was transferred to glass tubes containing 1 ml of 15% KOH in 90% (vol/vol) ethanol. The samples were heated at 80°C for 45 min and then cooled on ice. The sterols were extracted two times with 5 ml of heptane each time, and the extracts were evaporated to dryness under N<sub>2</sub> at 55°C. The extracts were dissolved in 0.25 ml of toluene, and 0.25 ml of the BSTFA reagent (T-5634; Sigma) was added. The samples were heated at 60°C for 1 h and then evaporated to dryness. The residues were dissolved in 0.25 ml of heptane and stored at -20°C prior to analysis by gas chromatography-mass spectrometry (GC-MS).

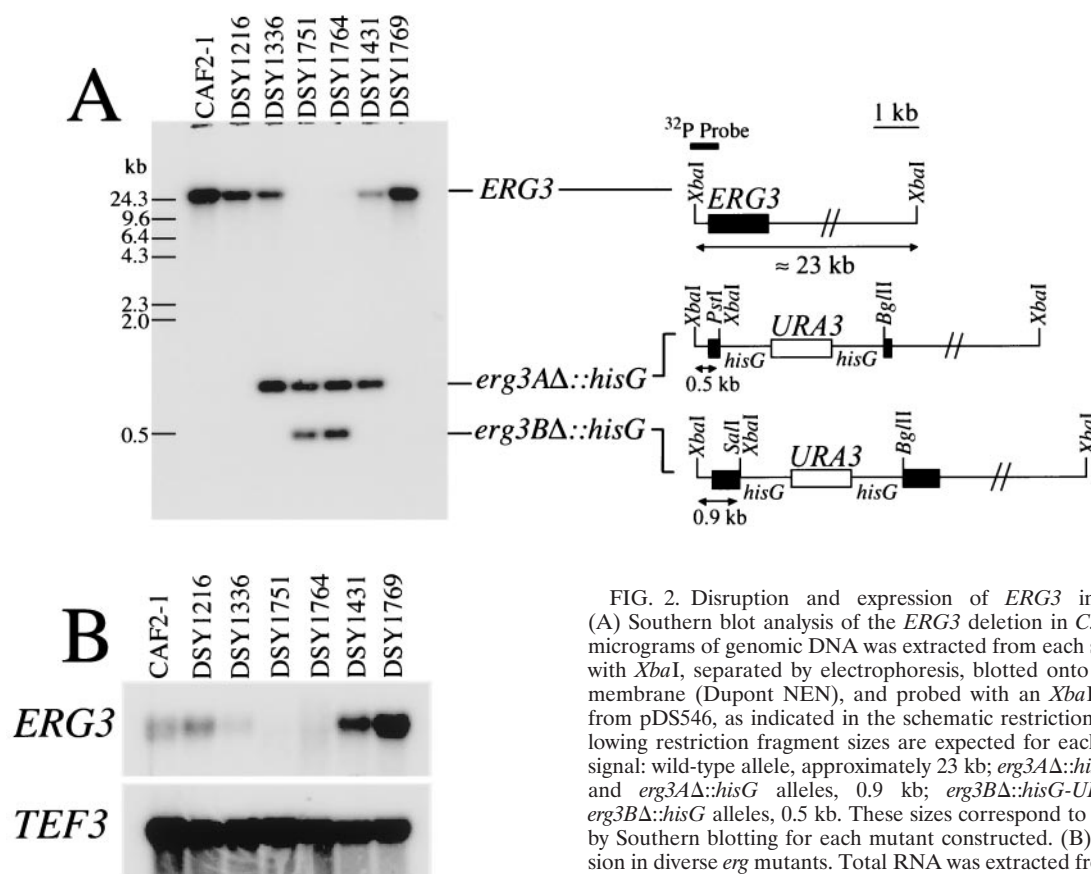


FIG. 2. Disruption and expression of *ERG3* in *C. albicans*. (A) Southern blot analysis of the *ERG3* deletion in *C. albicans*. Five micrograms of genomic DNA was extracted from each strain, digested with *Xba*I, separated by electrophoresis, blotted onto a GeneScreen membrane (Dupont NEN), and probed with an *Xba*I-*Pst*I fragment from pDS546, as indicated in the schematic restriction map. The following restriction fragment sizes are expected for each hybridization signal: wild-type allele, approximately 23 kb; *erg3ΔΔ::hisG-URA3-hisG* and *erg3AΔ::hisG* alleles, 0.9 kb; *erg3BΔ::hisG-URA3-hisG* and *erg3BΔ::hisG* alleles, 0.5 kb. These sizes correspond to those obtained by Southern blotting for each mutant constructed. (B) *ERG3* expression in diverse *erg* mutants. Total RNA was extracted from *C. albicans*, and 5  $\mu$ g of total RNA was separated by electrophoresis and blotted onto GeneScreen membranes. After hybridization with the same probe used for the experiment whose results are shown in panel A and washing steps, the membranes were exposed to Fuji X-ray film and were revealed after exposure to  $-80^{\circ}\text{C}$ . The *TEF3*-specific probe was hybridized to the same membrane after removal of the *ERG3*-specific probe, as described previously (31).

GC-MS analyses were performed with a Fison CE 8000 instrument with an AS800 autosampler and a Trio-1000-4559. The gas chromatograph was equipped with a Chrompak fused-silica column (25 m by 0.25 mm; CP-SIL 5CB). The following settings were used: source, positive electron ionization; scan, 50 to 650 Da in 0.9 s; interscan delay, 0.1 s; multiplier voltage, 450 V; solvent delay, 2.0 min (this may be extended up to 4.0 min); temperatures of zones 1 and 2, 220 and  $80^{\circ}\text{C}$ , respectively; gas chromatograph temperature ramp, 0.0 min at  $180^{\circ}\text{C}$ , 3.0 min at  $180^{\circ}\text{C}$ , 7.8 min at  $300^{\circ}\text{C}$  ( $25^{\circ}\text{C}/\text{min}$ ), 12.8 min at  $325^{\circ}\text{C}$  ( $5^{\circ}\text{C}/\text{min}$ ), and 14.8 min at  $325^{\circ}\text{C}$ ; analyzer pressure,  $-3.95$  mbar; filament current, 3.0 A; source current,  $1124 \mu\text{A}$ ; trap current,  $341 \mu\text{A}$ ; and He pressure, 10 lb/in<sup>2</sup>. Sterols were identified from their retention times and specific mass spectrometric patterns.

## RESULTS

**Phenotypic analysis of mutants with *ERG3* and *ERG11* mutations.** Given that most *erg* mutations in *C. albicans* are of clinical origin or have not been generated by targeted mutagenesis, we undertook the sequential disruption of *ERG3* and *ERG11* in *C. albicans* strain CAF4-2. The disruption of *ERG3* was performed with strain DSY1217, a *Ura*<sup>-</sup> derivative in which one *ERG11* allele had first been inactivated. The resulting *erg3/erg3* homozygous mutant, DSY1751, did not contain a copy of the wild-type *ERG3* gene, as deduced by Southern blotting analysis (Fig. 2A). Consistent with the deletion of both *ERG3* alleles, no *ERG3* mRNA could be observed in DSY1751 (Fig. 2B). Attempts to disrupt the second allele of *ERG11* in DSY1217 were not successful in the first phase. Since *erg11/erg11* homozygous mutants should lack ergosterol and therefore, in theory, should be resistant to amphotericin B,

we plated the *ERG11* *Ura*<sup>+</sup> heterozygote DSY1216 onto amphotericin B-containing medium. Amphotericin B-resistant colonies appeared at a high frequency ( $5 \times 10^{-5}$  to  $7 \times 10^{-5}$ ) after 3 days of incubation at  $30^{\circ}\text{C}$  compared to the frequency of appearance of wild-type parent CAF4-2 ( $1 \times >10^{-8}$ ). Southern blot analysis of an amphotericin B-resistant isolate, DSY1769, indicated that no wild-type *ERG11* alleles were present in this strain and that two alleles corresponding to *erg11Δ::hisG-URA3-hisG* alleles could be observed (Fig. 3A). This result suggests that the second allele disruption was obtained by mitotic recombination between the first disrupted allele and the remaining wild-type *ERG11* allele. Such events have been reported to occur in *C. albicans* at frequencies ranging from  $10^{-5}$  to  $10^{-6}$  (9), which are very similar to the values obtained by selection for amphotericin B resistance. Deletion of both *ERG11* alleles in DSY1769 was accompanied by the loss of signals corresponding to *ERG11* mRNA. The residual *ERG11* mRNA signal that was still detected with a labeled *ERG11* probe (probe A in Fig. 3B, including the first 400 bp of the *ERG11* open reading frame) does not likely correspond to intact mRNA, since no *ERG11* mRNA could be



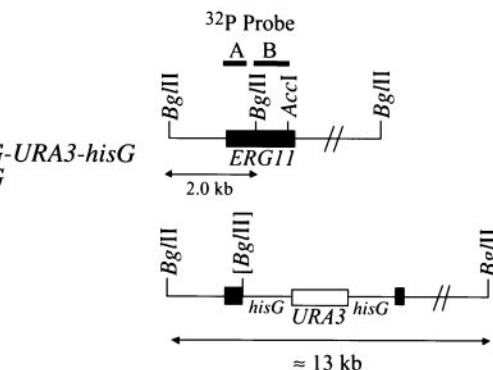
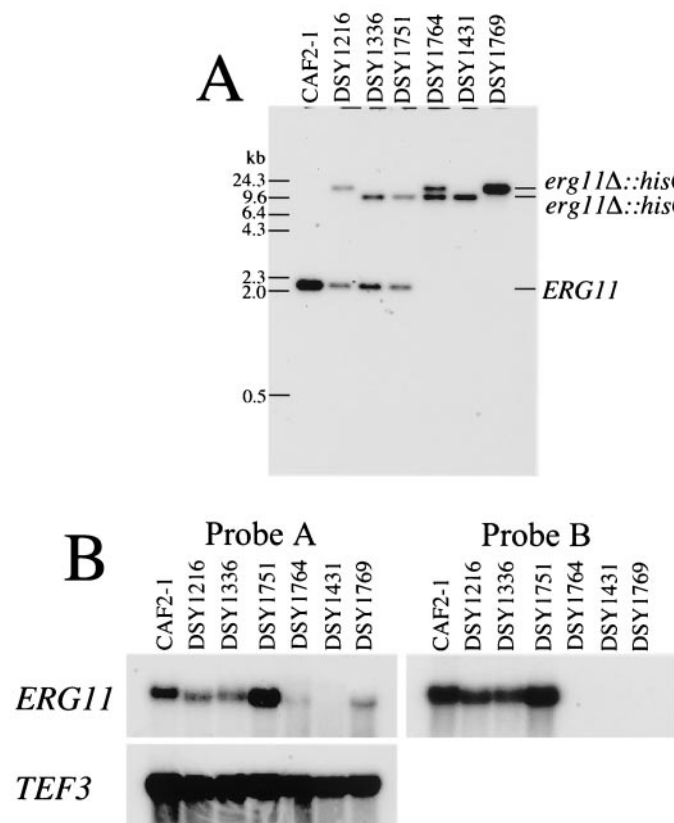


FIG. 3. Disruption and expression of *ERG11* in *C. albicans*. (A) Southern blotting analysis of *ERG11* deletion in *C. albicans*. Five micrograms of genomic DNA was extracted from each indicated strain, digested with *Bgl*III, separated by electrophoresis, blotted onto a GeneScreen membrane (Dupont NEN), and probed with a fragment (probe A) comprising the first 418 bp of the *ERG11* open reading frame, as indicated in the schematic restriction map. The following restriction fragment sizes are expected for each hybridizing signal: wild-type allele, 2 kb; *erg11* $\Delta$ :*hisG-URA3-hisG* and *erg11* $\Delta$ :*hisG* alleles, 13 and 10.3 kb, respectively. These sizes correspond to those obtained by Southern blotting for each mutant constructed. (B) *ERG11* expression in diverse *erg* mutants. Total RNA was extracted from *C. albicans*, and 5  $\mu$ g of total RNA was separated by electrophoresis and blotted onto GeneScreen membranes. After hybridization with labeled probe A and probe B as an 800-bp *Acc*I-*Bgl*III internal fragment corresponding to the *ERG11* deletion, the membranes were exposed to Fuji X-ray film and revealed after exposure to  $-80^{\circ}\text{C}$ . The *TEF3*-specific probe was hybridized to the same membrane after removal of the *ERG11*-specific probe, as described previously (31).

observed by use of a labeled probe whose sequence corresponded to that of the fragment deleted from the *C. albicans* *ERG11* genomic locus (Fig. 3B). Deletion of homozygous *ERG11* alleles was obtained in two other strains created in this study. Strain DSY1431 was obtained by selection on amphotericin B-containing medium and originated from DSY1336, which is heterozygous for *ERG3*. Strain DSY1764 was obtained from a homozygous *erg3/erg3* mutant (DSY1751) by targeted deletion of the remaining *ERG11* allele by the Ura blaster method. No wild-type *ERG11* allele could be detected in either DSY1431 or DSY1764 (Fig. 3A), and no signals corresponding to *ERG11* mRNA were observed with *ERG11*-specific probe B (Fig. 3B). The *ERG3* mRNA signals were markedly increased in *erg11/erg11* deletion mutants still carrying a functional *ERG3* allele (Fig. 2B). On the other hand, the *ERG11* mRNA signals were significantly increased only in the *erg3/erg3* homozygote, DSY1751 (Fig. 3B). Analysis of *ERG11* and *ERG3* expression by the mutants constructed in this study gave results that agree with the idea that, in the absence of ergosterol or when the ergosterol pathway is inhibited, *ERG* genes can be upregulated. Upregulation of both *ERG3* and *ERG11* in mutants lacking either gene had an effect comparable to that from the inhibition of sterol biosynthesis by the addition of azoles or other ergosterol biosynthesis inhibitors. This phenomenon has been described by the use of microarrays in *S. cerevisiae* (12) and in *C. albicans* exposed to itraconazole (4, 11).

The deletion of the *ERG3* and *ERG11* alleles affected the

growth of *C. albicans* in YEPD medium to different degrees. We calculated the doubling times ( $t_d$ s) to be between 102 and 106 min for strains CAF2-1, DSY1216, and DSY1336, all of which carry functional *ERG11* and *ERG3* alleles, and for the mutant with the homozygous *ERG3* mutation, DSY1751. The  $t_d$ s were 120 min for DSY1764 and 160 min for DSY1431 and DSY1769, thus showing that deletion of *ERG11* had a negative effect on the *C. albicans* growth rate. In addition to the fact that these differences in growth rates were similar in synthetic YNB medium, the ability of all strains to grow in this type of medium shows that these strains are not auxotrophic for ergosterol. In practical terms, the differences in the growth rates between these different strains means that if they are not under amphotericin B selection pressure, the mutants with higher  $t_d$ s will be not competitive in vitro or in vivo, unlike other normally growing *C. albicans* isolates.

The susceptibilities of these mutants to different classes of antifungal and metabolic inhibitors were first determined by a visual spotting assay on YEPD medium (Fig. 4) with different drug concentrations and were then determined by a regular NCCLS methodology, with the exception that YEPD medium was used in place of RPMI medium (Table 2). The results presented in Fig. 4 indicate that, in general, the mutants with

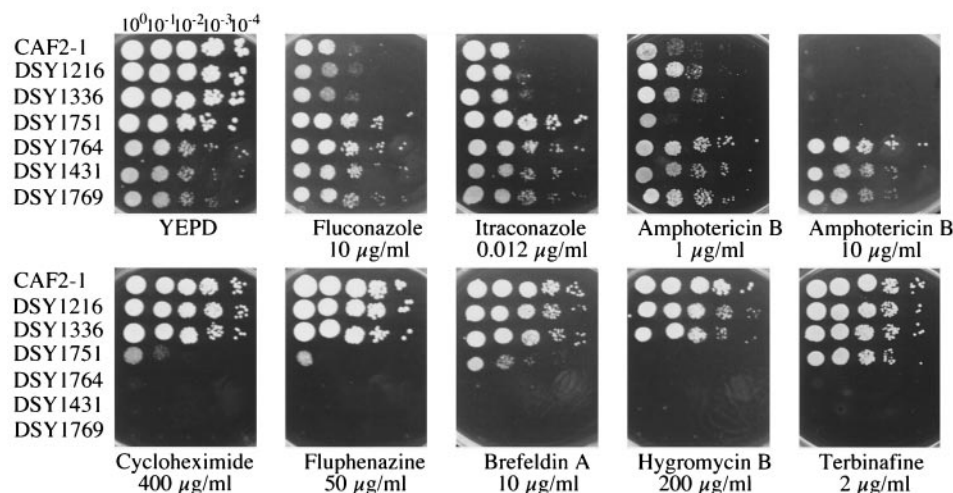


FIG. 4. Susceptibility assays with *C. albicans* *erg3* and *erg11* mutants. Each strain was diluted as indicated from a starting solution containing  $10^7$  cells per ml, and 5  $\mu$ l of each dilution was spotted onto the different agar plates containing the different drugs at the indicated concentrations. The plates were incubated for 48 h at 35°C.

single and double *ERG3* and *ERG11* mutations were more susceptible than the wild type to metabolic inhibitors (i.e., cycloheximide, fluphenazine, brefeldin A, or hygromycin B). The *erg3/erg3* mutant with the single mutation, DSY1751, was resistant to azoles but remained as susceptible to amphotericin B and terbinafine as the wild type (Fig. 4). DSY1751 was even slightly more susceptible to amphotericin B than the wild type, as judged by growth differences in spotting assays and the MICs measured by the standard NCCLS method (Table 2). The *erg11/erg11* mutants were resistant to azoles as well as to amphotericin B in either the wild-type or the *erg3/erg3* mutant background. The data shown in Fig. 4 were again consistent with the MICs measured by the NCCLS method: fluconazole MICs increased from 0.25  $\mu$ g/ml for the wild type to more than 128  $\mu$ g/ml for the *erg3/erg3* mutants, the *erg11/erg11* single mutants, and the *erg3/erg3 erg11/erg11* double mutants. The amphotericin B MICs increased from 0.25  $\mu$ g/ml for the wild type to more than 16  $\mu$ g/ml for the *erg11/erg11* single mutants and the *erg3/erg3 erg11/erg11* double mutants.

**Sterol analysis profiles.** The wild type and the mutants created during this work were subjected to GC-MS analysis for the identification of sterols. As summarized in Table 3, ergosterol was the major sterol in strains still carrying functional *ERG3* or *ERG11* alleles, i.e., strains DSY1216 and DSY1336.

The major difference between these two strains and the parent, CAF2-1, was the increase in the lanosterol fraction (from 6.7 to 22.3 and 18.2%, respectively) at the expense of the ergosterol fraction, which decreased from 67 to 60 and 51%, respectively. As expected, the *erg3/erg3* mutant accumulated large amounts of 14 $\alpha$ -demethylated sterols that lacked desaturation between the C-5 and C-6 sterol moieties. In this type of strain, ergosta-7,22-dienol (61% of all sterols), ergosta-7-enol (7% of all sterols), and also fecosterol or episterol (26.8% of all sterols) were identified. The detection of ergosta-7,22-dienol and ergosta-7-enol can be explained by the action of the C-24 sterol methyltransferase and C-24 sterol reductase (Fig. 1). The detection of these sterols is consistent with a blockage of  $\Delta^{5,6}$  desaturation of the sterol moiety. In the other mutants lacking *ERG11* alleles, 14 $\alpha$ -methylated sterols in the form of eburicol and lanosterol, 4,14-dimethylzymosterol, and 14 $\alpha$ -methyl-fecosterol were the dominant sterols. The last two sterol metabolites could be formed by removal of the C-4 methyl group by the actions of *ERG25*, *ERG26*, and *ERG27*. An additional methylation step (by the action of *ERG6*) is required for the formation of 14 $\alpha$ -methyl-fecosterol. The larger proportion of lanosterol or eburicol is consistent with the absence of 14 $\alpha$  demethylation (Fig. 1). Even if *ERG3* alleles were intact in strain DSY1769, the presence of a 14 $\alpha$ -methyl group was apparently inhibiting the further processing of sterol downstream of the action of *ERG6* in the ergosterol biosynthesis pathway.

TABLE 2. Susceptibilities of *C. albicans* *erg3* and *erg11* mutants to different antifungal agents

Strain	Genotype	MIC ( $\mu$ g/ml)	
		Fluconazole	Amphotericin B
CAF2-1	Wild type	0.25	0.5
DSY1216	<i>erg11/ERG11</i>	0.06	0.5
DSY1336	<i>erg11/ERG11 erg3/ERG3</i>	0.06	0.5
DSY1751	<i>erg11/ERG11 erg3/erg3</i>	>128	0.25
DSY1764	<i>erg11/erg11 erg3/erg3</i>	>128	>16
DSY1431	<i>erg11/erg11 erg3/ERG3</i>	>128	>16
DSY1769	<i>erg11/erg11</i>	>128	>16

DISCUSSION

In this study we created several mutants with mutations in the *ERG* pathway either by targeted mutagenesis or by selection in amphotericin B-containing medium. The drug susceptibility profile for *erg3/erg3* deletion mutant DSY1751 was resistance to fluconazole, as expected. This mutant was also more susceptible than the wild type to other categories of metabolic inhibitors, including, for example, cycloheximide, fluphenazine, brefeldin A, and hygromycin B. We did not systematically

TABLE 3. Sterol compositions of *C. albicans* sterol mutants

Sterol identified	Total sterol fraction (%) in strain:									
	CAF2-1 (wild type)	DSY1216 ( <i>erg11/ERG11</i> )	DSY1336 ( <i>erg11/ERG11</i> <i>erg3/ERG3</i> )	DSY1751 ( <i>erg11/ERG11</i> <i>erg3/erg3</i> )	DSY1764 ( <i>erg11/erg11</i> <i>erg3/erg3</i> )	DSY1431 ( <i>erg11/erg11</i> <i>erg3/ERG3</i> )	DSY1769 ( <i>erg11/erg11</i> )			
Lanosterol	6.7	22.3	18.2	2.4	26.9	21.4	57.0			
Eburicol (24-methylene-lanosterol)	ND	ND	ND	ND	37.9	29.6	18.0			
Zymosterol	7.2	5.7	6.2	ND	ND	ND	ND			
4,14-Dimethyl-zymosterol	ND	ND	ND	ND	27.6	31.5	15.1			
Episterol or fecosterol	3.8	ND	3.3	26.8	ND	ND	ND			
14-Methyl-fecosterol	ND	ND	ND	ND	3.9	2.5	4.1			
Ergosta-5,8,22-trienol	5.3	4.3	2.5	ND	ND	ND	ND			
Ergosta-7,22-dienol	7.0	4.8	10.8	61.0	ND	ND	ND			
Ergosta-7-enol	ND	ND	ND	7.1	ND	ND	ND			
Ergosterol	67.9	60.8	51.6	ND	ND	ND	ND			
Unidentified	2.1	2.1	7.4	2.7	3.7	15.0	5.8			

<sup>a</sup> ND, not detected.

test all types of *C. albicans* growth inhibitors in this study; however, the phenotypes of susceptibility to the growth inhibitors tested mirrored well the hypersusceptibility profiles observed for other *C. albicans* *erg* mutants in which mutations were constructed by targeted mutagenesis. For example, the *C. albicans* *erg6* and *erg24* mutants constructed by Jensen-Perkakes et al. (13) and Jia et al. (14), respectively, showed hypersusceptibility to substances identical to those used in this study, such as cycloheximide, fluphenazine, and brefeldin A. Only the *erg24* mutant showed a slight increase in azole resistance, while the *erg6* mutant was not affected by this class of antifungal agents (13, 14). The hypersusceptibilities to several unrelated drugs and metabolic inhibitors are believed to be the result of alterations in membrane fluidity, which thus contributes to enhanced drug permeability.

In our study, we observed that *erg3* mutant DSY1751 was not more resistant to amphotericin B, but it was even slightly more susceptible to this drug than the wild type (Fig. 4, agar plate with a concentration of 1 µg/ml). This suggests that amphotericin B could have additional cellular targets in *C. albicans*. This was unexpected, given that no ergosterol was found in this mutant and that this defect is generally linked to amphotericin B resistance (20). Moreover, comparisons with *erg3* mutants of other yeast species in which *erg3* mutations were constructed showed some discrepancies with the results obtained here. In *C. glabrata*, the deletion of *ERG3* does not lead to azole or amphotericin B resistance (8). In *S. cerevisiae*, targeted deletion of *ERG3* is coupled with resistance to both azoles and nystatin, the latter of which is structurally related to amphotericin B. The reason for these discrepancies remains unclear. It is possible that the methods used to assess drug susceptibilities were different between the studies, and therefore, a more careful comparison needs to be performed by identical susceptibility tests before further speculations can be made. A more direct comparison could be made between the *erg3/erg3* mutant constructed here and other clinical strains in which *ERG3* mutations have been constructed. In two separate studies, clinical strains resistant to both azoles and amphotericin B were subjected to sterol analysis. The sterol profiles observed in these strains were typical of those for strains with a mutation in *ERG3*, as they mainly contained ergosta-7,22-dienol and ergosta-7-enol. These sterol profiles therefore resemble those determined in DSY1751. However, no detailed genetic analyses are available for these resistant strains to confirm the basis of the hypothetical *ERG3* mutation (18, 25). Another azole-resistant *C. albicans* strain (the Darlington strain) has been analyzed carefully by Miyazaki et al. (21) and revealed that ergosta-7,22-dienol is a major sterol. This strain carries no functional *ERG3* alleles, thus enabling a link between the presence of this sterol and a defect in *ERG3*. The susceptibility of the Darlington strain to amphotericin B was almost similar to that established for CAF2-1 and shown in Table 2 (J. Bennett, personal communication). The phenotypes of the Darlington strain and the *erg3/erg3* homozygote DSY1751 are therefore similar and are in agreement with the idea that the Darlington strain is an *erg3* mutant.

A surprising result of this study was the generation of an *erg11/erg11* homozygous mutant in the presence of amphotericin B and under aerobic conditions. As inferred from the frequency of amphotericin B resistance and Southern analysis



of *erg11/erg11* deletion mutants DSY1431 and DSY1769, mitotic recombination was the likely event linked to the generation of amphotericin B resistance. Several studies have used the ability of *C. albicans* to undergo mitotic recombination to obtain a particular phenotype (9, 32). However, this method of raising homozygous mutants can be linked to additional compensatory mutations during the completion of mitotic recombination. One way to establish that no mutation other than the *ERG11* deletion was generated in DSY1769 or other amphotericin B-resistant isolates in the present study would be to restore wild-type amphotericin B susceptibility by reintroduction of an intact *ERG11* allele. This experiment was attempted in DSY1769; however, the transformation method used did not allow the recovery of any transformants. The poor transformation efficiency of strain DSY1769 could be due to its weak growth or its altered sterol profile. If compensatory mutations are possible in the *erg11/erg11* homozygous mutants produced in this study, their identification remains to be established. Given that the *erg11* mutants are viable only in the background of *erg3* mutants as mentioned above, we looked for possible loss-of-function mutations in *ERG3* alleles recovered from strains DSY1431 and DSY1769. However, the *ERG3* alleles from the parental wild type and these mutants were identical at the level of nucleotide sequence.

Different studies performed with *S. cerevisiae* have reported that strains with the *ERG11* mutation are not viable under aerobic conditions unless another suppression mutation that maps to *ERG3* is present (3, 16). An *erg11 C. glabrata* mutant can also be obtained under anaerobic conditions, and from these mutants, spontaneous revertants able to grow under aerobic conditions can be obtained (8). The sterol profiles of such mutants show that they contain high levels of lanosterol and obtusifolol. *erg11/erg11* mutant DSY1769 had a sterol profile similar to this sterol profile in terms of its lanosterol content, but its sterol profile differed with respect to its content of obtusifolol, a sterol not detected in DSY1769. Obtusifolol is mostly related to 4,14-dimethyl-zymosterol, a sterol metabolite found in DSY1769, but differs by the addition of an unsaturated methyl group at C-24. A *C. albicans* mutant called D10 was reported to lack the function conferred by *ERG11*, although no molecular evidence was given (2, 3). This mutant could grow aerobically and showed a sterol profile similar to those observed for the *erg11/erg11* mutants evaluated in this study, with the difference being that no obtusifolol was detected in our investigation. A common feature among *erg11* mutants of *C. albicans*, *C. glabrata*, and *S. cerevisiae* is that they can be generated in an *erg3* deletion background without the use of a specific selection procedure. However, the sterol profiles of the double *erg3/erg3 erg11/erg11* mutants and the *erg3/erg3* mutants of *C. albicans* are again different from those of both *C. glabrata* and *S. cerevisiae* mutants. In the last two yeast species the dominant sterol metabolites are 14 $\alpha$ -methyl-fecosterol, followed by lanosterol, while in *C. albicans* the major sterol is the lanosterol and eburicol fraction, followed by 4,4-dimethyl zymosterol.

In conclusion, this study clarifies the contribution of *ERG3* in azole resistance and also suggests that *erg11/erg11* mutants can spontaneously appear upon selective drug pressure in vitro when one *ERG11* allele is not functional. Whether this event can occur during treatment of patients with amphotericin

B is questionable. Experiments in which *ERG11* has been "switched off" have been performed with *C. glabrata* in animal models and showed that the lack of *ERG11* expression has only minor effects in animal models, probably because host sterols can compensate for the absence of *C. glabrata* sterols (22, 23). Therefore, positive selection of *erg11* mutants in the context of animal experiments can be envisaged.

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