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Deletion of the *Candida glabrata ERG3* and *ERG11* Genes: Effect on Cell Viability, Cell Growth, Sterol Composition, and Antifungal Susceptibility

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We have cloned and sequenced the structural genes encoding the $\Delta^{5,6}$ sterol desaturase (*ERG3* gene) and the 14α-methyl sterol demethylase (*ERG11* gene) from *Candida glabrata* L5 (*leu2*). Single and double mutants of these genes were created by gene deletion. The phenotypes of these mutants, including sterol profiles, aerobic viabilities, antifungal susceptibilities, and generation times, were studied. Strain L5D (*erg3*Δ::*LEU2*) accumulated mainly ergosta-7,22-dien-3β-ol, was aerobically viable, and remained susceptible to antifungal agents but had a slower generation time than its parent strain. L5LUD (*LEU2 erg11*Δ::*URA3*) strains required medium supplemented with ergosterol and an anaerobic environment for growth. A spontaneous aerobically viable mutant, L5LUD40R (*LEU2 erg11*Δ::*URA3*), obtained from L5LUD (*LEU2 erg11*Δ::*URA3*), was found to accumulate lanosterol and obtusifoliol, was resistant to azole antifungal agents, demonstrated some increase in resistance to amphotericin B, and exhibited a 1.86-fold increase in generation time in comparison with L5 (*leu2*). The double-deletion mutant L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*) was aerobically viable, produced mainly 14α-methyl fecosterol, and had the same antifungal susceptibility pattern as L5LUD40R (*LEU2 erg11*Δ::*URA3*), and its generation time was threefold greater than that of L5 (*leu2*). Northern (RNA) analysis revealed that the single-deletion mutants had a marked increase in message for the undeleted *ERG3* and *ERG11* genes. These results indicate that differences in antifungal susceptibilities and the restoration of aerobic viability exist between the *C. glabrata* ergosterol mutants created in this study and those sterol mutants with similar genetic lesions previously reported for *Saccharomyces cerevisiae*.

Candida glabrata is a pathogenic haploid yeast species which causes both mucocutaneous and deep infections in humans. The increasingly extensive use of topical and systemic antifungal drugs has increased the incidence of mucous-membrane colonization and infections with *C. glabrata* (35) and led to the appearance of azole-resistant infections with *Candida* species, including *C. glabrata* (22).

The mechanism(s) by which *Candida* species become resistant to azole antifungal agents is poorly understood. While these agents adversely affect a number of cellular functions, their primary mechanism of action is the inhibition of cytochrome P-450-dependent lanosterol 14α-demethylase (P-450_{DM}), encoded by the *ERG11* gene (15, 29, 31). Postulated mechanisms of resistance include changes in cell permeability (11, 13), overproduction or alteration of P-450_{DM} (3, 30), and modification of other enzymes involved in sterol synthesis (10). One such enzyme, the $\Delta^{5,6}$ sterol desaturase, encoded by the *ERG3* gene, has been associated with the development of resistance in two yeast species, *Saccharomyces cerevisiae* (16, 34) and *Candida albicans* (14), which are closely related to *C. glabrata*.

Mutations or disruptions of *ERG3* in *S. cerevisiae* result in the formation of ergosta-7,22-dien-3β-ol as the major sterol (2) and are associated with increased resistance to azole and polyene antifungal agents (34). Additionally, this gene has been implicated in the development of azole resistance because mutations of the *ERG3* gene exert a suppressor effect on the phenotype of strains containing *ERG11* mutations, allowing aerobic viability in the background of a normally lethal mutation (4, 16, 27). *S. cerevisiae* strains which harbor lethal mutations in the *ERG11* gene survive only in an anaerobic environment and require medium supplemented with ergosta-5,7,22-trien-3β-ol (ergosterol) for growth. However, spontaneous aerobically viable mutants derived from these strains accumulate primarily 14α-methylfecosterol and have been shown to carry a mutation in *ERG3* (4). The *ERG3* defect in these double mutants has been postulated to maintain aerobic viability by blocking the accumulation of toxic 3β,6α-diol sterols formed by an active desaturase enzyme on 14α-methylated sterols (33). These diol sterols have been shown to accumulate in wild-type strains of *S. cerevisiae* treated with azole antifungal agents (33) and in a *C. albicans* strain lacking P-450_{DM} activity (4).

Genetic studies of sterol biosynthesis in the pathogenic yeast *C. albicans* have been hindered by the organism's asexual diploid state. Mutations of genes involved in ergosterol biosynthesis have been inferred from sterol profile analyses of polyene- or azole-resistant strains without genetic confirmation. Current evidence suggests that lack of $\Delta^{5,6}$ desaturase activity can lead to azole resistance, as in the clinically derived *C. albicans* Darlington strain. This azole-resistant strain produces ergosta-8,24(28)-dien-3β-ol (fecosterol) as its major sterol and lesser but significant amounts of episterol (14). Evidence that *C. albicans* strains considered to be *erg11* mutants are aerobically viable in the absence of a suppressor mutation in the

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TABLE 1. *C. glabrata* strains used in this study

Strain	Genotype
L5	*leu2* mutation
L5L	*LEU2* prototroph
L5D	*erg3*Δ::*LEU2*
L59L	*LEU2 ura3* mutation
L59D	*erg3*Δ::*LEU2 ura3* mutation
L5LUD40	*LEU2 erg11*Δ::*URA3*
L5LUD40R	*LEU2 erg11*Δ::*URA3*; aerobically viable revertant
L5DUD61	*erg3*Δ::*LEU2 erg11*Δ::*URA3*
NCCLS 84	Wild-type azole-susceptible strain

ERG3 gene has been obtained from polyene-resistant strains lacking P-450_{DM} activity. These strains accumulate substantial amounts of 14α-methylated 3β,6α-diol sterols (4, 25). Nevertheless, an association between the occurrence of mutations in the *ERG3* and *ERG11* genes has been detected in a *C. albicans* strain, KD4952. *C. albicans* KD4952 is an ergosterol mutant obtained as a hypha-forming revertant of the nystatin-resistant strain KD4950, which lacks P-450_{DM} activity. The sterol profile of KD4950 suggests that the strain has mutations in both the *ERG11* and the *ERG3* genes, while its revertant KD4952 accumulates primarily ergosta-7,22-dien-3β-ol and is thought to retain only the *ERG3* mutation (25, 26). A comparison of in vitro triazole susceptibilities between KD4950, KD4952, and the wild-type KD14 has not been reported. Nystatin susceptibility of KD4952 is intermediate between those of KD4950 and KD14.

C. glabrata is a useful species for the study of sterol pathways and azole drug resistance because the fungus is haploid and because it is an important pathogen in its own right. We have cloned and sequenced the *C. glabrata ERG3* and *ERG11* genes and have deleted them alone and sequentially in the *C. glabrata* L5 (*leu2*) strain. Aerobic viability, sterol profiles, antifungal susceptibilities, and generation times of the deletion mutants were studied in order to better understand the sterol requirements and mechanism(s) of azole resistance of *C. glabrata.*

MATERIALS AND METHODS

Strains. The *C. glabrata* strains used in this study and their genotypes are listed in Table 1. *C. glabrata* L5 (*leu2*) was kindly supplied by D. R. Winge (20). Strain L5D (*erg3*Δ::*LEU2*) was created by disruption of the *ERG3* gene in strain L5 (*leu2*). *C. glabrata* L5L (*LEU2*) was created by transformation of L5 (*leu2*) with the *LEU2* gene of *S. cerevisiae*. The *URA3* auxotrophs, L59D (*erg3*Δ::*LEU2 ura3*) and L59L (*LEU2 ura3*), were obtained by plating strains L5D (*erg3*Δ::*LEU2*) and L5L (*LEU2*) on 5-fluoro-orotic acid-containing media as previously described (5). Strains L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*) and L5LUD40 (*LEU2 erg11* Δ::*URA3*) were obtained by disrupting the *ERG11* genes of L59D (*erg3*Δ::*LEU2 ura3*) and L59L (*LEU2 ura3*), respectively. Strain L5LUD40R (*LEU2 erg11*Δ::*URA3*) was a spontaneous aerobically viable mutant of L5LUD40 (*LEU2 erg11*Δ::*URA3*). *S. cerevisiae* genomic DNA was kindly supplied by A. Varma. Plasmid DNA was propagated in *Escherichia coli* DH10β (GibcoBRL, Gaithersburg, Md.) and *E. coli* XL1-blue cells (Stratagene, La Jolla, Calif.).

Media and growth. *C. glabrata* strains were maintained on 1% (wt/vol) yeast extract–2% (wt/vol) peptone–2% (wt/vol) dextrose (YEPD) media. Following transformation with the *erg3*Δ::*LEU2* deletion construct, transformants were plated on yeast nitrogen base media with 2% (wt/vol) glucose (MING). Strains transformed with the *erg11*Δ::*URA3* deletion construct were plated anaerobically on MING media supplemented with 35 μg of ergosterol per ml and 0.0875% (vol/vol) Tween 80. *E. coli* strains were grown in Luria-Bertini broth or agar with 50 μg of ampicillin per ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (United States Biochemical Corp., Cleveland, Ohio) per ml. Except as noted, yeast strains were grown at 30°C and *E. coli* was grown at 37°C. For long-term storage, yeast strains were placed at −80°C in 20% glycerol.

Vectors and deletion constructs. *C. glabrata ERG3* and *ERG11* genes were cloned into the plasmids pBluescript SKII (pBSK; Stratagene), pUC19, and pSPORT 1 (GibcoBRL). Restriction maps of these clones and deletion constructs are depicted in Fig. 1 and 2. The vector pGLEU contained the *S. cerevisiae LEU2* gene obtained from YEp13 as a 2.6-kb *Xho*I-*Bgl*II DNA fragment ligated into pUC19 digested with *Bam*HI-*Sal*I. The L5LUD40R (*LEU2 erg11*Δ::*URA3*) *ERG3* gene, obtained by PCR, was cloned into pBSK for sequence analysis.

Generation times. Overnight cultures of *C. glabrata* strains were inoculated into 50 ml of YEPD in 250-ml flasks and shaken at 200 rpm at 30°C. Cultures were initially adjusted to an optical density of 0.05 at 600 nm in a DU-64 spectrophotometer (Beckman Instruments, Columbia, Md.), and generation times were calculated from absorbance spectra measured at 2, 4, 6, and 8 h. These studies were performed in triplicate, and results reflect the average for these studies.

Nucleic acid isolation and hybridization. DNA was isolated and Southern analysis was performed as previously described (8). RNA was isolated from cells grown to mid-log phase, washed with 1 M sodium chloride, and resuspended in a lysis buffer (4% [wt/vol] sodium dodecyl sulfate, 0.15 M sodium acetate, and 0.05 M EDTA). An equal volume of 0.45-mm-diameter glass beads was added, and the cells were vortexed for a total of 3 min in 30-s intervals. The supernatant was removed and extracted three times with an equal volume of phenol-chloroform and a final time with chloroform. Northern (RNA) analysis was performed by standard procedures at high stringency (24). Radiolabeled probes were prepared by using the Prime-It II kit (Stratagene) according to the instructions of the manufacturer.

DNA transformations. *C. glabrata* was transformed by electroporation as described by Varma et al. (32). *E. coli* cells were transformed in a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) according to the instructions of Bio-Rad Laboratories.

PCR. PCRs were performed in a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer, Norwalk, Conn.) by using *Taq* DNA polymerase (Boehringer Mannheim Corporation, Indianapolis, Ind.). The oligonucleotides DSS1 and DSS2 (Table 2), which contain sequences complementary to the published *S. cerevisiae ERG3* sequence (2), were used to amplify a 1.083-kb DNA fragment from *S. cerevisiae* DNA. A 0.525-kb DNA fragment of the *ERG11* gene was obtained with primers DMS1 and DMS2 (Table 2). Both fragments were used to probe *C. glabrata* Southern blots. *C. glabrata* transformants were screened for homologous integration and gene deletion by PCR analysis with oligonucleotides which were complementary to deleted DNA sequences. The *ERG3* gene from strain L5LUD40R (*LEU2 erg11*Δ::*URA3*) was obtained by PCR with oligonucleotides complementary to sequences flanking the 3′ and 5′ regions of this gene.

A 0.83-kb DNA fragment of the *C. albicans* actin gene (19) was obtained by PCR with oligonucleotides ACT1 and ACT2 (Table 2).

DNA sequencing. Plasmid DNA was sequenced by the dideoxy chain termination method according to the instructions provided with the Sequenase kit (United States Biochemicals). Sequence analysis was performed by using the Genetics Computer Sequence Analysis Software Package (7) in a Convex C240 computer maintained by the Division of Computer Research and Technology at the National Institutes of Health.

Cloning of the *C. glabrata ERG3* and *ERG11* genes. The 1.083-kb *S. cerevisiae ERG3* fragment was used to probe a Southern blot of *C. glabrata* L5 (*leu2*) DNA digested with restriction endonucleases. On the basis of these results, a partial *Bgl*II-digested *C. glabrata* genomic DNA library containing fragments in the 3- to 4-kb range was constructed in pBSK. Following transformation into *E. coli*, clones were screened with the *S. cerevisiae* probe and further identified by sequence analysis. pCGDSB, obtained from this library, contained a 3.5-kb DNA fragment encoding part of the *C. glabrata ERG3* open reading frame (ORF), all except for 331 bp of the 3′-end sequence. To obtain a complete *C. glabrata ERG3* gene, pCGDSB was used to probe a partial DNA library containing *Xho*I-*Pst*I fragments in the 3- to 5-kb range. pCGDS-3P, a 3.2-kb DNA fragment ligated into pBSK, was obtained from this library. The two clones were ligated together with overlapping sequences deleted to obtain pCGDL5 (Fig. 1).

The *C. glabrata ERG11* gene was obtained in the manner described above. The 0.525-kb *S. cerevisiae ERG11* DNA fragment was used to probe a *C. glabrata* Southern blot. A partial genomic library containing 5- to 8-kb *Hin*dIII DNA fragments was screened. pDM1, a clone isolated from this library, contained a 6.5-kb DNA fragment encoding most of the *C. glabrata ERG11* sequences. The missing 5′ sequences were obtained by screening a *Pst*I-*Bam*HI genomic library composed of 2- to 3-kb DNA fragments ligated into pBSK. pDM2, a clone with a 2.7-kb insert encoding 187 bp of the 5′ end of the *ERG11* ORF and 5′ flanking sequences, was selected. pDM1 and pDM2 inserts were ligated together in pSPORT 1 to obtain pDM3 as depicted in Fig. 2.

The deletion constructs for the *ERG3* gene, pCGDL7, and the *ERG11* gene, pDM4, were obtained as illustrated in Fig. 1 and 2.

Transformation of *C. glabrata* L5 to leucine prototrophy. *C. glabrata* L5 (*leu2*) was transformed with *Sma*I-digested pCGLEU and grown on minimal media without leucine; transformants were stable when maintained on YEPD. One of these transformants, *C. glabrata* L5L (*LEU2*), was used as a control in the analysis of the *ERG3* deletion mutants.

Gene disruption. Disruption of the *ERG3* gene was accomplished by transforming *C. glabrata* L5 (*leu2*) with a linear *Sma*I-*Pst*I fragment from pCGDL7. The linearized deletion construct was not further purified from the remainder of the vector. Transformants were grown aerobically at 30°C. Disruption of the *ERG11* gene was achieved by transforming strains L59L (*LEU2 ura3*) and L59D (*erg3*Δ::*LEU2 ura3*) with *Avr*II-*Spe*I-digested pDM4. Transformants were grown anaerobically at 35°C, and transformants requiring an anaerobic environment

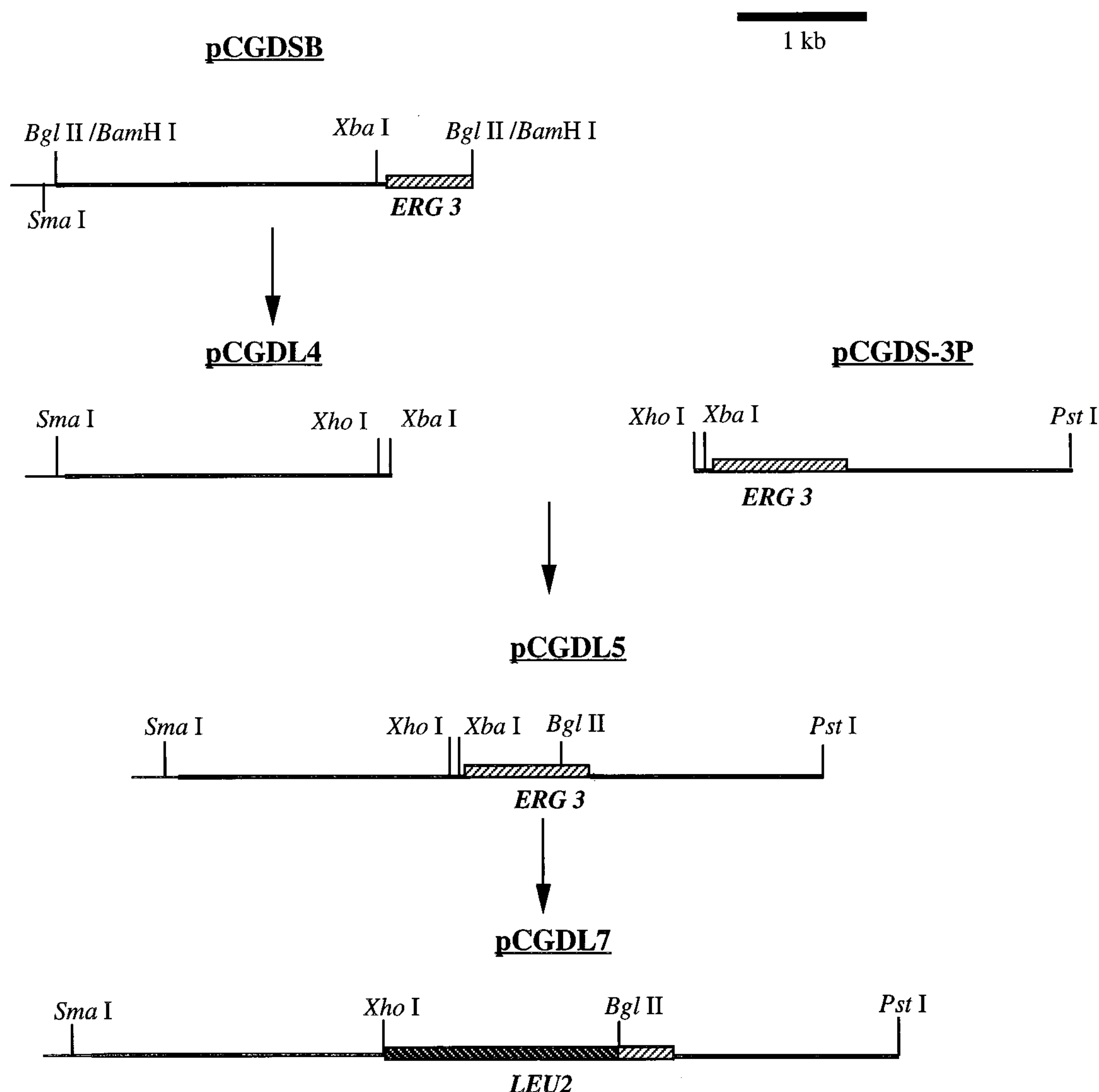

FIG. 1. Vector pCGDSB contains a 3.5-kb *Bgl*II DNA fragment ligated into the pBSK *Bam*HI site. pCGDL4 was made by subcloning a 2.7-kb *Sma*I-*Xba*I fragment from pCGDSB into pUC19. pCGDS-3P contains a 3.2-kb *Xho*I-*Pst*I *C. glabrata* DNA fragment cloned into pBSK. The same fragment was subcloned into *Xba*I-*Pst*I-digested pCGDL4 to create pCGDL5, producing a vector which contained the *ERG3* ORF and ~2.5 kb of 5′ and ~1.5 kb of 3′ flanking sequences. pCGDL7 was made by deleting an ~1-kb *Xho*I-*Bgl*II fragment of the *ERG3* gene and inserting a 2.6-kb *Xho*I-*Bgl*II DNA fragment of YEp13 containing the *S. cerevisiae LEU2* gene.

were subsequently maintained in a GasPak Anaerobic system (Becton Dickinson Microbiology Systems, Cockeysville, Md.).

Antifungal susceptibility. In vitro testing for susceptibilities to the triazole antifungal agents fluconazole (Pfizer, Central Research, Sandwich, United Kingdom) and itraconazole (Janssen Research Foundation, Beerse, Belgium) and the polyene antifungal agent amphotericin B (Bristol-Myers Squibb, Princeton, N.J.) was performed in microtiter plates. Antifungal agents were dissolved in dimethyl sulfoxide (Sigma Chemical Company, St. Louis, Mo.), and stock solutions were stored at −80°C. Dilutions of the drugs were made in RPMI 1640 with L-glutamine and 0.165 M MOPS (morpholinepropanesulfonic acid), without sodium bicarbonate, with 20 g of glucose per liter and with the pH adjusted to 7. Samples (100 μl) of the final drug dilutions were added to the microtiter plates. Yeast cells were plated on YEPD at 35°C overnight and resuspended in 0.85% (wt/vol) saline, and titers were adjusted spectrophotometrically. Further dilutions in RPMI 1640 were made, modified as described above. Inocula were verified by plating a dilution of the cells on YEPD agar and determining CFU per milliliter. Final inocula were in the range of 3×10^4 to 5×10^4 CFU/ml. Plates were incubated at 35°C and read at 24 and 48 h. Optical densities of 1:5 dilutions of the drug-free control well and drug-containing wells were read at 48 h in a Beckman DU spectrophotometer at 600 nm. At 48 h the amount of growth in each of the wells was measured by optical density. The MIC was defined as the concentration of drug which inhibited >75% of growth compared to the control well. These studies were performed with duplicate rows.

Sterol analysis. Nonsaponified lipids were extracted from late-exponential-phase cultures of *C. glabrata* L5 (*leu2*), L5D (*erg3*Δ::*LEU2*), L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*), L5LUD40R (*LEU2 erg11*Δ::*URA3*), and L5L (*LEU2*) as described previously for *C. albicans* (12). The nonsaponifiable lipid fraction was dissolved in a small volume of chloroform-methanol (2:1, vol/vol) and loaded on silica-gel H preparative thin-layer chromatography plates (Merck). The nonsaponifiable lipid fraction was chromatographed with light petroleum (bp, 60 to 80°C)-diethyl ether-acetic acid (85:15:1, vol/vol) as the developing solvent. Lipids were detected under UV light at 350 nm, and the sterol fraction was identified by comparison of its R_f value with that of an authentic ergosterol standard. The sterol fraction was eluted from the silica gel with chloroform-methanol (2:1, vol/vol), reduced to dryness under a stream of N_2, and converted to trimethylsilyl (TMS) derivatives (6). Sterol identification was made by gas chromatography (GC) and GC-mass spectrometry (GC-MS). For GC analyses, TMS-sterols were fractionated by using a Perkin-Elmer 8700 instrument equipped with a flame ionization detector and a Durabond DB5 fused silica capillary column (internal diameter, 0.3 mm), operated isothermally at 260°C. The injector and detector temperatures were 300°C, and the carrier gas was He. Sterols were identified by comparison of their retention times with those of authentic standards (ergosterol and 4,4,14α-trimethylcholesta-8,24-dien-3β-ol [lanosterol]), and 4,14α-dimethylergosta-8,24-dien-3β-ol (obtusifoliol), ergosta-7,22-dien-3β-ol, 4,14-dimethylzymosterol, and fecosterol were extracted from polyene-resistant strains of *C. albicans* (ATCC 38245, 38246, and 38248). The

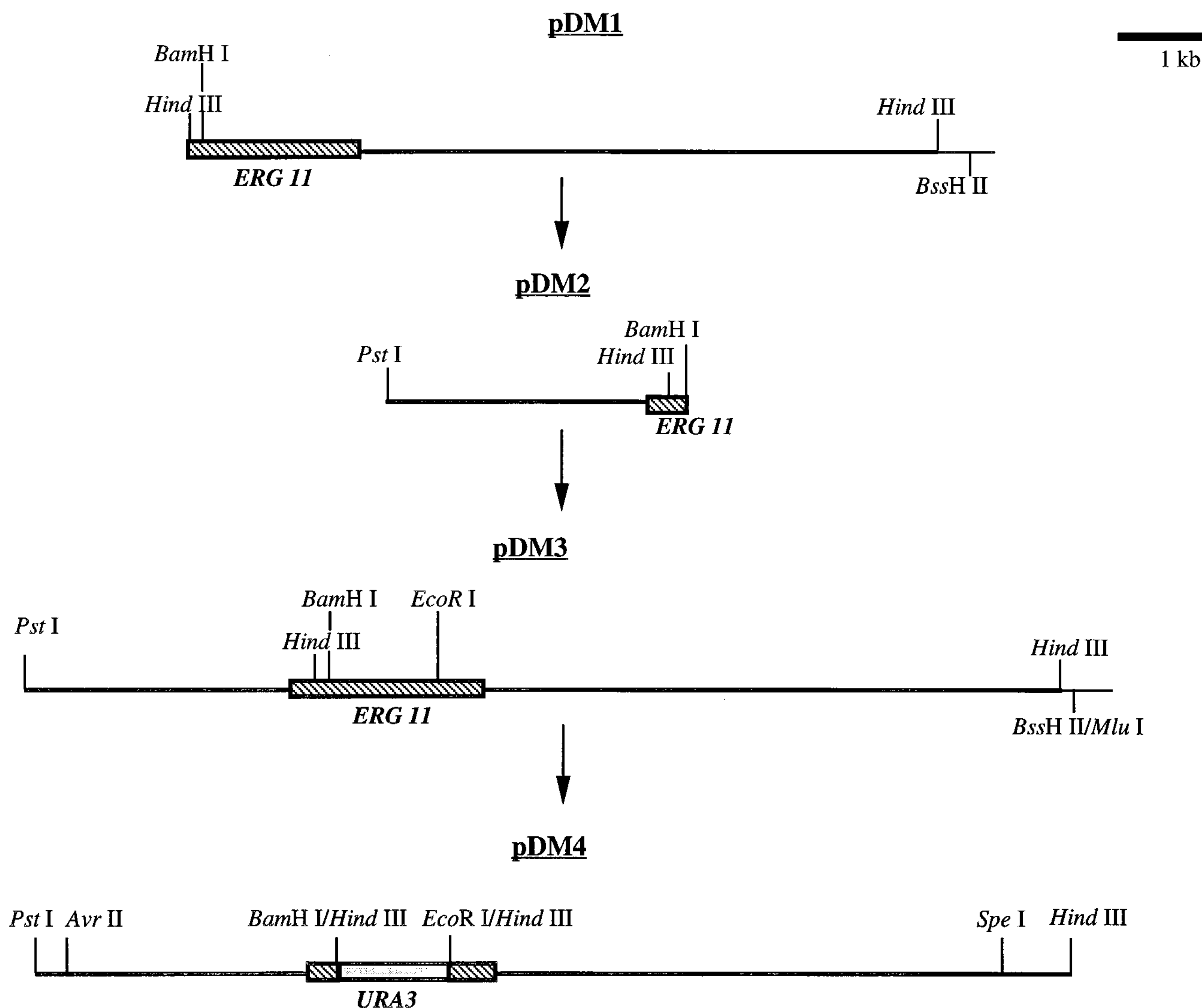

FIG. 2. Vector pDM1 contains a 6.5-kb *C. glabrata Hin*dIII fragment cloned into pBSK. This fragment contains sequences which encode all but 183 bases of the 5′ end of the *ERG11* gene ORF as well as 3′ flanking sequences. pDM2 is an ~2.7-kb DNA cloned into pSPORT 1. The fragment encodes the missing 5′ region of the *ERG11* ORF and 5′ flanking sequences. The *Bam*HI-*Bss*HII fragment of pDM1 was ligated into *Bam*HI-*Mlu*I-digested pDM2 to form pDM3. pDM3 was digested with *Bam*HI-*Eco*RI and blunt ended. The *S. cerevisiae URA3* gene, obtained as a 1.1-kb *Hin*dIII fragment from YEp24, was blunt ended and ligated into pDM3, forming the deletion construct pDM4.

sterols were quantified by electronic integration of chromatogram peaks, and the results are expressed as percent area. For GC-MS analyses, TMS-sterols were fractionated by using a Fisons MD800 dedicated GC-MS instrument with a 25-m fused silica column (internal diameter, 0.25 mm) of CPSil-8CB (Chrompak). The oven temperature ramp was 200°C for 2 min, rising to 300°C at a rate of 20°C/min. Injection (1 μl) was via a splitless injector (40:1) at 270°C, and the carrier gas was He. The MS electron energy was 70 eV, and the electron current was 100 μA. Sterols were identified by comparison of their fragmentation data with those in reference databases (National Institute for Standards and Technology, Gaithersburg, Md.) and publications (14, 34).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been submitted to the Genome Sequence Data Base. The nucleotide sequence accession number for the *C. glabrata ERG3* gene is L40390. The accession number for the nucleotide sequence of the *C. glabrata ERG11* gene is L40389.

RESULTS

Sequence analysis of the *C. glabrata ERG3* gene. The sequence of the *C. glabrata ERG3* gene and its nearby flanking sequence is shown in Fig. 3. An ORF beginning with the ATG codon at position +1 extends 1,092 nucleotides and encodes 364 amino acids. The derived amino acid sequence of the product of the *C. glabrata ERG3* gene and the sequence of the *S. cerevisiae ERG3* gene product have a 71.6% identity. A hydrophobicity plot generated by the Kyte and Doolittle algorithm (17) identifies a 23-amino-acid segment with characteristics of transmembrane domains. The segment is located near the amino terminus, beginning with amino acid 97, and has a hydropathy value of 1.9. This sequence has 83% identity with a 24-amino-acid sequence in the *S. cerevisiae* protein which has an average hydrophobicity of 2.0 and begins at amino acid position 95. This area has been postulated to serve as a region which binds the protein to the endoplasmic reticulum (2).

Analysis of the *ERG3* gene sequence of L5LUD40R (*LEU2 erg11*Δ::*URA3*) did not reveal the presence of any mutations.

Sequence analysis of the *C. glabrata ERG11* gene. The sequence of the *C. glabrata ERG11* gene and nearby flanking sequences is shown in Fig. 4. An ORF beginning with the ATG codon at position +1 extends 1,599 nucleotides and encodes

TABLE 2. Oligonucleotides used in PCRs

Oligonucleotide	Sequence	Description
DSS1	ATG GAT TTG GTC TTA GAA	*S. cerevisiae ERG3* primer
DSS2	CTT CTT GGT ATT TGG GTC	*S. cerevisiae ERG3* primer; antisense
DMS1	GTG TGA AGA TGA CTG ATC AAG AAA T	*S. cerevisiae ERG11* primer
DMS2	ATA CAT CTG TGT CTA CCA CCA CC	*S. cerevisiae ERG11* primer; antisense
CGDX2	TGG ACC ATT ACA TCT TCG	*C. glabrata ERG3* primer
CGDX8	CTC TAG CAT AAA CCA AGG	*C. glabrata ERG3* primer; antisense
CGDM3	GAC ATC TGG TCT TTC AGC	*C. glabrata ERG11* primer; antisense
CGDM13	GTA CCA AGC CAT ACG AGT	*C. glabrata ERG11* primer
CGDM21	GCC ATT TGT GTA CAC CAT	*C. glabrata ERG11* primer
ACT1	TAT CGA TAA CGG TTC TGG	*C. albicans CAACT1A* primer
ACT2	CAT CAC ACT TCA TGA TGG	*C. albicans CAACT1A* primer; antisense

533 amino acids. A second ATG codon is found in frame with this sequence starting at position 103. On the basis of sequence homology with the *S. cerevisiae ERG11* gene, the first ATG codon would appear to represent the initiation codon. The derived amino acid sequence of the *C. glabrata* P-450_{DM} is more homologous to the sequence of *S. cerevisiae* (15), with an 84.5% identity, than to that of *C. albicans* (18), with which it has a 65% identity. *S. cerevisiae* P-450_{DM} contains a region

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-560   GTGTGAGTGT ATGCAGTGTG AGTATATGCA GTGCGAGTGT ATGTAAAGAA TGCAAGTACC ATAAGAATGC AAGTGTACAA

-480   GCATATGTAT GTGTAGTACA ACTACAAGTA GTACAAGTAG TACAAGTATA GATGTTATGT ATATCATGTG ACAGCGTCTG

-400   CAACGATATA CTATACGACT TCGTATAGTA TTGAACGGAG ATAAGGTATA CGACCGTATA GAGACTATAC GAGTGTGCTC

-320   TTTGCGGATC CCCCCCCCAG CGAGAGCTGC TAGAGCTGAG AAAAGTCTCT CGAGAGCTGC GAGAGCCTTG AGGAGAGATG

-240   AGGCCTGGAA GAAGAGCTGA TCTCTCTAGA AGTGAAAAAT TTCATCCCAT TGTCTGGGCC CATACGACCG TTACATAATT

-160   GCCCAGTGCA GCCATCGGTT TTTACCTATA CGTGGGAACT ACGAGAACAA GAGCTAAGAG TATAAATATT GGGTACATTT

-80    GTCTTGCATT TCAGATAACC TACAGCCAGT AGAAGAAAAA AAAAAAAAAA CAGCACTAAG CTTTTATACA AAAACAAATA

+1     ATGGATTTGG TGTTGGAAAC TTTGGACCAT TACATCTTCG ACGATGTGTA TGCCAAGATT GCGCCTGTTG AGTTGCAACG
+81    TGGTATCGAC GACTCGTTGG TCAATGCTTT GTCTCTGAAT AAGATCGTCT CTAACTCTAC GCTGCTGCAT GAGACCTTGT
+161   CTATCACTAA CTCCTTGAAG CGTGTGAACA AGGACGTCTA CGGGTTGACT CCCTTCTTGT TTGACTTCAC AGAGAAGACC
+241   TACGCGTCTT TGCTGCCAAG AAACAACCTG ATCAGAGAGT TCTTCTCCCT GTGGGCTGTC GTCACCGTCT TCGGTCTGCT
+321   ATTGTACCTG ATCACCGCCT CTTTGTCCTA CGTTTTCGTC TTCGACAGAA CTATCTTCAA CCATCCAAAA TACTTGAAGA
+401   ACCAGATGTA CCTGGAGATC AAGTTGGCTG TCTCTGCCAT CCCTACTATG TCCCTCTTGA CTGTCCCTTG GTTTATGCTA
+481   GAGCTGAACG GATACTCTAA GCTGTACTAC GATGTCGACT GGGAGCACCA CGGTCTAAGG AAACTGCTGA TCGAGTACGC
+561   CACTTTCATC TTCTTCACCG ACTGTGGTAT CTACTTGGCT CACAGATGGC TGCACTGGCC TCGTGTCTAC AAGGCCTTGC
+641   ACAAGCCTCA CCACAAGTGG TTGGTCTGCA CTCCATTCGC CTCCCACGCC TTCCACCCAG TCGACGGTTA CTTCCAATCC
+721   TTGTCCTACC ACATCTACCC AATGATCCTG CCTCTACACA AGATCTCCTA CTTGATCCTG TTCACCTTCG TCAACTTCTG
+801   GTCCGTTATG ATCCACGACG GTCAACACAT GTCCAACAAC CCAGTTGTCA ACGGTACCGC CTGCCACACC GTCCACCACT
+881   TGTACTTCAA CTACAACTAC GGTCAGTTCA CCACCTTGTG GGACAGATTG GGTGGCTCCT ACAGAAGACC AGAGGACTCC
+961   CTGTTCGACC CTAAGCTAAA GATGGACAAG AAGGTCCTAG AAAAGCAAGC TAGAGAAACC GCCGCTTACA TCCAAGAGGT
+1041  GGAAGGTGAC GACACAGACA GAGTCTACAA CACCGACAAG AAGAAGACCA ACTAGACAGG AAACACCGGT GTCTCGCGCA

+1121  CACATTACTT TTCCTACATC ATAACTCTTT CATGACCCAT TTATTTTTGA TTTTTCACCT ACTAAATGAA CAATTACTAT

+1201  TTTTTTTTAT CATGGTTTAC CTTTGCAAAA CTATCAAAAA GGAAAAAGAA ATACAACCAC TATAGACTCT TTTTTTTTTC

+1281  AACTTATCTT TTTGTTAATT TATTCATTCA TTTCCAGAAT TATTTTCTTT ATGTCCATTA TTCTGATGAG TTATTTTTTT

+1361  CCTCAGAGAA AACTCAAGTG CTGACCTGGG TTCTCATATT TTTTATGGCA ACAAATTAAC GCTTATATAT GAAAGTCTCT

+1441  TACGTATCAA GAACTATACA CAATTTTTAT ATACCAATAT GAAATAGAAG TTCACTCAAG AACTATTTAC TCAAATAACG

+1521  GTCCTCTTTA ATCATGTACT GACCCTCCTA CACCAATCAT CGTATGCAGC TATGGCTTAC CAACCTTTGA TCTGCGATGC

+1601  GCATTACTTC TCAAAATGTG GTAAGCTAAA TTGAGTCAGG AACTTAGCTT GCATATAGAA GGTCACCCGG TAGTCCCGGC
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FIG. 3. Nucleotide sequence of the *C. glabrata ERG3* gene. The ORF begins at base +1 and extends to the end codon beginning at base 1093. Three putative TATA(A) boxes upstream of the initiation codon are double underlined. A tripartite sequence similar to the yeast transcriptional termination codon described by Zaret and Sherman (36) is identified in the 3′ noncoding sequences by a double line. The termination codon is underlined with a single line.

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-560  AAAAGCCTTG TTCCAACTAC AATCGAGTGA GCTTGAAGAG TTATAGTGAG ATGGGAGCAC AGGAGTACAA GGGTACAAAG

-480  AGCACTTCAC AGCACAGAAG CAGAAAACCC ACGTTCGCCT GCACCAAACG ACATGAGATA AGGATTTATC GCTTCTTCTC

-400  TAATCCAGTT GCACACATCG TATACCCGAG TGACATCTTT GCAAGCCCTT CCCATCACTG ATTAGACAAT GGAAACAGCT

-320  TAAACCCAAT TGCTGTGTCT TGTTTATCGG TCCATCTCTG TTTCTTTTTT TTTATGGCCT TTGCTCAGCA CAGTGAAAAC

-240  CCTTAACAAA CTGCAATTTT GAAAAAAAAA AATTGAAAAA AAATTTTATC ATCGTATATT CCACCTCGAA GAACCCGTAT

-160  ACTCATCTCG TATAATCAGT AGTCAAGACT TGTGCTAAAC ATCTTTACAA AAAAATGTAT ATAATGGGCG ATCCCTTCAT

-80   GTCCATTGTC TTGAATTTCT TGTTCTCATT GTCTATTTCG ATACTACAAA CCACATAAAT CAAAAAAAAA CTACAATAAC

+1    ATGTCCACTG AAAACACTTC TTTGGTCGTT GAACTATTGG AGTACGTGAA GCTTGGTCTT TCGTACTTCC AAGCTCTGCC
+81   ATTGGCGCAG AGAGTGTCTA TTATGGTCGC CTTGCCATTT GTGTACACCA TCACATGGCA ATTGCTTTAC TCCTTGAGAA
+161   AGGACAGACC ACCACTTGTG TTCTACTGGA TCCCATGGGT CGGCTCTGCT ATCCCATACG GTACCAAGCC ATACGAGTTC
+241   TTCGAAGACT GCCAAAAGAA ATACGGTGAT ATCTTCTCTT TCATGCTATT GGGTAGAATT ATGACTGTCT ACTTGGGTCC
+321   AAAGGGTCAC GAATTCATCT TCAACGCCAA GTTGGCCGAT GTTTCCGCTG AAGCTGCTTA CTCCCACTTG ACCACCCCAG
+401   TGTTCGGTAA AGGTGTTATC TACGATTGTC CAAACCACAG ACTAATGGAA CAAAAGAAGT TTGTCAAGGG TGCTTTGACT
+481   AAGGAAGCCT TTGTCAGATA CGTTCCATTG ATCGCTGAGG AAATCTACAA GTACTTCAGA AACTCCAAGA ACTTCAAGAT
+561   CAACGAAAAC AACTCCGGTA TCGTCGACGT TATGGTCTCC CAACCTGAAA TGACTATCTT CACTGCTTCC AGATCCTTGC
+641   TAGGTAAGGA AATGAGAGAC AAGTTGGACA CCGACTTCGC TTACTTGTAC AGTGACTTGG ACAAGGGTTT CACCCCAATT
+721   AACTTCGTCT TCCCTAACTT GCCTCTAGAA CACTACAGAA AGAGAGATCA TGCCCAACAA GCTATCTCTG GTACTTACAT
+801   GTCCTTGATT AAGGAAAGAC GTGAGAAGAA CGATATCCAA AACCGTGACT TGATTGATGA ATTGATGAAG AACTCCACTT
+881   ACAAGGATGG TACTAAGATG ACCGACCAAG AAATTGCCAA CCTATTGATT GGTGTCTTGA TGGGTGGTCA ACATACTTCC
+961   GCTGCTACCT CCGCTTGGTG TCTATTGCAT TTGGCTGAAA GACCAGATGT CCAAGAAGAA TTATACCAAG AACAAATGCG
+1041  CGTCTTGAAC AACGATACCA AGGAATTGAC TTACGATGAC CTACAAAACA TGCCTCTATT GAACCAAATG ATCAAGGAAA
+1121  CTTTGAGATT GCACCACCCA TTGCACTCTT TGTTCCGTAA AGTCATGAGA GATGTCGCTA TTCCAAACAC TTCCTACGTT
+1201  GTCCCAAGGG ACTACCACGT TCTAGTCTCC CCAGGTTACA CTCACTTGCA AGAAGAATTC TTCCCTAAGC CAAATGAATT
+1281  CAACATCCAC CGTTGGGACG GTGATGCTGC TTCTTCCAGT GCTGCTGGTG GTGACGAAGT TGATTACGGT TTCGGTGCTA
+1361  TCTCCAAGGG TGTTTCCTCT CCATACTTGC CATTCGGTGG TGGTAGACAC AGATGTATCG GTGAATTGTT CGCTTACTGT
+1441  CAATTGGGTG TGTTGATGTC CATTTTCATC AGAACCATGA AATGGCGTTA CCCAACTGAA GGTGAAACTG TCCCACCATC
+1521  TGACTTCACC TCCATGGTCA CCCTACCAAC TGCCCCTGCT AAGATCTACT GGGAAAAGAG ACATCCAGAA CAAAAGTACT

+1601  AGATGCTTTC AAAATATTAA ACATTTTCTT CCAATAATCA ATGGTCAAAA CCTACTTCAA ACAATATTGC TGTCTGGCTC

+1681  GTATACGGGA TATACGCTGA TTCATTAGCC TAATAGTAAG TCATCGTGAA TATCAACATG GATATTTCGG CATGACTTAA

+1761  GCTGGTTGTT TCGTTTAGAG GGCTTTGCTG CTAAACGAAC TATACGATGT CTTCATCCGT ATAACTAGCA ATGTATGCAG

+2041  TGCAAGCATA TGACAAAGTT GGTTGATCCC AAAGATTGTT TTAAAAAAAT TCATTTTCAT GATCAAAATT CAATGCTTCT

+2121  TATACTACAT TTTTTTCATT TTCATTCATC TTTATTTTGG TGCCATTAAC ATATAGACTT AAGTTTCATT TACTATTTTA

+2201  TATTTATTAT AAAATTTAAA CCGTGTATAC AATACAATAT TATTTAACAA ATCGAACTCT TGTATTATTA TCAAACTTTA

+2281  CCTTGGTAAG CCTAAAGAAA CCACGTTTT
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FIG. 4. Nucleotide sequence of the *C. glabrata ERG11* gene. The ORF begins at base +1 and extends 1,599 nucleotides to the end codon beginning at base 1600. Two putative TATA boxes in the 5′ flanking region and the yeast transcription termination signal in the 3′ flanking region are double underlined. The termination codon is underlined with a single line.

near the amino terminus which is 20 amino acids in length, has a hydropathy value greater than 1.6, and is followed immediately by cationic residues. These features are characteristic of membrane-anchoring peptides (23). While the *C. glabrata* amino terminus is mostly hydrophobic, no region with the above-mentioned characteristics could be identified. The putative heme-binding domain, or HR2 region, of P-450 proteins (9) can be identified in the *C. glabrata* protein and surrounds the cysteine residue at position 465. The cysteine residue has been postulated to provide the proximal thiolate ligand to heme and is surrounded by a number of invariant residues, a phenylalanine at residue −7, and glycine at residues −4 and +2 with respect to the cysteine. The HR2 regions of the *C. glabrata* and *S. cerevisiae ERG11*-derived proteins are 95% identical (15).

Deletion of the *ERG3* gene. Twenty-four colonies of *C. glabrata* L5 (*leu2*) which were transformed with pCGDL7 were initially screened by PCR, with oligonucleotides CGDX2 and CGDX8 (Table 2), to determine if homologous integration had taken place. Five of 24 transformants tested appeared to have undergone homologous integration with loss of the ORF. Gene deletion in one of these transformants, L5D (*erg3* Δ::*LEU2*), was confirmed by Southern blot analysis (Fig. 5).

Deletion of the *ERG11* gene. Following growth under anaerobic conditions, 29 of the L59L (*LEU2 ura3*) colonies transformed with the *erg11* deletion construct were plated on MING

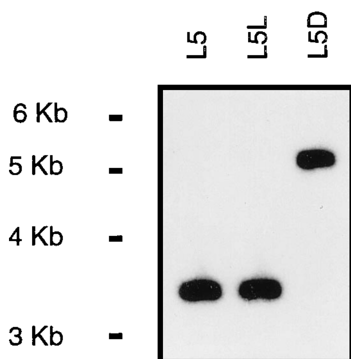

FIG. 5. Southern blot of *Eag*I-digested DNA extracted from L5 (*leu2*), L5L (*LEU2*), and an *ERG3* deletion mutant, L5D (*erg3*Δ::*LEU2*), probed with a 1.7-kb *Xho*I-*Nco*I DNA fragment of pCGDSB containing sequences 5′ relative to the *C. glabrata ERG3* gene. The probe hybridizes to a ~3.4-kb band in the L5 (*leu2*) and L5L (*LEU2*) lanes, whereas in the L5D (*erg3*Δ::*LEU2*) lane it hybridizes to a 5-kb band. The size difference is accounted for by the 1.6-kb difference in size between the deleted *ERG3* sequences in L5D (*erg3*Δ::*LEU2*) and the sequences encoding the *S. cerevisiae LEU2* gene which replaced them.

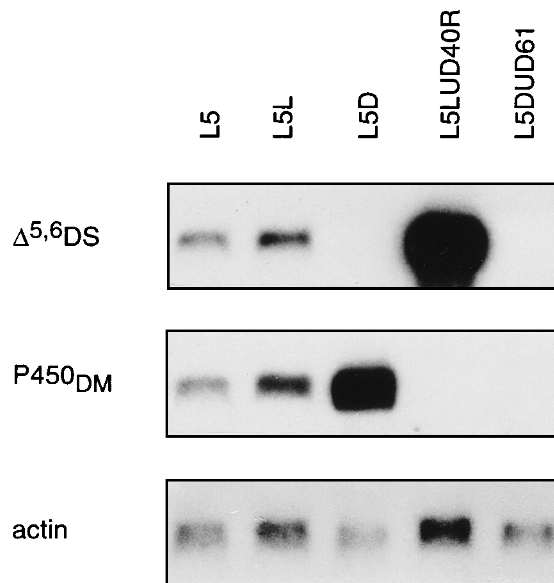

FIG. 7. Northern blot of RNA extracted from L5 (*leu2*), L5L (*LEU2*), L5D (*erg3*Δ::*LEU2*), L5LUD40R (*LEU2 erg11*Δ::*URA3*), and L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*). The blot was probed sequentially with sequences of the *ERG11* ORF, the *ERG3* ORF, and the *C. albicans* actin gene. No P-450_{DM} transcript is detected in *ERG11* deletion mutants, L5LUD40R (*LEU2 erg11*Δ::*URA3*) and L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*). However, a marked increase in P-450_{DM} message is seen in the *ERG3* deletion mutant, L5D (*erg3*Δ::*LEU2*). $\Delta^{5,6}$ desaturase ($\Delta^{5,6}$DS) transcript is not detected in L5D (*erg3*Δ::*LEU2*) and L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*), but the amount of $\Delta^{5,6}$ desaturase message is significantly increased in strain L5LUD40R (*LEU2 erg11*Δ::*URA3*). The actin probe was used as a control to quantitate relative amounts of mRNA in each lane.

media supplemented with ergosterol and Tween and grown under both aerobic and anaerobic conditions. All grew anaerobically, but only 14 of 29 grew aerobically. PCR amplification was done with oligonucleotides complementary to sequences of the *S. cerevisiae URA3* gene to confirm that transformation with pDM4 had occurred. The oligonucleotides CGDM3 and CGDM13 (Table 2) were used to screen for homologous transformation. All colonies were found to contain the *S. cerevisiae URA3* gene, indicating that transformation with pDM4 had occurred. The aerobically viable colonies contained the deleted sequences of the *C. glabrata ERG11* deletion construct, demonstrating a lack of homologous integration in these colonies (data not shown). Deletion of the *ERG11* gene in one of the aerobically nonviable colonies, L5LUD40 (*LEU2 erg11 Δ::URA3*), was confirmed by Southern blot analysis (Fig. 6). When *ERG11* deletion mutants were plated aerobically, spontaneous mutants which were aerobically viable were found to arise from a background lawn of colonies. An aerobically viable spontaneous mutant, L5LUD40R (*LEU2 erg11*Δ::*URA3*), recovered from strain L5LUD40 (*LEU2 erg11*Δ::*URA3*), was studied by Southern (Fig. 6) and Northern (Fig. 7) blot analyses, and its sterol profile was determined.

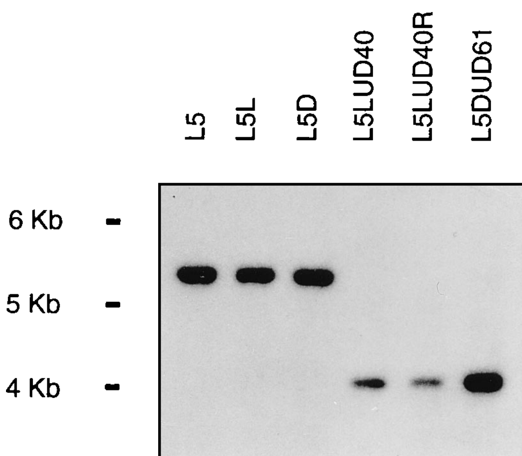

FIG. 6. Southern blot of *Ava*I-digested DNA extracted from L5 (*leu2*), L5L (*LEU2*), L5D (*erg3*Δ::*LEU2*), L5LUD40 (*LEU2 erg11*Δ::*URA3*), L5LUD40R, and L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*). The blot was probed with a 1.3-kb DNA fragment encoding sequences of the *ERG11* ORF which were not deleted from pDM4 and of 3′ flanking sequence. The probe was obtained with the oligonucleotides CGDM21 and CGDM3 (Table 2). The probe hybridizes to an ~5.3-kb band in the first three lanes, which contain DNA from strains with intact *ERG11* genes. In *ERG11* deletion mutants, the probe hybridizes to a band of ~4 kb. *Ava*I restriction sites are found in the 3′ and 5′ flanking sequences of the *ERG11* gene, but no sites are found within the ORF. In the deletion mutants, sequences from the *ERG11* ORF have been replaced by the *S. cerevisiae URA3* gene, which has one *Ava*I restriction site in its ORF. This accounts for the smaller band seen with *ERG11* deletion mutants.

Transformants of the L59D (*erg3*Δ::*LEU2 ura3*) strain were initially plated on MING supplemented with ergosterol and Tween and grown in an anaerobic environment. Forty-seven of these were then plated aerobically and anaerobically, in the same manner as for the L59L (*LEU2 ura3*) transformants. While all were aerobically viable, approximately one-third of the transformants grew at a much slower rate than the others. PCR analysis confirmed that homologous integration and deletion of the *ERG11* gene had taken place in these isolates (data not shown). Southern analysis confirmed deletion of the *ERG11* gene in one of these, L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*) (Fig. 6).

Northern blot analysis. A Northern blot of whole-cell RNA extracted from *C. glabrata* L5 (*leu2*), L5D (*erg3*Δ::*LEU2*), L5L (*LEU2*), L5LUD40R (*LEU2 erg11*Δ::*URA3*), and L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*) was probed with a radiolabeled *Xho*I-*Nco*I DNA fragment of pCGDSB, which encodes the *ERG3* ORF, and a DNA fragment of the *ERG11* ORF obtained by PCR with oligonucleotides CGDM3 and CGDM13 (Table 2). A fragment of the *C. albicans* actin gene was used as a control probe to quantify amounts of RNA. No *ERG3* transcript can be detected in the *ERG3* deletion mutants L5D

TABLE 3. Sterol compositions (percentage by weight) of various *C. glabrata* strains

Strain (genotype)	Sterol[a]	Sterol composition (% by wt)
L5 (*leu2*)	Ergosterol	100.0
L5L (*LEU2*)	Ergosterol	86.5
	Zymosterol	7.3
	4,4-Dimethyl-cholesta-5,7-dien-3β-ol	6.2
L5D (*erg3*Δ::*LEU2*)	Ergosta-7,22-dien-3β-ol	93.2
	Ergosta-8,22-dien-3β-ol	5.1
	Ergosta-7-en-3β-ol	1.7
L5LUD40R (*LEU2 erg11*Δ::*URA3*)	Lanosterol	81.0
	Obtusifoliol	16.5
	4,14-Dimethylzymosterol	2.5
L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*)	14α-Methylfecosterol	63.0
	Lanosterol	31.5
	Obtusifoliol	4.5
	4,14-Dimethylzymosterol	1.0

[a] Sterols were extracted from mid-exponential-phase cultures and analyzed by GC-MS.

(*erg3*Δ::*LEU2*) and L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*). Increased *ERG3* message is present in the *ERG11* deletion mutant L5LUD40R (*LEU2 erg11*Δ::*URA3*). On probing with the PCR fragment of the *ERG11* ORF, no transcript is detected in the *ERG11* deletion mutants, L5LUD40R (*LEU2 erg11*Δ::*URA3*) and L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*). Markedly increased amounts of *ERG11* transcript are detected in the *ERG3* deletion mutant L5D (*erg3*Δ::*LEU2*) (Fig. 7). L5 (*leu2*) RNA and serial dilutions of L5LUD40R (*LEU2 erg11*Δ::*URA3*) and L5D (*erg3*Δ::*URA3*) RNA were probed with the *ERG3* gene and the *ERG11* gene, respectively, again by using the actin gene as a control. These studies revealed a >20-fold increase in the amount of *ERG3* message in L5LUD40R (*LEU2 erg11*Δ::*URA3*) and the amount of *ERG11* message in L5D (*erg3*Δ::*LEU2*) in comparison with L5 (*leu2*) message (data not shown).

Sterol analysis. The sterol profiles of strains L5 (*leu2*), L5D (*erg3*Δ::*LEU2*), L5LUD40R (*LEU2 erg11*Δ::*URA3*), and L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*) are shown in Table 3. Ergosterol was the major sterol detected in the parent *C. glabrata* strains L5 (*leu2*) and L5L (*LEU2*), in common with other wild-type yeasts and fungi. However, it was replaced mainly by ergosta-7,22-dien-3β-ol in strain L5D (*erg3*Δ::*LEU2*), consistent with the deletion of *ERG3*, which encodes $\Delta^{5,6}$ sterol desaturase. Sterol analysis was not carried out on strain L5LUD40 (*ERG11*, encoding P-450_{DM}, deleted) because it is viable only in anaerobic medium supplemented with ergosterol. We reasoned that under these conditions, the sterol profile would reflect mainly exogenously supplied ergosterol rather than sterols synthesized de novo. Furthermore, exogenous ergosterol could influence the synthesis and composition of endogenous sterols, as might the absence of P-450_{DM} activity, thereby precluding a meaningful interpretation of the data. However, sterol analysis was carried out on strain L5LUD40R (*LEU2 erg11*Δ::*URA3*), a spontaneous mutant derived from L5LUD40 (*LEU2 erg11*Δ::*URA3*). Unlike L5 (*leu2*) and L5L (*LEU2*), which contained mainly ergosterol, L5LUD40R (*LEU2 erg11*Δ::*URA3*) biosynthesized the 14α-methylated sterols, lanosterol, obtusifoliol, and 4,14α-dimethylzymosterol. By contrast, the sterol fraction of the double mutant, L5DUD61 (*ERG3* and *ERG11* deleted), consisted of mainly 14α-methylfecosterol, with smaller amounts of lanosterol, obtusifoliol, and 4,14α-dimethylzymosterol.

Antifungal susceptibility. The results of antifungal susceptibility testing are shown in Table 4. Strain L5D (*erg3*Δ::*LEU2*) remained susceptible to azole and polyene antifungal agents. Strains L5LUD40R (*LEU2 erg11*Δ::*URA3*) and L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*) were resistant to the highest concentrations of azole antifungal agents tested and showed a two- to threefold increase in resistance to amphotericin B.

Generation times. The generation times at 30°C were as follows. Strains L5 (*leu2*) and L5L (*LEU2*), which accumulate mostly ergosterol, had generation times of 1.21 and 1.24 h, respectively. L5D (*erg3*Δ::*LEU2*), whose sterol fraction consisted of mostly ergosta-7,22-dien-3β-ol, had a generation time of 1.76 h. L5LUD40R (*LEU2 erg11*Δ::*URA3*), which contains

TABLE 4. Susceptibilities of *C. glabrata* strains to antifungal agents

C. glabrata strain (genotype)	MIC (μg/ml) of:					
	Fluconazole		Itraconazole		Amphotericin B	
	24 h	48 h	24 h	48 h	24 h	48 h
NCCLS 84 (wild type)	6.25	12.50	0.50	1.00	0.50	1.00
L5 (*leu2*)	3.13	6.25	0.25	0.50	0.25	0.50
L5L (*LEU2*)	3.13	6.25	0.25	0.50	0.25	0.50
L5D (*erg3*Δ::*LEU2*)	3.13	3.13	0.25	0.25	0.13	0.13
L5LUD40R (*LEU2 erg11*Δ::*URA3*)	>100.00	>100.00	>16.00	>16.00	2.00	2.00
L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*)	>100.00	>100.00	>16.00	>16.00	2.00	2.00

primarily lanosterol, had a generation time of 2.25 h. L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*), which accumulated mostly 14α-methylfecosterol, grew more slowly with a generation time of 3.73 h.

DISCUSSION

While mutations in the *S. cerevisiae ERG3* gene have been shown to be associated with azole resistance (34) and lack of $\Delta^{5,6}$ desaturase activity is associated with azole resistance in *C. albicans* (14), we have not shown a similar effect following disruption of the *ERG3* gene in *C. glabrata* L5 (*leu2*). These data reveal differences in the development of azole resistance between these closely related yeast species. It has been postulated that the *S. cerevisiae ERG3* gene may also play a role in azole resistance by exerting a suppressor effect on otherwise lethal mutations of the *ERG11* gene, which encodes P-450_{DM}, the target enzyme of azole antifungal agents (4, 27, 33). We have demonstrated the same protective result of the presence of a defective *ERG3* gene in *C. glabrata* L5DUD61 (*erg3* Δ::*LEU2 erg11*Δ::*URA3*), in which the *ERG11* gene was disrupted following disruption of the *ERG3* gene. This strain is aerobically viable, accumulates 14α-methylfecosterol, and is resistant to azole antifungal agents. In contrast, following the disruption of the *ERG11* gene in strain L5L (*LEU2*), which has a functional *ERG3* gene, L5LUD (*LEU2 erg11*Δ::*URA3*) strains required ergosterol and anaerobic conditions for growth. However, our studies also show that another mechanism unrelated to mutation or disruption of the *ERG3* gene allows *C. glabrata* to overcome the lethal effect of an *ERG11* gene disruption. L5LUD40R (*LEU2 erg11*Δ::*URA3*), an aerobically viable strain arising spontaneously from *C. glabrata* L5LUD (*LEU2 erg11*Δ::*URA3*), did not have a defective *ERG3* gene as determined by DNA sequence analysis and Northern blot analysis. Aerobically viable strains have also been spontaneously recovered from *S. cerevisiae erg11* mutants, but these strains have been shown to harbor mutations in the *ERG3* gene (4, 27, 34).

The *C. glabrata* strains with disrupted *ERG11* genes, L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*) and L5LUD40R (*LEU2 erg11*Δ::*URA3*), were resistant to azole antifungal agents and also showed increased resistance to the polyene antifungal agent amphotericin B. The resistance to azole antifungal agents is not surprising, since *ERG11* encodes the target enzyme of these agents. The increased amphotericin B resistance of these mutants is less striking: a two- to threefold increase was observed in comparison with resistances of the parent strains L5 (*leu2*), L5L (*LEU2*), and L5D (*erg3*Δ::*LEU2*). This difference, although small, may be of clinical significance and suggests that strains defective in removal of the C-14 methyl group are less suitable targets for this membrane-active agent than are strains with functional P-450_{DM} activity. This finding is in agreement with studies of *C. albicans* 6.4, which is resistant to both azole and polyene antifungal agents and accumulates only C-14-methylated sterols (11).

As in *S. cerevisiae*, disruption of the *ERG3* gene in *C. glabrata* results in the accumulation of ergosta-7,22-dien-3β-ol. *C. albicans* strains postulated to harbor mutations in the *ERG3* gene have been shown to accumulate either ergosta-7,22-dien-3β-ol (25, 26) or fecosterol (14) as their major sterol. Accumulation of the latter sterol suggests that desaturation of the $\Delta^{5,6}$ bond may be required for activity of $\Delta^{8,7}$ isomerase, Δ^{22} desaturase, or $\Delta^{24(28)}$ reductase. The difference in sterol composition between *C. albicans* $\Delta^{5,6}$ desaturase mutants may therefore reflect strain-to-strain variation or defects in sterol biosynthesis genes in addition to those encoding $\Delta^{5,6}$ sterol desaturase.

As in *S. cerevisiae*, sequential disruption of the *ERG3* and *ERG11* genes in *C. glabrata* produced a mutant which accumulates primarily 14α-methylfecosterol. Bard et al. (4) have demonstrated, by constructing strains with null mutations in both *ERG3* and *ERG11* genes, that *S. cerevisiae* does not require C-14-demethylated sterols for aerobic growth. We have shown that this is also the case with *C. glabrata* L5DUD61 (*erg3*Δ::*LEU2 erg11*Δ::*URA3*) and L5LUD40R (*LEU2 erg11* Δ::*URA3*).

The 14α-methylated sterol fraction of L5LUD40R (*LEU2 erg11*Δ::*URA3*) contained mainly lanosterol and smaller amounts of obtusifoliol and 4,14-dimethylzymosterol. This indicates that, as with *S. cerevisiae*, lanosterol appears to be the preferred substrate for the P-450_{DM} of *C. glabrata*. However, Aoyama and Yoshida (1), using P-450_{DM} purified from *S. cerevisiae*, have shown that the enzyme is equally active on 24-methylenedihydrolanosterol. The 14α-methylated sterol fractions of *C. albicans* and filamentous fungi contain primarily 24-methylenedihydrolanosterol (11, 21, 30). Hitchcock et al. (12) purified the *C. albicans* P-450_{DM} and found that the enzyme was 10- to 20-fold less active than that of *S. cerevisiae* when tested in a model membrane system with lanosterol as the substrate. One possible explanation given for this result was that the preferred substrate of *C. albicans* enzyme may be 24-methylenedihydrolanosterol. Further studies are needed to definitively identify the natural substrate of the *C. glabrata* P-450_{DM}.

We have demonstrated a marked increase in the amount of *ERG11* transcript in L5D (*erg3*Δ::*LEU2*). A similar increase in the amount of *ERG3* transcript was seen to occur in L5LUD40R (*LEU2 erg11*Δ::*URA3*) following deletion of the *ERG11* gene. These data suggest that ergosterol plays a role in the regulation of genes involved in its biosynthesis through a negative-feedback mechanism. Turi and Loper (28) have shown that *S. cerevisiae ERG11* message levels are increased during growth in glucose, in the presence of heme, and during both oxygen-limited and anaerobic growth conditions. Activity of P-450_{DM} requires the presence of oxygen, and the increased levels of *ERG11* message seen under anaerobic growth conditions may have resulted from the production of 14α-methylated sterols which were unable to function as suppressors of *ERG11* transcription.

Construction of mutants with null mutations of genes involved in the biosynthesis of ergosterol and analysis of their sterol profiles provide important information regarding the biosynthesis of ergosterol in pathogenic yeast species. This information should prove useful in determining the mechanism(s) of resistance in clinical isolates. The use of sterol profiles alone and the interpretation of results obtained from Northern analysis are not sensitive tools in defining the genetic defects which lead to the development of resistance in pathogenic fungi.

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