Candida albicans Sterol C-14 Reductase, Encoded by the *ERG24* Gene, as a Potential Antifungal Target Site

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The incidence of fungal infections has increased dramatically, which has necessitated additional and prolonged use of the available antifungal agents. Increased resistance to the commonly used antifungal agents, primarily the azoles, has been reported, thus necessitating the discovery and development of compounds that would be effective against the major human fungal pathogens. The sterol biosynthetic pathway has proved to be a fertile area for antifungal development, and steps which might provide good targets for novel antifungal development remain. The sterol C-14 reductase, encoded by the *ERG24* gene, could be an effective target for drug development since the morpholine antifungals, inhibitors of Erg24p, have been successful in agricultural applications. The *ERG24* gene of *Candida albicans* has been isolated by complementation of a *Saccharomyces cerevisiae* *erg24* mutant. Both copies of the *C. albicans* *ERG24* gene have been disrupted by using short homologous regions of the *ERG24* gene flanking a selectable marker. Unlike *S. cerevisiae*, the *C. albicans* *ERG24* gene was not required for growth, but *erg24* mutants showed several altered phenotypes. They were demonstrated to be slowly growing, with doubling times at least twice that of the wild type. They were also shown to be significantly more sensitive to an allylamine antifungal and to selected cellular inhibitors including cycloheximide, cerulenin, flufenazine, and brefeldin A. The *erg24* mutants were also slightly resistant to the azoles. Most importantly, *erg24* mutants were shown to be significantly less pathogenic in a mouse model system and failed to produce germ tubes upon incubation in human serum. On the basis of these characteristics, inhibitors of Erg24p would be effective against *C. albicans*.

Over the past decade a number of factors have contributed to the growing incidence of fungal infections. Advanced, invasive surgical techniques and induced immunosuppression resulting from transplantation protocols and chemotherapies, coupled with disease conditions associated with human immunodeficiency virus infection, have all played major roles (13). This increase in infection rates has necessitated additional and prolonged treatments with the available arsenal of antifungal drugs. This, in turn, has led to the emergence of drug-resistant strains of pathogenic fungi.

The two most commonly used classes of antifungal drugs are the polyenes and the azoles. The former act by binding to ergosterol, the fungal membrane sterol, which induces the formation of channels through which essential cellular components leak out, thereby causing cell death (7). The polyenes are restricted in their application due to their limited solubilities and toxic side effects (27). The azoles are fungistatic compounds which are effective by virtue of their ability to inhibit the C-14 lanosterol demethylase in the ergosterol biosynthetic pathway. Inhibition of this enzyme by azoles results in the accumulation of C-14 methyl sterols, which do not permit normal membrane function (31).

The growing problem with resistance to the azole class of antifungal agents, the drugs most commonly used to treat hu-

man infections, has been noted in a variety of pathogenic fungi including *Candida albicans*, a common human pathogen (6). Resistance has been the result of several types of mutations which lead to altered forms of the demethylase that have reduced affinities for azoles (19, 36) and overexpression of the demethylase gene (*ERG11*) (35) as well as genes for drug efflux pumps (9, 28). The threat of the loss of efficacy of the azoles has prompted concerted efforts to discover new drugs that might block fungal growth at different metabolic sites (33). Additional steps in ergosterol biosynthesis may be effective targets to be explored in this search.

The reaction catalyzed by the sterol C-14 reductase may be a good target for an antifungal suitable for use by humans. This enzyme, encoded by the *ERG24* gene, is inhibited by the morpholine class of antifungals, which has proved effective against fungal pathogens of plants (2). A second enzyme in the pathway, the C-8 sterol isomerase, encoded by the *ERG2* gene, is also subject to inhibition by the morpholines. The *ERG24* gene of *Saccharomyces cerevisiae* has been cloned and characterized (21–23) and has been found to be essential for growth under normal conditions. The *erg24* phenotype can be suppressed by mutations in *FEN1* and *SUR4* (5, 22, 30). These mutations did not alter the sterols produced by the *erg24* mutant but permitted conditions that would allow growth with ignosterol, the primary sterol intermediate that accumulates in *erg24* mutants. *FEN1* and *SUR4* were subsequently found to be identical to *ELO2* and *ELO3*, respectively (26), genes involved in fatty acid elongation and sphingolipid synthesis. More recently, *erg24* mutants have been shown to be rescued under certain condi-

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tions (10), including supplementation with calcium or magnesium (11).

In order to determine whether Erg24p would be a suitable drug target in *C. albicans*, the *erg24* phenotype would have to be characterized. We report here on the isolation of the *C. albicans* *ERG24* gene and the sequential disruption of both alleles. The resulting phenotype in terms of growth properties, drug sensitivity, and pathogenicity indicate that an Erg24p inhibitor could be an effective antifungal compound.

MATERIALS AND METHODS

Strains, plasmids, and libraries. *S. cerevisiae* strain AP2 (*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 erg24::LEU2*) (21) was used to isolate the *C. albicans* *ERG24* gene following complementation with a *C. albicans* genomic library. *S. cerevisiae* strain YPH499, obtained from the American Type Culture Collection, is the *ERG24* wild type for AP2. *C. albicans* strain BWP17 (*ura3Δ::ximm434/ura3Δ::ximm434 his1::hisG/his1::hisG -arg4::hisG/larg4::hisG*) was obtained from A. Mitchell (37). *C. albicans* strain SC5314, used as a wild type for calcium rescue studies, was obtained from W. Fonzi (12). *C. albicans* strain DAY185 was a gift from A. Mitchell. This strain was derived from BWP17 and has been restored to prototrophy at the *HIS1*, *URA3*, and *ARG4* loci (A. Mitchell, personal communication) to serve as a control for the mouse survival studies with *C. albicans* *erg24* mutants. *Escherichia coli* strain DH5α was used to amplify plasmid DNA. Plasmids pGEM-URA3, pGEM-HIS1, and pRS-ARG4 were obtained from A. Mitchell (37); and plasmid pAB-SK-ARG4 was obtained from N. Ishii (15). Plasmid pRS316 was obtained from P. Heiter (29), and plasmid pDBI52 was obtained from C. Kumamoto (8). *C. albicans* genomic library 655 was obtained from S. Scherer.

Media and growth. *E. coli* cultures were grown on Luria broth containing 10 g of tryptone per liter, 5 g of yeast extract per liter, and 10 g of NaCl per liter. Solid Luria broth medium was made by adding agar (Difco) at 20 g/liter. Ampicillin at 60 μg/ml was added after autoclaving for plasmid selection. Cultures were grown at 37°C.

S. cerevisiae and *C. albicans* were grown on complete synthetic medium (CSM) or YPAD, an enriched medium. CSM contained 0.17% yeast nitrogen base without amino acids (Difco), 0.5% ammonium sulfate, 2% D-glucose, and a mixture of amino acids plus adenine and uracil (Qbiogene, Carlsbad, Calif.). CSM lacking specific nutrients was used for selection. YPAD comprised 1% Bacto Yeast Extract, 2% Bacto Peptone, and 2% D-glucose. Adenine at 360 mg/liter was added to the media used for *C. albicans* to ensure optimal growth, and uridine at 80 mg/liter was added to the media used for *C. albicans* *ura3* strains. Ergosterol, when used, was added to autoclaved and cooled media at 20 μg/ml to rescue *erg24* mutants in *S. cerevisiae*. *S. cerevisiae* cultures were grown at 30°C, and *C. albicans* cultures were grown at 30 or 37°C. For anaerobic growth, brewer's jars or the GasPak system (BBL Microbiology Systems, Cockeysville, Md.) was used. *C. albicans* for inoculation in the animal model was prepared by growth on Sabouraud dextrose agar (BBL Microbiology Systems) at 35°C. For virulence assays, *C. albicans* strains were grown on Sabouraud dextrose agar plates (0.5% casein pancreatic digest, 0.5% animal tissue peptic digest, 2% dextrose, 1.7% agar). To characterize the ability to make germ tubes, wild-type strains and *erg24* mutants were pregrown overnight in YPAD and inoculated into 10% human serum (Irvine Scientific, Santa Ana, Calif.) at 10⁵ cells/ml. Germ tube formation was assessed at 3 and 12 h postinoculation.

Transformations. *E. coli* DH5α cells were transformed by standard transformation protocols (1).

For transformation of *S. cerevisiae*, the high-efficiency lithium acetate (LiAc) transformation method from the Gottsching Laboratory (<http://www.umanitoba.ca/faculties/medicine/-units/biochem/gietz/Trafo.html>) was used. The cells were grown overnight in 50 ml of YPAD. A total of 0.5 ml of the culture was transferred into fresh 50 ml of YPAD and allowed to grow for two or three cell divisions. The cells were then spun down at 4,000 rpm at room temperature, washed twice with 10 ml of sterile water and once with 1× TE (Tris-EDTA)-1× LiAc solution, and resuspended in 1× TE-1× LiAc solution to give a cell concentration of 2 × 10⁹ cells/ml. Next, the cell solution was incubated at 30°C for 15 min and 50 μl of the cell solution was transferred to a microcentrifuge tube. A total of 10 μl of carrier DNA (10 μg/μl) (herring testis carrier DNA; Clontech Laboratories, Palo Alto, Calif.), 1 μg of the transforming DNA, and 300 μl of 40% polyethylene glycol (PEG) 3350-1× TE-1× LiAc solution were added to the tube; and the contents were mixed by vortexing. After incubation at 30°C for 30 min, the cells were heat shocked at 42°C for 20 min. Finally, the cells

were spun down by centrifugation, resuspended in sterile water, and plated out onto selective medium. The plates were incubated at 30°C for 3 days under the appropriate aerobic or anaerobic conditions.

The *C. albicans* transformation procedure was provided by A. Mitchell (personal communication). A single *C. albicans* colony was selected and then inoculated into a 250-ml flask containing 50 ml of YPAD plus uridine and was grown at 30°C overnight. A total of 0.5 ml of the culture was transferred into 50 ml of YPAD plus uridine and incubated at 30°C for 4 to 7 h (four generations). The cells were centrifuged at 4,000 rpm (IEC Model CL) for 5 min, washed in 10 ml of sterile water, and pelleted at 4,000 rpm for 5 min. The cells were then resuspended in 0.5 ml of 1× TE-1× LiAc solution. One hundred microliters of this yeast cell suspension was transferred into a new microcentrifuge tube containing 5 μl of transforming DNA (at least 2 μg) and 10 μl of carrier DNA (10 μg/μl). After incubation of the tube at room temperature for 30 min, 700 μl of 40% polyethylene glycol 3350-1× TE-1× LiAc solution was added to the tube, the contents were gently mixed, and the mixture was incubated overnight at room temperature. The next morning, the cell solution was heat shocked at 42°C for 60 min and centrifuged for 30 s. Finally, the cell pellet was resuspended in sterile water and then plated onto selective medium.

DNA sequencing. DNA sequencing was performed at the Biochemistry Biotechnology Facility in the Indiana University School of Medicine. Once initial sequence data were obtained with primers T3 and T7, new primers were designed to facilitate acquisition of additional DNA sequence data. All new primers for sequencing were purchased through Sigma-Genosys, Ltd. (The Woodlands, Tex.).

PCR. PCRs were carried out on a Perkin-Elmer GeneAmp2400 thermocycler with the following parameters: 94°C for 3 min for the first denaturation of the DNA template, followed by 30 to 40 cycles with three identical steps: 94°C for 45 s, 55°C (5°C below the primer annealing temperature) for 30 to 60 s for the primers to anneal to the denatured DNA, and 72°C for elongation (for 2.5 to 3.5 min, depending on the length of the expected DNA product), followed by 72°C for 10 to 15 min to complete all possible reactions. To avoid mispriming, a touchdown PCR was applied. Initially, the annealing temperature was maintained at the same temperature as the primer's denaturing temperature for two cycles and was gradually dropped 1 to 2°C per cycle for an additional two cycles until a temperature 5°C below the primer's melting temperature was reached. The PCR was then run for another 25 to 35 cycles at an optimal annealing temperature.

Two kinds of thermostable DNA polymerases were used. *Taq* DNA polymerase in Buffer B (Promega, Madison, Wis.) was mainly used to screen genetic constructions or genetic insertions. The reaction buffer contained 10× buffer without MgCl₂ (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 1% Triton X-100) plus 1.5 to 2.0 mM MgCl₂ and 1.25 U of polymerase. A second polymerase, Expand High Fidelity *Taq* polymerase (2.6 U per reaction mixture; Roche, Indianapolis, Ind.) was used to amplify selectable marker genes. The 10× buffer with 15 mM MgCl₂ (20 mM Tris-HCl [pH 7.5] 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% [vol/vol] Tween 20, 0.5% [vol/vol] Nonidet P-40, 50% [vol/vol] glycerol) was used.

The following primers were used in this study (the boldface represents plasmid sequences): for preparation of *ERG24* disruption cassettes, primers NJ-*ERG24*-5cl (TGAAATCGTCAAAATTGAATCCAGTCACAAACCATAAGGAGTTCATAGTATTTCTGGGGGTTTCCCACTCAGCAGCTT) and NJ-*ERG24*-3cl (TAATATACATAAGGAATTATTTTAAAGGAACACTAGCTTTTCGTACTTTCCCAATCCTCGTGTGGAATTGTGAGCGGATA); for confirmation of *ERG24* disruptions, primers NJ-*ERG24*-5ck (CTTTTATCCGTCGTACCC) and NJ-*ERG24*-3ck (CGGTATTTGGTCAGGAATC); and for confirmation of *ARG4* integration at *ADE2*, primers NJ-*ADE2*-5ck2 (CTTATTCTCATCACACGCAT) and NJ-*pMAL*-3' (CATAGCAATCATGGAATACGG).

Sterol analysis. Sterols were isolated as described previously (24) and analyzed by gas chromatography (GC). A Hewlett-Packard HP5890 series II chromatograph equipped with the Hewlett-Packard CHEMSTATION software package was used to analyze the sterol contents. The capillary column (DB-1) was 15 m by 0.25 mm by 0.25 μm (film thickness) (J&W Scientific, Folsom, Calif.) and was programmed from 195 to 280°C (1 min at 195°C and then an increase at 20°C/min to 240°C, followed by an increase at 2°C/min until the final temperature of 280°C was reached). The linear velocity was 30 cm/s, nitrogen was the carrier gas, and all injections were run in the splitless mode.

GC-mass spectrometry analyses of sterols were done with a Thermoquest Trace 2000 gas chromatograph interfaced to a Thermoquest Voyager mass spectrometer. The GC separations were done on a fused silica column (DB-5MS; 20 m by 0.18 mm by 0.18 μm [film thickness]; J&W Scientific). The injector temperature was 190°C. The oven temperature was programmed to remain at 100°C for 1 min, followed by a temperature increase of 6.0°C/min to a final

temperature of 300°C. The final temperature was held for 25 min. Helium was the carrier gas, the linear velocity was 50 cm/s, and all injections were run in the splitless mode. The mass spectrometer was in the electron impact ionization mode at an electron energy of 70 eV, the ion source temperature was 150°C, and scanning was done from 40 to 850 atomic mass units at 0.6-s intervals.

Growth analysis. Doubling times for yeast cultures were determined with a Klett-Summerson photoelectric colorimeter. Cells were inoculated into nephelometer flasks containing 20 ml of medium and were incubated in a Lab-Line shaker at 200 rpm and 30 or 37°C. The cell density was measured every 1 to 2 h until the growth reached the stationary phase. Density-versus-time plots were made on semilogarithmic paper to determine the doubling time of each strain.

Drug susceptibility testing. Each strain to be tested was inoculated into 50 ml of liquid medium and incubated in a shaker overnight at 30°C. The cell concentration was determined with a hemacytometer and was then diluted to give a series of cell concentrations of 2×10^7 , 2×10^6 , 2×10^5 , and 2×10^4 cells/ml. A total of 5 μ l of each concentration was added to solid YAPD to give a series of spots (spot plate assay) containing 10^5 to 10^2 cells. To determine the drug concentration that would inhibit all growth for 48 h, the plates were prepared with various drug concentrations. The plates were prepared with YPAD media with the drug to be tested. The spot plate assay was then conducted with the cell concentrations listed above. The MICs were those concentrations at which no growth on plates with the highest inoculum was observed after 48 h of incubation. Clotrimazole, brefeldin A, cerulenin, cycloheximide, nystatin, and fluphenazine were obtained from Sigma (St. Louis, Mo.). Fenpropimorph and tridemorph were obtained from Crescent Chemical Co. (Hauppauge, N.Y.), and ketoconazole was obtained from ICN Pharmaceuticals Inc. (Costa Mesa, Calif.). Terbinafine was a gift from I. Hapala (Slovak Academy of Science). Stock solutions of terbinafine, tridemorph, brefeldin A, and cerulenin were prepared in ethanol. Clotrimazole, ketoconazole, and fenpropimorph stocks were prepared in dimethyl sulfoxide. Fluphenazine and cycloheximide solutions were prepared in distilled H₂O, while nystatin suspensions were prepared in *N,N*-dimethylformamide.

Animal model. Healthy female ND4 mice (age, 4 to 6 weeks; weight, approximately 25 g each; Harlan Sprague-Dawley, Indianapolis, Ind.) were used. Upon arrival at the animal facility, the mice were separated into groups of 10 mice each and were allowed to rest for 1 week before experimentation to reduce stress and behavior disturbances among the animals. Mice were given food and water ad libitum. Freshly grown cells were suspended in sterile saline, and the inoculum was adjusted to the desired density on the basis of hemacytometric cell counts. The viability of each inoculum was determined by plating dilutions onto Sabouraud dextrose agar plates, incubating at 35°C for 48 h, and counting the resulting colonies. To determine any differences in pathogenicity between *erg24* strains and the isogenic wild-type strains, survival following systemic infection with each strain was determined. To produce infection, each mouse in randomly selected groups of 10 mice each was intravenously injected (via the lateral tail vein) with 10^6 CFU of either strain. In a second set of experiments, the inoculum for *erg24* mice was increased fivefold to 5×10^6 CFU/mouse and the inoculum for wild-type mice remained the same (1×10^6 CFU/mouse). Survival was monitored for 21 days, and the mice were killed if signs of morbidity were observed. In these cases, death was recorded as occurring on the following day. Experiments were repeated twice, and similar results were obtained. The data presented here are those from one representative experiment. All animal care procedures were supervised and approved by the Animal Welfare Committee, Centers for Disease Control and Prevention.

Statistical analysis. To determine any significant differences in the rates of survival of animals infected with the *erg24* strain versus the isogenic wild-type strain, Student's *t* test was performed.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *ERG24* sequences from *S. cerevisiae* and *Arabidopsis thaliana* are M99419 and AF263244, respectively.

RESULTS

Isolation and characterization of *C. albicans* *ERG24* gene. The *C. albicans* *ERG24* gene was isolated by complementation of an *S. cerevisiae* *erg24* mutant (AP2) with *C. albicans* genomic library 655. After initial selection of transformants on CSM without uracil and with ergosterol (5,000 colonies), 15 colonies resulted from replica plating on CSM without uracil, with ergosterol. GC analysis of the sterol contents of five of the colonies indicated a wild-type sterol profile, with ergosterol

FIG. 1. Sterol profiles of an *S. cerevisiae* *erg24* (*S.c.erg24*) mutant and an *S. cerevisiae* *erg24* mutant containing the *C. albicans* *ERG24* gene in plasmid pIU2001 (*S.c.erg24*/pIU2001). Peak A, squalene; peak B, ergosta-5,8,14,22-tetraenol; peak C, ignosterol; peak D, lanosterol; peak E, ergosterol.

present as the major sterol. Figure 1 shows the sterol profile of AP2 grown with CaCl₂ supplementation, which permits growth of the *S. cerevisiae* *erg24* strain, with ignosterol as the primary sterol (11), and also shows the sterol profile of one of the AP2 mutants successfully transformed with the *C. albicans* *ERG24* gene.

A plasmid conferring the wild-type sterol profile in a complemented strain was extracted and amplified in *E. coli*. Restriction fragments of the extracted plasmid (pIU2001) were subcloned into yeast vector pRS316 and tested for *erg24* complementation by using AP2. The smallest complementing insert of 2.2 kb (pIU2005) was used for sequencing with primers T3 and T7. A 235-bp sequence obtained with primer T3 and a matching 467-bp sequence from the Stanford *C. albicans* DNA database were used to identify the entire *ERG24* gene in the 7 $\times$ contig *C. albicans* nonannotated DNA database. The *ERG24* sequence comprised 1,347 bp encoding a 448-amino acid polypeptide. The predicted amino acid sequence, shown in Fig. 2, is 58.2 and 37% identical to the predicted sequences

Figure 3 shows the growth of AP2 (*erg24*) and its wild type, YPH499, on CSM and YPAD supplemented with various levels of CaCl_2 . The wild type showed uniform growth across all

Disruption of the *C. albicans* *ERG24* gene. The procedure for the disruption of the two *C. albicans* alleles of the *ERG24* gene used short homologous regions of the *ERG24* gene flanking a selectable marker (37). PCR primers which contained 60 bp of the sequence of the *ERG24* gene and a short plasmid sequence that amplifies a *C. albicans* selectable marker were prepared. For the first allele, the primers amplified the *URA3* marker on pGEM-URA3. The PCR products were then used to transform strain BWP17 and transformants were selected on CSM-uracil. Two URA⁺ transformants were selected for PCR analysis to confirm disruption of one copy of the *ERG24* gene. PCR primers were constructed to amplify a 1.7-kb fragment that resulted from the wild-type *ERG24* sequence and a 2.1-kb

FIG. 3. Growth of *S. cerevisiae* wild type (YPH499) and an *erg24* (AP2) mutant on various concentrations of CaCl_2 . Plates with YPH499 were grown for 4 days, and plates with AP2 were grown for 10 days.

fragment in the *URA3*-disrupted *ERG24* allele. Figure 4 shows the 1.7-kb fragment in the wild-type strain (lane BWP17) and both fragments in NJ25 (lane NJ25), a heterozygous strain of *ERG24*.

A similar procedure was used to disrupt the second copy of the *ERG24* gene. In this case the *HIS1* marker from pGEM-HIS1 provided the disrupting selectable marker. The PCR products were then used to transform NJ25 with selection on CSM-histidine-uracil plus 200 mM CaCl_2 . Five colonies were selected from among the 367 colonies present, and each colony was confirmed by PCR to contain disruptions in both copies of the *ERG24* gene. In this case the primers would be expected to amplify the 2.1-kb fragment already identified in NJ25 and a 4.2-kb fragment that resulted from the *HIS1* disruption. All five isolates contained both the 2.1- and 4.2-kb fragments, indicating that both copies were disrupted in each isolate. Lane NJ51 of Fig. 4 shows both fragments in one of the isolates, isolate NJ51.

Sterol analysis of *C. albicans* strains with disrupted *ERG24* genes. Further confirmation of the successful disruption of the *C. albicans* *ERG24* gene emerges from sterol analysis of the disrupted strains. Figure 5 shows the sterol profiles of the wild-type strain, the heterozygous *ERG24/erg24* strain (NJ25), and the *erg24* strain (NJ51). The heterozygote had a profile very similar to that of the wild type, with ergosterol (peak B) as the predominant sterol. It also contained a small peak of ergosta-5,8,14,22-tetraenol (peak C) not seen in the wild-type strain. The homozygous *erg24* strain, NJ51, produced a profile in which ignosterol (ergosta-8,14-dienol; peak E) is a major

FIG. 4. PCR confirmation of the disruption of both copies of the *ERG24* gene of *C. albicans*. Lane BWP17, 1.7-kb fragment from the wild-type *ERG24* alleles of BWP17; lane NJ25, 1.7- and 2.1-kb fragments of the wild-type allele and the *URA3*-disrupted *ERG24* allele of NJ25; lane NJ51, 2.1- and 4.2-kb fragments of the *URA3*- and *HIS1*-disrupted *ERG24* alleles of NJ51.

FIG. 5. Sterol profiles of the wild type, the *ERG24* heterozygote (NJ25), and the *erg24* homozygote (NJ51) of *C. albicans*. Peak A, squalene; peak B, ergosterol; peak C, ergosta-5,8,14,22-tetraenol; peak D, lanosterol; peak E, ignosterol; peak F, ergosta-8,14,22-trienol.

sterol but is accompanied by larger amounts of a new sterol, ergosta-8,14,22-trienol (peak F). This sterol is not found in *erg24* mutants of *S. cerevisiae* (Fig. 1). In addition, the squalene contents of the *C. albicans erg24* mutants were much lower than those noted in *S. cerevisiae* mutants and were the same as those seen in the wild-type strains of both organisms.

Creation of a prototrophic *erg24* strain of *C. albicans*. A critical characteristic of a potential antifungal that would block the function of Erg24p is the pathogenic capability of the resulting strain with the *erg24* phenotype. In order to investigate this feature in an animal model, an *erg24* strain devoid of nutritional markers was used since the host organism would not be expected to provide sufficient levels of the missing nutrients to support growth. Strain NJ51 requires arginine, and

TABLE 1. Growth of wild-type and *erg24* strains of *C. albicans* with various media and temperature conditions

Strain	Doubling time (h) ^a			
	CSM + adenine + uridine		YPAD + uridine	
	30°C	37°C	30°C	37°C
BWP17	1.26	1.09	1.09	1.00
NJ51-1	2.03	2.88	2.28	2.15
NJ51-3	2.20	2.69	2.51	2.03
NJ51-3	2.80	2.79	2.81	2.07
NJ51-4	1.93	2.82	2.74	2.08
NJ51-5	2.32	3.11	2.66	1.89

^a Data represent one determination of four runs yielding essentially identical results.

the ARG⁺ phenotype must be restored. The *ARG4* gene was excised from plasmid pAB-SK-ARG with *Bam*HI as a 2.3-kb fragment. The *ARG4 Bam*HI fragment was ligated to pDBI52, an integrating plasmid, after deletion of the *URA3* gene. The resulting plasmid, pNJ02, was linearized at the *Ava*I site, which is located in the *ADE2* gene, and was used to transform strain NJ51. After appropriate selection, two of the three resulting colonies were confirmed by PCR to have the *ARG4* gene inserted at one of the *ADE2* loci. Primers were selected to amplify a 4.9-kb fragment at the *ADE2* locus containing the *ARG4* gene. Both isolates contained this fragment (data not shown).

Characteristics of *erg24* mutants of *C. albicans*. Surprisingly, transfer of the confirmed *erg24* mutants of *C. albicans* to media without CaCl₂ resulted in growth, although the rate of growth was reduced compared to that of the wild type. Growth rates, expressed as doubling times, were determined for the wild-type and *erg24* strains. As shown in Table 1 the doubling times are approximately twofold higher for the *erg24* mutants than for the wild type on both CSM and YAPD at 30 and 37°C.

The lack of a calcium requirement for growth indicates that the *ERG24* gene of *C. albicans* is not essential. It was of interest, however, to see if calcium had some effect on the growth of *erg24* mutants since it was able to restore growth to *erg24* mutants of *S. cerevisiae*. Figure 6 shows the effects of calcium on the growth of *C. albicans erg24* mutants on both medium types and in the presence of several concentrations of CaCl₂. Growth of the mutants actually showed greater inhibition with increased concentrations of CaCl₂ than did growth of the wild type. Thus, calcium has no ameliorating or enhancing effects on the growth of *C. albicans erg24* mutants.

Drug susceptibilities of *C. albicans erg24* mutants. The susceptibilities of *C. albicans erg24* mutants to a battery of antifungal compounds and cellular inhibitors were determined. The antifungal drugs used included terbinafine, an allylamine-inhibiting squalene epoxidase (16); fluconazole, clotrimazole, ketoconazole, and itraconazole, azoles that inhibit Erg11p (31); fenpropimorph and tridemorph, morpholines that inhibit Erg24p and Erg2p (2); and nystatin, a polyene antifungal that binds to ergosterol in membranes, causing cell leakage (7). The cellular inhibitors included cycloheximide, a common protein synthesis inhibitor; cerulenin, a fatty acid synthase inhibitor (25); fluphenazine, a calmodulin antagonist (14); and brefeldin A (32), a compound which interferes with the function of the

FIG. 6. Growth of *C. albicans* wild type (SC5314) and an *erg24* (NJ55) mutant on various concentrations of CaCl_2 . The plates were incubated for 4 days.

Golgi apparatus. Figure 7 shows the growth patterns of the wild type, the *ERG24* heterozygote, and three independently derived *erg24* mutants (NJ50, NJ51, and NJ55 containing various selectable markers) at four cell concentrations plated on media containing the compounds described above. The strains producing ergosterol (BWP17 and NJ25) were most sensitive to nystatin. The *erg24* mutants, however, were more resistant to the morpholine fenpropimorph than to tridemorph, another morpholine. In the spot plate assay the *erg24* mutants were uniformly resistant to the azoles and were more sensitive than the wild type to cycloheximide, fluphenazine, cerulenin, and brefeldin A. In another representation of drug susceptibility, shown in Table 2, the concentrations of these drugs necessary to completely prevent growth on plates for 48 h are shown. In the spot plate assay, at higher concentrations the azoles were about equally effective (fluconazole and ketoconazole) or slightly less effective (itraconazole and clotrimazole) against the *erg24* mutants than against the wild type. The concentrations of the cellular inhibitors required to suppress growth were at least 10-fold lower for the *erg24* mutant than for the wild type.

Survival studies. The results of survival studies for mice infected with the *erg24* or the wild-type strain at equal and different inoculum sizes are reported in Fig. 8A and B, respectively. The absence of the *ERG24* gene product resulted in the reduced pathogenicity of the mutant strain compared to that of the isogenic wild-type strain (DAY185), as demonstrated by the reduced mortality rate observed for mice infected with the

mutant (0% mortality at day 5 postinfection) compared to that for the mice infected with the wild type (100% mortality at day 5 postinfection) (Fig. 8A). By day 21, 90% of the animals infected with the *erg24* strain survived (data not shown). The same trend was observed in a second experiment in which the *erg24* inoculum was increased fivefold relative to that of the wild-type strain. By day 5 postinfection, 60% of the animals infected with the *erg24* strain survived, whereas 0% of the animals infected with the wild-type strain survived (Fig. 8B). Both results support the conclusion that inhibition or absence of Erg 24p activity compromises the pathogenicity of the mutant strain.

Ability to form germ tubes. Ninety percent germ tube formation was observed for wild-type cells after 3 h in human serum, whereas for two independent *erg24* mutants, no germ tube formation was observed after 3 or 12 h of incubation. For each cell type, a total of 400 to 500 cells were scanned.

DISCUSSION

The *ERG24* gene in the yeast sterol biosynthetic pathway is a potential site for the discovery and application of new antifungal agents. The morpholine class of antifungals targets this step, but no formulations of this class of drugs are suitable for systemic use in humans. The fact that the morpholines have been successfully used to combat fungal infections in agricultural settings (2) and the fact that this reaction is essential for growth under most conditions in *S. cerevisiae* imply that this

FIG. 7. Growth responses of *C. albicans* wild type (BWP17), the *ERG24* heterozygote (NJ25), and three *erg24* homozygotes (NJ50, NJ51, and NJ55) in the presence of sterol biosynthesis inhibitors and metabolic inhibitors.

step in the pathway may be an important one for fungal growth or pathogenicity. The first test in determination of whether *Erg24p* is a good target is definition of the characteristics of a fungal pathogen that is devoid of the *Erg24p* function.

The *C. albicans* *ERG24* gene has been isolated by complementation of an *S. cerevisiae* *erg24* mutant with a *C. albicans* library. The sequence of this gene was determined by initial sequencing of the complementing DNA, which was then used

TABLE 2. Inhibitory concentrations of cell inhibitors and antifungal compounds for wild-type (*ERG24*) and *erg24* strains of *C. albicans*

Compound	Inhibitory concn (mg/liter) ^a	
	<i>ERG24</i>	<i>erg24</i>
Brefeldin A	25	1.5
Cerulenin	10	1
Cycloheximide	>1,000	70
Fluphenazine	>150	50
Terbinafine	50	1
Fenpropimorph	0.12	1
Tridemorph	1	1
Nystatin	4	25
Clotrimazole	2	>5
Fluconazole	50	50
Itraconazole	10	50
Ketoconazole	>10	>10

^a Concentration at which no growth was evident after 48 h.

to identify the entire sequence in the *C. albicans* nonannotated database. The predicted amino acid sequence of the *C. albicans* gene was found to be 58.2 and 37% identical to those of *S. cerevisiae* and *A. thaliana*, respectively.

Sequential disruption of the two alleles of the *C. albicans* *ERG24* gene has been accomplished, and the *erg24* mutant is capable of growth under normal aerobic conditions on standard defined and enriched media. In contrast, *erg24* mutants of *S. cerevisiae* do not grow under standard conditions (21–23). In one study (22), two recessive mutations, *fen1* and *fen2*, were found to permit growth with ignosterol in the presence of fenpropimorph. In a second report (30), a pair of mutations, *fen1* (it is unclear whether the *fen1* mutations were the same in both of the studies) and *sur4*, were shown to permit growth in *erg24* mutants. Rather than allow alternate sterols to be synthesized and restore growth, these suppressor mutations permit the cell to grow with ignosterol, the major sterol accumulated by *erg24* mutants. Subsequently, *FEN1* and *SUR4* have been shown to be identical to *ELO2* and *ELO3*, respectively, genes involved in fatty acid elongation and sphingolipid synthesis (5, 26). Thus, alteration of other membrane lipids can modify the type of sterol that will satisfy membrane requirements and facilitate growth.

Recent reports have shown that *erg24* mutants of *S. cerevisiae* are capable of growth only on defined media under aerobic conditions (10) and on enriched media supplemented with Ca^{2+} or Mg^{2+} (11). The investigators hypothesized that ergosterol was responsible for the proper function of Pmrlp, a calcium pump. The pump was postulated to move calcium from the cytosol to vesicles, where it may be required for protein transport. A lack of ergosterol would inactivate the pump, and calcium transport into vesicles would be possible only in the presence of exogenous calcium supplements. The results obtained in this study would argue against this hypothesis since *erg24* mutants of *C. albicans* grow without calcium supplementation. Alternatively, ergosterol may not play a role in activating the Pmrlp pump in *C. albicans*.

In an examination of the sterol profiles generated by *erg24* mutants of *S. cerevisiae* and *C. albicans*, one striking difference is observed. *C. albicans* accumulates a significant level of ergosta-8,14,22-trienol, a sterol that is absent from the *S. cerevi-*

FIG. 8. Survival of mice infected with the wild-type strain and an *erg24* strain of *C. albicans*. (A) An inoculum of 10^6 CFU/mouse was used for each strain. ▲, *erg24* strain; ■, wild-type strain. (B) An inoculum of 5×10^6 CFU/mouse was used for *erg24*, and an inoculum of 1×10^6 CFU/mouse was used for the wild type. ■, *erg24* strain; ▲, wild-type strain. Ten mice were used in each group, and the results are from one representative experiment. By the use of pooled results from two independent experiments conducted with each strain, Student's *t* test indicated a significant difference in survival at days 3, 4, and 5 postinfection for both experiments ($P > 0.05$).

siae mutant. This intermediate may be the element that allows growth under normal conditions for *C. albicans* *erg24* mutants. This overall finding of different phenotypes for identical blocks in the pathways of *S. cerevisiae* and *C. albicans* is similar to that noted with the *ERG11* gene, which encodes the lanosterol C-14 demethylase. In this case, the *ERG11* gene has been shown to be essential for growth in *S. cerevisiae* but not in *C. albicans* (4). Thus, there are significant differences in sterol requirements for growth and in the way in which the ergosterol pathways adjust to blocked steps in these two organisms.

One of the major functions of sterol in membranes is to regulate permeability. Numerous reports have described ergosterol pathway mutants of *S. cerevisiae* with altered membrane permeability characteristics (3, 20, 34). A more recent study (18) with an *erg6* (*ERG6* encodes the sterol methyltransferase) mutant of *C. albicans* clearly showed that the *erg6* strain was significantly more sensitive to cellular inhibitors and some antifungal drugs. This finding is consistent with the increased permeability resulting from sterol substitution in the membrane. For example, the wild type was more than 50 times more

resistant than the *erg6* mutant to brefeldin A and terbinafine; the wild type was more than 100 times more resistant than the *erg6* mutant to the morpholines fenpropimorph and tridemorph. In the study described in this report an *erg24* strain of *C. albicans* has been shown to be at least 10 times more susceptible than the wild type to the metabolic inhibitors cycloheximide, cerulenin, fluphenazine, and brefeldin A. The *erg24* strain also showed a significantly increased level of susceptibility to an allylamine antifungal, terbinafine. Although the levels of susceptibility varied somewhat, the same pattern was noted in *erg6* mutants of *C. albicans* (18).

The susceptibilities of the *erg24* strains to morpholines did not change in the case of tridemorph, but resistance to fenpropimorph was seen. Morpholine resistance in *Saccharomyces* stems from overexpression of *ERG24* (21) and mutations in *FEN1* and *SUR4*. *fen1 erg24* double mutants are therefore morpholine resistant due to the *fen1* mutation and not the *erg24* mutation. It should also be noted that while both Erg2p and Erg24p are targets of the morpholine antifungals, treatment of the wild-type strain with growth-inhibitory concentrations of both drugs resulted predominantly in an *erg2* sterol profile, but higher fenpropimorph concentrations also resulted in the accumulation of ergosta-8,14 sterols, which are typically seen in *erg24* mutants (data not shown). These results indicate that fenpropimorph targets the C-14 reductase to a greater extent than tridemorph and helps to explain why *erg24* mutants are more resistant to this antifungal. In contrast, the *C. albicans erg6* mutant showed significantly elevated levels of susceptibility to both morpholines (18). Both *erg6* and *erg24* mutants were resistant to nystatin, a polyene which binds to ergosterol. The reduced affinity of nystatin for sterol intermediates in the membranes accounts for this observation.

The susceptibilities to the azoles vary with the particular azole used. The results presented in Fig. 7 show that the *erg24* mutants are more resistant than the wild type to all four azoles. Determination of the concentrations necessary to completely inhibit growth for 48 h, however, indicates low levels of resistance to clotrimazole and itraconazole only. Resistance in this case can be attributed either to the differential permeability to azoles in the *ERG24* and *erg24* membrane environments or to interactions among the enzymes and substrates involved in two adjacent steps in the ergosterol pathway. For example, a block at the *ERG24* step in the pathway (the *erg24* phenotype) would result in an accumulation of ergosterol and related sterols with a double bond at C-14. This, in turn, might alter the ability of Erg11p to process more substrate, thus reducing the function of this enzyme and diminishing the sensitivity of this step to azole inhibition. In contrast to this finding, *erg6* mutants of *C. albicans* were found to have the same levels of susceptibility to azoles as the wild type, although only clotrimazole and ketoconazole were tested (18). This was interpreted to reflect the fact that the sterol substitutions in the membrane did not alter the permeability characteristics in a way that had any impact on azole entry.

The most important and relevant characteristic of the *erg24* phenotype is its pathogenicity. Strains with this phenotype have already been found to be slowly growing under several conditions. When equivalent inocula of the *erg24* strain and the wild type are compared in a murine system, the *erg24* strain is significantly less pathogenic. Even when the inoculum of the

erg24 mutant strain was increased to five times that of the wild-type strain, the rates of survival of the *erg24*-infected animals were significantly greater than those of animals infected with the wild-type strain by day 3 postinfection and beyond. Although it is true that the doubling time of the *erg24* strain is twice that of the isogenic wild-type strain, this phenotype alone cannot entirely account for the significant difference in survival rates observed in these studies. While further testing, including studies with longer time frames and immunocompromised-animal models, would be required to specifically define the pathogenic potential of the *C. albicans erg24* mutant, it can at least be concluded that the mutant showed a greatly reduced pathogenicity compared to that of the wild-type strain.

The characteristics of the *erg24* phenotype in *C. albicans* indicate that Erg24p has potential as a target for new antifungals. The *erg24* phenotype has been shown to be susceptible to low concentrations of a variety of inhibitors and some antifungal compounds, most likely due to the increased membrane permeability resulting from altered membrane sterols. The *erg24* strain is slowly growing, indicating that its ability to proliferate in the host and invade tissue would be compromised, allowing more time for an immune response and drug treatment to control an infection. Finally, and most importantly, the *erg24* mutant has been shown to be far less able than the wild type of *C. albicans* to cause disease in the mouse model system, perhaps due to an inability to form hyphae.

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