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Isolation, characterization, and regulation of the *Candida albicans ERG27* gene encoding the sterol 3-keto reductase

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The *Candida albicans ERG27* gene which encodes the 3-keto reductase enzyme required for sterol C-4 demethylation was isolated and found to encode a 349 amino acid protein that is 60% identical at the amino acid level to the *Saccharomyces cerevisiae* Erg27p. A *C. albicans erg27* null was created in a strain containing an integrated *ERG27* rescue cassette under the control of the *pMAL2* inducible promoter. The *C. albicans erg27* strain was able to grow only in the presence of maltose indicating that the *ERG27* gene is essential. The *C. albicans erg27* null showed complete loss of both 3-keto reductase and oxidosqualene cyclase (Erg7p) activities compromising all sterol synthesis. These results suggest that Erg27p inhibitors might be effective antifungals. To explore *ERG27* regulation, an *erg11* null strain was generated. *C. albicans erg6* and *erg24* mutants were also employed along with the inhibitors, itraconazole and zaragozic acid A, to characterize *ERG27* expression using Northern analysis. Expression was increased two- to fourfold in *erg11, erg6* and *erg24* backgrounds. However, itraconazole which targets Erg11p (lanosterol demethylase) increased *ERG27* expression 10-fold and zaragozic acid A which targets the Erg9p (squalene synthase) increased *ERG27* expression fivefold. The azole and *erg11* results support other observations that azoles may affect non-sterol targets.

Keywords antifungal target sites, Candida albicans, ergosterol, sterol biosynthesis

Introduction

The incidence of fungal infections has been on the rise for a number of years with various circumstances acting as contributing factors. Among them have been the expanded and prolonged use of the currently available antifungal compounds, invasive medical procedures, and therapies and diseases that compromise the immune response [1,2]. Over-utilization of current antifungals compounds, especially in cases where the immune system responds inadequately, has acted as a strong selective pressure for the increased incidence of resistant forms of pathogenic organisms.

Although other classes of antifungal agents are currently available and new ones are in development, the azoles remain the major class of antifungal compounds used to treat human infection. The azoles are fungistatic drugs that inhibit the C-14 sterol demethylase encoded by the *ERG11* gene [3] (Fig. 1). Mechanisms of resistance to azoles have been shown to result from the increased expression of multi-drug transporters [4,5], mutations in the *ERG11* gene [6], and increased expression of the *ERG11* gene [7]. The latter mechanism of increased gene expression has been reported as a general response of fungi to ergosterol inhibitors and has been noted in many of the genes whose products function in the ergosterol biosynthetic pathway [8,9].

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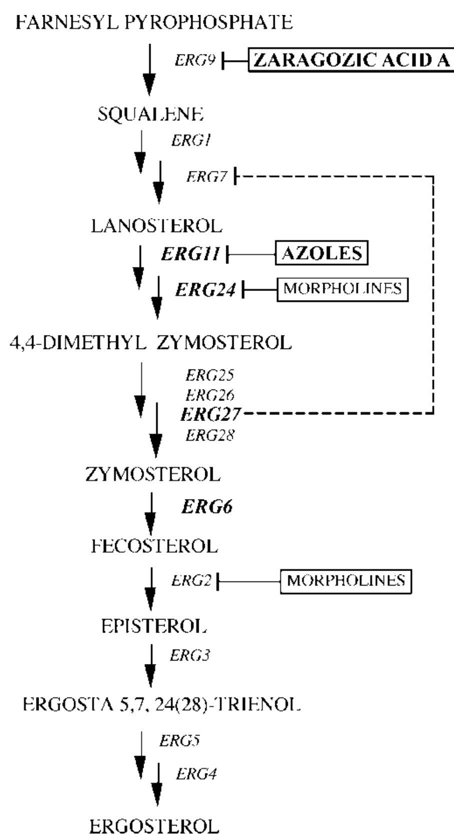

Fig. 1 The yeast sterol biosynthetic pathway indicating the genes encoding the biosynthetic enzymes and the sites of action of select inhibitors. Loss of Erg27p results in the absence of both 3-keto reductase and oxidosqualene cyclase (Erg7p) activity.

A limited number of sites for inhibition of ergosterol biosynthesis have been identified for the development of effective antifungal compounds. This restriction, coupled with the emergence of increasing drug resistance to available compounds has resulted in a real need for new classes of antifungal compounds [10].

The ergosterol biosynthetic pathway includes several enzymatic steps that may be excellent sites for the development of novel classes of antifungal compounds. To be a suitable candidate for inhibition, the biosynthetic step should be required for growth and/or pathogenicity. To explore these characteristics, the genes encoding each enzyme have been isolated and characterized in *Saccharomyces cerevisiae*, with the resulting information being used to repeat the procedure in *Candida albicans*, a common human fungal pathogen. The specific sterol requirements in these two organisms can differ thus requiring that each of the homologous genes be characterized. The *ERG11* and *ERG24* (encodes the sterol C-14 reductase) genes have been found to be essential in *S. cerevisiae* [11–14] but not in *C. albicans* [15–17]. In contrast, the *ERG6* gene encoding the sterol methyl transferase has been found to be non-essential in both organisms [18,19] while the *ERG25* gene encoding the C-4 sterol methyl oxidase [20–22] and the *ERG26* gene encoding the C-3 sterol dehydrogenase (C-4 carboxylase) [23,24] have been found to be essential in both organisms.

The final gene whose product participates in the C-4 sterol demethylation (along with Erg25p and Erg26p) is the *ERG27* gene encoding the sterol 3-keto reductase. Gachotte *et al*. [25] demonstrated that loss of *S. cerevisiae* Erg27p also results in a very marked diminution of lanosterol, the product of oxidosqualene cyclase activity (Erg7p). Therefore, the loss of Erg27p in *S. cerevisiae* results in the loss of the entire sterol pathway and is attributable to Erg27p being required to prevent digestion of Erg7p as it is transported to the lipid particles [26]. A fourth protein involved in C-4 demethylation is Erg28p [27] which has no pathway enzymatic function but plays a role in facilitating the functions of Erg26p and Erg27p, presumably by tethering these enzymes into a multi-enzyme complex in the ER [28]. *S. cerevisiae erg28* nulls are non-auxotrophic and produce ergosterol but also accumulate sterol intermediates like those seen in *erg26* and *erg27* nulls [27]. The *ERG28* gene is highly conserved across species and the *C. albicans* version is currently under study.

The three enzymes involved in the removal of the two C-4 methyl groups provide significant potential for the development of novel antifungal agents. Recently an effective Erg25p inhibitor has been isolated from a *Penicillium* broth [29]. Based on the characteristics of the *erg27* phenotype in S. *cerevisiae*, where a block at *ERG27* results in an inability of the pre-sterol pathway step at *ERG7* to function, Erg27p may have the best potential of the three as a target for development of a fungicidal drug targeting the sterol pathway.

Based on the phenotype of the *S. cerevisiae erg27* mutant, it was of interest to determine whether a mutation in the *C. albicans ERG27* gene would likewise result in a block prior to lanosterol formation. If so, this would make inhibition of this step a priority for the development of antifungal agents since inhibitors of Erg27p would be lethal to fungal cells since membrane sterol is essential for viability. In addition, delineation of some of the regulatory features of C-4 demethylation in *C. albicans* would be of interest from the basic perspective of pathway dynamics as well as the applied perspective of predicting whether up-regulation of *ERG27* expression could be a resistance mechanism that may emerge to Erg27p inhibitors. Analysis of *ERG27* gene expression in the presence of azole

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antifungals, other sterol inhibitors, and in particular in an *erg11* null strain would also be of wide interest in delineating general pathway responses to perturbation.

Materials and methods

Strains and plasmids

Saccharomyces cerevisiae strain SDG100 (*Mat α upc2 ade2 his3 ura3-52 erg27-1*) is described in Gachotte *et al*. [25]. *C. albicans* strain BWP17 (*ura3Δ::λimm434/ura3 Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*) was obtained from A. Mitchell [30]. *C. albicans* strains CAI4 (*ura3Δ::λimm434/ura3Δ::λimm434*) was obtained from W. Fonzi [31]. *C. albicans erg6* and *erg24* mutant strains, constructed in our lab, are described elsewhere [17,19]. *Escherichia coli* strain DH5α was used to amplify plasmid DNA. Plasmids pGEM-URA3, pGEM-HIS1 and pRS-ARG4ΔSpeI were obtained from A. Mitchell [30]. Plasmid pDBI52 was obtained from C. Kumamoto [32] while the *C. albicans* genomic library 655 (generated from *C. albicans* ATCC 56844) was obtained from S. Scherer [33].

Media and growth

E. coli cultures were grown in Luria broth (LB). Solid LB media were made by adding agar (Difco) at 20 g/l. Ampicillin was added after autoclaving at 60 μg/ml for plasmid selection. Cultures were grown at 37°C. *S. cerevisiae* and *C. albicans* were grown on either CSM (Complete Synthetic Medium) or YPAD, an enriched medium. CSM (Qbiogene, Carlsbad, CA, USA) 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 (360 mg/l) and uridine (80 mg/l). CSM media lacking specific nutrients were used in selection. YPAD was composed of 1% Bacto yeast extract, 2% Bacto peptone, and 2% D-glucose plus adenine (360 mg/l) and uridine (80 mg/l). Maltose was substituted for glucose at 2%. Ergosterol, when needed, was added to autoclaved and cooled media at 20 mg/l to rescue *erg27* mutants in *S. cerevisiae*. *S. cerevisiae* cultures were grown at 30°C and *C. albicans* cultures were grown at 30 or 37°C. Itraconazole was provided by Janssen Pharmaceutical. Stocks at 2 mg/ml were made in DMSO. Zaragozic acid A was obtained from Merck and stocks were made in distilled water at 5 mg/ml. For anaerobic growth, the Gas Pak System (BBL Microbiology Systems, Cockeysville, MD, USA) was used. Itraconazole-treated cells were grown in 0.2 mg/l itraconazole (results in 50% normal growth rate) to an OD_{660} of 0.7–1.0. The itraconazole concentration was raised to 1 mg/l for 6 h and the cells were harvested. Cells were treated with 30 μg/l zaragozic acid A until a concentration at OD_{660} of 0.7–1.0 was obtained and harvested.

Transformations

Escherichia coli DH5α cells were transformed using standard transformation protocols [34].

The transformation protocol for *S. cerevisiae* has been previously described [35]. *C. albicans* transformations were carried out as previously described [30].

DNA sequencing

DNA sequencing was performed at the Biochemistry Biotechnology Facility (BBF) in the Indiana University School of Medicine Indianapolis, IN, USA). Once initial sequence data were obtained using primers T3 and T7, new primers were designed to facilitate acquisition of additional DNA sequence data. All primers for sequencing were purchased through Sigma–Genosys (The Woodlands, TX, USA).

PCR

PCR reactions 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 30–40 cycles with three identical steps: 94°C for 45 s, 55°C (or 5°C below primer annealing temperature) for 30–60 s for primers to anneal to denatured DNA, and 72°C for elongation (for 2.5–3.5 min depending upon length of expected DNA product), followed by 72°C for 10–15 min to complete all possible reactions. To avoid mispriming, touch down PCR was applied. Initially, the annealing temperature was maintained at the same temperature as the primer's denaturing temperature for two cycles, and gradually dropped 1–2°C every two cycles until an annealing temperature 5°C below the primer's melting temperature was reached. The PCR reaction was then run for another 25–35 cycles at the final annealing temperature.

Two kinds of thermostable DNA polymerases were used. *Taq* DNA polymerase in Buffer B (Promega, Madison, WI, USA) was mainly used to screen genetic constructions or genetic insertions. Reaction buffer (50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 9.0, 0.1% Triton X-100) plus 1.5–2.0 mmol/l $MgCl_2$ and 1.25 U polymerase was used. A second polymerase, Expand High Fidelity *Taq* polymerase (EHF *Taq*) (Roche, Indianapolis, IN, USA) at 2.6 U per reaction, was used to amplify selectable marker genes. Reaction

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buffer with 15 mmol/l $MgCl_2$ (20 mmol/l Tris-HCl, pH 7.5, 100 mmol/l KCl, 1 mmol/l dithiothreitol (DTT), 0.1 mmol/l EDTA, 0.5% Tween 20 [v/v], 0.5% Nonidet P40 [v/v], and 50% glycerol[v/v]) was used.

PCR primers

The primers used in this study are listed in Table 1.

Sterol analysis

Sterols were isolated as previously described [36] and analyzed by gas chromatography (GC). A HP5890 series II equipped with the Hewlett-Packard CHEMSTATION software package was used to analyze sterol content. The capillary column (DB-1) was 15 m × 0.25 mm × 0.25-μm film thickness (J&W Scientific, Folsom, CA, USA) and was programmed from 195°C to 280°C (1 min at 195°C, then an increase at 20°C/min to 240°C, followed by 2°C/min until the final temperature of 280°C was reached). The linear velocity was 30 cm/s using nitrogen as the carrier gas, and all injections were run in the splitless mode.

Gas chromatography/Mass spectrometry (GC/MS) analyses of sterols were done using 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 × 0.18 mm × 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 ramp 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 with a linear velocity of 50 cm/s in the splitless mode. The mass spectrometer was in the electron impact ionization mode at an electron energy of 70 eV, an ion source temperature of 150°C, and scanning from 40 to 850 atomic mass units at 0.6 s intervals.

Spot-plate growth test

Yeast strains were inoculated into 50 ml of liquid media and incubated at 30°C in a shaker at 200 r.p.m. overnight. Cell concentrations were determined using a hemacytometer and diluted to give a series of cell concentrations of 2×10^7, 2×10^6, 2×10^5 and 2×10^4 cells/ml. 5 μl of each concentration was added onto solid media to give a series of spots containing 1×10^5–1×10^2 cells.

Rescue cassette for the erg27 phenotype

Since the *ERG27* gene has been shown to be essential in *S. cerevisiae* [25], it is likely to be essential in *C. albicans*. The disruption of the second copy of the *ERG27* gene would thus result in a non-viable cell. Since *C. albicans* cannot utilize exogenous sterol under aerobic or anaerobic conditions [37], a rescue cassette containing a third copy of the *ERG27* gene under the control of the inducible *pMAL2* promoter [28] was constructed. The *ERG27* ORF was amplified from BWP17 genomic DNA using primers 27-ampF and 27-ampR (Table 1). The former contained an *Eco*RV site at the 5′ end and the latter contained a *Xba*I site at the 5′ end. The 1.3-kb products were ligated into pDBI52 after both had been digested simultaneously with *Eco*RV and *Xba*I. A transformant with the correct ligations was isolated and designated pNJ01. The plasmid pNJ01 was linearized at the *Bam*HI site in the *ADE2* marker and used to transform NJ32. Transformants were selected on CSM-arg-ura+ade.

Disruption of the ERG27 alleles

Both copies of the *ERG27* gene were disrupted using a cassette comprised of short homology regions of the *ERG27* gene flanking a selectable marker [30]. The PCR primers 27-ARGF and 27-ARGR (Table 1) consisting of 50–60 bases of the *ERG27* gene and a short plasmid sequence were constructed to amplify the *ARG4* marker on pRS-ARG4Δ*Spe*I. The resulting 2.4-kb products were used to transform *C. albicans* strain BWP17 and transformants were selected on CSM-arg+ade+uri. Primers 27-HISF and 27-HISR containing 50–60 bp of *ERG27* sequence and plasmid sequence were used to amplify the *HIS1* gene on pGEM-HIS1. The *ERG27* sequences for the second disruption were distinct from those used for the *ARG4* amplified DNA employed in the disruption of the first allele to eliminate the possibility of insertion at the first disruption site. The PCR products were used to transform the *ERG27* heterozygote containing the rescue cassette, and transformants were selected on CSM-arg-his+ade with maltose as the carbon-energy source. Disruptions of both *ERG27* alleles were confirmed by PCR using appropriately designed primers.

Disruption of the ERG11 gene of C. albicans

PCR primers ERG11-URAF and ERG11-URAR (Table 1) containing 50–60 bases of the *ERG11* gene and a short plasmid sequence were used to amplify the *URA3* gene on plasmid pGEM-URA3. The resulting 1.4-kb products were used to transform *C. albicans* BWP17 and transformants were selected on CSM-ura. Confirmation of the first disruption was accomplished by using primers ERG11-disF and ERG11-disR (Table 1) which were designed to amplify a 1.4-kb

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Table 1 PCR Primers used in the disruptions of the *ERG27* and *ERG11* genes of *Candida albicans*.

Primer	Sequence (5′-3′)	Application
27-ARGF	GTCACTTTTAAAGGATTCTACAGTTGCAGTCATTACCGGGACATCTTCAAATTTAGGATGTTTTCCCAGTCACGACGTT	*ERG27 ARG4* disruption cassette
27-ARGR	CAAGGTTGACGTGTATTTACTATTTGATCCTTCAACTTTTCGTCCCACTCTTTAGTAAGTTGTGGAATTGTGAGCGGATA	*ERG27 ARG4* disruption cassette
27-HISF	TACGATCGCGTAACATTATTATTATTGTGTGAAAAATTAAATACTCAAGTCGGAAAAAAAGTTTTCCCAGTCACGACGTT	*ERG27 HIS1* disruption cassette
27-HISR	AAATTCAATTATCCTGAGCTGGAAAAAACGGAATTGACTACTAAAGATATTCACAAGTGTATGACCATGATTACGCCAAG	*ERG27 HIS1* disruption cassette
27-ampF	CCGATATCATGTCACTTTTAAAGGATTCTACAG	amplification of *ERG27* sequence
27-ampR	CCTCTAGACAAACTCATGGAATACAAGC	amplification of *ERG27* sequence
27-disF	GGTTGGAATGATCTCTGG	*ERG27* disruption confirmation
27-disR	ACTGGAAAAGAAAAGTAA	*ERG27* disruption confirmation
ADE2-F	CTTATTCTCATCACACACGCAT	*ADE2* integration
PMAL-R	CATAGCAATCATGGAATACGG	*ADE2* integration
PMAL-F	GCAGTTGAGAATGTTAGTTTTTG	*ADE2* integration
ADE2-intR	AGTATTCACGGATAGATCTGTTAGAG	*ADE2* integration
ERG11-URAF	GGATTCCTTGGTTTGGTTCTGCAGCTTCATATGGTCAACAACCTTATGAATTTTTCGAATGTTTTCCCAGTCACGACGTT	*ERG11 URA3* disruption cassette
ERG11-URAR	GGCAGCAGCAGTATCCCATCTAGTTGGATCAAAATCTTCAGGGTTATCAAAATATCTTTCTGTGGAATTGTGAGCGGATA	*ERG11 URA3* disruption cassette
ERG11-disF	GAAACTGTCATTGATGGC	*ERG11* disruption confirmation
ERG11-disR	CTATAATCAGGGTCAGGC	*ERG11* disruption confirmation
ERG27-probF	GTTCGTTTGTTGGAGGGGC	*ERG27* probe template
ERG27-probR	CAAGCCGAGGCAGTTTTGC	*ERG27* probe template

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fragment in the wild type allele and a 2.1-kb fragment in the *URA3*-disrupted allele. The primers were designed within the distal ends of the *ERG11* gene such that any *ERG11* sequence in the genome would be amplified. Since the *ERG11* gene has been shown to be non-essential in *C. albicans* [15,16], the second allele could be disrupted directly. The heterozygote was plated on YPAD supplemented with 15 mg/l nystatin to select for those cells that had undergone mitotic recombination and no longer produced ergosterol. Homozygous *erg11* mutants were confirmed as such by PCR using primers ERG11-disF and ERG11-disR.

RNA isolation

RNA extraction was performed using a standard protocol [30].

Northern blotting

Isolated RNA was resolved in 1.5% agarose, formaldehyde denaturing gels and then transferred to the membrane. Random radio-labeled probes were made using the *redi*prime^{TM}II Kit (Strategene, Cedar Creek, TX, USA) and PCR-generated DNA template from within the *ERG27* ORF generated by standard PCR using primers listed in Table 1 and pIU1717 (this publication). Hybridization was done in QuikHyb buffer (Strategene) at 65°C for 1 h. The membrane was washed with $1 \times$ SSC containing 0.1% SDS at 68°C until only specific probe was detected using a RPI GM-2 Rad-Monitor. Results were obtained by exposing a Molecular Dynamics Storage Phosphor screen to hybridized blots and developing on a Molecular Dynamics Strom 840 at 200-nm resolution. *ERG27* mRNA levels were determined relative to the constitutively expressed actin gene (*ACT1*).

Nucleotide sequence accession numbers

The GenBank accession numbers for the *ERG27* sequences from *S. cerevisiae* and *C. albicans* are NC 001144.2 and AY 140908, respectively.

Results

Isolation and characterization of the C. albicans ERG27 gene

Figure 1 depicts the ergosterol biosynthetic pathway and points of inhibitor action relevant to this study. The *C. albicans ERG27* gene was isolated by complementation of a *S. cervisiae erg27* mutant (strain SDG100) with the *C. albicans* genomic library 655. This procedure has been used to isolate several other genes of the *C. albicans* sterol pathway [17,19,22,24]. Transformed cells were plated onto CSM-ura+ergosterol plates and incubated at 30°C under anaerobic conditions for 3 days. More than 10 000 resulting colonies were replica plated onto CSM-ura medium plates and incubated at 30°C under aerobic conditions for 2 days. Colonies able to grow without ergosterol supplementation were analyzed by GC. The sterol profile of *S. cerevisiae* SDG100 (*erg27*) grown with limited ergosterol supplementation is shown (Fig. 2) along with profiles of a *C. albicans ERG27* transformant of SDG100 and a *S. cerevisiae* wild type. The sterol profile of the transformant is identical to that of the wild type indicating that the plasmid contains a *C. albicans* sequence that fully restores ergosterol synthesis. Elimination of the URA$^+$ plasmid by treatment with 5-fluoroorotic acid (FOA) confirmed that the plasmid-borne sequence from *C. albicans* was responsible for the wild type sterol profile.

From among eight colonies analyzed, a plasmid (pIU1717) which complemented *erg27* null strains and contained a 2.7-kb insert was extracted and both strands sequenced using T3 and T7 primers. Further

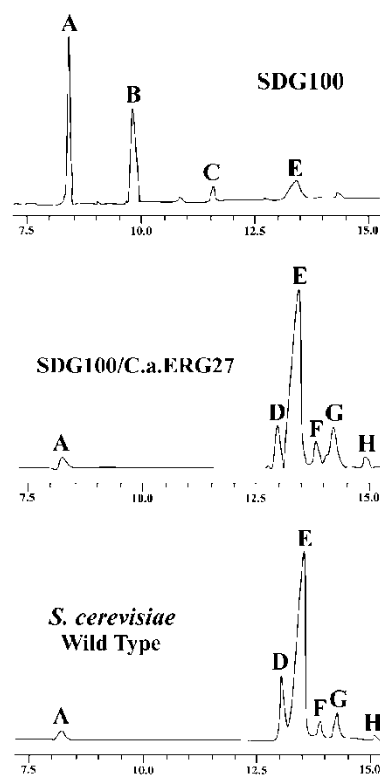

Fig. 2 The sterol profiles of a *Saccharomyces cerevisiae erg27* mutant (SDG100) supplemented with low levels of ergosterol to allow growth, SDG100 complemented with a *Candida albicans ERG27*, and a *S. cerevisiae* wild type. A, squalene; B, squalene epoxide; C, squalene dioxide; D, zymosterol; E, ergosterol; F, fecosterol; G, episterol; H, lanosterol.

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sequencing was accomplished using primers generated from revealed sequence. The putative *C. albicans ERG27* open reading frame was comprised of 1041 bp predicting a polypeptide of 346 amino acids. The two CTG codons at positions 318 and 323 code for serine rather than leucine in *C. albicans* [38]. *The ERG27* gene in *S. cerevisiae* had methionine and alanine at these positions, respectively [25]. In comparing this sequence with the *Candida* genomic sequence (10 × Contig, assembly 19), at the Stanford Genome Technology Center (www.sequence.stanford.edu/group/candida), ten differences were noted but only four of these resulted in an amino acid change (Table 2). The predicted amino acid sequence of the *C. albicans ERG27* ORF shows 60% identity with the Erg27p sequence reported for *S. cerevisiae* [25].

Creation of the erg27 null

Disruption of the first *ERG27* allele was accomplished using the *ERG27-ARG4* disruption cassette and confirmed by PCR using the primers 27-disF, 225 bp upstream of the *ERG27* start codon, and 27-disR, 654 bp downstream of the *ERG27* stop codon (Table 1). The primers amplified a 1.9-kb fragment for the *ERG27* wild type allele and a 2.8-kb fragment for the disrupted allele. Fig. 3a shows the presence of the 1.9-kb fragment in the wild type while both bands were present in the heterozygote (NJ32). NJ32 produced a wild type sterol profile. All heterozygous isolates tested yielded similar results (data not shown). Following transformation of the heterozygote with the linearized rescue cassette containing the *pMAL2-ERG27* construct, twelve resulting colonies were selected for PCR confirmation of integration at the *ADE2* locus. The integration was too large (~9 kb) to confirm with a single set of primers so two sets of primers were designed (Table 1). Primer ADE2F, located 40 bp upstream of the *ADE2* start codon, and primer pMALR, from the middle of *pMAL2*, were used to amplify 4.8-kb segment while primer pMALF, from the middle of *pMAL2*, and primer ADE2R, from 189 bp downstream of the *ADE2* stop codon, were used to amplify a 4.3-kb segment. Two isolates yielded the two predicted fragments and the results from one (NJ45) are shown in Fig. 3b. The same primers (Table 1) that were used to verify the *ARG4* disruption, for the first *ERG27* allele, were used to confirm that the second *ERG27* allele was disrupted. A 2.7-kb fragment was predicted to be amplified for *erg27::HIS1*. Fig. 3c shows the presence of both the 2.8-kb band from the *ARG4*-disrupted *ERG27* allele and the 2.7-kb band from the *HIS1*-disrupted *ERG27* allele in NJ61. Five NJ61 isolates and control strains were spot-plate tested for growth on maltose and glucose media. All strains, wild type and heterozygous, grew well at all dilutions on both glucose and maltose media (Fig. 4a). The one NJ61 isolate shown grew normally on maltose media but very poorly on glucose media (Fig. 4a). Northern analysis of strain NJ61 determined that *pMAL2-ERG27* expression on glucose was minimal (4%) relative to that noted for maltose grown cells (Fig. 4b). This low level of expression would predict a small accumulation of ergosterol in NJ61.

GC analysis of NJ61 grown on maltose and glucose showed a wild type sterol profile on the former and an *erg27* profile on the latter (Fig. 5). The sterol precursors (squalene, squalene epoxide, and squalene dioxide) that accumulated on glucose are the same intermediates produced by *S. cerevisiae* SDG100 when grown in the presence of low amounts of ergosterol. In the case of NJ61, the low amounts of ergosterol are the result of recombination of the *ERG27* gene in a small number of cells to a locus where it is not regulated by *pMAL2*.

Table 2 Sequences differences between the *ERG27* ORF in pIU1717 and 10 × Contig

Base Number	pIU1717 Base	10 × Contig Base	AAs Number	Codon	pIU1717 AA	10 × Contig AA
60	G	A	20	GG_	G	G
185	A	C	62	G_A	E	A
195	G	A	65	CC_	P	P
294	G	A	98	AA_	K	K
366	T	C	122	CT_	L	L
517	A	G	173	_AA	K	E
541	A	G	181	_TC	I	V
546	T	C	182	AG_	S	S
621	T	C	207	GG_	G	G
679	T	C	227	_AT	Y	H

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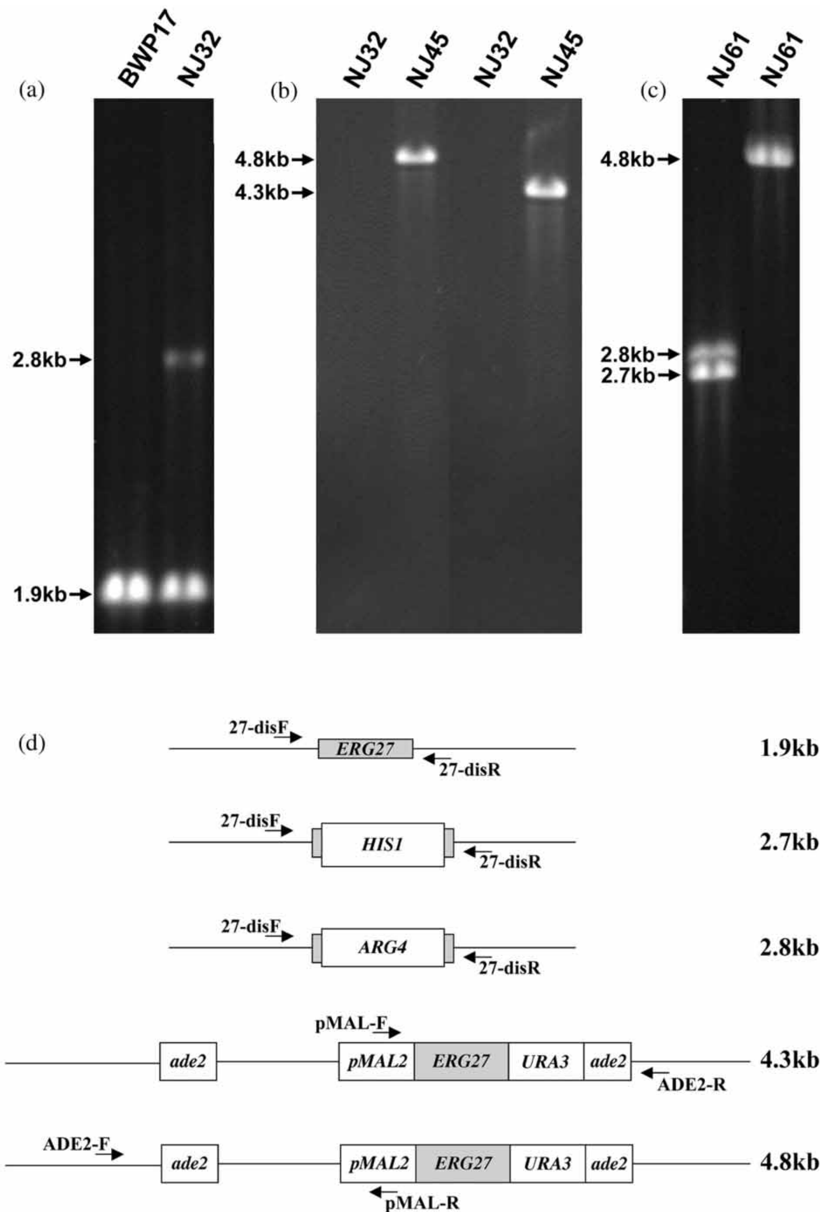

Fig. 3 PCR confirmation of the disruption of the two *ERG27* alleles of *Candida albicans*. (a) Confirmation of the disruption of the first *ERG27* allele showing the 1.9-kb fragment from the wild type allele and the 2.8-kb fragment from the *ARG4*-disrupted allele in NJ32. (b) Confirmation of the *ADE2* integration of the *pMAL2-ERG27* rescue cassette into NJ32 resulting in NJ45. NJ45 shows the predicted 4.3-kb and 4.8-kb fragments to confirm the integration. (c) Confirmation of the disruption of the second *ERG27* allele showing the 2.8-kb fragment from the *ARG4*-disrupted allele and the 2.7-kb fragment from the *HIS1*-disrupted allele in NJ61. NJ61 retains the 4.8-kb fragment from the *pMAL2-ERG27* rescue cassette. (d) Line diagrams indicating positions of the PCR primers used to confirm disruptions and integration sites.

Candida albicans *erg11 disruption*

A particularly important consideration in evaluating *erg* gene expression would be the alterations involving the Erg11p. Since azoles, the major class of antifungal drugs, target Erg11p, an *erg11* null strain was isolated by performing sequential disruptions of each *ERG11* allele in a wild type strain. The disruption of the first *ERG11* allele was confirmed by PCR that showed the presence of the predicted 1.4-kb wild type and 2.1-kb disrupted bands (Fig. 6) in the *ERG11* heterozygote, erg11H5. Analysis of the sterol content (Fig. 7) showed a wild type profile indicating that there were no other ergosterol mutations present in the heterozygote. Following mitotic recombination in the heterozygote and

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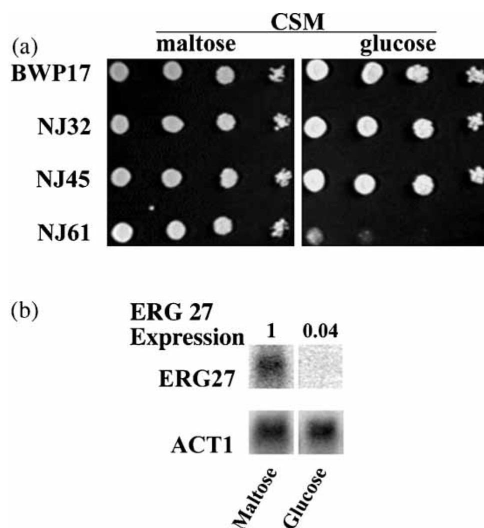

Fig. 4 (a) Growth of *Candida albicans* wild type (BWP17), *ERG27* heterozygote (NJ32), *ERG27* herterozygote with *pMAL2-ERG27* rescue cassette (NJ45), and *erg27* homozygote with *pMAL2-ERG27* rescue cassette (NJ61) on CSM medium with glucose or maltose. Identical patterns were noted on YPA medium. (b) Northern analysis of the expression of the *pMAL2-ERG27* cassette in cells grown on glucose and maltose. Levels of *ERG27* mRNA were quantified from the Northern blot signals and calculated relative to *ACT1* expression. *ERG27* expression values were normalized to the level of expression in maltose.

selection on nystatin, several homozygous *erg11* mutants were confirmed by PCR in which the 2.1-kb fragment of the disrupted *erg11* was observed but not the 1.4-kb wild type fragment (Fig. 6a). The *erg11*-disrupted strains generated here are the first non-clinical isolates of a *C. albicans erg11*. Sterol analysis of the *C. albicans erg11* strains (Fig. 7) indicated the presence of the predicted C-14 methyl sterols (24-methylene lanosterol {30%}, obtusifoliol {26%}, 14-methylergosta-8,24(28)-dien-3,6-diol {18%}, 14-methylfecosterol {15%}, and lanosterol plus some additional C14 methyl sterols {11%}) including significant levels of a C14 methylsterol diol (Fig. 7; peak N). This profile is consistent with those reported for clinical isolates of *erg11* [15,16]. The profile also indicates that (i) there is no functional Erg11p activity present (only C14 methyl and methylene sterols accumulate; (ii) that a functional Erg3p is present (sterol diol is present); and (iii) that the *ERG11* gene is not essential in *C. albicans*.

Northern analysis of C. albicans ERG27 expression

Based on the observation that lesions in the ergosterol pathway up-regulate expression of pathway genes [8,9], the expression of the *C. albicans ERG27* was determined using Northern analysis. To this point, no systematic investigation of the regulation of any of the C-4 demethylation genes has been undertaken.

To facilitate the analysis of *ERG27* regulation, several steps in the pathway (Fig. 1) were selected for inhibition either by known inhibitors or by utilizing mutants defective at those steps. Steps upstream and downstream of Erg27p action and steps that are the target sites for important antifungal drugs were included (Fig. 1). The isolation and sterol characterization of the *C. albicans erg6* [19] and *erg24* strains [17] have been reported elsewhere. Regulation of *ERG27* in an *erg11* background was also investigated using the *erg11* null as well as itraconazole, an azole that inhibits the Erg11p. The azole treatment protocol was chosen to saturate the Erg11p in order to eliminate all enzyme activity as would be the case in null mutant. In addition, the early pathway step catalyzed by squalene synthase (Erg9p) was inhibited using zaragozic acid A.

Figure 8a shows *ERG27* expression levels following treatment with itraconazole and zaragozic acid A. Relative to the untreated wild type strain, itraconazole and zaragozic acid A result in ten- and fivefold increases in *ERG27* expression, respectively. In a background where both *ERG11* alleles have been disrupted, a condition that would produce the same sterol profile as itraconazole treatment [3], *ERG27* expression increases three- to fourfold. Strains with disruptions in the upstream *ERG24* gene and the downstream *ERG6* gene (Fig. 1) show increases in *ERG27* expression in the twofold range (Fig. 8b).

Discussion

In order to determine if the *ERG27* gene is essential in *C. albicans* both copies were disrupted. Although disruption of *ERG27* in the haploid *S. cerevisiae* has shown this gene to be essential, there are significant differences in sterol requirements between the two organisms necessitating this verification. A prime example is the *ERG11* gene that encodes the C-14 demethylase. In *S. cerevisiae* the *erg11* phenotype results in no growth unless suppressed by a mutation in the downstream *ERG3* gene [39,40] while in *C. albicans* the *erg11* mutant is viable [15]. Similarly, the *ERG24* gene has been found to be essential in *S. cerevisiae* [12–14] but not in *C. albicans* [17].

The sterol profile in Fig. 2 shows that ergosterol synthesis has been restored in a *S. cerevisiae erg27* mutant by complementation with a *C. albicans* genomic library. The complementing DNA sequence has been confirmed as the *ERG27* gene by comparison with the *S. cerevisiae ERG27* sequence. The *C. albicans*

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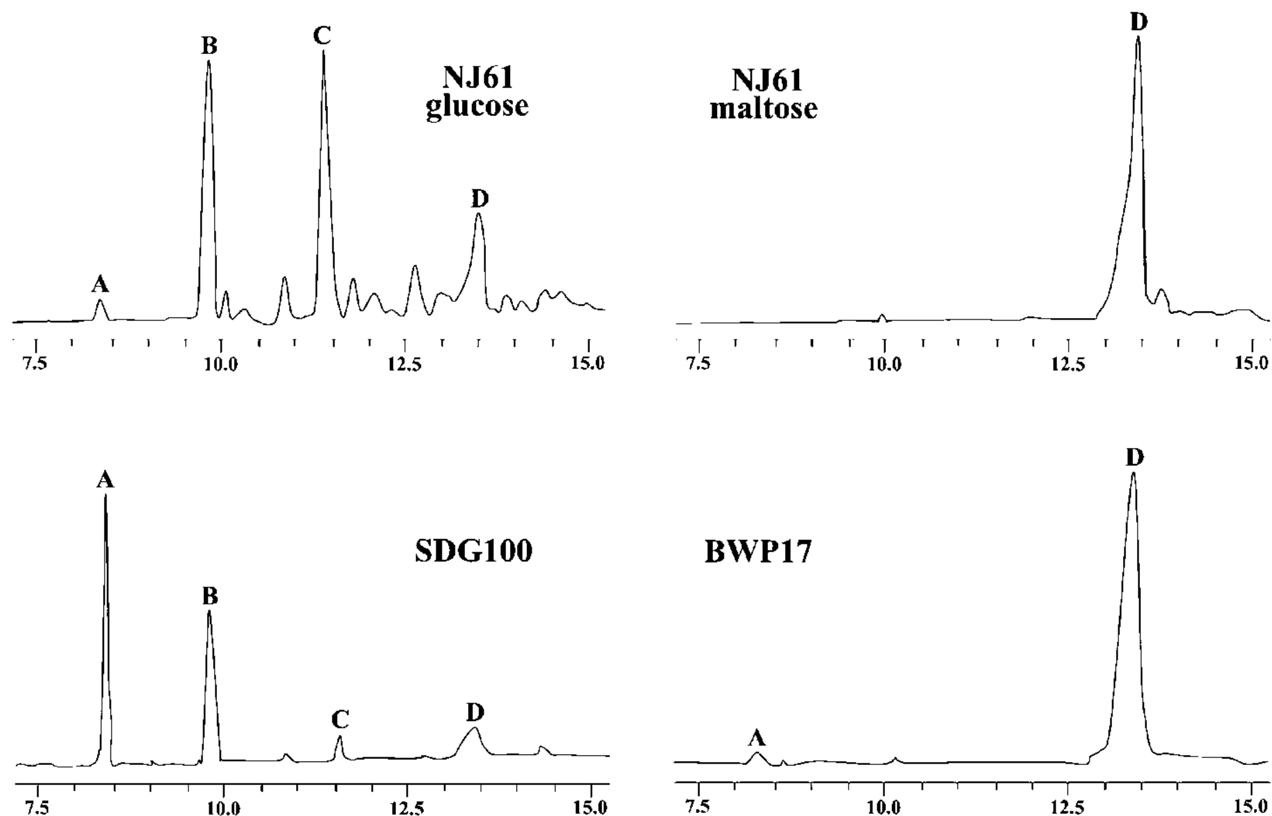

Fig. 5 The sterol profiles of *Candida albicans erg27* (NJ61) grown on glucose and maltose, *Saccharomyces cerevisiae erg27* (SDG100), and *C. albicans* wild type (BWP17). SDG100 was provided low levels of ergosterol to allow growth. Ergosterol accumulating in glucose-grown NJ61 is due to low level expression through the *pMAL2-ERG27* cassette. A, squalene; B, squalene epoxide; C, squalene dioxide; D, ergosterol.

ERG27 gene encodes a polypeptide that is 60% identical to the Erg27p reported for *S. cerevisiae*. The sequence of the *C. albicans ERG27* gene varies from that reported in the *C. albicans* sequence database (10 × Contig, assembly 19) by ten nucleotide substitutions (Table 2). Six do not change the encoded amino acid while the remaining four lead to amino acid substitutions that do not affect function. There are no identifiable phenotypes, including altered sterol phenotype, to distinguish this wild type *C. albicans* strain (BWP17) from others. The ten variations may be due to strain differences or to sequencing of an alternate allele.

In order to maintain viability where a likely essential gene was to be disrupted, a third copy of the *ERG27* was placed under the control of the *pMAL2* promoter and introduced into the *ERG27* heterozygote. The *pMAL2* promoter has been used to assess the essential function of other genes in C. *albicans*. In one case [41], gene *CaDPB2*, a potential homologue to the *S. cerevisiae DPB2* gene which encodes the α-subunit of DNA polymerase, was inserted under the control of *pMAL2* and a few colonies appeared after several days of growth on glucose. For this gene, basal transcription through the *pMAL2* was insufficient to permit growth. PCR analysis showed that the growing cells had regained, by recombination, the wild type gene at the original locus. In another study [24] using the *ERG26* gene of *C. albicans*, 2–3% of the cells showed growth on glucose and produce ergosterol. The study concluded that since the only cells able to grow synthesized ergosterol, the *ERG26* gene was essential.

Low levels of ergosterol were produced in the *erg27* strain grown on glucose. However, the abundant level of early pathway intermediates that also accumulate indicates that recombination of the *ERG27* wild type allele under *pMAL2* to another locus was occurring in a small number of the cells is the likely explanation for the limited growth. Northern analysis (Fig. 4b) of *ERG27* expression from a strain containing the *pMAL2-ERG27* construct grown on glucose supports this analysis in that low levels of expression resulting from the uncontrolled expression of the *ERG27* gene in a small number of cells is observed. Since growth is not possible without some ergosterol we conclude that the

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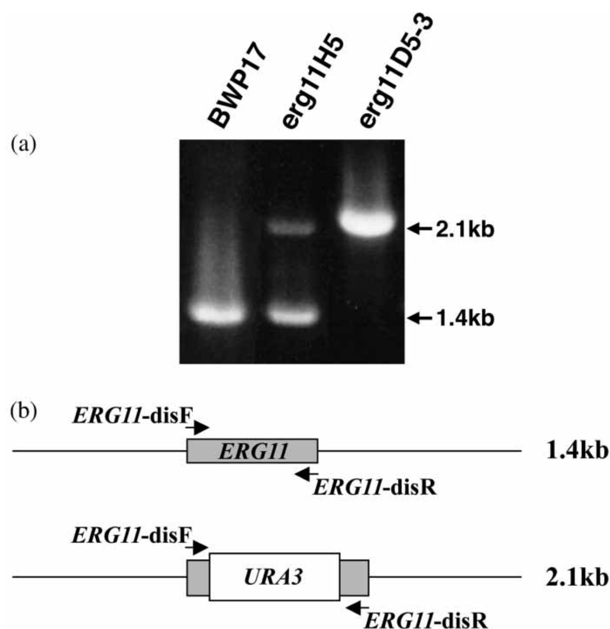

Fig. 6 PCR confirmation of the disruption of the two *ER11* alleles of *Candida albicans*. (a) Confirmation of the disruption of the first *ERG11* allele is demonstrated by the 1.4-kb fragment expected from the amplification of the *ERG11* wild type allele in BWP17 and in the *ERG11* heterozygote, erg11H5. The 2.1-kb *URA3* disrupted allele is also shown in erg11H5. Following mitotic recombination, the *erg11* homozygote, erg11D5-3, shows the presence only of the 2.1-kb disrupted allele. The PCR primers used were constructed to amplify within in termini of the *ERG11* gene such that *ERG11* genes anywhere in the genome would be detected. (b) Line diagrams indicating positions of the PCR primers used to confirm disruptions and integration sites.

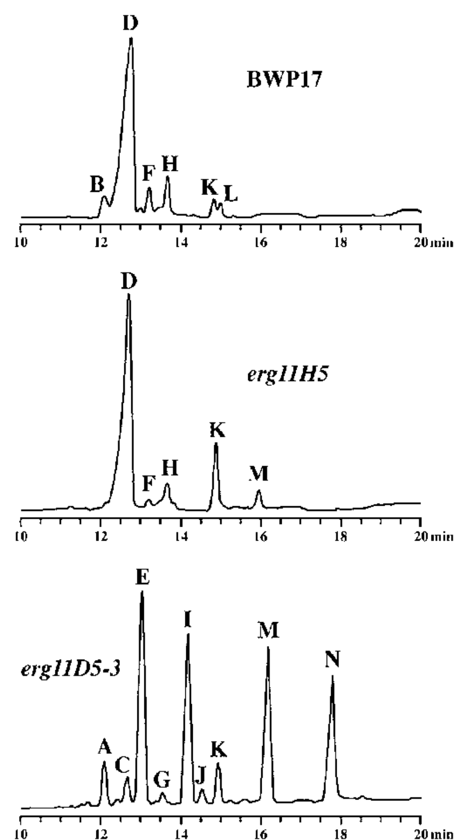

Fig. 7 The sterol profiles of the wild type, BWP17, the *ERG11* heterozygote, erg11H5, and the *erg11* homozygote, erg11D5-3. Peak identification: A, 14-methylergosta-5,8,22,24(28)-tetraen-3-ol; B, zymosterol; C, 14-methylergosta-5,8,22-trien-3-ol; D, ergosterol; E, 14-methylfecosterol; F, fecosterol; G, 14-methylergosta-5,8,24(28)-trien-3-ol; H, ergosta-dien-3-ol; I, obtusifoliol; J, lanost-8,24(28)-dien-3-one; K, lanosterol; L, 4,4-dimethylzymosterol; M, 24-methylene lanosterol; N, 14-methylergosta-8,24(28)-dien-3,6-diol (19%).

Erg27p function is essential in this organism. The compounds accumulating in the *erg27* strain are non-sterol pathway intermediates, squalene, squalene epoxide, and squalene dioxide. Although differing in proportion, these intermediates are identical to those reported for the *erg27* mutant in *S. cerevisiae* [25]. In contrast, lesions at other steps in the ergosterol pathway lead to the accumulation of sterol intermediates just prior to the mutation. Here, however, there are no C-4 demethylation-defective intermediates in *erg27* cells. Instead, no sterols at all accumulate suggesting a regulatory interaction between the 3-keto reductase and lanosterol synthase, the product of the *ERG7* gene (Fig. 1).

Disruption of the *ERG11* gene in *S. cerevisiae* results in non-viability unless a second mutation in the *ERG3* gene is also present. This is explained [39,40] by the formation of toxic sterol diol which accumulates as the result of the attempt of the Erg3p to desaturate the C5-6 position of C14-methyl fecosterol rather than its normal substrate, fecosterol. In the absence of Erg3p function, the lethal diol is not formed thus allowing *erg11 erg3* strains to grow. The situation in *C. albicans* has been less clear because of the exclusive dependence on clinical isolates that may contain other mutations. There have been reports of viable *erg11* mutants that accumulate sterol diol [15,16,39]. In one study [16], it was shown that the sterol diol disappeared in the presence of an *erg3* mutation indicating a direct role for *erg3* in preventing diol formation and indicating that the accumulation of diol reflects the presence of an *ERG3* genotype. The *erg11* null described here has been shown to have a functional *ERG3* gene based on the small accumulation of ergosterol intermediates containing the C-5 double bond and on the presence of significant levels of sterol diol. PCR has confirmed that there are no full-length *ERG11* genes in the strain.

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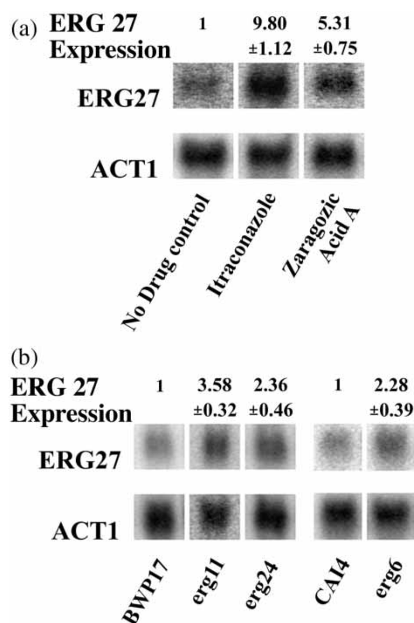

Fig. 8 Northern analysis of *ERG27* gene expression in *Candida albicans*. *ERG27* expression values were calculated relative to *ACT1* expression. (a) *ERG27* expression following treatment with itraconazole and zaragozic acid A. Values were normalized to the level of expression in the No Drug control. (b) *ERG27* expression in several mutant *ERG* gene backgrounds. Values were normalized to the level of expression in the appropriate wild type control strain.

There have been several reports where increased gene expression of the *ERG11* gene has been found to be a mechanism for resistance to the azole antifungals [7]. In addition, inhibitors of one step have also been shown to increase gene expression of several other steps in the pathway [8,9]. Microarray techniques have recently been employed to assess the regulation of genes in response to itraconazole [9]. In this study, a 2.4-fold increase in *ERG27* expression was reported in response to this azole. The availability of a *C. albicans erg11* null allows a comparison of azole treatment with a strain in which the Erg11p is absent. Under the azole treatment regimen used here, *ERG27* is up-regulated 10-fold. In contrast, expression of *ERG27* is increased only about one-third that amount in an *erg11* background. Since both itraconazole treatment and an *erg11* genotype produce the same sterol profile [3], this difference in expression may reflect the fact that azoles may have other cellular effects that can influence sterol gene expression. Alternatively, the difference may result from differences in the itraconazole treatment regimens (24 h exposure at 10 mg/l versus the protocol used here.) in the two studies. De Backer *et al.* [9] using microarray of itraconazole treated cells, found that over 276 non-sterol genes were responsive to itraconazole treatment in addition to the up-regulation of a number of sterol genes. Among these were 140 genes of unknown function. These results support the hypothesis that azoles may have non-sterol pathway targets. Zaragozic acid A, an inhibitor of the early *ERG9* step, results in a fivefold increase in *ERG27* expression.

ERG27 expression is increased about twofold in the disrupted *erg6* and *erg24* backgrounds. The data generated from drug treatments and *erg* gene disruptions support the suggestion that the general up-regulation of ergosterol gene expression is the result of ergosterol depletion [8,9]. In addition, the increase in expression of the *ERG27* gene in all backgrounds and treatments applied indicates that it may represent a key regulatory site in the ergosterol biosynthetic pathway.

The results presented here predict that the Erg27p would be a possible candidate for the development of antifungal compounds since this enzyme is required for growth in *C. albicans*. Further, the fact that the lack of the Erg27p results in a block at *ERG7*, in the pre-sterol part of the pathway, means that the cell cannot synthesize any sterol at all.

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