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Squalene epoxidase encoded by *ERG1* affects morphogenesis and drug susceptibilities of *Candida albicans*

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***Objectives*: Functional characterization of the *erg1* mutant of ergosterol biosynthesis of *Candida albicans*.**

***Methods*: We disrupted the *ERG1* gene of *C. albicans*, which encodes squalene epoxidase (EC 1.14.99.7). Since the disruption of both alleles of *ERG1* was lethal, the second allele of a heterozygous disruptant was placed under the control of a regulable promoter, *MET3p*, which is repressed by methionine and cysteine.**

***Results*: The reverse-phase HPLC analysis of sterol, extracted from the conditional mutant strain, showed a total lack of ergosterol and instead accumulation of squalene. This imbalance in sterol composition led to defects in growth and increased susceptibilities to drugs including fluconazole, ketoconazole, cycloheximide, nystatin, amphotericin B and terbinafine. Reduced drug efflux activity of the *erg1* mutant was associated with poor surface localization of Cdr1p, suggesting that enhanced passive diffusion and reduced efflux mediated by the ABC (ATP binding cassette) transporter Cdr1p increases drug susceptibility. Additionally, conditional *erg1* mutant strains were unable to form hyphae in various media.**

***Conclusions*: Taken together, our results demonstrate that the absence of ergosterol, which is one of the constituents of membrane microdomains (rafts), has a direct effect on drug susceptibility and morphogenesis of *C. albicans*.**

Keywords: regulable promoter, hyphae, drug resistance, Cdr1p, ergosterol, dimorphism

Introduction

The incidence of *Candida albicans* cells acquiring resistance to azoles has increased considerably in recent years, which has posed serious problems in successful chemotherapy of candidiasis. Current evidence indicates that multidrug resistance (MDR) is a multifactorial phenomenon comprising multiple mechanisms: including failure in drug export by extrusion pumps such as Cdr1p, Cdr2p [ATP binding cassette (ABC) family] and Mdr1p (major facilitator family), alterations in the azole-target Erg11p, as well as up-regulation of the encoding *ERG11* gene.[1–3] A combination of different resistance mechanisms has been reported to be responsible for fluconazole resistance in clinical isolates of *C. albicans*.[4]

The important role of lipids in drug susceptibilities of *Candida* cells has become apparent from recent studies.[5–7] Azole-resistant *C. albicans* isolates exhibit altered membrane phospholipid and sterol composition.[6,8–10] We and others have observed that ABC transporters Cdr1p of *C. albicans* and Pdr5p of *Saccharomyces cerevisiae* are particularly sensitive to changes in lipid composition where functions mediated by these drug extrusion pump proteins are affected.[5,11,12] Taken together, it appears that the associated changes in membrane lipid composition (phospholipid/ergosterol), its order (fluidity), and asymmetry could be important determinants of drug susceptibilities of yeast cells.[6,7]

Among various classes of lipids in *C. albicans*, membrane ergosterol is an important constituent, which is also the target of

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common antifungals like polyenes and azoles.[13–15] Ergosterol is also responsible for membrane rigidity, stability and resistance to physical stresses. In addition, ergosterol modulates membrane fluidity, permeability and the activities of membrane-bound enzymes.[16] Interestingly, the action of antifungals is affected by the changes in membrane lipid composition in general and by ergosterol in particular.[12,16] Because of the relationship between drug resistance and membrane ergosterol composition, many genes of the ergosterol biosynthetic pathway of *C. albicans* such as *ERG3, ERG6*, *ERG11*, *ERG24* and *ERG26* etc. have been analysed.[14,17–19]

ERG1 encodes squalene epoxidase (EC 1.14.99.7), which is a FAD-containing monooxygenase that converts squalene into 2,3-oxidosqualene.[13,20,21] Squalene epoxidase plays a key role in the synthesis of essential sterol compounds, hence homozygous disruption of *ERG1* was found to have deleterious effects in yeast cells.[22,23] In order to explore the direct involvement of ergosterol in morphogenesis and drug susceptibility of *C. albicans*, in this study, we disrupted one allele of *ERG1* and expressed the second allele under the control of the regulable *MET3* promoter.[24,25] We show that conditional suppression of *ERG1* of *C. albicans* increases its susceptibility to drugs and leads to defects in hyphae formation. The enhanced susceptibility of the conditional *erg1* mutant to various drugs is linked to increased passive diffusion and reduced efflux of drugs mediated by ABC transporter Cdr1p, which as a result of imbalance in sterol composition was poorly localized to the plasma membrane.

Materials and methods

Materials

Media chemicals were obtained from Difco (Detroit, MI, USA) and HiMedia (Mumbai, India). The drugs cycloheximide, terbinafine, nystatin, amphotericin B, rhodamine-6G and protease inhibitors (PMSF, leupeptin, pepstatin A, aprotinin) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Nourseothricin was obtained from Werner Bioagents (Jena, Germany). Ranbaxy Laboratories (New Delhi, India) kindly provided fluconazole, while ketoconazole was a gift from Dupont (Wilmington, DE, USA).

Bacterial and yeast strains and growth media

Plasmids were maintained in *Escherichia coli* DH 5α. *E. coli* was cultured in Luria–Bertani medium (Difco, BD Biosciences, NJ, USA), to which ampicillin was added (100 mg/L). *C. albicans* CAI4, CAF2-1 and the heterozygous disruptant strains were grown in Yeast Extract Peptone Dextrose (YEPD) broth or synthetic defined (SD) medium (Bio 101, Vista, CA, USA).[25] To repress the *MET3* promoter, the conditional *ERG1* mutants were grown in SD medium supplemented with 0.5 mM each of methionine and cysteine (M/C).

Deletion of ERG1 and chromosomal integration of MET3p-ERG1

ERG1 of *C. albicans* CAI4 was disrupted by employing the *URA*-Blaster method.[26,27] A cassette for the disruption of *ERG1* was constructed in several steps. Firstly, 690 bp of the 5′ sequences flanking the start of the *CaERG1* ORF were amplified using primers ERG1disA and ERG1disB (5′-CTAGAGCTC*GGATCC***GTGTTTACTCATATG**-3′ and 5′-CTAGAGCTC*GGATCC***GGACTGGCACTATTCTC**-3′: bold, regions of homology; italics, *Bam*HI). Similarly, 761 bp of the 3′ untranslated sequences were amplified by primers ERG1disC and ERG1disD (5′-CTAGAGCT*CTGCAG***ATTGTAAGATAGAGAG**-3′ and 5′-CTAGAGCT*CTGCAGG***TTCTTTCGTCCC**-3′: bold, regions of homology; italics, *Pst*I). PCR fragments were sub-cloned into pUC18, which resulted in plasmids pSKM3 and pSKM6, respectively. pSKM3 was digested with *Bam*HI and cloned into the *Bgl*II site of pUC18. The *Pst*I fragment of pSKM6 was inserted into the *Pst*I site of the resulting plasmid pSKM53. A plasmid with *ERG1* flanking sequences, in the correct orientation, was obtained (pSKM54). Its *Sac*I–*Sph*I fragment containing the *ERG1* disruption cassette was used to obtain heterozygous strain ΔE4 (*ERG1/erg1*Δ::*hisG-URA3-hisG*) by transformation. *URA3* was looped out from ΔE4 using 5-fluoroorotic acid (1 mg/mL) and 25 mg/L uridine to obtain ΔE4.2 (*ERG1/erg1*Δ::*hisG*).

To place *ERG1* under the control of the *MET3* promoter, we followed a previously described strategy.[25] First, a fragment of 543 bp corresponding to the 5′-end of the *ERG1* ORF was amplified by PCR using the primers ErgN-term (5′-**GCACACCAATAACTGTG**-3′) and ΔErgStp*Bgl*II–*Hin*dIII (5′-CTTAAGCTT*AGAT**CT***AGTCTCT-**CTATCTTAC**-3′) and cloned into pUC18, resulting in plasmid pSKM12. The *Bam*HI fragment of pSKM12 was inserted into the single *Bam*HI site of pCaDis, downstream of the *MET3* promoter.[24] The resulting plasmid pSKM14 was linearized by *Nco*I (which cuts within the *ERG1* fragment) and was used to transform the heterozygous strain ΔE4.2 (*ERG1/erg1*Δ::*hisG*) to obtain the conditional mutant ΔE4.2.7 (*MET3p-ERG1/erg1*Δ::*hisG*). Integration of heterozygous and conditional strains was checked by Southern blots.

Tagging of Cdr1p with GFP

To tag *CDR1*-ORF with *GFP*, its stop codon was mutated into a *Bam*HI site. For this, 250 bp of the C-terminal *CDR1* ORF was PCR-amplified using primers CDR29F and CDR30R (5′-ATTT*GGTACC***ATACATTAAATTTGCTGGTGGG**-3′ and 5′-GTTT*GGATCC***TTTCTTATTTTTTTTCTCTCTGTTACCC**-3′: bold, regions of homology; italics, *Kpn*I and *Bam*HI). The PCR amplicon was digested with *Kpn*I–*Bam*HI (sites introduced into forward and reverse primers during synthesis). *GFP* along with the *ACT1* termination sequence and the dominant selection marker gene *SAT1* were taken out by digesting vector pADH1G3 with *Bam*HI–*Pst*I.[28] The *Kpn*I–*Pst*I-digested vector backbone of pCPL51 (2.9 kb) was ligated with *Kpn*I–*Bam*HI-digested C-terminal *CDR1* along with *Bam*HI–*Pst*I-digested *GFP-SAT1* fragment to generate the final plasmid pCPG2. Its 3.5 kb *Kpn*I–*Sac*II fragment having CT-*CDR1-GFP-SAT1*-3′UTR was used to integrate CAF2-1 and ΔE4.2.7 at the *CDR1* locus. The transformants were selected on YEPD plates containing 200 mg/L of nourseothricin.[28] Transformants were designated as CAF2-1CDR1GFP and ΔE4.2.7-CDR1GFP for further study.

Growth of the mutant strains

C. albicans strains were grown for 20 h in SD medium with 0.5 mM methionine and 0.5 mM cysteine and their growth was monitored at 600 nm after regular time intervals. The growth of the conditional strains was found to be maximally suppressed at 14 h after the addition of M/C, and cells at this time point were collected and used for further analysis.

Identification of sterols by HPLC

Ergosterol formation and squalene accumulation were determined by reverse-phase HPLC using an HPLC system (Biologic

Duo-Flow, Bio-Rad, UK). Sterols were extracted by the alcoholic KOH method[7] and reverse-phase chromatography was carried out using a C-18 column fitted with a C-18 guard column. Elution was carried out using the previously described gradient program.[7,29] Samples were injected as solutions in 95% ethanol. Elution of compounds was automatically monitored by absorption at 214, 230, 260 and 282 nm. Ergosterol and squalene were used as standards (from Sigma) for comparison of their respective retention times.

Drug susceptibility testing in C. albicans strains

As a result of the exhaustion of methionine and cysteine after 18 h in the growth medium, it was not possible to determine the MICs of drugs and therefore only spot assays were performed for testing drug susceptibilities of the conditional *erg1* mutant strain. In spot assays, 5 μL of 5-fold serial dilutions of each yeast culture ($A_{600} = 0.1$) was spotted on to SD plates supplemented with and without 0.5 mM methionine and 0.5 mM cysteine, in the absence

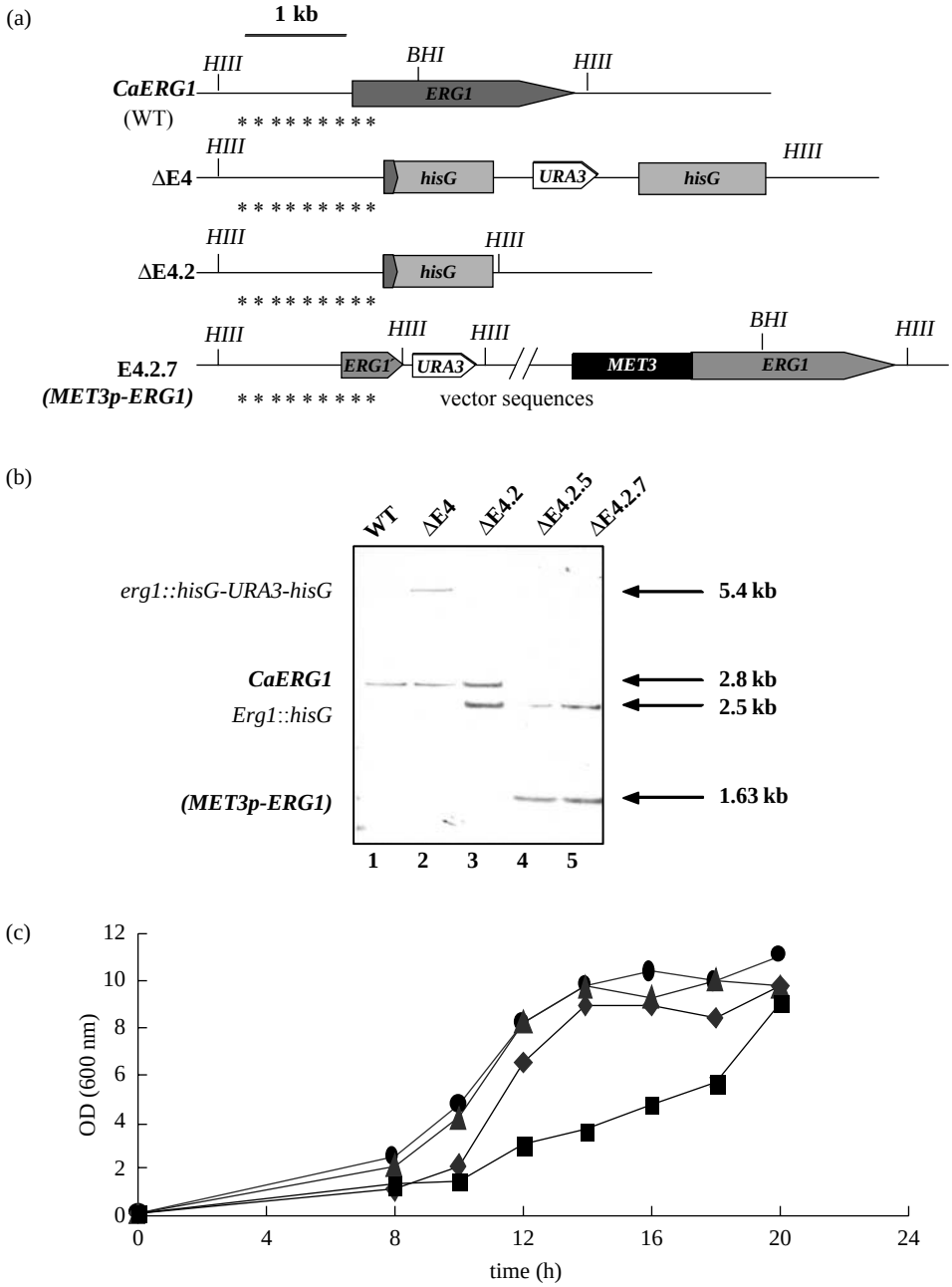

Figure 1. (a) Genomic configuration of the *C. albicans ERG1* wild-type locus and its deleted derivatives. (b) Southern blot to confirm the disruption of the first *ERG1* allele and orientation of the *MET3* promoter upstream of the second allele. Genomic DNA (2 μg) from each strain was extracted and digested with *Hin*dIII, blotted and probed with the 1 kb fragment indicated by asterisks in the figure. Strains tested were: lane 1, CAF2-1 (*ERG1/ERG1*); lane 2, ΔE4 (*ERG1/erg1*Δ::*hisG-URA3-hisG*); lane 3, ΔE4.2 (*ERG1/erg1*Δ::*hisG*); and lanes 4 and 5, ΔE4.2.5/ΔE4.2.7 (*MET3p*::*ERG1/erg1*Δ::*hisG*). (c) Growth of the *C. albicans* and its *erg1* mutant. Cells were tested for growth at 30°C in SD medium containing 0.5 mM of methionine and cysteine (M/C). Cells were inoculated to an initial OD_{600} of 0.1 in SD medium containing M/C. The growth was monitored at regular intervals at OD_{600}. Filled symbols represent growth of CAF2-1 without M/C (filled circles), CAF2-1 with M/C (filled diamonds), ΔE4.2.7 without M/C (filled triangles), and ΔE4.2.7 with M/C (filled squares).

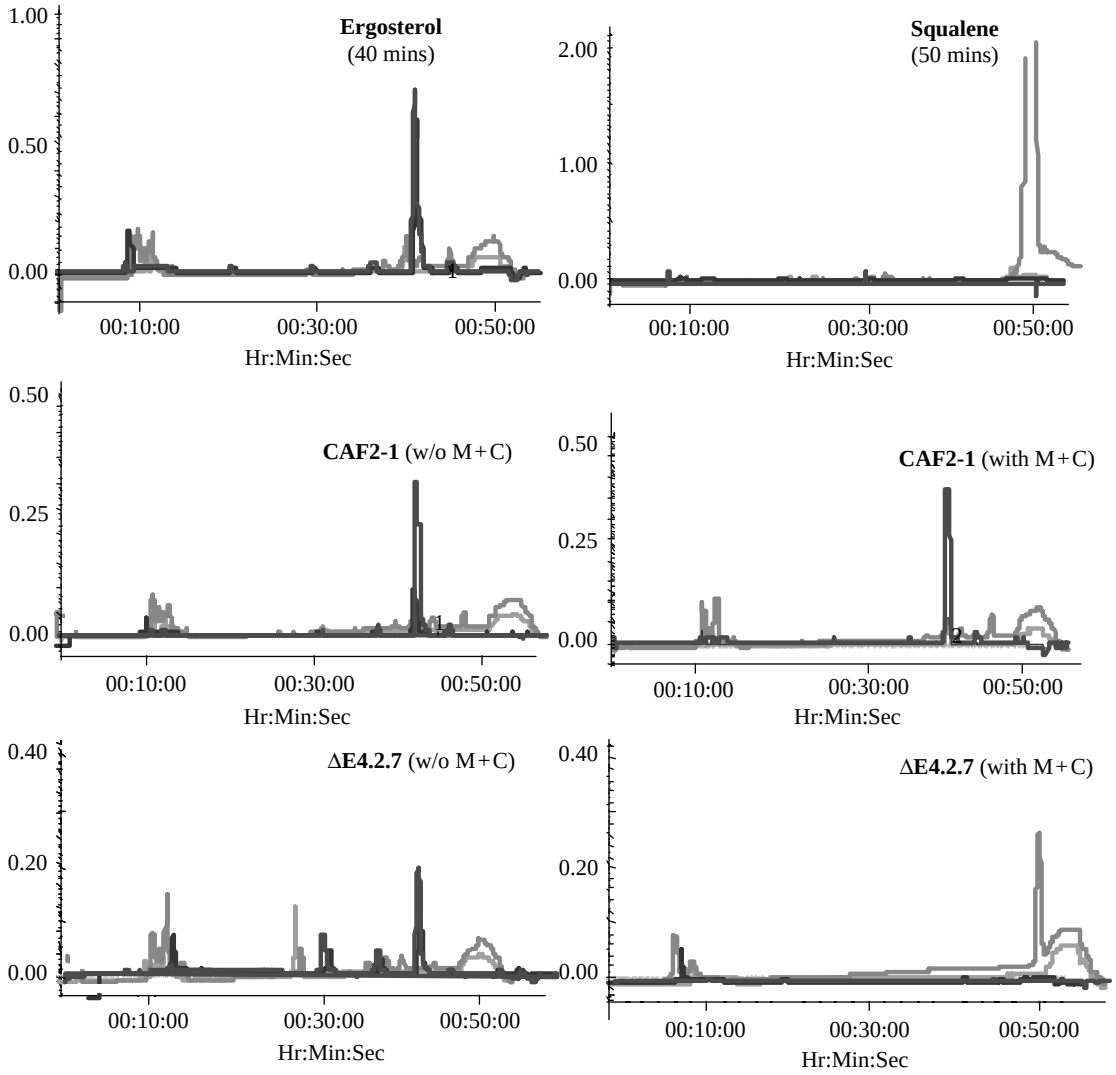

Figure 2. Sterol profiling of the wild-type strain and *ERG1/erg1* conditional mutants. Sterols were extracted by the alcoholic KOH method as described previously,[7] and were analysed by reverse-phase HPLC. Elution of compounds was automatically monitored by absorption at 214, 230, 260 and 282 nm.[7] Ergosterol and squalene were used as standards for comparison of their respective retention times with extracted sterols from *Candida* cells. The retention time of ergosterol is 40 min and that of squalene is around 50 min.

(control) and in the presence of the drugs. Growth differences were recorded following incubation of the plates for 2 days at 30°C.

Passive diffusion and rhodamine 6G (R6G) efflux

Passive diffusion and efflux of fluorescent R6G was determined using a protocol described previously,[6,7] except that SD medium supplemented with M/C was used for culturing cells. Cells were pelleted down and a 2% cell suspension was made in de-energization phosphate-buffered saline (PBS) containing 5 mM 2,4-dinitrophenol and 5 mM deoxy-D-glucose and incubated for 2 h at 30°C.[6,7,12] Cells were then washed and incubated in PBS containing 10 μM R6G. For passive diffusion, a 1 mL aliquot was taken out at different time intervals, centrifuged at 9000 **g** for 2 min and the optical density of the supernatant was determined at 527 nm.[6,7] Efflux was initiated by the addition of 1 mole of glucose and an aliquot of 1 mL was taken out at regular intervals for measuring optical density at 527 nm.

Plasma membrane preparation and western blotting

Plasma membranes (PM) were prepared as described previously,[30] except that SD medium without (control) and with M/C was used for cell culture. Western blotting was done with anti-Cdr1p and anti-Pma1p antibodies.[30] PM proteins were separated on an 8% SDS/polyacrylamide gel and electroblotted onto a nitrocellulose membrane and incubated with rabbit anti-Cdr1p antibody (diluted 1:500) and rabbit anti-Pma1p antibody (diluted 1:10 000). Proteins on immunoblots were detected using the chemiluminescence assay system (ECL kit; Amersham Biosciences).[30]

GFP fluorescence microscopy

The cells were grown in SD medium with and without M/C, placed on glass slides and directly viewed under a ×100 oil immersion objective of a confocal microscope (Radiance 2100, AGR, 3Q/BLD, Bio-Rad, UK).

Hyphal morphogenesis in mutant strains

Strains were grown for 5 days at 37°C on solid Lee's and Spider media.[25] Additionally, hyphal-inducing liquid media supplemented with 15% serum and *N*-acetyl glucosamine supplemented with 0.50 mM of M/C were also used.[31] Following incubation, the colonies and cells were checked for hyphae formation under a Zeiss Axioscope microscope.

Results and discussion

ERG1 is an essential gene

For the disruption of *ERG1*, which encodes squalene epoxidase, a key enzyme of ergosterol biosynthesis, *C. albicans* sequence data were obtained from the Stanford Genome Technology Centre (http://sequence.stanford.edu/group/candida). The deduced *C. albicans* Erg1 protein contains 496 residues, with a predicted molecular mass of 55.3 kDa.[20] The genomic region of *ERG1* was isolated by PCR using genomic DNA of the CAI4 strain. A *Sac*I–*Sph*I fragment from plasmid pSKM54 containing an *ERG1* disruption cassette was used to transform the CAI4 strain to obtain heterozygous strain ΔE4 (*ERG1/erg1*Δ::*hisG-URA3-hisG*). Heterozygous *ERG1/erg1* strains were generated without any difficulty but construction of the homozygous *erg1/erg1* strain using the same *hisG-URA3-hisG* blaster cassette repeatedly failed. This suggested that probably similar to the *ScERG1* gene of *S. cerevisiae*, *CaERG1* of *C. albicans* is an essential gene.[23]

To reconfirm this, we put the second allele under the control of the *MET3* regulable promoter, which is repressed by M/C.[24,25] The cells with one *ERG1* allele under the control of the regulable promoter were designated as ΔE4.2.7 (*MET3p-ERG1/erg1*Δ::*hisG*) (Figure 1a). Correct integrations in heterozygous and conditional strains were checked by Southern blot, as shown in Figure 1(b). Two independent clones were picked and analysed simultaneously for the phenotypes described below. Pairs of isogenic mutant strains were identical in all phenotypes - tested.

Growth of the conditional mutant strain is suppressed

The conditional mutant strain ΔE4.2.7 (*MET3p-ERG1/erg1*Δ::*hisG*) was grown in liquid SD medium with or without M/C. We observed that 0.5 mM of M/C was enough to considerably suppress the growth of the conditional mutant ΔE4.2.7, while growth of the wild-type CAF2-1 and heterozygous strain ΔE4 remained almost unaffected under similar repressing conditions (Figure 1c). However, growth suppression ceased after 24 h and conditional mutants with suppressor were found to regain their growth. Of note, strain ΔE4.2.7, when streaked on SD plates containing 0.5 mM M/C, continued to show suppression of growth and no regrowth was observed. The regrowth of conditional strain at 24 h could be because of the leakiness of the *MET3* promoter as was reported previously,[32] or partly could be due to exhaustion of M/C.

ERG1 conditional mutant strain lacks ergosterol

Mutants of the ergosterol biosynthetic pathway do not produce ergosterol and utilize sterol intermediates to compensate for the loss of ergosterol.[5,8] To confirm the status of ergosterol levels in the *erg1* mutant strain, reverse-phase HPLC was employed.[7,29] The HPLC analyses of extracted sterols revealed that ergosterol levels in the heterozygous strain ΔE4 were

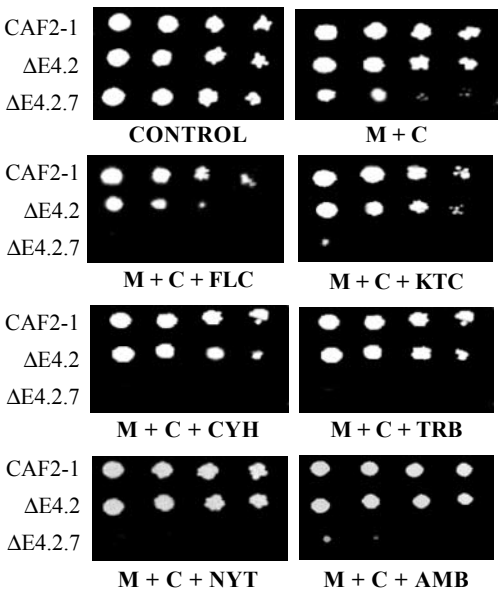

Figure 3. Drug susceptibility tests. In spot assays, 5 μL of 5-fold serial dilutions of each yeast culture (A_{600} = 0.1) was spotted onto SD plates containing the following drugs: fluconazole (FLC) (10 mg/L), cycloheximide (CYH) (500 mg/L), ketoconazole (KTC) (0.05 mg/L), terbinafine (TRB) (0.1 mg/L), nystatin (NYT) (1.25 mg/L), amphotericin B (AMB) (0.2 mg/L), in the presence or absence of M/C. Growth differences were recorded following incubation of the plates for 2 days at 30°C.

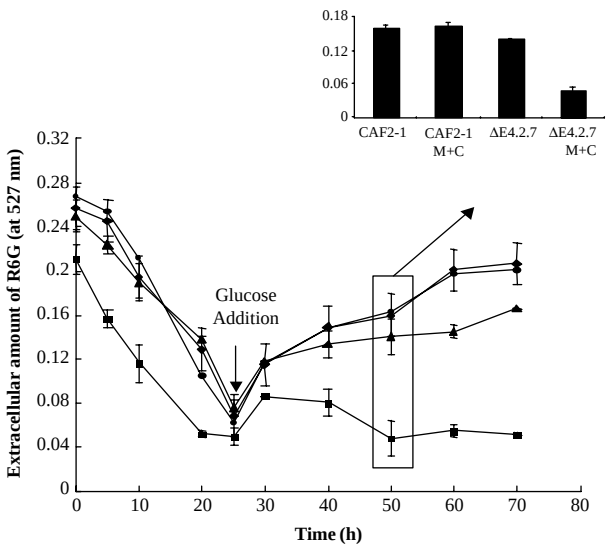

Figure 4. Transport of rhodamine 6G (R6G). For passive diffusion, de-energized cells were incubated with 10 μM R6G at 30°C, and at different time points, cells were rapidly centrifuged and the extracellular concentration of R6G in the supernatant was determined spectrophotometrically at 527 nm.[7] Active efflux was initiated by the addition of 1 mole of glucose at the indicated time to de-energize cells.[7] The symbols represent the extracellular amount of R6G in CAF2-1 (filled circles), CAF2-1 (M/C) (filled diamonds), ΔE4.2.7 (no M/C) (filled triangles) and ΔE4.2.7 (M/C) (filled squares). The inset shows R6G efflux at 25 min post-glucose addition.

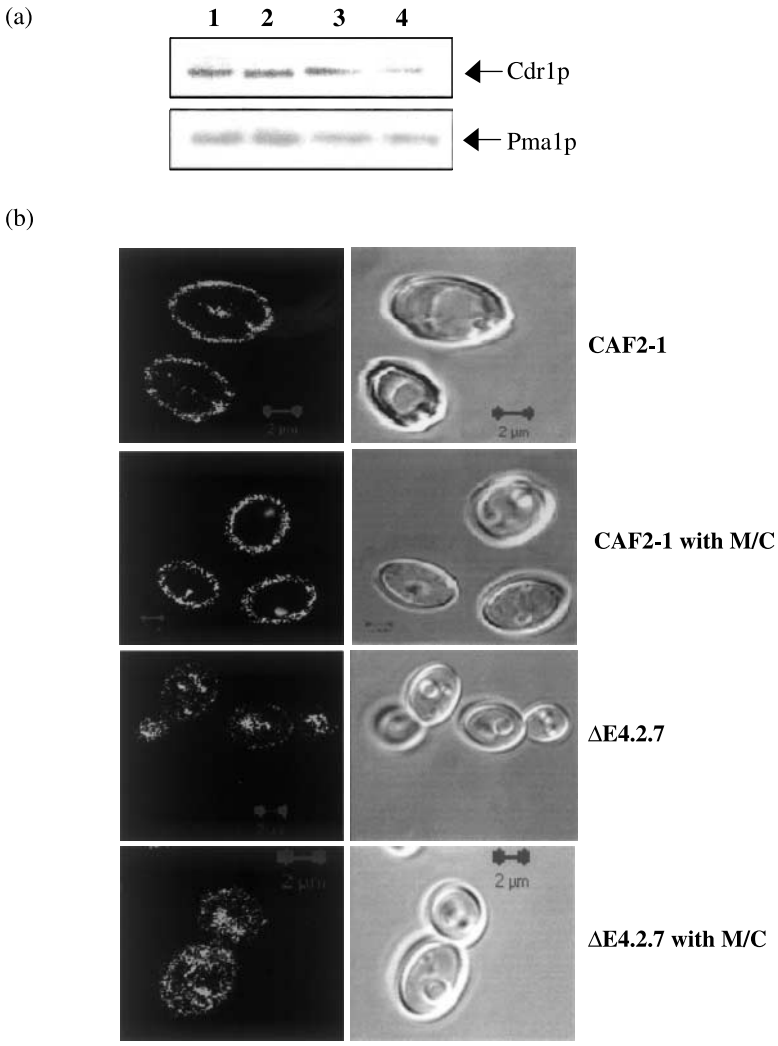

Figure 5. (a) Expression of Cdr1p. Plasma membrane (PM) fractions from CAF2-1, CAF2-1 (M/C), ΔE4.2.7 and ΔE4.2.7 (M/C) were prepared as described previously.[30] PM protein (40 μg) was separated on an 8% SDS/polyacrylamide gel, electroblotted onto nitrocellulose membrane and incubated with rabbit anti-Cdr1p antibody (diluted 1:500) and rabbit anti-Pma1p antibody (diluted 1:10 000). Proteins were detected using an ECL kit.[30] Fractions of strain CAF2-1 (lane 1), CAF2-1 with M/C (lane 2), ΔE4.2.7 without M/C (lane 3) and ΔE4.2.7 with M/C (lane 4) were analysed. Pma1p was used as the loading control. (b) ΔE4.2.7 cells producing Cdr1p-GFP were grown in SD medium with and without M/C and viewed directly by confocal microscopy. The left-hand panels show the fluorescence images and the right-hand panels show the corresponding phase contrast images. GFP fluorescence from CAF2-1/CDR1-GFP shows rimmed localization of Cdr1p on the plasma membrane both without and with methionine and cysteine, whereas those of ΔE4.2.7/CDR1-GFP without and with M/C result in a patchy appearance with more fluorescence inside the cells.

lower compared with the wild-type CAF2-1. Interestingly, the conditional disruptant strain (ΔE4.2.7), after growth in the presence of M/C, showed no detectable characteristic ergosterol peak with a retention time of 40 min, but instead showed a high peak of squalene at a retention time of 50 min (Figure 2). Thus, HPLC analysis revealed that ΔE4.2.7 strain, which conditionally lacks functional squalene epoxidase (Erg1p), is unable to synthesize ergosterol and instead accumulates squalene (Figure 2). The lack of ergosterol in the conditional strain was also confirmed by spectrophotometric analysis of extracted sterols (data not shown).[7] Both the quantitative methods thus confirmed that the conditional strain in the presence of M/C does not synthesize ergosterol and instead accumulates squalene.

ERG1 expression levels affect drug susceptibility

In view of the fact that alterations in lipid composition affect drug susceptibilities,[6,7,11,33] we examined whether the depletion of ergosterol and concomitant accumulation of squalene affected drug susceptibilities of conditional disruptant cells. Interestingly, under repressing conditions in serial dilution spot assays, the ΔE4.2.7 cells were hypersusceptible to all tested drugs (fluconazole, cycloheximide, ketoconazole, amphotericin B, nystatin and terbinafine) compared with CAF2-1 and heterozygous ΔE4.2 (Figure 3). The increased susceptibility of ΔE4.2.7 conditional mutant cells to terbinafine needs special mention. Terbinafine is known to target *ERG1*, therefore, its conditional disruption, as in ΔE4.2.7 cells, should result in resistance rather

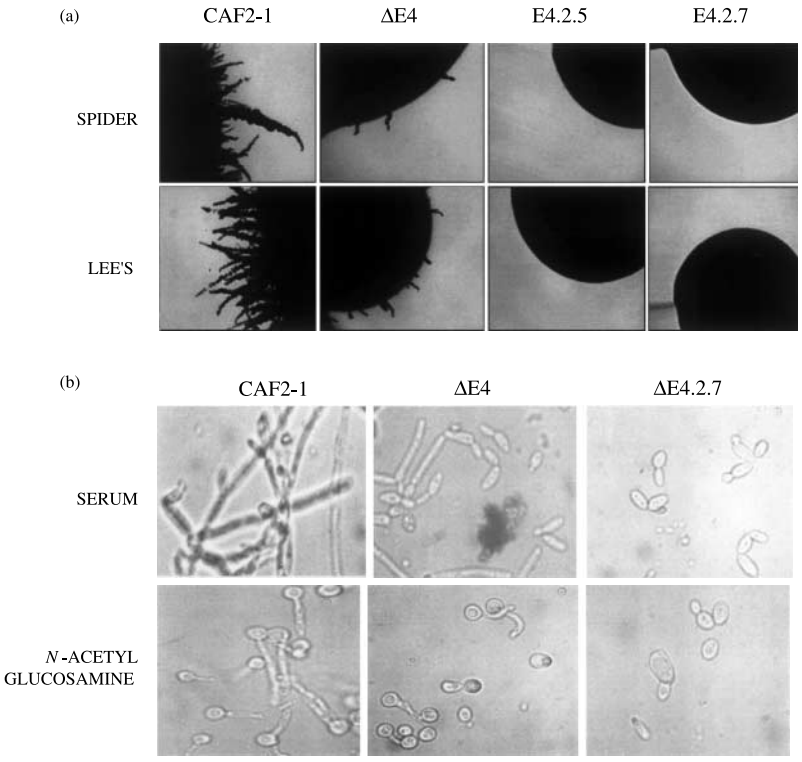

Figure 6. Filamentation of *erg1* mutants. Strains tested are designated as CAF2-1 (*ERG1/ERG1*), the heterozygous strain ΔE.4 *(ERG1/erg1Δ::hisG-URA3-hisG*) and two independent conditional mutants ΔE4.2.5 and ΔE4.2.7 (*MET3p::ERG1/erg1Δ::hisG*). (a) Strains were grown on solid Spider and Lee's media at 37°C for 5 days. Colony phenotypes were recorded microscopically (magnification 2.5-fold). (b) Hyphal induction in liquid media by using 15% serum and *N*-acetyl glucosamine (with M/C for the conditional mutant) and incubating for 12 h at 37°C.[31] Cells were seen microscopically at ×100 magnification.

that hypersusceptibility. However, our observations support previous reports in *Candida glabrata*,[22] where an *erg1* defect led to increased susceptibility to terbinafine. These results imply that various unidentified factors could also modulate the response of fungal cells to this allylamine.[13,23,34] Interestingly, the *erg1* conditional strain ΔE4.2.7 became susceptible to polyenes, nystatin and amphotericin B, even in the complete absence of detectable ergosterol (Figure 3). This finding was unexpected, because polyenes specifically interact with ergosterol and hence *erg1* mutants lacking ergosterol are expected to be resistant to these drugs. Our results support increasing evidence suggesting that membrane lipids other than ergosterol could also influence polyene susceptibility.[13,23,34]

erg1 conditional mutants show reduced passive diffusion and efflux of R6G

Both the entry (passive diffusion) and exit (efflux) of drugs affect the drug susceptibilities of *Candida* cells.[6,7] We therefore examined this aspect by measuring passive diffusion and energy-dependent efflux of R6G, which is a substrate of Cdr1p of *C. albicans*.[7] The conditional *erg1* knockout strain ΔE4.2.7, in the presence of M/C, displayed decreased amounts of extracellular R6G in de-energized cells, suggesting increased passive diffusion of the substrate (Figure 4). To determine active drug efflux, the fluorescent compound R6G was allowed to equilibrate in de-energized *Candida* mutant cells by passive diffusion and the energy-dependent extrusion of R6G was then initiated by the addition of glucose. As depicted in Figure 4, reduction in glucose-induced efflux, indicated by a decrease in extracellular concentration of R6G, was seen in conditional mutant strain ΔE4.2.7 (70% reduction in efflux of R6G at 25 min after the addition of glucose, compared with the CAF2-1 cells; see inset of Figure 4). Thus, the increase in passive diffusion and reduced ability to efflux, both probably contribute to hypersusceptibility of the conditional *erg1* mutant. Here we would like to mention that the conditional mutant without the suppressors, which is similar to the heterozygous strain, showed similar growth to the wild-type cells. This *erg1/ERG1* conditional strain without M/C could already show the difference in its ability to efflux R6G. This gene dosage-dependent reduction in efflux in *ERG1/erg1* implies that efflux changes are not growth-dependent. Thus addition of M/C which further reduces the efflux of the *erg1/erg1* strain is due to suppression of the second allele and not due to the slow growth.

Localization of membrane-bound transporter Cdr1p is impaired

We have previously observed that the ABC transporter Cdr1p is selectively sensitive to changes in lipid composition.[6,7] It is thus possible that the observed impaired efflux in the conditional mutant strain could be due to poor functionality of Cdr1p. Our immunoblot results revealed that in ΔE4.2.7 cells, which were collected after 14 h of M/C addition in SD medium, the expression of Cdr1p in PM was decreased compared with PM isolated from the wild-type strain (Figure 5a).

In another set of experiments, we expressed Cdr1p as a GFP-tagged protein by integrating the *CDR1-GFP* cassette at the *CDR1* locus by exploiting the *SAT1* marker,[28] in both wild-type and ΔE4.2.7 cells. Confocal microscopic images confirmed that GFP-tagged Cdr1p was poorly localized on the membrane of the conditional mutant strain, if grown under repressing conditions. Typical rimmed fluorescence appearance of Cdr1p-GFP was seen in CAF2-1, whereas ΔE4.2.7, under repressing conditions showed more intracellular fluorescence (Figure 5b). Localization of Cdr1p-GFP produced in strain CAF2-1 was not affected by the presence or absence of M/C (Figure 5b). These results indicate that the absence of ergosterol and/or the accumulation of squalene lead to poor surface localization of Cdr1p in *erg1* conditional mutants and, consequently, to poor efflux of drugs.

Hyphal morphogenesis requires wild-type ERG1 expression levels

The availability of a conditional strain, in which *ERG1* expression could be suppressed, provided an opportunity to examine if it affects morphogenesis of *C. albicans.* The ability of *erg1* disruptants to form hyphae was checked on solid Lee's and Spider media. Additionally, hyphal-inducing liquid media supplemented with 15% serum and *N*-acetyl glucosamine supplemented with 0.50 mM of M/C were also used.[31] As compared with CAF2-1, the heterozygous strain ΔE4 was found to have reduced formation of hyphae, while the conditional disruptant strain ΔE4.2.7 (*erg1/erg1*) appeared as colonies lacking filaments and thus had completely lost its ability to form hyphae under the conditions tested (Figure 6a and b). The inability of the conditional disruptant strain to form hyphae in all the tested media suggests that wild-type Erg1p-levels are critical for morphogenesis of *C. albicans* (Figure 6). Of note, both media used for hyphal induction already contained M/C and complex sources of nitrogen, therefore, no additional repressor to the hyphal media was necessary to partially suppress *ERG1* expression, while still permitting growth.[25]

Conclusions

In conclusion, we demonstrate that any imbalance in sterol composition severely increases drug susceptibility of *C. albicans* cells. The complete lack of ergosterol in the conditional *erg1* mutant and/or accumulation of squalene therein affect functioning of the ABC drug extrusion pump protein Cdr1p mainly because of its poor surface localization. There have been previous reports describing the role of ergosterol in the morphogenesis of *Candida*,[35,36] but in this study, we show a direct link between sterol composition and the ability of *Candida* cells to form hyphae. In this context, it is important to refer to a recent report, where the presence of polarized membrane domains, rich in ergosterol and sphingolipid in *C. albicans*, was demonstrated.[37] Both ergosterol and sphingolipids are two major constituents of membrane microdomains (membrane rafts) and blocking synthesis of either of the two results in defects in hyphae formation and drug susceptibilities.[7] Considering recent reports, it appears that Cdr1p of *C. albicans* may preferentially be associated with raft microdomains[6,7] that remain to be demonstrated experimentally.

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