PUBLICATIONS
Iron deprivation induces EFG1-mediated hyphal development in *Candida albicans* without affecting biofilm formation

Saif Hameed1, Tulika Prasad1, Dibyendu Banerjee1, Aparna Chandra2, Chinmay K. Mukhopadhyay2, Shyamal K. Goswami1, Ali Abdul Latti2, Jyotsna Chandra3, Pranab K. Mukherjee3, Mahmoud A. Ghannoum3 & Rajendra Prasad1

1School of Life Sciences, Jawaharlal Nehru University, New Delhi, India; 2Special Center for Molecular Medicine, New Delhi, India; and 3Center for Medical Mycology, University Hospitals Case Medical Center, Cleveland, OH, USA

**Correspondence:** Rajendra Prasad, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India. Tel.: +91 11 2670 4509; fax: +91 11 2674 1081; e-mail: rp47@mail.jnu.ac.in

Received 19 December 2007; revised 9 April 2008; accepted 17 April 2008
First published online 10 June 2008.

DOI:10.1111/j.1567-1364.2008.00394.x

**Editor:** Richard Calderone

**Keywords**
iron deprivation; hyphal development; EFG1; morphological regulators; membrane fluidity; biofilm.

**Abstract**

In this study, we investigated the role of cellular iron status in hyphae and biofilm formation in *Candida albicans*. Iron deprivation by a chelator, bathophenanthroline disulfonic acid, promoted hyphal development even in nonhyphal-inducing media without affecting growth of *C. albicans* cells. Iron-acquisition defective mutants, *Afr1* and *Delta1*, also showed hyphal formation, which was prevented by iron supplementation. Notably, most of the tested morphological mutants *Dcp1*, *Dfg1* and *Dtpk1* continued to form hyphae under iron-deprived conditions, except the *Dfg1* null mutant, which showed a complete block in hyphae formation. The role of *EFG1* in filamentation under iron-deprived conditions was further confirmed by Northern analysis, which showed a considerable upregulation of the *EFG1* transcript. Of notable importance, all the morphological mutants including *Dfg1* mutant possessed enhanced membrane fluidity under iron-deprived conditions; however, this did not appear to contribute to hyphal development. Interestingly, iron deprivation did not affect the ability of *C. albicans* to form biofilms on the catheter surface and led to no gross defects in azole resistance phenotype of these biofilms of *C. albicans* cells. Our study, for the first time, establishes a link between cellular iron, Efg1p and hyphal development of *C. albicans* cells that is independent of biofilm formation.

**Introduction**

Iron is a critical micronutrient required by almost all the organisms, especially as a cofactor in important metabolic functions (Weinberg, 1999a; Nyilassi *et al.*, 2005; Spacek *et al.*, 2005; Bullen *et al.*, 2006; Fischbach *et al.*, 2006). But at the same time, iron being a transition metal and because of its ability to donate and accept electrons, it can participate in the formation of toxic free radicals; therefore, availability of iron in host cells is tightly regulated (Radisky & Kaplan, 1999). Iron plays a vital role in conferring natural resistance to infections in humans (Bullen *et al.*, 2006). Because the pathogenic microorganisms have to scavenge iron from the host, therefore, the competition between pathogen and host for iron represents a critical aspect of many infectious diseases (Weinberg, 1999b; Schaible & Kaufmann, 2004). For this, the mammalian system is adapted to sequester iron within the host cells so that the availability of free iron itself becomes limited for the pathogens, which serves as a defense against infection (Emery, 1980; Weinberg, 1984; Kontoghiorghes & Weinberg, 1995).

For its survival in the host cells, *Candida* cells, like any other pathogen, have also adapted many complex strategies to scavenge the depicting iron from the host environment (Nyilassi *et al.*, 2005). In fact, the availability of iron can serve as a common adaptive signal for the pathogens to induce the expression of virulence traits (Mekalanos, 1992). Recent studies have already established a role for iron in systemic infections whereby the requirement of a high-affinity iron transporter (CaFtr1p) for infection in a mouse model was shown (Ramanan & Wang, 2000). Similarly, the requirement of a siderophore transporter (Arn1p) for epithelial invasion (Heymann *et al.*, 2002) and iron-dependent endothelial cell injury (Fratti *et al.*, 1998) suggests that iron plays a vital role in the virulence of *Candida albicans*.
In response to various environmental stimuli, C. albicans can switch from the unicellular yeast form into distinct filamentous forms. This ability of Candida to switch between two distinct morphological forms is considered as an important virulence trait (Ernst, 2000; Ernst & Schmidt, 2000; Kumamoto & Vince, 2005). Candida albicans morphology is directly related to environmental cues that trigger independent signal transduction pathways, many of which converge into two main signaling pathways regulating common targets required to initiate hyphal growth (Brown et al., 1997; Stoldt et al., 1997). The transcription factors such as EFG1 and CPH1 are primary regulators of morphogenesis and act via cyclic adenosine monophosphate (cAMP) and mitogen-activated protein kinase (MAPK) pathways, respectively (Ernst, 2000; Ernst & Schmidt, 2000). Thus, a double null mutant Δefg1Δefg1Δphi1Δphi1 is unable to form hyphae; however, single mutants are partially competent for hyphal differentiation under certain defined conditions (Lo et al., 1997; Stoldt et al., 1997). Hence, C. albicans cells display a variety of adaptive mechanisms in response to environmental cues; however, specific host factors that stimulate hyphal morphogenesis remain largely unidentified.

The dimorphic transition from yeast to hyphal form has also been described as a pivotal factor for C. albicans biofilm formation (Baille & Douglas, 1999). Biofilms are structured microbial communities that grow on the surface of indwelling prosthetic devices or catheters. Biofilm-associated infections are particularly important because the pathogenic organisms growing in the biofilms are often highly resistant to various drugs and to host innate defensive processes and thereby serve as an important attribute of their virulence (Ramage et al., 2006). The level of intracellular iron has already been known to serve as a signal for biofilm formation in pathogenic bacteria like Pseudomonas aeruginosa (Banin et al., 2005). Similarly, a number of proteins involved in iron acquisition and storage have been identified that were induced by biofilm growth in P. aeruginosa (Patraucan et al., 2007). Likewise, iron deprivation has been known to affect biofilm formation in Actinomyces naeslundii (Moelling et al., 2007).

We had observed earlier that iron deprivation led to enhanced drug sensitivity of C. albicans (Prasad et al., 2006). However, whether iron levels could also affect morphogenesis of Candida cells has not yet been demonstrated experimentally. In this study, we show that iron-deprived C. albicans cells or their mutants defective in iron acquisition show hyphal development in nonhyphal-inducing media and that this could be reversed by iron supplementation. The ability of C. albicans cells to develop hyphae under iron-deprived condition is specifically regulated by EfG1p. Interestingly, unlike hyphal development, biofilm formation and its drug resistance phenotype remained unaffected by the cellular iron status of C. albicans. Taken together, our findings for the first time show that under iron-limiting conditions the transcriptional regulator EFG1 plays a crucial role in mediating the hyphal development in C. albicans without affecting the ability of cells to form biofilms.

**Materials and methods**

**Materials**

Medium chemicals were obtained from HiMedia (Mumbai, India) and Difco (Detroit, MI). 1, 6-diphenyl-1,3,5-hexatriene (DPH), bathophenanthroline disulfonic acid (BPS) and ferric chloride (FeCl3) were obtained from Sigma Chemicals Co. (St Louis, MO). All chemicals used in this study were of analytical grade.

**Strains and growth media**

Candida albicans strains and the various mutant strains used in this study are listed in Table 1. All the strains were stored in 15% (v/v) glycerol stock at −80°C. The cells were freshly revived on 1% yeast extract, 2% peptone, 2% dextrose and 2.5% agar (YEPl) plates from this stock before each experiment. For all studies, the Candida cells were maintained on YEPl at 37°C. All the experiments were carried out on cells that were iron-replete to begin with. They were subjected to iron depletion only during the experiment. Strains were routinely grown in YEPl broth (1% yeast extract, 2% peptone, 2% dextrose), which served as an

---

**Table 1. List of Candida albicans strains used in this study**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Sources/ references</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAF2-1</td>
<td>URA3::ura3::imm434</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>Δftr1</td>
<td>Δura3::imm34/Δura3::imm34</td>
<td>Ramanan &amp; Wang (2000)</td>
</tr>
<tr>
<td>Δcph1</td>
<td>Δcph1::hisG/Δcph1::hisG</td>
<td>Weissman et al. (2002)</td>
</tr>
<tr>
<td>Δefg1</td>
<td>Δefg1::hisG/Δefg1::hisG</td>
<td>Stoldt et al. (1997)</td>
</tr>
<tr>
<td>Δefg1</td>
<td>Δefg1::hisG/Δefg1::hisG</td>
<td>Stoldt et al. (1997)</td>
</tr>
<tr>
<td>Δefg1</td>
<td>Δefg1::hisG/Δefg1::hisG</td>
<td>Lo et al. (1997)</td>
</tr>
<tr>
<td>Δftr1</td>
<td>Δftr1::hisG/Δftr1::hisG</td>
<td>Doedt et al. (2004)</td>
</tr>
<tr>
<td>Δupt1</td>
<td>Δupt1::hisG/Δupt1::hisG</td>
<td>Doedt et al. (2004)</td>
</tr>
<tr>
<td>Δupt1</td>
<td>Δupt1::hisG/Δupt1::hisG</td>
<td>Bockmuhl et al. (2001)</td>
</tr>
</tbody>
</table>
Table 2. List of primers used in this study

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EFG1</td>
<td>FP: 5'-TGCCCAGCAAACAACTGC-3'</td>
</tr>
<tr>
<td></td>
<td>RP: 5'-TTACCTGTTGCTGATCTTCGT-3'</td>
</tr>
<tr>
<td>CPH1</td>
<td>FP: 5'-AGCCACTGCCTCGAACGAAATTG-3'</td>
</tr>
<tr>
<td></td>
<td>RP: 5'-CAGTTAGAAGTATACGTACATCTTG-3'</td>
</tr>
<tr>
<td>EFH1</td>
<td>FP: 5'-GGATCCATGAAATGGTATGAC-3'</td>
</tr>
<tr>
<td></td>
<td>RP: 5'-GGATCCATTGGTATATGGAC-3'</td>
</tr>
<tr>
<td>TPK1</td>
<td>FP: 5'-ACATCCATGGAAACGACAC-3'</td>
</tr>
<tr>
<td></td>
<td>RP: 5'-TACCAATGACCACTCAAC-3'</td>
</tr>
</tbody>
</table>

iron-sufficient medium (ISM). Two percent (w/v) Bacto agar (Difco, BD Biosciences, NJ) was added to the YEUD broth to make solid media. Iron-poor medium (IPM) was prepared by adding 150 μM BPS to YEUD. Iron-rich medium (IRM) was prepared by adding 100 μM FeCl₃ to YEUD (Prasad et al., 2006).

Morphogenetic studies on solid and liquid media

Hyphal induction studies of wild-type (WT) and mutant cells of C. albicans were carried out on solid YEUD with 2.5% agar and liquid YEUD in the absence (control) and presence of 150 μM BPS (IPM). The exponentially growing log-phase cells were treated similar to the cells for hyphal induction as described in previous publication (Prasad et al., 2005) and then incubated at 37°C in the above mentioned media.

RNA isolation and hybridization

Northern blot analysis was performed essentially using standard protocols as described before (Sambrook et al., 1989; Prasad et al., 2006). Equal loading of RNA was checked by RNA bands. The relative intensities (RI) of EFG1, CPH1, EFH1 and TPK1 mRNA signals in Northern hybridizations were quantitated by exposure of the hybridized membrane in an FLAS000 Fuji Phosphoimager. RNA was electrophoresed on denaturing formaldehyde gel and blotted and UV cross-linked onto Hybond-N nylon membranes (Amerham Bipsiences). Membrane-bound RNA was stained with methylene blue before hybridization to check rRNA bands for equal loading. RI of EFG1, CPH1, EFH1 and TPK1 mRNA signals in Northern hybridizations were quantitated by exposure of the hybridized membrane in an FLAS000 Fuji Phosphoimager. The list of primers used for making the probes is given in Table 2.

Fluorescence polarization

The steady-state fluorescence polarization studies on the Candida cells were essentially carried out as described earlier (Kohli et al., 2002; Mukhopadhyay et al., 2002, 2004). Briefly, measurements were carried out in spheroplasts using the fluorescent probe, DPH with excitation and emission wavelengths of 360 and 426 nm, respectively, as described previously (Kohli et al., 2002; Mukhopadhyay et al., 2002, 2004).

Biofilm formation

Candida biofilms were formed and quantified as described previously (Chandra et al., 2001a). Briefly, C. albicans cells were grown overnight at 37°C in yeast nitrogen base (YNB) medium containing 0.73 μM of iron (Difco Laboratories) supplemented with 50 mM glucose. A standardized cell suspension was prepared from this culture by adjusting the cell density to 1 x 10⁷ cells mL⁻¹. Silicone elastomer (SE) disks (1.5 cm diameter; Cardiovascular Instrument Corp., Wakefield, MA) were placed in 12-well tissue culture plates and incubated in fetal bovine serum for 24 h at 37°C on a rocker. Next, the dishes were immersed in 3 mL of the standardized cell suspension (1 x 10⁷ cells mL⁻¹) and incubated for 90 min at 37°C. Subsequently, the disks were immersed in YNB medium with 50 mM glucose and incubated for different times at 37°C on a rocker. Fungal biofilms were quantified as described previously (Chandra et al., 2001a) by measuring their biomass and metabolic activity using dry-weight determination and tetrazolium dye [2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (XTT) reduction assays (by measuring absorbance at A₅₉₂ nm), respectively. Assays were carried out in four replicates on different days.

Fluorescence microscopy

SE disks with biofilms were transferred to microscope slides and stained for 1 min with 50 μL of Calcofluor-White (0.05% (v/v); Sigma Chemical Co.), which fluoresces in the UV range (λₘₐₓ = 432 nm). Stained biofilms were examined under a fluorescence microscope.

Confocal scanning laser microscopy (CSLM)

CSLM was used to evaluate the effect of disruption of FTR1 or CCC2 genes on the ability of C. albicans to form biofilms and to evaluate the architecture and thickness of biofilms formed by the respective mutant strains of C. albicans. Biofilms were grown on SE disks as described above and transferred to 12-well plates and incubated for 45 min at 37°C in 4 mL of phosphate-buffered saline containing the fluorescent stains FUN-1 (10 mM) and concanavalin A-Alexa Fluor 488 conjugate (ConA; 25 mg mL⁻¹). FUN-1 (excitation wavelength 543 nm and emission 560 nm long-pass filter) is converted to orange-red cylindrical intravacular structures by metabolically active cells, while ConA (excitation wavelength 488 nm and emission 505 nm long-
Iron deprivation promotes hyphal development in *Candida*

pass filter) binds to glucose and mannose residues of cell wall polysaccharides, resulting in green fluorescence. After incubation with the dyes, the biofilms were placed on a 35-mm-diameter glass-bottom Petri dish (MatTek Corp., Ashland, MA). Stained biofilms were observed with a Zeiss LSM510 confocal scanning laser microscope equipped with argon and HeNe lasers and mounted on a Zeiss Axiovert100 M microscope (Carl Zeiss Inc.). The objective used was a water immersion C-apochromat lens (403; numerical aperture of 1.2). Depth measurements were taken at regular intervals across the width of the device. For orthogonal analyses, a series of horizontal (xy) optical sections were obtained throughout the full length of the biofilm, and analyzed using LSM 510 software (Carl Zeiss). Confocal images of green (ConA) and red (FUN-1) fluorescence were conceived simultaneously using a multitrack mode. Planktonically grown *C. albicans* cells were used as comparators in these studies.

![Fig. 1](image_url)

Fig. 1. (a) Hyphal morphogenesis in the liquid (upper panel) and solid (lower panel) YEPD in the absence (control) of, presence of 150 μM BPS (IPM) and supplementation with 100 μM FeCl₃ (IRM) in the WT (A), iron acquisition mutants (B and C, for Δfrt1 and Δcccz2, respectively) and morphological mutant cells (D-I for Δehl, Δefg1, Δefg1Δehl, Δehl1, Δehl1Δefg1 and Δupk1, respectively). Magnification, ×100. (b) Northern blot analyses of EFG1, CPH1, EHF1 and TPK1. The upper panels show EFG1, CPH1, EHF1 and TPK1 transcript levels, respectively, in lanes (1) WT, (2) WT grown in the presence of 150 μM BPS, (3) Δfrt1, (4) Δcccz2, (5) iron-depleted WT strain after supplementation with 100 μM FeCl₃, (6) Δfrt1 after supplementation with 100 μM FeCl₃, (7) Δcccz2 after supplementation with 100 μM FeCl₃. The panel on the right side next to each of the transcript levels of the genes represents respective loading controls for indicating equal gel loading of total RNA for the respective gene transcripts.
Results

Iron deprivation promotes hyphal development in C. albicans

We explored whether levels of iron could affect hyphal development in Candida cells and for this we used BPS, a well-known iron chelator at a concentration that depleted iron from the media without affecting the growth of the Candida cells (Prasad et al., 2006). We measured intracellular iron levels by the enzymatic method and by using Calcein-AM, a fluorescent dye to establish iron-deficient conditions (data not shown) (Breuer et al., 1995; Cabantchik et al., 1996). Candida albicans cells when grown in iron-sufficient (ISM) liquid and solid YEPD media, showed no hyphal formation (Fig. 1a, A). However, in iron-poor media (IPM), cells could show hyphal development in the absence of any hyphae-inducing agent (Fig. 1a, A). This hyphal development could be prevented if BPS grown cells were supplemented with 100 μM FeCl₃ (Fig. 1a, A).

Iron-acquisition defective mutants also show hyphal formation

The role of iron in hyphal differentiation in C. albicans was further confirmed when we used two iron-acquisition defective mutants such as Δftr1 (defective in high-affinity iron uptake) (Ramanan & Wang, 2000) and Δcc2 [defective in copper transport, which is the essential component of the multicopper oxidase (FET3) and is required for high-affinity iron uptake] (Weissman et al., 2002). Interestingly, both the iron-transport defective mutants without the addition of an iron chelator showed hyphal development in solid as well as in liquid YEPD (Fig. 1a, B and C). The hyphal formation could be reversed if iron-transport defective mutants were supplemented with 100 μM FeCl₃ (Fig. 1a, B and C).

EFG1 mediates hyphal development under iron-depriving conditions

We evaluated whether the iron-regulated hyphal development is mediated by known signal cascades controlling
Iron deprivation promotes hyphal development in Candida

**Fig. 2.** Contribution of iron availability to the ability of Candida albicans to form biofilms on SE catheters. (a) Metabolic activity (black solid bars) and dry weight (gray hatched bars) of biofilms formed by different Candida mutants (Δefg1, Δatr1, Δccc2) and their isogenic WT strain. Data represent mean ± SD from three independent experiments (*P* = 0.000469, vs. WT isolate). (b-i) Fluorescence microscopic images of biofilms formed in the absence (b-e) or presence (f-i) of an iron-chelating agent (BPS). b, f – WT; c, g – Δefg1; d, h – Δatr1 and e, i – Δccc2. White arrows indicate the extracellular matrix. Magnification, ×20.

morphogenesis (Ernst, 2000; Ernst & Schmidt, 2000). For this, we exploited the Δcph1, Δefg1, Δefh1 and Δtpk1 null mutant strains that are defective in hyphal development in various hyphal-inducing media (Stoldt et al., 1997; Ernst, 2000; Ernst & Schmidt, 2000; Doedt et al., 2004). Similar to WT cells, all morphological null mutants such as Δcph1, Δefh1 and Δtpk1, with the exception of Δefg1 null mutant cells, exhibited filamentation, albeit to a lesser extent, on both iron-depleted solid and liquid media (Fig. 1a, D, E, G and I). Notably, a major transcription factor, CPH1, regulating hyphal transition and activated via the MAPK pathway did not appear to contribute to this iron starvation-mediated filamentation as the Δcph1 null mutant continued to show hyphal formation upon iron deprivation (Fig. 1a, D). On the other hand, unlike Δcph1 cells, Δefg1 null mutants cells were unable to form hyphae in both ISM and IPM liquid and solid YEPD media (Fig. 1a, E). The EFH1 transcription factor is a homolog of EFG1 that modulates some of the functions of the main regulator, EFG1 (Doedt et al., 2004). Interestingly, EFH1 did not appear to have any role in iron-regulated hyphal development because Δefg1 null mutant cells were able to make hyphae under IPM conditions on both solid and liquid media (Fig. 1a, G). Lack of filamentation under iron-deprived conditions in the double mutant cells, Δefg1Δcph1 (Fig. 1a, F) and Δefg1Δefh1 (Fig. 1a, H), further confirmed the role of EFG1 in hyphal development. To investigate the possibility that iron deprivation affects hyphal development through components upstream of EFG1 in the cAMP-signaling cascade, we took advantage of the Δtpk1 null strains. TPK1 encodes a protein kinase A, which acts upstream of EFG1 in the cAMP signaling pathway controlling morphogenesis (Ernst, 2000). The Δtpk1 null strains did not show any hyphal development in ISM solid and liquid YEPD media (Fig. 1a, I). However, Δtpk1 null mutant cells formed hyphae under IPM solid and liquid YEPD media (Fig. 1a, I). The observed
Table 3. Steady-state fluorescence polarization measurements (P value) in Candida albicans WT and morphological mutants in the absence (control) and presence of 150 μM BPS

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Fluorescence polarization</th>
<th>P values (mean ± SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>0.172 ± 0.001</td>
</tr>
<tr>
<td>WT + 150 μM BPS</td>
<td></td>
<td>0.095 ± 0.01</td>
</tr>
<tr>
<td>Δcph1</td>
<td></td>
<td>0.101 ± 0.008</td>
</tr>
<tr>
<td>Δcph1 + 150 μM BPS</td>
<td></td>
<td>0.089 ± 0.003</td>
</tr>
<tr>
<td>Δefg1</td>
<td></td>
<td>0.112 ± 0.005</td>
</tr>
<tr>
<td>Δefg1 + 150 μM BPS</td>
<td></td>
<td>0.092 ± 0.01</td>
</tr>
<tr>
<td>Δefg1Δcph1</td>
<td></td>
<td>0.098 ± 0.007</td>
</tr>
<tr>
<td>Δefg1Δcph1 + 150 μM BPS</td>
<td></td>
<td>0.073 ± 0.008</td>
</tr>
<tr>
<td>Δefhl</td>
<td></td>
<td>0.105 ± 0.01</td>
</tr>
<tr>
<td>Δefhl + 150 μM BPS</td>
<td></td>
<td>0.078 ± 0.009</td>
</tr>
<tr>
<td>ΔefhlΔefg1</td>
<td></td>
<td>0.095 ± 0.006</td>
</tr>
<tr>
<td>ΔefhlΔefg1 + 150 μM BPS</td>
<td></td>
<td>0.071 ± 0.01</td>
</tr>
<tr>
<td>Δtpkl</td>
<td></td>
<td>0.108 ± 0.01</td>
</tr>
<tr>
<td>Δtpkl + 150 μM BPS</td>
<td></td>
<td>0.093 ± 0.007</td>
</tr>
</tbody>
</table>

*The values are the mean fluorescence polarization P values of the cells (inversely proportional to membrane fluidity) ± the SD of the mean of the three independent sets of experiments (Kohli et al., 2002; Mukhopadhyay et al., 2002, 2004).

hyphal development under all iron-deprived conditions could be prevented if the media were supplemented with 100 μM FeCl₃ (Fig. 1a, D, F to I).

**EFG1 transcript is upregulated under iron-deprived conditions in C. albicans**

Northern analysis revealed considerable upregulation of EFG1 transcript levels under iron-deprived conditions (Fig. 1b). The transcript levels of EFG1 in iron-deprived growth conditions (IPM) could be restored to the WT levels when cells were supplemented with 100 μM FeCl₃ (IRM). The correlation between iron and EFG1 levels was further confirmed when we used two different iron-acquisition defective mutants: Δfrtl and Δccc2 cells. Both the mutants showed constitutively high levels of the EFG1 transcript as compared with the WT cells grown in ISM (Fig. 1b). Under iron-deprived growth conditions, no change in the transcript levels of other morphogenic regulators, namely, CPH1, EFH1 and TPK1, was observed (Fig. 1b).

**Iron deprivation enhances the membrane fluidity of Candida cells**

Our earlier data suggested an intricate relationship among membrane fluidity, iron deprivation and drug susceptibility of Candida cells, wherein we showed a causal relation between increased membrane fluidity of WT cells under iron-deprived conditions and drug susceptibility (Prasad et al., 2006). To explore whether membrane fluidity is also responsible for the enhanced filamentation under iron-deprived conditions, we used the steady-state fluorescence polarization technique using the fluorescent probe, DPH (Kohli et al., 2002; Mukhopadhyay et al., 2002, 2004) to examine the membrane order of the cells grown under ISM and IPM conditions. Of notable importance, a decrease in the 'P' value implies a decrease in membrane order or an increase in membrane fluidity. All the morphological mutant cells of Candida viz. Δcph1, Δefg1, Δefg1Δcph1, Δefhl, ΔefglΔefhl and Δtpkl showed lower 'P' values as compared with WT cells in ISM (Table 3). Interestingly, the 'P' values of all the above tested mutants and the WT strain were further lowered when these cells were deprived of iron under IPM conditions (Table 3). It should be noted that the addition of BPS in Candida cells did not affect membrane fluidity. The possibility of interference of BPS in membrane fluidity measurements was earlier ruled out by us (Prasad et al., 2006). We had shown that even in the absence of BPS, iron-acquisition defective mutants namely, Δfrtl (defective in high-affinity iron transport) and Δccc2 (copper transport mutant), show increased membrane fluidity as compared with the WT cells.

**Role of iron acquisition in biofilm-forming ability of C. albicans**

Earlier, Δefg1 mutant cells of C. albicans were shown to be defective in their ability to form biofilms (Ramage et al., 2002). Because catheter-associated fungal biofilms are rich in hyphal elements and also because the iron-acquisition defective mutant cells of Candida namely, Δfrtl and Δccc2, showed normal hyphal formation, we hypothesized that these iron-acquisition defective strains would form regular biofilms. To test this hypothesis, we determined the biofilm-forming ability of Δfrtl and Δccc2 mutant strains on catheter disks as described previously (Chandra et al., 2001a). We found that there was no significant difference in the ability of Δfrtl and Δccc2 mutant strains to form biofilms as compared with the WT strain (as measured by determining their metabolic activity and dry biomass, Fig. 2, A). As expected, the biofilm formed by the Δefg1 strain exhibited a significant decrease in metabolic activity, indicating reduced biofilm formation (P = 0.000469, vs. WT strain; Fig. 2, A). Fluorescence microscopy with the carbohydrate-specific dye Calcofluor White revealed that Δefg1 exhibited clusters of yeast cells and localized carbohydrate-specific staining (Fig. 2C). In contrast, the Δfrtl and Δccc2 mutant strains formed biofilms with diffuse Calcofluor White staining, indicating the presence of extracellular matrix (Fig. 2, D, E, white arrows). Because the main characteristic of fungal biofilms is their inherent resistance to azoles (Chandra et al., 2001a, b), we also determined whether disruption of FTR1 or CCC2 affects the azole susceptibility of Candida biofilms. Antifungal susceptibility...
testing revealed that biofilms formed by mutant strains lacking these genes were resistant against fluconazole (data not shown), suggesting that disruption of FTR1 and CCC2 genes did not lead to gross defects in fluconazole resistance although the actual minimum inhibitory concentration cannot be determined by current methodology. Notably, when we assessed the effect of BPS-induced iron deprivation on the ability of Candida strains to form biofilms, we observed that BPS affected fungal morphology of all the strains tested (Fig. 2, F–I), suggesting that the effect of BPS on Candida cells was not related to their ability to form biofilms.

Confocal analyses revealed no difference in the architecture of biofilms formed by iron uptake mutant strains

To confirm these findings and also to evaluate the architecture of the biofilms formed, we performed CSLM analyses, which showed that the ∆frt1 and ∆ccc2 mutants formed biofilms with a similar, hyphal-rich architecture as the WT parental strain (Fig. 3). As expected, only adhered cells were observed with the ∆efg1 mutant isolate, demonstrating that this isolate did not form robust biofilms. The mean thicknesses of biofilms formed by these strains, (1) WT, (2) ∆frt1, (3) ∆ccc2 and (4) ∆efg1, were 100 ± 10, 70 ± 23, 80 ± 15 and 40 ± 7.6 μm, respectively. No statistically significant difference was found between thicknesses of biofilms formed by the WT, ∆ccc2 or ∆frt1 mutants (P > 0.05), while the thickness of adhered cells of the ∆efg1 strain was significantly less than that of biofilms formed by the WT. Magnification, ×20 for each panel.

Fig. 3. Confocal microscopy analyses of biofilms formed by (a) WT, (b) ∆frt1, (c) ∆ccc2 and (d) ∆efg1 strains. Hyphae in the biofilms have been indicated by arrows. These images represent orthogonal sections, with a top–down image from a single plane shown in the central panel; the side and top views show the side views from two different planes. The mean thicknesses of biofilms formed by these isolates were calculated from the thickness measured at three different positions within the biofilm. The mean thicknesses of biofilms formed by these strains [(a) WT, (b) ∆frt1, (c) ∆ccc2 and (d) ∆efg1 strains] were 100 ± 10, 70 ± 23, 80 ± 15 and 40 ± 7.6 μm, respectively. No statistically significant difference was found between thicknesses of biofilms formed by the WT, ∆ccc2 or ∆frt1 mutants (P > 0.05), while the thickness of adhered cells of the ∆efg1 mutant strain was significantly less than that of biofilms formed by the WT strain. These results clearly showed that disruption of the FTR1 or the CCC2 gene did not affect the ability of C. albicans to form biofilms.

Discussion

In this study, we show that iron deprivation promotes hyphal development in C. albicans cells, which is exclusively mediated via the morphological regulator EFG1 without affecting biofilm formation. The role of EFG1-mediated hyphal development under iron deprivation was validated by Northern analysis, which revealed that in the presence of BPS (iron chelator), there was a considerable increase in the EFG1 transcript (Fig. 1b). The increase in EFG1 transcript under iron-depleted conditions of growth could be reversed by supplementing the iron-deficient cells with 100 μM FeCl3 (Fig. 1b). The mutants defective in iron acquisition such as ∆frt1 as well as copper transporter mutant ∆ccc2, which affects the high-affinity iron uptake in Candida, also showed enhanced hyphal formation on solid as well as in liquid YEPD without the addition of BPS (Fig. 1a, B and C).
Expectedly, both the null mutant cells also showed elevated levels of the *EFG1* transcript (Fig. 1b). Similar to the WT cells, the transcript of *EFG1* (Fig. 1b) and hyphal development exhibited under iron-depleted conditions of growth could be reversed by supplementing the iron acquisition mutant cells with 100 μM FeCl₃ (Fig. 1a, B and C). This reversion could be due to the presence of the low-affinity iron transporters, which become active in the IRM, and thereby compensates for the gene knockout effect of high-affinity iron transporters in the presence of excess iron in the surrounding media. Our observations are also well in agreement with the previous transcriptome analysis by Lan et al. (2004), who showed an inverse correlation between hyphal morphogenesis and iron availability in *C. albicans* cells. In our experiment, we used 150 μM of BPS to deplete the availability of cellular iron for maintaining homeostasis, a close condition described by Lan et al. (2004), which does not affect the growth. To further substantiate our finding with iron chelator BPS, we supplemented iron (FeCl₃) to reverse the phenomenon and also used high-affinity iron uptake mutants such as Δ*lfr1* and Δ*ace2*. Hyphal formation is one of the most important mechanisms of *Candida* to overcome the restricted growth conditions. Increased expression of *EFG1* and subsequent hyphal formation is the manifestation of the ability of *Candida* to overcome an adverse condition. Interestingly, to understand the role of iron in germ tube formation, earlier, Sweet & Douglas (1991) used a different approach, when they cultured strains of *Candida* with defined concentrations of iron ranging from 0.026 to 0.8 μM and checked germ tube formation by the addition of serum. Notably, they detected maximal germ tube formation between 0.2 and 0.4 μM iron concentrations. Given the inability of *Candida* to grow in a very low iron concentration, probably 0.2 to 0.4 μM of iron is the optimal range to express the filaments, which might be a condition similar to that achieved during our experiments or those by Lan et al.

*EFG1*, being a global regulator of morphology and metabolism, also plays a major role in gene regulation of *C. albicans* (Stoldt et al., 1997; Brown & Gow, 1999). We have earlier demonstrated (Prasad et al., 2006) that membrane fluidity correlates with azole sensitivity of *C. albicans* cells. Iron depletion of *Candida* cells increases membrane fluidity, resulting in enhanced passive diffusion of drugs, thereby increasing the drug susceptibility of the cells. However, in the current study, we observe that the hyphal development induced by iron starvation is independent of the membrane fluidity. Our conclusions are based on two observations: (1) all the morphological mutants (Δ*cph1*, Δ*eht1*, Δ*pkl1* and Δ*efg1*) showed an increase in membrane fluidity as compared with their WT host but showed no hyphal development. (2) When we deprived these mutants of iron, there was a further increase in membrane fluidity (Table 3). However, despite the increase in membrane fluidity of the morphological mutants on iron depletion, all the mutants formed hyphae, except Δ*efg1* null mutant, which was completely blocked in hyphae formation. This rules out any role of membrane fluidity in hyphal development by iron deprivation. Our results clearly show that iron-mediated hyphal development is *EFG1* mediated and independent of the changes in membrane fluidity. This also correlates well with an earlier study (Krishnamurthy et al., 2004), which reported that overall membrane fluidity did not appear to be a crucial factor for hyphal development; instead, levels of *OLE1* (stearoyl-CoA desaturase) were found to have a direct effect on specific components of the hyphal induction machinery because addition of oleic acid itself could promote hyphal development. Therefore, change in membrane fluidity even in cells overexpressing *OLE1* was not a contributing factor in hyphal development (Krishnamurthy et al., 2004). Iron-deprived transcriptome analysis by Lan et al. (2004) showed that *OLE1* is downregulated by 2.2-fold. While analyzing transcriptome of Δ*efg1* cells that were completely defective in hyphal morphogenesis, we observed a twofold increase in transcript levels of *OLE1*, suggesting that changes in fluidity are not related to the ability of cells to develop hyphae (T. Prasad et al., unpublished data). As reported earlier, enhanced membrane fluidity under iron-deprived conditions does sensitize *Candida* cells to various drugs (Prasad et al., 2006); therefore, the effect of iron on hyphal development and drug susceptibilities of *C. albicans* involve independent pathways.

Catheter-associated *Candida* biofilms are a major cause of device-associated nosocomial infections and consist of both yeast cells and hyphal forms (Ramage et al., 2002). *EFG1* has been identified as one of the genes involved in regulating the formation of a biofilm, probably mediated by its regulation of hyphae formation, a prerequisite for biofilm formation, and ruled out any role of the MAPK involving *CPH1* in the same (Ramage et al., 2002). In the present study, we found that the increased *EFG1* transcript under iron-deprived conditions did not correlate with any significant change in the ability of *C. albicans* to form a biofilm as compared with the WT cells (Fig. 2). Furthermore, BPS-induced iron deprivation of the growth medium did not affect biofilm-forming abilities of the mutant strains as compared with the WT strain.

Metabolic activities of biofilms formed by the tested mutant strains did not exhibit a statistically significant difference (*P* > 0.05). Although there was a trend toward reduced biomass in biofilms formed by the mutant strains, this trend was not statistically significant (*P* > 0.05). Biomass determination measures changes in weight because of live and dead fungal cells and hyphae, as well as the extracellular matrix. In contrast, XTT measurements evaluate the metabolic activity of a combined population of cells...
and hyphae. Because dry weight and XTT assays measure different variables of biofilms, a linear association between these assays is not always observed. Such differences have been reported earlier by our group as well as by other investigators (Kuhn et al., 2002). Also, iron-limiting conditions can induce an increase in XTT metabolism, similar to those reported earlier by Knight & Dancis (2006), who showed that iron-limiting conditions induce a fivefold increase in XTT reduc tase activity and that the activity of this enzyme in Candida cells grown in unbuffered (pH 4.0–4.4) medium was mediated by CaFRE10 protein. Our fluorescence and confocal microscopy analyses also revealed no difference in biofilm morphology and architecture between the iron uptake mutant strains and their isogenic WT parent. These results suggested that the ability of C. albicans to form a biofilm is not affected by disruption of the selected genes.

Antifungal drug susceptibility testing revealed that the biofilms formed by iron-acquisition mutant strains retained their resistance to fluconazole, thus indicating that targeted genes do not play a role in azole resistance of Candida biofilms. Notably, this observation differs from our previous study where it is shown that iron deprivation sensitized Candida cells to drugs (Prasad et al., 2006). This implies the fact that the resistance of sessile cells is a multifactorial phenomenon, which includes the effect of biofilm architecture and their different patterns of gene expression as compared with the planktonic cells (Ramage et al., 2002; Kathleen et al., 2007), and so cellular iron status probably does not play any role in the drug susceptibility of Candida biofilm cells. Because limiting the iron from the invading pathogen is one of the defense strategies adopted by the host (Emery, 1980; Weinberg, 1984; Kontoghiorghes & Weinberg, 1995), the biofilm development in C. albicans independent of the iron levels might serve as an evolutionary adaptation of eukaryotic pathogenic fungi over the pathogenic bacteria where iron levels do play a crucial role in forming biofilms (Banin et al., 2005; Moelling et al., 2007; Patrauchan et al., 2007).

Of notable importance, the signaling effect of hemoglobin which is also an effective inducer of hyphal development in C. albicans (Pendrak & Roberts, 2007), is independent of cellular iron status. Both heme and globin are unable to induce hyphal formation because the hemoglobin receptor requires an intact αβ hemoglobin dimer to function as a signal transducer for hyphal development. Interestingly, for hemoglobin signaling, Efg1p is also necessary for hyphal formation in C. albicans, whereas the other morphogenic regulator, Cph1p, has no role (Pendrak & Roberts, 2007). Thus, Efg1p-dependent hyphal development in C. albicans constitutes both iron-dependent and iron-independent pathways.

Hyphal morphogenesis is an integral part of the overall virulence strategy of C. albicans (Kumamoto & Vinces, 2005); the molecular cues controlling morphogenic transitions are still not well understood. Therefore, hyphal development under iron deprivation represents yet another unknown phenomenon that is specifically mediated by iron as other divalent cation chelators like EDTA inhibited hyphal development (Gil et al., 1994) and biofilm formation (Ramage et al., 2007). There might exist two possibilities for the mechanism of hyphal formation secondary to iron deficiency. Firstly, the cellular iron levels directly regulate the expression of the EFG1 transcript and, secondly, iron deficiency in the medium triggers yet another unknown signaling cascade that is regulated by the transcription factor, EFG1. In either of the two possibilities, the association between iron deficiency and hyphal formation is clinically relevant as it triggers an important adaptive virulent trait for acquiring iron from the host cells and manifest infection. This certainly merits further study for the development and improvement of targeted antifungal therapy and identification of potential attributes for the virulence of C. albicans.

Acknowledgements

S.H. and T.P. thank the Council of Scientific and Industrial Research for the award of Senior Research Fellowship and Research Associateship respectively. Morphological mutants and iron mutant strains provided by J. Ernst, Y. Wang and D. Kornitzer are also acknowledged. The work presented in this paper has been supported in part by grants to R.P. from the Department of Biotechnology, India (BT/PR3825/MED/14/488(a)/2003) and (BT/PR4862/BRB/10/360/2004), Council of Scientific and Industrial Research [38/(1122)/06/EMR-II], the Department of Science Technology (SR/SO/BB-12/2004), Indo-French (IFC/A/3403-2/2006), support to C.K.M. in the form of Welcome Trust Senior Research Fellowship, support to M.A.G. from the National Institutes of Health (RO1DE 13932-4) and the Bristol Myers Squibb Freedom to Discover Award and to P.K.M. from the American Heart Association (Scientist Development Grant 035313N).

Authors’ contribution

S.H. and T.P. contributed equally to this work.

References


Iron deprivation promotes hyphal development in *Candida*.


Morphogenic regulator EFG1 affects the drug susceptibilities of pathogenic Candida albicans

Tulika Prasad1, Saif Hameed2, Raman Manoharlal, Sudipta Biswas3, Chinmay K. Mukhopadhyay3, Shyamal K. Goswami2 & Rajendra Prasad2

1Advanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi, India; 2Membrane Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India; and 3Special Centre for Molecular Medicine, Jawaharlal Nehru University, New Delhi, India

Abstract
This study shows that the morphogenic regulator EFG1 level affects the drug susceptibilities of Candida albicans when grown on solid growth media. The Δefg1 mutant showed sensitivity particularly to those drugs that target ergosterol or its metabolism. Efg1Δ disruption showed a gene-dosage effect on drug susceptibilities and resulted in enhanced susceptibility to drugs in the homozygous mutant as compared with the wild type, heterozygous and revertant strains. The enhanced sensitivity to drugs was independent of the status of multidrug resistance (MDR, including an overexpression of membrane fluidity that coincided with the downregulation of ERG11 and upregulation of ELE1 and ERG3, leading to enhanced passive diffusion of drugs. Interestingly, Δefg1 mutant cells displayed enhanced levels of endogenous ROS levels. Notably, the higher levels of ROS in the Δefg1 mutant could be reversed by the addition of antioxidants. However, the restoration of ROS levels did not reverse the drug sensitivities of the Δefg1 mutant. Taken together, we, for the first time, establish a new role for EFG1 in affecting the drug susceptibilities of C. albicans cells, independent of ROS and known drug efflux mechanisms.

Introduction
The dimorphic opportunistic pathogen, Candida albicans, is normally a commensal organism in humans, but when the host is unable to mount an adequate immune response, as in AIDS, organ transplant, diabetes or in cancer patients, it results in mucosal, cutaneous or invasive mycoses (Odds, 1988; Calderone, 2002). Widespread and prolonged usage of antifungals in treating infections caused by C. albicans has led to the emergence of azole resistance. This acquired azole resistance in the clinical isolates of C. albicans mostly results in cross-resistance to many unrelated drugs, a phenomenon termed as multidrug resistance (MDR) (Franz et al., 1998, 1999; Kohli et al., 2002). Various mechanisms contribute toward the development of MDR, including an overexpression or mutations in ERG11, encoding the target enzyme of azoles lanosterol 14α-demethylase (Lamb et al., 1997; White et al., 1998, 2002; Prasad et al., 2004), an overexpression of the drug efflux pumps encoding genes such as CaCdr1 and CaCdr2 belonging to the ATP-binding cassette (ABC) pumps, and the drug efflux pumps encoding genes such as CaCdr1 and CaCdr2 belonging to the ABC cassette and MDR1 belonging to the major facilitator super-family (MFS) transporters (Ben-Yaacov et al., 1994; Gupta et al., 1998; Pao et al., 1998).

Although the mechanisms of antifungal resistance and the major factors that contribute to it are fairly well established, there is evidence to suggest that MDR is a multifactorial phenomenon that may be contributed by yet unknown mechanisms. For example, levels of a homolog of bacterial two-component response regulators SSK1 and iron display enhanced sensitivity to drugs in Candida cells (Prasad et al., 2006; Ruprich-Robert et al., 2008). Additionally, there are azole-resistant clinical isolates of C. albicans where mechanisms of resistance appear to be different from the commonly known strategies adopted by Candida (White et al., 1998).

Candida albicans can switch from the unicellular yeast form into either of the two distinct filamentous forms: cells with pseudohyphae or true hyphae in response to various environmental stimuli. This ability to switch is considered as
an important virulence trait and is also coregulated with other virulence factors, which are associated with the cellular morphology (Ernst, 2000; Ernst & Schmidt, 2000). Candida albicans morphology is directly related to environmental conditions and these cues trigger separate signal-transduction pathways, which regulate common targets required to initiate hyphal growth (Brown & Gow, 1999; Ernst, 2000; Ernst & Schmidt, 2000). The transcription factor Efg1p regulates the morphogenesis of C. albicans because it induces the yeast-to-hyphal transition and also regulates phenotypic switching and chlamydosporal formation of this pathogen (Sonneborn et al., 1999a,b). Interestingly, iron deprivation-induced hyphal morphogenesis in C. albicans is also mediated through the transcriptional regulator Efg1p (Hameed et al., 2008).

Considering the importance of the Efg1p regulator in morphogenesis, in this study, we have evaluated whether defects in morphogenic signaling pathways would also affect the MDR status of C. albicans cells. We observed that Δefg1 mutant cells are selectively sensitive to azoles and polyenes as compared with the wild-type (WT) strain when grown on solid agar media. The Δefg1 mutant cells display increased membrane fluidity, leading to enhanced passive diffusion of drugs. Taken together, our study establishes a convergence of EFG1 and MDR pathways and thus proposes an additional new role of this important morphogenetic regulator of C. albicans.

**Materials and methods**

**Materials**

Media chemicals were obtained from Difco (Detroit, MI) and HiMedia (Mumbai, India). Dichlororfluorescein di acetate (DCFDA), rhodamine 6G (R6G), the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) and the drugs O-nanthanol (O-PHE), nystatin (NYS), amphotericin B (AMB), cycloheximide (CYH), crystal violet (CV), methotrexate (MTX), ascorbic acid (AA) and pyrrolidinedithiocarbamate (PDTC) were obtained from Sigma Chemical Co. (St. Louis, MO). Fluconazole (FLC) was a kind gift from Ranbaxy Laboratories (New Delhi, India), while sulfomethuron methyl (SMM), ketoconazole (KTC) and itraconazole (ITR) were gifts from Dupont (Wilmington, DE).

**Growth media and strains used**

The Candida WT and the mutant strains (Table 2) were cultured in YEPD broth (Bio101, Vista, CA). For agar plates, 2% (w/v) agar (Difco, BD Biosciences, NJ) was added to the medium. All the C. albicans strains were stored in 15% (v/v) glycerol stock at −80°C. The cells were freshly revived on YEPD plates from this stock for each experiment. For the following studies, the Candida cells were grown on YEPD plates at 30°C and cells were harvested by adding a small amount of cold YEPD medium to plates and transferring the cells into a centrifuge tube. The harvested cells were used for the following experiments.

**Drug susceptibility tests**

Drug susceptibilities were measured using spot and filter disc assays. Spot assays for the strains were determined using a method as described previously (Mukhopadhyay et al., 2002; Prasad et al., 2006). The following stock solutions were used (the solvents used are given in parentheses): FLC, 1 mg mL⁻¹ (water); KTC, 1 mg mL⁻¹ (DMSO); ITR, 1 mg mL⁻¹ (DMSO); CYH, 20 mg mL⁻¹ (water); NYS, 1 mg mL⁻¹ (methanol); AMB, 1 mg mL⁻¹ (DMSO); O-PHE, 1 mg mL⁻¹ (ethanol); CV, 1 mg mL⁻¹ (ethyl alcohol); MTX, 1 mg mL⁻¹ (10 mM Tris-Cl); and SMM, 1 mg mL⁻¹ (methanol). The final drug concentrations used for this study are specified in the figure legends of Fig. 1a. In the spot assay, 5 µL of fivefold serial dilutions of each yeast culture (each with cells suspended in normal saline to an OD_{600 nm} of 0.1) was spotted onto YEPD plates in the absence (control) and presence of the drugs. Growth was not affected by the presence of the solvents used for the drugs (data not shown). Growth differences were recorded following incubation of the plates for 48 h at 30°C. A filter disc assay was performed as described earlier (Mukhopadhyay et al., 2002; Prasad et al., 2006). The drugs were spotted in a volume of 5–10 µL at the indicated amount in the figure legends of Fig. 1b and the diameters of the respective zones of inhibition were measured after incubation of the plates for 48 h at 30°C.

**Fatty acid composition**

Fatty acids were first extracted by alcoholic saponification of the Candida cells resuspended in 10 mL of 1 M ethanolic KOH (90% v/v ethanol), as essentially described earlier (Mishra & Prasad, 1987, 1989). Derivatized fatty acids using thionyl chloride were analyzed by gas-liquid chromatography (GLC) on a 2 M (15%) diethylene glycol succinate (DEGS) column (supported on Chromosorb W 100/120HP) at 180°C using a Shimadzu (Tokyo, Japan) GC9A instrument equipped with a flame ionization detector (Mishra & Prasad, 1987, 1989). The fatty acids were identified by comparing their retention times with simultaneously run authentic internal standards (Sigma Chemical Co.) and were quantified using a Chromatopac CR2A automatic integrator using area normalization method.

**Quantitation of ergosterol**

Sterols were extracted using the alcoholic KOH method and the percentage of ergosterol was calculated as described.
 previously (Arthington-Skaggs et al., 1999; Kohli et al., 2002; Mukhopadhyay et al., 2002; Prasad et al., 2006). The extracted sterols indicated a four-peak spectral absorption pattern produced by ergosterol and 24(28)-dehydroergosterol (DHE) contents. Both ergosterol and DHE absorb at 281.5 nm, whereas only DHE absorbs at 230 nm. The ergosterol content is determined by subtracting the amount of DHE (calculated from A_290) from the total ergosterol plus DHE content (calculated from A_281.5). The ergosterol content was calculated as a percentage of the wet weight of the cells using the following equations:

\[
\% \text{Ergosterol} + \% 24(28) \text{DHE} = [\frac{A_{281.5 \text{ nm}}}{290} \times F]/\text{pellet weight}
\]

\[
\% 24(28) \text{DHE} = \left[\frac{[A_{230 \text{ nm}}/518]}{F}\right]/\text{pellet weight}
\]

where \( F \) is the factor for dilution in petroleum ether and 290 and 518 are the \( E \) values (in percent per centimeter) determined for crystalline ergosterol and 24(28) DHE, respectively.

**Fluorescence polarization**

The steady-state fluorescence polarization studies on the *Candida* cells using the fluorescent probe, DPH, were essentially carried out as described earlier (Mukhopadhyay et al., 2002; Prasad et al., 2006). The extracted sterols indicated a four-peak spectral absorption pattern produced by ergosterol and 24(28)-dehydroergosterol (DHE) contents. Both ergosterol and DHE absorb at 281.5 nm, whereas only DHE absorbs at 230 nm. The ergosterol content is determined by subtracting the amount of DHE (calculated from A_290) from the total ergosterol plus DHE content (calculated from A_281.5). The ergosterol content was calculated as a percentage of the wet weight of the cells using the following equations:

\[
\% \text{Ergosterol} + \% 24(28) \text{DHE} = [\frac{A_{281.5 \text{ nm}}}{290} \times F]/\text{pellet weight}
\]

\[
\% 24(28) \text{DHE} = \left[\frac{[A_{230 \text{ nm}}/518]}{F}\right]/\text{pellet weight}
\]

where \( F \) is the factor for dilution in petroleum ether and 290 and 518 are the \( E \) values (in percent per centimeter) determined for crystalline ergosterol and 24(28) DHE, respectively.

**Fluorescence polarization**

The steady-state fluorescence polarization studies on the *Candida* cells using the fluorescent probe, DPH, were essentially carried out as described earlier (Mukhopadhyay et al., 2002; Prasad et al., 2006). The extracted sterols indicated a four-peak spectral absorption pattern produced by ergosterol and 24(28)-dehydroergosterol (DHE) contents. Both ergosterol and DHE absorb at 281.5 nm, whereas only DHE absorbs at 230 nm. The ergosterol content is determined by subtracting the amount of DHE (calculated from A_290) from the total ergosterol plus DHE content (calculated from A_281.5). The ergosterol content was calculated as a percentage of the wet weight of the cells using the following equations:

\[
\% \text{Ergosterol} + \% 24(28) \text{DHE} = [\frac{A_{281.5 \text{ nm}}}{290} \times F]/\text{pellet weight}
\]

\[
\% 24(28) \text{DHE} = \left[\frac{[A_{230 \text{ nm}}/518]}{F}\right]/\text{pellet weight}
\]

where \( F \) is the factor for dilution in petroleum ether and 290 and 518 are the \( E \) values (in percent per centimeter) determined for crystalline ergosterol and 24(28) DHE, respectively.
et al., 2002; Prasad et al., 2006). A 2 mM solution of DPH was prepared in tetrahydrofuran and 100 μL of this was added to 50 mL of rapidly stirring Tris-HCl (20 mM, pH 7.5). Excess of tetrahydrofuran was removed by flushing it with nitrogen. Spheroplasts were incubated for 1 h at 30 °C in a water bath shaker with gentle shaking at 90 r.p.m. in 2 μM DPH solution at a concentration of 4 × 10⁸ cells mL⁻¹.

Fluorescence polarization was measured by excitation with vertically polarized monochromatic light at 360 nm and emission intensity detected at 426 nm through an analyzer oriented either parallel or perpendicular to the direction of polarized excitation light. The degree of fluorescence polarization (P) was calculated according to the following:

\[
p = \frac{I_{VV} - (I_{VH} \times G)}{I_{VV} + (I_{VH} \times G)}
\]

where \(I_v\) is the corrected fluorescence intensity and subscripts V and H indicate the values obtained with a vertical or a horizontal orientation, respectively, of the polarizer and the analyzer (in that order). The corrected fluorescence was determined by subtracting the intensity of light measured with unlabeled control spheroplasts from the intensity observed with labeled cells, and the optical components used in the instrument are also corrected by calculating the grating factor G, which is denoted by \(I_{HV}/I_{HH}\).

**Passive diffusion of drug**

Passive diffusion of fluorescent compound rhodamine-6G (R6G) and radiolabeled [³H]-FLC was determined using a protocol described previously (Mukhopadhyay et al., 2002; Prasad et al., 2006). In a typical diffusion assay as described earlier, to achieve de-energization of exponentially grown Candida cells for depleting the intracellular ATP, cells were resuspended in de-energization buffer [phosphate-buffered saline (PBS) without glucose] with 5 mM deoxyglucose and 5 mM dinitrophenol at a cell density of 5 × 10⁸ cells mL⁻¹. Cells were centrifuged and the concentration of R6G in the supernatant and the intracellular concentration of radiolabeled FLC was determined using a filter disc assay, which indicated a marked increase in the susceptibility of the Δefg1 mutant to azoles such as FLC, KTC and ITR, which target lanosterol 14α-demethylase (Fig. 1a). The Δefg1 mutant also displayed enhanced sensitivity to polyenes such as AMB and NYS (Fig. 1a). The sensitivity to many other classes of drugs such as CYH, MTX, CV, PHE and SMM remained unaffected in Δefg1 mutant cells (Fig. 1a). The increase in drug sensitivity was the highest in the homozygous Δefg1 (Δefg1/Δefg1) mutant cells as compared with heterozygous (Δefg1/ΔEFG1) and revertant (Δefg1/Δefg1[ΔEFG1]) strains. The spot assays were validated using a filter disc assay, which indicated a greater diameter of the zone of inhibition with azoles and polyenes for the Δefg1 mutant cells (Fig. 1b). Notably, the liquid growth media-based NCCLS method of MIC determination revealed no change in the MIC₅₀ values of the Δefg1 mutant when tested with the same drugs as those used for solid assays (data not shown).

**RNA isolation and hybridization**

RNA isolation and Northern blot analysis was performed essentially using standard protocols (Sambrook et al., 1989). About 15 μg of total RNA was electrophoresed on a denaturing formaldehyde gel and blotted and UV cross-linked onto Hybond-N+ nylon membranes (Amersham Biosciences). Equal loading of RNA was checked by rRNA bands. The relative intensities (RI) of OLE1, ERG3, ERG11, CDR1, CDR2 and CaMDR1, and mRNA signals in Northern hybridizations were quantitated by exposure of the hybridized membrane in an FLA5000 Fuji Phosphoimager (data not shown).

**Results**

**The Δefg1 mutant shows enhanced susceptibility to drugs**

In this study, we have explored whether a link exists between the morphogenic regulator EFG1 and MDR regulatory pathways. For this, we exploited the Δefg1 mutant, which exhibited defective morphogenesis under various hyphae-inducing conditions (Ernst, 2000) and tested their susceptibility pattern to different drugs. For this, two independent methods, namely, spot and filter disc assays, were used to evaluate the drug susceptibilities of C. albicans cells on solid growth media. The drug testing, which was performed using serial dilution spot assays, revealed that there was a marked increase in the susceptibility of the Δefg1 mutant to azoles such as FLC, KTC and ITR, which target lanosterol 14α-demethylase (Fig. 1a). The Δefg1 mutant also displayed enhanced sensitivity to polyenes such as AMB and NYS (Fig. 1a). The sensitivity to many other classes of drugs such as CYH, MTX, CV, PHE and SMM remained unaffected in Δefg1 mutant cells (Fig. 1a). The increase in drug sensitivity was the highest in the homozygous Δefg1 (Δefg1/Δefg1) mutant cells as compared with heterozygous (Δefg1/ΔEFG1) and revertant (Δefg1/Δefg1[ΔEFG1]) strains. The spot assays were validated using a filter disc assay, which indicated a greater diameter of the zone of inhibition with azoles and polyenes for the Δefg1 mutant cells (Fig. 1b). Notably, the liquid growth media-based NCCLS method of MIC determination revealed no change in the MIC₅₀ values of the Δefg1 mutant when tested with the same drugs as those used for solid assays (data not shown).
Ergosterol levels of the \( \Delta \text{efg1} \) mutant are decreased

Considering the fact that Efg1p affects susceptibilities toward a selective class of drugs azoles and polyenes, and ergosterol being an important component of membrane, we estimated the level of ergosterol in the \( \Delta \text{efg1} \) mutant. It was observed that ergosterol levels were down (~24%) in the \( \Delta \text{efg1} \) mutant (Fig. 2a). Northern blot results revealed that the \( \Delta \text{efg1} \) mutant, when grown on solid media, displayed downregulation of \( \text{ERG11} \) and a simultaneous upregulation of \( \text{ERG3} \) genes (Fig. 2b).

Oleic acid levels are enhanced in the \( \Delta \text{efg1} \) mutant

We also evaluated the status of fatty acids (FAs) in the \( \Delta \text{efg1} \) mutant grown on solid media (Table 1). The results showed that in the \( \Delta \text{efg1} \) mutant, the content of 18:1 unsaturated oleic acid was significantly increased from 32.8% to 44.6%, while the contents of FAs 18:2 and 18:3 were reduced (Table 1). Interestingly, the \( \Delta \text{efg1} \) mutant cells showed considerable upregulation of \( \text{OLE1} \) gene encoding fatty acid desaturase (stereoyl CoA desaturase) that synthesizes oleic acid (Fig. 2b). Notably, the phospholipid composition remained unaffected between the \( \Delta \text{efg1} \) mutant and WT cells (data not shown).

\( \Delta \text{efg1} \) mutant show enhanced membrane fluidity and diffusion of the drug

We examined whether the observed changes in oleic acid and ergosterol levels affect the physical state of the membrane. The membrane order of cells grown on an agar surface was determined by the mobility of the fluorochrome, DPH, using fluorescence polarization measurements (Mukhopadhyay et al., 2002; Prasad et al., 2006). The lower polarization \( P \) value implies higher fluorochrome mobility and high membrane fluidity, whereas elevated \( P \) values indicate a decrease in membrane fluidity. As evident from the observed decrease in the \( P \) values (~43%), the \( \Delta \text{efg1} \) mutant membranes were more fluid as compared with WT cells (Fig. 3a). In the following experiment, the consequence of enhanced membrane fluidity was examined by monitoring passive diffusion of the drug. We blocked the contribution of the efflux pumps by de-energizing the cells to deplete the intracellular ATP (Mukhopadhyay et al., 2002; Prasad et al., 2006) and passive diffusion was then monitored in the de-energized cells using two drugs such as R6G and radiolabeled \( ^{3} \text{H} \)-FLC (Mukhopadhyay et al., 2002; Prasad et al., 2006). Figure 3b and c depicts the extracellular levels of R6G and the intracellular levels of \( ^{3} \text{H} \)-FLC (accumulation), in the \( \Delta \text{efg1} \) mutant as compared with the WT cells.

Table 1. Relative percentages of fatty acids* in the control strain CAF2-1 (WT) and \( \Delta \text{efg1} \) mutant (HLC52) calculated as described in Materials and methods

<table>
<thead>
<tr>
<th>Strains</th>
<th>( C_{14:0} )</th>
<th>( C_{16:0} )</th>
<th>( C_{16:1} )</th>
<th>( C_{18:0} )</th>
<th>( C_{18:1} )</th>
<th>( C_{18:2} )</th>
<th>( C_{18:3} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.3 ± 0.03</td>
<td>20.2 ± 0.9</td>
<td>11.4 ± 0.2</td>
<td>4.7 ± 0.11</td>
<td>32.8 ± 0.83</td>
<td>15.4 ± 0.09</td>
<td>2.3 ± 0.04</td>
</tr>
<tr>
<td>HLC52</td>
<td>1.2 ± 0.02</td>
<td>25 ± 0.99</td>
<td>6.1 ± 0.3</td>
<td>3.1 ± 0.10</td>
<td>44.6 ± 0.98</td>
<td>11.2 ± 0.09</td>
<td>1.1 ± 0.02</td>
</tr>
</tbody>
</table>

*The values are the mean ± SD of more than three independent sets of experiments.

\( \text{ERG3} \), \( \text{ERG11} \), \( \text{CDR1} \), \( \text{CDR2} \) and \( \text{CaMDR1} \) in the control strain CAF2-1 (WT) and HLC52 (\( \Delta \text{efg1} \) mutant) cells. Left panels show the transcript levels in lanes (1) WT, (2) HLC52. The panel on the right side next to each of the transcript represents the loading control to indicate equal gel loading of total RNA for the respective gene transcripts.
The disruption of EFG1 resulted in enhanced passive diffusion as was evident from a decrease (~26%) in the extracellular concentration of R6G and an increase (~41%) in the intracellular accumulation of [3H]-FLC (Fig. 3b and c). Notably, there was no appreciable difference in the passive diffusion in Δefg1 mutant cells when grown in liquid media (data not shown).

Enhanced drug susceptibility of the Δefg1 mutant is independent of multidrug efflux transporters

Overexpression of drug efflux pumps is one of the well-known mechanisms for the development of resistance in C. albicans (Ben-Yaacov et al., 1994; Albertson et al., 1996; Sanglard et al., 1997; Gupta et al., 1998; Pao et al., 1998; Kohli et al., 2002). To examine the role, if any, of major ABC efflux pumps such as CaCDR1, CaCDR2 and MFS pumps such as CaMDR1, in synergism with EFG1 disruption and drug sensitivity, we used the following experiments. Firstly, we checked the mRNA levels of the genes encoding major drug efflux transporters namely CaCDR1, CaCDR2 and CaMDR1 and found no considerable change in the transcript levels of these genes (Fig. 2b). The involvement of drug efflux pumps in influencing the drug sensitivity of the Δefg1 mutant was also ruled out by examining and comparing the efflux rates of drug substrates that remained unchanged irrespective of the status of Efg1p (data not shown).

Endogenous ROS levels are enhanced in the Δefg1 mutant

ROS elevation is associated with many metabolic statuses of Candida cells (Vinolo et al., 2009). We explored whether EFG1 modulates the ROS levels and affects drug susceptibility. For this, alteration in ROS production was measured using DCFDA fluorescence method, where increased fluorescence within cells implied enhanced ROS levels, which is depicted as a relative fluorescence value. Interestingly, our results demonstrated that the endogenous levels of ROS were considerably elevated (~54%) in the Δefg1 mutant as compared with the WT cells (Fig. 4a and b). Expectedly, the enhanced ROS levels could be reversed if media were supplemented with natural as well as synthetic antioxidants such as AA and PDTC, respectively (Fig. 4a and b). However, the restoration of ROS levels by antioxidants did not reverse the drug sensitivity of Δefg1 mutant.

---

Fig. 3. (a) Fluorescence polarization 'P values' of the control strain CAF2-1 (WT) and HLC52 (Δefg1 mutant) cells (x-axis), calculated as described in Materials and methods. Mean fluorescence polarization 'P' values of the cells ± SD of three independent sets of experiments as depicted on the y-axis. (b) Passive diffusion of R6G in the control strain CAF2-1 (WT) and HLC52 (Δefg1 mutant) cells (x-axis), calculated as described in Materials and methods. Mean of the OD_{507nm} of the supernatant ± SD of three independent sets of experiments as depicted on the y-axis. (c) Passive diffusion of [3H]-FLC in the control strain CAF2-1 (WT) and HLC52 (Δefg1 mutant) cells (x-axis), calculated as described in Materials and methods. Mean of the intracellular concentration of [3H]-FLC ± SD of three sets of experiments as depicted on the y-axis.
### Table 2. List of the *Candida albicans* strains used in this study

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Description name</th>
<th>Genotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAF2-1</td>
<td>WT</td>
<td>URA3::ura3:::imm434</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>BCa09-01</td>
<td>Heterozygous Δefg1</td>
<td>Δura3::imm434/Δura3:::imm434</td>
<td>Braun &amp; Johnson (2000)</td>
</tr>
<tr>
<td>HLC52</td>
<td>Homozygous Δefg1</td>
<td>Δura3::imm434/Δura3:::imm434</td>
<td>Lo et al. (1997)</td>
</tr>
<tr>
<td>HLC74</td>
<td>Retransformed Δefg1</td>
<td>Δura3::imm434/Δura3:::imm434</td>
<td>Lo et al. (1997)</td>
</tr>
</tbody>
</table>

**Fig. 4.** (a) ROS production in the control strain CAF2-1 (WT) and HLC52 (Δefg1 mutant) cells was measured using the DCFDA fluorescence method as described in Materials and methods. The upper panel depicts the fluorescence intensity (higher fluorescence depicts higher ROS production) as measured using a fluorescence microscope of WT, Δefg1 and Δefg1 treated with antioxidants viz. AA and PDTC with final concentrations of 5 mM and 10 μM, respectively. The lower panel depicts the phase-contrast micrographs of the upper panel (magnification × 63). (b) Quantitative measurement of ROS production in the control strain CAF2-1 (WT) and HLC52 (Δefg1 mutant) cells. ROS levels in WT, Δefg1 and Δefg1 treated with antioxidants viz. AA (5 mM) and PDTC (10 μM), respectively, depicted on the x-axis as measured by relative fluorescence (Rf) values. Mean of the Rf values ± SD of the three sets of experiments as depicted on the y-axis. (c) Drug resistance profiling of CAF2-1 (WT) and HLC52 (Δefg1 mutant) cells determined using a drug dilution spot assay in the presence of AA (5 mM) and PDTC (10 μM) as described in Materials and methods. The drug was used at the following concentration: FLC (1 μg mL⁻¹).

Discussion

In this study, we report that disruption of the morphogenic regulator *EFG1* enhances the drug sensitivity of *C. albicans* cells by a mechanism that is not dependent on the drug efflux pumps. Δefg1p disruption showed a gene-dosage effect on enhanced susceptibilities to drugs that was highest in the homozygous mutant (Δefg1/Δefg1) as compared with the WT, heterozygous (Δefg1/EFG1) and revertant (Δefg1/Δefg1[EFG1]) strains. We show that the Δefg1 mutant displayed sensitivity to azoles (FLC, ITR, KTC) and polyenes...
Interestingly, the increased drug susceptibility phenotype was selective because sensitivity to many other classes of tested drugs such as CTTH, MTX CV, PHE and SMM remained unaffected in the Δefgl mutant (Fig. 1a and b). Notably, the observed enhanced susceptibility of the Δefgl mutant was only confined to cells when grown on a solid agar surface and not when grown in liquid media (data not shown). It is reported by the Ernst group that the Δefgl fail to form hyphae when exposed to inducers such as serum or N-acetyl-D-glucosamine in liquid or solid media; however, under microaerophilic/embedded conditions, the Δefgl mutant displays enhanced filamentous growth (Brown & Gow, 1999; Doedt et al., 2004; Setiadi et al., 2006). This establishes the dual role of the Efglp regulator in controlling the same phenomenon of hyphal morphogenesis via different regulatory mechanisms owing to different growth conditions. Therefore, confinement of the drug sensitivity of the Δefgl mutant cells, grown only on a solid agar surface, does not reflect an artifactual effect due to a difference in the growth media. This was further validated by the fact that drug susceptibility assays that were performed on a solid surface via two independent methods, yielded a similar phenotype (Fig. 1a and b). Notably, C. albicans cells form biofilms on plastics and a host of biological surfaces that show elevated antifungal resistance (Mukherjee et al., 2005) and pose a major challenge for antifungal chemotherapy. Because Efglp levels are pivotal for the biofilm formation (Ramage et al., 2002), its role in antifungal resistance during surface growth may be of particular clinical relevance.

Our study supports that the MDR phenomenon in yeast is the result of culmination of several factors. Notwithstanding the involvement of any known MDR genes, Δefgl mutant cells consistently showed decreased resistance on solid growth media to a restricted class of drugs targeting ergosterol or its metabolism. Ergosterol is an essential and specific component of fungal membranes performing a variety of functions that modulate membrane fluidity and its permeability (Daum et al., 1998). Therefore, transcription factors regulating the ergosterol biosynthetic pathway represent an important target for antifungal agents. Interestingly, in an earlier study, the transcript level of ERG3 has been shown to be directly affected by the EFG1 levels (Lo et al., 2005). In this study, we observed a considerable downregulated transcript level of ERG11 (the rate-limiting step in ergosterol biosynthesis) and upregulated the ERG3 transcript in the Δefgl mutant in comparison with the WT cells (Fig. 2b). This was associated with a statistically significant decrease in ergosterol contents (~24%) in Δefgl mutant cells (Fig. 2a). Of note, ERG3 acts downstream of ERG11 in the ergosterol biosynthesis pathway and encodes the Δ5,6-desaturase (Chau et al., 2005). In anazole-inhibited pathway, Erg3p is responsible for converting the nontoxic 14-methyl intermediates (Chau et al., 2005) into the toxic sterol 14-methylergosta-8,24(28)-dien-3,6-diol. There have been reports (Kelly et al., 1997; Kakeya et al., 2003) that demonstrate that inhibition of 14a-demethylase by azoles results not only in ergosterol depletion but also in the accumulation of methylated sterol 14a-methylergosta-8, 22(28)-dien-3β,6α-diol. Moreover, even deletion of the ERG3 gene, in C. albicans, resulted in reduced susceptibility of Δerg3 to azoles (Kakeya et al., 2003) and amphotericin B (Kelly et al., 1997). Sterol analysis revealed that the Δerg3 mutant lost both ergosterol and the toxic diol product when grown in the presence of azoles (Kelly et al., 1997; Kakeya et al., 2003). In the present study, in Δefgl mutant cells where ERG3 is upregulated, azoles might act synergistically due to the accumulation of the toxic diol product; however, this remains to be experimentally validated.

Considering the fact that sensitivity to the azoles has often been linked to the changes in the membrane lipid composition (Kohli et al., 2002; Mukhopadhyay et al., 2002; Prasad et al., 2006) and that the enhanced drug sensitivity phenotype of Δefgl mutant cells observed presently was restricted only to azoles and polyenes, we speculated that probably EFG1 target membrane-associated genes, which affects its composition. Our Northern blot analysis revealed a marked increase in the OLE1 transcript in the Δefgl mutant (Fig. 2b) in comparison with the WT cells, coinciding with a significant increase in the oleic acid content of the membrane (Table I), leading to an increase in membrane fluidity (Fig. 3a), although it is expected that normally cells would cope with the fluctuations in oleic acid levels by compensatory adjustments in membrane lipids, which would maintain optimal membrane fluidity. However, despite the fact that no major changes in the overall phospholipid content were evident in Δefgl mutant cells, the observed increase in membrane fluidity implies a direct link between Ole1p and Efglp levels. Earlier, it has been shown that fatty acid biosynthesis genes require Efglp and, particularly, fatty acid unsaturation in C. albicans is dependent on Efglp levels under hypoxic conditions (Setiadi et al., 2006). In the present study, we observed a direct functional consequence of oleic acid increase as an increase in the membrane fluidity of Δefgl mutant cells. Consequently, the passive diffusion of drugs is increased in Δefgl mutant cells, which resulted in enhanced accumulation and sensitivity of drugs (Fig. 3b and c). Interestingly, these observations were confined to Δefgl mutant cells when grown in solid media because, whether grown in liquid media, there was no noticeable difference in membrane fluidity and passive diffusion of the drugs in Δefgl mutant cells (data not shown).

Among the novel mechanisms of drug resistance, the role of ROS is emerging, where an increase in ROS levels has been associated with several antifungals (Kobayashi et al., 2003).
Interestingly, \textit{Δefg1} mutant cells displayed enhanced levels of endogenous ROS (Fig. 4a and b). However, the enhanced ROS levels that could be reversed by the addition of antioxidants (Fig. 4a and b) could not reverse the drug sensitivity of \textit{Δefg1} mutant cells (Fig. 4c). There are reports to suggest that \textit{Candida} strains lacking \textit{ERG11} are susceptible to oxidative stress (Kan \textit{et al.}, 1996). It could therefore be possible that the inhibition of the \textit{ERG11} gene by \textit{EFG1} may produce a similar effect. Whether increased ROS levels in \textit{Δefg1} mutant acts as a signal for some regulatory cascade acting in parallel or in concert with the drug susceptibility mechanisms remains an open area of interest.

Taken together, our study indicates that apart from the well-established role of \textit{EFG1} as a morphogenetic regulator, it also functions as a regulator of antifungal resistance. Our data revealed that increased membrane fluidity leading to enhanced passive diffusion of drugs could be critical determinants of the drug susceptibilities of the \textit{Δefg1} mutant cells. Probably, the regulatory networks that control the morphogenesis in \textit{C. albicans} on solid media also control the genes involved in cellular functions not directly related to drug resistance. Certainly, more extensive analyses are required to elucidate the commonality between \textit{EFG1} and \textit{MDR} signaling cascades to find newer targets for antifungal chemotherapy.

**Acknowledgements**

We thank Joachim F. Ernst for providing strains and advice. We thank Ranbaxy Laboratories (New Delhi, India) for providing FLC. S.H. thanks the Council for Scientific and Industrial Research (CSIR) for the Senior Research Fellowship. T.P. acknowledges the support of the Ranbaxy Science foundation and the Department of Biotechnology in the form of the Ranbaxy Science Scholar Award, 2008, and the Innovative Young Biotechnologist Award, 2008. The work presented in this paper was supported in part by grants to R.P. from Indo-DFG (INT/DFG-P/05/2005), Department of Science and Technology (SR/SO/BB-12/2004), the Council of Scientific and Industrial Research (38(1123)/06/EMR-II), Department of Biotechnology (BT/PR9100/Med/29/03/2007, BT/PR9563/BRB/10/567/2009).

**Authors’ contribution**

Both T.P. and S.H. contributed equally to this paper.

**References**


