SECTION-I
3. Iron Deprivation induces hyphal development independent of biofilm formation in *C. albicans*

3.1 Background

Iron is a critical micronutrient required by almost all the organisms especially as a cofactor in important metabolic functions (Bullen *et al.*, 2006; Fischbach *et al.*, 2006; Nyilassi *et al.*, 2005; Spacek *et al.*, 2005; Weinberg *et al.*, 1999a). 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 providing natural resistance to infections in humans (Bullen *et al.*, 2006). Since the pathogenic microbes have to scavenge iron from the host, therefore the competition between pathogen and host for iron represents a critical aspect of many infectious diseases (Schaible & Kaufmann, 2004; Weinberg, 1999b). 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 serve as a defense against infection (Emery, 1980; Kontoghiorthes & Weinberg, 1995; Weinberg, 1984).

For its survival in the host cells, *Candida* cells like any other pathogen has also adapted many complex strategies to scavenge the depleting 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 high affinity iron transporter (CaFtr1p) for infection in mouse model was shown (Ramanan & Wang, 2000). Similarly the requirement of siderophore transporter (Arn1p) for epithelial invasion (Heyman *et al.*, 2002) and iron dependent endothelial cell injury (Fratti *et al.*, 1998) suggests that iron plays a vital role in the virulence of *C. 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 & Vinces, 2005). *C. albicans* morphology is directly related to environmental cues which trigger independent signal transduction pathways, many of which converge on two main signaling pathways regulating common targets required to initiate hyphal growth (Brown & Gow, 1999). The transcription factors such as *EFG1* and *CPH1* are primary regulators of morphogenesis and act via cAMP and MAP kinase pathways respectively (Ernst, 2000; Ernst & Schmidt, 2000). Thus a double null mutant Δ*efg1*Δ*efg1* Δ*cphl*Δ*cphl* 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 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, since 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 has been identified that were induced by biofilm growth in *P. aeruginosa* (Patrauchan *et al.*, 2007). Likewise iron deprivation has been known to affect biofilm formation in *Actinomyces naeslundii* (Moelling *et al.*, 2007).

*Candida* colonizes a variety of anatomical sites which are likely to be scarce in iron, especially under conditions of severe and chronic immunosuppression induced by HIV.
infection (Lan et al., 2004). It has been earlier observed 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 part of the study, it is demonstrated that iron deprived C. albicans cells or its mutants defective in iron acquisition show hyphal development in non-hyphal inducing media and that 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 remained unaffected by the cellular iron status of C. albicans. Taken together, these findings for the first time show that under iron limiting conditions the transcriptional regulator EFG1 plays a crucial role in affecting the hyphal development in C. albicans without affecting ability of cells to form biofilms.

3.2 RESULTS

3.2.1 Iron deprivation promotes hyphal development in C. albicans

It was explored if levels of iron could affect hyphal development in Candida cells and for this BPS, a well known iron chelator, was used at a concentration which depleted iron from the media without affecting the growth of the Candida cells (Prasad et al., 2006). C. albicans cells when grown in iron sufficient (ISM) liquid and solid YEPD media showed no hyphal formation (Fig. 10A). However, in iron poor media (IPM), cells could show hyphal development in the absence of any hyphae inducing agent (Fig. 10A). This hyphal development could be prevented if BPS grown cells were supplemented with 100 μM FeCl3 (Fig. 10A).

3.2.2 Iron acquisition defective mutants also show hyphae formation

The role of iron in hyphal differentiation in C. albicans was further confirmed when two iron acquisition defective mutants were used such as Δfir1 (defective in high-affinity iron uptake) (Ramanan & Wang, 2000) and Δccc2 [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 iron chelator showed hyphal development in solid as well as in liquid YEPD (Fig. 10B and C). The hyphal formation could be reversed if iron-transport defective mutants were supplemented with 100 μM FeCl₃ (Fig. 10B and C).

![Hyphal morphogenesis in the liquid (upper panel) and solid (lower panel) YEPD in the absence (control), presence of 150 μM BPS (IPM) and supplementation with 100 μM FeCl₃ (IRM) in the WT (A) and iron acquisition mutants (B and C for Δfrl1 and Δccc2 respectively). Magnification, 63X.](image)

**Fig. 10A to C.** Hyphal morphogenesis in the liquid (upper panel) and solid (lower panel) YEPD in the absence (control), presence of 150 μM BPS (IPM) and supplementation with 100 μM FeCl₃ (IRM) in the WT (A) and iron acquisition mutants (B and C for Δfrl1 and Δccc2 respectively). Magnification, 63X.

### 3.2.3 EFG1 mediates hyphal development under iron depriving conditions

It was evaluated if the iron mediated hyphal development is mediated by known signal cascades controlling morphogenesis (Ernst, 2000; Ernst & Schimdt, 2000).
Fig. 10D to F. Hyphal morphogenesis in the liquid (upper panel) and solid (lower panel) YEPD in the absence (control), presence of 150 μM BPS (IPM) and supplementation with 100 μM FeCl₃ (IRM) in the morphological mutant cells (D to F for Δcph1, Δefg1 and Δefg1Δcph1 respectively). Magnification, 63X.
Results and Discussion

Mutants such as Δcph1, Δefhl and Δtpkl with the exception of Δefgl null mutant cells, exhibited filamentation albeit to a lesser extent, on both iron depleted solid and liquid media (Fig. 10D, E, G and I). Notably, a major transcription factor CPH1, regulating hyphal transition and activated via MAPK pathway did not appear to contribute to this iron starvation mediated filamentation as Δcph1 null mutant continued to show hyphal formation upon iron deprivation (Fig. 10D). On the other hand, unlike Δcph1 cells, Δefgl null mutants cells were unable to form hyphae in both iron sufficient (ISM) and iron deprived (IPM) liquid and solid YEPD media (Fig. 10E).

EFH1 transcription factor is a homologue of EFG1 which 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 since Δefhl null mutant cells were able to make hyphae under IPM conditions on both solid and liquid media (Fig. 10G). Lack of filamentation under iron deprived conditions in the double ΔefglΔcph1 (Fig. 10F) and ΔefglΔefhl (Fig. 10H) mutant cells 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 the advantage of the Δtpkl null strains. TPK1 encodes a protein kinase A which acts upstream of EFG1 in the cAMP signaling pathway controlling morphogenesis (Ernst, 2000). The Δtpkl null strains did not show any hyphal development in iron sufficient (ISM) solid and liquid YEPD media (Fig. 10I). However, Δtpkl null mutant cells formed hyphae under iron deprived (IPM) solid and liquid YEPD media (Fig. 10I). The observed hyphal development under all iron deprived conditions could be prevented if the media was supplemented with 100 μM FeCl3 (Fig. 10, D, F to I).
Fig. 10G to I. Hyphal morphogenesis in the liquid (upper panel) and solid (lower panel) YEPD in the absence (control), presence of 150 μM BPS (IPM) and supplementation with 100 μM FeCl₃ (IRM) in the morphological mutant cells (G to I for,Δefh1, Δefh1Δefg1 and Δtkp1 respectively). Magnification, 63X.
3.2.4 *EFG1* transcript is upregulated in iron deprived conditions in *C. albicans* cells

Northern analysis revealed considerable upregulation of *EFG1* transcript levels under iron deprived conditions (Fig. 11). The transcript levels of *EFG1* in iron deprived growth conditions (IPM) could be restored to the wild type 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, Δftr1 and Δccc2 cells. Both the mutants showed constitutively high levels of *EFG1* transcript as compared to the WT cells grown in ISM (Fig. 11). Under iron deprived growth conditions, no change in the transcript levels of other morphogenic regulators, namely, *CPH1*, *EFH1* and *TPK1* was observed (Fig. 11).

![Fig. 11. Northern blot analyses of *EFG1*, *CPH1*, *EFH1* and *TPK1* respectively. Left panels show transcript levels in lanes (1) WT, (2) WT grown in presence of 150 μM BPS, (3) Δftr1, (4) Δccc2, (5) iron depleted WT strain after supplementation with 100 μM FeCl₃, (6) Δftr1 after supplementation with 100 μM FeCl₃, (7) Δccc2 after supplementation with 100 μM FeCl₃. Right panel represents loading controls for indicating equal gel loading of total RNA.](image-url)
3.2.5 Iron deprivation enhances the membrane fluidity of Candida cells

Our earlier data suggested intricate relationship between membrane fluidity, iron deprivation and drug susceptibility of Candida cells, wherein it a causal relation was showed between increased membrane fluidity of WT cells in iron deprived conditions and drug susceptibility (Prasad et al., 2006). To explore whether membrane fluidity is also responsible for the enhanced filamentation in iron deprived conditions, steady-state fluorescence polarization technique using the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) (Kohli et al., 2002, Mukhopadhyay et al., 2002; Mukhopadhyay et al., 2004) was used to examine the membrane order of the cells grown in ISM and IPM conditions. Of note, a decrease in the “p” value implies decrease in membrane order or increase in membrane fluidity. All the morphological mutant cells of Candida viz. Δcph1, Δefg1, Δefg1Δcph1, Δefh1, Δefg1Δefh1 and Δtpk1 showed lower “p” values as compared to WT cells in ISM (Table 5). Interestingly, the “p” values of all the above tested mutants and the WT strain were further lowered when these cells were deprived of iron in IPM conditions (Table 5). 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 (Prasad et al.; 2006) where it was shown that even in the absence of BPS, iron acquisition defective mutants namely, Δftr1 (defective in high affinity iron transport) and Δcc2 (copper transport mutant) show increased membrane fluidity as compared to the WT cells.
TABLE 5. Steady state fluorescence polarization measurements (p value) in *C. 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 “p” values (Mean ± SD)A</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.172 ± 0.001</td>
</tr>
<tr>
<td>WT+150 μM BPS</td>
<td>0.095 ± 0.01</td>
</tr>
<tr>
<td>Δcph1</td>
<td>0.101 ± 0.008</td>
</tr>
<tr>
<td>Δcph1+150 μM BPS</td>
<td>0.089 ± 0.003</td>
</tr>
<tr>
<td>Δefg1</td>
<td>0.112 ± 0.005</td>
</tr>
<tr>
<td>Δefg1+150 μM BPS</td>
<td>0.092 ± 0.01</td>
</tr>
<tr>
<td>Δefg1Δcph1</td>
<td>0.098 ± 0.007</td>
</tr>
<tr>
<td>Δefg1Δcph1+150 μM BPS</td>
<td>0.073 ± 0.008</td>
</tr>
<tr>
<td>Δefh1</td>
<td>0.105 ± 0.01</td>
</tr>
<tr>
<td>Δefh1+150 μM BPS</td>
<td>0.078 ± 0.009</td>
</tr>
<tr>
<td>Δefh1Δefg1</td>
<td>0.095 ± 0.006</td>
</tr>
<tr>
<td>Δefh1Δefg1+150 μM BPS</td>
<td>0.071 ± 0.01</td>
</tr>
<tr>
<td>Δpk1</td>
<td>0.108 ± 0.01</td>
</tr>
<tr>
<td>Δpk1+150 μM BPS</td>
<td>0.093 ± 0.007</td>
</tr>
</tbody>
</table>

A The values are the mean fluorescence polarization “p” values of the cells (inversely proportional to membrane fluidity) ± the standard deviation of the mean of the three independent sets of experiments.
3.2.6 Role of iron acquisition in biofilm forming ability of *C. albicans*

Earlier, \( \Delta efg1 \) mutant cells of *C. albicans* were shown to be defective in their ability to form biofilms (Ramage *et al.*, 2002). Since catheter-associated fungal biofilms are rich in hyphal elements and also that the iron acquisition defective mutant cells of *Candida* namely, \( \Delta ftr1 \) and \( \Delta ccc2 \) showed normal hyphal formation, it was hypothesized that these iron acquisition defective strains would form regular biofilms. To test this hypothesis, the biofilm forming ability of \( \Delta ftr1 \) and \( \Delta ccc2 \) mutant strains were determined on catheter discs as described previously (Chandra *et al.*, 2001a). It was found that there was no significant difference in the ability of \( \Delta ftr1 \) and \( \Delta ccc2 \) mutant strains to form biofilms compared to the WT strain (as measured by determining their metabolic activity and dry biomass, Fig. 12A). As expected, biofilm formed by the \( \Delta efg1 \) strain exhibited significant decrease in metabolic activity, indicating reduced biofilm formation \( (P=0.000469, \text{ vs. wild type strain}; \text{Fig. 12A}). \)

![Graph](https://example.com/graph.png)

**Fig. 12A.** Contribution of iron availability to the ability of *C. albicans* to form biofilms on silicone elastomer catheters. (A) Metabolic activity (black solid bars) and dry weight (grey hatched bars) of biofilms formed by different *Candida* mutants (\( \Delta efg1 \), \( \Delta ftr1 \), \( \Delta ccc2 \)) and their isogenic WT strain. Data represents mean ± SD from three independent experiments \( (*P= 0.000469, \text{ vs. wild type isolate}). \)
Fluorescence microscopy with the carbohydrate-specific dye Calcofluor White revealed that Δefg1 exhibited clusters of yeast cells and localized carbohydrate-specific staining (Fig. 12C). In contrast, the Δfir1 and Δccc2 mutant strains formed biofilms with diffuse Calcofluor White staining, indicating presence of extracellular matrix (Fig. 12, panels D,E, white arrows). Notably, when the effect of BPS induced iron deprivation was checked on the ability of Candida strains to form biofilms, it was observed that BPS affected fungal morphology of all strains tested (Fig. 12, F to I), suggesting that the effect of BPS on Candida cells was not related to their ability to form biofilms.

**Fig. 12 B to I. Contribution of iron availability to the ability of C. albicans to form biofilms on silicone elastomer catheters** (B to I) - Fluorescence microscopic images of biofilms formed in the absence (B to E) or presence (F to I) of iron chelating agent (BPS). (B, F) – WT; C, G – Δefg1; D, H– Δfir1 and E, I – Δccc2. White arrows indicate extracellular matrix. Magnification, 20X.
3.2.7 Confocal analyses revealed no difference in architecture of biofilms formed by iron uptake mutant strains

To confirm these findings and also to evaluate the architecture of formed biofilms, confocal scanning laser microscopy (CSLM) analyses was performed which showed that the Δftr1 and Δccc2 mutants formed biofilms with similar, hyphal-rich architecture as the WT parental strain (Fig. 13). As expected, only adhered cells were observed with the Δefg1 mutant isolate, demonstrating that this isolate did not form robust biofilms. Mean thickness of biofilms formed by these strains, (A) WT, (B) Δftr1, (C) Δccc2, and (D) Δefg1 were 100 ±10 μm, 70 ± 23 μm, 80 ± 15 μm, and 40 ± 7.6 μm, respectively. No statistically significant difference was found between thicknesses of biofilms formed by the WT, Δccc2, or Δftr1 mutants (P > 0.05), while thickness of adhered cells of Δefg1 mutant strain was significantly less than that of biofilms formed by the WT strain. These results clearly showed that disruption of FTR1 or CCC2 gene did not affect the ability of C. albicans to form biofilms.
Fig. 13. Confocal microscopy analyses of biofilms formed by (A) WT, (B) Δfrt1, (C) Δccc2, and (D) Δefg1 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 strips show the side views from two different planes. Mean thickness of biofilms formed by these isolates were calculated from thickness measured at three different positions within the biofilm. Mean thickness of biofilms formed by these strains ((A) WT, (B) Δfrt1, (C) Δccc2, and (D) Δefg1 strains) were 100 +/- 10 µm, 70 +/- 23 µm, 80 +/- 15 µm, and 40 +/- 7.6 µm, respectively. Magnification, 20X for each panel.
3.3 Discussion

In this part of the study, it was shown that iron deprivation promotes hyphal development in *C. albicans* cells which is exclusively mediated via 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. 11). The increase in *EFG1* transcript under iron depleted conditions of growth could be reversed by supplementing the iron deficient cells with 100 μM FeCl$_3$ (Fig. 11). The mutants defective in iron acquisition such as Δftrl as well as copper transporter mutant Δccc2, 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. 10 B & C). Expectedly both the null mutant cells also showed elevated levels of *EFG1* transcript (Fig. 11). Similar to the WT cells, the transcript of *EFG1* (Fig. 11) and hyphal development exhibited under iron depleted conditions of growth could be reversed by supplementing the iron acquisition mutant cells with 100 μM FeCl$_3$ (Fig. 10B and C). This reversion could be due to the presence of the low affinity iron transporters which become active in the iron rich media (IRM) and thereby compensates for the gene knockout effect of high affinity iron transporters in the presence of excess iron in the surrounding media. These 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 these finding with iron chelator BPS, we supplemented iron (FeCl$_3$) to reverse the phenomenon and also used high affinity iron uptake mutants such as Δftrl and Δccc2. Hyphae formation is one of the most important mechanisms of *Candida* to overcome the restricted growth conditions. Increased expression of *EFG1* and subsequent hyphae formation is the manifestation of the ability of *Candida* to overcome adverse condition. Interestingly, to understand the role of iron in germ tube formation,
earlier Sweet and Douglas (1991) employed 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 to 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 and which might be a similar condition achieved during these experiments or by Lan et al.

EFG1 being a global regulator of morphology and metabolism also plays a major role in gene regulation of C. albicans (Brown & Gow, 1999; Stoldt et al., 1997). It has been 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, it was observed 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 (Δcph1, Δefh1 and Δtpk1 and Δefg1) showed increase in membrane fluidity as compared to their wild type host but showed no hyphal development. 2) When these mutants were deprived of iron, there was further increase in membrane fluidity (Table 5). However, in spite of the increase in membrane fluidity of the morphological mutants upon iron depletion, all the mutants formed hyphae, except Δefg1 null mutant which was completely blocked in hyphae formation. This rule out any role of membrane fluidity in hyphal development by iron deprivation. These results clearly show that iron induced 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 since 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
Iron-deprived transcriptome analysis by Lan et al., 2004 showed that OLE1 is downregulated by 2.2 fold. While analyzing transcriptome of Δefg1 cells which were completely defective in hyphal morphogenesis, a 2 fold increase in transcript levels of OLE1 was observed suggesting that changes in fluidity are not related to the ability of cells to develop hyphae (Prasad et al., unpublished data). As reported earlier, enhanced membrane fluidity under iron deprived conditions do sensitize Candida cells to various drugs (Prasad et al., 2006), therefore, the effect of iron in 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 formation of biofilm, probably mediated by its regulation of hyphae formation, a pre-requisite for biofilm formation and ruled out any role of the mitogen-activated protein kinase (MAPK) involving CPH1 in the same (Ramage et al., 2002). In the current study, it was found that the increased EFG1 transcript in iron deprived conditions did not correlate with the significant change in the ability of C. albicans to form biofilm as compared to the wild type cells (Fig. 12). Furthermore, BPS induced iron deprivation of the growth medium did not affect biofilm forming abilities of the mutant strains as compared to 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 due to live and dead fungal cells and hyphae, as well as the extracellular matrix. In contrast, XTT measurements evaluate the metabolic activity of combined population of cells and hyphae. Since dry weight and XTT assays measure different variable of biofilms, a linear association between these assays is always not 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 increase in XTT metabolism, similar to these reported earlier by Knight & Dancis (2006), who showed that iron-limiting conditions induce
five-fold increase in XTT reductase activity, and that activity of this enzyme in *Candida* cells grown in unbuffered (pH 4.0-4.4) medium was mediated by CaFRE10 protein. Fluorescence and confocal microscopy analyses also revealed no difference in biofilm morphology and architecture between the mutant strains and their isogenic WT parent. These results suggested that the ability of *C. albicans* to form biofilm is not affected by disruption of the selected genes (Fig 13). Since limiting the iron from the invading pathogen is one of the defense strategies adopted by the host (Emery, 1980; Kontoghiorghes & Weinberg, 1995; Weinberg, 1984), 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 note, the signaling effect of hemoglobin (Hb) which is also an effective inducer of hyphaI development in *C. albicans* (Pendrak & Robert, 2007) is independent of cellular iron status. Both heme and globin are unable to induce hyphal formation since Hb receptor requires intact αβHb dimer to function as signal transducer for hyphal development. Interestingly, for Hb signaling, Efg1p is also necessary for hyphal formation in *C. albicans* whereas the other morphogenic regulator, Cph1p has no role (Pendrak & Robert, 2007). Thus Efg1p-dependent hyphal development in *C. albicans* constitutes both iron dependent and iron independent pathways.

Hyphal morphogenesis being 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 which is specifically mediated by iron as other divalent cation chelator 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 *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 virulence of C. albicans.