SUMMARY

Several *Candida* species are often present as benign commensals in healthy humans. However, in immunocompromised host, such as HIV-infected patients, patients who are under with immunosuppressive therapy, patients with natural immunodeficiency, and in diabetic patients, they become opportunistic pathogens causing superficial, as well as severe, life-threatening systemic infections, collectively called candidiasis infections (Andes et al., 2012; Miceli et al., 2011; Perez et al., 2013; Pfaller and Diekema, 2007; Pfaller et al., 2012; Sardi et al., 2013). Of the *Candida* species afflicting humans, *Candida albicans* is by far the most common. *C. albicans* infections are the fourth most common nosocomial bloodstream infection in the United States and associated with high mortality rate and prolonged hospital stay (Berman and Sudbery, 2002; Mallick and Bennett, 2013). There are relatively few classes of antifungal drugs available to combat invasive *Candida* infections such as azoles, polyenes, echinocandins, pyrimidines, and allylamines (Akins, 2005). Emergence of clinical strains resistant to the existing antifungal drugs has further compounded the problem (Boschman et al., 1998; Powderly, 1994). Moreover, formation of biofilm by several pathogenic *Candida* species has also rendered therapy ineffective, since biofilm cells are resistant to several antifungal drugs compared to planktonic cells (Walraven and Lee, 2013).

Amphotericin B (AmB), a frontline polyene macrolide antifungal, is considered the gold standard for the treatment of most life-threatening systemic fungal infections because of its broad antifungal spectrum and fungicidal activity (Anderson et al., 2014; Gray et al., 2012). *Candida* infections are becoming more common with increasing number of clinical isolates resistant to AmB and its lipid formulations, frequently resulting in treatment failures (Sterling and Merz, 1998). In addition to emergence of AmB resistant srains, association with dose-limiting severe side effects remains a challenge to use AmB as a first

choice of drugs for candidiasis therapies. AmB binds to ergosterol, the principal sterol in the fungal plasma membrane. Resistance strains against AmB are mainly associated with ergosterol biosynthetic pathway (Geber et al., 1995; Ghannoum et al., 1990; Kelly et al., 1996; Nolte et al., 1997; Peyron et al., 2002; Tsai et al., 2004; Young et al., 2003). Antifungal activity of AmB is correlated with sequestering of ergosterol, resulting aberration of several cellular functions (Anderson et al., 2014; Gray et al., 2012). Sphingolipids, a another class of lipids, physically as well as genetically interacts with ergosterol and ergosterol biosynthetic pathway respectively, and most importantly they are involved in numerous common cellular functions (Bagnat et al., 2001; Bagnat et al., 2000; Brown and London, 1998; Chung et al., 2003; Ikonen, 2001; Kim and Kwon-Chung, 1974; Simons and Ikonen, 1997; Simons and Sampaio, 2011; Zhang et al., 2010). Thus we speculated that sphingolipids could influence the AmB resistance/sensitivity. Thus, in this project attempts have been made to identify and characterize the sphingolipid biosynthetic pathway genes that are involved in the AmB resistance at the molecular level in *S. cerevisiae* and *C. albicans*.

In first part of work, *S. cerevisiae* sphingolipid biosynthetic pathway homozygous deletants of nonessential genes as well as heterozygous deletants of essential genes were screened for AmB resistance. Screening was performed by SC agar medium plate based assay with different concentrations of AmB. Two strains deleted in *FEN1* and *SUR4* genes were found to be sensitive by 2-fold and 5-fold respectively, to AmB as compared to parent strain (BY4743). True deletion were confirmed by diagnostic PCR, since only these two genes of this pathway modulated AmB resistance. AmB sensitivity of these deletants was further confirmed in haploid strain background (BY4741) of *S. cerevisiae*. *FEN1* and *SUR4* encode proteins belonging to elongase family, which synthesized C22 or C24 very long chain fatty acid (VLCFA) and C24 or C26 VLCFA respectively (Denic and Weissman,

2007; Jakobsson et al., 2006; Oh et al., 1997). In addition to these, protein encoded by *ELO1* also belongs to elongase family which synthesized C16 LCFA (Oh et al., 1997). However, the AmB sensitivity of *ELO1* gene deleted strain was comparable to parent strain. C26 VLCFA is an important constituent of naturally occurring sphingolipids of *S .cerevsiae*, because ceramide synthase has high affinity towards it during synthesis of ceramides. It has been well established that, sphingolipids synthesis is reduced upon deletion or mutation of *FEN1* and *SUR4* genes resulting in pleiotropic phenotypes such as disturbance in plasma membrane integrity, defects in secretory pathway, 1,3-beta-glucan synthase activity, V-ATPase and constitutive activation of autophagy activities (Abe et al., 2001; Chung et al., 2003; David et al., 1998; Dickson and Lester, 1999; Zimmermann et al., 2013). Most of the above individual phenotype are directly or indirectly associated with changes in sphingolipids constituents and compositions in the cells. Thus, it appears that aberration or regulation of sphingolipids synthesis in *FEN1* and *SUR4* deleted strains modulate AmB resistance.

To further confirm the role of these genes in pathogenic yeast for AmB resistance, we first searched their putative orthologs by sequence homology in *C. albicans* and found two uncharacterized *ORFs*, *Caorf19.6343* and *Ca.orf19.908* (*CaFen12*). Subsequently, these putative orthologs was confirmed by complementation in *S. cerevisiae* deletants with respect to AmB phenotype, and found that *Caorf19.6343* and *Ca.orf19.908* (*CaFen12*) *ORFs* are true orthologs of *FEN1* and *SUR4* genes respectively. In rest of the studies, *Caorf19.6343* and *Ca.orf19.908* are referred to as *CaFEN1* and *CaFEN12* respectively. Next, we characterized the *CaFEN1* and *CaFEN12* genes in terms of viability for *C. albicans*. For this, *CaFEN1* and *CaFEN12* genes were expressed under *MET3* regulatable promoter in SN95 strain of *C. albicans* and found that both are non essential for survival. After this result, strains deleted for individual (*CaFEN1* or *CaFEN12*) as well as

both the genes (*CaFEN1* and *CaFEN12*) were made to test AmB resistance and other phenotype. Though *ScFEN1* and *ScSUR4* genes are synthetic lethal in *S. cerevisiae* (Revardel et al., 1995), we found that double deletant of *CaFEN1* and *CaFEN12* is viable in *C. albicans*. The AmB resistance of these deletants were checked on both SC agar and RPMI 1640 media and found that, deletants of *CaFEN1* and *CaFEN12* were sensitive to AmB by 2-fold and 5-fold, respectively, as compared to the parent strain (SN95). The AmB sensitivity of these deleted strains is similar to that of *ScFEN1* and *ScSUR4*, confirming their role in AmB resistance in *C. albicans*. Moreover, double delete strain was found to be hypersensitive to AmB, which is almost 20-fold more sensitive than parent strain.

It has been reported that AmB leads to the oxidative stress by means of ROS production and as a consequences to this cells undergo apoptotic death (Phillips et al., 2003; Powderly et al., 1988; Sokol-Anderson et al., 1986). Therefore, ROS acts as a biological marker to test the oxidative stress condition as well as AmB resistance phenotype. We exploited this strategy to check oxidative stress, if any, and AmB resistance phenotype of *FEN1* and *SUR4* deletants of both *S. cerevisiae* and *C. albicans*. Thus, we quantified ROS production without and with treatment of AmB and found that without AmB, there was no significant difference in ROS production by mutants individually deleted in these genes in both *S. cerevisiae* and *C. albicans* as compared to the parent strains. However, double deletant strain of *C. albicans* immensely produced ROS by 16-fold compared to parent strain suggesting that, it is surviving under oxidative stress condition. Moreover, upon treatment with AmB, strains deleted in individual genes also produced higher ROS as compared to the parent strains, confirming the role of *FEN1* and *SUR4* in AmB resistance.

AmB resistance is correlated with ergosterol level (Sanglard et al., 2003; Vincent et al., 2013; Young et al., 2003). It has been reported that *ScFEN1* and *ScSUR4* genes genetically interact with ergosterol

biosynthetic pathway genes, perhaps increasing the ergosterol level upon deletion thereby sensitizing them to AmB. Thus, we quantified the ergosterol content of deletion strains of both the yeasts, and no significant increase in ergosterol level was seen compared to their parent strains. We also quantified the ergosterol level in haploid deletion strain and found the similar results. Moreover, there was low level of ergosterol in *ScSUR4* deleted strain, which should show AmB resistance phenotype. However this strain was found to be sensitive to AmB, suggesting that their AmB sensitivity is independent to ergosterol level which is also equally applicable to *ScFEN1*, *CaFEN1* and *CaFEN12* deleted strains. We further qualitatively analyzed the glycerophospholipids and sphingolipids for all deleted and parent strains. Profile of both class of lipids were found to be differed in deleted strains as compared to their parent strains, though, we could not directly connect the alteration in particular lipid molecule(s) to AmB sensitivity phenotypes of deleted strains.

Since, ergosterol and sphingolipids are physically as well as functionally interact, and are involved several cellular functions, we directly tested the AmB sensitivity upon compromised sphingolipids biosynthesis. AmB sensitivity of wild type strains of *S. cerevisiae* and *C. albicans* species were tested in the presence of myriocin, which inhibits the sphingolipids biosynthesis (Daum et al., 1998; Dickson, 2008; Miyake et al., 1995). We found that sub-lethal concentration of myriocin sensitized the cells to AmB, suggested the role of sphingolipids in AmB resistance. To further validate, sublethal concentration of PHS, an intermediate of sphingolipids upstream to IPC, when supplemented to myriocin-AmB treated cells rescued AmB sensitivity. This result confirmed that reduction in sphingolipids leads to the AmB sensitivity. Inhibition of complex sphingolipid synthesis by sublethal concentration of AbA (inhibitor of IPC synthase) did not enhance AmB sensitivity, suggesting that reduction of intermediates upstream to IPC rather than total sphingolipids leads to AmB

sensitivity. Surprisingly, addition of PHS to AbA-AmB, sensitized the cells, revealing accumulation of intermediate(s) upstream to IPC also mitigates AmB sensitivity. This result was validated by addition of higher sublethal concentration of PHS to the sublethal concentration of AmB, and found that PHS alone enhances the AmB sensitivity. Thus, there is an elegant balance of physiological concentration of intermediate(s), is required for maintaining the AmB resistance. Nevertheless, the exact sphingolipid intermediates upstream to IPC, which upon reduction or accumulation leads to AmB sensitivity remains to be identified. Perhaps, PHS seems to be better intermediate candidate which could regulate AmB resistance. This hypothesis is based on the involvement of PHS in many crucial cellular functions (Abe et al., 2001; Dickson, 2008), of which maintenance of cell wall integrity (CWI) by PHS is directly connected with AmB resistance phenotype. There are several reports which have shown the role of cell wall in AmB resistance (Bahmed et al., 2002; Brajtburg et al., 1990; Ramanandraibe et al., 1998; Seo et al., 1999), moreover PHS maintained the CWI (Dickson, 2008), requiring the elucidation of this connection if any. Accumulation of several fold PHS upon deletion of *FEN1* and *SUR4* (Ejsing et al., 2009), further strengthens our speculation, because deletion strain of these genes were found to be AmB sensitive. However, their role in CWI needs to be further examined, to validate that AmB sensitivity phenotype is due to aberration in cell wall integrity by alteration in sphingolipids synthesis. Therefore, we perused in-depth the role of sphingolipid biosynthetic pathway genes in cell wall integrity maintenance.

To gain better insight into the role of sphingolipids in modulation of CWI, we first screened deletants of sphingolipid biosynthetic pathway genes with cell wall perturbing agents calcofluor white (CFW) and congo-red (CR). These agents are usually employed to identify cell wall defective strain(s), by their sensitive phenotype. Interestingly, only *ScFEN1* and *ScSUR4* deletion strains were found to be sensitive to both

CFW and CR, among tested deletants of sphingolipid biosynthetic pathway genes. Cell wall defect of these deletants was further validated by several experiments like zymolyase sensitivity assay, SDS sensitivity assay and visualization of high chitin deposition on defective cell wall as compared to parent strain. Deletion strains for *CaFEN1* and *CaFEN12* genes of *C. albicans* respectively were also tested with above mentioned cell wall perturbing agents (CFW, zymolyase and SDS) and found to be sensitive. Moreover, double deletion strain of these genes was hypersensitive to all tested chemicals. Chitin deposition was also highly enriched at cell wall as compared to the parent strain. It was interesting to observe that deletion strain of *ScFEN1* was more sensitive to CFW, CR and zymolyase than the *ScSUR4* deletant, and same was true for their deletants of *C. albicans* orthologs. Since, CFW, CR and zymolyase sensitivity is correlated with β-1,3-glucan level, cells having less β-1,3-glucan in cell wall will be more sensitive to these cell wall perturbing agents and vice-versa (Ram and Klis, 2006), suggesting that *ScFEN1* and *CaFEN1* deletants have less β-1,3-glucan as compared to *ScSUR4* and *CaFEN12* deletants. However, the result was just opposite for SDS sensitivity, where *ScSUR4* and *CaFEN12* deletants were more sensitive than *ScFEN1* and *CaFEN1* deletants. SDS, a detergent is normally used to determine the compactness the cell wall structure (Delgado-Silva et al., 2014; Richard et al., 2002). Since, deletants of only two genes of sphingolipid pathway were showing cell wall defect, we inhibited sphingolipid biosynthesis by sublethal concentration of myriocin and tested for CFW sensitivity in wild type strains of *S.cerevisaie* and *C. albicans*. Addition of myriocin leads to the CFW sensitivity phenotype, which was further reversed by PHS supplementation confirming that, alteration of sphingolipid synthesis mediates cell wall defect.

After confirming the cell wall defect of *FEN1* and *SUR4* deletants in both the yeasts, we extended our study to correlate the mechanism of AmB sensitivity and cell wall defect. We speculated that, if cell wall

defect is the primary cause of AmB sensitivity in *FEN1* and *SUR4* deletants, then deletants of cell wall biogenesis genes should show AmB sensitivity phenotype. So, we tested the deletants of important cell wall biogenesis genes *FKS1*, *KRE6* and *GAS1* for AmB sensitivity. It is well known that deletion of these genes lead to the cell wall defect (Garcia-Rodriguez et al., 2000; Lesage and Bussey, 2006). Deletants of these genes were found to be AmB sensitive as compared to the parent strain confirming our hypothesis that cell wall defect leads to AmB sensitivity. Alteration in cell wall structural compositions such as β-1,3-glucan, β-1,6-glucan, mannoproteins and chitin leads to the cell wall defect, which can disturb the osmotic homeostasis of the cells. Instability in osmotic balance of the cells is usually alleviated by supplementation of osmotic stabilizers (Levin, 2005). To check whether AmB sensitivity of *FKS1*, *KRE6* and *GAS1* deletion strains are associated with cell wall structural changes or osmotic variation or both, we tested AmB sensitivity in the presence of sorbitol, an osmotic stabilizer. Indeed, sorbitol rescued the osmotic instability (Levin, 2005)), but it failed to reverse the AmB sensitivity of these deletants, suggesting that only structural changes in the cell wall are responsible for AmB sensitive phenotype. Further to this, AmB sensitivity of *FEN1* and *SUR4* deletants was also tested in the presence of sorbitol for both the yeasts. Sorbitol did not reverse the AmB sensitivity of *ScFEN1* and *ScSUR4* deletants, which is similar to cell wall mutants. However, sorbitol partially alleviates the AmB sensitivity of single deletants of *CaFEN1* and *CaFEN12* genes but not their double deletant. The partial restoration of AmB sensitivity by sorbitol of single deletants suggested that, their AmB sensitivity phenotype is due to both cell wall structural as well as and osmotic alteration. Therefore sorbitol cannot fully rescue the AmB sensitivity. However, failure of partial AmB sensitivity restoration of double deletant by sorbitol could be speculated interms of their strong cell wall defect and AmB hypersensitivity phenotype. These studies clearly revealed that deletion of *FEN1* and *SUR4* of both

the yeasts leads to the cell wall defect and it resulting in AmB sensitive phenotype.

Since, *CaFEN1* and *CaFEN12* are uncharacterized genes, we wanted to explore their role in *C .albicans*. As we have shown, these are nonessential genes for viability, but their deletion lead to AmB sensitivity as well as defective cell wall phenotypes. We have consistently found that double deletion strain of *CaFEN1* and *CaFEN12* genes was showing growth defect on SC agar medium, while the growth of single deletants was comparable to the parent strain. So, to study the role of these genes in growth, growth of single and double deletants was monitored in YPD, a rich medium. Single deletants grew at the same rate as parent strain, the growth of double deletant strain was slow and its doubling time was increased by 25% as compared to the parent strain. The reason(s) for slow growth of *C. albicans* upon double deletion of *CaFEN1* and *CaFEN12* genes is not yet clear. However reason can be speculated by their *S. cerevisiae* orthologs, *FEN1* and *SUR4*, which show genetic redundancy and compensate for each other's loss (Revardel et al., 1995). One of the crucial factors of *C. albicans* virulence is transition from the yeast form to the hyphal form, which leads to adhesion and invasion into host tissues and often involve biofilm formation (Baillie and Douglas, 2000; Chandra et al., 2001; Douglas, 2002, 2003; Mukherjee and Chandra, 2004). To check the role of *CaFEN1* and *CaFEN12* in hyphal formation, we grow deletant strains of these genes under hypha-inducing condition (10% FBS) in solid medium. We found that strain deleted in both genes was unable to form hyphae and lacked invasive growth; however the hyphal and invasive growth of individual deleted gene strains was comparable to the parent strain. Hyphal growth is associated with biofilm development, which is a major challenge for candidiasis therapy because of their intrinsic antifungal resistance properties (Mukherjee and Chandra, 2004; Sardi et al., 2013). Since we found that double deleted strain was deficient in hyphal growth, we tested its ability to

form biofilm. Double deletant strain was found to be deficient in biofilm formation, but such a defect was not observed in single deletants. Subsequently, we tested the AmB susceptibility of these deletant strains in preformed biofilm as well as during biofilm formation. Though, the AmB sensitivity of single deletants was same as parent strain in preformed biofilm, however double deletant strain was found to be 8-fold more sensitive compared to the parent strain. Moreover, during biofilm forming condition, single deletant and double deletant strains were 2-fold and 8-fold respectively, more sensitive to AmB as compared to the parent strain. Together these data suggest that *CaFEN1* and *CaFEN12* genes are involved in hyphal growth, biofilm formation and AmB resistance in both preformed biofilm as well as during biofilm forming condition.

To conclude, this study has led to the identification of a novel role of sphingolipids and sphnigolipid biosynthetic genes *FEN1* and *SUR4* in AmB resistance. We also showed that myriocin interacts synergistically with amphotercin B in *C. albicans* (SC5314), *C. glabrata* (CG462), *C. lusitaniae* (CL6) and *S. cerevisiae* (FY4) and enhances the sensitivity of AmB resistant strains such as deletant strains of *ERG6* and *ERG2* genes. Myriocin treatment also sensitized cells to echinocandin class of antifungal which specifically inhibits β-1,3-glucan synthesizing enzymes encoded by *FKS1* and *FKS2* genes (Healey et al., 2012), confirming the role of sphingolipids in modulation of antifungal resistance. Since, myriocin is an immunosuppressor and quite toxic to humans, non-toxic analogs of myriocin, if developed, can be used to sensitize pathogenic fungi to these antifungals, thereby enhancing their therapeutic efficacy. We first time characterized the *FEN1* and *SUR4* genes orthologs in *C. albicans* *CaFEN1* and *CaFEN12* respectively and found that these are nonessential genes for viability and involved in hyphal growth and biofilm formation. Apart from AmB sensitivity, we also showed that deletion of *FEN1* and *SUR4* genes in both *S. cerevisiae* and *C. albicans* leads to the cell defect and AmB sensitivity.