SUMMARY

There is a global increase in the incidence of *C. albicans* infections. This has been primarily due to a steep rise in the number of immunocompromised patients, extensive invasive surgical procedures, nosocomial acquired infections and an escalation of patients undergoing immunosuppresive therapies. But the above medical problem is complicated by the use of the currently existing limited antifungal agents that exhibit toxicity and severe side effects on the host. Over the last two decades, this problem is further augmented by the emergence and dominance of clinical strains that are resistant to antifungal drugs. Therefore, there is an urgent need to identify more drugs targets and develop new drugs with novel mechanisms of action. It is enviasged that gene products that are essential for growth and/or required for virulence would serve as ideal drug targets against this opportunistic pathogen.

Identification of genes essential for growth and virulence is cumbersome owing to the limitations associated with *C. albicans* genetics. Since this yeast exists as an obligate diploid under natural conditions, both the alleles of a gene have to be disrupted to obtain a functionally mutant phenotype. Multiple rounds of disruption in turn potentially increases the incidence of unwanted genetic changes such as trisomy, chromosome abnormalities or transformation induced mutations. Moreover, the inability of *C. albicans* to sporulate after forced mating in the laboratory has contributed to the lack of a direct method to determine essentiality of genes in this organism. Other limitations needed to be overcome in order to generate functional mutants in *C. albicans* is the low transformation efficiency coupled with a high frequency of non-homologous integration of DNA at non-targeted loci. It also has a non-canonical codon usage where CUG codes for serine instead of leucine so that it becomes necessary for heterologous genes to comply with the *C. albicans* genetic code before it can be expressed in this organism. Hence complementation analysis of homologous genes from related organisms such as *S. cerevisiae* is also not straightforward.

The primary objective of this study was to develop molecular genetic tools that would overcome some of the above limitations and aid in faster and easier identification of genes

important for survival and virulence of this opportunistic pathogen. The overall strategy was to get a phenotype only after a single transformation. Initially, an antisense approach was examined. Efficacy of antisense approach using regulatable and strong, constitutive promoters was tested under in vitro as well as in vivo conditions. This was accompanied by the development of transposons that were adapted to function in *C. albicans* for large scale identification of essential and virulence genes, taking advantage of haploinsufficiency and antisense phenomena. The alternative approach taken was to develop modular cassettes for deletion/regulated expression of both alleles of a gene after a single transformation. The above cassettes were used for *in vitro* and *in vivo* studies to verify their efficacy in the identification of genes crucial for survival and virulence, respectively.

Antisense phenomenon was initially studied *in vitro* using regulatable promoters, such as P_{MAL2}, P_{MET3} and P_{SAP2} at *ADE2* and *RHO1* locus. Heterozygous mutants generated with the promoters in antisense as well as in sense orientations were used to study antisense effect. Neither of the mutants exhibited any discernable phenotype on solid media under either promoter inducing or repressing conditions. Hence, all further studies were done in liquid broth cultures to check for subtle effects on growth. A transient slow growth was indeed observed in case of antisense heterozygous mutants at *ADE2* locus with all the three regulatable promoters tested. This transient antisense effect was, however, not observed at the *RHO1* locus. Instead, a transient slow growth was observed for both sense as well as antisense mutants, but only under promoter inducing conditions. Hence this was a phenomenon distinct from antisense effect. However, *SAP5* promoter induced antisense effect was not observed with the antisense heterozygous mutants of FAS2, a gene important for virulence, using a mice model of systemic candidiasis. When the effect of strong, constitutive *TDH1* and *TEF1* promoters was checked at *ADE2* locus in vitro, a more pronounced but transient antisense effect was observed. Though growth defect based on antisense effect was not prominent, different transposons were constructed with *C. albicans HIS1* marker gene that had the promoters *SAP2, SAP5, TDH1* or *TEF1* facing outwards from one of the termini of the transposon. This was done with the intention that they would serve as tools for large scale identification of essential and virulence genes based on phenotype contributed by haploinsufficiency phenomenon and/or antisense effect.

A more promising approach to study genes for their essentiality and virulence is if both alleles can be deleted or brought under the control of a regulatable promoter with ease, such as by a single round of transformation. The presence/absence of phenotype when the regulatable promoter is induced as opposed to when it is shut down would clearly indicate the role of the regulated gene in contributing to the phenotype. The effect of unintended changes elsewhere in the genome contributing to the phenotype can in this case be ruled out. To achieve this, a set of new modular cassettes named *HAH1/HAH1*-RP, modeled upon *UAU1* cassette, were developed, but by employing auxotrophic markers *HIS1* and *ARG4* that are not implicated in virulence. Moreover, *HAH1*-RP cassettes had the regulatable promoters P_{MAL2}, P_{MET3} or P_{TET} placed downstream of the *HAH1* cassette so that they can be integrated immediately upstream of the the coding sequence of the target gene to bring the latter under the control of the regulatable promoter.

It was found that *HAH1* cassette could efficiently bring about deletion of both alleles of *ADE2* gene after a single round of transformation. They were selected as His^+ Arg^+ segregants by outgrowing His^+ targeted integrants in rich medium. *HAH1*-RP cassettes could also effectively place both alleles of the target gene under the control of regulatable promoters, selected as His^+ Arg^+ segregants and obtained at a rate of ~3×10^{-9} cells per generation. The above cassettes helped in testing the essentiality of target genes. Test genes, such as *RHO1, PMA1* and *FAS1*, brought under the control of P_{MAL2}, P_{MET3} and/or P_{TET}, gave rise to double segregants that were dependent upon promoter activation for growth, thereby confirming the essentiality of these genes. However, many double segregants also arose that had wild type copy of the gene (as evident with white colonies as opposed to red colonies of *ADE2* double segregants as well as by PCR for detection of intact, wild type copy under endogenous promoter). These double segregants showing trisomy for the target gene were not dependent on the promoter for its growth. Such segregants were more prominent in case of *HAH1*-P_{TET} (>98%) in comparison to *HAH1*-P_{MAL2} and *HAH1*-P_{MET3} (<30%) cassettes. This indicated that P_{TET} was weaker in comparison to P_{MAL2} and P_{MET3}, also considering that double segregants without a third intact copy of the gene arose at a much higher frequency (>60%) from *HAH1*-P_{TET} when the target gene was non-essential, as in case of *CHK1* and

ICL1. It was also observed that the double segregants of *RHO1*, *FAS1* and *PMA1* under the control of P_{MAL2}, P_{MET3} and P_{TET}, respectively, showed a cidal rather than static effect when the promoter was shut off, thus behaving like null mutants under these conditions.

The regulatable promoters used in *HAH1*-RP cassettes, in particular P_{MET3} and P_{TET}, were also found to be shut off in vivo during infection of mice by *Candida* cells. These cassettes can hence be used to study cidal effect of essential genes and its ability to survive under in vivo conditions. This is an important aspect to be taken into consideration since recent reports suggest that genes essential in vitro need not necessarily be indispensable in vivo considering the disparity in their external milieu. It was also found that efficacy of regulation of P_{TET} in vivo by doxycycline was dependent on target gene expression level. But it may be possible to improve upon *TET* promoter based gene activation by modifying the tetracycline transactivator so that it can be used as a a generalised tool to regulate gene expression in vivo. It is envisaged that these cassettes can also be made amenable to large scale genetic analysis by introducing them as part of transposons for easy, rapid and routine screening of genes essential for survival and virulence, thus contributing to the hastening of drug target identification and validation.