Amit Kumar Yadav (2011). ***Studies on the sulphur assimilatory pathways of candida glabrata.*** **Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.**

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SUMMARY OF THE THESIS

Yeast and fungal infections in humans are an increasing source of concern, worldwide. Among the most frequently isolated species are *C. albicans* and *C. glabrata* (Schuman et al., 1998). *C. glabrata* is an opportunistic yeast pathogen which mainly affects immunocompromised patients and is found in the vaginal mucosa, skin and blood (Fidel *et al*, 1999). Phylogenetically *C. glabrata* is much closer to *S. cerevisiae* than to *C. albicans* despite inhabiting a different ecological niche (Kaur *et al,* 2005).

Sulphur is an essential nutrient of all living cells. However no information is available on how *C. glabrata* acquires its sulphur requirement. Sulphur assimilatory pathways have been studied in detail over the years in the yeast *S. cerevisiae* and to a lesser extent in the fission yeast *S. pombe* and the fungi *Neurospora crassa* and *Aspergillus nidulans*. These yeasts and fungi can fulfill their sulphur requirements either through the reduction of inorganic sulphate, or alternatively through the direct uptake and assimilation of reduced sulphur (such as cysteine, methionine, or the thiol containing tripeptide, glutathione). In the reductive assimilation of sulphate, first sulphate is taken up in the cells and then reduced by a series of enzymatic steps to sulphide followed by its incorporation into either homocysteine (via the enzyme o-acetyl homoserine sulphydrylase OAH-SH), or alternatively into cysteine (by combining o-acetyl serine and sulphide through the action of o-acetyl serine sulphydrylase (OAS-SH) or cysteine synthase). In *S. cerevisiae* only the OAH-SH pathway operates while in other yeasts the latter or both pathways have been found to be present. In addition to inorganic sulphate, yeasts can also utilize several other organic sulphur sources like methionine, glutathione, cysteine and homocysteine. These sulphur sources are taken up by specific transporters, and are then converted to the different sulphur compounds through the forward and reverse transulphuration pathways.

In this thesis I have initiated studies on the status of the sulphur assimilatory pathways in *C. glabrata*. To examine the sulphur assimilatory pathways in *C. glabrata*, we initially carried out an *in silico* analysis of orthologue identification where sulphur pathway proteins of *S. cerevisiae* were retrieved and their orthologues sought in the *C. glabrata* genome. Most of the sulphur assimilatory pathways were found to be similar to that of *S. cerevisiae.* Inorganic sulphur assimilation enzymes involving the OAH-SH pathway were found to be present in *C. glabrata* while the alternate cysteine synthase pathway was found to be absent similar to what is seen in *S. cerevisiae*. Both forward and reverse transsulphuration pathway orthologues could also be seen in *C. glabrata*. However, from the *in silico* predictions we

observed that redundancies were absent in the genome of *C. glabrata*, corresponding to the *SUL1* and *SUL2* sulphate transporters of *S. cerevisiae* only one orthologue could be found in *C. glabrata*. Similarly, the *MET31* and *MET 32* are homologus transcription factors of *S. cerevisiae* but in *C. glabrata* only one orthologue (*MET31*) could be found. These absences were reflective of the slimmer genome of *C. glabrata* (*C. glabrata* has 5283 ORFs while *S. cerevisiae* has 6200 ORFs). We also observed the absence of orthologues in *C. glabrata* of the *S. cerevisiae* high affinity cysteine transporter (*YCT1*) and high affinity glutathione transporter (*HGT1*). This suggested that perhaps cysteine utilization and glutathione may be defective in *C. glabrata*.

To validate these *in silico* pathway predictions we created a *met15*Δ deletion in *C. glabrata*. *C. glabrata met15* Δ strain was found to be a strict organic sulphur auxotroph like *S. cerevisiae* and it grew well on methionine or cysteine as the sole sulphur source thus showing that *C. glabrata* has both the forward and reverse transsulphuration pathways. When we examined the growth of *C. glabrata met15*Δ strain on low concentrations of cysteine we observed that *C. glabrata* could grow on low concentrations of cysteine (50μM) despite lacking a high affinity cysteine transporter (*YCT1*) orthologue suggesting that *C. glabrata* may have evolved some other high affinity cysteine transporter. When we evaluated the *C. glabrata met15Δ* strains for growth on glutathione we observed that they were unable to utilize glutathione. To examine if this was due to the absence of the orthologue of the *S. cerevisiae* glutathione transporter (*HGT1*) we introduced *S. cerevisiae HGT1* in *C. glabrata* and growth on glutathione was restored. In *S. cerevisiae* the glutathione transporter (*HGT1*) and the DUG pathway are both required for glutathione degradation. The DUG pathway is a recently identified alternate pathway for glutathione degradation and involves three proteins Dug1p, Dug2p and Dug3p. In spite of losing the glutathione transporter it was interesting to observe that the *C. glabrata* genome encodes the DUG proteins. To examine the functionality of the DUG pathway in *C. glabrata* we deleted one of the genes of the DUG pathway *CgDUG3,* and found that it disrupted growth on glutathione. These findings suggested that the DUG pathway was functional and that in addition to exogenous glutathione, the DUG pathway also has a likely role in intracellular glutathione homoeostasis. As glutathione is an essential metabolite in eukaryotes and *C. glabrata* lacks a glutathione transporter, endogenous glutathione biosynthesis becomes the sole source of glutathione. It was therefore of interest to know whether glutathione biosynthesis was essential in *C. glabrata*. To examine the essentiality of GSH biosynthesis we evaluated the effect of *CgGSH1* deletion in *C.*

glabrata by using plasmid shuffling. We observed that when the glutathione biosynthetic enzyme, *CgGSH1* was deleted in *C. glabrata*, the cells did not survive, suggesting that the enzyme is essential for survival in *C. glabrata*.

The lack of glutathione utilization in *C. glabrata* prompted us to examine growth on cystine (the oxidized form of cysteine), since cystine is more abundant in blood. We observed that *C. glabrata met15*Δ strains utilized cystine as a sulphur source while *S. cerevisiae met15*Δ strains lacked such ability. To elucidate the mechanism of cystine utilization in *C. glabrata* and to find the proteins responsible we employed a strategy where we searched for a membrane transporter of unknown function in *C. glabrata* lacking an orthologue in *S. cerevisiae*. Two such membrane proteins of *C. glabrata* which were unique to *C. glabrata* (*CAGL0I08613g* and *CAGL0F00209g*) and were absent in *S. cerevisiae* were identified. However complementation and deletion analysis failed to indicate a role for these ORFs in cystine utilization.

Subsequently, using a genomic library complementation approach an ORF *CAGL0M00154g* was identified complementing *S. cerevisiae met15*Δ strain for growth on cystine. ORF *CAGL0M00154g* belongs to the amino acid permease family of transporters and has 12 predicted transmembrane domains. Deletion of this ORF in *C. glabrata met15*Δ strain led to a null growth phenotype on cystine which was rescued when ORF *CAGL0M00154g* was reintroduced back. We renamed ORF *CAGL0M00154g* as *CgCYN1* (Cystine transporter) after confirmation by uptake experiments. To determine the kinetic parameters for *CgCYN1* we carried out the radioactive uptake experiments in a *S. cerevisiae yct1*Δ*met15*Δ strain because the radioactive cystine had a contamination of cysteine. From the uptake studies we determined a K_m of 18 μM for *CgCYN1* indicating that it is a high affinity transporter. From the inhibitor studies we could conclude that *CgCYN1* is a cystine specific transporter as the transport was found to be inhibited by cystine or other compounds which were structurally similar to cystine like cystathionine, selenocystine and lanthionine. In addition to these studies the transporter was also found to energy dependent.

Localization studies with *CgCYN1* revealed it to be localized to the plasma membrane. Orthologues of *CgCYN1* appeared to be present in other pathogenic yeasts like *C. albicans* and *H. capsulatum* (*CaCYN1* and *HcCYN1*). We cloned and expressed these ORFs in *S. cerevisiae met15*Δ strain and found them to complement in *S. cerevisiae met15*Δ strain for growth on cystine. Furthermore we also deleted both alleles of *CaCYN1* in *C. albicans met15*Δ strain and growth abrogation could be seen on cystine. However, when the apparent

S. pombe orthologue of *CgCYN1* was cloned and expressed in *S. cerevisiae met15Δ* strain it failed to complement for growth on cystine. Furthermore the *S. pombe* cysteine auxotroph *cys1aΔ* strain could not utilize cystine as a sulphur source. The prevalence of *CgCYN1* homologues among pathogenic yeasts and its absence among non-pathogenic yeasts prompted us to study its role in virulence. *CaCYN1* was deleted in *C. albicans* wild-type strain and virulence studies were done in a mice model of disseminated candidiasis. However no difference could be found between the virulence attributes of the wild type and mutant strains (*Cacyn1Δ* strain) suggesting the absence of a role in virulence in this model of Candidiasis.

In conclusion, the studies described in this thesis on *C. glabrata* sulphur pathways for the first time shed light on the status of sulphur assimilatory pathways in *C. glabrata.* Our studies revealed several aspects of *C. glabrata* sulphur metabolism. The *in silico* studies revealed assimilatory pathways very similar to *S. cerevisiae* that were also validated experimentally. *C. glabrata* however lacked the ability to utilize glutathione owing to the lack of a glutathione transporter and glutathione biosynthesis was found to be essential in this yeast. In addition we have described a high affinity plasma membrane cystine transporter of *C. glabrata* which represents the first plasma membrane cystine transporter of yeasts. The transporter was also shown to be prevalent among pathogenic yeasts, *C. glabrata* thus seems to have evolved several unique features of sulphur metabolism that are likely to play a key role in its natural habitat.