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SUMMARY OF THE THESIS

Debaryomyces hansenii is one of the most halotolerant and osmotolerant species of yeast. In *S. cerevisiae*, a signal transduction pathway involving the members of mitogen activated protein (MAP) kinase family i.e. high osmolarity glycerol (HOG) response pathway is responsible for adaptation to high osmolarity. Molecular genetic analysis in the yeast *S. cerevisiae* has identified several components of this pathway. However, not much information is available with regard to osmosensing signal transduction pathway in xerotolerant yeast *D. hansenii*. Earlier studies from our laboratory have established the presence of the HOG pathway in this species. The present study, therefore, aims towards the identification and characterization of other components, mainly osmosensors of the HOG pathway in *D. hansenii*.

Bioinformatics analysis of *D. hansenii* genome revealed the presence of the three putative osmosensores; two of them are homologues of *SLN1* (a hybrid histidine kinase) and *SHO1* (a transmembrane protein), the osmosensors present in *S. cerevisiae*. The third one was homologue of *NIK1*, a hybrid histidine kinase (HHK) which was originally identified as osmosensor in *N. crassa*. Interestingly, *NIK1* homologue is absent in *S. cerevisiae*. The present study deals with the identification and characterization of these osmosensors from the highly osmotolerant yeast *D. hansenii*.

One of the putative osmosensor *DhNIK1* was cloned and checked for complementation of *sln1* deletion in *S. cerevisiae* strain RJ1428. The lethality of *sln1* deletion in RJ1428 was suppressed by the presence of *PTP2* (a phosphatase) gene from a *URA3* based multi-copy plasmid. This strain therefore cannot grow on 5FOA plate. However, the introduction of a *DhNIK1*construct was able to support its viability on media containing 5-FOA. Additionally, functional complementation of *DhNIK1* was also checked in an *S. cerevisiae* strain TM299 carrying temperature sensitive allele of *SLN1 (sln1-ts)*. Therefore it can grow at permissive temperature (28°C) but not at high temperature (37°C). Interestingly, the introduction of *DhNIK1* construct in TM229 supported the growth at non-permissive temperature (37°C). *NIK1* orthologs from yeast and fungi have been assumed to be cytoplasmic proteins; however, this was not validated experimentally till date. To fill this gap, DhNik1p was fused with the GFP and the fusion construct was transformed both in *S. cerevisiae* and

D. hansenii. In these transformants, the GFP fluorescence was detected throughout the cytosol, thereby demonstrating, for the first time, that DhNik1p or its orthologues were indeed cytosolic protein. DhNik1p was a HHK as it contained both histidine kinase and receiver domains and the sequence alignment showed that H500 and D915 were the putative residues involved in the transfer of phosphate group from the kinase to the receiver domain. To probe the role of these residues two mutants H500Q and D915R were made. Inability of these two to complement *sln1* mutation in *S. cerevisiae,* verified their essential role in the functionality of DhNik1p. However, when these two mutants were transformed together in to TM229, the transformants could grow at non-permissive temperature. Therefore, like in a typical HHK, the phosphotransfer from H500 to D915 could occur *in trans*. Furthermore, bimolecular fluorescence complementation assay also demonstrated that DhNik1p existed as dimmer in vivo. To demonstrate that DhNik1p was an active kinase, the recombinant DhNik1p was purified and its activity was measured by using a non-radioactive luciferase based kinase assay. Taken together, these results clearly established that DhNik1p was an authentic HHK.

In *S. cerevisiae,* Hog1p is activated by two independent upstream branches. One is SLN1 branch involving redundant MAPKKK *SSK2/SSK22* while the other SHO1 branch acts through the promiscuous MAPKKK *STE11*. In order to check whether DhNik1p could activate HOG pathway in *S. cerevisiae* strain TM229 upon hyper osmotic shock, it was required to block the activation through Ste11p. Therefore *STE11* was deleted in TM299 by homologous recombination. The disruption was confirmed by PCR and the strain was named as NM2. Further, *DhNIK1* construct was introduced in to the NM2 strain and checked for Hog1 phosphorylation by western blotting under non permissive temperature. Interestingly, when the log phase cells were shifted from 28°C (permissive) to 37°C (non-permissive) temperature, Hog1p phosphorylation did not appear till 6 hr, while in contrast, in NM2 strain with vector only, the phosphorylation was detected even in 5 min. This result clearly demonstrated that DhNik1p could suppress inappropriate activation of Hog1p in absence of Sln1p. Furthermore, like Sln1p, DhNik1p could also negatively regulate the HOG pathway.

Next it was determined whether DhNik1p could respond to the high osmolarity stress by activating Hog1p. For this, *S. cerevisiae* strain NM2 expressing

DhNik1p was exposed 0.4 M NaCl at 37°C stress and Hog1p phosphorylation was detected by immunoblotting. Within 5 min of the application of the osmostress, the phosphorylated Hog1p appeared in these cells and it remained so even after sixty minutes. This was in contrast to the transient activation of Hog1p that was normally observed by exposing *S. cerevisiae* to 0.4M NaCl. It was presumed that the application of higher temperature and osmolarity simultaneously caused severe stress to the cells and this ultimately resulted in sustained Hog1p activation. Alternatively, DhNik1p could be requiring longer time to return to the active form. To be a bona fide osmosensor, DhNik1p should be reversibly activated upon withdrawal of the stress conditions. To test this, down shock experiment was conducted. The disappearance of phosphorylated Hog1p within 15 minutes of down shifting to SD medium clearly showed that DhNik1p functioned as an authentic osmosensor.

To study the function of DhNik1p in its native host i.e. *D. hansenii*, *DhNIK1* was disrupted by homologous recombination. The disruption was confirmed by PCR and western blotting by anti-DhNik1p antibody (raised in rabbit for this study). Since DhNik1p is an osmosensor, the growth of Δ*DhNIK1* was checked in presence of different osmolytes. No difference in growth could be observed in comparison to wild type strain of *D. hansenii*. Analysis of *D. hansenii* genome showed that both Sho1 and Sln1 orthogs were also present in this species. These pathways could be regulating HOG pathway independently and therefore Δ*DhNIK1* did not exhibit any osmosensitivity. Thus, additional disruptants are required to study the function of DhNik1p in *D. hansenii*.

DhNik1p had a unique N-terminal region consisting of five HAMP domain repeats. The presence of HAMP domain repeats is a characteristic feature of the group III HHK. HAMP domains are one of the most frequently reported domains associated with signalling proteins. However, there is limited knowledge regarding the role of these modules in signal transduction systems. Therefore the role of HAMP domain repeats in the functionality of DhNik1p was studied. Two lines of evidences indicated that they may be important for osmosensing by DhNik1p. Firstly, several osmo sensitive and fungicide resistant mutants were identified in earlier studies that carried point mutations in HAMP domain repeat in Nik1 of *N. crassa*. Secondly, it was observed that a chimeric construct where the N-terminal non kinase region (which was essential for its membrane localization and osmosensing) of DhSln1p (a Sln1p ortholog in *D. hansenii*) was swapped with the N-terminal region of DhNik1p

containing HAMP repeats could also complement the *sln1* mutation in *S. cerevisiae.* To characterize the role of HAMP domain in the functionality of DhNik1p, several mutants were created either by shuffling their positions or by deleting these domains systematically. The results obtained from the analysis of these mutants provided a novel insight into the functionality of DhNik1p which is presented as a model in Fig. 4.16. Under ambient osmolarity, the HAMP1, HAMP2 and HAMP3 domains held back HAMP4 from HAMP5 thereby leaving it free to form active kinase possibly upon dimerization. All the three domains appeared to be essential for this as deletion of any one of them (ΔH1, ΔH2 and ΔH3) led to a non-functional kinase.

The results with the mutant ΔH1-4 and ΔH4 corroborate well with this model as it posits that the absence of HAMP4 will form a kinase unresponsive to the changes in the external osmolarity. Thus, the alternative interactions among the HAMP domains, which are sensitive to changes in external osmolarity, creates an 'On-Off' switch in DhNik1p. In case of *N. crassa,* null mutation in *NIK1* (os-1) confers high level of resistance to fludioxonil where as point mutations in the fifth HAMP repeat resulted in low level of drug resistance but high level of osmosensitivity (Ochiai *et al,* 2001; Miller *et al,* 2002). Similar studies with *Botryotinia fuckeliana* (Cui et al, 2002) and *Cochliobolus heterostrophus* (Yoshimi et al, 2004) also identified point mutations in different HAMP domain repeats that exhibited varying degree of drug resistance and osmosensitivity. The structural insights about the HAMP domain repeats in these orthologs are required to understand these interesting mutants. Moreover, this information will definitely aid in developing novel fungicides in future.

HAMP domains are one of the very common structural modules associated with the signaling molecules from prokaryotes and lower eukaryotes. Our current knowledge about this domain is based on studying few transmembrane receptor histidine kinases of prokaryotic origin which possess single HAMP domain. In these examples, the HAMP domain mainly serves as a transducing module that inter-converts the mechanical signals from the transmembrane domain to the kinase module. The present study thus provides a novel paradigm about the functioning of the HAMP domains in proteins having multiple repeats of this domain.

Besides DhNik1p, in the present study characterization of other putative osmosensors *DhSLN1* and *DhSHO1* present in *D. hansenii* were also undertaken. *DhSLN1* was cloned and transformed into RJ1428. The viability of the transformants

was checked on 5-FOA plate. Like DhNik1p, the expression of *DhSLN1* could complement the *sln1* deletion in *S. cerevisiae*. Further, it was also established that DhSln1p was genuine hybrid histidine kinase since the mutation in conserved histidine (H496Q) and aspartate (D1153R) residues in the histidine kinase and receiver domains respectively, failed to complement the *sln1* function in *S. cerevisiae*. C-terminal half of *DhSLN1* comprising of the kinase and receiver domains showed high homology with that of the *ScSLN1* whereas in the N-terminal half of these two proteins there were considerable variation in primary sequence as well as in terms of the number of putative tans-membrane domains and presence of a intracellular region. In order to know the function of the N-terminus part of DhSln1p, a chimeric protein was made where the C-terminal region (comprised of histidine kinase and receiver domain) of DhSln1p was swapped with the C-terminal region of DhNik1p containing histidine kinase and receiver domain. Interestingly, the chimera thus generated could complement the *sln1* deletion in *S. cerevisiae* indicating that the N-terminus region of DhSln1p was important for functionality.

To dissect out the role of different domains predicted in the N-terminal region of DhSln1p, several deletion constructs were made. Firstly, individual transmembrane domain deletion constructs were generated and the activities of these constructs were checked *in vivo*. Interestingly, none of these constructs was active suggesting that all the predicted transmembrane domains are indispensable for the kinase function. However, our results of transmembrane deletions deviate from the previous findings with the homologous Sln1p from *S. cerevisiae* which possess two transmembrane domains. While the TMD1 deletion construct of ScSln1p is a constitutive kinase, the deletion of TMD2 inactivates the protein (Ostrander and Gorman, 1999).

The deletion study of ECD of ScSln1p has established its role in osmosensing and it has been found to be required for the dimerization (Ostrander and Gorman, 1999). In a separate study Reiser *et al* (2003) showed that the ECD is crucial for the kinase activity. Analysis of deletion mutants and chimeric constructs identified a stretch of the 13 amino acids in ECD of ScSln1p that was essential for function. DhSln1p was also predicted to have one extracellular domain (ECD) and therefore we also checked for the function of the ECD in DhSln1p. For this, a construct devoid of ECD was made and checked by phenotypic complementation study. Corroborating with the earlier finding from ScSln1p, the deletion of extracellular domain led to a loss in functionality of the protein. To further demonstrate the role of the ECD

another construct was generated, wherein both TMD1 and ICD were deleted yielding a protein with two TMD and one ECD; a topology similar to that of ScSln1p. Surprisingly, this construct was unable to complement the *sln1* deletion in *S. cerevisiae* but nevertheless, it again bolstered our earlier result that showed the essentiality of TMD1.

DhSln1p was also predicted to have an intracellular domain (ICD), flanked by TMD1 and TMD2. Since ScSln1p does not show the presence of this domain, it was tempting to envisage the function of the ICD. For this, an ICD deletion construct was generated and tested for the functionality. To our surprise, this construct was active as full length DhSln1p indicating that the intracellular domain is dispensable for the kinase function. Nevertheless, it could be argued that this domain could have role in sensing and possibly the regulation of the protein activity by changing the conformation of the protein from active to inactive form. All these possibilities need to be addressed systematically.

Finally, DhSho1p was indentified and predicted domain architecture revealed that its high similarity with ScSho1p. Functional complementation study clearly established that the DhSho1p complements the *sho1* deletion in *S. cerevisiae.* Furthermore, to know whether *DhSHO1* responded to the high osmolarity, the hog1 phosphorylation was monitored after exposing the cells to high osmolarity conditions (0.5 M NaCl). The western analysis showed that the hog1p can be detected within 5 minutes of salt shock and it remained up to 30 minutes. This result clearly proved that DhSho1p possibly acts as an osmosensor protein and complements the *sho1* function in *S. cerevisiae.*

Summing up, the current study establishes the presence of three putative osmosensors (DhSln1p, DhSho1p and DhNik1p) in *D. hansenii.* Among them DhSln1p and DhSho1p appeared to be membrane bound sensor whereas DhNik1p was present in the cytosol. Moreover, the present study not only illustrated the role of HAMP domain of DhNik1p in osmosensing but also showed how the alternative interactions among these domains could create an osmo-sensitive molecular switch to regulate the activity of DhNik1p.