The yeast *Debaryomyces hansenii* that has been isolated from saline environments such as seawater and concentrated brines, considered to be one of the most halotolerant and osmotolerant species of yeast. This species can grow in media containing 4 M NaCl, whereas growth of *Saccharomyces cerevisiae* is limited in media having more than 1.7 M NaCl. When exposed to high osmolar media, *D. hansenii* (like other yeast species) achieves osmotic balance by producing and accumulating primarily glycerol and, to some extent, arabitol as compatible solutes. However, it is not yet understood how *D. hansenii* senses osmotic stress and modulates glycerol production. Recently, an osmosensing signal transduction pathway has been identified in *S. cerevisiae*. A MAP kinase called *HOG1*, plays a central role in this pathway. Presence of such gene (s) in *D. hansenii* is not known. Thus, we attempted to identify and characterize homologous gene from this species of yeast as this may offer some clues regarding high osmotolerance and halotolerance in this yeast.

By low stringency Southern blot, we could initially detect presence of *HOG1* homologue in *D. hansenii*. In order to clone such gene, we looked for phenotypic complementation of *S.cerevisiae hog1* mutant by utilizing a *D. hansenii* genomic library constructed in yeast shuttle vector pRS425. Our screening effort resulted two plasmid clone pBH71 and pBH81. The osmoresistant phenotype was shown to be plasmid linked by retransformation of *S. cerevisiae* strain PB28. Restriction digestions of the plasmids pBH71 and pBH81 revealed that they could possibly be identical, therefore all further work was carried out with plasmid pBH81. Plasmid pBH81 was found to contain an insert of 6.5 kb length. A restriction map of the insert was carried out to localize the gene. In order to reduce the size of the fragment, different subcloning experiments were undertaken. It showed that a 2.5 kb *Hind*III-*Eco*RI fragment was capable of imparting osmoresistance phenotype.

To determine that the cloned fragment is a *HOG1* homologue, not an extragenic suppressor, it was subcloned into a yeast centromeric vector YCplac111 (Gietz RD and Sugino A, 1988). The yeast strain PB28 (*hog1Δ*) when transformed with putative *DHOG1* gene cloned in centromeric vector (pBH43) or in multi-copy vector (pBH42) showed comparable growth on various plates containing different osmolytes (Fig. 3.14).

Thus, the functional complementation observed during screening of the genomic library did not arise out of a multi-copy suppressor phenomenon. Increased accumulation of glycerol upon exposure to high osmolar media is a hallmark of *S. cerevisiae* osmoadaptive response and this is mediated through Hog1p. In *S. cerevisiae* strain PB28 harboring *hog1*Δ mutation, the enhancement of intracellular glycerol content during osmoadaptation was much lower than the parent strain whereas PB28/pBH42 (YBH42) and PB28/pBH43 (YBH43) strains when cultured in presence of 0.4 M NaCl showed increased accumulation in intracellular glycerol. Taken together these results suggested that this clone is capable of imparting full functional complementation of *S. cerevisiae* *hog1*Δ mutation, therefore could be a *D. hansenii HOG1* homologue and hence named *DHOG1*

Northern blot analyses were performed in order to determine whether expression of the *DHOG1* gene in *D. hansenii* is modulated by change in osmolarity of the media. Total RNA was isolated from cultures grown at various length of time in the media containing 1M NaCl and hybridized with a *DHOG1* probe. A single transcript of about 1.6 kb was detected in all samples (Fig. 4) and the size of the transcript correlated well with that of open reading frame identified. When the level of *DHOG1* mRNA expression was normalized with that of actin mRNA level, no significant difference in steady-state level was observed. Thus, our results suggest that the gene *DHOG1* is constitutively expressed in *D. hansenii.*

From the nucleotide sequence of the clone, an open reading frame of 1161 bp (encoding 387 amino acids) was identified. Comparison of sequences with previously identified *HOG1* homologous from different yeasts using CLUSTAL W program revealed that this sequence is homologous to Hog1p of *S. cerevisiae,* CaHog1p of *C. albicans*, Sty1p of *S. pombe.* The DHog1p also shows sequence similarity with MAP kinases from higher eukaryotes such as *Homo sapiens* Csbp1p or Csbp2p and *Xenopus laevis* Mpk2p. A TGY motif, characteristics of hyperosmolarity activated MAP kinases, is found at amino acids 174-176 in *D. hansenii* Hog1p. Similar motifs have been found to be present in homologous genes isolated from other yeast species, and also in MAP kinases from higher eukaryotes which have been shown to complement *S. cerevisiae*

*hog1*Δ mutants. Thus, we have cloned a *D. hansenii* gene that can complement *HOG1* function in *S. cerevisiae*. However, this clone did not improve salt tolerance in wild type *S. cerevisiae*. Thus, it appears that the high halotolerance of *D. hansenii* lies beyond osmosensing pathway. It could be possible that the proteins that are activated by this pathway might be directly responsible. Therefore, identification of these molecules could provide an insight of the mechanism of high halotolerance in *D. hansenii*.

Sudden exposure to high osmolar media results in the differential pattern of gene expression. Reprogramming of gene activity leads to the induction of several genes which are necessary to overcome the stress. In order to identify such genes and delineate their role in osmoloterance, a sensitive methodology, "cDNA Differential display" was utilized. Total RNA was isolated from the *S.cerevisiae* W303-1B induced and uninduced cultures with 0.5M NaCl for one hour. Several bands with varying intensity were detected in salt induced and uninduced samples. Among these two bands *sr1* and *sr2* were excised from the salt induced samples and re-amplified. Subsequently sr1 (0.3kb) and sr2(0.25 kb) were cloned into pGEM7Zf(+) vectors.

As yeast genome is fully sequenced, the partial sequencing of these fragment would be sufficient to know the complete sequence of the corresponding gene or ORF. Data base search showed that sr*1* sequence matched with ORF YMR291w and sr2 matched with ORF YOR086c. YPD report of these ORFs indicates that so far functions of these ORFs are not known. However, ORF YMR291 is a putative serine-threonine kinases. For further characterization these ORFs were clone by PCR and corresponding gene were named *SSR1* and *SSR2*.

Northern hybridization was carried out using these DNA as probes with RNA isolate from *S. cerevisiae* strain W303-1B that was induced with 0.5 M NaCl.. Induction of approx. four times in *SSR1* and three times were observed in *SSR2*, in 30 min, when the hybridization signal were compared with uninduced samples. By analysis of nucleotide sequence (retrived from data base) STRE element was detected in promoter region of both the genes *SSR1* and *SSR2*. STRE motifs are characteristics of genes

involved in adaptation to stress conditions. Three such elements (CCCCT or AGGGG) were detected in *SSR1* while, one such motif was found in *SSR2* promoter.

Presence of STRE motifs and induction of RNA with both *SSR1* and *SSR2* indicated their possible involvement in salt adaptation process. To determine their functional importance in this process, Two strains YBH31 (Fig.3.30 and 3.31) and YBH32 (Fig. 3.33), lacking *SSR1* and *SSR2* respectively, were constructed. When the growth of YBH31(*ssr1Δ*) and YBH32 (*ssr2Δ*) were observed in media with 1M NaCl or 1.5 M KCl or 1.5 M Sorbitol, no significant difference was observed in growth between these mutant strains and wild type (Fig. 3.35). Although deletion of these two genes does not seem to affect growth under stress condition, this does not necessarily rule out their possible involvement in adaptive responses. Redundancy of genes and overlap of functions are common in eukaryotic systems. Further investigation is required to find out the exact role(s) *SSR1* and *SSR2* play in adaptive response of the cells