MOLECULAR CLONING OF PBS2 HOMOLOGUE FROM Debaryomyces hansenii
The yeast *Debaryomyces hansenii* represents one of the most halotolerant and osmotolerant species of yeast. This species is capable of growing in media containing 4.0 M NaCl whereas growth of *S. cerevisiae* is limited in media having more than 1.7 M NaCl. Molecular basis of such high halotolerance and its species-specific mechanism remains unexplored. In yeast, the osmosensing signal transduction pathway (HOG pathway) plays cardinal role in regulating its growth and survival under high osmolar conditions. In baker's yeast *PBS2* encodes for the MAPKK of osmosensing signal transduction pathway. Apart from osmosensing, *PBS2* has been reported to participate in other physiological processes. It appears to have role in maintaining the integrity of cell membranes and plasticity of the cell wall. Although, previous work in our lab indicated the presence of an osmosensing signal transduction (MAPK) pathway in *D. hansenii*, the details of this pathway are yet to be unraveled. Such studies could provide molecular clues to understand the remarkable osmotolerance exhibited by this organism. In this part of work, a *PBS2* homologue was isolated from *D. hansenii*. Phenotypic characterization of gene was undertaken to obtain better insight into its complex physiological role.

### 3.1 Isolation and cloning of *PBS2* homologue from *D. hansenii*

*PBS2* deleted *S. cerevisiae* strain is osmosensitive and therefore unable to grow on salt plates containing beyond 0.3 M NaCl. It was speculated that the introduction of homologous gene from *D. hansenii* could restore its ability to grow on high osmolar media. Therefore, functional complementation strategy was followed to clone *PBS2* homologue from *D. hansenii*. A genomic library of *D. hansenii* constructed in 2μ based vector pRS425 (Bansal and Mondal, 2000) was employed for transformation into *S. cerevisiae* strain PB29 by lithium acetate method (Chapter 2, Section 2.2.8). Total of 3 μg DNA was used for transformation. The transformants were plated on salt plates containing 0.5 M NaCl and plates were incubated at 30°C for 4 days. Colonies appeared on the plates were selected and analyzed further. To confirm that the osmorersistant
phenotype shown by the clones was plasmid linked, DNA isolated from all 23 clones was transformed into DH5α strain of *E. coli* (Chapter 2, Section 2.2.7). Plasmid DNA isolated from these *E. coli* transformants (Chapter 2, Section 2.2.1) was retransformed in yeast strain PB29. Transformants were tested for their osmoresistant phenotype on SD (-Leu) plates supplemented with 0.5 M NaCl. After monitoring growth on plates for 4 days it was found that only two clones showed plasmid linked osmoresistance phenotype (Fig. 3.1). One of them, named as pDBS14.1 was selected for further studies.

### 3.1.1 Construction of restriction map of plasmid pDBS14.1

The plasmid pDBS14.1 was digested with several restriction enzymes. Digestion pattern is shown in Fig. 3.2. The insert present in pDBS14.1 had no sites for *BamHI*, *XhoI*, *PstI* and *SacII*. From the digestion pattern it appeared that the size of the insert in the clone was 4.0 kb. Based on various fragment sizes, a restriction map of the insert was prepared (Fig. 3.3).

### 3.1.2 Localization of putative PBS2 homologue on plasmid pDBS14.1

In order to localize the putative *DPBS2* gene on 4.0 kb insert, few subclones were made. Digestion of plasmid pDBS14.1 with *HindIII*, generated two fragments 9.2 kb and 1.6 kb. The larger fragment had vector backbone plus 2.4 kb of insert that was self ligated to obtain pDP1.1. Cloning of other 1.6 kb fragment into pRS425 resulted in plasmid pDP3.1. Similarly, pDBS14.1 was digested with *EcoRV* and the resulting 3.0 kb fragment was cloned in pRS425 to obtain plasmid pDP4.1.

These subclones were transformed into PB29 and growth of the transformants was checked on SD plates containing 0.7 M NaCl. The results of complementation analysis are shown in Fig. 3.4. The pDP4.1 was able to complement the *pbs2* mutation of strain PB29, while pDP3.1 and pDP1.1 could not confer functional complementation. Thus, 3.0 kb *EcoRV* fragment encoded the functional *DPBS2* gene that could complement the *pbs2* mutation.
Fig. 3.1 Complementation of pbs2Δ phenotype by D. hansenii genomic clones
Plates show growth of clones 16.1 and 14.1 on SD medium with (0.5 M NaCl) or without NaCl. PSY311 and PB29-M are W303.1B and PB29 (pbs2Δ) strains respectively transformed with vector pRS425.
Fig. 3.2 Restriction enzyme analysis of pDBS14.1
Plasmid DNA isolated from pDBS14.1 was digested with different restriction enzymes and resolved on 0.8 % agarose gel. λDNA digested with HindIII (lane 1); pPBS14.1 digested with BamHI (lane 2); ClaI (lane 3); EcoRI (lane 4); EcoRV (lane 5); KpnI (lane 6); HindIII (lane 7); HindIII/XhoI (lane 8); XhoI (lane 9); SacI (lane 10); PstI (lane 11); SacII (lane 12).
**Fig. 3.3 Plasmid map of pDBS14.1**
Plasmid with 4.0 kb *D. hansenii* genomic DNA fragment that complements *pbs2* mutation in *S. cerevisiae.*
Fig. 3.4 Restriction map of pDBS14.1 and results of the complementation analysis
Plasmids pDP1.1, pDP3.1 and pDP4.1 are subclones of pDBS14.1. These plasmids were transformed into PB29 (Apbs2) strain of W303.1B and transformants were streaked on NaCl (0.5 M) containing plates. Results of the growth complementation are summarized on the left; +, complemented; -, not complemented. For complementation test, the strains were streaked on Ura- and Leu- drop out plates (SD medium) containing 2% glucose and 0.5 M NaCl, and growth was observed after incubation at 30°C for 3 days. Restriction enzyme sites (HindIII, EcoRV) present in the insert are shown.
3.1.3 Analysis of putative DPBS2 gene by Southern hybridization

In order to confirm the cloned sequence was authentic *D. hansenii* gene and did not arise from cloning artifact, a Southern hybridization was performed. Genomic DNA of *D. hansenii* (MTCC234) and *S. cerevisiae* (W303.1B) were digested with three different restriction enzymes (*ClaI*, *HindIII* and *XhoI*) and resolved by (0.8 %) agarose gel electrophoresis. DNA from agarose gel was transferred to the nylon membrane and hybridization was carried out under stringent conditions (Chapter 2, Section 2.2.12) using $^{32}$P labeled 3.0 kb *EcoRV* fragment of pDBS14.1 as probe. With *ClaI* and *XhoI* digested genomic DNA, single bands of ~4.0 kb and ~20 kb respectively, were observed in the autoradiograph (Fig. 3.5, Panel B: lanes 2, 6). On the other hand two bands (2.5 and 5.5 kb) were highlighted with *HindIII* digested genomic DNA of *D. hansenii* (Fig. 3.5, Panel B: lane 4). There was no signal in lanes with *S. cerevisiae* genomic DNA (Fig. 3.5, Panel B: lanes 1, 3 and 5). Therefore, the cloned fragment is a bonafide *D. hansenii* gene. Our results also indicated that *S. cerevisiae* and *D. hansenii* genes exhibit significant divergence at the nucleotide level.

3.2 Cloned gene is not a multicopy suppressor

For cloning *DPBS2* gene, we have used genomic library constructed in a multicopy vector pRS425. Results reported above indicated that the *DPBS2* gene cloned in multicopy vector pRS425 (pDP4.1) could complement the osmosensitive phenotype of the *pbs2* mutation (Section 3.1.2). Therefore, it is also possible that the cloned gene could be a multicopy suppressor. To rule out this possibility, we determined whether the cloned gene could complement a *pbs2* mutation when present in a single copy plasmid. To study this, *DPBS2* was subcloned into a yeast centromeric vector, YCplacl11 (Geitz and Sugino, 1988) and the recombinant plasmid was transformed into the PB29 strain. Cultures were grown in SD medium (-Leu) till OD$_{600}$ of 0.5~1.0 and then serial dilutions were done ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$) and 3 µl of each dilution was spotted on SD plates
Fig. 3.5 Southern hybridization analysis of DPBS2 gene

Genomic DNA from *D. hansenii* (lanes 2, 4 & 6) and *S. cerevisiae* (lanes 1, 3 & 5) were digested with *Cul* (lanes 1 & 2) *HindIII* (lanes 3 & 4) and *XhoI* (lanes 5 & 6). *λ* DNA digested with *HindIII* was used as molecular size marker (lane M). Samples were resolved on 0.8 % agarose gel, transferred to nylon membrane and hybridized with DPBS2 probe. Panel A: gel picture of ethidium bromide stained gel. Panel B: Autoradiogram of the blot after hybridization.
supplemented with various osmolytes. Plates were then incubated for 3 days at 30°C. *S. cerevisiae* strain PB29 was highly osmosensitive and it did not grow in the plates containing more than 0.3 M NaCl. Our result showed that *DPBS2* gene cloned in centromeric vector (pDP7.1) could complement osmosensitive phenotype of PB29. Thus, the cloned gene could be a *PBS2* homologue and not an extragenic suppressor. In case of pDP7.1, relatively good growth was found in the plates containing 0.5 M NaCl but the strain bearing cloned gene in multicopy vector could grow even on the plates containing 0.7 M NaCl. However, unlike the wild type this strain also failed to grow in medium containing more than 1 M NaCl as indicated by our experiments in Section 3.6 of this chapter. With other osmolytes (KCl, sorbitol) similar growth pattern was observed (Fig. 3.6).

### 3.3 Effect of DPBS2 on altered calcofluor and polymyxin B sensitivity of PB29

Polymyxin B is an antibiotic known to damage the cell membranes (Storm *et al.*, 1977). Earlier studies have shown that *S. cerevisiae* cells harbouring a mutation in the *PBS2* gene are sensitive to polymyxin B (Boguslawski and Polazzi, 1987). Recently, it has been observed that *S. cerevisiae pbs2* mutant was resistant to calcofluor (Garcia-Rodriguez *et al.*, 2000). Calcofluor is a fluorochrome that exhibits antifungal activity and a high affinity for yeast cell wall microfibrils of cellulose and chitin. Dye interaction leads to abnormal chitin deposition, weakening of cell wall and subsequent cell lysis (Roncero *et al.*, 1998). We therefore studied whether *DPBS2* gene could complement the altered polymyxin B and calcofluor sensitivity showed by *pbs2* mutants. PB29 strain harbouring pDP4.1 were grown overnight in SD media (supplemented with leucine dropout mix), along with PB29-M (PB29/pRS425) and PSY311 (W303.1B/pRS425) to OD<sub>600</sub> of 0.5 to 1.0. Serial dilutions were made (10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup>, 10<sup>-4</sup>) and growth was checked on plates containing 0.5 mM polymyxin B or 0.5 mg/ml calcofluor. Our result (Fig. 3.7) indicated that *DPBS2* could complement the polymyxin B sensitivity of the
Fig. 3.6 Complementation of *S. cerevisiae* *pbs2Δ* mutant (PB29) by *DPBS2* gene

Serial 10-fold dilutions of saturated cultures were spotted on solid agar plates of minimal (SD) media supplemented with NaCl, KCl, Sorbitol or LiCl at the concentrations indicated. Plates were then incubated for 3 days at 30°C before photographs were taken. 1. W303.1B/pRS425, 2. PB29/pRS425, 3. PB29/pDP4.1, 4. PB29/pDP7.1. pDP4.1 and pDP7.1 are high-copy and low-copy number plasmids, respectively, containing the *DPBS2* gene.
Fig. 3.7 Growth pattern of PB29 carrying \textit{DPBS2} gene on plates containing polymyxin B (0.5 mM) and calcofluor (0.5 mg/ml)
Serial 10-fold dilutions of saturated cultures were spotted on solid agar plates of minimal (SD) media supplemented with drugs. The plates were then incubated for 3 days at 30°C before taking photographs. 1. W303.1B/pRS425; 2. PB29/pRS425; 3. PB29/pDP4.1. pDP4.1 is a pRS425 harbouring the \textit{DPBS2} gene.
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*pbs2* mutation in *S. cerevisiae*. However, no perceptible effect of *DPBS2* gene on the calcofluor resistance of this mutant was observed (Fig. 3.7).

### 3.4 *D. hansenii PBS2* is constitutively expressed

HOG pathway in *S. cerevisiae* is activated in response to high osmolarity. The cloned gene *DPBS2* is from highly halotolerant yeast *D. hansenii* and it could complement the *pbs2* mutation in *S. cerevisiae*, although partially. Thus, to determine whether the expression of *DPBS2* gene in *D. hansenii* is modulated by the change in osmolarity of media, Northern blot analysis was performed. *D. hansenii* strain MTCC234 was grown in YEPD media at 30°C up to an OD₆₀₀ of 1.0. Cells were harvested and induced by suspending in YEPD media supplemented with 1.0 M NaCl. Samples were withdrawn from cultures after 30 min and 60 min. Total RNA was isolated (Section 2.2.4) and approximately 20 µg of each sample was resolved by formaldehyde gel electrophoresis (Sambrook *et al.*, 1989). RNA was transferred to the nylon membrane and probed using a ~3.0 kb *EcoRV* fragment of *DPBS2*. Hybridization was carried out under stringent conditions (Chapter 2, Section 2.2.13). For internal control, following stripping of the blot (Chapter 2, Section 2.2.14), it was rehybridized with a °²P-labelled 1.6 kb *BamHI-HindIII* fragment carrying *S. cerevisiae ACT1* gene.

A single transcript of ~2.4 kb was detected in all samples (Fig. 3.8, panel A) and the size of the transcript correlated well with that of the open reading frame (ORF) identified. Size of transcript was determined approximately based on ribosomal RNA size. When the level of *DPBS2* mRNA expression was normalized with that of actin mRNA level (Fig 3.8, Panel B) no significant difference in the steady state level was observed. Thus, the *DPBS2* gene in *D. hansenii* was not modulated by changes in the osmolarity of the medium and is probably constitutively expressed under different salt concentration of the medium.
Fig. 3.8 Northern blot analysis of DPBS2 expression
Total RNA was isolated from *D. hansenii* strain MTCC234 treated with NaCl for different time intervals (in min) as indicated. Panel A: shows the Northern blot of the gel probed with radio-labeled *DPBS2* gene fragment. Panel B: autoradiograph of the blot probed with *S. cerevisiae* actin gene. Signals are indicated by arrows.
3.5 Sequencing of DPBS2 gene and Sequence Analysis

The putative DPBS2 gene was localized on the 3.0 kb EcoRV fragment (Section, 3.1.2). To determine the nucleotide sequence of this fragment, either plasmid pDP4.1 (carrying ~3.0 kb EcoRV fragment) or its subclones pDP2.1 (carrying ~1.4 kb EcoRV/HindIII fragment) and pDP3.1 (carrying ~1.6 kb HindIII fragment) were used. Sequencing was carried out by Sanger's dideoxy method (Sanger et al., 1997) using the Big Dye Terminator Cycle sequencing Kit (Version 3.0) in a ABI 310 DNA sequencer. Both universal primers (PT3 and PT7) and custom synthesized primers from commercial sources were used to carry out DNA sequencing for both strands of the DPBS2 coding sequence. The sequencing strategy is shown in Fig. 3.9.

3.5.1 Analysis of the nucleotide sequence of DPBS2 gene

The nucleotide sequence of DPBS2 gene along with its flanking regions and the derived amino acid sequence is shown in Fig. 3.10. From the nucleotide sequence of the clone, an ORF from bases 888 (ATG as start codon at +1) to 2939 (TAA as stop codon) encoding a protein of 683 amino acids was identified (Gene Runner program version 3.02 Hastings Software Inc, New York). The predicted molecular weight and pI of the protein are 74.7 kDa and 8.47, respectively. A profile scan search for this protein sequence showed the presence of a 'eukaryotic protein kinase domain' at amino acids 345-605 and a 'proline rich' region at amino acids 82-99.

3.5.2 Multiple sequence comparison of DPBS2 and other MAPK Kinases

Both the nucleotide and corresponding amino acid sequences were compared with the EMBL and GenBank databases using BLASTX program. The polypeptide showed high homology with S. cerevisiae Pbs2p (Score 473; expected =e^{-132}) and S. pombe Wislp (Score 410; expected =e^{-113}). The data base search also revealed that the cloned gene has substantial homology with MAP kinase kinases from various organisms. Recently D. hansenii was taken up for genome sequencing in the Genolevures
Fig. 3.9 The sequencing strategy for *DPBS2*
To obtain overlapping sequence-reads universal primer (PT3, forward; PT7, reverse) and custom designed primers were used for sequencing. Name of each primer along with the direction of sequence read is indicated.
Fig. 3.10 Continued on next page
Fig. 3.10. Nucleotide sequence and predicted amino acid sequence of the DPBS2 gene. Nucleotides are numbered on the left and amino acids are numbered on the right. An asterisk indicates the termination codon. A proline rich domain is underlined.
programme (Sherman et al., 2004). BLASTp search with the partially released database from this project also identifies a Dpbs2p sequence which showed 92% similarity and 83% identity with our sequence and the observed difference could be because of strain differences.

Nucleotide derived amino acid sequence of *D. hansenii DPBS2* was compared to previously identified homologous sequences from yeast, using the sequence alignment program CLUSTAL W. The predicted amino acid sequence of *D. hansenii DPBS2* shows 48% identity and 75% similarity with *S. cerevisiae* Pbs2p. With *S. pombe* Wis1p, it showed 42% identity and 65% similarity. When comparison was limited to only the 'eukaryotic protein kinase domain', Dpbs2p showed 73% identity and 88% similarity with Pbs2p. Comparison of phosphorylation site (Ser-X-X-X-Thr/Ser), showed 100% homology with that of Pbs2p (Fig. 3.11). Sequence comparison also indicated that *D. hansenii, S. cerevisiae, S. pombe*, have long N-terminal non-catalytic domains. On the contrary mammalian orthologues have comparatively short N-terminal non-catalytic domains.

3.5.3 Phylogenetic relationship

The MAPKKs belonging to yeast, fungi, mammalian, plant MAPK pathways were searched against the non-redundant protein database at NCBI using the BLAST program. From all the kinases, 19 sequences were selected and initial sequence alignment was produced by CLUSTAL W. A neighbour-joining tree was drawn for the entire data set using the CLUSTAL W tree joining option (Saitou and Nei, 1987). Tree was constructed with gaps excluded and corrected for multiple substitutions. Bootstrap values greater than 50% were taken as sufficient evidence for grouping. The tree consisted of two major groups. One group coincided with the MAPKKs from stress activated MAPK pathways from fungi and mammals. Other group included MAPKKs activated by other signals. *PBS2* homologues from *D. hansenii* and *S. cerevisiae* were grouped together along with homologues from *Neurospora crassa* and *S. pombe*. Although on sequence level *D. hansenii* and *S. cerevisiae* MAPKKs (Pbs2p and Dpbs2p) shows divergence in
Fig. 3.11 DPBS2 protein comparison.

Multiple alignment (CLUSTAL W software) of homologous proteins from *S. cerevisiae* (Pbs2p, 668 residues, 72 kDa, P08018) and *S. pombe* (Wislp, 605 residues, 64 kDa, P33886). Asterisks indicate perfectly conserved residue, two dots indicate very similar residues and full stops, indicate similar residues. The putative polyproline motif and phosphorylation sites are underlined.
their non-kinase domains but they were grouped most closely in the phylogenetic tree. It shows that they both are most closely related MAPKKs (Fig. 3.12).

3.6 Cloning of DPBS2 under S. cerevisiae promoter

Although DPBS2 gene has been isolated from a halotolerant organism but it could only partially complement the pbs2 mutation in S. cerevisiae. Hence, it was quite reasonable to assume that the partial complementation could be arising out of the differences in promoter strengths of DPBS2 and PBS2 in heterologous host S. cerevisiae. Therefore, to rule out this possibility DPBS2 was cloned under S. cerevisiae promoter.

PCR was employed to amplify promoter region of PBS2 gene from S. cerevisiae genomic DNA. For this purpose, the oligonucleotides (forward: AM155r, reverse: 156r) were designed (with incorporated XhoI and NcoI sites) (Chapter 2, Table 2.3). PCR was carried out for 30 cycles (denaturation: 95°C for 1 min per cycle; annealing: 55°C for 2 min per cycle; elongation: 72°C for 2 min per cycle) that amplified ~2.0 kb PBS2 upstream region. To incorporate ATG as the initiation codon of DPBS2, NcoI site was introduced in the oligonucleotide AM153r. In another reaction 0.5 kb N-terminus region of DPBS2 preceding HindIII site was amplified from pDBS14.1 using oligonucleotides (forward: AM153r, reverse 154r) (Chapter 2, Table 2.3). PCR was carried out in 30 cycles (denaturation: 95°C for 1 min per cycle; annealing: 55°C for 1 min per cycle; elongation: 72°C for 1 min per cycle). Amplified PBS2 promoter fragment was restriction digested with XhoI and NcoI and the 543 bp fragment was digested with NcoI and HindIII. A 1.7 kb HindIII/BamHI fragment was obtained by restriction digestion of plasmid pDBS14.1. These three fragments were ligated with XhoI BamHI digested pRS425 and the resultant plasmid containing DPBS2 gene cloned under S. cerevisiae promoter was named as pDPS21. The cloning strategy has been outlined in Fig. 3.13. To check phenotypic complementation, pDPS21 was transformed into PB29 strain and dilution spotting was done with other control strains on SD (-Leu) plates supplemented
Fig. 3.12 Phylogenetic tree for the MAPKKs
Numbers at branching points are bootstrap percentages for 1000 replicates. Branches supported for bootstrap of 50% or more are reliable. Each major clad is a unique group and its pathway is indicated in the Fig. The two major groups are distinguishable as indicated. Abbreviations and accession numbers for organisms are as follows: AT, Arabidopsis thaliana; CA, Candida albicans; DH, Debaryomyces hansenii; HS, Homo sapiens; LE, Lycopersicon esculentum; MM, Mus musculus; NC, Neurospora crassa; NT, Nicotiana tabacum; SC, Saccharomyces cerevisiae; SP, Schizosaccharomyces pombe; UM, Ustilago maydis; MEK_LL (CAA04261); MEK_NT (AAF67262); MKK7_HS (J18 AF014401), FUZ7_UM (GBU07801); PBS2_SC (NP_012407); WIS1_NP(595457); MKK_YL (CA45932); MAPKK_NC (BAC56235); STE7_CA (AAC49733); MKK4_HS (P45985); MKK3_HS (BAA13248); MEK_NT (NPK2 S53804); YR62_CE (AAAS85118); MAP2K_AT (CAA07281); BYR1_SP (P46734); MKK6_HS (P52564); MKK2_HS (AAH18645); MKK1_HS (NP002746); DPBS2_DH (AAK85200).
1. **S. cerevisiae** promoter amplified from W303.1B
2. Xhol and NcoI digestion

1. Amplified using Vent DNA polymerase
2. NcoI and HindIII digestion

1. 1.1 kb fragment obtained by digestion with HindIII and BamHI enzymes
2. Purified by gel extraction

**pDBS14.1**

**pDPS21**

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**Fig. 3.13 Strategy for cloning DPBS2 under S. cerevisiae promoter**

*S. cerevisiae* promoter region (~2.0 kb) and *NcoI/HindIII* fragment (0.4 kb) were amplified from W303.1B genomic DNA and pDBS14.1 respectively using Vent DNA polymerase. ~1.6 kb *HindIII/BamHI* fragment was excised from pDBS14 and gel purified. These three fragments were cloned in pRS425 vector as indicated. Restriction sites used for cloning are indicated.
with salt. Plates were incubated at 30°C for 3 days and it was found that the growth pattern was same for DPBS2 cloned gene under both its own promoter and S. cerevisiae promoter (Fig. 3.14). Therefore cloning of DPBS2 under S. cerevisiae promoter could not improve the level of complementation exhibited by DPBS2 in earlier experiments.

3.7 Analysis of DPBS2 growth pattern in Δssk2, Δssk22, Δpbs2 and Δste11, Δpbs2 strain background.

In S. cerevisiae PBS2 receives signal from two separate and functionally distinct osmosensors i.e. SLN1 and SHO1. Each branch alone is sufficient to activate Hog1p through same MAPKK, Pbs2p although they use different MAPKKK. Sho1p interact with Pbs2p via a proline-rich region around position 96 in the N-terminus of Pbs2p and an SH3 domain located in the hydrophilic C terminus of Sho1p. Sln1p does not directly interact with Pbs2p. In Sln1 pathway, two specialized redundant kinases carry out Pbs2p activation step. Sho1p signals through a promiscuous MAPKKK, Ste11p that is also essential for pheromone-response and pseudo-hyphal growth. Therefore, it would be interesting to see whether DPBS2 can recapitulate all these complex interactions in a heterologous host. Thus following experiment was done to ascertain whether DPBS2 could be activated by either of the upstream branches.

3.7.1 Construction of Δssk2, Δssk22, Δpbs2 and Δste11, Δpbs2 mutants

Previous experiments showed that DPBS2 could complement pbs2 mutation in S. cerevisiae. In order to examine whether Dpbs2p could also be activated independently by either of these two branches, strains were constructed carrying mutations in either of these pathways. For this two strains Y07195 (Δssk22) and Y05271 (Δste11) obtained from EUROSCARF were subjected to further mutagenesis by homologous recombination. A PBS2 disruption cassette was made by replacing 0.8 kb EcoRV region of PBS2 gene with 1.1 kb URA3 gene. A 2.3 kb fragment from this cassette comprising of
Fig. 3.14 Growth pattern of DPBS2 under S. cerevisiae promoter
Serial 10-fold dilutions of log phase cultures (OD$_{600}$ ~0.5-0.7) were spotted on solid agar plates of minimal (SD) media supplemented with NaCl. The plates were then incubated for 3 days at 30°C before taking photographs. 1. W303.1B/pRS425; 2. PB29/pRS425; 3. PB29/DP4.1; 4. PB29/pDPS21. DPS21 is DPBS2 gene cloned under S. cerevisiae promoter.
URA3 gene with flanking PBS2 sequence was transformed into Y07195 and Y05271 to obtain PSY412 (Δstell, Δpbs2) and PSY426 (Δssk22, Δpbs2) respectively. In order to introduce mutation in SSK2 gene, a disruption cassette with HIS5 marker was made. Two regions from SSK2 ORF, A (129-626 bp; PSP35: forward primer with HindIII linker and PSP36: reverse primer with XbaI linker;) and C (3512-4140 bp; PSP37: forward with BamHI linker and PSP38: reverse with EcoRI linker) were amplified. In a separate reaction, HIS5 (206-3072 bp) marker was amplified from pRS423 using oligonucleotides (forward: PSP33 with XbaI linker and reverse: PSP34 with BamHI linker). These fragments were cloned into pGEM3Z and the cloning strategy is depicted in Fig. 3.15. Finally this marker cassette was retrieved from the plasmid by EcoRI restriction digestion and transformed into PSY426 to delete SSK2 ORF by homologous recombination and PSY418 (Δssk2, Δssk22, Δpbs2) strain was obtained.

3.7.2 Dpbs2p is activated by both the Stellp and Ssk22p MAPKKKs in S. cerevisiae

In order to examine whether Dpbs2p could also be activated independently by either of these two branches, pDPS21 was transformed into PSY412 (Δstell, Δpbs2) and PSY418 (Δssk2, Δssk22, Δpbs2). Osmosensitivity of the transformants was checked by dilution spotting on plates containing salts at different concentrations. Cells were grown overnight and reinoculated into fresh SD (-Leu, -His, -Ura) media, cells were allowed to grow till log phase. Cell concentration was normalized to 1.0 OD600 for all the strains used before making serial dilutions. Serially diluted cultures were spotted on SD (-Leu, -His, -Ura) plates supplemented with different concentrations of NaCl. Plates were incubated at 30°C till growth was apparent. From dilution spotting it was indicated that Dpbs2p could be activated by signals from MAPKKKs of either of the upstream branches. However, on salt containing plates, cells with stell genetic background grew marginally better than those with ssk2, ssk22 genetic background. In the plates containing more than 0.7 M NaCl the transformants exhibited poor growth compared to control thus
Fig. 3.15 Construction of SSK2 disruption cassette
Gene encoding for HIS5 marker was amplified from pRS423 and ligated to fragment A and Cs of SSK2 gene. Oligonucleotides and restriction sites utilized for cloning are indicated.
confirming that Dpbs2p could only partially complement osmosensitivity of pbs2 mutation (Fig. 3.16).

Discussion

The yeast Debaryomyces hansenii was isolated from saline environments such as seawater (Norkrans, 1966) and concentrated brines (Onishi, 1963). It represents one of the most halotolerant and osmotolerant species of yeast. In S. cerevisiae a signal transduction pathway involving members of mitogen activated protein (MAP) kinase family i.e. high osmolarity glycerol (HOG) response pathway is responsible for adaptation to high osmolarity (Brewester et al., 1993). Molecular genetic analysis in the yeast S. cerevisiae has so far identified several components of this pathway. However, little information is available with regard to osmosensing signal transduction pathway in xerotolerant yeast D. hansenii. It was only recently that HOG1 homologue was isolated from this species (Bansal and Mondal, 2000). Our study, thus is an effort towards the identification of other components of this pathway in D. hansenii. Understanding such pathway and characterization of the components may shed light on the molecular basis of high halotolerance and osmotolerance in this species.

In S. cerevisiae, the PBS2 deletion leads to an osmosensitive phenotype (Brewester et al., 1993). To identify PBS2 homologues in D. hansenii, functional complementation strategy was used. The genomic DNA library of D. hansenii constructed previously in our lab, in yeast shuttle vector pRS425 (Christianson et al., 1992) was used. This genomic bank comprised of approximately 60,000 independent recombinants and was most likely representing complete genome of D. hansenii, assuming that the total genome length in this yeast is comparable to that in other yeasts known so far.

The pbs2Δ strain PB29 was transformed with D. hansenii genomic DNA library. On screening, 23 transformants were selected that grew on SD plates containing 0.7 M NaCl. To confirm the authenticity of the clones plasmid DNA from these clones was
**Fig. 3.16 Activation of DPBS2 in Δssk2, Δssk22, Δapbs2 (PSY418) and Δste11, Δapbs2 (PSY412) background in S. cerevisiae**

DPBS2 cloned under *S. cerevisiae* PBS2 promoter (pDPS22) was transformed into these strains. Cells were grown in SD medium till logarithmic phase. After adjusting OD<sub>600</sub> to ~1.0, serial dilutions (10 fold) were made in SD medium, 3 μl of each dilution was spotted on the plates with indicated salt concentrations. 0.5 M, 0.7 M plates were incubated for 3 days; 1.0 M, 1.2 M, 1.4 M plates were incubated for 5 days before taking photographs. 1. BY4741 + pRS315, 2. PSY412/PSY418 + pRS315, 3. PSY412/PSY418 + pDPS21.
isolated and retransformed into PB29 (Apbs2) strain. It was found that only in two clones complementation of osmosensitive phenotype was plasmid linked (Fig. 3.1). One of these clones, pDBS14.1 was selected for further studies. Through restriction analysis and subcloning the gene was localized to 3.0 kb EcoRV fragment (Fig. 3.3, 3.4). Southern hybridization suggests that it is a D. hansenii gene, probably present as single copy and has significant sequence divergence with S. cerevisiae at the nucleotide level (Fig. 3.5).

The cloned gene could complement osmosensitive phenotype of PBS2 mutation when cloned in a low copy vector. Thus, ruling out the possibility of its being a multicopy suppressor of pbs2 deletion (Fig. 3.6). However, the phenotypic complementation seems to be partial. One of the possible reasons for such partial complementation could be the level of expression of DPBS2 gene from its own promoter in a heterologous host. Cloning of DPBS2 under S. cerevisiae promoter did not improve its level of osmotolerance (Fig. 3.14). Pbs2p plays a very complex role in S. cerevisiae. Therefore, it is quite possible that Dpbs2p could not recapitulate all the functions in heterologous host.

Further confirmation that the cloned gene was a PBS2 homologue came from the sequence analysis. Both strands of the gene were sequenced (Fig. 3.10) using various custom synthesized oligonucleotides. Sequence analysis of the gene reveals that it has 2049 bp long ORF with an ATG as the start codon. A profile scan search showed the presence of ‘eukaryotic protein kinase domain’ at amino acids 345-605 and a ‘proline rich’ region at amino acids 82-99. Proline rich domains are known to play an essential role in the transmission of information in eukaryotic signal transduction pathways through specific protein-protein interactions (Kay et al., 2000). The proline rich region in Pbs2p was indicated as the region that binds to the SH3 domain present in the carboxy-terminal end of Sho1p (Maeda et al., 1995).

The DPBS2 sequence comparison with previously identified non osmotolerant yeasts S. cerevisiae and S. pombe using CLUSTALW program demonstrated that there was 48 % identity with S. cerevisiae and 42 % with S. pombe. In MAPK pathways the sequential activation of series of kinases takes place. Equivalent kinases in different
pathways have significant sequence homologies (Blumer and Johnson, 1994). The common feature of these kinases are that the upstream MAPKKK when activated in response to a signal, phosphorylates the downstream MAPKK on serine and threonine residues which in turn phosphorylates the further downstream MAPK on threonine and tyrosine in TXY motif. MAPKKs in mammals and yeast have highly conserved amino acid residues (Ser-X-X-X-Thr/Ser), located between sub-domains VII and VIII (Mansour et al., 1994; Ichimura et al., 1998). Comparison of this phosphorylation site showed 100% homology with that of Pbs2p (Fig. 3.11). A phylogenetic analysis of Dpbs2p with other MAPKKs indicate that D. hansenii is phylogentically close to S. cerevisiae and other fungal homologues. It also showed close relationship with MAPKKs from p38 and JNK (stress regulated pathways in mammals) pathways (Fig. 3.12).

Although the complementation shown by DPBS2 was partial but it is reasonably good indication towards its role in halotolerance of D. hansenii. To further check the pattern of expression of this gene in response to salt, Northern hybridization was done. Presence of 2.4 kb transcript (Fig. 3.8, Panel A) indicated that the cloned gene was functional in D. hansenii. Northern blotting also showed that there was no appreciable difference in the level of transcript when induced with salt for different time intervals. Thus, this experiment clearly indicated that DPBS2 gene was constitutively expressed in D. hansenii and expression was not modulated by changes in osmolarity of the media.

PBS2 gene in S. cerevisiae is involved in various other physiological processes. In fact, this gene was first isolated in a screen targeted to identify genes having a role in polymyxin B sensitivity, an antibiotic known to damage the integrity of cell membranes (Storm et al., 1977) and hence named as PBS2 (Boguslawski, 1992; Boguslawski and Polazzi, 1987). Overexpression of this gene leads to polymyxin B resistance, whereas its deletion results in polymyxin B sensitivity. Mutations in PBS2 also affects calcoflour sensitivity of S. cerevisiae. Our growth experiments showed that DPBS2 could complement the polymyxin B sensitive phenotype of PB29 strain. However, no perceptible effect of this gene on the calcofluor resistant phenotype of this mutant was found (Fig. 3.7). In this context it is interesting to note that a pbs2 mutant allele of S.
cerevisiae, showing altered sensitivity to calcofluor but having a normal level of osmotolerance, was recently isolated by Garcia-Rodriguez et al. (2000). It was concluded that role of Pbs2p in osmo-adaptation and calcofluor resistance, are two physiologically different phenomena. Our result could be construed as an extension of this idea. In the functional analysis, the DPBS2 could be viewed as a mutant allele of the PBS2. From this result, it is apparent that Pbs2p plays functionally distinct role in polymyxin B and calcofluor resistance.

In S. cerevisiae, activation of Pbs2p can occur via two different signal transduction branches. In one branch, Sln1p acts as an osmosensor while the other utilizes Sho1p to sense environmental signals (Maeda et al., 1994, 1995). Each branch alone is sufficient to activate Hog1p through same MAPKK, Pbs2p although they use different MAPKKK. In S. cerevisiae it was found that there lies the difference in the sensitivity of both the branches. In order to examine whether Dpbs2p could also be activated by either of these branches, the plasmid pDPS21 was transformed into strain PSY12 and PSY18. Osmo-sensitivity of the transformants was checked by dilution spotting on plates containing different salt concentrations. From the results, it was apparent that Dpbs2 was capable of receiving signals from MAPKKKs of either of the upstream branches. However, better growth could be observed in SSK2, SSK22 compared to STE11 genetic background (Fig. 3.16) indicating Ste11p could be less proficient in activating Dpbs2p. In the plates, containing more than 0.7 M NaCl the transformants exhibited little growth compared to control thus re-confirming that Dpbs2p could only partially complement osmosensitivity of pbs2 mutation.