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Essential role of HSP26 in stress tolerance of yeast during ethanolic fermentation

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ABSTRACT : Small heat shock proteins are a conserved protein family with members found in all organisms analyzed till date. Hsp26 of yeast Saccharomyces cerevisiae, a member of this family, is a temperature-regulated chaperone that forms large oligomeric complexes and dissociates into dimer to bind non-native proteins. Though detailed studies have been done on structure, regulation and chaperone activity of Hsp26, so far no biological role could be attributed in spite of extensive efforts. Here we show that Hsp26 is essential for stress tolerance of yeast cells during high temperature ethanolic fermentation.

Key words : small heat shock protein, biological role, chaperone, stress tolerance, ethanol production, high temperature.

INTRODUCTION

HSP26 is one of the major small heat shock proteins (hsp) of the yeast S. cerevisiae and lot of studies have been done to demonstrate its biochemical role. It is expressed during stress and at particular stages of development (Kurtz *et al*., 1986; Jiménez-Martí *et al*., 2009) shows significant amino acid sequence homology to the small hsps of other species (Susek *et al*, 1989). The Yeast Hsp26, a 24-subunit complex has been shown to undergo dissociation into dimers at elevated temperatures and such dissociation is required for the efficient interaction with non-native proteins, thus acting as a temperature regulated molecular chaperon (Haslbeck *et al.,* 2004). These structural changes are of functional importance as hsp26 is an efficient chaperone only at elevated temperatures. Thus, HSP26 is shown to be an intrinsic temperature sensor for detecting heat shock conditions (Stromer *et al*., 2004; Haslbeck et al., 1999). Another unusual property studied is their apparent ability to assemble into high molecular weight aggregates termed 'heat shock granules' following their expression under stress conditions (Bentley *et al*., 1992). It has been shown not to be important in unstressed vegetative cells but is strongly induced by heat shock, stationary phase arrest or nitrogen starvation (Kurtz *et al*., 1984; Kurtz *et al*., 1986; Susek *et al.,* 1990; Aranda et al., 2002). Till now no functional role could be attributed to this important hsp. However, it has been reported that the gene is not essential for the acquisition of thermotolerance in exponential or in stationary phase cells as well as in providing resistance to ethanol. Moreover, its role during spore development, thermoresistance during sporulation, spore germination, thermoresistance of mature or germinating spores or survival after long term storage in stationary phase or as spores in also unclear (Petko et al., 1986; Susek *et al*., 1989). Recently, another protein named the ETP1 (Ethanol Tolerance Protein 1) of yeast has been suggested to play a role in ethanol-induced turnover of the low-affinity hexose transporter Hxt3p for the ethanol-induced transcriptional activation of the ENA1 promoter and heat shock protein genes (HSP12 and HSP26). This again failed to correlate directly the observed phenotype to the actual role of HSP26 Snowdon *et al*., 2009. Cells deficient in cAMP-dependent protein kinase activity stop growth, arrest in G1, and show physiological changes normally associated with nutrient deprivation; these changes include the accumulation of trehalose and glycogen, enhanced expression of genes like hsp26, hsp12, ssa3 etc., and increased resistance to heat stress (Shin *et al*., 1987; tamai *et al*., 1994).

As the small heat shock protein hsp26 has been conserved for so many years, having no cellular role seemed unlikely although there could be several possible explanations for the lack of a phenotype in the hsp26 negative mutants. Firstly, the principal role of HSP26 may be to promote growth at high temperatures or enhance thermotolerance but its effect may be too subtle to be detected. Secondly, it may be a functional redundancy as HSP42 shares a high degree of homology with HSP26, particularly over a conserved hydrophobic stretch of about 35 amino acids located near the carboxyl terminus of the protein. Its expression was up regulated by increase in temperature and salt concentration as well as by conditions of limited growth and overgrowth of cell cultures (Wotten *et al*., 1996). Interestingly, although hsp26 expression is also up regulated under all these conditions there are differences in the response of these two genes to the various conditions of stress. The function of this highly conserved small HSP may be advantageous for growth only under very particular circumstances that have not been tested experimentally but have been utilized for selection throughout evolution.

S. cerevisiae is well known to have the ability to take up and ferment a wide range of sugars and is extensively used in ethanol production; finding the involvement of small heat shock proteins in stress survival of yeast during high temperature ethanolic fermentation will be very useful. Thus, finding thermotolerant yeast strain and also attributing a functional role to a highly conserved small hsp (hsp26)

would facilitate further development of better industrial strains. Though detailed studies have been done on structure, regulation and chaperone activity of Hsp26, so far no biological role could be attributed in spite of extensive efforts. Here we show that Hsp26 is essential for stress tolerance of yeast cells during high temperature ethanolic fermentation.

MATERIAL AND METHODS

Media and Growth conditions. For routine culture the strains were grown on YPD (1% w/v Yeast extract, 2% w/v peptone, 2% w/v glucose) at 30 C. The media used for selection of disruptants was YPDG418 (Geneticin was added at 200 g/ml concentration after the media was autoclaved and cooled to 55 C. The diploids were sporulated on presporulation (1% w/v yeast extract, 2% w/v peptone, 1% w/v Potassium acetate and 2%w/v agar) and sporulation media (0.5% w/v potassium acetate, 2% w/v agar; Bilinski and Casey, 1989). Fermentation experiments were carried out in YPD25 media (1% w/v yeast extract, 2% w/v peptone and 25% w/v glucose) at 38 C.

Construction of gene specific deletion cassettes. Routine DNA manipulations, including plasmid preparation, sub cloning, agarose electrophoresis and transformation were performed by standard methods (Sambrook *et al*.,1989). The dominant resistant module, KanMX4 containing the Kanr gene of the E. coli transposon Tn 903 and included in the vector pFA6a-KanMX4 was used to replace the yeast coding sequence and to select for S. cerevisiae transformants. The method employed to create the deletion cassettes is based on the polymerase chain reaction (PCR) targeting strategy. The deletion cassette contained KanMX4 flanked by 40 bp regions homologues to the target sequence from the promoter and the terminator of the yeast gene to be disrupted. The cassettes were generated by PCR with Taq DNA polymerase and Vent DNA polymerase using pFA6a-KanMX4 as template. For each ORF a pair of primers was designed to contain 40 nucleotides at the 5' end homologues to the target sequence followed by 20 nucleotides at the 3'end homologues to pFA6a-KanMX4 MCS. The deletion cassettes were designed to replace at least 80% of the target ORF (Wach *et al*., 1994).

Cloning strategy for hsp26 disruption with KanMX4 module:

Amplification of a fragment was done with H26-S primer (5'-ACATCCACAACCAACGAAG-3') and H26-A3 (5'-GAGGA TCCTGTTTTAATAGAGATCTGATATAT-3'). A second fragment corresponding to the other end of the ORF was amplified with H26-S3 (5' CGAATTCTGGCTCTATAGTGTTGT CC-3') and H26-A1 (5' AACGGTCATATATCGAAGCCAAAG-3'). Plasmid pFA6a-KanMX4 was digested with BamH1 and EcoR1 to yield a fragment of 1.476 Kb. The first fragment of hsp26 was digested with BamH1 and the second fragment of hsp26 was digested with EcoR1. Then all the three fragments were ligated and PCR amplified using hap26-S and hsp26-A1. Transformants were confirmed by restriction digestion (Fig. 1).

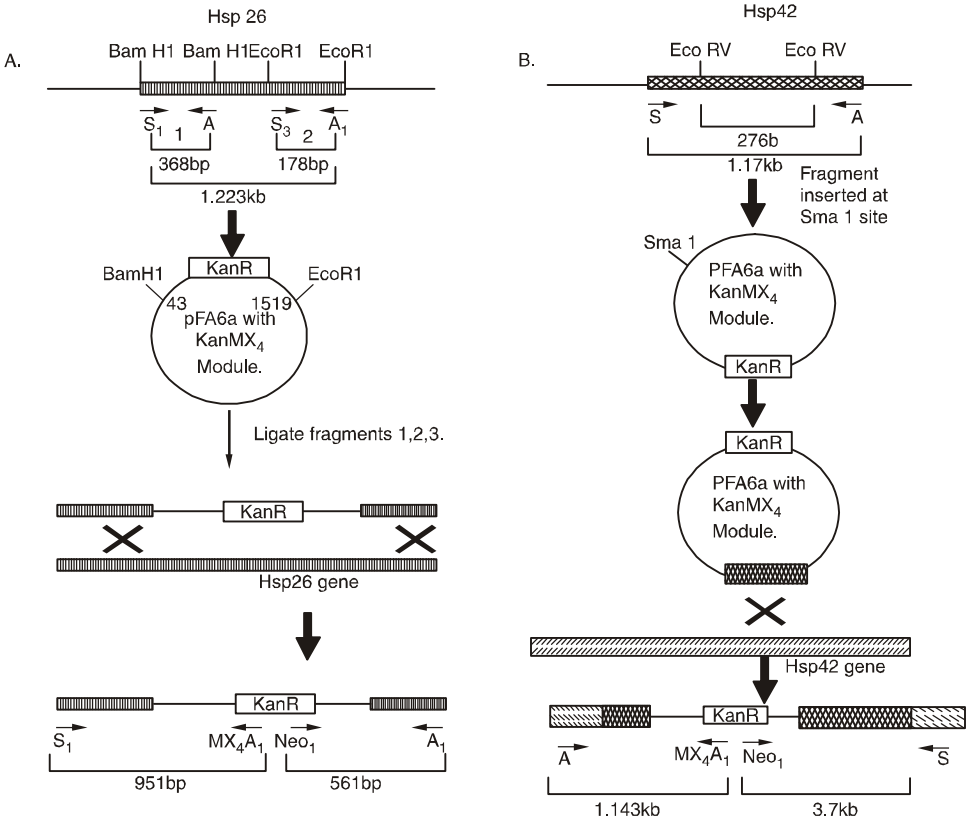

Fig. 1.

Cloning strategy for hsp42 disruption with KanMX4 module:

Hsp42 was PCR amplified from yeast genomic DNA with primers H42-S (5'-GTAGTGTACTGTCCAAACT-3') and H42-A (5'-TTTGGCTAATGGTGGTCTCGAC-3') and then digested with EcoRV. A band of 276bp corresponding to 652 to 927 bp of hsp42 ORF was gel purified. Plasmid pFA6a-KanMX4 was digested with Sma1 in presence of ligase buffer and hsp42 gene fragment was added along with ligase enzyme. Transformants were confirmed by restriction digestion (Fig. 1).

Transformation of Yeast Cell. Transformations of yeast cells were performed by a procedure modified from Ito et.al, (1983). Transformed cells were grown at 30 C in YPD for 12 hours and spread on YPD plates containing 200 g/ml geneticin. To purify transformants from background each large colony was streaked out on fresh YPDG418 plates. Only those clones which grew after the double selection were analyzed further.

Verification of yeast transformants. Correct replacements of the target gene at the genomic locus were verified by analytical PCR. Genomic DNA of the disrupted strain was used as template and appropriate primers (as given before) were used to check the two junctions corresponding to the replacement. Genomic DNA of wild type was used as PCR control and the predictable sizes of the disrupted PCR products in comparison to wild type were easily differentiated (Fig. 2).

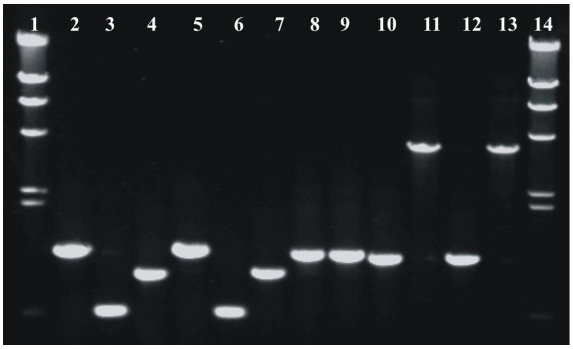

Fig. 2.

Micromanipulation to get haploids having various disruptions:

The diploid was sporulated and then the tetrads were dissected to obtain a segregation pattern among the spore clones for G418. Mating type was determined for the spore clones and then the type of disruption obtained was confirmed by nested and semi nested PCR using primers previously mentioned.

Growth experiment. Strains were pre grown in YPD at 38 C for – 16 hours. Then the disruptants and the wild type were equally mixed for the initial OD600 to be 0.1. The growth was monitored at various time points and plated by 10-fold serial dilution for percent survival (Fig. 3).

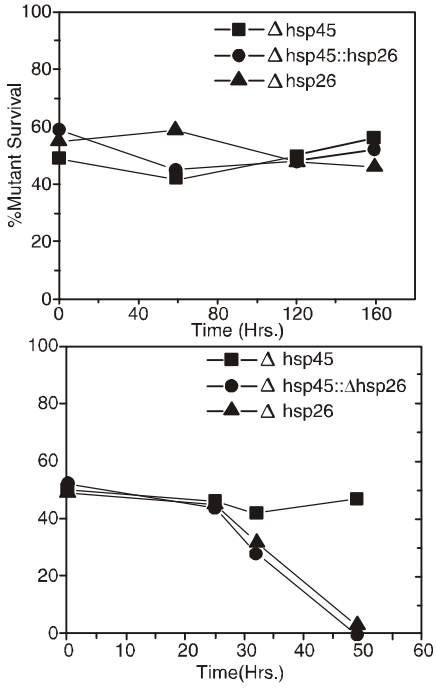

Fig. 3.

Fermentation analysis. Strains were pre-grown in YPD at 38 C for –16 hours. They were re-inoculated after disruptants were equalized and mixed with wild type according to 0.5% wet weight in YPD25 and incubated at 38 C for ~50 hours. Weight loss was monitored to get the percentage alcohol produced and simultaneously 10-fold serial dilutions were prepared and plated at different time points for percentage survival of the mutants (Fig.3B).

RESULTS AND DISCUSSION

It has been shown that neither the deletion nor the disruption of the unique hsp26 gene in yeast has any detectable effect on the ability of cells to grow and develop at various temperatures or to withstand the adverse effects of heat at any point during the lifecycle. Deletion of the hsp26 gene in hsp104 mutant background has been shown to have no effect on basal or induced thermotolerance (Sanchez et al., 1993). Cells lacking constitutive HSP70 exhibit a poor growth rate and are known to over-express HSP26 and some other HSPs. HSP26 over expression modulates the growth rate of yeast cells lacking constitutive HSP70, similar to mammalian cells (Unno et al., 1998). There are several possible explanations for the lack of a phenotype in the hsp26- mutants. First the primary role of hsp26 may be to promote growth at high temperatures or enhance thermotolerance but its effect may be too subtle to be detected (Petko and Lindquist, 1986). Secondly it may have a functional redundancy. Overlapping functions have been found for members of the hsp70 and hsp84 gene families, but only for closely related genes (Craig and Jacobsen, 1984). Keeping this in mind we wanted to see the effect in presence and absence of hsp42 which is regulated similarly by a range of stress conditions. HSP42 shares a high degree

of homology with HSP26 over a conserved hydrophobic stretch of about 35 amino acids located near the carboxyl terminus of the protein (Wotten et al.,1996). Its expression is up regulated by increase in temperature and salt concentration as well as by conditions of limited growth and overgrowth of cell cultures (Wotten *et al*., 1996). Interestingly although hsp26 expression is also up regulated under all these conditions there are differences in the response of these two genes to the various conditions of stress. The function of this highly conserved small HSP may be advantageous for growth only under very particular circumstances that have not been tested experimentally but have been utilized for selection throughout evolution. As S. cerevisiae is well known to have the ability to take up and ferment a wide range of sugars and is extensively used in ethanol production; we went on to check the role of hsp26 and hsp42 during ethanolic fermentation Considering the above-mentioned facts to be true, question remains as to why then hsp26 has remained conserved for more than a billion years as separate, abundantly induced protein in all eukaryotes.

Gene disruption is a powerful tool for determining the biological function of proteins encoded by uncharacterized ORFs. Information about the biological function can be inferred by monitoring the fitness of the resulting deletant strain under a variety of selective growth conditions. Simultaneous culture of a particular deletant along with wild type or other deletion mutants invokes a competitive environment, rendering subtle differences to show up, which otherwise would have been overlooked (Shoemaker et al., 1996). Ethanol fermentation is a natural phenomena occurring in yeast and hsp26 may be playing some role along with its homologue hsp42. Keeping this in view we went on to check the role of hsp26 in mixed population during ethanolic fermentation.

The haploids having ura+ background were disrupted with kanMX4 cassette to create single and double disruptants at the hsp26 and hsp42 locus. These disruptants did not result in any change in the growth phenotype. It was confirmed that the mating type does not cause any change in growth rate of the disruptants. It has been earlier reported (Petko & Lindquist, 1986) that hsp26 is expressed at very low levels during exponential growth at 23°C or 37°C, and that it is induced not only after heat shock but also when the cells approach stationary phase and under conditions of starvation and sporulation. We studied the effect of hsp26 disruption during stationary phase and found that hsp26 plays no direct role in survival of cells during stationary phase.

A vast array of treatments can induce synthesis of hsps (For review, see Nover *et al*, 1984). Among these, exposure to heat and ethanol have been shown to induce tolerance to both in many organisms (Neidhardt *et al.*, 1984; Plesset et.al., 1982; Li, 1983). We tested the ability of the mutants and the wild type to survive in these stress conditions in mixed culture fermentations both at 30°C and 38°C. While we found no effect at 30 C, a dramatic effect was observed at 38 C in mixed culture population, in the form of nearly 40-50 fold decrease in the survival of hsp26 disruptants as compared to wild type in 48 hours. Results were confirmed by repeating the experiment thrice [Fig. 3]. Therefore, through these experimental results we conclude that hsp26 does play a crucial role in the survival of yeast during high temperature fermentation.

This is in contrast to earlier reports where hsp26 has been shown to have no significant role (Susek and Lindquist, 1989; Petko and Lindquist, 1986). These results also explain the highly conserved pattern of expression of hsp26 indicating the involvement of small hsps in thermotolerance.

Fig. 1 Cloning strategy for gene disruptions using KanMX4 module: (A) Wild type hsp26 was digested with BamH1 and EcoR1 to get 2 fragments of size 368bp and 178bp resp. pFA6a with KanMX4 module was digested with BamH1 and EcoR1 to get a third fragment of size 1.4kb.The three fragments were ligated and transformed into yeast for disruption with homologues recombination. The disruption in the transformants showing successful integration was confirmed by PCR using hsp26 S1-KanMX4 A1 and hsp26 A1-KanMX4 neo1 primer pairs. (B) Similarly wild type hsp42 gene was digested with EcoRV and pFA6a with KanMX4 module was digested with Sma1 in presence of ligase buffer and then hsp42 fragment was added for ligation. Successful integration of marker will give PCR products using hsp42 A and KanMX4 A1, KanMX4 neo1 and hsp42 S primer pairs. Fig. 2 Agarose gel showing DNA products generated by diagnostic PCRs for various disruptions of hsp26 and hsp42: lane 1 and lane 14 are lambda Hind III marker ; lane 2, IG12 wt: hsp26 A1-hsp26 S1(1.223 kb) ; lane 3, IG 16(8C) hsp 26 : hsp26 A1-KanMX4 neo1 (0.561bp) ; lane 4, IG 16 (8C) hsp26 : hsp26 S1-MX4A1 (0.951bp) ; lane5, IG15 hsp42 : hsp26 A1-hsp26 S1 (1.223kb) ; lane6, IG17(5C) hsp26 & hsp42 : hsp26 A1-KanMX4 neo1 (0.561bp); lane7 IG17 (5C) hsp26 & hsp42 : hsp26 S1-MX4 A1 (0.951bp); lane 8, IG12 wt: hsp42 A-hsp42 S (1.17 kb); lane 9, IG 16(8C) hsp26 :hsp42 A-hsp42 S (1.17kb); lane 10, IG15 hsp42 : hsp42 A-MX4 A1 (1.143kb); lane11, IG15 hsp42 : hsp42 S-KanMX4 neo1 (3.7 kb); lane 12 IG17(5C)) hsp26 & hsp42 : hsp42 A-KanMX4 A1(1.143kb); lane 13, IG17(5C) hsp26 & hsp42 : hsp42S-KanMX4 neo1(3.7kb).

Fig. 3 showing survival of mutants in mixed culture during growth at 38° C in mixed culture growth of IG15-hsp42 with IG12 wt; IG17 (5C)-hsp42 & hsp26 with IG12 wt; IG16 (8C)-hsp26 with IG12 wt. (B) Percentage of mutant survival in mixed culture fermentation at 38° C in mixed culture fermentation of IG15-hsp42 with IG12 wt; IG17 (5C)-hsp42 & hsp26 with IG12 wt; IG16(8C)-hsp26 with IG12 wt.

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