LEA (late embryonic abundant)-like protein Hsp 12 (heat-shock protein 12) is present in the cell wall and enhances the barotolerance of the yeast *Saccharomyces cerevisiae*

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Yeast cells *Saccharomyces cerevisiae*, late embryogenic abundant-like stress response protein Hsp 12 (heat-shock protein 12) were found by immunocytochemistry to be located both in the cytoplasm and in the cell wall, from where they could be extracted with dilute NaOH solutions. Yeast cells with the Hsp 12 gene disrupted were unable to grow in the presence of either 12 mM caffeine or 0.43 mM Congo Red, molecules known to affect cell-wall integrity. The volume of yeast cells were less affected by rapid changes in the osmolality of the growth medium when compared with the wild-type yeast cells, suggesting a role for Hsp 12 in the flexibility of the cell wall. This was also suggested by subjecting the yeast cells to rapid changes in barometric pressure where it was found that wild-type yeast cells were more resistant to cellular breakage.

Key words: alkaline extraction, heat-shock protein 12 (Hsp 12), immunocytochemistry, late-embryonic-abundant-like protein (LEA-like protein), *Saccharomyces cerevisiae*, yeast.

INTRODUCTION

Barotolerance, the ability of organisms to remain viable after exposure to hydrostatic pressure, has been reported to be induced by heat shock treatment in yeast cells (*Saccharomyces cerevisiae*) [1]. Recently, it has been shown that yeast cells with the *TPS 1* (trehalose-6-phosphate phosphatase) gene deleted are sensitive to hydrostatic pressure [2], displaying an altered morphology owing to cytoskeletal deformation after the application of 200 MPa pressure. This altered morphology, which was ascribed to resultant changes in the cell wall, was not observed when the yeast cells were subjected to heat shock before the application of pressure. Rescue of the barosensitive Δ*tps* phenotype by heat shock suggests that a heat shock protein (Hsp) might fulfil some function in the cell wall.

Hsp 12 is a small hydrophilic LEA (late embryogenic abundant)-like protein, the concentration of which increases markedly after heat shock [2,3] on entry into stationary phase and when yeast cells are subjected to different forms of osmotic stress [4]. We have shown previously, using immunocytochemistry, that Hsp 12 is located in the vicinity of the plasma membrane [5]. Hsp 12 might be a potential candidate to rescue trehalose-deficient yeast cells from barosensitivity, since not only is Hsp 12 synthesized in response to heat stress [6,7], but we have also shown, using a model membrane system, that Hsp 12 acted in a manner analogous to trehalose in that Hsp 12 afforded protection of membrane integrity against desiccation- and ethanol-induced stress [5]. The immunocytochemical experiments reported previously were performed using thin sections embedded in epoxy resin [5]. The results showed that most of the gold particles were located on the external side of the plasma membrane, with a few particles present on the cytoplasmic side. In addition, a few particles were also seen in the area of the cell wall. Since cryosectioning has been reported to have a number of significant advantages over embedding into resin [8], notably, reduced antigen damage, increased antigen exposure and preservation of ultrastructure [9], we investigated whether this methodology might allow us to detect Hsp 12 in locations other than those surrounding the plasma membrane.

The present study reports that Hsp 12 was detected not only in the cytoplasm but also in substantial quantities in the vicinity of the cell wall. We therefore investigated the possible role of Hsp 12 in the cell wall. Our results indicated that the presence of this protein correlated with increased cell-wall flexibility coupled with a pressure-resistant phenotype.

METHODS

Yeast strains and growth conditions

The yeast strains used (gifts from Dr P. Meacock at the Department of Genetics, University of Leicester, Leicester, U.K.) were the haploid segregants from the diploid strain 842 (*a*/α, *ade2-1*/*ade2-1*, *trp1-1*/*trp1-1*, *leu2-3*/*leu2-112*, *his3-11*/*his3-15*, *ura3*/*ura3*, *canr1-100*/*CAN*). The Hsp 12 knockout was constructed and tested as described in [6]. Growth media and growth conditions were as described previously [4,5]. NaOH extraction of whole yeast cells was performed at 0 °C as described previously [10]. Packed yeast cells (100 mg) were washed with 100 mM Tris/HCl, 50 mM NaCl (pH 7.4) buffer before alkaline-soluble proteins were extracted with NaOH for 30 min on ice. The concentrations used were between 0.1 and 1 M NaOH.

Volume determination

Average yeast cell volume was determined by laser light scattering using a Malvern Mastersizer (Malvern Instruments, Malvern, Worcs., U.K.). Yeast cells were grown in YEPD (yeast extract peptone dextrose: 1 % yeast extract, 2 % peptone, 2 % glucose) medium (approx. 320 mosmol/l) to stationary phase, concentrated by centrifugation and washed with 10 mM Tris/HCl, 150 mM NaCl (pH 7.4) before being added to 1000 vol. of either 10 mM Tris/HCl and 1.15 M NaCl (pH 7.4) or distilled water. The average volume of 2000 cells was determined.

Abbreviations used: GFP, green fluorescent protein; Hsp, heat-shock protein; LEA, late embryogenic abundant; YEPD, yeast extract peptone dextrose.

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French press and ball mill

The effect of pressure changes on yeast cells was determined using a pressure cell (Aminco) of 25.4 mm diameter. Yeast cells (D_{600} 5.2) in 30 ml of 100 mM Tris/HCl and 50 mM NaCl (pH 7.4) were placed in a pressure cell at room temperature (20 °C). The cell was placed under a mechanical press and the pressure increased to the stated pressure. A part of the yeast suspension (1 ml) was released into an Eppendorf tube at atmospheric pressure while maintaining the pressure on the cell. The released yeast cells were centrifuged for 5 min at 10 000 ***g***, and the A_{280} of the supernatant, representing yeast cellular contents, was determined. The effect of abrasion on yeast cells was determined using a ball mill (Braun). Yeast cells (D_{600} 3.9) in 20 ml of 100 mM Tris/HCl and 50 mM NaCl (pH 7.4) were placed together with 6 g of glass beads of diameter 0.75–1.125 mm in a vessel at 4 °C. The vessel was then shaken while maintaining the temperature of the vessel at 4 °C with CO_2. The degree of yeast damage was determined from the A_{280} of the supernatant after centrifugation as before.

Immunochemical methods and electron microscopy

The anti-Hsp 12 antiserum used for Hsp 12 detection was from the same source as that used previously [5]. Antibodies to Hsp 12 were separated from contaminants by binding the antiserum to Hsp 12, itself bound to nitrocellulose, and released at low pH [11]. Immunocytochemistry was performed as described previously [12], except that the yeast cells were fixed using 0.5 % glutaraldehyde, 4 % (v/v) paraformaldehyde in K_2HPO_4 (pH 7.0) for 16 h at 4 °C, embedded in low-melting-point agarose, and infiltrated with 2.3 M sucrose as a cryoprotectant. Then they were mounted on small stubs and rapidly plunge-frozen in liquid nitrogen (− 196 °C). Ultrathin sections were cut at − 120 °C using a Reichert Ultra Cut S cryomicrotome. Sections were retrieved on to carbon-coated, glow-discharged nickel grids and immunolabelled as described previously [13].

RESULTS

Hsp 12 is present in the cell wall

We have shown that extraction of whole yeast cells with 0.2 M NaOH resulted in the extraction of a small, select group of proteins leaving the yeast cell intact [10]. This group of proteins included phosphoglycerate mutase, which was shown using immunocytochemistry to be located in the cell wall, and Hsp 12. To investigate the optimum extraction conditions for Hsp 12 from whole yeast cells, cells were incubated for 30 min at 0 °C with different concentrations of NaOH solutions between 0.1 and 1 M. We found that Hsp 12 was present in the extract irrespective of the strength of NaOH used, with optimum extraction observed with 0.6 M NaOH (Figure 1A). Western-blot analysis [5] and matrix-assisted laser-desorption ionization–time-of-flight MS were used to verify that the 12 kDa protein present on the gel (Figure 1B) was indeed Hsp 12 after trypsin digestion [10]. Since extraction of yeast cells with 0.2 M NaOH had no visible effect on the cells when treated cells were examined by electron microscopy [10], we concluded that Hsp 12 was present in the cell wall. Extraction at high pH might suggest an ionic interaction between Hsp 12 (pI ∼ 5) and cell-wall mannoprotein phosphate groups in YEPD medium, which also has a pH ∼ 5. To investigate the presence of such an interaction, the extraction was performed using 2 M NaCl, 10 mM Tris/HCl (pH 7.4) for 30 min at 0 °C; no Hsp 12 was found to be extracted using this buffer (results not shown). We next investigated whether loss of disulphide bridges between cell-wall proteins might allow the extraction of Hsp 12. Yeast cells were therefore extracted in 10 mM Tris/HCl, 50 mM NaCl, 2 % (v/v) 2-mercaptoethanol (pH 7.4) for 30 min at 0 °C, and the extracted protein analysed by SDS/PAGE. Again no Hsp 12 was observed on the gel (results not shown). Similarly, extraction under acidic conditions, e.g. 1 M HCl, failed to extract any Hsp 12.

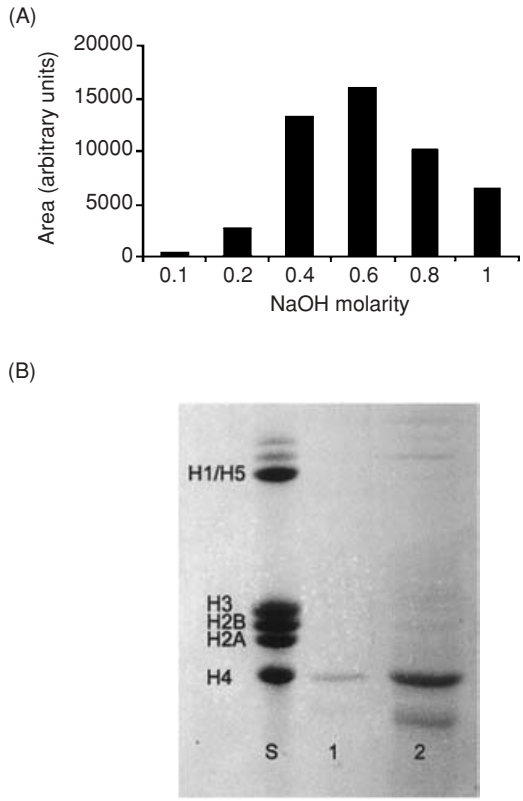

Figure 1 Relationship between Hsp 12 extraction and NaOH molarity

(**A**) Whole yeast cells were extracted with NaOH ranging between 0.1 and 1 M and the extracts separated by SDS/PAGE (cf. **B**). Hsp 12 was quantified by densitometry after Coomassie Brilliant Blue staining. The results shown are typical of three separate experiments. (**B**) SDS/PAGE of a 0.8 M NaOH extract of whole yeast cells (lane 2). A total heat-soluble fraction of yeast cells is also shown (lane 1). The standard (S) is a total extract of chicken erythrocyte histones, their approximate molecular masses (kDa) being H1, 22.5; H3, 15.3; H2B, 13.7; H2A, 14.0, H4, 11.2.

To confirm that Hsp 12 was indeed present in yeast cell walls, thin sections, prepared by cryosectioning yeast cells, were probed with an antiserum specific for Hsp 12. The location of this antibody was determined by electron microscopy using a colloidal gold-labelled goat anti-rabbit antibody. Examination of the sections (Figure 2) showed that gold particles were present not only close to the plasma membrane as reported previously [5], but also both in the cytoplasm and in the cell wall. Most of the particles were found to be in the area of the cell wall. Control sections of the yeast cells failed to show labelling (results not shown, but see [5]).

Since Hsp 12 has no features associated with cell-wall proteins, we next investigated whether the presence of Hsp 12 in the cell wall was an artifact of the experimental conditions. Since Hsp 12 is an abundant protein, it is possible that the presence of Hsp 12 in the cell wall might be brought about by lysis of some yeast cells releasing Hsp 12 into the medium, followed by binding of the protein to the cell wall. We initially investigated this possibility by using a commercial yeast preparation grown in a batch

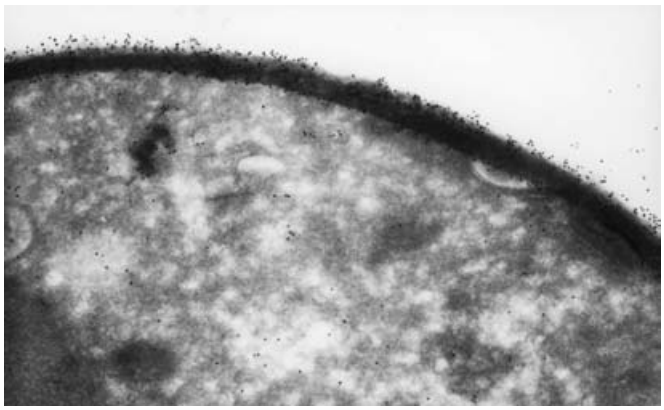

Figure 2 Electron micrograph of an immunogold-labelled yeast cell probed with rabbit anti-Hsp 12 antiserum

The magnification used was × 30 000. The gold particles used were of 10 nm diameter.

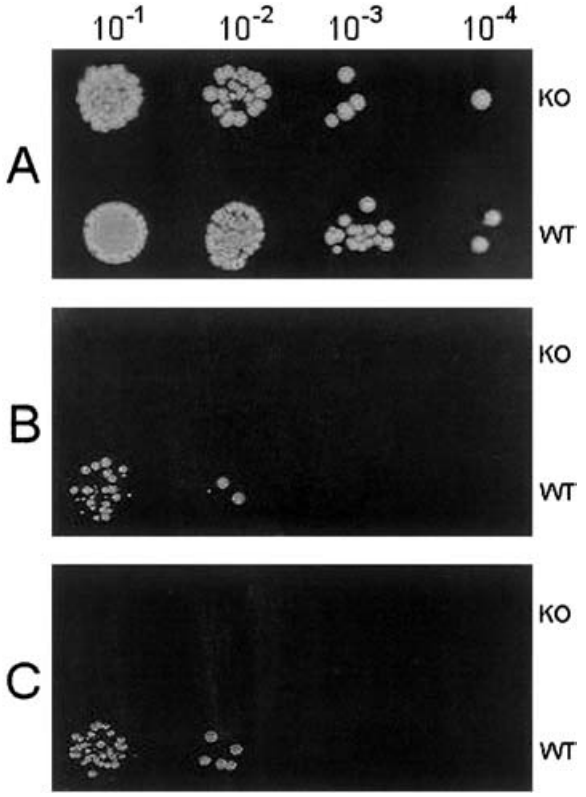

Figure 3 Growth at 30 °C for 48 h of wild-type (WT) yeast and yeast with the Hsp 12 gene disrupted (KO)

(**A**) On plain YEPD agar plates, (**B**) YEPD agar plates incorporating 12 mM caffeine or (**C**) 0.43 mM Congo Red. Values above the panels indicate the dilution of the yeast culture applied to the plate.

fermenter and not subjected to any mechanical stress. Hsp 12 was extracted from this strain with NaOH in an identical manner (results not shown). We next investigated release of protein into the medium during growth in a shaking culture by determination of the fluorescence of *Aequoria* GFP (green fluorescent protein) in the medium of recombinant yeast cells expressing GFP in the cytosol. These yeast cells were grown in YEPD medium in shaking culture at 250 rather than at 50 oscillations/min to enhance any possible mechanical damage, and the fluorescence of the cells and the medium determined after 16 h of growth. Although the cells fluoresced brightly when viewed using a fluorescent microscope, no GFP was detected in the medium (results not shown). It would therefore appear that neither mechanical damage to the yeast cells nor cell lysis was responsible for releasing significant quantities of cytosolic proteins into the medium. Since Hsp 12 might interact differently with proteins not native to yeast, we investigated whether Hsp 12 was present in the medium during yeast growth. YEPD medium from yeast cells grown to early stationary phase was concentrated by ultrafiltration, and proteins in the retained solution analysed by SDS/PAGE. No Hsp 12 was present on the gel (results not shown). Since lysis and secondary binding might have been a rapid events, we next cultured yeast cells in YEPD medium buffered to pH 7.4 with Tris/HCl and investigated the presence of Hsp 12 in the medium as well as in the cell wall by alkaline extraction. Hsp 12, which has a pI of approx. 5.0, calculated from the amino acid composition [5], would be negatively charged at this pH and therefore unlikely to interact electrostatically with negatively charged cell-wall mannoprotein phosphate groups. SDS/PAGE analysis of the concentrated medium failed to detect Hsp 12, and Hsp 12 was extracted from the cell wall by 1 M NaOH in a manner similar to the Hsp 12 extracted from yeast cells grown in unbuffered YEPD medium (results not shown). We therefore concluded that Hsp 12 naturally occurs in the cell walls of yeast rather than 'artifactually' associating with the cell walls during experimental manipulation.

Caffeine and Congo Red have been used to screen for yeast mutants with altered cell-wall architecture [12,14]. These molecules affect the stability of the cell wall and thereby enhance any defects brought about by unidentified mutations to cell-wall proteins and/or structure. We therefore compared the growth of wild-type yeast and yeast with the Hsp 12 gene disrupted [6] on YEPD plates containing either 12 mM caffeine or 0.43 mM Congo Red. Whereas growth of the wild-type yeast was inhibited by the presence of either caffeine or Congo Red, yeast cells with the Hsp 12 gene disrupted failed to grow in the presence of either (Figure 3), suggesting that Hsp 12 plays some role in maintaining the stability of the cell wall.

On account of their high hydrophilicity, it has been proposed that the function of LEA proteins in plant seeds might be to act as water-replacement molecules in the desiccated state [14a]. The stability of the yeast cell wall is in part due to the hydrogen-bonded interactions between the various polysaccharides. Hsp 12 is synthesized in response to the presence of mannitol, salt or ethanol in the medium [4,5], all of which result in osmotic stress and a decreased water activity. Since hydrogen-bonded interactions between polysaccharides increase in response to a decreased water activity, we investigated whether the presence of Hsp 12 affected the flexibility of the cell wall.

To investigate the effect of Hsp 12 on cell-wall flexibility, we compared the response of wild-type yeast and yeast with the Hsp 12 gene disrupted to rapid changes in the osmotic strength of the environment (Figure 4). Yeast cells were grown in YEPD medium (approx. 320 mosmol/l) to stationary phase, concentrated by centrifugation and the concentrated yeast added to 1000 vol. of high-osmolality buffer (approx. 1320 mosmol/l). The volume of the yeast cells was monitored by laser light scattering immediately after addition to the high-osmolality buffer and at 15 s intervals thereafter (Figure 4A). The volume of yeast cells in YEPD buffer and immediately after addition to the high-osmolality buffer were identical, demonstrating that no change in cell volume

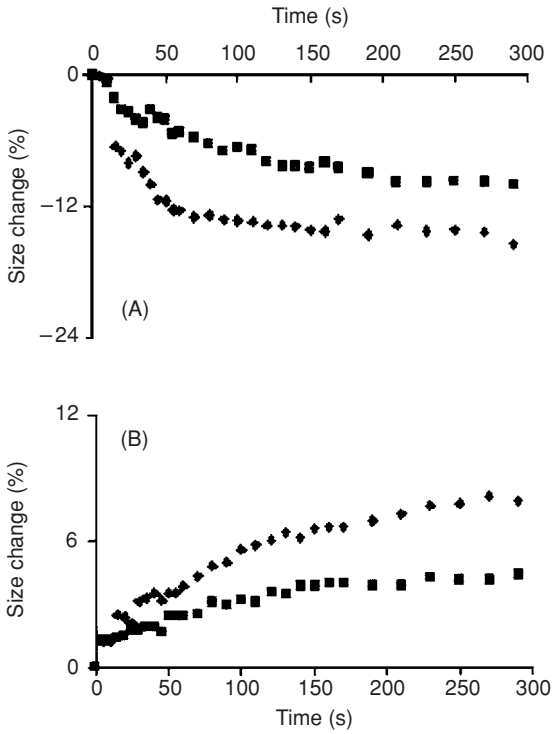

Figure 4 Changes in yeast cell volume as a function of time

(**A**) Changes after an increase of approx. 1000 mosmol/l in the osmolality of the medium. Wild-type (◆) and yeast with the Hsp 12 gene disrupted (■) were grown in YEPD medium to stationary phase, washed with 10 mM Tris/HCl, 150 mM NaCl (pH 7.4) and then added to 1000 vol. of 10 mM Tris/HCl, 1.15 M NaCl (pH 7.4). The average volume of 2000 cells was determined by laser light scattering. The results shown are typical of three separate experiments. (**B**) As for (**A**) above except that the cells were added to 1000 vol. of distilled water.

occurred between addition of the cells to this buffer and the time required for the volume of 2000 cells to be determined (results not shown). Immersion of wild-type yeast cells in high osmolality buffer resulted in a rapid decrease in the volume of the cells with an approx. 13 % decrease observed after 1 min. Thereafter, the cells continued to decrease in volume but at a far slower rate, with an approximate volume decrease of 17 % being observed after 10 min. Note that the data shown in Figure 4 only reflect changes in the size of yeast cells for 5 min after changing the osmolality of the medium. In contrast, the rate of decrease in the volume of yeast cells with the Hsp 12 gene disrupted was markedly slower, with approx. 5 % decrease observed after 60 s and approx. 8 % decrease observed after 10 min. No change in cell volume was observed when cells of either wild-type yeast or yeast with the Hsp 12 gene disrupted were immersed in a solution of equal osmolality as a control.

Since the change in the volume of wild-type yeast cells was approximately double that of yeast with the Hsp 12 gene disrupted in response to an increase of 1000 mosmol/l in the environment, we next investigated whether a similar response would be observed when the osmotic strength of the environment decreased. Accordingly, the same concentrated yeast cells were added to distilled water and the volume of the cells determined as a function of time as before. We found (Figure 4B) that immersion of yeast cells in distilled water resulted in a rapid increase in the volume of the cells with the response of the wild-type cells approximately double that of yeast with the Hsp 12 gene disrupted. Whereas the volume of the latter cells increased by approx. 4.5 % after

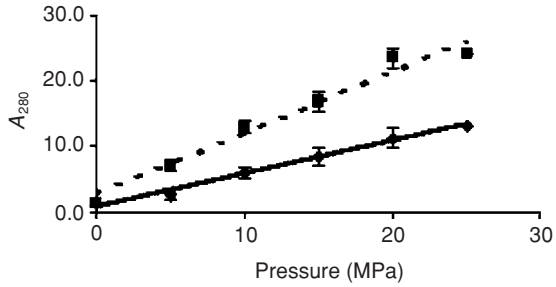

Figure 5 Effect of increasing hydrostatic pressure on cell breakage

Hydrostatic pressure was applied using a French press. Yeast cells were grown in shaking culture to early stationary phase in YEPD medium and cell disruption was determined by the A_{280} of the supernatant fraction. ◆, Wild-type yeast; ■, yeast with the Hsp 12 gene disrupted. The results shown are from four separate experiments.

7.5 min, wild-type cells increased by approx. 8.8 % over the same period. Although these volume increases were approximately half of those seen when the yeast cells were suddenly exposed to increased osmolality, it must be noted that the change in the osmolality between YEPD medium and distilled water was one-third of that between YEPD medium and high-osmolality buffer.

The changes in yeast cell volume observed in response to changes in the osmolality of the environment could either be ascribed to Hsp 12 affecting the ingress or egress of water or alternatively to an altered flexibility of the cell wall brought about by Hsp 12. We would consider that the former is quite probably governed by the difference in osmolality of the cytoplasm and that of the external environment, which was identical for wild-type yeast and yeast with the Hsp 12 gene disrupted. The latter hypothesis was therefore tested by comparing the sensitivity of wild-type yeast and yeast with the Hsp 12 gene disrupted on subjecting yeast to increased barometric pressure. Both yeast strains were subjected to a maximum of 25 MPa pressure using a French press pressure cell, and then returned rapidly to atmospheric pressure. Cell disruption was assayed by determination of the A_{280} of the supernatant fraction after centrifugation. Approximately double the amount of cell breakage occurred in yeast cells with the Hsp 12 gene disrupted compared with wild-type yeast irrespective of the pressure to which the preparations were subjected (Figure 5).

We next compared the abrasion resistance of wild-type yeast and yeast with the Hsp 12 gene disrupted, since the abrasion resistance of plastics is inversely proportional to the elasticity [15]. Yeast cells were disrupted using a ball mill for increasing time at 4 °C and cell breakage was again assessed by determining the release of soluble material at A_{280}. We found that the presence of Hsp 12 slightly decreased the abrasion resistance of yeast cells (Figure 6). Approx. 10 % less abrasion was observed if Hsp 12 was not present when yeast cells were treated for 120 s.

DISCUSSION

In this study, we have demonstrated that considerable quantities of the stress-response protein, Hsp 12, are present in the cell walls of *S. cerevisiae*. Immunocytochemistry after cryo-sectioning rather than after embedding in resin [5] showed considerably more Hsp 12 to be present, particularly in the cell wall. This was probably because antigenic determinants present on Hsp 12 are affected by the latter process, as reported previously

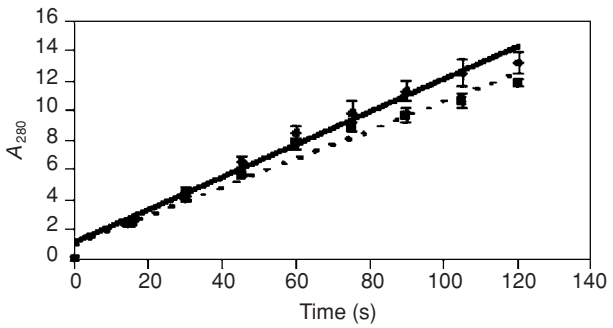

Figure 6 Effect of abrasion time on cell breakage

Cells were abraded in the ball mill at 4 °C by the action of 0.75–1.125 μm glass beads. Yeast cells were grown in shaking culture to early stationary phase in YEPD medium and cell disruption was determined by the A_{280} of the supernatant fraction. ◆, Wild-type yeast; ■, yeast with the Hsp 12 gene disrupted. The results shown are from four separate experiments.

[8,9]. Circumstantial evidence in favour of an exterior location of Hsp 12 in the yeast cell has been suggested by other authors [16], who have reported that Hsp 12 is essential for biofilm formation in a Sardinian wine strain of *S. cerevisiae*.

Our results indicate that disruption of the Hsp 12 gene results in yeast cells with less ability to respond to increased pressure. This therefore suggests that Hsp 12 plays some function in the cell wall that results in a wall with increased flexibility. Evidence in favour of Hsp 12 playing a crucial role in the flexibility of the cell wall is that the application of hydrostatic pressure to trehalose-deficient yeast mutants resulted in cytoskeletal deformation. However, if these mutants were heat-shocked before the application of hydrostatic pressure, no effects were observed [2]. A brief heat shock is known to result in transcription of the Hsp 12 gene [6,7].

It is clearly important for the yeast cell to be able to respond rapidly to changes in its environment. The cell wall largely comprises polymers of uncharged sugar molecules with the β-1,3 glucans primarily responsible for the mechanical strength of the cell wall [17,18]. These polysaccharides interact with one another via H bonding and, for charged polysaccharides, ionic interactions. These interactions would result in stable inflexible structures akin to that of long-chain starch molecules, since the β-1,3 glucans have been reported to exist as helical structures [19]. Yeast cells, when subjected to stress, have been shown to express a variety of cell-wall proteins including components of the 1,3-β-glucan synthase Fks1p and Fks2p [3,20]. Whereas these enzymes would allow the insertion of residues into or deletion of residues from the cell wall, a requirement for growth and bud development, they would not allow the yeast cells to adapt to environmental changes that caused alterations in wall elasticity. Our hypothesis is that Hsp 12, which is a highly hydrophilic protein, interrupts the H bonding and ionic interactions between adjacent glucan chains. Such a role for Hsp 12 is supported by the results presented in the present study, which demonstrated that yeast cells with the Hsp 12 gene disrupted were unable to grow in the presence of Congo Red. This bi-functional dye has been proposed to target the helical components of the glucan network [21], presumably by interacting with adjacent helices via the 4-amino-1-naphthalenesulphonic acid residues. We would envisage that Congo Red competes with Hsp 12 for the same binding sites thereby preventing yeast growth.

We would postulate that hyperosmolar environmental conditions would result in increased hydrogen bonding between adjacent polysaccharide polymers in the cell wall as the water activity of the environment would be reduced. Hydrogen bonding between adjacent polysaccharide polymers via Hsp 12 would allow adjacent chains to move relative to one another in a manner analogous to the function of plasticizers in plastic polymers, which decrease the glass transition temperature of the polymer. This hypothesis would explain the synthesis of Hsp 12 in response to environmental stress. Although we have investigated the relationship between Hsp 12 and the adaptation of the yeast cell wall to environmental stress, Hsp 12 might not be the only protein involved in this process. A number of other proteins expressed after heat shock have been reported to be present in the cell wall [22,23]. The observed decreased abrasion resistance of yeast cells expressing Hsp 12 compared with the knockout strain is similar to the decreased abrasion resistance found with plastic polymers, where an inverse relationship between hardness/flexibility and abrasion resistance has been reported [15].

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