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Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H⁺-ATPase

Peter. W. Piper[1], Claudia Ortiz-Calderon[1], Caroline Holyoak[1,2], Peter Coote[2] and Martin Cole[2]

[1]Department of Biochemistry and Molecular Biology, University College London, London WC1E 6BT, UK
[2]Microbiology Division, Unilever Research, Colworth Laboratory, Sharnbrook, Bedford MK44 1LQ, UK

Abstract *Saccharomyces cerevisiae* has a single integral plasma membrane heat shock protein (Hsp). This Hsp30 is induced by several stresses, including heat shock, ethanol exposure, severe osmostress, weak organic acid exposure and glucose limitation. Plasma membrane H⁺-ATPase activities of heat shocked and weak acid-adapted, *hsp30* mutant and wild-type cells, revealed that Hsp30 induction leads to a downregulation of the stress-stimulation of this H⁺-ATPase. Plasma membrane H⁺-ATPase activity consumes a substantial fraction of the ATP generated by the cell, a usage that will be increased by the H⁺-ATPase stimulation occurring with several Hsp30-inducing stresses. Hsp30 might therefore provide an energy conservation role, limiting excessive ATP consumption by plasma membrane H⁺-ATPase during prolonged stress exposure or glucose limitation. Consistent with the role of Hsp30 being energy conservation, Hsp30 null cultures give lower final biomass yields. They also have lower ATP levels, consistent with higher H⁺-ATPase activity, at the glucose exhaustion stage of batch fermentations (diauxic lag), when Hsp30 is normally induced. Loss of Hsp30 does not affect several stress tolerances but it extends the time needed for cells to adapt to growth under several stressful conditions where the maintenance of homeostasis will demand an unusually high usage of energy. *hsp30* is the first yeast gene identified as both weak organic acid-inducible and assisting the adaptation to growth in the presence of these acids.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* presents several advantages for studying the stress responses of a model eukaryotic cell. Many genes of stress protection have been characterized in this organism and loss-of-function mutants analysed. Such studies have revealed certain major yeast heat shock proteins (Hsps) as having roles in the chaperoning, reactivation or degradation of partially unfolded or aggregated protein. Still others contribute to antioxidant defences or are without any clearly demonstrable function (reviewed in Mager and Moradas-Ferreira 1993; Parsell and Lindquist 1993; Piper 1993; Moradas-Ferreira et al 1996). Discrete gene promoter elements responsive to stress have also been identified (Sorger 1991; Marcher et al 1993; Moradas-Ferreira et al 1996) and stress-responsive signal transduction pathways are beginning to be unravelled, some of which influence the activity of these promoter elements (reviewed by Thevelein 1994).

While studies on the functions of most *S. cerevisiae* Hsps have now been reported, this is not the case for the single Hsp associated with plasma membrane. This Hsp30 is a highly hydrophobic integral membrane protein,

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Correspondence to: P. W. Piper, Tel: +44 171 387 7050; Fax: + 44 171 380 7193; E-mail: piper@bsm.bioc.ucl.ac.uk

shown in earlier work to be induced by heat shock, ethanol and carbon source limitation (Panaretou and Piper 1992; Régnacq and Boucherie 1993; Piper et al 1994). Hsp30 has been found to be primarily plasma membrane-associated in both membrane fractionation (Panaretou and Piper 1992) and immunofluorescence studies (M. Régnacq, unpublished data). Stress proteins localizing to the plasma membrane have also been identified in other eukaryotic systems, for example a 30 kDa Hsp in soybean roots (LaFayette and Travis 1990) and an osmostress-induced 60 kDa protein in the alga *Dunaliella* (Fisher et al 1994). However, for none of these proteins and for no eukaryotic integral plasma membrane Hsp has a role yet been established. We have therefore conducted detailed studies on an *hsp30* mutant yeast and its wild-type parent in order to establish a function for yeast Hsp30.

MATERIALS AND METHODS

Yeast strains

This study employed *S. cerevisiae* strains with a multiply protease-deficient genetic background in order to avoid the problems of proteolysis that can arise when purifying plasma membranes with intact proteins from stressed yeast cells (Panaretou and Piper 1992). These strains were BJ2168 (**a** *leu2–3, 112, trp1, ura3–52, prb1–1122, pep4–3, prc407, gal2*) and KT3 (isogenic to BJ2168 except for a *ura3* gene fragment insertional inactivation of the *hsp30* gene, at the *Hin*dIII site (+18) within the *hsp30* open reading frame). KT3 was generated by:

1. HindIII digestion of pSP-Hsp30 (a vector comprising the 1.4 kb Pst1–BamH1 fragment of pUC19–Hsp30 (Régnacq and Boucherie 1993) inserted into Pst1 plus BamH1-digested plasmid pSP46 (Melton et al 1984) in which the polylinker *Hin*dIII site had been inactivated.
2. Infill of this DNA, then tailoring with Bcl1 termini using Bcl1 linkers.
3. Ligation to the 1.1 kb *ura3*-containing fragment released by BamH1 digestion of YDpU (Berben et al 1991), thereby yielding phsp30::URA3.
4. Creation of strain KT3 by transformation of BJ2168 to uracil prototrophy with the 1.1 kb Kpn1 plus BamH1 fragment of phsp30::URA3.

The *ura3* fragment disruption of the *hsp30* locus of strain KT3 (*hsp30::ura3*) was confirmed by Southern analysis.

Yeast culture

Shake-flask cultures for plasma membrane isolation were grown with aeration to mid-exponential phase at 28°C (5×10^6–1×10^7 cells ml^{-1}) on liquid YPD medium (2% bactopeptone, 1% yeast extract, 2% glucose) previously titrated with HCl to pH 5.74, pH 4.5 or pH 3.8. Sodium chloride or sorbic acid (added from a pH 7.0 1 M potassium sorbate stock solution), where present, were added to media prior to autoclaving. To heat shock cells cultures were shifted to 40°C for the stated times prior to harvesting.

Plasma membrane purification and plasma membrane H^+-ATPase assay

Plasma membranes for plasma membrane H^+-ATPase analysis were purified by banding on isopycnic sucrose gradients and their purity assessed as described by Panaretou and Piper (1992). Protein determinations were performed using the Bio-Rad Protein Assay Kit and bovine serum albumin as standard. The plasma membrane H^+-ATPase activities shown were determined on sucrose gradient-purified plasma membrane preparations, according to the procedure of Serrano (1988), with the inclusion of molybdate, nitrate and azide in the assay mixture (to inhibit any residual contamination by acid phosphatase, mitochondrial ATPase and vacuolar ATPase respectively). Throughout this study, plasma membrane H^+-ATPase activities are expressed as units mg^{-1} of total plasma membrane protein, 1 unit being 1μmol Pi released min^{-1} under the assay conditions of Serrano (1988).

Fermentation and ATP analysis

For study of the effects of low pH, sorbic acid or salt on glucose batch fermentation a starter culture of each strain was initially grown to exponential phase (OD_{600nm} 0.6) in YPD medium containing no stress agent. This was then used to inoculate pH 5.74, pH 4.5 or pH 3.8 YPD (with or without the stated sorbic acid or NaCl levels) at 5×10^3 cells ml^{-1}. Care was taken to ensure identical numbers of wild-type and mutant cells were used as inocula for each of the fermentations, these fermentations comprising two identical 100 ml portions of each culture at 30°C; a 100 ml flask culture for growth and ATP measurements and a 100 ml Duran bottle culture for respiratory data determinations. Growth was followed spectrophotometrically (OD_{600nm}). Total culture ATP measurements, determined using the Celsis High Sensitivity Bioluminescence kit (Celsis, Cambridge Science Park, Cambridge, UK), in conjunction with a Labsystems Luminoscan luminometer programmed to operate at 30°C (Labsystems, Basingstoke, UK); also O_2 consumption, CO_2 production and respiratory quotients, measured every 60 min using a MicroOxymax 'closed-circuit' respirometer (Columbus Instruments, Columbus, OH, USA), were all as described by Holyoak et al (1996).

Biomass yields were determined after the cultures had attained stationary phase, 10 ml of each culture being filtered onto a 0.45-μm pore nitrocellulose filter (Gelman Sciences, USA), which was washed with sterile water and dried in an oven before being weighed.

Protein pulse-labelling

Exponential phase cultures, growing on pH 4.5 minimal defined medium supplemented with leucine, tryptophan and uracil, were labelled for 40 min with [^{3}H]leucine as detailed by Cheng and Piper (1994). Immediately after the labelling period, plasma membranes were purified as above and SDS-PAGE analysis of plasma membrane proteins conducted as in Panaretou and Piper (1992).

RESULTS

Hsp30 is induced by weak organic acid treatment of low pH cultures

Hsp30 has previously been reported to be induced by heat shock, by ethanol, and with the entry to stationary phase caused by glucose limitation (Panaretou and Piper 1992; Régnacq and Boucherie 1993; Piper et al 1994). More recently we have shown the 1.3 kb transcript of the *hsp30* gene is weakly induced by either acidification of cultures or exposure to 0.5 M NaCl, and induced more strongly by exposure to weak organic acids at low pH and the severe osmostress caused by 2 M sorbitol, 1 M KCl or 1 M NaCl (manuscript in preparation). In order to measure Hsp30 protein induction we have resorted to pulse-labelling of proteins in vivo, followed by purification of plasma membranes and separation of the proteins of these membrane preparations on one-dimensional 12.5% SDS gels (Fig. 1). This is because the extreme hydrophobicity of Hsp30 has, in our hands, prevented quantitative transfer of this protein to nylon or nitrocellulose membranes during conventional Western blotting procedures. Pulse-labelling of plasma membrane proteins of pH 4.5 cultures either treated for 40 min with a growth-inhibitory concentration of a weak organic acid (9 mM sorbic acid), or adapted to the highest level of sorbic acid that still permitted pH 4.5 growth (2 mM), revealed Hsp30 to be one of two plasma membrane proteins strongly induced by this acid, a compound used extensively as a food preservative (Fig. 1). Hsp30 labelling is absent in cells of the *hsp30* mutant strain KT3 that are either exposed to sorbic acid at pH4.5 (Fig. 1) or heat shocked (data not shown) indicating *hsp30* (an open reading frame on chromosome III alternatively designated YCR21C) as the only strongly stress-inducible gene for this protein in yeast. Partial peptide sequence data on the larger sorbic acid-induced plasma

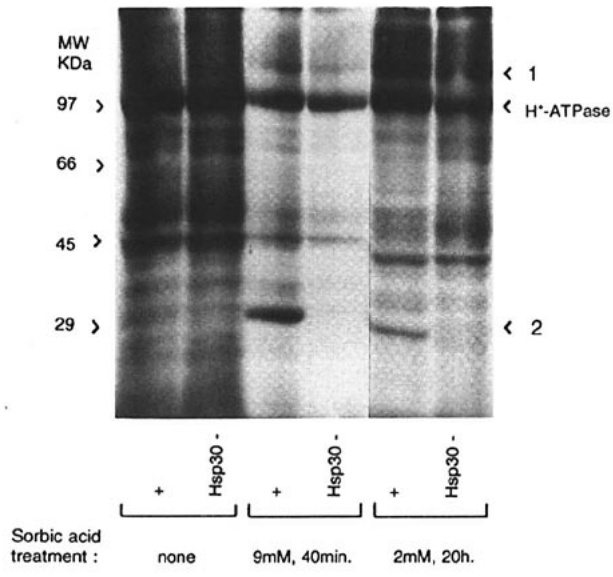

Fig. 1 One-dimensional gel analysis of labelled proteins in purified plasma membranes from vegetative pH 4.5 cultures of wild-type (+) and *hsp30* mutant (Hsp30 -) cells. Cultures were pulse labelled for 40 min with [^{3}H]leucine, either in the absence of sorbic acid; with sorbic acid (9 mM) present for just for the 40 min labelling period; or with sorbic acid (2 mM) present for 20 h prior to and during the period of labelling. Immediately after the labelling period plasma membranes were purified and samples, each corresponding to 20 000 d.p.m. [^{3}H]-labelled total plasma membrane protein, analysed in each gel lane (see Materials and Methods). Flurographic exposure of the gel was for 20 days. Positions of MW markers are indicated to the left of the figure; H$^+$-ATPase and two proteins (1, 2) induced by sorbic acid are indicated to the right. Protein 1 corresponds to a Snq2p homologue and protein 2 is Hsp30 since its labelling is lost in the *hsp30* mutant.

membrane protein in Figure 1 has shown that this protein has high homology to the product of the *snq2* gene (manuscript in preparation). *snq2* encodes an ABC-transporter membrane protein whose overexpression confers resistance to 4-nitroquinoline-N-oxide (Servos et al 1993).

Sorbic acid was employed as the weak organic acid inducer of *hsp30* in this study since it is not metabolized by yeast (Holyoak et al 1996), enabling a single sorbic acid addition to a culture to provide continuous weak acid stress during the subsequent fermentation. *S. cerevisiae* is known to metabolize certain other weak organic acids (e.g. propionate; Pronk et al 1994). This paper also emphasizes the effects of Hsp30 loss on cells exposed to weak organic acid stress since this is the first study to identify a yeast gene (*hsp30*) that is both weak acid-induced and which assists the adaptation that allows yeast growth in the presence of this acid (see Discussion).

Hsp30 does not increase the thermal stability of the plasma membrane H⁺-ATPase of isolated plasma membranes

The induction of Hsps by the heat shock response is known to protect against cellular damage caused by supraoptimal temperatures. Some of these proteins (e.g. Hsp70, Hsp104) help to sequester and reactivate proteins that have suffered limited thermal denaturation or aggregation, while others (e.g. components of the ubiquitination pathway for protein turnover in eukaryotic cells) assist in the degradation of aberrant or denatured proteins (reviewed in Mager and Moradas-Ferreira 1993; Parsell and Lindquist 1993). We initially investigated the possibility that Hsp30 might be thermoprotective towards plasma membrane H⁺-ATPase, an important enzyme activity for both the survival of heat stress (Panaretou and Piper 1990; Coote et al 1994) and adaptation to growth in the presence of weak organic acids (Holyoak et al 1996). Plasma membranes were purified from wild-type (strain BJ2168) and *hsp30* mutant (strain KT3) cells that had been either grown at 28°C, then heat-shocked for 40 min at 40°C, or had adapted to pH 4.5 28°C growth in the presence of 2 mM sorbic acid. Both treatments result in Hsp30 induction in the BJ2168 strain (Fig. 1; also data presented in Panaretou and Piper 1992). In vitro inactivation of plasma membrane H⁺-ATPase at 40°C was then measured in all four membrane preparations. Rates of in vitro H⁺-ATPase inactivation were essentially identical in wild-type and *hsp30* mutant membranes (Fig. 2), indicating that H⁺-ATPase thermal stability is not appreciably influenced by the presence or absence of Hsp30.

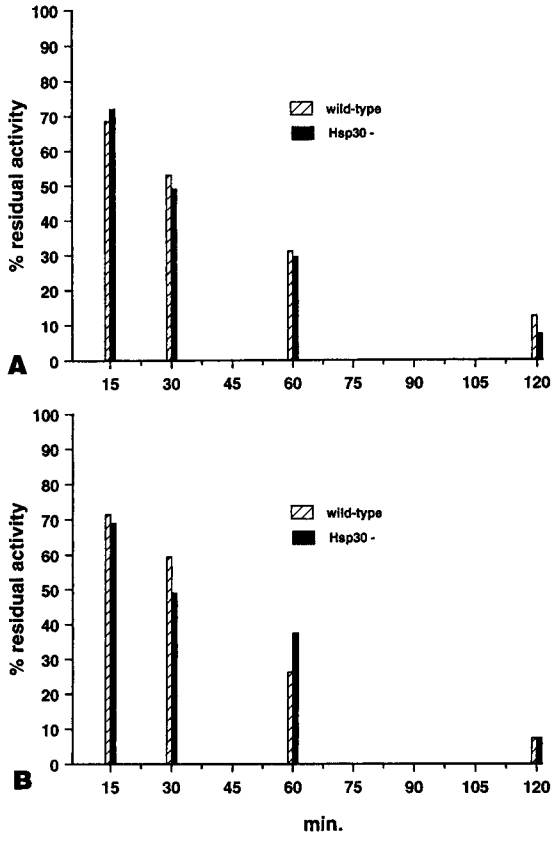

Fig. 2 Measurements of the loss of H⁺-ATPase activity in purified *hsp30* mutant (Hsp30 –) and wild-type plasma membranes maintained at 40°C. The cells had, prior to membrane preparation, been either heat shocked at 40°C for 40 min (A) or grown overnight at pH 4.5 in the presence of 2 mM sorbic acid (B).

Hsp30 is a negative regulator of plasma membrane H⁺-ATPase activity in both heat shocked and sorbic acid-adapted cells

In studying the effects of Hsp30 loss on rates of thermal inactivation of H⁺-ATPase (Fig. 2), we unexpectedly found higher initial levels of H⁺-ATPase activity in the membranes lacking Hsp30. Thus for the membrane preparations used to generate the data in Figure 2 the initial H⁺-ATPase-specific activity was, in the purified plasma membranes from the heat shocked or sorbate-grown *hsp30* mutant cells, 1.2 and 1.65 units per mg total membrane protein, respectively. In contrast, for the equivalent fractions of wild-type cells it was only 0.55 and 0.4 units per mg total membrane protein. We therefore investigated whether Hsp30 induction might be linked to plasma membrane H⁺-ATPase regulation. If this is the case, the presence or absence of a functional *hsp30* gene should influence H⁺-ATPase activity under those stress conditions that cause strong Hsp30 induction.

Vegetative wild-type and *hsp30* mutant cultures were subjected to 0, 40, 80 and 120 min of 40°C heat shock treatment. It had previously been shown that Hsp30 is acquired by the plasma membrane of the wild-type strain BJ2168 within 40 min at 40°C (Panaretou and Piper 1992). Plasma membranes were then purified from these eight cell samples and the H⁺-ATPase-specific activity of each preparation determined relative to total membrane protein content. This experiment was performed identically three times; on each occasion the specific activity of plasma membrane H⁺-ATPase from the *hsp30* mutant showing dramatic increases with heat shock, while the H⁺-ATPase-specific activity in the membranes from the parent strain showed relatively little change (Fig. 3A). The *hsp30* mutation had no major influence over H⁺-ATPase activity in plasma membranes from unstressed vegetative cells (the zero time sample; Fig. 3A), which is to be expected since Hsp30 is not strongly induced in such cells (Panaretou and Piper 1992; Régnacq and Boucherie 1993; Piper et al 1994).

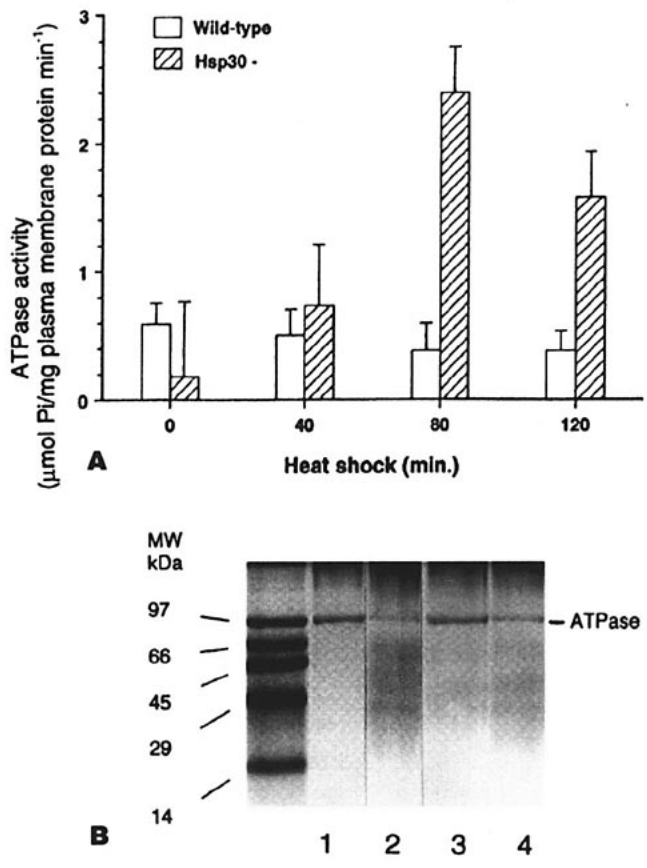

Fig. 3 (A) Plasma membrane H+-ATPase activity in purified plasma membranes from *hsp30* mutant and wild-type cells heat shocked at 40°C for 0, 40, 80 and 120 min prior to membrane preparation. Error bars indicate SD of data obtained from three sets of identically-prepared membrane preparations. (B) Analysis by Coomassie blue staining of H+-ATPase protein levels in plasma membranes purified from wild-type (1, 2) and *hsp30* mutant (3, 4) cells that, prior to membrane preparation, were in 28°C growth (1, 3) or had been heat shocked for 40 min at 40°C (2, 4). Each gel sample was 20 µg total membrane protein. Here, and in Figure 4B, Hsp30 is not visible since its staining requires higher protein loading of the gel.

When cells of the BJ2168 wild-type strain are heat shocked at 40°C the levels of H+-ATPase *protein* in the plasma membrane actually decline (Panaretou and Piper 1992). Taking this into consideration the specific activity measurements for the wild-type strain (BJ2168) in Figure 3A almost certainly reflect activation of that fraction of the H+-ATPase that still remains in the plasma membrane 40–120 min into the heat shock. Proton extrusion measurements on intact cells of another Hsp30+ yeast strain also indicate that heat shock does indeed activate plasma membrane H+-ATPase (Coote et al 1991). The levels of H+-ATPase protein in the *hsp30* mutant (readily measurable on gels of total plasma membrane proteins as the most abundant protein) also showed this rapid decline to a new steady state level after heat shock (Fig. 3B), the kinetics and extent of this decline being indistinguishable from that seen in wild-type cells (data not shown). Therefore, although the H+-ATPase-specific activity measurements on membranes purified from both the *hsp30* mutant and wild-type in Figure 3A under-represent the true activation level of the H+-ATPase remaining in these membranes 40–120 min into the heat shock, this under-representation influences both sets of data equally and does not negate the finding that heat shocked cells of the *hsp30* mutant have much higher H+-ATPase activity. In addition, the observation that Hsp30 null cells still display a decline in H+-ATPase protein levels with heat shock (Fig. 3B) shows that this decline does not require Hsp30 synthesis.

The dramatic differences in H+-ATPase activity between heat shocked cells of *hsp30* and wild-type strains (Fig. 3A) indicate Hsp30 induction acting to downregulate a heat-induced stimulation of plasma membrane H+-ATPase activity. To see whether Hsp30 loss might have a similar effect on H+-ATPase activity during another Hsp30-inducing stress we determined how Hsp30 loss influences plasma membrane H+-ATPase activity in cells adapted to growth in the presence of weak acid stress (Fig. 1).

Plasma membranes were purified from wild-type and *hsp30* mutant cells adapted to growth at pH 4.5 in the presence of 0, 0.5 and 2 mM sorbic acid. In three identically performed experiments H+-ATPase assays again revealed the loss of Hsp30 results in higher plasma membrane H+-ATPase activities, confirming that Hsp30 induction confers downregulation of the H+-ATPase (Fig. 4A). Even though growth in the presence of weak organic acids requires considerably higher plasma membrane H+-ATPase activity (Viegas and Sa-Correia 1991; Holyoak et al 1996), adaptation to growth in the presence of sorbic acid involved appreciable reduction in H+-ATPase protein levels (Fig. 4B). This reduction in H+-ATPase was similar for both wild-type and *hsp30* mutant cells (Fig. 4B). Thus, as with the assays of H+-ATPase in membranes from heat shocked cells (Fig. 3A), the H+-ATPase assay data in Figure 4A under-represents to similar extents for both the wild-type and mutant the activation of that fraction of H+-ATPase remaining in the plasma membrane of cells adapted to growth in the presence of sorbic acid.

Hsp30 inactivation does not affect several stress tolerances of yeast

Comparing BJ2168 and KT3 cultures in vegetative growth we could find no effect of *hsp30* inactivation on thermotolerance (survival at 50°C), irrespective of whether or not the cells had been heat shocked for 40

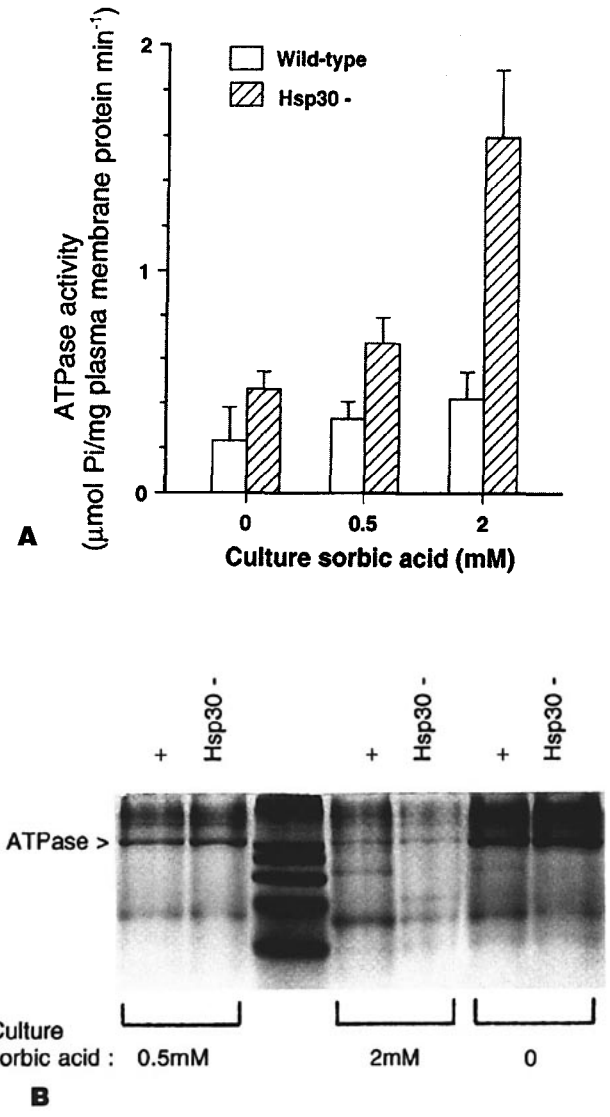

Fig. 4 (A) Plasma membrane H⁺-ATPase activity in plasma membranes purified from *hsp30* mutant and wild-type cells that had been grown for 20 h at pH 4.5 in the presence of 0, 0.5 or 2 mM sorbic acid. Error bars indicate SD of data from three sets of identically-prepared membrane preparations. (B) Analysis by Comassie blue staining of H⁺-ATPase protein levels in these plasma membrane preparations from *hsp30* mutant (Hsp30 –) and wild-type (+) cells. Each gel sample was 20 μg total membrane protein.

min at 40°C to induce the heat shock response (and Hsp30 in the BJ2168 strain). We were also unable to identify any difference in the *survival* of vegetative cells of these two strains following short-term exposure to: 15% (v\v) ethanol; 500 mM or 750 mM potassium sorbate at pH 4.5; 6 μM 4-nitroquinoline-N-oxide; 0.25 mM or 0.5 mM hydrogen peroxide; ultraviolet light; 2.5 M NaCl; and a single freeze–thaw cycle (data not shown). Hsp30 is also induced with carbon limitation (Panaretou and Piper 1992; Régnacq and Boucherie 1993). We therefore investigated the survival of BJ2168 and KT3 cells transferred to glucose-free minimal medium and maintained at 28°C over 14 days. There was no difference in the survival of these two strains over this period. There were also no differences in the 50°C thermotolerances or freeze–thaw tolerances of cultures of BJ2168 and KT3 that had entered stationary phase after growth on rich (YPD) medium (not shown).

Hsp30 inactivation reduces biomass yields and extends the period needed for cells to adapt to growth at low pH, growth in the presence of weak organic acid stress or growth under conditions of severe osmostress

Although loss of Hsp30 appears to have no major impact on several of the stress tolerances of *S. cerevisiae* cells, fermentation analysis revealed that there is a phenotype associated with loss of this protein. Glucose-grown cultures lacking Hsp30 consistently displayed reduced final biomass yields (Table). They also took longer to adapt to enable growth under many stressful conditions of fermentation, manifested as an extended lag phase in batch culture (Figs 5–7).

The minimum amounts of weak organic acid or salt (NaCl) that caused complete inhibition of growth were the same for the *hsp30* mutant as for its wild-type parent (2–3 mM sorbic acid on pH 4.5 YPD plates and 1.2–1.4 M NaCl on pH 5.74 YPD plates, respectively). Also the minimum pH for growth of both strains in liquid YPD medium was the same (pH 2.3–2.5). In contrast the *hsp30* mutant took longer than the wild-type to adapt to enable subsequent growth when exposed to lower, non-inhibitory

Table Biomass yields of wild-type and *hsp30* mutant cells grown on rich glucose media of different pH; also conditions providing weak acid or NaCl stress. Each value is the mean of three separate determinations (SD < 5%)

Culture medium	Biomass yield ($g.l^{-1}$) wild-type	*hsp30*
YPD pH 3.8, no addition	2.08	1.68
YPD pH 3.8, 0.5mM sorbic acid	1.51	1.10
YPD pH 3.8, 0.9mM sorbic acid	1.37	0.78
YPD pH 3.8, 1.8mM sorbic acid	no growth	no growth
YPD pH 4.5, no addition	2.08	1.75
YPD pH 4.5, 0.5mM sorbic acid	1.74	1.44
YPD pH 4.5, 0.9mM sorbic acid	1.55	1.34
YPD pH 4.5, 1.8mM sorbic acid	1.48	1.24
YPD pH 5.74, no addition	2.09	1.81
YPD pH 5.74, 0.75M NaCl	0.84	0.75
YPD pH 5.74, 1M NaCl	0.54	0.36

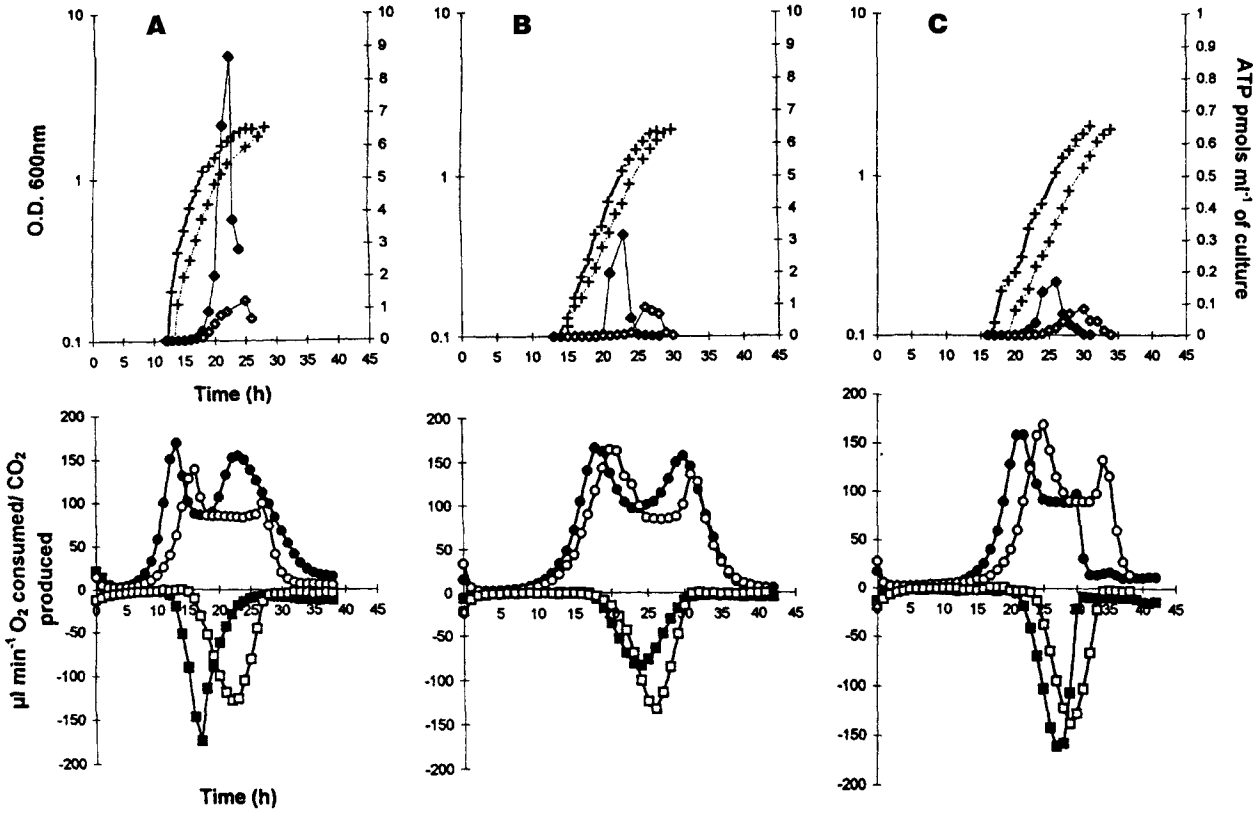

Fig. 5 The effects of low pH stress on growth of *hsp30* mutant and wild-type cells. Strains BJ2168 (filled symbols) and KT3 (open symbols) inoculated into (A) pH 5.74 YPD (effectively negligible acid stress); (B) pH 4.5 YPD; and (C) pH 3.8 YPD. The + symbols in the upper diagrams show OD_{600nm} (BJ2168, thick line; KT3, thin line) and total culture ATP levels (expressed as pmol\ml culture; ◆, BJ2168; ◇, KT3), both of which were followed to the stage of the fermentation where oxygen consumption started to decline. The lower diagrams show CO_2 evolution (●, BJ2168; ○, KT3) and O_2 consumption (■, BJ2168; □, KT3).

levels of these same stresses. When exposed to progressively lower pH values (Fig. 5), subjected to weak organic acid stress (Fig. 6), or subjected to severe osmostress (Fig. 7) the mutant displayed a longer lag relative to the wild-type. This extension to lag was not due to any difference in the number of viable cells in the cultures (data not shown). It was very small under conditions providing essentially very little stress (growth on pH 5.74 YPD; Fig. 5A), but increased with lowering of the culture pH (Fig. 5C). The additional presence of a non-metabolizable weak acid (0.9 mM sorbic acid, chosen as a concentration that still permits growth at both pH 3.8 and pH 4.5) resulted in only slight increases in lag with wild-type cells, but a considerable extension to the lag displayed by the *hsp30* mutant (compare the cultures in Fig. 6A, B with cultures identical but for the lack of sorbic acid, Fig. 5B, C). These effects of sorbic acid, also of the *hsp30* mutation, in extending lag phase were greater at low pH when weak organic acids have considerably greater inhibitory effects on microbial growth (see Discussion). However, despite Hsp30 loss extending the times needed for adaptation to both low pH stress and weak organic acid stress, both the mutant and wild-type cultures displayed essentially identical maximal growth rates *after they had adapted* to enable growth under these conditions (Figs 5 and 6).

Hsp30 loss was also found to extend the time needed for adaptation to severe osmostress. High levels of sodium chloride caused considerable lag extensions even with wild-type cells (compare BJ2168 cultures in Fig. 7 with BJ2168 grown identically but for the absence of salt, Fig. 5A). When cells were innoculated into medium containing 0.75 M NaCl, Hsp30 loss resulted in only a small extension to lag (Fig. 7A). In contrast it caused a considerable lag extension in 1 M NaCl medium (Fig. 7B), a salt concentration close to the maximum permitting growth of these strains. Cultures of both the *hsp30* mutant and wild-type that had adapted so as to enable growth in the presence of 0.75 M or 1 M NaCl subsequently attained the same maximal growth rates as cultures grown in the absence of salt (Figs 5A and 7).

Together Figures 5–7 show that loss of Hsp30 extends lag under a variety of stress conditions, but that it neither

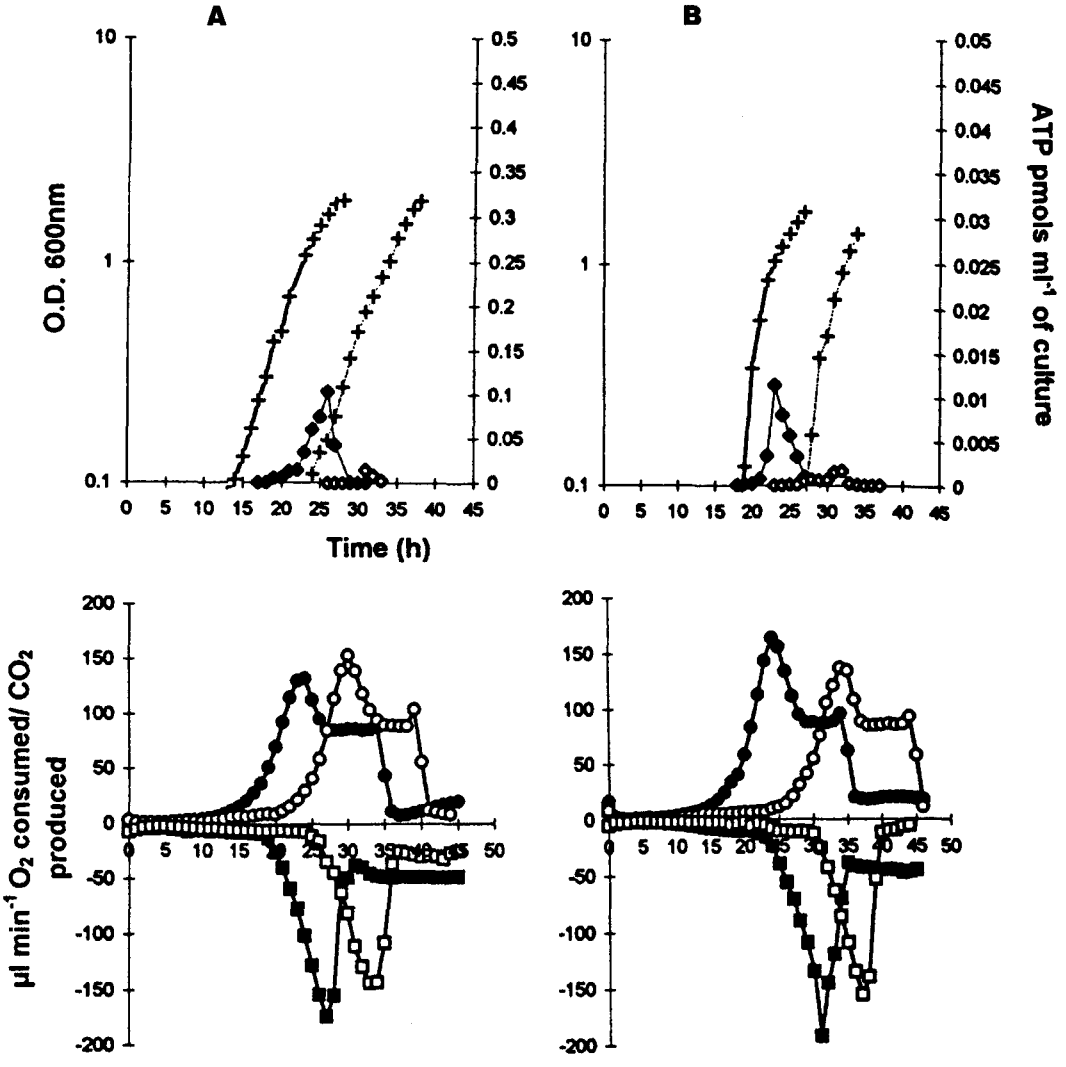

Fig. 6 The effects of weak organic acid stress on growth of the *hsp30* mutant and wild-type. Strains BJ2168 (filled symbols) and KT3 (open symbols) inoculated into (A) pH 4.5 and (B) pH 3.8 YPD medium containing 0.9 mM sorbic acid. The control cultures grown identically but for the absence of sorbic acid are Figure 5B and C, respectively. The symbols in the upper diagrams showing OD_{600nm} and total culture ATP levels; also those in the lower diagrams showing CO_2 evolution and O_2 consumption, are as described in the legend to Figure 5.

prevents growth under these conditions nor lowers maximal growth rates. The induction of Hsp30 therefore *assists* adaptation to growth in cells exposed to these stresses, but is not essential for this adaptation. These are all stresses which demand considerable energy expenditure for the maintenance of homeostasis (see Discussion).

Energy-demanding stresses lower ATP levels during the late exponential and diauxic lag phases of batch fermentation, an effect enhanced by Hsp30 loss

To measure ATP levels during fermentation we employed a rapid analysis technique that measures ATP level per unit of culture volume rather than per unit of cell mass (see Materials and Methods). This was to mimimize effects of sample manipulation on the determined values, although it is an approach that only provides ATP measurements when cultures have reached fairly high cell densities. Under our conditions of fermentation this approximated to the point where CO_2 production starts to decline, indicative of glucose exhaustion and the onset of the diauxic shift phase of batch fermentation when cells are starting to adapt to respiratory growth on the ethanol produced by the earlier fermentation of glucose (Figs 5 and 6).

Upon sudden exposure to non-inhibitory levels of low pH stress, weak organic acid stress or osmostress, homeostasis will be disturbed. Re-establishment of homeostasis to levels that can allow subsequent growth will almost certainly necessitate considerable energy expenditure

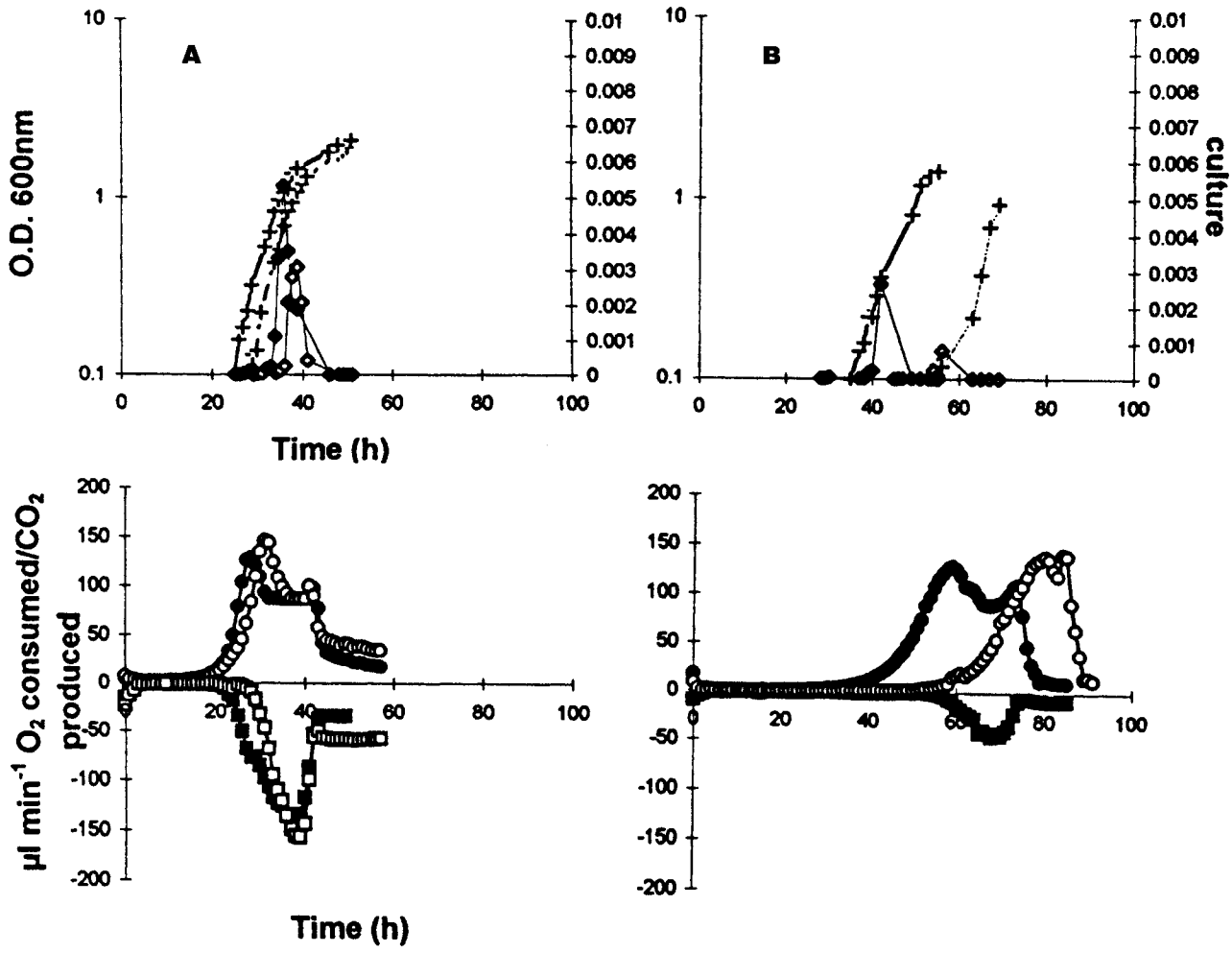

Fig. 7 The effects of severe salt stress on growth of *hsp30* mutant and wild-type cells. Strains BJ2168 (filled symbols) and KT3 (open symbols) were inoculated into pH 5.74 YPD medium containing (A) 0.75 M NaCl and (B) 1 M NaCl. The control culture grown identically but for the absence of salt is shown in Figure 5A. The symbols in the upper diagrams showing OD_{600nm} and total culture ATP levels; also those in the lower diagrams showing CO_2 evolution and O_2 consumption, are as described in the legend to Figure 5. Note that ATP was virtually undetectable in almost all samples by the rapid ATP analysis procedure used in this study. indicating dramatic culture ATP reduction with severe osmostress (compare ATP levels in these figures with those of the control lacking osmostress, Fig. 5A).

(see Discussion). Figure 5 shows that ATP levels are lowered simply by growth at lower pH. ATP level is dramatically reduced still further in cultures that have resumed growth after inoculation into media providing weak organic acid stress (compare ATP of BJ2168 in Fig. 6A, B with that of BJ2168 grown identically but for the absence of sorbic acid in Fig. 5B, C). This considerable reduction in ATP due to sorbic acid must represent lowering of the intracellular ATP/ADP+AMP ratio since weak organic acids do not alter the total adenine nucleotide pool size in yeast (Krebs et al 1983). In cultures containing 0.75 M or 1 M NaCl ATP levels were so low as to be virtually undetectable by the rapid analysis procedure we employed (compare ATP determinations for BJ2168 cultures in Fig. 7 with BJ2168 cells grown identically but for the absence of salt, Fig. 5A). We interpret this as indicating dramatic reduction of cellular ATP with the energy demands of severe osmostress.

Cultures of the *hsp30* mutant and wild-type showed the same *total* CO_2 evolution and O_2 consumption as pH was lowered (compare Fig. 5A with 5C), or in the presence or absence of sorbic acid stress (compare Fig. 6A, B with Fig. 5B, C), or in presence or absence of severe osmostress (compare Fig. 7A, B with Fig. 5A). This suggests that the overall levels of carbon flux were similar during all of these fermentations (although occurring over different time periods) irrespective of the presence of additional stress agents in some of these cultures. It is probable therefore that the depletion of cellular ATP due to growth under conditions of low pH, weak organic acid

treatment at low pH, or osmostress (Figs 5–7) reflects increased ATP usage, not reduced metabolic activity. Another major consequence of the high energy demands of growth under these conditions is a considerable lowering of biomass yield (apparent from the data for wild-type cells in the Table).

The point where glucose became limiting in the fermentations in Figures 5–7 is indicated by the sudden reversal of the increasing CO_2 production. Soon after the cultures reached diauxic lag, as indicated by the sudden reduction in CO_2 production and increased O_2 consumption. At this point ATP levels also decline (Figs 5 and 6) since the yeast can now no longer generate ATP by glucose fermentation and is not yet adapted to efficient respiratory maintenance on the ethanol generated during the earlier fermentative growth phase. At this diauxic lag the ATP levels of wild-type cultures were usually significantly higher than those of the *hsp30* mutant, in essentially unstressed cells (Fig. 5A), cells subject to low pH stress (Fig. 5C) and cells subject to weak organic acid stress (Fig. 6). This is also apparent from plots of ATP versus OD_{600nm} for these same cultures (Fig. 8). In the osmostressed cultures (Fig. 7) the ATP measurements are so low as to prevent firm conclusions as to the effects of Hsp30 loss on ATP.

DISCUSSION

Hsp30 downregulates the activity of plasma membrane H⁺-ATPase, an enzyme stimulated by several stresses

Induction of Hsp30 and a reduction of plasma membrane H⁺-ATPase protein levels are two prominent changes to yeast plasma membrane protein composition with sublethal heat or ethanol stress (Panaretou and Piper 1992; Piper et al 1994), as well as sorbic acid treatment (Figs 1 and 4B). Studies on the *end4* mutant have indicated that the reduction in H⁺-ATPase levels in heat shocked cells is due to stimulation of endocytosis (R. Braley and P.W. Piper, unpublished data). That it is not a consequence of *hsp30* expression is shown by the lack of any effect of the *hsp30* mutation on this decline in H⁺-ATPase protein in purified plasma membranes from heat shocked or sorbate-treated cultures (Figs 3B and 4B). Instead Hsp30 induction in heat shocked or sorbate-adapted *S. cerevisiae* leads to a downregulation of the stress-induction of plasma membrane H⁺-ATPase activity (Figs 3 and 4). Although not shown by direct H⁺-ATPase assay, *hsp30* induction in diauxic shift and in severely osmostressed cultures probably serves the same purpose.

Several stresses that act to dissipate the proton motive force across the plasma membrane and/or cause intracellular pH decline have been found to stimulate yeast plasma membrane H⁺-ATPase activity. They include heat shock (Coote et al 1991); growth under acid conditions (Eraso and Gancedo 1987); ethanol exposure (Cartwright et al 1987; Rosa and Sa-Correia 1991, 1992) and weak lipophilic acid treatment (Viegas and Sa-Correia 1991; Holyoak et al 1996). By stimulating H⁺-ATPase these diverse stresses increase *catalysed* proton efflux. This may, under the appropriate physiological conditions, help to counteract the *passive* proton influx to the cell that is a consequence of the same stresses increasing the proton permeability of the plasma membrane.

Hsp30 may be part of a system for conservation of ATP under conditions of prolonged stress and during diauxic shift

Plasma membrane H⁺-ATPase activity influences tolerances of heat and of ethanol (Panaretou and Piper 1990; Rosa and Sa-Correia 1992; Coote et al 1994). It is also important for growth both at low pH and in the presence of weak organic acids, *S. cerevisiae* mutants with reduced plasma membrane H⁺-ATPase activity being defective in growth under these conditions (McCusker et al 1987; Holyoak et al 1996). Thus it is paradoxical that the levels of H⁺-ATPase protein should decline in cells exposed to these stresses (Panaretou and Piper 1992; Piper et al 1994; also Figs 3B and 4B). However, this enzyme hydrolyses much of the ATP generated by the cell, with the result that maximal H⁺-ATPase activity is rare and energetically expensive to maintain (Serrano 1991). Maintaining maximal H⁺-ATPase activity over long periods of such stresses as a weak acid exposure (at low pH) or severe osmostress (Figs 6 and 7) might indeed be unsustainable. Although the *immediate* response of the cell to these stresses may be a rapid stimulation of H⁺-ATPase, this may have to be followed by a *delayed* response in which H⁺-ATPase enzyme levels are lowered (Figs 3B and 4B) and Hsp30 is induced. The role of Hsp30 might be to downregulate stress-stimulation of that fraction of the H⁺-ATPase that remains (Figs 3 and 4). These two events may limit excessive H⁺-ATPase action, thereby helping to conserve ATP during extended stress exposure. This is a hypothesis that can be tested in future investigations. It can be argued that a yeast cell that has *totally* exhaused its ATP reserves will be inviable. Such a cell will not be able to phosphorylate glucose by the hexokinase reaction and thus generate energy by fermentation of glucose. It will also be incapable of regulating metabolism, in view of the importance of protein kinase reactions in such regulation. Stress response-induced limitations to excessive H⁺-ATPase action may be a failsafe mechanism to help prevent such an occurrence.

Plasma membrane H⁺-ATPase action is known to consume a considerable fraction of all the ATP generated in

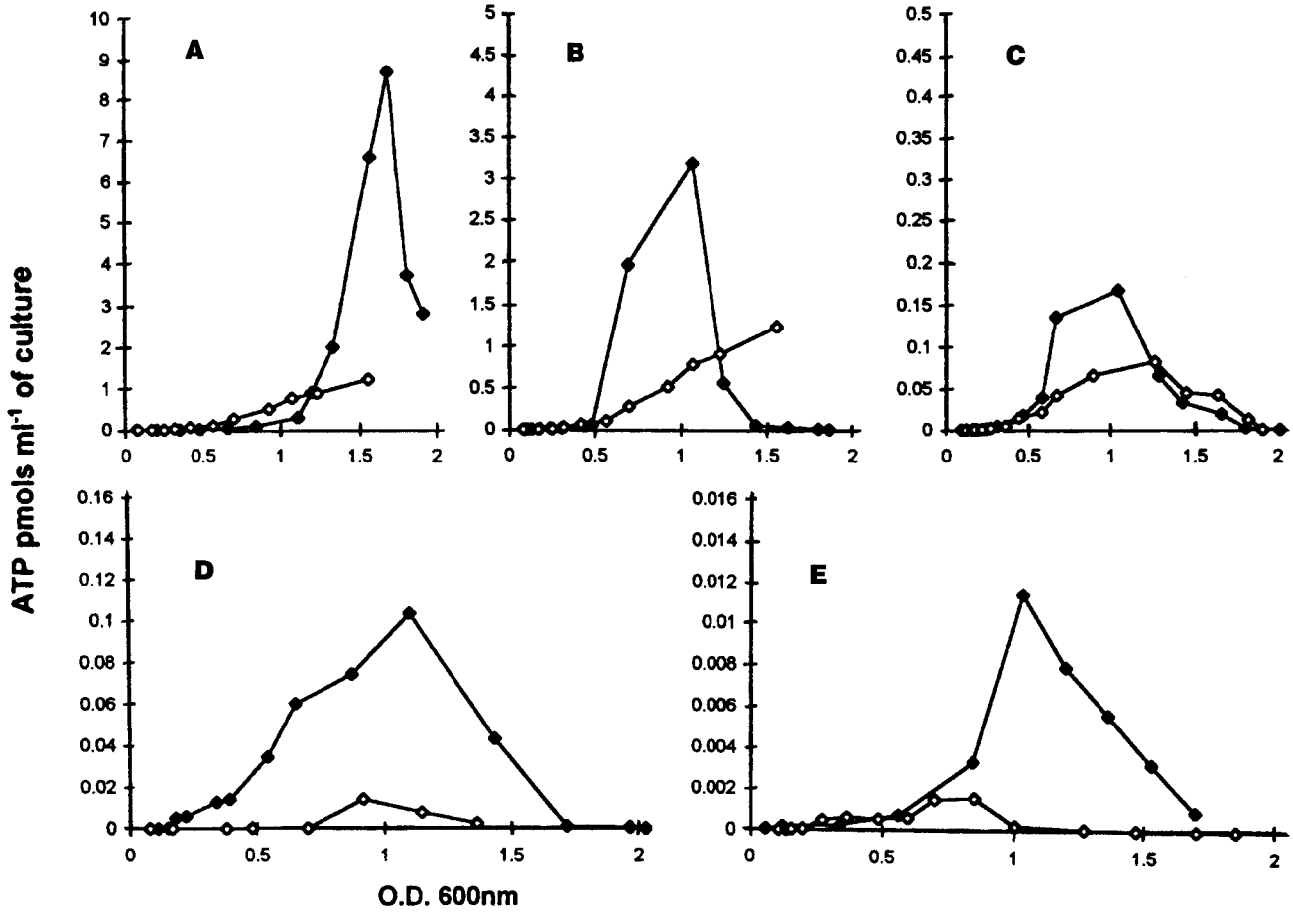

Fig. 8 Plots of culture ATP level versus OD_{600nm} for BJ2168 (filled symbols) and KT3 (open symbols) growing on (A) pH 5.74 YPD, (B) pH 4.5 YPD, (C) pH 3.8 YPD, (D) pH 4.5 YPD medium containing 0.9 mM sorbic acid and (E) pH 3.8 YPD containing 0.9 mM sorbic acid.

both yeast (Serrano 1991) and plant cells (Sussman 1994). In the *hsp30* mutant the lack of Hsp30 downregulation of this H^+-ATPase (Figs 3A and 4A) will enhance ATP usage. This is consistent with the lower ATP levels of this mutant at diauxic shift (Figs 5, 6 and 8). Glucose exhaustion induces Hsp30 (Panaretou and Piper 1992; Régnacq and Boucherie 1993), with the result that Hsp30 will be present in even an essentially unstressed diauxic culture such as that in Figure 5A. Diauxic cells, having exhausted the available glucose and not yet being adapted to respiratory growth on ethanol, probably also need to conserve ATP. Lack of Hsp30 downregulation of H^+-ATPase, leading to higher ATP consumption in the *hsp30* mutant, is also consistent with the reduced biomass yields of this mutant under several conditions of fermentation (Table). These yield reductions are in addition to the normal reductions in biomass yield that result from growth in the presence of sorbic acid or salt (compare *hsp30* mutant and wild-type; Table). We therefore propose that Hsp30 has an energy conservation function, acting to limit excessive H^+-ATPase activity during situations of glucose exhaustion (diauxic lag) and energy-demanding stress. While the decreased ATP levels (Figs 5, 6 and 8) and reduced biomass yields of *hsp30* cultures (Table) indicate such an energy conservation role for Hsp30, further work is needed to establish it unequivocally.

Growth under conditions of low pH, weak organic acid stress and osmostress is energically-expensive, largely due to the high energy demands of maintaining homeostasis

Figures 5–7 show the considerable reductions to ATP level in cultures subject to low pH stress, weak organic acid stress or severe osmostress. As far as we are aware this is the first time this has been demonstrated. It is noteworthy that sizeable reductions in the ATP pool do not prohibit growth. Exposure to low pH (effectively a high concentration of protons) increases passive proton entry to the cell. Maintenance of intracellular pH under

these conditions necessitates increased plasma membrane H^+-ATPase-catalysed proton extrusion, a probable reason that ATP levels fall with exposure to more acid pH values (Figs 5A–C). Weak organic acids (e.g. sorbic, benzoic, salicylic and propionic) are most effective inhibitors of microbial growth at low pH since their rapid penetration into cells and their membrane-disruptive properties both require the uncharged, protonated form of the acid (Brown and Booth 1991). At pH > 5–6 they exist mainly in the non-liposoluble charged form and, as such, are far less cytotoxic. Following entry to cells of low pH cultures as the undissociated acid, they subsequently dissociate in the higher pH environment of the cytosol, resulting in intracellular proton release and accumulation of the anionic form of the acid. The released protons, if not actively extruded by increased plasma membrane H^+-ATPase action or absorbed by the buffering capacity of the cell, will cause intracellular acidification. This fall in intracellular pH is a major cause of growth inhibition by weak acids (Brown and Booth 1991). It is partly counteracted, thereby allowing intracellular pH to rise to levels possibly compatible with growth, by the increased plasma membrane H^+-ATPase action that is a major energy demand of weak acid stress. Growth under conditions of severe osmostress is also energetically expensive. To survive osmostress yeast cells must restore their turgor pressure through the accumulation of high levels of glycerol, one ATP being consumed for each glycerol molecule synthesised from glucose. In addition conditions of high sodium ion concentration, as employed for the cultures in Figure 7, also necessitate much higher levels of ATP-driven Na^+ ion extrusion by the sodium pump (Haro et al 1993).

Hsp30 assists adaptation to growth in the presence of energy-demanding stresses

Although Hsp30 is a stress protein it does not have a major impact on several stress tolerances. However, it assists the adaptation that allows growth at low pH, or growth in the presence of severely stressful levels of weak acid or salt, since these adaptations are slower in Hsp30-deficient cells. Non-inhibitory levels of sorbic acid or osmostress cause considerable lag phase extensions even in wild-type cells, yet loss of Hsp30 causes this lag to be increased still further (Figs 6 and 7). This suggests that the mutant, following its inoculation into media containing these stress agents, is less efficient at re-establishing the levels of homeostasis that will support subsequent growth. Lack of a Hsp30 downregulation of the plasma membrane H^+-ATPase in sorbate-adapted *hsp30* cells (Fig. 4) should commit these cells to a higher ATP usage, consistent with their decreased ATP levels relative to the wild-type (Figs 6 and 8).

Yeasts undergo a slow adaptation process that enables growth in the presence of low levels of weak organic acid (Brown and Booth 1991). Apart from increased proton extrusion by enhanced plasma membrane H^+-ATPase action (Viegas and Sa-Correia 1991; Holyoak et al 1996), the events leading to this weak acid adaptation are almost totally uncharacterized. Gene expression changes are almost certainly involved, yet *hsp30* is the first gene shown to be both weak acid inducible (Fig. 1) and assisting weak acid adaptation (Figs 5 and 6). Certain lipophilic weak acids (notably octanoic and decanoic acids) are natural by-products of yeast fermentation (Stevens and Hofmeyer 1993). This may be the reason that low pH yeast cultures have a discrete stress response induced by weak organic acids, the two major plasma membrane proteins induced by this response being Hsp30 and a Snq2p homologue (Fig. 1). Another effect of weak organic acids on stress gene expression in low pH yeast cultures (also other treatments that lower intracellular pH) is the complete inhibition of a different, more widely-studied stress response, the response to heat shock (Cheng and Piper 1994).

This study has shown that Hsp30 induction reduces the rapid stimulation of plasma membrane H^+-ATPase that is an immediate physiological response of yeast cells subjected to heat shock and other stresses. Since all fungal and plant cells use plasma membrane H^+-ATPase for homeostasis regulation (Serrano 1991; Sussman 1994), it will be interesting to determine if a stress-inducible protein of equivalent function to yeast Hsp30 exists in diverse plants and fungi. We propose that the role of Hsp30 may be primarily one of energy conservation, ensuring that cellular ATP levels do not become too depleted. This is the first time that such a function has been suggested for a stress protein, although we accept that further work is needed to prove this role unequivocally. Currently we are investigating how Hsp30 regulates H^+-ATPase at the molecular level. Initial results indicate that deletion of the C-terminal 11 amino acids of plasma membrane H^+-ATPase, an important site of H^+-ATPase regulation (Serrano 1991), abolishes the Hsp30 effect on H^+-ATPase activity (R. Braley and P.W. Piper, in preparation). This indicates that the Hsp30 regulation of the H^+-ATPase occurs through a specific mechanism involving signal transduction, not a non-specific effect whereby H^+-ATPase is responding to structural alterations of the plasma membrane resulting from the insertion of Hsp30 at this membrane.

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