

# Stress-induced production, processing and stability of a seripauperin protein, Pau5p, in *Saccharomyces cerevisiae*

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## Keywords

Pau5 protein; stress; stability;  
O-mannosylation; wine.

## Abstract

*PAU* genes comprise the largest multiple gene family in *Saccharomyces cerevisiae* with 24 members whose sequence homology ranges from 82% to 100%. Although transcriptional regulation for some of the *PAU* genes has been reported, none of the Pau proteins has been characterized. We constructed yeast strains encoding a C-terminal tandem affinity purification-tagged Pau5 in the *PAU5* locus to study Pau5 production and properties *in vivo*. Pau5 is highly induced by low temperature, low oxygen and wine fermentation conditions. It is unstable in cells grown under laboratory conditions and is temporarily stabilized by low oxygen, osmotic and ethanol stresses. Pau5 degradation is accompanied by an unknown modification with a gradual increase in molecular mass by 3 kDa. Furthermore, Pau5 is O-mannosylated mainly by Pmt1; mannosylation enhances stability of the protein. The mannosylated Pau5 is soluble whereas the nonmannosylated proform Pau5 is an integral membrane protein. Our findings suggest that the intracellular concentration of Pau5 is regulated by wine making stress both at transcriptional and posttranslational levels; Pau5 might play a role in adaptation of yeast cells during alcoholic fermentations.

## Introduction

The *PAU* genes comprise the largest gene family in *Saccharomyces cerevisiae* with 24 members; 15 members have not officially been annotated and *PAU8–PAU22* were recently reserved with *SGD*. *PAU* genes are highly homologous and share 82–100% sequence homology. Systematic analyses of the yeast transcriptome by microarray and Northern blot analyses revealed that *PAU* genes are upregulated by wine-making conditions (Rachidi *et al.*, 2000a; Rossignol *et al.*, 2003), i.e. anaerobiosis (Rachidi *et al.*, 2000b; Lai *et al.*, 2005) and low temperatures (Homma *et al.*, 2003; Schade *et al.*, 2004). Expression of *PAU* genes is also highly induced by histone depletion (Wyrick *et al.*, 1999) and by inactivation of some essential genes, especially those involved in ergosterol synthesis (Mnaimneh *et al.*, 2004). However, due to cross-hybridization between highly homologous *PAU* genes, e.g. at least nine chromosomes were found to bind a 247-bp fragment from *PAU1* when probed under high stringency conditions (Viswanathan *et al.*, 1994), the assignment of the transcripts to particular *PAU* genes in the analyses mentioned above is unreliable. Rachidi *et al.*

(2000b) used plasmid-borne copies of *PAU–lacZ* fusions to find a differential expression of *PAU3*, *PAU4* and *PAU5* genes induced by anaerobiosis with *PAU5* highly upregulated.

Pau proteins are often referred to as seripauperins, based on their serine-poor composition compared with their relatives, the Tir/Dan proteins (Viswanathan *et al.*, 1994). Pau proteins, which are generally 120–124 amino acids (aa) in length, show 85–100% identity and share a conserved domain (PF00660, PAU domain) of about 90 residues with the N-terminal region of Tir/Dan proteins (Abramova *et al.*, 2001a). Tir/Dan proteins have been well studied in terms of their induction, modification and localization, yet no function has been ascribed for them; Tir/Dan proteins are cell wall mannoproteins which are induced by anaerobiosis and cold and/or heat shock (Kondo & Inouye, 1991; Kowalski *et al.*, 1995; Sertil *et al.*, 1997, 2003; Fujii *et al.*, 1999; Abramova *et al.*, 2001a; Cohen *et al.*, 2001), and might play a role in maintaining cell wall integrity (Bourdineaud *et al.*, 1998) or sterol uptake (Alimardani *et al.*, 2004) during stress.

Pau proteins are predicted to be glycosylated. Glycosylation is an essential posttranslational modification in yeast and higher eukaryotes affecting protein folding, solubility,

stability and localization, as well as function (Varki, 1993; Hounsell *et al.*, 1996; Sanders *et al.*, 1999; Harty *et al.*, 2001; Helenius & Aebi, 2001). Two main types of glycosylation exist; *N*-linked glycosylation and *O*-linked glycosylation. In yeast, *O*-mannosylation is initiated in the endoplasmic reticulum (ER) by addition of dolicholphosphate-activated mannose to serine or threonine residues. This reaction is catalyzed by distinct mannosyl transferases (Gentsch & Tanner, 1996; Strahl-Bolsinger *et al.*, 1999; Ernst & Prill, 2001). Tir1/Srp1 and Tip1, both rich in serine and threonine residues, are *O*-mannosylated and linked to the cell wall through glycosyl-phosphatidyl inositol (GPI) anchoring (van der Vaart *et al.*, 1995; Donzeau *et al.*, 1996; Kitagaki *et al.*, 1997; Bourdineaud *et al.*, 1998; Fujii *et al.*, 1999). Unlike Tir/Dan proteins, Pau proteins that lack the long C-terminal serine and threonine-rich stretches and the GPI anchor signal are predicted to be secreted into the culture medium (Viswanathan *et al.*, 1994).

A complete understanding of gene functions requires knowledge of protein properties; however, none of the Pau proteins has been experimentally characterized. In this study, we report on the production and properties of Pau5 under various stress conditions using a Pau5-tandem affinity purification (TAP) fusion.

## Materials and methods

### Yeast strains and culture conditions

The haploid lab strain BY4742 (*MAT $\alpha$* , *his3 $\Delta$ 1*, *leu2 $\Delta$ 0*, *lys2 $\Delta$ 0*, *ura3 $\Delta$ 0*) and a diploid industrial wine strain S92 were used (Table 1). Yeasts were cultured in YPD (DIFCO, containing 1% yeast extract, 2% peptone, 2% dextrose) or synthetic complete (SC) medium [0.67% yeast nitrogen base

(DIFCO), 2% dextrose plus amino acids]. Aerobic cultures were grown in test tubes or flasks with shaking at 180 r.p.m. For hypoxic growth, cells were grown in 5 mL medium in tightly capped 5-mL tubes without shaking at 25 °C. For other stresses, cells were grown in YPD to mid-log phase at 25 °C, collected by centrifugation, washed twice with water and then resuspended in fresh YPD or YPD supplemented with additional dextrose (up to 5%, 20% or 40%), sorbitol (1 M), ethanol (7% v/v) or rapamycin (1  $\mu$ g L<sup>-1</sup>) to a final cell density of OD<sub>600 nm</sub> = 0.5. Cells were then grown aerobically at 25 or 10 °C, or statically at 25 °C for 2 h or the times indicated. For wine fermentations, yeast cells were pregrown aerobically in YPD to OD<sub>600 nm</sub> = 0.5, harvested by centrifugation and resuspended in grape must. Chardonnay grape must (100 mL in 120-mL glass bottles fitted with a vapour lock) was inoculated to a final concentration of 1  $\times$  10<sup>6</sup> cells mL<sup>-1</sup>. Fermentations were carried out at 20 °C without stirring, and weight loss was monitored to determine the fermentation stage and sampling points.

### DNA manipulation and plasmid construction

Standard procedures were used for all DNA manipulations as described by Ausubel *et al.*, (1989). Oligonucleotide sequences are listed in Table 2. The following plasmids were constructed: (1) pUG6-TAP. pBS1539 containing the TAP cassette was digested with SmaI–BamHI (Rigaut *et al.*, 1999) and then inserted into Sall (blunted)–BamHI sites of pUG6 (Guldener *et al.*, 1996). This plasmid contains a TAP-kanMX cassette used for TAP-tagging in wine strains. (2) pHVX2-PAU5-TAP. PAU5-TAP sequences were amplified using primers p46 and p47 from *S. cerevisiae* strain LY6. The PCR product was blunted, digested with XhoI and then ligated into EcoRI (blunted)–XhoI sites of pHVX2, a 2  $\mu$  vector in which the *PGK1* promoter drives expression of the cloned genes (Grobler *et al.*, 1995). (3) pMET25p-PAU5-TAP. pUG36, a single copy vector in which the *MET25* promoter drives expression of yeast enhanced green fluorescent protein (yEGFP), was used to express TAP-tagged proteins. PAU5-TAP sequences were amplified from strain LY6, using reverse primer p47 and forward primer p89. PCR products were digested with SpeI and XhoI and ligated into the SpeI–XhoI sites of pUG36, yielding pUG36-PAU5-TAP. The XbaI fragment containing GFP sequences was then removed from this plasmid, yielding pMET25p-PAU5-TAP. (4) pMET25p-TAP. This plasmid was generated by removing PAU5 sequences from pMET25p-PAU5-TAP through SpeI and NcoI digestion, end blunting and religation. (5) pUG23-PAU5. This plasmid was generated by ligating SpeI and XhoI-digested PCR products, which were amplified from pHVX2-PAU5-TAP using primers p89 and p148, into the SpeI–XhoI sites of pUG23.

**Table 1.** Yeast strains used in this study

Strain	Genotype	Resource
BY4742	<i>MAT<math>\alpha</math></i> . <i>his3<math>\Delta</math>1</i> , <i>leu2<math>\Delta</math>0</i> , <i>lys2<math>\Delta</math>0</i> , <i>ura3<math>\Delta</math>0</i>	Invitrogen
LY6	BY4742, <i>PAU5-TAP-URA3</i>	This study
LY19	BY4742, <i>pau5::TAP-loxP-kanMX4-loxP</i>	This study
LY30	BY4742, pHVX2	This study
LY32	BY4742, pHVX2-PAU5-TAP	This study
LY68	BY4742, <i>pmt1::loxP-kanMX4-loxP</i>	This study
LY74	LY6, <i>pmt1::loxP-kanMX4-loxP</i>	This study
LY75	LY6, <i>pmt2::loxP-kanMX4-loxP</i>	This study
LY76	LY6, <i>pmt3::loxP-kanMX4-loxP</i>	This study
LY77	LY6, <i>pmt4::loxP-kanMX4-loxP</i>	This study
LY78	LY6, <i>pmt5::loxP-kanMX4-loxP</i>	This study
LY79	LY6, <i>pmt6::loxP-kanMX4-loxP</i>	This study
LY98	LY68, pMET25p-PAU5-TAP	This study
LY190	BY4742, pMET25p-PAU5-TAP	This study
LY280	LY68, pMET25p-TAP	This study
S92	<i>MAT<math>\alpha</math>a</i>	BioSpringer, USA
LY15	S92, <i>PAU5-TAP-loxP-kanMX4-loxP</i>	This study

**Table 2.** Oligonucleotides used in this study

No. & name	Sequence
p18. PAU5-up	ctctagcgtctatccgcagacggatctacactattgcaaat <i>tccatgaaaagagaag</i>
p23. PAU5-down	ttctgcctagtgatttctcagggcgaaccttcggatagc <i>tacgactactataggg</i>
p28. PAU5-f	gtgaccagtaatagcctgtttggg
p33. PAU5-r	tccaacatttgctgaagaatg
p37. TEV-r	caaattctcgtagcagtagttgg
p38. Kan-r	atgttcagaacaactctgg
p46. PAU5-N	gaattcatgggtcaaattaactcaatcg
p47. TAP-C	attgtactcagtcagggtgactccccg
p54. pmt1-d-up	agaagagcctatcaagaaacagtaacagctacaagcacggtc <i>agctgaagcttcgtacg</i>
p55. pmt1-d-down	agcttatagaagactttacataaaaaatgtggagagtactggga <i>tacgactactataggg</i>
p56. pmt1-f	tctaccaacatttgccacc
p57. pmt2-d-up	acatcgctactgtcattctgtttcgaaccgtggtccagcaatc <i>agctgaagcttcgtacg</i>
p58. pmt2-d-down	aattctgtttttactatgtacttactacgatgatcttaac <i>tacgactactataggg</i>
p59. pmt2-f	tcatttacagtcgtgatacg
p60. pmt3-d-up	aatatttggtgctcacaataaccggcaataaaaaaacagaca <i>agctgaagcttcgtacg</i>
p61. pmt3-d-down	atTTTTgtgtggtgaagaaaagTTTTgatcattctatatat <i>tacgactactataggg</i>
p62. pmt3-f	tgacttgacgaatgTTTgCG
p63. pmt4-d-up	catattttaccaagatatttctgacagctccacagaagtagc <i>agctgaagcttcgtacg</i>
p64. pmt4-d-down	gaggaaggcgttcagttcgtttgtggttagtgaacagaaaaag <i>tacgactactataggg</i>
p65. pmt4-f	tcatcgtagcgtgatcttc
p66. pmt5-d-up	tttaacaattaagcagggtgtaagatcataactgctttaaag <i>agctgaagcttcgtacg</i>
p67. pmt5-d-down	tctttgtcaaacgaatgtccaatcgaatacgggtgatgga <i>tacgactactataggg</i>
p68. pmt5-f	tctgcaagtagttcagttgg
p69. pmt6-d-up	ccaacgatagtcctaataactgttttctattattacca <i>agctgaagcttcgtacg</i>
p70. pmt6-d-down	cattattttacaattttcgtgattctgtgtaaaggagttgg <i>tacgactactataggg</i>
p71. pmt6-f	ttgacgttattagttacc
p89. PAU5-spel	ctgcagactagtatggccaattaactcaatcg
p148. PAU5-xhoI	attgtactcagattgcaatagtgtagatacc

### TAP-tagging and gene disruption

TAP-tagging was performed as described previously (Rigaut *et al.*, 1999). A targeting cassette encoding the TAP tag and a selectable marker was amplified by PCR from plasmid pBS1539 or pUG6-TAP with primer pairs which have 17 nt common sequences at 3' ends for amplification of TAP cassette, and 43 nt gene specific sequences at 5' ends for precise integration into the genome. The cassettes were then transformed into *S. cerevisiae* BY4742 or S92 using the modified lithium acetate method described by Gietz *et al.* (1992). Correct in-frame integrations were confirmed by colony PCR and Western blotting. Gene disruption was done as described by Guldener *et al.* (1996).

### Preparation of cell lysates

Yeast cells were harvested, washed with cold water and used for immediate protein extraction or stored at  $-80^{\circ}\text{C}$ . Total yeast proteins were prepared by resuspending cells in  $1 \times$  precold sodium dodecyl sulphate (SDS) sample buffer [15% (v/v) glycerol, 0.125 M Tris-HCl pH 6.8, 5 mM EDTA, 2% (w/v) SDS, 1%  $\beta$ -mercaptoethanol,  $1 \times$  protease inhibitor cocktail (Roche)] at  $10 \mu\text{L}$  per  $1 \text{ OD}_{600 \text{ nm}}$  cells, and

then heated at  $98^{\circ}\text{C}$  for 10 min. After adding an equal volume of glass beads, the samples were vortexed for 5 min and centrifuged at  $16\,000 \text{ g}$  for 5 min.

### Immunoblotting

Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to  $0.22 \mu\text{M}$  nitrocellulose membranes using a Bio-Rad Mini-PROTEAN 3 system. The nitrocellulose membrane with transferred proteins was stained by the MemCode reversible protein stain kit (Pierce), photographed, destained and then blocked for 1 h with 3% nonfat dried milk powder prepared with wash buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20). A 1:5000 or 1:10 000 diluted peroxidase anti-peroxidase soluble complex (PAP, Sigma) was used for probing TAP tag or Pau5-TAP fusion proteins. In some cases, the blots were also blotted with antibodies against Pgk1p or ALP (Molecular Probes) as a reference. Super-Signal<sup>®</sup> West Pico or Femto Chemiluminescent Substrate kits (Pierce) and CL-Xposure film (Pierce) were used for visualization of protein-antibody complexes. Films were scanned with HP Scanjet 3970 and photos were adjusted with PHOTOSHOP 6.0 (Adobe).

### Cycloheximide chase experiments

To test the effect of oxygen on Pau5 stability, yeast strain LY6 was grown aerobically at 25 °C to mid-log phase in YPD, then shifted to low oxygen growth at 25 °C for 16 h. Cycloheximide was then added to a final concentration of 100 µg mL<sup>-1</sup> without disturbing the cultures. For aerobic chasing, cultures were shifted to a 20-mL test tube and grown at 25 °C with shaking for various times. For low oxygen chasing, 5-mL cultures were kept in the closed tubes and grown statically at 25 °C for various times. Equal samples of cells (1 OD<sub>600 nm</sub>) were collected, added to 10 mM NaN<sub>3</sub> and incubated on ice for 10 min. Cell lysis and immunoblotting were performed as previously described. To test the effect of sorbitol and ethanol on Pau5 stability, yeast strain LY190 (carrying pMET25p-PAU5-TAP) was grown to mid-log phase in SC without uracil and methionine, then 2 OD<sub>600 nm</sub> cells were collected, washed and resuspended in 3 mL YPD, YPD plus 7% (v/v) ethanol or YPD plus 1 M sorbitol. Cycloheximide was then added to a final concentration of 100 µg mL<sup>-1</sup>. At various intervals (0, 15, 30 and 45 min) following cycloheximide addition, equal samples of cells (0.5 OD<sub>600 nm</sub>) were collected and analyzed as above.

### ConA-Sepharose binding

Logarithmically growing cells expressing Pau5-TAP or TAP in chromosomes or on a *CEN* plasmid (pMET25p-PAU5-TAP) were harvested, washed and resuspended in 1 mL cold ConA buffer [1.6% (v/v) Triton X-100, 0.1% (w/v) SDS, 0.5 M NaCl, 20 mM sodium phosphate pH 7.6] (Weber *et al.*, 2004) and lysed by vortexing with glass beads at 4 °C for 5 min and centrifugation at 3000 g for 5 min. Crude extracts (700 µL) were incubated with 200 µL of ConA-Sepharose beads at 4 °C for 4 h and then centrifuged at 15 700 g for 1 min to separate beads (P) from the supernatant (S). The beads were washed once with ConA buffer and split into three portions: the first portion was directly resuspended in 150 µL 1 × SDS sample buffer for protein extraction; the remaining two portions were resuspended in 1 mL of ConA buffer and ConA buffer plus 10% (w/v) methyl α-D-mannopyranoside (MMP), respectively, and incubated at room temperature (RT) for 45 min, and then separated into beads fractions and supernatants by centrifugation. The resulting beads were resuspended in 150 µL 1 × SDS sample buffer. The supernatant fractions were precipitated by 10% (w/v) tricarboxylic acid (TCA) and washed with cold acetone and then resuspended in 150 µL 1 × SDS sample buffer, heated at 98 °C for 5 min and centrifuged for 5 min. 10 µL of sample was applied to SDS-PAGE and immunoblotting.

### Differential centrifugation

Logarithmically growing cells were collected and resuspended in extraction buffer (50 mM Tris pH7.5, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenyl-

methylsulphonyl fluoride and 1 × protease inhibitor cocktail) and vortexed with glass beads for 5 min at 4 °C. After centrifugation at 500 g for 2 min to remove the unbroken cells and cell debris, the crude extract was centrifuged at 10 000 g for 10 min to yield the pellet (P10) and the supernatant (S10), which was then centrifuged at 100 000 g for 1 h to yield the P100 pellet and S100 supernatant. To test solubility, the crude extract was split into four portions which were treated with buffer, 1 M NaCl, 0.1 M Na<sub>2</sub>CO<sub>3</sub> (pH11), or 1% (v/v) Triton X-100 for 30 min on ice, respectively, before centrifugation at 100 000 g for 1 h. Centrifugation was performed at 4 °C. Proteins in the supernatants were precipitated using 10% (w/v) TCA. All protein pellets were resuspended in 1 × SDS sample buffer, heated for 5 min at 98 °C and centrifuged at 10 000 g for 5 min. Equal portions of the fractions were applied to 12% SDS-PAGE and immunoblotting.

### Microscopy

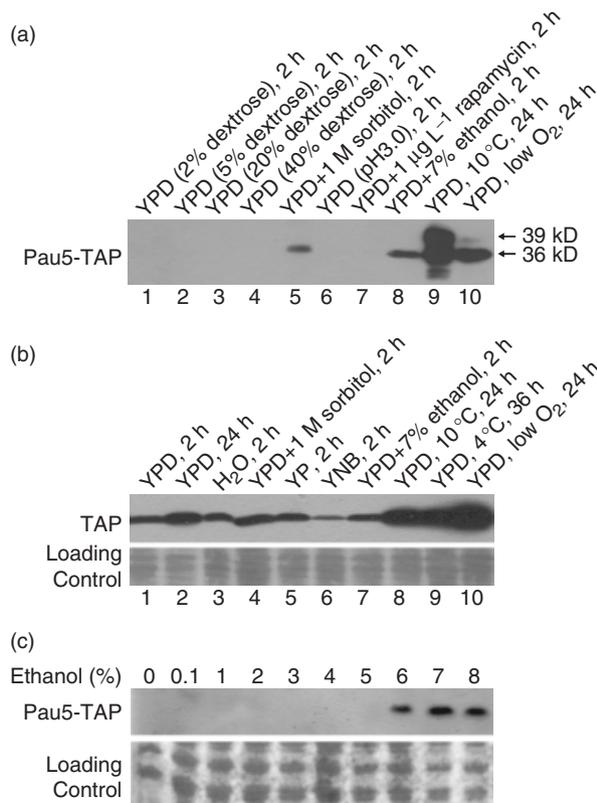
For green fluorescent protein (GFP) microscopy, cells transformed with pUG23 or pUG23-PAU5 constructs were grown to mid-log phase in liquid SD-His medium. Cells were washed and resuspended in fresh medium, mounted on slides (Guthrie & Fink, 1991) and immediately analyzed at RT with a Zeiss Pascal Confocal microscope.

## Results

### Pau5 is induced by low temperature and low oxygen conditions

To detect Pau5 under physiological conditions, we constructed a yeast strain encoding a Pau5-TAP fusion at the genomic *PAU5* locus. Knowing that expression of *PAU* genes is induced during wine fermentation, we grew yeast cells encoding Pau5-TAP fusion under different stress conditions normally met in wine making practices: osmotic stress, low pH, nitrogen starvation, high ethanol concentration, low temperature and low oxygen. Immunoblot analysis revealed that Pau5-TAP was not induced in cells grown in YPD without any stresses (Fig. 1a, lane 1), with 5%, 20% and 40% glucose (Fig. 1a, lanes 2, 3 and 4), low pH (Fig. 1a, lane 6) or in the presence of rapamycin (Fig. 1a, lane 7); Pau5-TAP was highly induced in cells grown at 10 °C or under low oxygen condition (Fig. 1a, lanes 9 and 10). A low level of Pau5p-TAP was observed in cells grown in YPD containing 1 M sorbitol or 7% (v/v) ethanol (Fig. 1a, lanes 5 and 8), which could reflect stabilization of the protein. To test this hypothesis, we measured *PAU5* promoter activity by replacing the *PAU5* ORF with a TAP tag in the genome and detecting TAP production under different growth conditions. As expected, up-regulation of TAP expression at the *PAU5* locus in response to low temperature and low oxygen was observed

by the presence of large amount of TAP (Fig. 1b, lanes 8, 9 and 10). In contrast to the Pau5-TAP fusion shown in Fig. 1a, TAP was also detected in cells grown in YPD without any



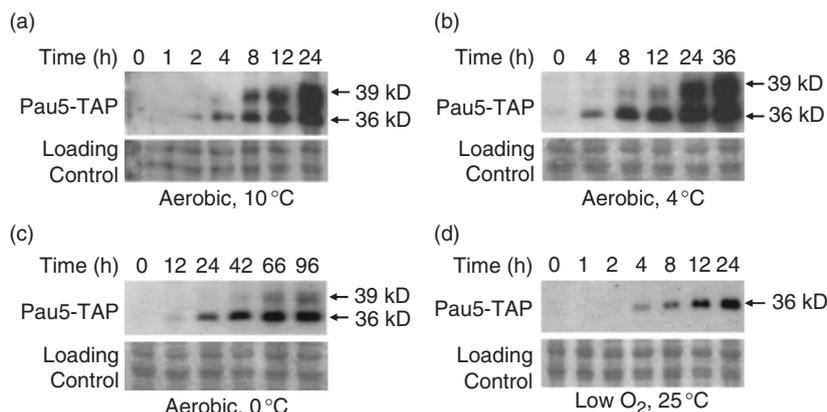
**Fig. 1.** Induction of Pau5-TAP by stresses. (a) Immunoblotting of cell lysates from strain LY6 (chromosomally encoding Pau5-TAP) grown under the conditions indicated. (b) Immunoblotting of cell lysates from strain LY19 (encoding TAP in *PAU5* locus) grown under the conditions indicated. YP, 1% yeast extract and 2% bactopectone. YNB, yeast nitrogen base without ammonium sulphate and amino acids. (c) Immunoblotting of cell lysates from strain LY6 grown in YPD supplemented with various concentrations of ethanol for 2 h. Loading control was shown by nitrocellulose membrane staining.

stresses (Fig. 1b, lane 1), indicating a basal level activity of the *PAU5* promoter under laboratory growth conditions. A similar level of TAP in cells grown in YPD without any stresses (Fig. 1b, lane 1) and YPD containing 1 M sorbitol (Fig. 1b, lane 4) or 7% (v/v) ethanol (Fig. 1b, lane 7) indicated that osmotic and ethanol stresses have no effect on the activity of *PAU5* promoter. Therefore, the low level of Pau5-TAP accumulated in cells grown in sorbitol or ethanol-containing media is a result of increased stability of Pau5, which was later confirmed by cycloheximide chase experiments (Fig. 5b).

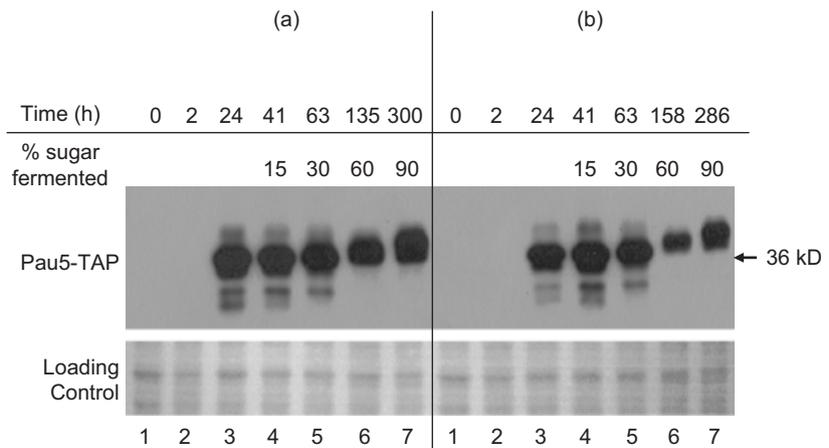
We further examined the steady state level of Pau5-TAP in cells treated with various concentrations of ethanol for 2 h. As shown in Fig. 1c, no accumulation of Pau5-TAP was detected in cells treated with < 6% (v/v) ethanol. It is interesting that sorbitol (1 M) and high concentrations of ethanol stabilize Pau5; both compounds bind free water and increase the osmotic stress in the medium. Pau5, therefore, might play a role in the adaptation of yeast cells to osmotic stress.

### Changes of Pau5 levels in response to low temperature, low oxygen and wine fermentation conditions

In addition to the above steady-state analyses, we further examined changes in Pau5 levels in response to low temperature, low oxygen and wine fermentation conditions. Immunoblotting revealed that a 36-kDa band first appeared after 2 h at 10 °C, 4 h at 4 °C, and 12 h at 0 °C; a 39-kDa band was first detected after 4 h at 10 °C, 8 h at 4 °C and 42 h at 0 °C (Fig. 2a–c). The intensity of the two bands gradually increased with time at all three temperatures. It seems that at low temperature and aerobic growth conditions, part of the 36 kDa form of Pau5-TAP gradually evolved into a 39 kDa form through an unknown posttranslational modification. In cells grown under low oxygen conditions, only the 36-kDa band was detected; it appeared after 4 h and then



**Fig. 2.** Immunoblotting of cell lysates from strain LY6 (chromosomally encoding Pau5-TAP) grown aerobically at different low temperatures (a–c) or under low oxygen at 25 °C (d) for various times.



**Fig. 3.** Immunoblotting of cell lysates from wine yeast strain LY15 (chromosomally encoding Pau5-TAP). Cells were collected at various time points as indicated during Chardonnay must fermentations. (a) and (b) are results from two independent fermentations. Loading controls were visualized by staining as described in 'Materials and methods'.

continued to accumulate with time (Fig. 2d). The increase in cellular content of Pau5 with exposure time to low temperature and low oxygen may reflect both increased production and slowed turnover in response to these conditions.

The production of Pau5 during wine fermentation using Chardonnay grape must and wine yeast strains encoding Pau5-TAP fusion protein was also investigated. The results (Fig. 3) showed that Pau5-TAP appeared somewhere between 2 and 24 h after inoculation, peaked after 24 h or at the beginning of the stationary phase (41 h) and subsequently remained constant or declined a little. This pattern of Pau5-TAP levels is in agreement with transcriptional data obtained during alcoholic fermentations using synthetic must (Rachidi *et al.*, 2000b; Rossignol *et al.*, 2003). The molecular mass of Pau5-TAP increased during the later stages of fermentation (Fig. 3a and b, lanes 6 and 7).

### Pau5 is unstable in aerobically grown cells

To investigate the stability of Pau5, we first conducted induction and chase experiments. Yeast cells were grown aerobically at 10 °C or under low oxygen conditions to induce the expression of Pau5-TAP, and then shifted to normal conditions to stop induction. In yeast cells grown aerobically at 10 °C, multiple bands of Pau5-TAP were detected that decreased in concentration when cells were shifted to 25 °C for 10 and 30 min (Fig. 4a). The 36 kDa form of Pau5-TAP that accumulated both at 10 °C (Fig. 4a) and under low oxygen conditions (Fig. 4b) decreased in concentration with a concomitant increase in size after cells were shifted to 25 °C or aerobic growth conditions for 10 and 30 min. Pau5-TAP disappeared after 60 min (Fig. 4a and b). Cycloheximide chase experiments showed that half-life of Pau5-TAP is about 10 min in aerobically grown cells and between 30 and 60 min in cells cultured in the presence of low oxygen (Fig. 5a). Given that the TAP tag by itself is stable

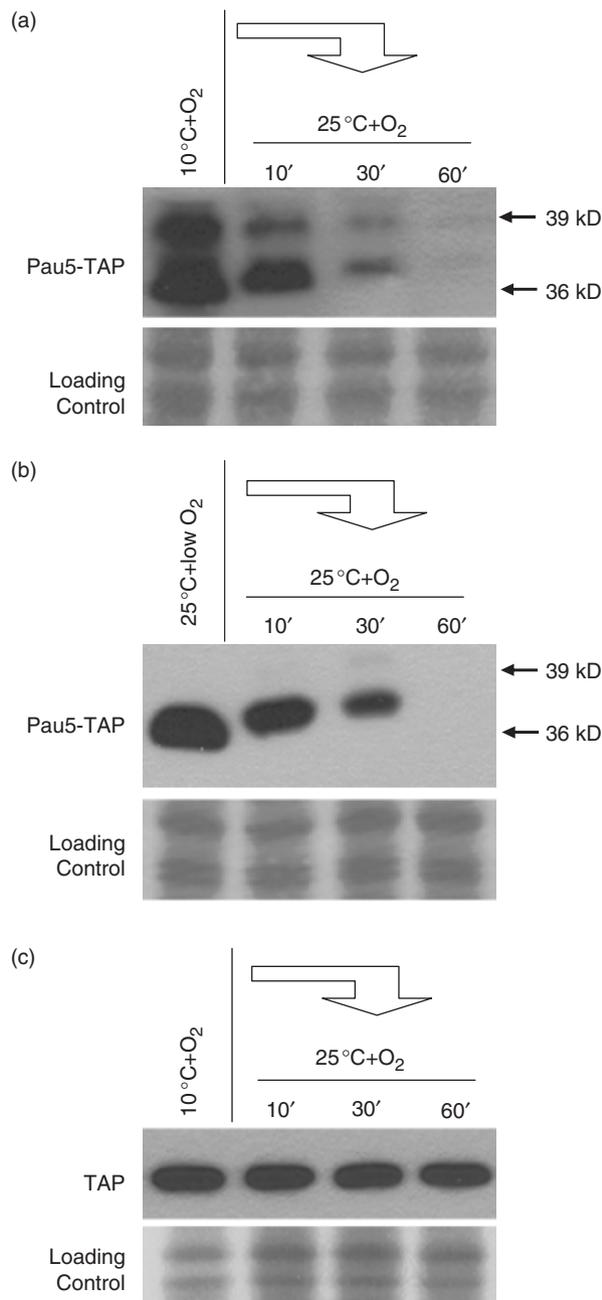
and does not change its molecular mass (Fig. 4c), these findings suggested that Pau5 is rapidly degraded in cells grown aerobically at room temperature with a gradual gain of molecular mass by about 3 kDa.

### Pau5 is temporarily stabilized by ethanol and sorbitol

The effects of ethanol and sorbitol on stability of Pau5 were next investigated by cycloheximide chase experiments. Pau5-TAP fusion was overexpressed in a *CEN* plasmid driven by the *MET25* promoter. After protein synthesis was stopped by cycloheximide, Pau5-TAP was rapidly degraded in YPD with a half-life < 15 min. Ethanol [7% (v/v)] and sorbitol (1 M) treatment substantially slowed the turnover of Pau5-TAP; the half-life was longer than 45 min (Fig. 5b). In addition, the increase in size of Pau5-TAP was clearly observed during its degradation process.

### Pau5 is O-mannosylated mainly by Pmt1

Sequence data predicted that Pau proteins are likely to be O-mannosylated. We therefore investigated the mannosylation state of Pau5 *in vivo* using ConA-Sepharose binding methods. Immunoblotting showed that multiple forms of Pau5-TAP induced by 10 °C and the single 36 kDa form of Pau5-TAP induced by low oxygen were bound by ConA beads (Fig. 6a, lanes 5, 6 and 7); treatment with buffer alone had no effect on the binding of Pau5-TAP to the ConA beads (Fig. 6a, lanes 8 and 9), but treatment with methyl  $\alpha$ -D-mannopyranoside released Pau5-TAP to the supernatant (Fig. 6a, lanes 10 and 11). Only the TAP tag was found in the supernatant fraction (Fig. 6a, lanes 2, 3 and 4). These data indicated that Pau5 was mannosylated. Given that Pau5 contains no N-linked glycosylation sites but 23 serine/threonine residues, this mannosylation is highly likely to be O-linked.



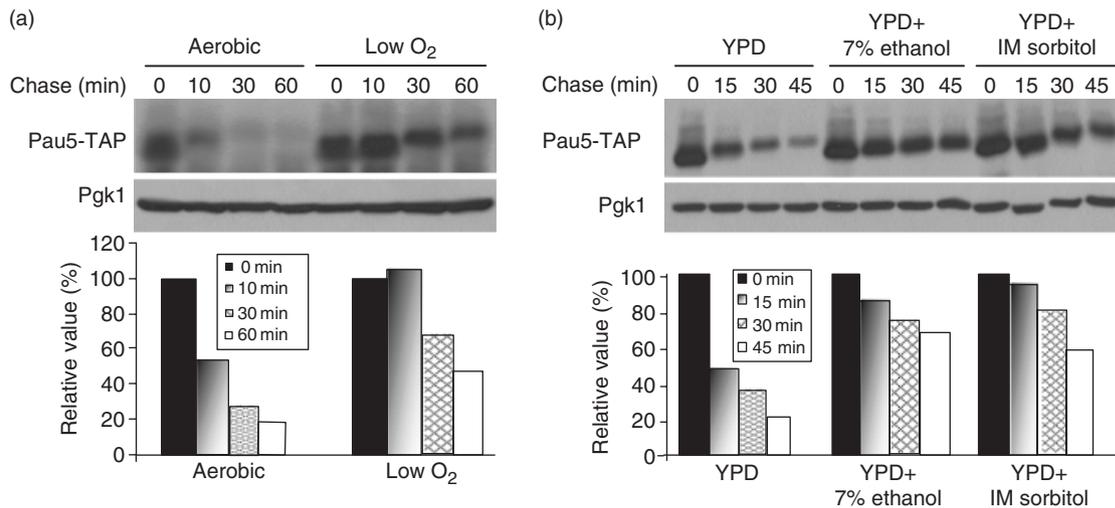
**Fig. 4.** Degradation of Pau5-TAP. (a) Immunoblotting of cell lysates from strain LY6 (chromosomally encoding Pau5-TAP) grown at 10 °C for 16 h and shifted to 25 °C for various times. (b) Immunoblotting of cell lysates from strain LY6 grown under low oxygen conditions for 16 h and shifted to aerobic growth for various times. Loading controls were visualized by staining as described in 'Materials and methods'. (c) Immunoblotting of cell lysates from strain LY19 (chromosomally encoding TAP in *PAU5* locus) grown at 10 °C for 16 h, and then shifted to 25 °C for various times. Loading control is shown by membrane staining.

In yeast, *O*-mannosylation is initiated in the ER (Haselbeck & Tanner, 1983). Six *O*-mannosyltransferases (PMTs) that are classified into three subfamilies have been identified

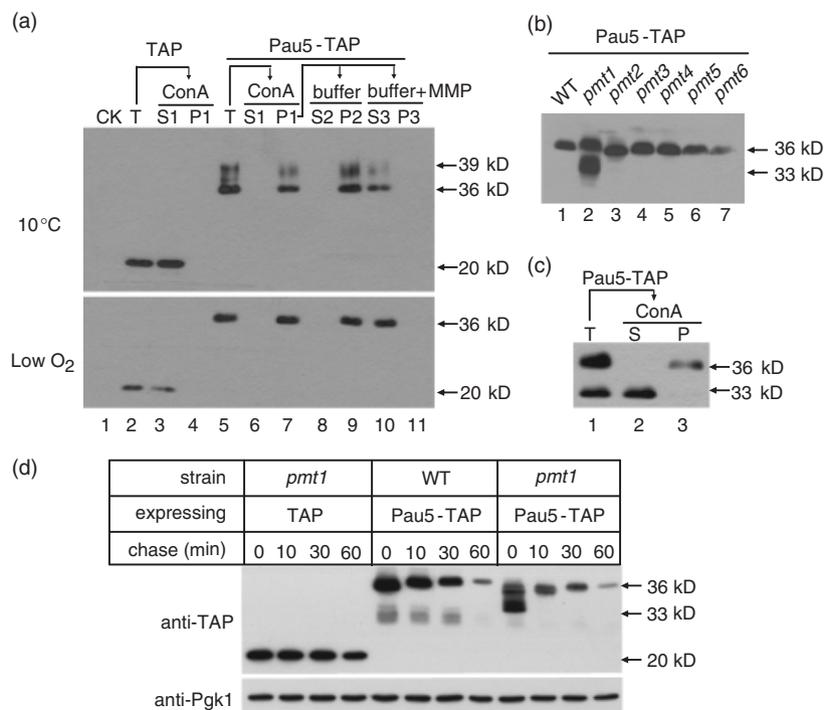
(Gentzsch & Tanner, 1996; Strahl-Bolsinger *et al.*, 1999) and are responsible for mannosylation of specific proteins (Gentzsch & Tanner, 1997; Girrbach & Strahl, 2003). To determine which PMT was involved in Pau5 *O*-mannosylation, we deleted six *PMT* genes to generate six different *PAU5-TAP* tagged yeast strains, and expressed Pau5-TAP under low oxygen conditions. In wild-type (WT), *pmt2*, *pmt3*, *pmt4*, *pmt5* and *pmt6* deletion mutants, Pau5-TAP was present almost exclusively as a 36 kDa form; in *pmt1* deletion cells an additional 33 kDa band was detected (Fig. 6b). This 33 kDa form was confirmed not to be mannosylated by its presence in the supernatant fraction in a ConA binding experiment (Fig. 6c). These data indicate that *Pmt1p* is necessary for efficient Pau5 mannosylation. We then examined the effects of *O*-mannosylation on stability of Pau5-TAP in *pmt1* deletion cells by cycloheximide chase experiments. The nonmannosylated and under-mannosylated forms of Pau5-TAP produced in *Pmt1* deficient strains disappeared after protein synthesis was stopped for 10 min, while the mannosylated Pau5-TAP both in WT and *pmt1* deletion strains was still detected after 60 min (Fig. 6d), indicating that deficiency of Pau5 mannosylation accelerated its degradation. As a control, the TAP tag itself showed high stability in *pmt1* deletion strains.

#### The nonmannosylated proform Pau5 is an integral membrane protein

Sequence data predicted a potential transmembrane domain (7–24 aa) in Pau5. We examined whether Pau5-TAP is a membrane protein using subcellular fractionation. After centrifugation of total protein extracted from *pmt1* deletion cells at 100 000 *g* for 1 h, the mannosylated 36 kDa form of Pau5-TAP cofractionated with the soluble marker Pgl1 in the supernatant fraction (Fig. 7a), suggesting that it might localize to the lumen of organelles or the cytoplasm. The 33 kDa form of Pau5-TAP that was shown to be nonmannosylated (Fig. 6b) was almost exclusively found in the membrane-containing P10 pellet (Fig. 7a) and is most likely an integral membrane protein as exemplified by alkaline phosphatase (ALP), as it was partially stripped off by 1% Triton X-100, but not by 1 M NaCl and 0.1 M Na<sub>2</sub>CO<sub>3</sub> (pH11), reagents that are typically used for solubilization of peripheral membrane proteins (Fig. 7b). In order to determine the subcellular localization of Pau5, we constructed and expressed Pau5-GFP fusion proteins in wild-type cells from a *CEN* plasmid under control of *MET25* promoter. Microscopy showed that the fluorescence in cells expressing Pau5-GFP seems to be distributed in the cytoplasm, but not as uniformly and strongly as that in cells expressing GFP (Fig. 7c). Given that Pau5 is not stable in the presence of oxygen, we cannot exclude the possibility that the observed fluorescence in Pau5-GFP cells was affected by degradation of Pau5-GFP.



**Fig. 5.** Stabilization of Pau5-TAP in the presence of low oxygen, ethanol and sorbitol stresses. (a) Immunoblotting of cell lysates from strain LY6 (chromosomally carrying *PAU5-TAP* fusion) which were grown under low oxygen conditions at 25 °C for 16 h, exposed to cycloheximide and chased under aerobic or low oxygen conditions for the times indicated. (b) Immunoblotting of cell lysates from strain LY190 (expressing Pau5-TAP under control of *MET25* in a *CEN* plasmid). Logarithmically growing cells in YPD were collected and suspended in YPD, YPD plus 7% ethanol and YPD plus 1 M sorbitol, respectively, and then treated with cycloheximide for the indicated times. The blots were probed with anti-Pgk1 antibodies as a reference. Charts on the bottom of (a) and (b) show the relative level of Pau5-TAP normalized by the respective Pgk1 band signal.



**Fig. 6.** Mannosylation of Pau5. (a) ConA binding of Pau5-TAP with cell lysates from strain LY19 expressing TAP and strain LY6 expressing Pau5-TAP. Proteins extracted from BY4742 were used as control (CK). (b) Immunoblotting of cell lysates from strains chromosomally expressing Pau5-TAP in WT or in different *pmt* deletion strains. Pau5-TAP was induced by growing cells under low oxygen conditions at 25 °C for 16 h. (c) ConA binding analysis of Pau5-TAP with cell lysates from strain LY98 (*pmt1*, carrying *pMET25p-PAU5-TAP*). Cells were grown in SC without uracil and methionine to mid log phase. (d) Immunoblotting of cell lysates from strains LY280 (*pmt1*, *pMET25p-TAP*), LY 190 (WT, *pMET25p-PAU5-TAP*) and LY98 (*pmt1*, *pMET25p-PAU5-TAP*). Logarithmically growing cells in SC media without uracil were exposed to cycloheximide and chased for various times.

**Disruption and overexpression of *PAU5* do not affect cell growth**

In an attempt to determine the function of *PAU5*, we analyzed disruption and overexpression variants. No growth defects were found in either case, whether plated on medium supplemented with 0.001% SDS, 1 M sorbitol or 7% etha-

mol, or following incubation at low temperature or under anaerobic conditions (Fig. 8).

**Discussion**

We report three major findings in this study. First, Pau5 is highly induced by low temperature and low oxygen

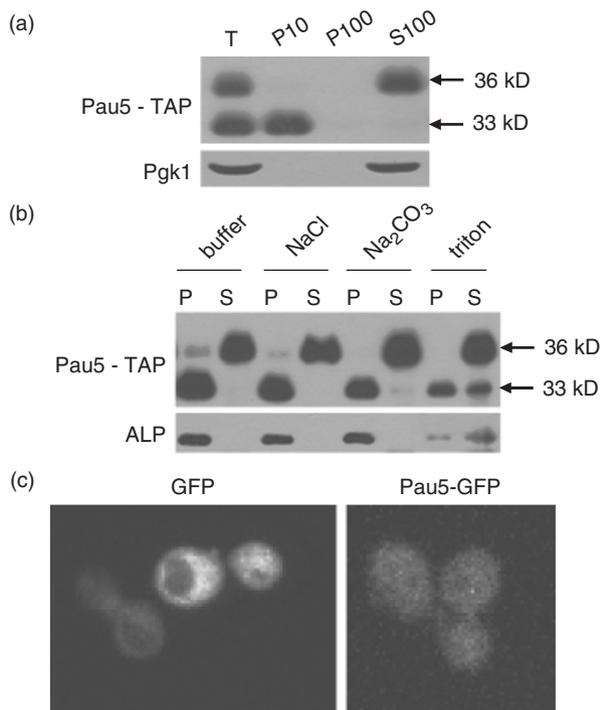
conditions. Secondly, Pau5 in yeast cells grown under laboratory conditions is unstable; certain stresses temporarily stabilize Pau5. Thirdly, *O*-mannosylation of Pau5 enhances its stability.

Low temperature and hypoxia/anaerobiosis have been reported to upregulate expression of *PAU* genes at the mRNA level (Schade *et al.*, 2004; Lai *et al.*, 2005), and the protein data presented here support these findings. Further-

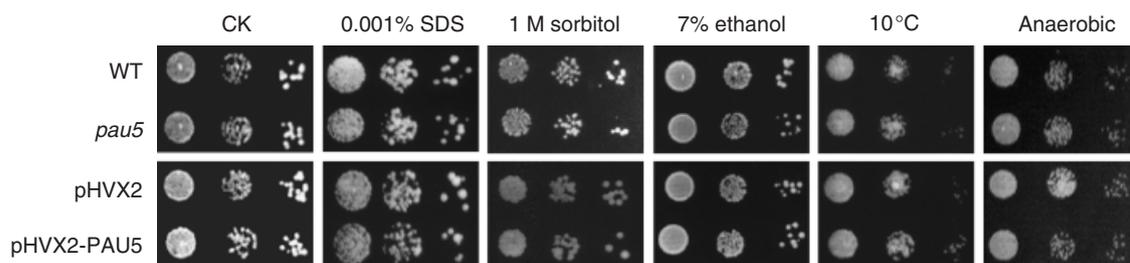
more, multiple forms of Pau5 protein with molecular mass larger than that calculated from amino acid sequences were detected, suggesting that posttranslational modification events occurred, which could be essential for its stability and function.

*Saccharomyces cerevisiae* adapts to low temperature and hypoxia/anaerobiosis by expressing a large group of specific cold responsive genes and hypoxic/anaerobic genes (Homma *et al.*, 2003; Schade *et al.*, 2004; Lai *et al.*, 2005), among which are those required for biosynthesis of unsaturated fatty acids (*OLE1*) or sterols (*HMG1*, *HMG2* and most members of *ERG* genes), as well as *PAU* genes and their relatives *DAN/TIR* genes. One of the conserved changes in yeast cells grown under low temperature and hypoxic/anaerobic conditions is the decrease of membrane fluidity which is counteracted by increasing production of unsaturated fatty acids or uptake of sterols during adaptation. Pau proteins and Pau domain-containing proteins (Dan/Tir family) might be involved in this adaptation process, because: (1) expression of *PAU* genes, *DAN/TIR* genes and most of the sterol biosynthetic genes are under control of the same transcription activators, Upc2 and Ecm22 (Abramova *et al.*, 2001a, b; Vik & Rine, 2001); (2) inactivation of the ergosterol synthesis pathway highly induces the expression of *PAU* genes (Mnaimneh *et al.*, 2004); (3) integrated analysis of highly heterogeneous genome-wide data predicted functional annotation of *PAU* genes into sterol metabolism (Tanay *et al.*, 2004); (4) Dan1 is involved in the promotion of sterol uptake during anaerobic stress (Alimardani *et al.*, 2004).

Oxygen not only negatively regulated the expression of *PAU* genes, but also accelerated the degradation of Pau proteins. Similar regulation by oxygen at both transcriptional and posttranslational levels occurs in the  $\alpha$ -subunit of hypoxia-inducible factor (HIF) in mammalian cells. HIF- $\alpha$  is induced by anaerobiosis and rapidly degraded in the proteasome in the presence of oxygen (Huang *et al.*, 1998), through a pathway involving hydroxylation of specific proline residues which allows binding of HIF- $\alpha$  to pVHL, the



**Fig. 7.** Subcellular fractionation and localization of Pau5. (a) Immunoblotting of cell lysates from strain LY98 (*pmt1*, *pMET25p-PAU5-TAP*) after being fractionated by centrifugation at 10 000 and 100 000 *g* as described in 'Materials and methods'. (b) Immunoblotting of cell lysates from strain LY98 treated with buffer, NaCl, Na<sub>2</sub>CO<sub>3</sub> and Triton X-100 before centrifugation and probed. (c) Microscopy of wild-type cells transformed with pUG23 (expressing GFP) and pUG23-PAU5 (expressing Pau5-GFP).



**Fig. 8.** Growth of cells on agar plates under various stresses. Wild-type (BY4742), LY19 (BY4742, *pau5*Δ), LY30 (BY4742, pHVX2) and LY32 (BY4742, pHVX2-PAU5-TAP) strains were grown in SC or SC minus leucine broth overnight at 25 °C to mid log phase and spotted with 10-fold serial dilution on SC or SC-Leu plates, without or with supplement of 0.001% SDS, 7% (v/v) ethanol and 1 M sorbitol. Anaerobic conditions were obtained by BBL GasPak Anaerobic Systems (Becton Dickinson).

recognition component of an ubiquitin ligase (Maxwell *et al.*, 1999; Ivan *et al.*, 2001; Jaakkola *et al.*, 2001). A similar combination of transcriptional and posttranslational regulation has also been reported in the glucose signal transduction pathway in *S. cerevisiae*; for example, glucose not only represses the expression of *MTH1* gene, but also induces rapid degradation of Mth1 protein (Flick *et al.*, 2003; Moriya & Johnston, 2004). In this case, Mth1 is phosphorylated before ubiquitin-mediated degradation (Spielewoy *et al.*, 2004). We observed degradation of Pau5-TAP accompanied by 3 kDa increase in its molecular mass, which might be as a result of hyperphosphorylation. To define this modification and the cellular machinery required for Pau5 turnover will be of great significance in understanding the mechanisms of oxygen-regulated protein degradation in *S. cerevisiae*. Besides the development of systems in selective removal of 'unwanted' proteins, yeast has also evolved mechanisms to stabilize proteins by some specific stresses under which these proteins are 'wanted'. For example, Cln3, a key cell cycle target for osmotic stress, is temporarily accumulated in osmotically stressed cells as a result of increased stability (Bellí *et al.*, 2001). We observed stabilization of Pau5 by osmotic and ethanol stresses which, together with hypoxia/anaerobiosis and low temperature, all point to the fermentative life style of *S. cerevisiae*. Therefore, we speculate that Pau proteins might be involved in adaptation of yeast cells to alcoholic fermentations.

Pau5 is O-mannosylated, but we have not confirmed which of the 23 Ser/Thr residues are mannosylated. We observed fully mannosylated Pau5 in cells lacking one of the six O-mannosyltransferases, but only in the *pmt1* deletion cells was mannosylation incomplete, indicating that Pmt1 is the main player in Pau5 mannosylation; either the role of Pmt1 could be partially accomplished by other Pmt member(s), or the defects of other Pmt member(s) are rescued by Pmt1. O-mannosylation has been reported to be required for stability and localization of some cell wall mannoproteins, such as Axl2 (Sanders *et al.*, 1999), Wsc1, Wsc2 and Mid2 (Lommel *et al.*, 2004). The Pau relative Tir1p/Srp1 was also absent from both the membrane and cell wall fractions in *pmt1* deletion cells (Bourdineaud *et al.*, 1998). Our data indicates that O-mannosylation enhances stability of Pau5. We further present evidence that the mannosylated mature form Pau5 is soluble, whereas the nonmannosylated pro-form Pau5 is an integral membrane protein. There are two possible explanations for the differential localization of mannosylated vs. nonmannosylated Pau5; either O-mannosylation causes release of the protein from the membrane, or the membrane anchor sequence is cleaved. Given that mature Tir1 is missing its signal sequence (Kitagaki *et al.*, 1997), and Tir1 has N-terminal with regions that are highly homologous to the N-terminal of Pau proteins, the latter possibility would seem the most probable. Experiments on

subcellular localization of Pau5-GFP described in this paper were inconclusive because of the instability of Pau5 in the presence of oxygen. Indirect immunofluorescence microscopy with yeast cells grown under anaerobic conditions might be useful in future studies on Pau5 localization.

No growth phenotype was observed by deletion or over-expression of *PAU5*. This is not surprising, as other Pau members may overlap with Pau5 in their functions. The elucidation of functions for Pau proteins might need to wait until identification of their binding partners and an approach to simultaneously delete all 24 copies of *PAU* genes. Nevertheless, the current study on production and properties of Pau5 provides important clues for future functional characterization.

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