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Full Paper

Proline transport and stress tolerance of ammonia-insensitive mutants of the *PUT4*-encoded proline-specific permease in yeast

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The imino amino acid, proline, has roles in both cellular nutrition and response to stress. Proline uptake in *Saccharomyces cerevisiae* is largely mediated by a high affinity, specific permease, Put4p, and a low affinity general amino acid permease, Gap1p. Both are subject to nitrogen catabolite repression (NCR) and nitrogen catabolite inactivation (NCI). In order for proline to be fully exploited, its transport must be derepressed, as occurs upon depletion of preferred nitrogen sources, and molecular oxygen must be present to allow the first step of catabolism via proline oxidase. This study focuses on the isolation of variants of Put4p, which are insensitive to repression by a preferred nitrogen source (ammonia) and their subsequent effect on proline transport and stress tolerance. Specific amino acid residues in the carboxy-terminal region of Put4p were targeted by site-directed mutagenesis. Substitution at Serine^{605}, a potential phosphorylation target, led to the amelioration of ammonia-induced down-regulation of Put4p. When combined with a promoter mutation (−160), the S^{605}A mutation resulted in increased proline uptake and accumulation. This increase in proline accumulation was associated with increased cell viability in conditions of high temperature and osmotic stress raising possible benefits in industrial fermentation applications.

Key Words——proline uptake; proline-specific permease; *PUT4*; *Saccharomyces cerevisiae*; site-directed mutagenesis; stress tolerance

Introduction

Beverage fermentations often involve the growth of yeast in a nutritionally complex and harsh medium. In grape must, the nitrogen compounds present include proteins, peptides, amino acids, ammonium ions and trace levels of vitamins and nucleotides (Henschke and Jiranek, 1993). Of these, yeast can utilize most α-amino acids, small peptides and ammonium under typical enological conditions. Assimilable nitrogen in grape must varies from 60 to 2,400 mg L^{-1} (Ough and Amerine, 1988) with low levels limiting yeast growth rate and total biomass formation resulting in a sluggish fermentation (Bely et al., 1990). Of the sources of nitrogen available to yeast in grape juice, proline often predominates (Ough and Amerine, 1988); however, unlike other amino acids, only small amounts of proline are utilized throughout enological fermentations

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(Ingledew et al., 1987; Ough and Stashak, 1974). This is due to the prevailing conditions, which effectively prevent the utilization of significant amounts of this amino acid.

Before it can be catabolized and utilized as a nitrogen source, proline must be transported into the cell. This occurs by a non-specific low affinity (K_m = 13 mM; Lasko and Brandriss, 1981) transport system encoded by the *GAP1* (General Amino-acid Permease) gene, and a high affinity (K_m = 0.025 mM; Magana-Schwencke and Schwencke, 1969) proline specific permease encoded by the *PUT4* (Proline UTilization) gene (Jauniaux et al., 1987). In addition, two SPS sensor regulated amino acids permeases, Agp1p and Gnp1p, with broad substrate specificities (Regenberg et al., 1999) have recently been shown to facilitate proline uptake (Andréasson et al., 2004). In contrast to *AGP1* and *GNP1*, both *GAP1* and *PUT4* are subject to nitrogen catabolite repression (NCR) and their protein products to nitrogen catabolite inactivation (NCI). Proline is catabolized within the mitochondrion in a two-step conversion to glutamate (Krzywicki and Brandriss, 1984; Wang and Brandriss, 1987), in which the first enzyme, proline oxidase (*PUT1*) is oxygen dependent (see Fig. 1). During the early stages of a wine fermentation, when oxygen may be present (depending on the extent of reductive handling of the juice), the high levels of preferred nitrogen sources, such as ammonia and glutamine, result in repression of transporter synthesis and inactivation of existing Gap1p and Put4p (Courchesne and Magasanik, 1988; Roberg et al., 1997; Soetens et al., 2001; Stanbrough and Magasanik, 1995). In subsequent stages of fermentation when levels of preferred nitrogen compounds decline and proline transport is derepressed, oxygen instead is no longer available (Poole, 2002).

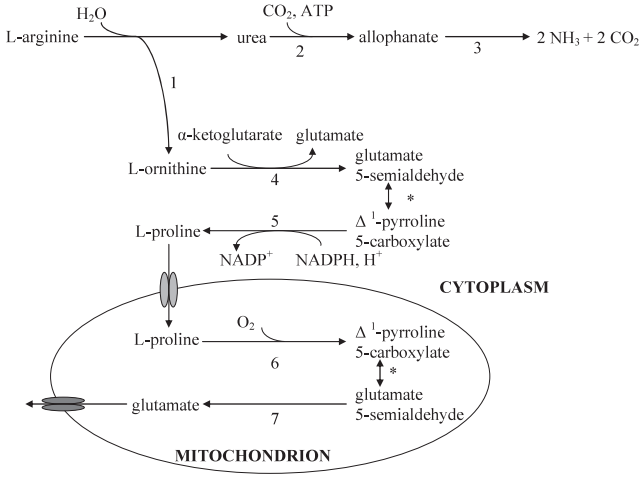

Fig. 1. Arginine and proline degradation in *Saccharomyces cerevisiae* adapted from Henschke and Jiranek (1993).

Enzymes: 1, arginase; 2, urea carboxylase; 3, allophanate hydrolase; 4, ornithine 5-aminotransferase; 5, Δ-pyrroline-5-carboxylate reductase; 6, proline oxidase; 7, Δ-pyrroline-5-carboxylate dehydrogenase; ∗, spontaneous.

The potential usefulness of proline as a nitrogen source is highlighted through work on the degradation of arginine, the third most prevalent amino acid in grape must (Ough and Amerine, 1988). Under anaerobic conditions, yeast is able to assimilate only three of the four nitrogen atoms of arginine with the fourth, as proline, being unassimilated. Martin et al. (2003) were however able to modify the anaerobic degradation of arginine, to allow for the assimilation of all four nitrogen atoms. That is, the NADPH-dependent P5C reductase (*PRO3*) and the *URE2* negative regulator were deleted, while a *PUT2* (encoding NAD(P) linked Δ^1-pyrroline-5-carboxylate dehydrogenase) devoid of a mitochondrial targeting sequence was strongly expressed (see Fig.1). In being able to utilize the fourth (proline) nitrogen in arginine, the recombinant strain was able to produce twice the biomass of the unmodified strain.

Given the typical predominance of proline in grape must and yet the frequent need for nitrogen supplementation of fermentations (Ough and Amerine, 1988), there is an obvious interest in yeast which can more efficiently utilize proline as a nitrogen source under enological conditions. Exploitation of this largely unassimilable but abundant nitrogen source could allow ferments to proceed more reliably and without supplementation. Problem fermentations may so be eliminated, thereby increasing production efficiency and wine quality.

Beyond offering a nutritional advantage, enhanced proline uptake may also convey a broad protective effect. Proline confers survival and growth advantages in the presence of herbicides (Chen and Dickman, 2005) and hydrogen peroxide (Terao et al., 2003), and acts as a cryoprotectant (Morita et al., 2002) in yeast, as well as compatible solute in plants (Delauney and Verma, 1993) and bacteria (Csonka, 1981). As such the ability of yeast to accumulate higher intracellular concentrations of proline, even without significant catabolism, may offer benefits in the high osmolarity environment of the grape juice fermentation.

This study aims to complement other studies by

overcoming the metabolic restrictions imposed by ammonia on proline utilization, through the constitutive expression of Put4p permease in yeast. Site-directed mutagenesis targeted amino acid residues in the C-terminal region of Put4p that are potentially involved in the ammonia-induced down-regulation of the permease. In addition to considering proline uptake and accumulation, tolerance of such strains to osmotic and thermal stress was also considered in light of the potential role of this amino acid in such attributes (Morita et al., 2002; Takagi et al., 2000).

Materials and Methods

Strains and media. The wild-type strain used in this study, KP2 (a haploid prototroph of W303**a**), was derived by mating W303**a** with a lys-2 marker yeast by classical genetics (Guthrie and Fink, 1991). The prototrophic haploid strain was backcrossed several times with VJ23, (a strain isogenic to W303 except for the *his3* and *leu2* mutations) in order to reconstitute the W303 genetic background of KP2. Deletion strains derived from KP2, including those harboring plasmid borne copies of *PUT4*, are listed in Table 1. Rich media (YPD) was used for routine culture maintenance. Yeast nitrogen base (YNB) media lacking ammonium sulfate and amino acids, was supplemented with the required amino acids and glucose at 20 g L^{-1}. The nitrogen source was 5 g L^{-1} ammonium sulfate or proline (Sherman, 1991). *Escherichia coli* were grown on Luria Bertani (LB) broth supplemented with ampicillin at 100 mg L^{-1} for plasmid selection, unless otherwise stated.

Transformations. Yeast cells were transformed by treatment with lithium acetate (Gietz et al., 1992). Geneticin (Astral Scientific) was used at 400 mg L^{-1} where indicated for selection of *kanMX* based gene disruptions (Walker et al., 2003). *E. coli* transformation was according to Ausubel et al. (1994).

Nucleic acid techniques. Plasmid DNA preparations from *E. coli* were made using the Promega Wizard *Plus* SV plasmid miniprep kit according to the manufacturer's instructions. Yeast genomic DNA and RNA was isolated as described by Adams et al. (1997) and Schmitt et al. (1990) respectively. DNA manipulations were performed using standard procedures (Maniatis et al., 1982). DNA was isolated and purified with an UltraClean 15 DNA purification kit (MO BIO Laboratories).

PCR amplification of DNA. PCR amplification was performed in 50 μl reactions containing 1 U DyNAzyme EXT DNA polymerase (Finnzymes), DyNAzyme EXT

Table 1. Microbial strains used in this study.

Strain	Genotype	Source
Saccharomyces cerevisiae		
W303a	*ade2-1*, *can1-100*, *his3-11*, *-15*, *leu2-3*, *-112*, *trp1-1*, *ura2-1*, *MAT***a**	Susan Henry
Lys2 marker	*lys2*, *MAT*α	Susan Henry
VJ23	*his3-11*, *-15*, *leu2-3*, *-112*, *MAT*α	This laboratory
KP2	W303 (prototrophic), *MAT*α	This study
KP30	W303, *MAT*α, *npr2*Δ*::kanMX4*	This study
KP31	W303, *MAT*α, *rcy1*Δ*::kanMX4*	This study
KP10	W303, *MAT*α, *put4*Δ*::kanMX4*	This study
KP12	W303, *MAT*α, *put4*Δ*::kanMX4*, *ura3*Δ*::kanMX4*	This study
KP21	KP12 harboring pTEF-PUT4	This study
KP13	KP12 harboring pFL38-PUT4	This study
KP52	KP12 harboring pFL38-PUT4(−160)	This study
KP61	KP12 harboring pFL38-PUT4($K^{604}R$)	This study
KP62	KP12 harboring pFL38-PUT4(LVAA)	This study
KP63	KP12 harboring pFL38-PUT4(LLAA)	This study
KP64	KP12 harboring pFL38-PUT4($S^{605}A$)	This study
KP65	KP12 harboring pFL38-PUT4($S^{605}E$)	This study
TW3	KP12 harboring pFL38-PUT4(−160, $S^{605}A$)	This study
Escherichia coli		
DH5α		Tony Focareta
Epicurian Coli XL1-Blue		Stratagene

PCR buffer, 100 pmol of each primer, 50 ng of plasmid DNA or 200 ng of genomic DNA and 0.5 mM dNTPs. $MgCl_2$ concentrations and cycling parameters were optimized for each PCR reaction. Primers used for PCR amplification are listed in Table 2.

Site-directed mutagenesis. Mutations in specific codons of Put4p and nucleotides in the *PUT4* promoter were introduced with the aid of the QuikChange Site-Directed Mutagenesis kit (Stratagene). The PCR reaction was according to the supplier's instructions, except that the extension time was 3 min/kb (24 min). Individual reactions contained pFL38-PUT4 (10 ng) as template and specific sets of primers as listed in Table 2. PCR products of the correct size were digested with *Dpn*I prior to transformation of Epicurian Coli XL1-Blue competent cells. DNA sequencing (in triplicate) of the isolated plasmids using primers SDMSEQ and MutSEQ1, confirmed the presence of specific point mutations. Plasmids pFL38 and p416TEF (Bonneaud et al., 1991; Mumberg et al., 1995) were the source material for plasmids described in Table 1, which (with the exception of pFL38-PUT4 and those containing single point mutations) were generated in vivo using the gap-repair method (Ma et al., 1987). A 1 : 5 ratio of linearized plasmid and amplified DNA (*PUT4* gene or *TEF2* promoter sequences), was used to transform yeast KP12. Plasmid constructs were verified by PCR analysis and DNA sequencing (IMVS, Adelaide, Australia).

PCR labeling of probes for Northern slot blot analysis. DNA probes for the detection of RNA were labeled with Digoxigenin-11-dUTP (DIG) by incorporation into PCR products. The PCR DIG Probe Synthesis Kit (Roche) was used as per the manufacturer's instructions. The DIG labeled products were gel purified before use. Primers used for the synthesis of DIG labeled probes are listed in Table 2.

Northern slot blot analysis. RNA slot blotting: Aliquots containing 5 μg of RNA were diluted in 100 μl of RNA dilution buffer (5 : 3 : 2 ratio of MQ water : 20×

Table 2. Sequences of the primers used in this study.

Primer	Sequence (5′→3′)
TEFPUTf[a]	GGAGTACTTGTTTTTAGAATATACGGTCAACGAAGTATAATTAACTAAACCCATCTAGAAATAAATCATGG
Put4r[a]	TTGCTGCAGTGGTAAAGATCA
Put4f[a]	AAGATTGTAGAAAGGATCCGC
P4UTRr[a,c]	ATTGTTCTTGTGGAAGGGCA
P4probeF[a,c,d]	TGCCCTTCCACAAGAACAAT
P4probeR[a,c,d]	AACAAGGCGTCCAAGAACTT
M1-160f[b]	CAGACGCATAAACATATATGCATATACATACTTATACACTCG
M1-160r[b]	CGAGTGTATAAGTATGTATATGCATATATGTTTATGCGTCTG
K604Rf[b]	GTCGAGATCGAGGAGAGATCAAGAGAAATTGAGGAGATGAG
K604Rr[b]	CTCATCTCCTCAATTTCTCTTGATCTCTCCTCGATCTCGAC
LV-AAf[b]	GATCGATGTTACTACAGGGGCAGCCGAGATCGAGGAGAAATC
LV-AAr[b]	GATTTCTCCTCGATCTCGGCTGCCCCTGTAGTAACATCGATC
Dileuf[b]	CAAAGACAAGTTCTTGGACGCCGCGGCGTAACGCTTTATGAAC
Dileur[b]	GTTCATAAAGCGTTACGCCGCGGCGTCCAAGAACTTGTCTTTG
S605Af[b]	GTCGAGATCGAGGAGAAAGCAAGAGAAATTGAGGAGATGAG
S605Ar[b]	CTCATCTCCTCAATTTCTCTTGCTTTCTCCTCGATCTCGAC
S605Ef[b]	GGCTAGTCGAGATCGAAGAGAAAGAAAGAGAAATTGAGGAG
S605Er[b]	CTCCTCAATTTCTCTTTCTTTCTCTTCGATCTCGACTAGCC
MutSEQ1[c]	AGGGTGTAAAGTGCGTGTG
MutSEQ2[c]	TACTGCAATGTGAATTCCC
MutSEQ3[c]	TATGACGTTTGGGTGGCCT
MutSEQ4[c]	TTATGTACATGCCCCAATC
SDMSEQ[c]	GTTCGGCCATAAGCTGTA
PDAf[d]	TCGCACCTGTATCTTCACAAA
PDAr[d]	AATCCCTAGAGGCAAAACCTT

[a] Primers used in PCR amplification of *PUT4* gene. [b] Primers used in site-directed mutagenesis of *PUT4* gene. [c] Primers used in DNA sequencing of *PUT4* gene and its variants. [d] Primers used for PCR synthesis of DIG-labeled probes.

SSC : formaldehyde) before being transferred to Hybond N+ nylon membrane (Amersham Life Scientific) using a slot blot apparatus (Hoeffer 648, Amersham Pharmacia Biotech). RNA was fixed to the membrane by UV crosslinking using the Amersham Life Sciences UV crosslinker apparatus (254nm @ 70,000 μJ/cm^2).

Hybridization and detection of DIG-labeled DNA probe bound to specific RNA: Prehybridization using DIG EASy Hyb buffer (Roche) was conducted at 50°C for 2 h. Hybridizations were carried out at 50°C overnight using 25 ng/ml of denatured DIG-labeled probe and washed to a stringency of 0.5× SSC/0.1% SDS at 68°C. Chemiluminescent detection of RNA was conducted according to Van Miltenburg et al. (1995). Briefly, the membrane was washed at room temperature, followed by incubation in 1× blocking solution (Roche) prior to incubation with anti-Digoxigenin-AP antisera (1 : 100,000 in blocking solution; Roche). The membrane was washed before equilibration in detection buffer and then incubated in the dark with 0.5 ml ECF substrate (Amersham Life Sciences) per 10 cm^2 of membrane. Fluorescent bands were detected using a Storm 860 phosphoimager (Molecular Dynamics).

Proline assays. Proline uptake: Measurement was as described by Lasko and Brandriss (1981) with minor variations. Cells (5–20 ml) were harvested by filtration, washed in YNB (lacking carbon and nitrogen sources), and incubated for 5 min in pre-warmed aerated YNB lacking a nitrogen source. [^{14}C]-proline (0.5 μCi, specific activity 260 mCi/mmol, Amersham Pharmacia, CFB71) was added to a final concentration of 0.2 mM and 500 μl samples were taken at 15, 60, 105 and 150 s after addition. The sampled cells were filtered, washed with YNB lacking a carbon source and containing 0.1% proline, and dried (microwave at 300 W, 2 min), before addition of 4 ml scintillation fluid (Starscint, 6013248). [^{14}C]-proline incorporation was determined as counts per min (CPM) using a Packard scintillation counter. Values were normalized (nmol proline min^{-1} mg^{-1} cells) by dry weight determination (in duplicate) of an identical sample at the time of sampling.

Proline accumulation: YNB media containing 150 g L^{-1} glucose, 1 g L^{-1} proline, and 0.427 mM [^{14}C]-proline (Amersham Pharmacia, CFB71) was inoculated with yeast cells at 1 × 10^6 cells ml^{-1}. Samples (500 μl) taken at 0, 3, 6, 9, 12 and 24 h intervals were harvested on 0.22 μm nitrocellulose filters, and washed twice with sterile MQ water before addition of 4 ml of scintillation fluid. [^{14}C]-proline incorporation was determined as described above. Values for the mean (including standard deviation) were from duplicate determinations of triplicate cultures.

Stress treatments. Osmotolerance: Strains precultured in YNB media containing 1 g L^{-1} proline as nitrogen source, were transferred (at 5 × 10^6 cells ml^{-1}) to YNB media of high osmolarity (1.1 M NaCl, 1.1 M glucose or 1.1 M sorbitol) and grown for 24 h. Conical flasks (250 ml) fitted with a septum sealed side port for sampling and side arm (to allow direct measurement of growth) were used in the experiment. Growth was monitored over 24 h, and measured directly in the culture flasks by inserting the side-arm (filled with culture) into a Hach 2100AN Turbidimeter fitted with a 610 nm filter. Culture density was expressed as nephelometric turbidity units (NTU). The percentage of viable cells present at the end of fermentation was measured by comparison of the number of colony forming units after 2–3 days of growth on YPD from appropriately diluted cultures (in phosphate buffered saline) to a total cell count of the same time point, using a hemocytometer.

Thermotolerance: Precultured cells were washed in fresh media and subjected to heat (20 min, 45°C). Viability measurements were performed prior to cells being transferred to YNB plus 1 g L^{-1} proline (5 × 10^6 cells ml^{-1}), and growth monitored as turbidity units (NTU). Values for the mean (including standard deviation) were from duplicate determinations of triplicate cultures.

Results

Generation of strains non-responsive to nitrogen catabolite repression

Strain KP2 (W303 prototroph, *MAT*α), in which *PUT4* expression is subject to NCR, was chosen as the wild type strain for this study (Poole, 2002). Gene-replacement of *PUT4* with the *kanMX4* selection marker was achieved by homologous recombination with a PCR-generated DNA fragment consisting of the *kanMX4* cassette with flanking *PUT4* sequences (Wach et al., 1994) and resulted in the construction of strain KP10 (*put4*Δ*::kanMX4*). This strain served as background for introduction through gap-repair and homologous recombination (Ma et al., 1987; Mumberg et al., 1995) of *PUT4* under the control of the constitutive *TEF2* promoter (i.e. *pTEF-PUT4*) to yield strain, KP21. A strain

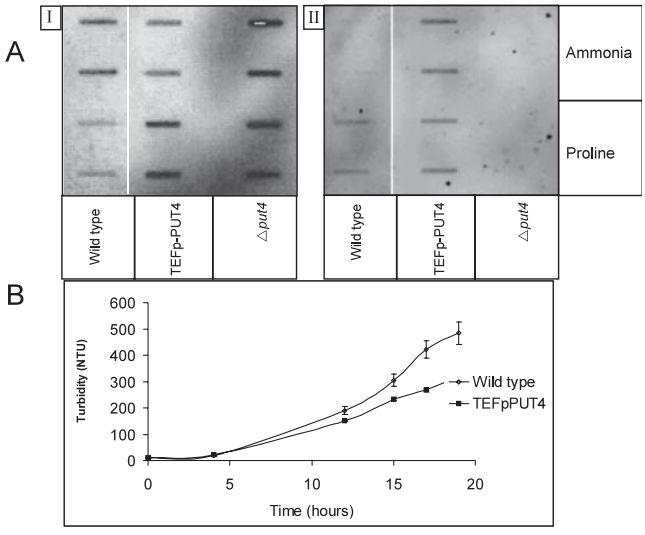

Fig. 2. Northern slot blot analysis of *PUT4* expression.

A: Strains KP2 (wild type) and KP21 (*TEFp-PUT4*) were grown in minimal media containing either proline or ammonium as the sole source of nitrogen. Cells were harvested at 5×10^7 cells/ml and cellular RNA was extracted and analyzed in duplicate by Northern slot-blot analysis as described in MATERIALS AND METHODS. Panel I: detection of *PDA* mRNA with DIG-labeled DNA probe corresponding to the *PDA* gene was the loading control of RNA samples. Panel II: *PUT4* mRNA detected with a DIG-labeled DNA probe corresponding to the *PUT4* gene. B: Strains KP2 (wild type) and KP21 (*TEFp-PUT4*) were grown (in triplicate) in minimal media containing ammonium as the sole source of nitrogen. Growth of the culture was measured directly using a Turbidimeter, and expressed as nephelometric turbidity units (NTUs).

with constitutive *PUT4* expression was constructed in order to study the effects of a nitrogen source on this proline-specific permease. This strain gave a constitutive pattern of *PUT4* expression (defined as presence of *PUT4* mRNA under ammonia-repressive conditions); however, its growth was typically of the order of 65% of the wild type (Fig. 2), presumably due to the metabolic load imposed on this strain by the relatively high strength of the *TEF2* promoter (Piruat et al., 1997).

Post-translational down-regulation of the proline-specific permease, Put4p

The presence of preferred nitrogen sources not only leads to the repression of *PUT4*, but also to the inactivation of the Put4p permease (Jauniaux et al., 1987). Therefore, to determine whether *PUT4* expression equates to Put4p activity, direct determination of Put4p transport activity by one of the constitutive strains (KP21, *pTEF-PUT4*) was carried out by isotopic assay (Lasko and Brandriss, 1981). To ensure transport occurred via Put4p and not Gap1p, radiolabeled [^{14}C]-proline was included in the assay at a final concentration of 0.2 mM, to exceed the K_m of Put4p (0.025 mM; Magana-Schwencke and Schwencke, 1969), whilst falling well below that of Gap1p (13 mM; Lasko and Brandriss, 1981). The contribution of the two SPS-regulated amino acid permeases, Agp1 and Gnp1, was considered negligible based on previous studies. Thus Agp1 has low affinity and broad specificity for various amino acid substrates, is not significantly expressed in cells grown in urea or proline alone whilst no detectable uptake of [^{14}C]-proline is observed at concentrations of 0.1 mM (Andréasson et al., 2004; Iraqui et al., 1999). Similarly *GNP1* is not functionally expressed when cells are grown in proline alone, with over-expression of *GNP1* failing to increase proline uptake relative to the parental strain (Regenberg et al., 1999; Schreve et al., 1998).

Compared to KP2 (wild type), Put4p activity was higher in the constitutive *PUT4* strain KP21, both in the absence and presence of ammonium (Fig. 3). Proline uptake was, however, lower in the presence of ammonium than when grown with proline alone, an expected consequence of the post-translational inactivation of Put4p, occurring with preferred nitrogen sources (Grenson, 1983). In keeping with early reports that Put4p activity decreases with progression through the growth phase (Horak and Rihova, 1982), we also found proline uptake to decline in all strains as the culture moved through the phases of growth.

In the shorter term, inactivation of Put4p was partial and transient. Thus, proline uptake activity was shown to decrease rapidly over the first 30 min after ammonium addition, declining from 35 $\pm$ 4 nmol proline min^{-1} mg^{-1} cells to 2 $\pm$ 2 nmol proline min^{-1} mg^{-1} cells (Fig. 4). At 60 and 120 min after the addition of ammonium, Put4p activity recovered to approximately 40% of that measured at the zero time point. No recovery of this sort is evident in similar studies of the Gap1p response to the addition of ammonium (Hein and André, 1997; Springael and André, 1998). Such post-translational regulation of Put4p is likely to involve several genes and factors. Two of these are *NPR2* and *RCY1*. Suggested to be a protein kinase that stabilizes amino acid transporters by antagonizing their ubiquitin-mediated degradation (De Craene et al., 2001) when deleted, *NPR2*, was expected to increase the loss of proline uptake activity upon exposure to am-

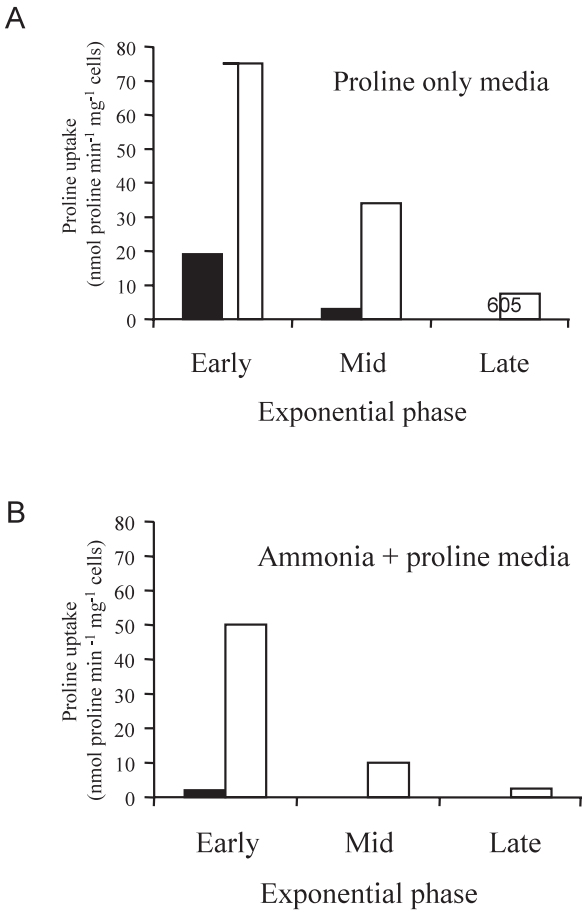

Fig. 3. The effect of growth phase on Put4p activity.

Cultures of KP2 (wild type, ■) and KP21 (*TEFp-PUT4*, □) were grown in media containing either (A) proline alone or (B) ammonia and proline as nitrogen sources. Cells were harvested at three points during growth: early (1×10^7 cells ml^{-1}), mid (5×10^7 cells ml^{-1}) and late (1×10^8 cells ml^{-1}) exponential phase, and assayed for Put4p activity using [^{14}C]-proline. Values are the mean of duplicate determinations from duplicate cultures. SD values for such experiments ranged from 2% to 12%.

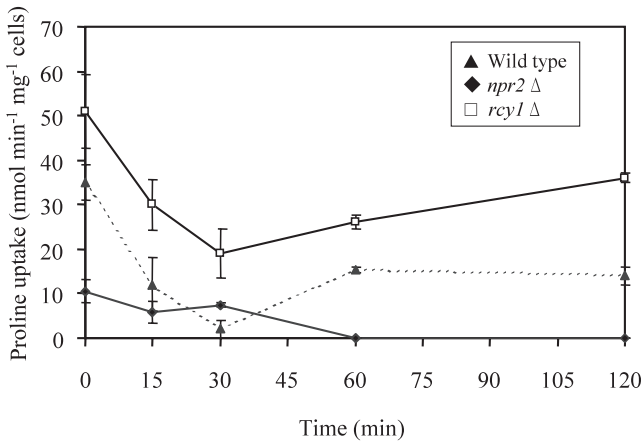

Fig. 4. The effect of ammonium addition on Put4p activity in wild type strain KP2, *npr2*Δ strain KP30 and *rcy1*Δ strain KP31.

YNB medium containing proline (as the nitrogen source), was inoculated with the yeast strains (at 5×10^6 cells ml^{-1} from a stationary phase culture) and grown until early exponential phase (1×10^7 cells ml^{-1}). Ammonium sulfate (5 g L^{-1}) was added and Put4p activity was measured using [^{14}C]-proline at 0, 15, 30, 60, 120 min post addition. Wild type strain KP2 (▲); *npr2*Δ strain KP30 (◆) and *rcy1*Δ strain KP31 (□). All values derived from the mean of duplicate determinations from triplicate cultures ± SD.

monium or else reduce the extent of recovery of lost activity. Both outcomes were evident (Fig. 4). In accordance with previous findings (Rousselet et al., 1995) the initial activity of Put4p was ca. 30% of the activity of the wild-type strain and by the 60 min time point no activity was detected and no recovery was observed.

The observations of Wiederkehr et al. (2000) suggest the *RCY1* gene product is involved in endocytic membrane traffic and recycling of early endosomes to the membrane in a process involving ubiquitin. Although not deficient in internalization of membrane fractions, the *rcy1* mutation blocks the intersection of the endocytic pathway with the vacuolar protein sorting pathway, thereby reducing degradation of internalized proteins. Initial Put4p activity was higher in the *rcy1*Δ strain than in the wild-type strain, yet followed a similar pattern of down-regulation and partial recovery of Put4p activity. As anticipated, however, a greater proportion (i.e. approx. 70% vs. 40%) of proline uptake activity was recovered by the 120 min time point. To further explore the role of Put4p inactivation, we sought to determine the consequences of key sequence changes to the ORF of *PUT4* through determination of short-term uptake activities and in longer term proline accumulation assays.

Characterization of PUT4 mutants resistant to nitrogen catabolite inhibition of Put4p

Ubiquitination of a number of plasma membrane proteins results in their endocytosis followed by vacuolar degradation (Galan et al., 1996; Hicke and Riezman, 1996; Omura et al., 2001; Springael and André, 1998). Previous reports have demonstrated specific lysine residues within the N-terminal domains of Gap1p (Soetens et al., 2001) and Put4p (Omura et al., 2005) to be ubiquitinated, and essential for down-regulation and direct sorting to the vacuole. Omura and coworkers (2005) further speculate that additional lysine resi-

dues, besides the N-terminal nine investigated, may be responsible for ubiquitination and subsequent degradation of the Put4p permease. Interestingly, a Lys to Arg substitution in the DXKSS sequence within the C-terminal domain of Ste2p resulted in the elimination of both ubiquitination and internalization of the variant (Hicke and Riezman, 1996). Further, replacement of a lysine residue within a similar motif in Gap1p (EEKAI) with glutamate, rendered the permease resistant to ammonium-induced inactivation (Hein and André, 1997).

Based on such studies, we targeted by site-directed mutagenesis specific amino acid residues in the Put4p protein identified as being analogous to those involved in the nitrogen catabolite inactivation of Gap1p (Hein and André, 1997; Springael and André, 1998). Put4p variants, with specific amino acid changes in the C-terminal domain, were analyzed for Put4p activity. A lysine residue within the EEKSR motif of Put4p was mutagenized ($K^{604}R$) using primers K604Rf/r (Table 2) to yield strain KP61. The resulting substitution did not significantly alter the uptake of proline, other than delaying recovery of uptake activity from the 30 min point after ammonium addition (Fig. 5). Hein and André (1997) demonstrated by site-directed mutagenesis that the replacement of a di-leucine motif (L^{575}, L^{576}) within a predicted α helix in the C-terminus of Gap1p

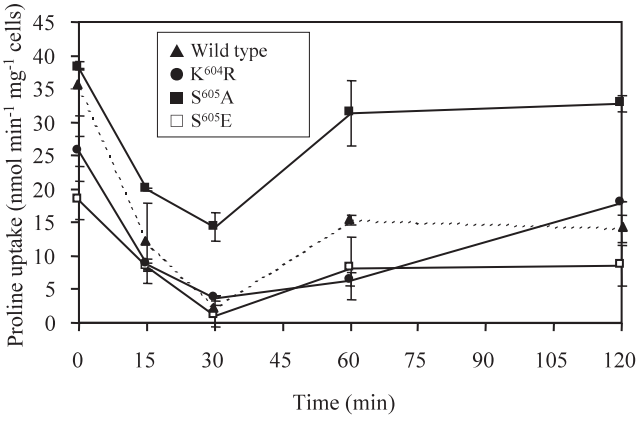

Fig. 5. The effect of ammonium addition on the activity of endogenous Put4p and variant Put4p permeases.

Strains were grown in media containing proline as the sole source of nitrogen. At time 0, ammonium sulfate (5 g L^{-1}) was added to the growth medium, and uptake of [^{14}C]-proline was assayed at the intervals shown, over a 120 min period. Values are the mean of duplicate determinations from triplicate cultures. Endogenous Put4p (▲) and Put4p $K^{604}R$ (●), Put4p $S^{605}A$ (■), Put4p $S^{605}E$ (□) activities found in KP2, KP61, KP64, and KP65 respectively.

with two alanines protected the permease against ammonium-induced inactivation. Two similar motifs in the C-terminal region of Put4p, a leucine-valine motif at residues 598 and 599 and a di-leucine motif at residues 626 and 627, were replaced with two alanines (using primers LV-AAf/r and Dileuf/r, respectively; Table 2), in the strains KP62 (Put4p $L^{598}A$, $V^{599}A$) and KP63 (Put4p $L^{626}A$, $L^{627}A$). Both substitutions resulted in no detectable Put4p activity (data not shown), possibly attributed to either misfolding or incorrect sorting of the Put4p permease.

Interestingly, two types of di-leucine motifs are found in mammalian cells; one is dependent on the phosphorylation of a proximal residue, while the other is constitutively active and includes an acidic amino acid (Giesler et al., 1998). A single serine residue, S^{605}, the only predicted phosphorylation site in the C-terminal region of Put4p (Blom et al., 1999), was replaced with alanine ($S^{605}A$), using primers S605Af/r (Table 2) to yield strain KP64. Based on the supposition that the negative charge of glutamate could mimic the negative charge of a phosphorylated site (Stark, 1998), another substitution, serine to glutamate ($S^{605}E$), was created using primers S605Ef/r (Table 2) to produce strain KP65.

Proline grown cells expressing the Put4p $S^{605}A$ variant (KP64) displayed similar rates of proline uptake to the wild type (38 $\pm$ 0.1 nmol proline min^{-1} mg^{-1} cells vs. 35 $\pm$ 4 nmol proline min^{-1} mg^{-1} cells; Fig. 5). When ammonium was added, the Put4p $S^{605}A$ permease appeared resistant to inactivation, displaying higher activity (14 $\pm$ 0.3 nmol proline min^{-1} mg^{-1} cells) compared with wild type (2 $\pm$ 2 nmol proline min^{-1} mg^{-1} cells). The rate of proline uptake by Put4p $S^{605}A$ not only recovered between the 30 and 120 min time points, but in fact achieved a value of 32 $\pm$ 0.1 nmol proline min^{-1} mg^{-1} cells or 86% of the activity found in the ammonium-free condition. In comparison, the $S^{605}E$ modification led to a lower rate of proline uptake after growth in proline (18.3 $\pm$ 2.9 nmol proline min^{-1} mg^{-1} cells), but one which declined more slowly after ammonium addition. The proportion recovered by the 120 min time point was similar to that of the wild type (ca. 40%).

Proline accumulation in strains constitutively expressing Put4p $S^{605}A$

The longer term consequences of modifications intended to increase the half-life of the permease were

examined by combining these with those which resulted in ammonia insensitive over-expression. The *PUT4* gene with and without the $S^{605}A$ mutation, was driven by the *PUT4* promoter mutation (T→C at −160). The −160 mutation represents an example of the minimum modification needed in order for over-expression of *PUT4* under repressive conditions as occurs during growth in minimal media containing ammonia as the nitrogen source (data not shown). Proline accumulation was measured by monitoring the incorporation of the radiolabeled amino acid over a 24 h incubation. The two constitutive strains, KP52 (T→C at −160) and TW3 (T→C at −160, $S^{605}A$), were compared with KP13, a *put4*Δ strain harboring plasmid-borne *PUT4* of unmodified sequence and therefore subject to NCR.

The wild-type strain showed low accumulation with all over-expression strains accumulating more proline throughout the experiment (Fig. 6A). The $S^{605}A$ mutation resulted in a marked increase (up to 50-fold) in accumulation of proline within 3 h by TW3 (T→C at −160, $S^{605}A$) compared with KP52 (T→C at −160). These results indicate a stabilized Put4p permease which is insensitive to NCI, perhaps through disruption of phosphorylation and ubiquitination (Springael and André, 1998). The comparable growth of all three strains in YNB media (Fig. 6B) suggests that increased accumulation of proline was not detrimental in the time frame of this experiment. Further, since the first step in proline catabolism, mediated by the product of *PUT1*, proline oxidase, is repressed by ammonium (Xu et al., 1995), most proline that was accumulated would be expected to be present as the free amino acid, with minor amounts having been directly incorporated into new proteins.

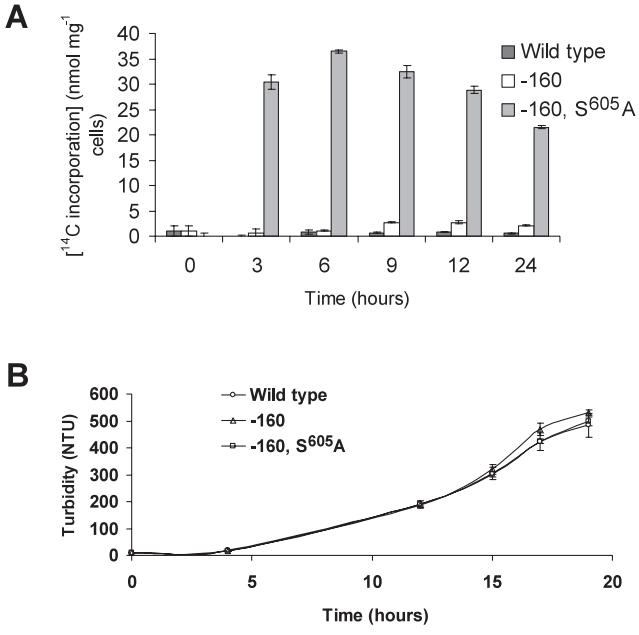

Fig. 6. Accumulation of proline from media by wild type and *PUT4* variant strains.

A: Proline accumulation assay: YNB media containing ammonium sulfate (5 g L^{-1}), proline (1 g L^{-1}) and 0.427 mM [^{14}C]-proline was inoculated in duplicate with 5×10^6 cells ml^{-1} and incubated at 30°C over a 24 h period. Proline incorporation (as [^{14}C] radioactivity) was measured in duplicate for each strain at three hourly intervals for 12 h and at 24 h. B: Comparative growth of strains to ascertain any pleiotrophic growth effects of the introduced plasmid-borne mutation(s). Parallel cultures were grown in media as described above (without [^{14}C]-proline). Growth was measured as described in MATERIALS AND METHODS. Data points represent means of triplicate cultures, standard error bars are shown. KP13 (Wild type), KP52 (−160), TW3 (−160, $S^{605}A$).

Consequences of enhanced proline accumulation

Proline is an osmoprotectant in plants and bacteria (Le Rudulier et al., 1984) and increased intracellular proline content confers increased viability after freezing and desiccation (Takagi et al., 2000). Therefore, the overexpression strains KP52 (T→C at −160) and TW3 (T→C at −160, $S^{605}A$), which are capable of increased proline accumulation, were evaluated for increased tolerance to stress. Strain TW3 (*PUT4* T→C −160, $S^{605}A$) exhibited significantly greater final viability following heat shock, and during growth in a medium containing 1.1 M NaCl but not 1.1 M sorbitol (Table 3). No significant differences were found in growth behavior between the strains when grown on medium containing 1.1 M glucose.

Discussion

In the presence of oxygen, *S. cerevisiae* is capable of growth on proline as the sole nitrogen source. However, proline is a poor nitrogen source; thus proline transport and catabolism are repressed and inhibited in the presence of superior nitrogen sources such as ammonium and glutamine (Courchesne and Magasanik, 1988; Lasko and Brandriss, 1981; Xu et al., 1995). The aim of this study was to isolate mutants of the *PUT4*-encoded proline-specific permease insensitive to ammonia repression, and investigate the effects on proline transport and stress tolerance in *Saccharomyces cerevisiae*.

Table 3. Percentage viability of wild type and constitutive *PUT4* strains exposed to heat shock or high osmolarity.

	KP13 (PUT4 wild type)	KP52 (−160)	TW3 (−160, $S^{605}A$)
Glucose	56.00 ± 7.00	51.00 ± 4.36	50.67 ± 9.70
Sorbitol	38.67 ± 6.23	47.00 ± 14.57	33.67 ± 3.84
NaCl	32.67 ± 6.74	40.00 ± 1.53	**63.67** ± 5.36
Heat	40.00 ± 3.61	38.67 ± 7.13	**63.67** ± 10.75

The average mean values (±SD) of triplicate determinations of cell viability reported. Values of significance highlighted in bold.

While over-expression of *PUT4* using the strong ammonia insensitive promoter *TEF* (considered constitutive) does lead to an increased rate of proline uptake, inactivation of the permease is apparent over the longer term (e.g. Fig. 3). Examination of the kinetics of inactivation revealed rapid loss of uptake activity over the initial 30 min after a shift into an ammonium-containing medium, followed by a partial recovery of activity in the subsequent 90 min. By comparison, no such recovery was observed during the ammonium-induced down-regulation of Gap1p (Hein and André, 1997). Deletion of the *RCY1* and *NPR2* genes confirmed the involvement of these genes in the inactivation of Put4p. In *rcy1* strains, degradation of internalized proteins has been shown to be reduced (Wiederkehr et al., 2000), an observation which might explain the higher initial activity and greater recovery of lost Put4p activity in an *rcy1* mutant (KP31) in this study (Fig. 4). Conversely, the fact that elimination of Npr2p, an antagonist of ubiquitin-based permease degradation, results in low and short-lived Put4p activity highlights the importance of this gene to maintenance of proline uptake activity. This latter observation also provides justification for investigation of ubiquitination targets in Put4p that are analogous to those found in Gap1p (Soetens et al., 2001).

The down-regulation (and inactivation) of Gap1p is dependent on the localization of the permease (De Craene et al., 2001; Roberg et al., 1997; Soetens et al., 2001; Springael and André, 1998). Ammonium-induced loss of measurable Gap1p activity thus reflects the progressive removal of Gap1p from the plasma membrane. During growth on proline Gap1p is ubiquitinated at two lysine residues upon the addition of a preferred nitrogen source, which leads to poly-ubiquitination and subsequent down-regulation (Springael et al., 1999b). This ubiquitination of Gap1p is dependent on the function of Npi1p and Npi2p (Springael et al., 1999a, b). The insensitivity of the $Gap1p^{LL\rightarrow AA}$ mutant to ammonium-induced down-regulation, despite the fact that it is ubiquitinated (Hein and André, 1997), suggests that ubiquitination is not solely responsible for Gap1p inactivation, and that multiple motifs are involved in Gap1p regulation.

The lysine residue within the EEKSR motif in the C-terminal region of Put4p was modified to arginine in an attempt to disrupt ubiquitination of Put4p. The $K^{604}R$ mutation did not ameliorate the ammonium-induced down-regulation of Put4p in comparison to the wild type, which indicates that this lysine residue is either not a ubiquitination site, or its ubiquitination has little effect on Put4p down-regulation. Interestingly, sequence identity does not necessarily correlate with function, as in the case of ubiquitination of the Gap1p and Put4p permeases. Recent studies have identified specific lysine residues, namely K^9 and K^{16} in Gap1p (Soetens et al., 2001) and K^9, K^{34}, K^{35}, K^{60}, K^{68}, K^{71}, K^{93}, K^{105}, and K^{107} in Put4p (Omura et al., 2005), as ubiquitination sites within the N-terminal sequences, in which there is only limited homology.

The phosphorylation state of Gap1p has been implicated in the ubiquitination and down-regulation of Gap1p. The kinetics of Gap1p down-regulation and dephosphorylation mirror each other (Stanbrough and Magasanik, 1995), implying that dephosphorylation of Gap1p is required for internalization. Recently, it was shown that Gap1p phosphorylation is decreased on inactivation of Npr1p (De Craene et al., 2001). On addition of ammonium to a proline-grown culture or the inactivation of Npr1p, neosynthesized Gap1p is targeted from the Golgi to the vacuole and Gap1p is internalized at the membrane (De Craene et al., 2001). It is interesting to note that the N-terminal region of Put4p contains 14 predicted phosphorylation sites (Blom et al., 1999), compared with the single phosphorylation site predicted in the C-terminal region (S^{605}). This prevalence of potential phosphorylation sites is consistent with evidence that the N-terminal region of Put4p is involved in ammonium-induced down-regulation, and ubiquitination (Omura et al., 2005).

It is also noteworthy that other determinants are likely to play a role in post-translational control, as in the case of Gap1p, where a di-leucine motif in the C-terminal region functions as an internalization signal (Hein and André, 1997). Modification of two potential internalization signals, $L^{558}V^{559}$ and $L^{626}L^{627}$ motif in the C-terminal region of Put4p, rendered the permease inactive. This might result either from mis-localization of Put4p or inactivation of the permease activity.

Our findings and others indicate that the intracellular levels of proline and arginine are correlated to the stress resistance of yeast (Morita et al., 2002; Takagi et al., 2000). The disruption of *PUT1* caused proline accumulation and led to increased viability in comparison to the control strain following exposure to freezing and desiccation (Takagi et al., 2000). Further, the survival rate of both wild type and *put1* strains increased in proportion to their arginine contents (Morita et al., 2002). Proline as a highly soluble compound functions as an osmoprotectant, and is accumulated in the cytoplasm and transported to the vacuole when in excess (Morita et al., 2002). The specific role of proline in osmoprotection and cryoprotection is, however, unclear.

We report on the isolation of variants of proline-specific permease, Put4p, which are able to accumulate proline, even when cells are grown in the presence of ammonia in the media. Furthermore, some of these mutants are able to withstand both osmotic and thermal stress. These findings raise the possibility that through an increased uptake of proline, which is potentially of nutritional as well as stress-tolerance significance, such strains may find use in high gravity, low nitrogen fermentations. Further work is now required to test this notion.

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