Enhancing yeast performance under oenological conditions by enabling proline utilisation

by

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Thesis summary

Assimilable nitrogen, which is typically lacking in grape juice, is an important nutritional requirement of *Saccharomyces cerevisiae*. As such, fermentations frequently become protracted, terminate prematurely or develop undesirable aroma profiles. Amino acids and ammonium are the main sources of assimilable nitrogen in grape juice. The amino acid proline often predominates. Proline uptake is mediated by a high affinity, proline-specific permease, Put4p, and a low affinity general amino acid permease, Gap1p. The expression and activity of these transporters is subject to nitrogen catabolite repression (NCR) and nitrogen catabolite inactivation (NCI). That is, in the presence of a preferred nitrogen source, the expression of *PUT4* and *GAP1* is repressed and the permeases are inactivated. For yeast to fully exploit proline, its transport must be derepressed by depletion of other (preferred) amino acids and molecular oxygen must be present to allow proline catabolism by proline oxidase. Consequently, as oxygen is typically depleted well before the other amino acids in grape juice are reduced to non-repressive concentrations, proline is largely un-utilised by yeast during oenological fermentation. This study aims to overcome these metabolic restrictions on proline utilisation.

A preliminary study was conducted to determine the potential for proline transport-capable strains to utilise proline during the initial stages of fermentation when oxygen may be present, particularly in red grape must. Initially, the transcriptional regulation of the *PUT4* gene was targeted to generate strains capable of proline transport under normally repressive conditions. In the first case, the *URE2* gene, encoding a negative regulator involved in nitrogen discrimination, was deleted. In the second case, *PUT4* was expressed from the constitutive *TEF2* promoter. It was observed that both strains express *PUT4* in the presence of a preferred nitrogen source. This expression led to Put4p activity during the initial stages of growth and fermentation, with Put4p activity declining over the course of the growth phase. Proline removal from the media, however, was limited to the initial stages of fermentation while oxygen was available. It seems that the rapid depletion of oxygen limits the amount of proline transported into the yeast cell.

The two proline transport-capable mutants were analysed for growth and fermentation characteristics. It was found that the deletion of the *URE2* gene led to a slow initial growth and the formation of a larger biomass. The *ure2* delete strain also utilised significantly more nitrogen during fermentation than the wild type. Consequently, a *ure2* delete strain would not be suitable for industrial use. The expression of *PUT4* from a constitutive promoter did lead to an increase in nitrogen assimilation during fermentation when compared with the wild type. However, this observed increase was significantly less than that observed in the *ure2* delete strain. In an effort to produce a proline transport-capable strain with potential industrial benefit, strains constitutive for *PUT4* specifically were isolated using random, *in vitro* mutagenesis of the *PUT4* promoter region. Four point mutations were identified that, when introduced singly into the *PUT4* promoter, led to expression of *PUT4* in the presence of a preferred nitrogen source.

The rapid depletion of oxygen observed in the preliminary study will limit the potential usefulness of strains capable of proline transport. Micro-oxygenation is rapidly becoming an accepted practice during oenological fermentation. The potential benefit of the controlled addition of oxygen during fermentation is restricted by the timing of any oxygen addition. Oxygen additions made at the onset of the stationary phase are the most beneficial. During the preliminary study, it was noted that Put4p activity decreased during the growth phase to low levels at the onset of the stationary phase. To ensure that sufficient active Put4p is present at the onset of the stationary phase, the post-translational control of the Put4p was investigated.

Site-directed mutagenesis was used to target residues in the carboxy-terminal region of Put4p that are potentially involved in the ammonia-induced down-regulation of the permease. The substitution, S605A, lead to the amelioration of ammonia-induced down-regulation of Put4p. The activity of the Put4p S605A variant decreased over the course of the growth phase, but not to the same extent observed in the wild type. Furthermore, a recovery seen after down-regulation restored a greater percentage of the original activity compared with the wild type.

To determine whether such a strain proved better able to ferment media in the presence of micro-oxygenation, the fermentation kinetics of a strain constitutively expressing

PUT4(S605A) were compared with the wild type. Micro-oxygenation of ferments did not result in an increase in fermentation rate nor a decrease in fermentation time in the mutant. However, the cell viability of the strain capable of proline transport was increased in comparison with the wild type, suggesting a role for proline in stress responses within the yeast cell.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge, contains no material previously published or written by another person, except where due reference has been made in the text.

This thesis may be made available for loan or photocopying

Kathryn Poole

April, 2002.

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I would like to dedicate this thesis to my parents, Lyn and John, who have given me everything but asked for nothing in return. You have my respect, my admiration and my love.

Chapter 1 Literature Review

1.1 Introduction

Wine is the fermented product of the fruit from various *Vitis* species, in most cases *V. vinifera*. The production of wine involves the conversion of sugars in the grape must to ethanol and carbon dioxide. This fermentation is mediated by microorganisms that metabolise the grape sugars for energy. If left, grape must would ferment without the addition of yeast, as the indigenous yeast and bacteria on the grapes would grow. Traditional ferments are carried out by indigenous yeast, but the use of selected strains with desirable attributes affords greater control over oenological fermentation. Wine yeast strains also produce organoleptically important metabolites that contribute to the overall quality of the wine. Typically, *Saccharomyces cerevisiae* strains with proven characteristics are employed. These characteristics include an ability to grow in a low pH environment, and an ability to tolerate the high sugar content of must at the beginning of fermentation and high concentrations of ethanol at the end. Yeast strains must also be capable of effectively metabolising all sugars available under anaerobic conditions. All of these traits typically provide the yeast with the ability to dominate the indigenous yeast present on the grapes during fermentation.

One of the most important goals in wine making is to ensure that the fermentation progresses to the desired residual sugar concentration. For a dry table wine, fermentation is considered complete when residual sugars are less than 2-4 g/L (Alexandre and Charpentier, 1998). Slow (sluggish) or prematurely terminated (stuck) fermentations can occur for several reasons, including a lack of sufficient assimilable nitrogen or a lack of oxygen during fermentation. Stuck fermentations are difficult to restart (Rankine, 1989; Ough, 1992) and wines that have become stuck are often of inferior quality to those that have not (Ough, 1992). Stuck wines are susceptible to contamination with other microorganisms, such as acetic acid bacteria and lactic acid bacteria, that can utilise the residual sugars. Acetic acid bacteria produce acetic acid from glucose, which will lead to an increase in volatile acidity in the wine (Boulton *et al.*, 1996). The growth of lactic acid bacteria can lead to complex changes to the flavour of the wine, apparently due to the formation of mannitol, butanol, propanol and acetic acid (Boulton *et al.*, 1996). Grape must typically contains all the nutrients required for

the growth of *S. cerevisiae* and consequent fermentation. However, these nutrients may not be sufficient to ensure that the end point of fermentation is reached.

1.2 Nitrogen requirements for fermentation

1.2.1 Nitrogen in grape must

The nitrogen in grape must is present in the form of amino acids, proteins, peptides and ammonium, with trace levels present as vitamins and nucleotides (Henschke and Jiranek, 1993). The amino acids comprise between 60-90% of the total juice nitrogen, representing the main source of nitrogen for yeast during oenological fermentation. Proline, arginine, alanine and glutamate usually predominate with glutamine, serine, threonine and γ-amino butyric acid being present to a lesser extent (Table 1.1). *S. cerevisiae* is not capable of utilising all the available nitrogen sources, with large peptides and proteins not accumulated or hydrolysed (Henschke and Jiranek, 1993). Similarly, proline is not thought to be utilised under oenological conditions, even though it is typically present at high concentrations.

Levels of assimilable nitrogen in grape must can vary from 60 to 2400 mg/L (Ough and Amerine, 1988). This concentration is affected by many factors, including the region, grape variety and the stage of berry maturation (Henschke and Jiranek, 1993; Alexandre and Charpentier, 1998). Low levels of initial assimilable nitrogen will limit the yeast growth rate and biomass formation, such that a low rate of sugar catabolism results. A lack of nitrogen leads to an arrest in protein synthesis, resulting in a rapid decrease in sugar transport as the sugar transporters are irreversibly inactivated (Lagunas *et al.*, 1982; Busturia and Lagunas, 1986; Salmon, 1989). This leads to a sharp decline in fermentation as the intracellular levels of fermentable sugars are depleted.

Nitrogen is considered limiting if it cannot sustain fermentation at a satisfactory rate nor allow completion of fermentation within a satisfactory time period. This minimum amount of nitrogen required for successful fermentation has been estimated to be 120-140 mg N/L, depending on the yeast strain (Bely *et al.*, 1990a). Nevertheless, concentrations of assimilable nitrogen above this minimal level may still result in fermentation problems. If the nitrogen concentration is sub-optimal (less than about 400 mg/L), hydrogen sulfide (H_2S) is likely to be produced (Jiranek *et al.*, 1995a), leading to

Table 1.1 Range of amino acid concentrations in 53 juices[a]

Amino acids	Min. (mg/L)	Max. (mg/L)	Mean (mg/L)
Aspartic acid	5.0	54.4	23.0
Threonine	9.6	117.2	41.9
Serine	11.3	198.0	48.4
Asparagine/glutamic acid	134.5	742.3	378.3
Glutamine	7.2	455.5	77.2
Proline	42.9	1782.2	743.1
Glycine	0.0	11.5	3.0
Alanine	10.2	297.3	84.8
Citrulline	0.0	14.8	1.5
Valine	3.7	50.8	21.6
Methionine	0.0	12.5	3.6
Isoleucine	1.6	34.3	12.9
Leucine	1.8	34.0	15.3
Tyrosine	0.0	60.2	11.8
Phenylalanine	3.2	36.6	13.7
γ-Aminobutyric acid	1.2	163.4	67.3
Tryptophane	0.0	30.0	3.6
Histidine	2.8	100.1	21.3
Ornithine	0.4	18.8	5.3
Lysine	1.2	23.0	6.5
Arginine	6.6	649.2	179.8

[a] The information contained in this table was taken from Ough and Amerine (1988).

unpleasant "off" odours that reduce the quality of the wine (Jiranek *et al.*, 1995b). H_2S is produced by yeast as part of the synthesis of the sulphur containing amino acids, cysteine and methionine, via the sulfate assimilation pathway. When sufficient nitrogen is present nitrogenous precusors of the sulphur-containing amino acids combine with the sulphide that is produced from sulphite by sulphite reductase. However, when nitrogen is lacking, the sulphide diffuses out of the cell as H_2S. H_2S production is exacerbated by the ready availability of sulphite during fermentation, which is often added to grape must to inhibit the growth of non-*Saccharomyces* yeast. Sulphite can freely diffuse into the cell, where it is converted to sulphide. Consequently, excess nitrogen is required not only to ensure that fermentation is completed at a satisfactory rate, but also to minimise H_2S production.

To avoid sluggish or stuck fermentations resulting from a lack of nitrogen in the must, wine makers have added diammonium phosphate (DAP), a readily assimilable nitrogen source. The amount of DAP that can be added during fermentation is regulated by law. In Europe 300 mg/L DAP (corresponding to 63 mgN/L) may be added. Under American law the limit is 950 mg/L DAP (199 mgN/L). In Australia, the amount of inorganic phosphate that can be added during fermentation is limited to 400 mg/L Pi, which corresponds to 556 mg/L of DAP or 116 mgN/L (Henschke and Jiranek, 1993). The use of DAP has its drawbacks. The addition of DAP during the later stages of fermentation can result in a high residual ammonium concentration in the wine, which may lead to microbial instability. A mechanism by which the naturally occurring nitrogen compound, proline, could be utilised during oenological fermentation may, in part, alleviate the need to use additives, allowing production of wine that is additive free, an image that the Australian wine industry is striving for.

1.2.2 Proline as a nitrogen source

Although proline can act as a sole source of nitrogen for *S. cerevisiae*, it is not considered an assimilable nitrogen source in wine making, as it is not thought to be utilised under oenological conditions. In contrast to the other amino acids, the amount of proline typically remains unchanged during or after fermentation (Ough, 1968; Ough and Stashak, 1974; Sponholz, 1991). Proline is not transported into the yeast cell if preferred nitrogen sources are present (Duteurtre *et al.*, 1971; Horak and Rihova, 1982). During fermentation, ammonium and amino acids such as glutamine and arginine in the

grape must inhibit the transport of proline until they are depleted. This depletion of the preferred nitrogen sources leads to the activation of a proline specific permease and transport of proline into the cell (Duteurtre *et al.*, 1971). However, molecular oxygen is generally absent by this stage of the fermentation. The proline oxidase enzyme, which catalyses the first step in proline utilisation, requires oxygen to function (Duteurtre *et al.*, 1971) and, as such, proline is not catabolised under these conditions. The ability to utilise proline as a nitrogen source would be beneficial to wine making, as proline is often the most abundant amino acid in grape must (Ough and Stashak, 1974; Ough and Amerine, 1988). Although the levels are quite variable, the average concentration of proline in grapes has been reported to be 743 mg/L, corresponding to about 90 mgN/L, which amounts to 12% of total must nitrogen (Ough and Stashak, 1974; Ough and Amerine, 1988). In addition to the proline present in grape must, proline is also an intermediate in arginine degradation (Brandriss and Magasanik, 1980). The catabolism of arginine leads to the production of ammonium via urea formation and, in aerobic conditions, glutamate via proline formation (Figure 1.1).

Under laboratory conditions, proline was shown to be utilised during fermentations when the headspace was flushed with oxygen (Ingledew and Kunkee, 1985; Ingledew *et al.*, 1987). The uncontrolled addition of oxygen throughout the fermentation is impractical for the industrial fermentation of wine, as high concentrations of oxygen have a deleterious affect on the quality and stability of wine flavour and can result in an excessive increase in nitrogen assimilation (Ingledew and Kunkee, 1985; Jiranek *et al.*, 1995a; Sablayrolles *et al.*, 1996). Ingledew and colleagues (1987) suggested that oxygen be added during the early stages of fermentation. This, however, would not result in the utilisation of proline, as preferred nitrogen sources are present and proline uptake by the cell would be inhibited. The dynamics of oxygen utilisation during oenological fermentation has not been determined to date, nor has the amount of oxygen required for proline oxidase function. Such information might enable the judicious addition of oxygen during fermentation to allow the catabolism of proline without the undesirable effects associated with excessive oxygenation.

Molecular techniques may be of benefit in producing strains capable of proline utilisation during wine making. One option is to generate recombinant yeast containing proline utilisation genes, derived from another organism, which do not require oxygen

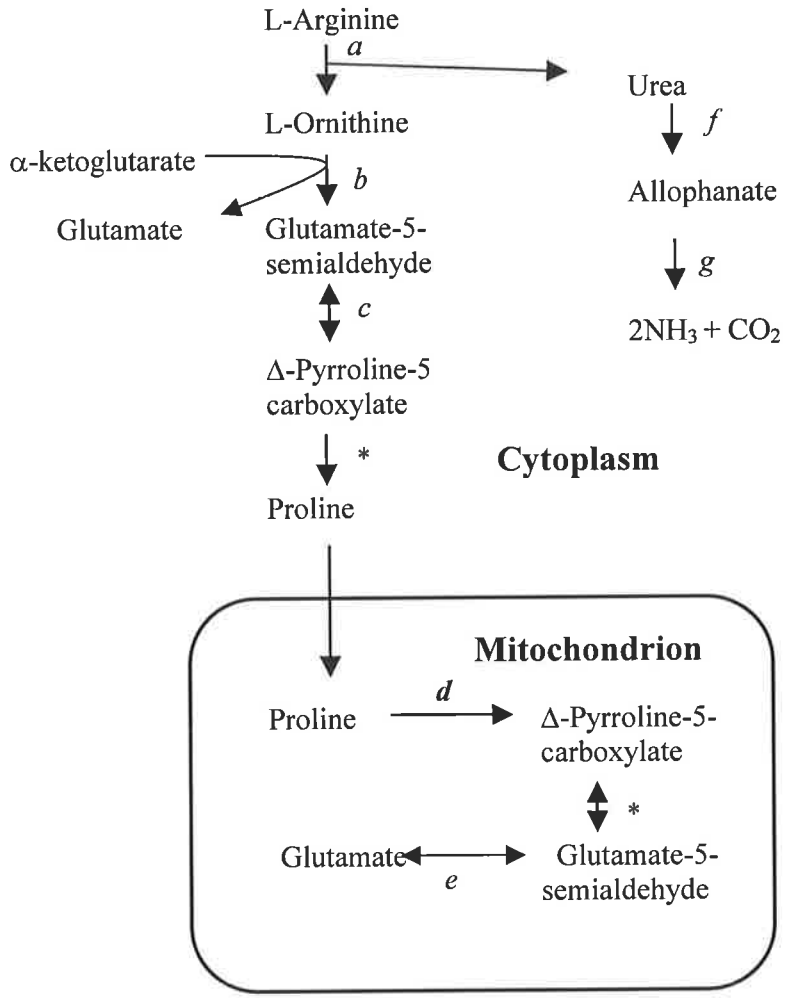


Figure 1.1 Arginine and proline degradation in *S. cerevisiae*

Enzymes: (a) arginase; (b) ornithine aminotransferase; (c) Δ-pyrroline-5-carboxylate reductase; (d) proline oxidase; (e) Δ-pyrroline-5-carboxylate dehydrogenase; (f) urea carboxylase; (g) allophanante hydrolase; (*) spontaneous.

for proline catabolism. Proline catabolism in *S. cerevisiae* is mediated by an electron transport chain (ETC) linked proline oxidase, which converts proline to Δ-pyrroline-5-carboxylate (P5C). P5C is then converted to glutamate by a mitochondrial NAD(P)-linked P5C dehydrogenase (Brandriss, 1979; Brandriss and Magasanik, 1979; Wang and Brandriss, 1986). In some plants and certain anaerobic bacteria, it has been found that proline catabolism is not dependent on oxygen and the conversion of proline to glutamate is mediated by a cytosolic NAD(P)-linked bifunctional proline dehydrogenase (PDH/P5CR) (Costilow and Laycock, 1969; Mazelis and Fowden, 1971; McNamer and Stewart, 1974; Costilow and Cooper, 1978). When expressed in *S. cerevisiae,* the PDH/P5CR enzyme from soybean allowed the anaerobic utilisation of proline as the sole nitrogen source (Smyl, 2000). However, the growth rate and cell numbers of the transgenic strain were greatly reduced. This may suggest that the anaerobic utilisation of proline is inefficient. Alternatively, as the enzyme is NAD(P)-linked, the redox balance of the cell may be disrupted by this catabolic pathway. An alternative strategy to produce wine yeast that can utilised proline is to modify the proline specific permease, such that it is no longer inactive during the initial stages of fermentation, allowing the proline oxidase enzyme access to proline when oxygen still may be available.

1.3 Proline uptake and utilisation by *Saccharomyces cerevisiae*

1.3.1 Proline transport

Nitrogen starved cells accumulate proline until a steady state level is reached in a process that is temperature dependent and enhanced by glucose (Magana-Schwencke and Schwencke, 1969). This suggests that the transport of proline is energy dependent. Transport of proline into the cell occurs only in the presence of poor nitrogen sources or when cells are nitrogen-starved, suggesting that the genes encoding proline transporters are subject to nitrogen catabolite repression (NCR) (Duteurtre *et al.*, 1971; Woodward and Cirillo, 1977; Lasko and Brandriss, 1981). The transport of proline when cells are grown on a poor medium is maximal during the early exponential phase. It has also been found that proline transport occurs during stationary phase after growth on rich media (Horak and Rihova, 1982), suggesting that the quality of available nitrogen is not the only important regulatory factor in the control of proline transport.

Proline uptake in *S. cerevisiae* is mediated by two transporters (Horak and Rihova, 1982). A low affinity transporter, which is not specific for proline, transports proline when present at high concentrations. When the concentration of proline in the medium is low, proline is transported into the cell via a specific permease of high affinity. The apparent affinity constant (K*m*) of the proline specific permease is 0.025 mM (Magana-Schwencke and Schwencke, 1969) and 13 mM for the low affinity transport system (Lasko and Brandriss, 1981).

The high affinity proline specific permease is encoded by the *PUT4* gene (for Proline UTilisation). This gene contains an open reading frame of 1881 nucleotides in length. It has a low codon bias, indicative of a protein translated at low levels (Vandenbol *et al.*, 1989). The low affinity transport system is encoded by the *GAP1* gene (for General Amino-acid Permease). *GAP1* has an open reading frame of 1803 nucleotides in length (Jauniaux and Grenson, 1990). The predicted polypeptide sequences of both genes are strongly hydrophobic. The hydropathy plots of both sequences are very similar, predicting 12 membrane-spanning regions for each protein (Vandenbol *et al.*, 1989; Jauniaux and Grenson, 1990). Neither seems to have a cleavable signal sequence at the amino-terminus. The sequences of *PUT4* and *GAP1* have regions of homology with *HIP1* (a histidine permease) and *CAN1* (a constitutive arginine permease), with major differences between sequences occurring at the termini (Vandenbol *et al.*, 1989). This suggests that there may have been a common origin for these amino acid permeases.

1.3.2 Regulation of the permease genes

The proline specific permease and the general amino acid permease are subject to NCR at the transcriptional and post-translational level (Grenson, 1983a), yet neither is induced by proline (Lasko and Brandriss, 1981). That is, when preferred nitrogen sources are available, existing Gap1p and Put4p are inactivated and the synthesis of new protein is repressed. Preferred nitrogen sources are those that allow the fastest doubling times in minimal media and include ammonium and amino acids such as glutamine and asparagine (Table 1.2). When grown on a preferred nitrogen source, the levels of *PUT4* and *GAP1* mRNA are repressed by the complex interplay of a number of regulatory factors. Growth on poor nitrogen sources results in the derepression and activation of these genes.

Table 1.2 Mean doubling times for the growth of five laboratory strains of *S. cerevisiae* on various compounds as sole source of nitrogen

Nitrogen compound	Doubling time (min)
Glutamine	137
Ammonia	142
Asparagine	146
Urea	158
Glutamate	159
Serine	163
Arginine	169
Aspartate	170
Alanine	187
Phenylalanine	190
Ornithine	203
Citrulline	208
Allantoin	208
Valine	225
Leucine	257
Proline	260
Tyrosine	265
Isoleucine	280
Threonine[1]	298
Tryptophan	298
Allantoate	322
Methionine[a]	349
Histidine[2]	475
Glycine[3b]	608
Lysine	no growth
Cysteine	no growth

This table is reproduced from Henschke and Jiranek (1993). Superscripted numbers indicate the number of strains that failed to grow on the respective nitrogen compound. [a]Mean of four strains only. [b]Limited growth.

1.3.2.1 Transcriptional regulation of the permease genes

Saccharomyces cerevisiae has four proteins that are members of the GATA family, which contain characteristic C_4 zinc finger motifs that bind DNA sequences with a GATA sequence at their core (Cunningham and Cooper, 1992; Cunningham and Cooper, 1993; Coffman *et al.*, 1997; Blinder and Magasanik, 1995; Stanbrough *et al.*, 1995; Soussi-Boudekou *et al.*, 1997). These proteins are Gln3p, Nil1p, Dal80p and Nil2p. Gln3p and Nil1p also have highly acidic amino-terminal regions, which are characteristic of transcriptional activators (Stanbrough and Magasanik, 1996), and have been shown, *in vitro*, to be capable of binding *cis*-acting upstream activating sequences present in nitrogen catabolic genes and activating transcription (Cunningham *et al.*, 1996; Stanbrough and Magasanik, 1996).

The ability of Gln3p and Nil1p to activate transcription depends on the quality of the nitrogen source. When glutamine is present, neither Gln3p nor Nil1p can activate transcription. When the nitrogen source is glutamate, as opposed to glutamine, Gln3p upregulates the transcription of genes such as *GAP1* and *PUT4* (Stanbrough and Magasanik, 1995). The increased expression of these genes on poor nitrogen sources such as proline, however, is not dependant on Gln3p (Courchesne and Magasanik, 1988). *GLN3* is not required for the maximal expression of all nitrogen-regulated genes (Daugherty *et al.*, 1993), which suggests that the regulation of permease expression by Gln3p is linked to the maintenance of glutamine levels within the cell, as opposed to nitrogen assimilation under conditions of nitrogen starvation.

Nil1p is unable to activate transcription on glutamine or glutamate, but does so in the presence of poor nitrogen sources (Stanbrough and Magasanik, 1995). Nil1p is capable of autogenous activation, binding GATA motifs upstream of the *NIL1* gene (Rowen *et al.*, 1997). When the yeast is grown on either glutamine or glutamate, the product of the *NIL2* gene, another GATA protein, blocks the GATA sites upstream of the *NIL1* gene (Soussi-Boudekou *et al.*, 1997). When glutamate and glutamine are absent, Nil1p is capable of initiating expression of the *NIL1* gene even in the presence of Nil2p (Rowen *et al.*, 1997). The resulting increase in Nil1p overcomes any residual effect of Nil2p on *NIL1* gene expression and any blockage of expression by Nil2p of other genes, such as *GAP1* and *PUT4*. Interestingly, Gln3p and Nil1p recognise the same site in the promoter of *GAP1* for activation of expression (Stanbrough and Magasanik, 1996).

The ability of the activators Gln3p and Nil1p to activate gene expression is mediated by the product of the *URE2* gene. Deletion of *URE2* leads to upregulation of the genes subject to NCR. It has been shown that, during growth on preferred nitrogen sources, Gln3p and Nil1p are localised to the cytoplasm. When the nitrogen source is poor, these activators are translocated to the nucleus, where they activate transcription. The Ure2p is believed to be responsible for the cytoplasmic sequestration of Gln3p and Nil1p on poor nitrogen sources and, in the case of Gln3p, a direct interaction has been shown with Ure2p (Beck and Hall, 1999). Although such an interaction is yet to be shown for Nil1p, both Gln3p and Nil1p have been shown to be present in the nucleus in a *ure2*Δ strain, as when cells are grown on poor nitrogen sources. This suggests that Ure2p is also involved in the cytoplasmic localisation of Nil1p in the presence of preferred nitrogen sources. A proposed model for the GATA factor-dependent regulation of nitrogen catabolite sensitive genes is presented in Figure 1.2.

Dal80p, a multi-pathway regulatory gene, is also involved in the regulation of the *GAP1* gene (Cunningham and Cooper, 1992). Dal80p down-regulates the expression of a number of genes involved in nitrogen metabolism, including *GAP1* and *PUT4* (Cunningham and Cooper, 1993), and, under nitrogen-derepressing conditions, Dal80p negatively regulates the expression of Nil2p (Soussi-Boudekou *et al.*, 1997).

The expression of the GATA factors is highly regulated. Interestingly, GATA factor gene expression is regulated by the GATA factors themselves. As mentioned above, Nil1p and Nil2p regulate *NIL1* gene expression (Rowen *et al.*, 1997). In addition, *DAL80* expression is Gln3p and Nil1p dependent and negatively regulated by Dal80p. GATA sequences also appear in the promoter region of the *NIL2* gene, which is largely independent of Gln3p but is partially dependent on Nil1p and negatively regulated by Dal80p (Coffman *et al.*, 1997). Some nitrogen regulated genes, including *GAP1*, are still partially subject to NCR even in the absence of Ure2p, Nil2p and Dal80p (Soussi-Boudekou *et al.*, 1997). This suggests that there are as yet undiscovered negative regulators involved in NCR.

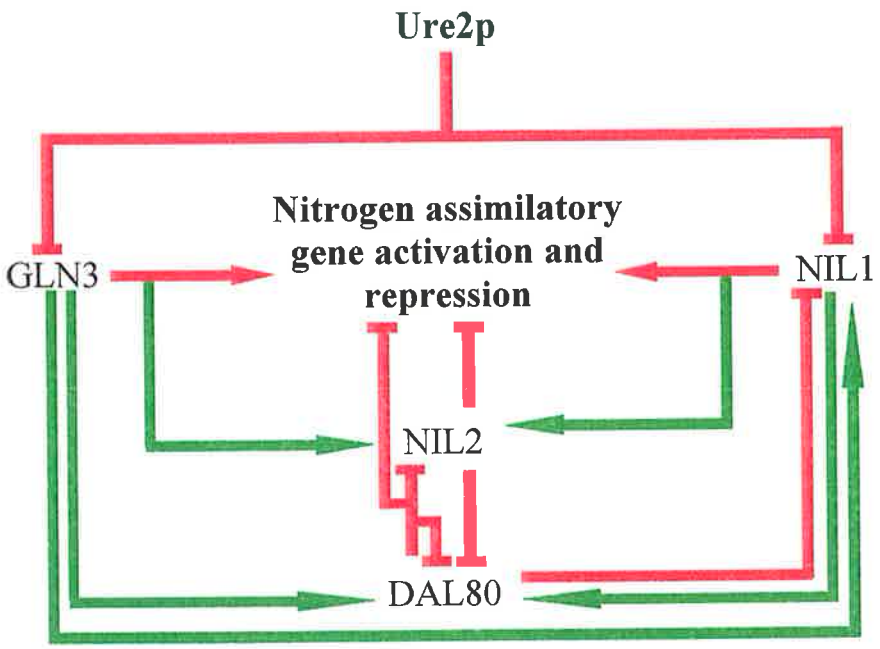


Figure 1.2 A proposed regulatory circuit of GATA factor-dependent transcription in the yeast *S. cerevisiae*

This figure has been adapted from Cunningham *et al.* (2000). Green arrows indicate positive regulation and red bars indicate repression of transcription.

1.3.2.2 Post-translational control of Put4p and Gap1p

During growth on a preferred nitrogen source, existing proline specific permease and general amino acid permease proteins are reversibly inactivated. This inactivation is mediated by the products of the *NPI1* and *NPI2* genes (for Nitrogen Permease Inactivation). *npi1* and *npi2* mutants show an increase in Put4p and Gap1p activity in the absence of increased *PUT4* and *GAP1* mRNA (Grenson, 1983a; Jauniaux *et al.*, 1987). It has recently been shown that Npi1p plays a role in ubiquitination (Springael and Andre, 1998; Springael *et al.*, 1999a). Ubiquitin may function as a signal for some cell surface proteins to be endocytosed and then degraded in the lysosome. In the presence of proline, the Gap1p transporter is ubiquitinated, but the addition of ammonium results in increased conversion of Gap1p to ubiquitinated forms, leading to an increase in degradation (Springael and Andre, 1998). The product of the *NPR1* gene (for Nitrogen Permease Reactivation) is believed to oppose the action of Npi1p and Npi2p when the nitrogen source is poor. An *npr1* mutation leads to a decrease in Put4p and Gap1p activity when growth occurs on proline, in comparison with the wild type (Grenson, 1983b). The loss of Npr1p leads to the complete loss of general amino acid permease activity, but only a 50% decrease in the activity of the proline specific permease (Grenson, 1983a; Jauniaux *et al.*, 1987). This adverse affect of the *npr1* mutation can be offset by mutations in *NPI1* and *NPI2*, suggesting that these proteins are antagonistic. *NPR1* is homologous to protein kinases (Vandenbol *et al.*, 1990), suggesting that phosphorylation/dephosphorylation may regulate the ubiquitination and subsequent inactivation of Put4p and Gap1p. This is supported by the fact that the kinetics of dephosphorylation and inactivation of Gap1p have been found to parallel each other (Stanbrough and Magasanik, 1995).

An additional protein, the product of the *NPR2* gene, has recently been discovered to be involved in the post-translational regulation of Put4p (Rousselet *et al.*, 1995). Npr2p has no significant homology to other known proteins. Its expression is not subject to NCR, but is induced by proline. Mutations in *NPR2* result in deficient proline transport, with no concomitant decrease in *PUT4* mRNA. The mode of action of Npr2p is unknown, but it may be involved in the putative phosphorylation of Put4p, or it may play a role in targeting Put4p to the plasma membrane. It has been shown that Gap1p transport to the plasma membrane is dependent on the quality of the nitrogen source. On poor nitrogen sources, Gap1p is transported from the Golgi complex to the plasma membrane,

whereas on glutamate, Gap1p is transported from the Golgi to a vacuole in a sorting process controlled by *SEC13* (Roberg *et al.*, 1997).

GAP1 and *PUT4* gene expression and protein activity are very tightly controlled by a complex interplay of many regulatory factors operating at differing levels in response to differing nitrogen sources. Many of the control mechanisms involved are pleiotropic and affect other permeases and nitrogen assimilatory genes, as well as *PUT4* and *GAP1* (Courchesne and Magasanik, 1983; Cunningham and Cooper, 1992; Daugherty *et al.*, 1993). Tight control of the permease genes is necessary, as the intracellular presence or absence of functional amino acid permeases is in itself a control mechanism for the expression of substrate-inducible amino acid catabolic genes.

1.3.3 Proline catabolism in *Saccharomyces cerevisiae*

The genes responsible for the catabolism of proline can be induced to high levels in *S. cerevisiae* when proline is the sole nitrogen source (Brandriss and Magasanik, 1979). The proline utilisation pathway involves two distinct enzymes. Proline is first converted to Δ^1-pyrroline-5-carboxylate (P5C) by proline oxidase, which is encoded by the *PUT1* gene. P5C is then converted to glutamate by P5C dehydrogenase, which is encoded by the *PUT2* gene (Brandriss, 1979; Brandriss and Magasanik, 1979; Brandriss, 1983; Wang and Brandriss, 1986).

Sequence analysis of the *PUT1* gene reveals that there is little codon bias, which is indicative of a moderately expressed protein. *PUT1* is a nuclear encoded gene (Wang and Brandriss, 1986) and the polypeptide is then transported into the mitochondria (Wang and Brandriss, 1987). The *PUT2* gene encodes a 575 amino acid polypeptide, synthesised as a precursor protein in the cytoplasm. A 2 kDa signal sequence is cleaved from the polypeptide on entry into the mitochondria (Krzywicki and Brandriss, 1984; Kaput *et al.*, 1989).

1.3.4 Regulation of the *PUT1* and *PUT2* genes

The expression of *PUT1* and *PUT2* is induced by proline (Brandriss and Magasanik, 1979; Brandriss, 1987; Wang and Brandriss, 1986) and repressed by preferred nitrogen sources (Daugherty *et al.*, 1993; Xu *et al.*, 1995). Steady state levels of *PUT1* RNA are

30- to 50- fold higher when cells are grown on proline as opposed to ammonium (Wang and Brandriss, 1986) and the amount of *PUT2* RNA is 10- to 15- fold higher during growth on proline than during growth on ammonium (Brandriss, 1983). In the presence of a preferred nitrogen source, i.e. ammonium, and the inducer, i.e. proline, *PUT1* and *PUT2* transcription occurs with transcription increasing on transfer to proline alone (Brandriss and Magasanik, 1979).

The growth of *S. cerevisiae* on proline as a sole nitrogen source requires a functional *PUT3* gene product (Marczak and Brandriss, 1991). The *PUT3* gene encodes a protein that is 979 amino acids in length (Marczak and Brandriss, 1991). The codon bias is low, which is typical of transcriptional activators in yeast, and the gene is expressed relatively poorly, at about one molecule per cell. Expression of the *PUT3* gene is, however, constitutive (Marczak and Brandriss, 1989).

The Put3 protein is constitutively bound to upstream activating sequences found in the *PUT1* and the *PUT2* promoters, but can only activate transcription when the inducer, proline is present (Marczak and Brandriss, 1989; Siddiqui and Brandriss, 1989; Axelrod *et al.*, 1991). The DNA-binding domain of Put3p, a C_6 zinc finger, is in the amino-terminal region (Marczak and Brandriss, 1991). A fragment of the protein, consisting of residues 31-126, retained the ability to specifically bind the DNA sequence $CGGN_{10}CCG$ (Reece and Ptashne, 1993). The Put3 protein has been shown to form homodimers by virtue of a coiled coil between residues 69 and 100 (Des Etages *et al.*, 1996). The activation region of the Put3 protein is in the carboxy-terminal 89 residues, as shown by the fact that a fusion protein containing this portion of Put3p fused to the Gal4p DNA-binding domain can activate transcription of the *GAL4* gene (Des Etages *et al.*, 1996). This fusion protein was not responsive to proline, however, hence the proline-responsive domain does not lie in the carboxy-terminal region of Put3p.

The proline utilisation pathway is not induced only by proline, but is also repressed by preferred nitrogen sources (Daugherty *et al.*, 1993; Xu *et al.*, 1995). However, it has been shown that Put3p mediates only the induction by proline, not the repression by preferred nitrogen sources, nor the subsequent derepression when transferred to a poor nitrogen source, such as γ-amino butyric acid (GABA) (Des Etages *et al.*, 1996). The

increase in *PUT2* transcription on poor nitrogen sources is not mediated by the Gln3p transcriptional activator, yet the expression of *PUT1* and *PUT2* increases in a *URE2* null mutant (Xu *et al.*, 1995). The negative regulator Dal80p also represses the expression of *PUT1* and *PUT2* on preferred nitrogen sources, but only to a mild extent (Daugherty *et al.*, 1993). This suggests that Nil1p, or an as yet unidentified factor, may be involved in the regulation of *PUT1* and *PUT2*.

This information suggests a number of strategies for enabling proline utilisation by wine yeast during oenological fermentation. Specifically, *PUT4* expression could be derepressed specifically by targeting the *PUT4* promoter, or indirectly by the disruption of a negative regulator such as *URE2*. Elevated expression of *PUT4* may not be sufficient to ensure proline transport, due to the post-translational down-regulation of Put4p in the presence of preferred nitrogen sources. As such, the target sequences in Put4p involved in down-regulation might also need to be modified. Ultimately, the availability of sufficient oxygen will also become important.

1.4 Oxygen requirements of *Saccharomyces cerevisiae*

The yeast *S. cerevisiae* can grow in either aerobic or anaerobic conditions and is capable of deriving energy from either respiration or fermentation. Fermentation is very active even in the presence of oxygen and particularly in the presence of glucose, whereas respiration occurs slowly under these conditions (DeDeken, 1966; Lagunas *et al.*, 1982; Lagunas, 1986). The presence of fermentable sugars in the medium (namely glucose, maltose and fructose) leads to the repression of the genes involved in respiration (Lagunas *et al.*, 1982). Under anaerobic conditions, energy is derived solely from fermentation.

Growth under anaerobic conditions is limited, however, and exogenous anaerobic growth factors are required for continued growth. For example, the formation of sterols and unsaturated fatty acids is dependent on the presence of molecular oxygen (Aries and Kirsop, 1978). Oxygen acts as the hydrogen acceptor in the first step of sterol synthesis (Henry, 1982) and as the hydrogen acceptor during the introduction of double bonds in the desaturation of fatty acids (Kirsop, 1982). Sterols and unsaturated fatty acids are important to the fluidity and permeability of the yeast plasma membrane, particularly in the presence of ethanol (Rattray *et al.*, 1975). During anaerobic growth, the

concentration of these essential membrane components is halved with each cell division until they become growth limiting (Aries and Kirsop, 1977). Thus, the concentration of unsaturated fatty acids and sterols in the initial, aerobic stages of oenological fermentation determines the final biomass that is generated. An investigation of the sterol content in an active dried wine yeast found that its initial sterol content was 0.59% of dry weight. Growth of this yeast for 48 hours under aerobic conditions led to an increase in the sterol content of the cells to 2.17%. When these aerated yeast were then used during anaerobic fermentation, the sterols decreased to 0.14%, compared with 1.83% for an aerobic fermentation (Giovanelli *et al.*, 1996).

Molecular oxygen is also involved in other metabolic reactions in *S. cerevisiae*, including the conversion of proline to P5C by proline oxidase (Duteurtre *et al.*, 1971). Proline is not catabolised by *S. cerevisiae* under anaerobic conditions (Duteurtre *et al.*, 1971; Ingledew *et al.*, 1987), yet can sustain growth as the sole nitrogen source in the presence of oxygen. Duteurtre and colleagues (1971) showed that, in the presence of oxygen, proline degradation is blocked by the addition of Antimycin A at a concentration that temporarily blocks respiration. Antimycin A specifically inhibits mitochondrial electron transport between cytochromes *b* and *c1*. It has also been observed that vegetative petite cells (*rho*$^-$ cells) cannot utilise proline as a sole nitrogen source under aerobic conditions (Wang and Brandriss, 1987). The expression of proline oxidase is also upregulated by ten-fold in the presence of oxygen (Wang and Brandriss, 1987). These results suggest that a functional electron transport chain is required for proline oxidase function.

1.4.1 Role of oxygen during oenological fermentation

A lack of oxygen can contribute to stuck or sluggish fermentation. The final biomass generated during fermentation is, in part, dependent on the amount of sterols and unsaturated fatty acids formed during the aerobic phase of fermentation. Oxygen can be added to an oenological fermentation to improve biomass yield and, subsequently, fermentation rate. The timing of any addition of oxygen is very important. For example, in the fermentation of wort, the addition of varying amounts of oxygen at the time of inoculation had no effect on the overall biomass, nor on the fermentation rate. It was found that the optimal time for oxygen addition during brewing was 12 hours post inoculation (O'Connor Cox and Ingledew, 1990). At this point in fermentation, the

cells had undergone one to two divisions and the authors suggest that this extent of lipid dilution leads to cells more efficient in the use of oxygen for unsaturated fatty acid synthesis.

During the fermentation of wine, Sablayrolles and Barre (1986) found that the optimal time for the addition of oxygen (5 mg/L) was at the end of the growth phase, after the maximal rate of carbon dioxide production had been achieved. The poor response to an addition of oxygen during the initial stages of fermentation was attributed to the large fraction of oxygen consumption during the growth phase that was antimycin-sensitive. This antimycin sensitive utilisation of oxygen suggests that the cells retain some respiratory capability, even in the presence of high concentrations of fermentable sugars during the growth phase (Sablayrolles and Barre, 1986). An increase in cell viability is observed after an addition of oxygen due to an increase in ethanol tolerance. This is associated with the changes in membrane composition, as unsaturated fatty acids and sterols are produced, while the level of toxic fatty acids decreases. Combined additions of nitrogen and oxygen were more beneficial than individual additions (Sablayrolles *et al.*, 1996), probably due to the increased demand for nitrogen arising from oxygen stimulation of biomass formation.

The addition of oxygen to oenological fermentation does not lead solely to the synthesis of anaerobic growth factors. Salmon *et al.* (1998) investigated oxygen uptake by yeast harvested from oenological fermentation conditions and discovered that some synthesis of mitochondrial cytochromes occurs under these conditions, despite glucose repression of the respiratory enzymes. These results indicate that, even during anaerobic fermentation, the yeast retains the capacity to utilise oxygen in mitochondrially-related activities. The superfluous oxygen consumption observed when oxygen is added to a ferment has thus been attributed to the partial functioning of several mitochondrial pathways. It is not known what function the continued presence of mitochondrial cytochromes plays in oenological fermentation. Under brewing conditions, dissolved oxygen enhanced yeast growth and the rate of nitrogen uptake, despite the inhibition of ergosterol and unsaturated fatty acid synthesis by chemical inhibitors (O'Conner Cox *et al.*, 1993). These results support the findings of Salmon and colleagues (1998) that lipid biosynthesis is not the only role for oxygen after growth in anaerobic conditions.

Oxygen-dependent ergosterol biosynthesis was shown to utilise only 15% of the oxygen added to a culture at the beginning of the stationary phase. Maximal sterol biosynthesis was estimated to require 0.75-1.5 mg/L of oxygen from an addition of 5 mg/L of oxygen in total (Salmon *et al.*, 1998). It is not known what proportion of oxygen is used for the degradation of proline, nor how much oxygen is required overall for this to occur. The only study to show degradation of proline during fermentation used conditions in which the headspace was flushed with oxygen for the entire experiment (Ingledew *et al.*, 1987).

The presence of oxygen during fermentation not only affects the process rate, but may also influence the sensory characteristics of the resulting wine. Aerobic fermentation leads to increases in cell numbers, but also to a decrease in the production of glycerol, which may have a detrimental affect on the mouth-feel of the resulting wine, and higher levels of acetaldehyde and acetic acids, which may also be undesirable (Giovanelli *et al.*, 1996). Again, this study was not conducted with controlled additions of oxygen, but under aerobic conditions for the duration of the fermentation. The controlled addition of oxygen during alcoholic fermentation might not result in oxidative changes in the final product. It has been suggested that the presence of actively fermenting *S. cerevisiae* leads to the consumption of any oxygen dissolved in the fermenting must by the yeast (Cheynier *et al.*, 1997), thereby sparing the must components from undergoing chemical oxidation to undesirable end products.

Hyperoxidation of the must before yeast inoculation has been proposed as an alternative approach to protecting wine from oxidative instability. The hyperoxidation of white must involves the exclusion of sulphur dioxide and the introduction of oxygen to the must before inoculation. Must hyperoxidation leads to the formation of insoluble brown pigments due to the precipitation of phenolic compounds. The clarification of hyperoxidised must removes these insoluble brown pigments (Schneider, 1998). Thereby, hyperoxidation ultimately results in more colour-stable wine, as the oxygen consumption capacity of these wines is reduced (Schneider, 1998).

1.5 Summary

Proline is an untapped nitrogen source present in grape must. Although *S. cerevisiae* is capable of utilising proline as a sole nitrogen source, oenological conditions inhibit the

uptake of proline until oxygen is depleted, thus the catabolism of proline does not occur. The hypothesis for this study is that strains capable of transporting proline in the presence of preferred nitrogen will be capable of utilising proline as a nitrogen source during the initial, aerobic stages of fermentation. The utilisation of proline as an additional nitrogen source may reduce the incidence of fermentation problems associated with a lack of nitrogen during fermentation. The aims of this study, therefore, are to generate strains capable of transporting proline in the presence of preferred nitrogen sources and investigate the potential for these strains to utilise proline under model oenological conditions. The availability of oxygen during the initial stages of fermentation will also be investigated.

Chapter 2 Materials and methods

2.1 Strains used in this study

2.1.1 Yeast strains

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 2.1. The parental W303 strain and the lys2 strain were obtained from Susan Henry. The auxotrophic variants and deletion strains, plus those harbouring mutations in the *PUT4* gene were generated in this study.

2.1.2 Bacterial strains

Escherichia coli was utilised in this study for the amplification of plasmid DNA and for nick repair after DNA ligation. The DH5α strain was obtained from Tony Focareta of The University of Adelaide, Molecular Biosciences Department. DH5α was used as it is endonuclease negative, and consequently suitable for the introduction of plasmid DNA.

The site-directed mutagenesis of plasmid DNA conducted in this study produced non-methylated plasmid DNA. In this case, the plasmids generated were introduced into the competent *E. coli* cells provided by the manufacturer (Epicurian Coli XL1-Blue Supercompetent Cells, Stratagene).

2.2 Culture media

All media were sterilised before use by autoclaving at 121°C (20 min), except those containing >20 g/L glucose, which were filter sterilised (0.45 μm).

2.2.1 Bacterial culture media

E. coli was cultured in Luria Broth (LB) (Appendix 1) To maintain plasmids, ampicillin was added to a concentration of 50 μg/L, as suggested in Maniatis *et al.* (1982).

2.2.2 Media for yeast cultures

A rich medium, YEPD, was used for the maintenance of *S. cerevisiae* strains that did not contain plasmid DNA, and for the growth of strains containing auxotrophic markers (Appendix 1).

Table 2.1 ***Saccharomyces cerevisiae*** **strains used in this study**

Strain	Genotype	Source
W303a	*ade2-1 can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura2-1 MAT***a**	S. Henry
W303α	*ade2-1 can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura2-1 MAT*α	S. Henry
VJ23	*his3-11,-15 leu2-3,-112 MAT*α	This Laboratory
KP2	W303, *MAT*α	This study
KP3	W303, *MAT***a**	This study
KP10	W303, *put4*Δ*::KanMX4*, *MAT*α	This study
KP11	W303, *ura3* Δ*::KanMX4*, *MAT*α	This study
KP12	W303, *put4* Δ*::KanMX4 ura3* Δ*::KanMX4 MAT*α	This study
KP13	W303, *put4* Δ*::KanMX4 ura* Δ *3::KanMX4 MAT*α harbouring pFL-PUT4 (Appendix 2)	This study
KP20	W303, *ure2* Δ*::KanMX4 MAT*α	This study
KP21	KP12 harbouring pTEF-PUT4 (Appendix 2)	This study
KP30	W303, *npr2* Δ*::KanMX4*, *MAT*α	This study
KP31	W303, *rcy1* Δ*::KanMX4*, *MAT*α	This study
KP41	KP12 harbouring pFL-put4M1	This study
KP42	KP12 harbouring pFL-put4M2	This study
KP43	KP12 harbouring pFL-put4M3	This study
KP51	KP12 harbouring pFL-put4(-90)	This study
KP52	KP12 harbouring pFL-put4(-160)	This study
KP53	KP12 harbouring pFL-put4(-213)	This study

Table 2.1 continued

KP54	KP12 harbouring pFL-put4(-500)	This study
KP55	KP12 harbouring pFL-put4(-708)	This study
KP61	KP12 harbouring pFL-put4K604R	This study
KP62	KP12 harbouring pFL-put4LVAA	This study
KP63	KP12 harbouring pFL-put4LLAA	This study
KP64	KP12 harbouring pFL-put4S605A	This study
KP65	KP12 harbouring pFL-put4S605E	This study
KP71	KP12 harbouring pFL-(-90put4S605A)	This study

S. cerevisiae strains containing plasmid DNA were grown in minimal, yeast nitrogen base media (YNB, Appendix 1). This medium contained 20 g/L glucose as a source of carbon and 5 g/L $(NH_4)_2SO_4$ as the source of nitrogen. Media for the removal of nitrogen catabolite repression contained proline (5 g/L) in place of $(NH_4)_2SO_4$.

2.2.3 Chemically defined grape juice medium

During the preliminary study, described in Chapter 3, a chemically defined grape juice medium (CDGJM, Henschke and Jiranek, 1993) whose composition resembles that of grape juice was used, with some modifications (Appendix 1). Glucose was present at 180 g/L, as opposed to 200 g/L. The complex mixture of amino acids was omitted, and nitrogen was supplied in the form of 5 g/L $(NH_4)_2SO_4$. In some experiments proline was added to 200 mg/L. Cells used for the inoculation of CDGJM were cultured overnight in starter media, which was identical to CDGJM with the exception of the glucose concentration (100 g/L), and the presence of ergosterol (10 mg/L) and oleic acid (2 ml, Tween-80).

2.2.4 Synthetic medium

A second synthetic medium (Milieu Synthetique, MS) was utilised to mimic oenological conditions (Salmon and Barre, 1998) for the oxygenation experiments. The composition of this medium is described in Appendix 1.

2.3 Growth and fermentation

Bacterial liquid cultures were incubated at 37°C with shaking at 160 rpm. Bacteria grown on solid media were incubated statically at 37°C.

Yeast grown on solid media were incubated statically at 30°C. Liquid yeast cultures were incubated at 30°C with shaking at 160rpm.

2.3.1 Fermentation protocol

Yeast cells from a single colony on a fresh solid minimal YNB plate were transferred to 100 ml of synthetic grape juice starter medium in 500 ml Erlenmeyer flasks and loosely fitted with a screw cap lid. The cultures were incubated overnight until the early stationary phase was reached (>1 x 10^8 cells/ml). This culture was used to inoculate the fermentation media.

Fermentations were conducted in either 100 ml or 1L of media. For 100 ml fermentations, 250 ml modified Erlenmeyer flasks were used. These flasks were fitted with sidearms (1 cm light path) to allow the non-invasive monitoring of culture density using a Turbidimeter (Hach, 2100AN). Flasks were also fitted with fermentation locks to allow the release of CO_2, whilst precluding the introduction of oxygen. A sample port fitted with a rubber seal allowed the extraction of samples over the course of fermentation. Before inoculation, flasks were sparged with either high purity nitrogen or oxygen to generate either anaerobic or aerobic conditions, respectively. Media was inoculated with 5 x 10^6 cells/ml and cultures were then incubated at 30°C, shaking at 160 rpm.

Fermentations in 1L were conducted in 2L Braun Biostat B fermenters. Before inoculation the media was sparged with air until the dissolved oxygen content reached 100% (by internal dissolved oxygen probe). The ferment vessel was then sealed to prevent the introduction of additional oxygen. Yeast cells were introduced via a septum port to a final concentration of 5 x 10^6 cells/ml. A thermal jacket maintained a temperature of 30°C and ferments were stirred via an internal 6-blade impeller at 160 rpm.

In some cases the headspace was flushed with nitrogen to ensure anaerobic conditions during fermentation. At these times nitrogen was introduced via copper tubing fitted to an inlet port in the top of the vessel. High purity nitrogen (>99.99%, Linde) was piped through an Alltech Gas-Trap®, an Alltech Oxy-Trap® and an Alltech Indicating Oxy-Trap®, to remove any oxygen.

For both 100 ml and 1 L cultures, fermentation progress was monitored initially by means of a hand held refractometer (Atago). This method of estimating glucose concentrations is limited to the initial stages of fermentation, as the presence of alcohol in a sample will affect the refractive index. The glucose concentration at the end of fermentation was estimated using Clinitest indicator tablets (Bayer Diagnostics).

2.3.2 Micro-oxygenation

Micro-oxygenation experiments were carried out at the Institut National de la Recherche Agronomiquc, Montpellier, France. A calibrated micro-oxygenation device

was used to introduce a known amount of dissolved oxygen during fermentation as previously described (Blayteron *et al.*, 1998). Fermentations of 1L of MS (Appendix 1), sparged with Argon gas for 20 min to remove oxygen, were conducted in custom 1.2 L fermentation vessels. The medium was inoculated at 1 x 10^6 cells/ml and fermentation locks were then fitted to maintain anaerobic conditions. Ferments were stirred by means of an internal stirring rod. The progress of fermentation was monitored by determining vessel weight loss, indicative of glucose catabolism and concomitant carbon dioxide evolution (Sablayrolles *et al.*, 1987).

2.3.3 Analysis of fermentation parameters

2.3.3.1 Dry cell weight determination

Millipore filters (0.22 μm, GSWG, 47 mm diameter) were dried in a microwave oven on medium power setting (300W) for 10 min. The filters were then cooled in a desiccator before being weighed on an analytical balance. Culture samples (10-20 ml depending on cell density) were filtered through weighed filters and the cells washed with an equal volume of MilliQ water. The filters were then dried as above, cooled in the desiccator and weighed to allow determination of the dry cell weight.

2.3.3.2 Viable cell counts

The percentage of viable cells present at the end of fermentation was measured by epifluorescence, as described by King *et al.* (1981). Reactive Solution was prepared by dissolving 30 mg of 8 anilino 1 napthalene sulphonic acid in 10 ml of Ringer solution (Appendix 1). One volume of Reactive Solution was mixed with 1 volume of cells and incubated at room temperature for 5 min. Cells were counted using a haemocytometer. The dead cells were counted during visualisation by fluorescence microscopy and the percentage viability was calculated from the two determinations.

2.3.3.3 Determination of ammonia concentration

An ammonia probe (Corning Ion Selective Electrode, 476130) was used to determine the concentration of ammonia in the ferment samples, as per the manufacturer's instructions.

2.3.3.4 Determination of glucose concentration in culture samples

Residual glucose concentrations in appropriately diluted fermentation samples were determined using an enzymatic, UV-method as described in the Boehringer Mannheim biochemical analysis protocol for D-Glucose/D-Fructose. Briefly, 1 ml of Working Solution (see Appendix 1) was placed in a 1 ml cuvette (1 cm light path) and A_{340} was measured. 33 µl of sample was added, mixed in the cuvette and incubated at room temperature for 15 min. The A_{340} was measured again. The concentration of glucose present in the sample was determined using the following formula:

$$C(g/L) = \Delta A_{340} \times 0.89514 \times Df$$

where C is the concentration of glucose and Df is the dilution factor.

2.3.3.5 Determination of proline concentration

The proline concentration was determined using isatin. Samples were diluted (if necessary) to proline concentrations of <200 mg/L. The sample (40 µl) was mixed with 0.5M citrate buffer, pH 4.1 (40 µl), 0.075% isatin in acetone (250 µl) and 100% ethanol (500 µl). The samples were heated at 100°C for 5 min and the resulting blue residue resuspended in a 3 ml mixture of acetone and water (2:1 v/v). The absorbance of the solution was measured at 595 nm and the proline concentration was determined by comparison with standards of known concentration.

2.4 Classical yeast genetics

Haploid yeast strains of opposing mating type were mixed in a droplet of sterile water on a thin YEPD plate and incubated for 2 hours at 30°C. Diploid zygotes, distinguishable by their characteristic morphology, were then isolated on the plate, using a micromanipulator and a bright field microscope (Olympus), before incubation for a further 48 hours at 30°C. Diploid cells were plated on solid sporulation media (Appendix 1) and incubated for 48 hours at 30°C. Ascus formation was monitored microscopically in wet mounts of a small sample of cells by phase-contrast microscopy. A loopful of cells from sporulation plates was resuspended in 1 ml of 100 units/ml β-glucuronidase (Sigma G-7770) and incubated for 30 min at room temperature. The progress of ascus wall digestion was monitored microscopically and the incubation was extended if required. A loopful of β-glucuronidase-treated spore suspension was gently streaked onto a thin YEPD plate. Intact asci containing four spores were isolated and

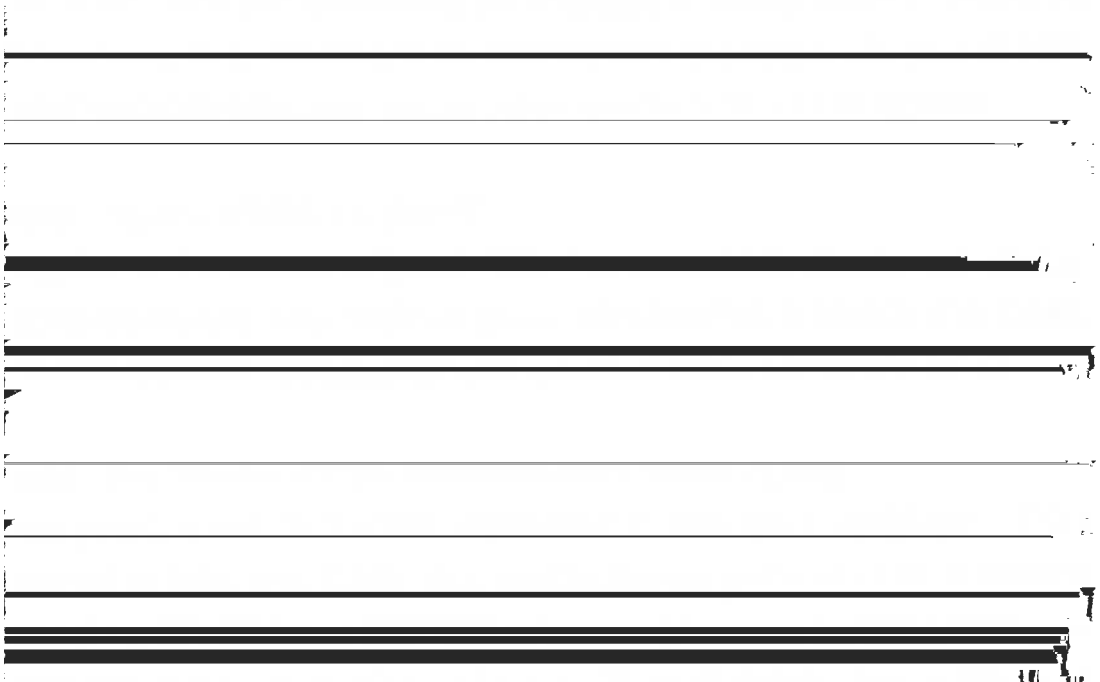
Vector DNA (1-10 μg), digested with appropriate restriction enzymes and gel purified, was transferred to a 1.5 ml eppendorf tube (routinely 10 μl). Calf Intestinal Phosphatase (CIP) (1 μl), and 10 x CIP buffer (2 μl) (CIP and CIP buffer from Boehringer Mannheim, 713 023), were added to the vector DNA, and the total volume of the reaction was brought to 20 μl by the addition of MilliQ water. The reaction was incubated at 37°C for 60 min. The enzyme was heat inactivated by incubation at 65°C for 10 min. DNA was isolated using the UltraClean 15 DNA purification kit (Mo Bio Laboratories, Inc.), according to the manufacturer's instructions. The plasmid DNA pellet was briefly dried under vacuum and resuspended in 10 μl of MilliQ water.

2.5.3 Ligation of DNA into plasmid

Ligation of the restriction-digested DNA into appropriately digested and alkaline phosphatase-treated vector DNA was performed as described in Maniatis *et al.* (1982). T4 DNA ligase and the appropriate buffer were obtained from Geneworks (TL-1).

2.5.4 Amplification of DNA-Polymerase Chain Reaction (PCR)

The procedure used for the PCR amplification of DNA was a modification of that reported by Saiki *et al.* (1988). PCR amplification was performed in 50 μl reactions containing 1U DYNAzyme EXT DNA polymerase (Finnzymes, F-505S), DYNAzyme



EXT PCR buffer, 100 pmol of each primer, 50 ng of plasmid DNA or 250 ng of genomic DNA and 0.5 mM dNTPs. $MgCl_2$ concentrations and cycling parameters were optimised for each PCR reaction. Primers used for PCR amplification are listed in Table

2.2. Cycling reactions were conducted using an Eppendorf *Mastercycler Gradient* thermocycler.

2.5.5 Error-prone PCR

Error-prone PCR was used to generate random mutations in a specific region of DNA. The conditions used were the same as those described for PCR, with the exception of the DNA polymerase: 1U of DyNAzyme II recombinant enzyme (Finnzymes, F-501S), in conjunction with DyNAzyme buffer, was used for error-prone reactions.

2.5.6 Colony-cracking PCR for rapid screening of transformants

A rapid screen for transformants containing a specific DNA sequence was performed using a variation of the standard PCR protocol. In this case, template DNA was not isolated before PCR was conducted. Half of a single colony from a transformation plate (either *E. coli* or *S. cerevisiae*) was suspended in a PCR mix containing 1U DyNAzyme EXT DNA polymerase (Finnzymes, F-505S), DyNAzyme EXT PCR buffer, 100 pmol primer and 0.5 mM dNTPs. PCR was then conducted as normal.

2.5.7 PCR labelling of probes for Northern blot analysis

DNA probes for the detection of RNA were labelled with Digoxigenin-11-dUTP (DIG), by incorporation into PCR products. The PCR DIG Probe Synthesis Kit (Roche 1 636 090) was used as per the manufacturer's instructions. The DIG-labelled products were gel purified before use. Primers used for the production of DIG-labelled probes are listed in Table 2.3.

2.5.8 Site-directed mutagenesis

Selected codons of Put4p and nucleotides in the *PUT4* promoter were mutated using the Stratagene QuikChange Site-Directed Mutagenesis kit. The template DNA was pFL-PUT4 (10 ng) isolated using the Promega Wizard *Plus* SV Miniprep plasmid purification system, as per the manufacturer's instructions. The primers used to introduce mutations are listed in Table 2.4.

The mutagenic PCR was carried out as described in the QuikChange manual, with one modification. The extension time was increased to 3 min/kb of template DNA (24 min). An aliquot of the PCR product was analysed on a 1% agarose gel to confirm that the

Table 2.2 Primers used for PCR amplification throughout this study

Primer	Sequence (5'-3')
urekanf	TTGTTTTAAGCTGCAAATTAAGTTGTACACCA AATGATGACGTACGCTGCAGGTCGA
urekanr	AAGCAGCCTTCATTCACCACGCAATGCCTTGA TGACCGCGGATGAATTCGAGCTCG
Ure2f	ATCCCCCGTACGAACTT
Ure2r	GCCTATATACATACCCTTA
TEFPUTf	GGAGTACTTGTTTTTAGAATATACGGTCAACGAAGTA TAATTAACTAAACCCATCTAGAAATAAATCATGG
Put4r	TTGCTGCAGTGGTAAAGATCA
Put4f	AAGATTGTAGAAAGGATCCGC
P4UTRr	ATTGTTCTTGTGGAAGGGCA
Npr2f	TTGTTGCCTGTATCTCCGAA
Npr2r	TGGATTCGTGTGTACTATCTT
Rcy1f	GAAAAATAGAGCCCGCGTAA
Rcy1r	TCAAGGCTAACTTTTGCCGA
Screenf	TGCCCTTCCACAAGAACAAT
Screenr	AACAAGGCGTCCAAGAACTT

Table 2.3 Primers for the PCR synthesis of DIG-labelled probes

Primer	Sequence (5'-3')
P4probeF	TGCCCTTCCACAAGAACAAT
P4probeR	AACAAGGCGTCCAAGAACTT
PDAf	TCGCACCTGTATCTTCACAAA
PDAr	AATCCCTAGAGGCAAAACCTT

Table 2.4 PCR primers used in site-directed mutagenesis

Primer	Sequence (5'-3')
M1-160f	CAGACGCATAAACATATATGCATATACATACTTATACACTCG
M1-160r	CGAGTGTATAAGTATGTATATGCATATATGTTTATGCGTCTG
M1-500f	GTTAGTTTTTTTCATTGCGCTAACTATGACGTTTGGGTGGCC
M1-500r	GGCCACCCAAACGTCATAGTTAGCGCAATGAAAAAAACTAAC
M1-708f	CCGCGCGGTTCAAATCTTGTCGCAAGTTTGTCAAAATTGC
M1-708r	GCAATTTTGACAAACTTGCGACAAGATTTGAACCGCGCGG
M2-90f	ATATATATAGGGGGTTTGTGTCCCTCTTCCTTTCCTTTTT
M2-90r	AAAAAGGAAAGGAAGAGGGACACAAACCCCCTATATATAT
M2-213f	CTGAATATTCCATGAATGCAGGCACAGCATATCTCCATCC
M2-213r	GGATGGAGATATGCTGTGCCTGCATTCATGGAATATTCAG
K604Rf	GTCGAGATCGAGGAGAGATCAAGAGAAATTGAGGAGATGAG
K604Rr	CTCATCTCCTCAATTTCTCTTGATCTCTCCTCGATCTCGAC
LV-AAf	GATCGATGTTACTACAGGGGCAGCCGAGATCGAGGAGAAATC
LV-AAr	GATTTCTCCTCGATCTCGGCTGCCCCTGTAGTAACATCGATC
Dileuf	CAAAGACAAGTTCTTGGACGCCGCGGCGTAACGCTTTATGAAC
Dileur	GTTCATAAAGCGTTACGCCGCGGCGTCCAAGAACTTGTCTTTG
S605Af	GTCGAGATCGAGGAGAAAGCAAGAGAAATTGAGGAGATGAG
S605Ar	CTCATCTCCTCAATTTCTCTTGCTTTCTCCTCGATCTCGAC
S605Ef	GGCTAGTCGAGATCGAAGAGAAAGAAAGAGAAATTGAGGAG
S605Er	CTCCTCAATTTCTCTTTCTTTCTCTTCGATCTCGACTAGCC

PCR had generated a product of correct size. The remainder of the PCR product was digested with the *DpnI* restriction enzyme provided before transformation of the Epicurian Coli XL1-Blue supercompetent cells provided. Plasmid DNA isolated from the transformants was sequenced to confirm the introduction of specific mutations, using either sequencing primer SDMSEQ or MutSEQ1.

2.5.9 Sequencing reactions

The primers used for sequencing are listed in Table 2.5. Templates for the sequencing reactions were always plasmid DNA prepared as described in section 2.7.2.2. The sequencing reactions complied with the recommendations of Applied Biosystems (ABI). Briefly, 30 pmol of primer, 200 ng of template DNA and 8 µl of ABI Big Dye sequencing reagent (obtained from the Molecular Pathology Sequencing Service, Institute of Medical and Veterinary Science, Adelaide) were used in a 20 µl reaction.

Sequencing products were precipitated with 60% isopropanol during a 30 min incubation at room temperature. The sequencing products were harvested by centrifugation for 20 min at 12000*g*. The supernatants were aspirated carefully and the pellets were washed with 250 µl of 75% isopropanol. Air-dried pellets were supplied to the Molecular Pathology Sequencing Service for separation and analysis using an ABI automated sequencer.

2.6 Yeast molecular methods

2.6.1 Yeast transformation

2.6.1.1 Preparation of carrier DNA

Single stranded carrier DNA was prepared by boiling salmon sperm DNA (Sigma D-1626), 2 mg/ml in TE buffer, for 10 min before cooling rapidly on ice.

2.6.1.2 Lithium acetate transformation - colony method

This method of transforming yeast cells was utilised for the routine introduction of plasmids. One large colony (or several small colonies) was resuspended in 1 ml of MilliQ water and then harvested by centrifugation (3000*g*, 1 min). The cells were resuspended in 100 mM LiAc, 1 x TE (Appendix 1), pH 7.5 (50 µl) and 1 µg of plasmid DNA and 50 µg of carrier DNA were added. A freshly prepared solution (300 µl) of

Table 2.5 Primers used in sequencing reactions

Primer	Sequence (5'-3')
MutSEQ1	AGGGTGTAAAGTGCGTGTG
MutSEQ2	TACTGCAATGTGAATTCCC
MutSEQ3	TATGACGTTTGGGTGGCCT
MutSEQ4	TTATGTACATGCCCCAATC
SDMSEQ	GTTCGGCCATAAGCTGTA

40% PEG 3400 (ICN 151916), buffered with 1 x TE and 100 mM LiAc was added and the cells were incubated for 30 min at 30°C before heat shocking at 42°C for 45 min. Cells were harvested in a microfuge (5 sec pulse) before resuspension in TE and plating on selective media.

2.6.1.3 High efficiency transformation of *Saccharomyces cerevisiae* using lithium acetate

High efficiency transformation of *S. cerevisiae* was conducted essentially as described in Gietz *et al.* (1992). Cells were grown overnight in YEPD from a single colony. A second overnight culture was inoculated from this stationary phase culture and grown to a density of 1 x 10^7 cells/ml. This culture was diluted to 2 x 10^6 cells/ml and grown for 2 hours to allow another cell division. Cells were then harvested at 4000*g* for 5 min, washed in 10 ml of MilliQ water and resuspended in 1.5 ml of freshly prepared Buffered Lithium Solution (Appendix 1). For each transformation, 15 µl of carrier DNA was mixed with up to 5 µg of transforming DNA in a volume of less than 20 µl. This DNA mix was added to 200 µl of yeast suspension and 1.2 ml of PEG solution (Appendix 1). The cells were incubated at 30°C for 30 min then heat shocked at 42°C for 45 min. Cells were harvested and resuspended in 1 x TE before plating on selective media. Transformants were incubated at 30°C and appeared after at least two days of incubation.

2.6.1.4 High efficiency transformation - selection on geneticin

Transformants containing the *kanMX4* marker were selected on YEPD plates containing 400 mg/L of geneticin. The transformation procedure was as that described above for high efficiency transformation (2.6.1.3), with the following exception: cells were harvested after heat shock and resuspended in 1 ml of YEPD medium, without selection, and incubated for 2 hours before plating on selective solid media.

2.6.2 Isolation of *Saccharomyces cerevisiae* genomic DNA

As described by Adams *et al.* (1997), YEPD (10 ml) was inoculated with a single colony of *S. cerevisiae* and incubated overnight. Cells were collected by centrifugation and washed in 0.5 ml of sterile MilliQ water. The pellet was then resuspended in 0.2 ml of cell lysis solution (Appendix 1) before the addition of 0.2 ml of

phenol:chloroform:isoamylalcohol (25:24:1) and 0.3 g of acid-washed glass beads. Samples were then vortexed for 4 min and 0.2 ml of TE was added before the samples were centrifuged for 2 min (12000*g*). The aqueous layer was transferred to a fresh tube and 1 ml of 100% ethanol was added. Samples were centrifuged (12000*g*) for another 2 min and the supernatant was discarded. The pellet was resuspended in 0.4 ml of TE, an RNase cocktail was added (Amicon, 2286) and the samples were incubated for 30 min at 30°C. To re-precipitate the DNA, 10 µl of 4 M ammonium acetate and 1 ml 100% ethanol were added. DNA was harvested by centrifugation for 2 min at 12000*g*. The pellet was air dried and redissolved in TE buffer (pH 7.5).

2.6.3 RNA preparations from yeast

All solutions used for the preparation and analysis of RNA were made in DEPC-treated MilliQ water. Sterile MilliQ water (1 L) was treated with 1 ml of DEPC and incubated at 37°C overnight. The treated MilliQ was then autoclaved for 20 min to inactivate the DEPC. The method used to isolate RNA from the yeast was based on the method of Schmitt *et al.* (1990). Yeast cells were harvested at different points over the growth phase by centrifugation at 3000*g* for 5 min. The cells were resuspended in 400 µl of AE buffer (Appendix 1). All subsequent steps were performed on ice. SDS (10%) (40 µl) was added to the cell suspension and the tube was vortexed. Phenol saturated with AE buffer (440µl) was added and the tube was vortexed again. The samples were then incubated at 65°C for exactly 4 minutes. The mixture was cooled rapidly in a dry ice/ethanol bath and allowed to thaw on ice before centrifugation (12000*g*) for 2 minutes. The upper layer was transferred to a fresh microfuge tube, 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added and the samples were vortexed. The samples were subsequently incubated at room temperature for 5 min, centrifuged for 2 min and the upper layer was transferred to a fresh microfuge tube. The RNA was precipitated by the addition of 0.1 volumes of 3M sodium acetate and 2.5 volumes of ice-cold ethanol, and stored at –20°C overnight. The nucleic acid was then pelleted by centrifugation at 12000*g* for 15 min at 4°C. The supernatant was discarded and the pellet was washed with 1 ml of ice cold 70% ethanol. The supernatant was removed and the pellet was dried briefly under vacuum. The RNA was resuspended in DEPC-treated MilliQ water (100 µl) and then stored at – 80°C.

2.6.4 Plasmid construction by gap-repair

Plasmids were constructed by the gap-repair method (Ma *et al.*, 1987), which exploits the ability of yeast to effect homologous recombination. A centromeric yeast vector containing the *URA3* gene was digested with restriction enzyme as described in section 2.5.1. The linearised vector was co-transformed into *S. cerevisiae* with insert containing ends sharing at least 30 bp of homology with the free ends of the vector (vector:insert, 1:5). Yeast transformation was conducted as described in section 2.6.1.3. Strains carrying gap-repaired plasmids were selected on YNB media lacking uracil.

2.6.5 Northern blot analysis of *Saccharomyces cerevisiae* RNA

2.6.5.1 Determination of RNA concentration

The solutions used for the analysis of RNA were prepared in DEPC-treated water. RNA was isolated from *S. cerevisiae* as described in section 2.6.3. To determine the concentration of RNA, 5 µl of each sample was diluted 1/200 in sterile DEPC-treated water and the A_{260} was measured. The concentration of RNA in the original sample (in µg/L) was calculated by multiplying the A_{260} reading by 40 and then by the dilution factor, i.e. 200 (Maniatis *et al.*, 1982)

2.6.5.2 Electrophoresis of RNA samples

Agarose gels (1.5%) containing formaldehyde (2.2M) were used for the electrophoresis of RNA samples as described previously (Maniatis *et al.*, 1982). Samples of RNA (5-10 µg) were prepared in Formaldehyde Gel Loading Buffer (Appendix 1) and electrophoresed through a Formaldehyde Gel (Appendix 1) at 100V in 1 x MOPS buffer (Appendix 1).

2.6.5.3 Blotting of RNA to nylon membranes

2.6.5.3.1 RNA slot blotting

Aliquots containing 5 µg of RNA were diluted in 100 µl of RNA Dilution Buffer (Appendix 1) before being transferred to nylon membrane (Hybond-N+, Amersham Life Science) using a slot blot apparatus (Hoeffer 648, Amersham Pharmacia Biotech). The RNA was fixed to the membrane by UV crosslinking using an Amersham Life Sciences UV crosslinker (254 nm @ 70,000 µJ/cm^2).

2.6.5.3.2 Transfer of electrophoresed RNA to nylon membranes

Electrophoresed RNA samples were transferred to nylon membranes by upward capillary transfer. Before the assembly of the transfer system, the formaldehyde gel was washed (2 x 15 min) with 20 x SSC (Appendix 1). The transfer system was then assembled as described previously (Maniatis *et al.*, 1982), with 20 x SSC as the transfer buffer.

2.6.5.4 Hybridisation of DIG-labelled probe

Hybridisations were conducted in hybridisation bags (Roche, 1 666 649) at the appropriate temperature in a hybridisation oven. Nylon membranes were pre-hybridised in 30 ml of hybridisation buffer (DIG Easy Hyb, Roche) for 2 hours at 50°C. The pre-hybridisation buffer was then replaced with 30 ml of fresh hybridisation buffer containing 25 ng/ml of the denatured (10 min at 100°C, 1 min at 0°C) DIG-labelled probe. The probe was hybridised to RNA on the membrane at 50°C overnight. The unbound probe was removed by washing the membrane (2 x 15 min) in 2 x SSC/0.1% SDS at room temperature. This was followed by further washes (2 x 15 min) in 0.5 x SSC/0.1% SDS at 68°C.

2.6.5.5 Detection of bound DIG-labelled probe

All washing steps used during the detection of Northern blots were conducted at room temperature. After the post-hybridisation washes, the membrane was equilibrated in Wash Buffer (Appendix 1) for 1 min. The membrane was then incubated in 1 x Blocking Solution (Appendix 1) for 30 min. Anti-Digoxigenin-AP antisera (Roche 1 093 274) was diluted 1/100 000 in Blocking Solution and the membrane was transferred to this solution and incubated for 30 minutes with gentle agitation. The membrane was then washed (2 x 15 min) in Wash Buffer before equilibration in Detection Buffer (Appendix 1). The membrane was sealed in a plastic bag containing 0.5 ml of ECF substrate/100 cm^2 (Amersham Life Sciences, 1067873) and incubated in the dark for 1-16 hours. Fluorescent bands were detected using a Storm 860 phospho-imager (Molecular Dynamics), using a blue chemifluorescent filter setting.

2.7 *Escherichia coli* molecular methods

2.7.1 Rapid size screen of plasmid DNA

This method was used as a rapid screen for the successful insertion of DNA fragments into plasmids. Single transformants were isolated with a sterile yellow (200 µl) pipette tip and streaked onto an LB plate containing 50 µg/ml of ampicillin. The remainder of the colony was resuspended in 15 µl of Cracking Solution (Appendix 1). The pipette tip was left in the solution and the sample was incubated at 65°C for 15 min. Any sample remaining in the tip was then expelled, the tubes were closed and incubated for a further 10 min at 65°C. The samples were centrifuged at 12000*g* for two minutes before being loaded onto a 1% agarose gel in 1 x TAE (Appendix 1). The DNA was resolved on the agarose gel at 120V. Recombinant plasmids ran higher on the gel than the vector alone.

2.7.2 Plasmid preparation from *Escherichia coli*

2.7.2.1 Small scale plasmid preparation

Plasmid DNA was routinely isolated by an alkaline lysis method similar to that described in Maniatis *et al.* (1982). A single *E. coli* colony was grown overnight in selective media (LB containing 50 µg/L ampicillin). Cells were harvested from 1 ml of culture by centrifugation and resuspended in 100 µl of Solution 1 (Appendix 1). Solution 2 (200 µl, Appendix 1) was added and the tube was inverted four times. Solution 3 (150 µl, Appendix 1) was added and the tube immediately was inverted four times before a 15 min incubation on ice. The samples were centrifuged at 12000*g* for 15 min and the supernatant was transferred to a fresh microfuge tube. Ammonium acetate (250 µl, 7.5 M) was added and the tube was inverted before incubation for 15 min on ice. The samples were centrifuged for another 15 min and the supernatant was transferred to a fresh tube. To precipitate plasmid DNA, 1 ml of isopropanol was added and the samples were incubated for 15 min on ice before centrifugation at 12000*g* for 15 min. The pellets were washed with ice cold 70% ethanol and air-dried. Plasmid DNA was resuspended in 50 µl of TE buffer and stored at –20°C.

2.7.2.2 Preparation of plasmid for sequencing and site-directed mutagenesis

High purity plasmid DNA preparations were made using the Promega Wizard *Plus* SV plasmid miniprep kit. A single colony of *E. coli* containing plasmid was incubated overnight in LB medium containing 50 µg/L of ampicillin. Cells (1 ml) were harvested

by centrifugation at 12000*g* for 1 min and plasmid DNA was prepared as per the manufacturer's instructions. Plasmid DNA was resuspended in 100 ml of nuclease-free water and stored at –20°C.

2.7.3 Preparation of competent *Escherichia coli* cells for transformation

Competent cells were prepared and stored as described in Ausubel *et al.* (1994). An overnight culture of *E. coli* DH5α was diluted 1:10 in LB and grown to an A_{580} of 0.4. The cells were chilled on ice for 10 min and then pelleted by centrifugation (2700*g*, 7 min, 4°C). The pellet was gently resuspended in cold $CaCl_2$ solution (60 mM $CaCl_2$, 15% glycerol, 10 mM PIPES, pH 7.0) and left on ice for 30 min. The cells were then washed twice by pelleting at 2700*g* for 5 min at 4°C and resuspending in 10 ml of cold $CaCl_2$ solution. After the second wash, the cells were resuspended in $CaCl_2$ solution and stored in 200 µl aliquots at –70°C.

2.7.4 Transformation of chemically competent *Escherichia coli* cells

An aliquot of competent cells was thawed at room temperature. Once thawed, the cells were mixed gently. Transforming DNA (<50 ng in less than 10 µl) was added to 200 µl of competent cells in 1.5 ml reaction tubes and this mixture was incubated on ice for 30 min. The cells were then heat shocked at 42°C for 60 sec and returned to the ice bath for 5 min. Luria broth (800 µl) was added to the cells, which were then incubated at 37°C for 45 min. Transformed cells were spread onto solid LB media (Appendix 1) containing 50 µg/ml of ampicillin and incubated overnight at 37°C. Potential recombinant colonies were identified by a rapid size screen protocol (2.7.1), or by colony-cracking PCR (2.5.6).

2.8 Analysis of protein in *Saccharomyces cerevisiae*

2.8.1 SDS-Polyacrylamide gel electrophoresis

Polyacrylamide gels (12%) were obtained from Gradipore (NG21-012). Gradipore iGels do not contain SDS; however, the samples were electrophoresed under denaturing conditions. Protein samples were prepared in SDS Sample Buffer (Appendix 1) and the Running Buffer also contained SDS (Appendix 1). Samples were electrophoresed for 1.5 hours at 100V in the Bio-Rad Miniprotean II gel apparatus. The molecular weight

markers used were Amersham Pharmacia High-Range Rainbow Molecular Weight Markers (RPN 756).

2.8.2 Transfer of proteins to nitrocellulose

The Bio-Rad mini Protean II gel system was used to transfer proteins from mini gels to nitrocellulose (Hybond-C Extra, Amersham Life Sciences, RPN203E). The polyacrylamide gel was gently removed from the plastic support and the wells were removed. The gel was equilibrated by soaking in Western Transfer Buffer (Appendix 1) for 30 min, along with Whatman paper, nitrocellulose and sponges. The transfer 'sandwich' was set up as described by the manufacturer. Proteins were transferred in Western Transfer Buffer at 100V for 90 min.

2.8.3 Western blotting

Western blotting was conducted as described by Towbin *et al.* (1979), with minor variations. Nitrocellulose was removed from the transfer apparatus and rinsed (2 x 5 min) in TBS (Appendix 1). The membrane was covered with Blocking Buffer (Appendix 1) and incubated at room temperature for 1 hour with gentle agitation. The blocking solution was then drained and the membrane was incubated in TBST (Appendix 1), containing a 1/500 dilution of primary antibody (anti-Put4p polyclonal antisera). The nitrocellulose membrane was incubated at room temperature with gentle agitation for 1 hr in the presence of primary antibody, before washing (3 x 5 min) in TBST. Secondary antibody was then added (1/7500 dilution in TBST) and the blot was incubated for 30 min at room temperature with gentle agitation. The nitrocellulose membrane was then washed (3 x 5 min) in TBST before rinsing with TBS to remove Tween-20 detergent.

Secondary antibodies used in this study were conjugated to alkaline phosphatase. The Western blot was developed by the addition of Alkaline Phosphatase Buffer (Appendix 1) containing 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT- Promega S3771), as per the manufacturers instructions. Once bands were coloured sufficiently, the reaction was stopped by rinsing with MilliQ water.

2.8.4 Proline uptake assay

Proline uptake was measured as described in Lasko and Brandriss (1981), with minor variations. Radio-labelled proline was obtained from Amersham Pharmacia (CFB71). Between 5 and 20 ml of cells (depending on culture density) were harvested by filtration and the cells were washed with YNB lacking carbon and nitrogen sources. The cells were then incubated for 5 min in pre-warmed, aerated YNB lacking a nitrogen source. Radio-labelled proline (0.5 μCi, specific activity 260 mCi/mmol) was added to a final concentration of 0.2 mM and 500 μl samples were taken at 15, 60, 105 and 150 sec after the addition. The sampled cells were filtered and washed with YNB lacking a carbon source and containing 0.1% proline. The filters were dried by heating in a microwave oven on a medium power setting (300W) for 2 min, before the addition of 4 ml of scintillation fluid (Starscint, 6013248). The amount of radio-labelled proline retained by the harvested cells was determined using a Packard scintillation counter, counting ^{14}C CPM for 1 min. To determine CPM/mg cells at the time of sampling, a second sample of cells was filtered and the dry cell weight was determined (2.3.3.1).

2.8.5 Determination of the degree of Put4p down-regulation in the presence of ammonium

Cell cultures were grown to stationary phase in YNB, then subcultured at 5 x 10^6 cells/ml in YNB containing proline (5 g/L) as the sole nitrogen source. These cultures were then incubated until they reached 1 x 10^7 cells /ml, at which point the proline uptake was measured. After the addition of methylamine or ammonium to 4.8 g/L or 5 g/L, respectively, proline uptake was again measured at 15, 30, 60 and 120 min, with concurrent samples taken for the determination of dry cell weight at each time point.

2.8.6 Antisera generation

2.8.6.1 Peptide generation

Rabbit polyclonal antisera was generated against peptides corresponding to sequences found in Put4p. Each peptide contained a terminal cysteine to facilitate conjugation to a carrier protein. Peptide 1 corresponded to residues 54-65 of Put4p (His-Arg-Ser-Asp-Asn-Glu-Lys-Asp-Asp-Ala-Leu-Cys) and Peptide 2 corresponded to residues 617-627 (Cys-Gly-Phe-Lys-Asp-Lys-Phe-Leu-Asp-Ala-Leu-Leu). These peptides were custom made by Auspep (Parkville, Australia).

2.8.6.2 Peptide conjugation to a carrier protein

Each custom peptide was conjugated to a carrier protein (Imject Maleimide Activated mcKLH), obtained from Pierce. Conjugation was carried out as per the manufacturer's instructions. Briefly, 2 mg of peptide was reconstituted in 500 µl of PBS (Appendix 1) and the Maleimide Activated mcKLH was reconstituted at 10 mg/ml in the solution provided. Peptide (2 mg) was conjugated to 2 mg of carrier protein by mixing the two solutions and leaving them at room temperature for 2 hours.

2.8.6.3 Purification of peptide-carrier protein conjugates

A precipitate was formed during the conjugation reaction, as some of the mcKLH-peptide conjugate came out of solution. This precipitate was isolated by centrifugation. An Amicon centricon YM-100 from Millipore was used to remove EDTA from the buffer containing the rest of the conjugated protein. The samples were centrifuged at 1000*g* until 200 µl of retentate was left. The retentate was combined with the precipitated conjugate, then diluted to 2ml with PBS.

2.8.6.4 Inoculation of rabbits

All animal handling was carried out by the staff at the University of Adelaide Central Animal House, Waite Campus. Two 12 week old lop-eared rabbits were used to generate antisera, one for each peptide-carrier conjugate. Each inoculum was prepared with 500 µl of conjugate in PBS emulsified in 500 µl of Freund's complete adjuvant. The rabbits received intramuscular injections with boosts at 2, 6 and 10 weeks post primary injection. A terminal bleed of each rabbit was obtained 12 weeks post primary injection.

2.8.6.5 Serum preparation from terminal bleed

After collection, the blood was allowed to clot for 1 hour at 37°C. The clot was separated from the walls of the collection tube using a pasteur pipette. The serum sample was placed at 4°C overnight to allow the clot to contract. The serum was then isolated and centrifuged at 10 000*g* for 10 min to remove contaminating solid material.

2.8.6.6 Purification of peptide-specific antibodies

The immunoglobulin G fraction was purified from crude rabbit serum by ammonium sulphate precipitation as described in Ausubel *et al.* (1994). Briefly, one volume of saturated ammonium sulphate solution (Appendix 1) was added to two volumes of sera, with constant mixing at 4°C. A precipitate was allowed to form over 4 hours at 4°C. The precipitate was collected by centrifugation at 12000*g* for 20 min. The precipitate was then washed by vortexing in two volumes of cold 33% saturated ammonium sulfate. The pellet was resuspended in 1 ml of cold PBS. A SulfoLink Kit (Pierce) was used to affinity purify the antisera according to the manufacturer's instructions. The purified antisera were stored at –20°C.

Chapter 3 Preliminary study: construction of mutants capable of proline transport

3.1 Introduction

Saccharomyces cerevisiae utilises only small amounts of proline, if any, during oenological fermentation (Ough and Stashak, 1974; Ingledew *et al.*, 1987). Proline initially is excluded from the yeast cell because the presence of preferred nitrogen sources, such as ammonia and glutamine, inhibits proline transport (Duteurtre *et al.*, 1971; Woodward and Cirillo, 1977; Horak and Rihova, 1982). After the depletion of these preferred nitrogen sources, proline can gain access to the cell, but it is not catabolised as the oxygen has been depleted. Oxygen is required for the function of proline oxidase, the first enzyme in the proline catabolic pathway (Duteurtre *et al.*, 1971), and is encoded by *PUT1* (Wang and Brandriss, 1986). There is little data to indicate how much oxygen is dissolved in must and the concentration will depend on how the juice is handled. However, a significant concentration of oxygen may be present and, as such, a constitutive proline-transporting strain could not only transport proline, but catabolise it when oxygen is, in fact, present.

The purpose of this initial stage of work was i) to construct and exploit strains constitutively expressing *PUT4*, the high affinity proline permease, as a means of quantifying the amount of proline utilisation that might be achieved in the pre-anaerobic phase of fermentation and ii) to obtain an indication of the amount of oxygen actually present in grape juice fermentations. In recognition of the multi-factorial regulation of *PUT4* expression, two strategies were adopted as a means of producing constitutive *PUT4* strains.

Proline uptake is mediated by two transporters (Horak and Rihova, 1982; Regenberg *et al.*, 1999), a proline specific permease of high affinity, Put4p, and a general amino acid permease with low affinity for proline, Gap1p. The expression of both *PUT4* and *GAP1* is controlled by a complex interplay of regulators. The Ure2p repressor blocks expression of a number of nitrogen assimilatory genes, including *PUT4* and *GAP1*, during growth in the presence of preferred nitrogen sources (Courchesne and Magasanik, 1988; Coschigano and Magasanik, 1991; Coffman *et al.*, 1994; Stanbrough

and Magasanik, 1995; Xu *et al.*, 1995). Deletion of the *URE2* gene results in upregulation of these nitrogen assimilatory genes under normally repressive conditions (Courchesne and Magasanik, 1988). As such, one approach that was used to produce a strain constitutively expressing the proline transport proteins was to delete the *URE2* gene. However, the upregulation of *GAP1*, in addition to *PUT4*, has potentially detrimental pleiotropic effects. Specifically, amino acids transported by Gap1p bring about substrate induction of their own catabolic pathways, which may lead to inefficient use of nitrogen. Therefore, to avoid any possible complications of a *ure2Δ* strain, a second strain was constructed in which *PUT4* was placed under the control of a constitutive promoter. In this way, constitutive expression was limited to *PUT4*.

3.2 Selection of strains

Industrial wine yeast strains are not easily manipulated at a genetic level. Typically, they are unstable as haploids and do not contain convenient genetic markers. For this reason, a well-characterised laboratory yeast, W303, was selected for use in this study. Although not as efficient as industrial strains in the fermentation of high concentrations of sugar, W303 is still capable of completely fermenting a medium containing 180g/L of glucose (data not shown) and consequently is appropriate for use in this study.

W303 (*ade2-1 can1-100 his3-11,-15 leu2-3,-112 trp1-1 ura2-1*) was obtained from Susan Henry. A strain with no markers for use as a control was prepared by crossing W303**a** with a lys-2 marker yeast before sporulation, dissection and selection of a prototrophic haploid. This strain was then back-crossed against a W303-derivative VJ23 (*his3-11,-15 leu2-3-112 MAT***a**) five times to reconstitute the W303 genetic background (Table 2.1).

3.3 Selection of nitrogen sources

The *PUT4* permease gene is subject to nitrogen catabolite repression (Grenson 1983a). Published data show that *PUT4* expression is repressed in the presence of glutamine or ammonium (Jauniaux *et al.*, 1987). The derepression of *PUT4* occurs when cells are grown on poor nitrogen sources, for example proline (Jauniaux *et al.*, 1987). Proline does not act as an inducer (Lasko and Brandriss, 1981), but proline is poor as a sole nitrogen source, thereby resulting in an upregulation of all the genes subject to nitrogen

catabolite repression. Given this information, it was decided that high concentrations of ammonium sulphate (5 g/L) would be used as the preferred nitrogen source, to cause nitrogen catabolite repression. To generate non-repressive conditions, proline was used as sole nitrogen source (starting concentration 5 g/L).

3.4 Construction of constitutive *PUT4* strains

3.4.1 Deletion of *URE2*

The *URE2* gene was deleted using the gene-replacement strategy reported by Wach and colleagues (Wach *et al.*, 1994; Wach *et al.*, 1997), utilising the *kanMX4* selection marker. The *kanMX4* marker contains the kanamycin resistance gene *kan*^{r} from transposon Tn903 (Oka *et al.*, 1981), flanked by the *Ashbya gossypii TEF* gene promoter and terminator (Steiner and Philippsen, 1994), and confers resistance to the broad-spectrum antibiotic, geneticin. A PCR product was generated using primers urekanf and urekanr (Table 2.2). This PCR product corresponds to the *kanMX4* module flanked by >30 bp of sequence homologous to sequences flanking the *URE2* gene (-33 to 6 and 1034 to 1073). Strain KP2 was transformed with this PCR fragment and transformants were selected on YEPD containing 400 mg/L of geneticin. Geneticin-resistant colonies were screened for *ure2Δ* using primers Ure2f and Ure2r (Table 2.2). The isolated *ure2Δ* strain was designated KP20.

3.4.2 Cloning of *PUT4* under the control of a constitutive promoter

Plasmid-borne *PUT4*, under the control of a constitutive promoter, was introduced into a *put4*Δ background to generate a strain in which *PUT4* could be expressed in the presence of repressive nitrogen sources. The *PUT4* gene was cloned into a centromeric vector downstream of the *S. cerevisiae TEF2* promoter. The cloning of the *PUT4* gene into this vector by traditional restriction enzyme digestion and ligation proved difficult. Consequently, an alternative approach to plasmid construction was used. The *PUT4* gene was amplified using primers TEFPUTf and Put4r (Table 2.2). The pTEF-PUT4 plasmid (Appendix 2) was then constructed by gap-repair (Ma *et al.*, 1987). The pTEF-PUT4 plasmid was generated in a *put4Δ::kanMX4 ura3Δ::kanMX4* haploid yeast (strain KP12) to create strain KP21 (Table 2.1). Given the difficulties encountered in attempting to construct the plasmid by conventional cloning methods, some of the

following experiments were variously conducted with KP20 and/or KP21, depending on when these strains became available.

3.5 Results

3.5.1 Analysis of *PUT4* expression in response to the quality of available nitrogen

The presence of ammonium ions in culture media leads to the repression of *PUT4* expression, mediated in part by the *URE2* gene product (Xu *et al.*, 1995). Northern blot analysis was used to confirm that the deletion of the *URE2* gene leads to upregulated *PUT4* expression in the presence of a preferred nitrogen source. A DIG-labelled DNA probe amplified using primers P4probeF and P4probeR (Table 2.3), corresponding to the *PUT4* coding region, was used to detect *PUT4* RNA. The probe was confirmed to detect a band of the correct molecular weight (approximately 2 kb) by Northern blot analysis of electrophoretically resolved total cellular RNA (data not shown). A second, larger band was also detected in proline-grown wild type cells that was not present in a *put4*Δ strain. This band was inferred to correspond to ribosomally-associated *PUT4* RNA. The specificity of the probe allowed further analysis of *PUT4* expression to be conducted by Northern slot blot analysis.

In the case of the wild type strain, KP2, the expected pattern of *PUT4* expression was observed. That is, *PUT4* RNA was detected after growth in media containing proline as the sole source of nitrogen (Figure 3.1). After extended development of the blot, a faint signal was also detected in cells grown in the presence of ammonium. To confirm that this faint band was indeed low-level *PUT4* expression, a *put4*Δ negative control (KP10) was included. No band was detected in the RNA prepared from this negative control strain after growth under either repressive or derepressive conditions. Analysis of the *ure2*Δ strain, KP20, showed that *PUT4* is expressed under both repressive and derepressive conditions. As previously shown in the literature, this highlights the role of Ure2p in the control of *PUT4* expression (Jauniaux *et al.*, 1987). The constitutive *PUT4* strain, KP21, also contained *PUT4* RNA, regardless of the nitrogen source, as expected for a gene under the control of the constitutive *TEF* promoter.

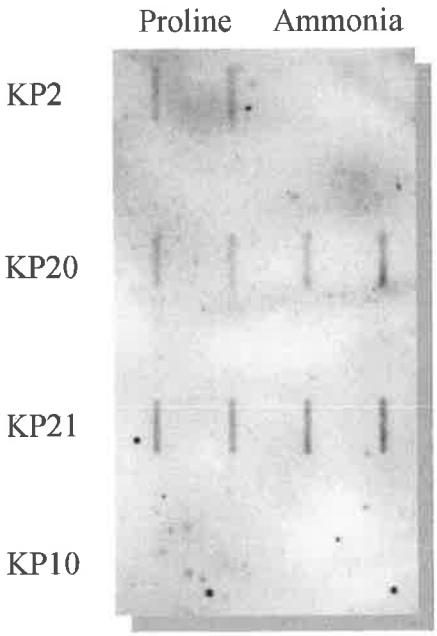


Figure 3.1 Northern slot-blot analysis of *PUT4* expression

Strains were grown in media containing either proline or ammonium as the sole source of nitrogen. Cells were harvested during exponential phase (5×10^7 cells/ml) and total cellular RNA was extracted and analysed, in duplicate, by Northern slot-blot analysis. *PUT4* mRNA was detected with a DIG-labelled DNA probe corresponding to the *PUT4* gene.

3.5.2 Investigation of Put4p activity in constitutive *PUT4* strains

The presence of preferred nitrogen sources not only leads to the repression of *PUT4* expression, but also to the inactivation of the Put4 permease (Grenson, 1983a). Therefore, to determine whether *PUT4* expression equates to Put4p activity, direct determination of Put4p transport activity was done. An isotopic Put4p assay (Lasko and Brandriss, 1981) was used to determine whether functional Put4p was present when strains constitutively expressing *PUT4* were grown in the presence of ammonia. In this assay, the proline was added to a final concentration of 0.2 mM, as described in Lasko and Brandriss (1981), to exceed the K*m* of Put4p (0.025 mM), while falling well below that of Gap1p (13 mM), ensuring transport via Put4p and not Gap1p.

Previously published studies have shown that Put4p activity is not responsive solely to the quality of available nitrogen sources, but that it also decreases with progression through the growth phase (Horak and Rihova, 1982). Accordingly, Put4p activity was assayed at three points during growth in KP2, KP20 and KP21 cultures to i) establish whether *PUT4* expression leads to Put4p activity in the presence of ammonium and ii) investigate whether the reported growth phase-dependent decrease in Put4p activity is affected by the constitutive expression of *PUT4*. Strains were grown in parallel cultures in YNB containing either proline as the sole source of nitrogen, or ammonia and proline as nitrogen sources. Cells were harvested at the early, mid and late exponential phases of growth and assayed for Put4p activity using ^{14}C-proline.

When KP2 was grown in YNB containing proline as the nitrogen source, the Put4p activity was highest during the initial stages of exponential growth (19 nmol proline/min/mg cells) and decreased over the course of the growth phase to become undetectable in the late exponential phase (Figure 3.2A), in agreement with a previous report (Horak and Rihova, 1982). The KP20 and KP21 strains displayed higher rates of proline uptake (initially 33 and 75 nmol proline/min/mg cells respectively) Nevertheless, Put4p activity also decreased with progression through the growth phase. At all three sample points during the exponential phase, Put4p activity was highest in KP21 and lowest in the wild type (Figure 3.2A).

Put4p activity was also higher in KP20 and KP21 cultures grown in the presence of ammonium (Figure 3.2B). However, the activity was lower than that observed in the

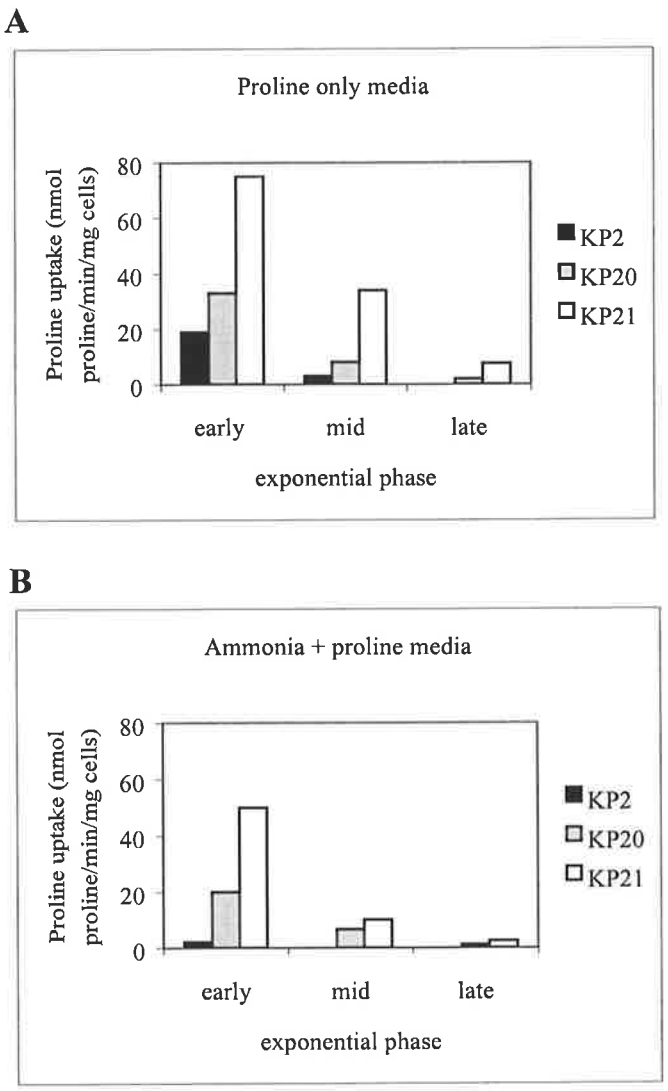


Figure 3.2 Put4p permease activity at different points of the growth phase

Cultures of KP2, KP20 and KP21 were grown in media containing either proline alone (A) or ammonia and proline (B) as nitrogen sources. Cells were harvested at three points during growth: early (1 x 10^7 cells/ml), mid (5 x 10^7 cells/ml) and late (1x 10^8 cells/ml) exponential phase, and assayed for Put4p activity using an isotopic assay. Values are the mean of duplicate measurements from duplicate cultures.

strains growing in proline, an expected consequence of the proposed post-translational inactivation of Put4p that has been reported to occur during growth on a preferred nitrogen source (Grenson, 1983b). Interestingly, the KP2 culture exhibited a low level of Put4p activity (2 nmol proline/min/mg cells) in the presence of ammonium, at the onset of the exponential phase, before becoming undetectable. This activity may be a reflection of the limited *PUT4* transcription observed when KP2 is grown under similarly repressive conditions (Figure 3.1). Use of the *put4Δ* strain, KP10, confirmed that, after growth in media containing either proline or ammonium as sole nitrogen source, radio-labelled proline was not transported via the Gap1 permease under the conditions of the assay (data not shown).

3.5.3 Removal of proline from chemically defined grape juice media under model oenological conditions

Model fermentations were utilised to investigate whether increased Put4p activity in KP20 and KP21 leads to the removal of proline from a chemically defined grape juice medium (CDGJM). Duplicate 1L aliquots of CDGJM containing 5 g/L of ammonium sulphate and 200 mg/L of proline were saturated with air, inoculated with 5 x 10^6 cells/ml and monitored for the first 30 hours of fermentation. The proline concentrations of the samples were determined using the isatin method (Section 2.3.3.5). Data obtained from this experiment revealed that a limited amount of proline was removed from the media by KP2 (23 mg/L) and KP20 (24 mg/L). The KP21 strain removed 55 mg/L of proline (Figure 3.3A). The removal of proline by the KP2 and KP20 strains was limited to the initial 6 hours of fermentation, whereas removal of proline from the media by KP21 lasted 18 hours. The culture density was also monitored during this experiment. Strains KP21 and KP2 had similar growth kinetics and attained densities of ca. 1200 NTU. In contrast, the *ure2Δ* strain, KP20, grew markedly slower during the first 30 hours of fermentation and only attained a density of ca. 200 NTU (Figure 3.3B).

3.5.4 Depletion of oxygen from a chemically defined grape juice medium

Proline utilisation by *S. cerevisiae* is not dependent solely on transport of proline into the cell. Proline acts as an inducer of the genes involved in the proline catabolic pathway, *PUT1* and *PUT2* (Brandriss and Magasanik, 1979; Wang and Brandriss, 1987; Brandriss, 1983). However, the activity of proline oxidase (encoded by *PUT1*) is

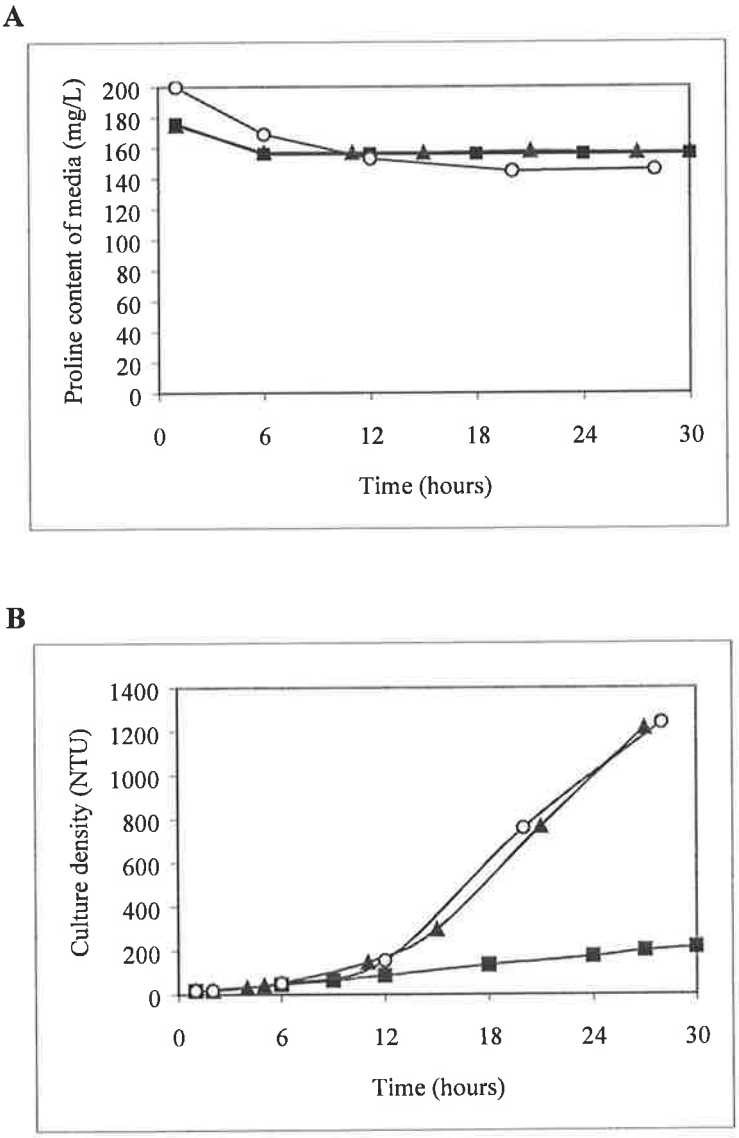


Figure 3.3 Proline removal from Chemically Defined Grape Juice Medium

Chemically Defined Grape Juice Medium was inoculated with either KP2 (▲), KP20 (■) or KP21 (○) at 5 x 10^6 cells/ml and the ferment was monitored for 30 hours for residual proline concentration in the medium (A) and cell density (B). Results represent the average from duplicate ferments. The experiment involving KP21 (○) was conducted on a separate occasion.

dependent on the presence of oxygen and a functional electron transport chain (Duteurtre *et al.*, 1971). Consequently, the amount of proline catabolised will be determined by the amount of oxygen available during fermentation.

As stated, little data has been reported on the kinetics of oxygen availability during oenological fermentation. Therefore, the removal of oxygen from air-saturated CDGJM was monitored to determine the maximal amount of oxygen that potentially could be available during the initial stages of fermentation. Duplicate 1L aliquots of CDGJM were inoculated with KP2 or KP20 at 5 x 10^6 cells/ml in 2L fermentation vessels (Braun Biostat-B), which were then sealed to preclude the further introduction of oxygen. The dissolved oxygen concentrations of the inoculated media were monitored by means of an in-place dissolved oxygen probe. Oxygen was depleted from the media within 6 and 7 hours post inoculation for strains KP2 and KP20 respectively. Both time points of depletion preceded the onset of the exponential phase of growth (Figure 3.4A). Again large differences again were seen in the growth kinetics of the *ure2*Δ strains in comparison to the wild type (Figure 3.4B).

3.5.5 Oxygen availability during oenological fermentation

Grape must is generally not saturated with air at inoculation. New World wine makers, in particular, actively exclude the introduction of oxygen to white grape must, and the addition of sulphur dioxide is recommended in an effort to inhibit the enzymatically induced oxidation of white must and wine. Red wine is not as susceptible to oxidation, due to its high phenol content. As such, oxygen is not as actively excluded from coming into contact with red must. Recently, hyperoxidation has been suggested as an alternative approach to the handling of white must (see review by Schneider, 1998). Hyperoxidation of white must involves the omission of sulphur dioxide and the introduction of oxygen to the must before inoculation. This leads to the formation of insoluble brown pigments due to the precipitation of phenolic compounds. These insoluble complexes can be removed before inoculation by traditional clarification processes. The hyperoxidation of must produces wines which are more colour stable, since the resulting wines have a reduced capacity for oxygen consumption (Schneider, 1998).

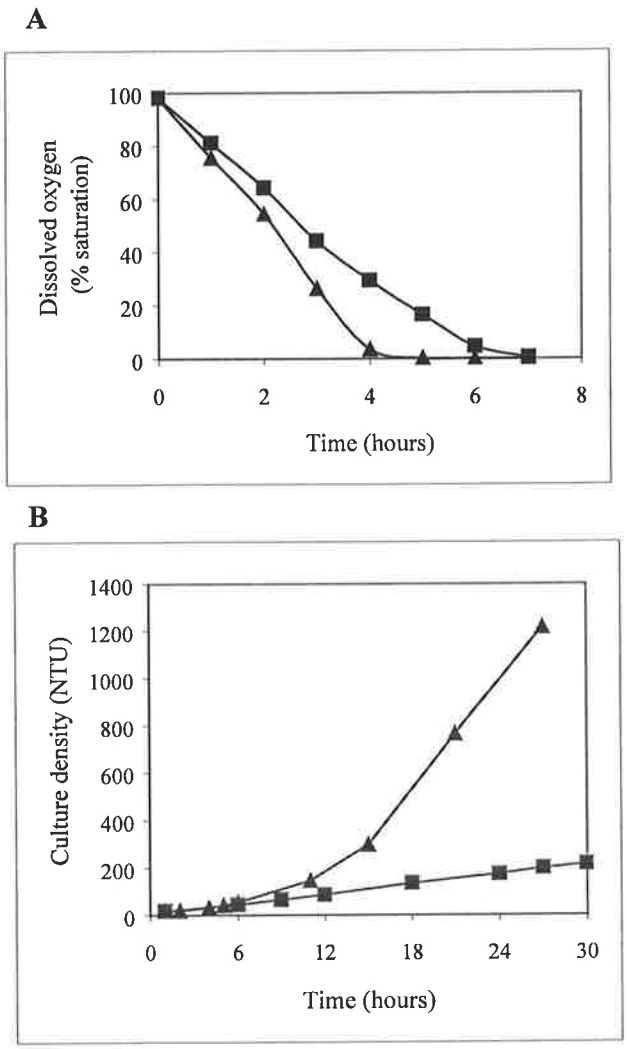


Figure 3.4 Oxygen removal from air-saturated Chemically Defined Grape Juice Medium after inoculation.

Chemically Defined Grape Juice Medium was saturated with air before inoculation with either KP2 (▲) or KP20 (■) at 5 x 10^6 cells/ml. Dissolved oxygen concentration (A) was monitored using an in-place dissolved oxygen probe, and culture density (B) was monitored for the initial 30 hours of fermentation. Results are the average of duplicates.

To gain insight into the amount of oxygen that may be available at the time of must inoculation, a cursory survey involving 10 small-scale fermentations was conducted. A dissolved oxygen probe was used to monitor the oxygen content of juices from the point of inoculation until oxygen was no longer detectable (Table 3.1).

The dissolved oxygen content varied between the different musts, as would be expected due to differences in grape variety, juice handling, temperature and the yeast utilised for fermentation. However, despite these differences, some general statements may be made. Dissolved oxygen content in the four white musts handled reductively was less than 1% of saturation at the time of inoculation. Where non-reductive handling was used, the oxygen that was present (<42% saturation) was depleted from all the ferments within 4 hours of inoculation.

What can be established from this small survey is that the available oxygen in the initial stages of fermentation is limited and is depleted rapidly after inoculation, reflecting the results obtained for oxygen saturation in the model ferments.

3.5.6 Comparative growth kinetics and nitrogen utilisation of KP2, KP20 and KP21

The deletion of the Ure2 repressor is expected to have pleiotropic consequences, some of which may impact on the growth kinetics and nitrogen utilisation of the yeast (Courchesne and Magasanik, 1988; Coschigano and Magasanik, 1991). Growth curves of triplicate KP2, KP20 and KP21 cultures were plotted to determine whether the deletion of *URE2* or the constitutive expression of *PUT4* had a negative impact on growth. YNB containing ammonia as the sole source of nitrogen was inoculated at 5 x 10^6 cells/ml and incubated at 30°C. The cell density of the cultures was measured at numerous points during growth. Again, as shown earlier (Figure 3.3B; Figure 3.4B), KP20 had a longer lag phase than KP2, but, upon extended incubation, appeared to form a higher final biomass. KP21 had similar growth kinetics to KP2 (Figure 3.5A).

To evaluate the impact of constitutive *PUT4* expression on overall nitrogen consumption, model fermentations were conducted in triplicate in CDGJM containing 180 g/L glucose as the carbon source and 5 g/L of ammonium chloride as the source of nitrogen. In order to mimic oenological conditions, the medium was sparged with

Table 3.1 A survey of oxygen availability during the initial stages of grape juice fermentation

Type of must	Dissolved oxygen at inoculation (%)	Hours to depletion
red[a]	40	4.00
red[a]	36	3.75
red[a]	47	4.25
red[a]	23	2.75
red[a]	33	3.50
white[a]	42	4.25
white[b]	<1	NA
white[b]	<1	NA
white[b]	<1	NA
white[b]	<1	NA

500 L musts were prepared from various sources of fruit, inoculated with various yeasts and fermented under typical industrial conditions. A dissolved oxygen probe was used to quantify the initial oxygen concentration and to follow its disappearance after inoculation.

[a] Juices were handled oxidatively , i.e. CO_2 gas was not used to protect the must from oxygen.

[b] Juices were handled reductively, i.e. oxygen was excluded by protecting the must with a blanket of CO_2 gas.

NA: not applicable

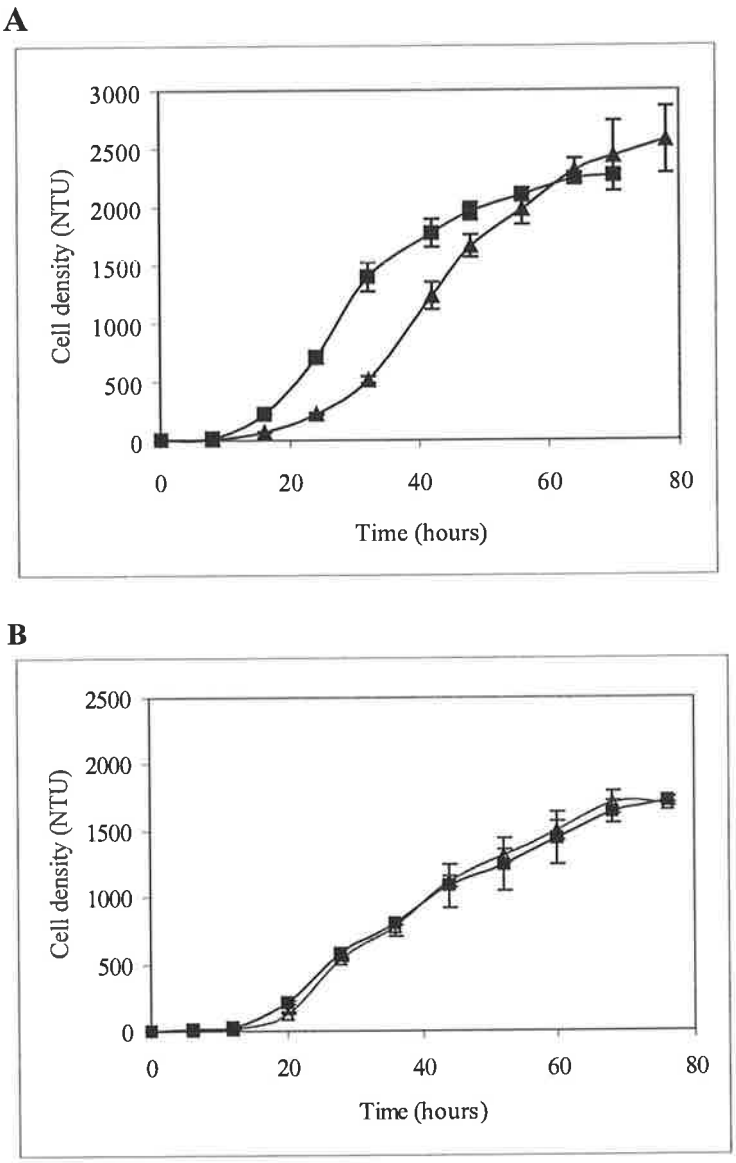


Figure 3.5 Comparative growth of strains KP2, KP20 and KP21 under model oenological conditions.

A comparison of KP20 (▲) and KP2 (■) (Panel A) and of KP21 (Δ) and KP2 (■) (Panel B), performed on separate occasions. Cultures were grown in chemically defined grape juice medium, containing ammonium as the sole source of nitrogen, until the depletion of glucose (initial concentration 180 g/L). Culture density was measured in 10 cm light path tubes with a turbidimeter. Values are the mean of three determinations (± standard error).

nitrogen before inoculation to generate near anaerobic conditions. Flasks were fitted with fermentation locks to exclude air, while allowing the release of fermentation gases. The defined medium was inoculated with either KP2, KP20 or KP21 at 5 x 10^6 cells/ml. Once glucose was depleted, samples were taken for the determination of residual ammonium, using an ammonium probe (Table 3.2).

The KP20 culture used significantly more nitrogen (407 ± 2.5 mgN) than both the KP21 strain (393 ± 4.9 mgN) and the wild type control (376 ± 3.0 mgN). The nitrogen consumed by KP21 was significantly higher than the wild type, yet this increased nitrogen consumption was still significantly less than for KP20.

3.6 Conclusions

The deletion of the *URE2* gene leads to the expression of *PUT4* in the presence of ammonium. Similarly, cloning of the *PUT4* coding sequence downstream of the *TEF* promoter leads to non-repressible *PUT4* expression.

In both cases (KP20 and KP21), constitutive *PUT4* expression results in increased Put4p activity. Like the wild type strain, however, this activity decreases over the course of the growth phase. In each strain, Put4p activity was lower in the culture grown in ammonium. However, Put4p activity of KP20 and KP21 relative to KP2 was enhanced in cells grown in ammonia, being as much as 10 and 25 times higher respectively, compared with 1.7 and 4 times higher in proline respectively.

The inoculation of air-saturated media leads to the depletion of oxygen within 7 hours. For a limited sample of small-scale fermentations of commercially-handled must, initial dissolved oxygen concentrations ranged from <1% to 42% saturation, depending on juice handling and fermentation conditions. Again, inoculation led to the rapid depletion of oxygen, in this case within 4 hours.

Proline was observed to be used by the constitutive *PUT4* strains; however, the deletion of *URE2* leads to an increased overall nitrogen utilisation, slower initial growth and increased biomass formation. Increased nitrogen utilisation is also observed for a strain constitutively expressing *PUT4* (KP21), yet this increased nitrogen utilisation is not as

Table 3.2 Nitrogen utilisation by proline transport capable strains

Strain	Nitrogen utilised (mg)
KP2	376 (± 3.0)[a]
KP20	407 (± 2.5)[a,b]
KP21	393 (± 4.9)[a,b]

Strains were cultured in triplicate in CDGJM containing ammonium as the sole source of nitrogen and 180 g/L of glucose. Upon depletion of the glucose, samples were taken and the amount of ammonium removed from the medium was calculated. Values marked with the same superscript were significantly different from each other as determined by a Student t-test.

high as for the *ure2Δ* strain. Accordingly, a *ure2Δ* strain is unlikely to be suitable for industrial use.

3.7 Discussion

Whilst this preliminary study was being completed, Salmon and Barre (1998) published findings on the fermentative properties of a *ure2Δ* strain (UV9). They found that UV9 exhibited an increase in proline uptake via Gap1p and Put4p under both repressive and non-repressive conditions. A slower rate of growth and higher final biomass were also observed, along with an increased removal of proline from synthetic media containing a complex amino acid mixture. Analysis of the amino acid content of the synthetic media at the end of fermentation showed that UV9 utilised significantly more nitrogen than the parental strain. All of these results are in accordance with information derived from the preliminary study undertaken here. However, it is difficult to make direct comparisons between the work conducted by Salmon and Barre (1998) and this study, as there were differences in experimental design, strain background and measured parameters.

Salmon and Barre (1998) conducted experiments in media containing a complex amino acid mix, whereas, in this study, the focus was on expression of the *PUT4* gene and the activity of Put4p under repressive conditions, generated by the presence of ammonium alone. In media containing 300 mgN/L, Salmon and Barre (1998) found that UV9 had a final biomass of 5.2 g dry weight/L, compared with 3.4 g dry weight/L for the parental strain. Biomass formation and fermentation rate are related to the initial nitrogen concentration, as increased nitrogen allows the formation of greater biomass as well as positively affecting the rate of sugar utilisation (Bisson, 1991). In addition, previous studies have shown increased biomass formation when cells are grown on complex amino acid mixtures (1.75 ± 0.09 C-mol/mol glucose), compared with either glutamate (1.53 ± 0.06 C-mol/mol glucose) or ammonium (1.48 ± 0.05 C-mol/mol glucose) as the sole source of nitrogen (Albers *et al.*, 1996), where 1 C-mol is the amount of compound containing 1 mol of carbon. It also has been shown that biomass formation from glutamate (0.99 ± 0.06 C-mol/C-mol) is greater than the contribution of glucose to biomass formation (0.1 ± 0.005 C-mol/C-mol), reflecting the utilisation of carbon skeletons from nitrogenous compounds (Albers *et al.*, 1998). The access of a *ure2Δ* strain to additional nitrogen sources, as well as to additional carbon sources, during the

initial stages of fermentation may explain the increase in biomass formation and the increased fermentation rate described by Salmon and Barre (1998). In this study, a *ure2*Δ strain also achieved a higher final culture density than the wild type parental strain in a medium containing ammonium as the sole source of nitrogen. This can be attributed to the derepression of the *GDH1* gene encoding the NADPH-GDH enzyme, which converts ammonium to glutamate. *GDH1* is a nitrogen catabolite-repressed gene (Daugherty *et al.*, 1993; Ter Schure *et al.*, 1998) that would be upregulated as a consequence of the deletion of the *URE2* gene. An increased concentration of glutamate would, therefore, be available for biosynthetic reactions within the yeast cell.

Salmon and Barre (1998) suggest that a *ure2*Δ strain may have potential industrial benefits, citing the increased assimilation of amino acids and the increase in fermentation rate observed for UV9, when grown in media containing 300 mgN/L. When strains were grown in nitrogen-limited media (80 mgN/L), the fermentation rates of UV9 and the wild type did not differ. An increase in biomass formation was still observed (2.5 g dry weight/litre for UV9 compared with 2.3g dry weight/litre for the parental) (Salmon and Barre, 1998). At this low concentration, nitrogen is presumed to be limiting and, as such, the increased nitrogen assimilation observed for UV9 is insufficient to stimulate an increased fermentation rate, although the increased biomass formation is presumably a result of an influx of amino acids at the onset of fermentation. The preliminary study conducted here did not measure the rate of fermentation; however, no significant difference was noted between the time required for the complete fermentation of 180 g/L of glucose by the wild type as compared to the *ure2*Δ strain. Presumably, this difference between the results obtained here and the work of Salmon and Barre (1998) is a reflection of the difference in the culture media used.

The modification of strains to utilise alternate nitrogen sources is of little value if the overall nitrogen requirement of the resulting strains is increased. Under conditions in which nitrogen is in excess, there is little need for the yeast to assimilate poor nitrogen sources, while under nitrogen-limiting conditions, an increased nitrogen requirement may lead to additional fermentative problems instead of alleviating them. In the light of

this, the conclusion drawn here is that *ure2Δ* strains are unlikely to have great industrial relevance.

The original aims of this study were to generate strains capable of proline transport and to investigate their potential to utilise proline during the initial, aerobic stages of fermentation. It can be seen from this study that manipulation of the expression of *PUT4* leads to proline transport in the initial stages of growth. However, oxygen is depleted rapidly. Proline removal from the medium during growth under model oenological conditions was also limited to the initial stages of fermentation. The anaerobic conditions that persist for the rest of the fermentation preclude any further utilisation of proline. Proline, however, may still be directly incorporated during protein synthesis. The proteinaceous fraction of the yeast cell has been reported to be 10-30% of dry cell weight (Salmon, 1989), of which proline corresponds to 35 + 4 mg/g protein (Albers *et al.*, 1996). Given the example of a final biomass of 3.4 g dry weight/L, as reported for V5 (Salmon and Barre, 1998), a maximum of 35 mg of proline would be required for *de novo* protein synthesis. The accumulation of proline for direct incorporation may, therefore, represent some advantage for a constitutive *PUT4* strain. Obviously, a functional proline oxidase is needed to fully exploit proline as a nitrogen source - the key to any significant benefit of such yeasts. For this reason, oxygenation during oenological fermentation is likely to be required to maximise the potential of these strains capable of proline transport to utilise proline.

The use of oxygen during fermentation is not widespread in Australia. Oxidation reactions in wine can be detrimental to the quality of the finished product, particularly for white wine. However, metabolically-active yeast can protect the fermenting must from detrimental oxidation reactions (Cheynier *et al.*, 1997). Therefore, the controlled addition of oxygen while yeast is fermenting actively is, theoretically, a realistic industrial practice.

The addition of oxygen during oenological fermentation has been shown to increase the maximal fermentation rate and to decrease the length of fermentation (Sablayrolles and Barre, 1986). The timing of oxygen additions is critical. Additions that are made too early during fermentation can lead to increased biomass and excessive nitrogen

utilisation and concomitant fermentation problems resulting from insufficient nitrogen. Oxygen additions that are made too late during fermentation have little positive effect. The optimal time for the addition of oxygen is shortly after the maximal fermentation rate has been reached (Sablayrolles and Barre, 1986). It also has been found that combined additions of oxygen and nitrogen are more beneficial during oenological fermentation than additions of either nitrogen or oxygen alone (Sablayrolles, 1996). Therefore, the use of micro-oxygenation to increase proline utilisation may lead to a similar improvement in fermentation parameters.

The expression of *PUT4* in the presence of ammonium leads to Put4p activity during the initial stages of fermentation. It was noted, however, that Put4p activity decreases over the course of the growth phase. The addition of oxygen at the onset of the stationary phase would not result in proline utilisation if the continued presence of a preferred nitrogen source resulted in the post-translational inactivation of Put4p. Therefore, before the benefits of oxygen additions can be evaluated, such inactivation must be eliminated or reduced. This highlights the need to investigate the ammonium-induced down-regulation of Put4p as part of an effort to generate strains in which active Put4p is present at a point in the growth phase at which oxygen additions would have the greatest potential benefit for fermentation.

Chapter 4 Generation of *PUT4* mutants no longer responsive to nitrogen catabolite repression

4.1 Introduction

The constitutive expression of *PUT4* leads to Put4p activity during the initial, aerobic stages of fermentation (Chapter 3). Such a strain capable of proline transport may prove beneficial for oenological fermentation, since it has the potential to utilise proline as an additional source of nitrogen. The development of a proline-utilising strain for industrial use must take into consideration the methodology used to generate the desired phenotype. The use of genetically modified organisms in food and beverage production is not, as yet, a socially acceptable practice. There also are strict guidelines in Australia, and around the world, which restrict the types of genetically modified organisms that may be utilised industrially. In order to generate strains that are more likely to be accepted, it was decided that genetic modifications must be reduced to a minimum.

The preliminary study described in Chapter 3 revealed that expression of *PUT4* from a constitutive promoter produced the desired phenotype (Put4p activity in the presence of preferred nitrogen sources) without the undesirable, pleiotropic consequences seen in the *ure2*Δ strain. The constitutive expression of *PUT4* was achieved by cloning the *PUT4* gene downstream of the promoter of the *TEF2* gene. The aim of this section of work therefore was to produce the same outcome with fewer modifications. Specifically, it was to bring about constitutive *PUT4* expression by mutation of the native *PUT4* promoter sequence in such a way that *PUT4* expression is not repressed in the presence of preferred nitrogen sources.

The control of *PUT4* expression is complex. *PUT4* is subject to nitrogen catabolite repression involving multiple regulatory factors (Jauniaux *et al.*, 1987; Daugherty *et al.*, 1993). The nitrogen catabolite repressors, Ure2p and Dal80p, are involved in the regulation of a number of genes. The preliminary study showed that the deletion of such a regulator, Ure2p, affected nitrogen utilisation and the growth of the resulting strain (KP20), as well as resulting in the up-regulation of *PUT4*.

Ure2p represses gene expression by binding to the Gln3 activator and localising it to the cytoplasm (Beck and Hall, 1999; Kulkarni *et al.*, 2001), in such a manner that Gln3p is not available for the transcriptional activation of nitrogen catabolite-responsive genes. As such, there is no readily identifiable means of modifying a strain so that this repression can be eliminated for *PUT4* alone. The second repressor involved in the nitrogen catabolite repression of *PUT4*, Dal80p (Cunningham and Cooper, 1992), is a GATA factor. GATA factors are DNA-binding proteins that bind to sequences with a GATA motif at their core. The activators that mediate *PUT4* expression on poor nitrogen sources, Gln3p and Nil1p, are also GATA factors (Minehart and Magasanik, 1991; Stanbrough *et al.*, 1995; Cunningham *et al.*, 1996). The recognition sequences of the GATA factors are similar and in the *GAP1* promoter, the Gln3p activator binds to the same GATAAG sequences as Nil1p (Stanbrough and Magasanik, 1996). In the case of other genes, the activator, Gln3p, and the repressor, Dal80p, bind to the same sequences (Cunningham *et al.*, 1994). Consequently, the duality of function of the GATA sequences complicates their being targeted for modification with the specific goal of producing a constitutive *PUT4* strain.

The SCPD promoter database (Zhu and Zhang, 1999) was used to search the *PUT4* promoter region for consensus-binding sequences of other transcriptional regulators. No regulatory sequences have been mapped in the *PUT4* promoter region, the binding sites of the GATA regulatory proteins included (T. Cooper, personal communication). Based on previously identified consensus sequences, however, a number of putative regulatory factor binding sites were predicted within the *PUT4* promoter region (Table 4.1). However, none of these regulatory sequences suggest a possible mutation strategy to upregulate *PUT4* expression. As such, random mutagenesis of the *PUT4* promoter was used in an attempt to generate strains capable of *PUT4* expression in the presence of ammonia.

4.2 Random mutagenesis of the *PUT4* promoter region

Mutagenesis of the *PUT4* promoter region was conducted in an effort to generate strains in which *PUT4* expression was no longer repressed by the presence of a preferred nitrogen source. Error-prone PCR was utilised to introduce random mutations. A DNA polymerase lacking proof-reading activity (DyNAzyme II, Finnzymes) was used in the amplification reaction. The error rate of DNA polymerase can be increased during PCR

Table 4.1 Putative regulatory sequences in the *PUT4* 5' untranslated region

Strand	Position	Regulatory factor	Consensus binding sequence
+	(-61,-56)	GCN4	TGAATA
-	(-76,-71)	GCN4	TGATTC
+	(-82,-74)	HSTF	TTCGAAGAA
+	(-109,-105)	GCR1	CTTCC
+	(-114,-110)	GCR1	CTTCC
+	(-137,-132)	UASPHR	CTTCCT
+	(-137,-133)	GCR1	CTTCC
+	(-154,-150)	STRE	AGGGG
-	**(-160,-154)**	**TBP**	**TATATAT**
+	(-161,-155)	TBP	TATATAT
-	(-162,-156)	TBP	TATATAT
+	(-163,-157)	TBP	TATATAT
-	(-164,-158)	TBP	TATATAT
+	(-165,-159)	TBP	TATATAT
+	(-247,-243)	GCR1	CATCC
+	(-251,-247)	ADR1	TCTCC
+	(-270,-265)	GCN4	TGAATG
+	(-275,-267)	HSTF	TTCCATGAA
+	(-280,-272)	HSTF	GAATATTCC
+	(-281,-276)	GCN4	TGAATA
+	(-367,-362)	SWI5	TGCTGA
+	(-374,-370)	GCR1	CATCC
-	(-380,-376)	STRE	AGGGG
-	(-445,-441)	STRE	AGGGG
+	(-461,-459)	ECB	GGAAAAT
+	(-523,-518)	MCB	TCGTGA
-	(-547,-542)	ATF	ACGTCA
-	(-564,-559)	STE12	ATGAAA
-	(-582,-578)	GCR1	CTTCC
+	(-626,-621)	GC/FAR	CCCGGG
+	(-723,-719)	ADR1	TCTCC
+	(-787,-782)	GCN4	TGAATT
+	(-790,-782)	UASINO	ATGTGAATT
+	(-790,-782)	NBF	ATGTGAATT

The SCPD promoter database (Zhu and Zhang, 1999) was used to search the 5' untranslated region of *PUT4* (+1 to –800) for predicted regulatory sequences.

by adding $MnCl_2$ or by adding disproportionate amounts of dNTPs, and the number of errors can be increased by increasing the number of cycles during the reaction (Zhou *et al.*, 1991). However, it was decided that the error rate of the selected polymerase in a standard reaction would generate sufficient errors, i.e. 1 error in 10^4 bases (information provided by manufacturer). To ensure that mutations were restricted to the 5' non-coding sequence upstream of *PUT4*, primers Put4f and P4UTRr (Table 2.2) were used to amplify 1.2 kb of DNA corresponding to the promoter region of *PUT4*.

Conventional cloning methods, utilising the ligation of restriction-digested DNA, require the occurrence of appropriate restriction sites in the sequence of interest. There were no such recognition sequences adjacent to the *PUT4*-coding region. The introduction of a restriction site within the amplifying primers was avoided, as the modification to the sequence might have affected the expression of *PUT4*. Consequently, plasmid construction by gap-repair was used to introduce modified promoter sequences upstream of the *PUT4* open reading frame (Figure 4.1). Specifically, the centromeric plasmid, pFL-PUT4 (Appendix 2), was digested with *Nhe* I to generate a linearised plasmid. The *Nhe* I enzyme cuts the pFL-PUT4 plasmid once, within the 5' non-coding sequence of *PUT4*. The linearised plasmid and error-prone PCR reaction products (in a ratio of 1:5) were then transformed into a *ure3Δ::kanMX4 put4 Δ::kanMX4* haploid yeast (KP12). In addition, KP12 was transformed with pFL-PUT4 for use as a negative control (strain KP13).

4.3 Selection of strains capable of *PUT4* expression under repressive conditions

A number of strategies were considered for the selection of strains expressing *PUT4* in the presence of a preferred nitrogen source. A negative selection strategy using a toxic proline analogue was trialed. L-azetidine-2-carboxylate can be transported into the cell via Put4p (Lasko and Brandriss, 1981), whereupon it can be incorporated directly into proteins *in lieu* of proline, thereby disrupting the protein structure and function and leading to cell death (Fowden and Richmond, 1963). The benefit of this strategy lies in the fact that L-azetidine-2-carboxylate can be added at a concentration that ensures its transport via Put4p (0.5 mM) (Lasko and Brandriss, 1981), as opposed to Gap1p, due to the differing affinities of the two permeases for this proline analogue. The presence of ammonium at a repressive concentration in solid media containing L-azetidine-2-

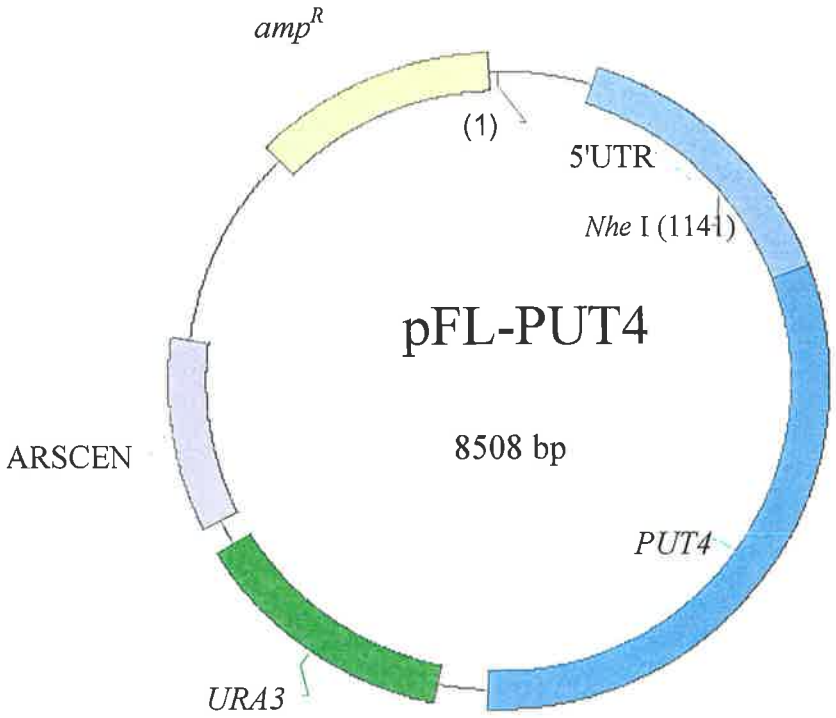


Figure 4.1 Generation of plasmids containing mutations within the 5' untranslated region (UTR) of the *PUT4* gene

The pFL-PUT4 plasmid was digested using the restriction enzyme *Nhe I,* for which there is a unique restriction site within the 5'UTR of *PUT4*. This linearised plasmid was co-transformed into a *put4Δ::kanMX4 ura3 Δ::kanMX4* haploid yeast (KP12) with DNA fragments corresponding to the 5'UTR of *PUT4*, generated by PCR with a DNA polymerase-lacking proof-reading activity. Plasmids containing mutagenised promoter regions were expected to occur through gap-repair (Ma *et al.*, 1987).

carboxylate would, hypothetically, lead to the growth of strains in which *PUT4* was subject to NCR, but to the death of those strains capable of *PUT4* expression in the presence of ammonium. Ultimately, the use of L-azetidine-2-carboxylate was avoided, as the growth of the constitutive *PUT4* control strain, KP21, was indistinguishable from the growth of a wild type strain on such media (data not shown). However, in this case mutagenesis was limited to the *PUT4* promoter, obviating the need to choose a selection strategy that would discriminate between upregulation of *PUT4* and that of *GAP1*.

A second selection strategy was chosen to allow positive selection of strains expressing *PUT4* in the presence of a preferred nitrogen source. The selection media contained methylamine, an ammonium analogue, at repressive concentrations (4.8 g/L), and proline (2.5 g/L) as the sole source of nitrogen in YNB minimal media (Salmon and Barre, 1998). Methylamine cannot be utilised as a nitrogen source by *S. cerevisiae* (Van Dijken and Bos, 1981), but is transported into the cell via the ammonium transport systems (Roon *et al.*, 1975) and is presumed to lead to nitrogen catabolite repression. To confirm *PUT4* repression by the presence of methylamine, KP2 cultured with proline as the sole source of nitrogen was treated with methylamine (4.8 g/L), ammonium (5 g/L) or proline (5 g/L) when the culture had reached the early stage of exponential growth (1 x 10^7 cells/ml). Total cellular RNA was isolated at 15, 30, 60, 90, 120 and 240 min and probed separately with a DNA probe corresponding to the *PUT4* coding region or the coding region of the constitutively-expressed *PDA* gene (amplified using primers PDAf and PDAr, Table 2.3). The addition of proline did not affect the expression of *PUT4* (Figure 4.2). *PUT4* expression was down-regulated after the addition of either methylamine or ammonium. Conversely, *PDA* expression was not affected by the addition of proline, methylamine or ammonium.

The repression of *PUT4* expression by methylamine allows the selection of strains in which the proline permease is expressed, since proline is the sole available source of nitrogen. The validity of this selection strategy was confirmed using strain KP21, in which *PUT4* is expressed from the constitutive *TEF2* promoter. Strain KP21 grew on this selection medium, whereas the wild type control, KP2, did not. From multiple gap-repair transformations, five individual colonies were isolated with the selective media. Of these, three were viable when subcultured on fresh, selective media, and were designated KP41, KP42 and KP43.

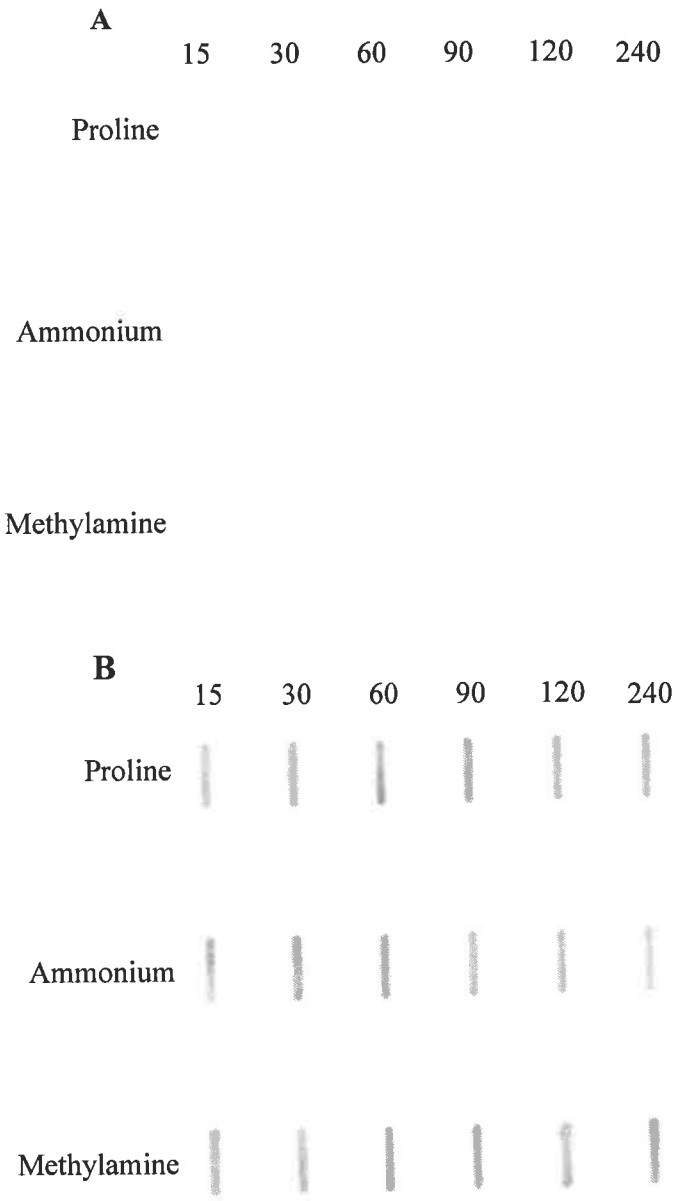


Figure 4.2 Down-regulation of *PUT4* expression in response to methylamine

Proline (5 g/L), ammonium (5 g/L) or methylamine (4.8 g/L) was added to a proline-grown culture of the wild type strain, KP2. Total cellular RNA was isolated 15, 30, 60, 90, 120 and 240 min after the addition for Northern slot-blot analysis. Blots were probed with DIG-labelled DNA probes corresponding to the *PUT4* gene (A) or the *PDA* gene (B).

4.4 Confirmation of *PUT4* expression in the presence of ammonium

The three strains selected by growth on methylamine and proline were analysed using Northern slot-blot analysis to confirm *PUT4* expression in the presence of ammonium. Strains were cultured to the mid-exponential phase of growth in minimal YNB containing ammonium as the sole source of nitrogen, and total RNA was isolated and probed for *PUT4* RNA. No *PUT4* RNA was detected in the wild type after growth in the presence of ammonium (Figure 4.3). The positive control, with RNA isolated from KP21 containing the *PUT4* gene driven by a constitutive promoter, contained *PUT4* RNA when grown in ammonium. Interestingly, of the three strains isolated by the original phenotypic selection strategy, *PUT4* RNA was detected in only two strains, KP41 and KP42, after growth in ammonium-containing media.

4.5 Identification of mutations in the *PUT4* promoter region of KP41 and KP42

Random mutagenesis led to the generation of strains in which *PUT4* was no longer repressed in the presence of ammonium. The mutagenised region of DNA was sequenced to elucidate how this change in transcriptional regulation had been achieved. DNA from KP41, KP42 and the control strain KP2 was amplified using primers Put4f and P4UTRr in a PCR reaction containing a high fidelity DNA polymerase (DyNAzyme EXT, Finnzymes). The amplified DNA was then introduced into the high copy number, bacterial vector pGEM-T Easy (Promega). This allowed isolation of high purity plasmid containing a DNA insert corresponding to the mutagenised promoter regions. For each strain, plasmids were purified from three separate colonies and sequenced in duplicate to ensure that the modifications detected did not result from the high fidelity PCR reaction. The primers used for sequencing were MutSEQ1, MutSEQ2, MutSEQ3 and MutSEQ4 (Table 2.5).

The entire *S. cerevisiae* genome has been sequenced, however, and the strain sequenced was S288c, not W303. Sequencing of the *PUT4* promoter region of KP2 revealed that there were no differences in the sequence of this region when compared with the corresponding region of S288c. It was found that strain KP41 contained three point mutations and that there were two mutations in KP42. The mutations identified from KP41 were: T→C at position –160, G→A at position –500 and A→G at position –708. The following substitutions were identified in KP42: T→C at position –90 and T→C at

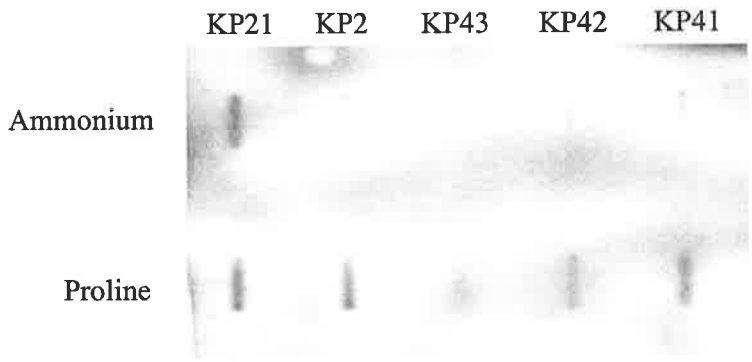


Figure 4.3 Northern slot-blot analysis of *PUT4* expression in strains capable of growth on methylamine and proline

The three strains isolated in a screen using methylamine and proline were grown to mid-exponential phase in media containing either ammonium or proline as the sole source of nitrogen. Total cellular RNA was probed with a *PUT4*-specific DNA probe. The KP21 strain, containing *PUT4* under the control of a constitutive promoter was included as a positive control. 10 μg of RNA was loaded in each well.

position –213. It is interesting that, in each case, a purine has been replaced with a purine or a pyrimidine replaced with a pyrimidine, suggesting that PCR mutagenesis with error-prone polymerase leads to more transitions than transversions, an effect also noted by Ling and Robinson (1997). In addition, it is noteworthy that each mutant contained at least two modifications.

4.6 Identification of single point mutations resulting in *PUT4* expression in the presence of ammonium

One of the aims of this project is to generate strains capable of proline transport with minimal genetic modification. Consequently, each mutation identified in the *PUT4* promoter region of strains KP41 and KP42 was introduced as a single point mutation upstream of native *PUT4* in order to determine if the desired phenotype, expression of *PUT4* in the presence of ammonium, could be attributed to the modification of a single base pair. Point mutations were introduced using the Stratagene Quikchange site-directed mutagenesis kit. This kit introduces point mutations into plasmid-borne sequences, allowing the rapid generation of specific mutations. The primers used were M1-160f/r, M1-500f/r, M1-708f/r, M2-90f/r and M2-213f/r (Table 2.4), and the template was the pFL-PUT4 plasmid. Point mutations were confirmed by sequencing in triplicate. The modified plasmids were then introduced into a *put4::kanMX4 ura3Δ::kanMX4* haploid yeast (KP12), to yield strains KP51 through KP55 (Table 2.1).

The effect of each point mutation on *PUT4* expression in the presence of ammonium was assessed using Northern blot analysis. Strains were grown in YNB media containing ammonium as the sole source of nitrogen. Total cellular RNA was isolated and a *PUT4*-specific DNA probe (amplified with primers P4probeF and P4probeR, Table 2.3) was used to detect *PUT4* RNA. Of the five single point mutations, four resulted in *PUT4* expression in the presence of ammonium; specifically, these were T→C at position –90 (KP51), T→C at –160 (KP52), T→C at –213 (KP53) and A→G at –708 (KP55) (Figure 4.4). When the amount of *PUT4* RNA detected in the four strains was adjusted for the amount of total RNA loaded on the gel, there was no significant difference between these strains in *PUT4* RNA after growth in media containing proline. (Lanes 3 and 4 were slightly overloaded, Figure 4.4). After growth in ammonium, faint bands corresponding to *PUT4* RNA were detected in the wild type

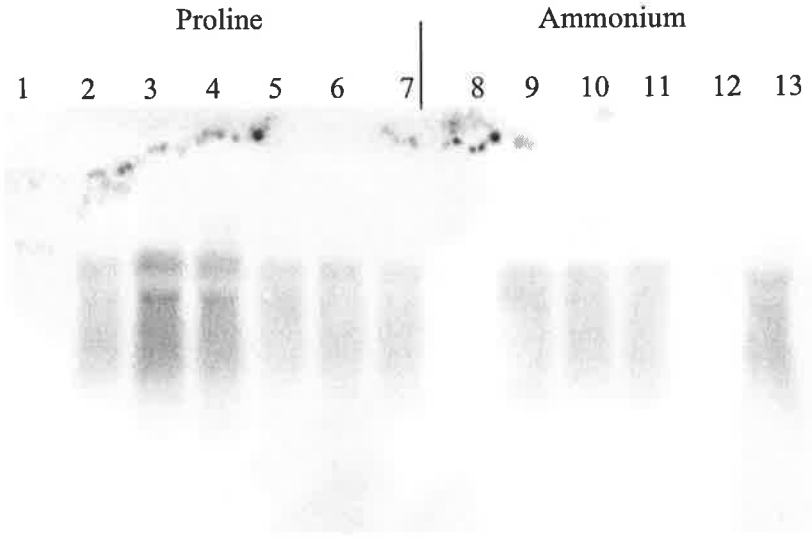


Figure 4.4 Expression of *PUT4* in strains containing point mutations within the *PUT4* promoter, under derepressive and repressive conditions

Strains were cultured in media containing either proline (lanes 1-7) or ammonium (lanes 8-13) as the sole source of nitrogen. Lane 1 contains RNA isolated from strain KP10, which lacks the *PUT4* gene. RNA was analysed from strains KP13 (lanes 2 and 8), strain KP51 (lanes 3 and 9), KP52 (lanes 4 and 10), KP53 (lanes 5 and 11), KP54 (lanes 6 and 12) and KP55 (lanes 7 and 13). Lanes 3 and 4 were overloaded.

and in the strain containing the G→A mutation at –500. No difference was detected in the level of *PUT4* RNA in ammonium-grown cultures compared with proline-grown cultures of the strains containing T→C at –90 (KP51), T→C at–160 (KP52), T→C at –213 (KP53) and A→G at –708 (KP55).

It is interesting that four of the five sequenced mutations individually result in *PUT4* expression in the presence of ammonium. Strains KP51, KP52, KP53 and KP55 were incubated on solid selective media to investigate whether a combination of two mutations leading to an increase in *PUT4* expression is required for growth under these conditions (Figure 4.5). The wild type KP2 strain did not grow on this media, in comparison with the strains KP21 (*ure2Δ::kanMX4*), KP41 (containing two point mutations) and KP42 (containing three point mutations). The strains containing single point mutations, KP51, KP52 and KP55, did not exhibit growth, whereas KP53 grew slowly in comparison with the KP21, KP41 and KP42 strains. This indicates that although each point mutation leads to *PUT4* expression in the presence of ammonium, three of the point mutations did not lead to sufficient Put4p activity to enable growth on the selective media. This suggests that, at least in the case of strain KP41, the two mutations were required for initial selection.

4.7 Analysis of regulatory sequences in the *PUT4* promoter region

A proposed model for transcription by RNA polymerase II in *S. cerevisiae* describes a system in which basal elements are located in and downstream of the TATA box region, with upstream elements binding activators or repressors (Struhl, 1987). In higher eukaryotes, the transcription initiation site is almost always situated 25-30 base pairs downstream of the TATA box. In *S. cerevisiae*, however, the distance between the TATA box and the mRNA initiation site can range from 40-120 bases.

The vast quantity of information available on regulatory sequences has allowed the development of computer software for the prediction of consensus sequences for a number of known DNA-binding proteins. As stated, the SCPD promoter database of *S. cerevisiae* (Zhu and Zhang, 1999) was used to search the *PUT4* promoter region for consensus sequences for the binding of regulatory proteins, revealing 34 putative sites (Table 4.1). Analysis was conducted for both the native *PUT4* promoter and the 4 *PUT4* promoter sequences containing the four point mutations described above. The T→C

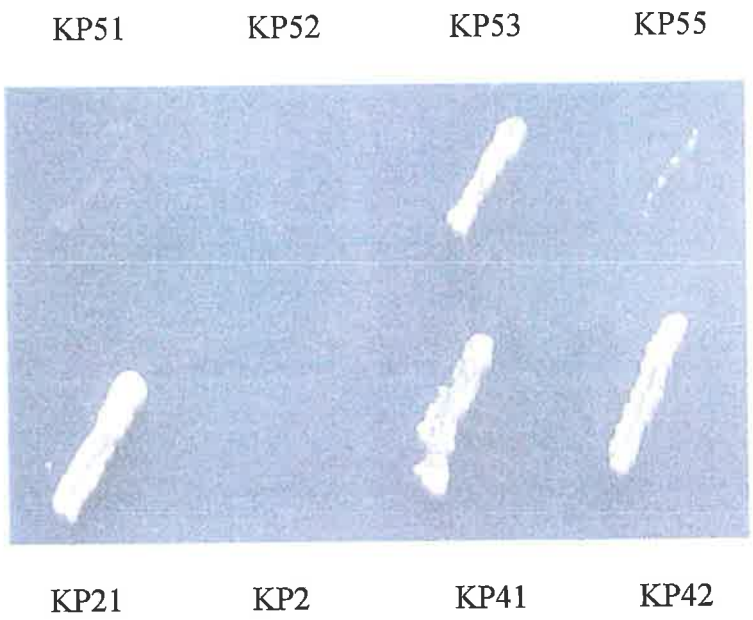


Figure 4.5 Comparative growth of *PUT4* promoter mutants on media containing proline as sole nitrogen source and methylamine

Strains were sub-cultured from fresh YNB solid media onto plates containing proline as the sole source of nitrogen (2 g/L) and methylamine at repressive concentrations (4.8 g/L) and incubated for 48 hours at 30°C.

mutation at –160 lies within a predicted TATA-binding factor (TBF) sequence and, as such, this consensus sequence was not detected in the analysis of the promoter containing this point mutation. Binding of TBF to TATA sequences is required for the formation of the basal transcription machinery and the subsequent initiation of transcription. Therefore, unless the change to the putative TATA sequence unexpectedly enhances binding of TBF, it is unlikely that disruption of a TBF-binding site would lead to the upregulation of *PUT4* expression. In addition, there are other predicted TATA motifs in the promoter of *PUT4* (Table 4.1).

Investigation of expression from a truncated *GAP1* promoter found that a 3' deletion to –388 led to an increase in expression in the presence of the preferred nitrogen source glutamine (Stanbrough and Magasanik, 1996). These workers concluded that this expression resulted from the deletion of a negative control site for basal expression. Since the mutations reported here at positions –90 and –160 lie downstream of the predicted TATA sequences, their increased expression of *PUT4* under repressive conditions may be the result of the disruption of an analogous control site for basal expression. The mutations at positions –213 and –708, leading to *PUT4* expression under normally repressive conditions, lie upstream of the predicted TATA sequences and, as such, are likely to affect the binding of a transcriptional activator or a transcriptional repressor.

Daugherty and colleagues (1993) predict four GATA motifs in the *PUT4* promoter region, at positions –333 to –325 (non-coding strand), –304 to –296, –206 to –198 (non-coding strand) and –187 to –179 (Figure 4.6). None of the mutations generated in this study lie within these predicted GATA-recognition sequences. The T→C mutation at position –213 lies 8 bases upstream of one of these predicted GATA motifs. However, published data (Cunningham and Cooper, 1993) suggest that the minimal binding site for Dal80p is 9 base pairs in length, with GATAA at its core (Figure 4.6), indicating that it is unlikely that a mutation at –213 would affect Dal80p binding. The Gln3p activator requires the dodecanucleotide sequence, 5'-TTNCTGATAAGG-3', for transcriptional activation of the allantoin pathway genes (Bysani *et al.*, 1991). The mutation at position –213 also lies outside this sequence, suggesting that Gln3p binding and activation have not been modified.

-720	TCAAATCTTG TC(A→G)CAAGTTT GTCAAAATTG CTTGTCATGT	-671
-670	CTGCCAAATC TCCAGGGTCC TAAATCTGAG GTAGCACGGC ACGGCACAAG	-621
-620	ATGGTACCCA TGGTGCGTGA CGACAGCCGC TACTTGCACG GCTTTGTTCC	-571
-570	GGCAGCCCGG GTACCCGCGC TGCGAGTTGA AACATCATCA AACCAAAAGG	-521
-520	GAAGCGACGT TAGTTTTTTT CATTGCGCTA GCTATGACGT TTGGGTGGCC	-471
-470	TAGCCGGTTC GCGTGTGCCT GTCGCTTTTG TCGCTTTTCA ACTTCTGCCC	-421
-420	GATATTTCCT ATCAAAGGAA AATGGGACGT TTTCAACCCC TCGCTATCAT	-371
-370	CGTGCCTGCA CTCTGCCTAT CGCCAACTAC ACCGGGGTTT TATCTGCTTC	-321
-320	ACCCCTCCAT CCAGTGCTGA TAACAAGAAG AACCTTGCAG GGTAGGGCAG	-271
-270	GACCTACGGC CAAAATACTA ATTATGTCTG TTTATGTACA TGCCCCAATC	-221
-220	TGAATATTCC ATGAATG(T→C)AG GCACAGCATA TCTCCATCCA	-171
-170	TGTACTGATA CAGACGCATA AACATATATG (T→C)ATATACATA	-121
-120	CTTATACACT CGAATATTTG TAGACTGATG TACTTCTATA TATATATAGG	-71
-70	GGGTTTGTGT (T→C)CCTCTTCCT TTCCTTTTTT TTTCTCTCTT CCCTTCCAGT	-21
-20	TTCTTTTATT CTTTGCTGTT TCGAAGAATC ACACCATCAA TGAATAAATC	-1

Figure 4.6 The 5' untranslated region of *PUT4*.

Mutations leading to the expression of *PUT4* in the presence of ammonium are represented in black. Predicted stress elements (Zhu and Zhang, 1999) are highlighted in red, and predicted GATA factor-binding motifs (Daugherty *et al.*, 1993) are highlighted in blue.

The Gln3p- and Nil1p-mediated activation of the *GAP1* gene involves TTG(G/T)T sites located near GATAA-binding sequences, in addition to GATAA-containing motifs (Stanbrough and Magasanik, 1996). The *PUT4* promoter region does not contain such TTG(G/T)T motifs and the upregulation of *PUT4* in the mutants generated here cannot be explained by the introduction of such sequences. The mutations generated in this study do not lie within predicted regulatory sequences, leading to difficulties in explaining the mechanism of increased expression. It is possible that, since none of the mutations lie within upstream activating or repressing sequences responsive to nitrogen sources, the modifications described here might moderate transcriptional control mediated by alternate factors.

4.8 Conclusions

Random mutagenesis of the 5' untranslated region of *PUT4* generated two strains capable of *PUT4* expression in the presence of ammonium.

PUT4 expression under normally repressive conditions was observed in four strains, containing a single base pair substitution, derived from the above pair of strains.

The basis for the constitutive expression could not be provided from an analysis of putative regulatory consensus sequences.

4.9 Discussion

Proline is a poor nitrogen source for the yeast *S. cerevisiae*. It supports a slow doubling time when present as the sole source of nitrogen, requiring more energy for conversion to glutamate than other, preferred nitrogen sources. As such, proline transport is repressed in the presence of preferred nitrogen sources. The transport of proline into the cell leads to induction of the proline catabolic genes, *PUT1* and *PUT2*, despite the fact that these genes are also repressed by nitrogen catabolites (Brandriss, 1983; Wang and Brandriss, 1986; Siddiqui and Brandriss, 1988). An interesting conclusion can be drawn from the growth of the constitutive *PUT4* strain, KP21, on methylamine and proline. Methylamine cannot be utilised by *S. cerevisiae* as a nitrogen source; however, it is presumed to lead to nitrogen catabolite repression. Previously, this selection strategy resulted in the selection of a strain lacking a functional *URE2* gene product (Salmon and

Barre, 1998). In such a strain, the nitrogen catabolite repression of *PUT1* and *PUT2* would be alleviated. The growth of strains in which *PUT4* alone is up-regulated in the presence of methylamine highlights the fact that induction of *PUT1* and *PUT2* by the presence of intracellular proline is sufficient for proline catabolism and cell growth, despite the fact that these genes are not fully derepressed. However, two point mutations were required for efficient growth on the selective media.

The nitrogen catabolite repression of *PUT4* involves the regulatory factors, Gln3p and Nil1p (activators) and Dal80p and Ure2p (repressors). Of these, the Gln3p, Nil1p and Dal80p regulators are DNA-binding proteins, which recognise sequences with a GATA motif at their core. The mutations generated above do not lie within the predicted GATA sequences in the 5' untranslated region upstream of the *PUT4* gene.

The recent development of micro-array analysis of total cellular RNA, combined with the availability of the *S. cerevisiae* genome sequence, has generated information about the genes regulated by a variety of environmental signals. Data from various micro array experiments indicate that *PUT4* expression is not controlled solely by nitrogen catabolite repression. It has been shown in two separate studies that *PUT4* is up-regulated in response to salt stress (Posas *et al.*, 2000; Yale and Bohnert, 2001). The up-regulation of genes in response to salt stress is, in part, mediated by STREs (stress responsive elements), the consensus for binding being C_4T or AG_4. Three such elements appear in the promoter of *PUT4* (Figure 4.6). The mutation at position -90 generates a C_3T motif. However, it seems unlikely that such a mutation would lead to up-regulation of transcription in the presence of ammonium, as the regulators Msn2p and Msn4p, which mediate transcription from STREs, are tightly regulated themselves and, in addition, may not recognise this C_3T motif.

The expression of *PUT4* has also been shown to respond to DNA damage induced by ionizing radiation (Gasch *et al.*, 2001), ethanol stress and the stationary phase (Gasch *et al.*, 2000). This indicates that there are multiple regulatory influences on *PUT4* gene expression, in addition to its control by nitrogen catabolite repression, and suggests that there may be, as yet, unidentified regulatory sequences in the *PUT4* promoter.

This study aims to generate strains capable of proline transport, with minimal modifications to the genome. Four mutations have been identified, which, when present as single point mutations, lead to the expression of the *PUT4* gene in the presence of the preferred nitrogen source, ammonium. It also has been shown that the transport of proline into the yeast cell, via Put4p, leads to growth on proline as the sole source of nitrogen, despite the presence of the ammonia analogue methylamine. In the light of the industrial scope of this project, these are positive results, as *PUT4* expression has been enabled under normally repressive conditions and it has been shown that the transport of proline into the cell leads to proline catabolism when oxygen is available.

Chapter 5 The post-translational down-regulation of the proline specific permease

5.1 Introduction

The transport of proline in *S. cerevisiae* is tightly regulated, as proline is a poor nitrogen source requiring more energy for catabolism than other, preferred nitrogen sources. Proline transport is mediated by the high affinity, proline specific permease, Put4p, and the general amino acid permease, Gap1p (Horak and Rihova, 1982). The regulation of proline transport via the *GAP1* and *PUT4* gene products occurs at the transcriptional and post-translational level in response to the quality of the available nitrogen (Courchesne and Magasanik, 1983)

The aim of this project was to develop strains capable of significant proline utilisation under oenological conditions. The expression of *PUT4* from a constitutive promoter leads to Put4p activity in the early stages of the growth phase, despite the presence of the preferred nitrogen source ammonium (Chapter 3). The amount of proline utilised by such a constitutive *PUT4* strain is limited by the rapid depletion of oxygen from the media or must following inoculation. An addition of oxygen is expected to maximise proline utilisation. The effect of an addition of oxygen during oenological fermentation varies with the timing of the addition. Additions made at the onset of fermentation lead to increased biomass, which would be detrimental when nitrogen is limiting (Sablayrolles and Barre, 1986). An addition of oxygen too late during fermentation has little effect on the fermentation rate or time. The most beneficial time for an oxygen addition is after the maximal fermentation rate has been reached, which is late in the exponential phase of growth (Sablayrolles and Barre, 1986). Put4p activity is low at this point of the growth phase in strains expressing *PUT4* in the presence of ammonium (Figure 3.2B), presumably due to the post-translational inactivation of Put4p. To generate strains in which Put4p is active late in the exponential phase, the nitrogen catabolite inactivation of this permease was investigated.

5.2 Nitrogen catabolite inactivation of Put4p and Gap1p

The *NPI1* and *NPI2* gene products are involved in nitrogen catabolite inactivation of Put4p and Gap1p (Grenson and Acheroy, 1982; Grenson, 1983a, Jauniaux *et al.*, 1987).

The *NPI1* gene encodes an ubiquitin protein ligase (also known as Rsp5p) (Hein *et al.*, 1995). Npi1p attaches the ubiquitin polypeptide to lysine residues within certain permease proteins (Galan *et al.*, 1996). *NPI2* encodes Npi2p (Doa4p), an ubiquitin hydrolase, which is involved in the maintenance of the pool of free ubiquitin within the cell (Springael *et al.*, 1999b). The *NPR1* gene product antagonises the action of *NPI1* and *NPI2* and is required for the maintenance of Gap1p and Put4p activity during growth on proline (Grenson, 1983a). Npr1p has homology to serine/threonine kinases, suggesting that the phosphorylation state of the permease affects post-transcriptional regulation (Vandenbol *et al.*, 1990). This is supported by the finding that Gap1p dephosphorylation and inactivation have similar kinetics (Stanbrough and Magasanik, 1995). The *NPI1*, *NPI2* and *NPR1* gene products do not control the activity of Put4p exclusively. A second gene, *NPR2*, whose product is involved in maintaining Put4p activity in the absence of preferred nitrogen sources, has been identified (Rousellet *et al.*, 1995). Npr2p does not affect Gap1p activity, but is implied in the control of urea uptake, as it was identified in a screen designed to select strains with decreased urea transport. The deletion of *NPR2* results in a slow growth phenotype (Winzeler *et al.*, 1999), suggesting that Npr2p has other roles within the yeast cell that are yet to be elucidated. Interestingly *NPR2* is not subject to NCR and is induced by proline (Rousellet *et al.*, 1995).

The aim of this section of research was to generate strains in which Put4p is active at a time when the addition of oxygen would be most beneficial for fermentation, i.e. the late exponential or early stationary phase. In order to achieve this, the nitrogen catabolite inactivation of Put4p was targeted for modification. The deletion of *NPI1* was not considered, since *NPI1* is an essential gene (Hein *et al.*, 1995). While *NPI2* is not an essential gene, its deletion would affect the ubiquitination of any number of proteins, as Npi2p is involved in maintaining the intracellular pool of ubiquitin (Springael *et al.*, 1999b). The overexpression of *NPR1* would also have pleiotropic consequences, which could be detrimental for the cell. Npr1p is involved in the maintenance of Put4p and Gap1p activity in the absence of preferred nitrogen sources (Grenson, 1983b). Npr1p is also involved in the down-regulation of Tat2p, the tryptophan permease (Schmidt *et al.*, 1998). The function of Npr2p has not been characterized, and this protein does not share homology with any proteins of known function. As such, over-expression of *NPR2* may

lead to unforeseen consequences, potentially detrimental during oenological fermentation.

The nitrogen catabolite inactivation of Gap1p has been characterized partially (Hein and Andre, 1997; Springael and Andre, 1998; Springael *et al.*, 1999b) and residues in the C-terminal region of Gap1p that are required for ammonia-induced inactivation have been identified (Hein and Andre, 1997; Springael and Andre, 1998). Gap1p and Put4p are similar proteins (Vandenbol *et al.*, 1990), allowing the selection of residues in the C-terminal region of Put4p to be specifically modified in an attempt to generate Put4p variants that are resistant to ammonia-induced inactivation.

5.3 The ammonium-induced down-regulation of Put4p

PUT4 expression is down-regulated and Put4p is inactivated on addition of ammonium to a culture grown in the presence of proline (Courchesne and Magasanik, 1983; Grenson, 1983a; Jauniaux *et al.*, 1987). In order to investigate the dynamics of Put4p inactivation, the *PUT4* gene must be down-regulated rapidly, so that *de novo* Put4p synthesis does not occur. Cycloheximide blocks protein synthesis, but it cannot be used here, since the addition of cycloheximide to a proline-grown culture leads to the rapid inactivation of Put4p (Courchesne and Magasanik, 1983). Transient expression of *PUT4* was observed in the wild type strain, KP2, after inoculation of YNB containing ammonium (Figure 3.1). Conversely, the addition of ammonium or methylamine to KP2 cultured in YNB containing proline led to a rapid decrease in *PUT4* RNA, which became undetectable 30 min post addition (Figure 4.2). Consequently, the methodology used to investigate the post-translational down-regulation of Put4p required that ammonium be added to cultures containing proline as the sole source of nitrogen.

Initially, the response of Put4p to the addition of ammonium in the wild type strain, KP2, was characterised. YNB medium containing proline as the sole source of nitrogen was inoculated with 5 x 10^6 cells/ml from a stationary phase culture. When the culture had reached 1 x10^7 cells/ml, ammonium sulphate was added (5 g/L). The specific rate of proline uptake via Put4p was measured at 0, 15, 30, 60 and 120 min post ammonium addition. Put4p activity decreases rapidly over the first 30 min after ammonium addition, declining from 35 ± 4 nmol proline/min/mg cells to 2 ± 2 nmol proline/min/mg cells. Interestingly, at 60 and 120 min after the addition of ammonium, Put4p activity is restored to approximately 40% of that measured at the zero time point

(Figure 5.1). Such a recovery is not observed in similar studies of the Gap1p response to the addition of ammonium (Hein and Andre, 1997; Springael and Andre, 1998).
The effect of ammonium additions on Put4p activity in an *NPR2* deletion strain was investigated in order to further characterise this interesting pattern of post-translational down-regulation. *NPR2* was replaced with the *kanMX4* cartridge by homologous recombination in the wild type haploid strain, KP2, to produce strain KP30 (Table 2.1). The deletion of the *NPR2* gene was confirmed by PCR analysis (data not shown), using primers Npr2f and Npr2r (Table 2.2). Put4p activity in the *npr2*Δ strain was measured after the addition of ammonium to a proline-grown culture (Figure 5.2). The initial activity of Put4p was ca. 30% of the activity of the wild type strain, KP2, in accordance with the findings of Rousselet and colleagues (1995). The nitrogen catabolite inactivation of Put4p in the *npr2*Δ strain did not have the same kinetics seen in the wild type strain. A slower inactivation was observed, but Put4p activity was undetectable 60 min after the addition of ammonium, at which time the activity was increasing in the wild type strain.

The post-translational regulation of Put4p and Gap1p has been described using the terms "inactivation" and "reactivation" (Courchesne and Magasanik, 1983; Grenson, 1983a). These terms are perhaps misleading, as they could imply active and inactive permease forms. In actuality, the activity of Gap1p appears to depend on the localisation of the permease. The down-regulation of Gap1p in response to an ammonium addition is the result of Gap1p endocytosis and subsequent degradation in the vacuole (Springael and Andre, 1998). In addition, Gap1p is not down-regulated in response to ammonium in an *act1-1* strain that is deficient in endocytosis (Springael and Andre, 1998). Recently, it was shown that Npr1p is required for the stabilisation of Gap1p in the membrane and it regulates the trafficking of newly synthesised Gap1p to the membrane (De Craene *et al.*, 2001). These findings suggest that the down-regulation and subsequent up-regulation of Put4p activity (Figure 5.1) after the addition of ammonium may be due to the endocytosis of Put4p, followed by recycling to the membrane.

The product of the *RCY1* gene is involved in endocytic membrane traffic and recycling of early endosomes to the membrane in a process involving ubiquitin (Wiederkehr *et al.*, 2000). Although not deficient in internalisation of membrane fractions, an *rcy1*

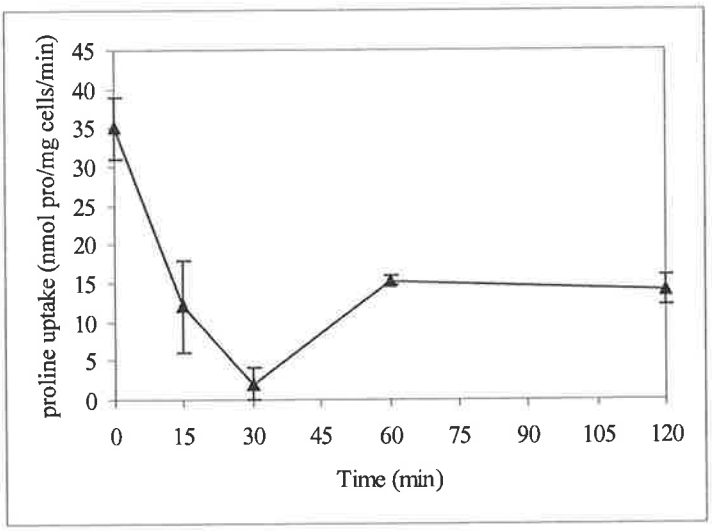


Figure 5.1 The effect of ammonium addition on Put4p activity

Wild type cells (KP2) were cultured in YNB containing proline as the sole nitrogen source until the early exponential phase (1 x 10^7 cells/ml). Ammonium sulphate (5 g/L) was added and the Put4p activity was measured using ^{14}C proline at 0, 15, 30, 60, 120 min post addition. Values are the means of duplicate determinations from triplicate cultures.

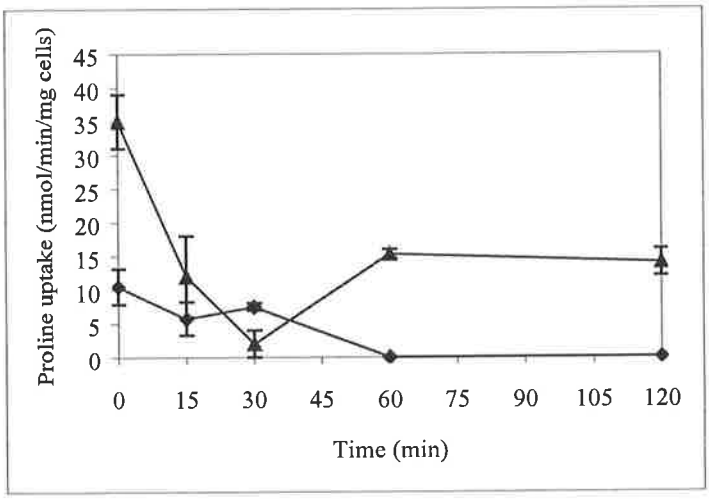


Figure 5.2 The effect of ammonium addition on Put4p activity in an *npr2*Δ strain

An *npr2*Δ strain (KP30) was cultured to the early exponential phase (1 x 10^7 cells/ml) in media containing proline as the sole source of nitrogen. Ammonium sulphate (5 g/L) was added and Put4p activity was measured at 0, 15, 30, 60 and 120 min post addition. Values are the means of duplicate determinations from triplicate cultures. Results for the wild type (▲) were plotted against results obtained for KP30 (♦).

mutation blocks the intersection of the endocytic pathway with the vacuolar protein sorting pathway. Additionally, the internalised material is not recycled to the membrane. The *RCY1* gene was deleted in the haploid wild type strain, KP2, by homologous recombination with the *kanMX4* cartridge, flanked by sequences corresponding to the 5' and 3' untranslated region of the *RCY1* gene. The deletion of the *RCY1* gene in strain KP31 was confirmed by PCR (data not shown), using primers Rcy1f and Rcy1r (Table 2.2). The down-regulation of Put4p activity by the addition of ammonium to an exponential culture of this *rcy1*Δ strain was investigated. Interestingly, Put4p activity, after growth in a derepressive medium, was 51 ± 8 nmol proline/min/mg cells compared with 35 ± 4 nmol proline/min/mg cells in the wild type strain, KP2 (Figure 5.3). Put4p activity decreased to 19 ± 6 nmol proline/min/mg cells after 30 min, before increasing to 26 ± 1 nmol proline/min/mg cells and 36 ± 1 nmol proline/min/mg cells after 60 min and 120 min, respectively. At each time point, Put4p activity was higher in the *rcy1*Δ strain than in the wild type strain, yet followed a similar pattern of down-regulation followed by an increase in Put4p activity.

The possible recycling of Put4p to the membrane could be investigated by localisation studies using Green Fluorescent Protein (GFP)-tagged Put4p. To this end, the *PUT4* gene was cloned in frame with *GFP* in the p416MET25HDEL vector (obtained from Jeff Eglinton, Australian Wine Research Institute, Adelaide, Australia). Plasmids, one containing *GFP* cloned upstream of *PUT4* and a second with *GFP* cloned downstream of *PUT4,* were constructed by gap-repair in a *put4*Δ*::kanMX4 ura3* Δ*::kanMX4* haploid strain (KP12) to allow both N-terminal and C-terminal tagging of Put4p. The successful construction of these plasmids was determined by PCR (data not shown), using primers Screenf and Screenr (Table 2.2). Unfortunately, Put4p activity was not detected in strains expressing GFP-tagged Put4p. The strains expressing Put4p-GFP were analysed by fluorescent microscopy to investigate whether this lack of function was due to a lack of Put4p in the plasma membrane. Fluorescence in these strains was at a much lower intensity than in a control strain expressing GFP alone. Unfortunately, the low intensity of fluorescence made it impossible to photograph the cells using the facilities currently available in the laboratory.

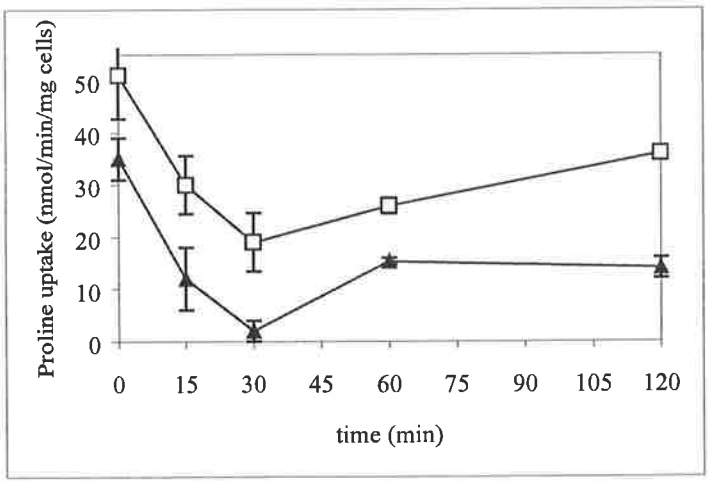


Figure 5.3 The effect of ammonium addition on Put4p activity in an *rcy1*Δ strain

An *rcy1*Δ delete strain (KP31) was cultured to the early exponential phase (1 x 10^7 cell/ml) in media containing proline as the sole nitrogen source. Ammonium sulphate (5 g/L) was added and the Put4p activity was measured at 0, 15, 30, 60, 120 min post addition. Values are the means of duplicate determinations from triplicate cultures. KP31 (□) is compared here with KP2 (▲).

5.4 Site-directed mutagenesis of *PUT4*

The activity of Put4p was increased in an *rcy1*Δ strain in comparison to the wild type. However, deletion of the *RCY1* gene resulted in a slow growth phenotype (Winzeler *et al.*, 1999). Consequently, this strain would not be appropriate for use in wine making. Site-directed mutagenesis was employed to generate Put4p variants with minimal changes to the genome. This approach allows the inactivation of Put4p to be targeted specifically. The residues targeted were identified by comparing the amino acid sequence of Put4p with information published on residues involved in the nitrogen catabolite inactivation of Gap1p (Hein and Andre, 1997).

Ubiquitination of Put4p is probably involved in its ammonia-induced inactivation, as mutations in *NPI1* (encoding an ubiquitin-protein ligase) and *NPI2* (encoding an ubiquitin hydrolase) affect the post-translational regulation of Put4p (Grenson and Acheroy, 1982; Grenson, 1983a; Jauniaux *et al.*, 1987). Ubiquitin is a 76 amino acid polypeptide that is covalently linked to lysine residues in its substrate through its C-terminal glycine (Reviewed in Hicke, 1999). Internalisation via the endocytic pathway, which is triggered by the conjugation of ubiquitin, is observed in plasma membrane proteins, including the uracil permease, Fur4p (Galan *et al.*, 1996), the α-factor receptor, Ste2p (Hicke and Reizman, 1996), the **a**-factor receptor, Ste3p (Roth and Davis, 1996), the maltose permease, Mal61p (Medintz *et al.*, 1998) and Gap1p (Springael and Andre, 1998). A single lysine to arginine substitution in the C-terminal region of the α-factor receptor leads to a Ste2p variant that is neither ubiquitinated nor endocytosed on ligand exposure (Hicke and Reizman, 1996). This lysine residue lies in a DXKSS motif and a similar motif, EEKSR, is found in the C-terminal region of Put4p. In addition, the substitution of lysine for the first glutamate residue in a similar motif in Gap1p (EEKAI) results in a Gap1p mutant that is not down-regulated by ammonium. Therefore, it was reasoned that the lysine within the EEKSR motif of Put4p (K604) could be an ubiquitination site within Put4p (Figure 5.4). Hence this lysine was replaced with arginine (K604R) by site-directed mutagenesis using primers K604Rf/r (Table 2.4). All site-directed mutagenesis was conducted using pFL-PUT4 as a template and the introduction of mutations was confirmed by sequencing, using the SDMSEQ primer (Table 2.5).

1 MVNIL PFHKN NRHSA GVVTC ADDVS GDGSG GDTKK EEDVV QVTES PSSGS 50
51 RNNHR SDNEK DDAIR MEKIS KNQSA SSNGT IREDL IMDVD LEKSP SVDGD 100
101 SEPHK LKQGL QSRHV QLIAL GGAIG TGLLV GTSST LHTCG PAGLF ISYII 150
151 ISAVI YPIMC ALGEM VCFLP GDGSD SAGST ANLVT RYVDP SLGFA TGWNY 200
201 FYCYV ILVAA ECTAA SGVVE YWTTA VPKGV WITIF LCVVV ILNFS AVKVY 250
251 GESEF WFASI KILCI VGLII LSFIL FWGGG PNHDR LGFRY WQHPG AFAHH 300
301 LTGGS LGNFT DIYTG IIKGA FAFIL GPELV CMTSA ECADQ RRNIA KASRR 350
351 FVWRL IFFYV LGTLA ISVIV PYNDP TLVNA LAQGK PGAGS SPFVI GIQNA 400
401 GIKVL PHIIN GCILT SAWSA ANAFM FASTR SLLTM AQTGQ APKCL GRINK 450
451 WGVPY VAVGV SFLCS CLAYL NVSSS TADVF NWFSN ISTIS GFLGW MCGCI 500
501 AYLRF RKAIF YNGLY DRLPF KTWGQ PYTVW FSLIV IGIIT ITNGY AIFIP 550
551 KYWRV ADFIA AYITL PIFLV LWFGH KLYTR TWRQW WLPVS EIDVT TGLVE 600
601 IEEKS REIEE MRLPP TGFKD KFLDA LL 627

Figure 5.4 The amino acid sequence of Put4p

The amino acid sequence of Put4p is presented, with residues targeted for site-directed mutagenesis presented in enlarged font, predicted phosphorylation sites (Blom *et al.*, 1999) presented in blue text and predicted trans-membrane spanning domains (Vandenbol *et al.*, 1989) are underlined. The sequences on which peptides were designed for site-directed mutagenesis are highlighted in red.

The rate of proline uptake by the Put4p K604R variant (KP61) was analysed as described for the wild type strain. On comparison with the wild type, the only significant difference in the rate of proline uptake by Put4p K604R occurred 60 min after the addition of ammonium, when it was approximately half of that of the wild type (Figure 5.5).

The substitution of a di-alanine motif for di-leucine (A575LA576L) results in a Gap1p mutant that is not inactivated upon addition of ammonium (Hein and Andre, 1997). A di-leucine motif has also been implicated in the internalisation and endosomal targeting of some membrane proteins in mammalian cells, including the ligand-induced endocytosis of the IL-6 receptor (Dittrich *et al.*, 1996) and the internalisation of the epidermal growth factor receptor (Kil *et al.*, 1999). There are 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 (Figure 5.4). Both of these motifs were converted to di-alanine by site-directed mutagenesis (L598A V599A and L626A L627A), using primers LV-AAf/r and Dileuf/r respectively (Table 2.4).

The effect of the addition of ammonium to proline-grown cultures of strains KP62 and KP62 expressing the Put4p L598A V99A and Put4p L626A L627A respectively was analysed as described for the wild type strain. Interestingly, proline uptake was not detected in either strain (data not shown). This could be the result of misfolding of the Put4p protein, disruption of Put4p activty, or, alternatively, the permease may not be transported to the membrane.

Two types of di-leucine motifs have been identified in mammalian cells. One type is dependent on the phosphorylation of a proximal residue, while the other is constitutively active and includes an acidic amino acid (Geisler *et al.*, 1998). As such, the Put4p sequence was analysed for putative phosphorylation sites (Blom *et al.*, 1999). The serine residue, S605, was the only predicted phosphorylation site in the C-terminal region of Put4p (Figure 5.4). Consequently, site-directed mutagenesis was used to replace this residue with alanine (S605A), using primers S605Af/r (Table 2.4) and hence produce strain KP64. In addition, S605 was replaced with glutamate (S605E), using primers S605Ef/r (Table 2.4) to produce strain KP65, as the negative charge of a

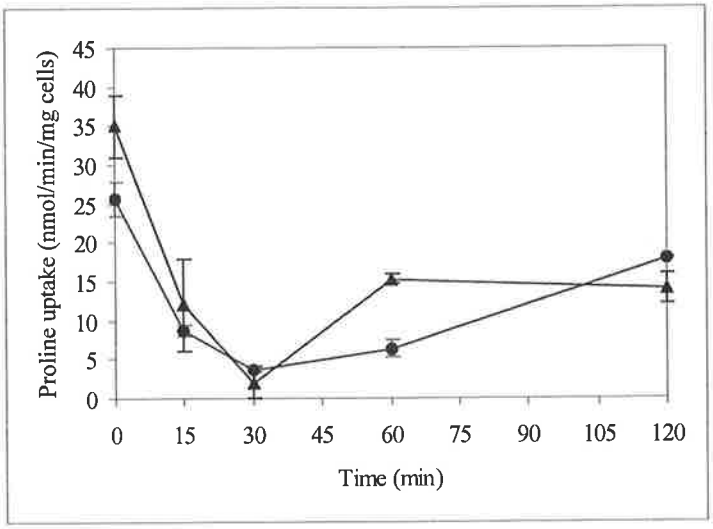


Figure 5.5 The effect of ammonium addition on the activity of Put4p K604R

Strain KP61 was cultured to the early exponential phase (1 x 10^7 cells/ml) in media containing proline as the sole source of nitrogen. Ammonium sulphate (5 g/L) was added and Put4p K604R activity was assayed at 0, 15, 30, 60 and 120 min post addition. Values are the means of duplicate determinations from triplicate cultures. Strain KP61 (●) is plotted with KP2 (▲) to allow comparison with the wild type.

glutamate residue may mimic the negative charge of a phosphorylated site (Stark, 1998).

After growth in proline, the Put4p S605A mutant had a similar rate of proline uptake as the wild type (38 ± 0.1 nmol proline/min/mg cells compared with 35 ± 4 nmol proline/min/mg cells) (Figure 5.6). The addition of ammonium to this proline-grown culture resulted in the down-regulation of Put4p S605A. However, 30 min post ammonium addition, the activity was 14 ± 0.3 nmol proline/min/mg cells compared with 2 ± 2 nmol proline/min/mg cells in the wild type. The rate of proline uptake by Put4p S605A then increased and, 120 min after the addition of ammonium, it had reached 32 ± 0.1 nmol proline/min/mg cells, or 86% of the activity after growth in proline (Figure 5.6). In comparison, the S605E modification led to a lower rate of proline uptake after growth in proline (18.3 ± 2.9 nmol proline/min /mg cells). The same pattern of down-regulation over the first 30 min followed by up-regulation was observed, with the rate of proline uptake reaching 8.5 nmol proline/min/mg cells 120 min after the addition of ammonium (Figure 5.6). When expressed as a percentage of the rate of proline uptake after growth in proline, this restored rate is similar to that of the wild type, i.e. ca 40%.

To investigate the affect of each of these mutations on Put4p ubiquitination and degradation, a polyclonal anti-Put4p antiserum was generated. The generation of an antisera to full-length Put4p was avoided due to the homology between Put4p, Can1p (constitutive arginine permease) and Hip1p (histidine permease) (Vandenbol *et al.*, 1989). In addition, the purification of membrane proteins can be a time-consuming and frustrating exercise, due to their tendency to associate with other proteins by virtue of their hydrophobic membrane-spanning regions. The Gap1p, Hip1p and Can1p permeases differ mostly at their termini. Accordingly, peptides corresponding to regions within the N- and C-termini of Put4p were conjugated to the immunogenic Keyhole Limpet Haemocyanin protein for the generation of antisera (Figure 5.4). Unfortunately, neither antisera detected bands corresponding to the predicted molecular weight of Put4p (69 kDa, Vandenbol *et al.*, 1989) in whole cell protein preparations resolved using SDS-PAGE (data not shown), and further attempts at antisera generation were not possible.

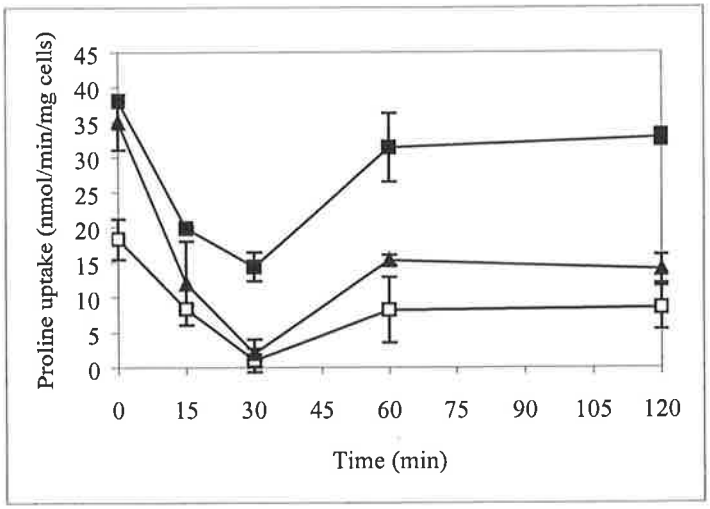


Figure 5.6 The effect of ammonium additions on the activity of Put4p S605A and Put4p S605E

Strains KP64 (■) and KP65 (□) were cultured to the early exponential phase of growth (1 x 10^7 cells/ml) in media containing proline as the sole source of nitrogen. Ammonium sulphate (5 g/L) was added and Put4p S605A (■) and Put4p S605E (□) activity was assayed at 0, 15, 30, 60, 120 min post addition. Values are the means of duplicate determinations from triplicate cultures. The KP2 strain (▲) is plotted to allow comparison with the wild type.

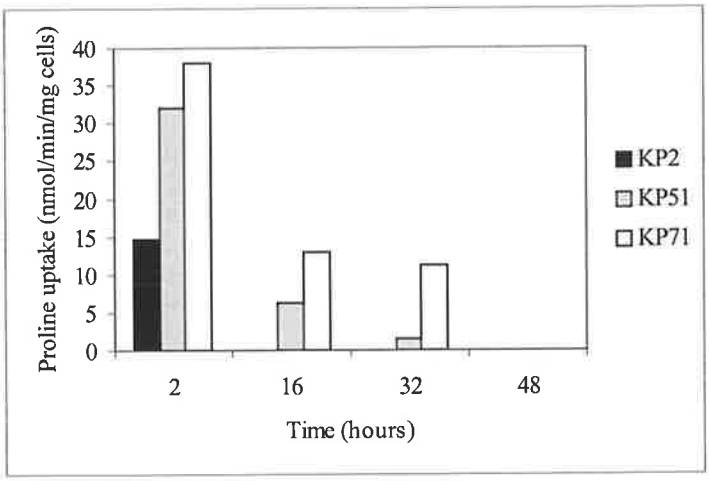
5.5 Proline uptake activity in a strain constitutively expressing Put4p S605A

The aim of this section of work was to generate a strain in which Put4p is active late in the exponential phase of growth. To this end, a strain was constructed in which a point mutation (C→T at –90) that results in *PUT4* expression in the presence of ammonium was combined with the point mutation (T→G at 1813) that results in the S605A substitution in the C-terminal region of Put4p. Site-directed mutagenesis was used to introduce both point mutations into the *PUT4* gene in the pFL-PUT4 centromeric vector (primers M2-90f/r and S605Af/r, Table 2.4). The resulting plasmid (pFL–(–90put4S605A) was transformed into the *put4Δ::kanMX4 ura3 Δ::kanMX4* haploid yeast, creating strain KP71.

To investigate whether Put4p S605A expressed from a promoter that is not responsive to ammonium leads to increased proline uptake, Put4p activity was monitored during growth in YNB containing ammonium sulphate (5 g/L) and proline (5 g/L). The wild type control, strain KP2, was compared with strain KP51 (containing the C→T mutation at –90) and strain KP71 (C→T, -90; T→G, 1813). Duplicate aliquots of media were inoculated with 5 x 10^6 cells/ml and the rate of proline uptake via Put4p was measured at three points during the growth phase and at a fourth time point as the cultures entered the stationary phase.

Put4p activity was detected in the early stage of exponential growth in all three strains, decreasing over the course of the growth phase. Strain KP51 had a higher Put4p activity than the wild type strain. The highest Put4p activity was observed in strain KP71 (Figure 5.7A). Interestingly, the rate of proline uptake in KP51 correlates with Put4p activity in the *ure2Δ* strain under similar conditions (Figure 3.2B). Strain KP71 had the highest Put4p activity at 2, 16 and 32 hours post inoculation. However, no proline uptake was detected after 48 hours of growth. The culture density of the strains was monitored over the course of this experiment (Figure 5.7B). The growth kinetics of all three strains were comparable during the first 26 hours, but KP51 and KP71 formed a higher final biomass. This is presumably due to proline utilisation by these two strains, as the experiment was conducted under aerobic conditions.

A



B

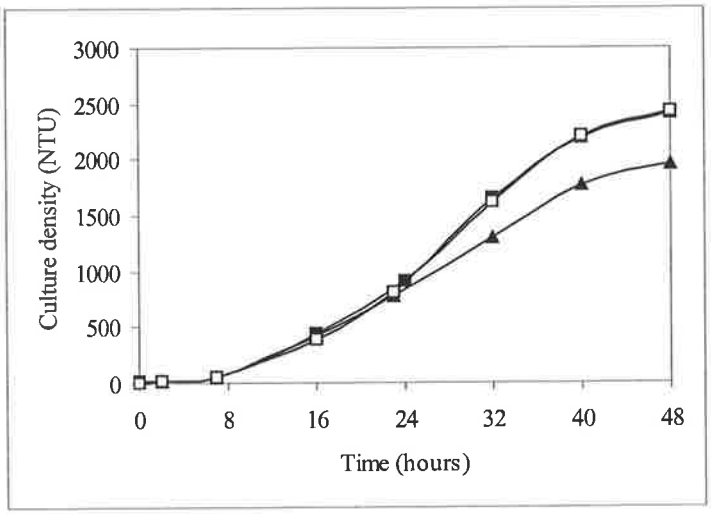


Figure 5.7 Put4p activity at different points during growth in strains KP2, KP51 and KP71

Duplicate aliquots of YNB media containing proline (5 g/L) and ammonium (5 g/L) were inoculated with 5 x 10^6 cells/ml of either KP2, KP51 or KP71 and incubated at 30°C. Duplicate samples from each culture were taken at 2, 16, 32 and 48 hours and proline uptake was measured (A). The culture density was monitored over the course of the experiment for each strain, KP2 (▲), KP51 (■) and KP71 (□) (B).

5.6 Conclusions

The expression of Put4p from a promoter containing a C→T at position –90 leads to increased Put4p activity, in comparison to the wild type, during growth on ammonium. The level of the Put4p activity measured correlates with the Put4p activity measured in a *ure2*Δ mutant under the same conditions.

The addition of ammonium to proline-grown cultures of *S. cerevisiae* leads to the down-regulation of Put4p activity for 30 min. The Put4p activity then increases, reaching 40% of the activity measured before ammonium addition.

The S605A substitution in the C-terminal region of Put4p ameliorates the ammonium-induced down-regulation. After 30 min of exposure to ammonium, Put4p S605A activity was approximately 7 times that of the wild type cells. After 120 min of exposure to ammonium, Put4p S605A activity had recovered to 86% of the activity measured before the addition of ammonium.

The combination of the C→T mutation at position –90 with the S605A substitution in the C-terminal region of Put4p leads to increased Put4p activity in comparison to the wild type and to a strain constitutively expressing *PUT4*. In such a strain, Put4p is active late in the exponential phase of growth, which is the optimal time for oxygen addition.

5.7 Discussion

There are many parallels between the control of Gap1p and Put4p. The mechanism by which the activity of Gap1p is regulated has been investigated intensively and the possibility of analogous mechanisms in Put4p warrants consideration. The ammonium-induced down-regulation of Gap1p leads to a progressive reduction in Gap1p activity after the addition of a preferred nitrogen source (Hein and Andre, 1997). In contrast, Put4p activity initially decreases in response to the addition of ammonium, followed by partial recovery of Put4p activity 60 min post addition, with no concomitant increase in mRNA (Figure 4.2). Clearly, the post-translational regulation of Gap1p and Put4p differs. However, the Npi1p, Npi2p and Npr1p regulatory proteins are involved in the regulation of both Gap1p1 and Put4p, suggesting a partially conserved regulatory strategy.

Although originally described as inactivation and reactivation, the down-regulation of Gap1p has been shown to depend on the localisation of the permease (Roberg *et al.*, 1997; Springael and Andre, 1998; De Craene *et al.*, 2001; Soetens *et al.*, 2001). Gap1p is ubiquitinated at two lysine residues during growth on proline, with the addition of a preferred nitrogen source leading to poly-ubiquitination and subsequent down-regulation (Springael *et al.*, 1999b). This ubiquitination of Gap1p is dependent on the function of Npi1p (Springael *et al.*, 1999a) and Npi2p (Springael *et al.*, 1999b). The Gap1p $^{LL\rightarrow AA}$ mutant is not down-regulated by the addition of ammonium, despite the fact that it is ubiquitinated (Hein and Andre, 1997). This highlights the fact that ubiquitination does not produce an inactive form of Gap1p, and that multiple motifs are involved in Gap1p regulation.

Ubiquitination of the Ste2p receptor occurs at a lysine residue in a DXKSS motif (Hicke and Riezman, 1996). A similar motif appears in the C-terminal region of both Gap1p and Put4p. Consequently, 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 K604R mutation did not ameliorate the ammonium-induced down-regulation of Put4p in comparison to the wild type, suggesting that this lysine residue is either not an ubiquitination site, or that its disruption has little involvement in Put4p down-regulation. Recently, the ubiquitination sites within Gap1p were identified. The lysine residues (K9 and K16) at which Gap1p is ubiquitinated are found in the N-terminal region of Gap1p (Soetens *et al.*, 2001). The homology between the Put4p and Gap1p sequences does not extend to the termini, where the most variation between the two sequences is found. Consequently, it is not possible to predict which of the 8 lysine residues in the N-terminal region of Put4p may act as ubiquitination sites.

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 internalisation. 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,

neosynthesised Gap1p is targeted from the Golgi to the vacuole and Gap1p is internalised 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 (S605). This prevalence of potential phosphorylation sites supports the postulation that the N-terminal region of Put4p may be involved in its ammonium-induced down-regulation, and possibly also ubiquitination.

The ubiquitination and dephosphorylation of Gap1p are involved in its ammonium-induced down-regulation. However, there are other determinants that also play a role in post-translational control. The di-leucine motif in the C-terminal region of Gap1p functions as an internalisation signal (Hein and Andre, 1997). Modification of two potential internalisation signals in the C-terminal region of Put4p rendered the permease inactive. This may be a result of either mis-localisation of Put4p or inactivation of its transport activity. Unfortunately, the antisera developed could not be used to determine where the Put4p$^{LL\rightarrow AA}$ and Put4p$^{LV\rightarrow AA}$ mutants were localised. In addition, GFP labelling of Put4p rendered Put4p inactive. Fluorescence microscopy suggests that Put4p-GFP is not localised to the plasma membrane (Figure 5.4).

A residue in the C-terminal region of Put4p that is involved in ammonium-induced down-regulation has been identified. The substitution of alanine for the serine 605 residue results in a Put4p mutant with a reduced susceptibility to ammonium-induced down-regulation. The rationale for the construction of the Put4p S605A mutant was that di-leucine motifs in mammalian cells sometimes require a proximal phosphorylated serine to function as an internalisation signal. However, without localisation data it is difficult to explain how the S605A substitution results in increased Put4p activity post ammonium addition in comparison to the wild type. Assuming a process of internalisation and subsequent recycling to the membrane, it is also difficult to distinguish between a slower internalization or a more rapid recycling of S605A with the information at hand.

The difference between the ammonium-induced down-regulation of Gap1p and Put4p is that Put4p is initially down-regulated, followed by a partial recovery of Put4p activity 60 min after the addition of ammonium. This recovery of Put4p may be a reflection of

the presence of proline in the media. It was found that sub-culturing of W303 into fresh media containing ammonium as the sole source of nitrogen does not lead to the rapid down-regulation of *PUT4* expression. Instead, *PUT4* is transiently expressed during the initial stages of growth. Consequently, this study has focused on the affect of the addition of ammonium to proline-grown cultures, since such an addition led to rapid down-regulation of *PUT4* expression. The *NPR2* gene is induced by proline, and Npr2p is involved in the maintenance of Put4p activity (Rousellet *et al.*, 1995). It has been reported previously that Put4p activity in an *npr2*Δ strain is 30% of that measured in the wild type after growth on proline (Rousellet *et al.*, 1995). This level of activity correlates with the level of Put4p activity in an *npr2*Δ strain measured under the same conditions in this study (Figure 5.2). The function of Npr2p has not been defined and there is no homology between Npr2p and any protein of known function. It may be that Npr2p is involved in the putative recycling of Put4p to the membrane, as Put4p activity in an *npr2*Δ strain activity is down-regulated, with no recovery of activity after the addition of ammonium to a proline-grown culture. The deletion of *NPR2* has pleiotropic consequences, as this strain has a slow growth phenotype.

The *RCY1* gene was deleted to investigate the possible role of recycling in the post-translational control of Put4p. Rcy1p is involved in recycling to the membrane as well as the intersection of the endocytic and vacuolar pathways (Weiderkehr *et al.*, 2000). In a *rcy1*Δ strain, the α factor receptor, Ste2p, accumulates in an enlarged compartment after internalisation in response to ligand binding, instead of being degraded in the vacuole. In addition, the recycling of endocytosed material to the plasma membrane was down-regulated strongly in a *rcy1*Δ strain (Wiederkehr *et al.*, 2000). Interestingly, the pattern of Put4p down-regulation followed by a recovery of activity that was observed in the wild type was also seen in the *rcy1*Δ strain. However, the activity of Put4p initially was higher in the *rcy1*Δ strain and at all time points after exposure to ammonium when compared with the wild type strain. A possible explanation for the observed differences in Put4p activity in the *rcy1*Δ strain is that the basal turnover of Put4p is decreased, since Put4p is internalised but cannot be degraded in the vacuole due to the accumulation of endocytosed material in the enlarged endocytic compartments observed in such a strain (Weiderkehr *et al.*, 2000). The recovery of Put4p activity may either be a reflection of an alternate recycling pathway not

dependent on Rcy1p or, alternatively, it could indicate that the recovery of Put4p activity is not a result of a recycling process.

In terms of the industrial scope of this project, a suitable point mutation has been identified that leads to increased Put4p activity in the presence of the preferred nitrogen source ammonium. The presence of the C→T point mutation at position –90 leads to increased Put4p activity when compared to the wild type, and comparable to Put4p activity measured in the *ure2*Δ strain. Combining this mutation with a second point mutation, T→G at position 1813 (leading to the S605A substitution), results in a strain with increased Put4p activity compared with that of the wild type and the constitutive *PUT4* strain. This strain (KP71) is capable of proline transport late in the exponential phase of growth, a time when oxygen additions are most beneficial for the avoidance of stuck fermentation, without resulting in inefficient use of nitrogen as a result of increased biomass formation (Sablayrolles *et al.*, 1996).

Chapter 6 Fermentation by a strain capable of proline transport

6.1 Introduction

A deficiency of nitrogen or a lack of oxygen during oenological fermentation can lead to a prematurely terminated (stuck) or protracted (sluggish) fermentation. If nitrogen becomes depleted, the sugar transport systems will be irreversibly inactivated (Lagunas *et al.*, 1982; Salmon, 1989), which in turn results in the cessation of fermentation. Concentrations of nitrogen sufficient for the completion of fermentation may still be insufficient to avoid a sluggish fermentation or the production of hydrogen sulphide (Jiranek *et al.*, 1995a). The addition of diammonium phosphate (DAP) to supplement the available nitrogen of grape must is a common oenological practice. The addition of DAP is regulated by law. In Europe, 300 mg/L DAP (corresponding to 63 mgN/L) may be added and, under American law, the limit is 950 mg/L DAP (199 mgN/L). In Australia, the amount of inorganic phosphate that may be added during fermentation is limited to 400 mg/L Pi, which corresponds to 556 mg/L of DAP or 116 mgN/L. In comparison, the average concentration of proline in grape must corresponds to 90 mgN/L. Clearly, the use of proline as a nitrogen source during fermentation may prove beneficial in the avoidance of fermentative problems and may also negate the need for nitrogen additions in the form of DAP.

Proline is not considered an assimilable nitrogen source during oenological fermentation. Proline transport is inhibited by the presence of preferred nitrogen sources (Horak and Rihova, 1982) and proline catabolism is dependent on the presence of oxygen (Duteurtre *et al.*, 1971). The proline catabolic genes, *PUT1* and *PUT2*, are induced by intracellular proline and are also subject to nitrogen catabolite repression (Wang and Brandriss, 1986; Siddiqui and Brandriss, 1988; Xu *et al.*, 1995). The growth of strains expressing *PUT4* on media containing methylamine and proline (Chapter 4) confirms the induction of *PUT1* and *PUT2* by proline, despite the repression of these genes by methylamine. Consequently, the availability of oxygen during oenological fermentation may be a key restriction to the utilisation of proline as a nitrogen source in a strain capable of proline transport.

Oxygen is an important requirement, since the first enzyme in the proline catabolic pathway, proline oxidase, requires oxygen and a functional electron transport chain for the conversion of proline to Δ-pyrroline-5-carboxylate (Duteurtre *et al.*, 1971). Components of the electron transport chain of *S. cerevisiae* are down-regulated in response to high concentrations of fermentable carbon sources (Perlman and Mahler, 1974) and under anaerobic conditions (Groot *et al.*, 1971). However, it has been shown that some respiratory capacity is retained by *S. cerevisiae* when it is cultured under oenological conditions (Sablayrolles *et al.*, 1996; Salmon *et al.*, 1998). Antimycin A specifically inhibits electron transfer between cytochromes *b* and *c*. The addition of Antimycin A inhibits proline catabolism (Duteurtre *et al.*, 1971) and also partially inhibits oxygen consumption by *S. cerevisiae* that has been cultured under oenological conditions (Salmon *et al.*, 1998). The Antimycin A-sensitive oxygen consumption during oenological fermentation is greatest during cell growth, in the initial stages of fermentation (Sablayrolles *et al.*, 1996). In addition, aerobic fermentation leads to proline utilisation despite the presence of fermentable carbon sources (Ingledew *et al.*, 1987). This data suggests that the components of the electron transport chain required for the catabolism of proline may be present despite carbon catabolite repression and anaerobic conditions.

The addition of oxygen or nitrogen during fermentation can have variable effects, depending on the timing of the addition. The addition of oxygen during the initial stages of fermentation has little effect on reducing fermentation time (Sablayrolles and Barre, 1986). Oxygen additions (5 mg/L) made as the cells entered the stationary phase are more beneficial for the reduction of fermentation time and the avoidance of stuck or sluggish fermentation than additions made at the start of fermentation or after the catabolism of half the available sugar (Sablayrolles and Barre, 1986). The addition of nitrogen (300 mg/L DAP) when half of the available sugar has been fermented is more effective in reducing fermentation time while avoiding excess biomass formation compared with earlier nitrogen additions (Bely *et al.*, 1990a; Sablayrolles *et al.*, 1996). The combined addition of nitrogen (300 mg/L DAP) and oxygen (5 mg/L) as the cells enter the stationary phase enables the completion of fermentation of nitrogen-deficient must within a satisfactory time frame (Sablayrolles *et al.*, 1996).

Constant rate fermentation has been used to compare the effectiveness of different nitrogen sources for the maintenance of the fermentation rate during the stationary phase. Interestingly, the concentration of a known poor nitrogen source, glycine, required to maintain a constant fermentation rate did not significantly differ from that of glutamine, a preferred nitrogen source (Manginot *et al.*, 1997). Unfortunately, Manginot and colleagues (1997) did not investigate the use of proline as an additional nitrogen source, so no data is available on its usefulness as a nitrogen source during the stationary phase. It has long been known that proline is a poor nitrogen source during growth, as it supports slower doubling times than other, preferred nitrogen sources. Fermentation experiments were performed to begin to determine the effect and nature of any benefits for a yeast with an ability to constitutively transport proline. In particular, model fermentations were conducted in order to investigate whether oxygen additions made as the cells entered the stationary phase would lead to an improvement in the fermentation kinetics of the strain capable of proline transport (KP71) compared with the wild type control (KP2). The experiments presented in this section of work were conducted at the Institut des Produits de la Vigne, l'Institut National de la Recherche Agronomique, Montpellier, France.

6.2 Results

6.2.1 Fermentation kinetics of KP2 and KP71 in the absence of oxygen

The rate of CO_2 evolution is correlated to fermentation progress and can be used as an indicator of nitrogen deficiencies during fermentation (Bely *et al.*, 1990b). A higher concentration of available nitrogen on inoculation leads to a higher rate of CO_2 production over the course of fermentation and a concomitant decrease in fermentation times (Bely *et al.*, 1990a). CO_2 evolution at the end of a sluggish fermentation, resulting from insufficient assimilable nitrogen, is characterised by a slow rate of decrease. A problem-free fermentation is characterised by a sharp decline in the rate of CO_2 evolution at the end of fermentation (Bely *et al.*, 1990a). The effect of proline transport on fermentation was investigated using the rate of CO_2 production as an indicator of fermentation rate, and the duration of fermentation was also monitored.

The synthetic medium (Milieu Synthetique, MS) used for these experiments simulated grape juice (pH 3.3) and differs from the Chemically Defined Grape Juice Medium used previously in this study. The concentration of assimilable nitrogen in the medium was

300 mgN/L, in the form of ammonium and a complex mixture of α-amino acids. Proline was present at a concentration of 950 mg/L (corresponding to 114 mgN/L).

A synthetic medium (MS, 1.1L) in small fermentors (1.2L capacity) was sparged with argon to remove oxygen. The medium was inoculated with 5 x 10^6 cells/ml taken from an overnight culture that had reached stationary phase. Fermentation locks were fitted to exclude the introduction of oxygen. The evolution of CO_2 was monitored automatically by measurement of fermentor weight loss every 20 min (Sablayrolles *et al.*, 1987). The production rate of CO_2 (dCO_2/dt) was calculated by polynomial smoothing of sets of 10 measurements of weight loss due to CO_2 evolution. The numerous time points at which weight loss was measured allowed the calculation of the rate of CO_2 production with good precision (Bely *et al.*, 1990b). The duration of fermentation was taken as the time point at which the CO_2 production curve intersected the time axis.

Initially, the fermentation kinetics of strain KP2 were compared to strain KP71 during a fermentation in which no oxygen was added. There was no observable difference in the rate of CO_2 evolution between duplicate samples over the course of the fermentation. However, there was some variability in the fermentation time (Figure 6.1). This slight difference noted in fermentation time could be attributed to the variation observed between the duplicate samples. The kinetics of CO_2 evolution were the same for both KP2 and KP71, with the maximum rate of fermentation being 1.65 g/L/h.

6.2.2 Fermentation kinetics of KP2 and KP71 when oxygen is available during the initial stages of fermentation

Must hyperoxidation can be used to produce wines with a reduced capacity for oxygen consumption (Schneider, 1998). A secondary benefit of such a technique is an increased availability of oxygen for *S. cerevisiae* during the initial stages of fermentation. Consequently, the kinetics of CO_2 evolution during the fermentation of hyperoxidised media by KP2 and KP71 were compared in order to determine if the increased proline transport by KP71 could improve the kinetics of fermentation when oxygen is available at the time of inoculation.

A synthetic medium (MS, 1.1L) in small fermentors was sparged with high purity oxygen until the dissolved oxygen content had reached saturation. The anaerobic

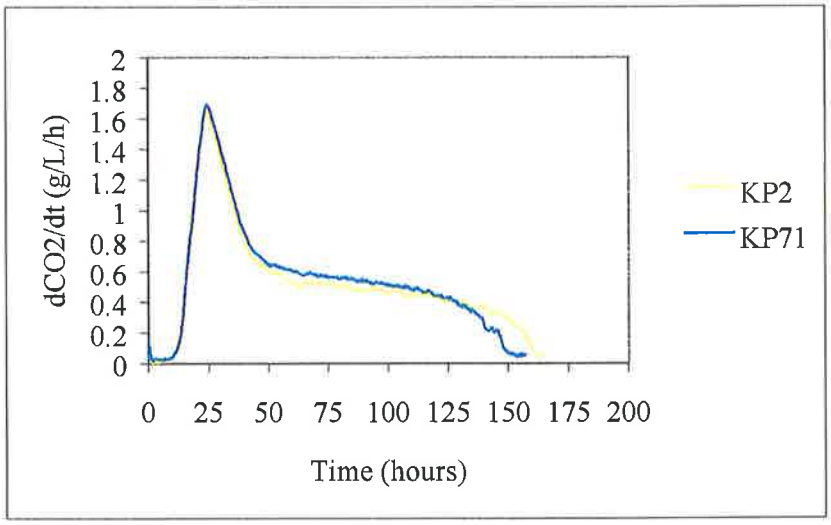


Figure 6.1 Rate of CO_2 evolution during fermentation by KP2 and KP71

Argon-sparged medium (MS) was inoculated (at 5 x 10^6 cells/ml) with either KP2 or KP71. Weight loss due to the evolution of CO_2 was automatically monitored by continuous weighing of the vessels, and was used to monitor fermentation progress. The curves are derived from a typical experiment.

factors, oleic acid and ergosterol, were included in the medium despite the presence of oxygen. The aerated medium was inoculated (at 5 x 10^6 cells/ml) from a stationary phase culture of either KP2 or KP71. The kinetics of CO_2 evolution over the course of fermentation was monitored as above. Again, there was no difference in fermentation rate between the KP2 and the KP71 cultures (Figure 6.2). However, the presence of oxygen during the initial stages of fermentation led to an increased maximal rate of fermentation (1.85 g/L/h compared to 1.65 g/L/h) and a decreased fermentation time for both strains (120-130 h, Figure 6.2) compared with fermentation in which conditions initially were anaerobic (150-165 h, Figure 6.1).

6.2.3 The effect of oxygen additions during the stationary phase

The best time for the addition of oxygen to an oenological fermentation is when the cells enter the stationary phase of growth (Sablayrolles *et al.*, 1996). Such an addition of oxygen results in an increase in fermentation rate and a decrease in the time required for fermentation, without resulting in an excessive increase in biomass. When combined with an addition of nitrogen, such an oxygen addition can prevent stuck or sluggish fermentation (Sablayrolles *et al.*, 1996). The KP71 strain contains two point mutations in the *PUT4* gene, C→T at position –90 and T→G at 1813. The mutations in strain KP71 led to *PUT4* expression and increased Put4p activity during the course of growth, despite the presence of ammonium, a preferred nitrogen source. Consequently, the addition of oxygen to KP71 when the cells are in the late stationary phase may lead to an increase in fermentation rate resulting from the utilisation of proline as a supplementary nitrogen source.

A synthetic medium (MS, 1.1L), in small fermentors designed for the addition of oxygen (Blateyron *et al.*, 1998), was sparged with argon to remove any dissolved oxygen. This anaerobic medium was inoculated (at 5 x 10^6 cell/ml) with either KP2 or KP71 from stationary phase cultures. The rate of fermentation was monitored by the calculation of CO_2 evolution, as described above. An oxygen addition (7 mgO_2/L, 14 mgO_2/L or 28 mgO_2/L) was made after 46 hours of fermentation. This time point was selected as it occurred after the rate of fermentation had passed its maximum and the cells were in the late exponential phase. The addition of oxygen led to an increase in the fermentation rate (Figure 6.3); however, the small variation between the increase observed for KP71 and that for KP2 can be accounted for by the variation between the

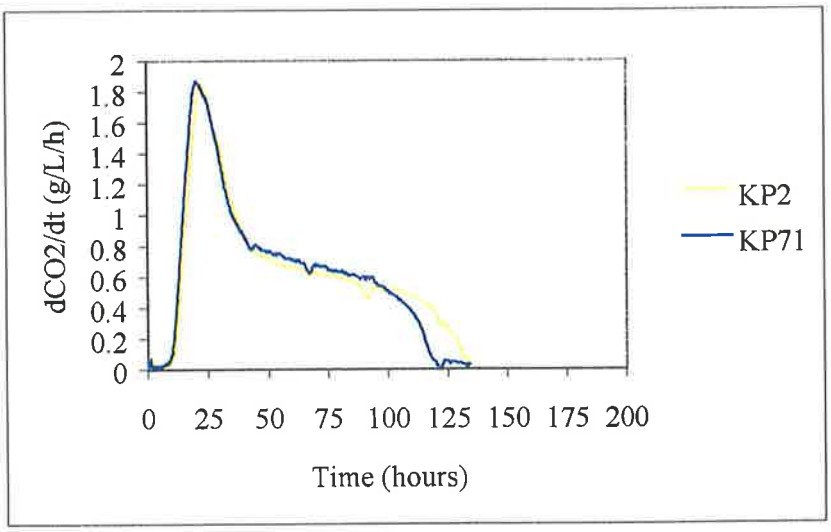


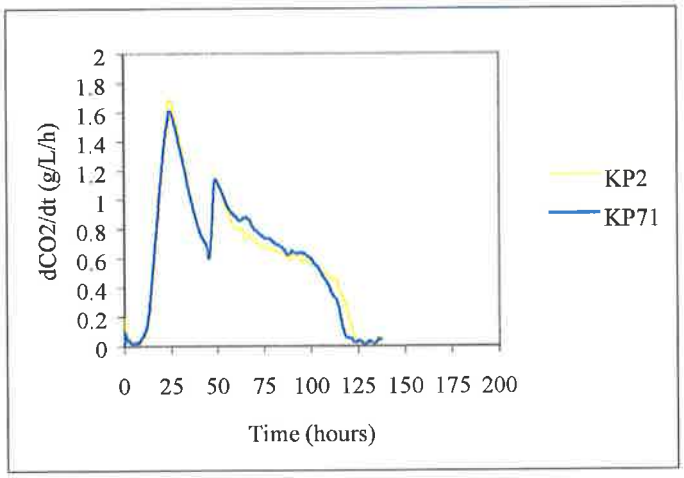
Figure 6.2 The rate of CO_2 evolution during fermentation of hyperoxidised media

Oxygen-saturated synthetic medium (MS) was inoculated (at 5 x 10^6 cells/ml) with either KP2 or KP71. Weight loss due to the evolution of CO_2 was automatically monitored by continuous weighing of the vessels, and was used to monitor fermentation progress. The curves are derived from a typical experiment.

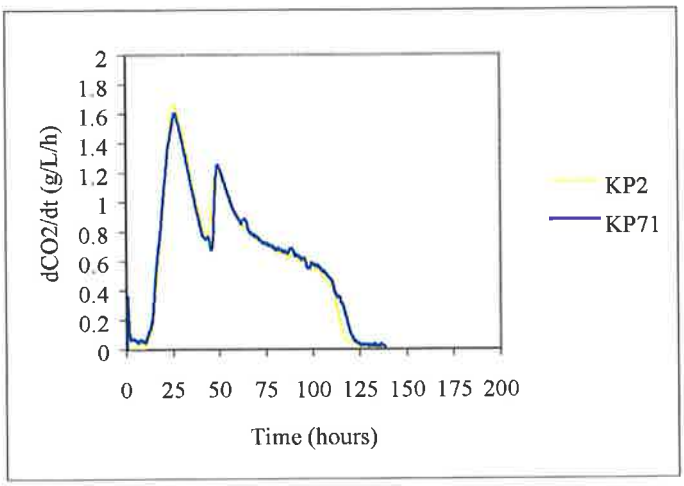
Figure 6.3 Addition of oxygen during fermentation

Argon-sparged medium was inoculated with KP2 or KP71 (5 x 10^6 cells/ml). The rate of CO_2 evolution was monitored by automatic logging of the weight change of the ferment vessel. After 46 hr of fermentation, a controlled oxygen addition was made: 7 mgO_2/L (A), 14 mgO_2/L (B), 28 mgO_2/L (C). The curves are derived from a typical experiment.

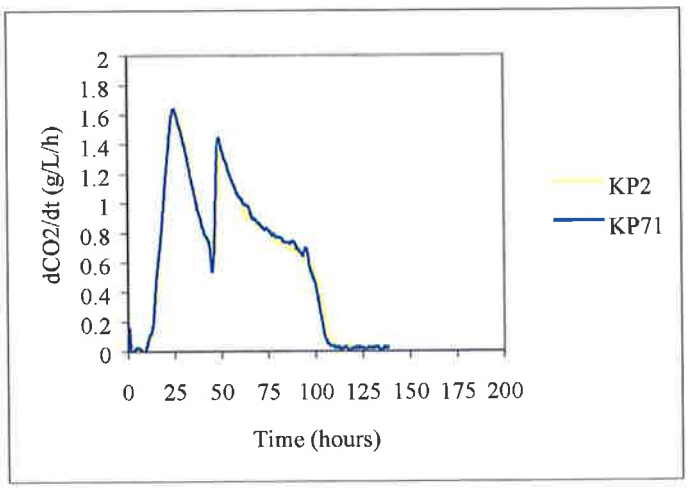
A


dCO2/dt (g/L/h)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 25 50 75 100 125 150 175 200
Time (hours)
KP2
KP71

B


dCO2/dt (g/L/h)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 25 50 75 100 125 150 175 200
Time (hours)
KP2
KP71

C


dCO2/dt (g/L/h)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 25 50 75 100 125 150 175 200
Time (hours)
KP2
KP71

samples. When 7 mgO_2/L was added, the rate of fermentation increased to 1.1 g/L/h for both strains. The addition of 14 mgO_2/L led to an increase in fermentation rate to 1.2 g/L/h and the addition of 28 mgO_2/L resulted in an increase in fermentation rate to 1.4 g/L/h.

6.2.4 Viability of KP2 and KP71 cultures at the end of fermentation

KP71 would presumably accumulate more proline during the course of fermentation. Consequently, we sought to measure parameters that may be influenced by such an increase. The disruption of the proline oxidase gene in *S. cerevisiae* leads to an increase in cell viability of 2-5% in response to freezing and desiccation stress, presumably due to the accumulation of proline (Takagi *et al.*, 2000). The supplementation of a high-gravity fermentation (i.e. 350 g/L glucose) with proline (30 mM) increased viability over the course of fermentation (Thomas *et al.*, 1994). In addition, proline is a known osmoprotectant in plants and bacteria (reviewed by Le Rudulier *et al.*, 1984). Consequently, the percentage of viable cells at the end of fermentation was calculated for strains KP2 and KP71. Viable cell numbers in each culture were determined by epifluorescence (King *et al.*, 1981) and were found to be increased to a small but reproducible degree in KP71 ($98 \pm 0.6\%$) compared with KP2 ($95.3 \pm 0.6\%$).

6.3 Conclusions

The addition of oxygen to a W303 mutant with increased Put4p activity (KP71) during fermentation did not lead to an increase in fermentation rate.

At the end of fermentation, KP71 cultures contained a higher percentage of viable cells in comparison to the wild type.

These results confirm those of others (Sablayrolles and Barre, 1986) that the addition of oxygen increases the rate of fermentation and leads to a decrease in fermentation time.

6.4 Discussion

The aim of this study was to increase fermentation reliability by enabling proline to be utilised as a nitrogen source during oenological fermentation. Such utilisation of proline does not occur ordinarily (Ough and Stashak, 1974), as the catabolism of proline requires the presence of oxygen and a functional electron transport chain. During the

initial stages of fermentation, when oxygen may be available, the transport of proline into the cell is repressed by the presence of preferred nitrogen sources, such as ammonium and glutamine. After the depletion of these nitrogen sources, oxygen is no longer available and proline consequently is not utilised. The initial hypothesis was that enabling proline transport during the initial stages of fermentation would lead to proline utilisation if oxygen is available at the point of inoculation. However, oxygen is rapidly depleted from the medium and the must on inoculation. Consequently, the addition of oxygen during fermentation was investigated in an effort to increase proline utilisation. Strain KP71 was developed to produce a yeast in which the proline specific permease, Put4p, is active during the course of the exponential phase of yeast growth. This strain was compared with the wild type control, KP2, in order to determine if an addition of oxygen would lead to an increase in fermentation rate in a strain capable of proline transport.

During the fermentation of de-aerated media, there was no significant difference in the rate of fermentation in KP2 cultures compared with that in KP71. The kinetics of fermentation of air-saturated media by KP2 and KP71 also did not differ. In this study, transient *PUT4* expression and Put4p activity have been observed when fresh media are inoculated, despite the presence of preferred nitrogen sources. This proline transport capability may be strain specific, as it would be expected that, if such a pattern of expression existed in wine yeast strains, some proline would be removed from the grape must during the initial stages of fermentation. Despite the widely-held view that proline is not utilised during oenological fermentation, some proline (300 mg/L from a starting concentration of 2.6 g/L) was removed during fermentation in a study conducted by Ingledew and colleagues (1987), in contrast with other studies, in which proline was either not removed from the must or was seen to increase (Ough and Stashak, 1974; Salmon and Barre, 1998).

The addition of varying levels of oxygen as the cells entered the stationary phase of growth led to an increase in fermentation rate in both KP2 and KP71. However, there was no difference in the increase in fermentation rate between the two strains. Data on the levels of proline in the media are not available, therefore it is difficult to determine why the mutant does not differ from the wild type. It is possible that Put4p activity in KP71 was too low to transport a significant amount of proline at this point of

fermentation. Alternatively, if the preferred nitrogen sources had been depleted from both cultures, *PUT4* would be upregulated in KP2 as well as in KP71. It is also possible that, even if strain KP71 was capable of proline transport at the point of oxygen addition, the electron transport chain may not have been functional. This would have precluded proline catabolism, as the proline oxidase enzyme requires both molecular oxygen and a functional electron transport chain (Duteurtre *et al.*, 1971). Alternatively, the utilisation of proline may not lead to an increase in fermentation rate as the degradation of proline may be energetically unfavourable. Proline is a poor nitrogen source, supporting a slow rate of growth when present as the sole source of nitrogen. Presumably, more energy is required for the conversion of proline to glutamate than for the conversion of other nitrogen sources to components of the central nitrogen metabolism (i.e. ammonium, glutamine or glutamate).

The percentage viability of KP71 cultures at the end of fermentation was increased in comparison to that of KP2. There are diverse data that suggest that proline plays a role in the stress response of *S. cerevisiae*. The disruption of *PUT1* caused proline accumulation and led to a 2-5% increase in percentage viability compared with the control strain after exposure to freezing and desiccation stress (Takagi *et al.*, 2000), and the addition of proline (30 mM) to a fermentation containing high concentrations of glucose (350 g/L) led to an increase in viability over the course of fermentation (Thomas *et al.*, 1994). In addition, the *PUT4* promoter contains stress-responsive elements and the *PUT4* gene is upregulated in response to salt stress (Posas *et al.*, 2000; Yale and Bonhert, 2001) and osmotic stress (Rep *et al.*, 2000).

Proline is a highly soluble compound and its accumulation is presumed to allow cells to balance the osmolarity of the cytoplasm with the surrounding medium (Le Rudulier *et al.*, 1984). However, proline has other properties that may contribute to a role in the response to stress. Proline in solution is able to increase the solubility of proteins, reducing protein precipitation by ethanol and ammonium sulphate (Schobert and Tschesche, 1978). Proline has also been shown to prevent the aggregation of proteins during refolding (Samuel *et al.*, 2000).

Proline has long been known to act as an osmoregulator in plants and bacteria. In model organisms for both gram-negative (*E. coli*) and gram-positive (*Bacillus subtilis*)

bacteria, proline is accumulated within the cell during osmotic stress (Reviewed in Kempf and Bremer, 1998). Proline accumulation in response to stress in plants is due to the down-regulation of the proline catabolic genes and the induction of the components of the proline synthesis pathway (Yoshiba *et al.*, 1997). However, proline accumulation in the gram-positive bacteria, *Bacillus subtilis*, is facilitated by a strong increase in proline uptake via the OpuE permease, in high-osmolarity environments (Von Blohn *et al.*, 1997). There are two proline transport systems in *E. coli*, one of which is upregulated by osmotic stress (Grothe *et al.*, 1986). The increase in cell viability observed in KP71 may be due to the accumulation of proline via the increased Put4p activity over the course of fermentation.

An increase in the percentage of viable cells at the end of oenological fermentation may be beneficial, as sluggish fermentation might be avoided in some cases. The use of a strain capable of proline transport, such as KP71, might also be useful in the brewing process, where viability is important as the yeasts are typically harvested at the end of fermentation and used to inoculate successive fermentations. In order to further investigate the affect of increased proline transport on viability in such industrial processes, the modifications identified in this study need to be introduced into an industrial strain. The W303 strain was selected for use in this study, because it can completely ferment high concentrations of glucose (180 g/L) under anaerobic conditions, and because an industrial yeast appropriate for use in genetic studies was not available. Such an industrial wine yeast derivative has recently been developed within the laboratory, by deletion of the *HO* gene and the *URA3* gene from the wine yeast, L2056 (M. Walker, personal communication). Work is now under way to introduce the modifications generated through this study into such a strain and to use it to produce industry-relevant data on the fermentative and viability properties of such a strain.

Chapter 7 General Discussion

Nitrogen availability is a determinant of oenological fermentation reliability. Nitrogen depletion leads to the cessation of fermentation (Salmon, 1989), and nitrogen deficiency leads to sluggish fermentation (Bisson, 1991) and may also result in the production of hydrogen sulphide (Jiranek et al., 1995a). The amount of assimilable nitrogen (ammonium and alpha amino acids) present in grape must varies and is often insufficient for the avoidance of such fermentation problems.

In the wine industry, the supplementation of nitrogen in grape must is usually achieved by the addition of diammonium phosphate (DAP). The regulation of DAP addition in various countries limits the amount of nitrogen that can be added in this form (Europe - 63 mgN/L; Australia - 116 mgN/L; North America - 199 mgN/L). In comparison, the average concentration of proline (749 mg/L), a naturally occurring and yet unutilised nitrogen source in grape must, equates to 90 mgN/L. As such, the construction of yeast strains capable of utilising proline as a nitrogen source under oenological conditions provides an alternate strategy to increase fermentation reliability by increasing nitrogen availability.

The yeast *S. cerevisiae* is capable of growth on proline as the sole nitrogen source. However, this growth is dependent on the presence of oxygen (Dutuertre *et al.*, 1971). During oenological fermentation, conditions rapidly become anaerobic, precluding proline utilisation after the first few hours of fermentation. Proline is generally not utilised during the initial, aerobic stages of fermentation, as the presence of preferred nitrogen sources inhibits proline transport into the cell. It is widely believed that no proline is utilised during oenological fermentation and, in some cases, the concentration of proline is seen to increase (Ough and Stashak, 1974). This increase in proline is presumably due to the export of proline (an intermediate in the arginine degradation pathway) from the cell by Put4p, the only amino acid permease that can mediate bi-directional flux of its substrate (Horak and Rihova, 1982; Horak, 1997).

Even when it is utilised, proline is a poor nitrogen source. When provided as the sole source of nitrogen, the slower doubling time observed suggests that the conversion of

proline to glutamate requires more energy than the conversion of preferred nitrogen sources to components of the central nitrogen metabolic pool (ammonium, glutamine or glutamate). This is likely due to the structure of the imino acid proline, which contains a secondary amino group in the proline ring. Consequently, the catabolism of proline to glutamate requires ring cleavage instead of a transamination reaction producing glutamate or a direct deamination reaction yielding ammonium, which are involved in the catabolism of other amino acids. In addition to a requirement for oxygen for the reaction catalysed by proline oxidase, one NAD^+ molecule is required for the generation of glutamate catalysed by the pyrroline-5-carboxylate dehydrogenase.

The yeast *S. cerevisiae* discriminates between good and poor nitrogen sources, preferentially accumulating and catabolising those that support the fastest doubling times (Cooper, 1982). As proline is a poor nitrogen source, its transport and catabolism is repressed by the presence of preferred nitrogen sources such as ammonium and glutamine (Lasko and Brandriss, 1981; Courchesne and Magasanik, 1983; Couchesne and Magasanik, 1988; Xu *et al.*, 1995). The catabolism of proline is regulated, in part, by the exclusion of proline, which is the inducer of the proline catabolic genes *PUT1* and *PUT2* (Brandriss and Magasanik, 1979). Hypothetically, then, modifications allowing the transport of proline into the yeast cell, in addition to the presence of oxygen, should lead to the utilisation of proline as a nitrogen source.

Proline transport in *S. cerevisiae* occurs via the general amino acid permease, Gap1p, and the proline specific permease, Put4p (Regenberg *et al.*, 1999). Both of these permeases are subject to control in response to the quality of available nitrogen at the transcriptional and post-translational level (Courchesne and Magasanik, 1983; Grenson, 1983a; Courchesne and Magasanik, 1988; Stanbrough and Magasanik, 1995; Xu *et al.*, 1995). The aim of this study was to produce strains capable of proline transport under repressive conditions. However, the genetic modification to achieve this phenotype was to be reduced to a minimum in order to facilitate the long-term goal of this project, which is to generate strains more likely to be accepted for use in industry. It proved possible to generate a strain (KP71) capable of increased proline transport over the course of the growth phase by the modification of two base pairs in the *PUT4* gene. The modifications entailed one mutation in the promoter region, resulting in expression in the presence of the preferred nitrogen source, ammonium, and one in the coding

sequence, which reduces the post-translational down-regulation of the permease in response to ammonium.

The increase in proline transport activity over the course of the growth phase became important, as it was discovered that the removal of oxygen from a hyperoxygenated must post-inoculation is rapid, with the depletion occurring before the onset of the exponential phase of growth. Such rapid depletion of oxygen restricts the amount of proline that can be catabolised. Consequently, oxygen additions were investigated as a means to increase the fermentation rate in a strain capable of proline transport, in comparison to the wild type. Whilst an increase in fermentation rate and a decrease in fermentation time were observed if oxygen was added during fermentation, there was no improvement in the fermentation kinetics of the strain capable of proline transport in comparison with the wild type. The lack of detectible benefit in terms of fermentation kinetics in the KP71 mutant may have several explanations: i) depletion of preferred nitrogen sources results in an upregulation of Put4p activity in the wild type making this strain analogous to the transport-capable strain, ii) a functional electron transport chain is lacking, or iii) the degradation of proline may be energetically unfavourable. More exhaustive oxygenation experiments need to be conducted in order to further investigate any potential benefit of proline transport during yeast growth in an oenological fermentation.

The strain W303 was selected on the basis of its ability to ferment high concentrations of glucose, combined with the ease of genetic manipulation of such a laboratory isolate. However, during the course of this study, differences were noted between published data and the results obtained here. There is no mention in the literature of a transient expression of *PUT4* after inoculation into fresh media. This expression and the resulting Put4p activity account for the proline removal from a chemically defined grape juice medium during the initial stages of fermentation, in contrast to the widely held belief that proline levels do not decrease over the course of fermentation. In one notable exception, Ingledew and Kunkee (1985) noted a decrease in proline levels during fermentation, suggesting that, in some *S. cerevisiae* strains, nitrogen catabolite repression of the proline specific permease is less stringent. Any improvements in the fermentation kinetics of a constitutive *PUT4* strain might only be apparent in a strain background where *PUT4* is not expressed in the wild type during the initial stages of

fermentation. Consequently, the absence of a transient expression of *PUT4* in an industrial wine yeast should be confirmed before the mutations that lead to increased proline transport are transferred into this more appropriate strain for further investigation of the potential benefits of such a phenotype.

To date, the key advantage of the strain capable of proline transport, KP71, is that it exhibited an increased viability at the end of fermentation in comparison with the wild type. Proline is an osmoprotectant in plants and bacteria (Reviewed in Le Rudulier *et al.*, 1982), and evidence exists that it may be involved in the stress response of *S. cerevisiae*. The expression of *PUT4* is upregulated under stressful conditions, including in response to salt stress (Yale and Bohnert, 2001) and osmotic shock (Rep *et al.*, 2000), in the presence of ethanol and from 2 days into the stationary phase (Gasch *et al.*, 2000). Increased intracellular proline levels increase viability after freezing and desiccation (Takagi *et al.*, 2000) and during high-gravity fermentation (Thomas *et al.*, 1994). The modification of the *PUT4* gene to allow increased expression and decreased inactivation within a wine yeast isolate may help avoid fermentation problems arising at the end of fermentation even in the absence of proline catabolism. The presence of greater amounts of proline within the cell would not only help osmotic balance, but also would help prevent protein aggregation (Samuel *et al.*, 2000) that occurs in conditions of stress.

The finding that increased proline transport has an impact beyond the scope of nitrogen metabolism highlights the need for a fuller evaluation of the strains capable of proline transport than was afforded within this study. The consequences for processing of the sensory properties of wine produced with such a yeast might also be examined. However, even before the full benefits of this novel approach to dealing with fermentation problems that arise out of a deficiency of assimilable nitrogen are defined, new approaches to dealing with these problems now also seem feasible. For example, the efficiency of nitrogen utilisation by *S. cerevisiae* could also be targeted. This is possible because the demand for nitrogen during fermentation varies between strains (Manginot *et al.*, 1997) and thus must be able to be manipulated. Therefore, the adaptive evolution of strains in continuous culture (Ferea *et al.*, 1999) under nitrogen-limiting conditions, for example, may enable the isolation of a strain with a significantly decreased requirement for nitrogen during fermentation. Strains isolated in this way

have the advantage of not having arisen out of genetic manipulation, thereby preventing possible consumer backlash to their industrial application. The availability of microarray technology and the complete *S. cerevisiae* genome sequence would further allow comparison of the evolved strain with its parental, providing insight into the mechanisms of nitrogen efficiency.

Appendix 1 Solutions

AE Buffer

50 mM sodium acetate
10 mM EDTA
pH 4.8

Alkaline Phosphatase Buffer

100 mM Tris-HCl, pH 9.0
100 mM NaCl
5 mM $MgCl_2$

Blocking Buffer

5 % (w/v) non-fat dry milk in 1 x TBST

Blocking Solution

10 % (w/v) blocking reagent
in 10 x Maleic Acid Buffer

Buffered Lithium Solution

100 mM lithium acetate
1 x TE, pH 7.5

Cell Lysis Solution

2 % Triton X-100
1 % SDS
100 mM NaCl
10 mM Tris-Cl (pH8)
1 mM Na_2EDTA

Chemically Defined Grape Juice Medium

Component		Amount/litre
Carbon	Glucose	180 g
Salts	potassium tartrate	2.5 g
	L-malic acid	3 g
	K_2HPO_4	1.14 g
	$MgSO_4 \cdot 7H_2O$	1.23 g
	$CaCl_2 \cdot 2H_2O$	0.44 g
Trace Minerals	$MnCl_2 \cdot 2H_2O$	198.2 µg
	$ZnCl_2$	135.5 µg
	$FeCl_2$	32.0 µg
	$CuCl_2$	13.6 µg
	H_3BO_3	5.7 µg
	$Co(NO_3)_2 \cdot 6H_2O$	29.1 µg
	$NaMoO_4 \cdot 2H_2O$	24.2 µg
	KIO_3	10.8 µg

Vitamins	myo-inositol	100 mg
	pyridoxide·HCl	2 mg
	nicotinic acid	2 mg
	calcium pantothenate	1 mg
	thiamine·HCl	0.5 mg
	ρ-amino benzoic acid	0.2 mg
	riboflavin	0.2 mg
	biotin	0.125 mg
	folic acid	0.2 mg
Nitrogen	$(NH_4)_2SO_4$	5 g

Cracking Solution

50 mM NaOH
0.5 % SDS
0.1 M EDTA
5 mM glycerol
0.005 % bromophenol blue

Detection Buffer

100 mM Tris-HCl, pH 9.5
100 mM NaCl

6 x DNA Loading Buffer

0.25 % bromophenol blue
0.25 % xylene cyanol
30 % glycerol

Formaldehyde gel

For 100 ml gel

1.5 g agarose
72 ml DEPC-treated MilliQ water
10 ml 10x MOPS Electrophoresis Buffer
18 ml formaldehyde

Formaldehyde gel-Loading Buffer (10x)

50 % glycerol
10 mM EDTA (pH 8.0)
0.25 % (w/v) bromophenol blue
0.25 % (w/v) xylene cyanol FF

LB Medium

For 1L

10g tryptone
10g NaCl
5g yeast extract

Maleic Acid Buffer, 10 x

0.1 M maleic acid
0.15 M NaCl
pH 7.5 (pH adjusted with 10 M NaOH)

Milieu Synthetique (MS)

Component		Amount/L
Sugar	glucose	180
Acids	malic acid (DL)	6
	citric acid	6
Minerals	KH_2PO_4	0.75
	K_2SO_4	0.5
	$MgSO_4 \cdot 7H_2O$	0.25
	$CaCl \cdot 2H_2O$	0.155
	NaCl	0.2
Nitrogen	NH_4Cl	0.46
	Stock solution-amino acids	20.36 ml
Trace elements	Stock solution (1000x)	1 ml
Vitamins	Stock solution (100x)	10 ml
Anaerobic factors	Stock solution (1000x)	1 ml

Amino acid stock solution (MS)

Amino Acid	concentration (g/L)
tyrosine	1.4
tryptophan	13.7
isoleucine	2.5
aspartic acid	3.4
glutamic acid	9.2
arginine	28.6
leucine	3.7
threonine	5.8
glycine	1.4
glutamate	38.6
alanine	11.1
valine	3.4
methionine	2.4
phenylalanine	2.9
serine	6

histidine	2.5
lysine	1.3
cysteine	1
proline	46.8

Vitamin stock solution (MS)

Vitamin	Concentration (g/L)
myo-inositol	2.0
pantothenic acid	0.15
thiamine·HCl	0.025
nicotinic acid	0.2
pyridoxine	0.025
biotin	0.0003

Trace elements stock solution (MS)

Element	Concentration (g/L)
$MnSO_4 \cdot H2O$	4
$ZnSO_4 \cdot 7H2O$	4
$CuSO_4 \cdot 5H2O$	1
KI	1
$CoCl_2 \cdot 6H2O$	0.4
H_3BO_3	1
$(NH4)_6Mo_7O_{24}$	1

Anaerobic factor stock solution (MS)

ergosterol	1.5 g
oleic acid	0.5 g
ethanol	50 ml
Tween-80	50 ml

MOPS Electrophoresis Buffer (10x)

0.2 M MOPS, pH 7.0
20 mM sodium acetate
10 mM EDTA, pH 8.0

PBS

For 1L

0.2 g KCl
8 g NaCl
0.2 g KH_2PO_4
1.15 g Na_2HPO_4

PEG Solution

40 % polyethylene glycol 3400
1 x TE, pH 8.0
100 mM lithium acetate

Ringer Solution
For 1L
- 8.5 g NaCl
- 0.25 g KCl
- 0.2 g $NaHCO_3$
- 0.3 g $CaCl_2$
- 10 g glucose

RNA Dilution Buffer
- MilliQ water:20 x SSC:formaldehyde (5:3:2)

Saturated Ammonium Sulphate Solution
- 450 g $(NH_4)_2SO_4$
- H_2O to 500ml

Solution heated whilst stirring until ammonium sulphate completely dissolved

SDS Electrophoresis Running Buffer
For 1L
- 30.2 g Tris base
- 144 g glycine
- 10 g SDS

SDS Sample Buffer (2x)
- 100 mM Tris-Cl (pH 6.8)
- 4 % (w/v) SDS
- 0.2 % (w/v) bromophenol blue
- 20 % (v/v) glycerol
- 200 mM β-mercaptoethanol
- 10 mM Tris-Cl (pH 7.5)
- 1 mM EDTA

Solution 1
- 50 mM glucose
- 25 mM Tris-HCl, pH 8.0
- 10 mM EDTA

Solution 2
- 0.2 N NaOH
- 1 % SDS

Solution 3
- 3 M potassium acetate
- 2 M acetic acid
- pH 4.8

Sporulation Medium
For 1L
- 10 g potassium acetate
- 2.5 g yeast extract
- 1 g glucose

20 x SSC

3 M NaCl
300 mM sodium citrate, pH 7.0

50 x TAE

For 1L

242 g Tris base
57.1 ml glacial acetic acid
0.05 M EDTA

TBS

For 1L

0.01 M Tris-HCl, pH 8.0
8.8 g NaCl

TBST

0.05 % Tween-20 in TBS

Tris EDTA buffer (TE)

10 mM Tris-Cl (pH 7.5)
1 mM EDTA

Wash Buffer, 10 x

0.3 % Tween-20
in 10 x Maleic Acid Buffer

Washing Solution, 2 x

2 x SSC
0.1 % SDS

Washing Solution, 0.5 x

0.5 x SSC
0.1 % SDS

Western Transfer Buffer

25 mM Tris
192 mM glycine
20 % methanol

Working Solution

16.65 ml Buffer/NADP/ATP
330 µl hexokinase
330 µl phosphoglucose isomerase
33.35 ml MilliQ water

Buffer/NADP/ATP

0.75 mol/L triethanolamine, pH 7.6
10 mmol/L $MgSO_4$
1.2 mmol/L NADP
8 mmol/L ATP

Yeast Nitrogen Base (YNB) medium
For 1 L

100 g glucose
1.7 g salts
1 mL of 1.84 mg/mL Trace components
5 g $(NH_4)_2SO_4$
10 mL vitamin stock

YNB Salts:
For 1 L
0.1 g KH_2PO_4
0.05 g M_2SO_4
0.01 g $CaCl_2$

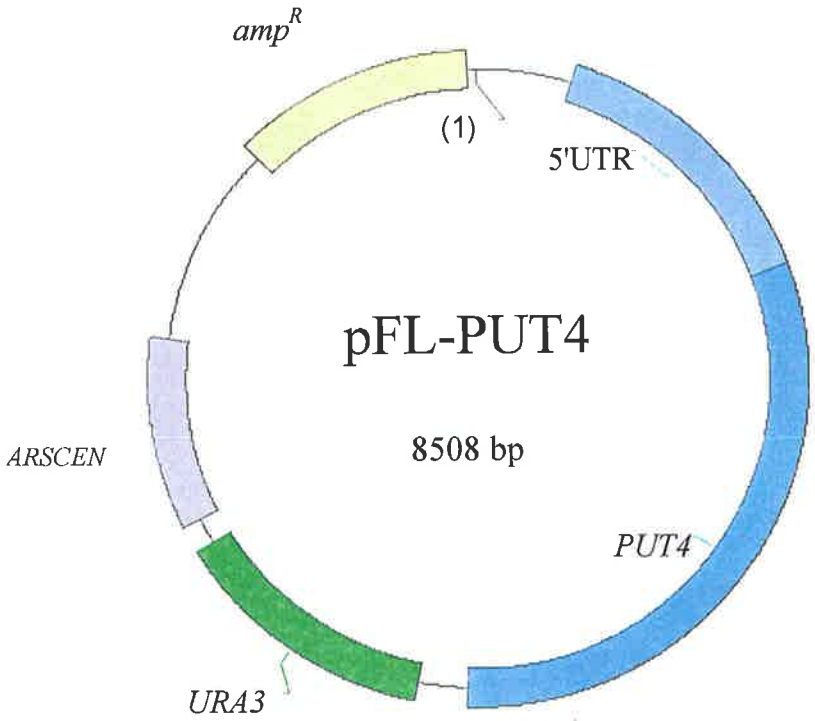
YNB trace components:
For 10,000 L
5 g H_3BO_3
0.4 g $CuSO_4 \cdot 5H_2O$
1 g KI
2 g $FeCl_3 \cdot 6H_2O$
4 g $MnSO_4$
2 g $Na_2MoO_4 \cdot 2H_2O$
4 g $ZnSO_4 \cdot 7H_2O$

YNB Vitamin Stock Solution:
For 1 L
2 μg biotin
0.4 μg calcium pantothenate
2 μg folic acid
0.4 μg nicotinic acid
0.2 μg ρ-aminobenzoic acid
0.4 μg pyridoxine·HCl
0.2 μg riboflavin
0.4 μg thiamine·HCl

YEPD medium
For 1L

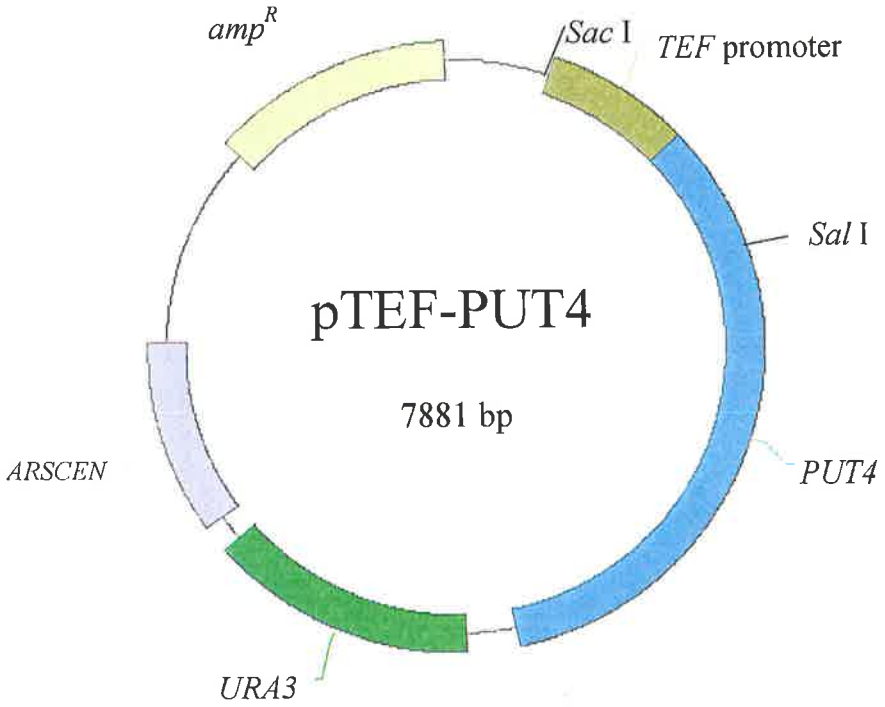
20g glucose
10g yeast extract
20g bacto-peptone

Appendix 2 Plasmid construction



Construction of pFL-PUT4

A 3.9 kb fragment generated with PCR primers, Put4f and Put4r, corresponding to *PUT4* with 1.2 kb 5'untranslated region (UTR), was digested with *Bam* HI and *Hind* III and ligated into the multiple cloning site of similarly digested pFL38 (Bonneaud *et al.*, 1991).



Construction of pTEF-PUT4.

The *TEF2* promoter was excised from the p416TEF vector (Mumberg *et al.*, 1995) using *Sac* I and *Sal* I restriction enzymes. An interim vector (pFL-put4') was constructed by ligating this 600 bp promoter fragment into the 6.7 kb fragment generated by a *Sac* I, *Sal* I digestion of pFL-PUT4. This interim vector was digested with *Sal I*. A *put4Δ::kanMX4 ura3 Δ::kanMX4* strain was co-transformed with the linearised pFL-put4' and a PCR product generated with primers TEFPUTf and Put4r (Table 2.2), allowing generation of the pTEF-PUT4 vector by gap-repair.

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