



# **Identification of genes affecting glucose catabolism in nitrogen-limited fermentation**

**By**

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**A thesis submitted for the degree of Doctor of Philosophy,  
in the Faculty of Sciences**

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The University of Adelaide  
Australia**

**December 2005**



**THE UNIVERSITY  
OF ADELAIDE  
AUSTRALIA**

## **Thesis Summary**

When assimilable nitrogen becomes limiting in fermentation processes such as winemaking, sugar transport systems of the inoculated yeast are inactivated and biomass formation is restricted. As a consequence such fermentations may fail to catabolise all available sugar leaving the product out of specification, at greater risk of spoilage and deterioration and needing greater input to rectify. In recognition of this critical importance of assimilable nitrogen in the successful completion of several fermentation processes, this study has sought to develop yeast strains that utilise this typically limited nutrient group more efficiently. Wine strains, usually of *Saccharomyces cerevisiae*, are known to differ in the efficiency with which they exploit nitrogen. As a consequence, so-called 'nitrogen efficient' strains may offer greater prospects for reliable completion of fermentation.

With the aid of transposon mutagenesis together with a high throughput method for analysis of multiple micro-fermentations, nitrogen efficient mutants were identified that were able to catabolise more sugar for a given amount of utilised nitrogen. Mutants displaying improved nitrogen efficiency were further characterised in shake-flask fermentations and the affected genes were identified with the assistance of Inverse-PCR.

As wine and laboratory yeast strains can be phenotypically different, especially in terms of their ability to affect oenological fermentations, a haploid derivative of the wine yeast strain L-2056 was developed, such that it could be easily genetically manipulated.

Of the identified genes, disruption of *NGR1* and *GID7*, lead to an enhanced catabolism of sugar in both a laboratory strain and a haploid derivative of a wine strain of *Saccharomyces cerevisiae*, during growth in a chemically defined grape juice medium with limiting nitrogen. Deletion of *NGR1* or *GID7* also resulted in minor changes to the amounts in which selected metabolites

were produced (determined by HPLC). Biomass yield (measured as dry weight) was also decreased in *NGR1* mutants.

Previous studies have demonstrated a strong link between assimilable nitrogen and fermentation rate, when other nutrients are not limiting. The total nitrogen utilised and the timing of nitrogen uptake of *ngr1Δ* and *gid7Δ* strains was found to be very similar to the parent strain. Thus it was hypothesised that *ngr1Δ* and *gid7Δ* strains could be using the available nitrogen differently to enable enhanced glucose catabolism.

Deletion of either *NGR1* or *GID7* was found to affect the expression of genes involved in the core pathway for the utilisation of non-preferred nitrogen sources, known as Central Nitrogen Metabolism (CNM). The transcriptional abundance, measured by Real-Time PCR, of *GDH1*, *GDH2*, *GLT1* and *GLN1* was altered in these mutants. This distorted expression of CNM genes could translate to a re-modelling of enzyme quantities and thus re-distribution of the core nitrogen-containing compounds, and thereby the cellular response under nitrogen-limiting conditions.

## **Declaration of Authorship**

This thesis 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 reference has been made in the text.

Essentially all of the work detailed in Chapters 3 and 4 has been published in the form of two scientific articles. These are included in Appendix II. And the references are:

**Gardner, J. M., McBryde, C. M., Vystavelova, A., de Barros Lopes, M., Jiranek, V.** (2005). Identification of genes affecting glucose catabolism in nitrogen-limited fermentation. *FEMS Yeast Research* **5**, 791-800.

**Walker, M. E., Gardner, J. M., Vystavelova, A., McBryde, C., de Barros Lopes, M. and Jiranek, V.** (2003). Application of the re-useable, *KanMX* selectable marker to industrial yeast: construction and evaluation of heterothallic wine strains of *Saccharomyces cerevisiae*, possessing minimal foreign DNA sequences. *FEMS Yeast Research* **4**, 339-347.

This thesis may be made available for loan or photocopying.

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December 2005.

## **Acknowledgements**

First of all, my sincere thanks to my principle supervisor Vladimir Jiranek for his tremendous support, enthusiasm and patience through out this study. It has been a pleasure to learn from you. Also, thanks to my co-supervisor Miguel de Barros Lopes, for sharing his knowledge and for help with many technical aspects of this study.

Thank you to the past and present members of the Microbial Biotechnology Laboratory, it has been a joy to work alongside such talented people. In particular, Michelle Walker, for the keen sharing of ideas and Kate Poole for your warm friendship, laughter and all the beer.

I would like to thank my family, my parents, Elaine and Will, for their never-ending love and encouragement, my brother, Andrew and adopted sister, Sonia for their joyousness and my aunt and uncle, Jennifer and Douglas for our friendship, lively discussions and all the magnificent food and wine.

Finally, I would like to thank Colin for sharing all the things we love and his courageous persistence that have made our dreams a reality.

This project was supported financially by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research and Development Corporation (GWRDC). Further support was kindly provided by Lallemand Australia. I would also like to thank the Australian Postgraduate Award Programme and the GWRDC for the generous scholarship.

**Abbreviations**

|                  |  |
|------------------|--|
| A                | adenine  |
| ABP              | anchor bubble primer                           |
| AP1              | activating protein 1                           |
| ARE              | activating protein 1 responsive element        |
| bp               | base pair                                      |
| °C               | degrees centigrade                             |
| C                | cytosine                                       |
| cAMP             | cyclic AMP                                     |
| CDGJM            | chemically defined grape juice medium          |
| CER              | carbon dioxide evolution rate                  |
| cm               | centimetre                                     |
| CNM              | central nitrogen metabolism                    |
| CO <sub>2</sub>  | carbon dioxide                                 |
| C <sub>T</sub>   | crossing threshold                             |
| DAP              | diammonium phosphate                           |
| DIG              | digoxigenin-11-dUTP                            |
| DNA              | deoxyribonucleic acid                          |
| dNTPs            | deoxynucleotide triphosphates                  |
| EMS              | ethylmethanesulfonate                          |
| FAN              | free amino nitrogen                            |
| G                | guanosine                                      |
| g                | gram   |
| x g              | x gravity                                      |
| GABA             | 4-amino butyrate                               |
| GCN              | general control of amino acid synthesis        |
| GDH              | glutamate dehydrogenase                        |
| GFP              | green fluorescent protein                      |
| GOGAT            | glutamate synthase                             |
| GS               | glutamine synthetase                           |
| h                | hour   |
| H <sub>2</sub> S | hydrogen sulphide                              |
| HNE              | high nitrogen efficiency                       |
| hnRNPs           | heterogeneous nuclear ribonuclear proteins     |
| HOG              | high osmolarity glycerol                       |
| HPLC             | high performance liquid chromatography         |
| HSE              | heat shock elements                            |
| IMVS             | Institute for Molecular and Veterinary Science |
| IPCR             | inverse PCR                                    |
| L                | litre  |
| LB               | Luria-Bertani                                  |
| M                | molar  |
| MAP              | mitogen activated protein                      |
| min              | minute   |
| mL               | millilitre                                     |
| mg               | milligram                                      |
| mM               | millimolar                                     |
| mol              | mole   |
| mRNA             | messenger RNA                                  |

|                    |  |
|--------------------|--|
| N/M/P              | asparagine / methionine / proline                        |
| NAD <sup>+</sup>   | nicotinamide adenine dinucleotide                        |
| NADH               | nicotinamide adenine dinucleotide reduced form           |
| NADP               | nicotinamide adenine dinucleotide phosphate              |
| NADPH              | nicotinamide adenine dinucleotide phosphate reduced form |
| NCR                | nitrogen catabolite repression                           |
| nmol               | nanomole   |
| NOPA               | ophtaldialdehyde/n-acetyl-L-cysteine                     |
| NRD                | negative regulatory domain                               |
| OD                 | optical density  |
| PCR                | polymerase chain reaction                                |
| PKA                | protein kinase A   |
| pmol               | picomole   |
| QRTPCR             | quantitative real time polymerase chain reaction         |
| RNA                | ribonucleic acid   |
| rpm                | revolutions per minute                                   |
| RRM                | RNA recognition motif                                    |
| sec                | second   |
| SDS                | sodium dodecyl sulphate                                  |
| snRNPs             | small nuclear ribonuclear proteins                       |
| STRE               | stress responsive element                                |
| TCA                | tri-carboxylic acid                                      |
| Tn                 | transposon   |
| TNBS               | ninhydrin,2,4,6-trinitrobenzene sulfonic acid            |
| TOR                | target of rapamycin                                      |
| U                  | units  |
| UAS                | upstream activating sequence                             |
| UAS <sub>NTR</sub> | nitrogen regulated upstream activating sequence          |
| μL                 | microlitre   |
| μM                 | micromolar   |
| URS                | upstream regulatiry sequence                             |
| V                  | volt   |
| v/v                | volume per volume  |
| VA                 | volatile acidity   |
| w/v                | weight per volume  |
| YAN                | yeast assimilable nitrogen                               |
| YEPD               | yeast extract peptone dextrose                           |

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**Table of Contents**

|                           |     |
|---------------------------|-----|
| Thesis Summary            | i   |
| Declaration of Authorship | iii |
| Acknowledgements          | iv  |
| Abbreviations             | v   |

**Chapter 1 Literature Review**

|      |  |    |
|------|--|----|
| 1.1  | Introduction   | 1  |
| 1.2  | Stuck fermentation   | 2  |
| 1.3  | The effect of nitrogen on fermentation dynamics                      | 6  |
| 1.4  | The physiological effects of nitrogen starvation during fermentation | 8  |
| 1.5  | Nitrogen compounds in wine   | 9  |
| 1.6  | The nitrogen demand of yeast   | 10 |
| 1.7  | Yeast sense nitrogen sources in the extracellular environment        | 12 |
| 1.8  | Yeast cells monitor their intracellular nitrogen pools               | 14 |
| 1.9  | Nitrogen catabolite repression                                       | 15 |
| 1.10 | Nitrogen import into the cell  | 18 |
| 1.11 | Central Nitrogen Metabolism  | 20 |
| 1.12 | Links between nitrogen and carbon metabolism                         | 21 |
| 1.13 | The effect of yeast strain and nitrogen utilisation on wine aroma    | 22 |
| 1.14 | Concluding statement   | 24 |

**Chapter 2 Materials and Methods**

|     |   |    |
|-----|---|----|
| 2.1 | Yeast strains and maintenance   | 26 |
| 2.2 | Bacterial strains and maintenance   | 26 |
| 2.3 | Culture media   | 26 |
|     | 2.3.1 <i>Media for yeast cultures</i>   | 26 |
|     | 2.3.2 <i>Chemically defined grape juice media</i>   | 26 |
| 2.4 | Growth and fermentation   | 27 |
|     | 2.4.1 <i>Mini-fermentations</i>   | 27 |
|     | 2.4.2 <i>Laboratory scale anaerobic fermentations</i>                                     | 27 |
|     | 2.4.3 <i>Dry cell weight determination</i>  | 28 |
|     | 2.4.4 <i>Determination of glucose and ammonia by enzymatic analysis</i>                   | 28 |
|     | 2.4.5 <i>Determination of other metabolites by HPLC</i>                                   | 29 |
|     | 2.4.6 <i>Viable cell counts</i>   | 30 |
| 2.5 | Yeast classical genetics  | 30 |
|     | 2.5.1 <i>Yeast mating</i>   | 30 |
|     | 2.5.2 <i>Yeast rare mating</i>  | 30 |
|     | 2.5.3 <i>Yeast sporulation</i>  | 30 |
|     | 2.5.4 <i>Ascus microdissection</i>  | 31 |
|     | 2.5.5 <i>High efficiency transformation of <i>S. cerevisiae</i> using lithium acetate</i> | 31 |
| 2.6 | Nucleic acid isolation  | 32 |
|     | 2.6.1 <i>Isolation of genomic DNA from <i>S. cerevisiae</i></i>                           | 32 |

---



|       |   |    |
|-------|---|----|
| 2.6.2 | <i>RNA preparations from yeast</i>  | 32 |
| 2.6.3 | <i>Determination of DNA or RNA concentration</i>                                  | 33 |
| 2.6.4 | <i>Plasmid preparation from E. coli</i>   | 33 |
| 2.6.5 | <i>Rapid determination of plasmid DNA size</i>                                    | 34 |
| 2.7   | Molecular cloning techniques  | 34 |
| 2.7.1 | <i>Restriction endonuclease digestion of DNA</i>                                  | 34 |
| 2.7.2 | <i>Dephosphorylation of vector DNA</i>  | 35 |
| 2.7.3 | <i>Ligation of DNA into plasmid</i>   | 35 |
| 2.7.4 | <i>Preparation and use of competent E. coli cells for transformation</i>          | 35 |
| 2.8   | Nucleic acid amplification procedures   | 36 |
| 2.8.1 | <i>Polymerase Chain Reaction (PCR)</i>  | 36 |
| 2.8.2 | <i>Vectorette PCR</i>   | 36 |
| 2.8.3 | <i>Sequencing reactions</i>   | 37 |
| 2.8.4 | <i>Direct genomic sequencing</i>  | 37 |
| 2.8.5 | <i>Colony cracking PCR for rapid screening of transformants</i>                   | 38 |
| 2.8.6 | <i>PCR labelling of probes for Southern Blot analysis</i>                         | 38 |
| 2.8.7 | <i>Quantitative Real Time PCR</i>   | 38 |
| 2.9   | Southern Blot analysis of genomic DNA   | 39 |
| 2.10  | Random chemical mutagenesis   | 40 |
| 2.11  | Determination of flocculation status  | 40 |
| 2.12  | Disruption and selection of mitochondrial mutants by ethidium bromide mutagenesis | 41 |

### **Chapter 3 Construction of a wine yeast derivate suitable for research using classical and molecular genetic techniques**

|       |   |    |
|-------|---|----|
| 3.1   | Introduction  | 42 |
| 3.2   | Results   | 43 |
| 3.2.1 | <i>Analysis of industrial wine yeast strains suitable for genetic manipulation</i>    | 43 |
| 3.2.2 | <i>Construction of haploid L-2056 strains</i>   | 44 |
| 3.2.3 | <i>Production of non-flocculant L-2056 1B and L-2056 1D</i>                           | 45 |
| 3.2.4 | <i>Comparison of the fermentation properties of L-2056, L-2056 1Ba and L-2056 1Da</i> | 46 |
| 3.2.5 | <i>Generation of a uracil auxotrophy into L-2056 1B</i>                               | 47 |
| 3.3   | Conclusions   | 48 |
| 3.4   | Discussion  | 48 |

### **Chapter 4 Identification of genes affecting glucose catabolism in nitrogen-limited fermentation**

|       |   |    |
|-------|---|----|
| 4.1   | Introduction  | 52 |
| 4.2   | Results   | 53 |
| 4.2.1 | <i>Construction of JMG2 and JMG3</i>  | 53 |
| 4.2.2 | <i>Preparation of the mTn3xHA/GFP/URA3 library</i>                          | 53 |
| 4.2.3 | <i>Selection of mutants with a high nitrogen efficiency (HNE) phenotype</i> | 54 |

|   |  |    |
|---|--|----|
| 4.2.4   | <i>Identification of genes influencing nitrogen efficiency</i>   | 57 |
| 4.2.5   | <i>Deletion of NGR1 or GID7 confers nitrogen efficiency upon KP2</i>   | 59 |
| 4.3   | Conclusions  | 60 |
| 4.4   | Discussion   | 60 |
| <br>  |  |    |
| <b>Chapter 5 Deletion of NGR1 or GID7 from a haploid wine yeast increases glucose catabolism and also affects other aspects of fermentation</b> |  |    |
| 5.1   | Introduction   | 66 |
| 5.2   | Results  | 67 |
| 5.2.1   | <i>Deletion of HNE genes from C9</i>   | 67 |
| 5.2.2   | <i>Determination of the glucose utilisation profiles of ngr1Δ and gid7Δ strains</i>  | 67 |
| 5.2.3   | <i>Determination of ammonia utilisation by ngr1Δ and gid7Δ strains</i>   | 68 |
| 5.2.4   | <i>Analysis of the biomass yield of ngr1Δ and gid7Δ strains</i>  | 68 |
| 5.2.5   | <i>Analysis of the cellular morphology of ngr1Δ and gid7Δ strains during fermentation</i>  | 70 |
| 5.2.6   | <i>Analysis of the major fermentation metabolites of ngr1Δ and gid7Δ strains</i>   | 70 |
| 5.2.7   | <i>Construction of KP2 ngr1 gid7</i>   | 72 |
| 5.2.8   | <i>Determination of the glucose utilisation of KP2 ngr1 gid7</i>   | 72 |
| 5.3   | Conclusions  | 73 |
| 5.4   | Discussion   | 73 |
| 5.4.1   | <i>Fermentation profiles of ngr1Δ and gid7Δ strains</i>  | 73 |
| 5.4.2   | <i>Combination of ngr1Δ and gid7Δ mutations in a single strain</i>   | 74 |
| 5.4.3   | <i>Mechanisms of nitrogen efficiency of ngr1Δ and gid7Δ strains</i>  | 75 |
| 5.4.4   | <i>Cellular growth of ngr1Δ and gid7Δ strains</i>  | 75 |
| 5.4.5   | <i>The production of cellular metabolites by ngr1Δ and gid7Δ strains</i>   | 77 |
| <br>  |  |    |
| <b>Chapter 6 The effect of deletion of NGR1 and GID7 on the transcription of genes involved in Central Nitrogen Metabolism.</b>                 |  |    |
| 6.1   | Introduction   | 81 |
| 6.2   | Results  | 83 |
| 6.2.1   | <i>Validation and controls used for Quantitative Real Time PCR</i>   | 83 |
| 6.2.2   | <i>Quantification of the transcription from genes involved in Central Nitrogen Metabolism: The effect of deletion of NGR1 or GID7 from KP2</i> | 83 |
| 6.2.3   | <i>NGR1 and GID7 expression</i>  | 84 |
| 6.3   | Conclusions  | 84 |
| 6.4   | Discussion   | 85 |

|                     |   |    |
|---------------------|---|----|
| <b>Chapter 7</b>    | <b>General Discussion and Future Directions</b> | 91 |
| <b>Appendix I</b>   | <b>Solutions</b> (additional to those in text)  | 95 |
| <b>Appendix II</b>  | <b>Papers arising from this study</b>           | 98 |
| <b>Bibliography</b> |   | 99 |

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## **Chapter 1 Literature Review**

### ***1.1 Introduction***

The rapid growth of the Australian Wine Industry has led to an increase in the demand for winemaking tools and techniques that enable winemakers to have greater control of fermentation. With so many wine brands now available in the market place, it is increasingly important to not only produce a higher quality product, but also one that is discernibly different. Access to novel microbial strategies will aid winemakers to not only preserve the positive characteristics of the grape must, but also to change and enhance wine products with additional aroma, flavour, mouth-feel and colour characteristics. Acceptance of such products is thought to be likely, as interest in new microbial strategies by the New World winemaking community has been well proven by the now commonly utilised purified yeast and malolactic bacterial cultures. This study has provided information that may be adapted to produce new yeast strains for industrial use, as well as personnel trained as suitable points of contact for the wine industry, so that these microbiological procedures can translate to a reliable outcome.

Yeast are responsible for the fermentation of grape must into wine. Under mostly anaerobic conditions yeast ferment monosaccharides (glucose and fructose) into ethanol, carbon dioxide and other minor compounds. Traditionally wine makers have relied upon the indigenous yeasts present on the surface of grapes and on winery equipment. Uninoculated fermentations are initially dominated by non-*Saccharomyces* species, such as those belonging to the genera of *Hanseniaspora*, *Candida* or *Kloeckera* (Fleet, 1993; Fugelsang, 1997), but is typically completed by *Saccharomyces cerevisiae*. Modern winemaking practices, especially in New World wine producing regions, now incorporate the purposeful inoculation of selected *S. cerevisiae* with favourable oenological characteristics. Even though the utilisation of specific yeast strains allows winemakers better control over the characteristics of fermentation, problem fermentations, specifically those which are sluggish or fail to complete, are still common. With the recognition of the central role of juice

nitrogen deficiencies in many of these problem ferments, the use of nitrogen supplements has become widespread and routine. Nevertheless this strategy is not always successful. Nitrogen additions are a valuable tool for prevention and control of stuck fermentations, yet the timing of addition is critical and the presence of residual nitrogen can favour microbial spoilage and formation of a carcinogen, ethyl carbamate. At any rate, it is also desirable to reduce additives made to premium wines.

Another option for the prevention of stuck fermentation, which is only recently beginning to be employed, is the use of “nitrogen efficient” wine yeast strains. The aim of this study was to develop new and alternative strategies for dealing with low nitrogen juices based on such strains. Specifically, genes were identified that when altered, conferred a greater efficiency of nitrogen utilisation during fermentation.

### ***1.2 Stuck fermentation***

The completion of fermentation is highly desirable and necessary to ensure that a finished wine is within industrial specifications (Iland and Gago, 2002). When fermentations are incomplete, high levels of residual sugar and low levels of alcohol can encourage microbial spoilage and the formation of undesirable aromas and flavours. It is well known that stuck fermentations are one of the major problems in the winemaking industry. Even with the use of preventative treatments approximately 5-10 % of industrial fermentations experience some degree of sluggishness or become stuck (P. Leske, Nepenthe wines; T. Adams, Tim Adams Wines and N. Bourke, McLaren Vale Vitners; pers comm). A sluggish fermentation is one which exceeds the average time taken to reach a desired sugar concentration (usually less than 0.4 %) (Bisson, 1999). Typically, fermentations will take 7-10 days to reach dryness, whereas sluggish fermentations may exceed 55-85 days (Henschke and Jiranek, 1993). Stuck fermentations are those that completely arrest leaving a higher than desirable residual sugar content in the wine.

Management of sluggish or stuck fermentations is expensive and laboursome. For example, long duration fermentations tie up fermentor space and require extra antioxidant and antimicrobial operations due to the loss of the protective

carbon dioxide blanket typically found over an active fermentation. Furthermore, re-starting a stuck fermentation requires additional resources and operations, and even if successful the wine may already be tainted by the development of “stuck fermentation/long fermentation characters”, such as the presence of hydrogen sulphide or volatile acidity.

Several factors are known to contribute to the occurrence of stuck fermentation, most commonly they are (in no particular order): restricted availability of oxygen, improper inoculum preparation, excessive clarification, aggressive heating/chilling regimes, high concentrations of ethanol and/or nutritional or vitamin deficiencies (Houtman and du Plessis, 1986; Kunkee, 1991; Sablayrolles, 1996; Alexandre and Charpentier, 1998; Bisson, 1999). The presence of sufficient assimilable nitrogen in particular is a key factor in the prevention of stuck fermentation as it is necessary for yeast biomass formation and the maintenance of fermentation activity (Agenbach, 1977; Ingledew and Kunkee, 1985; Bely, et al., 1990a; 1990b). Wine yeast that are starved for nitrogen have limited growth and the catabolism of sugar is greatly reduced. Nitrogen starvation results in a depletion of cytosolic amino acids and their precursors, making them unavailable for protein synthesis. A shortage in the supply of amino acids results in a slowing of protein synthesis, thereby arresting cells in the G1 phase.

Nutritional starvation (including nitrogen starvation) also activates stress signalling cascades which are mediated by positive transcriptional control elements such as stress signalling responsive elements (STRE), activating protein 1 (AP1) responsive elements (ARE) and heat shock elements (HSE) (Ruis and Schuller, 1995). These elements mediate the activation of stress responsive genes. Consequently cells under nitrogen limitation enter stationary phase, which characteristically includes the accumulation of reserve carbohydrates glycogen and trehalose to aid in prolonged survival in a nutrient limited environment.

Nitrogen starvation also causes a decline in fermentation rate as the slowing of protein synthesis retards the replacement of short lived sugar transporters

(Busturia and Lagunas, 1986; Salmon, 1989; Bely, et al., 1994). The half-life of such transporters is of the order of 5 hours compared to around 70 hours for other proteins in the cell. However, this decline in fermentation rate is not solely due to sugar transport inactivation, since nitrogen starvation also brings about direct inactivation of the glycolytic pathway (Alexandre and Charpentier, 1998). Most nitrogen sources supply the central nitrogen pool, comprised of ammonium, glutamate and glutamine. Ammonium is an allosteric activator of phosphofructokinase, a key rate-limiting step in the glycolytic pathway catalysing the addition of a phosphate to fructose-6-phosphate to create fructose-1,6-bisphosphate (Ramaiah, 1974). During nitrogen starvation, the concentration of ammonium is low, thus phosphofructokinase is not activated and the flux through the glycolytic pathway is reduced.

As stated already, a cessation of sugar catabolism and a reduction in biomass formation are not the only cellular consequences of a nitrogen limitation. Stuck or sluggish fermentations may also develop “stuck fermentation characters” and other undesirable aromas. Hydrogen sulphide (H<sub>2</sub>S) formation, which is particularly prevalent in low nitrogen juices, is a key example of the latter. Under nitrogen-excess conditions the sulphide ion formed by sulphite reductase is utilised in the synthesis of sulphur containing amino acids. When nitrogen is limiting, amino acid precursors for this biosynthesis are unavailable, leaving sulphide to diffuse from the cell in the form of H<sub>2</sub>S (Stratford and Rose, 1985; Jiranek, et al., 1995b).

To prevent stuck fermentations, the must needs to contain all the necessary nutrients including nitrogen for the formation of sufficient biomass and the maintenance of sugar transporters and catalytic enzymes. At present in the wine making industry, nitrogen deficiencies are addressed both in the vineyard and during the wine making process. In the vineyard, fertilisation practices have attempted to increase the content of assimilable nitrogen with some success (Ough and Bell, 1980; Bell, 1991). During fermentation nitrogen compounds, usually diammonium phosphate, are added to the must. This addition is effective, although its timing is critical. It is considered that the most appropriate moment for the addition of nitrogen is after the completion of

the growth phase and close to the halfway point of the fermentation progress (in terms of sugar utilisation) (Bely, et al., 1990a). Nitrogen additions that are made in the second half of the fermentation, that is after the maximum biomass is formed and during the stationary phase, will have little affect due to the decline in the rate of protein synthesis, particularly as the glucose transport systems are already in the process of inactivation (Salmon, 1989). Earlier additions will most likely increase the total biomass formation (Bely, et al., 1990a), which may lead to a premature depletion of other essential nutrients, or else a fermentation that may become excessively vigorous and will perhaps depend heavily on refrigerative control. As nitrogen is not typically measured at present in industry, winemakers may be tempted to make excessive additions. This can lead to residual nitrogen compounds such as urea in the finished wine leaving it prone to formation of the carcinogen ethyl carbamate and also at risk of being microbially unstable (Monteiro, et al., 1989; Ough, 1991).

Another factor that influences nitrogen utilisation by yeast is the availability of oxygen. Oxygen is a key factor for yeast biomass formation, and a strong stimulant of fermentation (Ingledeew and Kunkee, 1985; Sablayrolles, 1996). Wine yeast differ in their oxygen demand and the most appropriate timing for addition is thought to be at the end of the growth phase (Julien, et al., 2000 and pers. comm.). Access to oxygen allows cells to desaturate some fatty acids as well as produce sterols, thereby maintaining optimal proportions of these membrane components (Ratray, et al., 1975). In this way, membrane associated proteins, including nitrogen compound transporters, are able to function correctly. In place of molecular oxygen, the so-called “survival factors”, or oxygen substitutes are also able to contribute to good membrane function, and thus the reliability of fermentation (Larue, et al., 1980; Bisson, 1999). Such compounds can be supplemented during fermentation, for instance with the use of yeast hulls or inactivated yeast extracts (Munoz and Ingledeew, 1989).



### ***1.3 The effect of nitrogen on fermentation dynamics***

The amount of assimilable nitrogen available to be utilised by yeast has a direct affect on the dynamics of fermentation. The initial concentration of assimilable nitrogen is found to be linearly related to the maximum fermentation rate, inversely proportional to the duration of fermentation, and exponentially related to biomass formation (Cantarelli, 1957; Agenbach, 1977; Bely, et al., 1990a). The measurement of fermentation rate, most conveniently evaluated by the carbon dioxide evolution rate (CER), can assist a wine maker in predicting the risk of a fermentation becoming sluggish or stuck. This risk is proportional to the decline of the CER during the second half of fermentation. Insa et al. (1995) attempted to predict this decline by measurement of the CER during the first half of the fermentation, and reported that 74 % of fermentations were predicted correctly. Surprisingly the decline of the CER and the concentration of assimilable nitrogen had no correlation, suggesting that although nitrogen is one of the major limiting factors in stuck fermentations, when considered alone it is poorly related to the kinetics at the end of the fermentation.

Nitrogen additions made to fermentation will increase the CER; the precise effect depending on the timing of the addition. The earlier nitrogen is added, the higher the maximum CER (Bely, et al., 1990a). Nitrogen additions have also been found to be more effective when added together with oxygen, for example 5 mg L<sup>-1</sup> (Sablayrolles, et al., 1996). The initial concentration of assimilable nitrogen also influences how the CER will be affected by further additions of nitrogen. As shown by Bely et al. (1990a) when the maximum CER is plotted against the concentration of assimilable nitrogen, a change of slope occurs around 140 mg FAN (Free amino nitrogen) L<sup>-1</sup>. Interestingly this correlates with values proposed for the minimal nitrogen requirements or threshold of nitrogen deficiency (Agenbach, 1977). Increasing the initial nitrogen content beyond 140 mg FAN L<sup>-1</sup> will still increase the maximum fermentation rate, yet not to the same extent as initial increases. It is unclear how the fermentation rate impacts on the composition and sensory aspects of the finished wine. A yeast population with maximum fermentation capacity will complete fermentation quickly, yet this may not reflect optimal conditions for wine making. High CERs may not be conducive for the formation or

retention of desirable aroma compounds. However, the timing of nitrogen additions has been shown to affect the formation of some aroma contributing compounds. For example nitrogen additions, particularly ammonium, made in the early stages of fermentation, contributed to biomass formation and correlated with an increased glycerol and acetate production (Beltran, et al., 2005).

Fermentation duration is also affected by the concentration of assimilable nitrogen, the time to complete fermentation being inversely proportional to the amount of nitrogen available (Bely, et al., 1990a). As with the CER, the earlier the nitrogen addition, the shorter the duration of fermentation. The fermentation rate can be related to the fermentation duration by:

$$\log t_{\max} = -0.877 \log d\text{CO}_2 / dt_{\max} + 2.289 \quad (\text{at } 24^\circ\text{C})$$

where  $t_{\max}$  = duration of fermentation (hours) and  $d\text{CO}_2 / dt_{\max}$  = maximum fermentation rate (Bely, et al., 1990a; Kunkee, 1991).

Biomass formation is exponentially rather than directly related to the concentration of initial assimilable nitrogen (Agenbach, 1977). This demonstrates that the concentration of nitrogen affects yeast growth and yeast fermentation differently (Cantarelli, 1957). The initial concentration of nitrogen does not affect the maximum growth rate of yeast, but rather the duration of the growth phase, and subsequently the rate of growth decline (Benzenger and Navarro, 1987). The timing of nitrogen addition certainly affects this nutrient's fate; early additions made before the stationary phase result in maximum cell populations, whereas later additions have a reduced effect (Bely, et al., 1990a). This has been disputed by (Mendes-Ferreira, et al., 2004), as they reported that addition of ammonium was effective in increasing biomass during stationary phase. However in this study, the time point for stationary phase was determined to be at the end of the growth phase rather than after a period of growth cessation, normally considered as one of the markers of stationary phase (Herman, 2002). In practical terms the differential impact of initial assimilable nitrogen levels upon biomass, fermentation rate

and fermentation duration means that at low levels of nitrogen (less than approximately 140 mg FAN L<sup>-1</sup>), growth and fermentation rates are commercially inadequate, while at higher concentrations, and/or with nitrogen additions in the first half of fermentation, fermentation rate and biomass yield are strongly stimulated, thus allowing sugars to be catabolised in an acceptable time.

#### ***1.4 The physiological effects of nitrogen starvation during fermentation***

When yeast are starved of nitrogen they undergo numerous physiological changes so as to cope with the stressful nutrient limited environment. For instance, they enter stationary phase and their cellular content of RNA and protein decreases from 16 % and 60 % to 4 % and 22 % (w/w), respectively (Schulze, et al., 1996). The dynamics of metabolite production are also affected. For example, intracellular carbohydrates, such as trehalose and glycogen increase (Schulze, et al., 1996) and in continuous culture experiments when nitrogen was limiting, less glycerol and more ethanol are produced (Liden, et al., 1995).

Several studies have analysed the transcript dynamics of commercial wine yeast strains, both throughout fermentation and in varying nitrogen concentrations (Backhus, et al., 2001; Rossignol, et al., 2003; Varela, et al., 2005). In regards to nitrogen, Rossignol et al. (2003) found that after nitrogen exhaustion, many genes subject to nitrogen catabolite repression, known to be controlled by the TOR (target of rapamycin) pathway, were de-repressed. This highlighted the vital role of the TOR pathway in signalling nutrient depletion. During fermentation when nitrogen was supplied in the initial must at low concentrations as arginine (16 mg L<sup>-1</sup>), in comparison to high concentrations (1240 mg L<sup>-1</sup>), expression of genes involved in nitrogen recycling, general translation and oxidative phosphorylation were increased (Backhus, et al., 2001). Interestingly, transcription of the cAMP/PKA inhibitor encoded by *RCO2* was increased in conditions of low nitrogen. The authors suggest that the up-regulation of this cAMP/PKA inhibitor and thus the restriction of the cellular response to glucose in the later stages of fermentation may be one

explanation as to why nitrogen supplied in the second half of fermentation has a limited effect on sugar catabolism.

The effect of a nitrogen addition on the transcriptome has also been evaluated during fermentation of a Riesling must by the wine yeast strain, VIN13 (Marks, et al., 2003). In this study, a diammonium phosphate (DAP) addition (200 mg FAN L<sup>-1</sup>) was made after approximately 30 % of sugars had been catabolised, after cells had already entered stationary phase. Originally the medium contained 242 mg FAN L<sup>-1</sup>, and at the time of DAP addition 51 mg FAN L<sup>-1</sup> still remained in the must. Consequently, the change in the transcript profile was in response to an increase in nitrogen availability (as ammonium) rather than a relief from nitrogen depletion. The expression patterns of 350 genes were altered; in general genes involved in amino acid metabolism, sulphate assimilation and protein synthesis were up-regulated, whereas genes encoding small molecule transporters, nitrogen catabolic enzymes and protein degradation enzymes were down-regulated. Interestingly in this strain, the addition of DAP also decreased the expression of genes involved with transport and utilisation of arginine, which most likely would lead to a decrease in the formation of urea within the cell, and thus a decreased likelihood of ethyl carbamate formation (Ough, et al., 1988).

### ***1.5 Nitrogen compounds in wine***

Nitrogen sources and their concentration within grape must can vary widely (60-2400 mg FAN L<sup>-1</sup>) (Amerine, et al., 1980; Ough and Amerine, 1988). The majority of nitrogen in grape must comes from amino acids, polypeptides and ammonium, but also from protein and minor amounts of amines/polyamines, nucleic acid derivatives and vitamins. The total nitrogen concentration of a grape must depends upon a number of variables including climate, grape ripeness, variety and fertilisation practices (Sponholz, 1991; Henschke and Jiranek, 1993). The nitrogen content present within a grape must can be chemically determined using the ninhydrin, 2,4,6-trinitrobenzene sulfonic acid (TNBS), o-phthaldialdehyde/N-acetyl-L-cysteine (NOPA) spectrophotometric assay, or the Formol titration methods, amongst others (Lie, 1973; Crowell, 1985; Dukes and Butzke, 1998; Gump, et al., 2002). These analyses determine

the amount of free alpha amino nitrogen (FAN) group present. However, these determinations may not equate to the amount of assimilable (biologically available) nitrogen present, often described as yeast assimilable nitrogen (YAN). Assimilable nitrogen is the sum total of amino acids and ammonium minus proline (as proline is not utilised by wine yeast under anaerobic conditions (Ingledeew, et al., 1987)). The determination of FAN does not always represent the amount of YAN present in a medium, as some nitrogen rich amino acids contain non-alpha-amino nitrogen (Henschke and Jiranek, 1993). These methods also only provide a chemical value for nitrogen content rather than how much assimilable nitrogen is available to yeast. The concentration of assimilable nitrogen within a must can also be measured by monitoring the CER during fermentation. Bely et al. (1990a) used this method to analyse 40 grape musts and found that the amount of assimilable nitrogen highly correlated with both the fermentation duration and the maximum CER.

Within the first 20-30 hours of fermentation, the majority of nitrogen-containing compounds are transported into the yeast cell (Salmon, 1989; Jiranek, et al., 1995a). Once inside the cell, nitrogen-containing compounds can be directly incorporated into biosynthetic pathways or alternatively degraded to become part of the central nitrogen pool (Large, 1986). The intracellular nitrogen pool is distributed between vacuoles for storage and the cytoplasm for immediate use. The import, distribution and the subsequent mobilisation of nitrogen compounds are the major methods of regulating how and when nitrogen is utilised and will be explained later in greater detail (Cooper, 1980).

### ***1.6 The nitrogen demand of yeast***

The amount of assimilable nitrogen utilised by yeast in the satisfactory completion of fermentation is dependent on the yeast strain and conditions of the fermentation, such as oxygen availability, the concentration of glucose, ethanol and vitamins, as well as the temperature and the pH of the must (Jones, 1989; Jiranek, et al., 1990; 1991; 1995a; Bisson, 1991; Henschke and Jiranek, 1993; Sablayrolles, et al., 1996; Manginot, et al., 1998; Julien, et al., 2000).

The fermentation characteristics of a single yeast strain can also vary under nitrogen-limited or -excess conditions (Jiranek, et al., 1991; 1995a).

Previous studies have concentrated on the minimum amount of nitrogen required to complete fermentation. Determined under nitrogen-limiting conditions, in sugar concentrations of 160 - 258 g L<sup>-1</sup>, these values range between 70 and 140 mg FAN L<sup>-1</sup>, rather than that which will support maximum fermentation rates. (Agenbach, 1977; Bely, et al., 1990a; Henschke and Jiranek, 1993). The minimum nitrogen requirement is considered to be below which the duration or rate of fermentation is unsatisfactory. Maximum requirement is the amount required for maximum growth and rate of sugar utilisation. When only the minimum requirement is supplied, it is completely depleted from the medium and it is more than likely that H<sub>2</sub>S will be liberated (Jiranek, et al., 1995b). The suppression of H<sub>2</sub>S production is most likely achieved when the amount of nitrogen present exceeds the minimum requirement. For this reason the maximum amount of assimilable nitrogen is perhaps the more practical measure of nitrogen demand from an industrial perspective, as it takes into account both completion of fermentation and suppression of H<sub>2</sub>S production due to limited nitrogen.

The maximum amount of nitrogen utilised by yeast is found to vary between strains. In a study of nine strains a natural variation between strains in maximum nitrogen utilisation of 140 mg FAN L<sup>-1</sup> was found (Jiranek, et al., 1995a). This difference is most obvious during the stationary phase of fermentation (Manginot, et al., 1998). The average maximum nitrogen usage of wine yeast strains has been reported to be 300, 400 and 429 mg FAN L<sup>-1</sup> (Bely, et al., 1990a; Ough, et al., 1991; Jiranek, et al., 1991; 1995a). Importantly, these values exceed some estimates of amino acid content in grape juices (Sponholz, 1991; Henschke and Jiranek, 1993) thereby offering a possible explanation as to why some fermentations suffer from sluggishness or even attenuate before all sugar is catabolised. Also the range in values of maximum nitrogen usage (that is 140 mg FAN L<sup>-1</sup>) exceeds some of the legal limits for nitrogen additions (64, 200 and 360 mg FAN L<sup>-1</sup> as diammonium phosphate in Europe, USA and Australia). Thus the use of a strain with a high nitrogen

demand can potentially mean that nitrogen additions exceeding the legal limit would be needed to satisfy the strain's requirements for this nutrient class.

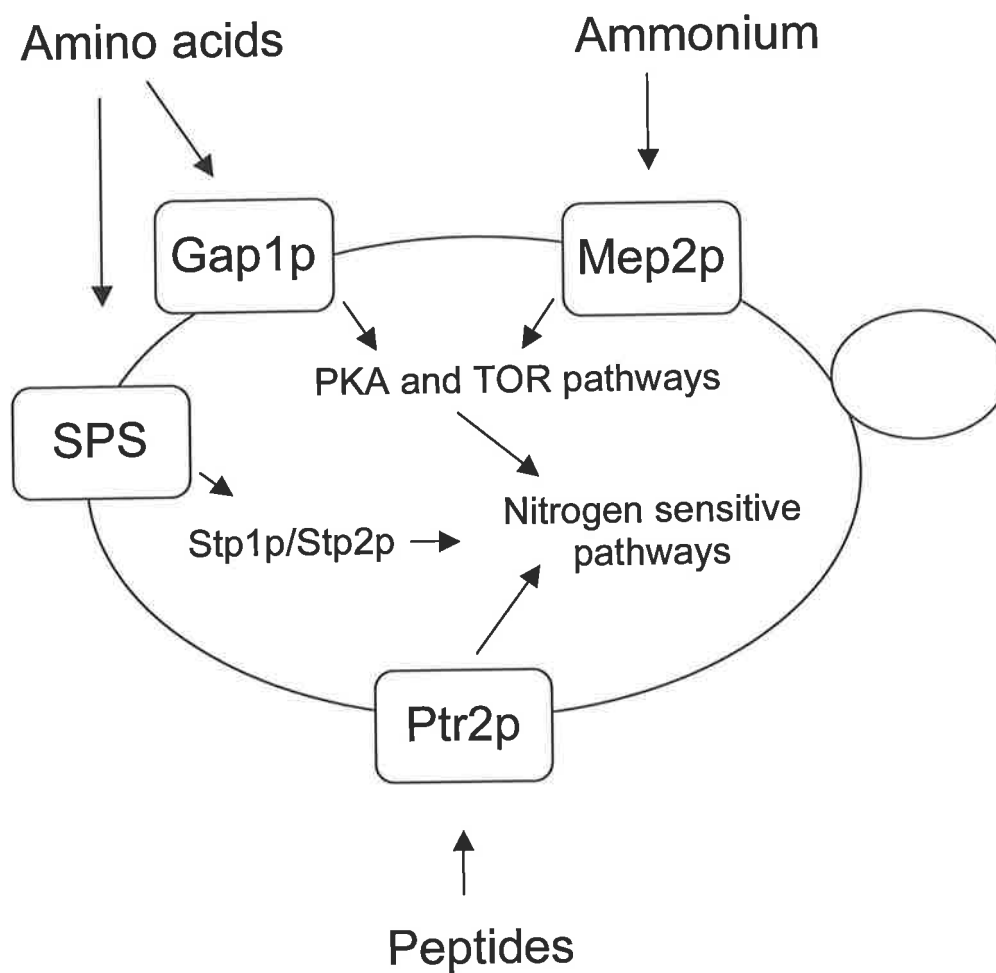
The nitrogen usage of several wine yeast strains during the stationary phase was investigated by Manginot et al. (1998). They found that strains with the highest specific fermentation rates corresponded to those that utilised the least nitrogen during the stationary phase. These "nitrogen efficient strains" were hypothesised to direct nitrogen towards protein synthesis reactivation, in particular for sugar transport systems, rather than to the formation of biomass. The strains with the highest nitrogen requirement were indeed found to synthesise the most biomass.

Yeast nitrogen demand also varies depending on the type of nitrogenous compounds available, and consequently what metabolites are formed (Albers, et al., 1996). For example, when yeast is provided with amino acids, in comparison to solely ammonium, less glycerol is synthesised as there is less of a requirement to recycle NADH formed during amino acid biosynthesis.

### ***1.7 Yeast sense nitrogen sources in the extracellular environment***

There are several mechanisms discovered to date that enable yeast to sense the availability of nutrients. In regards to assimilable nitrogen sources: amino acids, ammonium and peptides, the proposed sensors include the SPS complex, Gap1p, Mep2p and Ptr2p respectively (Figure 1.1).

An increase in extracellular amino acid concentrations triggers a signalling cascade via the SPS complex, which is composed of Ssy1p, Ptr3p, and Ssy5p (Forsberg and Ljungdahl, 2001). These three proteins interact physically and are localised at the plasma membrane (Klasson, et al., 1999; Bernard and Andre, 2001; Forsberg and Ljungdahl, 2001). In the presence of extracellular amino acids, the SPS complex activates a number of transcription factors, including Spt1p and Spt2p (Andreasson and Ljungdahl, 2002). These factors influence the transcription of genes of numerous processes, including amino acid transport, the upper part of glycolysis, trehalose and glycogen synthesis



**Figure 1.1** A diagrammatic representation of the main systems of extracellular sensing of potential nitrogen sources of *S. cerevisiae*, the SPS system, Gap1p, Mep2p and Ptr2p. Their key (known) downstream targets are also shown.



and also the glyoxylate cycle and the pentose phosphate pathway (Eckert-Boulet, et al., 2004).

More recently, the general amino acid permease Gap1p has also been shown to act as an amino acid sensor (Donaton, et al., 2003). Nitrogen starved cells, in the presence of a fermentable carbon source rely on Gap1p to detect nitrogen sources and initiate the activation of protein kinase A (PKA) targets which are generally involved in metabolic control and stress resistance (Thevelein and de Winde, 1999).

Yeast are also responsive to the concentration of extracellular ammonium, not simply the flux of ammonium across the membrane (ter Schure, et al., 1995a; 1995b; Palkova, et al., 1997). *MEP2*, encodes the highest affinity ammonium permease ( $K_m$  1.4 – 2.1  $\mu$ M) and is thought to also function as an ammonium sensor (Marini, et al., 1997; Lorenz and Heitman, 1998). *MEP2* differs from the other members of the ammonium permease family, *MEP1* and *MEP3*, by appearing to have increased expression when the surrounding medium has limited ammonium and also by its product being N-terminally glycosylated (Marini and Andre, 2000). All three genes are subject to nitrogen catabolite repression (NCR, which is discussed in greater detail later), consequently expression is repressed in the presence of good nitrogen sources (Marini, et al., 1997). Similar transcription profiles have also been observed in fermentations that are more alike to those in the wine industry (Beltran, et al., 2004). Mep2p similar to Gap1p has also been suggested to trigger the PKA pathway (Thevelein, et al., 2005).

Peptides can be transported by the PTR system, which is specified by three genes, *PTR1-3*. *PTR2* encodes a peptide transporter, and has been suggested to be a sensor of peptide uptake. *PTR2* is regulated by the products of *PTR1*, *PTR3*, *CUP9* and *SSY1* (Byrd, et al., 1998; Barnes, et al., 1998; Didion, et al., 1998). Imported di-peptides that contain N-terminal amino acids (according to the N end-rule, Bachmair, et al., 1986) have been shown to enable a positive feed-back loop to enhance their import. *PTR2* is transcriptionally repressed by Cup9p, yet imported peptides can bind to Ptr1p, also known as Ubr1p, which

increases its ability to promote degradation of Cup9p via ubiquitination (Turner, et al., 2000).

### ***1.8 Yeast cells monitor their intracellular nitrogen pools***

The general control of amino acid biosynthesis (GCN pathway) is activated when low concentrations of amino acids are sensed by the accumulation of uncharged tRNAs (Hinnebusch, 1997). Activation of the GCN pathway is facilitated by Gcn2p bound to these tRNAs, which phosphorylates the  $\alpha$  subunit of eukaryotic initiation factor-2 (eIF2), ultimately down regulating the initiation of protein synthesis and in turn activating the transcription of *GCN4*. Gcn4p has been shown to be a master regulator during amino acid starvation, activating over 1,000 genes, such as those involved in amino acid biosynthesis (Natarajan, et al., 2001).

As mentioned above, the TOR kinase pathway is also known to be directly involved in the translational response to nitrogen availability, as well as other cellular stresses (Beck and Hall, 1999; Cardenas, et al., 1999; Hardwick, et al., 1999; Komeili, et al., 2000; Shamji, et al., 2000). The essential TOR kinases, Tor1p and Tor2p are completely inhibited by a complex of rapamycin (a compound naturally secreted by some bacteria as a defence mechanism) and Fpr1p, a small peptidyl-prolyl isomerase (Heitman, et al., 1991). Exposure of cells to rapamycin causes a response similar to nutrient starvation, such that protein synthesis is reduced until growth is ultimately arrested in  $G_0$ . Tor1/2p are thought to elicit their action by triggering a response resulting in nutrient responsive factors being sequestered in the cytoplasm (Beck and Hall, 1999). The relationship between Tor1/2p and these factors, in particular, Gln3p (a positive transcriptional regulator of nitrogen-regulated genes, described in detail below) is still in debate (Cooper, 2002; Cox, et al., 2004). Recently it has been proposed that TOR signals arise in response to amino acid availability through Sapp-Sit4p complexes (Rohde, et al., 2004). For instance Sit4p has been shown to be a positive regulator of *GLN3* when nutrients are limiting (Di Como and Arndt, 1996; Beck and Hall, 1999). Alternately in nutrient sufficient situations, TOR is active and promotes the binding of Sit4p to Tap42p rendering it inactive.

The specific activation of TOR is unknown, though recently it has been suggested that at least a subset of TOR activity is initially mediated by the internal concentrations of glutamine (Crespo, et al., 2002). Depletion of glutamine activates the transcription factors Gln3p, Rtg1p and Rtg3p; known to be controlled in part by TOR. Although, Dilova et al. (2004) have shown that at least TOR and glutamine mediated RTG gene expression can be separated, showing that TOR and glutamine can act independently to inactivate this pathway.

### ***1.9 Nitrogen catabolite repression***

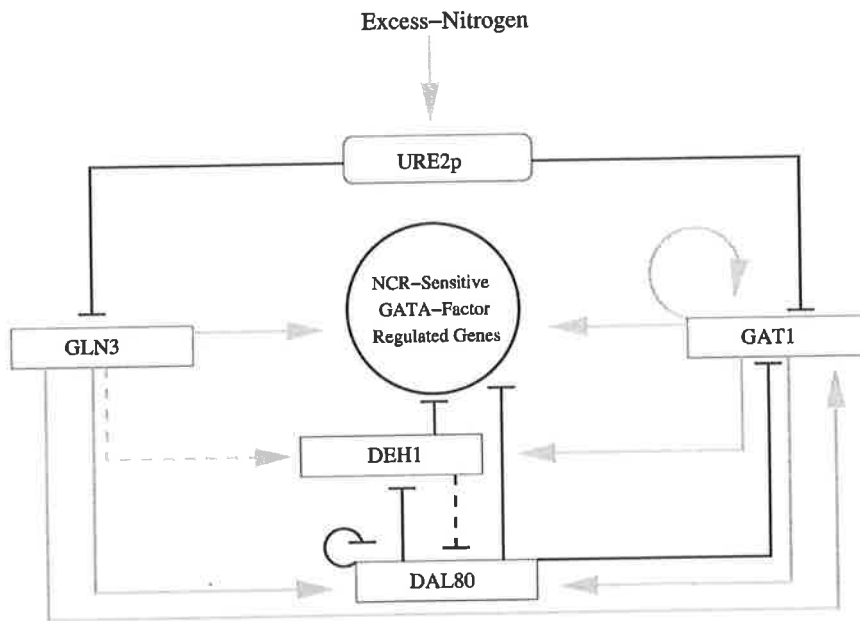
As a wide variety of nitrogen compounds can be utilised by *Saccharomyces cerevisiae*, nitrogen metabolism is regulated so as to maximise the catabolism of preferred nitrogen sources. For example, glutamate, asparagine and ammonium, in some strains, are preferred sources and consequently support rapid growth. Nitrogen sources such as these are transported into the cell and utilised preferentially for biosynthetic pathways (Castor, 1953), whilst nitrogen catabolite pathways for secondary, or 'poor' nitrogen sources (e.g. proline or urea) are not activated until required. This phenomenon is commonly known as nitrogen catabolite repression (NCR), and is regulated at both the level of gene expression and of enzyme activity (Wiame, et al., 1985; Hofman-Bang, 1999; Cooper, 2002; Magasanik and Kaiser, 2002).

The regulation of NCR is controlled by the presence of a preferred nitrogen source such as glutamine and ammonium (Mitchell and Magasanik, 1984a; Stanbrough and Magasanik, 1995; Coffman, et al., 1996). The balance between these two compounds/metabolites is complex, and the effect of each on gene expression differs according to the growth medium. For example, NCR induced by ammonium is known to act via Ure2p, and is independent of the glutamine concentration (ter Schure, et al., 1998). ter Schure et al. (1995b) also found that the concentration of ammonium rather than the ammonium flux is responsible for its effect on nitrogen metabolism, highlighting the potential importance of a yeast ammonium sensor, putatively Mep2p, such as that found in bacteria (Magasanik, 1988).

The repression caused by ammonium and glutamine is known not to be caused by a general stress response, but by specific NCR signals (ter Schure, et al., 1998; 2000). The gene expression of enzymes used in the secondary nitrogen utilisation pathways is mainly influenced by the interplay of four transcriptional regulators of the GATA family, and one non-GATA factor Ure2p (Figure 1.2). Regulators of this family have C4 zinc finger motifs which bind to DNA sequences containing a GATA DNA sequence at their core (Omichinski, et al., 1993), in yeast these include Gln3p, Gat1p/Nil1p, Dal80p/Uga43p, Nil2p/Deh1p/Gzf3p, (Cunningham and Cooper, 1992; 1993; Blinder and Magasanik, 1995; Stanbrough and Magasanik, 1995; Coffman, et al., 1997; Soussi-Boudekou, et al., 1997). The GATA factors involved in NCR all recognise the UAS<sub>NTR</sub> (nitrogen regulated upstream activation sequence), located in the promoter of nitrogen regulated genes. The UAS<sub>NTR</sub> is known to be necessary and sufficient for NCR regulation (Cooper, et al., 1989), the core of which contains two GATA factor binding sequences.

Gln3p and Gat1p are transcriptional activators, and under nitrogen derepressing conditions will initiate expression of a wide range of nitrogen metabolic genes by binding to GATA sequences or (GATA/TAG in the case of Gln3p) (Blinder, et al., 1996). On poor nitrogen sources Gln3p is not required for maximum expression of nitrogen metabolic genes (Daugherty, et al., 1993), but is thought to be responsible for maintenance of cellular glutamine levels, since *GLN3* is expressed by cells grown on glutamate. Interestingly *GLN3* has homology to *AREA* from *Aspergillus nidulans* and *NIT2* of *Neurospora crassa*, each of these being positive regulators of nitrogen metabolism. Unlike Gln3p, Gat1p is only synthesised on poor nitrogen sources (Stanbrough and Magasanik, 1995). The expression of *GAT1* is also subject to NCR; on rich nitrogen sources Nil2p interferes with *GAT1* transcription and on poor nitrogen sources Gat1p can activate its own transcription.

During growth on ammonium or glutamine, Ure2p inhibits the transcriptional activators Gln3p and Gat1p, causing repression of several nitrogen metabolic genes such as *GAP1*, *PUT4*, and *GLN1* (Glutamine synthetase) (Mitchell and Magasanik, 1984b; Miller and Magasanik, 1991), although the exact



**Figure 1.2** The NCR circuit, as referred to in the text. Arrows indicate up-regulation, blunted arrows represent down-regulation (not always at the level of transcription). Dashed lines indicate a weaker response. Figure and text adapted from Boczko, et al., 2005.

mechanism by which this occurs is still unclear. There is convincing evidence that Ure2p binds to Gln3p, and probably also to Gat1p, retaining them in the cytoplasm, and thus blocking their transactivating capabilities (Beck and Hall, 1999). The expression of *URE2* is constitutive, thus Ure2p is abundant within the cytoplasm (Cardenas, et al., 1999), yet Gln3p must be phosphorylated to interact with Ure2p. Beck and Hall (1999), have shown that TOR kinases Tor1p and Tor2p prevent the nuclear accumulation of Gln3p most probably by controlling its phosphorylation state, thus controlling the ability of Ure2p to sequester Gln3p in the cytoplasm. Mks1p (multicopy compensator of A kinase suppression) has also been implicated in this interaction by being a negative regulator of Ure2p function (Edskes, et al., 1999); yet it seems that Mks1p only affects Gln3p dependent and not Gat1p dependent gene expression (Shamji, et al., 2000).

To summarise, in the presence of good nitrogen sources, the positive activators of NCR, Gln3p and Gat1p are located in the cytoplasm and most probably in a complex with Ure2p (Blinder, et al., 1996; Cooper, 2002), and consequently there is no activation of NCR genes which would otherwise enable the utilisation of poor nitrogen sources (Cardenas, et al., 1999; Hardwick, et al., 1999; Bertram, et al., 2000; Cox, et al., 2000). But, when poor nitrogen sources are present Gln3p and Gat1p are targeted to the nucleus and NCR sensitive genes are transactivated (Blinder and Magasanik, 1995; Coffman, et al., 1995).

Another type of nitrogen related metabolic gene regulation occurs via Dal80p, which unlike NCR, actively represses transcription and is active under different circumstances to NCR. Dal80p, functions as a repressor of Gln3p by forming a dimer with itself, and then binding to Gln3p binding sites within the promoter of NCR sensitive genes (Cunningham and Cooper, 1992). Dal80p is not required for NCR, yet is sensitive to NCR itself (Daugherty, et al., 1993). Gat1p is also capable of forming heterodimers with Dal80p via their C-terminal leucine zipper motifs (Svetlov and Cooper, 1998) and these heterodimers have been hypothesised to form another DNA binding negative regulatory unit.

Transcriptional activation generally requires the combination of activators, basal transcription machinery and co-activators. Co-activators form large complexes on the promoter and are required for the remodelling of the chromatin structure. A co-activator linking Gln3p and Gat1p to the transcriptional machinery has been described: Gan1p (also known as Ada1p) is required for full expression of various nitrogen utilisation genes (Horiuchi, et al., 1997; Soussi-Boudekou and Andre, 1999) and interestingly its activity is also affected by the nitrogen source present.

### ***1.10 Nitrogen import into the cell***

Regulation of nitrogen utilisation also occurs at the level of nitrogen import. In a fermenting must the factors having greatest influence on nitrogen transport capabilities are pH, ethanol concentration, temperature, degree of aeration and carbon dioxide pressure (reviewed by Henschke and Jiranek, 1993). The main nitrogen sources, ammonium ion, amino acids and peptides are transported by membrane permeases. Specifically, the ammonium ion is transported into the cell via at least the three Mep permeases which function by active transport and possibly also by simple diffusion (Marini, et al., 1997). Amino acids can be imported by the general amino acid permease, Gap1p (Grenson, et al., 1970; Jauniaux and Grenson, 1990) or by specific amino acid permeases, and peptides are transported by proteins of the ATP-binding cassette (ABC), PTR or the recently identified OPT systems of peptide transporters (Steiner, et al., 1995; Hauser, et al., 2000).

The three known ammonium permeases are encoded by the genes *MEP1*, *MEP2* and *MEP3*. Mep1p and Mep3p are low affinity but high capacity transporters, whereas Mep2p is a high affinity and low capacity transporter (Dubois and Grenson, 1979; Marini, et al., 1994; Lorenz and Heitman, 1998). All three *MEP* genes are subject to NCR (Marini, et al., 1997) and are expressed when there are low concentrations of ammonium present in the media. *MEP* genes are all activated by the Gln3p transcription factor. Nil1p also activates *MEP2*, yet in contrast *MEP1* and *MEP3* are down regulated by Nil1p. Thus, in the presence of a rich nitrogen source all *MEP* genes are repressed. On poor nitrogen sources *MEP2* is activated by both Gln3p and

Nil1p, whereas *MEP1* and *MEP3* are activated by Gln3p but down regulated by Nil1p.

Most of the specific amino acid transport systems are constitutively activated, thus the type and amounts of nitrogen sources in the medium do not affect their expression. Nitrogen sources within the medium are found to influence the expression and activity of the general amino acid permease Gap1p and the proline specific permease Put4p (Wiame, et al., 1985). For example, Put4p is subject to NCR, thus is only present and functional in the absence of a rich nitrogen source such as glutamine or ammonium. Gap1p transports more than 20 substrates including all of the L-amino acids (biological amino acids), some D-amino acids, and several metabolic intermediates such as citrulline, ornithine and alpha amino adipic acid (Wiame, et al., 1985; Jauniaux and Grenson, 1990). During growth on poor nitrogen sources the activity of Gap1p is high whereas in the presence of rich nitrogen sources such as glutamine or ammonium the activity of Gap1p is low (Courchesne and Magasanik, 1983). Regulation of Gap1p occurs at three levels, permease expression, activity and compartmentalisation (Grenson, 1983a; Grenson, 1983b). Specifically, *GAP1* is positively activated by Gln3p and Nil1p, and repressed by Ure2p, depending on the conditions of the medium. At the permease level, Gap1p activity is regulated by the products of the nitrogen permease inactivator genes *NPI1* and *NPI2* (Grenson, 1983b). *NPI1* encodes a ubiquitin protein ligase Rsp5p (Huibregtse, et al., 1995). In the presence of the ammonium ion, Rsp5p ubiquitinates Gap1p, targeting it for inactivation and degradation in the vacuole. Inactivated Gap1p can be reactivated by the product of the nitrogen permease reactivator gene *NPRI*. Having homology to protein kinases, Npr1p is thought to reactivate Gap1p and other nitrogen permeases by phosphorylation (Grenson, 1983a; Grenson, 1983b).

Another source of nitrogen from the environment are peptides. *S. cerevisiae* possesses two peptide transport systems. Di- and tri-peptides are transported across the plasma membrane by the PTR system (Naider, et al., 1974; Island, et al., 1991) whereas, tetra- or penta-peptides are transported by the OPT system (Hauser, et al., 2000). Both of these systems are enhanced by limiting or poor

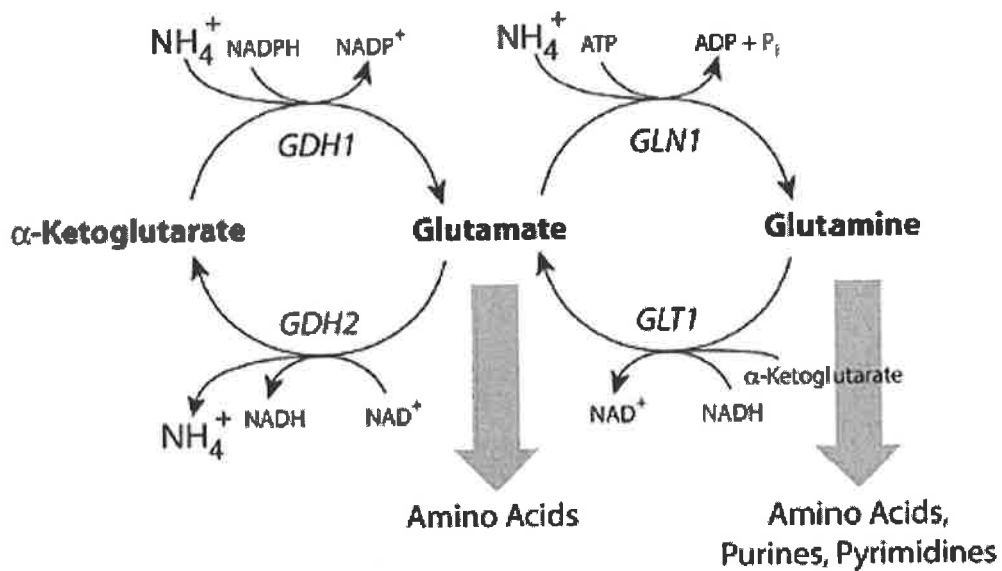


nitrogen sources and by the presence of micromolar concentrations of extracellular amino acids (Island, et al., 1987; Hauser, et al., 2001). The OPT system is probably not relevant to industrial fermentations since its expression seems to be limited to sporulation (Chu, et al., 1998).

### **1.11 Central Nitrogen Metabolism**

During yeast growth the medium will not always supply all of the necessary amino acids required for protein synthesis, consequently yeast cells must synthesise them from available precursors. The core pathway for the utilisation of all non-preferred nitrogen sources is known as Central Nitrogen Metabolism (Figure 1.3). Nitrogen containing compounds are fed into the CNM to ultimately produce glutamate and glutamine. The production of glutamate is of utmost importance as the amino nitrogen of glutamate is estimated to provide around 85 % of the total cellular nitrogen (Cooper, 1982). The remaining 15 % is provided by glutamine, which is particularly important as a precursor of purines and pyrimidines. The major pathway of glutamate production is normally through the action of NADPH dependent glutamate dehydrogenase (NADPH-GDH), encoded by *GDH1*. NADPH-GDH catalyses the formation of glutamate from  $\alpha$ -ketoglutarate (from the citric acid cycle) and ammonium. This reaction consumes NADPH and generates  $\text{NADP}^+$ . Interestingly, when cells are growing on ammonium, a significant proportion of NADPH used by the cell is consumed by this single enzymatic reaction (Bruinenberg, et al., 1983). *S. cerevisiae* also has a second NADPH-GDH encoded by *GDH3*, which has distinctly different allosteric properties (DeLuna, et al., 2001). It is thought that the co-ordinated expression of these two isoenzymes, together with their varying biological stability (DeLuna, et al., 2005) allows a balanced utilisation of  $\alpha$ -ketoglutarate and production of glutamate under various conditions.

Glutamate can also be produced by the coupling of glutamine synthetase (GS), encoded by *GLN1* and glutamate synthase (GOGAT), encoded by *GLT1*. GS catalyses the formation of glutamine from glutamate, ammonium and ATP, whereas GOGAT catalyses the formation of glutamate from glutamine,  $\alpha$ -ketoglutarate and NADH. Thus the net reaction of GS/GOGAT is the



**Figure 1.3** A diagrammatic representation of the major pathways of Central Nitrogen Metabolism, together with the redox and energy co-factors that are involved in each reaction, as explained in the text. The *S. cerevisiae* gene for each of the enzymatic steps is designated in italics. Figure and text are adapted from Magasanik and Kaiser (2002).

formation of glutamate from  $\alpha$ -ketoglutarate and ammonium, whilst NADH and ATP are converted to  $\text{NAD}^+$  and ADP.

The *GDH2* gene encodes NAD-linked glutamate dehydrogenase (NAD-GDH) (Miller and Magasanik, 1990), which is the major, but not sole, enzyme required for the formation of ammonium from glutamate. The expression of *GDH2* increases on glutamate and decreases in the presence of both glutamine and ammonium (Miller and Magasanik, 1991; Tate and Cooper, 2003), being particularly sensitive to glutamine. This glutamine sensitivity has been reported to be due to a complex regulation involving six sites of the *GDH2* promoter; two upstream activator sites (UAS) and 4 negative regulatory domains (NRD). GS and NAD-GDH share a somewhat co-ordinated expression. They are both highly expressed in the presence of glutamate and reduced on glutamine, yet NAD-GDH has reduced expression on ammonium whereas GS does not (Mitchell and Magasanik, 1984a). The sensitivity of *GDH2* to ammonium in comparison to *GLN1* seems to be due to an extra URS, not present in *GLN1*.

The control of these enzymes of CNM is key to the processing of nitrogen containing compounds and as already described, are subject to control by NCR factors, amongst others. For instance, the expression of *GDH1* has been found to be affected by the following transcription factors, Gln3p, Leu3p, the HAP complex, Gcn4p and Mrg19p (Daugherty, et al., 1993; Hu, et al., 1995; Dang, et al., 1996; Riego, et al., 2002; Das and Bhat, 2005).

### ***1.12 Links between nitrogen and carbon metabolism***

The control of carbon and nitrogen metabolism is not independent, and has been eloquently reviewed by Cooper (2004). The integrated regulation of involved pathways would help to ensure the greatest chance of efficient growth and thus survival.

There have been a number of examples of transcription factors which are involved in both nitrogen and carbon regulation, for example the HAP complex and Snf1p (Dang, et al., 1996; Bertram, et al., 2002), as well as examples of where pathways are linked. In regards to fermentation, when ammonium is

present as a major source of nitrogen, the supply of  $\alpha$ -ketoglutarate for the formation of glutamate is a particularly important link between carbon and nitrogen metabolic pools. The enzymes involved in this response are encoded by the Retrograde genes; *CIT2*, *ACO1*, *IDH1/2* and *DLD3*, these function together with early parts of the oxidative branch of the TCA cycle to bring about the re-supply of mitochondria with oxaloacetate and acetyl-CoA. Intracellular ammonium is thought to positively regulate Retrograde transcription (Tate and Cooper, 2003) and mediate its effect through the transcription factors Rtg1p, Rtg2p, Rtg3p and Mks1p (Liao and Butow, 1993; Chelstowska and Butow, 1995; Liu and Butow, 1999).

### ***1.13 The effect of yeast strain and nitrogen utilisation on wine aroma***

In a wine fermentation, the profile of nitrogen utilisation by yeast is not simply of importance in terms of the growth and fermentative capacity, but also in regards to the effect upon wine aroma. *S. cerevisiae* produces a multitude of compounds, many of which contribute to the aroma of the finished wine. The presence and relative abundance of these compounds are influenced by many factors, including the particular wine yeast strain(s) present and the type and quantity of nitrogen source (Houtman and du Plessis, 1986; Herriaiz, et al., 1990; Vila, et al., 1998; Antonelli, et al., 1999). Thus, wine yeast strains that utilise nitrogen differently have the potential to yield different aroma profiles, and consequently impact the quality of wine.

Wine is described as possessing three classes of aromas; the grape or varietal aromas, and the fermentation and maturation aromas. The varietal aromas consist of both the primary grape aromas from undamaged fruit as well as the secondary grape aromas, which arises as a result of berry crushing. Fermentation derived aromas are formed by yeast and bacteria during the alcoholic fermentation and maturation aroma is formed during the aging of the wine. Aroma formation as a consequence of fermentation is of most interest with regards to must nitrogen levels and the effect of yeast. The major volatiles in this class are ethanol, glycerol and carbon dioxide. Each is indirectly affected by nitrogen but their contribution to aroma complexity is, in most cases, minimal. Compounds more directly affected by nitrogen and having

greater impact on aroma are the higher alcohols, diols, esters, organic acids, aldehydes, ketones and sulphur compounds.

Higher alcohols are an important part of the complexity of wine aroma, their sensory contribution has been found to be pleasant at concentrations below 300 mg L<sup>-1</sup>, yet becomes undesirable above 400 mg L<sup>-1</sup> (Rapp and Versini, 1991). The major higher alcohols produced by yeast are the aliphatic alcohols, amyl alcohol, isobutanol, n-propanol, isoamyl alcohol and hexanol, and the aromatic alcohol 2-phenylethanol which usually has the greatest aromatic impact (Antonelli, et al., 1999). Yeast produce higher alcohols by decarboxylation and reduction of alpha-keto acids. These alpha-keto acids are mainly produced anaerobically from carbohydrates, but can also come from the corresponding amino acid via the Ehrlich mechanism (Crowell, et al., 1961; Watson and Hough, 1969). Accordingly, attempts have been made to relate the presence of certain amino acids to the formation of their corresponding volatile compounds in wines (Sablayrolles and Ball, 1995; Guitart, et al., 1999). For example, the addition of valine, leucine and isoleucine, increases the production of the corresponding higher alcohols; isobutanol, isoamyl alcohol and amyl alcohol.

The formation of higher alcohols is also affected by must nitrogen content as the total concentration of higher alcohols can be increased by decreasing assimilable nitrogen (Ayrapaa, 1971; Ough and Bell, 1980; Rapp and Versini, 1991). The production of higher alcohols is also dependent on other components of the medium, for example concentrations of sugar, pH, temperature, aeration and the yeast strain utilised (Guymon, et al., 1961; Ough and Amerine, 1966; Rankine, 1967; Giudici, et al., 1989).

Fatty acid esters are also of great importance to wine aroma, as they are the dominant esters formed and usually impart pleasant odours. In wine, fatty acid esters are formed by yeast from acyl Coenzyme A. It has been shown that the amount of esters formed is positively correlated with the nitrogen content of the must and both the amount and type are dependent upon the yeast strain (Bell, et al., 1979).

Organic acids such as succinic, butanoic, propanoic and lactic acid also contribute to wine complexity. With the exception of acetic acid, they are usually only present in levels below the perception threshold.

The production of acetic acid by yeast is thought to be affected by nitrogen composition (as reviewed by Radler, 1993). Elevated acetic acid occurs when growth is generally limiting, presumably this applies when growth is protracted due to nitrogen limitation. The type of nitrogen source also has an affect, under conditions of nitrogen excess, methionine and valine promote acetic acid formation whereas ammonium, glutamic acid, glutamine and asparagine inhibit formation.

Succinic acid is linked to nitrogen metabolism, as it is produced as part of the TCA and Retrograde response and has been shown to form almost exclusively from glutamate (Albers, et al., 1998), which occurs from 2-oxoglutarate, via succinyl CoA by  $\text{NAD}^+$  utilising 2-oxoglutarate dehydrogenase (Nissen, et al., 1997).

Aldehydes and ketones, with the exception of acetaldehyde, have relatively little impact on the sensory profile of the fermentation aroma. Aldehydes are at detectable levels in the initial stages of fermentation, but then presumably are converted to alcohols (Rapp and Versini, 1991). The liberation of the sulfur containing compounds such as  $\text{H}_2\text{S}$ , organic sulphides and thiols is a major problem for wine aroma, as they have pungent off odours and extremely low sensory thresholds (Rankine, 1963). As previously mentioned, the formation of hydrogen sulphide is a particular problem in nitrogen limited wine fermentations. A clear understanding of the precise impact of juice nitrogen composition and yeast strain variation on the aroma profile of wine is lacking. In part this is attributable to the complexity and variability of both of these parameters.

#### ***1.14 Concluding statement***

Nitrogen is a vital yeast nutrient during fermentation and its impact on metabolism is beginning to be understood. Protracted fermentations due to

nitrogen deficiency are still a common problem in the wine industry. As wine yeast strains vary in the requirement and thus efficiency of nitrogen utilisation to maintain and complete fermentation there is scope to understand how these phenotypes occur and how they can be manipulated. The aims of this study are therefore to find genes that affect nitrogen efficiency, to better understand this process and then engineer strains that are able to reliably complete fermentation in low nitrogen musts.

## Chapter 2 Materials and Methods

### 2.1 *Yeast strains and maintenance*

The wine strains used in this study were *Saccharomyces cerevisiae* or *Saccharomyces bayanus*, as listed in Table 2.1. Laboratory strains of *Saccharomyces cerevisiae* are shown in Table 2.2. KP2 and KP3 (derivatives of W303) were chosen as laboratory strains, as they undergo fermentation in the presence of high concentrations of glucose (200 g L<sup>-1</sup>) (Poole, 2002). Strains were cultured in YEPD (yeast extract, 10 g L<sup>-1</sup>, bacteriological peptone, 20 g L<sup>-1</sup>, D-glucose, 20 g L<sup>-1</sup>) at 30°C with shaking at 160 rpm and maintained at 4°C on YEPD-agar (with the addition of 20 g L<sup>-1</sup> bacteriological agar), or at -80°C as glycerol stocks. (An aliquot of culture grown overnight in YEPD supplemented with glycerol to a final concentration of 15% v/v.)

### 2.2 *Bacterial strains and maintenance*

*Escherichia coli* was utilised in this study for the amplification of plasmid DNA. *E. coli* was cultured in Luria-Bertani (LB) broth (tryptone, 10 g L<sup>-1</sup>, yeast extract, 5 g L<sup>-1</sup>, NaCl, 10 g L<sup>-1</sup>) at 37°C with shaking at 160 rpm, and maintained on LB-Agar (LB with the addition of 20 g L<sup>-1</sup> bacteriological agar). To maintain plasmids during growth (where required) 100 mg L<sup>-1</sup> of ampicillin or 40 mg L<sup>-1</sup> of kanamycin was added to LB.

### 2.3 *Culture media*

#### 2.3.1 *Media for yeast cultures*

*S. cerevisiae* strains were routinely grown in YEPD. Minimal, Yeast Nitrogen Base medium was also used where highlighted. This medium contained 20 g L<sup>-1</sup> glucose, 5 g L<sup>-1</sup> ammonium sulphate and the salts, trace elements and vitamins, listed in Appendix I.

#### 2.3.2 *Chemically Defined Grape Juice Medium*

A chemically defined medium was used to imitate oenological conditions, with known concentrations of all components. This Chemically Defined Grape Juice



**Table 2.1** Wine yeast strains used in this study

| <b>Wine yeast strain</b> | <b>Source</b>     |
|--------------------------|-------------------|
| <b>L-2056</b>            | Lallemand Pty Ltd |
| <b>CY-3079</b>           | Lallemand Pty Ltd |
| <b>EC 1118</b>           | Lallemand Pty Ltd |
| <b>Burgundy</b>          | Lallemand Pty Ltd |
| <b>AWRI R2</b>           | Lallemand Pty Ltd |
| <b>S6U</b>               | Lallemand Pty Ltd |
| <b>AWRI 796</b>          | Lallemand Pty Ltd |
| <b>Vitileuvre</b>        | Lallemand Pty Ltd |
| <b>CEG</b>               | Lallemand Pty Ltd |
| <b>016</b>               | Lallemand Pty Ltd |
| <b>Simi white</b>        | Lallemand Pty Ltd |

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Table 2.2 Laboratory yeast strains used in this study.

| Yeast Strain         | Genotype <sup>a</sup>                                  | Source <sup>b</sup> |
|----------------------|--|---------------------|
| L-2056 S1            | L-2056 <i>HO/ho::kanMX3 MATa/α</i>                     | This study          |
| L-2056 1B            | L-2056 <i>ho::kanMX3 MATa</i>                          | This study          |
| L-2056 1D            | L-2056 <i>ho::kanMX3 MATα</i>                          | This study          |
| L-2056 1Ba           | L-2056 <i>ho::kanMX3 MATa rf</i>                       | This study          |
| L-2056 1Da           | L-2056 <i>ho::kanMX3 MATα rf</i>                       | This study          |
| 41a2                 | L-2056 <i>ura3::kanMX4/ura3::kanMX4 MATa/α</i>         | Michelle Walker     |
| 1Ba mit <sup>-</sup> | L-2056 <i>ho::kanMX3 MATa rf mit<sup>-</sup></i>       | This study          |
| 1Da mit <sup>-</sup> | L-2056 <i>ho::kanMX3 MATα rf mit<sup>-</sup></i>       | This study          |
| 1Ba <i>ura3</i>      | L-2056 <i>ura3::kanMX4 ho::kanMX3 MATa</i>             | This study          |
| 1Da <i>ura3</i>      | L-2056 <i>ura3::kanMX4 ho::kanMX3 MATα</i>             | This study          |
| W303                 | <i>ade2-1 can1-100 his 3-11,-15 leu2-3,-112 trp1-1</i> | S. Henry            |
| KP2                  | Prototrophic derivative of W303 <i>MATa</i>            | Poole, 2002         |
| KP3                  | Prototrophic derivative of W303 <i>MATa</i>            | Poole, 2002         |
| JMG2                 | KP2 <i>ura3::kanMX3 MATα</i>                           | This study          |
| JMG3                 | KP3 <i>ura3::kanMX3 MATa</i>                           | This study          |
| JMG2.82              | JMG2 <i>tif4631::mTn-3xHA/GFP MATα</i>                 | This study          |
| JMG2.98              | JMG2 <i>put4::mTn-3xHA/GFP MATα</i>                    | This study          |
| JMG2.113             | JMG2 <i>ngr1::mTn-3xHA/GFP MATα</i>                    | This study          |
| JMG2.120             | JMG2 <i>gid7::mTn-3xHA/GFP MATα</i>                    | This study          |
| 4417                 | BY4741 <i>tif4632Δ::kanMX4</i>                         | SGD                 |
| 7284                 | BY4741 <i>tif4631Δ::kanMX4</i>                         | SGD                 |
| 3352                 | BY4741 <i>ngr1Δ::kanMX4</i>                            | SGD                 |
| 3446                 | BY4741 <i>gid7Δ::kanMX4</i>                            | SGD                 |
| KP2 <i>tif4632</i>   | KP2 <i>tif4632Δ::kanMX4 MATa</i>                       | This study          |
| KP2 <i>tif4631</i>   | KP2 <i>tif4631Δ::kanMX4 MATa</i>                       | This study          |
| KP2 <i>ngr1</i>      | KP2 <i>ngr1Δ::kanMX4 MATa</i>                          | This study          |
| KP2 <i>gid7</i>      | KP2 <i>gid7Δ::kanMX4 MATa</i>                          | This study          |
| KP3 <i>ngr1B</i>     | KP3 <i>ngr1Δ::kanMX4 MATa</i>                          | This study          |
| KP2/3 <i>ngr1</i>    | KP2/3 <i>ngr1Δ::kanMX4 gid7Δ::kanMX4 MATa</i>          | This study          |
| C9                   | L-2056 <i>hoΔ MATa</i>                                 | Walker, et al.,     |
| C9D                  | L-2056 <i>hoΔ MATα</i>                                 | Walker, et al.,     |
| C9 <i>ngr1</i>       | L-2056 <i>hoΔ ngr1Δ::kanMX4 MATa</i>                   | This study          |
| C9 <i>gid7</i>       | L-2056 <i>hoΔ gid7Δ::kanMX4 MATa</i>                   | This study          |

<sup>a</sup> rf, retarded flocculation mutant; <sup>b</sup> SGD, *Saccharomyces cerevisiae* Deletion Project, [http://www.sequence.stanford.edu/group/yeast\\_deletion\\_project/deletions3.html](http://www.sequence.stanford.edu/group/yeast_deletion_project/deletions3.html)

Medium (CDGJM) was described in Henschke and Jiranek (1993) and the composition is listed in Appendix I.

## **2.4 Growth and fermentation**

### **2.4.1 Mini-fermentations**

Starter cultures (1 mL) of selected yeast strains were grown overnight in 48 well microtitre plates (Corning, Costar, cat # 3548) in YEPD at 30°C with shaking at approximately 150 rpm. Fermentations were carried out aerobically in a fresh 48 well microtitre plate in 1 mL of CDGJM containing 75-100 mg FAN L<sup>-1</sup> as ammonium, in each well. Inoculation from starter cultures was achieved by use of a 48-pinned replicator (manufactured by Will Gardner). Fermentations were incubated at 30°C with shaking at 100 rpm in sealed plastic containers, which included a water reservoir to prevent excess evaporation. In the secondary screens, triplicate fermentations were performed in 10-50 mL tubes containing 5-20 mL of CDGJM with 75-750 mg FAN L<sup>-1</sup> as ammonium or as a mixture of amino acids and ammonium (as defined in the text).

### **2.4.2 Laboratory scale anaerobic fermentations**

Fermentations were carried out under anaerobic conditions, in triplicate cultures of 150 mL of CDGJM where the concentration of nitrogen varied from low (75 mg FAN L<sup>-1</sup>) to high (750 mg FAN L<sup>-1</sup>) supplied either as ammonium alone or as a mixture of amino acids and ammonium (Henschke and Jiranek, 1993), and as listed in Appendix I.

50 mL starter cultures (in CDGJM Starter Medium, Appendix I) were inoculated from overnight YEPD cultures of the appropriate yeast strain in 250 mL Erlenmeyer flasks loosely fitted with a screw cap lid and incubated overnight at 30°C with shaking at 160 rpm. Fermentations were performed in 150 mL of CDGJM in 250 mL modified Erlenmeyer flasks. An anaerobic environment was maintained as these flasks were fitted with fermentation locks to allow the release of CO<sub>2</sub>. Samples were extracted with a 1 mL syringe through a septum sealed port (Suba seal®). Upon inoculation with cells at 5 x

$10^6$  cells  $\text{mL}^{-1}$ , starter culture flasks were sparged for 3 minutes with high purity nitrogen at approximately  $500 \text{ mL min}^{-1}$ . Flasks were then incubated at  $30^\circ\text{C}$ , shaking at 160 rpm. Samples were regularly collected, clarified by centrifugation and stored at  $-20^\circ\text{C}$ . Yeast growth was estimated from optical densities at 600 nm of appropriately diluted samples. Measures determined from fermentation samples are typically presented as an average of triplicate determinations with standard deviations included. Student's t-test was also used to determine the probability that two sets of data are different (represented as p values and considered significantly different when  $p < 0.05$ ).

#### ***2.4.3 Dry cell weight determination***

Millipore filters ( $0.22 \mu\text{m}$ , GSWG, 47 mm in diameter) were prepared by drying in a microwave oven on medium power setting (approximately 400 Watts) for 10 minutes, cooling in a desiccator and weighing with an analytical balance. Culture samples (2.5 - 10 mL, depending on cell density) were collected on prepared filters under vacuum and collected cells were washed with an equivalent volume of deionised water (Elga Maxima<sup>®</sup> purified water), microwaved, cooled and the filters re-weighed (as above) to determine dry cell weight.

#### ***2.4.4 Determination of glucose and ammonium by enzymatic analysis***

An estimation of glucose concentration, and thus the progress of fermentation was obtained during the initial stages of fermentation with a hand held refractometer (Atago), and during the final stages of fermentation using indicator tablets (Clinitest<sup>®</sup>, Bayer). The refractive index was measured in Brix units, a common measurement of density used in the wine industry, of which 1 Brix equates to roughly  $10 \text{ g L}^{-1}$  of sugar when no ethanol is present. Accurate determination of glucose and ammonium concentration of clarified culture supernatants was performed with Roche spectrophotometric enzymatic analysis kits (Arrow Scientific, cat # 0139106 and cat # 1112732, respectively). In some cases analysis of residual ammonium was also achieved using an ammonium electrode (Corning, cat # 476130), as per the manufacturer's instructions.

Spectrophotometric enzymatic assays, were performed as described by the manufacturer, with some modifications. Briefly, 300  $\mu\text{L}$  of working solution for glucose determination (0.25 M triethanolamine, 3.3 mM magnesium sulphate, 0.37 mM  $\beta$ -nicotinamide adenine dinucleotide phosphate (NADP), 2.67 mM adenosine 5'-triphosphate disodium salt (ATP) and 13.2  $\mu\text{g mL}^{-1}$  of hexokinase) or ammonium determination (0.15 M triethanolamine, 11 mM 2-oxoglutarate, 0.2 mM nicotinamide adenine dinucleotide (NADH) and 7.9 U  $\text{mL}^{-1}$  of glutamate dehydrogenase) were placed in a well of a 48 well microtitre plate and absorbance at 340 nm ( $A_{340}$ ) was measured with a  $\mu\text{Quant}$  micro-titre plate spectrophotometer (BioTek Instruments) set with an automatic correction to a 1 cm light path. 10  $\mu\text{l}$  of sample was added and incubated at room temperature for 15 minutes. The  $A_{340}$  was measured again, and the concentration of glucose or ammonium present in the sample determined using one of the following formulae:

$$C_{\text{glucose}} = (\Delta A_{S340} - \Delta A_{B340}) \times 0.89514 \times \text{Df}$$

$$C_{\text{ammonium}} = (\Delta A_{S340} - \Delta A_{B340}) \times 0.0843 \times \text{Df}$$

Where C is the concentration of the metabolite ( $\text{g L}^{-1}$ ),  $\Delta A_{S340}$  is the change in absorbance of the sample and  $\Delta A_{B340}$  is the change in absorbance of an assay performed with water instead of sample (blank). Df is the dilution factor.

#### ***2.4.5 Determination of other metabolites by HPLC***

Ethanol, glycerol, acetic acid, succinic acid and acetaldehyde were analysed from terminal samples by high performance liquid chromatography (HPLC). Samples were filtered through 0.45  $\mu\text{m}$  PVDF syringe filters (Millipore). HPLC analysis was performed on undiluted and diluted (1:20) samples on an Aminex HPX-87H column (300 mm x 7.8 mm) (BioRad). Elution was performed at 60°C with 2.5 mM  $\text{H}_2\text{SO}_4$  at a flow rate of 0.5  $\text{mL min}^{-1}$ . Detection was achieved by means of a RID-10A refractive index detector

(Shimadzu). Analytes were quantified by comparison with prepared standards in CDGJM using Delta integration software (Dataworks).

#### **2.4.6 *Viable cell counts***

The percentage of viable cells present during fermentation was measured by comparison of the number of colony forming units after 2-3 days of growth on YEPD from appropriately diluted cultures (in PBS) to a total cell count of the same time point, achieved with the use of a hemocytometer.

### **2.5 *Yeast classical genetics***

#### **2.5.1 *Yeast mating***

A loop full of fresh overnight culture from YEPD agar was mixed thoroughly with a yeast strain of the opposite mating type on a thin YEPD plate and incubated for 2 hours at 30°C. Mating cells were then identified by their characteristic barbell shape and separated from the mixed population using a micromanipulator (Olympus). Isolated diploid strains were then incubated for a further 48 hours at 30°C.

#### **2.5.2 *Yeast rare mating***

Strains were grown in 5 mL of YPAD (YPED with an addition of 100 mg L<sup>-1</sup> adenine) for 48 hours, at 27°C. 5 mL of fresh YPAD was then inoculated with 200 µL of culture of each strain, mixed and incubated statically at 27°C for 4 days. 100 – 1000 µL aliquots of the mating mixture were then plated on selective media.

#### **2.5.3 *Yeast sporulation***

Cells were plated on solid potassium acetate sporulation medium (1 % potassium acetate, 0.1 % yeast extract, 0.05 % glucose, 2 % bacto-agar) and incubated for up to 10 days at 30°C. The formation of asci was monitored by examination of a wet mount using an Olympus phase contrast microscope.

Spores were prepared for spore-cell mating experiments by digestion of the ascus wall with  $\beta$ -glucuronidase, as below, yet suspensions were vortexed for 1 minute and incubated for 8-12 hours at 30°C. The suspension was then vortexed for 2 minutes, centrifuged for 2 minutes at 2,000 g, washed in 1 mL of TE Buffer (Appendix I), and finally resuspended in 50  $\mu$ L of TE Buffer.

#### **2.5.4 *Ascus microdissection***

To disrupt the ascus wall, a loop full of culture from sporulation plate was resuspended in 1 mL of 100 U mL<sup>-1</sup>  $\beta$ -glucuronidase (Sigma G-7770) and incubated for 15 – 60 minutes at 30°C. The spore suspension was then streaked onto a thin YEPD plate, and asci containing 4 spores were isolated and dissected using a micromanipulator (Olympus).

#### **2.5.5 *High efficiency transformation of *S. cerevisiae* using lithium acetate***

A single colony from a YEPD plate was used to inoculate YEPD broth, grown overnight, subcultured and grown to a density of  $2 \times 10^7$  cells mL<sup>-1</sup> (an absorbance of approximately 0.4 at an optical density of 600 nm (OD<sub>600</sub>)). Cells from 50 mL of culture were then harvested at 2,700 g for 5 min and resuspended in 25 mL of deionised water. Cells were harvested and resuspended in 1 mL of freshly prepared buffered lithium solution (100 mM lithium acetate, 100 mM Tris base (pH 8.0), 10 mM ethylenediaminetetraacetic acid (EDTA)). 200  $\mu$ L of the cell suspension was then mixed with 200  $\mu$ g of carrier DNA (Sigma, cat # D-1626; prepared by boiling a 2 mg mL<sup>-1</sup> stock in TE Buffer for 10 minutes, and then cooling on ice for 5 minutes), 2-5  $\mu$ g of transforming DNA (in a volume of less than 20  $\mu$ l), and 1.2 mL of PEG solution (40 % w/v polyethylene glycol 4000 (Fluka) in buffered lithium solution). Each transformation was then incubated at 30°C for 30 minutes followed by a heat shock at 42°C for 45 minutes. Cells were harvested at 2,700 g for 5 minutes and resuspended in 200  $\mu$ L of TE Buffer before plating on selective media. Where geneticin was used as a selection, heat-shocked cells were cultured without selection for 1-2 hours before plating on media with 200-1000 (typically 400) mg L<sup>-1</sup> of geneticin.

## **2.6 Nucleic acid isolation**

### **2.6.1 Isolation of genomic DNA from *S. cerevisiae***

Approximately  $2 \times 10^9$  cells from a 10 mL YEPD stationary phase culture were collected by centrifugation at 20,000 g and then washed in 1.0 mL of sterile deionised water. Cell pellets were resuspended in 0.2 mL of Cell Lysis Solution (2 % w/v Triton X-100, 1 % w/v sodium dodecyl sulphate (SDS), 100 mM sodium chloride (NaCl), 10 mM Tris base (pH 8.0) and 1 mM EDTA). Following this, 0.2 mL of phenol:chloroform (5:1) and 0.3 g of acid washed glass beads were added. Samples were vortexed for 4 minutes, 0.2 mL of TE Buffer was added and samples were centrifuged at 20,000 g for 2 minutes. The aqueous layer was collected in a fresh tube and 1 mL of absolute ethanol was added. Samples were centrifuged for another 2 minutes at 20,000 g and the supernatant discarded. The pellet was resuspended in 0.4 mL of TE buffer, containing 3  $\mu$ L of RNase cocktail (1.5 U of RNase A and 60 U of RNase T1) and samples were incubated for 30 minutes at 30°C. Genomic DNA was precipitated with 10  $\mu$ L of 4 M ammonium acetate and 1 mL of absolute ethanol, and collected by centrifugation at 20,000 g for 2 minutes. The pellet was air-dried and resuspended in deionised water.

### **2.6.2 RNA preparations from yeast**

Approximately  $5 \times 10^8$  yeast cells were harvested by centrifugation at 12,800 g for 5 minutes. The pellet was washed in 1 mL Phosphate Buffer Solution (PBS, Appendix I), followed by resuspension in 0.5 mL of TRIzol® Reagent (Invitrogen, Cat# 15596-026), in a 2 mL screw capped centrifuge tube. Samples were plunged into liquid nitrogen for 20 seconds, and then allowed to thaw slowly on ice. Acid-washed glass beads (0.3 g) were then added and samples were vortexed for 2 minutes. Following this, samples were incubated at 65°C for 3 minutes, and with a 100  $\mu$ L addition of chloroform, were shaken vigorously by hand for 15 seconds. After incubation at room temperature for 5 minutes, cell debris was pelleted by centrifugation at 12,800 g, for 10 minutes at 4°C. The top clear phase was collected in a new tube, 250  $\mu$ L of isopropanol



was added, the tube was gently mixed by inversion 6 times and incubated at room temperature for 10 minutes. RNA was collected by centrifugation at 12,800 g for 10 minutes at 4°C. Pellets were washed in 75 % v/v ethanol (vortexed and re-centrifuged for 5 minutes at 12,800 g at 4°C). After washing, the pellet was air-dried for 10 minutes, ensuring that ethanol was completely removed. Finally, RNA was dissolved in 100 µL of RNase-free water (deionised water; treated overnight at room temperature with 0.1 % v/v Diethyl pyrocarbonate (DEPC) and autoclaved to degrade residual DEPC), and incubated at 65°C for 5 minutes, with occasional vortexing, to resuspend the pellet. RNA samples were stored at -80°C.

RNA preparations used in Real-Time PCR were treated with DNase utilising the DNA-free kit (Ambion), as per manufacturer's instructions, to ensure the complete removal of contaminating DNA.

### **2.6.3 Determination of DNA or RNA concentration**

The concentration of DNA and RNA preparations was determined from the absorbance of a suitable dilution at 260 nm ( $OD_{260}$ ), given that the  $OD_{260}$  of a solution of double-stranded DNA at 50 µg mL<sup>-1</sup> or RNA at 40 µg mL<sup>-1</sup> is approximately 1. Purity of nucleic acids (from protein) was also estimated by determination of the absorbance at 280 nm ( $OD_{280}$ ), and comparison of the ratio of  $OD_{260}:OD_{280}$ . A ratio of 1.8-2.0 was deemed adequate.

### **2.6.4 Plasmid preparation from *E. coli***

Plasmids were routinely isolated by alkaline lysis, similar to that described in Sambrook et al. (2001). *E. coli* cultures were grown overnight from a single colony in LB broth with the appropriate antibiotics. Cells from 1.5 mL of culture were harvested by centrifugation and thoroughly resuspended in 100 µL of Solution 1 (50 mM D-glucose, 25 mM Tris base (pH 8.0) and 10 mM EDTA (pH 8.0)) and then 200 µL of Solution 2 (0.2 M sodium hydroxide (NaOH) and 1 % w/v SDS) was added and the tube inverted 4 times. Following this, 150 µL of Solution 3 (3 M potassium acetate and 11.5 % v/v glacial acetic acid) was

added and the tube was vortexed in an inverted position for 10 seconds, before 15 - 30 minutes incubation on ice. Each sample was centrifuged for 10 minutes at 1,800 g. The supernatant was transferred to a fresh tube and RNase treated with 1  $\mu$ L of RNase cocktail (0.5 U of RNase A and 20 U of RNase T1) for 30 - 60 minutes at 37°C. Following this, samples were extracted with an equal volume of phenol:chloroform (5:1), the upper phase was collected and ethanol precipitated with 250  $\mu$ l of 7.5 M ammonium acetate and 0.7 volumes of isopropanol. Samples were incubated for 15 minutes on ice before centrifugation at 18,000 g for 15 minutes. The pellets were washed with ice-cold 70 % v/v ethanol and air-dried. Plasmid DNA was resuspended in 50  $\mu$ l of deionised water or TE Buffer and stored at -20°C.

High purity plasmid DNA preparations were prepared using the Promega Wizard *Plus* SV plasmid miniprep kit, or the MO BIO-Ultra clean plasmid mini-prep kit, as per the manufacturers instructions.

#### ***2.6.5 Rapid determination of plasmid DNA size***

Single bacterial colonies were selected with a pipette tip, patched onto an LB agar plate (containing 50  $\mu$ g mL<sup>-1</sup> ampicillin), and then resuspended in 15  $\mu$ l of Cracking Solution (50 mM NaOH, 0.5 % w/v SDS, 0.1 M EDTA, 5 mM glycerol and 0.005 % w/v bromophenol blue). With the pipette tip still in the solution, each sample was incubated at 65°C for 15 minutes, and the sample was loaded onto a 1 % w/v TAE agarose gel (Appendix I). Initially, only minimal TAE Running Buffer was present in the chamber so as to avoid dispersal of the sample in the TAE buffer. Samples were run into the gel at low voltage (2 V cm<sup>-1</sup>), and then the gel was covered with TAE buffer and the voltage increased to 6 V cm<sup>-1</sup>. Plasmids containing inserts were identified as larger products compared to the vector alone.

## **2.7 Molecular cloning techniques**

### **2.7.1 Restriction endonuclease digestion of DNA**

DNA (1-10 µg) was incubated with 5-50 U of restriction enzyme in a final volume of 50 µl at 37°C overnight, with the appropriate buffer, as directed by the manufacturer. When required, DNA was cleaned following with the MO BIO-Ultra Clean DNA purification kit, as per manufacturer's instructions.

### **2.7.2 Dephosphorylation of vector DNA**

Approximately 2 µg of restriction-digested plasmid DNA was agarose gel purified, with the use of the Perfect Prep DNA purification kit (Eppendorf), and then de-phosphorylated with typically 0.1-0.5 U of calf intestinal phosphatase per mole of DNA ends and 2 µL of 10 x CIP Buffer (Roche, Cat # 713 023) in a total volume of 20 µL. The reaction was incubated at 37°C for 60 minutes, and heat inactivated at 65°C for 10 minutes. DNA was cleaned using the MO BIO-Ultra clean DNA purification kit according to the manufacturer's instructions. The pellet was briefly dried under vacuum and resuspended in 10 µl of deionised water.

### **2.7.3 Ligation of DNA into plasmid**

Ligation of restriction-digested DNA fragments into appropriately digested and alkaline phosphatase treated vector DNA was performed with (0.1-50 Weiss Units) T4 DNA ligase (Gene Works) and the associated buffer, as directed by the manufacturer, in a maximum total volume of 15 µL. The molar ratio of insert to vector ends was prepared at 5:1 whenever possible, and in some instances, polyethylene glycol, magnesium chloride and ATP were also added, typically in the concentrations of 9.3 % v/v, 3.33 mM and 0.33 mM respectively.

### **2.7.4 Preparation and use of competent *E. coli* cells for transformation**

An overnight culture of *E. coli* DH5α cells was diluted 1:10 in LB broth and grown to an absorbance of 0.4 at 580 nm. The cells were chilled on ice for 10

minutes and pelleted at 4°C at 1,300 g for 7 minutes. The pellet was gently resuspended in cold calcium chloride (CaCl<sub>2</sub>) solution (60 mM CaCl<sub>2</sub>, 15 % v/v glycerol, 10 mM PIPES pH 7.0) and left on ice for 30 minutes. Cells were washed twice by pelleting at 1,300 g for 5 minutes at 4°C and resuspending in 10 mL of cold CaCl<sub>2</sub> solution. Finally, cells were resuspended in CaCl<sub>2</sub> solution and stored in individual aliquots at -80°C.

Previously prepared competent cells were thawed at room temperature and gently mixed. 200 µl of cells were added to no more than 15 µL of ligation mix and incubated on ice for 30 minutes. Cells were heat-shocked at 42°C for 60 seconds and returned to an ice bath for 5 minutes. 2 mL of LB was added and samples were incubated at 37°C for 45 minutes on a rotating wheel. Transformed cells were spread onto LB plates containing the appropriate antibiotic, and incubated overnight at 37°C.

## **2.8 Nucleic acid amplification procedures**

### **2.8.1 Polymerase Chain Reaction (PCR)**

PCR amplification was performed in 12.5 - 50 µl reactions typically containing 1 U DyNAzyme EXT DNA polymerase (Finnizymes F-505S), DyNAzyme EXT PCR Buffer, 100 pmol primer, 50 ng plasmid DNA or 200 ng genomic DNA, 0.5 mM deoxy nucleotide triphosphates (dNTPs). Magnesium chloride concentrations and cycling parameters were optimised for each PCR reaction. Primers used for PCR amplification are listed in Table 2.3. Cycling reactions were conducted using an Eppendorf *Mastercycler gradient* thermocycler, or a Corbett thermocycler.

### **2.8.2 Vectorette PCR**

Vectorette PCR was undertaken, essentially as is outlined by Carl Friddle (<http://genome-www.stanford.edu/group/botlab/protocols/vectorette.html>).

Genomic DNA (1-3 µg) was digested with 10 U of *DraI* in a total volume of 20 µL. Samples were inactivated at 65°C for 20 minutes, and then 3 µL of *DraI* restriction digestion buffer, 1 µL (400 U) of T4 DNA ligase, 0.5 µL of 5

Table 2.3 Primers used in this study

| Primer       | Sequence (5'→3')   |
|--------------|--|
| HOFga        | TGAGCTGTTGCTTACGGTGC   |
| HORga        | CAAACCTAATGTGACCGTCGCT   |
| HOFwd        | GTCACGGCTAACTCTTACGTTATGTGCGCA   |
| HOcRev       | GCTTCACGAACTTCTATATGCTCGCCGTAC   |
| ura3kanF     | TTCATTGGATGTTTCGTACCACCAAGGAATTACTGGAGTTA-<br>-GTTGAAGCATTAGGTCCCAGACATGGAGGCCAGAATAC  |
| ura3kanR     | ATAGTCCTCTTCCAACAATAATAATGTCAGATCCTGTAGA-<br>-GACCACATCATCCACGTTTCAGTATAGCGACCAGCATTCC |
| MAT          | AGTCACATCAAGATCGTTTATGG  |
| MATa         | ACTCCACTTCAAGTAAGAGTTTG  |
| MAT $\alpha$ | GCACGGAATATGGGACTACTTCG  |
| URA3F1       | AGGGAAGACAAGCAACGAAA   |
| URA3R        | TTGGTTCTGGCGAGGTATTG   |
| IPCR3        | GTTTTTCGTTCCACTGAGCGT  |
| IPCR4        | ACAAGACACGTGCTGAAGTCA  |
| GFPprobeF    | ACGCTCAGTGGAACGAAAAC   |
| GFPprobeR    | TGACTTCAGCACGTGTCTTG   |
| THG.SEQ1     | AGCGGCCTTCTTTCTTTGGAAGTAC  |
| NGR1A        | AAAACCATCTGCGTGTATATCCTA   |
| NGR1D        | GACTTTGTAGCGATTTTCACATTTT  |
| GID7A        | GTTTCAGATCTATGCTGAGACACG   |
| GID7D        | CCATTTGGTATGGATTATCACTAGG  |
| G418F        | AAAAGACTCACGTTTCGAGGC  |
| G418R        | CGAGCATCAAATGAAACTGC   |
| ABPa         | GAAGGAGAGGACGCTGTCTGTCGAAGGTAAGGAA-<br>-CGGACGAGAGAAGGGAGAG                            |
| ABPb         | GACTCTCCCTTCTCGAATCGTAACCGTTCGTACGAG<br>-AATGCGTGTCTCTCCTTC                            |
| GFP          | CATCACCTTACCCTCTCCACTGAC   |
| Xa           | CTTCTACCTTCAATGGCCGCC  |
| UV           | CGAATCGTAACCGTTCGTACGAGAATCGCT   |
| THG.SEQ1     | AGCGGCCTTCTTTCTTTGGAAGTAC  |

mM ATP, 24.5  $\mu$ L of deionised water and 1  $\mu$ L of annealed anchor bubble was added. Annealed anchor bubble was prepared by heating a mixture of ABPa and ABPb, each at a concentration of 4  $\mu$ M, at 65°C for 5 minutes, adding magnesium chloride to 2 mM, and slowly cooling the solution to room temperature. The ligation mixture was then incubated at 16°C for 24 hours. Following this, a 100  $\mu$ L PCR reaction was assembled typically from the following components, 5  $\mu$ L of the ligation reaction, 2.5  $\mu$ L of 20  $\mu$ M Xa or GFP primers (anneals to mTn3xHA/GFP) and 2.5  $\mu$ L of 20  $\mu$ M UV primer (anneals to the anchor bubble), 8  $\mu$ L of 2.5 mM deoxy-nucleotides, 10  $\mu$ L of Dynazyme EXT buffer, 71  $\mu$ L of deionised water and 0.5  $\mu$ L (5U) of Dynazyme-EXT. Hot start amplification was performed with 35 cycles of 92°C, 20 seconds, 67°C, 30 seconds and 72°C for between 45 and 180 seconds, depending on the size of the expected product. Putative fragments containing mTn-3xHA/GFP and adjacent sequences were gel separated and purified with the Perfect-prep gel clean up kit (Eppendorf), according to the manufacturer's instructions. Purified fragments were then sequenced with the Xa primer.

### **2.8.3 Sequencing reactions**

Sequencing cycle reactions were set up as recommended by Applied Biosystems (ABI), with a 20  $\mu$ L volume containing 3.2 pmol of primer, 50 - 200 ng of template DNA and 4  $\mu$ L of ABI Big Dye sequencing reagent. PCR reactions were as follows, 25 cycles of 96°C, 10 seconds, 50°C, 5 seconds and 60°C, 4 minutes. Sequencing products were precipitated with 80  $\mu$ L of 75 % v/v isopropanol, where reactions were vortexed and incubated for 30 minutes at room temperature, before centrifugation for 20 minutes at 20,000 g. Supernatants were carefully aspirated and pellets were washed with 250  $\mu$ L of 75 % v/v isopropanol. Air-dried pellets were sent to the IMVS Molecular Pathology sequencing service for separation and analysis using an ABI automated sequencer.

#### **2.8.4 Direct genomic sequencing**

Genomic DNA was prepared with the Qiagen Yeast gDNA (100/G) extraction kit. Genomic DNA (10 µg) was ethanol precipitated and resuspended in 16 µl of Big Dye ready reaction mix (Applied Biosystems), and 4 µL of THG.SEQ1 primer (3.5 pmol µL<sup>-1</sup>). PCR was then undertaken, with the following cycles; 5 min at 95°C followed by 90 cycles of 95°C, 25 seconds and 60°C, 2 minutes. PCR reactions were then purified on a Centricon<sup>®</sup> column, isopropanol precipitated and sequenced via the IMVS Molecular Pathology sequencing service as above.

#### **2.8.5 Colony cracking PCR for rapid screening of transformants**

In some cases putative positive transformants were tentatively identified by taking advantage of the cell lysis that occurs at extreme temperatures (>80°C) by using a variation of the standard PCR protocol. In this case template DNA was not isolated before PCR was conducted. A sample 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 (Geneworks), DyNAzyme EXT PCR Buffer, 100 pmol of appropriate primer and 0.5 mM dNTPs. PCR was then conducted as previously outlined.

#### **2.8.6 PCR labelling of probes for Southern Blot analysis**

DNA probes were labelled with Digoxigenin-11-dUTP (DIG), by incorporation into PCR products using Taq DNA polymerase. The PCR DIG Probe Synthesis Kit (Roche Cat#1 636 090) was used as per manufacturer's instructions.

#### **2.8.7 Quantitative Real Time PCR**

Quantitative Real Time PCR was performed on a BioRad Iq5<sup>®</sup>. Reactions were prepared with the Qiagen one step RTPCR kit (final volume 12.5µl), as per the manufacturer's instructions, with the addition of 100 ng of template RNA and 20 pmol of primers (Table 2.4). Each experimental reaction was evaluated in triplicate from two separate triplicate fermentations; that is, each

**Table 2.4** Primers used for Quantitative Real Time PCR, designed with the aid of the Qiagen primer design program available freely at <https://customassays.qiagen.com/design/inputsequences.asp>

| Primer    | Sequence (5'-3')       |
|-----------|------------------------|
| QiaNGR1F  | CCAAAGACAGGAAAATCCATA  |
| QiaNGR1R  | CTGGGGGTTCAGTAGAAGT    |
| QiaGID7F  | CAAATGTGGGACTATAAAGAAA |
| QiaGID7R  | ACCGTAGGCGAAGCAAGA     |
| QiaGDH1F  | GTAAGCACCGAACAGGTAA    |
| QiaGDH1R  | CATGAGAGAATTGAGCAGACA  |
| QiaGDH2F  | CCCAGGATTGGTAGAACA     |
| QiaGDH2R  | ATGGATCGTTGATTAGAATG   |
| QiaGDH3F  | GACGGGCATTACTTCTGA     |
| QiaGDH3R  | CAACGATTCCTCTAACGACT   |
| QiaGLT1F  | GGTGTCCAACCAAGTTC      |
| Qia GLT1R | GGTAAGCCACGATCCAAAGTA  |
| QiaGLN1F  | CAATGACGGTACTCCAAAC    |
| QiaGLN1R  | ACCAGATTTCTTCATCCTTA   |
| QiaHXT6F  | TCAACACCATGTGGGAAGAA   |
| QiaHXT6R  | ATGGCTTATCATCGTGAGCC   |
| QiaHXT7F  | TCAACACCATGTGGGAAGAA   |
| QiaHXT7R  | ATGGCTTGTCATCGTGAGTC   |
| QiaACTF   | GTTACTCACGTCGTTCCA     |
| QiaACTR   | GTCAAATCTCTACCGGCCAAA  |



measure was an average of 18 data points. Transcripts purified from mutant strains were quantitated by comparison to those generated from the parent strain, and also checked against an actin control (*ACT1*).

Standard curves were also generated with each primer pair, from control strain RNA (from five or six dilutions ranging from  $5 \times 10^2$  ng  $\rightarrow$   $1 \times 10^{-3}$  ng). Amplification from mutant strain RNA was also evaluated to check for template bias.

At the completion of each QRT-PCR, a melt curve analysis was undertaken, to determine the number of major PCR products present. The following cycle was performed; 80 cycles commencing at 60°C for 10 seconds, with each subsequent cycle increasing by 0.5°C.

## **2.9 Southern Blot analysis of genomic DNA**

Appropriately digested yeast genomic DNA was heat treated at 65°C for 20 minutes, and was combined with gel loading buffer (30 % v/v glycerol, 0.25 % w/v bromophenol blue, and 0.25 % w/v xylene cyanol) before separation on a 1 – 2 % w/v TAE agarose gel. DIG-labelled molecular weight markers were also included (Roche DNA Molecular Weight III, Cat # 1 218 603). After separation of the DNA the gel was depurinated by incubation in Depurination Buffer (250 mM hydrochloric acid) for 10 minutes. The gel was rinsed with deionised water before incubation in Denaturation Solution (0.5 M NaOH, 1.5 M NaCl) twice for 15 minutes. Again the gel was rinsed with deionised water, and submerged in Neutralisation Solution (1.5 M Tris-Cl, 1.5 M NaCl, pH 7.5) twice for 15 minutes.

DNA was transferred from the gel to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) by capillary transfer using 20 X SSC buffer (3 M NaCl, 0.3 M tri-sodium citrate). After transfer DNA was cross-linked to the nylon membrane using an Amersham Life Sciences UV cross-linker (254 nm @ 70,000  $\mu$ J/cm<sup>2</sup>).

DIG Easy Hyb buffer (Roche) was used for pre-hybridisation and hybridisation. Nylon membranes were placed in glass hybridisation tubes with 30 mL of DIG Easy Hyb Buffer and pre-hybridised for 2 hours at 42°C. Probes (PCR labelled with DIG) were boiled for 10 minutes followed by rapid cooling in an ice bath, and the denatured probe diluted in DIG Easy hyb buffer was hybridised to the membrane bound DNA overnight at 42°C. Following this, the membrane was washed twice in 2 X SSC, 0.1 % w/v SDS at room temperature for 15 minutes, followed by two more 15 minute washes in 0.5 X SSC, 0.1 % w/v SDS at 68°C. The membrane was equilibrated in maleic acid buffer (1 M Maleic acid, 1.5 M NaCl, pH 7.5) for 1 minute. All subsequent washes were performed at room temperature. The membrane was blocked by incubation in 1 X Blocking Solution (Roche) dissolved in Maleic Acid Buffer. Anti-Digoxigenin-AP antisera (Roche) was diluted 1:10,000 in fresh Blocking Solution and the membrane was incubated in this solution for 30 minutes with gentle agitation. The membrane was washed twice for 15 minutes in Maleic Acid Wash Buffer (Maleic Acid Buffer with 0.3 % v/v Tween-20) before equilibration in Detection Buffer (0.1 M Tris-Cl, 0.1 M NaCl, pH 9.5). The membrane was sealed in a plastic bag containing 0.5 mL of ECF substrate/ 100 cm<sup>2</sup> (Amersham Life Sciences, Cat # 1067873) and incubated in the dark for 16 hours. Fluorescent bands were detected using a Storm<sup>®</sup> phosphoimager with a blue chemifluorescent filter.

### ***2.10 Random chemical mutagenesis***

Ethyl methanesulfonate (EMS) was used to randomly mutagenise the yeast genome, essentially as described by Lindegren et al. (1968). Wild type cells were grown until stationary phase in YEPD. Cells were harvested by centrifugation and washed with deionised water before resuspension in 9.7 mL of 0.1 M sodium phosphate buffer at  $5 \times 10^7$  cells mL<sup>-1</sup>. Cultures were heated at 30°C for 5 minutes, and then 300 µL of EMS was added and mixed. Culture samples (100 µl) were removed at 10 minute intervals and diluted with 9.9 mL of 6 % w/v sodium thiosulfate to inactivate the EMS. Cells were cooled on ice for 5 minutes and were diluted ten-fold in 15 % v/v glycerol and stored at -80°C. The cellular death rate was examined by plotting the percentage of

viable cells against time. The cells subjected to EMS for 75 minutes had a 97 % death rate, these were selected for the selection of non-flocculant mutants.

### ***2.11 Determination of flocculation status***

Flocculation status was determined by a modified Helm sedimentation test, as described by Soares et al. (1992). This method relies on the fact that non-flocculant cells remain suspended longer than flocculant cells. Approximately  $2 \times 10^9$  cells from an overnight culture were washed in 250 mM EDTA, and then in 250 mM NaCl, pH 2. Cells were resuspended in a measuring cylinder with 24 mL of  $15 \text{ g L}^{-1}$  NaCl at pH 4.0, which was subsequently adjusted to 4 mM  $\text{CaCl}_2$  and inverted and mixed to promote flocculation. The suspension was allowed to settle for 2 minutes after which 100  $\mu\text{L}$  of cell suspension was collected from 2 cm under the meniscus. Selected cells were diluted in YEPD, re-grown until saturated and following this the flocculation selection was repeated, so as to selectively amplify the non-flocculant mutants. Re-growth and selection was repeated a total of four times.

### ***2.12 Disruption and selection of mitochondrial mutants by ethidium bromide mutagenesis***

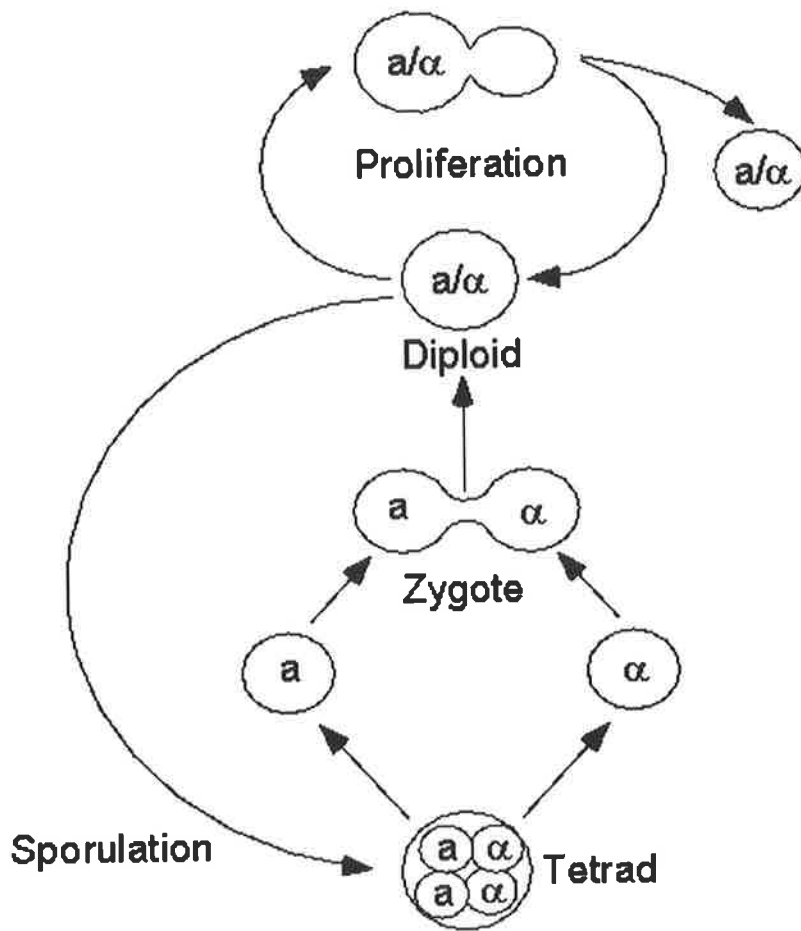
A selected overnight YPAD culture (100  $\mu\text{L}$ ) was inoculated into 5 mL of minimal medium containing  $10 \mu\text{g mL}^{-1}$  of ethidium bromide. Flasks were wrapped in foil and incubated at  $30^\circ\text{C}$  for 24 hours. A sample was taken from each flask, diluted ten-fold in PBS and incubated on YPDG (1 % w/v yeast extract, 2 % w/v bacto-peptone, 3 % v/v glycerol, 0.1 % w/v glucose and 2 % w/v bacto-agar) at  $30^\circ\text{C}$  for 3 days. Petite colonies were selected and their inability to respire was confirmed by lack of growth on YEPGE (0.67 % w/v yeast extract, 2 % w/v peptone, 2 % v/v glycerol, 2 % v/v ethanol and 2 % w/v bacto-agar).

## **Chapter 3 Construction of a wine yeast derivative suitable for research using classical and molecular genetic techniques**

### **3.1 Introduction**

Traditionally, wine fermentations were performed by indigenous yeast found upon grapes or winemaking equipment. As knowledge flourished of the significant impact that selected strains can have on the fermentation characteristics and consequently the final attributes of a finished wine, New World winemaking embraced the availability of purified wine yeast strains. Of the wine yeast that have been isolated, considerable differences between their fermentation characteristics have been noted (Manginot, et al., 1998; Carrasco, et al., 2001; Gardner, et al., 2002; Berthels, et al., 2004). This highlights the scope for further strain development, and one method of achieving this is through genetic manipulation.

Industrial wine yeast strains are typically difficult to manipulate genetically. Application of classical genetic techniques to industrial wine yeast can be troublesome as they are often genetically diverse and can have complex life cycles (Bakalinsky and Snow, 1990). The life cycle of budding yeast can include transitions between ploidy (as outlined in Figure 3.1), further complicated by the fact that they are commonly homothallic. Compounding this, wine strains can be polyploid or aneuploid, they do not contain convenient genetic markers and, since many are flocculant, estimates of cell numbers can be troublesome and inaccurate. Most current industrial strains have been isolated from nature and consequently their origins are complex and mostly unknown. Thus the occurrence of sequence divergence between mated populations can be common and this, together with the possibility of chromosomal rearrangements during meiosis, can result in poor spore viability (as reviewed by Wolfe, 2003). Industrial yeast strains can be made heterothallic simply by deletion of the *HO* gene (Tamai, et al., 2001). The *Ho* protein enables the cell to switch mating type, facilitating mating of cells within a population originally of one mating type.



**Figure 3.1** A diagrammatic representation of the transitions in ploidy as reviewed by Herskowitz (1988). *S. cerevisiae* can proliferate through a mitotic cell cycle in either a haploid or diploid state, but most often given that nutrients are not limiting, exist as diploids. Under certain circumstances, such as starvation, diploids can undergo sporulation, consisting of both meiosis and spore formation, to form haploid spores contained in an ascus and these spores are capable of germination to produce haploid cells capable of mitotic proliferation. Haploid cells of opposite mating type can mate through cell fusion to form a zygote and consequently a diploid cell. This process is enhanced in most yeast strains as they are homothallic, meaning that cells can switch their mating type through the action of the Ho endonuclease and mate with cells originating from the same parent.

The purpose of this initial work was to develop a wine yeast strain that could be easily manipulated by classical and molecular genetic techniques as part of a genetically based strain modification. Objectives included an analysis of sporulation, mating and flocculation in a selection of commercial wine strains. Then, upon selection of a suitable strain, construction of a heterothallic version with the addition of a selectable marker was required. Having been found to have the desired attributes, a selectable heterothallic derivative of L-2056 was constructed by disruption of both the *HO* and *URA3* genes.

### **3.2 Results**

#### **3.2.1 Analysis of industrial wine yeast strains suitable for genetic manipulation**

The sporulation patterns of eleven commonly used wine yeast strains were evaluated (Table 3.1). L-2056 was selected as it sporulated quickly (within 3 days) and efficiently (approximately 60 % of cells formed asci, and 89 % of these contained 4 spores). Importantly, in 75 % of dissected tetrads all four of these spores were viable, as determined by growth after micro-dissection on YEPD agar (Table 3.2). Spore products derived from L-2056 were allowed to re-diploidise and then were subjected to another round of sporulation and dissection. These strains again displayed the same desirable sporulation pattern as the parent (data not shown). The strains Burgundy and S6U also produced viable four-spore tetrads (67 % and 83 %), yet four spore tetrads were scarce within the sporulated Burgundy population (5 %) and upon sporulation and dissection of re-diploidised daughter strains of S6U, no viable tetrads were found. Using these methods a range of phenotypic variation was seen between these wine yeast strains at this level, both in terms of ease of sporulation, the number of spores and their viability.

L-2056 was originally isolated from vineyards in the Côtes du Rhône. This strain is reported to have a moderate fermentation rate, a relatively high nutrient requirement, good alcohol tolerance as well as low SO<sub>2</sub> and VA production (Lallemand Pty Ltd). Re-diploidised spores derived from two L-2056 tetrads were analysed during fermentations performed in CDGJM with 771 mg FAN L<sup>-1</sup>. The fermentation duration, total nitrogen utilised, final cell

**Table 3.1** Sporulation properties of industrial wine yeast strains.

| Yeast strain | Time taken to sporulate (days) | Proportion of sporulated cells (%) | Percentage of asci with |          |          |
|--------------|--------------------------------|------------------------------------|-------------------------|----------|----------|
|              |                                |                                    | 2 spores                | 3 spores | 4 spores |
| L-2056       | 3                              | 60                                 | 0                       | 11       | 89       |
| CY-3079      | 4                              | 65                                 | 0                       | 0        | 100      |
| EC 1118      | 4                              | 35                                 | 50                      | 0        | 50       |
| Burgundy     | 1                              | 70                                 | 70                      | 5        | 5        |
| R2           | 4                              | 65                                 | 40                      | 1        | 1        |
| S6U          | 3                              | 35                                 | 10                      | 5        | 10       |
| 796          | 3                              | 30                                 | 10                      | 5        | 0        |
| Vitileuvre   | 6                              | 1                                  | 0                       | 2        | 1        |
| 016          | 3                              | 1                                  | 0                       | 1        | 0        |
| Simi white   | 3                              | 70                                 | 40                      | 30       | 1        |
| CEG          | ND <sup>a</sup>                | N/A                                | N/A                     | N/A      | N/A      |

Sporulation was induced by inoculation of liquid sporulation medium with overnight YEPD cultures of each of the given yeast.

<sup>a</sup> ND, not detected after 14 days incubation. N/A not applicable.

**Table 3.2** The number of viable spores within four spore tetrads.

| Yeast strain | Viable Spores |     |     |     |     | Dissected tetrads giving 4/4 viability (%) |
|--------------|---------------|-----|-----|-----|-----|--|
|              | 0/4           | 1/4 | 2/4 | 3/4 | 4/4 |  |
| L-2056       | 0             | 0   | 0   | 2   | 6   | 75   |
| CY 3079      | 0             | 1   | 3   | 10  | 0   | 0  |
| EC 1118      | 3             | 4   | 5   | 0   | 0   | 0  |
| Burgundy     | 0             | 0   | 0   | 1   | 2   | 67   |
| S6U          | 0             | 0   | 1   | 0   | 5   | 83   |

Four spore tetrads were dissected on YEPD agar. The proportion of dissected tetrads where all four spores were viable is shown in the far right hand column.

count and dry cell weight were examined (Table 3.3). Strains derived from dissected spores displayed very similar, if only slightly increased fermentation durations (an average of 144 hr) to the L-2056 parental strain (139 hr). The degree to which nitrogen was utilised, ranging between 198 and 413 mg L<sup>-1</sup> for the re-diploidised spores, a decrease from the parent strain (486 mg L<sup>-1</sup>). This variation in nitrogen utilisation, particularly in that of L-2056 #1 which assimilated only half of the ammonium in comparison to the parent strain, was surprising. Interestingly, this culture also produced less cells and dry weight per litre. This variation reveals that L-2056 may not be homozygous. KP2 was included as a control as this strain has been used routinely in this laboratory as an example of a laboratory yeast strain with a reasonable fermentation rate. Even so the inferior fermentation ability of laboratory strains is highlighted here, as the duration of fermentation (195 hr) is increased by 56 hours, representing an increase of 40 %, when compared to L-2056. The amount of nitrogen assimilated, cell count and dry cell weight were all similar to that of L-2056.

### **3.2.2 Construction of haploid L-2056 strains**

The *HO* gene was disrupted by the one-step gene disruption method (Rothstein, 1983), where the *kanMX3* cassette was utilised as a dominant selection marker (Wach, et al., 1994). The *kanMX3* cassette confers resistance to the antibiotic geneticin. Attainment of geneticin resistance was chosen as most industrial yeast are sensitive to this aminoglycoside antibiotic, and the use of the *kanMX3* gene cassette is well proven (Wach, et al., 1994). Geneticin elicits its action by interfering with yeast ribosomes, consequently blocking protein synthesis. The insertion of the *kanMX3* cassette causes the expression of a bacterial aminoglycoside phosphotransferase enabling the yeast cell to inactivate aminoglycoside antibiotics. Strains vary in their sensitivity to geneticin, a fact also dependent on the growth medium, wherein the presence of high concentrations of salt can interfere with the action of the antibiotic (Webster and Dickson, 1983).



**Table 3.3** Evaluation through fermentation by L-2056 and its derivatives (#1-#8), from two separate spore dissections.

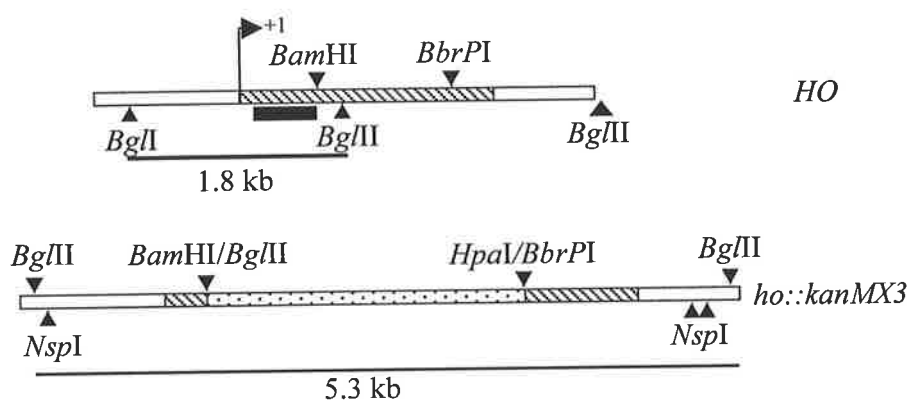
| Yeast Strain | Nitrogen assimilated (mg FAN L <sup>-1</sup> ) | Fermentation duration (hours) | Cell count (cells mL <sup>-1</sup> x 10 <sup>8</sup> ) | Cellular dry weight (g L <sup>-1</sup> ) |
|--------------|--|-------------------------------|--|--|
| L-2056 #1    | 197.6 +/- 22.9                                 | 144.5 +/- 5.7                 | 1.49 +/- 0.0   | 5.2 +/- 0.5                              |
| L-2056 #2    | 328.5 +/- 8.4                                  | 146.5 +/- 5.7                 | 1.99 +/- 0.45  | 5.8 +/- 0.4                              |
| L-2056 #3    | 391.4 +/- 22.9                                 | 150.2 +/- 2.5                 | 3.60 +/- 0.23  | 5.8 +/- 0.7                              |
| L-2056 #4    | 412.7 +/- 57.7                                 | 141.5 +/- 1.4                 | 3.03 +/- 0.01  | 5.5 +/- 0.2                              |
| L-2056 #5    | 380.4 +/- 0.0                                  | 136.0 +/- 0.0                 | 1.83 +/- 0.07  | 4.6 +/- 0.1                              |
| L-2056 #6    | 388.8 +/- 50.7                                 | 143.2 +/- 10.2                | 2.28 +/- 0.0   | 5.0 +/- 0.1                              |
| L-2056 #7    | 360.0 +/- 108.1                                | 148.0 +/- 17.0                | 1.15 +/- 0.0   | 5.4 +/- 0.2                              |
| L-2056 #8    | 383.8 +/- 14.4                                 | 142.5 +/- 0.0                 | 1.67 +/- 0.09  | 6.1 +/- 1.1                              |
| L-2056       | 485.8 +/- 38.5                                 | 139.3 +/- 4.6                 | 2.07 +/- 0.10  | 5.9 +/- 0.4                              |
| KP2          | 420.3 +/- 25.2                                 | 195.2 +/- 18.0                | 2.10 +/- 0.04  | 5.7 +/- 0.2                              |

Duplicate 100 mL fermentations were performed in CDGJM with 771 mg FAN L<sup>-1</sup>, supplied as ammonia. The prototrophic laboratory yeast KP2 was included as a control. Analyses were performed directly after the completion of fermentation.

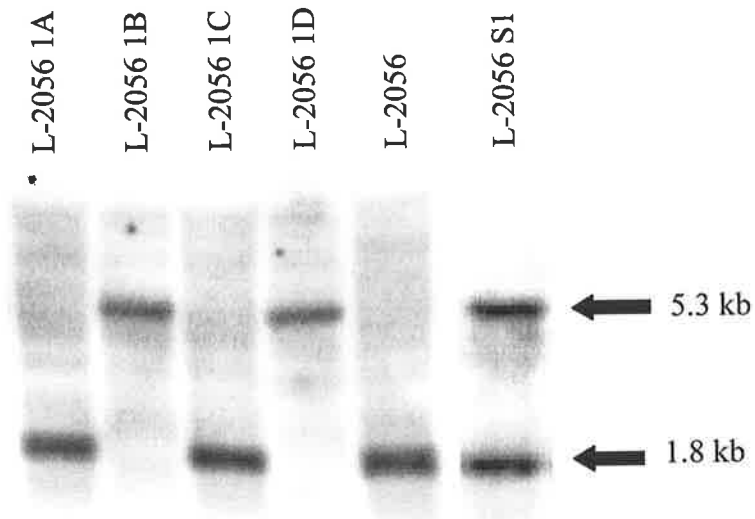
Primers HOFga and HORga (Table 2.3) were used to amplify 3,704 bp of genomic sequence containing the 1,760 bp *HO* gene (-547 → +3157, Figure 3.2). The fragment was inserted into pGEMT (Promega) by “TA cloning”, to generate plasmid pGEMT-HO. The 1245 bp *BbrPI* - *BamHI* fragment (containing the majority of the *HO* coding sequence) was excised from pGEMT-HO and replaced with the 2,562 bp *HpaI* - *BglIII* *kanMX3* cassette from pFA6-lackanMX3 (Wach, et al., 1994) to yield the plasmid pGEMTho::kanMX3. Strain L-2056 was then transformed with the 5 kb *NspI* fragment from pGEMTho::kanMX3. This fragment corresponds to the *kanMX3* cassette flanked by -664→+705 and +1593→+2662 of sequence homologous to the *HO* gene and adjacent regions.

Positive transformants were selected on YPD containing geneticin (Sigma) at 500 µg mL<sup>-1</sup>, and disruption of the chromosomal *HO* gene was confirmed by Southern Blot hybridisation (Figure 3.3). Primers HOFwd and HOCrev (Table 2.2) were used to generate a 564 bp Dioxygenin-labelled fragment corresponding to +60→ +624 of *HO*. Positive transformants were identified by the presence of a 5.3 kb band corresponding to a *kanMX3* insertion and a 1.8 kb band corresponding to wild-type *HO*. Diploid *HO/ho::kanMX3* transformants were then sporulated, dissected, and the presence of *ho::kanMX3* in strains derived from each spore was analysed. The haploid status of strains L-2056 1B and L-2056 1D was also confirmed by their failure to sporulate and their ability to mate with each other and mating type tester strains, KP2 or KP3. L-2056 1B was determined to be of mating type a and L-2056 1D of mating type α by the PCR mating type assay developed by Huxley et al. (1990) using *MAT*, *MATa* and *MATα* primers (Table 2.3).

To determine an appropriate concentration of geneticin for further studies with these strains, growth of L-2056 and the daughter strains from L-2056 *ho::kanMX3* transformants was monitored on YEPD-agar with increasing concentrations of geneticin (250 - 2000 µg mL<sup>-1</sup>). It was found that concentrations above 4-500 µg mL<sup>-1</sup> were sufficient to select for geneticin resistance. Further studies indicated that different batches and/or suppliers of



**Figure 3.2** A diagrammatic representation of the cloned *HO* gene and the *ho::kanMX3* construct. The *HO* coding sequence (contained within the PCR amplified -547 → +3157 segment) is indicated by the hatched regions, *kanMX3* sequence by the stippled region and the solid bar indicates the 564 bp 3' *HO* probe. The fragments liberated from the *HO* and *ho::kanMX3* alleles upon *Bgl*II digestion, and contributing to the banding pattern seen in the Southern blot analysis are indicated with labelled (1.8 kb or 5.3 kb) solid lines. The key restriction enzyme sites referred to in the text are shown above. The *HO* gene is encoded on the Crick strand. This figure is adapted from that published by Walker, et al. (2003).



**Figure 3.3** Southern Blot hybridization with the *HO* probe to confirm the genotype of strains during the construction of haploid L-2056 *ho::kanMX3* strains. Genomic DNA was digested with *Bgl*II. L-2056 1A-1D are strains derived from the four spores of one ascus of L-2056 S1, a diploid of the genotype *HO/ho::kanMX3*. L-2056 1B and L-2056 1D are haploids as the presence of a 5.3 kb band, confirms the presence of a *kanMX3* insert in the *HO* locus.

geneticin had variable activities (data not shown). For instance, 400  $\mu\text{g mL}^{-1}$  supplied from Astral Scientific (cat# G-1033) had an equivalent activity to 500  $\mu\text{g mL}^{-1}$  of geneticin from Sigma Aldrich (cat# G9516).

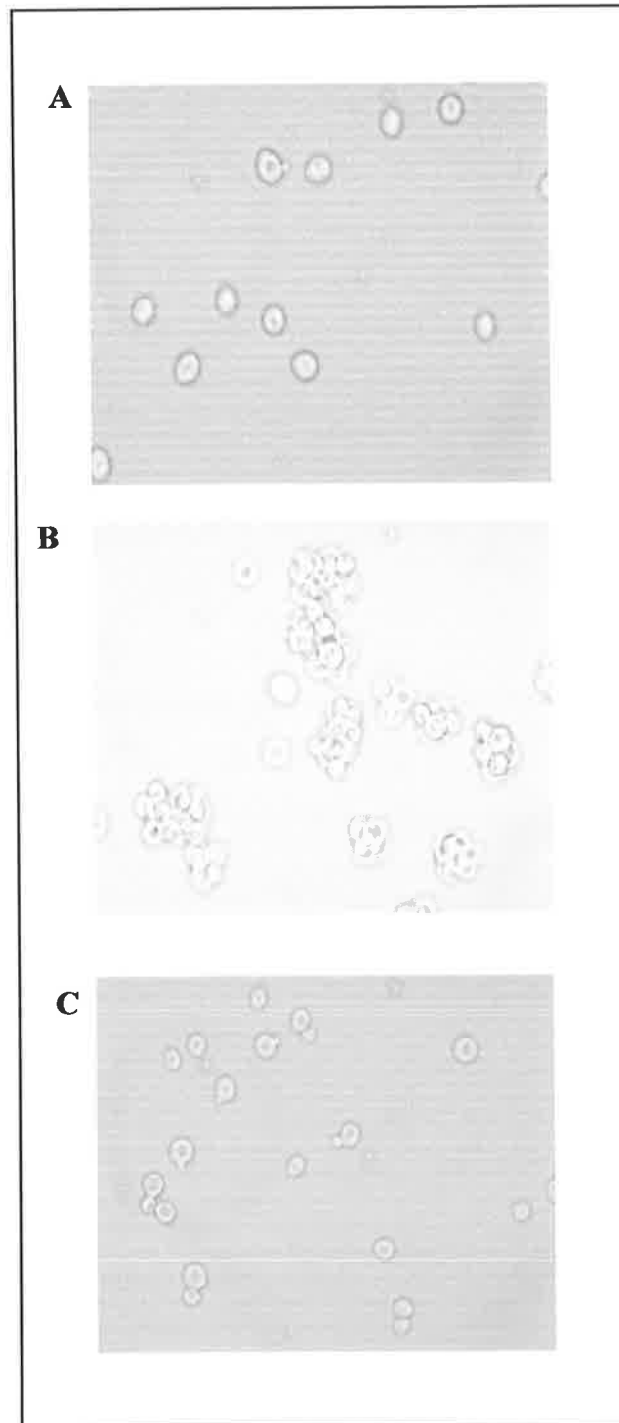
### ***3.2.3 Production of non-flocculant L-2056 1B and L-2056 1D***

Haploid cell populations are known to form cell aggregates, also recognized as flocculation (Kron, 1997). As this characteristic is readily observable under a microscope it also provides a quick method for checking putative haploid status. Strains L-2056 1B and L-2056 1D were indeed found to flocculate whereas the parental L-2056 did not (Figure 3.4).

Flocculation is not desirable in a research strain as aggregated cells are difficult to count accurately and effective mixing to re-suspend cells during fermentation trials becomes important. Attempts were therefore made to produce a non-flocculant derivative of L-2056 1B and L-2056 1D. Overnight cultures were subjected to chemical mutagenesis (EMS) (Adams, 1997) and non-flocculant cells were selected from the top fraction of a cell suspension in a column containing a solution of sodium and calcium salts (Soares, et al., 1994). The final selection of cells was re-plated on minimal medium and colonies were individually tested for flocculation in liquid YEPD. Strain L-2056 1Ba was found not to flocculate under these conditions (Figure 3.4). Thirty strains were selected, but unfortunately each strain displayed some degree of reversion to flocculation upon repeated cultivation. Strains L-2056 1Ba and L-2056 1Da were selected for further manipulation as these appeared the least flocculant.

### ***3.2.4 Comparison of the fermentation properties of L-2056, L-2056 1Ba and L-2056 1Da***

As L-2056 was isolated from a vineyard the exact heritage of this strain is unknown. It is possible that strains derived from the isolated spores may lose some of their desirable fermentation qualities. It has been reported that ploidy can affect fermentation kinetics (Salmon, 1997), consequently the performance of these haploid strains within 100 mL fermentations of CDGJM was evaluated. Fermentation kinetics were found to be similar to that of the

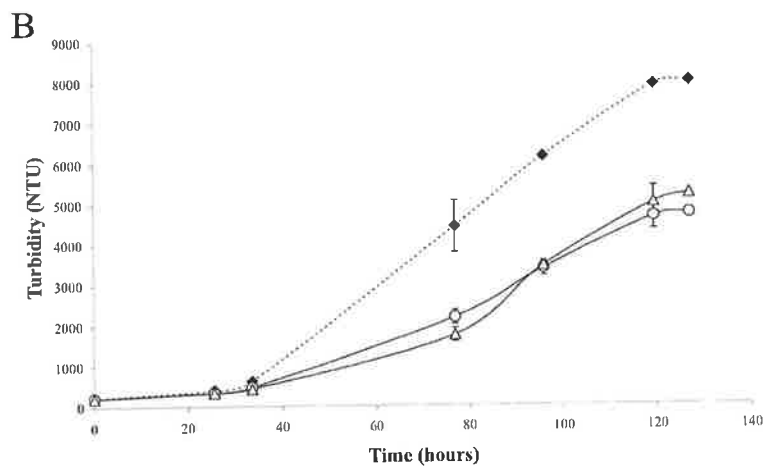
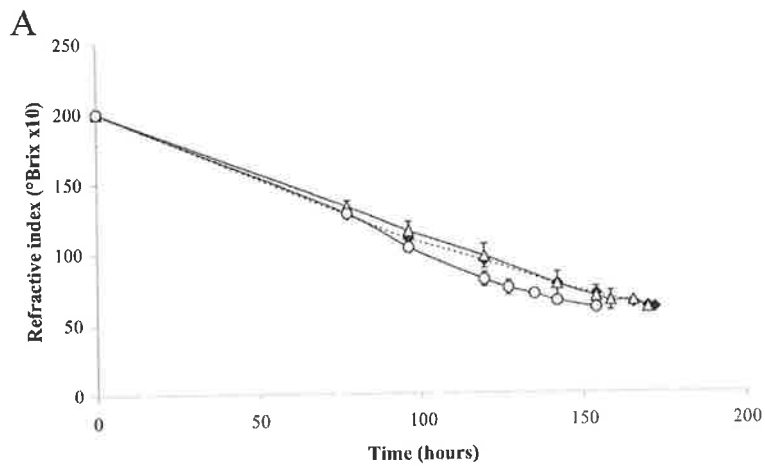


**Figure 3.4** Photomicrographs highlighting flocculation phenotypes of (A) L-2056 (diploid), (B) L-2056 1B (haploid) and (C) L-2056 1Ba (haploid-nonflocculant). Cells were grown for 16 hours in YEPD, and viewed at 40 X magnification by phase contrast microscopy.

parental, such that fermentation duration of haploids was 92 - 99 % of that of the parental (*ca* 171hrs) (Figure 3.5). These data show that L-2056 1Ba but not L-2056 1Da haploid strain has a shorter fermentation duration than the diploid (Student's t-test  $p < 0.05$ ), similar to what has been reported previously. Other attributes examined included biomass formation and degree of nitrogen utilisation, with no major differences being observed (Figure 3.5). Turbidity of haploids was approximately half that of the diploid, which is expected, given that cell size has been previously shown to be influenced by ploidy (Salmon, 1997; Galitski, et al., 1999). Notably, the variation observed in nitrogen utilisation of the diploid derivatives (Table 3.3) was not reflected in these haploid derivatives, suggesting that the polymorphic nature of this phenotype is confined to the diploid L-2056 parent, however many more derivatives would need to be examined to draw any firm conclusions. We therefore believed that differences between the haploid and parent strains were not deleterious to fermentation behavior and thus proceeded to utilise the strain in this project.

### **3.2.5 Generation of a uracil auxotroph into L-2056 1Ba**

A number of methods were employed in attempts to produce versions of L-2056 1Ba or L-2056 1Da that were auxotrophic for uracil. In the first instance, attempts were made to loop-out the *kanMX3* cassette in order to enable its re-use in disruption of *URA3*. Unfortunately, these efforts were unsuccessful in the time allocated within this project. This laboratory has since achieved loop-out of the marker through meiotic recombination, generating strain C9, albeit too late for the initial part of this project (Walker, et al., 2003). The alternate strategies used in this project eventually necessitated trialing of three transforming fragments to disrupt *URA3*. Disruption constructs with i) 387 bp (5') and 228 bp (3') of sequence either side of the 280 bp deletion from the open reading frame of *URA3*, ii) alike to i) yet with a 308 bp insertion in place of the 280 bp deletion to simulate a similar size fragment and iii) the *kanMX3* cassette flanked by the first and last 30 bp of the *URA3* coding sequence, were generated by PCR and selection of uracil auxotrophs was attempted on 5-fluoro-orotic acid (Boeke, et al., 1984). Each of these approaches was found to be unsuccessful. The desired strains was successfully constructed by classical genetics using a diploid L-2056 *ura3::kanMX4 / ura3::kanMX4* (41a2, M.



**C**

| Yeast Strain | Fermentation Duration |             | Total Nitrogen Utilised<br>(mg FAN L <sup>-1</sup> ) |
|--------------|-----------------------|-------------|--|
|              | (hours)               | % of parent |  |
| L-2056       | 171 +/- 1             | 100 +/- 1   | 438.50 +/- 20.29                                     |
| L-2056 1Ba   | 157 +/- 19            | 92 +/- 11   | 445.95 +/- 26.66                                     |
| L-2056 1Da   | 170 +/- 0             | 99.4 +/- 0  | 451.05 +/- 16.19                                     |

**Figure 3.5** The fermentation characteristics of L-2056 (◆) L-2056 1Ba (○) and L-2056 1Da (△). (A) Glucose catabolism, as estimated by refractive index, (B) cell growth, measured by turbidity, (C) fermentation duration and the total nitrogen utilised as measured with a Corning ion selective electrode. Duplicate 100 mL fermentations were performed in CDGJM with 1,060 mg FAN L<sup>-1</sup> as ammonia. Note that approximately 60 Brix (60) indicates depletion of glucose.



Walker, this laboratory) produced by utilising a *URA3* knockout construct (*KpnI* / *SacI* of pDR1123) that was kindly donated by D. Riviers (Replogle, et al., 1999). This construct contained *kanMX4* flanked by -740→-223 and +879 →+1321 of *URA3*. Strain 41a2 was crossed with haploid strains of L-2056 (1Ba and 1Da) to introduce the uracil auxotrophy. As each of these strains were already resistant to geneticin, a mitochondrial mutant based selection was employed to enable the selection of a L-2056 *ura3* haploid. Mitochondrial mutants of L-2056 1Ba and L-2056 1Da (1Ba *mit*<sup>-</sup> and 1Da *mit*<sup>-</sup>) were generated by ethidium bromide mutagenesis (Deutsch, et al., 1974), petite colonies were selected on YPDG and confirmed by their inability to grow on YEPGE.

Either 1Ba *mit*<sup>-</sup> or 1Da *mit*<sup>-</sup> was mated *en masse* using the rare mating protocol (Guthrie and Fink, 1991) with 41a2. L-2056 *URA3/ura3::kanMX4 mit*<sup>-</sup>/*mit*<sup>+</sup> *HO/ho::kanMX3* diploids were selected on minimal YEPGE medium. Upon sporulation and dissection of tetrads, 1Ba *ura3* and 1Da *ura3* were determined to be of genotype *ura3::kanMX4 ho::kanMX3 mit*<sup>+</sup> by Southern Blot analysis with the HO probe and their failure to sporulate. *URA3* status was determined by PCR with primers URA3F1 and URA3R (Table 2.3) and failure to grow on minimal media. Mitochondrial status was determined by the ability to grow on YEPGE medium (data not shown).

Finally 1Ba *ura3* (*MATa*) and 1Da *ura3* (*MATa*) were shown to mate to each other and also to the appropriate laboratory strains KP2 and KP3 using classical yeast mating approaches (data not shown).

### **3.3 Conclusions**

- a) L-2056 was found to be suitable for genetic manipulation.
- b) Two haploid strains were constructed in L-2056 (1B and 1D) by deletion of the *HO* gene using a PCR derived *kanMX3* cassette inserted by long flanking homologous recombination.
- c) Less flocculant derivative strains L-2056 1Ba and L-2056 1Da were selected.

- d) L-2056 1Ba and L-2056 1Da are suitable for use for further studies as their fermentation attributes are similar to the parent strain L-2056.
- e) Uracil auxotrophs of L-2056 1Ba and L-2056 1Da were constructed.

### **3.4 Discussion**

We have successfully constructed haploid derivatives of the wine yeast strain L-2056, allowing further studies to be performed where recombinant genetics is required. The haploid nature of 1Ba *ura3* or 1Da *ura3* allows simpler genetic manipulations, such as knockout procedures, where only a single copy gene deletion is required, also facilitated by the addition of the auxotrophic marker *ura3*. The ease of mating of these two strains further shows their suitability for classical genetic manipulations.

Haploid wine yeast have been constructed by others, (Bakalinsky and Snow, 1990), yet these particular strains were generated by spore cell mating and consequently a number of back crosses were required in an attempt to regain a genetic composition similar to the original industrial parent strain. These manipulations could potentially cause loss of the positive fermentation properties originally displayed by these commercial strains. In this study, we chose to perform as few manipulations as possible in an attempt to retain these positive characteristics. Importantly the strains produced here did retain the desirable fermentation kinetics of their parent, perhaps differing only by their ability to complete fermentation in a shorter duration. Bakalinsky and Snow (1990) and Baganz et al. (1997) also examined the effect of deletion of *HO* upon fermentation and finding that there was little effect, suggested that fundamental fermentative properties are independent of heterozygosity or even complete loss of function of *HO*. Ploidy and fermentation was also evaluated by Salmon (1997) where he found that fermentation duration and cell growth was related to ploidy. The internal activity of alcohol dehydrogenase on a per cell basis was found to be directly proportional to ploidy, thus it was hypothesised that the gene copy for other metabolic processes involved with fermentation would also be maintained. If indeed this is the case, then the

slower fermentation dynamics, observed by this group, of higher ploidy cells could be due to a bottle neck of activity at the plasma membrane, particularly as cells with higher ploidy are larger and have a lower surface area to volume ratio. Also, in regards to fermentation, the maintenance of sugar transport systems at the plasma membrane has been previously identified as one of the limiting factors of fermentation (Salmon, et al., 1993). Higher ploidy cells also require more energy for replication due to the nature of their chromosomal complement, thus it would be expected that metabolic processes would be slower than in haploid cells when in nutrient limiting conditions (Mable, 2001). With these studies taken into consideration, the potential of haploids to have an increased fermentation rate could certainly be advantageous in industrial applications. However if desired, diploid *ho* strains can be easily generated, as homothallic cells of opposite mating type, such as those generated in this study can be conveniently mated together.

The introduction of an auxotrophic marker was particularly important for this project as we wished to be able to introduce foreign DNA and select strains which contain the insertion. UV mutagenesis or homologous recombination can be used to engineer an auxotrophy into the genome (Hashimoto, et al., 2005). Homologous recombination is typically performed with flanking sequences of 30 - 60 bp, which are usually conveniently included on PCR primers. However this approach was unsuccessful in L-2056. Inclusion of 300 - 400 bp of flanking sequence solved this problem, suggesting that long flanking sequences are required for homologous recombination in this strain and highlighting another potential strain-specific characteristic.

The evaluation of this strain within this project finished at this point. It is of course important to determine if there are any other differences between 1Ba *ura3* or 1Da *ura3* and L-2056. Due to the difficulties and thus delays in the development of these strains the screening component of this project (Chapter 4) was continued in a derivative of the laboratory strain W303 (KP2). The derivatives of L-2056 generated here were however used in a subsequent phase of this project; where *HNE* genes of interest were deleted in the haploid wine

yeast strains to determine if the phenotype of nitrogen efficiency could be conferred in such a background.

As mentioned, there are many known differences between laboratory and wine yeast as highlighted by the longer fermentation duration of W303 (Table 3.3). The use of W303 instead of the haploid derivatives of L-2056 at this point may have limited the potential of the screening section of this study. However, some other laboratory strains have been found to be suitable for fermentation studies involving spirit production, as found by Schehl et al. (2004), where a diploid laboratory strain (HHD1) was constructed from a cross between CEN.PK113-5D and CEN.PK113-16B.

The difficulties of recombinant technologies in industrial yeast have been highlighted previously in this document. The usefulness of the development of these strains and the protocols to generate them has certainly been confirmed by their central use in this project and also in other projects in this laboratory.

## **Chapter 4 Identification of genes affecting glucose catabolism in nitrogen-limited fermentation**

### **4.1 Introduction**

Nitrogen efficiency is defined as the ability to catabolise an increased amount of sugar when supplied with a defined amount of nitrogen (Jiranek, et al., 1995a; Manginot, et al., 1998). Nitrogen deficiency is known to lead to an arrest of protein synthesis, a restriction of biomass formation and also a rapid inactivation of sugar transport systems, ultimately resulting in a retarded or even stuck fermentation. An option for the prevention of retarded or stuck fermentation which has yet to be widely employed is the use of “nitrogen efficient” wine yeast strains. That is, strains able to catabolise more sugar when supplied with a limited amount of nitrogen. In order to determine the basis for differences in nitrogen efficiency, this study aimed to identify and characterise genetic mutations that confer a greater efficiency of nitrogen utilisation, or “high nitrogen efficiency” (HNE) during fermentation. As this phenotype must be evaluated during fermentation, a screening method was developed where the relative fermentation progress of multiple mutants was easily measured.

Ideally this study was to be conducted in a wine yeast background, to increase the probability of identification of genes or their regulatory circuits which influence nitrogen efficiency in industrial wine fermentations. Yet, due to delays incurred during the construction of such a strain (Chapter 3), the initial isolation and in turn screening for *HNE* genes was undertaken in JMG2 and JMG3, derivatives of the laboratory strain W303. W303 was chosen as it is known to be able to ferment high concentrations of glucose under anaerobiosis (this laboratory). It is presumed that there are numerous genetic differences between wine and laboratory yeast strains, particularly given the heightened fermentation performance of wine strains observed during fermentation. Recently Dunn and colleagues (2005) analysed the karyotype of four commercial wine strains by comparative genomic hybridisation to micro-arrays derived from the typical laboratory strain S288C. Differences were detected in Ty elements, hexose transporters and metal ion transporters amongst others.

Therefore, following their identification in JMG2 and JMG3, *HNE* gene deletions were, in turn, introduced into a derivative wine yeast strain for a more thorough characterisation in the preferred genetic context (Chapter 5).

This chapter describes the identification of three genes that influence the nitrogen efficiency of JMG2 and therefore are candidates for manipulation as a new and alternative strategy for dealing with low nitrogen juices.

## **4.2 Results**

### **4.2.1 Construction of JMG2 and JMG3**

JMG2 (*ura3*, *MAT $\alpha$* ) was constructed by deletion of *URA3* from KP2 via the *kanMX* gene replacement strategy (Wach, et al., 1994). A PCR product corresponding to the *kanMX4* module flanked with 59 and 60 bp of *URA3* sequences (+102 → +160 and +653 → +712) was amplified from the vector pBS418 (Jeff Eglinton, AWRI) with primers *ura3kanF* and *ura3kanR* (Table 2.3). PCR was performed in 1 X Dynazyme EXT Buffer with 0.5 mM dNTPs, 0.5 mM MgCl<sub>2</sub> and 1 U of Taq (Sigma). Cycling conditions were as follows: 30 cycles of 94°C, 30 seconds, 52°C, 1 minute, 72°C, 2 minutes. KP2 was transformed with this PCR product and positive transformants were selected on YEPD-geneticin. Deletion of *URA3* in JMG2 was confirmed by non-growth on minimal medium and PCR with primers *URA3F1* and *URA3R*. PCR was performed in 1X Dynazyme EXT Buffer with 0.5 mM dNTPs, 1 mM MgCl<sub>2</sub> and 1 U of Dynazyme EXT. Cycling conditions were as follows: 30 cycles of 94°C, 30 seconds, 62°C, 1 minute, 72°C, 3 minutes. JMG2 was crossed with KP3, diploids were sporulated, dissected and JMG3 (*ura3*, *MAT $\alpha$* ) was identified by PCR.

### **4.2.2 Preparation of the *mTn3xHA/GFP/URA3* library**

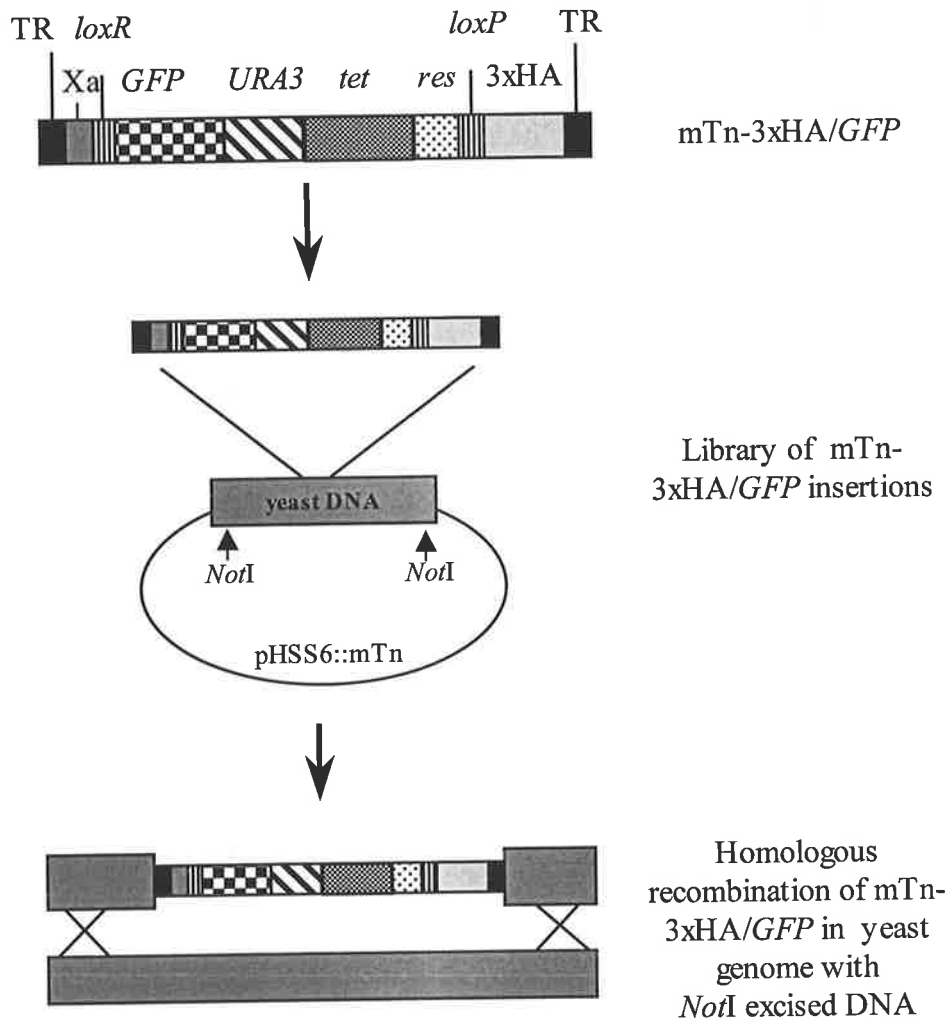
A transposon mutagenesis system was used in preference to chemical mutagenesis as the former allows efficient mutant tracking via PCR identification of the inserted transposon (Tn) tag (Ross-Macdonald, et al., 1997). Several other characteristics of this system made it suitable for this study, such as the introduction of single insertion mutations that are close to

random and are themselves non-toxic to the yeast cell. The inserted transposon also contains useful tools such as the *GFP* reporter gene, three copies of the HA epitope (from the influenza haemagglutinin protein; Wilson, et al., 1984) and the ability to reduce the size of the insertion, via the *cre-lox* system, to a minimal HA tag. The mTn-3xHA/*GFP*-mutagenised library came in the form of 18 pools of pHSS::mTn plasmid DNA containing the mutagenic transposon inserted randomly into fragments of a *S. cerevisiae* S288C genomic library (Figure 4.1). The use of a genomic library from S288C rather than from wine yeast may have limited the potential for finding genes that affect nitrogen efficiency that are specific to wine yeast. However, since it was not of interest to discover all mutations that would display a HNE phenotype, this approach was determined to be adequate.

Eighteen aliquots of the mTn-3xHA/*GFP*-mutagenised library were received that were of approximately one microgram of DNA each. One microlitre of each pool was transformed into the *E. coli* strain DH5 $\alpha$ , and transformants were selected on LB with 40 mg L<sup>-1</sup> kanamycin and 3 mg L<sup>-1</sup> tetracycline. Approximately 50,000 transformants were obtained from each pool. Mini-prep DNA was prepared from each library pool, and the mTn-3xHA/*GFP* mutagenised genomic library fragments, excised from the pHSS6 vector using *NotI* restriction enzyme. The library fragments were then transformed into JMG2 and JMG3. The amount of DNA in transformations was kept as low as possible, that is without compromising transformation efficiency, (typically 0.5  $\mu$ g per transformation) so as to minimize the occurrence of double integration events. Transformants were selected on minimal medium. The use of minimal media also selected against and therefore avoided, the complication of mutations that would lead to nitrogen related amino acid auxotrophies.

#### ***4.2.3 Selection of mutants with a high nitrogen efficiency (HNE) phenotype***

A fermentation screen was designed to select nitrogen efficient strains from the pool of mutants containing the transformed genomic library. Preliminary work by Wenk (1999) suggested that nitrogen efficient strains could be identified



**Figure 4.1** Diagrammatic representation of the transposon mutagenesis strategy. The mTn-3xHA/*GFP* library was obtained from Dr M. Snyder, Yale University (Ross McDonald et al., 1997). *NotI* restriction fragments of yeast genomic DNA containing the Tn3 transposon were transformed into KP2.

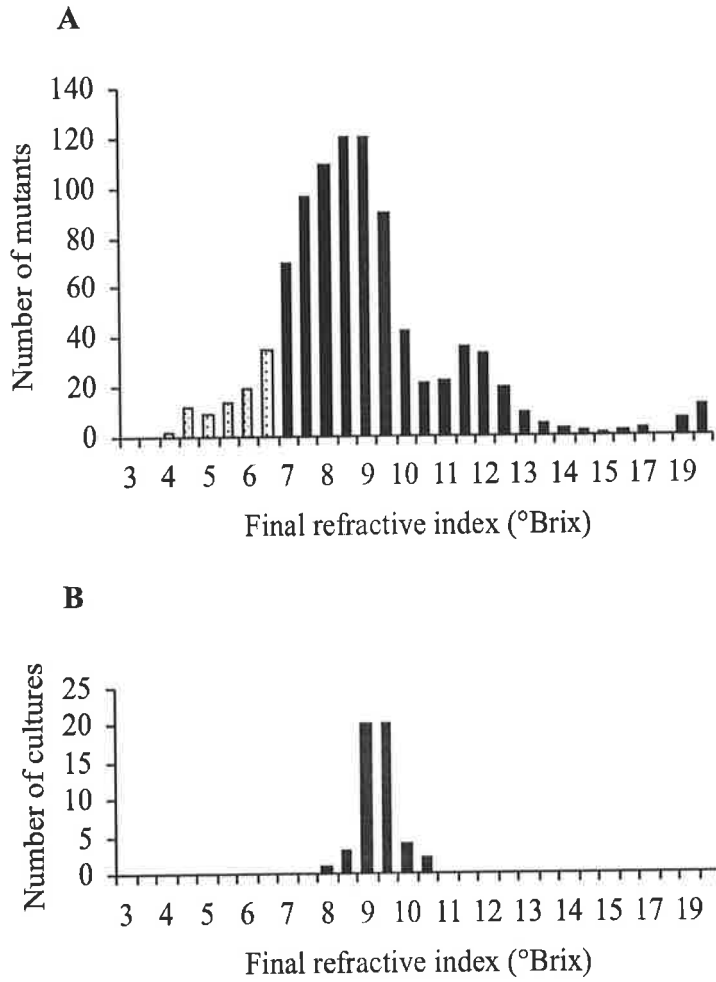
TR (terminal inverted repeats), Xa (Factor a cleavage recognition site), *GFP* (green fluorescent protein), *tet* (tetracyclin resistance gene), *HA* (haemagglutinin epitope tag), *loxR* and *loxP* (lox sites, target for Cre-recombinase).



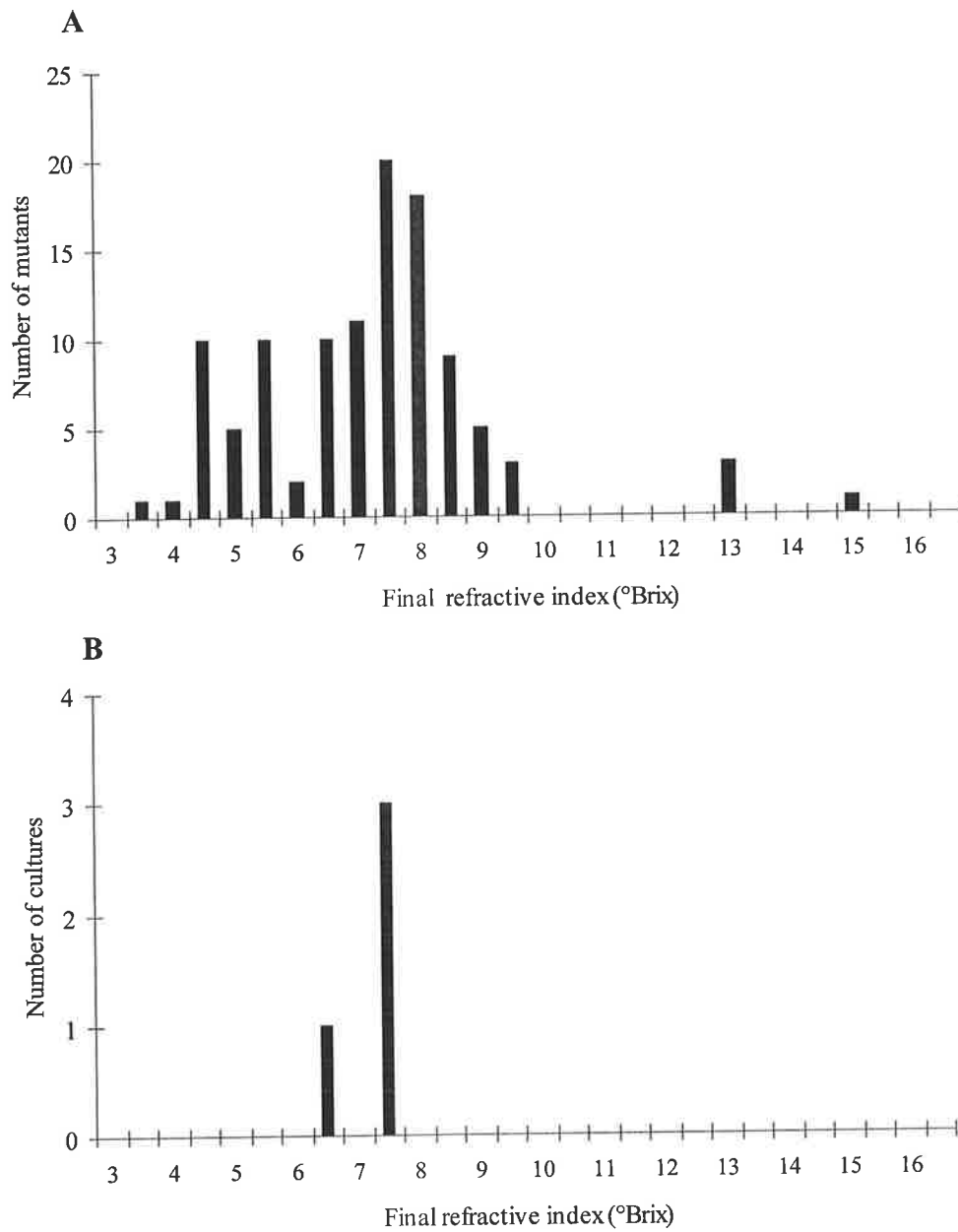
either from their reduced nitrogen consumption in nitrogen-excess : carbon-limited media (e.g. 750 mg FAN L<sup>-1</sup>, 200 g glucose L<sup>-1</sup>) or else from their greater glucose catabolism in nitrogen-limited : carbon-excess media (~75 mg FAN L<sup>-1</sup>, 200 g glucose L<sup>-1</sup>). Therefore the latter medium formulation was adopted for the large numbers of mutants evaluated in the primary screen since the extent of glucose catabolism was easily estimated using a refractometer. Nitrogen efficient strains were identified by selecting those able to catabolise more carbon than the parent strain under nitrogen-limiting conditions.

During the primary screen, 1 mL YEPD starter cultures of 5,000 randomly selected transposon mutants were grown overnight at 30°C in 48-well plates (Corning, Costar) with shaking (~150 rpm). A 48-pinned replicator (manufactured in house) was used to inoculate screening fermentations, performed aerobically, comprising 1 mL of CDGJM (75-100 mg FAN L<sup>-1</sup> as ammonium) in new 48-well plates, before incubation at 30°C with shaking (100 rpm). Fermentation progress was determined after 10 - 14 days by estimation of the residual sugar content of each culture using a handheld refractometer. Mutants that catabolised the greatest amount of sugar were selected for evaluation in the secondary screen. In this way, from the 5,000 mutants assessed, 110 (2 %) were selected as the most nitrogen efficient and retained for further investigation (Figure 4.2). These mutants catabolised at least 10 % more glucose than the parent strain, KP2. The original aim was to examine 12,000 mutants in the primary screen, to enhance the chance of examining most of the *ca* 6,000 genes in the yeast genome. Nevertheless, screening of 5,000 mutants yielded sufficient candidates for investigation within the time-frame and scope of this project.

In a secondary screen using the same medium but performed in triplicate 5 mL fermentations, 40 of these mutants (36 %) were again shown to catabolise at least 10 % more glucose than the parent strain (Figure 4.3). This 18-fold (2 % vs. 36 %) enrichment for nitrogen efficient mutants supported the suitability of the method.



**Figure 4.2** Histograms showing the distribution of final refractive index values for individual micro-fermentations, taken after 14 days performed by approximately 1,000 transposon mutants (A) or 50 separate cultures of KP2 (B) in CDGJM containing 100 mg FAN L<sup>-1</sup> as ammonia. Values are derived from a typical experiment for a subset (1,000) of the total 5,000 mutants examined. The stippled bars (A) correspond to the mutants selected for further investigation.

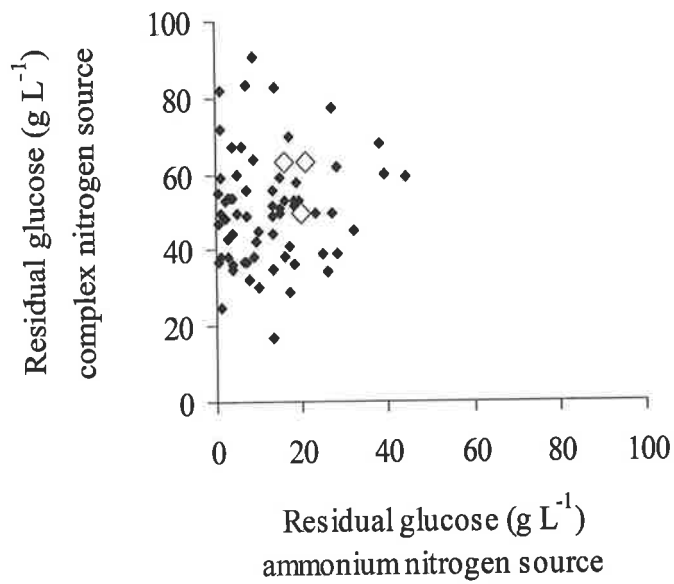


**Figure 4.3** Histograms showing the distribution of average final refractive index values for triplicate 5 ml fermentations by each of the selected 110 mutants selected from the primary screen (A) or KP2 (B) in CDGJM containing 75 mg FAN L<sup>-1</sup> as ammonia. Standard deviations were within 10 %.

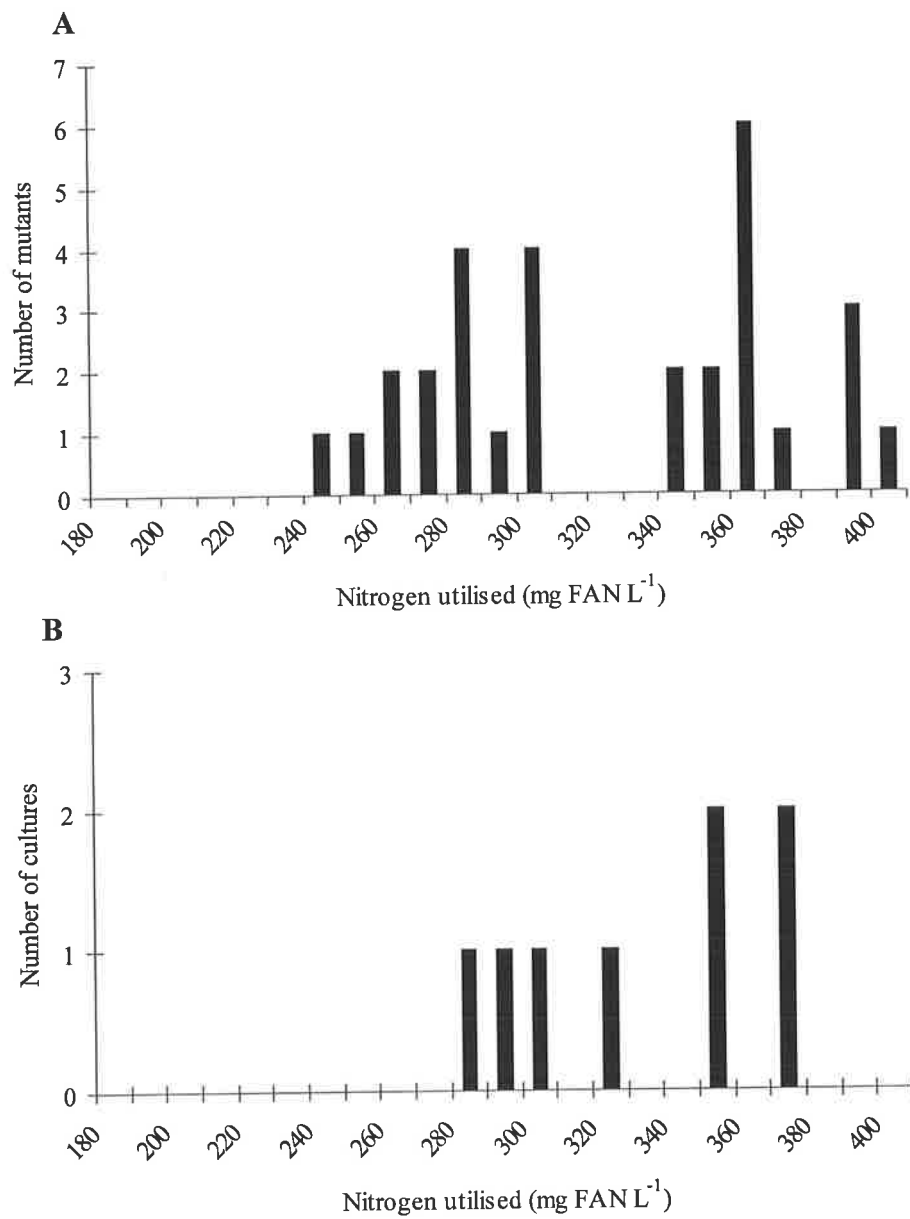
Grape juices typically contain assimilable nitrogen as a complex mixture of compounds, primarily amino acids and ammonium (Amerine, et al., 1980; Ough and Amerine, 1988; Henschke and Jiranek, 1993). All screening of mutants to this point, however, had been conducted using ammonium alone. The relative nitrogen efficiency of the selected mutants was therefore examined during growth in a limited-nitrogen medium (75 mg FAN L<sup>-1</sup>) comprised of a mix of nitrogen compounds. In general, the extent of glucose catabolism was decreased when a mixed limiting source of nitrogen was provided, yet the majority of mutants still proved superior to the parent strain (Figure 4.4). For a small number of mutants there was little change in efficiency across the two nitrogen sources.

The amount of nitrogen utilised in CDGJM containing excess nitrogen (750 mg FAN L<sup>-1</sup> as ammonium) was also evaluated (Figure 4.5). As ammonium was the sole nitrogen source the residual nitrogen was measured with an ammonium electrode. Ten of the thirty strains examined used less nitrogen than the average nitrogen consumption (329 +/- 36 mg FAN L<sup>-1</sup>) by the parent strain. The best strain utilised 27 % (~52 mg FAN L<sup>-1</sup>) less than the parent.

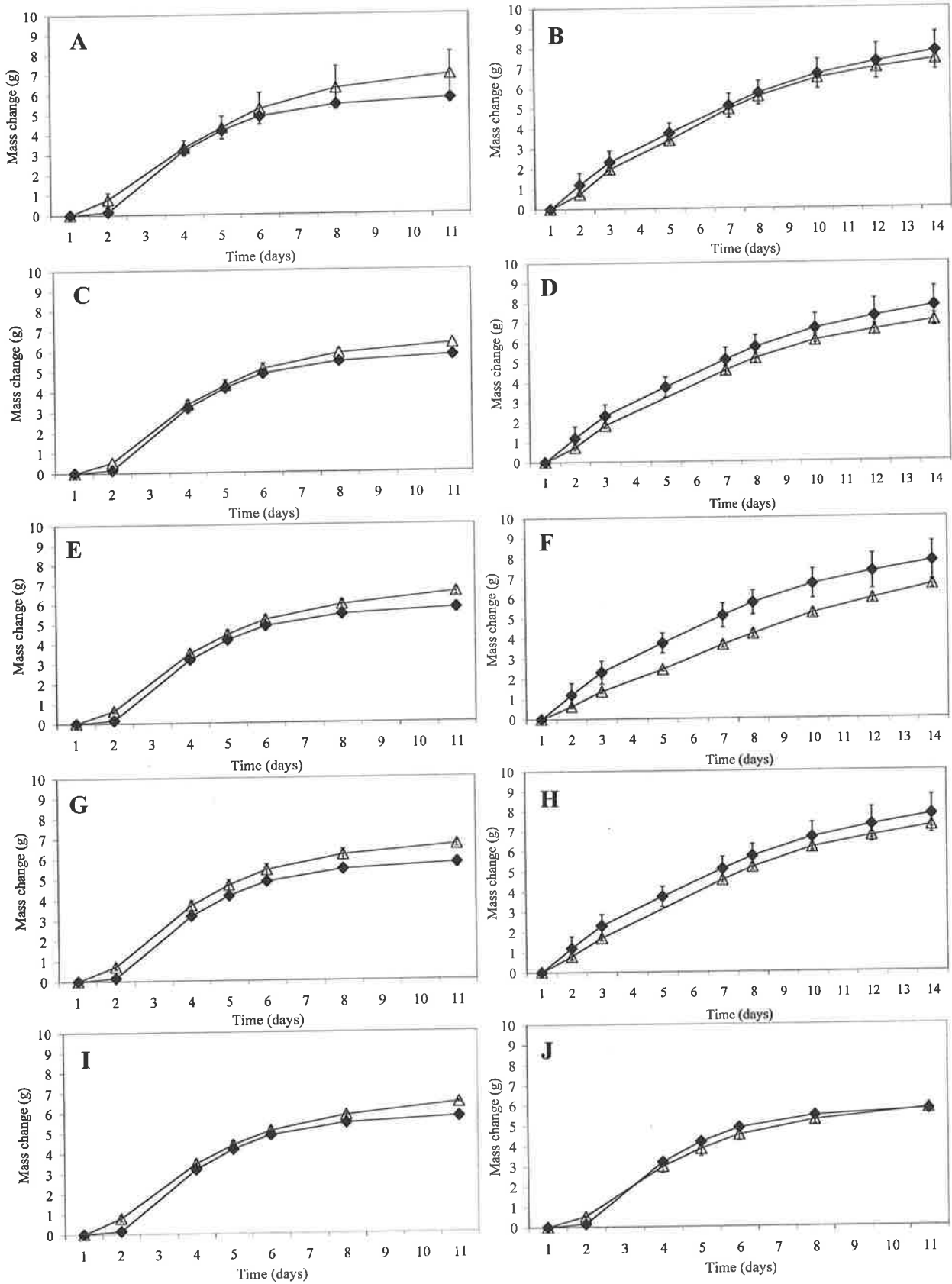
Ten strains with the most enhanced performance were selected from the above screens and the extent of glucose catabolism by these was determined in two separate experiments performed in triplicate 100 mL fermentations in CDGJM containing 75 mg FAN L<sup>-1</sup> as ammonium. The progress of each fermentation was routinely monitored by weight loss, revealing that half of the mutants, JMG2.63, JMG2.77, JMG2.82, JMG2.113 and JMG2.125 performed better than the parent (Figure 4.6: A, C, E, G, I). Fermentations did not reach dryness, and were deemed "stuck" when the weight of the culture had not changed in 12 hours. Accumulated weight loss due to carbon dioxide evolution is related to glucose catabolised (El Haloui, et al., 1989), and while convenient may also be a function of strain. Accordingly, the total glucose catabolised was also determined (Table 4.1) and revealed some inconsistencies. For instance, JMG2.125 had a 12.2 % increase in the total weight loss (compared to the parent), yet no measured difference in total glucose consumed. Likewise,



**Figure 4.4** Comparison of residual glucose after 14 days of fermentation by selected transposon mutants (◆) and KP2 (◇) when grown in 5 mL of CDGJM containing 75 mg FAN L<sup>-1</sup> supplied as ammonium or as an ammonium and amino acid (complex) nitrogen source.



**Figure 4.5** Histograms showing the distribution of quantities of nitrogen utilised by selected transposon mutants (A) and KP2 (B, Average = 329 +/- 36 mg FAN L<sup>-1</sup>) after fermentation of CDGJM containing 750 mg FAN L<sup>-1</sup> as ammonia.



**Figure 4.6** Mass change during fermentation of mutant strains ( $\triangle$ ), JMG2.63 (A), JMG2.72 (B), JMG2.77 (C), JMG2.78 (D), JMG2.82 (E), JMG2.98 (F), JMG2.113 (G), JMG2.120 (H), JMG2.125 (I) and JMG2.130 (J) in comparison to ( $\blacklozenge$ ) KP2. Fermentation was carried out anaerobically in 100 mL of CDGJM with 75 mg FAN L<sup>-1</sup> as ammonia. Data points are the average of triplicate fermentations and standard errors are included.

**Table 4.1** The total mass change and residual glucose of fermentations performed with selected transposon mutants.

|   | Yeast Strain | Total mass change (g) | Increased mass change (%) | Residual glucose (g L <sup>-1</sup> ) | Increased glucose consumption (%) | Affected gene  |
|---|--------------|-----------------------|---------------------------|---------------------------------------|-----------------------------------|----------------|
| A | KP2          | 5.75 +/- 0.23         | 0 %                       | 46.2 +/- 0.8                          | 0 %                               | -              |
|   | JMG2.63      | 6.93 +/- 1.12         | 20.5 %                    | 36.8 +/- 3.8                          | 6.1 %                             | <i>RDN37-1</i> |
|   | JMG2.77      | 6.32 +/- 0.12         | 9.9 %                     | 43.1 +/- 1.0                          | 2.0 %                             | <i>RDN37-1</i> |
|   | JMG2.82      | 6.55 +/- 0.19         | 13.9 %                    | 41.5 +/- 5.7                          | 3.1 %                             | <i>TIF4632</i> |
|   | JMG2.113     | 6.65 +/- 0.14         | 15.9 %                    | 35.0 +/- 4.7                          | 7.3 %                             | <i>NGR1*</i>   |
|   | JMG2.125     | 6.45 +/- 0.05         | 12.2 %                    | 46.3 +/- 2.2                          | 0 %                               | <i>RDN37-1</i> |
|   | JMG2.130     | 5.80 +/- 0.18         | 0.87 %                    | 49.7 +/- 5.8                          | -2.3 %                            | <i>RDN37-1</i> |
| B | KP2          | 7.79 +/- 0.95         | 0 %                       | 76.1 +/- 4.3                          | 0 %                               | -              |
|   | JMG2.72      | 7.38 +/- 0.18         | -5.3 %                    | 63.9 +/- 5.5                          | 9.9 %                             | Not identified |
|   | JMG2.78      | 7.05 +/- 0.32         | -9.5 %                    | 72.1 +/- 7.4                          | 3.3 %                             | <i>YDL133W</i> |
|   | JMG2.98      | 6.92 +/- 0.20         | -11.1 %                   | 65.7 +/- 3.5                          | 8.4 %                             | <i>PUT4</i>    |
|   | JMG2.120     | 7.22 +/- 0.11         | -7.3%                     | 48.7 +/- 4.0                          | 22.1 %                            | <i>GID7*</i>   |

Fermentations were performed in two separate experiments (A and B) in 100 mL of media containing limiting nitrogen as ammonia (75 mg FAN L<sup>-1</sup>). The identity of the affected genes is also included. \*Genes chosen for further evaluation.

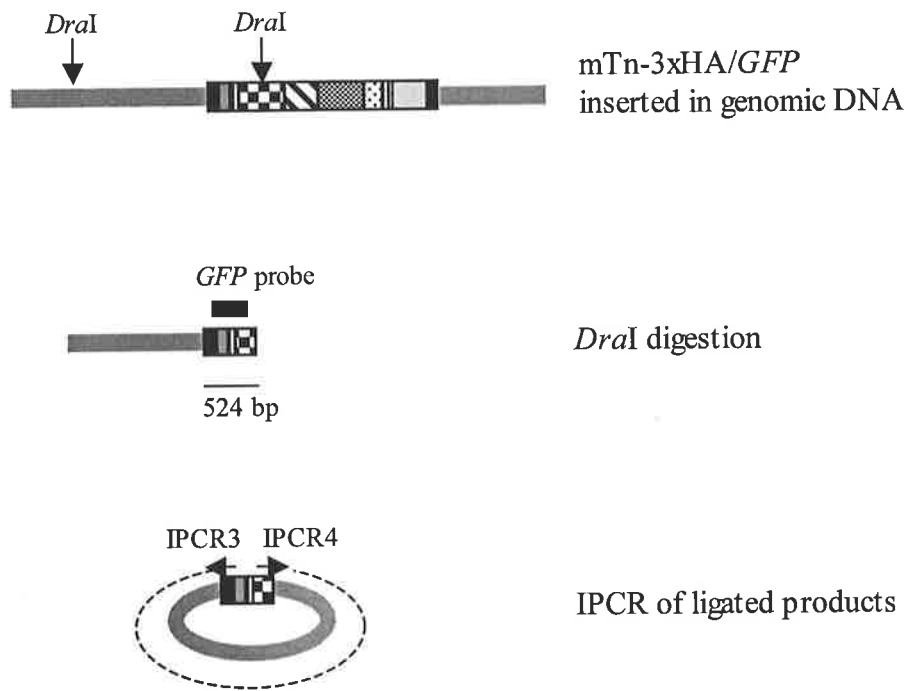


JMG.120 catabolised 22.1 % (*ca.* 29.2 g L<sup>-1</sup>) more glucose than the parent, yet actually showed as lower weight loss (by 7.3 %) than the parent. Weight measurements made during these fermentations were crude (collected approximately daily) in comparison to those reported by Sablayrolles, et al. (1987) and El Haloui, et al. (1989) (collected every 20 minutes) which may explain why a reliable correlation between mass change and glucose consumption did not occur.

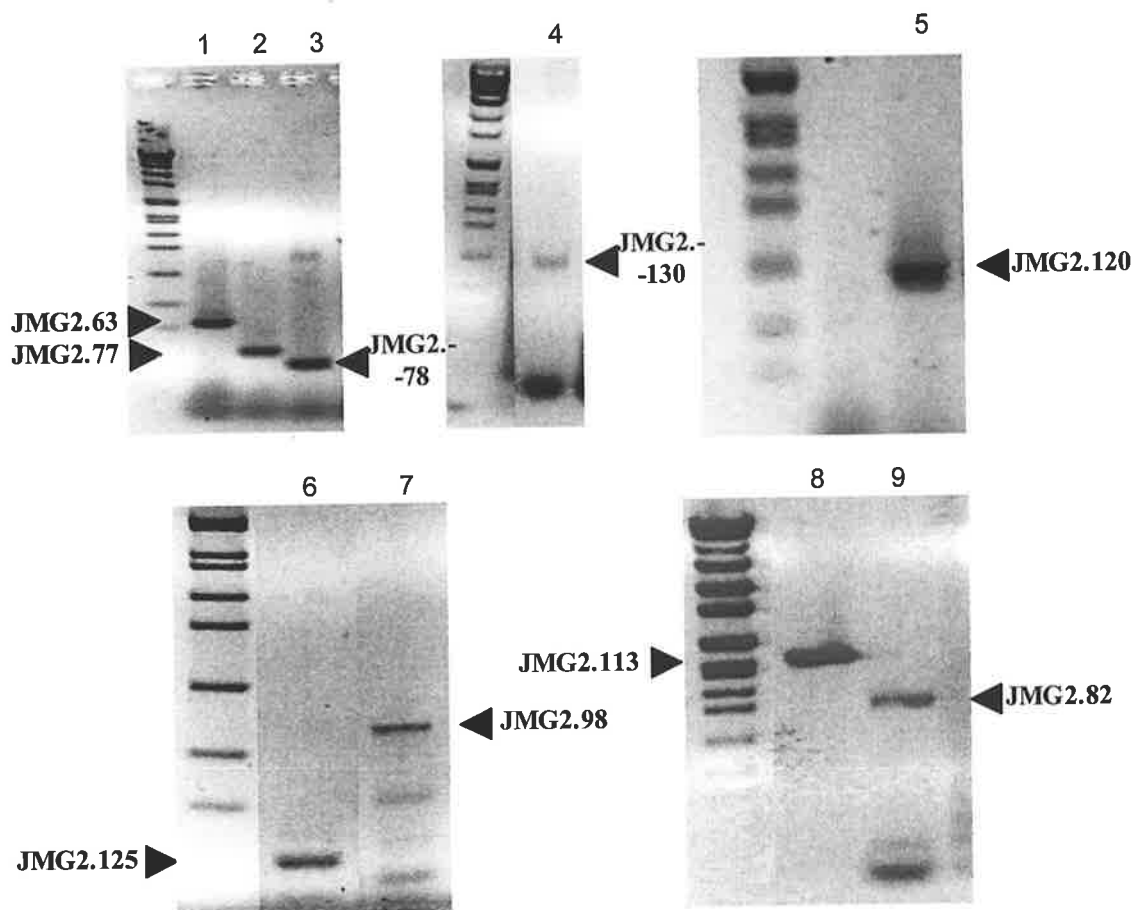
#### **4.2.4 Identification of genes influencing nitrogen efficiency**

The location of the transposon and therefore the identity of the affected gene of nine of the ten strains of interest was determined from the sequence homology of the insertion site to the *Saccharomyces* genome database (<http://www.yeastgenome.org/>) (Table 4.1). Regions adjacent to and including the inserted transposon were amplified by Inverse PCR (Martin and Mohn, 1999; Huang, et al., 2000) (Figure. 4.7). To achieve this, a *DraI* genomic digestion of the transposon mutant was ligated at a low DNA concentration (approximately 1.0 µg mL<sup>-1</sup>) in the presence of T4 DNA ligase (5 U mL<sup>-1</sup>) to obtain a high proportion of self-annealed products. Ligation reactions were purified with a *Perfect Prep* kit (Eppendorf) to a final volume of 30 µL and PCR was then performed with primers IPCR3 and IPCR4. The PCR reaction was performed in 1 X Dynazyme EXT Reaction Buffer with 100 pmol of each primer, 1 µL of purified ligation reaction, 2 mM MgCl<sub>2</sub>, 100 µM dNTPs and 1 U of Dynazyme EXT. The PCR amplification conditions were as follows: 10 min at 94°C followed by 30 cycles of 94°C, 30 sec, 56°C, 1 min, 72°C, 4 min. The presence and approximate size of PCR products was established by electrophoresis (Figure. 4.8).

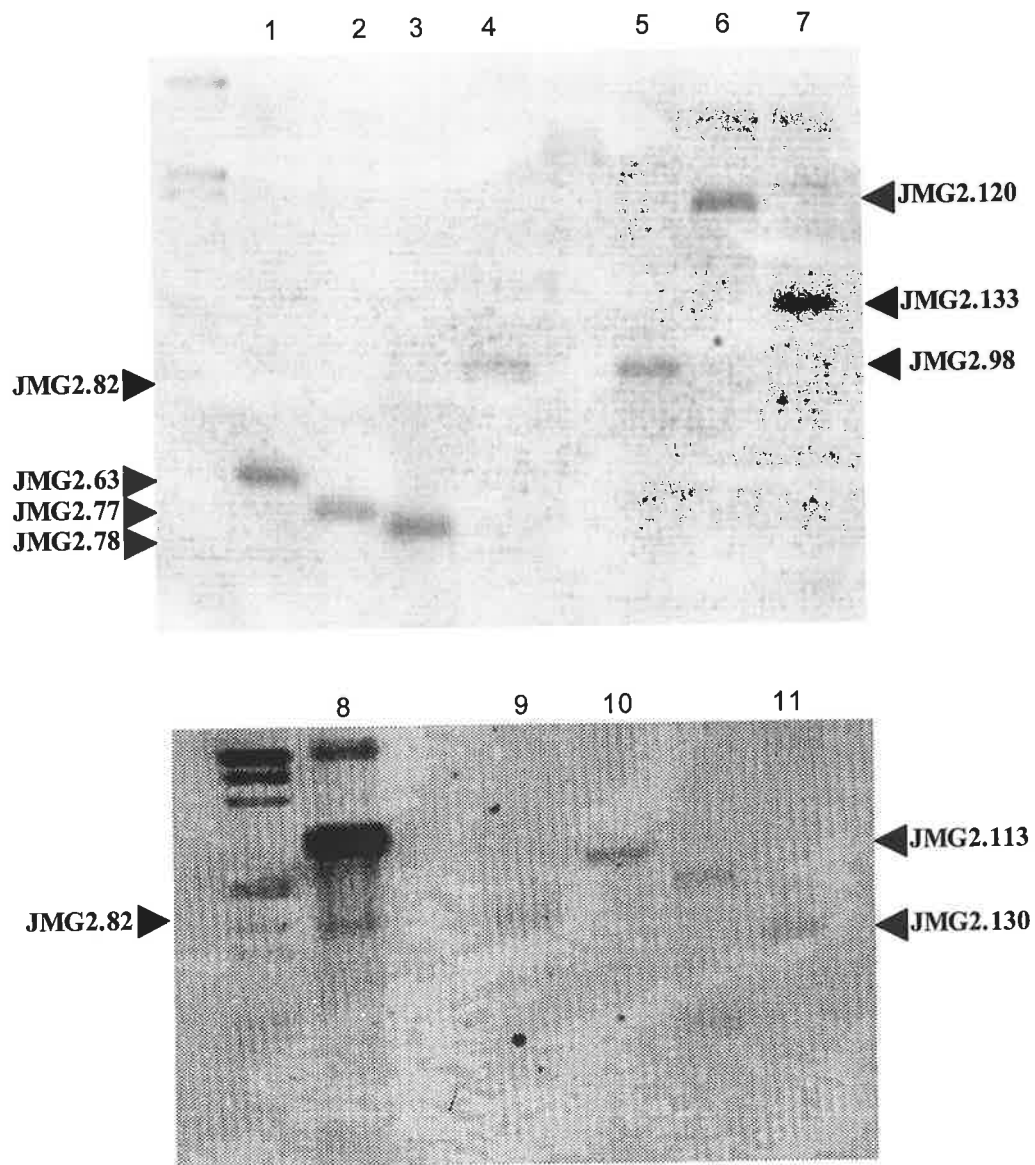
Amplification of the correct fragments was confirmed by comparing the size of the IPCR fragments with the size of fragments detectable by Southern Blot analysis of *DraI* digested mutant DNA hybridised with a probe specific to *GFP* within mTn-3xHA/*GFP* (Figure. 4.9). The latter was generated by PCR using transposon mutant genomic DNA (200 ng), primers GFPprobeF and GFPprobeR (Table 2.3) (100 pmol each), 1 mM MgCl<sub>2</sub>, 50 µM dNTPs, 50 µM



**Figure 4.7** A diagrammatic representation of the Inverse PCR methodology. Purified genomic DNA from the strain of interest was digested with *DraI*, which cuts within the *GFP* sequence of the transposon and in the yeast genomic DNA. Digested products are ligated and PCR is performed with IPCR3 and IPCR4, amplifying yeast genomic DNA adjacent the transposon.



**Figure 4.8** Inverse PCR of transposon mutants, with approximate fragment sizes, JMG2.63 ~360bp (1), JMG2.77 ~150 bp (2), JMG2.78 ~100 bp (3), JMG2.130 ~1100 bp (4), JMG2.120 ~3856 bp (5), JMG2.125 ~1000 bp (6), JMG2.98 ~1601 bp (7), JMG2.113 ~2116 bp (8), JMG2.82 ~1600 bp (9). *DraI* digested genomic DNA was self ligated, and PCR was performed with primers IPCR3 and IPCR4.



**Figure 4.9** Southern Blot of transposon mutants JMG2.63 (1), JMG2.77 (2), JMG2.78 (3), JMG2.82 (4), JMG2.98 (5), JMG2.120 (6), JMG2.133 (7), GFP vector positive control (8), JMG2.82 (9), JMG2.113 (10), JMG2.130 (11). Genomic DNA was digested with *DraI* and hybridised with a probe specific to the *GFP* gene of the inserted transposon.

DIG labelled dNTPs (Roche cat # 1277065) and 1 U of Dynazyme EXT DNA polymerase (Finnzymes). The PCR reaction was performed in 1 X Taq Reaction buffer (Sigma) over 30 cycles at 94°C, 30 sec, 56°C, 1 min, 72°C, 1 min. Sequencing of the amplified genomic fragments (automated sequencing with primers IPCR3 and IPCR4) and comparison to the yeast genome utilising the BLAST homology search engine (<http://seq.yeastgenome.org/cgi-bin/SGD/nph-blast2sgd>) revealed the identity of the affected genes.

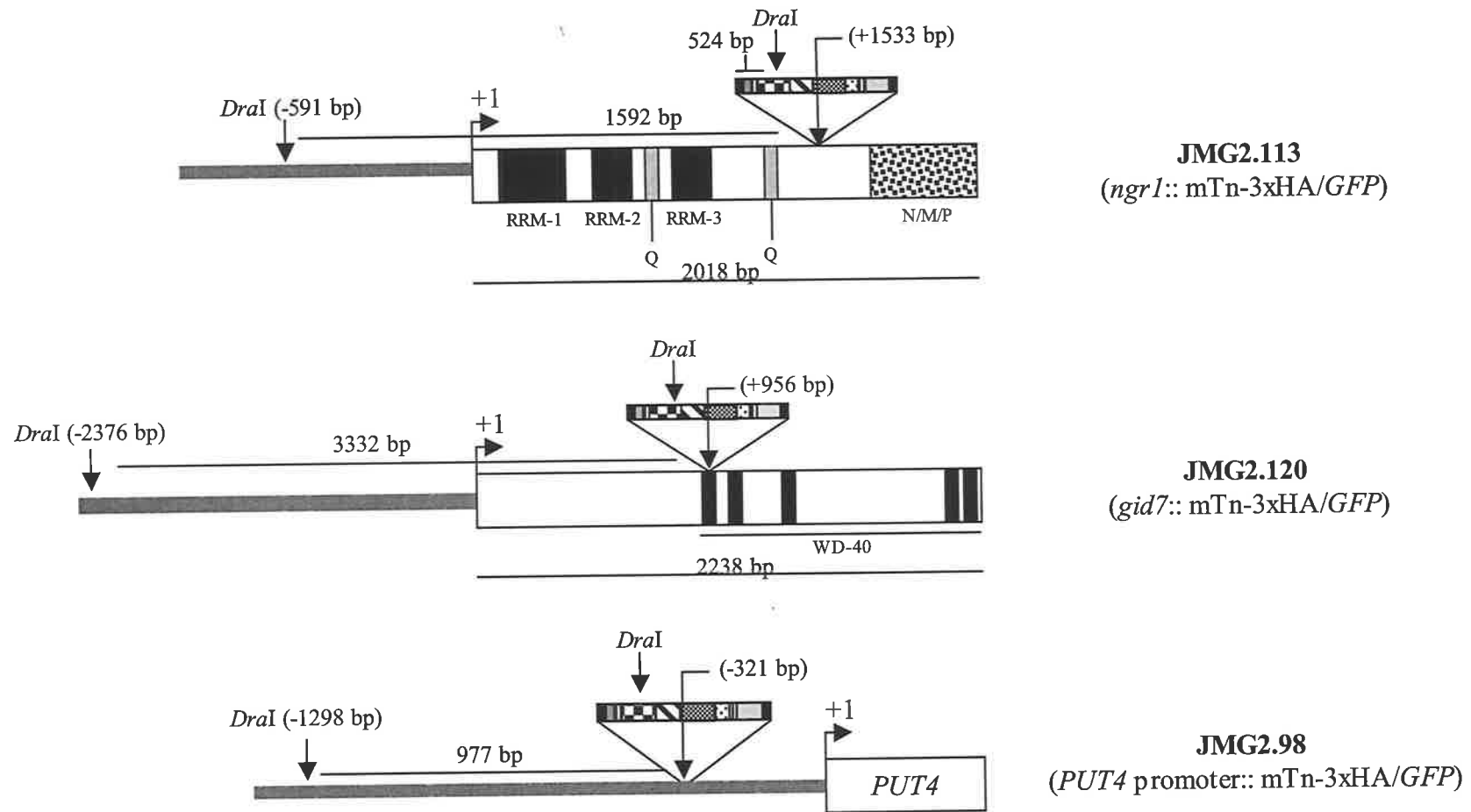
Localisation of the transposon was also attempted by Vectorette PCR (Riley, et al., 1990) and direct genomic sequencing (Horecka and Jigami, 2000), but without success.

Transposons were located within the coding regions of *TIF4632* (+1054 bp), *NGR1* (+1533 bp), *GID7* (+956 bp), *YDL133W* (+435 bp), *RDN37-1* (various locations) and in the promoter of *PUT4 (YOR348C)* (- 321 bp) (Table 4.2). For *NGR1*, *GID7* and *PUT4*, insertions are shown in Figure 4.10. The transposon location within strain JMG2.72 has not yet been determined as all attempts to produce a product from this strain with Inverse PCR failed. Four of the nine identified insertion sites were located in *RDN37-1*, which encodes the ribosomal RNA of which there are 100 - 200 tandem repeats in a 1 - 2 Mb region on the right arm of chromosome XII. For reasons outlined in Section 4.4, these mutants were considered false positives and were removed from further consideration.

*NGR1* and *GID7* were chosen for further analysis as the corresponding mutant strains, JMG2.113 and JMG2.120, catabolised 10.5 g L<sup>-1</sup> (7.3 %) and 27.4 g L<sup>-1</sup> (22.2 %) more glucose than the parent strain, when 75 mg FAN L<sup>-1</sup> as ammonium was supplied. In the context of winemaking, the increased glucose catabolism shown here is quite significant, especially considering that in most cases the catabolism of essentially all sugar from grape juice can be critical: a residual of >7.5 g L<sup>-1</sup> will put a wine out of specification as a dry table wine (Iland and Gago, 2002). JMG2.98, having an increased total glucose catabolism of 8.4 % was also of interest, however, given that this strain harboured a

**Table 4.2** The known functions of genes identified from transposon mutants considered 'nitrogen efficient'.

| <b>Affected gene</b>                   | <b>Known function(s)</b>   | <b>Reference</b>  |
|--|--|---|
| <i>TIF4632</i><br>( <i>YGL049C</i> )   | Translation initiation factor eIF4G, one of the subunits of the mRNA cap-binding protein complex (eIF4F).  | Goyer, et al., 1993   |
| <i>NGR1</i><br>( <i>RBP1/YBR212W</i> ) | RNA binding protein that negatively regulates growth rate. Interacts with the 3' UTR of the mitochondrial porin ( <i>POR1</i> ) mRNA and enhances its degradation. Overexpression impairs mitochondrial function. Expressed in stationary phase. | Akada, et al., 1997<br>Lee and Moss , 1993<br>Buu, et al., 2004<br>Martinez, et al., 2004                     |
| <i>GID7 (YCL039W)</i>                  | Involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase.   | Regelmann, et al., 2003   |
| <i>YDL133W</i>                         | Hypothetical ORF of unknown function   | <i>Saccharomyces</i> genome database<br><a href="http://www.yeastgenome.org/">http://www.yeastgenome.org/</a> |
| <i>RDN37-1</i>                         | 35S ribosomal RNA transcript.  | Venema and Tollervey, 1999  |
| <i>PUT4 (YOR348C)</i>                  | Proline permease, required for high-affinity transport of proline.   | Jauniaux, et al., 1987  |



**Figure 4.10** A diagrammatic representation of the major features of HNE mutants JMG2.113, JMG2.120 and JMG2.98. The insertion sites of mTn-3xHA/GFP are shown for each strain, along with *DraI* sites, RNA recognition motifs (RRM), poly-glutamine region (Q), asparagine / methionine / proline rich region (N/M/P) and WD40 regions.

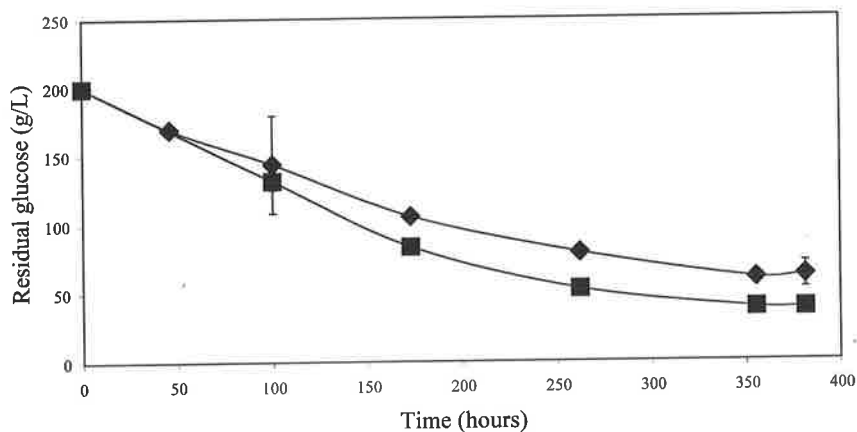
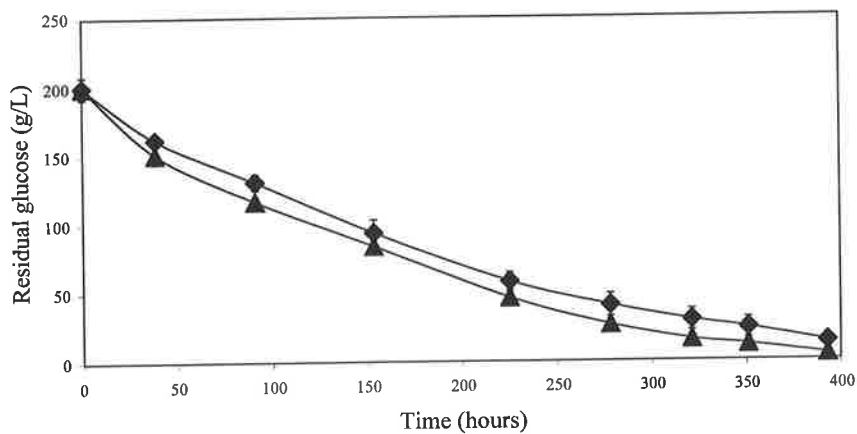
transposon in the promoter of *PUT4* it was appropriate that it became part of a project already underway in this laboratory, which focussed on *PUT4* expression in yeast. Strains JMG2.82 and JMG2.78, and the affected genes *TIF4632* and *YDL133W* were not pursued further as they had minimal impact on glucose catabolism (3.1 % and 3.3 %, respectively).

#### **4.2.5 Deletion of *NGR1* or *GID7* confers nitrogen efficiency upon KP2**

The entire open reading frames of *NGR1* and *GID7* were disrupted in KP2, using the *kanMX* gene replacement strategy, to confirm that the nitrogen efficient phenotype displayed by JMG2.113 and JMG2.120 was indeed due to a disruption of these genes. We took advantage of the readily available yeast deletion strains from the Yeast Deletion Project ([http://www-sequencce.stanford.edu/group/yeast\\_deletion\\_project/deletions3.html](http://www-sequencce.stanford.edu/group/yeast_deletion_project/deletions3.html)). A PCR product corresponding to the *kanMX4* module flanked with *NGR1* or *GID7* sequences (- 260 → - 1 and +2020 → +2329; - 445 → - 1 and +2239 → +2613, respectively) was amplified from genomic DNA purified from the corresponding yeast deletion mutants, 3352 (BY4741 *ngr1Δ::kanMX4*) and 3446 (BY4741 *gid7Δ::kanMX4*), using the primer pairs NGR1A, NGR1D and GID7A and GID7D. KP2 was transformed with these PCR products and transformants were selected on YEPD-geneticin (400 μg mL<sup>-1</sup>). Deletion from KP2 of *NGR1* to yield KP2 *ngr1* (*ngr1Δ::kanMX4*), and *GID7* to yield KP2 *gid7* (*gid7Δ::kanMX4*) was confirmed by Southern Blot hybridization with a probe specific for *kanMX4*. The *kanMX4* probe was PCR amplified from pFA6-lackanMX4 (Wach, et al., 1994) using primers G418F and G418R (Table 2.3) . PCR conditions employed were the same as those for the GFP probe.

Triplicate fermentations were performed in 100 mL of CDGJM with 75 mg FAN L<sup>-1</sup> of nitrogen as ammonium. Deletion of *NGR1* from KP2 resulted in an increased rate of glucose catabolism. For example, over 400 hours, KP2 *ngr1* was found to catabolise 195.7 g L<sup>-1</sup> of glucose whereas KP2 only catabolised 186.6 g L<sup>-1</sup>, representing a 4.9 % increase in glucose catabolism (Figure 4.11). Similarly KP2 *gid7* catabolised 161.8 g L<sup>-1</sup> of glucose whereas KP2 only





**Figure 4.11** Catabolism of glucose by deletion strains KP2 *ngr1* (▲), KP2 *gid7* (■) compared to the parent strain, KP2 (◆). Values are the mean of triplicate 100 mL fermentations performed in CDGJM with 75 mg FAN L<sup>-1</sup> supplied as ammonia. The two graphs represent two independent experiments and standard deviations are included.

catabolised  $137.9 \text{ g L}^{-1}$ , that is, an increase of 17.3 % more glucose catabolised than the parent strain.

It is worth noting that the duration of fermentation performed with severely limited amounts of nitrogen (e.g.  $75 \text{ mg FAN L}^{-1}$ ) was prone to some variation between experiments, particularly where durations were in the order of 400 hours. Such variations (in absolute duration) between experiments were minimised through standardisation of methodologies, reagents and fermentation vessels. However variation was not eliminated, thereby highlighting the limitation of shake flask technologies under critical conditions. Since no alternate approach was available, it was resolved that all treatments were conducted in triplicate and that the parent strain be always included as an internal reference in each experiment. Accordingly, fermentation data are presented in relation to this internal reference, and is often expressed as a percentage of the parent strain.

#### **4.3 Conclusions**

- a) Nitrogen efficiency of a laboratory yeast can be altered through transposon mutagenesis.
- b) The mutagenic and screening strategies developed in this study were successfully used to identify three genes that significantly affect glucose catabolism in nitrogen limited fermentations; *NGR1*, *GID7* and *PUT4*.
- c) Deletion of the entire open reading frame of *NGR1* or *GID7* conferred a nitrogen efficient phenotype to KP2.

#### **4.4 Discussion**

A transposon-based system was successfully used to identify genes involved in determining the efficiency with which nitrogen is utilised during fermentation of a wine-like medium. Mutants with increases or decreases in nitrogen efficiency were both considered potentially valuable to this study. Nevertheless, given that most mutations probably resulted from a loss of function in the target gene (Seifert, et al., 1986; Daignan-Fornier and Bolotin-

Fukuhara, 1988), those yielding an increase in nitrogen efficiency were considered more likely to be linked with changes to nitrogen metabolism. Whereas, mutants with reduced nitrogen efficiency might arise from any mutation that debilitates growth and/or any stage of sugar catabolism, mutants with increased nitrogen efficiency were hypothesised to come about through disruption of a gene or metabolic outcome normally antagonistic to efficient exploitation of nitrogen.

An increase in the frequency of nitrogen efficient mutants through the various stages of screening confirmed the effectiveness of the screening procedure. This success aside a number of shortcomings were encountered, for instance, four of the nine sites identified were within *RDN37-1*, encoding ribosomal RNA, of which there are 100 - 200 tandem repeats in a 1 – 2 Mb region on the right arm of chromosome XII. As these mutants have been isolated in our laboratory through other unrelated screens we believe them to be false positives, a feature apparently not uncommon to these libraries (M. Snyder pers. comm.). In other cases, initial identification of transposon insertion sites as attempted by Vectorette PCR, the method proposed by manufacturers of the transposon library, was not straightforward. Direct genomic sequencing by this method as used by Horecka and Jigami (2000) was also unsuccessful, with Inverse PCR ultimately proving effective (Martin and Mohn, 1999; Huang, et al., 2000).

The mTn-3xHA/*GFP* library is made with S288C as the source of genomic yeast sequences, and as this project was specifically targeted at finding genes that would affect wine fermentations, a wine yeast transposon library inserted into a wine yeast would have been more appropriate. The potential genetic differences between wine yeast and laboratory yeast are presumably reflected by their quite different fermentation profiles. However, as explained these tools were not available at the time the screen was carried out. Nonetheless, as an outcome of this screening, the *NGR1*, *GID7* and *PUT4* genes were identified as having a significant impact on glucose catabolism during nitrogen-limited fermentation.

The specific biological function(s) of *NGR1* (negative growth regulator 1) is unknown. *NGR1* has been implicated in the regulation of growth rate, particularly on non-fermentable carbon sources (Lee and Moss, 1993; Ikeda, et al., 1996), as deletion of the open reading frame confers a 30 % increase in growth rate in the early exponential phase during growth on glucose, or an increase of 60 % or 75 % during growth on galactose or glycerol, respectively. Supporting this *NGR1* (*RBPI*) was independently isolated from a screen selecting for genes that negatively regulate growth when over-expressed (Akada, et al., 1997). *NGR1* has also been shown to be glucose repressible (Lee and Moss, 1993), yet has also been suggested to be involved in the response to high sugar stress, as *NGR1* mRNA was up-regulated 3.7 fold when cells were exposed to high (400 g L<sup>-1</sup>) sugar (Erasmus, et al., 2003). This feature highlights the potential importance of *NGR1* during fermentation. Recently *NGR1* has been shown to be potentially important in survival during stationary phase at 37°C (Martinez, et al., 2004). Stationary phase cultures were maintained at 37°C for up to 16 days and *ngr1Δ* strains had some loss of viability. These researchers also reported that the proper function of mitochondria was important for survival during stationary phase, and many of the 32 genes, critical for this phase were also found to be functionally associated with mitochondria.

The six regions of Ngr1p (three RNA recognition motifs (RRM), two glutamine rich regions and a carboxyl terminal asparagine, methionine and proline rich region (N/M/P)) hypothesised to be structurally important for function are quite separate and varied, suggesting that Ngr1p may have multiple roles within the cell. The transposon insertion of JMG2.113 was located between a polyglutamine region and the N/M/P rich region of *NGR1* (Figure 4.10), which corresponds to a region of low complexity, located 16 amino acids from the carboxyl terminal. This insert possibly disrupts the function of Ngr1p in JMG2.113 completely, or otherwise partially by disabling the N/M/P rich region. This carboxyl region of Ngr1p (from amino acid 566 - 572), is comprised of 25.5 % asparagine, 12.2 % methionine and 11.3 % proline. Asparagine and glutamine rich regions are common to transcriptional

activators (Tjian and Maniatis, 1994), suggesting a potential role in transcription for Ngr1p. Asparagine rich regions have also been implicated in RNA binding. There are three other known RNA binding motifs (RRMs) in Ngr1p. RRM motifs are found in eukaryotic proteins that bind to RNA or single stranded DNA, such as heterogeneous nuclear ribonucleoproteins (hnRNPs), proteins involved in alternative splicing, and protein components of small nuclear ribonucleoproteins (snRNPs) (Burd and Dreyfuss, 1994). The motif also appears in a few single stranded DNA binding proteins. Structurally, RRM motifs are normally comprised of four strands and two helices arranged in an alpha/beta sandwich (Nagai, et al., 1990; Hoffman, et al., 1991). Due to the presence of these RRM motifs, if Ngr1p is involved in transcription, then it most likely binds RNA or locally un-wound single stranded DNA in the transcription bubble. Recent evidence suggests that Ngr1p negatively regulates mitochondrial porin expression post-transcriptionally (Buu, et al., 2004), as it was shown to interact with and subsequently accelerate turnover of porin mRNA. Mutants of *NGR1* were also shown to have increased mitochondrial outer membrane porins. The involvement of Ngr1p in the mitochondria may also help to explain the *ngr1* growth phenotypes, reported by Lee and Moss (1993), as deletion enhances growth rate particularly on non-fermentable carbon sources such as glycerol. This is also supported by Buu et al (2004), where cells that over-expressed *NGR1* grew very poorly on glycerol.

The human homologues of *NGR1* encode two lymphocyte proteins, thought to be involved in apoptosis (46 % identical at the protein level, NCBI BLASTP, <http://www.ncbi.nlm.nih.gov/BLAST/>). The products of *TIA-1* and *TIAR* have been shown to bind to uridine rich motifs via one of their three RRM domains (Dember, et al., 1996; Piecyk, et al., 2000). *TIA-1* and *TIAR* have also been implicated in the general arrest of translation during an environmental stress response. It is thought that Tia-1 and Tiar recruit particular poly(A)<sup>+</sup> RNAs to stress granules to prevent them from being transcribed (Kedersha, et al., 1999). Perhaps Ngr1p has a similar function in yeast, where in *ngr1* mutants transcription during fermentation is not as heavily down-regulated due to the

mounting environmental stresses such as decreasing nutrients and increasing ethanol, thus enabling, as we have observed, an extended catabolism of sugar. *GID7* is known to be involved in catabolite degradation of Fructose-1,6-bisphosphatase (FBPase). Ordinarily, FBPase is inactivated and degraded when cells are shifted to a glucose containing medium, however, in *GID7* mutants, or other *GID* mutants, this is not the case (Regelmann, et al., 2003). Interestingly, the expression of another of the *GID* genes, also known as *VID30*, is regulated by ammonium, in particular being up-regulated on low ammonium (van der Merwe, et al., 2001). The action of Vid30p in low ammonium has also been shown to direct nitrogen metabolism towards glutamate formation. Perhaps *GID7* has a similar function. That is, in a *gid7Δ* strain, if the Central Nitrogen Metabolism is altered, perhaps this strain can re-direct nutrients towards maintenance of fermentation.

Gid7p is predicted to contain five WD40 domains (SMART, <http://smart.embl-heidelberg.de/>). The WD40 domain is thought to mediate protein-protein interactions, and is found in many regulatory proteins such as Ste4p, the  $\beta$  subunit of the heterotrimeric G-protein GTPase (Fong, et al., 1986). It is thought that through this domain Gid7p is able to facilitate interaction with other, if not multiple proteins. WD40 domains are also found in some proteins involved in mRNA degradation such as Ski8p (Madrona and Wilson, 2004), suggesting a possible common function to Ngr1p. The transposon insertion of JMG2.120 is located at +956 of the *GID7* coding sequence, corresponding to an insertion that is six amino acids inside the most amino terminal WD40 domain, possibly disrupting this domain, or indeed the rest of the protein's function.

The transposon in mutant JMG2.98 is inserted at position -321 in the promoter of the *PUT4* gene. *PUT4* encodes the proline permease. It is possible the inserted transposon disrupts binding of upstream regulators, such as neighboring putative sites for Swi5p (-362, -367) or Gcn4p (-281, -276) (Zhu and Zhang, 1999) although none is particularly close to the inserted transposon (> 40 bp away). Disruption of the regulation of *PUT4* might also lead to

constitutive expression. Work in this laboratory has located sequences within the 5' region of *PUT4* (-90, -160, -213 and -708) of which disruption allows constitutive expression under normally repressing conditions (Poole, 2002). Ordinarily *PUT4* is nitrogen catabolite repressed in the presence of a rich nitrogen source such as ammonium. Derepression of *PUT4* should allow the cell to utilise more of the abundant supply of proline present in CDGJM and thus have access to a greater supply of nitrogen. In work outside the scope of this study, the expression of *PUT4* and subsequent activity of Put4p in JMG2.98 under the experimental conditions used is being determined in an attempt to explain the high nitrogen efficiency of this mutant.

Other genes identified from the screening of the transposon library include *TIF4632* and *YDL133W*, which were not pursued because of the modest impact of their deletion on glucose consumption. JMG2.82 has a transposon inserted at position +351 of the 914 bp *TIF4632* gene sequence, encoding the eIF4G (eukaryotic initiation factor 4 gamma), a global translation initiation factor. eIF4G is known to mediate the binding of several factors to the yeast nuclear cap binding complex as a part of cap-dependent mRNA translation initiation. There are two isoforms of eIF4G in yeast, eIF4G1 and eIF4G2, encoded by *TIF4631* and *TIF4632* respectively. A 320-amino acid stretch in the carboxyl terminal half of these genes is 80 % identical, thought to make their essential functions redundant (Goyer, et al., 1993). However, these two proteins do have functional differences (Tarun and Sachs, 1997). eIF4G1 is proposed to be affected by the nutritional status of the cell, as it is rapidly degraded when cells enter the diauxic growth phase or when treated with rapamycin (Berset, et al., 1998). It is thought that this effect is mediated by the TOR signal transduction pathway by regulating the stability of eIF4G1. Perhaps eIF4G2 has a minor regulatory effect on glucose catabolism and deletion of *TIF4632* disrupts the cells' ability to recognise nutrient depletion, thus protein synthesis and consequently glucose catabolism could be maintained in this mutant.

YDL133W is a hypothetical open reading frame located on chromosome IV, the transposon insertion in this mutant is located at position +435, and no function has been associated with this putative gene.



## **Chapter 5 Deletion of *NGR1* or *GID7* from a haploid wine yeast increases glucose catabolism and also affects other aspects of fermentation.**

### **5.1 Introduction**

The use of nitrogen efficient strains in nitrogen deficient musts is an innovative way to decrease the likelihood of residual sugar in a finished wine. For instance, the deletion of *NGR1* or *GID7* from a laboratory yeast (KP2) increases the total glucose consumption in a nitrogen limited fermentation by 4.9 % and 17.3 % respectively (Chapter 4). The use of selected wine yeast strains is known to impact upon the dynamics and the final aroma and flavour compounds produced during industrial wine fermentation (Manginot, et al., 1998; Lambrechts and Pretorius, 2000). Wine yeast are valued for their ability to rapidly ferment sugars without formation of significant amounts of undesirable flavours and aromas, their tolerance to ethanol, and their resistance to sulfur dioxide (Rankine, 1977). All such attributes will be present in wine yeast due to differences in the genetic constitution of such strains compared to laboratory strains. For these reasons it was desirable to evaluate whether deletion of *NGR1* or *GID7* has effects of oenological consequence in a wine yeast background.

Deletion strains were constructed in C9, a haploid derivative of the wine yeast strain L-2056 which lacks the *kanMX* cassette. In short, C9 was derived from 1B (Chapter 3); in C9 the *HO* disruption construct containing the *kanMX* cassette has been removed by loop out of this marker through meiotic recombination (Walker, et al., 2003). The use of C9 allowed for the re-application of the convenient *kanMX* selection system in the construction of these deletion strains. The fermentation performance of such deletion strains was assessed alongside that of the already described laboratory strain based yeast deletions KP2 *ngr1* and KP2 *gid7* (Chapter 4).

Grape juices are known to vary widely in the amount, for example 60 – 2400 mg L<sup>-1</sup> (Ough and Amerine, 1988), and type of yeast assimilable nitrogenous compounds that they contain. Thus these strains were evaluated in media with

both limiting and excess nitrogen, supplied as ammonium or to mimic a typical grape must, a complex mixture of amino acids and ammonium (Henschke and Jiranek, 1993).

Characteristics of fermentation that were hypothesised to be affected by a change in nitrogen utilisation were examined to aid in determining how the deletion of these genes i) confers the nitrogen efficient phenotype and ii) affects other characteristics of oenological fermentation so as to reveal potential pitfalls of the industrial use of such deletants. Therefore, glucose catabolism, ammonium uptake, biomass production, cell morphology and accumulation of major metabolites were investigated.

## **5.2 Results**

### **5.2.1 Deletion of HNE genes from C9**

*NGR1* and *GID7* were deleted from C9, to determine if a similar nitrogen efficient phenotype could be transferred to a derivative of an industrial strain. C9 *ngr1* (*ngr1Δ::kanMX4*) and C9 *gid7* (*gid7Δ::kanMX4*) were constructed in a similar approach to KP2 *ngr1* and KP2 *gid7* (Chapter 4). Briefly the open reading frames of *NGR1* and *GID7* were replaced with *kanMX4* by homologous recombination. Transforming fragments were generated by PCR amplification with primer sets NGR1A, NGR1D, GID7A and GID7D (Table 2.3) from deletion library strains 3352 and 3446 respectively. This method allowed generation of long flanking homologous regions, which seemed to be a requirement for efficient homologous recombination in C9. Positive transformants were selected on geneticin (400 μg mL<sup>-1</sup>) and deletion strains were verified by Southern Blot hybridisation with the *kanMX* specific probe.

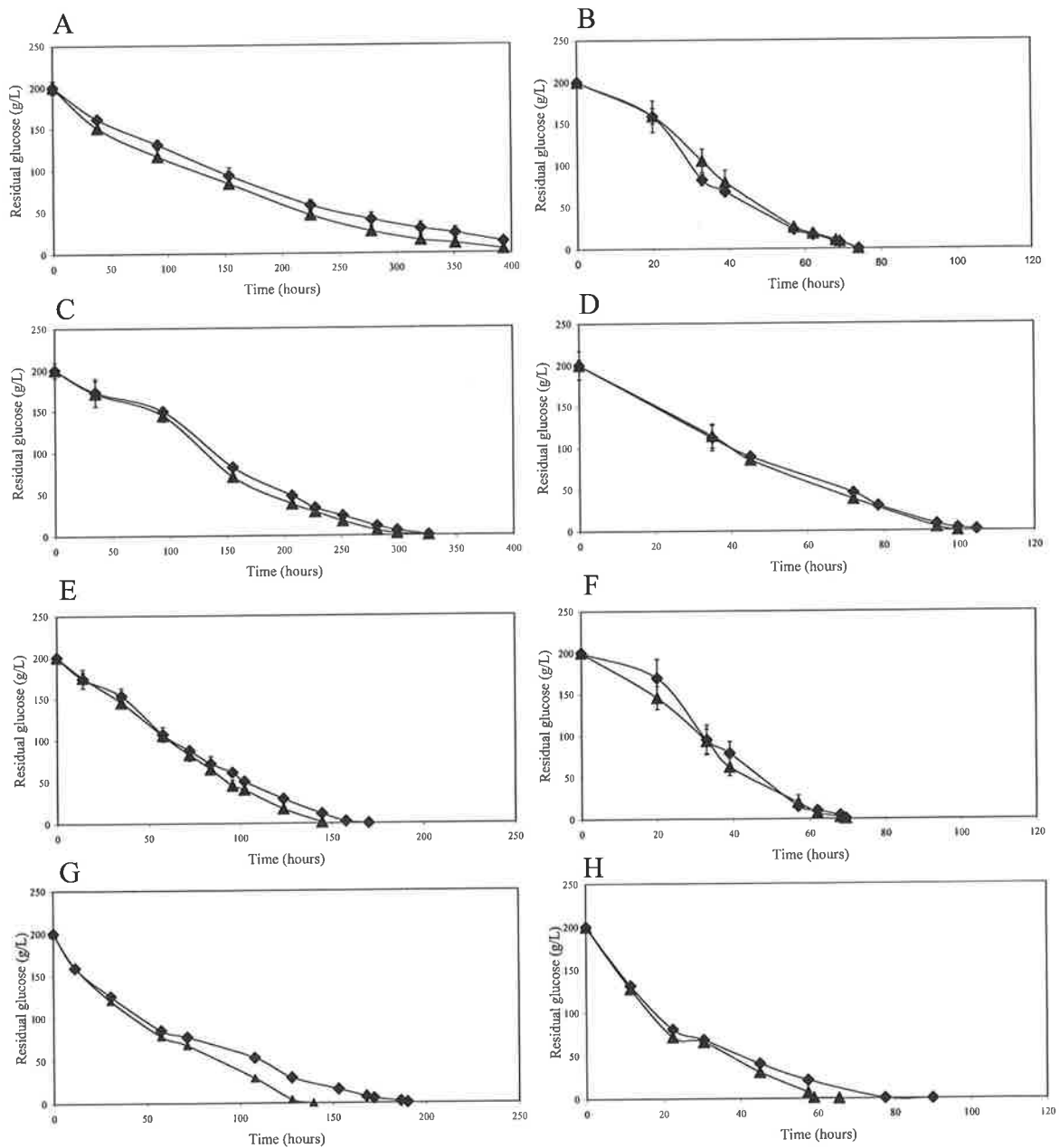
### **5.2.2 Determination of the glucose utilisation profiles of *ngr1Δ* and *gid7Δ* strains**

To determine the effect of deletion of *NGR1* and *GID7* on glucose utilisation, fermentations were performed under anaerobic conditions with nitrogen supplied in moderate or low amounts (300 or 75 mg FAN L<sup>-1</sup>) as complex

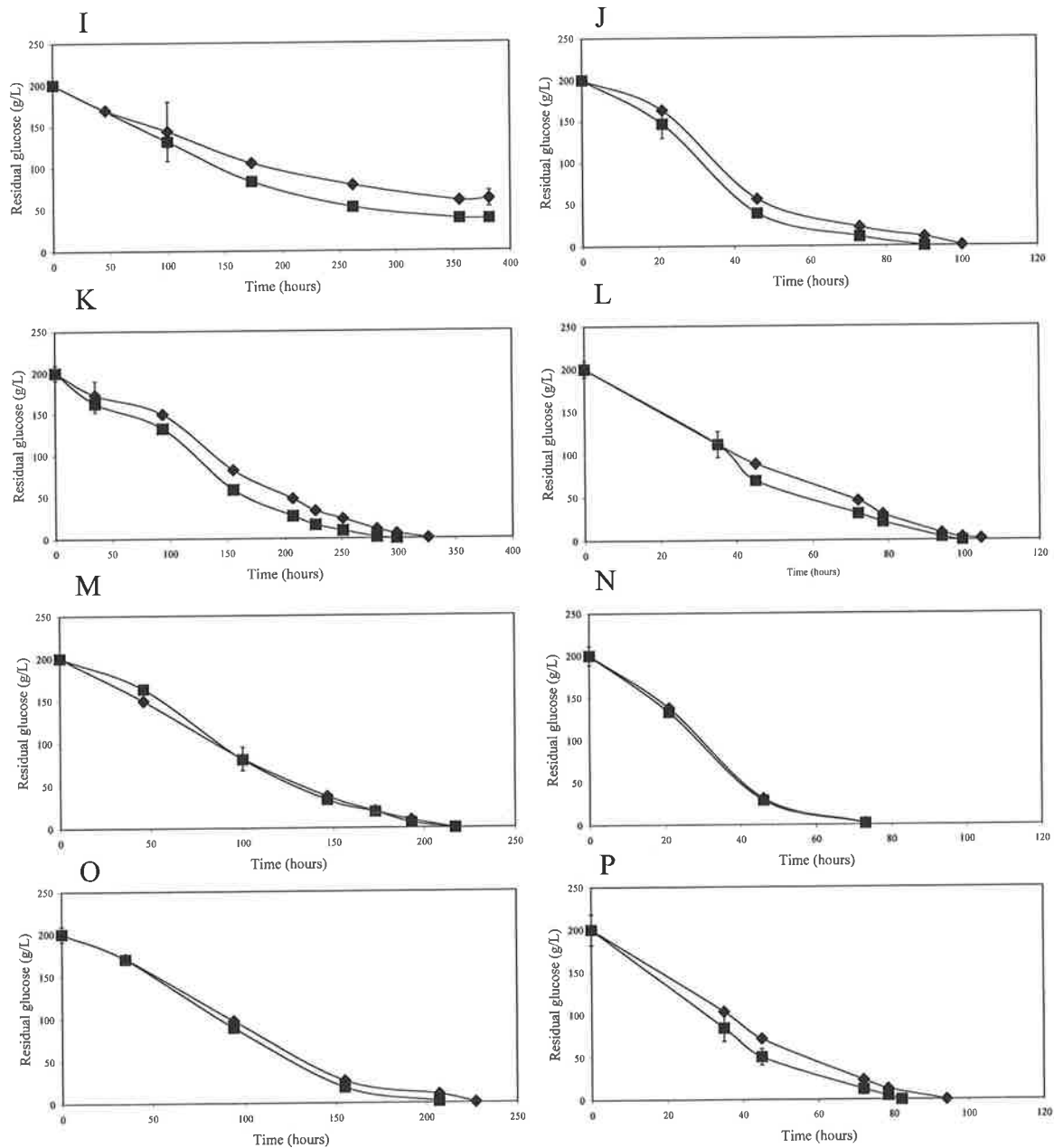
nitrogen (a mix of amino acids and ammonium) or with solely ammonium (Figures 5.1 and 5.2).

As reported in Chapter 4, deletion of *NGR1* from KP2 resulted in increased glucose catabolism in a medium of limiting nitrogen supplied as ammonium (Figure 5.1 A). Over 400 hrs, KP2 *ngr1* was found to catabolise 195.7 g L<sup>-1</sup> of glucose whereas the parent strain, KP2 only catabolised 186.6 g L<sup>-1</sup> representing a 4.9 % increase in glucose catabolism. When complex nitrogen is present, deletion of *NGR1* from KP2 appears to have very little if any affect upon the rate of glucose catabolism (Figure 5.1 C, D). Deletion of *NGR1* from C9 resulted in a significant shortening of total fermentation time in the presence of both limiting and non-limiting amounts of complex nitrogen and limiting amounts of ammonium (Figure 5.1 E, G, H). More specifically, when low amounts of ammonium were available, C9 *ngr1* catabolised 200 g L<sup>-1</sup> of glucose in 85 % (*ca.* 145 h) of the time required by C9 (*ca.* 170 h; Table 5.1). Similarly, when low amounts of a mixture of amino acids and ammonium were available, C9 *ngr1* catabolised 200 g L<sup>-1</sup> of glucose in 73 % (*ca.* 140 h) of the time required by C9 (*ca.* 190 h). Also, when moderate nitrogen is available, C9 *ngr1* completed fermentation in 85 % (*ca.* 65.5h) of the time of C9 (*ca.* 77.5 h). Surprisingly though, when moderate ammonium is available, fermentation by KP2 *ngr1* is not significantly different to KP2.

Deletion of *GID7* from KP2 confers the ability to catabolise glucose faster when nitrogen is present as ammonium or as complex nitrogen (Figure 5.2 I-L). As described in Chapter 4, KP2 *gid7* is able to catabolise 17.3 % more glucose than the parent strain when ammonium is present in low amounts. Similarly, when a low concentration of complex nitrogen is present, deletion of *GID7* from KP2 shortens the time required for fermentation of 200 g L<sup>-1</sup> of glucose by 33.5 hrs; that is the total fermentation duration is 91.6 % of the parent strain strain (*ca.* 298.5 h vs *ca.* 326 h). In media with a high concentration of ammonium or complex nitrogen, fermentation duration is also reduced (to 90 % and 95.2 % respectively). Surprisingly deletion of *GID7* from C9 has no affect on glucose catabolism when ammonium is supplied, yet when complex nitrogen



**Figure 5.1** Catabolism of glucose by yeast deletion strains of *NGR1* (▲) and parent (◆). *NGR1* was deleted from KP2 (A-D) and C9 (E-H). Fermentations were performed in CDGJM with 75 (A,C,E,G) or 300 mg FAN L<sup>-1</sup> (B,D,F,H) supplied either as ammonia (A,B,E,F) or as a mixture of amino acids and ammonia (C,D,G,H).



**Figure 5.2** Catabolism of glucose by yeast deletion strains of *GID7* (■) and parent (◆). *GID7* was deleted from KP2 (I-L) and a wine yeast strain C9 (M-P). Fermentations were performed in CDGJM with 75 (I,K,M,O) or 300 mg FAN L<sup>-1</sup> (J,L,N,P) supplied either as ammonia (I,J,M,N) or as a mixture of amino acids and ammonia (K,L,O,P).

**Table 5.1** The fermentation duration (FD) of yeast strains or, where all glucose was not catabolised, the increase in glucose consumption (<sup>G</sup>), and the biomass yield (BY).

|                        | 75mg FAN L <sup>-1</sup><br>ammonia |                      | 300mg FAN L <sup>-1</sup><br>ammonia |                      | 75mg FAN L <sup>-1</sup><br>complex N |                      | 300mg FAN L <sup>-1</sup><br>complex N |                      |
|------------------------|-------------------------------------|----------------------|--------------------------------------|----------------------|---------------------------------------|----------------------|--|----------------------|
|                        | FD                                  | BY                   | FD                                   | BY                   | FD                                    | BY                   | FD                                     | BY                   |
|                        | (hours)                             | (g L <sup>-1</sup> ) | (hours)                              | (g L <sup>-1</sup> ) | (hours)                               | (g L <sup>-1</sup> ) | (hours)                                | (g L <sup>-1</sup> ) |
| <b>KP2</b>             | -                                   | 2.2 +/- 0.1          | 105                                  | 5.6 +/- 0.1          | 326                                   | 3.4 +/- 0.1          | 74                                     | 6.1 +/- 0.2          |
| <b>C9</b>              | 170                                 | 3.1 +/- 0.1          | 70                                   | 6.9 +/- 0.4          | 190                                   | 2.9 +/- 0.1          | 78                                     | 6.9 +/- 0.4          |
|                        | (%)                                 | (%)                  | (%)                                  | (%)                  | (%)                                   | (%)                  | (%)                                    | (%)                  |
| <b>KP2 <i>ngr1</i></b> | 4.9 <sup>G*</sup>                   | 104 +/- 3.9          | 100                                  | 92 +/- 5.1*          | 100                                   | 101 +/- 5.2          | 95*                                    | 107 +/- 7.2          |
| <b>KP2 <i>gid7</i></b> | 17.3 <sup>G*</sup>                  | 100 +/- 3.1          | 90*                                  | 98 +/- 2.1           | 92*                                   | 82 +/- 4.4*          | 95*                                    | 110 +/- 2.0*         |
| <b>C9 <i>ngr1</i></b>  | 85*                                 | 85 +/- 2.0*          | 100                                  | 77 +/- 7.2*          | 73*                                   | 78 +/- 2.1*          | 85*                                    | 87 +/- 1.3*          |
| <b>C9 <i>gid7</i></b>  | 100                                 | 100 +/- 2.0          | 100                                  | 98 +/- 1.65          | 91*                                   | 97 +/- 1.5           | 87*                                    | 108 +/- 9.7          |

Fermentations were performed in CDGJM with 75 or 300 mg FAN L<sup>-1</sup> as ammonia or a mix of ammonia and amino acids (complex N). Mutant values are expressed as a percentage of the parent strain.\* Values that are significantly different to the parent i.e. p < 0.05 (Student's t-test).

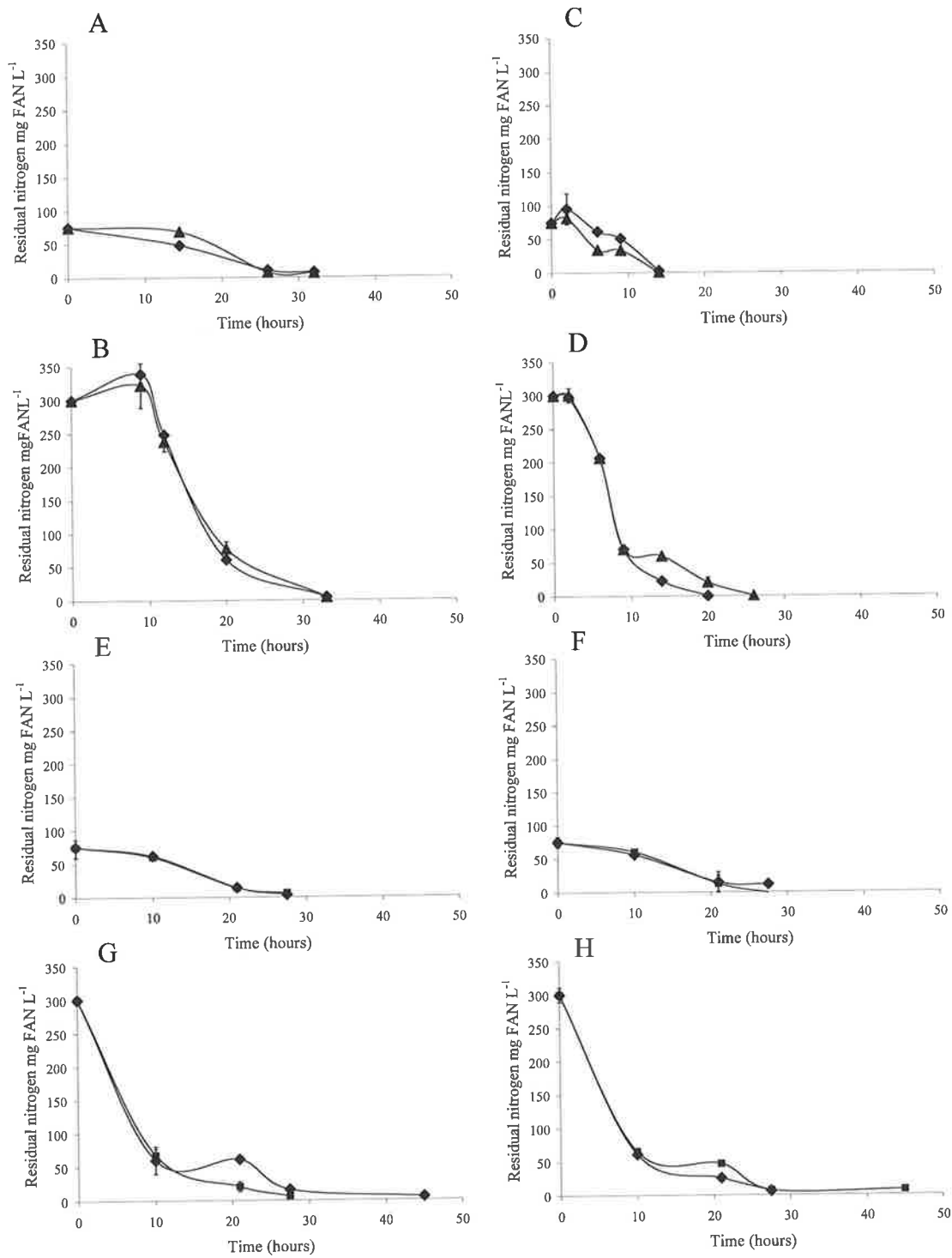
is present fermentation duration is reduced to 87-91.2 % of the parent strain (Figure 5.2 M-P).

### **5.2.3 Determination of ammonium utilisation by *ngr1Δ* and *gid7Δ* strains**

The uptake of ammonium by mutant strains during fermentation (when nitrogen was supplied as ammonium) was examined, and found to be very similar to that of the parent strain (Figure 5.3). It was found that the majority of ammonium was depleted from the medium within 35 hours, when the medium contained either 75 or 300 mg FAN L<sup>-1</sup>. As nitrogen efficiency is also known to vary between yeast strains when excess nitrogen is available (Jiranek, et al., 1995a), and deletion of *NGR1* and *GID7* had certainly altered the ratio of nitrogen utilisation to glucose catabolism, it was of great interest to determine if less total nitrogen was utilised by strains with these genes deleted when excess nitrogen was available. The total nitrogen utilisation was examined when excess nitrogen was supplied (750 mg FAN L<sup>-1</sup>, Table 5.2). Mutant strains did not utilise significantly different amounts of nitrogen to the parent strain (the average utilised by KP2 and C9 based strains, was 273.6 and 220.4 mg FAN L<sup>-1</sup> respectively). Interestingly, when supplied with 750 mg FAN L<sup>-1</sup>, these strains utilised less nitrogen than when they were supplied with 300 mg FAN L<sup>-1</sup>, where all available nitrogen was utilised.

### **5.2.4 Analysis of the biomass yield of *ngr1Δ* and *gid7Δ* strains**

C9 *ngr1* produced a smaller final dry cell weight of between 77-87 % of that of the wild-type C9 (Table 5.1 and 5.2), independent of the amount or type of nitrogen supplied. This reduction in dry weight is not explained by the measurement of cell number yield or cell size determined here, which both appear very similar to the parent strain (Table 5.3). Cell size was measured with a micrometer by visual inspection of live cells at 100 X magnification, yet this method may not have been sensitive enough to describe the differences noted in dry cell weight, and a Coulter Counter or FACS analysis would have been more appropriate. This phenotype of C9 *ngr1* seems to be specific to the C9 strain background as KP2 *ngr1* was not found to significantly affect the final dry weight. Finally the cellular viability of C9 *ngr1* was examined, as it was



**Figure 5.3** Removal of nitrogen from the media during fermentation by yeast deletion strains of *NGRI* (▲)(A-D) or *GID7* (■)(E-H), in KP2 (A, B, E, G) or C9 (C, D, F, H). Triplicate fermentations were supplied with either 75 (A, C, E, F) or 300 mg FAN L<sup>-1</sup> (B, D, G, H) of ammonia, in comparison to the parent strain (◆) KP2 or C9. Standard deviations are included.



**Table 5.2** The total nitrogen utilised and final dry weight produced by mutant strains (also expressed as a percentage of the parent strain) during fermentation where 750 mg FAN L<sup>-1</sup> was supplied as ammonia.

| <b>Yeast strain</b>    | <b>Total nitrogen utilised (mg L<sup>-1</sup>)</b> | <b>Total nitrogen utilised (%)</b> | <b>Final Dry Weight (%)</b> |
|------------------------|--|------------------------------------|-----------------------------|
| <b>KP2</b>             | 276.4 +/- 17.5                                     | 100 +/- 6.3                        | 100 +/- 8.3                 |
| <b>KP2 <i>ngr1</i></b> | 271.4 +/- 12.5                                     | 98 +/- 4.5                         | 106 +/- 8.42                |
| <b>KP2 <i>gid7</i></b> | 273.2 +/- 7.7                                      | 99 +/- 7.7                         | 104 +/- 1.0                 |
| <b>C9</b>              | 217.5 +/- 13.7                                     | 100 +/- 13.7                       | 100 +/- 3.4                 |
| <b>C9 <i>ngr1</i></b>  | 197.7 +/- 57.2                                     | 91 +/- 26.3                        | 87 +/- 1.0                  |
| <b>C9 <i>gid7</i></b>  | 246.1 +/- 8.9                                      | 113 +/- 8.9                        | 104 +/- 6.7                 |

**Table 5.3** The cell number, cell viability and cell size of strains, sampled at the end of fermentation directly after the utilisation of all glucose.

| Yeast strain    | Cell number                                |               | Cell viability                             |               | Cell size  |                |                |
|-----------------|--|---------------|--|---------------|------------|----------------|----------------|
|                 | Cells (x10 <sup>8</sup> mL <sup>-1</sup> ) | %             | Cells (x10 <sup>8</sup> mL <sup>-1</sup> ) | %             | µM         | %              |                |
| <b>C9</b>       | 2.27 +/- 0.19                              | 100.0 +/- 8.5 | 1.74 +/- 0.27                              | 76.8 +/- 11.9 | <b>SC</b>  | 11.25 +/- 1.58 | 100.0 +/- 14.0 |
|                 |  |               |  |               | <b>EOF</b> | 11.75 +/- 1.95 | 100.0 +/- 16.6 |
| <b>C9 ngr1</b>  | 2.17 +/- 0.05                              | 95.9 +/- 2.4  | 1.60 +/- 0.04                              | 73.6 +/- 1.95 | <b>SC</b>  | 12.08 +/- 1.48 | 107.4 +/- 12.3 |
|                 |  |               |  |               | <b>EOF</b> | 11.88 +/- 1.78 | 101.1 +/- 15.0 |
| <b>KP2</b>      | 1.85 +/- 0.18                              | 100.0 +/- 9.9 | -  | -             | -          | -              | -              |
| <b>KP2 gid7</b> | 1.79 +/- 0.04                              | 96.8 +/- 2.4  | -  | -             | -          | -              | -              |

The initial medium contained 75 mg FAN L<sup>-1</sup> in complex form. Cell size was determined visually with the use of a micrometer from both starter cultures (SC) and at the end of fermentation (EOF).

hypothesised that the change in cellular biomass may affect cell health at the end of fermentation. This was found not to be the case, with viabilities being very similar to the parent strain (73.6 % vs 76.8 %, Table 5.3).

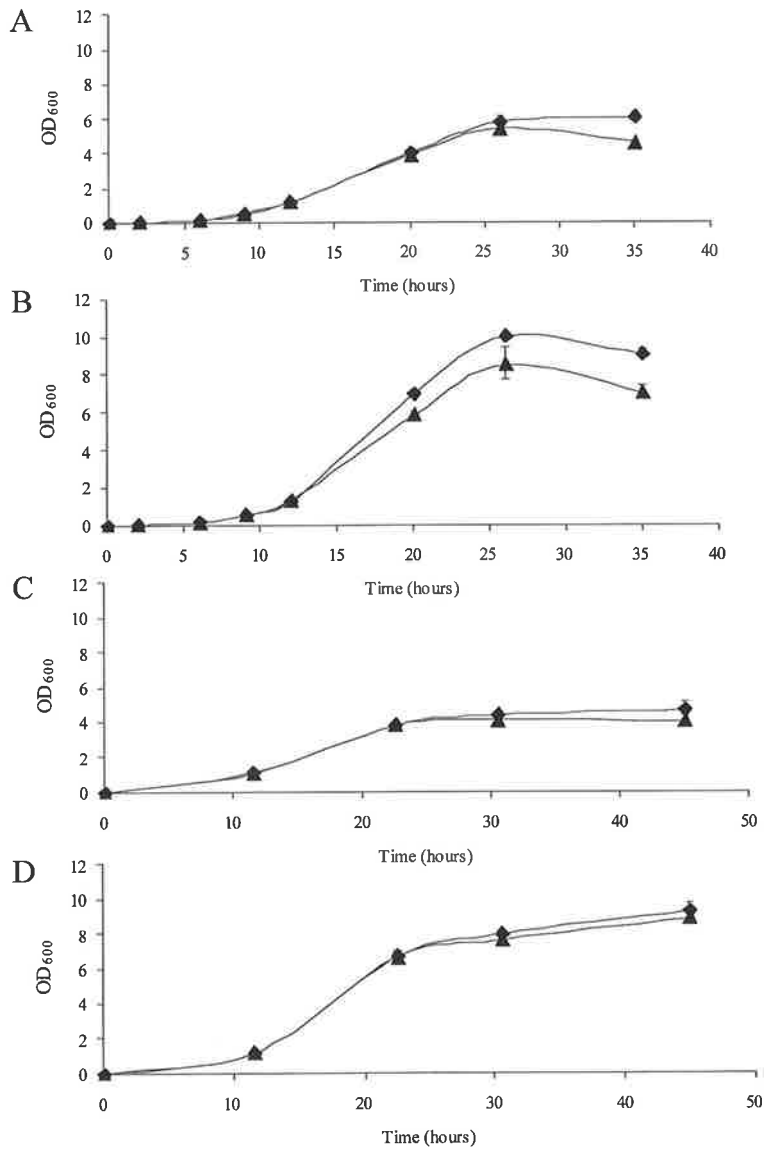
Interestingly, KP2 *gid7* also produces a smaller final dry weight (82 % of the parent strain) when grown in media containing low concentrations of complex nitrogen, yet under other conditions examined no differences were found (Table 5.1). The final cell number of KP2 *gid7* was also not significantly different to the parental. The growth of strains was examined in the early part of fermentation by measurement of OD<sub>600</sub> and cell numbers until cells were in stationary phase. There were only small differences between the growth curves of mutant and parent strains (Figures 5.4 and 5.5). For example, the largest difference was observed when C9 *ngr1* was supplied with 300 mg FAN L<sup>-1</sup> as ammonium, where the OD<sub>600</sub> is reduced (Figure 5.4 B), correlating with a decreased biomass yield, (measured as dry weight), of 77 % of that of the parent strain (Table 5.1).

#### ***5.2.5 Analysis of the cellular morphology of ngr1Δ and gid7Δ strains during fermentation***

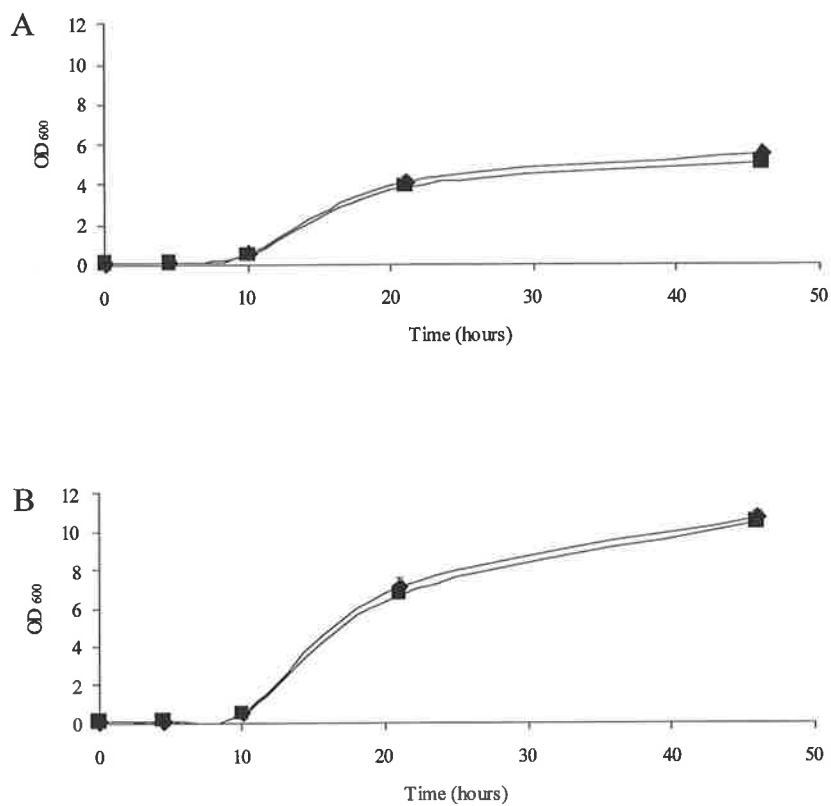
The cellular morphology of deletion strains was analysed microscopically during fermentation. Wet mounted cells were viewed with a Olympus BHA, phase contrast microscope at 40 X magnification. There were no morphological differences observed between mutant and parent strains (an example is presented in Figure 5.6).

#### ***5.2.6 Analysis of the major fermentation metabolites of ngr1Δ and gid7Δ strains***

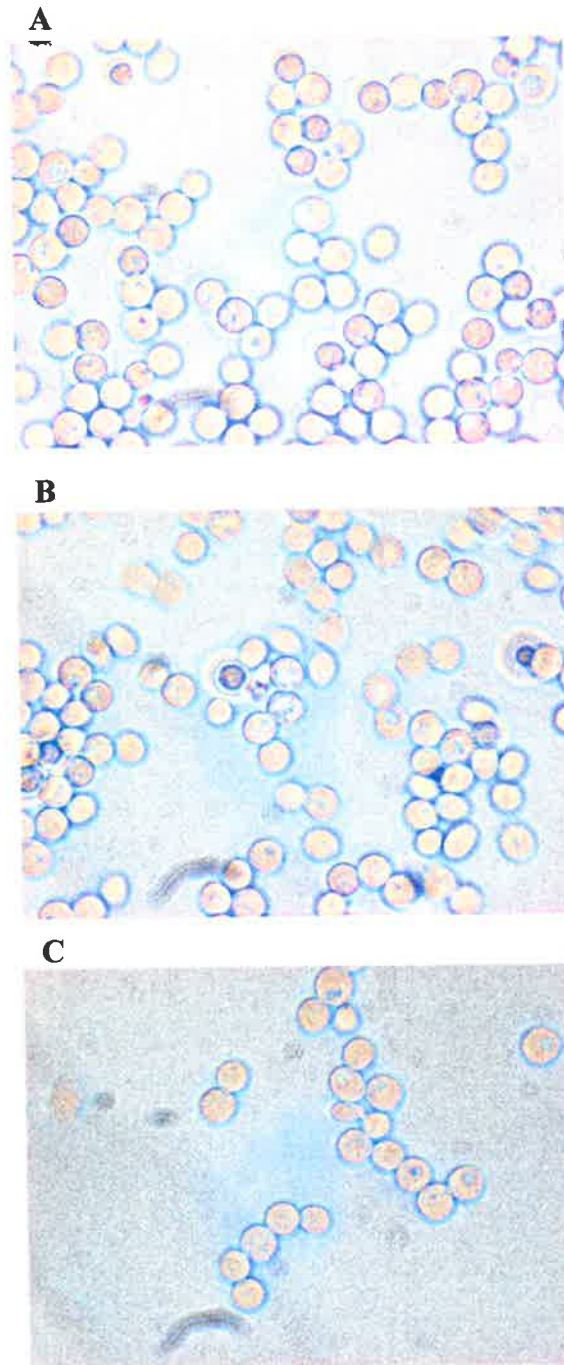
The accumulation of fermentation metabolites at the end of fermentation, some of which are flavour active, was examined. Ethanol, glycerol, acetic acid, succinic acid, citric acid, tartaric acid, lactic acid and acetaldehyde were analysed by high performance liquid chromatography (Table 5.4). The



**Figure 5.4** Growth during fermentation, measured by optical density at 600 nm by yeast deletion strain C9 *ngr1* (▲), or the parent strain C9 (◆). Triplicate fermentations were supplied with 75 (A, C) or 300 mg FAN L<sup>-1</sup> (B, D) of nitrogen as either ammonia (A, B) or as a complex mix of amino acids and ammonia (C, D). Standard deviations are included.



**Figure 5.5** Growth during fermentation, measured by optical density at 600 nm by yeast deletion strain KP2 *gid7* (■), or the parent strain KP2 (◆). Triplicate fermentations were supplied with 75 (A) or 300 mg FAN L<sup>-1</sup> (B) of nitrogen as ammonia. Standard deviations are included.



**Figure 5.6** Cell photos of KP2 (A), KP2 *ngr1* (B) and KP2 *gid7* (C) at a late stage of fermentation, that is 87.5 % of glucose catabolised (25 g L<sup>-1</sup> of residual glucose). Fermentations were supplied with 75 mg FAN L<sup>-1</sup> as ammonia. Wet mount slides were photographed at 40 X.

**Table 5.4** The concentration of some major cellular metabolites from CDGJM fermented by *NGR1* or *GID7* deletants with ammonia or complex nitrogen at two concentrations.

|   | mg FAN L <sup>-1</sup> | Citric acid | Tartaric acid | Succinic acid | Lactic acid    | Glycerol       | Acetic acid    | Acetaldehyde   | Ethanol        |               |
|---|------------------------|-------------|---------------|---------------|----------------|----------------|----------------|----------------|----------------|---------------|
| KP2 <i>ngr1</i>                                 | A                      | 75          | B/T           | B/T           | 102.1 +/- 6.7  | 100.0 +/- 10.8 | 105.9 +/- 3.5  | 115.3 +/- 4.5  | 118.1 +/- 8.3  | 104.9 +/- 5.7 |
|   |                        | 300         | B/T           | 96.3 +/- 1.7  | 95.6 +/- 2.0   | 107.7 +/- 37.6 | 99.3 +/- 1.7   | 104.8 +/- 1.5  | 112.8 +/- 5.9  | 103.4 +/- 3.1 |
|   | CN                     | 75          | 100.9 +/- 2.4 | 108.0 +/- 3.7 | 68.6 +/- 6.3   | 252.2 +/- 23.2 | 117.0 +/- 4.1  | 279.6 +/- 45.4 | 117.2 +/- 27.0 | 97.5 +/- 4.3  |
|   |                        | 300         | 102.3 +/- 3.8 | B/T           | 104.0 +/- 7.0  | 101.8 +/- 0.0  | 97.8 +/- 0.6   | 88.3 +/- 3.7   | 95.4 +/- 2.1   | 105.3 +/- 3.3 |
| C9 <i>ngr1</i>                                  | A                      | 75          | B/T           | B/T           | 106.0 +/- 2.1  | 271.4 +/- 9.1  | 103.2 +/- 0.8  | 92.4 +/- 7.7   | 101.9 +/- 15.3 | 102.7 +/- 2.4 |
|   |                        | 300         | B/T           | 93.9 +/- 4.7  | 61.0 +/- 4.3   | 216.3 +/- 10.7 | 104.9 +/- 2.2  | 104.1 +/- 24.3 | 116.0 +/- 13.8 | 100.3 +/- 1.0 |
|   | CN                     | 75          | 99.1 +/- 4.5  | B/T           | 80.3 +/- 13.6  | 155.9 +/- 31.2 | 116.9 +/- 4.1  | 102.9 +/- 4.2  | 110.4 +/- 21.3 | 100.5 +/- 8.4 |
|   |                        | 300         | B/T           | B/T           | 92.5 +/- 17.0  | 303.7 +/- 27.7 | 106.4 +/- 1.5  | 119.3 +/- 0.7  | 101.7 +/- 2.6  | 105.9 +/- 2.8 |
| KP2 <i>gid7</i>                                 | A                      | 75          | 91.4 +/- 1.3  | B/T           | 92.2 +/- 6.4   | 114.3 +/- 12.5 | 109.1 +/- 2.1  | 145.6 +/- 7.3  | 107.0 +/- 13.1 | 117.7 +/- 6.7 |
|   |                        | 300         | B/T           | B/T           | 95.3 +/- 3.2   | 72.4 +/- 8.2   | 99.5 +/- 0.6   | 108.4 +/- 1.8  | 131.5 +/- 24.4 | 96.1 +/- 11.6 |
|   | CN                     | 75          | B/T           | B/T           | 48.4 +/- 6.1   | 225.0 +/- 5.6  | 103.7 +/- 15.9 | 98.9 +/- 1.3   | 108.3 +/- 10.0 | 109.2 +/- 3.5 |
|   |                        | 300         | 101.3 +/- 7.1 | B/T           | 93.3 +/- 6.1   | 96.4 +/- 5.6   | 90.9 +/- 15.9  | 98.1 +/- 1.3   | 105.2 +/- 10.0 | 102.5 +/- 6.8 |
| C9 <i>gid7</i>                                  | A                      | 75          | 96.2 +/- 1.5  | 96.8 +/- 4.7  | 92.7 +/- 0.0   | 98.4 +/- 6.7   | 103.7 +/- 5.0  | 97.1 +/- 4.2   | 92.0 +/- 0.0   | 100.7 +/- 0.0 |
|   |                        | 300         | B/T           | B/T           | 121.8 +/- 14.0 | 122.0 +/- 8.0  | 101.5 +/- 1.0  | 101.6 +/- 6.2  | 100.8 +/- 3.7  | 96.2 +/- 12.7 |
|   | CN                     | 75          | B/T           | B/T           | 91.5 +/- 3.8   | 64.1 +/- 25.0  | 98.8 +/- 1.6   | 103.9 +/- 6.9  | 121.6 +/- 9.8  | 99.9 +/- 4.4  |
|   |                        | 300         | 101.4 +/- 3.1 | N/S           | 99.0 +/- 10.8  | 105.3 +/- 30.3 | 97.8 +/- 2.9   | 93.5 +/- 11.1  | 91.7 +/- 29.9  | 102.9 +/- 1.7 |
| <b>Ranges of metabolites (g L<sup>-1</sup>)</b> |                        |             |               |               |                |                |                |                |                |               |
| KP2   |                        | 0 - 0.8     | 0 - 0.03      | 0.5 - 2.4     | 0.02 - 0.4     | 6.7 - 9.9      | 0.9 - 1.45     | 0.25 - 0.6     | 82.5 - 98.7    |               |
| C9  |                        | 0 - 2.0     | 0 - 0.06      | 0.5 - 1.9     | 0.06 - 0.18    | 5.9 - 9.8      | 0.5 - 1.3      | 0.47 - 0.66    | 92.6 - 107.5   |               |

Values are expressed as a percentage of the concentration determined from fermentation with the parent strain (ranges shown below in g L<sup>-1</sup>) in the same experiment. Initial fermentation media either contained 75 or 300 mg FAN L<sup>-1</sup> as ammonia (A) or complex nitrogen (CN). Some concentrations were below the measurement threshold for that experiment (B/T). Conditions where the fermentation duration was 95 % or less than the parent strain are highlighted in green. The metabolites which had an increased or decreased abundance by >5 % and were significantly different to the parent, i.e. p < 0.05 (Student's t-test) are highlighted in yellow or orange respectively. The values are the average of single measures from triplicate fermentations.

concentration of citric acid and tartaric acid was often too low to be detected by this system.

There was no clear association between conditions that yielded differences in major metabolites to conditions where *ngr1* or *gid7* mutants decreased fermentation duration (Table 5.4, compare conditions highlighted in green to metabolite concentrations highlighted in red or yellow). Consequently, these metabolite studies do not contribute to an explanation of the mechanism by which these mutants can enable nitrogen efficiency. The differences in metabolite production found here, however, are useful, as they highlight other oenological consequences of the use of these strains.

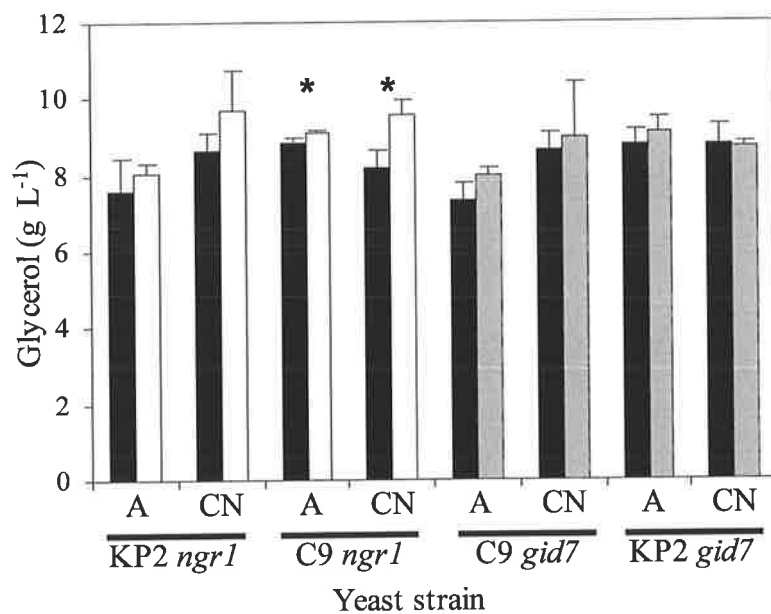
There were no major differences in citric acid, tartaric acid or ethanol detected between parent and mutant strains. The final concentration of succinic acid produced by both *ngr1Δ* and *gid7Δ* strains in KP2 was decreased to approximately half (*ca.* 0.92 – 1.22 g L<sup>-1</sup>) of that produced by the parent strain when 75 mg FAN L<sup>-1</sup> was supplied in a complex form. Similarly when C9 *ngr1* is supplied with 300 mg FAN L<sup>-1</sup> as ammonium, only 61 % (0.53 g L<sup>-1</sup>) of the citric acid was produced. When grown under other conditions the succinic acid production of mutants was very similar to the parent strain.

The lactic acid production of C9 *ngr1* was increased to around 1.5 – 3 times that of the parent strain under all conditions tested. In the same way, both KP2 *ngr1* and KP2 *gid7* produced around twice as much lactic acid when supplied with 75 mg FAN L<sup>-1</sup> in complex form.

A small but reproducible increase in glycerol accumulation (3.2 - 17.0 %) occurred for both *ngr1Δ* and *gid7Δ* compared to the corresponding parental strains when 75 mg FAN L<sup>-1</sup> was available in either form (Table 5.4 and Figure 5.7). The exception was C9 *gid7* which for this deletion also had minimal effect on fermentation duration.

In four of the five conditions / strain combinations where acetaldehyde concentration differed from the parent strain, it was slightly increased.





**Figure 5.7** The concentration of glycerol at fermentation completion or arrest (in the case of KP2, KP2 *ngr1* and KP2 *gid7* supplied with ammonium), when conducted by wildtype (■) KP2 and C9 or their derivatives bearing deletions of *NGR1* (□) or *GID7* (■). Triplicate fermentations were performed in CDGJM containing low nitrogen (75 mg FAN L<sup>-1</sup>) as either ammonium (A) or as a complex mixture of complete amino acids and ammonium (CN) as indicated. Values shown in the first 8 bars were derived from a separate experiment to that which yielded the values shown in the last 8 bars. \*Differences in glycerol yield of mutants compared to the corresponding parent strain that were significant, ie  $p < 0.05$  (Student's t-test).

The acetic acid production of KP2 *ngr1* and KP2 *gid7* was also seen to be increased when 75 mg FAN L<sup>-1</sup> was supplied in complex form when *NGR1* is deleted and as ammonium when *GID7* is deleted.

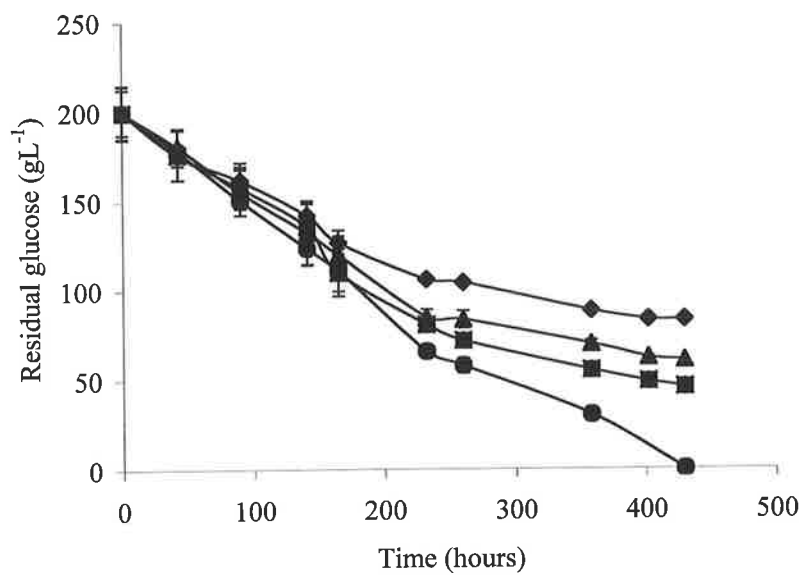
### **5.2.7 Construction of KP2 *ngr1 gid7***

To evaluate whether a strain further enhanced for nitrogen efficiency could be made by a combination of both deletions of *NGR1* and *GID7*, a double mutant was constructed. KP2/3 *ngr1 gid7* was generated by crossing KP2 *ngr1* with KP3 followed by sporulation and dissection to generate KP3 *ngr1B*. KP3 *ngr1B* was mated with KP2 *gid7*, sporulated and dissected to generate the double mutant KP2/3 *ngr1 gid7* (*ngr1Δ::kanMX4, gid7Δ::kanMX4*). Mating type was determined by PCR, as described by Huxley et al. (1990) and gene disruptions by Southern Blot hybridization, as performed previously (data not shown).

It was also of interest to evaluate a double mutation in C9, yet all attempts to produce this mutant were unsuccessful, suggesting that this combination of mutations is lethal in C9. Construction of C9 *ngr1 gid7* was attempted as above and included the mating combinations; C9 *ngr1 MATa* x C9 *gid7 MATa* and C9 *ngr1 MATa* x C9 *gid7 MATa* (a total of forty tetrads were dissected from four separate mating reactions). Preliminary efforts to create a double in a haploid derivative of the wine yeast strain CY-3079 have also failed (data not shown).

### **5.2.8 Determination of the glucose utilisation of KP2 *ngr1 gid7***

Deletion of both *NGR1* and *GID7* from KP2 was found to significantly enhance glucose catabolism when 75 mg FAN L<sup>-1</sup> nitrogen was supplied as ammonium (Figure 5.8). In this experiment KP2 *ngr1* and KP2 *gid7* catabolised 19.4 % (139.2 g L<sup>-1</sup>) and 32.2 % (154.3 g L<sup>-1</sup>) more glucose than the parent strain (116.52 g L<sup>-1</sup>) by 430 hrs, whereas KP2/3 *ngr1 gid7* depleted all glucose from the medium (200 g L<sup>-1</sup>), which equated to 72 % more glucose than the parent strain.



**Figure 5.8** Catabolism of glucose by yeast deletion strains KP2/3 *ngr1 gid7* (●) KP2 *ngr1* (▲), KP2 *gid7* (■) and KP2 (◆). Fermentations were performed in CDGJM with 75 mg FAN L<sup>-1</sup> as ammonia. Data points are the mean from triplicate fermentations. Standard deviations are included.

### 5.3 Conclusions

- a) The nitrogen efficiency phenotype was conferred on a haploid wine yeast strain.
- b) The fermentation duration of a haploid wine yeast strain (C9) was shortened by deletion of the entire open reading frame of *NGR1*.
- c) Deletion of *GID7* from C9 shortened fermentation duration when supplied with nitrogen as a complex mix.
- d) A double deletion of both *NGR1* and *GID7* from KP2 dramatically shortens fermentation duration when 75 mg FAN L<sup>-1</sup> is supplied as ammonium.
- e) Deletion of *NGR1* from C9 decreased the cell dry weight, decreased succinic acid and increased lactic acid production.
- f) Deletion of *GID7* decreased succinic acid and increased lactic acid production.
- g) Both *NGR1* and *GID7* deletion strains tend to produce slightly more glycerol during fermentation.

### 5.4 Discussion

#### 5.4.1 Fermentation profiles of *ngr1Δ* and *gid7Δ* strains

The fermentation profiles of two strains, KP2 (a derivative of the commonly used laboratory strain W303) and C9 (a haploid derivative of the commonly used wine yeast strain L-2056), with deletions of the complete open reading frame of *NGR1* and/or *GID7* have been elucidated. Deletion of either or both of these genes is found to significantly increase the total amount of glucose catabolised or shorten the fermentation duration under various amounts and sources of supplied nitrogen. Eleven of the 16 deletant : media combinations resulted in more glucose catabolised or a shorter fermentation duration than the parent strain. For the remaining five combinations, mutants were found to behave equivalently to the parent strain (Table 5.1).

More specifically, deletion of *NGR1* from C9 enhances glucose catabolism, reducing fermentation duration by up to 27 % (to be 73 % of that of the parent strain) under three of the four nitrogen conditions tested. Deletion of *NGR1*

from KP2 has a reduced effect, yet still enhances glucose catabolism when supplied with low concentrations (75 mg FAN L<sup>-1</sup>) of ammonium or medium concentrations (300 mg FAN L<sup>-1</sup>) of complex nitrogen. Fermentation duration is also reduced by up to 23 % when *GID7* is deleted from KP2 or C9. Only when C9 *gid7* is supplied with ammonium as the sole nitrogen source is there no measured effect on fermentation duration.

The different effects observed between varying nitrogen sources may reflect the original mutant selection criteria (ie on low ammonium), that is a nitrogen efficient phenotype is apparent when low nitrogen is supplied as ammonium but not necessarily when more nitrogen is present or when complex nitrogen is supplied. The utilisation of complex nitrogen involves more intricate pathways than that of solely ammonium (as reviewed by Hofman-Bang, 1999); accordingly it is surprising that deletion of *GID7* from KP2 or C9, and *NGR1* from C9 decreases the fermentation duration when complex nitrogen is supplied. For this reason it is hypothesized that these genes affect pathways active during fermentation whether nitrogen is supplied as ammonium or as a complex mixture of nitrogen compounds. It is important to remember that all of these nitrogen sources feed into the Central Nitrogen Metabolic pathway, suggesting that *NGR1* and *GID7* are active within these processes. However, the effect of these gene deletions on fermentation varies depending on the strain analysed. For example, deletion of *GID7* from C9 has no measured affect when ammonium is present, yet KP2 *gid7* displays a significant increase in glucose catabolised in this same medium. With this in consideration, it seems that the function(s) of these proteins must vary, at least slightly, between these two yeast strains. The genetic differences between KP2 and C9 is unknown. In our experience, and as shown here, the fermentation profiles are dramatically different; as expected, C9 has a faster fermentation rate.

#### **5.4.2 Combination of *ngr1Δ* and *gid7Δ* mutations in a single strain**

As both genes influence nitrogen efficiency in KP2 when low ammonium is supplied, a double deletion was constructed to determine if deletion of both *GID7* and *NGR1* would even further enhance nitrogen efficiency. KP2/3 *ngr1 gid7* was found to significantly enhance fermentation performance,

catabolising all glucose in the media by 430 hrs, whereas the KP2, KP2 *ngr1* and KP2 *gid7* strains become protracted, leaving 83.5, 60.8 and 45.7 g L<sup>-1</sup> in the medium respectively. Accordingly, these genes, under these conditions, display an additive effect on nitrogen efficiency, and consequently are likely to function in quite separate pathways. Due to the superior nitrogen efficiency of the double deletion, this strategy could be a very good candidate for a genetic approach to decrease the likelihood of fermentation difficulties encountered with low nitrogen fermentations. For this reason it was of great interest to construct a double deletion in a wine yeast background. The apparent lethality of C9 *ngr1 gid7* revealed difficulties with this strategy, and it is of great interest to determine if a double deletion could be constructed in a different wine yeast background. So far attempts to do so in another wine yeast background have also failed.

#### **5.4.3 Mechanisms of nitrogen efficiency of *ngr1Δ* and *gid7Δ* strains**

It is difficult to predict how deletion of *NGR1* or *GID7* allows the cell to catabolise glucose faster, or more efficiently. Certainly the transport of ammonium does not seem to be affected, as the rate and time of uptake is very similar to that of the parent strain. Presumably the end use of the available nitrogen has been modified. Also these strains do not alter the total amount of nitrogen utilised when excess nitrogen is available (750 mg FAN L<sup>-1</sup>); in fact the total nitrogen utilised was significantly less than if supplied with moderate (300 mg FAN L<sup>-1</sup>). This could be attributed to a cell's ability to sense low concentrations of extracellular nitrogen and subsequently transport more nitrogen to cope in a potentially limiting environment. Both amino acid and ammonium sensors have been described; the SPS complex (Forsberg and Ljungdahl, 2001) and Mep2p (Lorenz and Heitman, 1998) and yeast have been shown to be responsive to extracellular concentrations of ammonium (ter Schure, et al., 1995a; 1995b).

#### **5.4.4 Cellular growth of *ngr1Δ* and *gid7Δ* strains**

Previous studies indicate the importance of Ngr1p in growth control, as deletion of *NGR1* has been shown to increase growth rate, particularly on non-fermentable carbon sources (Lee and Moss, 1993). In this study there was no

detectable increase in the growth rate of *ngr1Δ* however, C9 *ngr1* consistently produced a reduced dry cell weight (77 – 87 %), at the end of fermentation, across all conditions tested. The most dramatic effect on dry cell weight (77-78 % of the parent strain) occurred when 300 mg FAN L<sup>-1</sup> as ammonium or 75 mg FAN L<sup>-1</sup> as complex nitrogen was available. The initial growth curve, when ammonium was supplied (Figure 5.4 A and B) reflected this result, as a lower absorbance suggests a slightly reduced biomass production in the early stages of fermentation. In all probability this result reflects cell size as there was no difference in cell number. Cell size was measured by visual inspection at 100 X using a micrometer, but these results need to be confirmed. There was no significant difference observed between strains despite a 30 % change in dry cell weight. Assuming that the intracellular content concentration remains constant, and remembering that yeast cells are generally 70 % water, a difference of only 1.0 μm would reflect a difference of 30 % of dry cell weight. Clearly a better procedure, such as the use of a Coulter Counter or image-analysed microscopy should be used in future characterisation of these strains.

The reduction in biomass infers that the nitrogen efficiency of C9 *ngr1* might arise as a result of a sacrifice of the production of particular cellular metabolites, for instance, reserve compounds, such as trehalose and glycogen, thereby enabling a faster or extended fermentative activity. The trigger for trehalose accumulation during alcoholic fermentation is thought to be reliant upon the stage of cell growth, in particular entry to stationary phase (Novo, et al., 2005), which depends on nutrient availability (Werner-Washburne, et al., 1996). Reserve compounds are known to enhance cell viability by conferring stress tolerance (Plourde-Owobi, et al., 2000). For instance, trehalose helps to maintain cell membranes and preserve proteins in their native state (Lucero, et al., 2000). The viability of C9 *ngr1* once all glucose was depleted was examined, and this strain was found to have very similar viabilities to the parent strain. This suggests that if there were a difference in cell size, it may not be due to a smaller store of reserve compounds. Yet it is also possible that cells taken from the end of fermentation were not under sufficient stress to enable the detection of such a difference.

When KP2 *gid7* was supplied with low concentrations of nitrogen in complex form, not only was fermentation time decreased to 92 % of the parent strain, but alike to C9 *ngr1*, the dry cell weight at the end of fermentation was also reduced. Again, no reduction in cell size was detectable with the methods utilised here. Genes influencing cell size have also been found to be involved in cell cycle progression. For instance, another of the *GID* genes, *GID8*, has been shown to be involved in promotion of START (Pathak, et al., 2004), and a double deletion of both *GID8* and *DCR2*, a potential phosphoesterase, is found to increase cell size by 10 – 15 %.

#### **5.4.5 The production of cellular metabolites by *ngr1Δ* and *gid7Δ* strains**

A small increase in the production of glycerol by both *ngr1Δ* and *gid7Δ* strains has been noted (up to a 17 % increase, Table 5.4 and Figure 5.7). After ethanol and carbon dioxide, glycerol is one of the major products of fermentative metabolism. It is of great interest to the wine industry as, although it does not have any aromatic properties, it can contribute to a desirable fullness and sweetness of wine. In particular, sweetness contributed by glycerol has a relatively low threshold of 5.2 g L<sup>-1</sup> (Noble and Bursick, 1984). Glycerol is produced during the regeneration of NAD<sup>+</sup> from NADH, a requirement of anabolic metabolism, particularly important during anaerobiosis, to equilibrate the intracellular oxidation-reduction (redox) potential. It is also known to be important in the balance of osmotic pressure when the cell is under stress (Taherzadeh, et al., 2002). Presumably the increase in glycerol produced when *NGR1* and/or *GID7* is deleted reflects that the redox balance of the cell has been altered. Interestingly, Remize et al. (1999) noted that a glycerol overproducing strain, produced by overexpressing *GPD1*, had a faster fermentation rate at stationary phase. They hypothesise that this could be due to an enhanced release of inorganic phosphate. The production of glycerol has also been related to the timing of nitrogen additions, for instance earlier additions translate to increased glycerol and also higher biomass (Beltran, et al., 2005).

Measurement of the major organic acids produced at the end of fermentation revealed that in seven of the sixteen conditions examined, mutant strains



produce more lactic acid than the wildtype, and in four of these conditions succinic acid was decreased. Production of organic acids during fermentation yields a net formation of NADH. This NADH, must be regenerated to NAD<sup>+</sup> to be available for glycolysis. During fermentation this is achieved when acetaldehyde is reduced to ethanol and, as stated above, by the production of glycerol. Reflecting this; in six of the seven conditions where the final concentration of lactic acid is increased, so has glycerol, albeit marginally. Most notably, C9 *ngr1* consistently produced 1.5 - 3.0 times more lactic acid than C9. In *S. cerevisiae* D-lactate is produced from methyl-glyoxal (2-oxopropanal) by two pathways. Firstly, by the Methyl-glyoxal pathway, which is thought to be a defence mechanism to protect the cellular contents from methyl-glyoxal, a toxic compound produced during glycolysis (Racker, 1951; Ponces Freire, et al., 2003). S-lactoyl-glutathione is produced from methyl glyoxal and glutathione, which is then converted to D-lactate by glyoxalases (glyoxyalase I; lactyl-glutathione lyase and glyoxylase II; hydroxyacylglutathione hydrolase) (Penninckx, et al., 1983). The second pathway, thought to be of lesser importance, occurs where methylglyoxal is reduced to lactaldehyde by NADPH-dependent methylglyoxal reductase and then NAD<sup>+</sup>-dependent lactaldehyde dehydrogenase oxidizes lactaldehyde to lactic acid (Maeta, et al., 2005). Methyl-glyoxal is produced during the triphosphate reaction by the β-elimination reaction from an enediolate phosphate intermediate (Richard, 1991) which is produced during the fermentation of glucose. Presumably accumulation of the substrates for this reaction could occur in *NGR1* mutants due to an increased flux through glycolysis. Recently, methyl-glyoxal has been proposed to function as a signal initiator, involved in the oxidative stress response via the high osmolarity glycerol (HOG)-mitogen-activated-protein (MAP) kinase (Maeta, et al., 2005). The down regulation of this pathway in *ngr1Δ* and *gid7Δ* strains may represent an example of how an HNE strain could sacrifice other pathways, allowing it an enhanced fermentation profile.

Succinate, is another of the major by-products of ethanol fermentation. Four of the sixteen conditions, resulted in decreased succinic acid production, and each of these also produced more lactic acid, yet only three of the four conditions

also decreased fermentation duration. As there is no clear link between these conditions, it is difficult to speculate what the mechanism is which has produced this effect. The majority of succinic acid formed during fermentation occurs via the reductive branch of the Tri-carboxylic acid pathway (TCA cycle) during fermentation (Camarasa, et al., 2003), except where glutamate is present in high concentrations. When nitrogen is supplied as ammonium, glutamate production during fermentation relies on the Retrograde genes (The Retrograde Response) together with early parts of the oxidative branch of the TCA cycle so as to re-supply mitochondria with oxaloacetate and acetyl-CoA. Intracellular ammonium positively regulates retrograde transcription, thus the enzymes that synthesize  $\alpha$ -ketoglutarate, a precursor of glutamate (Tate and Cooper, 2003), can be assimilated. A decrease in glutamate catabolism leads to  $\alpha$ -ketoglutarate being scarcely available to be oxidatively de-carboxylated to succinic acid via acetyl CoA (Albers, et al., 1998). Thus a decrease in succinic acid production in *ngr1Δ* and *gid7Δ* strains in these four conditions may suggest that less glutamate is available. It is recognised that glutamate was supplied in three of the four conditions, yet each of these conditions had only low concentrations ( $< 20 \text{ mg L}^{-1}$ ), that would have been depleted from the media in the early parts of fermentation (Jiranek, et al., 1995).

Succinic acid can also be formed by the GABA degradation pathway, yet the significance of this pathway during fermentation is unclear (Coleman, et al., 2001). Again dependent on glutamate, GABA (4-amino butyrate) can be produced from glutamate by a glutamate decarboxylase followed by transamination to form succinate semialdehyde and dehydrogenation to produce succinate. One purpose of GABA formation is thought to be temporary nitrogen storage; this mechanism may allow *GID7* and/or *NGR1* mutant strains to have access to more nitrogen from the central nitrogen pool. Otherwise, as above, glutamate may not be available to form GABA which would in turn lead to reduced succinic acid.

There are a number of other yeast metabolites not measured in this study that are influenced by nitrogen metabolism and are also important to the aroma and flavour of wine, for instance, higher alcohols, aldehydes and ketones. Clearly a

comprehensive analysis of the production of these types of compounds in these strains would be beneficial.

The deletion of *NGR1* and *GID7*, affects a number of other facets of fermentation, rather than just enhancing glucose catabolism, such as dry cell weight formation and metabolite production. However none of these has a strong correlation with the nitrogen efficient phenotype and thus they appear to be consequential effects. Because of the pleiotropic effects of these mutations and as glutamate availability is implicated in a number of the metabolite differences noted, it is thought that these genes probably affect a pathway involved in Central Nitrogen Metabolism. Further to this, the original isolation of *GID7* by Regelman and co-workers (2003) yielded other *GID* genes including *GID1*, also known as *VID30*. The affect of Vid30p in low ammonium has been shown to direct nitrogen metabolism towards glutamate formation. Perhaps *GID7* and/or *NGR1* may have a similar function.

## **Chapter 6 The effect of deletion of *NGR1* and *GID7* on the transcription of genes involved in Central Nitrogen Metabolism.**

### **6.1 Introduction**

Previous studies have demonstrated a strong link between assimilable nitrogen, fermentation rate and consequently, fermentation duration, when other nutrients are not limiting (Agenbach, 1977; Bely, et al., 1990a; Sablayrolles, 1996). The total nitrogen utilised and the timing of nitrogen uptake of *ngr1* $\Delta$  and *gid7* $\Delta$  strains was found to be very similar to the parent strain (Chapter 5). Thus it was hypothesised that *ngr1* $\Delta$  and *gid7* $\Delta$  strains could be using the available nitrogen in a modified way to enable enhanced glucose utilisation.

The core pathway for the utilisation of all non-preferred nitrogen sources is known as Central Nitrogen Metabolism (CNM). Nitrogen containing compounds are fed into the CNM to ultimately produce glutamate and glutamine (Cooper, 1982). The major pathway of glutamate production is normally through the action of NADPH-dependent glutamate dehydrogenase (NADPH-GDH), encoded by *GDH1* (Figure 1.2). NADPH-GDH catalyses the reaction of  $\alpha$ -ketoglutarate, from the TCA cycle, and ammonium to form glutamate. This reaction consumes NADPH and generates  $\text{NADP}^+$ . Glutamate can also be produced by the combination of glutamine synthetase, encoded by *GLN1*, and glutamate synthase, encoded by *GLT1* (the GOGAT pathway). Glutamine synthetase catalyses the formation of glutamine from glutamate, ammonium and ATP, and glutamate synthase catalyses the formation of glutamate from glutamine,  $\alpha$ -ketoglutarate and NADH. Thus the net reaction of the GOGAT pathway is the formation of glutamate from  $\alpha$ -ketoglutarate and ammonium, whilst NADH and ATP are converted to  $\text{NAD}^+$  and ADP.

Genes involved in CNM are mainly regulated at the transcriptional level. They all contain numerous upstream activating sequences affected by nitrogen ( $\text{UAS}_{\text{NTR}}$ ) amongst other regulatory sequences, which act in conjunction to execute their characteristic expression pattern. Accordingly, it is appropriate to

evaluate transcript quantities of genes involved in CNM as an indicator of the effect of *NGR1* or *GID7* deletions.

As stated previously, a large scale systematic search for protein interactions has shown that there are putative interactions of Gid7p with other Gid/Vid proteins, but also with Hxt7p. Hxt7p is a high affinity hexose transporter, thought to be important at the end of fermentation for maintenance of hexose transport (Luyten, et al., 2002; Perez, et al., 2005). Yet Hxt7p and an almost identical transporter Hxt6p have been previously shown to be rapidly degraded upon the depletion of nitrogen when in the presence of glucose (Krampe, et al., 1998; Krampe and Boles, 2002). The inconsistencies between these experiments may be a consequence of different strain backgrounds. The studies finding that *HXT6/7* were expressed and functional at the end of fermentation where nitrogen was limiting were performed in a wine yeast background (V5), and therefore are probably more relevant to this study. However, it highlights the potential complication of strain differences. Perhaps the absence of Gid7p prevents deactivation of Hxt7p, alike to its action on FBPase (Regelmann, et al., 2003), and is still able to maintain its glucose catabolic activity under conditions of nitrogen starvation.

To evaluate the effect of a deletion of *NGR1* or *GID7* upon the transcriptional activity of genes involved in central nitrogen metabolism (*GDH1*, *GDH2*, *GDH3*, *GLT1* and *GLN1*) and of one hexose transporter, *HXT7*, were evaluated by Quantitative Real-Time PCR.

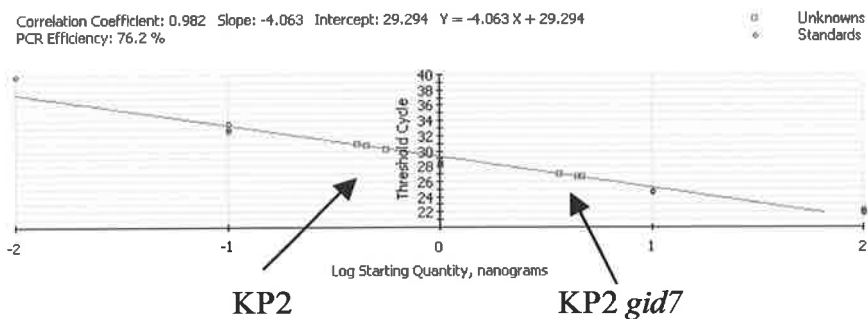
## 6.2 Results

### 6.2.1 Validation and controls used for Quantitative Real Time PCR (QRTPCR)

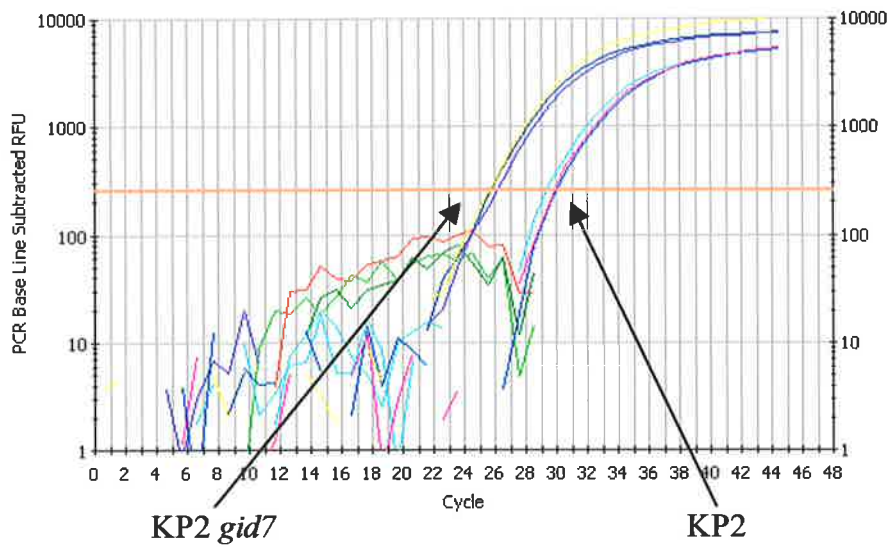
RNA was isolated from each of the triplicate fermentations of two separate experiments and analysed by QRTPCR in quadruplicate reactions. Primers were designed using the Qiagen custom probe design software (<https://customassays.qiagen.com/design/inputsequences.asp>) (Table 2.4). Amplicons were <150 bp so as to increase the probability of efficient amplification. QRTPCR was performed with the one step Quantitect Probe RT-PCR kit (Qiagen, #204443) spiked with SYBR green (Molecular Probes, #S7567) and analysed with a Bio-Rad icycler<sup>®</sup>. Standard curves were generated from calibrator RNA purified from the parent strain with primers from the gene of interest and also from *ACT1* (Figure 6.1). The amplification efficiencies of the target and reference genes were also analysed and taken into consideration (data not shown). The effect of a deletion of *NGR1* or *GID7* on the relative transcript abundance of genes of interest was determined by comparison of the Crossing Threshold ( $C_T$ ) values of QRTPCR of the mutant strain to the corresponding parent strain under the same conditions (Figure 6.2). A melt curve was also performed at the completion of each QRTPCR, so that the integrity of each generated fragment was confirmed to be of the same sequence and that there were no contaminating products present, in particular primer dimer (data not shown). Controls were included in every reaction, including a reaction lacking template to check for general contamination, and a reaction lacking reverse transcriptase to validate that QRTPCR products were not amplified from contaminating genomic DNA.

### 6.2.2 Quantification of the transcription from genes involved in Central Nitrogen Metabolism: The effect of deletion of *NGR1* or *GID7* from *KP2*.

Total RNA was isolated from a late stage of a nitrogen limited fermentation (75 mg FAN L<sup>-1</sup> as ammonium) when only 25 g L<sup>-1</sup> glucose remained (for example, 175 g L<sup>-1</sup>, or 87.5 % already catabolised). This correlated to a



**Figure 6.1** An example of one of the standard curves of Crossing Threshold ( $C_T$ ) values generated by QRT-PCR from reference RNA with QiaACT1 primers. Unknown  $C_T$  values were generated from RNA purified from triplicate fermentations (sampled near the end of fermentation, 87.5 % of glucose catabolised) of KP2 *gid7*, and KP2 with QiaGDH2 primers.



**Figure 6.2** The transcript abundance of *GDH2*, measured by fluorescence of SYBR green during QRT-PCR of RNA template from triplicate fermentations (sampled at the end of fermentation) of KP2 *gid7* (average  $C_T = 29.7 \pm 0.3$ ) or KP2 (average  $C_T = 25.7 \pm 0.3$ ). QRT-PCR controls, - template and - reverse transcriptase are included. The Crossing Threshold is indicated by the orange line



fermentation duration of around 192 hours for mutant strains, and around 212 hours for the parental strain, that is the mutant strain reached this final concentration in 90.5 % of the duration of the parent strain.

During the final stages of a nitrogen limited fermentation the absence of *NGR1* from KP2 caused a slight decrease in the expression of *GDH1* (to 70 +/- 4 % of the parent strain,  $p = 0.041$ ), and an increase in *GLT1* (to 250 +/- 50 % of the parent strain,  $p = 0.002$ ), *GLN1* (to 157 +/- 31% of the parent strain) and a very small increase in *HXT7* (123 +/- 11.5 %,  $p = 0.025$ ; Figure 6.3). Significant differences in transcript abundance were not detected for *GDH2* or *GDH3*.

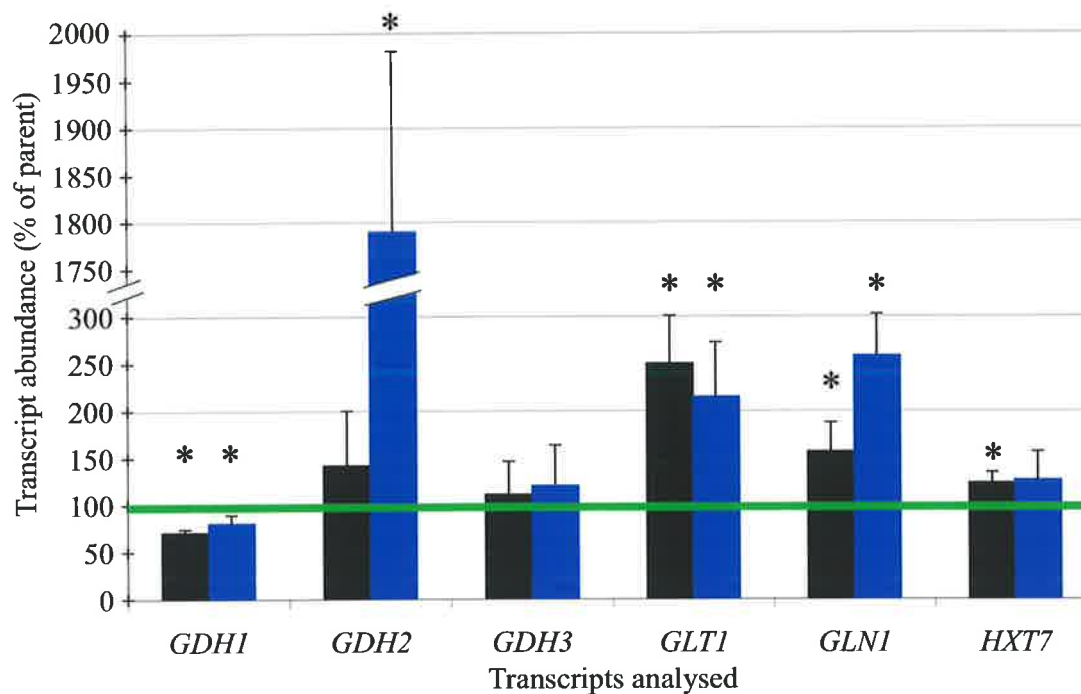
Deletion of *GID7* from KP2 caused a similar shift in the transcription of genes of CNM. Transcription from *GLT1* and *GLN1* increased (to 215 +/- 58 %,  $p = 0.008$  and 258 +/- 44,  $p = 0.001$ , of the parent strain respectively). *GDH1* was decreased (to 80 +/- 9 % of the parent strain,  $p = 0.005$ ) and *GDH2* was dramatically up-regulated (to 1790 +/- 191 % of the parent strain,  $p = 0.001$ ). There were no significant differences observed for *GDH3* or *HXT7*.

### **6.2.3 *NGR1* and *GID7* expression**

Analysis of the transcription of *NGR1* and *GID7* was attempted from KP2 cells grown in YEPD, minimal media with high or low ammonium, or proline or glutamine and from CDGJM during early and late stages of fermentation. Yet, unfortunately transcripts from either gene were undetectable in all of these media. Positive PCR controls (of cloned and purified DNA) were performed with success (data not shown), so it is possible, yet improbable, that the expression of these genes in these media is lower than that detectable within this system. Time restraints prevented further attempts being made.

## **6.2 Conclusions**

- a) In the later stages of fermentation by KP2 *ngr1*, the transcript abundance of *GDH1* is less than the parent strain, whereas the transcript abundance of *GLT1*, *GLN1* and *HXT7* is increased (albeit marginally).



**Figure 6.3** The abundance of six transcripts detected by Quantitative Real time PCR near the end of fermentation (87.5 % of glucose catabolised) from KP2 *ngr1* (black bars) or KP2 *gid7* (blue bars), represented as a percentage of that found from the parent strain KP2. The initial fermentation medium was CDGJM with 75 mg FAN L<sup>-1</sup> supplied as ammonia. \*Values that are significantly different to the parent ie  $p < 0.05$  (Student's t-test). The y axis is broken to accommodate the high values for *GDH2* expression. The expression level in the parent (100 %) is highlighted in green

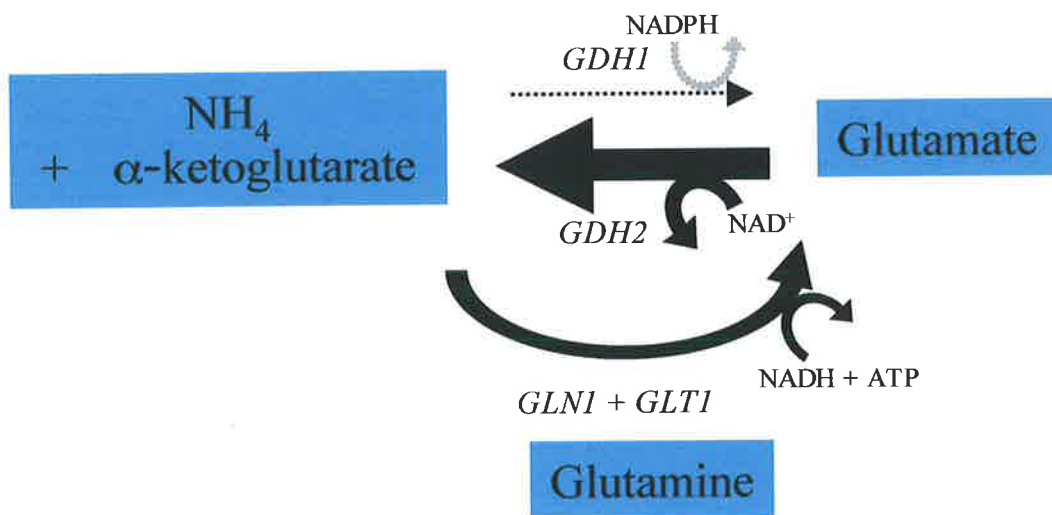
- b) In the later stages of fermentation by KP2 *gid7* the abundance of the *GDH1* transcript is decreased, whereas *GLT1*, *GLN1* and *GDH2* are increased, the *GDH2* transcript level being almost 18-fold higher than that in the parent strain.

### **6.3 Discussion**

Deletion of either *NGR1* or *GID7* affects the expression of genes involved in Central Nitrogen Metabolism (CNM) during fermentation. The transcriptional abundance of *GDH1*, *GLT1* and *GLN1* in both KP2 *ngr1* and KP2 *gid7* is partially altered, whereas the expression of *GDH2* is dramatically increased when *GID7* is deleted from KP2, being almost 18-fold more than the parent strain (Figure 6.3). This distorted expression of CNM genes is expected to translate to a remodeling of enzyme quantities and thus a re-distribution of the core nitrogen-containing compounds, namely glutamate, glutamine,  $\alpha$ -ketoglutarate and ammonium, as summarised in Figure 6.4. Cellular nutrient status is thought to be regulated partly through the relative abundance of these core compounds, thus the putative change of this status may result in the increased nitrogen efficiency of *ngr1* $\Delta$  and *gid7* $\Delta$  strains. Evaluation of expression profiles and at other stages of fermentation and in C9 may provide further insight into the mechanism.

The main function of NADPH-dependent glutamate dehydrogenase (NADPH-GDH, encoded by *GDH1*) is to convert ammonium to glutamate when cells are supplied with ammonium as a nitrogen source. Down regulation of this enzyme may result in NADPH being more readily available for anabolic reactions such as the synthesis of biomass and lipids, which may confer greater stress resistance and consequently prolonged glucose catabolism toward the end of fermentation. Gene expression was analysed late in fermentation, and at this stage all nitrogen was depleted from the medium, thus the action of incorporation of ammonium by NADPH-GDH or the GOGAT pathway would be due to a re-shuffling of the pre-existing nitrogen compounds.

In the presence of glucose, the expression of *GDH1* is controlled by Gln3p (Daugherty, et al., 1993), the HAP complex, a CCAAT box binding factor,



**Figure 6.4** Schematic diagram representing the alteration of the expression of genes (shown in bold italics), the conversion of compounds in CNM their products and the redox co-factors utilised during these reactions. For simplicity the net redox co-factors utilised by the GOGAT pathway (*GLN1 + GLT1*) are shown. This represents a time point late in fermentation (CDGJM with 75 mg FAN L<sup>-1</sup>) as shown by Real time PCR in KP2 *ngr1* and KP2 *gid7*. Thicker arrows represent an increase in transcript abundance, dotted arrows a decrease, compared to the parent strain. *GDH2* was only significantly up-regulated in KP2 *gid7*.

also involved in carbon metabolism (Dang, et al., 1996) and Leu3p, a transcriptional regulator of branched-chain amino acids (Hu, et al., 1995). Apart from the results shown here there is no direct evidence implicating either *NGR1* or *GID7* in the regulation of this gene.

The increase of *GDH2* expression in *gid7Δ* cells most likely leads to an increase in NAD-GDH enzyme activity, as *GDH2* has been previously shown to be mainly regulated at the transcriptional level (ter Schure, et al., 1995b). The predominant function of this enzyme is to release ammonium and  $\alpha$ -ketoglutarate from glutamate. NAD-GDH is functional when cells are grown on glutamate or nitrogen sources that are glutamate precursors, thereby making ammonium available for the formation of glutamine. Expression of *GDH2* is repressed when in the presence of ammonium or glutamine. The reverse reaction of NAD-GDH also occurs, particularly when over-expressed, and this activity has been suggested to be dependent on the concentration of  $\alpha$ -ketoglutarate (Miller and Magasanik, 1990; Bro, et al., 2004). The putative increase in NAD-GDH activity would lead to an increase in the concentration of intracellular ammonium, presumably resulting in signaling an altered nitrogen metabolism (ter Schure, et al., 1995b). Cells are thought to be able to detect the intracellular concentration of ammonium and glutamine, possibly in a similar fashion to the bacterial nitrogen sensor, PII (Ninfa and Atkinson, 2000) and the concentrations of these metabolites presumably serve as primary cues that are capable of signaling the nutrient status of the cell (ter Schure, et al., 1998). The putative release of ammonium by this overactive NAD-GDH in *gid7Δ* cells could have delayed the onset of the cellular stress response to limiting nutrients, thus allowing a prolonged fermentation duration. As discussed in Chapter 5, *ngr1Δ* and *gid7Δ* strains produce slightly increased glycerol when nitrogen is limiting and this is most likely due to increased  $\text{NAD}^+$  utilisation by an over-active NAD-GDH.

The interconversion of pyridine nucleotide co-factors,  $\text{NAD}^+$ , NADH,  $\text{NADP}^+$  and NADPH is described as redox metabolism (as reviewed by van Dijken and Scheffers, 1986). Maintenance of cellular redox balance in both the cytosol

and mitochondria is vital for cell health. During fermentation  $\text{NAD}^+$ , an electron acceptor, is reduced to NADH in most anabolic and catabolic pathways.  $\text{NAD}^+$  is mainly regenerated during the conversion of pyruvate to ethanol. Whereas, most anabolic reductive reactions require NADPH.

Recently a link between Ngr1p and NADH was discovered. Originally it was believed mitochondria were impermeable to nicotinamide adenine dinucleotides, and thus redox states were only connected by the shuttling of oxidized or reduced equivalents (van Dijken and Scheffers, 1986). It is now suggested that NADH is able to move through the outer mitochondrial membrane through the porin channel (Averet, et al., 2002), and this may serve to regulate the distribution of NADH. Mitochondrial porin mRNA has been shown to be de-stabilised by Ngr1p (Buu, et al., 2004), thus in *ngr1* mutants, perhaps mitochondrial porins are in greater abundance, and regulation due to the compartmentalisation of NADH in the mitochondria has been lost or altered.

The complex transcriptional regulation of *GDH2* is influenced by nitrogen sources. There have been five regions shown to be important for a normal expression profile, two upstream activating regions ( $\text{UAS}_N$  and  $\text{UAS}_G$ ) and three upstream repression regions (URS) (Miller and Magasanik, 1991). Gln3p binds to  $\text{UAS}_N$  and is repressed by glutamine, and  $\text{UAS}_G$  appears to be induced by glutamate, yet the activating factor(s) are unknown. Miller and Magasanik (1991) reported that at least two of the three URS were required to repress expression when cells were grown with ammonium. They showed that *GLN1* has a co-ordinated expression to *GDH2*, except when grown in the presence of ammonium, where *GDH2* is repressed, but *GLN1* is not. Comparison of the *GLN1* and *GDH2* promoter regions revealed that *GLN1* lacked the URS of *GDH2*. In this study, the increase in transcript abundance of *GDH2* is more pronounced than that of *GLN1* in *gid7* $\Delta$  cells, thus it is probable that the loss of *GID7* has caused a disruption of the negative regulation mediated by URSs in *GDH2*. *GID7* also appears to negatively regulate transcription of *GLN1* and *GLT1*, as there is approximately twice as much transcript of these genes than in the parent strain in *gid7* $\Delta$  cells. However, as the net reaction of glutamine

synthetase and glutamate synthase (the GOGAT pathway) is the production of glutamate from  $\alpha$ -ketoglutarate and ammonium (Magasanik and Kaiser, 2002), whilst utilising NADH and ATP as co-factors, the increase in expression of *GLT1* and *GLN1* may simply be a response to the overexpression of *GDH2*, to utilise released ammonium, replace the depleted glutamate pool, and possibly recycle some of the excess NADH. This scenario has also been suggested in relation to restoration of growth of phosphoglucose isomerase mutants by overexpression of *GDH2* so as to restore redox imbalance (Boles, et al., 1993).

An increase in the GOGAT pathway results in increased glutamate production via this pathway, resulting in a net increase in the use of NADH and ATP, as opposed to the use of NADPH by NADPH-GDH. The intracellular concentration of ATP has been shown to be inversely proportional to the cells glycolytic flux (Larsson, et al., 1997). Thus an enhanced activity of glutamine synthetase in these mutants may stimulate glucose catabolism so as to replace depleted ATP, and perhaps also NADH (Flores-Samaniego, et al., 1993; Larsson, et al., 1997). Alternatively an enhanced glucose catabolism could be stimulating the GOGAT pathway due to more available ATP. NADPH is chiefly involved in anabolic processes such as the assimilation of sugars into biomass (van Dijken and Scheffers, 1986). The two important sources of NADPH during anaerobic growth are from the hexose monophosphate pathway and the NADP<sup>+</sup> dependent isocitrate dehydrogenase, which converts isocitrate to  $\alpha$ -ketoglutarate, which is then consumed by pathways such as lipid and amino acid synthesis (Briunenbergh, et al., 1983). The *ngr1* $\Delta$  and *gid7* $\Delta$  strains may produce or utilise an altered amount of NADPH. For instance, *ngr1* $\Delta$  and *gid7* $\Delta$  cells may have a reduced availability of NADPH, which may explain the down-regulation of NADPH-GDH and also the reduced biomass observed in *ngr1* $\Delta$  mutants (Chapter 4).

The increased expression of *GDH2*, *GLT1* and *GLN1* in *ngr1* $\Delta$  and *gid7* $\Delta$  cells may also lead to decreased glutamine. The intracellular concentration of glutamine has been suggested to influence the expression of retrograde (RTG) genes via the action of TOR proteins (Crespo, et al., 2002) and thus affect the

activity of the TCA cycle. If this is indeed the case, the decreased intracellular glutamine may explain why in these mutant strains, in some circumstances, there is less succinic acid produced (Chapter 5).

It is quite plausible that Gid7p may not elicit its effect on these genes directly, as there is no evidence of it having DNA binding ability, however Gid7p may bind to other protein(s), via its WD40 domains, to retard transcription. Conversely, Gid7p may affect mRNA stability, in a similar way to *NGR1*, particularly as other WD40-containing proteins, such as Ski8p (Madrona and Wilson, 2004), have also been implicated in the control of mRNA stability.

Ngr1p could function as a transcription factor upon genes involved in CNM, particularly as it has been shown to possess putative nucleic acid binding abilities. Isolation of Ngr1p by Ikeda and co-workers (1996) revealed its ability to bind to the autonomous replicating sequence ARS, for example, ATTTTATGTTT. Interestingly a number of T/G rich sequences are located in the promoters of *GLT1* and *GLN1*. For example the sequence TTGTATTT is located at in the promoter of *GLT1* (-22 → -29), which is directly adjacent to a GATA binding sequence. This is evidence that Ngr1p may act in concert with GATA factors to regulate the expression of *GLT1*. Alternatively Ngr1p may bind to these sequences post-transcriptionally to regulate the stability of the *GLT1* and *GLN1* mRNA, in a similar way to that of the mitochondrial porin mRNA.

Another of the *GID* genes, (*GID1* / *VID30*), is also involved in central nitrogen metabolism, directing nitrogen metabolism towards glutamate formation, particularly in conditions of low ammonium (van der Merwe, et al., 2001). Gid7p may have a similar function to Gid1p, which suggests that the family of *GID* proteins may also be functionally related during fermentation, as they are during respiration. A large scale systematic search for protein interactions has shown that there are putative interactions of Gid7p with other Gid/Vid proteins, but also with Hxt7p (Ho, et al., 2002). As mentioned previously, Hxt7p is a high-affinity hexose transporter, thought to be important at the end of fermentation for maintenance of hexose transport (Perez, et al., 2005) and



known to be rapidly degraded upon the depletion of nitrogen (Krampe and Boles, 2002). Hypothetically, the absence of Gid7p could prevent deactivation of Hxt7p, alike to its role in the regulation of FBPase (Regelmann, et al., 2003) and thus a *gid7Δ* strain could still maintain its glucose catabolic activity under conditions of nitrogen starvation. However, there was very little difference in the expression of *HXT7* in KP2 *gid7Δ* or KP2 *ngr1Δ* strains late in fermentation. These results are somewhat inconclusive. If Gid7p does affect Hxt7p in a similar fashion as it does FBPase, then the longevity of transcription, together with the integrity and location of the Hxt7p transporter needs to be evaluated during fermentation.

Gid7p is predicted to contain five WD40 repeats (predicted by SMART, the Simple Modular Architecture Research Tool). The WD40 domain is thought to mediate protein-protein interactions, and is found in many regulatory proteins such as Ste4p, encoding the  $\beta$  subunit of the heterotrimeric G-protein GTPase (Fong, et al., 1986). This domain of Gid7p may facilitate interaction with other, if not multiple proteins. Interestingly, another WD40 domain containing protein, Ski8p, is essential for the functioning of 3' to 5' mRNA decay mediated by the exosome (Madrona and Wilson, 2004). Given this, it is possible that Gid7p alike to Ngr1p could regulate CNM genes by affecting mRNA stability.

Determination of gene expression in response to environmental conditions is a valuable tool in the determination of gene function. Attempts were made to evaluate the normal expression of *NGR1* and *GID7* when cells were grown in different carbon and nitrogen sources and during fermentation. Unfortunately there was no expression detected with this system. Presumably, the primers selected or the RNA preparations were inadequate for detection of these transcripts, and further studies are required to evaluate the transcriptional profiles of these genes.

## **Chapter 7 General Discussion and Future Directions**

The catabolism of essentially all sugars from an industrial wine fermentation is vital to the successful production of a 'dry' wine within stylistic specifications (Iland and Gago, 2002). Problem fermentations, which often arise due to an insufficient supply of yeast assimilable nitrogen (Ingledeew and Kunkee, 1985; Bely, et al., 1990a; Stratford and Rose, 1985; Pierce, 1987; Alexandre and Charpentier, 1998) are typically addressed through vineyard or winery practices that increase the amount of available nitrogen. The exploitation of yeast strains that are more nitrogen efficient (Jiranek, et al., 1991; 1995a; Julien, et al., 2000; Gardner, et al., 2002) is becoming a more common solution.

We sought to increase the understanding of the basis for differences in nitrogen efficiency as a prelude to manipulating this attribute in industrial strains for greater fermentation reliability. A haploid wine yeast derivative was constructed to enable relatively straight-forward genetic manipulations in an appropriate background (Chapter 3). A transposon-based system and multi-well plate format fermentations were successfully used to generate and identify mutants whose capacity to catabolise sugar had been altered (Chapter 4). Other techniques such as the continuous culture of strains in conditions of increased stress could have been employed to achieve a similar outcome, yet the method used was considered as one that allowed direct identification of affected genes. Mutants with increased or decreased nitrogen efficiency were both considered potentially valuable to this study. Nevertheless, given that most mutations probably resulted from a loss of function in the target gene (Seifert, et al., 1986; Daignan-Fornier and Bolotin-Fukuhara, 1988), those yielding an increase in nitrogen efficiency were considered more likely to be directly linked with changes to nitrogen metabolism.

Deletants of *NGR1* and / or *GID7* in a laboratory and a wine strain derivative generally yielded superior fermentation performance in media of solely ammonium or mixed nitrogen sources (Chapter 5). Quantitation of the nitrogen

requirement of deletants revealed that unlike previously characterised nitrogen efficient wine yeasts (Jiranek, et al., 1991; Jiranek, et al., 1995a), deletion of *GID7* or *NGR1*, typically did not reduce the amount of nitrogen removed from the medium. Fermentations of nitrogen-deficient media by such strains might therefore be expected to become nitrogen depleted, even though fermentation should progress and complete ahead of the wild type. The usefulness of these strains as a means of effecting fermentation of nitrogen-deficient juices remains high, although the potential for development of other problems, such as liberation of hydrogen sulfide (Jiranek, et al., 1995b), needs to be assessed under such conditions.

The second criterion for evaluation of deletants was their pattern of metabolite production. As outlined in Chapter 5 there were some minor differences in metabolite production such as glycerol, succinic and lactic acid production, however the significance of each of these on the final aroma of wine still remains to be evaluated. It is also important to evaluate other sensorially important compounds, particularly those affected by nitrogen metabolism, such as higher alcohols. Also, a comprehensive check for the introduction of any detrimental changes, such as over-production of undesirable metabolites (de Barros Lopes, et al., 2000), is yet to be carried out. A metabolomics approach would be ideal for such future work. Examination of the changes in metabolite production of these mutants has given some insight into the function of *Ngr1p* and *Gid7p*. For example, an increased production of glycerol may indicate an alteration in the redox balance of deletants, an increase in lactic acid the involvement of the methyl-glyoxal pathway and decreased succinic acid production, suggests a lower intracellular concentration of glutamate.

To determine precisely how deletion of *NGR1* or *GID7* increases nitrogen efficiency will obviously require further investigation. No clear explanations of a link to the high nitrogen efficiency phenotype determined here can be derived from reports to date on the characterisation of these genes. *NGR1* has already been shown to be involved with growth, high sugar stress and stability of mitochondrial porin mRNA (Lee and Moss, 1993; Ikeda, et al., 1996; Akada, et al., 1997; Erasmus, et al., 2003; Buu, et al., 2004) The presence of RNA

binding domains highlights its potential importance in processes involving translation such as RNA recruitment, similar to its human homologues (*TIA-1* and *TIAR*), or RNA stability. *GID7* is involved in catabolite degradation of fructose-1,6-phosphatase (Regelmann, et al., 2003), yet as shown here it is likely to have other functions. For instance, systematic searches for protein interactions revealed a putative interaction of Gid7p with the high affinity hexose transporter Hxt7p (Ho, et al., 2002), possibly linking *GID7* with sugar transport and hence fermentation.

As *NGR1* and *GID7* affect fermentation dynamics in media of both ammonium alone and mixed nitrogen sources it was hypothesised that these genes may affect Central Nitrogen Metabolism. Indeed this was the case, in both deletants the transcript abundance of *GDH1* was decreased and *GLT1* and *GLN1* were increased. *GDH2* transcript levels were also dramatically increased in KP2 *gid7*. Whether these variations in transcript abundance translate to an altered corresponding protein profile and remodeling of Central Nitrogen Metabolism remains to be proven. A remodeling of these pathways could certainly alter the use and re-cycling of redox intermediates such as NADH, which presumably would change metabolite production, such as glycerol, lactic and succinic acid as seen here. It is certainly also of interest to determine whether other pathways have also been affected by the deletion of these genes and this is most appropriately carried out with a global analysis of the genome and/or proteome utilising such techniques as DNA microarray and two dimensional SDS polyacrylamide gel electrophoresis.

Regardless of how deletion of *NGR1* and *GID7* enhances nitrogen efficiency during fermentation, these findings provide new strategies for the construction or selection of optimised strains with greater fermentation reliability. While the superior nitrogen efficiency of the double deletion strains is attractive, it currently seems inapplicable for producing improved wine yeasts, at least in the C9 derivative of the wine strain, L-2056.

With the current move towards a reduction in the use of additives for both food beverage manufacturing, the use of nitrogen additions as a preventative

measure for stuck fermentation is becoming less desirable. The use of nitrogen efficient strains is fast becoming an alternative approach. This study has advanced the understanding of what makes a particular yeast nitrogen efficient, and how indeed to construct them. In spite of this, there are still many issues surrounding the use of genetically engineered organisms in food and beverage production and thus the use of such strains may be limited. However, the occurrence of natural variation in nitrogen efficiency between existing yeast, together with the understanding of the mechanisms behind such traits will certainly aid the identification or development of natural variants in this field.

## Appendix I Solutions (additional to those outlined in text)

### Media

#### Yeast Minimal Media

|                             |                        |
|-----------------------------|------------------------|
| <i>D-glucose</i>            | 2.0 g L <sup>-1</sup>  |
| <i>YNB salts</i>            |                        |
| Potassium phosphate         | 1.0 g L <sup>-1</sup>  |
| Magnesium phosphate         | 0.5 g L <sup>-1</sup>  |
| Sodium chloride             | 0.1 g L <sup>-1</sup>  |
| Calcium chloride            | 0.1 g L <sup>-1</sup>  |
| <i>YNB trace components</i> |                        |
| Boric acid                  | 500 µg L <sup>-1</sup> |
| Cupric sulphate             | 40 µg L <sup>-1</sup>  |
| Potassium iodide            | 100 µg L <sup>-1</sup> |
| Ferric chloride             | 200 µg L <sup>-1</sup> |
| Manganese sulfate           | 400 µg L <sup>-1</sup> |
| Sodium molybdate            | 200 µg L <sup>-1</sup> |
| Zinc sulfate                | 400 µg L <sup>-1</sup> |
| <i>Ammonium sulphate</i>    | 5 g L <sup>-1</sup>    |
| <i>Vitamins</i>             |                        |
| Calcium pantothenate        | 400 µg L <sup>-1</sup> |
| Nicotinic acid              | 400 µg L <sup>-1</sup> |
| Pyridoxine hydrochloride    | 400 µg L <sup>-1</sup> |
| Thiamine hydrochloride      | 400 µg L <sup>-1</sup> |
| ρ-Amino benzoic acid        | 200 µg L <sup>-1</sup> |
| Riboflavin                  | 200 µg L <sup>-1</sup> |
| Folic acid                  | 2 µg L <sup>-1</sup>   |
| Biotin                      | 2 µg L <sup>-1</sup>   |

#### Chemically Defined Grape Juice Medium

|                 |   |                                |
|-----------------|---|--------------------------------|
| <i>Carbon</i>   | D-Glucose   | 200 g L <sup>-1</sup>          |
| <i>Nitrogen</i> |   |                                |
| -Ammonium       | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>   | 0.354 – 3.54 g L <sup>-1</sup> |
| -Amino acid mix | (a total of 785 mg N L <sup>-1</sup> , media varied between 78.5 – 785 mg N L <sup>-1</sup> ) |                                |
|                 | Alanine   | 100 mg L <sup>-1</sup>         |
|                 | Arginine  | 750 mg L <sup>-1</sup>         |
|                 | Asparagine  | 150 mg L <sup>-1</sup>         |
|                 | Aspartic acid   | 350 mg L <sup>-1</sup>         |
|                 | Glutamic acid   | 500 mg L <sup>-1</sup>         |
|                 | Glutamine   | 200 mg L <sup>-1</sup>         |
|                 | Glycine   | 50 mg L <sup>-1</sup>          |
|                 | Histidine   | 150 mg L <sup>-1</sup>         |
|                 | Isoleucine  | 200 mg L <sup>-1</sup>         |
|                 | Leucine   | 300 mg L <sup>-1</sup>         |
|                 | Lysine  | 250 mg L <sup>-1</sup>         |
|                 | Methionine  | 150 mg L <sup>-1</sup>         |

|                       |                                |                          |
|-----------------------|--------------------------------|--------------------------|
|                       | Phenylalanine                  | 150 mg L <sup>-1</sup>   |
|                       | Proline                        | 500 mg L <sup>-1</sup>   |
|                       | Serine                         | 400 mg L <sup>-1</sup>   |
|                       | Threonine                      | 350 mg L <sup>-1</sup>   |
|                       | Tryptophan                     | 100 mg L <sup>-1</sup>   |
|                       | Tyrosine                       | 20 mg L <sup>-1</sup>    |
|                       | Valine                         | 200 mg L <sup>-1</sup>   |
| <i>Salts</i>          |                                |                          |
|                       | Potassium sodium tartrate      | 2.5 g L <sup>-1</sup>    |
|                       | L-malic acid                   | 3.0 g L <sup>-1</sup>    |
|                       | Potassium phosphate, dibasic   | 1.14 g L <sup>-1</sup>   |
|                       | Magnesium sulphate             | 1.23 g L <sup>-1</sup>   |
|                       | Calcium chloride               | 0.44 g L <sup>-1</sup>   |
| <i>Trace Minerals</i> |                                |                          |
|                       | Manganese chloride monohydrate | 189.2 µg L <sup>-1</sup> |
|                       | Zinc chloride                  | 135.5 µg L <sup>-1</sup> |
|                       | Ferric chloride                | 32.0 µg L <sup>-1</sup>  |
|                       | Copper chloride                | 13.6 µg L <sup>-1</sup>  |
|                       | Boric acid                     | 5.7 µg L <sup>-1</sup>   |
|                       | Cobaltous nitrate hexahydrate  | 29.1 µg L <sup>-1</sup>  |
|                       | Sodium molybdate dihydrate     | 24.2 µg L <sup>-1</sup>  |
|                       | Potassium iodide               | 10.8 µg L <sup>-1</sup>  |
| <i>Vitamins</i>       |                                |                          |
|                       | Myo-inositol                   | 100 mg L <sup>-1</sup>   |
|                       | Pyridoxide-HCl                 | 2 mg L <sup>-1</sup>     |
|                       | Nicotinic acid                 | 2 mg L <sup>-1</sup>     |
|                       | Calcium pantothenate           | 1 mg L <sup>-1</sup>     |
|                       | Thiamine-HCl                   | 0.5 mg L <sup>-1</sup>   |
|                       | p-amino benzoic acid           | 0.2 mg L <sup>-1</sup>   |
|                       | Ribovlavin                     | 0.2 mg L <sup>-1</sup>   |
|                       | Biotin                         | 0.125 mg L <sup>-1</sup> |
|                       | Folic acid                     | 0.2 mg L <sup>-1</sup>   |

### ***CDGJM Starter Medium***

Starter cultures were grown in a medium identical to CDGJM, except that it contained only 100 g L<sup>-1</sup> D-glucose and had the addition of 2 mL of an anaerobic growth factor mix (5 g L<sup>-1</sup> ergosterol and 25 % Tween-20 were dissolved in hot 100% ethanol).

**Miscellaneous Buffers**

**TE Buffer**

100 mM Tris base (pH 7.4 or pH 8.0, adjusted with hydrochloric acid)  
10 mM EDTA (pH 8.0)  
pH either 7.4 or 8.0

**PBS**

0.2 g L<sup>-1</sup> KCl  
8 g L<sup>-1</sup> NaCl  
0.2 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>  
1.15 g L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>

**TAE Running Buffer**

4.84 g L<sup>-1</sup> Tris Base (Sigma 7-9)  
1.142 mL glacial acetic acid  
1 mM EDTA (pH 8.0)

TAE agarose gels were made in TAE Running Buffer with the appropriate addition of Agarose 1 (Amresco), typically 0.8-2.0 %.



**Appendix II      Papers arising from this study**

**Gardner, J. M., McBryde, C. M., Vystavelova, A., de Barros Lopes, M., Jiranek, V.** (2005). Identification of genes affecting glucose catabolism in nitrogen-limited fermentation. *FEMS Yeast Research* **5**, 791-800.

**Walker, M. E., Gardner, J. M., Vystavelova, A., McBryde, C., de Barros Lopes, M. and Jiranek, V.** (2003). Application of the reuseable, *KanMX* selectable marker to industrial yeast: construction and evaluation of heterothallic wine strains of *Saccharomyces cerevisiae*, possessing minimal foreign DNA sequences. *FEMS Yeast Research* **4**, 339-347.

## Identification of genes affecting glucose catabolism in nitrogen-limited fermentation

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Received 5 December 2004; received in revised form 19 January 2005; accepted 16 February 2005

First published online 16 April 2005

### Abstract

In recognition of the importance of assimilable nitrogen in the successful completion of several fermentation processes, we have sought to develop yeast strains that utilise this typically limited nutrient group more efficiently. With the aid of transposon mutagenesis together with a high-throughput method for analysis of multiple fermentations, we have identified 'nitrogen-efficient' mutants that catabolise more sugar for a given amount of nitrogen utilised. In this way we have identified two genes, *NGRI* and *GID7*, whose disruption leads to an enhanced catabolism of sugar in an industrial strain and/or a laboratory strain, during growth in a chemically defined grape juice medium with limiting nitrogen. Deletion of *NGRI* or *GID7* also resulted in minor changes in metabolites produced, and biomass yield, measured as dry weight, was also decreased in *NGRI* mutant strains.  
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**Keywords:** Assimilable nitrogen; Wine; Transposon mutagenesis; Yeast; Fermentation; Industrial biotechnology

### 1. Introduction

The natural substrates for many fermented beverages, including wine, are frequently deficient in assimilable nitrogen. This fact is recognised to be a common cause of fermentation difficulties [1–5], and in turn the reason for the widespread and routine use of nitrogen supplements. Nevertheless, supplementation is not always successful. The timing of additions can be critical [2] and over-addition may increase the risk of microbial instability, that is increase the risk of contaminating micro-

bial growth post-fermentation, of dramatic changes in the sensory profile, and of formation of carcinogens and biogenic amines [6–9]. Moreover consumers are calling for fewer additives to foods and beverages. An ideal solution therefore is to enable the inoculated yeast to more efficiently exploit the naturally occurring assimilable nitrogen [10]. This approach would both facilitate reliable completion of fermentation and eliminate or minimise the need for additives.

Wine yeast strains are known to differ in the efficiency with which they exploit nitrogen sources [11,12]. As a consequence, so called 'nitrogen-efficient' strains may offer greater fermentation reliability [12–14]. We sought to identify genes that could be deleted to improve nitrogen efficiency as a prelude to improving this attribute in industrial strains with otherwise desirable properties. A transposon mutagenesis system was used together

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with a high-throughput, micro-fermentation method for mutant evaluation. Mutants displaying improved nitrogen efficiency were further characterised in shake-flask fermentations and their affected genes identified by PCR amplification and determination of sequences adjacent to the transposon insert. Subsequent investigations were undertaken with disruptants in both laboratory and wine yeast backgrounds.

## 2. Materials and methods

### 2.1. Yeast strains and culture conditions

The *Saccharomyces cerevisiae* strains W303, C9 and their derivatives (Table 1) were used for this study. The laboratory strain W303 was selected as it is able to ferment chemically defined grape juice medium (CDGJM) [15] containing sugar concentrations alike to those found in typical grape must (approximately 200 g l<sup>-1</sup>). Except where indicated in Table 1, all strains were derived from the prototrophic strains KP2 and KP3, which were produced by mating W303 with P49 [16] followed by five back-crosses with W303. Strain C9 was produced in this laboratory [17] and is a haploid derivative of the common industrial wine yeast strain, L-2056 (Lallemand). Yeast deletion strains 3352 (BY4741 *ngr1::KANMX4*) and 3446 (BY4741 *gid7::KANMX4*), were acquired from the *S. cerevisiae* Deletion Project, [http://www-sequence.stanford.edu/group/yeast\\_deletion\\_project/deletions3.html](http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html) (Invitrogen, Carlsbad, CA).

Yeast were routinely grown in complete (YPD: 1% yeast extract, 2% Bactopeptone and 2% glucose) or minimal [18] medium, with an addition of 2% Bacto-agar where necessary. Experimental cultures were grown in

CDGJM [15]. Where appropriate, low to moderate concentrations of total nitrogen (typically 75–100 mg FAN l<sup>-1</sup> as either amino acids and ammonium, or ammonium alone) were present in CDGJM to simulate a limiting-nitrogen environment. Potassium acetate medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 2% Bacto-agar) was utilised for yeast sporulation before spore dissection and mating, carried out as described [19]. Yeast transformation was performed following lithium acetate treatment [20], and positive transformants were selected on YPD-geneticin (400 µg ml<sup>-1</sup>).

### 2.2. Construction of JMG2 and JMG3

JMG2 was constructed by deletion of *URA3* from KP2 via the *KANMX* gene replacement strategy [21]. A PCR product corresponding to the *KANMX4* module flanked with 59 and 60 bp of *URA3* sequences (+653 → +712 and +102 → +160) was amplified from pBS418 with primers *ura3KANF* and *ura3KANR* (Table 2). PCR was performed in 1X Dynazyme EXT buffer with 0.5 mM dNTPs, 0.5 mM MgCl<sub>2</sub> and 1 U of Taq (Sigma, St. Louis, MO). Cycling conditions were as follows: 30 cycles of 94 °C, 30 s; 52 °C, 1 min; 72 °C, 2 min. KP2 was transformed with this PCR product and positive transformants were selected on YPD-geneticin (400 µg ml<sup>-1</sup>). Deletion of *URA3* in JMG2 was indicated by non-growth on minimal medium and confirmed by PCR with primers *URA3F1* and *URA3R*. PCR was performed in 1X Dynazyme EXT buffer with 0.5 mM dNTPs, 1 mM MgCl<sub>2</sub> and 1 U of Dynazyme EXT. Cycling conditions were as follows: 30 cycles of 94 °C, 30 s; 62 °C, 1 min; 72 °C, 3 min. Strain JMG2 was crossed with KP3, diploids were sporulated, dissected and JMG3 was identified by PCR.

Table 1  
Yeast strains used in this study

| Yeast strain           | Relevant genotype                           | Source           |
|------------------------|---|------------------|
| KP2                    | Prototrophic derivative of W303 <i>MATα</i> | This laboratory  |
| KP3                    | Prototrophic derivative of W303 <i>MATα</i> | This laboratory  |
| JMG2                   | <i>ura3::KANMX3 MATα</i>                    | This study       |
| JMG3                   | <i>ura3::KANMX3 MATα</i>                    | This study       |
| JMG2.113               | <i>ura3::KANMX3 ngr1::mTn-3xHA/GFP</i>      | This study       |
| JMG2.120               | <i>ura3::KANMX3 gid7::mTn-3xHA/GFP</i>      | This study       |
| 3352                   | BY4741 <i>ngr1::KANMX4</i>                  | SGD <sup>a</sup> |
| 3446                   | BY4741 <i>gid7::KANMX4</i>                  | SGD <sup>a</sup> |
| KP2 <i>ngr1</i>        | <i>ngr1::KANMX4 MATα</i>                    | This study       |
| KP2 <i>gid7</i>        | <i>gid7::KANMX4 MATα</i>                    | This study       |
| KP3 <i>ngr1 B</i>      | <i>ngr1::KANMX4 MATα</i>                    | This study       |
| KP2/3 <i>ngr1 gid7</i> | <i>MATα ngr1 gid7</i>                       | This study       |
| C9                     | <i>MATα ho</i>                              | [17]             |
| C9 <i>ngr1</i>         | <i>MATα ho ngr1::KANMX4</i>                 | This study       |
| C9 <i>gid7</i>         | <i>MATα ho gid7::KANMX4</i>                 | This study       |

<sup>a</sup> SGD, *Saccharomyces cerevisiae* Deletion Project, [http://www-sequence.stanford.edu/group/yeast\\_deletion\\_project/deletions3.html](http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html).

Table 2  
Oligonucleotides used for PCR

| Primer    | Sequence (5'–3')   |
|-----------|--|
| ura3KANF  | TTCATTGGATGTTCGTACCACCAAGGA<br>ATT<br>ACTGGAGTTAGTTGAAGCATTAGGTCC<br>CAG<br>ACATGGAGG CCCAGAATAC |
| ura3KANR  | ATAGTCCTCTCCAACAATAATAATGT<br>CAG<br>ATCCTGTAGAGACCACATCATCCACGG<br>TTC<br>AGTATAGCGACCAGCATT C  |
| URA3F1    | AGGGAAGACAAGCAACGAAA   |
| URA3R     | TTGGTTCTGGCGAGGTATTG   |
| IPCR3     | GTTTTCGTTCCACTGAGCGT   |
| IPCR4     | ACAAGACACGTGCTGAAGTCA  |
| GFPprobeF | ACGCTCAGTGGAAACGAAAAC  |
| GFPprobeR | TGACTTCAGCACGTGTCTTG   |
| NGR1A     | AAAACCATCTGCGTGTATATCCTA   |
| NGR1D     | GACTTTGTAGCGATTTTCACATTTT  |
| GID7A     | GTTTCAGATCTATGCTGAGACACG   |
| GID7D     | CCATTTGGTATGGATTATCACTAGG  |
| G418F     | AAAAGACTCACGTTTCGAGGC  |
| G418R     | CGAGCATCAAATGAAACTGC   |

### 2.3. Generation of yeast mutants using a transposon insertion genomic library

A yeast genomic library containing random transposon insertions was obtained from Dr Michael Snyder, Yale University [22]. A library of mutants was generated by transformation of JMG2 or JMG3 with genomic DNA fragments containing *mTn-3xHA/GFP URA3* insertions. Briefly,  $5 \times 10^8$  cells were transformed [20] with  $1 \mu\text{g}$  of *NotI* digested and purified mutagenised DNA. Transformants were selected on minimal medium (to eliminate auxotrophic mutants).

### 2.4. Selection of nitrogen-efficient strains

During the primary screen, 1 ml YPD starter cultures of 5000 randomly selected transposon mutants were grown overnight at 30 °C in 48-well plates (#3548, Corning Costar, Rochester, NY) with shaking (~150 rpm). A 48-pinned replicator was then used to inoculate screening fermentations comprising 1 ml of CDGJM ( $75\text{--}100 \text{ mg FAN l}^{-1}$  as ammonium) in fresh 48-well microtitre plates, before aerobic incubation at 30 °C with shaking (100 rpm). Fermentation progress was determined after 10–14 days from the residual sugar content of each culture as estimated using a handheld refractometer. Mutants that catabolised the greatest amount of sugar were selected for further evaluation in the form of a secondary screen, performed in triplicate 5–20 ml cultures of CDGJM containing  $75\text{--}750 \text{ mg FAN l}^{-1}$  as ammonium or as a mix of

nitrogen compounds (a combination of amino acids and ammonium, as described in [15]). Fermentation progress was again monitored by refractive index. Mutants achieving the greatest degree of sugar catabolism after incubation for 10–14 days were selected for further investigation.

### 2.5. Identification of *mTn3* × *HA/GFP/URA3* insertion points in nitrogen-efficient strains

The location of the transposon and therefore the identity of the affected gene of each strain of interest was determined from sequence homology of the insertion site to the *Saccharomyces* Genome Database (<http://www.yeastgenome.org/>). Regions adjacent to and including the inserted transposon were amplified by inverse PCR [23,24] and sequenced. To achieve this, a *DraI* genomic digestion of the transposon mutant was ligated at a low DNA concentration (approximately  $1 \mu\text{g ml}^{-1}$ ) in the presence of T4 DNA ligase ( $5 \text{ U ml}^{-1}$ ) to obtain a high proportion of self-annealed products. Ligation reactions were purified with a *Perfect Prep* kit (Eppendorf) to a final volume of  $30 \mu\text{l}$  and PCR was then performed with primers IPCR3 and IPCR4. The PCR reaction was performed in  $1 \times$  Dynazyme EXT (Finnzymes, Espoo, Finland) reaction buffer with 100 pmol of each primer,  $1 \mu\text{l}$  of purified ligation reaction, 2 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  dNTPs and 1 U of Dynazyme EXT. The PCR amplification conditions were as follows: 10 min at 94 °C and then 30 cycles of 94 °C, 30 s; 56 °C, 1 min; 72 °C, 4 min. The size of the generated IPCR fragment was confirmed by comparison to a Southern blot [25,26] of *DraI* digested mutant DNA hybridised with a probe specific to *GFP* (data not shown). The latter was generated by PCR using transposon mutant genomic DNA (200 ng), primers GFPprobeF and GFPprobeR (100 pmol each), 1 mM  $\text{MgCl}_2$ , 50  $\mu\text{M}$  dNTPs, 50  $\mu\text{M}$  DIG labelled dNTPs (Roche, Rotkreuz, Switzerland) and 1 U of Dynazyme EXT DNA polymerase (Finnzymes). The PCR reaction was performed in  $1 \times$  Taq reaction buffer (Sigma) over 30 cycles at 94 °C, 30 s; 56 °C, 1 min; 72 °C, 1 min. Sequencing of the amplified genomic fragments (automated sequencing by IMVS Adelaide with primers IPCR3 and IPCR4) and comparison to the yeast genome utilising the BLAST homology search engine (<http://seq.yeastgenome.org/cgi-bin/nph-blast2sgd>) revealed the identity of the affected gene.

### 2.6. Construction of *NGR1* and *GID7* deletants in *KP2* and *C9*

*NGR1* and *GID7* were disrupted in strains *KP2* and *C9* [17] using the *KANMX* gene replacement strategy. We took advantage of the readily available yeast

deletion strains from the Yeast Deletion Project by amplifying a PCR product corresponding to the *KANMX4* module flanked by *NGR1* or *GID7* sequences ( $-260 \rightarrow -1$ ,  $+2020 \rightarrow +2329$  and  $-445 \rightarrow -1$ ,  $+2239 \rightarrow +2613$ , respectively) from genomic DNA purified from the corresponding yeast deletion mutants 3352 and 3446, using the primer pairs *NGR1A*, *NGR1D* and *GID7A* and *GID7D*. Strains KP2 and C9 were then transformed with these PCR products and transformants were selected on YPD-geneticin. Deletion of each gene was confirmed by Southern blot hybridisation with a probe specific for *KANMX4*. The *KANMX4* probe was PCR-amplified from pFA6-lacKanMX4 [21] using primers G418F and G418R. PCR conditions employed were the same as for the GFP probe. KP2/3 *ngr1 gid7* was generated by back-crossing KP2 *ngr1* with KP3 followed by sporulation and dissection to generate KP3 *ngr1B*. Subsequently KP3 *ngr1B* was mated with KP2 *gid7*, sporulated and dissected to generate the double mutant KP2/3 *ngr1 gid7*. Mating type was determined by PCR (as described by [27]), and gene disruptions by Southern-blot hybridisation, as above.

### 2.7. Small-scale fermentations

Fermentations were carried out (according to [17]) under anaerobic conditions, in triplicate cultures of 150 ml of CDGJM containing  $200 \text{ g l}^{-1}$  of glucose, where the concentration of nitrogen varied from low ( $75 \text{ mg FAN l}^{-1}$ ) to high ( $750 \text{ mg FAN l}^{-1}$ ) and was supplied either as ammonium only or as a mixture of amino acids and ammonium [15]. Samples were regularly collected, clarified and fermentation progress was evaluated by enzymatic determination (Roche kit #0139106) of residual glucose. Residual ammonium was also determined enzymatically (Roche kit #1112732). Ethanol, glycerol, acetic acid, succinic acid and acetaldehyde were quantified by HPLC analysis in undiluted and diluted (1:20) terminal samples, clarified through syringe filters ( $0.45 \mu\text{m}$  PVDF, Millipore, Bedford, MA). HPLC analysis utilized an Aminex HPX-87H column ( $300 \text{ mm} \times 7.8 \text{ mm}$ ; BioRad) and was performed at  $60^\circ\text{C}$  with  $2.5 \text{ mM H}_2\text{SO}_4$  at a flow rate of  $0.5 \text{ ml min}^{-1}$ . Peaks were detected with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan) and quantified by comparison with prepared standards in CDGJM using Delta integration software (DeltaWare Dataworks, Brisbane, Australia).

Optical density ( $600 \text{ nm}$ ) of appropriately diluted culture samples was used as an estimate of yeast cell growth. Biomass yield was determined from the weight of washed (10 ml deionised water) and dried cells ( $600 \text{ W}$  microwave oven, medium setting, 10 min) collected from 5 to 10 ml culture samples on  $0.22 \mu\text{m}$  filter disks (pre-dried and pre-weighed).

## 3. Results

### 3.1. Screening for nitrogen-efficient yeast mutants

Transposon insertion mutants were generated in the genome of strains JMG2 and JMG3. Selection on minimal medium eliminated auxotrophic mutations. Preliminary work (data not shown) suggested that nitrogen-efficient strains could be identified either from their reduced nitrogen consumption in nitrogen-excess:carbon-limited media (e.g.  $750 \text{ mg FAN l}^{-1}$ ,  $200 \text{ g glucose l}^{-1}$ ) or else from their greater glucose catabolism in nitrogen-limited:carbon-excess media ( $\sim 75 \text{ mg FAN l}^{-1}$ ,  $200 \text{ g glucose l}^{-1}$ ). The later formulation was adopted for the medium in which large numbers of mutants were evaluated in the primary screen, since the extent of glucose catabolism was easily estimated using a refractometer. In this way, from the 5000 mutants assessed, 110 (2%) preliminary isolates were selected as the most nitrogen-efficient and retained for further investigation (Fig. 1). These mutants catabolised at least 10% more glucose than the parental strain. In a secondary screen using the same medium but performed in triplicate 5 ml fermentations, 40 of these mutants (36%) were again shown to catabolise at least 10% more glucose than the parental strain (data not shown). This 16.5-fold enrichment for nitrogen-efficient mutants supported the suitability of the method.

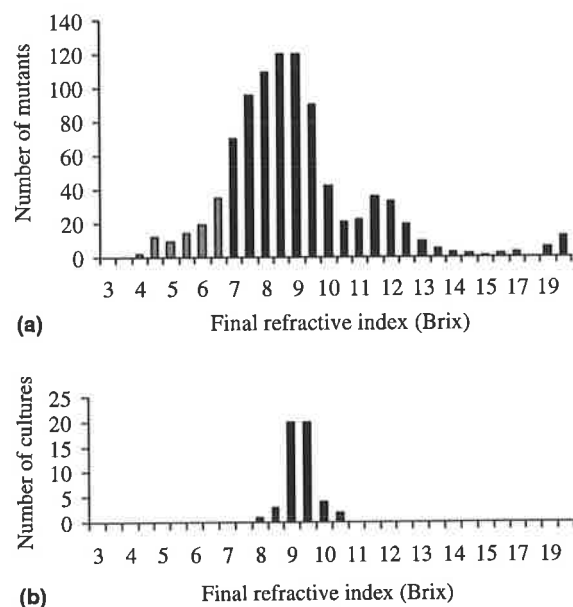


Fig. 1. Histograms showing the distribution of final refractive index values for individual micro-fermentations performed by approximately 1000 transposon mutants (a) or 50 separate cultures of the wild type KP2 (b). All cultures were grown for 14 days in CDGJM containing  $100 \text{ mg FAN l}^{-1}$  as ammonium. Values are derived from a typical experiment for a subset of the total 5000 mutants examined. The grey bars (Panel a) correspond to the mutants selected for further investigation.

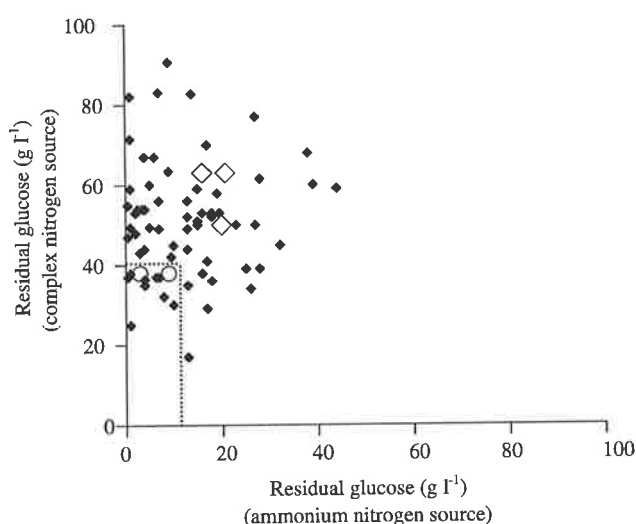


Fig. 2. Comparison of the residual glucose concentrations after 14 days of fermentation by selected transposon mutants ( $\blacklozenge$ ), in particular JMG2.113 and JMG2.120 ( $\circ$ ), and the wild type, KP2 ( $\diamond$ ), when grown in 5 ml of CDGJM containing  $75 \text{ mg FAN l}^{-1}$  supplied as ammonium or as an ammonium and amino acid (complex) nitrogen source. Mutant strains that catabolised the most glucose, that is those fermentations with less residual glucose than the selection threshold (dotted lines) were chosen for further analysis.

Grape juices typically contain assimilable nitrogen as a complex mixture of compounds, primarily amino acids and ammonium [15,28]. All screening of mutants up to this point, however, had been conducted using ammonium alone. The relative nitrogen efficiency of the selected mutants was therefore examined during growth in a limited-nitrogen medium ( $75 \text{ mg FAN l}^{-1}$ ), instead comprised of a mix of nitrogen compounds. In general the extent of glucose catabolism was decreased when a mixed, limiting source of nitrogen was provided, yet the majority of mutants still proved superior to the

parental strain (Fig. 2). For a small number of mutants there was little change in efficiency across the two nitrogen sources.

### 3.2. Identification and characterisation of genes influencing nitrogen efficiency

Ten mutants with the most enhanced performance, that is where significantly more glucose is catabolised when nitrogen is supplied as a complex mix or as solely ammonium, were selected from the above screens and assessed in 150 ml fermentations of CDGJM containing  $75 \text{ mg FAN l}^{-1}$  as amino acids and ammonium. Such volumes permitted a more comprehensive sampling regime and hence more accurate determination of fermentation kinetics, yield of biomass and key metabolites. Under these conditions mutants JMG2.113 and JMG2.120 were chosen for genetic analysis because in comparison to the parental strain, after glucose fermentation had become protracted they had catabolised 7.3% and 22.2% more glucose, respectively, under the nitrogen-limited conditions (data not shown). The genomic location of the transposon was therefore determined for both strains by IPCR amplification and sequencing of genomic fragments containing the insert. JMG2.113 bore an insertion at +1533 bp of *NGRI/YBR212W* (Negative Growth Regulatory protein) [29], and JMG2.120 at +956 bp of *GID7/YCL039W* (glucose-induced degradation process of FBPase) [30].

The entire open reading frame of each gene was in turn deleted in strain KP2 and the wine yeast derivative, C9, and the resultant deletants cultured in media of various nitrogen contents. Deletion of *NGRI* or *GID7* from the laboratory strain KP2 increased the amount of glucose catabolised under most conditions examined. In media containing limiting ammonium these strains failed to support completion of fermentation, that is

Table 3

Duration of glucose fermentation and the yield of biomass for wild type and mutants during growth at two nitrogen concentrations, supplied as either ammonium or ammonium and amino acids

|                 | Ammonium                   |                               |                             |                               | Complex N                  |                               |                             |                               |
|-----------------|----------------------------|-------------------------------|-----------------------------|-------------------------------|----------------------------|-------------------------------|-----------------------------|-------------------------------|
|                 | $75 \text{ mg FAN l}^{-1}$ |                               | $300 \text{ mg FAN l}^{-1}$ |                               | $75 \text{ mg FAN l}^{-1}$ |                               | $300 \text{ mg FAN l}^{-1}$ |                               |
|                 | Duration (h)               | Biomass ( $\text{g l}^{-1}$ ) | Duration (h)                | Biomass ( $\text{g l}^{-1}$ ) | Duration (h)               | Biomass ( $\text{g l}^{-1}$ ) | Duration (h)                | Biomass ( $\text{g l}^{-1}$ ) |
| KP2             | — <sup>a</sup>             | $2.2 \pm 0.1$                 | 105                         | $5.6 \pm 0.1$                 | 326                        | $3.4 \pm 0.1$                 | 74                          | $6.1 \pm 0.2$                 |
| C9              | 190                        | $3.1 \pm 0.1$                 | 70                          | $6.9 \pm 0.4$                 | 190                        | $2.9 \pm 0.1$                 | 78                          | $6.9 \pm 0.4$                 |
|                 | (%)                        | (%)                           | (%)                         | (%)                           | (%)                        | (%)                           | (%)                         | (%)                           |
| KP2 <i>ngr1</i> | 4.9 <sup>a</sup>           | $104 \pm 3.9$                 | 100                         | $92 \pm 5.1$                  | 100                        | $101 \pm 5.2$                 | 95                          | $107 \pm 7.2$                 |
| KP2 <i>gid7</i> | 17.3 <sup>a</sup>          | $100 \pm 3.1$                 | 90                          | $98 \pm 2.1$                  | 92                         | $82 \pm 4.4$                  | 95                          | $110 \pm 2.0$                 |
| C9 <i>ngr1</i>  | 85                         | $85 \pm 2.0$                  | 100                         | $77 \pm 7.2$                  | 73                         | $78 \pm 2.1$                  | 85                          | $87 \pm 1.3$                  |
| C9 <i>gid7</i>  | 100                        | $100 \pm 2.0$                 | 100                         | $98 \pm 1.7$                  | 91                         | $97 \pm 1.5$                  | 87                          | $108 \pm 9.7$                 |

Values for the mutant strains are expressed as a percentage of the parental strain grown under the same conditions. Biomass is expressed as  $\pm$  SD. Fermentation durations are the average of triplicate fermentations and were within 3% of the reported figure.

<sup>a</sup> Complete catabolism of glucose did not occur, thus values reported are the percentage increase in glucose catabolism compared to that of the parental strain, after 450 h.

they became “stuck” (Table 3). Nevertheless strains KP2 *ngr1* and KP2 *gid7*, grown in the media used for the primary and secondary screen (i.e. 75 mg FAN l<sup>-1</sup>, supplied as ammonium), still consumed more glucose than the wild type, KP2, before fermentation arrested. In media of higher ammonium content or else complex nitrogen-limited media, KP2 *gid7* completed fermentation sooner, that is in approximately 90% of the time of the wild type. In complex media of 300 mg FAN l<sup>-1</sup> both deletants in KP2 displayed a fermentation duration similar to that of the parent strain.

Where deletions were introduced into the C9 background and the strains cultured in media containing ammonium as sole nitrogen source, improvements in fermentation performance were seen for C9 *ngr1* cultured in nitrogen-limited media (Table 3). In this case, fermentation duration was reduced to 85% of the wild type. By comparison, deletion of either *NGR1* or *GID7* in C9 reduced the duration of fermentation in all cases where media comprising mixed nitrogen sources were used. Most notably, C9 *ngr1* grown in complex nitrogen-limited media was able to reduce fermentation duration by 27% compared with the wild type grown under the same conditions.

Biomass yields by all cultures were determined to establish whether any improvements in nitrogen efficiency were attributable to a reduced formation of biomass. There was, however, no correlation between biomass yield and fermentation duration.

### 3.3. Glucose utilisation by KP2/3 *ngr1 gid7*

The double-deletion strain KP2/3 *ngr1 gid7* was constructed as described in Section 2. Deletion of both

genes was confirmed by Southern-blot hybridization (data not shown). In fermentation trials utilising nitrogen-limited media containing ammonium as sole nitrogen source, the rate of glucose catabolism was markedly increased in KP2/3 *ngr1 gid7* such that only this strain completed the fermentation of all the glucose (200 g l<sup>-1</sup>) within the duration of the experiment (Fig. 3). In so doing the double mutant catabolised 71% more glucose than the wild-type strain (KP2, 116.5 g l<sup>-1</sup>), 44% more glucose than KP2 *ngr1* (139.3 g l<sup>-1</sup>) and 30% more than KP2 *gid7* (154.3 g l<sup>-1</sup>). Attempts were made to construct an *NGR1* and *GID7* double mutant in C9, however, the combination of both mutations in this background appeared to be lethal.

### 3.4. Nitrogen utilisation, biomass and glycerol yields by deletants

Differences in nitrogen efficiency have previously been suggested through determinations of the maximum amount of nitrogen that is utilised by a strain in conditions of nitrogen excess [11,12]. Therefore the maximum nitrogen utilisation of the deletants was determined using media containing ammonium as sole nitrogen source and supplied in excess to the requirements of these strains (750 mg FAN l<sup>-1</sup>). Interestingly, nitrogen utilisation was not generally seen to be reduced for these deletants compared with the parental strains (276 ± 18 mg l<sup>-1</sup> for KP2 and 218 ± 14 mg l<sup>-1</sup> for C9). In fact, strain C9 *gid7* tended toward a higher consumption of ammonium (246 ± 9 mg l<sup>-1</sup>).

Biomass yields and cell numbers were also determined. No differences were seen in the latter between the mutants and corresponding wild type (data not shown). However, just as biomass yield had not correlated with fermentation duration (Table 3), there was no clear link between biomass yield and degree of nitrogen consumption determined under the conditions of this experiment (data not shown). Strain C9 *ngr1* was perhaps the sole exception, showing comparable reductions in biomass yield and nitrogen utilisation. Reduced biomass yields were a recurring feature of C9 *ngr1*, such that across all experiments biomass yields ranged between 77% and 87% of that of the wild type, independent of the amount or type of nitrogen supplied. Reduced biomass yields combined with unchanged cell numbers suggest that C9 *ngr1* produce cells that were smaller than the wild type.

The final parameter determined at the conclusion of fermentation was the yield of gross metabolites, namely, ethanol, glycerol, acetic acid, succinic acid, citric acid and acetaldehyde. Most analytes were unchanged in their concentration across the strains examined (data not shown). Glycerol yields appeared to increase in most cases, but significant increases of up to ~17% were limited to strain C9 *ngr1* in both media (Fig. 4).

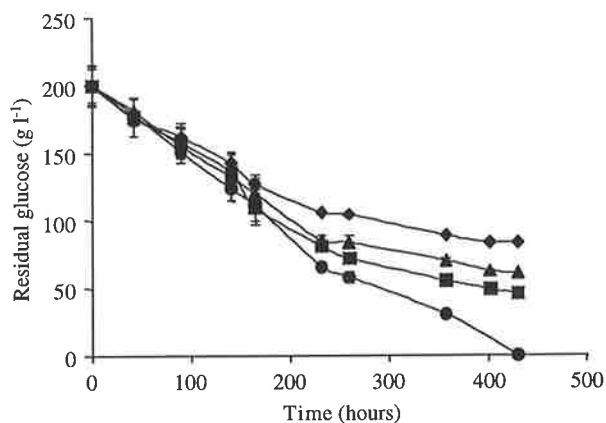


Fig. 3. Catabolism of glucose by wild-type strain KP2 (◆) and deletion strains KP2 *ngr1* (▲), KP2 *gid7* (■) and KP2/3 *ngr1 gid7* (●). Fermentations were performed in CDGJM with 75 mg FAN l<sup>-1</sup> as ammonium. Data points are the mean from triplicate fermentations ±SD. NB: smaller error bars are concealed behind the data symbols.

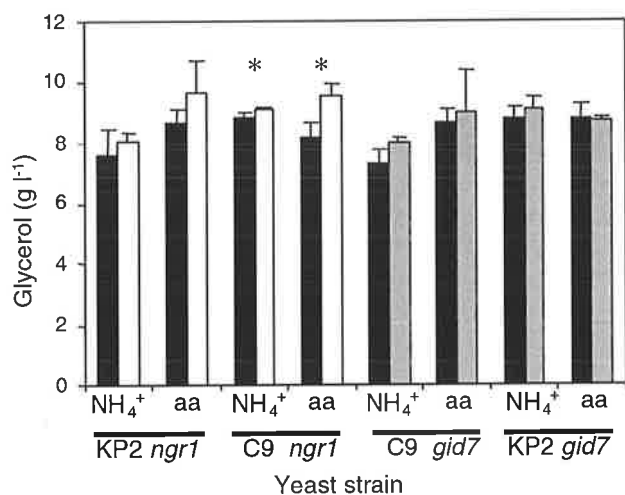


Fig. 4. The concentration of glycerol at fermentation completion or arrest (in the case of KP2, KP2 *ngr1* and KP2 *gid7* supplied with ammonium), when conducted by wild type (■) KP2 and C9 or their derivatives bearing deletions of *NGR1* (□) or *GID7* (▣). Triplicate fermentations were performed in CDGJM containing low nitrogen (75 mg FAN l<sup>-1</sup>) as either ammonium or as a mixture of complete amino acids and ammonium (aa) as indicated. Values shown in the first eight bars were derived from a separate experiment to that which yielded the values shown in the last eight bars. Glycerol yields by mutants which were significantly different ( $p < 0.05$ , Student's *t*-test) from the corresponding wild type are indicated (\*).

#### 4. Discussion

The catabolism of essentially all sugars from an industrial wine fermentation is vital to the successful production of a 'dry' wine within stylistic specifications [31]. Problem fermentations, which often arise due to an insufficient supply of yeast-assimilable nitrogen [1–5] are typically addressed through vineyard or winery practices that increase the amount of available nitrogen. The exploitation of yeast strains that are more nitrogen-efficient [10,13,14] is becoming a more common solution. Conceptually, increased nitrogen efficiency might come about either with or without a net reduction in the amount of nitrogen assimilated. Such improvements could presumably arise through one or a combination of (i) maintenance of sugar transport systems, catabolic and associated enzymology and processes or (ii) a reduced expenditure of nitrogen on non-essential processes, thereby disengaging nitrogen to support core fermentative activities. Highly nitrogen-efficient strains that are a manifestation of either scenario would be useful to industry.

We sought to increase the understanding of the basis for differences in nitrogen efficiency, as a prelude to manipulating this attribute in industrial strains for greater fermentation reliability. A transposon-based system and multi-well plate format fermentations were successfully used to generate and identify mutants whose capacity to catabolise sugar had been altered (see Fig.

2). Mutants with increases or decreases in nitrogen efficiency were both considered potentially valuable to this study. Nevertheless, given that most mutations probably resulted from a loss of function in the target gene [32,33], those yielding an increase in nitrogen efficiency were considered more likely to be linked with changes in nitrogen metabolism. Whereas mutants with reduced nitrogen efficiency might arise from any mutation that debilitates growth and/or any stage of sugar catabolism, mutants with increased nitrogen efficiency were hypothesised to come about through disruption of a gene or metabolic outcome normally antagonistic to efficient exploitation of nitrogen.

Up to 40 transposon mutants as well as deletants of *NGR1* and *GID7* in a laboratory and a wine strain derivative were evaluated through several fermentations. The transposon mutants, shown to have increased nitrogen efficiency in aerobic fermentations of media with limited nitrogen supplied as ammonium, typically catabolised less glucose when grown on a complex nitrogen source (Fig. 2). Subsequent trials with the deletants conducted under semi-anaerobic conditions generally yielded superior performance in media with mixed nitrogen sources (Table 3). These apparently discrepant results in fact agree with previous reports and may be attributed to the different availability of oxygen in each case. Ammonium is a more efficient nitrogen source than most amino acids in aerobic cultures of laboratory strains [34] whereas, under self-anaerobic oenological conditions, supplementation of grape juice fermentations yields higher fermentation rates from mixed nitrogen sources rather than ammonium [35,36].

Quantitation of the nitrogen requirement of deletants revealed that unlike previously characterised nitrogen-efficient wine yeasts [10,11], deletion of *GID7* or *NGR1* typically did not reduce the amount of nitrogen removed from the medium. Fermentations of nitrogen-deficient media by such strains might therefore be expected to become nitrogen-depleted, even though fermentation should progress and complete ahead of the wild type. The usefulness of these strains as a means of effecting fermentation of nitrogen-deficient juices remains high, however, the potential for development of other problems, such as liberation of hydrogen sulfide [37], would need to be assessed under such conditions.

As already described, two genes were isolated and deleted from a laboratory and a wine strain derivative to allow more detailed characterisation. That deletion of *NGR1* or *GID7* has benefits for nitrogen efficiency was made clear by the fact that out of the 16 deletant:media combinations trailed, eleven resulted in more glucose catabolised than by the parent strain. For the remaining five combinations, deletants performed equivalently to the wild type (Table 3). The most rapid fermentation of a single-gene deletion was observed for C9 *ngr1* grown in a medium of limited, mixed nitrogen, wherein



total fermentation duration was reduced by up to 27% (approx. 50 h). In the context of an industrial fermentation process like winemaking, such a reduction in the residence time in expensive fermenter infrastructure can have significant benefits for plant throughput and productivity. The deletion of both *NGRI* and *GID7* from KP2 seemed also to significantly enhance fermentation, increasing the total glucose catabolised by up to 71% of the parent strain. Unfortunately, this combination of deletions appears lethal in the wine yeast strain C9. Further work with other industrial strains will help determine whether lethality of the *ngr1/gid7* deletions was specifically related to these modifications or else arose through unrelated effects such as a randomization of chromosomes.

A second criterion for evaluation of deletants was their pattern of metabolite production. It is noteworthy that not only did deletion of *NGRI* or *GID7* fail to produce detrimental consequence for the parameters examined, in some cases potential improvements were noted. Production of glycerol, a metabolite conferring desirable 'mouth-feel' properties to wine when in concentrations above its taste threshold level of  $5.2 \text{ g l}^{-1}$  [38], was in fact produced in increased amounts (by up to ~17%) by some deletants (Fig. 4). Apart from any sensory contributions, increased production of glycerol may indicate an alteration in the redox balance of deletants. Glycerol is produced during the regeneration of  $\text{NAD}^+$ , a requirement for anabolic metabolism, particularly during anaerobiosis. It is also known to be important in the balance of osmotic pressure [39]. Previously, a glycerol-overproducing strain, generated by over-expressing *GPDI*, was shown to have a higher fermentation rate at stationary phase [40]. Researchers hypothesise that this could be due to an enhanced release of inorganic phosphate or a reduced production of ATP [41]. It is therefore possible that any benefit for fermentation seen in *NGRI/GID7* deletants is a secondary effect of elevated glycerol production or fluxes of inorganic phosphate or ATP.

Determining precisely how deletion of *NGRI* or *GID7* increases nitrogen efficiency will require further investigation. No clear explanations can be derived from reports to date on the characterisation of these genes. Nonetheless, the insights offered by this study do allow for some speculation as to the basis for the influence of *NGRI* and *GID7* on nitrogen efficiency. A laboratory strain bearing a deletion of both genes was capable of catabolising a greater amount of glucose during fermentation than either single deletant under equivalent nitrogen-limited conditions (Fig. 3). It therefore seems likely that in KP2 the genes function in separate pathways in order to produce an additive effect. However, this combination of deletions in C9 appears to confer lethality suggesting a functional link between these two genes.

The precise biological function(s) of *NGRI* is unknown but the gene is glucose-repressible [29], is linked to the response to high-sugar stress [42] and, most recently, *NGRI* (*RBPI*) has been shown to promote degradation of mitochondrial porin mRNA [43], possibly by binding to its 3'UTR via its RNA recognition motifs [29]. Of relevance to the present study is the fact that *NGRI* (negative growth regulator (1) was originally isolated based on its influence on growth rate [29,44,45]. Deletion of the open reading frame increased early log-phase growth rate by 30% during growth on glucose, or by 60% or 75% during growth on galactose or glycerol, respectively. Conversely, over-expression of *NGRI* (*RBPI*) resulted in reductions in growth rate [45]. We have not detected any difference in growth rate of *ngr1* strains in this study (data not shown). The only impact on a growth attribute is that deletants consistently yield a reduced biomass with little change to cell number. Cells of *ngr1* strains are therefore smaller and/or lighter. The proposed reduction in cell size did not alter the uptake of ammonium (data not shown), as suggested for other oenological strains of reduced size [46]. However, it is possible that the basis for the increased nitrogen efficiency seen in such mutants is related to a reduction in nitrogen-demanding syntheses, perhaps those involved in formation of cellular reserve compounds or cell enlargement, thereby setting free nitrogen to support faster or extended fermentative activity.

*GID7* is one of several *GID* genes, known to be involved in catabolite degradation of fructose-1,6-phosphatase, since this activity is still present in *gid7* strains that have been shifted to a glucose-containing medium [30]. The putative *GID7* protein is predicted to contain five WD40 repeats (SMART, <http://smart.embl-heidelberg.de/>), which are thought to mediate protein-protein interactions and are found in many regulatory proteins such as the  $\beta$ -subunit of the heterotrimeric G-protein GTPase [47]. Systematic searches for protein interactions have highlighted putative interactions of Gid7p with other *GID* proteins [48], but also with the high-affinity hexose transporter encoded by *HXT7*. This transporter is important at the end of fermentation for maintenance of hexose transport [49] and known to be rapidly degraded upon the depletion of nitrogen [50]. Perhaps, therefore, the absence of Gid7p prevents deactivation of Hxt7p, alike to FBPase, enhancing the mutants ability to maintain its glucose-catabolic activity under conditions of nitrogen starvation. An alternative possibility for the impact on nitrogen efficiency of deletion of *GID7* comes from work on another *GID* gene. *VID30* (*GID1*), is up-regulated on low ammonium [51], resulting in a channeling of nitrogen metabolism towards glutamate formation. It might therefore be speculated that *GID7* functions in a similar manner to *VID30* such that disruption of *GID7*

brings about a re-direction of nitrogen which ultimately benefits maintenance of fermentation.

Regardless of how deletion of *NGRI* and *GID7* enhances nitrogen efficiency during fermentation, these findings provide new strategies for the construction or selection of optimised strains with greater fermentation reliability. While the superior nitrogen efficiency of the double-deletion strains is attractive, it currently seems inapplicable for producing improved wine yeasts, at least in the C9 derivatives of the wine strain, L-2056. Also, a comprehensive check for the introduction of any detrimental changes, such as over-production of undesirable metabolites [52], is yet to be carried out. Further work is therefore required not only to determine if this strategy can be applied to other wine yeast strains, but also to define the metabolic basis for the enhancement in these strains as well as any further mutants isolated using the method set-out in this report.

### Acknowledgements

This project is supported by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research and Development Corporation (GWRDC), with matching funds from the Australian Government (projects UA 00/5 and UA 01/04). Further support was kindly provided by Lallemand Australia. We wish to thank Kate Poole for strains KP2 and KP3, Michael Snyder for a gift of the transposon insertion library, Will Gardner for manufacture of the 48-pinned replicator, and Sakkie Pretorius and Michelle Walker for critical reading of the manuscript. Jennifer Gardner is a holder of an Australian Postgraduate Award. Colin McBryde is a holder of a Postgraduate Award from The University of Adelaide as well as a supplementary scholarship from the GWRDC.

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# Application of the reusable, *KanMX* selectable marker to industrial yeast: construction and evaluation of heterothallic wine strains of *Saccharomyces cerevisiae*, possessing minimal foreign DNA sequences

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Received 11 April 2003; received in revised form 3 July 2003; accepted 8 July 2003

First published online 12 August 2003

## Abstract

The characterisation of wine yeasts and the complex metabolic processes influencing wine fermentation and the quality of wine might best be achieved by exploiting the standard classical and recombinant genetic techniques which have been successfully used with laboratory strains. However, application of these techniques to industrial strains has been restricted because such strains are typically prototrophic and often polyploid. To overcome this problem, we have identified commercial wine strains with good mating and sporulation properties from which heterothallic derivatives were constructed by disruption of the *HO* gene. Consequently, these haploids are amenable to genetic analysis, whilst retaining desirable wine-making properties. The approach used was an adaptation of a previously published gene disruption procedure for laboratory yeast and is based on the acquisition of geneticin resistance from a removable *KanMX* marker. The present work is the first report of the application of a construct of this type to the disruption of the *HO* gene in wine yeasts that are in common commercial use. Most of the 4.9-kb disruption construct was successfully removed from the genome of the haploid derivative strains by loop-out of the *KanMX* marker through meiotic recombination. Sequencing of the *HO* region confirmed the reduction of foreign sequences to a 582-bp fragment comprised largely of a single direct repeat at the target gene. The removal of the active foreign gene (conferring antibiotic resistance) allows the application of other constructs based on the *KanMX* module without the need to resort to other selectable marker systems. Laboratory-scale fermentation trials typically showed minimal differences between the *HO* disruptants and the parental wine strains in terms of fermentation kinetics and formation of key metabolites.

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**Keywords:** *KanMX*; Industrial biotechnology; Wine yeast; Recombinant; Haploid; Heterothallic

## 1. Introduction

Laboratory strains of *Saccharomyces cerevisiae* stably exist as haploids, usually exhibit good mating ability, readily take up exogenous DNA and contain convenient selectable (auxotrophic) markers. As such, classical and recombinant genetic techniques (e.g. refer to [1,2]) have

been highly effective for the delineation of metabolic processes, their genetic basis and its regulation, and have allowed the precise tailoring of these processes. In comparison, industrial strains lack many of these properties making their characterisation and optimisation by these techniques more difficult. Thus in investigations of properties with a complex genetic basis, such as flavour compound formation, the non-availability of stable haploid industrial strains for use in deletion analyses analogous to those used for laboratory strains [3], is a disadvantage. The alternative of using laboratory strains as a model for industrial strains is not always appropriate, given the disparity in the properties of these groups of strains [4,5].

Homothallism and polyploidy in wine yeast are major

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obstacles to applying the knowledge gained through studies of laboratory yeast to industrial yeast. In the haploid state, as with laboratory strains, single genes can be disrupted or introduced and the metabolic consequences studied. This is more difficult in diploid or polyploid wine strains since multiple copies of the same gene typically mask such genetic modifications. Although wine strains can be induced to sporulate and haploid spores of each mating type ( $a$  and  $\alpha$ ) isolated, the population is unstable. A mating switch (mediated by *HO* gene) occurs in some daughter cells, thereby allowing re-diploidisation through mother:daughter mating or else general mating between cells of opposite mating type within the population.

Stable heterothallic haploid derivatives of homothallic wine strains, which are unable to undergo a mating type switch, have been achieved through the introduction of the dysfunctional *ho* allele by pure breeding and cell-spore hybridisation methodologies [6], or else through isolation of naturally occurring mutants such as those of the champagne wine strain, V5 [7]. While successful, these approaches can be time-consuming, or as in the case of breeding, result in the generation of a strain of a hybrid genetic makeup. A more targeted approach involving gene replacement [8] to specifically disrupt the *HO* gene, originally reported for the laboratory diploid strain, MT13 [9], has since been applied to industrial yeasts [10]. Nevertheless, such strategies still have the disadvantage of yielding heterothallic haploid progeny that retain several kilobases of foreign sequences, including the dominant selectable *neo* gene, which confers G418 resistance.

A gene disruption construct with a removable version of a G418 resistance gene (*KanMX*) based on that reported by Wach et al. [11] represents a further refinement since, unlike an auxotrophic marker, the *KanMX* selectable marker has little impact on growth rate [12]. Puig and coworkers [13] have demonstrated the utility of such a *KanMX* construct in the disruption of the *URA3* gene and hence introduction of a uracil auxotrophy directly into a range of higher-ploidy industrial strains. In the present study we set out to produce haploid wine strain derivatives for use as tools for oenological research and as vehicles for the generation of optimised strains with potential for large-scale trials or commercial use. Accordingly, we identified from amongst a collection of wine yeast strains, in common commercial use, those with good mating and sporulation properties as candidates for the generation of stable haploid derivatives. Conversion to heterothallism was achieved through the use of a *HO* gene disruption construct comprising a *KanMX* selection, which can be looped-out through meiotic recombination. Sequencing of the target gene confirmed the precise nature of the construct residue after loop-out, and fermentation experiments and key metabolite analysis defined the impact of these modifications on the broad properties of the haploid derivatives.

## 2. Materials and methods

### 2.1. Strains, media and transformation procedures

Eleven commercial wine yeast strains (seven of which were kindly provided by Lallemand Australia) were used in this study. Two mating type tester strains, KP2 (*MAT $\alpha$* ) and KP3 (*MATa*), were derived from other studies in this laboratory. All strains were grown in YPD media (1% yeast extract, 2% Bacto-peptone, 2% D-glucose), which was solidified with 2% Bacto-agar when necessary. Yeasts were sporulated on potassium acetate (KOAc) medium (1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 2% bacteriological agar) for 8 days at 30°C. When required, asci were digested, spores dissected and mating carried out using standard classical genetic techniques [1].

*Escherichia coli* strain DH5 $\alpha$  was used for plasmid propagation. *E. coli* transformation was according to Mandel and Higa [14]. Ampicillin (100  $\mu\text{g ml}^{-1}$ ) was used for plasmid selection in *E. coli*.

### 2.2. Nucleic acid manipulation

Genomic DNA was isolated from yeast grown overnight in 10 ml YPD according to Adams et al. [2]. Plasmid DNA extraction was accomplished using commercial DNA purification kits (Wizard<sup>®</sup> Plus Mini-preps, Promega, Madison, WI, USA). DNA manipulations were performed according to Maniatis et al. [15], using commercially available restriction enzymes, and T4 DNA ligase (Geneworks, Adelaide, Australia). Restriction digests and isolated fragments were purified with *UltraClean* (MioBio, Solana Beach, CA, USA) or *Perfect Prep* (Eppendorf) kits. *Dynazyme EXT* DNA polymerase (Finnzymes, Espoo, Finland) used in PCR reactions was obtained from Geneworks, Adelaide.

### 2.3. Construction of *ho::KanMX* disruptants

*HO* was disrupted by the one-step gene disruption method [8]. Primers HOFga (5'-TGAGCTGTTGCT-TACGGTGC-3') and HORga (5'-CAAACCTAATGT-GACCGTCGCT-3'), designed according to the S288c genomic sequence from the *Saccharomyces* Genome Database (SGD, accession number 9169867) [16], were used to amplify 3704 bp of genomic sequence containing the *HO* gene. Each reaction was performed in 1  $\times$  *Dynazyme EXT* reaction buffer and contained 100 pmol of each primer, 200 ng genomic DNA (from L2056), 1 mM MgCl<sub>2</sub>, 100  $\mu\text{M}$  dNTPs, and 1 unit of *Dynazyme EXT* DNA polymerase (Finnzymes). Hot-start amplification was initiated with a 2-min 94°C denaturation, followed by 30 amplification cycles (94°C, 30 s; 64°C, 1 min; 72°C, 4 min) and terminated with a 60-min extension at 72°C. The fragment was inserted by 'TA cloning' into

pGEMT (Promega), yielding plasmid pGEMT-HO. The 1245-bp *Bbr*PI–*Bam*HI fragment (containing the *HO*-coding sequence) was excised from pGEMT-HO and replaced with the 2562-bp *Hpa*I–*Bgl*II *KanMX3* module from pFA6-lacKanMX3 [11] to give the recombinant plasmid pGEMTho::KanMX. The plasmid was cut with *Nsp*I and the isolated 4.9-kb fragment used to transform [17] L2056, O16 and CY3079. The transformation mixture was pre-incubated for 2–4 h in YPD, prior to plating on selective medium (YPD containing geneticin (Astral Scientific, Gympie, N.S.W., Australia) at 300 µg ml<sup>-1</sup> for L2056 or 400 µg ml<sup>-1</sup> for CY3079 and O16). After initial selection, transformants were re-streaked onto YPD-plus-geneticin plates.

The disruption of the chromosomal *HO* gene in the wine strains as well as their sporulation products was confirmed by Southern blot hybridisation [15] using a PCR-generated DIG-labelled *HO* probe (primers: HOFwd (5'-GTCACGGCTAACTCTTACGTTATGTGCGCA-3') and HOCrev (5'-GCTTCACGAACCTCTATATGCTCGCCGTAC-3')) and chemiluminescent detection [18]. The wild-type *HO* gene was indicated by a 1.8-kb band, a single and tandem insertion of the *ho*::*KanMX* module by the presence of a 5.3-kb and 10.1-kb band, respectively. The haploid state of spores which had not re-diploidised due to successful disruption of *HO* was verified by their failure to sporulate, their ability to mate with one of the tester strains KP2 (*MAT*α) and KP3 (*MAT*α), and by PCR determination of their *MAT* locus constitution using *MAT*-, *MAT*α- and *MAT*α-specific primers [19].

#### 2.4. Removal of *KanMX* sequences from the genome of haploid *ho* wine yeast

Individual colonies of the haploid wine yeast strains, 1B (*ho*::*KanMX*, *MAT*α) and 1D (*ho*::*KanMX*, *MAT*α), arising after 16 h growth on YPD, were mixed on a fresh plate and incubated for 4 h. Diploids were isolated by micro-dissection, incubated for a further 2 days, sporulated and treated with β-glucuronidase (Sigma) overnight at 30°C. The cells were vortexed (2 min), collected by centrifugation (1 min, 14 000×g), washed with TE (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) and re-suspended in sterile water. The diploid vegetative cells were killed by diethyl ether treatment [20], and the remaining haploid spores grown on YPD plates. Small spore colonies were picked at random and candidate strains, no longer carrying *KanMX*, identified by their sensitivity to geneticin (400 µg ml<sup>-1</sup>) on YPD plates. Geneticin-sensitive colonies were characterised by Southern blot analysis (*ho* disruption and loss of *KanMX* gene) and PCR (mating type) as described above. The probe for the *KanMX* gene was prepared by PCR using primers G418 Fwd (5'-AAAAGACTCAGTTTCGAGGC-3') and G418 Rev (5'-CGAGCATCAAATGAACTGC-3').

#### 2.5. DNA sequencing of the *HO* gene after removal of *KanMX* sequences

The *HO* genes from Δ*ho* strains C9 and F3 were amplified using primers HOFga and HORga. The PCR products from individual reactions were isolated and either pooled together (for each strain), or kept separate and purified using a *Perfect Prep* kit (Eppendorf) before use.

DNA sequencing (IMVS, Adelaide, Australia) was performed on products from the individual and pooled PCR reactions using primers spanning the *HO* gene. Sequence analysis was performed using the BLASTN program [21]; <http://genome-www.stanford.edu/Saccharomyces/>; or <http://www.ncbi.nlm.nih.gov/blast/>.

#### 2.6. Small-scale fermentations

The impact on fermentation performance of the various genetic modifications described was evaluated in laboratory-scale fermentations. Strains were cultured according to Jiranek et al. [22]. Yeasts were inoculated into 10 ml YPD and grown overnight prior to inoculation (at 2.5 × 10<sup>6</sup> cells ml<sup>-1</sup>) into 25 ml of Chemically Defined Grape Juice Medium (CDGJM) [22] as starter culture and grown for 24 h. Fermentations were conducted in duplicate in 250-ml conical flasks fitted with a fermentation lock and a septum-sealed sidearm port for aseptic and anaerobic sampling and supplementation. CDGJM (100 ml) was inoculated with yeast to 5 × 10<sup>6</sup> cells ml<sup>-1</sup> from the overnight starter culture and fermentation locks fitted. In order to mimic the near-anaerobic conditions of an industrial grape juice fermentation, the head space of each flask was flushed for 3 min with sterile filtered (0.45 µm) high-purity nitrogen delivered through the flask septum via a 21-gauge needle. Flasks were incubated at 30°C with shaking. Fermentation progress was monitored by determination of the cumulative weight loss of the flasks that occurred through the evolution of CO<sub>2</sub> [23]. Samples were collected regularly and analysed for residual glucose and/or fructose content using enzymatic test kits (Boehringer, Mannheim, Germany). Terminal samples (1 ml and 50 ml) were collected, clarified by centrifugation and frozen for subsequent metabolite determination by high-performance liquid chromatography (HPLC) and total SO<sub>2</sub> determination by aspiration.

#### 2.7. Chemical analysis

Residual glucose, fructose, succinic acid, acetic acid, acetaldehyde, glycerol and ethanol were determined by HPLC analysis. Frozen ferment samples were thawed and 500-µl aliquots filtered through 0.45-µm PVDF syringe filters (Millipore). HPLC analysis was performed on undiluted and diluted (1:20) samples, on either a Benson Carbohydrate SS H<sup>+</sup> column (300 mm × 7.8 mm) (Alltech, Deerfield, IL, USA) or an Aminex HPX-87H column

(300 mm × 7.8 mm) (Bio-Rad, Hercules, CA, USA). Elution was performed at 60°C with 2.5 mM H<sub>2</sub>SO<sub>4</sub> at a flow rate of 0.5 ml min<sup>-1</sup>. Detection was performed by means of a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). Quantitation was achieved by comparison with prepared standards in CDGJM using Delta integration software (Deltaware, Charlottetown, Canada). Total SO<sub>2</sub> determinations were performed in duplicate according to Iland et al. [24] on thawed samples (20 ml).

### 3. Results

#### 3.1. Construction and characterisation of haploid wine yeast

Eleven wine yeast strains were subjected to two to three cycles of sporulation, dissection and re-diploidisation. Populations of strain L2056, O16 and CY3079 displayed good rates of sporulation (ca. 40%) and yielded a high proportion of four-spore asci with 100% viability (Table 1). As a result of their amenability to classical genetic manipulation, these strains were considered suitable candidates from which to construct haploid (*ho*) derivatives. A comparison of L2056 and the re-diploidised derivatives generated through this initial selective phase, revealed similar fermentative properties (data not shown), thereby agreeing with earlier findings [6] that fundamental fermentative properties are not dependent on genetic heterogeneity of the strain.

The prototrophic and diploid (or higher ploidy) nature of wine strains precludes the use of traditional selection systems based on the complementation of recessive auxotrophic markers. Therefore, disruptions of the *HO* gene were carried out by the one-step gene disruption technique using a customised *ho::KanMX3* construct derived from that reported by others [11], and incorporating the dominant selectable *KanMX3* marker to confer geneticin resistance. The predicted heterozygosity of the diploid transformants with regard to the *HO* gene was confirmed by

Southern blot analysis (Fig. 1A,B). A 1.8-kb band, detected by the *HO* probe in the parental strains and transformants, was consistent with the wild-type *HO* gene predicted from the sequence published on the *Saccharomyces* Genome Database [16]. A second larger band, corresponding to the disrupted *ho* gene (*ho::KanMX*), was detectable only in the transformants and varied in size according to whether a single (~5.3 kb) or tandem (~10.1 kb) insertion of the 4.9-kb *NspI* fragment at the *HO* locus had occurred. The number of insertions varied across strains, implying no strain dependence of the single versus tandem insertional event (data not shown). We randomly selected O16 transformant, #5, CY3079 transformant, #8, and 1B, a transformant of L2056 for use in further studies.

Haploid *ho* derivatives of the identified transformants were obtained after sporulation and tetrad dissection. The expected 2:2 pattern of segregation of *HO* and *ho::KanMX* genes was observed in the dissected spores by Southern blot analysis (data not shown). Determination of the mating type of the isolated spores by PCR using *MAT-*, *MATα-* and *MATa-*specific primers (Fig. 1C) allowed identification of non-switching haploid wine yeast derivatives of both mating types and also demonstrated the presence of both *MAT* bands in two spores from each tetrad being indicative of their possession of a functional *HO* gene and having already switched mating type and re-diploidised [25]. This experiment clearly demonstrated the effectiveness of the *HO* disruption as well as the switching efficiency of the wild-type *HO* spores.

Further indications of behaviour corresponding to the haploid state were the failure of the *HO* disruptants to sporulate, ready mating with the appropriate tester strains KP2 (*MATα*) and KP3 (*MATa*), and subsequent sporulation (data not shown).

#### 3.2. Removal of foreign sequences from the yeast genome

Initial efforts to induce loop-out of the functional *KanMX* marker by growth under non-selective conditions

Table 1  
Sporulation behaviour and spore viability of 11 commercial wine yeast strains

| Strains        | Manufacturer | Percentage of population sporulated <sup>a</sup> | Percentage of asci with four spores | Percentage of tetrads with four viable spores <sup>a</sup> |
|----------------|--------------|--|-------------------------------------|--|
| Premier Cuvee  | Red Star     | 45.5   | 17.0                                | 0  |
| Pasteur Red    | Red Star     | 15.0   | 7.0                                 | 0  |
| Burgundy       | Lallemmand   | 27.8   | < 0.1                               | 40   |
| L2056          | Lallemmand   | 35.8   | 27.9                                | 62   |
| Flor Sherry    | Red Star     | 14.9   | 1.7                                 | 10   |
| R2             | Lallemmand   | 16.3   | 2.0                                 | 13   |
| EC1118         | Lallemmand   | 46.0   | 2.0                                 | 0  |
| CY3079         | Lallemmand   | 42.9   | 1.7                                 | 40   |
| O16            | Lallemmand   | 49.4   | 3.0                                 | 83   |
| CEG            | Lallemmand   | 0  | ND                                  | ND   |
| Cote des blanc | Red Star     | 0  | ND                                  | ND   |

ND, not determined.

<sup>a</sup>Sporulation was induced on solid media and the viability of spores was determined as described in the text.

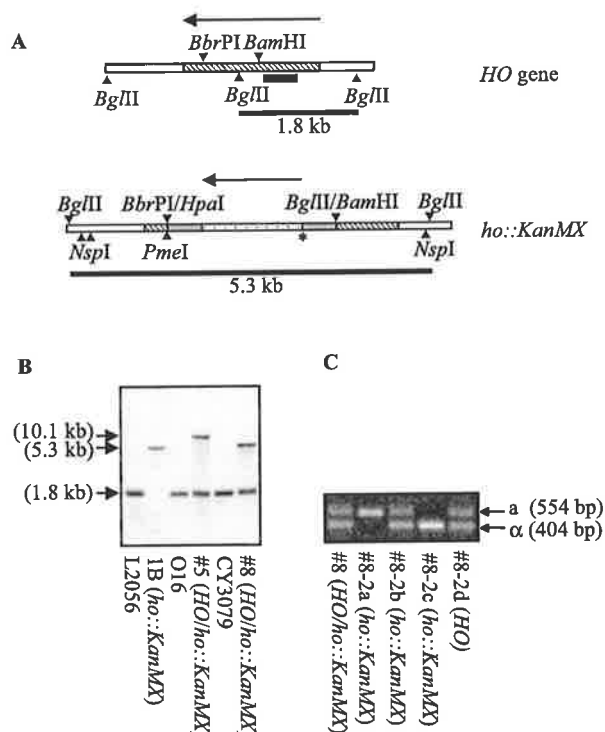


Fig. 1. Construction and characterisation of *HO* disruptant strains. A: Block diagram depicting the main features of the cloned *HO* gene and the *ho::KanMX* disruption construct. Key restriction enzyme sites used in the cloning of *HO* and construction of the *ho::KanMX* disruption module are indicated. Untranslated 5' and 3' *HO* sequences are indicated by open bars, *HO*-coding sequence by hatched regions, *KanMX* sequence by a stippled region, DR sequences (DR1, left; DR2, right) by grey regions. Arrows give the direction of gene transcription and solid bars indicate the 564-bp 3' *HO* probe. Location of a *PmeI* site and a 3' nucleotide sequence (\*), which are suggested to be involved in the loop-out event (see text), are also indicated. Labelled (1.8 kb, 5.3 kb) solid lines indicate the fragments liberated from the *HO* and *ho::kanMX* alleles upon *BglII* digestion, and contributing to the banding pattern seen in the Southern blot analysis (B) of genomic DNA from *HO* disruptant strains. C: PCR determination of mating type of CY3079 *HO* disruptant #8 and haploid progeny showing the presence or absence of the *MATa* (554 bp) or *MATα* (404 bp) amplification products.

failed, even with extended (18) subculturing steps. Consequently, the isolation of strains 1B and 1D, *HO* disruptants of L2056 of each mating type, allowed their mating and sporulation to encourage marker loop-out by meiotic recombination. A diethyl ether-enriched spore suspension was plated *en masse* on non-selective media and resultant colonies patched onto YPD media containing and devoid of geneticin, respectively. From a pool of 1470 such colonies, two geneticin-sensitive colonies (F3 and C9) were identified (Fig. 2A), thereby giving a loop-out frequency of between  $10^{-3}$  and  $10^{-4}$ . F3 and C9 were shown to be haploid (*MATa*) by MAT PCR (data not shown).

The loss of a large part of the disruption construct (essentially one direct repeat (DR) and *KanMX*) from F3 and C9 was confirmed by Southern blot analysis (Fig. 2B). On application of the *HO* probe, a 5.3-kb band corre-

sponding to the *ho::KanMX* allele was observed in the haploid and diploid *ho* disruptants in place of the 1.8-kb band seen in the wild-type strain, L2056. Moreover, a 3.4-kb band was detected in the geneticin-sensitive strains F3 and C9, which is consistent with the predicted fragment size arising from removal of the *KanMX* gene and a single DR sequence. The loop-out of these sequences was confirmed by the failure of the *KanMX* probe to detect such sequences in the candidate strains. A 5.3-kb band detectable using the *KanMX* probe and corresponding to the intact *ho::KanMX* allele was detected only in the original *HO* disruptants, 1B, 1D and their resulting diploid. No such bands were visible in L2056 or the F3 and C9 strains.

DNA sequencing of the *HO* gene amplified from the  $\Delta ho$  strains C9 and F3 was undertaken to verify the loss of the *KanMX* gene and single DR, and to determine the precise nature of any residual sequences. The *HO* gene sequences from both C9 and F3 were found to be identical and are shown in Fig. 3. Recombination at the *ho::KanMX* locus resulted in the loss of the *KanMX* gene (1619 nucleotides) and one DR sequence of 465 nucleotides. The second 465-nucleotide DR is retained at the target site as part of a 582-nucleotide sequence which includes the multiple cloning site (110 nucleotides, bases 5882–5940). An additional seven-base insert (CCGTTTA)

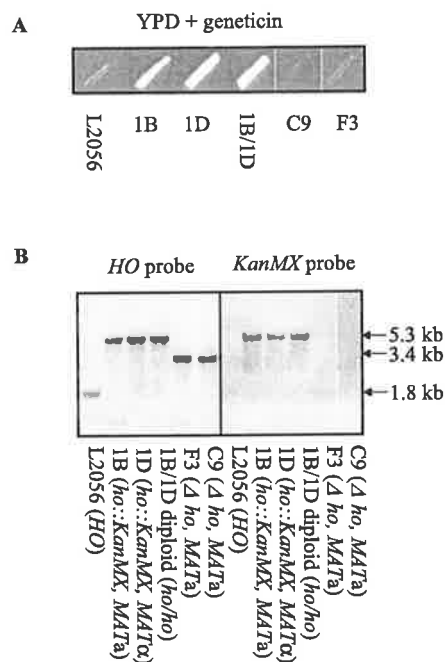


Fig. 2. Geneticin sensitivity of strains C9 and F3 (A) and Southern blot analysis confirming the appropriate distribution of *HO* and *KanMX* sequences and hence loop-out of *KanMX* and DR sequences from the genome thereof (B). Experimental controls include the parental wine strain L2056, *ho* haploid derivative 1B (*MATa*), *ho* haploid derivative 1D (*MATα*), *holho* 1B/1D diploid and geneticin-sensitive strains C9 and F3. Total DNA isolated from the strains was digested with *BglII* and subjected to Southern blot analysis using separate probes for *HO* and *KanMX*.



```

1  TCTCTGAGGAAAGTTGATCAAGAGCCAATAATAATCTATATACTGGTTAA
51  AACAAATCAGTGCCGGTAAACGCTTTTGTATCTTGAGATGGCGTATACT
101  ACTCCAGCATTCGAGTTAAGAAAAAGCTAAAAATGGTTTTTTCATCCA
151  AAATATTAATTTTACTTTTATTACATACAACCTTTTAAACTAATATACA
201  CATTTTAGCAGATGCGCGCACCTGCGTTGTACCACAACCTTATGAGGG
251  CCGCGGACAGCATCAAACCTGTAAGATTCCGCCACATTTTATACACTCTGG
301  TCCTTTAACTGGCAAACCTTCGGGCGTAATGCCCAATTTTCGCCTTTGT
351  CTTTTGCCTTTTTCACTTCACAACCTGGCTTATCGAAATTAATACGACTC
401  ACTATAGGGAGACCCGCGATCCCGGGCCGATAGGCCACTAGTGGATCT
451  GATATCATCGATGAATTCGAGCTCGTTTCCGTTTACCGTACAAGATG
501  GCATCGACCTAAACACCATAGATGGTCCGGCCATTGACTGCCTATTAA
551  CGCCAAACGTTGCGAGATATACACTCGAAATGTGTATGTAGATGTAACA
601  GACGGCTATATGAATGCCACTAGCCGGTTATTTTACTCTAGTAAATACA
651  TATTTCCCTAGAATTTTTTACGTGCATGCATAAGAGTAGAATAGTCTAAAG
701  AGAAGGGTCCCTCGAGGCTAGCTCAGACCAGCTGCGGATTGCCCTGCCAAG
751  GCTTCTTAACTCGAAGCCAAATGGCGTCGCCAACCTCCGAAGTGGAGCT
801  CGAGCCGACGAGTCCCGCTTCTGACTCCCGAGTCCAACACCTCCTGCA
851  TCGCTGCTCGACCCGCTCGCCGCGCAACCATGTTCAACGACAACCTC
901  AGCATCATGGCAGCAGAGGATGCATCCGATCGGGTTCGCCTCCCGGC
951  GGCGAGTCCAAGCTATCTACTGAGATTTCTGGCTCTTTTGTGTACG
1001  CACCTAACCCACAGACCAAGCATCCAAGCCATACCTTTTACAGCAGGAGTT
1051  ACRAAGTCACTACGTCAGTGAAGAAATTTAGATAAAACACCATTTCCCTG
1101  GAGTACTGGACCAATCTTATGCAGCTAGAAATCTCAATTGAGCATCA
1151  GATAATCCAAATCTCTAATCTCAATGTCAAAGTTGAAATATTCTCCTTA
1201  GAGCGCTCCATTTCTTATGAAGCGTTTTGGGGAAAACCTCACCTTCAA
1251  TGTCATTGGGAATGCTTATGATGGTTTTTGGAAATATATATATCCAC
1301  CATCAACGCTCTGACATTTGCTGCAGATTTCTCCATCTCACTTTATATTG
1351  GTGGCATTCTACCACTTTTTTCCAAAGTGGTTTTGGTAGGACCCCTGAC
1401  TGACAATTTATGACCTGAGTACATTTGTAATGCAAGACGCTGATAAACTG
1451  TTCTACGCTGGGATCAACCTACCAGGTTCCACTCAAAGCTCTGTGT
1501  TTGGTTTTTGTGTATATATAGATTTTCTGATAGCCCTGTGTGACATT
1551  TATGACGCGGGCAGCGGAGCCATCTGCGCACATAACGTAAGAGTTAGCCG
1601  TGACGTTGCGATGCTTTAATTCACCGTTAGCCATCAGAATAGTCGTG
1651  TTTTCAGAAACATTTTAAAGTATAGATCGAATTGATGCTGCTTATGAG
1701  GATATGGATTTAGAGCTAATAGTTGTAATAGAAATAAAAGCCATTTAGAA
1751  TAGGAATGAATACATTTTATGAGCTTTAAATATTAATGATGAATAGT
1801  CGGTTTGAACGCTTCATCATGCTTCAACAAGCTTCTGCAACCTCATT
1851  GAGTAGCAATAGCTGTTTACTTTTCCCGGACTTTATTCGAAGTTATG
1901  TGAACCTTTCGTGGTTTACACCTAATAACGCCAGCGGTAGTACAATTC
1951  AAAGTAGTA

```

Fig. 3. DNA sequence analysis of the *HO* gene after loop-out of *KanMX* and DR sequences from the yeast genome. The residual disruption construct sequence is represented in bold (multi-cloning site in italics) whilst *HO* sequence (+1965 to +1594 and +705 to -295) is in regular font. The cloning sites *BbrpI*-*HpaI* (*CACAAC*) and *BglII*-*Bam*HI (*AGATCC*) which originate from the disruption construct pGEM  $\Delta$ ho::*KanMX3* are underlined. The disrupted *PmeI* site is shown in grey and the extraneous seven nucleotides therein are boxed. Differences between the determined sequence and the corresponding sequence reported in the *Saccharomyces* Genome Database are highlighted against a black background. The sequence depicted is that of the Crick strand.

within the *PmeI*-cloning site (GTTTAAA) adjacent to the DR sequence was noted and suggests the locus of the cross-over event. Also, in comparison with the *HO* gene sequence reported on the *Saccharomyces* Genome Database [16], the corresponding sequence remaining in C9 and F3 differed at 10 nucleotides.

The low frequency of loss of the *KanMX* module prompted the decision to isolate *MAT $\alpha$*  haploid derivatives of *KanMX* loop-out strains, C9 and F3, by classical genetics. Strains C9 and F3 were individually crossed with the isogenic *ho*::*KanMX MAT $\alpha$*  strain, 1D. Diploids were isolated by micro-dissection, induced to sporulate, the resulting tetrads dissected and spores grown on YPD prior to replica streaking onto YPD in the presence and absence of geneticin. The segregation of the geneticin-sensitive phenotype in the various spores matched the segregation pattern of the  $\Delta$ ho (geneticin-sensitive) and *ho*::*KanMX* (ge-

neticin-resistant) genes as visualised through Southern blot analysis (data not shown).

### 3.3. Small-scale fermentation trials of parental and haploid wine yeast

Our earlier studies using the wine strain L2056, and homogenous derivatives isolated through repeated spore re-diploidisation, revealed no large differences in a comparison of fermentation kinetics and key metabolite formation (data not shown). Small-scale fermentation trials were also carried out with the parental strains and their haploid derivatives to determine whether the genetic modifications described above adversely affected the key properties of fermentation kinetics and the formation of major metabolites. Close agreement was seen in terms of fermentation kinetics for wine yeast L2056 and both types of *HO* disruptants (*ho*::*kanMX* and *KanMX* loop-out) (Fig. 4A), and wine yeast O16 and derivatives (data not shown). The retention of the *KanMX* marker, as in the case of strain 1B, did not appear to adversely affect the fermentative ability of the haploid derivatives of L2056. Greater differences in fermentation kinetics were, however, observed between wine yeast CY3079 and derivatives (Fig. 4B). Thus, while the maximal rate of fermentation appeared not to be less than that of the parental strain, a greater

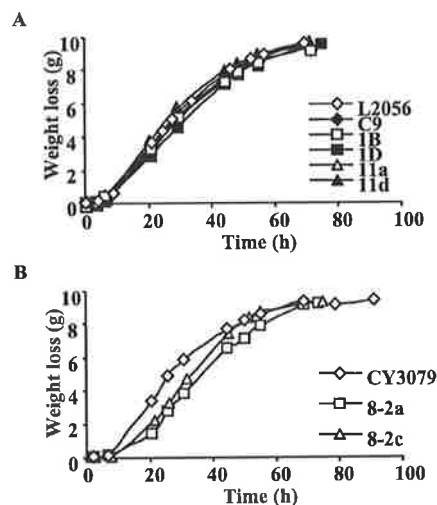


Fig. 4. Fermentation performance of wine yeasts and their haploid, *ho*::*KanMX* or *KanMX* loop-out derivatives. Fermentations were performed over the course of several experiments and their progress monitored by measurement of the cumulative weight loss of fermentation flasks due to CO<sub>2</sub> evolution. Wine yeast L2056 and the haploid, *ho*::*KanMX*, and  $\Delta$ ho derivatives (A) were cultured as described in the text, with some exceptions. Ferments (100 ml) of modified CDGM contained 1.87 g l<sup>-1</sup> ammonium sulphate and 100 g l<sup>-1</sup> each of glucose and fructose and were incubated at 28°C. The starter cultures were prepared without the reported anaerobic growth factors [22]. Wine yeast CY3079 and derivatives 8-2a ( $\Delta$ ho, *MAT $\alpha$* ) and 8-2c ( $\Delta$ ho, *MAT $\alpha$* ) were cultured as described in the text. All values are the mean of four to six replicate fermentations per strain.

lag preceded attainment of this rate by the derivative strains 8-2a and 8-2c.

The residual sugar content of terminal fermentation samples was found to be within the range for all ferments to be considered complete (Table 2). HPLC analysis allowed the yield of a limited range of metabolites to be also defined. Of greatest interest was the influence of the described genetic modifications and the resulting haploid state of the wine yeast derivatives on the yield of the predominant metabolites, ethanol and glycerol. Ethanol yields were in close agreement, with haploids differing from the corresponding parental strain by no more than 5%. Haploid glycerol yields were within 10% of the amounts formed by the parental strains. Other metabolites to be quantified included acetic acid, acetaldehyde, succinic acid and sulphur dioxide (SO<sub>2</sub>). Acetaldehyde and SO<sub>2</sub> were most variable, with the yield of these compounds by some derivatives differing from those of the parental strains by up to ~50 and ~75%, respectively.

#### 4. Discussion

Wine strains are mostly prototrophic, homothallic yeasts that sporulate poorly, and produce few viable spores. They are often heterozygous and possess ill-defined chromosomal constitutions [26,27], generally making them unsuited to classical and molecular genetic manipulation. To date, most progress in terms of the construction of new industrial yeast strains has been achieved via traditional breeding techniques [28]. These methods are time-consuming and, since they involve the fusion of genomes from two strains, lack the precision of molecular genetic techniques.

Few previous studies have utilised genetic techniques as part of a characterisation of yeast physiology as seen under wine-making conditions. Where these techniques have been used, they have been applied largely to laboratory strains and not to wine strains. Similarly, many recent reports detailing the construction of improved recombinant yeasts have predominantly been carried out in laboratory strains using traditional bacterial/yeast shuttle vec-

tors [29,30], with very few using wine strains [31,32]. Although such studies have made significant contributions towards an understanding of wine yeast physiology, it is unlikely that the resulting recombinant strains will be acceptable for commercial use. The reasons are two-fold; firstly, the episomal plasmids are unstable and readily lost under non-selective conditions [33,34]; and more importantly, the presence of bacterial sequences, antibiotic resistance genes and selectable markers within the existing plasmid constructs currently may impede granting of regulatory approval to exploit such organisms in commercial food processes.

In this study we have sought to overcome the limitations of using laboratory strains to study yeast physiology during oenological fermentations, by generating haploid derivatives of wine yeast strains that are amenable to classical and recombinant genetic manipulation. Accordingly, from three widely used commercial wine strains, selected for their desirable mating and sporulation efficiencies and high tetrad viability, we constructed stable haploids that retain the key fermentative properties of the originating strains. The ability of the generated haploid strains to be readily manipulated by classical genetic techniques with predictable outcomes demonstrated the efficacy of these haploid strains as the basis for a genetic investigation in a wine yeast background. Furthermore, in contrast to recently described haploid wine strains [10], those produced in this study possess only minimal foreign or non-*Saccharomyces* DNA sequences within their genomes. As such, the usefulness of the present haploid derivatives may be extended beyond that of tools for oenological research to include their use as more acceptable vehicles for the construction of optimised wine yeasts.

The disruption of *HO* in all three strains was based on a previously described system [11], which was chosen because of the ability of the selectable marker to be removed when no longer required. The present work represents the first report of the use of the removable *KanMX*-based gene disruption system to commonly used commercial wine strains to generate stable haploid derivatives. Importantly, sequencing of the target region has revealed the precise

Table 2  
HPLC analysis of supernatants from fermentation trials using wine strains L2056 and CY3079, and haploid *ho::KanMX* and  $\Delta ho$  derivatives

| Metabolite (g l <sup>-1</sup> )             | Strain      |             |             |             |             |             |             |             |             |
|---|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|   | L2056       | 1B          | 1D          | C9          | 11a         | 11d         | CY3079      | 8-2a        | 8-2c        |
| Glucose                                     | 0           | 0           | 0           | 0           | 0           | 0           | 0           | 0           | 0           |
| Fructose                                    | 1.95 ± 0.59 | 0           | 0           | 0           | 0           | 0           | ND          | ND          | ND          |
| Ethanol                                     | 100.8 ± 2.4 | 103.1 ± 2.6 | 104.1 ± 1.6 | 102.1 ± 2.0 | 100.6 ± 3.0 | 100.1 ± 4.2 | 105 ± 0.94  | 106.7 ± 3.2 | 106.7 ± 4.3 |
| Glycerol                                    | 10.4 ± 0.1  | 9.8 ± 0.1   | 10.7 ± 0.2  | 10.1 ± 0.4  | 9.8 ± 2.1   | 10.4 ± 0.1  | 7.0 ± 0.2   | 6.3 ± 0.1   | 6.8 ± 0.2   |
| Acetic acid                                 | 1.4 ± 0.1   | 1.5 ± 0.1   | 1.8 ± 0.1   | 1.6 ± 0.3   | 1.7 ± 0.1   | 1.8 ± 0.1   | 0.6 ± 0.03  | 0.6 ± 0.1   | 0.7 ± 0.1   |
| Acetaldehyde                                | 0.61 ± 0.07 | 0.32 ± 0.05 | 0.49 ± 0.05 | 0.49 ± 0.06 | 0.52 ± 0.08 | 0.38 ± 0.06 | ND          | ND          | ND          |
| Succinic acid                               | 0.42 ± 0.03 | 0.36 ± 0.04 | 0.39 ± 0.03 | 0.35 ± 0.03 | 0.47 ± 0.16 | 0.33 ± 0.01 | 0.22 ± 0.02 | 0.25 ± 0.03 | 0.26 ± 0.02 |
| Total SO <sub>2</sub> (mg l <sup>-1</sup> ) | 33.9 ± 1.7  | 30.8 ± 3.5  | 37.3 ± 3.2  | 32.4 ± 4.0  | 29.1 ± 1.7  | 44.8 ± 0.8  | 73.2 ± 3.07 | 21.2 ± 6.8  | 18.8 ± 1.3  |

HPLC analysis was carried out in duplicate in each of two separate runs and was performed for each of the two to six replicates of the fermentation cultures and the resulting average values for a given determination (± S.E.M.) are reported. Total sulphur dioxide determinations (in duplicate per replicate) were carried out according to Iland et al. [24]. ND, not determined.

nature of the sequences left after loop-out of the marker (see Fig. 3). The DR sequences in the disruption construct did in fact enable effective removal of the intervening selectable marker and one of the DR sequences. The presence of an extraneous seven bases (CCGTTTA) adjacent to the remaining DR suggests that the recombination event centred on a 5-bp sequence (CCGTT) 3' of DR1 and the *PmeI* recognition sequence 3' of DR1 (see Fig. 1). The loop-out resulted in the retention of DR2 (originating from pFA6-lacKanMX3; accession no: ASAJ2684 (bases 3431–3896)) and the multi-cloning site 3' thereof.

In our hands, loop-out of the selectable marker under non-selective conditions [13] did not occur and was only achieved by meiotic recombination. For the *ho::KanMX* derivatives of L2056 chosen as a test case, 1B (*MAT $\alpha$* ) and 1D (*MAT $\alpha$* ), mating and sporulation yielded marker removal at a rate of  $\sim 10^{-3}$ , which is in agreement with previous findings ([11], P. Philippsen, personal communication). Although screening of this recombination event is somewhat time-consuming, once one haploid  $\Delta ho$  null is isolated, congenic partners can be easily constructed by classical genetic techniques. The removal of the *KanMX* marker also has the benefit of simplifying the design of subsequent gene disruption vectors and transformant screening, since the same selectable marker cassette can be used. The flanking sequences of new disruption constructs need only be changed to provide homology to the target gene, thereby correctly targeting the integration event. The need to establish parameters for selection according to an alternate antibiotic is also avoided.

The second group of important outcomes from this work relates to the similarity of the fermentative performance of the haploid derivatives compared to the parental wine yeast strains. Generally, fermentation proceeded at highly comparable rates (see Fig. 4). The exception, an extended lag seen for derivatives of CY3079, is likely to have arisen from inoculation inconsistencies caused by the flocculant nature of these haploids (data not shown). Nevertheless, the failure of any of the haploids to ferment faster than the diploid parental strains is at odds with Salmon's findings for genetically reduced and hence physically smaller cells such as haploids [35]. Such cells are suggested to ferment more effectively, due to their larger surface area-to-volume ratio and presumably improved transmembrane movement of nutrients and wastes, compared to larger (e.g. diploid and triploid) cells. Such benefits, however, were not apparent in our study. Future work must accurately determine cell sizes and define any differences in membrane functionality to resolve this issue.

In addition to comparable fermentation kinetics, some of the haploid strains produced major metabolites in amounts closely matching those of the parental strains. Notably glycerol and particularly ethanol yields were little changed between strains. Such findings are in keeping with the indications that the *HO* locus is a phenotypically neutral site [12,36]. Where larger differences were seen be-

tween the parental strains and their derivatives, especially in terms of acetic acid, acetaldehyde, succinic acid and  $SO_2$ , the differences are unlikely to be sensorially significant per se [37]. Whether such variations might be important through the involvement of these metabolites in reactions leading to other sensorially important products remains to be determined.

The findings of this study indicate that the reduced genetic makeup of most of the haploid strains studied here is sufficient to produce a close approximation of the gross fermentative properties of the parental strain. Future work will provide a fuller evaluation of such haploid wine strains and their metabolic relevance compared to the originating commercial strains.

#### Acknowledgements

This project is supported by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research Development Corporation, with matching funds from the Federal Government (Project UA99/1). We thank Lallemund for providing commercial wine yeast strains, and Kate Poole for strains KP2 and KP3.

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