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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Effects of nitrogen catabolite repression and di-ammonium phosphate addition during wine fermentation by a commercial strain of *S. cerevisiae*

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Abstract Two deletion mutants expected to be defective in nitrogen catabolite repression (NCR) were constructed in a commercial wine yeast background M2: a *ure2* mutant and a *dal80 gzf3* double mutant. Wild-type and both mutant strains were fermented in Sauvignon Blanc grape juice with and without addition of di-ammonium phosphate (DAP). The *dal80 gzf3* double mutant exhibited a long fermentative lag phase, which was offset by DAP addition (corresponding to 300 mg/L of N). Neither the NCR mutations nor DAP addition affected the content of volatile thiols in the final wine. Microarray analyses of transcripts in the wild-type and *dal80 gzf3* double-mutant strains were performed after 2% and 70% sugars were fermented. Of 80 genes previously identified as NCR-regulated, only 13 were upregulated during fermentation of the *dal80 gzf3* double-mutant strain in grape juice. Following DAP addition, 34 of the known NCR genes were downregulated, including 17 that were downregulated even in the NCR mutant strain. The results demonstrate an unexpected complexity of the NCR response that may reflect differences between strains of yeast or differences in gene regulation during alcoholic fermentation compared with standard aerobic growth.

Keywords *Saccharomyces cerevisiae* · Wine · Fermentation · Nitrogen catabolite repression · Microarrays · Varietal thiols

Introduction

Nitrogen supply strongly influences yeast growth and metabolism during fermentation (Beltran et al. 2005). The concentration and ratio of nitrogenous compounds present in grape juice depend on grape variety, harvest time, and vineyard management factors. Yeast assimilable nitrogen (YAN) includes ammonium and the α-amino nitrogen of amino acids (excluding proline, which is not used by yeast as a nitrogen source under anaerobic conditions). Proline and arginine are usually the most abundant nitrogenous compounds in grape juice (30–65% of total amino acid content) and ammonium makes up ca 40% of grape juice YAN (Beltran et al. 2004).

Growth of yeast cells under enological conditions is often considered to be nitrogen limited, and nitrogen deficiency is a major cause of stuck or sluggish fermentations. This deficiency can be ameliorated by supplementation with di-ammonium phosphate (DAP), an ammonium source (Salmon 1989). However, it is also known that high nitrogen (e.g. 480 mg L^{-1}) can result in undesirable aromas (Bell and Henschke 2005). The mechanisms by which DAP additions affect fermentation kinetics and wine sensory properties are not well understood.

DAP is a preferred nitrogen source for yeast that induces nitrogen catabolite repression (NCR), inhibiting the transcription of genes required for the use of poor nitrogen sources (Magasanik and Kaiser 2002). Once preferred sources become limited, the genes for uptake and utilisation of alternative sources are subsequently de-repressed.

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NCR involves transcriptional regulation by four members of the GATA family of transcription factors, as well as the regulatory protein Ure2p (Cooper 2002). Tight control of activator and repressor levels is necessary to regulate NCR, and three of the GATA factors regulate their own expression of themselves and that of each other. *GLN3* is an exception, as transcriptional regulation of this gene has not been demonstrated (Cooper 2002). Gat1p and Gzf3p are the main regulators in NCR conditions, whereas Gln3p and Dal80p are the main regulators in derepressed conditions (Hofman-Bang 1999).

The stimulus to induce NCR is the presence of a preferred nitrogen source in the medium (Magasanik and Kaiser 2002). A glutamine signal activates Ure2p, which binds Gln3p, retaining it in the cytoplasm and preventing the activation of nitrogen-regulated genes (Beck and Hall 1999; Blinder et al. 1996). This activation occurs in the presence of ammonium, glutamine or asparagine, but not with glutamate, as the glutamine concentration is too low when glutamate is the sole nitrogen source (Courchesne and Magasanik 1988). A glutamate signal retains Gat1p in the cytoplasm, allowing Gzf3p to repress nitrogen-regulated genes (Stanbrough et al. 1995). *GZF3* deletion results in inappropriate activation of nitrogen-responsive genes by Gat1p with glutamate or glutamine (Rowen et al. 1997). This signal occurs when glutamate and glutamine are the nitrogen source. However, with ammonium, the glutamate concentration is too low to repress Gat1p, since $NADP^+$-linked glutamate dehydrogenase (encoded by *GDH2*) catalyzes the rate-limiting step to produce glutamate, which is rapidly converted to glutamine via glutamine synthetase (Magasanik and Kaiser 2002). Therefore, neither ammonium nor glutamate induces full NCR, since they repress only Gln3p or Gat1p, respectively.

Gln3p and Gat1p become active upon depletion of preferred nitrogen sources and enter the nucleus to activate *GAT1* expression. *GAT1* expression is also autogenously stimulated and overcomes inhibition by *GZF3*, which is expressed constitutively at low level (Rowen et al. 1997). Gln3p and Gat1p together activate nitrogen-regulated genes. However, they also induce *DAL80* expression, resulting in down-regulation of GATA factor-regulated genes including *GAT1*. This quickly brings the system to a steady state (Cooper 2002). Therefore, Dal80p seems to serve as a negative feedback loop in de-repressed conditions for setting an appropriate maximum level of nitrogen-regulated gene expression (Magasanik and Kaiser 2002). A *dal80* mutant has greatly elevated expression of Gln3p- and Gat1p-activated genes in de-repressed conditions, but not in NCR conditions.

Nitrogen regulation based on nitrogen source affects the expression of almost 10% of yeast genes (Godard et al. 2007). GATA factor target genes are induced during growth on poor nitrogen sources and include core nitrogen metabolism genes (*GDH2*, *GLN1* and *GLT1*), permease genes (*GAP1* and *MEP2*) and catabolic pathway genes, including those for utilisation of proline, arginine and urea (Hofman-Bang 1999; Beltran 2005). Loss of NCR is linked to the activation of amino acid biosynthetic pathways, known as "general control of amino acid biosynthesis". This process is mediated by the transcription factor Gcn4p, which is regulated by NCR (Godard et al. 2007).

Although NCR regulation during growth on many different nitrogen sources has been investigated in depth (Godard et al. 2007), the regulation of this process during fermentation is not well characterised. During wine fermentation, the change from a nitrogen-repressed to a nitrogen-de-repressed condition occurs as nitrogen is consumed, affecting ammonium and amino acid consumption (Beltran et al. 2004). Beltran et al. (2004) investigated wine fermentation and nitrogen permease gene expression in control (300 mg L^{-1}), high (1,200 mg L^{-1}) and low (60 mg L^{-1}) nitrogen conditions (nitrogen consumption during control (CNC), high (HNC) and low (LNC), respectively). The LNC and CNC fermentations shifted from a nitrogen-repressed to a nitrogen-de-repressed condition as nitrogen was consumed, whereas NCR was maintained throughout the HNC fermentation. During NCR, arginine and alanine uptake was inhibited and consumption of glutamate, aspartate and glutamine was reduced. Therefore, arginine and alanine must be predominantly transported by Gap1p or other specific permeases repressed during NCR. Ammonium uptake was higher in the HNC fermentation despite higher repression of ammonium permease (*MEP*) genes. Marini et al. (1997) proposed two hypotheses to explain this effect: either yeast possess an unknown alternative ammonium transport system, or ammonium may simply diffuse into yeast cells.

The effect of DAP addition on gene expression during different stages of wine fermentation has been investigated using microarrays (Marks et al. 2003). DAP addition after 30% sugar consumption, affected the expression of 350 genes in the commercial wine yeast strain VIN13. Nitrogen catabolite and small molecule transporter genes were down-regulated (e.g. *GAP1*), whereas genes involved in amino acid metabolism, sulfate assimilation, purine biosynthesis and ribosomal protein biosynthethesis were upregulated. These gene expression changes have implications for fermentation kinetics and wine aroma.

Thiol production has been linked to juice nitrogen levels and high nitrogen during fermentation is thought to reduce the thiol content of the wine, possibly via NCR. Volatile thiols are key aroma compounds in Sauvignon Blanc wine. Three major thiols provide the distinctive character of Sauvignon Blanc wine: 4-mercapto-4-methylpentan-2-one (4MMP) (cat's pee or broom), 3-mercaptohexanol (3MH)

(grapefruit) and 3-mercaptohexyl acetate (3MHA) (passionfruit) (Tominaga et al. 1998a; Darriet et al. 1995; Dubourdieu et al. 2006). NCR occurs during DAP addition, and appears to directly influence thiol production. Thibon et al. (2008) and Subileau et al. (2008b) found evidence that NCR represses thiol release in synthetic medium with added *S*-cysteine conjugate thiol precursors. However, recent evidence suggests that this compound may not be the major precursor (Subileau et al. 2008a; Grant-Preece et al. 2010; Kobayashi et al. 2010). The role of NCR with respect to thiol release in grape juice remains unclear.

This study investigated the influence of DAP addition on yeast nitrogen metabolism, nitrogen-regulated gene expression and thiol production during Sauvignon Blanc wine fermentation. Deletion mutants of a commercial wine yeast strain were constructed in nitrogen regulatory genes, in order to assess their effect on thiol release, and nitrogen regulation and metabolism, during fermentation. Global gene transcription was analysed during fermentation via microarray profiling.

Materials and methods

Strains and culture conditions

The *Saccharomyces cerevisiae* strains used in this study are listed in Table 1. Two yeast backgrounds were used: the standard lab yeast (based on S288C), and a widely used commercial wine yeast called Enoferm M2. A homosporic derivative of M2, called M2h, was obtained by two rounds of tetrad dissection (H. Niederer, unpublished). A haploid *ura3* line of this derived M2 was obtained by two standard homologous replacements, firstly of the *HO* gene with a hygromycin-resistant HGM-MX2 cassette, and subsequently of the *URA3* gene with the *ura3-del0* mutation from BY4743 (H. Niederer, unpublished).

Standard rich medium, yeast-peptone-dextrose (YPD; 1% yeast extract, 2% peptone, 2% glucose and 2% agar for solid medium), was used for routine culture. Minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose and 2% agar) was used to test for auxotrophies or to select for prototrophs. For antibiotic selection, 300 μg mL^{-1} hygromycin or 200 μg mL^{-1} G-418 was added to YPD, or to minimal medium without ammonium sulfate (0.17% yeast nitrogen base without amino acids and without ammonium sulfate, 0.1% sodium glutamate, 2% glucose and 2% agar). Fermentations were conducted in 2007 Sauvignon Blanc grape juice, obtained from a Pernod Ricard vineyard located at Rapaura in Marlborough, New Zealand (kindly supplied by Andy Frost). Frozen 2-L bottles of juice were thawed at room temperature and sterilised with an overnight incubation with 200 μL L^{-1} dimethyl dicarbonate (DMDC) at 25°C with shaking. Initial starter cultures of yeast were grown in YPD at 28°C with shaking for 24 h. Cell counts were conducted for these cultures using a haemocytometer. Yeast was inoculated into 200 mL of grape juice at a final concentration of 1×10^6 yeast cells mL^{-1}, in 250-mL flasks with side-ports and sealed with air locks. Three diploid derivatives of a commercial wine yeast, Enoferm M2 (wild type, *dal80 gzf3* double mutant and *ure2* mutant), were fermented in three conditions (juice, juice with added DAP (300 mg L^{-1} N) and juice with added urea (300 mg L^{-1} N). Fermentations were conducted in triplicate, resulting in a total of 27 samples and three uninoculated controls. Ferments were conducted at 25°C with 100 rpm shaking for up to 7 days. Fermentation progress in each flask was monitored via weight loss. Weight loss curves were corrected for sampling, by weighing the flasks before and after the aliquots were taken.

Cell samples were taken from wild-type and *dal80 gzf3* double-mutant ferments in juice and juice with DAP, at two time points, corresponding to (1) rapid nitrogen consumption in exponential growth (defined as 2% of total weight loss, referred to as "exponential" samples) and (2) nitrogen depletion of standard juice when cell growth had stopped (defined as 70% of total weight loss, referred to as "stationary" samples). Cell counts were conducted with a haemocytometer and showed no significant differences between the ferments either at exponential growth (4.53±

Table 1 List of *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
BY4743 (S288c) deletion strains	Three diploid deletion strains homozygous for either *URE2*::*kanMX4*, *DAL80*::*kanMX4* or *GZF3*::*kanMX4*	(Giaever et al. 2002)
M2h	Wild-type diploid (homosporic derivative of commercial wine yeast Enoferm M2)	H. Niederer (this laboratory)
Haploid M2h	*HO*::hgmMX4; *ura3Δ0; MATα*	H. Niederer (this laboratory)
ure2 mutant M2h	M2h diploid homozygous for *URE2*::*kanMX4*	This work
dal80 gzf3 double mutant M2h	M2h diploid homozygous for *DAL80*::*kanMX4*, *GZF3*::*kanMX4*	This work

0.05×10^7 cells/mL; mean±SD) or at stationary phase ($2.23\pm0.09\times10^8$ cells/mL). Duplicate samples of 1×10^8 cells were harvested by centrifugation, snap frozen in liquid nitrogen and stored at −80°C for microarray analysis. When the fermentation was complete, the resulting wines were harvested via centrifugation of the yeast and storage of the supernatant at −20°C.

Oligonucleotides

Sequences of primers used in this work are provided in Table 2. Pairs of forward (F) and reverse (R) oligonucleotide primers were used to amplify from 300 bp upstream and downstream of the NCR regulatory genes *DAL80*, *GZF3* and *URE2* (integration primers). Primers flanking the integration primer pairs (test primers), were used to confirm insertion. Internal kanamycin resistance (Kan^R) gene primers (F and R) were used in combination with test primers to confirm the presence of the correct junction fragments.

Construction of the *ure2* and *dal80 gzf3* deletion strains

Specific BY4743 deletion strains (*ure2*, *dal80*, *gzf3*) were the source of DNA for polymerase chain reactions (PCRs) to generate knock-out constructs in the wine yeast. The yeast strain used for primary transformation was a marked haploid strain of M2h (*MATα*, *ho*::hgm^R and *ura3Δ0*), which had been previously backcrossed to the M2h strain to remove any potential secondary mutations introduced during strain construction. Transformants were selected on YPD medium with G-418. Kan^R gene insertion at the expected locus was confirmed via PCR, using an internal Kan^R gene primer (F) and a test primer external to the introduced fragment (R).

Table 2 Oligonucleotide primers used in this study

Name	Sequence (5′ to 3′)
DAL80 integration	
F	TCACCCGTTTGTCGTTGATA
R	TGAGGCTTTATTGGCTTGCT
DAL80 test	
F	CGGTCGTGCCTATAATGTCC
R	TGTCCAGTTTTGCACTTTGC
GZF3 integration	
F	CTGCCCCTTTCTGAGTTTGA
R	GAGGGAGAGGTATGCCAATG
GZF3 test	
F	CCCGTCATCGCTGAGTTATT
R	GCTTTGAACAGTGCCACAGA
URE2 integration	
F	ACCATAGAACGCCGAAACAG
R	TGAACAAAAACCGTTTGCAT
URE2 test	
F	GCACTACATGCGGAGAATCA
R	TCGGGAAATCTTGCCTTAAA
Kan^R	
F	GGTCGCTATACTGCTGTC
R	CCTTGACAGTCTTGACGTGC

F forward, *R* reverse

Putative *ure2*, *dal80* and *gzf3* mutants of haploid M2h with Kan^R insertions (Kan^R, Hgm^R, *ura3*) were crossed with the homozygous wild-type diploid strain M2h (Kan^s, Hgm^s and Ura^+). The crosses were streaked onto minimal medium (without ammonium sulfate) with 300 μg mL^{-1} hygromycin and 200 μg mL^{-1} G-418, to select for F1 hybrids (Kan^R, Hgm^R and Ura^+).

To generate a homozygous *ure2* mutant in an otherwise wild-type M2h background, the F1 hybrid (*URE2 ure2*::KAN, *HO ho*::HGM, *URA3 ura3Δ0*) was sporulated, random spores were plated at low density onto solid YPD medium to allow auto-diploidizing of HO progeny, and colonies that were Kan^R, Hgm^S and Ura^+ were selected by replica plating. PCR was used to confirm the presence of a homozygous *ure2*::KAN locus and to exclude the presence of the wild-type gene in the final lines.

To generate a *dal80 gzf3* double mutant, the F1 crosses of *dal80* and *gzf3* were similarly sporulated and random spore progenies isolated. In the case of *DAL80*, a diploid Ura^+ progeny carrying a homozygous *dal80*::KAN mutant was isolated, while for *GZF3* a Ura^- haploid (Hgm^R) progeny with the *gzf3*:KAN mutant was isolated. These two progeny were then crossed (with selection for Hgm^R Ura^+ hybrids), sporulated, and colonies that were Kan^R, Hgm^S and Ura^+ were screened by PCR to identify double mutants and to exclude the presence of the wild-type genes. The resulting strain was homozygous diploid for both the *gzf3* and *dal80* mutations in an otherwise wild-type M2h strain.

YAN measurement

YAN was quantified using the "ammonium" and "1° amino nitrogen-$_{BL}$" Unitab™ reagent kits (Unitech Scientific, CA), following the manufacturer's instructions. Measurements were taken of the starting juice, as well as at the end of fermentation using wine clarified by centrifugation.

Thiol measurement

Thiol extraction followed a modified method of Tominaga et al. (1998a,b), as described by Lund et al. (2009). Thiols were quantified using a gas chromatograph (Agilent 6890N) with a 5973-inert mass selective detector (Agilent, Santa Clara, CA). Internal standards of deuterated 4MMP,

3MHA and 3MH were added to all samples prior to extraction, and the sample thiol concentration (ng L^{-1}) was assessed using a standard curve.

Microarrays

Total RNA was extracted from frozen yeast samples of 1×10^8 cells using a modification of the hot phenol method (Collart and Oliviero 1995). Pelleted yeast cells were resuspended in 400 μL TES solution (100 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.5% SDS). Buffer-saturated phenol (400 μL) was added to each sample. Samples were vortexed vigorously for 10 s and incubated at 65°C for 1 h, with further vortexing for 10 s at 15-min intervals. Samples were placed on ice for 5 min, centrifuged for 10 min at 12,000×*g*, 4°C, and the aqueous layer was transferred to a new tube and extracted with chloroform (400 μL) in the same fashion. A 1/10 volume of 3 M sodium acetate pH 5.2 was added, followed by 2.5 volumes of ice-cold 100% ethanol. Samples were precipitated at −80°C for 45 min and centrifuged for 30 min at 12,000×*g*, 4°C. The supernatant was removed and the pellet was washed in 200 μL of ice-cold 70% ethanol. The samples were centrifuged for 10 min at 12,000×*g*, 4°C and pellets were resuspended in 80 μL of 100% formamide.

The RNA concentration of the extracted samples was assessed using the NanoDrop® spectrophotometer ND-1000, following the manufacturer's instructions (NanoDrop products, Wilmington, USA). The RNA samples were subsequently analysed using the Agilent 2100 Bioanalyzer, following the manufacturers' instructions (Agilent, Santa Clara, CA), to further assess the RNA quality. In order to remove the formamide prior to microarray analysis, a subset of each RNA sample was reprecipitated using ethanol precipitation and resuspended in DMDC-treated water (15 μL). The RNA concentration of each sample was reassessed using the NanoDrop® spectrophotometer ND-1000 and the samples were stored at −80°C.

The RNA samples were subjected to poly $(A)^+$ RNA purification, cDNA synthesis and biotin-labelled cRNA synthesis and fragmentation procedures, as described in the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). The cRNA samples were hybridised to Affymetrix GeneChip® Yeast Genome 2.0 Arrays (Affymetrix, Santa Clara, CA) and processed as described by the manufacturer (Eukaryotic Arrays GeneChip Expression Analysis and Technical Manual, Affymetrix, Santa Clara, CA).

Microarray data analysis

Differential expression analysis of the microarray data (Smyth 2004; Smyth et al. 2005) was conducted using the Limma package from Bioconductor (Smyth 2005; Gentleman et al. 2004). Normalisation of the data via robust multiarray averaging and computation of a linear model fit was applied using this programme. Various contrasts between pairs of conditions were conducted in order to identify a list of differentially expressed genes for each contrast. A gene was considered to show significant differential expression if the *M* value was ≥1 or ≤−1, and the *P* value (adjusted for false discovery rate using the Benjamini and Hochberg (1995) method) was <0.01. The probe sets were linked to their target gene descriptions using NetAffx (www.affymetrix.com/analysis/index.affx) and the *Saccharomyces* Genome Database (http://www.yeastgenome.org).

Results

Experiments were conducted with a widely used commercial wine strain of yeast. Enoferm M2 has the same DNA fingerprint as, and is likely very similar or identical to, other commercial strains known as WE372 (Anchor), W15 (Lalvin), Tipico (Oenoferm) (Bradbury et al. 2006; Richards et al. 2009) as well as AWRI796 (Maury). All these strains have homozygous microsatellite fingerprints, so that the homosporic derivative used here is expected to be isogenic with, and show similar fermentation properties to, the original commercial line. The fermentation kinetics of, and the wine produced by, the derivative strain M2h were indistinguishable from that of the commercial parent (data not shown).

NCR mutants were constructed in a commercial wine yeast strain

Two NCR mutants were constructed in the M2h background (Table 1). *S. cerevisiae ure2* mutants have previously been analysed during fermentation (Salmon and Barre 1998; Thibon et al. 2008). We also constructed a *dal80 gzf3* double mutant, which was expected to show constitutive expression of NCR genes at high levels (Godard et al. 2007). Both mutants were obtained in an otherwise wild-type, diploid, commercial wine strain by transferring deletion constructs from the laboratory strain and subsequent back-crossing to wild-type M2h (see "Materials and methods"). This back-crossing process also helped to remove phenotypic noise associated with transformation, which can be mutagenic.

DAP affected the fermentation behaviour of an NCR mutant strain

Fermentation was undertaken with three M2h strains: wild-type, *ure2* and the *dal80 gzf3* double mutant. Three nitrogen conditions were used: Sauvignon Blanc grape juice (YAN=262 mg L^{-1}, see below), juice with added DAP (+300 mg L^{-1} of N, to give a final YAN of

562 mg L^{-1}) and juice with added urea (+300 mg L^{-1} of N; urea is not expected to induce NCR, Godard et al. 2007).

Figure 1 shows the average fermentation kinetics for the three strains grown in grape must with different nitrogen conditions. The addition of 300 mg L^{-1} N as DAP or urea caused only a slight improvement in the fermentation rates for wild-type M2h and the *ure2* mutant. The most significant difference in fermentation kinetics was that the *dal80 gzf3* double mutant displayed an extended lag phase (40–45 h, compared with ~18 h for wt and ~22 h for the *ure2* mutant). The addition of DAP, but not urea, largely reversed the lag phase difference in the double deletion strain, producing a lag phase similar to that of the *ure2* mutant.

Nitrogen uptake in NCR mutants

Figure 2 shows the final 1° amino nitrogen and ammonium concentrations for the fermentations. The initial concentrations in the juice were measured as 176 mg L^{-1} of 1° amino nitrogen and 86 mg L^{-1} of ammonium nitrogen, so that the initial YAN content of the juice was estimated to be 262 mg L^{-1}.

By the end of the M2h fermentation, all ammonium had been consumed in juice without DAP. Therefore, lifting of NCR is predicted to occur during this fermentation. In contrast, DAP addition resulted in some ammonium remaining at the end of the fermentation; therefore, NCR is expected to be maintained throughout the fermentation. Some 1° amino nitrogen remained in the standard M2h fermentation (around 10% of the initial concentration). DAP addition decreased usage of amino nitrogen, so that around 50% of the starting concentration remained in the medium at the end of fermentation.

In contrast, the results for the *ure2* mutant and the *dal80 gzf3* double mutant of M2h did not follow simple predictions. In normal juice, both mutant strains took up slightly less 1° amino nitrogen than wild type (Fig. 2), rather than the predicted increase in uptake (assuming that the 1° amino nitrogen pool has a significant contribution from non-preferred N sources). The addition of DAP to juice resulted in their being more 1° amino nitrogen left in the medium by the two mutants, but their uptake after DAP addition was greater than that of wild-type M2h (Fig. 2). As expected, with DAP addition, both NCR mutants took up less ammonium than wild type, particularly the *ure2* mutant (Fig. 2).

Thiol production

We measured the production of aromatic varietal thiols in the wine made from Sauvignon Blanc juice, as thiol yields have previously been linked to nitrogen supply (Thibon et al. 2008; Subileau et al. 2008b).

Figure 3 shows the 3MH and 3MHA content of wines produced by wild-type, *ure2* mutant and *dal80 gzf3* double-mutant M2h strains in different nitrogen conditions. There were only minor differences in thiol production between the three strains and the three nitrogen conditions. The 3MHA and 3MH concentrations ranged from 558 to 691 and 4,285 to 5,731 ng L^{-1}, respectively. These concentrations are well above the sensory thresholds for 3MHA and 3MH (4 and 60 ng L^{-1}, respectively) (Tominaga et al. 1998b). No 4MMP was detected in any of the samples (data not shown).

DAP added to juice caused slight decreases in total 3MH production for the three strains; however, a similar decrease was also observed with the addition of urea, which does not

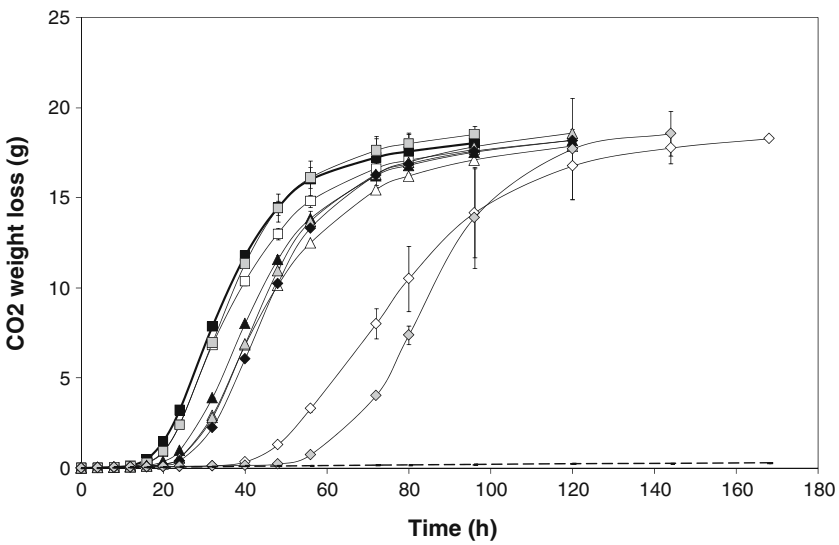

Fig. 1 The *dal80 gzf3* double mutant displayed a long lag phase which was offset by DAP addition. This graph shows weight loss versus time of wild-type (*square symbols*), *ure2* mutant (*triangle symbols*) and *dal80 gzf3* double-mutant (*diamond symbols*) M2h strains during fermentation in three nitrogen conditions: juice (*open symbols*), juice+DAP (300 mg/L of N) (*black symbols*) and juice+urea (300 mg/L of N) (*grey symbols*). The uninoculated control is shown in a dotted line. (n=3, *error bars* show SEM)

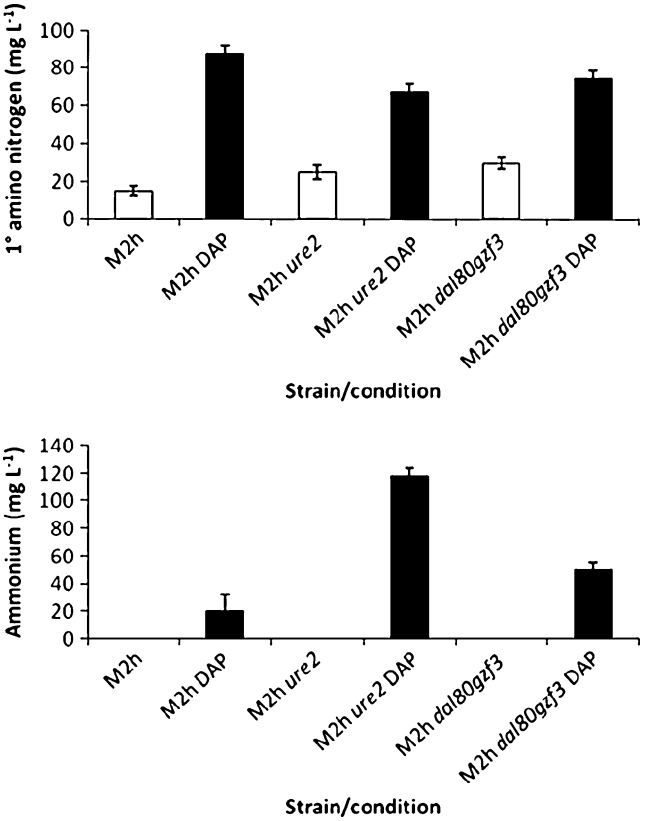

Fig. 2 The strains differed in usage of 1° amino nitrogen and ammonium. The figure shows the concentration of 1° amino nitrogen and ammonium present in wine at the end of fermentation by three yeast strains (M2h, M2h *ure2* and M2h *dal80gzf3*), each with (*filled bars*) and without (*open bars*) the addition of 300 mg L^{-1} nitrogen as DAP. Values are the average of triplicate ferments, with standard error indicated. Ammonium was below the limit of detection at the end of ferments without added DAP

induce NCR (Magasanik and Kaiser 2002). We conclude that, for the M2h yeast strain, NCR does not appear to affect production of 3MH and 3MHA during fermentation in Sauvignon Blanc grape juice.

Regulation of gene expression during fermentation

The M2h transcriptome was assessed by microarray analysis at two time points, representing 2% and 70% of sugar conversion, respectively, representing exponential and stationary phases of cell growth. Both wild-type and the *dal80 gzf3* double mutant were assessed, fermented in juice with or without added DAP. In total, eight samples were analysed in triplicate, except for two samples (*dal80 gzf3* double mutant at exponential phase, and *dal80 gzf3* double mutant with added DAP at exponential phase) for which only two replicates gave acceptable signal:noise ratios (data not shown).

Comparison of gene expression data were undertaken between the eight sample conditions, using the criteria of adjusted P value<0.01 and log(2)fold change >1 or <−1. As expected, the *dal80 gzf3* double mutant showed complete loss of expression of both deleted genes in all conditions (see Table S1 in the Electronic supplementary material), combined with very high expression of the kanamycin resistance gene (the kanMX cassette was used to replace both genes).

The comparisons showing the greatest changes were between the exponential and stationary phase samples—within strain comparisons showed 1,970, 2,546, 2,010 and 1,801 genes with altered expression for the wt, wt+DAP, *dal80gzf3*and *dal80gzf3*+DAP fermentations, respectively (Tables S3, 4, 5 and 6, respectively, in the Electronic supplementary material). A total of 1,314 gene changes were common to all four comparisons (Table S7 in the

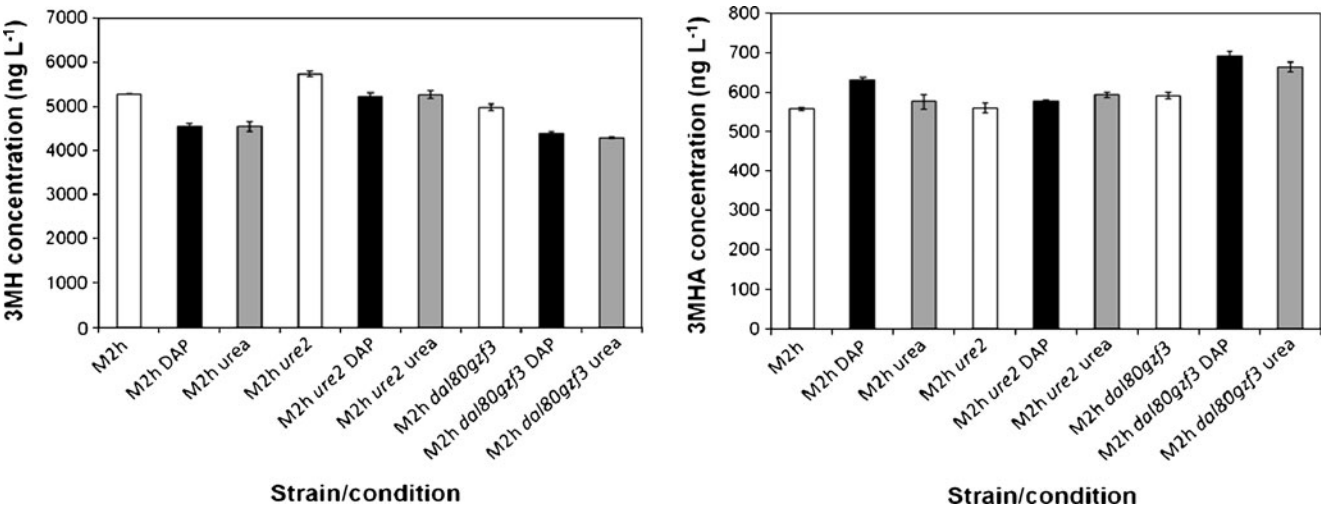

Fig. 3 Thiol production showed no significant differences in NCR mutants or after the addition of DAP (*dark bars*) or urea (*grey bars*) to 300 mg L^{-1}. Strains and treatments are as in Fig. 1

Electronic supplementary material). This result is consistent with previous findings that the switch from exponential growth to non-growing cells represents the largest shift in gene expression during fermentation (Rossignol et al. 2003; Marks et al. 2008).

Consistent with previous results (Rossignol et al. 2003; Marks et al. 2008), the most highly downregulated genes at stationary phase were the rRNA genes, a large number of ribosomal protein transcripts and other genes involved in protein synthesis. Cell cycle genes and several genes in the sulphur pathway were also downregulated. Genes that were upregulated late in fermentation (apart from nitrogen-related genes considered separately below) included many fermentation stress genes (Marks et al. 2008), 37 of 74 genes that have been identified as upregulated at stationary phase of cultures grown in YPD broth (Martinez et al. 2004), and several genes for thiamine biosynthesis (*THI4*, *THI13*, *THI22* and *THI73*).

Regulation of gene expression by NCR

To identify NCR-related changes in gene expression during fermentation, we monitored 80 transcripts from the list of 85 known NCR-regulated genes compiled by Godard et al. (2007) (see Table S2 in the Electronic supplementary material and Table 3). To test whether NCR effected changes in expression of these 80 genes, the 'null' hypothesis of no change was tested against three specific predictions:

1. H_0: Expression of NCR-regulated genes in the *dal80 gzf3* double mutant would not differ significantly from that of the wild-type strain, in exponential phase when ammonium is still present for both strains.

This comparison showed 27 genes differentially expressed, with 18 upregulated in the double mutant (Table S8 in the Electronic supplementary material). Of these 18, 12 were known NCR-regulated genes (shown in Table 3, left results column), including all 11 of the most highly upregulated genes (from *DCG1* [upregulated by a factor of $\log_2$=5.24] to *MEP2* [2.22]). No known NCR-regulated genes were among the nine genes repressed in the *dal80 gzf3* double mutant. The enrichment of NCR-regulated genes in the upregulated subset led us to conclude that the double mutant had altered NCR regulation during the early stages of fermentative growth, and this null hypothesis was therefore rejected.

2. H_0: Ammonium addition to the wild-type strain would not significantly down-regulate the expression of known NCR-regulated genes late in the ferment. Stationary phase comparisons of the wild-type strain with and without DAP showed 70 genes changed in expression level, of which 29 were downregulated (Table S9 in the Electronic supplementary material). The top five genes downregulated by DAP addition were all known NCR-related genes, ranging from *GAP1* (−4.97) to *DIP5* (−3.46). In all, 16 known NCR-regulated genes were repressed by DAP addition to the wild-type M2h strain, whilst none were induced (Table 3). This difference led us to conclude that DAP addition does affect NCR-regulated genes late during fermentation, and this null hypothesis was also rejected.
3. H_0: Expression of known NCR-regulated genes would not change in comparisons between exponential and stationary phases of the wild-type ferment. In total, 1,991 genes were regulated in stationary phase in the wild-type strain, with 1,003 induced and 988 repressed. Table 3 shows that this number included 34 known NCR-regulated genes that were upregulated and six that were downregulated. This imbalance is also significant, and this null hypothesis was therefore rejected.

These three comparisons all resulted in rejection of the null hypothesis, and led to the conclusion that at least some components of NCR regulation are operating during fermentation. One NCR-regulated gene, *GAP1*, fulfilled all three expectations described by the hypotheses above; that is, transcripts were upregulated in the NCR mutant strain, downregulated by DAP addition to the wt strain, and induced at stationary phase in the wt strain (Table 3, top row). A further five genes showed the expected regulation in all these three comparisons, but in addition were down-regulated by DAP even in the *dal80 gzf3* double mutant (Table 3, next five rows; discussed in the following section). Another 36 NCR-regulated genes showed various combinations of transcriptional changes consistent with NCR regulation in one or more comparisons. However, six known NCR genes (bottom of Table 3) showed transcript regulation in the opposite direction to that predicted—they were downregulated late in the ferment when nitrogen was exhausted from the medium. In addition, a further 33 previously identified NCR genes showed no significant changes in transcript levels in any of these four comparisons.

A stationary phase comparison between the *dal80 gzf3* double mutant and wild-type strains revealed 141 genes showing a strain difference, including eight NCR-regulated genes that were upregulated in the *dal80 gzf3* double mutant compared with wild type at stationary phase (*DAL2*, *DAL3*, *DAL5*, *DAL7*, *DCG1*, *DUR3*, *NPR2* and *UGA4*) (data not shown). These changes may reflect the extra upregulation expected in the *dal80 gzf3* double mutant compared with wild type, because the DAL80 and GZF3 proteins normally act to dampen expression levels of genes expressed when NCR is lifted.

Table 3 Expression changes in genes whose transcript levels are affected by NCR and/or DAP (numbers refer to $\log_2$ change in expression)

Gene[a]	Strain difference	DAP repression (stat)		Stat phase (wt)	
	dg/wt (exp)[b]	wt-dap/wt	dg-dap/dg	Up	Down
GAP1	2.53	−4.97		2.05	
DAL3	5.03	−1.79	−3.31	3.05	
DAL5	2.78	−1.40	−3.56	2.95	
MEP2	2.22	−2.62	−1.54	2.52	
PUT1	4.20	−4.76	−3.80	6.57	
UGA4	2.44	−2.23	−3.38	4.72	
DAL2	3.04		−2.22	2.04	
DAL7	5.03		−3.49	1.72	
DCG1	5.24		−3.26	3.18	
DUR3	1.76		−3.49		
DAL4	2.98			2.25	
DAL1	4.74				
DAL80		−3.64		7.18	
PTR2		−3.21		1.97	
PUT2		−2.65		2.33	
PUT4		−4.54		3.30	
YGK3		−2.40		2.38	
OPT2			−2.70	3.69	
DIP5		−3.46	−2.57		
YGR125W		−2.52	−2.58		
CAN1		−3.00			
GLT1		−2.19			
VBA1		−2.31			
ATG14				2.53	
AVT4				1.38	
ECM37				2.54	
GAT1				2.24	
GDH2				2.21	
GDH3				3.45	
HXK1				2.02	
LEE1				3.93	
MOH1				5.57	
PRB1				2.13	
UGA3				1.26	
UGX2				4.71	
VID30				2.37	
YHR138C				5.04	
YJR011C				1.57	
YLR149C				4.26	
YLR257W				2.18	
YOR052C				2.86	
AGP1					−4.47
GDH1					−3.71
GGC1					−2.19
OPT1					−2.58
RPS0B					−3.76
SLX9					−1.47

[a] The 47 genes listed were classified by Godard et al. (2007) as NCR-regulated either type A (bolded) or type P. A further 33 NCR-regulated genes showed no changes in any of the four comparisons above: *AAH1*, *ALD4*, *ARG4*, *AVT1*, *AVT7*, *BAT2*, *CAR1*, *CHA1*, *CPS1*, *DSD1*, *DUR1,2*, *ECM38*, *GCN4*, *GLN1*, *GUD1*, *LAP4*, *LRC1*, *MEP1*, *MEP3*, *MIG2*, *NIT1*, *NPR2*, *NRK1*, *NRT1*, *PEP4*, *PMP1*, *RNY1*, *RSM10*, *RTS3*, *UGA1*, *URK1*, *YDR090C* and *YHI9*. Expression of *SDL1* was not analysed since it is not on the microarray, and *ASP3-1*, *ASP3-2*, *ASP3-3*, *ASP3-4* are deleted in the M2 strain (Schacherer et al. 2009)

[b] The numbers in each column show the $\log_2$ ratios of expression for each gene in the following comparisons (left to right): the *dal80 gzf3* double mutant (dg) compared with wild type (wt), both measured at 2% weight loss (exponential phase (exp)); the wild-type strain with and without added DAP (both measured at stationary phase=70% weight loss, stat); the *dal80 gzf3* double mutant with and without DAP (both at stationary phase); and wild type at exponential compared with stationary phase. Blank squares indicate comparisons where the changes in expression were not significant (see "Materials and methods")

The effect of adding ammonium

Comparison of the wild-type strain at exponential phase with and without DAP addition, showed no significant differences in gene expression for any NCR genes (or indeed for any other genes, data not shown). The double-mutant strain was also unaffected by DAP addition during exponential growth. These results are not surprising, since good levels of ammonium were present in the grape juice at this time (data not shown).

Unexpectedly, DAP addition proved to have a significant effect on gene regulation even in the *dal80 gzf3* double mutant at stationary phase, confirming the conclusion above that some component of nitrogen regulation was retained in this strain. In total, 12 NCR-regulated genes were repressed by DAP in this mutant strain, seven of which were also repressed by DAP addition in wild-type M2h (Table 3).

To examine whether ammonium addition induced full NCR, we looked at the relative induction of transcript levels in the wild-type M2h strain at stationary phase compared with exponential phase, with and without DAP addition. At 70% weight loss, the M2h fermentation had taken up all of the available ammonium and 1° amino nitrogen, whereas the ferment with added DAP had both nitrogen sources remaining in the medium (Fig. 2). Yet, four known NCR-regulated genes (*DAL2*, *DAL4*, *DAL7* and *DCG1*) were upregulated to a similar degree in M2h with DAP, as they were without added DAP (data not shown). In addition, six further NCR genes still retained some upregulation at stationary phase with DAP addition (*DAL80*, *DAL3*, *DAL5, MOH1*, *PUT1* and *UGA4*), even though they exhibited greater upregulation without DAP.

Discussion

Although NCR during aerobic growth on many different nitrogen sources has been investigated in depth (Godard et al. 2007), nitrogen catabolite repression of gene expression during wine fermentation is only beginning to be characterised. Wine fermentation provides stressful conditions for yeast growth, with high osmotic pressure, anaerobiosis, low pH and ethanol production. In addition, grape juice has different nutritional composition to standard lab media; growth is usually limited by the supply of nitrogen or vitamins, rather than carbon. Because of the importance of nitrogen in wine fermentations, we investigated the effect of NCR mutants and added ammonium on a commercial yeast strain during fermentation.

We fermented a homosporic derivative a widely used commercial wine yeast, M2, in Sauvignon Blanc grape juice containing 262 mg L^{-1} YAN. The lack of a major difference in the maximal fermentation rate following DAP and urea addition suggests that fermentation by M2h was not nitrogen-limited in this juice. It is generally considered that 140 mg L^{-1} YAN is required to avoid stuck fermentations (Beltran 2005), although nitrogen requirements do depend on the yeast strain (Jiranek et al. 1995). Unexpectedly the *ure2* mutant showed a slight lag phase in fermentation, and the *dal80 gzf3* double mutant showed a very long lag phase. The mechanism of the delayed initial fermentation in these mutants is not known, but the fact that it could be overcome by the addition of DAP but not urea suggests that it involves NCR and is a direct result of the deletion of the two NCR genes.

The relatively high level (300 mg L^{-1}) of nitrogen added as ammonium was sufficient to cause incomplete uptake of both 1° amino nitrogen and ammonium at the end of the fermentation (Fig. 2). The DAP addition therefore served the experimental purpose of changing NCR. DAP addition resulted in an overall more ammonium uptake and less 1° amino nitrogen uptake for all three strains, similar to the results of Beltran et al. (2005). In addition, the *dal80 gzf3* mutant consumed less ammonium than wild-type M2h. Of the three known genes for ammonium uptake, only *MEP2* showed significant transcript regulation in these experiments. *MEP2* transcription was induced in the double mutant compared with the wild type during exponential growth and this difference was not affected by DAP addition; at stationary phase both wild-type and the double mutant strains showed very high *MEP2* transcript expression, with DAP repressing this induction equally in both strains (data not shown). These transcriptional differences are consistent with expectations for NCR regulation of *MEP2*, but are in the wrong direction to explain the ammonium uptake differences. Moreover the MEP2 protein has the lowest capacity of the three characterised ammonium transporters (Marini et al. 1997) so is an unlikely candidate in any case. An alternative option is that the reduced ammonium uptake in the *dal80 gzf3* double mutant may represent the metabolic cost of expression of the deregulated NCR genes; however, this seems unlikely to have caused such a large effect. Currently, the only characterised system for high-velocity, low-affinity ammonium uptake by yeast involves Mep1p, which showed no significant transcript changes in these experiments. However, there is an additional uncharacterised ammonia uptake system (Marini et al. 1997) that may be affecting our results, as it may have done for Beltran et al. (2005). One option for such an uptake system may be the aquaporins, which in mammals have been shown to transport ammonia (Saparov et al. 2007).

The uptake of 1° amino nitrogen in grape juice would be predicted to be enhanced in the two NCR mutants, since the uptake pathways for poor nitrogen sources should be de-repressed. However, in our experiments, no enhancement

of amino nitrogen uptake was observed in both NCR mutants in normal grape juice; if anything more residual amino acids remained at the end of the ferment (Fig. 2). This result is in contrast to previous work of Salmon and Barre (1998), who showed that a *ure2* mutant took up more amino acids than wild type, during fermentation in a synthetic grape juice medium. The lack of a difference in our measured uptake is also in contrast to our transcriptional data from the *dal80 gzf3* double mutant, which showed a clear de-repression of several nitrogen permeases during the early exponential phase of fermentation, including those for uptake of amino acids (*GAP1*), allantoin (*DAL4*, *DAL5*), urea (*DUR3*) and γ-aminobutyric acid (*UGA4*) (Table 3). At stationary phase, *DAL4*, *DAL5*, *DUR3* and *UGA4* each remained upregulated by around fourfold in the double mutant. A possible explanation for these differences is that we measured total 1° amino nitrogen, which includes both preferred and non-preferred nitrogen sources. It may be that non-preferred nitrogen is a relatively small component of the Sauvignon Blanc grape juice used here, so that strain differences in the uptake of this fraction were not apparent in our experiments.

Our results for varietal thiol production showed no effect of either NCR mutation or added DAP, and do not support previously proposed links between NCR and production of 3MH and 3MHA, at least for the M2h strain of yeast. Previously Subileau et al. (2008a) found that addition of DAP to both synthetic medium and to Sauvignon Blanc grape juice decreased yeast 3MH production. Using synthetic grape juice medium, Thibon et al. (2008) found that a *ure2* mutant of a wine yeast VL3 produced wines with a 3-fold increase in thiols, and also showed that transcripts of a thiol-producing gene, IRC7, were upregulated in the *ure2* mutant at the later stages of fermentation. In our experiments, the IRC7 gene showed no significant differences in any comparisons (although in all four ferments there was a minor down-regulation at stationary phase compared with exponential, see Table S1 in the Electronic supplementary material). These differences between the different researchers may be the result of yeast strain differences in response to nitrogen.

The transcriptome data provided strong support for regulation of known NCR genes during fermentation of M2h yeast in three separate comparisons.

1. The *dal80 gzf3* double mutant showed upregulation of NCR-regulated genes compared with wild-type M2h, during exponential growth in the presence of preferred nitrogen sources. These NCR-regulated genes encode nitrogen permeases for uptake of amino acids (*GAP1*), ammonium (*MEP2*), allantoin (*DAL4* and *DAL5*), urea (*DUR3*) and GABA (*UGA4*), plus genes involved in metabolism of allantoin (*DAL1*, *DAL2* and *DAL3*) and proline (*PUT1*). Previous studies have shown that many NCR-regulated genes are upregulated in single *dal80* and *gzf3* mutants (Coffman et al. 1997; Rowen et al. 1997), as well as in a *dal80 gzf3* double mutant strain (Godard et al. 2007). Deletion of both *DAL80* and *GZF3* in M2h resulted in elevated expression of many of these nitrogen-regulated genes during fermentation, independent of nitrogen source. However, some known NCR-regulated genes maintained aspects of regulation in the strain, both differentially between stationary and exponential phases as well as following DAP addition (Table 3). This remaining nitrogen regulation may be partly due to Ure2p activity. The presence of ammonium at stationary phase would retain Ure2p activity, which would maintain inactivation of Gln3p and Gat1p and therefore prevent activation of nitrogen-regulated genes.
2. NCR genes were downregulated in the wild-type strain in the presence of added ammonium, compared with the same strain when preferred nitrogen is exhausted. DAP addition to the wild-type strain affected the expression of 70 genes and repressed the expression of 16 known NCR-regulated genes late in the ferment. There was down-regulation of genes which encode nitrogen permeases for uptake of amino acids (*GAP1* and *DIP5*), ammonium (*MEP2*), proline (*PUT4*) and GABA (*UGA4*), plus genes involved in metabolism of allantoin (*DAL3*) and proline (*PUT1* and *PUT2*).

   Marks et al. (2003) found 350 genes affected by DAP addition late in the ferment (after 30% sugar consumption) in the commercial wine yeast strain VIN13. Similar to our results, the largest changes were in NCR-regulated genes, including *DAL80*, *DAL3*, *GAP1*, *MEP2* and *UGA4*. However, DAP addition did not induce full NCR in the wild-type M2h strain, since there was further upregulation of some NCR-regulated genes when cells reached stationary phase. These results are consistent with previous data from aerobic cultures suggesting that ammonium does not induce full NCR in yeast, since glutamate levels are too low to repress Gat1p (Magasanik and Kaiser 2002). For example, Godard et al. (2007) found that ammonium induced a weaker NCR effect compared with asparagine, glutamine and serine.
3. There was upregulation of 34 known NCR-regulated genes in the wild-type strain at stationary phase, when nitrogen is exhausted from the medium compared with exponential phase, consistent with previous studies (Beltran et al. 2004; Rossignol et al. 2003). Many of these NCR-regulated genes were also found to be upregulated in the EC1118 yeast strain at stationary phase of fermentation by Rossignol et al. (2003), including *PUT1, PUT2, DAL2, DAL3, DAL4, DAL5, DAL7, GAP1, MEP2, PTR2* and *GDH2*. However, there are also some differences—for example *UGA1*

was upregulated in Rossignol et al. (2003) but not in this study, and the inverse for *PUT4*. These differences could be due to the media used (synthetic media versus grape juice) or to strain variation in NCR regulation. Such variation has been reported in previous studies. For example, yeast strains Σ1278b and S288c exhibit differences in their NCR response when they are grown on ammonium (Takagi et al. 2000).

Two aspects of NCR regulation during fermentation differed from expectations derived from aerobic growth experiments. Firstly, only a small proportion of known NCR genes altered expression completely as expected. Of the 47 NCR-regulated genes affected in our experiments, only six of these changed expression in all three predicted comparisons. Moreover, 33 of the 80 known NCR genes showed no regulation under any of the comparisons (Table 3, legend).

Secondly, six NCR genes were downregulated in the wild-type strain at stationary phase, rather than being upregulated, as was expected and as was found for another 34 NCR genes. It is possible that alternative regulatory controls of these genes over-ride NCR control. For example, the NCR-regulated gene *AGP1* is regulated by the SPS plasma membrane amino acid sensor system along with *MUP1* and *GNP1* (Iraqui et al. 1999), and all three genes are strongly downregulated at stationary phase. The NCR-regulated gene *GDH1* is also regulated by carbon sources (Riego et al. 2002).

In summary, our results demonstrated that two regulatory proteins, Dal80p and Gzf3p, are important for control of some NCR genes during fermentation, as is the case during growth. The data confirmed that ammonium addition regulated known NCR genes during fermentation, but did not induce full NCR, as defined previously for aerobic growth (Godard et al. 2007). The results also confirmed that many known NCR-regulated genes were regulated during the switch from exponential to stationary phases of fermentative growth. However, we found major differences between NCR as it applies to a commercial wine yeast in fermentative conditions and laboratory yeast grown under standard aerobic conditions. Further work is needed to establish the role of the strain and various aspects of the fermentation conditions in this differential regulation.

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