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Functional genomic analysis of a commercial wine strain of *Saccharomyces cerevisiae* under differing nitrogen conditions

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Abstract

DNA microarray analysis was used to profile gene expression in a commercial isolate of *Saccharomyces cerevisiae* grown in a synthetic grape juice medium under conditions mimicking a natural environment for yeast: High-sugar and variable nitrogen conditions. The high nitrogen condition displayed elevated levels of expression of genes involved in biosynthesis of macromolecular precursors across the time course as compared to low-nitrogen. In contrast, expression of genes involved in translation and oxidative carbon metabolism were increased in the low-nitrogen condition, suggesting that respiration is more nitrogen-conserving than fermentation. Several genes under glucose repression control were induced in low-nitrogen in spite of very high (17%) external glucose concentrations, but there was no general relief of glucose repression. Expression of many stress response genes was elevated in stationary phase. Some of these genes were expressed regardless of the nitrogen concentration while others were found at higher levels only under high nitrogen conditions. A few genes, *FSP2*, *RGS2*, *AQY1*, YFL030W, were expressed more strongly with nitrogen limitation as compared to other conditions.

Keywords: Microarray; Nitrogen limitation; Wine yeast

1. Introduction

A vast amount of research on the model eukaryote, *Saccharomyces cerevisiae*, has been conducted using laboratory strains under laboratory conditions. Laboratory media were designed to facilitate genetic analysis of this yeast and bear little resemblance to the natural environment of this organism. *Saccharomyces* is found in the wild associated with grape surfaces, but is generally in minor proportions to other yeasts [1]. *S. cerevisiae* evolved to dominate anaerobic growth on high-sugar substrates, for example, crushed grapes. A typical environment in which *S. cerevisiae* thrives is rich in sugar (20–25%, equimolar mixture of glucose and fructose), low in pH (pH 3.0–3.8) and nitrogen is most frequently the limiting nutrient for growth [2]. Many juices, however, contain ample amounts of all essential macro- and micronutrients. Under these conditions, populations generally enter stationary phase due to attainment of maximal cell density rather than to limitation for any given nutrient. Cells retain high rates of metabolic activity and are competent for further growth upon reduction of the cell number. In contrast to laboratory strains, natural and commercial isolates of *Saccharomyces* are highly ethanol tolerant.

Nitrogen limitation imposes a nutritional stress on metabolic activities of non-growing as well as growing cultures. Stationary phase rates of fermentation are not sustained under nitrogen limitation [3] and it has been suggested that this is due to reduced viability [3]. Physiological analysis of this 'typical' energy-sufficient, nutrient-limited stationary phase has been limited. Several genes controlled by the STRE element involved in response to general stress were shown to be expressed in commercial strains during stationary phase in the absence of nutritional limitation [4].

The completion of the sequence of the genome of *S. cerevisiae* allowed development of tools for the evaluation of expression of the entire complement of genes encoded

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in the genome [5,6,7]. The goal of this study was to use DNA microarray hybridization analysis to compare mRNA expression profiles in cultures of a commercial wine yeast grown in a synthetic grape juice medium under nutrient-sufficient and nitrogen-limited conditions in order to analyze the physiological status of the two cultures at different stages of growth. An aligned goal was the identification of genes that might be associated with nutrient deficiency in stationary phase.

2. Materials and methods

2.1. Yeast strain and growth conditions

An isolate from the commercial wine strain of *S. cerevisiae* known as 'French White' (UCD2100) was used for this study (Red Star Yeast, Milwaukee, WI, USA). UCD2100 was selected for this analysis because it had a median rate of fermentation in comparison to several other strains that were tested in a pilot study. The medium used was MMM 'Minimal Must Medium' developed originally by Giudici and Kunkee [8]. The medium was modified as follows: proline, Casamino acids, ammonium phosphate and ammonium hydroxide were excluded in order to limit nitrogen. Medium pH was adjusted using potassium hydroxide. Arginine was added as sole nitrogen source to a level of 1.24 g l^{-1} for the high-nitrogen condition and to 0.165 g l^{-1} for the low-nitrogen fermentation. MMM is somewhat unstable and tends to develop a tartrate precipitate upon aging of the components, so the medium was made and sterilely filtered immediately prior to inoculation.

Cells were pre-grown in 5 ml of MMM to saturation ($OD_{580\,nm} = 10$) on a roller drum at room temperature to early stationary phase (approximately 48 h). The cells were harvested by centrifugation, rinsed once and resuspended in MMM with the appropriate nitrogen concentration. Cultures for subsequent total RNA preparations were grown in 1 l of medium (in a 2-l flask), and inoculated with approximately 10^6 cells ml^{-1}. Replicate fermentations were conducted and the cells pooled for the mRNA isolation. Cultures were grown at 30°C with moderate agitation (100 rpm). Under typical industrial conditions, carbon dioxide evolution maintains cells in suspension and there is very little settling. Moderate agitation was used to mimic this situation. At the times indicated, portions of the cultures were harvested for mRNA preparation. Flasks were swirled to resuspend settled biomass, transferred to centrifuge tubes and centrifuged at 3000 rpm for 5 min at room temperature (approximately 27°C) to avoid a temperature shock to the culture. Cell pellets were resuspended, transferred to 1.5-ml Eppendorf tubes and frozen immediately in liquid nitrogen and then transferred to −80°C until used for mRNA preparation. A bench top refractometer (Fisher) was used to determine total sugar present at time of sampling. A standard curve using MMM with decreasing amounts of sugar and increasing amounts of ethanol was created in order to correct for deviation due to ethanol. Samples were clarified by centrifugation prior to analysis.

2.2. Preparation of mRNA and microarray analysis

Total RNA was extracted using standard protocols [9] and mRNA purified using Pharmacia Biotech oligo(dT) cellulose columns following manufacturer's instructions with the following modifications as suggested by C. Siedel: mRNA was selected with one pass over the column rather than two, linear acrylamide was used as a carrier for the mRNA rather than glycogen, and samples were resuspended in DEPC-treated water instead of TE buffer.

Fabrication of DNA microarrays, synthesis of fluorescently labeled cDNA and hybridization was as previously described [5–7]. The data were quantified using the program Scanalyze 1.1 [5–7]. An initial cluster analysis was performed to identify classes of genes displaying common patterns of expression [10]. Scanalyze 1.1 and the clustering software are both available on the Internet at rana.stanford.edu/software. The complete microarray data set is available at http://genome_www4.stanford.edu/MicroAway/SMD/

3. Results and discussion

3.1. Growth conditions and experimental design

The commercial strain, French White, was selected for these studies because it presented median values for several fermentation parameters in an initial screen of wine strains. To evaluate gene expression patterns under native conditions, cultures were prepared in a synthetic juice medium. Cells were grown in high (1.24 g l^{-1}) or low (0.165 g l^{-1}) arginine as sole nitrogen source. Arginine is generally present in high concentration in grape juice and serves as principle nitrogen source. Proline is also present at equal or greater concentrations in grape juice, but is not utilized as a nitrogen source under anaerobic conditions. Since limited aeration was necessary in order to harvest samples for subsequent analysis, proline was omitted from the synthetic juice medium to more closely replicate actual fermentation conditions.

Cultures were monitored by absorbance and by glucose and fructose concentration (Fig. 1). Extensive preliminary experiments were conducted to define the nitrogen conditions limiting for growth and fermentation. A minimal concentration of 0.33 g l^{-1} arginine was needed for complete utilization of sugar. The value of 0.165 g l^{-1} was selected for this study as being half of the concentration required for consumption of sugar present. The high-nitrogen concentration was selected as one that greatly exceeds

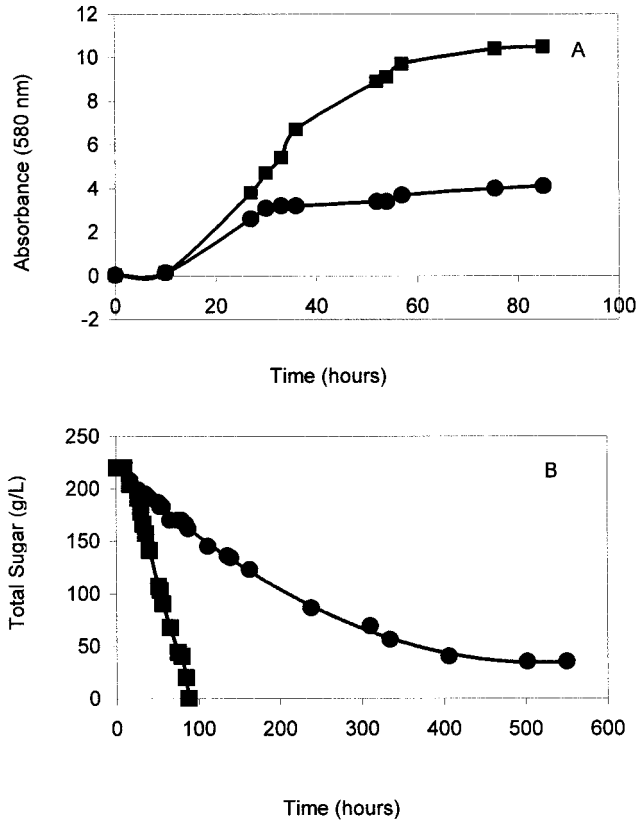


Fig. 1. Growth and sugar utilization patterns of cells grown in low (0.165 g l^{-1}) and high (1.24 g l^{-1}) arginine concentrations. Data represent the average of duplicate fermentations. A: Growth as measured by absorbance at 580 nm. B: Total sugar (sum of glucose and fructose concentrations). Low-nitrogen, 0.165 g l^{-1} (○); high-nitrogen (■)

the amount of this nutrient needed for maximal biomass production and rapid sugar consumption.

Samples were taken at three time points for each nitrogen condition. The 33-h time point represents late-exponential/early stationary phase for the low-nitrogen condition (78% of maximal absorbance attained) while the equivalent time point for the high-nitrogen condition was at 51% of maximal absorbance or one doubling away from stationary phase. The two 58-h time points were both at roughly 90% of final maximal absorbance. Significantly more total sugar was consumed in the high-nitrogen time course (roughly 2% remaining at 85 h) as compared to the low-nitrogen condition (17% sugar remaining at 85 h) (Table 1).

At the time points indicated, mRNA was harvested from replicates of each culture and used in microarray analysis. The following pair-wise comparisons were conducted: high-nitrogen early time point to mid and late time points (H33H58 and H33H85); low-nitrogen early time point to mid and late time points (L33L58 and L33L85); and high-nitrogen to low-nitrogen at early (H33L33) and late (L85H85) time points. In each case the sample indicated first in each comparison was labeled with Cy3 (green fluorescence) and the other labeled with Cy5 (red fluorescence) during reverse transcription. Two types of filters were used for data reliability. To be considered usable, data points needed to exceed a 150-pixel minimum and display a regression correlation higher than 0.6. Data points not meeting both of these criteria are listed as an 'O' in the tables. If the regression correlation was higher than 0.6, but the pixel value below 150, the data point is designated by a subscript 'B', with B indicating the spot on the original array would appear black. The colors indicate relative change of expression, green meaning two-fold or higher expression values for the sample labeled with green fluorescence and likewise red indicating two-fold or higher expression in the mRNA sample labeled with red fluorescence. Yellow indicates equivalent (less than two-fold difference) in expression. Y_B indicates no expression in either condition, or failure to detect expression.

The regression correlation quantifies the correlation for pixel distribution for both colors relative to each other on a given spot on the microarray. A poor regression correlation can mean that neither condition is represented very well on the spot even if it meets the 150-pixel minimum or that there is something unsuitable about the distribution of pixels on the spot. In this case the data are unusable. Alternately, a low correlation may reflect that one color is under-represented while the other is highly represented. A low correlation value was also observed in some situations in which both spots were highly represented. In this case, the data are meaningful, but the relative quantitation of expression may not be. The latter two situations yield informative data. All data displaying a poor regression correlation coefficient are indicated by a subscript 'X' in the tables.

An initial cluster analysis [10] was performed to identify pathways displaying a difference in level of expression across one or more time courses. Pathways so identified were then examined in detail. The level of expression of co-regulated genes or those from the same or an aligned pathway were evaluated. For greater confidence in the data, multiple genes from a common pathway or that are co-regulated or otherwise known to be co-expressed, were evaluated and the data are presented in the following tables. In some cases, only a lone gene was more highly expressed in one condition; such data, while potentially useful, are not presented. This is because replicate micro-

Table 1
Growth parameters

Nitrogen (Arg g l^{-1})	Time (h)	Total sugar (g l^{-1})	$OD_{580\ nm}$
1.24	33	166	5.4
	58	90	9.7
	85	20	10.5
0.165	33	195	3.2
	58	183	3.7
	85	166	4.1

Table 2
Analysis of cyclin genes

Gene	H33H58	H33H85	H33L33	L85H85	L33L58	L33L85
CLN1	Y	G	G	Y	G	G
CLN2	G	G	G	Y_B	G	Y
CLN3	Y	Y	Y	Y	Y	R
CLB1	G	G	G	O	Y	Y
CLB2	Y_B	O	G	O	R	Y
CLB3	Y	R	Y	Y	Y	Y
CLB4	Y	R	Y	Y	R	Y
CLB5	Y	Y	Y	Y_B	Y	Y
CLB6	Y	Y_B	Y	Y_B	Y	Y

O indicates unusable data; Y indicates no difference in level of expression and Y_B means expression below detection limit of 150 pixels. G indicates two-fold or higher expression under conditions labeled with green fluorescence and R means two-fold or higher expression under conditions labeled with red fluorescence. Condition labeling color is indicated by the color of shading around the time point.

arrays were not run which are necessary to differentiate between a spurious spike and a real trend for a single gene. Except where indicated in the text, a 2.5-fold difference in expression or greater was used as the criterion of differential expression. This is greater than the value of 2 determined from statistical analysis to indicate a difference at the 99.75% confidence level. However, replication is necessary for confidence in levels of expression at the lower limit of significance.

3.2. Relative replicative status of the samples

The growth data (Table 1) suggest that the earliest time point in high-nitrogen is in exponential growth while the others are in late-exponential or stationary phase. To confirm this assessment, levels of mRNA of the cyclin genes were evaluated (Table 2). Cyclins are expressed at specific time points during the cell cycle (reviewed in [11]). Differences in the appearance of cyclin mRNA in one population versus another would indicate corresponding differences in the relative numbers of cells of the population at that stage of the cell cycle. The late G1 cyclins [11], *CLN1* and *CLN2*, showed higher expression in the H33 sample, especially as compared to the late time point (H85) and to the early time point of the low-nitrogen culture (L33). Likewise, the earliest time point of the low-nitrogen culture displayed higher expression of the G1 cyclins than did the later time points. Comparison of the latest time points for both nitrogen conditions (L85H85) showed no difference in cyclin expression. Thus, G1 cyclin gene expression suggests that more cells are entering the cell cycle in the nutrient sufficient cultures and at earlier time points.

CLN3 expression was equivalent across most comparisons as expected since this cyclin is thought to be synthesized constitutively [12], but was elevated in the L85 time point as compared to the earlier time point (L33). This may indicate that a higher percentage of this population is in G0/early G1. Alternately, it could mean that a higher level of mRNA is needed to maintain Cln3p levels under these starvation conditions. *CLB1* expression was also higher in the earlier time points and at higher nitrogen concentration. The late S phase cyclins [11], *CLB3* and *CLB4*, showed an interesting trend of being more highly expressed at the midpoint of both the high- and low-nitrogen time course. This may indicate that under these conditions S phase is elongated or that higher mRNA levels are needed to maintain Clb3p and Clb4p levels. The cyclin data fully supported the assessment of culture replicative status from the absorbance measurements.

An alternate method to evaluate the relative replicative activities of two samples is to compare genes involved in synthesis of cytoplasmic components that would be required for distribution to growing buds. The *SEC* genes define many components required for net cell wall and membrane synthesis as well as for synthesis of endoplasmic reticulum and Golgi components [13]. Similarly, the

Table 3
Expression of genes for protein synthesis, degradation and localization

Gene	H33H58	H33H85	H33L33	L85H85	L33L58	L33L85
PRE	Y	Y	Y	Y	Y	Y
RPL	Y	G	Y to R	G	Y	Y
RPS	Y	G	Y to R	G	Y	Y
SEC	Y	Y	Y to G	Y	Y	Y

See legend to Table 2. Y to R indicates that genes of the family displayed no differences in expression (Y) or tended to be elevated under R-labeling conditions. Similarly, Y to G indicates no differences with a tendency of several genes to be elevated in the G-labeling conditions.

yeast cytoplasm contains a proteolytic particle, the proteasome, involved in protein turnover, the components of which are encoded by the *PRE* genes [14]. Evaluation of *PRE* and *SEC* gene expression (Table 3) indicated very little difference across the comparisons. The only exception was a tendency for *SEC* gene expression to be greater in the H33 time point as compared to the same time point from the low-nitrogen case (L33). The Y to G designation for the H33 to L33 comparison indicates that while several *SEC* genes showed an increase in expression in the H33 sample, the fold difference was near 2, the limit of significance.

Ribosomal protein expression (*RPL* and *RPS* genes [15]) was evaluated to determine relative translational activity of the time points. It was expected that relative translational activity would correlate with relative replicative activity, but this was not the case (Table 3). Instead, there was higher expression of the *RPL* and *RPS* genes in the low-nitrogen time points as compared to the high-nitrogen time course. The *RPL* and *RPS* genes displaying a 2.4-fold increase or greater in the H33 versus H85 samples were: *RPL2A, RPL2B, RPL4A, RPL5A, RPL6A, RPL9B, RPL13, RPL14A, RPL15A, RPL16B, RPL17, RPL17B, RPL19A, RPL27B, RPL30A, RPL30B, RPL41A, RPL45, RPS5, RPS10A, RPS13C, RPS18B, RPS21, RPS24A, RPS26A, RPS26B, RPS28A, RPS31A* and *RPS33B*. In the case of the H33 to L33 comparison, several ribosomal genes were expressed at or around a two-

Table 4
Carbon metabolism genes

Gene	H33H58	H33H85	H33L33	L85H85	L33L58	L33L85
Glycolysis						
ADH1	Y_X	Y	R_X	G_X	Y	Y
CDC19	Y_X	G	Y_X	G	Y	Y
ENO1	Y_X	G_X	G_X	G_X	R	Y
ENO2	Y_X	G_X	Y_X	G_X	R	Y
FBA1	Y_X	Y	Y_X	G_X	Y	Y
GPD1	G_X	G_X	G_X	G	G	G
GPD2	Y_X	Y	Y_X	Y	Y	Y
GPM1	Y_X	Y	G_X	G_X	R	Y
HOR2	G_X	G_X	G_X	Y	Y	Y
HXK1	G_X	G_X	Y_X	Y_X	G	Y
HXK2	G_X	Y	Y_X	Y	Y	Y
PDC1	Y	Y_B	G	Y_B	Y	Y
PDC5	Y_X	G_X	G_X	G_X	Y	Y
PDC6	Y	G_X	G	R	Y	Y
PDX1	Y	Y	Y_X	R_B	Y	Y
PFK1	Y_X	Y	Y_X	G	Y	Y
PFK2	Y_X	G	Y_X	G	Y	Y
PGI1	Y_X	G_X	G_X	G_X	R	O
PGK1	Y_X	G_X	Y_X	G_X	R	Y
PGM1	G	Y	G	Y	Y	Y
PGM2	Y	Y	Y_X	G	Y	Y
PYC1	Y	O	R	Y	Y	Y
PYC2	Y	G_B	Y	Y	Y	Y
PYK2	Y	Y	Y	Y	G	Y
TDH1	Y_X	Y_B	G_X	G_X	R	Y
TDH2	Y_X	R	Y	G_X	R	Y
TDH3	Y	Y	Y_X	G_X	R	Y
Pentose Phosphate Pathway						
GND1	Y	O	G_X	G	Y	Y
GND2	Y	Y_X	R	G	Y	Y
RKI1	Y	Y_B	G	Y	Y	Y
RPE1	Y	Y	Y	O	Y	Y
TAL1	Y	Y	G	Y	R	Y
TLK1	G_X	Y_B	G	G	Y	Y
TLK2	Y	Y	R	G	G	G
ZWF1	Y	Y	Y	Y	Y	Y

See legend to Table 2. Subscript 'x' indicates poor correlation coefficient (< 0.6).

Table 5
TCA cycle, glyoxylate and respiration genes

GENE	H33H58	H33H85	H33L33	L85H85	L33L58	L33L85
ACO1	Y	Y	R_X	G	G	Y
ATP1	Y	Y	Y	G	Y	Y
ATP3	Y_X	Y	Y_X	Y	Y	Y
ATP5	Y	Y_X	Y	G	Y	Y
CIT1	Y	Y	Y_X	Y	Y	Y
CIT2	R_X	R	Y	G	R	R
COX4	Y	G	Y	G	Y	Y
COX5A	Y	R	Y_X	G	Y	Y
COX5B	Y	Y	Y_X	G	Y	Y
COX9	Y	Y	Y	Y	Y	Y
COX12	Y	Y	R_X	G	Y	Y
COX13	R	G	R_X	G	Y	Y
CYC1	Y	Y	R	Y	Y	Y
CYC2	Y	Y	Y	Y	Y	Y
CYC3	Y	Y	Y	Y	O	Y_B
CYC7	Y_X	Y	R_X	Y	G	Y
FUM1	G	G	Y	G	Y	Y
ICL1	Y	Y	Y	Y	Y	Y
ICL2	R	Y	R	Y	Y	Y
IDH1	Y	Y	Y	Y	Y	Y
IDH2	Y	Y	R_X	Y	G	Y
IDP1	G	G	G	Y	Y	Y
IDP2	Y	Y	Y	G	O	Y
KGD1	Y_X	Y_X	Y	G	G	Y
KGD2	Y	R	Y	Y	Y	Y_X
MDH1	Y	Y	R_X	G	G	Y
MDH2	R	Y	R	G	Y	Y
QCR2	Y	Y	Y	G	R	Y
QCR8	Y	G	R	Y_B	Y	Y
SDH1	Y_X	G_X	R_X	G	Y	Y
SDH2	R	Y	R	G	Y	Y
SDH3	Y	R	R_X	G	Y	Y

See legends to Tables 2 and 4.

fold difference, higher in L33. Because of nearness to the lower limit of significance this is indicated as 'Y to R' in Table 3. The genes showing an increase in L33 are: *RPLA3, RPL14B, RPL17, RPL17A, RPL25, RPL27, RPL32, RPL35B, RPL35A, RPL43A, RPL46, RPS12, RPS16A, RPS24EA, RPS24EB, RPS25*, and *RPS25B*. Interestingly, with the exception of *RPL17*, the ribosomal protein genes showing a difference in expression are not the same. In contrast, at the later time point comparison (L85 H85), the fold difference in gene expression was greater (2.5 or above) in the L85 sample for the following genes: *RPL1, RPL2A, RPL2B, RPLA1, RPLA2, RPLA3, RPL5A, RPL6A, RPL6B, RPL9A, RPL9B, RPL13, RPL13A, RPL14A, RPL14B, RPL15A, RPL15B, RPL17, RPL17A, RPL17B, RPL19A, RPL19B, RPL25, RPL27, RPL27B, RPL30A, RPL30B, RPL32, RPL35A, RPL35B, RPL37A, RPL37B, RPS7A, RPS7B, RPS8A, RPS8B, RPS10A, RPS12, RPS13C, RPS16A, RPS16B, RPS18A, RPS18B, RPS21, RPS24A, RPS24EA, RPS24EB, RPS25, RPS26A, RPS26B, RPS27B, RPS28A, RPS28B, RPS30, RPS31, RPS31A* and *RPS31B*.

An increase in mRNA levels of ribosomal genes under conditions of nitrogen limitation has been noted before. Carbon source upshift of nitrogen-starved cells leads to a two-fold increase in expression of the *RPL* and *RPS* genes [13]. The increase in *RPL* and *RPS* gene expression observed here suggests that an increase in expression of these may be sustained under conditions resembling the yeast natural environment. In the low-nitrogen time course sugar levels are still quite high while this substrate is near depletion in the high-nitrogen culture at this time.

3.3. Genes of carbon metabolism

The sugar consumption data suggested greater fermentative activity in the high-nitrogen time course as compared to the low-nitrogen condition. In general, mRNA levels for many of the glycolytic genes were higher in the H33 condition as compared to later time points of high-

Table 6
Genes controlled by glucose repression

Gene	H33H58	H33H85	H33L33	L85H85	L33L58	L33L85
ACH1	Y	Y	R	Y	Y	Y
ADH2	Y_X	Y	R	G_X	R	Y
FBP1	Y	Y_B	Y	Y	Y	Y
GAL1	Y	Y_B	Y	Y	Y	Y
GAL7	Y	G_X	Y	Y	Y	Y
GAL10	Y	Y	Y	Y	Y	Y
MAL31	Y	R	R_X	Y	Y	Y
MAL32	Y	R	Y	Y_X	Y	Y
PCK1	Y	Y_B	R	Y_B	Y	Y
SUC2	Y	Y	R	R	G	Y

See legends to Tables 2 and 4.

nitrogen and to the early time point of the low-nitrogen sample (Table 4). Interestingly, at the late time point, 85 h, expression of the glycolytic genes appears to be greater for the low-nitrogen condition (L85), as compared to high-nitrogen (H85). While much of the data show a low correlation coefficient, many of the messages showing the same trend are fully usable data. While surprising, this observation is very consistent with the higher translational

Table 7
Amino acid biosynthesis genes

Gene	H33H58	H33H85	H33L33	L85H85	L33L58	L33L85
ARO3, ARO7	Y	Y	Y	Y	Y	Y
ARO4	G	G	Y	Y	Y	Y
ARO8	G_X	G	G_X	Y	Y	Y
ARG1	G_X	G_X	G_X	Y	Y	Y
ARG4	Y_X	Y	G_X	R	Y	Y
ARG2, ARG3	Y	Y	Y	Y	Y	Y
ARG5,6	Y_X	Y	Y	R	Y	Y
ARG8	Y	Y	Y	Y	G	Y
CPA1	Y_X	Y	Y_X	Y_X	Y	Y
CPA2	Y	G	G	Y_B	Y	Y
HIS1	G	Y	Y	Y	Y	Y
HIS2	Y	Y	G	Y	Y	Y
HIS3	Y	Y_B	Y	Y	Y	Y
HS4	G	Y	G	Y	Y	Y
HIS5	G_X	G	G_X	Y	Y	Y
HOM2	G_X	G	G_X	Y	Y	Y
HOM3	Y	Y	Y	R	Y	Y
HOM6	Y	Y	Y	Y	Y	Y
ILV1, ILV2	Y	Y	Y	Y	Y	Y
ILV5	Y	Y	Y_X	Y	Y	Y
ILV6	Y_X	G	Y_X	G	Y	Y
LEU1	R_X	G	Y	Y	Y	Y
LEU2	R_X	Y	G_X	G	R	Y
LEU3	R_X	Y	Y	Y	Y	Y
LEU4	Y_X	Y	G_X	R	Y	Y
LYS1	G	G	G	Y	Y	Y
LYS2	Y	G	Y	Y	Y	Y
LYS5	Y	Y	Y	Y	Y	Y
LYS9	Y	R	G	G_B	Y	Y
LYS14	Y	G	Y	Y	Y	Y
LYS21	Y_X	G_X	G_X	G	Y	Y
PRO2	R	R	G	Y	Y	Y
THR1	Y	R	Y	R	Y	Y

See legends to Tables 2 and 4.

activity of the low-nitrogen late time point, and suggests that the translational capacity is needed for continued synthesis of enzymes engaged in carbon catabolism.

Genes of the pentose phosphate pathway showed a similar trend (Table 4), with no differences in expression in the high-nitrogen time course, but with greater expression in the late time point of the low-nitrogen sample. The relative activities of the TCA cycle and of respiration were also evaluated (Table 5). In general, level of expression of these genes did not vary consistently across the high-nitrogen time course. The differences noted were generally at the limit of significance (at or around a two-fold difference in expression). Some were elevated at the early time point (*FUM1*, *IDP1*), while others were elevated later (*CIT2*), or increased at the mid time point, but higher in the early time point than in the latest time point (*COX13*). There was likewise very little difference in expression of these genes in low-nitrogen. Comparison of the low- to high-nitrogen time points indicated greater expression of several genes in low-nitrogen. Many of these genes are under glucose repression control and there is a significant amount of glucose present in the media (17% at the latest time point). This suggests that one response to low-nitrogen is to switch from a fermentative mode of metabolism to respiration. This is reminiscent of the 'Pasteur effect': the inhibition of fermentation at low-nitrogen conditions by the presence of oxygen [17]. The Pasteur effect is considered to be largely irrelevant to yeast growth conditions in laboratory media since it is only observed under very specific and somewhat unphysiological conditions [17], but these findings suggest that it is relevant to the native environment in batch growth conditions. Respiration may allow better conservation of nitrogen resources than fermentation. *CIT2* (peroxisomal citrate synthetase) expression was consistently higher in the 'poorer' growth conditions, later time points or lower nitrogen content, suggesting that operation of the glyoxylate cycle might be associated with stationary phase. The pattern of *ICL2* expression is also consistent with this observation as is the expression of the *FOX2* gene (see Table 11).

Expression of the TCA and respiration genes may simply indicate a general relief of glucose repression rather than a specific adaptation to limiting nitrogen. To test this possibility, the relative levels of expression of other genes known to be repressed in the presence of glucose were evaluated (Table 6). The *ACH1, ADH2, MAL31, PCK1* and *SUC2* genes were expressed at the early time point in low-nitrogen, but other glucose repressed genes (*GAL*, *FBP1*) showed no change. Only *ADH2* expression was greater under low-nitrogen conditions at the later time point. This suggests that general relief of all glucose repression is not occurring and the effect is selective for genes involved in respiration. This finding is not surprising since it is well known that multiple transcriptional mechanisms regulate glucose repression/derepression.

3.4. Genes of nitrogen, sulfate and phosphate metabolism

Since nitrogen is the limiting nutrient in one of the time courses, the levels of mRNA for genes involved in amino

Table 8
Genes involved in nitrogen source degradation

Gene	H33H36	H33H85	H33L33	L85H85	L33L58	L33L83
AGP1	Y	Y	Y	R	Y	R
AGP2	R	Y	R	Y	O	O
AGP3	Y	G	Y	Y	Y	Y
CAR1	G	O	G	G	R	R
CAR2	G_X	O	Y	Y_B	Y	R
DAL1	G	G	G_X	G_B	Y	Y
DAL2	G	G	G_X	Y_B	Y	Y
DAL3	Y	Y	G	Y	Y	Y
DAL4	Y_X	G	G_X	G	Y	R
DAL5	G_X	G_X	G_X	G	Y	R
DUR1,2	Y	O	Y	Y_B	Y	Y
DUR3	Y_X	G_X	R_X	G	Y	Y
GAP1	Y	Y	Y_X	Y	Y	Y
GDH1	Y	G	G	G	Y	Y
GDH2	Y	Y	Y	Y	Y	Y
GLN1	Y	Y	G_X	Y_B	Y	Y
GLN3	Y	Y	Y	Y	Y	Y
PUT1	G_X	G_X	Y	Y	Y	Y
PUT2	G_X	Y	G_X	Y	Y	Y
UBP13	G_X	G	G_X	R	Y	Y
URE2	Y	Y_B	R	Y_B	Y	Y

See legends to Tables 2 and 4.

Table 9
Genes involved in methionine biosynthesis and sulfur metabolism

Gene	H33H58	H33H85	H33L33	L85H85	L33L58	L33L85
CYS3	G	Y	G	R	Y	Y
CYS4	Y	G_X	Y	Y	Y	Y
GSH1	Y	Y	Y_X	Y	Y	Y
GSH2	Y	G	G	Y	Y	Y
MET2	Y	Y	Y	Y	Y	Y
MET3	Y	Y	Y	O	Y	Y
MET6	Y_X	Y	G_X	R	Y	Y
MET10	Y_X	Y	Y_X	Y	Y	Y
MET14	Y	Y_B	G	R	Y	Y
MET16	Y	R	Y	R	Y	Y
MET17	G_X	Y	G_X	R	Y	Y

See legends to Tables 2 and 4.

acid biosynthesis were evaluated (Table 7). In the low-nitrogen time course, there was little to no difference in expression levels for the entire time course. Genes indicated in bold are under general nitrogen control [18] and show similar patterns of expression. For the high-nitrogen time course, expression was equivalent or higher at the earlier time point, with the exception of *PRO2* and genes for leucine biosynthesis, which were higher at the mid time point (*LEU*) or at the later time point as well (*PRO2*). In general, expression was greater in the high-nitrogen condition as compared to the low-nitrogen at the early time point and similar at the late time point. Thus amino acid biosynthesis gene expression correlates with cyclin expression. Cells displaying greater replicative activity also display greater levels of expression of the amino acid biosynthetic genes. Biosynthesis does not correlate with translational activity (*RPL*, *RPS* gene expression), suggesting that these cultures are efficiently recycling nitrogen reserves.

The expression of genes involved in degradation of nitrogen sources [19] was also evaluated. As expected, the culture with more arginine (H33) displayed higher levels of expression of the genes involved in arginine degradation, *CAR1, CAR2, PUT1, PUT2* (Table 8). There was higher expression at the high-nitrogen early time point as compared to the lower nitrogen condition. However, genes for arginine and allantoin (*DAL1*) degradation were elevated for the low-nitrogen condition late time point, consistent with active amino acid recycling. Expression of genes (*DUR1,2*) involved in urea degradation, an intermediate in arginine catabolism, was uniform across the comparisons. For the low-nitrogen time course, most genes did not display any variation in expression but the *CAR1, CAR2, DAL4* and *DAL5* genes were elevated at the late time point. *DAL4* and *DAL5* encode allantoin and allantoate permeases, respectively. Expression of the general amino acid permease (*GAP1*) did not vary. Expression of the amino acid transporter *AGP* genes did not vary in the same way. *AGP2* is present in higher levels in the H58 and L33 cultures but not in the later time points of the low-nitrogen time course. *AGP1* is expressed to a greater extent in the H85 as compared to L85 condition. *AGP3* showed little variation in expression with the exception of being elevated in the early high-nitrogen time point as compared to the late time point. The mRNA of the gene encoding the high-affinity urea transporter, *DUR3*, was more highly expressed in the H33 sample, but higher in the low-nitrogen time course as compared to the high-nitrogen conditions (H33L33 and L85H85).

NADP-glutamate dehydrogenase (*GDH1*) expression was elevated in H33 as compared to H85 and L33, but was higher in L85 than in H85. *GDH2* expression did not

Table 10
Genes of phosphate metabolism

Gene	H33H58	H33H85	H33L33	L85H85	L33H58	L33H85
PHO3	G	G	Y	Y_X	Y	Y
PHO5	Y	G	Y	Y	Y	Y
PHO11	Y	Y	Y	Y	Y	Y
PHO13	G	G	Y	G	Y	Y
PHO80	Y_B	Y_B	Y	Y_X	Y	Y
PHO81	Y	R	Y	R	Y	Y
PHO84	Y	Y	Y	R	Y	Y

See legends to Tables 2 and 4.

vary, and the *URE2* gene, which is involved in regulation of *GDH1* [16], shows a difference only in the H33L33 pairwise comparison. Glutamine synthetase (*GLN1*, *GLN3*) gene expression did not vary significantly. Expression of *UBP13*, which encodes ubiquitin carboxyl-terminal hydrolase was elevated in H33 across all three comparisons, but was higher in L85 than in H85. The data for genes of nitrogen degradation generally indicate higher levels of expression in the H33 time point, with elevated levels in L85 as compared to H85. This suggests that the L85 time point may be more biosynthetically active than H85, consistent with previous data described above.

With respect to methionine biosynthesis and sulfate reduction (Table 9), expression of genes was roughly equivalent across the high and low-nitrogen time course. Comparison of the two conditions to each other indicated higher expression in H33 and H85. Phosphate metabolism gene expression did not vary in the low-nitrogen time course (Table 10). There was also no difference observed between levels of expression in the comparison of the two early time points. *PHO81* and *PHO84* were more highly expressed in the H85 sample as compared to the equivalent low-nitrogen time point. These genes are both induced by limitation for phosphate. This suggests that the high-

Table 11
Analysis of fatty acid, phospholipid and sterol metabolism genes

Gene	H33H58	H33H85	H33L33	L85H85	L33L58	L33L85
Fatty Acid Metabolism						
ACS1	R	R	Y	R	Y_B	Y
ACS2	Y	Y	G_X	Y	G	Y
CAT2	R	G	R	G	Y	Y
ELO2	Y	Y	Y	Y	Y	Y
FAA1	G_X	Y	G_X	Y	Y	Y
FAA3	Y	Y	G	O	G	Y
FAA4	Y_X	R	G_X	R	G	Y
FAS1	Y	G	G	R	Y	Y
FAS2	Y	Y	Y	Y	Y	Y
FOX2	R	R	R	Y	Y	Y
OLE1	Y_X	G	Y_X	R	G	Y
Phospholipid Metabolism						
CHO1	Y	Y	G	Y	Y	Y
CHO2	Y	Y	G_X	R	Y	Y
CKI1 ,CPT1	Y	Y	G	R	Y	Y
OPI3	Y	G	G_X	Y	R	Y
PLB1	Y_X	Y	G_X	R	Y	Y
PIS1	Y_B	G	R_X	O	Y	Y
PSD1	Y	Y	G	Y	Y	Y
PSD2	Y	Y	Y	Y	Y	Y
Sterol Biosynthesis						
ERG1	G_X	Y	G_X	Y	Y	Y
ERG2	G_X	R	G_X	Y	Y	Y
ERG3	Y	G	G_X	O	Y	Y
ERG4	G	G	G	Y	Y	Y
ERG5	G_X	G_B	G_X	G	Y	Y
ERG6	G_X	G_X	G_X	Y	Y	Y
ERG7	G	G	G	Y	Y	Y
ERG8	G_X	G	G	Y	Y	Y
ERG9	G_X	Y	G_X	Y	Y	Y
ERG10	Y	Y	G	Y	Y	Y
ERG11	G_X	Y	Y	Y	Y	Y
ERG12	G_X	Y	G_X	Y	Y	Y
ERG20	G_X	R	G_X	Y	Y	Y
ERG25	Y	Y	Y	O	Y	O
MVD1	G_X	G	G_X	Y	Y	Y

See legends to Tables 2 and 4.

Table 12
Analysis of genes involved in one carbon metabolism

Gene	H33H58	H33H85	H33L33	L85H85	L33L58	L33L85
ADE3	G_X	G_X	G_X	R	Y	Y
DFR1	Y	Y	Y	Y	Y	Y
GCV1	G	R	G	Y_B	Y	Y
GCV3	G	G	G	Y_B	Y	Y
MET11	Y_X	G	G_X	R	Y	Y
MIS1	G	G_X	Y	Y	Y	Y
MTD1	Y	Y	G_X	R	Y	Y
RNR1	G_X	R	G	R	Y	Y
RNR2	Y_X	G	Y_X	G	Y	Y
SAH1	G_X	G	G_X	Y	Y	Y
SAM1	G_X	G_X	G_X	Y	Y	Y
SAM2	G_X	Y_X	G_X	R_X	Y	R
SER2	G	Y	G	Y	Y	Y
SHM2	O	Y_B	G_X	R_B	Y	Y

See legends to Tables 2 and 4.

nitrogen culture has consumed more phosphate than the low-nitrogen sample, consistent with greater biomass production and more complete utilization of substrate.

3.5. Fatty acid, phospholipid and ergosterol synthesis

Phospholipids and sterols are required for net membrane synthesis. In addition, alterations in fatty acid, phospholipid and sterol levels are needed to maintain ethanol tolerance [3]. Therefore expression of the genes involved in biosynthesis of these components would be expected to be greater in the high-nitrogen time course. This is exactly the pattern observed for the sterol (*ERG*) genes (Table 11). Genes of phospholipid metabolism were equivalent within the two time courses, but were expressed to a greater extent in the high-nitrogen as compared to the low-nitrogen condition. A similar trend was seen with fatty acid biosynthesis, but the data were not as clear-cut. The *ACS1* (acetyl CoA synthetase) and *FOX2* (fatty acid oxidation also yielding acetyl CoA) genes were expressed more strongly at later time points. In general as with amino acid biosynthesis, fatty acid and phospholipid synthesis is greater in the more replicatively active cultures.

3.6. Genes involved in one-carbon transfers

Another index of relative growth is the level of expression of genes involved in one-carbon transfers. Such transfers are generally associated with biosynthesis (amino acids, phospholipids, nucleotides). There was a clear trend in expression of this family of genes. Expression was greater in the more actively growing culture and at the higher nitrogen level (Table 12). The *RNR* (ribonucleotide reductase) genes were included in Table 12 as an indication of deoxynucleotide synthesis providing precursors for DNA synthesis. In general, expression of *RNR1* was higher under nutrient-sufficient conditions as compared to low-nitrogen (H33L33 and L85H85), consistent with earlier observations of the replicative activity of these samples. *RNR1* gene expression appeared to be higher at the later time point of the high-nitrogen time course (H33H85). This observation is consistent with the higher levels of expression of the S phase cyclins in this culture and suggests an elongated S phase under these conditions (no nutrient limitation, growth limitation caused by attainment of maximal cell density and presence of ethanol).

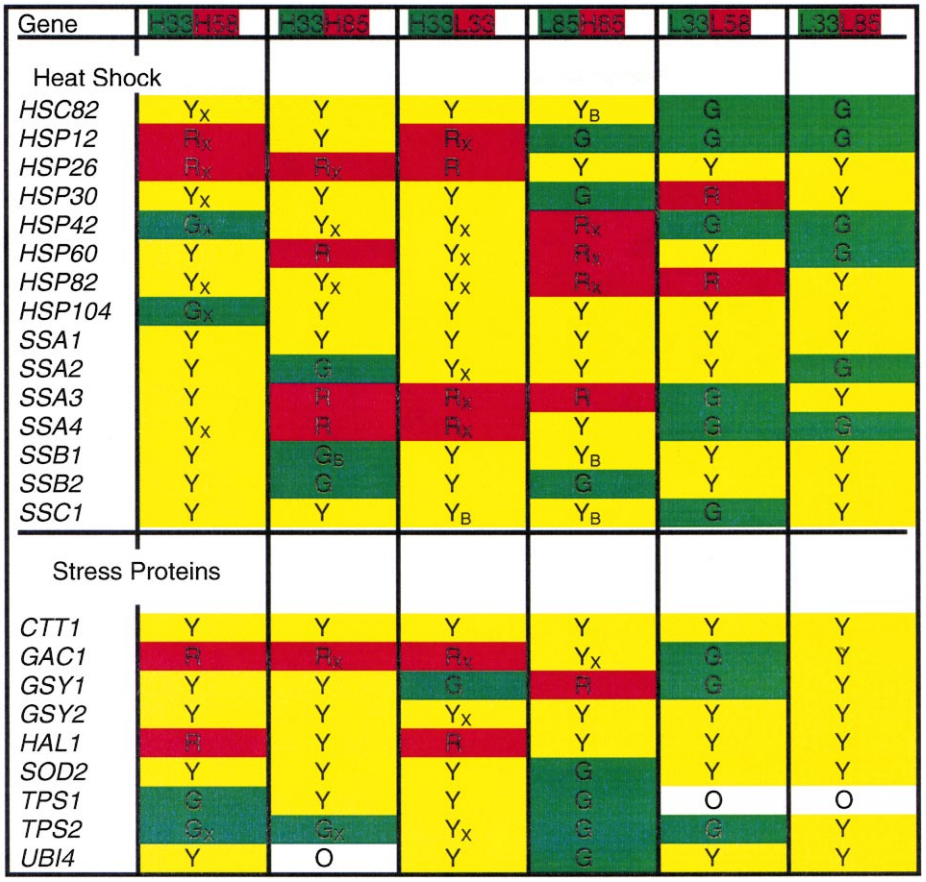
One interesting trend was observed with the *BIO* genes, which are involved in uptake of intermediates in the bio-

Table 13
Biotin synthesis and uptake

Gene	H33H58	H33H85	H33L33	L85H85	L33L58	L33L85
BIO2	Y	Y	Y_X	R	G	G_X
BIO3	G	Y	G	R	G	G_X
BIO4	Y	Y	G	Y_B	G	G_X
BIO5	Y	R	G	R	G	G_X

See legends to Tables 2 and 4.

Table 14
Genes involved in stress response



Gene	H33H58	H33H85	H33L33	L85H85	L33L58	L33L85
Heat Shock						
HSC82	Y_X	Y	Y	Y_B	G	G
HSP12	R_X	Y	R_X	G	G	G
HSP26	R_X	R_X	R	Y	Y	Y
HSP30	Y_X	Y	Y	G	R	Y
HSP42	G_X	Y_X	Y_X	R_X	G	G
HSP60	Y	R	Y_X	R_X	Y	G
HSP82	Y_X	Y_X	Y_X	R_X	R	Y
HSP104	G_X	Y	Y	Y	Y	Y
SSA1	Y	Y	Y	Y	Y	Y
SSA2	Y	G	Y_X	Y	Y	G
SSA3	Y	R	R_X	R	G	Y
SSA4	Y_X	R	R_X	Y	G	G
SSB1	Y	G_B	Y	Y_B	Y	Y
SSB2	Y	G	Y	G	Y	Y
SSC1	Y	Y	Y_B	Y_B	G	Y
Stress Proteins						
CTT1	Y	Y	Y	Y	Y	Y
GAC1	R	R_X	R_X	Y_X	G	Y
GSY1	Y	Y	G	R	G	Y
GSY2	Y	Y	Y_X	Y	Y	Y
HAL1	R	Y	R	Y	Y	Y
SOD2	Y	Y	Y	G	Y	Y
TPS1	G	Y	Y	G	O	O
TPS2	G_X	G_X	Y_X	G	G	Y
UBI4	Y	O	Y	G	Y	Y

See legends to Tables 2 and 4.

synthesis pathway of this vitamin and subsequent biotin synthesis [20]. Wild-type yeast strains are only capable of performing the last three steps of biotin synthesis, so must be provided with this vitamin or an immediate precursor [20]. This vitamin is a cofactor required for many biosynthetic carboxylation and transcarboxylation reactions as well as in carbon metabolism and respiration. Most other biosynthetic pathways showed no difference in expression across the low-nitrogen time course. In a cluster analysis, the *BIO* genes emerge as one group of genes displaying significantly higher expression in the earliest time point of the low-nitrogen time course (Table 13). In general there is no difference in levels in the high-nitrogen conditions, but expression is greater as compared to low-nitrogen (H33L33, L85H85). We are intrigued by this result as the MMM medium contains ample biotin from the yeast nitrogen base component. These observations suggest that demands for biotin might be higher than previously expected under these growth conditions.

3.7. Stress response and heat shock genes

Several genes are induced by environmental and nutritional stress in *S. cerevisiae* (reviewed in [21]). Previous studies of the expression of stress-related genes under the control of the STRE element during wine production conditions identified several heat shock proteins that are expressed upon entry into stationary phase [4]. Expression of these and other stress and heat shock proteins [21] was therefore evaluated. *HSP12* and *HSP26* encode small proteins of unknown function that were found to be induced upon entry into stationary phase (Table 14), consistent with an earlier study [4]. *HSP12* and *HSP26* are more highly expressed at later time points in the high-nitrogen culture. *HSP12* mRNA levels were elevated in the early time point for the low-nitrogen time course while *HSP26* shows no further induction. The mRNAs for both *HSP12* and *HSP26* were present in higher concentration in the early low-nitrogen time point as compared to the early high-nitrogen time point, confirming that a greater proportion of this population has entered stationary phase. *HSP12* is also more highly expressed in the late time point of the low-nitrogen culture as compared to the late time point of the nitrogen-sufficient culture. Thus, expression of these two genes is consistent with the expected replicative status of the cultures, as both contain a significant proportion of cells in stationary phase.

For the Hsp70 family (*SSA1* to *SSA4*, *SSB1*, *SSB2*,

SSC1), *SSA1* and *SSB1* show little to no change and *SSA2* is higher in the early time points as compared to both late time points regardless of the nitrogen status of the culture. *SSA3* and *SSA4* are more highly expressed at the latest time point in the high-nitrogen culture, and in the early time point of the nitrogen-deficient culture versus the same point of the early culture. Both also tend to show higher expression at the early time point of the low-nitrogen culture as opposed to later time points. *SSA3* is higher in the nitrogen-rich late time point, H85, as compared to the late time point from the nitrogen-deficient culture. Thus, expression of these two genes seems to be associated with a nutritionally sufficient stationary phase. They are high in H85 and in L33, two samples that are likely in an equivalent stationary phase.

Similar results, higher expression in a nutrient-sufficient stationary phase, are seen for *HSP60* and *HSC82*. *HSP42* expression also appears to be higher in the more replicatively active cultures, H33 versus L33 and in H85 versus L85. The *HSP30* mRNA, also reported to be higher in stationary phase during grape juice fermentation, was elevated in the low-nitrogen time course as compared to high-nitrogen, but this trend was not consistent in other samples suggesting that expression of this gene might be restricted to a particular stage of stationary phase. Other heat shock genes did not show a clear trend.

Several other genes are also associated with entry into stationary phase. The *GAC1* gene which is involved in activation of glycogen synthesis [22] was expressed at both the mid and late time point of the high-nitrogen sample as compared to the early time point (Table 14). This gene was also expressed more strongly in the early time point of the low-nitrogen culture, in agreement with the literature [23]. Expression of *GSY2*, which encodes the major isozyme of glycogen synthetase, did not vary much across the samples. On the other hand, *GSY1* showed greater expression in the high-nitrogen time course as compared to low-nitrogen. Several genes, *SOD2, TPS1, TPS2* and *UBI4*, also associated with stress response [21], showed higher expression in the low-nitrogen late time point as compared to the high-nitrogen sample, and the *TPS* genes also show greater expression at the earlier time point of the high-nitrogen time course. This pattern of expression is similar to that observed for the glycolytic genes.

The stress and heat shock genes examined seemed to be indicators of stress or entry into stationary phase, but either did not distinguish between the nutritional status of the cultures or were more highly expressed under nutrient-sufficient situations where entry into stationary phase can be considered to be caused by attainment of maximal cell density as opposed to nutrient limitation. The YER150W gene (*SPI1*) has been reported to be expressed in stationary phase in wine fermentations [4]. This gene was found to be more highly expressed in the L33 sample, as compared to all other conditions (Table 15). Expression did not appear to increase at later time points or under nutrient-sufficient conditions. We therefore wanted to search for genes that would be more highly expressed in nutritionally poor stationary phase (Table 15). Eight genes showed a high level induction in the late time point of the low-nitrogen culture as compared to the high-nitrogen culture. One, YJL221C (*FSP2*, homology to α-D-glucosidase) was highest in both late time points, but was higher in the low-nitrogen condition. This gene is induced in limiting conditions and displays high identity to the *MAL62* protein [24]. YML128C, YNL200C and YPR195C, encoding proteins of unknown function, were more highly expressed in the low-nitrogen culture than the high-nitrogen condition, but showed no difference in expression in the high-nitrogen condition. YML128C and YNL200C were previously identified as genes expressed under stationary phase conditions during wine fermentation [4]. Thus these proteins might be an indicator of nutritional stress. YOR107W expression was also high in the lower nitrogen condition at both time points. The fold expression increase in H85 versus H33 was 2.0, at the lower limit of significance. This gene has been designated *RGS2* and is a negative regulator of glucose-induced cAMP signaling pathway [25], and a higher level of expression may indicate that a larger percentage of the population is in a true G0 state. Rgs2p plays a role in regu-

Table 15
Genes expressed under nitrogen stress

See legends to Tables 2 and 4.

lation of STRE gene expression [25]. The fact that ribosomal protein synthesis increases at this time point suggests that the cAMP/PKA pathway is functional as the increase in expression of the *RPL* and *RPS* genes under nitrogen-limiting conditions is mediated by PKA [16]. The role of the Rgs2p may be to limit response of the cAMP/PKA pathway to external nutrients but allow response to internal signals. Thus the appearance of this protein might be an excellent indicator of the nutritional status of the culture.

YPR192W (*AQY1*), which has homology to plant and animal aquaporins [26], is more highly expressed in the low-nitrogen time course and in both the mid and late time points of the high-nitrogen condition. However, the differences in expression in the high-nitrogen time course are at the lower limit of significance, 2.13 for H58 versus H33 and 2.15 for H85 versus H33. Both comparisons show a difference in the same direction. This gene is expressed more highly during nitrogen limitation [26], consistent with our data. Interestingly, it is believed to be non-functional in laboratory strains of *Saccharomyces* [26]. The role of the *AQY1* protein in yeast is unknown but it has been shown to induce water uptake in *Xenopus* oocytes [26].

The *GRE1* gene was originally identified as a gene expressed under hyperosmotic conditions [27]. Gre1p resembles the late embryogenesis abundant protein of higher plants [27]. The *GRE1* gene is negatively regulated by the cAMP-*PKA* pathway and is induced upon nitrogen limitation [27]. The appearance of *GRE1* mRNA is consistent with inhibition of the cAMP signal transduction pathway by Rgs2p.

YFL030W encodes an alanine:glyoxylate aminotransferase which is strongly glucose-repressed but expressed during gluconeogenic growth on acetate and ethanol [28]. The observation of expression under these conditions suggests it is not required for gluconeogenesis so much as for amino acid biosynthesis. Its presence may be indicative of more versatile amino acid recycling.

Analysis of this set of genes has revealed several that may be excellent indices of the physiological status of fermenting cultures. Expression of *RGS2* would indicate exit from permissive growth conditions, while several of the *HSP* genes would indicate entry into stationary phase. The presence of both would indicate nutrient deficiency of stationary phase. *GRE*, which is induced by nitrogen limitation, may be a reporter specific for nitrogen starvation along with YFL030W. Further studies will define the role of these genes in nutrient-limited energy-sufficient stationary phase as well as characterize the status of the cAMP/PKA signal transduction cascade.

4. Conclusions

A clear view of the relative physiological status of nutrient-sufficient and nitrogen-limited populations has emerged from this analysis. Nutrient-sufficient cultures that enter a stationary phase based upon attainment of maximal cell density display higher levels of expression of genes involved in biosynthesis of precursors of macromolecules, and appear to retain competence for cell division. In contrast, energy-sufficient cultures entering stationary phase due to nitrogen limitation display greater expression of genes involved in translation and in oxidative carbon metabolism, suggesting that respiration is more conserving of nitrogen resources than fermentation. The low-nitrogen culture also displayed higher mRNA levels for genes involved in nitrogen compound recycling. Expression of the stress genes examined in general indicated higher levels of expression upon entry into stationary phase or was higher in the high-nitrogen time course, which may reflect the added stress of high ethanol concentrations. Several genes were identified that appeared to increase in expression only during nitrogen-limited stationary phase. One of these genes encodes the *RGS2* protein, which inhibits the cAMP/PKA signal transduction cascade thus restricting responses to glucose. This observation explains the characteristics of arrested fermentations during wine production. Such arrested cultures do not respond to simple nutrient addition, as would be expected from inhibition of the cAMP/PKA cascade. However analysis of other genes repressed by the cAMP/PKA cascade did not reveal any changes in pattern of expression (to be reported) suggesting that only a subset of PKA-regulated genes is being derepressed under these conditions.

Some cautionary notes in interpretation of these data are warranted. First, it is not clear if mRNA levels of non-growing cells will reflect differences in protein levels. That is, transcriptome analysis might not reflect the proteome. However, the information on transcriptional activity is clearly important and reflects a need for the gene product. The absence of a transcriptional signal does not imply the converse, that the gene product is not required under the conditions analyzed. Higher levels of expression may simply be needed to maintain a 'status quo' level of protein, especially under conditions of accelerated mRNA and protein turnover. Further, it is important to bear in mind that the data are being generated across a population, and certain expression levels may be due to only a percentage of the population being in a specific physiological state. Alternately, mRNA levels might indeed reflect the status of the entire population. Finally, the mRNAs of genes with a significant homology can cross-hybridize, impacting assessment of true changes in the level of expression of individual members of gene families.

The data presented here are from unreplicated microarrays. Internal controls were used to evaluate reproducibility and data quality. The trends discussed occur across a pathway or across a time course and are therefore significant. Except in a few cases indicated in the text, the change in expression was greater than 2.5-fold, replicate

arrays would allow greater confidence of differences in expression at the lower limit of expression. Analysis of replicate samples may reveal further trends in gene expression not considered herein.

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References

[1] Fleet, G.H. (1993) The microorganisms of winemaking: isolation, enumeration and identification. In: Wine Microbiology and Biotechnology (Fleet, G.H., Ed.), pp. 1–25. Harwood Academic Publishers, Chur.

[2] Boulton, R.B., Singleton, V.L., Bisson, L.F. and Kunkee, R.E. (1996) Principles and Practices of Winemaking, 604 pp. Chapman and Hall, New York.

[3] Bisson, L.F. (1999) Stuck and sluggish fermentations. Am. J. Enol. Vitic. 50, 107–119.

[4] Puig, S. and Perez-Ortin, J.E. (2000) Stress response and expression patterns in wine fermentations of yeast genes induced at the diauxic shift. Yeast 16, 139–148.

[5] Schena, M., Shalon, D., Davis, R.W. and Brown, P.O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270, 467–470.

[6] Shalon, D., Smith, S.J. and Brown, P.O. (1996) A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res. 7, 639–645.

[7] DeRisi, J.L., Iyer, V.R. and Brown, P.O. (1997) Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 278, 680–686.

[8] Giudici, P. and Kunkee, R.E. (1994) The effect of nitrogen deficiency and sulfur containing amino acids on the reduction of sulfate to hydrogen sulfide by wine yeasts. Am. J. Enol. Vitic. 45, 107–112.

[9] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1995) Current Protocols in Molecular Biology. Wiley Interscience, New York.

[10] Eisen, M.B., Spellman, P.T., Brown, P.O. and Botstein, D. (1998) Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA 95, 14863–14868.

[11] Lew, D.J., Weinert, T. and Pringle, J.R. (1997) Cell cycle control in *Saccharomyces cerevisiae*. In: The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Cell Cycle and Cell Biology (Pringle, J.R., Broach, J.R. and Jones, E.W., Eds.), pp. 607–695. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[12] Tyers, M., Tokiwa, G. and Futcher, B. (1992) The Cln3–Cdc28 kinase complex of *S. cerevisiae* is regulated by proteolysis and phosphorylation. EMBO J. 11, 1773–1784.

[13] Kaiser, C.A., Gimeno, R.E. and Shaywitz, D.A. (1997) Protein secretion, membrane biogenesis, and endocytosis. In: The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Cell Cycle and Cell Biology (Pringle, J.R., Broach, J.R. and Jones, E.W., Eds.), pp. 91–227. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[14] Finley, D., Tanaka, K., Mann, C., Feldmann, H. and Hochstrasser, M. et al. (1998) Unified nomenclature for subunits of the *Saccharomyces cerevisiae* proteasome regulatory particle. Trends Biochem. Sci. 23, 244–245.

[15] Planta, R.J. and Mager, W.H. (1998) The list of cytoplasmic ribosomal proteins of *Saccharomyces cerevisiae*. Yeast 14, 471–477.

[16] Klein, C. and Struhl, K. (1994) Protein kinase A mediates growth-regulated expression of yeast ribosomal protein genes by modulating *RAP1* transcriptional activity. Mol. Cell. Biol. 14, 1920–1928.

[17] Lagunas, R. (1986) Misconceptions about the energy metabolism of *Saccharomyces cerevisiae*. Yeast 2, 221–228.

[18] Hinnenbusch, A. (1992) General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in *Saccharomyces cerevisiae*. In: The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Gene Expression (Jones, E., Pringle, J.R. and Broach, J.R., Eds.), pp. 319–414. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[19] Cooper, T.G. (1982) Nitrogen metabolism in *Saccharomyces cerevisiae*. In: The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression (Strathern, J.N., Jones, E.W. and Broach, J.R., Eds.), pp. 39–100. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[20] Phalip, V., Kuhn, I., Lemoire, Y. and Jeltsch, J.M. (1999) Characterization of the biotin biosynthesis pathway in *Saccharomyces cerevisiae* and evidence for a cluster containing BIO5, a novel gene involved in vitamin uptake. Gene 232, 43–51.

[21] Craig, E. (1992) The heat-shock response of *Saccharomyces cerevisiae*. In: The Molecular and Cellular Biology of the Yeast *Saccharomyces*: Gene Expression (Jones, E., Pringle, J.R. and Broach, J.R., Eds.), pp. 501–538. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[22] François, J.M., Thompson-Jaeger, S., Skroch, J., Zellenka, U., Spevak, W. and Tatchell, K. (1992) *GAC1* may encode a regulatory subunit for protein phosphatase type 1 in *Saccharomyces cerevisiae*. EMBO J. 11, 87–96.

[23] Tadi, D., Hasan, R.N., Bussereau, F., Boy-Marcotte, E. and Jacquet, M. (1999) Selection of genes repressed by cAMP that are induced by nutritional limitation in *Saccharomyces cerevisiae*. Yeast 15, 1733–1746.

[24] Vandenbol, M., Durand, P., Bolle, P.A., Dion, C., Portelle, D. and Higler, F. (1994) Sequence analysis of a 40.2 kb DNA fragment located near the left telomere of yeast chromosome X. Yeast 12, 1657–1662.

[25] Versele, M., de Winde, J.H. and Thevelein, J.M. (1999) A novel regulator of G protein signaling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2. EMBO J. 18, 5577–5591.

[26] Bonhivers, M., Carbrey, J.M., Gould, S.J. and Agre, P. (1998) Aquaporins in *Saccharomyces*. J. Biol. Chem. 273, 27565–27572.

[27] Garay-Arroyo, A. and Covarrubias, A.A. (1999) Three genes whose expression is induced by stress in *Saccharomyces cerevisiae*. Yeast 15, 879–892.

[28] Takada, Y. and Noguchi, T. (1985) Characteristics of alanine:glyoxylate aminotransferase from *Saccharomyces cerevisiae*, A regulatory enzyme in the glyoxylate pathway of glycine and serine biosynthesis from tricarboxylic acid-cycle intermediates. Biochem. J. 231, 157–163.