Nitrogen-regulated transcription and enzyme activities in continuous cultures of *Saccharomyces cerevisiae*

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Variations in the transcription of nitrogen-regulated genes and in the activities of nitrogen-regulated enzymes of the yeast *Saccharomyces cerevisiae* were studied by changing the carbon and nitrogen fluxes. *S. cerevisiae* was grown in continuous culture at various dilution rates (D) under nitrogen limitation with NH₄Cl as sole nitrogen source. With an increase in D from 0.05 to 0.29 h⁻¹, both the glucose and the ammonia flux increased sixfold. The activities of the two ammonia-incorporating enzymes, NADPH-dependent glutamate dehydrogenase (NADPH-GDH) and glutamine synthetase (GS), encoded by GDH₁ and GLN₁, respectively, increased with increasing D, while the activity of the glutamate-degrading enzyme, NAD-dependent glutamate dehydrogenase (NAD-GDH), decreased. Surprisingly, no changes were observed in the transcription of GDH₁ and GLN₁; however increased D was accompanied by an increase in GAP₁ transcription. At the metabolite level, the increase in the glucose and nitrogen flux did not result in changes in the intracellular 2-oxoglutarate, glutamate or glutamine concentrations. It is shown that growth on ammonia alone is not sufficient to cause repression of GAP₁ and GLN₁ transcription and that the regulation of GAP₁ transcription and both NADPH-GDH and GS activity is not an on/off switch, but is gradually modulated in correlation with the ammonia concentration.

**Keywords:** *Saccharomyces cerevisiae*, nitrogen regulation, nitrogen limitation, nitrogen metabolism, continuous culture

INTRODUCTION

The main nutrients, with respect to quantity, used by the yeast *Saccharomyces cerevisiae* are carbon and nitrogen. This implies that the carbon and nitrogen fluxes as well as their mutual interaction constitute important parameters in the regulation of cell growth. The coordination of the carbon and nitrogen fluxes may be regulated at the level of uptake or at the level of metabolism, depending upon the growth conditions and the nature of the carbon and nitrogen source. Glucose and ammonia can be used as sole carbon and nitrogen sources, respectively; their uptake is mediated both by specific low- and high-affinity permeases (Roon *et al.*, 1977; Dubois & Grenson, 1979; Bisson & Fraenkel, 1983; Marini *et al.*, 1994).

Intracellularly, carbon and nitrogen fluxes interact at the level of glutamate. During growth on ammonia as sole source of nitrogen, NH₄⁺ is incorporated into 2-oxoglutarate by the NADPH-dependent glutamate dehydrogenase (NADPH-GDH) to produce glutamate. Subsequently, glutamate is converted to glutamine by the incorporation of another ammonium ion at the expense of ATP by glutamine synthetase (GS). On the other hand, during growth on glutamate as sole nitrogen source, glutamate is degraded to 2-oxoglutarate and NH₄⁺ by the NAD-dependent glutamate dehydrogenase (NAD-GDH), to produce NH₄⁺ for the synthesis of glutamine.

The regulation of the intracellular fluxes of carbon and nitrogen may be at substrate level or at transcription level. At substrate level it has been shown that glutamine exhibits strong allosteric inhibition of NAD-GDH and GS activity (Coschigano & Magasanik, 1991). At transcriptional level the regulation is more complicated. The expression of the structural gene for NAD-GDH, GDH₂,
is regulated by the activity of the transcriptional activator Gln3p (Mitchell & Magasanik, 1984a; Miller & Magasanik, 1991). In the presence of ammonia or glutamine, the Gln3p activity is modulated by Ure2p. Ure2p causes an inactivation of Gln3p and therefore repression of GDH2 (Coschigano & Magasanik, 1991). Also, the transcription of the structural gene for GS, GLN1, is regulated by Gln3p and consequently by Ure2p. Therefore Ure2p represses the transcription of both the GDH2 and the GLN1 gene by inhibiting the Gln3p activity. In addition the transcription of GLN1 is subject to general amino acid control, in contrast to the expression of GDH2. Prolonged amino acid starvation causes an increase in the transcription of GLN1 via an increased translation of the transcriptional activator Gcn4p (Dever et al., 1992). Besides GDH2 and GLN1 transcription, the transcription of other genes, e.g. GAP1 (encoding general amino acid permease), is also regulated by the Gln3p protein. Furthermore, Gln3p seems to be necessary for full gene expression of GDH1, encoding NADPH-GDH, although GDH1 transcription is supposed to be constant (Daugherty et al., 1993). A further illustration of the regulation between the carbon and nitrogen fluxes is the observation that the promoter region of GDH2 contains a carbon-responsive upstream activation site in addition to a nitrogen-responsive element (Coschigano et al., 1991).

To gain insight into the intracellular coordination of the carbon and nitrogen fluxes, we have grown S. cerevisiae in a nitrogen-limited continuous culture. By using low carbon (glucose) concentrations under nitrogen (ammonia)-limiting conditions, the carbon flux and the nitrogen flux can be modulated by changing the dilution rate (D). Ammonia was used because of its incorporation into carbon metabolites at well-defined steps. Firstly, the influence of the changes in the fluxes of nitrogen and carbon on the transcription of Gln3p-regulated genes was analysed. Secondly, under the same conditions several intracellular metabolites, such as 2-oxoglutarate and amino acids, were analysed to study whether a correlation exists between the metabolite concentrations and the expression of Gln3p-regulated genes. It is demonstrated that variations within a narrow range of the carbon and nitrogen fluxes lead to changes in the activities and transcription of nitrogen-regulated enzymes and genes, respectively.

**METHODS**

**Strain and growth conditions.** S. cerevisiae strain SU32 was grown at 30°C in a 21 BiofloIII fermenter (New Brunswick Scientific) connected to a computer controller unit running with Advanced Fermentation Software (New Brunswick Scientific). S. cerevisiae SU32 was inoculated in a medium previously described by Sierksra et al. (1992), and after overnight growth a continuous feed was connected. Subsequently the yeast was grown at D of 0.05, 0.1, 0.15, 0.19 and 0.29 h⁻¹ with 1.5 g NH₄Cl I⁻ and 9.5 g glucose I⁻ in the feed. During batch and nitrogen-limited growth the pH was automatically controlled at 5 by the addition of 5 M KOH. The airflow was 1.5 l min⁻¹ and the oxygen tension was kept above 50% by controlling the stirrer speed. Carbon dioxide production [rCO₂ in mmol (g dry wt)⁻¹ h⁻¹] and oxygen consumption [rO₂ in mmol g⁻¹ h⁻¹] were measured on-line by connection of the fermenter headspace to an Uras3G CO₂ analyser and a Magnos4G O₂ analyser (Hartmann & Braun).

**Sampling and preparation of samples.** When steady state was reached, samples were taken in duplicate for three successive days, and immediately frozen in liquid nitrogen and stored at -80°C. The total sample volume was approximately 50 ml. Preparation of cell-free extracts for metabolite determination, enzyme assays, mRNA isolation and dry weight determination were performed as described by Sierksra et al. (1992). Free amino acids were isolated by resuspending 3 ml frozen culture sample into 15 ml boiling ethanol. The samples were boiled for 5 min and concentrated threefold by evaporation of approximately 12 ml suspension in a vacuum dryer. Sampling of the culture medium for the analyses of extracellular metabolites (acetate, glycerol, ethanol, glucose and NH₄⁺ concentrations) was performed by quick filtration (within 10 s after sampling) of the culture liquid through a 0.22 μm membrane filter.

**Determination of metabolites and free amino acids.** Extracellular metabolites were measured by means of HPLC analysis or enzymically, essentially as described by Bergmeyer (1974). All metabolites of glycolysis and the citric acid cycle were measured spectrophotometrically as described previously by Sierksra et al. (1993) using a Hitachi U2000 spectrophotometer. All values are given as an average of three independent samples ± SEM. Free amino acid analysis was performed on an ALPHAPLUS amino acid analyser, series 2 (Pharmacia LKB). Glutamate concentrations were also measured using the l-glutamic acid determination kit (Boehringer Mannheim).

**Labelling of oligonucleotides and Northern blot analysis.** For the detection of the ACT1 and H2A/H2B mRNA, labelled oligonucleotides were used as described by Sierksra et al. (1992). The other oligonucleotides used for the detection of mRNAs in Northern analyses were: 5'-CAGGGAGACATT-TGAGGAAATGGTATTG-3' (GAPI); 5'-TGTAGTGA-GTCCATCATGCTCTGGCAT-3' (GLNI); 5'-AAAG-AGGAGAAACTTCTCTGTTAAGCTTTAGTTAATT-CGCGCTCTGACAT-3' (GDH1); 5'-GATCAATCGGG- GTCTGGATTCTCAAAT-3' (ILV5); and 5'-ACCAGCT-AGAACAATCGAGGAGGCAAAC-3' (HIS4). Northern analyses were performed as described by Sierksra et al. (1992). Northern blots for detection of the GAP1, HIS4 and ILV5 mRNA levels were probed with ACT1 as internal control for the amount of RNA blotted, for the detection of GLN1 and GDH1 mRNA levels, H2A/H2B was used as an internal control.

**Enzyme assays.** The activities of NADPH-GDH and NAD-GDH were measured under Vmax conditions essentially as described by Boles et al. (1993). GS activity was measured as described by Mitchell & Magasanik (1983). Reaction velocities were proportional to the amount of enzyme added. The protein content of the cell extracts was determined by the method of Petterson (1977).

**RESULTS**

**Growth conditions and cell morphology.** S. cerevisiae SU32 was grown in nitrogen-limited continuous culture using ammonia as sole nitrogen source and glucose as sole carbon source. To determine the minimum medium concentrations of glucose and ammonia at which the culture was growing nitrogen-limited, S. cerevisiae was grown at D 0.1 h⁻¹ with a feed containing 1.5 g NH₄Cl I⁻ and increasing glucose concentrations,
starting at 7.0 g l⁻¹. At glucose concentrations higher than 8.5 g l⁻¹ no further increase in the biomass was observed (data not shown), indicating that with 1.5 g NH₄Cl l⁻¹ and 8.5 g glucose l⁻¹ in the feed, the culture was nitrogen-limited. To ensure nitrogen limitation the glucose concentration used was 9.5 g l⁻¹ and subsequently the yeast was grown at D values of 0.05, 0.1, 0.15, 0.19 and 0.29 h⁻¹. The cells exhibited a pseudohyphal-like morphology at all growth rates (Fig. 1). Gimeno et al. (1992) described a similar morphology of S. cerevisiae when growing on nitrogen-limited agar plates. Therefore, these data indicate that S. cerevisiae was nitrogen-limited under the conditions employed.

Physiological parameters

To be able to correlate the expression of genes involved in nitrogen and carbon metabolism to the fluxes of nitrogen and carbon, a number of physiological parameters were determined to establish the growth conditions of the yeast in detail. As shown in Fig. 2(a), with an increase in D from 0.05 to 0.29 h⁻¹, the specific CO₂ production increased sixfold from 1.6 to 9.8 mmol g⁻¹ h⁻¹; O₂ consumption also increased sixfold, from 1.5 to 9.0 mmol g⁻¹ h⁻¹. Consequently the respiration quotient was constant at around 1.1 at all D values. This indicates that no major changes occurred in the carbon metabolism under these conditions, a finding which is supported by the observation that no ethanol, glycerol or acetate could be detected in the medium (detection limits 0.05 g l⁻¹) (data not shown). The biomass remained constant at about 4.4 g l⁻¹ (Fig. 2b). As shown in Fig. 2(c), the residual

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Fig. 1. Morphology of pseudohyphae of S. cerevisiae in a nitrogen-limited continuous culture. Bar, 20 μm.

Fig. 2. (a) Production of CO₂ (□), consumption of O₂ (△) and respiratory quotient (○); (b) biomass (○), yield on glucose (*) and on ammonia (●); (c) residual concentrations of glucose (■) and ammonia (▲); (d) flux of glucose (●) and of ammonia (+) into the cells. All values are given as the mean of three independent samples ± SEM.
Fig. 3. Concentrations of glutamine, glutamate and 2-oxoglutarate in a nitrogen-limited continuous culture at different $D$ values. All values are given as the mean of three independent samples ± SEM.

Table 1. Activities of NADPH-GDH, NAD-GDH and GS in proline-, glutamate- and glutamine-grown batch cultures

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>NADPH-GDH</th>
<th>NAD-GDH</th>
<th>GS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proline</td>
<td>1.23±0.06</td>
<td>0.018±0.001</td>
<td>0.93±0.17</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.37±0.03</td>
<td>0.550±0.016</td>
<td>1.65±0.16</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.97±0.03</td>
<td>0.006±0.003</td>
<td>0.14±0.002</td>
</tr>
</tbody>
</table>

* Specific activity in μmol substrate min$^{-1}$ (mg total soluble cellular protein)$^{-1}$. Values are given as the mean of three independent samples ± SEM.

Because both the ammonia and glucose consumption increased linearly with $D$ and no changes in the biomass were observed, the yields on ammonia and glucose in the whole range of $D$ values remained constant at about 3.0 g(g NH$_4$Cl)$^{-1}$ and 0.47 g(g glucose)$^{-1}$ (Fig. 2b). Altogether, the data presented clearly demonstrate that, under the conditions employed, the fluxes of both ammonia and glucose increased linearly with $D$ and no changes occurred in the carbon metabolism.

Levels of glycolytic metabolites

Although the physiological parameters indicated no major changes in the carbon metabolism, the levels of glycolytic intermediates and ATP were determined. The glucose uptake was shown to increase linearly with $D$ and consequently the glycolytic flux also increased with $D$. With increasing $D$ the levels of the glycolytic intermediates glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate remained constant at 3.19±0.25, 0.51±0.05 and 4.52±0.39 pmol g$^{-1}$, respectively. The ATP concentration also did not change significantly and remained at 4.25±0.60 pmol g$^{-1}$.

2-Oxoglutarate and amino acid concentrations

2-Oxoglutarate, glutamate and glutamine play an essential role in the interaction between carbon and nitrogen metabolism. Therefore, their respective concentrations were analysed to investigate if changes in these concentrations could be linked to the regulation of the nitrogen metabolism. As is shown in Fig. 3 the 2-oxoglutarate, glutamine and glutamate concentrations did not change significantly and remained at about 5, 27 and 180 μmol g$^{-1}$, respectively.

Expression of nitrogen-regulated genes

To investigate the regulation of gene expression as a function of the nitrogen and carbon fluxes, mRNA was isolated from cells grown at various $D$ values, and the expression of various genes was determined by Northern blotting. These genes included GDH1 (encoding

NH$_4^+$ concentration was 2.7 mM at $D$ 0.05 h$^{-1}$, and decreased rapidly to 1.0 mM at $D$ 0.1 h$^{-1}$, followed by a more gradual decrease to 0.05 mM at $D$ 0.29 h$^{-1}$. The nitrogen and carbon fluxes were determined respectively from the residual ammonia and glucose concentration in the medium in combination with the $D$ value, and as shown in Fig. 2(d), the nitrogen flux increased linearly approximately sixfold with increasing $D$ from 0.05 to 0.29 h$^{-1}$. In contrast to the residual ammonia concentration, the residual glucose concentration increased from 0.01 to 0.4 mM with an increase in $D$ from 0.05 to 0.29 h$^{-1}$ (Fig. 2c). Nevertheless the glucose flux calculated from these data also increased linearly approximately sixfold with an increase in $D$ from 0.05 to 0.29 h$^{-1}$ (Fig. 2d). Because the residual concentrations of ammonia and glucose are small compared to their concentrations in the feed (< 1%), these variations do not significantly influence the respective fluxes.
Nitrogen-regulated metabolism in *S. cerevisiae*

The transcription of *GAP1* was used as a marker for Gln3p activity. *ILV5* and *HIS4* are both under the control of the Gcn4p transcriptional activator.

Expression of these genes was analysed in batch cultures grown on glutamine, glutamate and proline as the sole nitrogen source to compare these data with those determined from the continuous culture experiments. In agreement with previously presented data (Benjamin et al., 1989; Jauniaux & Grenson, 1990; Minehart & Magasanik, 1992; Daugherty et al., 1993), expression of the *GAP7*, *GLN7* and *GDH7* was repressed during growth on glutamine and derepressed during growth on proline and glutamate, while no differences in the expression of the *ILV5* and *HIS4* genes were observed during growth on the various nitrogen sources. (Fig. 4).

In a nitrogen-limited continuous culture with a small excess of glucose, the amount of *GAP7* mRNA increased with *D* (Fig. 4). The increase of *GAP1* transcription correlates with the increase in the nitrogen and carbon fluxes, as well as the decrease in the extracellular ammonia concentration. The level of *GLN1*, *GDH1*, *ILV5* and *HIS4* mRNAs did not change with increasing *D*. From these data we conclude that growth on ammonia alone is not sufficient to cause repression of *GAP1* and *GLN1* gene expression.

**Enzyme activities**

Glucose and ammonia fluxes increased linearly with *D*. To investigate whether the yeast reacted to the increase of these fluxes by increasing the amount or activities of the enzymes involved in the incorporation of ammonia into 2-oxoglutarate and glutamate, the maximum activities of these enzymes were analysed in crude extracts.

First their activities were determined in glutamine-, glutamate- and proline-grown batch cultures to compare these data with those found in continuous culture. In proline- and glutamate-grown cultures, the activity of NADPH-GDH was slightly higher than in glutamine-grown cultures (Table 1). As has been shown previously (Mitchell & Magasanik, 1984a), the activities of both NAD-GDH and GS were higher during growth on proline and glutamate than during growth on glutamine.

and the levels of the *GDH1* and *GLN1* transcripts were compared with *H2A/2B*. Northern blots are shown in the upper panels. The data were quantified by calculating the intensity ratio between the gene of interest and the reference gene; these ratios are shown in the lower panels. Transcription levels in batch-grown cultures with glutamine (Gln), glutamate (Glu) or proline (Pro) as the sole nitrogen source are shown in the left panels. Transcription levels in nitrogen-limited continuous cultures at *D* 0·05, 0·1, 0·15, 0·19 and 0·29 h⁻¹ are shown in the right panels.
As shown in Table 2, in a nitrogen-limited continuous culture with a small excess of glucose, the activity of the NADPH-GDH increased from 1.10 to 2.03 μmol min⁻¹ (mg protein)⁻¹, and the activity of GS increased from 0.36 to 1.02 μmol min⁻¹ (mg protein)⁻¹, when D was increased from 0.05 to 0.29 h⁻¹; the NAD-GDH activity decreased from 0.033 to 0.012 μmol min⁻¹ (mg protein)⁻¹. Because the mRNA level of the GDH1 and GLN1 genes did not change by the same amount as the activities of NADPH-GDH and GS we conclude that the enzymes are also regulated at a post-transcriptional level. Comparison with proline-, glutamate- and glutamine-grown batch cultures reveals that the NADPH-GDH and GS activities at lower D are similar to glutamine-grown cultures, and at higher D are similar to proline- or glutamate-grown cultures.

### DISCUSSION

An ammonia-limited continuous culture of *S. cerevisiae* with a small excess of glucose was used to investigate the influence of the carbon and nitrogen fluxes on the regulation of transcription and activities of enzymes involved in the interaction of these fluxes. By varying the concentration of glucose in the feed it was determined that, with 1.5 g NH₄Cl l⁻¹ and 9.5 g glucose l⁻¹, the culture was nitrogen-limited at D 0.1 h⁻¹ (data not shown). The pseudohyphal-like morphology also indicated that the culture was nitrogen-limited at all other D values.

Both the ammonia (nitrogen) and glucose (carbon) flux increased linearly with D, and no significant changes in the carbon metabolism occurred, as can be concluded from the observation that the increase in glucose uptake was equal to the increase in CO₂ production and O₂ consumption, and that no ethanol, glycerol or acetate were detected in the culture medium. Nevertheless, significant changes were observed in the residual concentrations of ammonia and glucose. With increasing D the residual ammonia concentration decreased and the residual glucose concentration increased. An increase in the carbon and nitrogen flux did not result in changes in the concentrations of glycolytic intermediates, 2-oxoglutarate, glutamate or glutamine, which was in line with the linear increase in both the glucose and ammonia flux with D.

The expression of genes encoding nitrogen-regulated enzymes and the activity of these enzymes is known to be dependent upon the nature of the nitrogen source used by the yeast (Mitchell & Magasanik, 1984b; Miller & Magasanik, 1991). The expression of the GLN1 and GAP1 genes is low during growth on a repressible nitrogen source, e.g. glutamine, and high during growth on a derepressible nitrogen source, e.g. proline. These differences in mRNA expression are caused by an altered activity of the transcriptional activator Gln3p (Mitchell & Magasanik 1984a). Except for the fact that full expression of the GDH1 gene is dependent upon the presence of the GLN3 gene product (Daugherty et al., 1993), not much is known about its transcriptional regulation. The influence of the nitrogen and carbon flux on the regulation of the GDH1 and GLN1 gene expression was analysed by mRNA detection. With increasing D and hence increasing fluxes, no change in their expression was observed. However, the expression of another Gln3p-regulated gene, GAP1, increased with D, indicating increased activity of the transcriptional activator Gln3p. In contrast to GAP1, the expression of GLN1 is also regulated by the transcriptional activator Gcn4p. The expression of two other Gen4p-regulated genes, ILV5 and HIS4, was constant at all D values, indicating no change in the activity of Gcn4p. Therefore the constant expression of GLN1 can be explained by the constant activity of Gcn4p which overrules the increased activity of Gln3p. Alternatively, the increased in GAP1 mRNA levels might also be caused by a Gln3p-independent mechanism, e.g. a decreased activity of a PER1-regulated pathway (Couchesne & Magasanik, 1983), caused by the lower residual ammonia concentrations at higher D.

From the increased GAP1 expression at high D it can be concluded that the use of ammonia as nitrogen source alone is insufficient to cause repression of the GAP1 transcription; moreover, no change in the concentrations of glutamate and glutamine could be observed, and it is therefore more likely that the concentration of ammonia is causing the repression.

With increasing D and thus decreasing residual NH₄⁺ concentrations, the activities of the enzymes using NH₄⁺ for the synthesis of glutamate and glutamine, NADPH-GDH and GS, respectively, increased whereas the activity of the glutamate-degrading and NH₄⁺-producing enzyme, NAD-GDH, decreased with increasing D. At low D the NADPH-GDH and GS activities were comparable to glutamine-grown batch cultures. At higher D, however, their activities became similar to proline- or glutamate-grown batch cultures. The increased activity of NADPH-GDH and GS is not caused by elevated transcription, as shown by constant GDH1 and GLN1 mRNA levels, and...
Three regulatory systems control expression of glutamine synthetase in and M. Suijker for the free amino acid analysis.

REFERENCES

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REFERENCES


