A nitrogen-limited, glucose-repressed, continuous culture of *Saccharomyces cerevisiae*

Laurens N. Sierkstra,¹ Eelko G. ter Schure,¹ John M. A. Verbakel²
and C. Theo Verrips^{1,2}

Author for correspondence: Laurens N. Sierkstra. Tel: +31 30 533349. Fax: +31 30 513655.

¹ Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

² Unilever Research Laboratorium Vlaardingen, Olivier van Noortlaan 120, 3133 AT Vlaardingen, The Netherlands

Glucose-repressed growth of *Saccharomyces cerevisiae* was analysed in a nitrogen-limited continuous culture at different dilution rates (D). The glucose consumption of the yeast decreased from $3.4 \text{ g g}^{-1} \text{ h}^{-1}$ to $3.0 \text{ g g}^{-1} \text{ h}^{-1}$ when D was decreased from 0.3 h^{-1} to 0.15 h^{-1} . No transcripts of the *SUC2* and *HXK1* genes, encoding, respectively, invertase and hexokinase isoenzyme 1, could be detected. Because both genes are regulated by glucose repression at the transcriptional level, this confirmed that the culture was glucose repressed at every D . During the decrease in D , no change in the activities or mRNA levels of key enzymes in carbon metabolism was observed, except for alcohol dehydrogenases I and II and phosphoglucomutase. These enzymes increased in activity and/or mRNA level when D was decreased, which was also observed in glucose- and galactose-limited continuous cultures. This demonstrates that the expression levels of alcohol dehydrogenases I and II, and also phosphoglucomutase, are coupled to the growth rate of the organism. A comparison between the alcohol dehydrogenase II activity in glucose- and nitrogen-limited continuous cultures demonstrated that the growth rate contributes as much to repression of alcohol dehydrogenase II activity as does glucose. Both the glucose consumption and the activity of the glycolytic enzymes were relatively constant when D was decreased and, as a consequence, the concentrations of intracellular metabolites remained constant. A slight decrease in the glucose 6-phosphate concentration was observed, which could be caused by the slight decrease in glucose consumption at low D values. The 2-oxoglutarate and cAMP concentrations increased twofold when D was decreased. The first probably reflects the increased NH_4 consumption at high D values, while the latter is caused by the high amount of extracellular cAMP compared with the amount of intracellular cAMP. The decrease in growth rate raised the amount of biomass, while nitrogen was limiting in all cases. Analysis of the biomass composition at the different D values revealed that the amount of nitrogen per gram dry weight was constant, while the amounts of carbon, hydrogen and oxygen were higher at low D values. In addition, higher concentrations of trehalose and glycogen were found at low D values. This demonstrates that the glucose which is used at high D values for the production of biomass is converted into storage carbohydrates, e.g. trehalose and glycogen, at low D values. A nitrogen pulse did not result in a dramatic response of *S. cerevisiae* except for a rapid change in the free amino acid pool. Thus *S. cerevisiae* is not able rapidly to enhance growth upon a nitrogen pulse, which is in contrast with the response to a glucose pulse of a carbon-limited continuous culture of *S. cerevisiae*.

Keywords: *Saccharomyces cerevisiae*, glucose repression, nitrogen limitation, continuous culture, carbon metabolism

INTRODUCTION

Glucose repression regulates the transcription of a number of genes in *Saccharomyces cerevisiae* dependent upon the availability of glucose or fructose as carbon sources. The presence of these carbon sources represses the transcription of, amongst others, genes whose products are involved in the utilization of carbon sources other than glucose or fructose (for a recent review, see Gancedo, 1992). The signal transduction pathway leading to transcriptional regulation of glucose-repressible genes has not yet been elucidated, but it has been shown that the mere presence of glucose is not sufficient to cause glucose repression of the *SUC2* and *HXX1* transcripts (Sierkstra *et al.*, 1992a). This suggests that a threshold concentration of glucose or a glucose metabolite is involved in the generation of the initial signal. A number of proteins have been isolated which are involved in the repression of *SUC2* transcription (Gancedo, 1992), and one of these, hexokinase II, was suggested to play a key role in the formation of the initial signal for glucose repression (Entian & Frohlich, 1984; Ma *et al.*, 1989; Rose *et al.*, 1991). Recently the transcription factor MIG1 has been identified, which seems to specifically regulate glucose-repressible genes. MIG1 binding site consensus sequences have been identified in a number of glucose-repressible genes (Nehlin & Ronne, 1990; Mercado *et al.*, 1991; Sierkstra *et al.*, 1992b). In addition, the repression of the *GAL* genes encoding the enzymes of the Leloir pathway is also accomplished, in part, by MIG1-dependent regulation of *GAL4* transcription (Nehlin *et al.*, 1991; Griggs & Johnston, 1991). ADR1, a transcription factor regulated by the cAMP-dependent protein kinase A (Cherry *et al.*, 1989), has been implicated in the regulation of the transcription level of the glucose-repressible *ADH2* gene, encoding alcohol dehydrogenase II. Recent results, however, have suggested that *ADH2* is not solely regulated by glucose repression, and these data also showed that the expression level of this gene is dependent upon the growth rate or glycolytic flux of the yeast (Sierkstra *et al.*, 1992a, 1993). Moreover, ADR1 seems to specifically regulate genes which are directly or indirectly involved in peroxisomal metabolism (Simon *et al.*, 1991). So, although transcription factors have been identified as components of the glucose-repression pathway, the upstream regulation of these factors in the signal transduction cascade and the formation of the initial signal have not yet been elucidated.

Data concerning glucose repression are limited to shake-flask experiments performed under non-defined physiological conditions. Recently we have initiated a study to investigate glucose repression, and the signal transduction pathway involved, under defined physiological conditions in continuous cultures. A requirement for such a study is to first describe the exact physiological state of the yeast under glucose-derepressed and glucose-repressed growth. Therefore the levels of glycolytic intermediates, ATP and cAMP, and the activity and mRNA levels of key enzymes in carbon metabolism as well as physiological parameters, such as CO₂ production and oxygen consumption, have

been determined under glucose-derepressed conditions (Sierkstra *et al.*, 1992a). In this study, glucose-repressed growth was analysed by cultivation of a commercial bakers' yeast under nitrogen limitation with an excess of glucose as carbon source.

METHODS

Strain and growth conditions. *S. cerevisiae* strain SU32 was grown at 30 °C in a 2 litre fermenter connected to an Applikon ADI 1020 controller unit. *S. cerevisiae* was inoculated into the medium previously described (Sierkstra *et al.*, 1992a) and, after overnight batch growth, a continuous feed with the same composition (resulting in a glucose-limited continuous culture) was connected. The dilution rate (*D*) was increased and, at *D* 0.3 h⁻¹, a nitrogen-limited feed was connected, which was identical to glucose-limited medium except that the amount of NH₄Cl was decreased from 7.63 g l⁻¹ to 1.4 g l⁻¹ and the glucose concentration was 103 g l⁻¹ instead of 20 g l⁻¹. Subsequently the yeast was grown at *D* 0.3 h⁻¹, 0.26 h⁻¹, 0.22 h⁻¹, 0.18 h⁻¹ and 0.15 h⁻¹. During batch and glucose-limited growth, the pH was automatically controlled at pH 5 by the addition of 5 M NH₄OH, while during nitrogen-limited growth, the pH was controlled at pH 5 with 5 M KOH. The air flow rate was 2 litres min⁻¹ and the oxygen tension was kept above 30% by regulation of the stirrer speed. CO₂ production [rCO₂ mmol h⁻¹ per gram dry wt (g)], oxygen consumption (qO₂, mmol g⁻¹ h⁻¹) and ethanol production were measured on line by connection of the headspace of the fermenter to a VG gas analysis mass spectrometer MM 8-80.

Preparation of samples. Preparation of cell-free extracts for enzyme assays, mRNA isolation for Northern blot analysis, extracts for metabolite determination and the determination of gram dry weight (abbreviated as g) per litre were performed as described previously (Sierkstra *et al.*, 1992a). Sampling of culture liquid for the determination of extracellular metabolites (acetate, acetaldehyde, glycerol, pyruvate, ethanol and the residual glucose concentrations) was performed by quick filtration (within 10 s after sampling) of the culture liquid through a 0.22 µm pore-size Millipore filter.

Determination of metabolites, free amino acids and composition of dry weight. Extracellular metabolites were measured by means of HPLC analysis and/or enzymically on the Hoffmann La Roche Cobas Mira autoanalyser essentially as described by Bergmeyer (1974). Trehalose was measured according to Van Aelst (1991), with the modification that the glucose formed was measured by incubating the mixture with hexokinase, glucose-6-phosphate dehydrogenase and ATP. The amount of glycogen in the extracts was estimated with the α-amylase kit (MA-KIT 30 Roche, no. 07 2062 3) of Hoffmann La Roche. Determination of the amount of nitrogen, carbon and hydrogen present in the dry yeast was performed on a Heraeus CHN RAPID analyser. Analysis of the oxygen content was performed according to Römer *et al.* (1972). All glycolytic metabolites were measured spectrophotometrically as described previously (Sierkstra *et al.*, 1992a) on a Philips UV/VIS spectrophotometer series PU8700. Measurement of total cAMP was done with the Amersham cAMP kit according to the instruction manual. Analysis of free amino acids was performed on an ALPHA PLUS amino acid analyser, series 2 (Pharmacia/LKB).

Enzyme assays. Enzyme activities were measured under *V*_{max} conditions essentially as described by Bergmeyer (1974).

Fig. 1. (a) Consumption of glucose (●) and flux of carbon into biomass (○), ethanol (▲) and CO_2 (△) (mol C-atoms $\text{l}^{-1} \text{h}^{-1}$); (b) flux of carbon into acetaldehyde (◇), glycerol (■) and pyruvate (□) (mmol C-atoms $\text{l}^{-1} \text{h}^{-1}$). Results for the extracellular metabolites are the means of three independent samples. The standard error in this calculation is $\leq 10\%$.

Reaction velocities were proportional to the amount of enzyme added. Activities are in U (mg total cellular soluble protein) $^{-1}$, in which 1 U is defined as the conversion of 1 μmol substrate min^{-1} at 30 °C at pH 7.0. All enzyme assays were performed on the Cobas Mira autoanalyser of Hoffmann La Roche. The protein content of the cell extracts was measured by the method of Bradford (1976) using the Bio-Rad reagent with BSA as a standard.

Labelling of oligonucleotides and Northern blot analysis. For the detection of specific mRNAs, labelled oligonucleotides described previously were used (Sierkstra *et al.*, 1992a, b). Separation of 3 μg total RNA, blotting of the gel, hybridization conditions and washing procedures have been previously described (Sierkstra *et al.*, 1992a, b). In all experiments, actin or histone was used as an internal control for the amount of mRNA blotted. The amount of specific mRNA was measured on an LKB Ultrascan XL densitometer.

RESULTS

Physiological parameters

S. cerevisiae SU32 was grown under nitrogen limitation with 103 g glucose l^{-1} in the feed at steady-state D values of 0.3 h^{-1} , 0.26 h^{-1} , 0.22 h^{-1} , 0.18 h^{-1} and 0.15 h^{-1} . This succession of different D values was employed to avoid flocculation, which was noticed at low D values in initial experiments. Under nitrogen limitation, the cells were elongated, as observed by Gimeno *et al.* (1992), while the shape of the cells was normal under glucose limitation. At all D values, the residual glucose and NH_4^+ concentrations were $> 100 \text{ mmol l}^{-1}$ and $< 0.1 \text{ mmol l}^{-1}$, respectively, while the oxygen consumption was constant at 3.8 mmol $\text{g}^{-1} \text{h}^{-1}$ (results not shown). The glucose consumption was constant at 3.4 $\text{g g}^{-1} \text{h}^{-1}$ when D was decreased from 0.3 h^{-1} to 0.26 h^{-1} and then gradually decreased to 3.0 $\text{g g}^{-1} \text{h}^{-1}$ at D 0.15 h^{-1} . The flux of carbon into biomass should have decreased twofold when D was decreased from 0.3 h^{-1} to 0.15 h^{-1} ; however a 1.4-fold decrease was observed (Fig. 1a). The flux of carbon into

ethanol and CO_2 followed the pattern of glucose consumption, although a slight increase in the ethanol and CO_2 flux could be observed when D decreased from 0.3 h^{-1} to 0.26 h^{-1} (Fig. 1a). The flux into glycerol and acetaldehyde increased (Fig. 1b), although the latter decreased again at D 0.15 h^{-1} . The carbon flux into pyruvate remained constant when D was decreased, although a slight increase at D 0.23 h^{-1} was observed. No acetate could be detected in the medium ($\leq 0.05 \text{ g l}^{-1}$).

The composition of the biomass at different D values was analysed and the amounts of carbon, hydrogen, oxygen and nitrogen were determined in the total amount of biomass (Table 1). A decrease in D resulted in an increase in the amount of hydrogen, carbon and oxygen converted into biomass, whereas the amount of nitrogen remained constant.

mRNA analysis

The levels of the transcripts of the *HXK2*, *PDC1*, *PDA1* and *PGI1* genes were constant with decreasing D values. No *SUC2* and no *HXK1* mRNA could be detected (results not shown). The *ADH1/2* transcripts remained constant when D was decreased from 0.3 h^{-1} to 0.23 h^{-1} . A subsequent decrease in D to 0.15 h^{-1} resulted in a ninefold increase in *ADH1/ADH2* mRNA level (Fig. 2).

Enzyme levels

The activities of phosphoglucoisomerase, glucose-6-phosphate dehydrogenase and pyruvate decarboxylase were constant at every D at approximately 3.2, 0.22 and 0.5 U (mg protein) $^{-1}$, respectively (Fig. 3a). An increase in alcohol dehydrogenase I and II activity from, respectively, 0.012 and 0.025 U (mg protein) $^{-1}$ at D 0.3 h^{-1} to 0.17 and 0.24 U (mg protein) $^{-1}$ at D 0.15 h^{-1} was observed. The phosphoglucomutase activity increased

Table 1. Amounts of nitrogen, carbon, oxygen and hydrogen as a percentage of the total amount of biomassResults are the means of two independently isolated samples, $\pm$ the range.

<i>D</i> (h ⁻¹)	N (%)	C (%)	O (%)	H (%)	Biomass (g l ⁻¹)
0.3	8.11 $\pm$ 0.01	43.68 $\pm$ 0.06	34.68 $\pm$ 0.50	6.24 $\pm$ 0.06	4.2 $\pm$ 0.1
0.26	7.81 $\pm$ 0.06	43.78 $\pm$ 0.05	33.42 $\pm$ 0.84	6.17 $\pm$ 0.08	4.7 $\pm$ 0.1
0.23	7.21 $\pm$ 0.06	43.56 $\pm$ 0.12	35.93 $\pm$ 0.64	6.01 $\pm$ 0.28	5.0 $\pm$ 0.1
0.18	6.81 $\pm$ 0.19	43.77 $\pm$ 0.30	35.09 $\pm$ 0.79	6.39 $\pm$ 0.03	5.4 $\pm$ 0.1
0.15	6.07 $\pm$ 0.01	43.60 $\pm$ 0.07	35.39 $\pm$ 0.22	6.24 $\pm$ 0.10	5.9 $\pm$ 0.3

Fig. 2. Northern blot analysis of the *ADH1/ADH2* transcript at different *D* values. The amount of specific mRNA is given beneath each lane as a percentage of the initial value at *D* 0.3 h⁻¹ and corrected for the internal standard *H2A/H2B*.

from 0.024 U (mg protein)⁻¹ at *D* 0.3 h⁻¹ to 0.112 U (mg protein)⁻¹ at *D* 0.15 h⁻¹ (Fig. 3b). The decrease from *D* 0.3 h⁻¹ to *D* 0.15 h⁻¹ resulted in a slight increase in the

hexokinase activity from 0.9 U (mg protein)⁻¹ to 1.1 U (mg protein)⁻¹.

Metabolite levels

The levels of glycolytic intermediate of the first part of glycolysis, ATP and cAMP were measured (Table 2). Glucose 6-phosphate decreased slightly during the decrease in *D* in spite of the increase in hexokinase activity, while glucose 1-phosphate, fructose 1,6-diphosphate and fructose 6-phosphate remained constant. The level of ATP was constant at different *D* values although it was slightly lower at *D* 0.15 h⁻¹. Because of its importance as a metabolite at the branch point between carbon and nitrogen metabolism, 2-oxoglutarate was also measured. The concentrations of 2-oxoglutarate and cAMP were raised twofold when *D* was decreased from 0.3 h⁻¹ to 0.15 h⁻¹. At all *D* values, the amount of extracellular cAMP was > 80 % of the total amount of cAMP.

The concentration of the reserve/stress carbohydrates trehalose and glycogen increased from 0.0055 and 0.093 μ mol g⁻¹, respectively, at *D* 0.3 h⁻¹, to 0.36 and 1.16 μ mol g⁻¹ at *D* 0.15 h⁻¹.

Fig. 3. Specific activities of the following enzymes as a function of *D*: (a) HXK (hexokinase, ●), PGI (phosphoglucose isomerase, ○), PDC (pyruvate decarboxylase, □) and G6PDH (glucose-6-phosphate dehydrogenase, ■); (b) GAL5 (phosphoglucose mutase, ◇), ADHI (alcohol dehydrogenase I, ▲) and ADHII (alcohol dehydrogenase II, △). Results shown are the means of three independently isolated samples assayed in duplicate. Bars indicate SE.

Table 2. Concentrations of glucose 6-phosphate (g6p), fructose 6-phosphate (f6p), fructose 1,6-bisphosphate (f1,6bp), glucose 1-phosphate (g1p), ATP, cAMP and 2-oxoglutarate (2-Oxo) in $\mu\text{mol g}^{-1}$ at different D values

The concentrations are corrected for the increased amount of biomass produced at lower D values. Results are the means of three independently isolated samples assayed in duplicate, $\pm$ SE.

D (h^{-1})	g6p	f6p	f1,6bp	g1p	ATP	cAMP	2-Oxo
0.3	2.33 ± 0.36	0.33 ± 0.09	20.4 ± 2.1	0.25 ± 0.02	5.72 ± 0.43	8.0 ± 0.9	50.6 ± 6.0
0.26	2.42 ± 0.21	0.28 ± 0.18	17.0 ± 2.5	0.24 ± 0.12	6.28 ± 0.55	8.8 ± 0.6	61.8 ± 7.5
0.23	2.33 ± 0.30	0.36 ± 0.06	18.5 ± 2.1	0.31 ± 0.10	5.10 ± 0.25	9.4 ± 0.9	78.3 ± 6.9
0.18	2.05 ± 0.03	0.16 ± 0.01	21.5 ± 1.1	0.22 ± 0.12	5.32 ± 0.24	13.0 ± 0.7	87.3 ± 6.1
0.15	1.91 ± 0.32	0.33 ± 0.20	21.9 ± 1.8	0.24 ± 0.13	4.25 ± 0.56	14.2 ± 1.8	102.0 ± 1.6

Nitrogen pulse

A pulse of 0.1 M NH_4^+ was given to the nitrogen-limited continuous culture at $D 0.3 \text{ h}^{-1}$ in order to investigate the effect on carbon utilization and growth. An increase in the biomass could be seen 200 min after the nitrogen pulse. No significant change in the concentrations of intracellular metabolites, cAMP and ATP, or in the activities and mRNA levels of key enzymes in carbon metabolism, was observed after the pulse (results not shown). Only a slight decrease in the 2-oxoglutarate concentration could be detected ($46 \mu\text{mol g}^{-1}$ at $t = 0$ to $30 \mu\text{mol g}^{-1}$ at $t = 120$). Analysis of the concentration of the free amino acids revealed that there were four patterns for the changes in amino acid concentration after a nitrogen pulse. No change in the concentrations of cysteine, leucine, methionine, isoleucine, valine and tyrosine could be observed. The concentrations of glutamate, arginine, proline, serine, lysine, glycine and phenylalanine increased 30 min after the pulse, while the concentrations of threonine and histidine increased 7.5 min after the addition of nitrogen. A direct increase in the concentrations of alanine, glutamine and aspartate was observed, although the concentration of aspartate decreased again 30 min after the pulse. Within 60 min after nitrogen addition, the total amino acid pool was increased from 300 to $700 \mu\text{mol g}^{-1}$ and thereafter remained constant (results not shown).

DISCUSSION

Repression of respiration

At all D values the residual glucose concentration was $> 100 \text{ mmol l}^{-1}$, while that of the limiting substrate, NH_4^+ , was $< 0.1 \text{ mmol l}^{-1}$. No *SUC2* or *HXK1* mRNA could be detected. Both *SUC2* and *HXK1* are subject to glucose repression at the transcriptional level (Carlson, 1987; Sierkstra *et al.*, 1992a), and this result demonstrates that the cells were glucose repressed at every D . The specific oxygen consumption was $3\text{--}3.8 \text{ mmol g}^{-1} \text{ h}^{-1}$ at every D . The maximum specific oxygen consumption observed under glucose-derepressed conditions for this strain is $7 \text{ mmol g}^{-1} \text{ h}^{-1}$ (Sierkstra *et al.*, 1992a, 1993). Thus half of the maximum amount of oxygen is still consumed under glucose-repressed conditions. It has been reported that the enzymes of the citric acid cycle and oxidative

phosphorylation are regulated by glucose repression (Holzer, 1976; Gancedo, 1992), and the difference in the specific oxygen consumption between glucose-derepressed and glucose-repressed cultures could reflect this repression of enzyme synthesis. However, when D was increased in carbon-limited continuous cultures and ethanol production occurred, a decrease in the respiration to $3\text{--}3.5 \text{ mmol g}^{-1} \text{ h}^{-1}$ was observed (Sierkstra *et al.*, 1992a, 1993). This decrease was not caused by glucose repression of respiration but by the altered metabolic state of the yeast at this D , which changed from completely respirative to respiro-fermentative; this could also explain the difference in respiration between glucose-derepressed and glucose-repressed cultures.

Carbon fluxes

The consumption of glucose was almost constant at $3\text{--}3.4 \text{ g glucose g}^{-1} \text{ h}^{-1}$ when the growth rate was decreased, which demonstrates that the maximal glucose consumption of yeasts is independent of the growth rate. The flux of carbon into biomass was only 1.4-fold higher at $D 0.3 \text{ h}^{-1}$ as compared with $D 0.15 \text{ h}^{-1}$ instead of the theoretical twofold increase. This was caused by the fact that the biomass concentration in the fermenter was higher at $D 0.15 \text{ h}^{-1}$. Analysis of the biomass composition showed that the amount of nitrogen was constant but that the amount of hydrogen, carbon and oxygen was higher at low D values. Thus more carbon-, hydrogen- and oxygen-containing compounds are produced at lower D values, which was confirmed by the measurement of trehalose and glycogen. This demonstrates that the glucose which is used at higher D values for the formation of biomass is, at least in part, converted into reserve carbohydrates at low D values. The flux of carbon into ethanol and CO_2 decreased at low D values, while the acetaldehyde and glycerol production increased. Increased glycerol production at low D values could have the same function as trehalose and glycogen production. The decreased ethanol flux is in contrast to the higher alcohol dehydrogenase I activity at low D values. This result suggests that the conversion of acetaldehyde into ethanol in *S. cerevisiae* is, under these conditions, regulated by the availability of co-factors such as NADH and NAD, instead of the amount of enzyme. No acetate could be

detected (detection limit $< 0.05 \text{ g l}^{-1}$). The strain is capable of acetate production (Sierkstra *et al.*, 1992a), but in this case either the acetyl-CoA synthetase bypass (Postma *et al.*, 1989) is not used or the affinity of acetyl-CoA synthetase for acetate is greatly enhanced under these conditions, resulting in undetectable residual acetate levels. The decrease in D left the concentrations of the glycolytic intermediates constant, which is in accordance with the virtually constant uptake of glucose and constant glycolytic enzyme levels. Only a slight decrease in glucose 6-phosphate concentration was observed, which coincided with the slight decrease in glucose consumption. In contrast, the concentrations of total cAMP (intra- and extracellular) and 2-oxoglutarate both increased twofold when the dilution was decreased. Because cAMP is mostly found extracellularly, the twofold decrease in D will result in a twofold increase in the total cAMP concentration. The lower intracellular 2-oxoglutarate concentration at high D values is probably caused by the increased formation of glutamine because more NH_4^+ is available per hour at higher D values.

Expression of key enzymes in carbon metabolism

The change in D under glucose-repressed conditions had no effect on the activities and mRNA levels of key enzymes in carbon metabolism, apart from phosphoglucomutase and alcohol dehydrogenases I and II, which increased with decreasing dilution rates. This was also observed in glucose- and galactose-limited continuous cultures (Sierkstra *et al.*, 1992a, 1993), which demonstrates that alcohol dehydrogenase II is not exclusively regulated by glucose but that the expression level of this gene is coupled to the growth rate of the organism. In addition, the growth rate significantly contributes to the regulation of alcohol dehydrogenase I and phosphoglucomutase.

Comparison of the expression levels of key enzymes during glucose-repressed versus glucose-derepressed growth

Activity measurements in both glucose- (Sierkstra *et al.*, 1992a) and nitrogen-limited continuous cultures showed that phosphoglucosomerase and glucose-6-phosphate dehydrogenase have identical activities under both growth conditions. Pyruvate decarboxylase activity is induced two- to threefold, while the total hexose phosphorylating capacity is twofold lower under glucose repression, probably reflecting the absence of the *HXK1* transcript. Phosphoglucomutase activity is 10–20-fold lower under repressed growth conditions, indicating that, in contrast to other reports (Johnston, 1987), this gene is also regulated by carbon source. Alcohol dehydrogenase II activity was reduced tenfold under glucose-repressed conditions as compared with glucose-derepressed conditions (at $D 0.3 \text{ h}^{-1}$), while a tenfold increase in activity was also observed when D was decreased from 0.3 h^{-1} to 0.15 h^{-1} . This demonstrates that the contribution of the growth rate to the regulation of alcohol dehydrogenase II levels is at least as important as the regulation of alcohol dehydrogenase II by glucose repression.

Nitrogen pulse

A nitrogen pulse had limited effects upon the carbon metabolism and growth rate of *S. cerevisiae*. No direct change in CO_2 production, oxygen consumption and biomass could be detected. Moreover, no effect of the nitrogen pulse on the concentrations of intracellular metabolites and on the expression of the above mentioned genes (translational or transcriptional level) could be detected. Only the intracellular amino acid pool changed: from low to high amino acid levels. After glucose pulses to carbon-limited continuous cultures, increased cAMP production was observed and, furthermore, the mRNA level of glucose-regulated genes was altered (Sierkstra *et al.*, 1992b). The measurements performed in this study did not reveal a nitrogen-induced signal transduction cascade involved in the regulation of carbon utilization in *S. cerevisiae*. Additional experiments are currently being performed to further investigate nitrogen-specific signal transduction pathways in yeast with emphasis on inositol 1,4,5-phosphate metabolism (Schomerus & Kuntzel, 1992), GCN4-mediated transcription (Hinnebusch, 1988) and NCR-regulated transcription (e.g. Cooper *et al.*, 1992).

REFERENCES

- Bergmeyer, H. U. (1974). *Methods of Enzymatic Analysis*. Weinheim: Verlag Chemie.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Carlson, M. (1987). Regulation of sugar utilization in *Saccharomyces* species. *J Bacteriol* **169**, 4873–4877.
- Cherry, J. R., Johnson, T. R., Dollard, C., Shuster, J. R. & Denis, C. L. (1989). cAMP dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator ADR1. *Cell* **56**, 409–419.
- Cooper, T. G., Kovari, L., Sumrada, R. A., Park, H. D., Luche, R. M. & Kovari, I. (1992). Nitrogen catabolite repression of arginase (*CAR1*) expression in *Saccharomyces cerevisiae* is derived from regulated inducer exclusion. *J Bacteriol* **174**, 48–55.
- Entian, K. D. & Frohlich, K. U. (1984). *Saccharomyces cerevisiae* mutants provide evidence of hexokinase PII as a bifunctional enzyme with catalytic and regulatory domains for triggering catabolite repression. *J Bacteriol* **158**, 29–35.
- Gancedo, J. M. (1992). Carbon catabolite repression in yeast. *Eur J Biochem* **206**, 297–313.
- Gimeno, C. J., Ljungdahl, P. O., Styles, C. A. & Fink, G. R. (1992). Unipolar cell divisions in the yeast *Saccharomyces cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell* **68**, 1077–1090.
- Griggs, D. W. & Johnston, M. (1991). Regulated expression of the *GAL4* activator gene in yeast provides a sensitive switch for glucose repression. *Proc Nat Acad Sci USA* **88**, 8597–8601.
- Hinnebusch, A. G. (1988). Mechanism of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. *Microbiol Rev* **52**, 248–273.
- Holzer, H. (1976). Catabolite inactivation in yeast. *Trends Biochem Sci* **1**, 178–181.

- Johnston, M. (1987). A model fungal gene regulatory mechanism: the *GAL* genes of *Saccharomyces cerevisiae*. *Microbiol Rev* **51**, 458–476.
- Ma, H., Bloom, L. M., Walsh, C. T. & Botstein, D. (1989). The residual enzymatic phosphorylation activity of hexokinase II mutants is correlated with glucose repression in *Saccharomyces cerevisiae*. *Mol Cell Biol* **9**, 5643–5649.
- Mercado, J. J., Vincent, O. & Gancedo, J. M. (1991). Regions in the promoter of the yeast *FBP1* gene implicated in catabolite repression may bind the product of the regulatory gene *MIG1*. *FEBS Lett* **291**, 97–100.
- Nehlin, J. O. & Ronne, H. (1990). Yeast *MIG1* repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *EMBO J* **9**, 2891–2898.
- Nehlin, J. O., Carlberg, M. & Ronne, H. (1991). Control of yeast *GAL* genes by *MIG1* repressor; a transcriptional cascade in the glucose response. *EMBO J* **10**, 3373–3378.
- Postma, E., Verduyn, C., Scheffers, W. A. & Van Dijken, J. P. (1989). Enzymic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **55**, 468–477.
- Römer, F. G., van Osch, G. W., Buis, W. J. & Griepink, B. (1972). Automation of carbon determinations. II. Automatic determination of mg amounts of carbon dioxide and its application to oxygen determination in organic substances. *Mikrochim Acta*, 674–679.
- Rose, M., Albig, W. & Entian, K. D. (1991). Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexose phosphorylation by hexokinases PI and PII. *Eur J Biochem* **199**, 511–518.
- Schomerus, C. & Küntzel, H. (1992). *CDC25*-dependent induction of inositol 1,4,5-triphosphate and diacylglycerol in *Saccharomyces cerevisiae* by nitrogen. *FEBS Lett* **307**, 249–252.
- Sierkstra, L. N., Verbakel, J. M. A. & Verrips, C. T. (1992a). Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of *Saccharomyces cerevisiae*. *J Gen Microbiol* **138**, 2559–2566.
- Sierkstra, L. N., Nouwen, N. P., Verbakel, J. M. A. & Verrips, C. T. (1992b). Analysis of glucose repression by pulsing glucose to a galactose limited continuous culture of *Saccharomyces cerevisiae*. *Yeast* **8**, 1077–1087.
- Sierkstra, L. N., Nouwen, N. P., Verbakel, J. M. A. & Verrips, C. T. (1993). Regulation of glycolytic enzymes and the Crabtree effect in galactose limited continuous cultures of *Saccharomyces cerevisiae*. *Yeast* (in press).
- Simon, M., Adam, G., Rapatz, W., Spevak, W. & Ruis, H. (1991). The *Saccharomyces cerevisiae ADR1* gene is a positive regulator of transcription of genes encoding peroxisomal proteins. *Mol Cell Biol* **11**, 699–704.
- Van Aelst, A. A. (1991). *Glucose control of the RAS-adenylate cyclase pathway in the yeast S. cerevisiae*. PhD thesis, Katholieke Universiteit Leuven, Belgium.

Received 24 June 1993; accepted 2 December 1993.