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Dynamic Responses of Reserve Carbohydrate Metabolism Under Carbon and Nitrogen Limitations in *Saccharomyces cerevisiae*

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The dynamic responses of reserve carbohydrates with respect to shortage of either carbon or nitrogen source was studied to obtain a sound basis for further investigations devoted to the characterization of mechanisms by which the yeast *Saccharomyces cerevisiae* can cope with nutrient limitation during growth. This study was carried out in well-controlled bioreactors which allow accurate monitoring of growth and frequent sampling without disturbing the culture. Under glucose limitation, genes involved in glycogen and trehalose biosynthesis (*GLG1*, *GSY1*, *GSY2*, *GAC1*, *GLC3*, *TPSI*), in their degradation (*GPH1*, *NTH1*), and the typical stress-responsive *CTTI* gene were coordinately induced in parallel with glycogen, when the growth has left the pure exponential phase and while glucose was still plentiful in the medium. Trehalose accumulation was delayed until the diauxic shift, although *TPSI* was induced much earlier, due to hydrolysis of trehalose by high trehalase activity. In contrast, under nitrogen limitation, both glycogen and trehalose began to accumulate at the precise time when the nitrogen source was exhausted from the medium, coincidentally with the transcriptional activation of genes involved in their metabolism. While this response to nitrogen starvation was likely mediated by the stress-responsive elements (STREs) in the promoter of these genes, we found that these elements were not responsible for the co-induction of genes involved in reserve carbohydrate metabolism during glucose limitation, since *GLG1*, which does not contain any STRE, was coordinately induced with *GSY2* and *TPSI*. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — growth phases; nutrient limitation; reserve carbohydrates; stress; gene expression; *Saccharomyces cerevisiae*

INTRODUCTION

In the yeast *Saccharomyces cerevisiae*, cell division is tightly controlled at the level of the G₁ phase of the cell cycle by environmental conditions including nutrient availability (Werner Washburne *et al.*, 1993, 1996; Thomas and Hall, 1997). Before engaging in a subsequent round of division, cells must sense and integrate both intracellular and extracellular signals. The most usual experimental approach to investigate nutrient signalling pathways in growth control is the sudden removal of a nutrient essential to exponentially growing cells. As a consequence, haploid cells exit the mitotic

cycle, enter the quiescent G₀ state and develop stationary-phase characteristics which include accumulation of reserve carbohydrates and acquisition of thermotolerance (for reviews, see Werner Washburne *et al.*, 1993, 1996). Diploid cells starved for nitrogen on a non-fermentable carbon source undergo meiosis and sporulation, whereas they sometimes develop pseudohyphal growth on a glucose-based medium (Gimeno *et al.*, 1992; Kron, 1997).

The way in which yeast cells respond to the sudden removal of nutrient from the growth medium (i.e. nutrient starvation) is probably different from the way that they assess the progressive depletion of an essential nutrient during batch cultures (i.e. nutrient limitation), even though both conditions eventually lead to growth arrest. Indeed, Lillie and Pringle (1980) observed that as

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cells were depleting their supply of exogenous glucose, they started to accumulate glycogen when approximately half of the original glucose had been consumed. Other modifications probably occurred at the same time, including a reduction of the glycolytic flux and of the overall level of protein synthesis (François *et al.*, 1987; Boucherie, 1985) and induction of heat shock and stress proteins (Werner Washburne *et al.*, 1989; Bissinger *et al.*, 1989; Boorstein and Craig, 1990; Varela *et al.*, 1995). Using the DNA microarrays technique, DeRisi *et al.* (1997) illustrated the wide changes in expression patterns of known and uncharacterized genes during diauxic growth on a glucose-based rich medium. These physiological adaptations suggest that sophisticated dynamic sensing processes must exist to inform the cell of shortage of glucose and perhaps of other essential nutrients.

A critical step towards the characterization of these sensing processes was to define precisely the timing of the physiological responses with respect to the dynamic consumption of the limiting nutrient during growth. Such a study can be accurately performed in well-equipped fermenters which allow monitoring of growth parameters (temperature, pH, gas-transfer, etc.) and high-frequency sampling without disturbing the culture, procedure which should avoid any misinterpretations from a two-sample analysis, i.e. one point in the exponential phase and the second one in the 'stationary' phase. Taking into account that glycogen and trehalose are hallmarks of changing nutritional conditions (Lillie and Pringle, 1980; Thompson-Jaeger *et al.*, 1991; François *et al.*, 1991; Hardy *et al.*, 1994; Winderickx *et al.*, 1996), we reinvestigated the dynamic changes of these two aspects of metabolism during growth of yeast under carbon- or nitrogen-limiting conditions. Since it was recently claimed that genes possessing one or several stress-responsive elements (STREs, Kobayashi and McEntee, 1993) share the same expression profile during growth on glucose (De Risi *et al.*, 1997), the wide panel of genes investigated in this study allowed us to reconsider the requirement of STREs in gene induction.

MATERIALS AND METHODS

Construction of yeast strains

The *Saccharomyces cerevisiae* strains used in this work are listed in Table 1. The *snf1Δ::HIS3*

allele was constructed from pBSK-SNF1 which contains a 3.3 kb *EcoRI/BamHI* genomic fragment from *SNF1* locus. Partial *BsmI* deletion resulted in the loss of the 3' promoter region and most of the *SNF1* catalytic domain (pJL23). Subsequent insertion of a *HIS3* 1.1 kb *BamHI* fragment into the *BclI* site of the *SNF1* regulatory domain yielded the final construct, pJL24. Construction of the *hxx2Δ::HIS3* allele as carried out from pMR124, which contains a 2.43 kb *KpnI/EcoRI* genomic fragment with the 5' part of the *HXX2* gene (gift from M. Rose and K. D. Entian, University of Frankfurt, Germany). The *KpnI/EcoRI* fragment was subcloned into *KpnI/EcoRI* digested pUC18 for convenient use in the following cloning steps (pJL9). As for *SNF1*, the *HIS3* 1.1 kb *BamHI* fragment was introduced into the unique *BclI* site of *HXX2* (pJL10) and further deletion of the 120 bp *EcoRV* fragment from pJL10 yielded the final construct (pJL15). Gene disruption was carried out in the diploid strain JF326 according to the one-step replacement procedure (Rothstein, 1983), using the 3.5 kb *EcoRI/BamHI* fragment from pJL24 (*SNF1* disruption) and the 3.4 kb *EcoRI/BamHI* fragment from pJL15 (*HXX2* disruption). Correct disruption of *SNF1* and *HXX2* genes in *HIS*⁺ colonies was confirmed by Southern blot analysis. Sporulation and dissection of diploids yielded the haploid strains JLP74-10A (*snf1Δ::HIS3* mutant) and JLP11-1A (*hxx2Δ::HIS3* mutant).

Construction and integration of plasmids bearing tradunctional fusions between *GSY2*, *GAC1*, *GPH1* or *TPS1* and the *E. coli lacZ* gene, and the pNL1 episomic plasmid carrying the *NTH1-lacZ* tradunctional fusion gene, have been described previously (Parrou *et al.*, 1997). The centromeric plasmid pWB204Δ-236 carrying a *SSA3-lacZ* fusion gene has been described by Boorstein and Craig (1990). Reinsertion of wild type *LEU2* and *HIS3* genes in JLP5-1A strain yielded the prototroph JLP48-3B strain. Crosses between haploid strains bearing *lacZ* fusion genes and strains with *SNF1*, *HXX2* or *NTH1* null mutation were performed to obtain heterozygous diploid strains. These latter were sporulated and dissected, and haploid null mutant strains carrying a fusion gene were selected. Targeted integration of *NcoI* linearized pJL5 (*GSY2-lacZ* fusion gene) at the *ura3* locus of RS13-58A-1 (*tpk^w bcy1* mutant) yielded the strain JF908.

Table 1. Yeast strains used in this work.

Yeast strain	Genotype	Source
KT1112	a <i>leu2 ura3 his3</i>	K. Tatchell
JF326	diploid from KT1112	This study
X2180-1A	a prototroph	YGSC ¹
CENPK133-7D	a prototroph	P. Kötter
JLP74-10A	a <i>leu2 ura3 his3 snf1Δ::HIS3</i>	This study
JLP11-1A	a <i>leu2 ura3 his3 hxxk2Δ::HIS3</i>	This study
YSN1	a <i>leu2 ura3 his3 can1 gal⁻ nth1Δ::LEU2</i>	H. Holzer ²
RS13-58A-1	a <i>leu2 ura3 his3 trp1 ade8</i> <i>tpk1^w tpk2::HIS3 tpk3::TRP1 bcy1::LEU2</i>	M. Wigler ³
11a-1A	a <i>leu2 his3 trp1 ade2 ade8 URA3::CTT1-lacZ</i>	K. Tatchell
JLP1-1D	a <i>leu2 his3 trp1 ade2 can1 URA3::GSY2-lacZ</i>	This study
JF908	a <i>leu2 ura3 his3 trp1 ADE8 URA3::GSY2-lacZ</i> <i>tpk1^w tpk2::HIS3 tpk3::TRP1 bcy1::LEU2</i>	This study
JLP5-1A	a <i>leu2 his3 URA3::GSY2-lacZ</i>	This study
JLP44-8A	a <i>leu2 his3 URA3::GPH1-lacZ</i>	This study
JLP51-3D	a <i>leu2 his3 URA3::GAC1-lacZ</i>	This study
JLP48-3B	a <i>LEU2 HIS3 URA3::GSY2-lacZ</i>	This study
JLP75-4A	a <i>leu2 his3 URA3::GSY2-lacZ snf1Δ::HIS3</i>	This study
JLP81-1C	a <i>leu2 his3 URA3::GSY2-lacZ hxxk2Δ::HIS3</i>	This study
JLP87-8A	a <i>leu2 his3 URA3::TPS1-lacZ</i>	This study
JLP88-21A	a <i>leu2 his3 GAL URA3::TPS1-lacZ NTH1</i>	This study
JLP88-21B	a <i>leu2 his3 GAL URA3::TPS1-lacZ nth1Δ::LEU2</i>	This study

¹Mortimer and Johnston (1986). *Genetics* **113**, 35–43; ²Nwaka *et al.* (1994). *FEBS Lett.* **344**, 225–228;

³Cameron *et al.* (1988). *Cell* **53**, 555–566.

Reagents, media and culture conditions

Yeast nitrogen base without amino acids and ammonium sulphate, Bacto peptone and yeast extract were from Difco laboratories. Chemicals for media preparation were from Merck or Prolabo and were of the purest grade available. Rich medium (YEP) contained (per litre) 10 g yeast extract, 20 g Bacto peptone and carbon sources at the indicated concentrations. Mineral medium (MIN) was prepared according to Johnston *et al.* (1977) and, unless otherwise stated, it contained 10 g/l ammonium sulphate and 20 g/l glucose. Mineral medium (YNB) contained 1.7 g/l yeast nitrogen base without amino acids and ammonium sulphate, 5 g (NH₄)₂SO₄ and 10 g glucose. It was supplemented with 100 µg/l of auxotrophic requirements. Both MIN and YNB were buffered at an initial pH of 5.8 by addition of 10 g succinic acid and 6 g NaOH/litre. The 'cell factory medium' (CFM) was prepared according to Verduyn *et al.* (1992). In order to avoid any cross-reaction during autoclaving, stock solutions of YNB (20 ×), vitamins and oligoelements (1000 ×) were sterilized by filtration (Millipore

nitrocellulose filters, 0.2 µm pore size, 45 mm diameter). Stock solution of glucose (500 g/l) was sterilized by autoclaving for 30 min at 110°C. Yeast precultures were carried out in half-litre flasks containing 100 ml of the medium used in the subsequent batch fermenter. Cultures were performed at 30°C in 2 l fermenters (Inceltech-SGI, France) with a 1.7 l working volume, a 500 rpm impeller speed and a 0.5 l/min airflow in order to maintain dissolved oxygen near saturation. For nitrogen starvation experiments, yeast cells grown in either YEP or YNB (20 g/l glucose and 5 g/l ammonium sulphate) were rapidly collected by filtration at OD₆₀₀ 0.5 (Millipore nitrocellulose filters, 0.45 µm pore size, 45 mm diameter), washed once with 10 ml of YNB without nitrogen source and resuspended in the same volume of this nitrogen-free medium. Growth was monitored by measuring the absorbance of the culture at 600 nm with an Easyspec IV spectrophotometer (Safas SA, France). Growth curves were fitted using an exponential regression analysis (Excel 5.0 software), taking into account for calculation experimental points within the time window indicated

by the arrows on the axis, i.e. during the apparent exponential phase. The prerequisite for this time window was to reach a correlation coefficient (r^2) higher than 0.998. The μ constant from the equation $OD=b \exp(\mu.t)$ of the fitting curve corresponds to the specific growth rate ($\mu=\ln 2/T_G$) where T_G =the doubling time.

Northern and Western blot analysis

Western blot analysis of Tps1p was performed as described by Parrou *et al.* (1997). Signal quantification of autoradiographic film from Western analysis was performed using the Tina 2.0 PC Bas software. Total yeast RNAs were isolated following the procedure described in Siderius *et al.* (1997). Probes (656 bp *EcoRI/BamHI* fragment from *GSY2*, 825 bp *XmnI/NcoI* fragment from *GLG1*, 1245 bp *DraIII/NdeI* fragment from *NTH1*, 1985 bp *SacI/SacI* fragment from *LacZ* and 1562 bp *BamHI/HindIII* fragment from *ACT1*) were $\alpha^{32}\text{P}$ -labelled using the random-priming method (Amersham Megaprime Kit).

Enzymatic assays and analytical procedures

For enzymatic assays, 10^9 cell samples were quickly collected by filtration on nitrocellulose filters and frozen into liquid nitrogen. Preparation of crude extracts was performed as described by Parrou *et al.* (1997). Tre6P synthase activity was measured by the method of UDP formation (Vandercammen *et al.*, 1989), except that the reaction was carried out at 42°C to reduce glycogen synthase interference. Glycogen synthase activity was measured in the presence of 0.25 mM UDP[U- ^{14}C]glucose without (active form) or with 10 mM Glc6P (total form) (François *et al.*, 1988), and neutral trehalase activity was assayed as in Neves and François (1992). The total amount of proteins in the extracts was determined by the method of Bradford (1976) using bovine serum albumin as a standard. $2 \cdot 10^8$ cell samples were collected by centrifugation for the measurement of β -galactosidase activity (Rose and Botstein, 1983) and the quantitative determination of glycogen and trehalose (Parrou and François, 1997). Glucose and ammonium from the culture medium were measured using the Glucose Oxydase Reaction Kit (Sigma) and the Ammonium Electrode Analyser (Orion), respectively.

RESULTS

Establishment of growth conditions and terminology

In order to investigate the effects of specific nutrient limitations on the dynamics of glycogen and trehalose metabolism, an essential prerequisite was to define culture media limited respectively by carbon, nitrogen, sulphur or phosphorus source. With respect to terminology, we considered one of these four essential elements as 'limiting' for growth under discontinuous batch cultures, when it became exhausted from the medium while the others remained in large excess. The exhaustion of the limiting nutrient led to either a transient growth arrest (e.g. diauxic shift under glucose limitation, see Figure 1) or a sudden and continuous decay of the specific growth rate giving rise to a linear growth curve, followed by the entrance of cells into stationary phase (e.g. ammonium [see Figure 6], sulphur or phosphorus limitation). At this stage of growth, the cell biomass had to be roughly proportional to the initial concentration of the limiting nutrient.

We therefore found that the initial glucose concentration had to be lower than or equal to 10 g/l in minimal media to ensure that it was limiting for growth of auxotrophic KT1112 and derivative strains. In contrast, the biomass of prototroph JLP48-3B, X2180-1A and CENPK133-7D strains increased almost proportionally with rising sugar concentrations, at least up to 40 g/l. From this observation, it was concluded that growth defects of auxotroph strains could result from impaired metabolic activities linked to their auxotrophies. As regards nitrogen source, nitrogen limitation on glucose-based MIN medium was obtained with an initial ammonium sulphate concentration lower than or equal to 2 g/l. In contrast, it was difficult to get proportional limitations for either phosphorus or sulphur sources, probably because of high intracellular stores of these compounds, which rendered yeast cells poorly sensitive to P_i - or S-limitation. Hence, we did not investigate these nutritional conditions in this present work.

Coordinated induction of genes but differential accumulation of glycogen and trehalose under glucose limitation

Lillie and Pringle (1980) originally observed that yeast cells undergoing diauxic growth on glucose limited medium began to accumulate glycogen

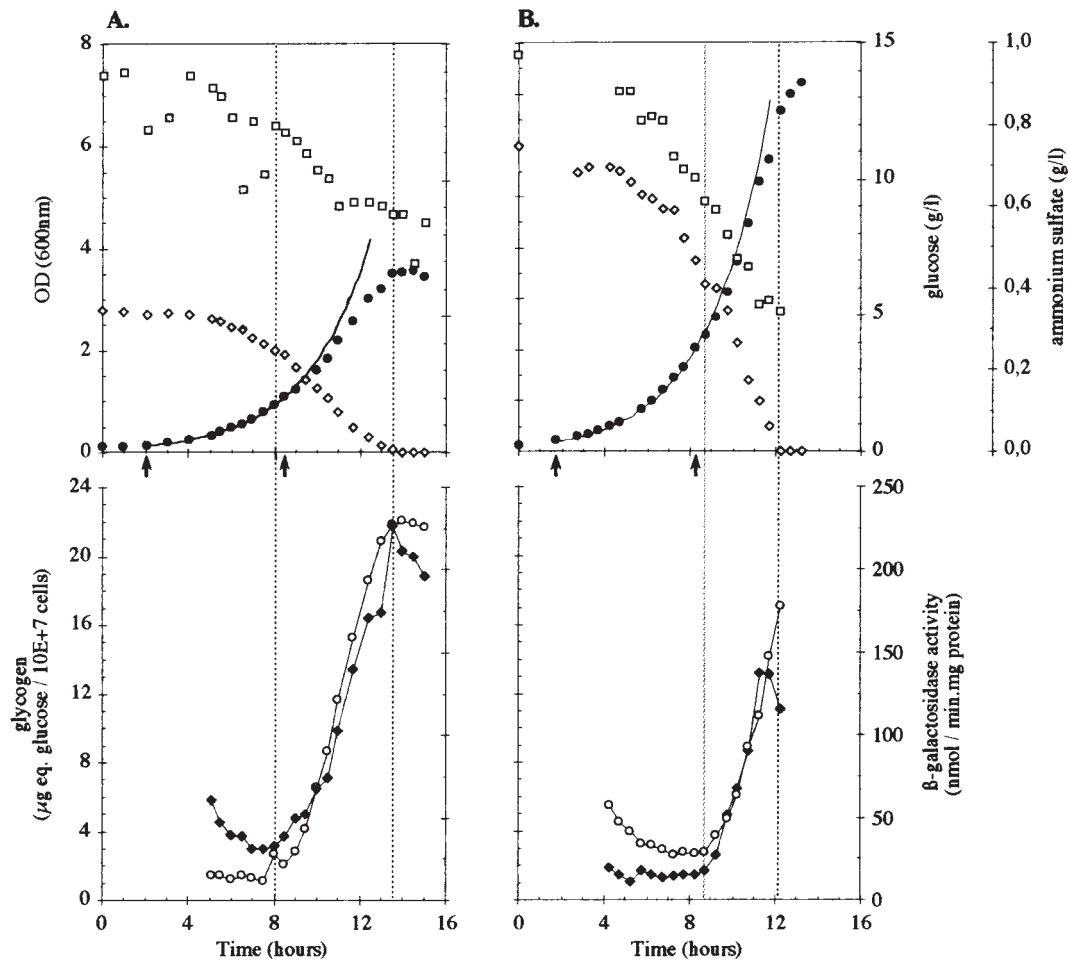


Figure 1. Glycogen levels and β -galactosidase specific activity from the *GSY2-lacZ* fusion gene during diauxic growth of the JLP48-3B strain on MIN with 5 g (A) or 10 g (B) glucose per litre. The culture medium was inoculated at initial $\text{OD}_{600}=0.05$ (about 10^6 cells/ml) from an overnight shake flask preculture in the same medium. Cell density (●); glycogen (○); β -galactosidase activity (◆); glucose (◇); ammonium sulphate (□). Parameters from the equation of the fitting curve calculated from points between the arrows: $\mu=0.34$, $r^2=0.9983$ (A) and $\mu=0.35$, $r^2=0.9989$ (B).

when approximately 50% of glucose remained to be consumed. We confirmed their results and further showed that glycogen accumulation coincided with transcriptional induction of *GSY2*, as determined by β -galactosidase activity from the *GSY2-lacZ* fusion gene, both reaching a maximum at the diauxic shift (Figure 1). This pattern was confirmed by Northern blot analysis of *GSY2* transcripts, which peaked at the onset of glucose exhaustion from the medium (see Figure 3), suggesting a decline of transcription after this point. Identical expression patterns from *lacZ* fusion genes (results not illustrated) were obtained in this study with genes encoding Glc3p (glycogen

branching enzyme; Rowen *et al.*, 1992; Thon *et al.*, 1992), Gac1p (a regulatory protein of glycogen synthase; François *et al.*, 1992), Gph1p (glycogen phosphorylase; Hwang *et al.*, 1989) and with *YPR184w* encoding the glycogen debranching enzyme (unpublished results). It is also shown in Figure 3 that transcripts of *GLG1* encoding glycogenin (Cheng *et al.*, 1995) increased in parallel with *GSY2* mRNAs.

With respect to genes involved in trehalose metabolism, we found that β -galactosidase activity from the *TPS1-lacZ* construct sharply rose in parallel with glycogen accumulation, despite a weak and steady increase during the exponential

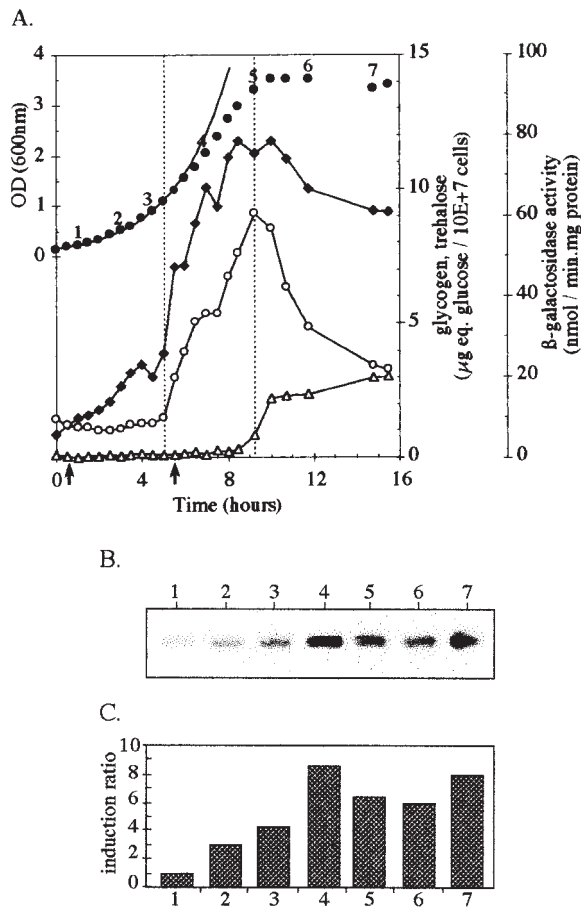


Figure 2. Glycogen and trehalose levels, β -galactosidase specific activity from the *TPS1-lacZ* fusion gene and Western blot analysis of Tps1p during diauxic growth of the JLP87-8A strain on YNB with 10 g glucose/litre. (A) Procedure and symbols as in Figure 1: trehalose (Δ). Parameters of the fitting curve: $\mu=0.39$; $r^2=0.9984$. (B) Samples (1-7) were taken during growth for immunoblot analysis with the primary antibody KH-1142 raised against the 56 kDa subunit of Tre6P synthase (gift from J. Londesborough). (C) Induction factor relative to sample 1 after signal quantification from (B).

phase of growth (Figure 2A). The rise of *TPS1* expression was quantitatively related to the accumulation of its product as revealed by Western blot analysis (Figures 2B, 2C). These results complemented previous data showing that Tre6P synthase/phosphatase complex activity significantly increased before the diauxic shift (François *et al.*, 1991). Northern blot analysis additionally revealed an accumulation of *NTH1* transcripts similar to that found for *GSY2* mRNAs (Figure 3). This study was extended to genes that have been

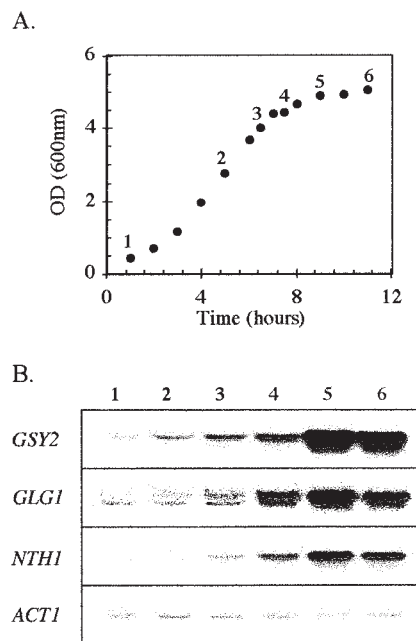


Figure 3. Northern blot analysis of *GSY2*, *GLG1*, *NTH1* and *ACT1* mRNAs during diauxic growth of KT1112 on YEP with 10 g glucose/litre. (A) The culture was performed in a 500 ml shake flask containing 100 ml of medium. (B) $2 \cdot 10^8$ cell samples (1-6) were taken during growth for Northern blot analysis. The *ACT1* mRNA level was used as loading control.

considered to be induced between the diauxic shift and the entrance into stationary phase (Werner Washburne *et al.*, 1996), namely the stress-responsive gene *CTT1* encoding the cytosolic catalase, and *SSA3* encoding an Hsp70p. We found that the expression profile from the *CTT1-lacZ* fusion gene perfectly matched that of *TPS1*, while *SSA3-lacZ* expression clearly increased after the diauxic shift (results not shown), as was previously reported for the *SSA3-lacZ* construct and *SSA3* transcripts (Boorstein and Craig, 1990; Fuge *et al.*, 1994).

As shown in Figure 2, trehalose began to accumulate only at the onset of the diauxic shift after the induction of *TPS1*, which is involved in its biosynthesis. Since *NTH1* was coordinately induced with *TPS1*, we investigated whether the delay of trehalose accumulation was not responsible for the delay of trehalose accumulation using a *nth1* null mutant. This mutant strain exhibited wild-type *TPS1* expression, but it accumulated trehalose in parallel with glycogen in contrast to wild-type strains (Figure 4).

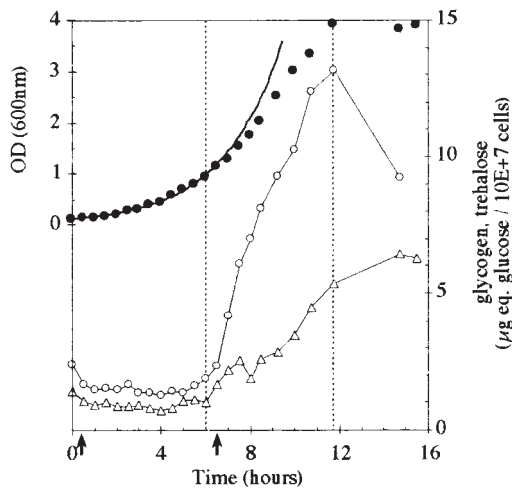


Figure 4. Glycogen and trehalose levels during diauxic growth of JLP88-21B (*nth1* null mutant) on YNB with 10 g glucose/litre. Procedure and symbols as in Figure 1; trehalose (Δ). Parameters of the fitting curve: $\mu=0.38$, $r^2=0.9989$.

Glucose effect on gene expression

It can be seen in Figure 1 that the higher the initial glucose concentration, the lower the level of *GSY2* expression and the content of glycogen at the diauxic shift of growth in MIN. Note that such an inhibitory effect was previously reported for *CTT1-lacZ* expression on YEP with 20 and 100 g glucose/litre (Bissinger *et al.*, 1989). As illustrated

in Table 2, this inhibitory effect was also observed in other media (YNB and YEP) and in null-mutants for *SNF1* and *HXK2*, two genes involved in glucose repression (for a review, see Ronne, 1995). It also occurred, although to a lower extent, in a strain with lowered cAMP-dependent protein kinase (*tpk1^w tpk2 tpk3 bcy1* mutant). In contrast, the inhibitory effect was not observed in galactose-based medium, indicating that it was glucose-specific.

The medium pH is not the trigger of glycogen accumulation under glucose-limited conditions

Throughout these studies, we systematically found that glycogen accumulation occurred during a transition period between the exit from an exponential phase and the entrance into the diauxic shift (Figures 1, 2, 4). This period corresponded to a decay of specific rate of both growth and glucose consumption (not shown). Since the acidification of the culture medium is known to cause inhibition of cell proliferation (Jones *et al.*, 1981), the possibility that pH could be a common denominator between glycogen accumulation and the growth inhibition observed during this transition period was examined. It is, however, unlikely that a drop in pH could be the trigger of this phenomenon, since a decrease of only 0.4 unit was recorded during the growth in a medium initially buffered at pH 5.8 (Figures 1, 2, 4, parameter not plotted).

Table 2. Glucose effect on β -galactosidase activity from *GSY2-lacZ* fusion gene.

Lane	Strain	Genotype	Medium	Sugar concentration (g/l)					
				5	10	20	40	60	100
				β-Galactosidase activity (nmol/min.mg protein)					
1	JLP48-3B	WT	MIN	210	150	90	–	–	–
2	JLP5-1A	WT	YNB	125	70	60	50	–	–
3	JLP5-1A	WT	YEP	175	140	100	40	–	–
4	JLP5-1A	WT	YEP	–	85	48	24	–	12
5	JLP81-1C	<i>hxx2</i>	YEP	–	100	70	40	–	32
6	JF908	<i>tpk^w, bcy1</i>	YEP	–	190	170	150	–	130
7	JLP5-1A	WT	YEP	–	–	64	48	36	–
8	JLP5-1A	WT	YEPGal	–	–	256	260	264	–

Glucose was used in all experiments except in lane 8, where it was replaced by galactose. Because of variability of β -galactosidase activity between independent experiments, the wild-type strain JLP5-1A was systematically included as a control in the groups of experiments reported in lanes 4–6 and 7–8. For one specific condition, the value reported in the Table is the mean of five consecutive points performed in 1 h intervals during the diauxic shift, with a standard deviation less than 10%.

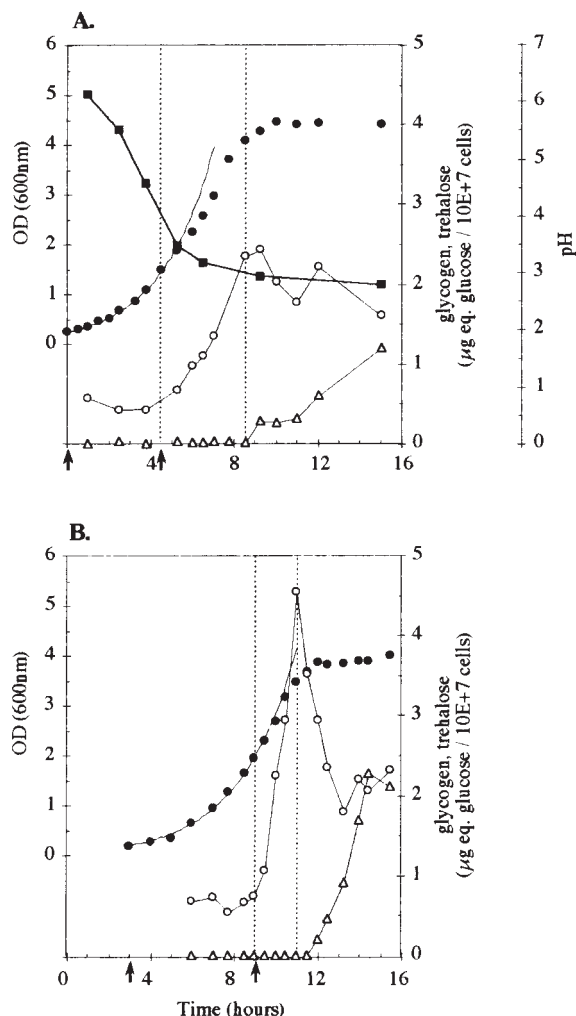


Figure 5. Effect of medium pH on glycogen induction during diauxic growth of X2180-1A strain on CFM with 10 g glucose/litre. The pH of the medium was uncontrolled (A) or maintained constant at 5.0 (B). Procedure and symbols as in Figure 1; trehalose (Δ); pH (\blacksquare). Parameters of the fitting curve: $\mu=0.40$, $r^2=0.9984$ (A) and $\mu=0.37$, $r^2=0.999$ (B).

Nonetheless, experiments were performed using a mineral medium (CFM) in which the pH was either not controlled or kept at a constant value during growth. In a preliminary experiment, we confirmed that the kinetics of reserve carbohydrate accumulation in CFM initially buffered at pH 5.8 was the same as in buffered MIN and YNB (not illustrated). Under uncontrolled pH, glycogen accumulation began very early as cell proliferation ceased to be exponential, when the external pH decreased below 4.0 (Figure 5A). When the pH

of the medium was kept at a constant value of 5.0, glycogen started to accumulate before the diauxic shift, while the growth was still exponential; in this case, the rupture in the growth pattern was clearly delayed (Figure 5B). This latter growth condition also resulted in a four-fold increase in the cells' capacity to accumulate glycogen, e.g. 2 $\mu\text{g eq. glucose per } 10^7 \text{ cells per hour}$ as compared to 0.5 μg under uncontrolled pH. These results suggest that extracellular pH negatively affects the rate of activities involved in glycogen metabolism. However, it is unlikely that the pH affects glycogen accumulation indirectly by influencing the growth rate of the population, since cells growing slowly on gluconeogenic substrates display a very strong accumulation of reserve carbohydrates (unpublished results).

Reserve carbohydrates induced in response to nitrogen limitation

In contrast to glucose limitation, exponentially growing cells on a nitrogen-limited medium coordinately induced *GSY2* and glycogen accumulation at the precise moment when the exogenous nitrogen source was exhausted (Figure 6). Identical results were obtained with media limited by 1 or 2 g ammonium sulphate/litre (not shown). Interestingly, yeast cells accumulated high levels of glycogen, while the transcriptional activation of *GSY2* was lower than under glucose limitation (cf. Figures 1 and 6). Another notable peculiarity under nitrogen limitation was the concomitant accumulation of trehalose and glycogen.

Our results suggest that the sudden lack of nitrogen which arose during growth could trigger these genetic and metabolic changes. Similar results could therefore be expected when exponential growing cells on glucose experience nitrogen starvation. Indeed, it can be seen in Figure 7 that glycogen and trehalose accumulation started almost immediately after the shift to nitrogen-free medium, with a kinetic profile quantitatively comparable to that found under nitrogen limitation (Figure 6). This was accompanied by an increase of β -galactosidase from the *GSY2-lacZ* fusion gene (Figure 7), by an accumulation of Gsy2p and its activation, as indicated respectively by a rise of the total form of glycogen synthase activity and an increase in the ratio between the active and total form (not shown). Similar increases for *TPS1* expression from the *TPS1-lacZ* fusion gene and for Tre6P synthase activity (not shown) were

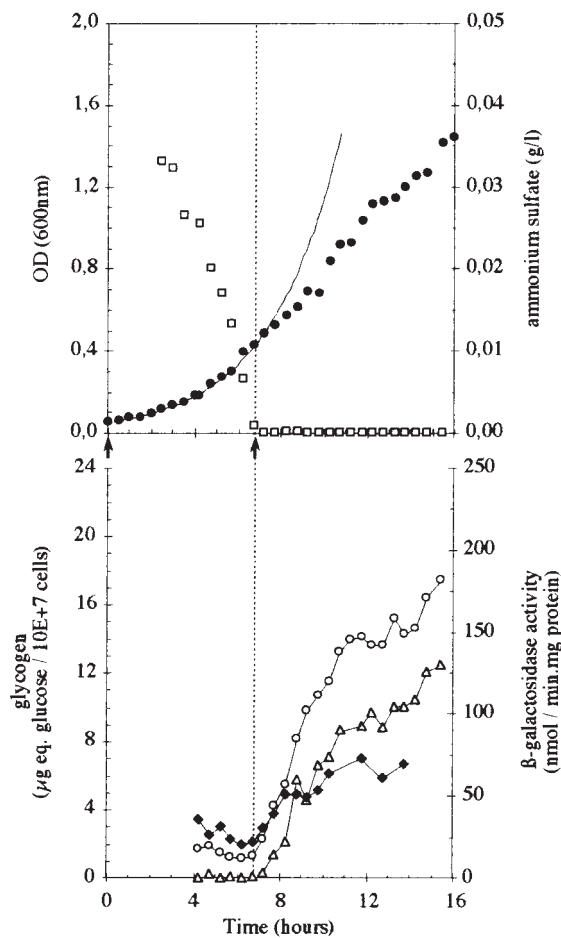


Figure 6. Glycogen and trehalose levels and β -galactosidase specific activity from the *GSY2-lacZ* fusion gene during growth of the JLP48-3B strain on MIN with 0.05 g ammonium sulphate/litre. Procedure and symbols as in Figure 1; trehalose (Δ). Parameters of the fitting curve: $\mu=0.31$, $r^2=0.9956$.

observed. In addition, a sudden 30–50% drop of neutral trehalase activity was seen 10 min after the shift, followed by a recovery to its initial value after 2 h (Figure 7). This subsequent rise may be explained by the transcriptional activation of *NTH1*, which occurred in parallel with *GSY2* and *TPS1* (not illustrated).

DISCUSSION

Using glycogen and trehalose as typical markers of changing environmental conditions, we report that reserve carbohydrate metabolism in *Saccharomyces cerevisiae* is differentially controlled by nitrogen and glucose limitations. While we did not find

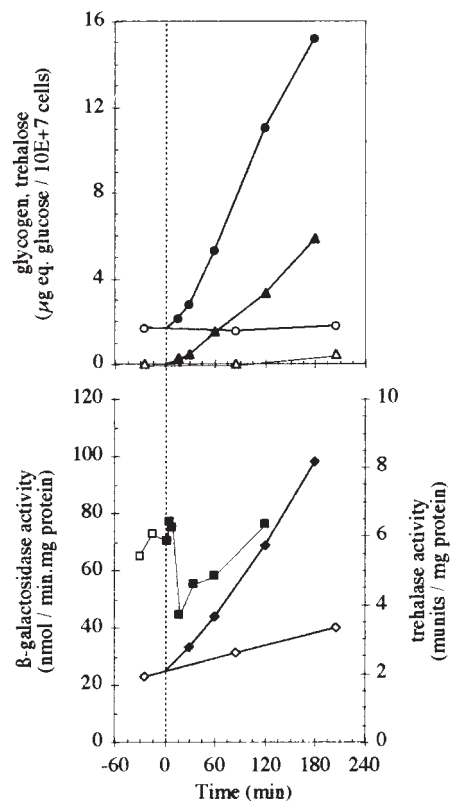


Figure 7. Effect of nitrogen starvation on glycogen and trehalose levels, and on specific activities of β -galactosidase (*GSY2-lacZ* fusion gene) and trehalase. Exponential growing cells of JLP48-3B strain were transferred at time zero from YNB to nitrogen-free medium, as described in Materials and Methods. Symbols: glycogen (\circ , \bullet); trehalose (Δ , \blacktriangle); specific activities of β -galactosidase (\diamond , \blacklozenge) and trehalase (\square , \blacksquare); control (empty symbols), nitrogen starvation (full symbols).

any obvious factor responsible for the activation of this metabolism under glucose limitation (see below), complete exhaustion of the nitrogen source under nitrogen limitation appeared to be the primary condition to trigger the activation of both glycogen and trehalose metabolism. As compared to glucose limitation, other differences were a lower transcriptional activation of genes, despite a large and concomitant accumulation of both reserve carbohydrates. This large accumulation could not be solely accounted for by the moderate induction of *GSY2* and *TPS1*, especially as there was a simultaneous activation of *GPH1* and *NTH1*, which probably resulted in recycling of glycogen and trehalose, as previously reported under other stress conditions (Parrou *et al.*, 1997).

The net accumulation of reserve carbohydrates therefore implies additional mechanisms, including activation of glycogen synthase and inactivation of neutral trehalase, and increased availability of glucose-6P, the substrate of Tre6P synthase and a positive effector of glycogen synthase (François *et al.*, 1988; Huang *et al.*, 1997).

An astonishing feature of cellular responses to glucose limitation reported some 20 years ago by Lillie and Pringle (1980), was that glycogen accumulation began when approximately half of the glucose remained to be consumed, regardless of its initial concentration in the medium. Interestingly, we observed in most of the experiments reported in this work that glycogen began to accumulate almost coincidentally with a premature exit from the exponential phase, suggesting a link between growth inhibition and the triggering of glycogen accumulation. However, in some experiments (e.g. controlled pH) or during growth in galactose-limited medium (unpublished results), glycogen accumulation was initiated while the growth was still perfectly exponential. Therefore, these two events do not appear necessarily to be linked and the common idea that reduction of the specific growth rate causes accumulation of glycogen should not be considered as a general rule (Lillie and Pringle, 1980; François *et al.*, 1987; Larsson *et al.*, 1997). This work and previous reports (Lillie and Pringle, 1980; François *et al.*, 1987, 1991; San Miguel and Argüelles, 1994) have shown that trehalose accumulation only started at the diauxic shift. Using a *nth1* null mutant, we could show that this delay is due to high trehalase activity which overcomes Tre6P synthase/phosphatase complex activity during the transition phase of growth. In addition, the lack of neutral trehalase also resulted in measurable amounts of trehalose during the exponential phase, revealing the basal activity of Tre6P synthase, which is probably necessary for the steady-state formation of trehalose-6P, a key regulatory molecule controlling the glycolytic flux in yeast (Blazquez *et al.*, 1993; Thevelein and Hohmann, 1995). Since we observed that the transcription of *NTH1* is enhanced during this transition and maximal at the entrance of the diauxic shift, the drop of neutral trehalase activity (François *et al.*, 1987; San Miguel and Argüelles, 1994) suggests a strong inactivation of this enzyme, by a mechanism which remains to be clarified. A potential physiological significance of high levels of 'inactive' trehalase, as well as glycogen phosphorylase, during and after the diauxic

shift of growth, is to allow yeast cells to rapidly resume growth under favourable conditions by mobilization of their endogenous stores.

The coordinated induction of genes involved in glycogen metabolism was previously inferred from the coordinated expression of one specific gene with glycogen accumulation (Hwang *et al.*, 1989; François *et al.*, 1992; Thon *et al.*, 1992; Rowen *et al.*, 1992; Hardy *et al.*, 1994; Ni and LaPorte, 1995). The use of fermentation technology for accurate monitoring of growth and sampling allowed us to further reinforce this observation for the wide panel of genes involved in glycogen metabolism, i.e. *GSY2*, *GPH1*, *GLC3*, *GAC1*, *GLG1* and *YPR184w*, encoding the debranching enzyme (M. A. Teste and J. François, unpublished results). A coordinated induction during the diauxic growth, never reported so far, was also observed for *TPS1*, *NTH1* and *CTT1*, respectively involved in trehalose biosynthesis, trehalose degradation and the general stress response. Notable discrepancies between our results and literature data need to be pointed out. The first concerns the absence of *GPH1* induction in the work of De Risi *et al.* (1997), while this gene has been shown in many reports to be induced before the diauxic shift (Hwang *et al.*, 1989; Thon *et al.*, 1992; François *et al.*, 1992). The most striking difference was the decrease of *TPS1* mRNAs from the exponential to the 'stationary' phase reported by Winderickx *et al.* (1996), which is in deep contradiction with a strong increase of β -galactosidase activity from the *TPS1-lacZ* fusion gene (Figure 2A; Winderickx *et al.*, 1996), of *TPS1* transcripts and of Tps1p between the exponential and the diauxic shift of growth (Figures 2B, 2C; François *et al.*, 1987; Vuorio *et al.*, 1993).

The term 'stationary phase' should be definitely avoided in designating this panel of genes (Werner Washburne *et al.*, 1996), since they were clearly induced much earlier than the 'post-diauxic shift' *SSA3* gene (Boorstein and Craig, 1990) and the truly 'stationary phase' *SNZ1* gene (Braun *et al.*, 1996). Moreover, the specific induction of this class of genes while glucose was still abundant in the medium, and their maximal expression at the onset of the diauxic shift, argued against the claim that these genes are under 'catabolic repression' (Vuorio *et al.*, 1993; Kopp *et al.*, 1993; Nwaka *et al.*, 1995a, b; Hardy *et al.*, 1994; Winderickx *et al.*, 1996), since typical glucose-repressed genes are solely derepressed after the complete exhaustion of glucose from the medium (Ronne, 1995;

De Risi *et al.*, 1997). Even though catabolite repression is indeed inappropriate, we noticed a strong negative correlation between initial glucose concentration and reserve carbohydrate metabolism, but we do not yet have a clear explanation for this specific 'glucose effect'.

A common denominator for most of the genes studied in this work was the presence of at least one STRE in their promoter region. This *cis*-acting element was considered to be necessary to mediate transcriptional activation of genes in response to various stress conditions (for reviews, see Ruis and Schüller, 1995; Siderius and Mager, 1997), including nutrient starvation, temperature, oxidative, osmotic and ethanol shocks. In particular, this STRE-dependent activation was well illustrated for *CTTI* and *GSY2* in response to nitrogen starvation (Marchler *et al.*, 1993; Ni and LaPorte, 1995; unpublished results). It was also proposed that *GSY2* and *HSP12* induction during diauxic growth on glucose was dependent on STREs present in their promoter (Ni and LaPorte, 1995; Varela *et al.*, 1995). De Risi *et al.* (1997) extended this statement to 'STRE-containing' genes, since they showed their co-induction before the diauxic shift of growth. However, at least with respect to genes involved in reserve carbohydrate metabolism, we do not share this assertion because *GLG1*, which does not harbour any STRE, was induced in parallel with *GSY2* and *TPS1*. Furthermore, preliminary data based on identical experimental procedures showed that *GSY2* was still properly induced after removal of the two STREs by site-directed mutagenesis. The co-induction of this panel of genes is not triggered by inactivation of the cAPK pathway (Parrou *et al.*, manuscript in preparation) and work is currently under way to identify the molecular mechanisms underlying this specific induction during the diauxic growth on glucose.

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