Energetic limits to metabolic flexibility: responses of *Saccharomyces cerevisiae* to glucose–galactose transitions

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Glucose is the favoured carbon source for *Saccharomyces cerevisiae*, and the Leloir pathway for galactose utilization is only induced in the presence of galactose during glucose-derepressed conditions. The goal of this study was to investigate the dynamics of glucose–galactose transitions. To this end, well-controlled, glucose-limited chemostat cultures were switched to galactose-excess conditions. Surprisingly, galactose was not consumed upon a switch to galactose excess under anaerobic conditions. However, the transcripts of the Leloir pathway were highly increased upon galactose excess under both aerobic and anaerobic conditions. Protein and enzyme-activity assays showed that impaired galactose consumption under anaerobiosis coincided with the absence of the Leloir-pathway proteins. Further results showed that absence of protein synthesis was not caused by glucose-mediated translation inhibition. Analysis of adenosine nucleotide pools revealed a fast decrease of the energy charge after the switch from glucose to galactose under anaerobic conditions. Similar results were obtained when glucose–galactose transitions were analysed under aerobic conditions with a respiratory-deficient strain. It is concluded that under fermentative conditions, the energy charge was too low to allow synthesis of the Leloir proteins. Hence, this study conclusively shows that the intracellular energy status is an important factor in the metabolic flexibility of *S. cerevisiae* upon changes in its environment.

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INTRODUCTION

Glucose and fructose are preferred sugars for *Saccharomyces cerevisiae*. Apparently, evolution of this yeast in natural environments that are rich in these sugars (e.g. fruit and nectar) has led to a complicated, multi-layered regulatory programme that only enables metabolism of alternative carbon sources (e.g. maltose, ethanol and galactose) when these preferred carbon sources are dwindling.

In the case of galactose, a four-enzyme metabolic route, the Leloir pathway, has to be expressed to enable its catabolism. The structural genes for galactose permease (*GAL2*), galactokinase (*GAL1*), galactose-1-phosphate uridylyltransferase (*GAL7*) and uridine-diphosphoglucose 4-epimerase (*GAL10*) all belong to the *GAL* regulon, which is subject to tight transcriptional regulation. Glucose causes a nearly complete transcriptional repression of the *GAL* genes, mediated by the non-phosphorylated form of Mig1, and thereby effectively shuts down galactose utilization

(Johnston *et al.*, 1994). Induction of the *GAL* genes is initiated by interaction of galactose and ATP with Gal3, which then forms a complex with the negative regulator Gal80 (Platt & Reece, 1998). This releases the positive transcriptional regulator Gal4 from Gal80 control and allows it to activate transcription of the *GAL1*, *GAL2*, *GAL7* and *GAL10* genes, which contain upstream activation sequences (UAS_{GAL}) in their promoter regions (Leuther & Johnston, 1992; Wu *et al.*, 1996). Consequently, *S. cerevisiae* cells grown in batch cultures on glucose/galactose mixtures show diauxic utilization of these sugars (Ostergaard *et al.*, 2001b; Raamsdonk *et al.*, 2001).

To investigate the adaptive mechanisms involved in the transition between glucose and galactose metabolism, we used well-controlled, glucose-limited chemostat cultures that were switched to galactose-excess conditions. Unexpectedly, the first results showed that, under anaerobic conditions, *S. cerevisiae* could not switch from glucose-limited growth to galactose utilization. Still, *S. cerevisiae* does not exhibit a Kluyver effect for galactose and is therefore able to ferment galactose in the absence of oxygen (Fukuhara, 2003) [the Kluyver effect has been

Supplementary data files are available with the online version of this paper.

defined as the inability to grow on and ferment sugars under anaerobic conditions that do support aerobic, respiratory growth (Barnett *et al.*, 1990)]. This unexpected behaviour could reflect different metabolic problems: (i) lack of transcriptional induction of the *GAL* regulon, (ii) absence of Gal protein synthesis, (iii) accumulation or depletion of low-molecular-mass metabolites and effectors that affect *in vivo* activity of the Leloir pathway. For example, the glucose-to-galactose switch might cause an impaired flux through the Leloir pathway by insufficient substrate levels for galactokinase (e.g. ATP) (Ostergaard *et al.*, 2001a) or through metabolite toxicity (e.g. galactose 1-phosphate) (de Jongh *et al.*, 2008).

The goal of the present study was to elucidate the cause of the observed impaired galactose consumption after a switch from anaerobic, glucose-limited chemostat cultivation to anaerobic, galactose-excess conditions. To dissect the involvement of different levels of cellular information and regulation, we analysed the dynamics of glucose–galactose transitions via a multi-level approach.

METHODS

Strain and media. The *Saccharomyces cerevisiae* strains used in this study were the prototrophic haploid reference strain CEN.PK113-7D (*MATa*) (van Dijken *et al.*, 2000), a wild-type strain CBS8066 (van Dijken *et al.*, 2000), a *hxx2::KanMX4* mutant (Diderich *et al.*, 2002), a *reg1::KanMX4* mutant (Raghevedran *et al.*, 2004) and a *rip1::KanMX4* mutant (this study). All three mutant strains were derived from the CEN.PK113 background. Stock cultures were grown at 30 °C in shake flasks containing 100 ml synthetic medium with 20 g glucose per litre. The synthetic medium contained, per litre of demineralized water, 5 g (NH₄)₂SO₄, 3 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.15 ml silicon antifoam (BDH) and trace element concentrations according to Verduyn *et al.* (1992). After heat sterilization of the medium for 30 min at 120 °C, a filter-sterilized vitamin solution (Verduyn *et al.*, 1992) was added. Ergosterol (10 mg l⁻¹) and Tween 80 (420 mg l⁻¹) for the anaerobic cultivation were dissolved in pure ethanol and steamed at 80 °C before addition to the medium. The concentration of glucose in the reservoir medium was 7.5 g l⁻¹ or 25 g l⁻¹, respectively, for the aerobic and anaerobic cultivation. Glucose was added to the synthetic medium after separate heat sterilization at 110 °C.

Chemostat cultivation and perturbation experiment. The *S. cerevisiae* strains were grown at 30 °C in 2 litre bioreactors (Applikon) with a working volume of 1.5 litres that was controlled via an electrical level sensor. Removal of effluent from the centre of the culture ensured that biomass concentrations in the effluent line differed by less than 1 % from those in the culture (van den Berg *et al.*, 1996). The dilution rate was set at 0.10 h⁻¹. The pH was measured online and kept constant at 5.0 by the automatic addition of 2 M KOH using an Applikon ADI 1030 Biocontroller. A stirrer speed of 800 r.p.m. and air flow of 0.75 l min⁻¹ were applied to keep the dissolved oxygen concentration, as measured with an oxygen electrode, above 60 % of air saturation in all aerobic chemostat cultivations. In the anaerobic cultivation, the bioreactor and medium vessel were sparged with pure nitrogen gas (<5 p.p.m. O₂; Linde Gas Benelux) and Norprene tubing was used to minimize oxygen diffusion into the fermenters. Steady-state samples were taken after ~10 volume changes to avoid strain adaptation due to long-term cultivation (Ferea *et al.*, 1999; Jansen *et al.*, 2004; Mashego *et al.*,

2005). Biomass dry weight, extracellular metabolite, dissolved oxygen and gas profiles were constant over at least three volume changes.

Galactose-pulse experiments were started by simultaneously switching the medium pump off and injecting the galactose. The pulse consisted of 60 g galactose in 60 ml demineralized water and was injected aseptically through a septum. The galactose concentration immediately after the pulse was approximately 200 mM.

Analytical methods. The exhaust gas was cooled by a condenser connected to a cryostat set at 2 °C and dried with a Perma Pure dryer (Inacom Instruments) before analysis of the O₂ and CO₂ concentrations with a Rosemount NGA 2000 analyser. The gas flow rate was determined with an Ion Science Saga digital flow meter. Culture dry weights were determined as described by Postma *et al.* (1989). Ethanol, galactose, glycerol and glucose concentrations in supernatants were determined by HPLC analysis with a Bio-Rad Aminex HPX-87H column at 60 °C. The column was eluted with 5 mM sulfuric acid at a flow rate of 0.6 ml min⁻¹. All relevant metabolites were detected by a Waters 2410 refractive index detector.

Intracellular metabolites. Samples for intracellular metabolite analysis were obtained by withdrawing 1 ml broth from the fermenter via a rapid sampling set-up (Lange *et al.*, 2001) into 5 ml 60 % (v/v) methanol/water at -40 °C to immediately quench all metabolic activities. The samples were then processed according to a previously published intracellular sampling processing method (Wu *et al.*, 2005) to give 0.5 ml intracellular metabolite solution ready for further analysis.

Intracellular concentrations of galactose 1-phosphate, glucose 1-phosphate and glucose 6-phosphate were measured enzymically according to Bergmeyer & Michal (1974). The assay mixture contained 100 mM glycylglycine (pH 8.7), 10 mM MgCl₂, 0.01 mM glucose 1,6-biphosphate, 0.2 mM NADP⁺, and sample. The reaction to measure glucose 6-phosphate was started with 17 mU glucose-6-phosphate dehydrogenase. The reaction to measure glucose-1-phosphate was started with 7 mU phosphoglucomutase. Finally, the reaction to measure galactose 1-phosphate was started with 0.45 mU galactose-1-phosphate uridylyltransferase.

Intracellular concentrations of UDP-glucose and UDP-galactose were measured enzymically according to Bergmeyer & Michal (1974). The assay mixture contained 100 mM glycylglycine (pH 8.7), 10 mM MgSO₄·7H₂O and 0.25 mM NAD⁺. The reaction to measure UDP-glucose was started with UDP-glucose-6-phosphate dehydrogenase (0.01 U ml⁻¹). The reaction to measure UDP-galactose was started with galactose-1-phosphate uridylyltransferase (0.1 U ml⁻¹, Sigma).

The assays were performed in black flat-bottomed Costar 96-well microtitre plates (Corning) by following NADH or NADPH fluorescence in a Tecan Genios microtitre plate reader. The excitation wavelength was 340 nm and the emission wavelength was 465 nm.

Quantitative real-time PCR. Sampling of cells from the bioreactors and total RNA extraction were performed as described previously (Piper *et al.*, 2002). Isolated RNA was DNase I treated according to the Qiagen RNeasy mini kit protocol for RNA cleanup. First-strand cDNA synthesis was carried out using 2 µg DNase-treated RNA, random hexamer primers and Moloney murine leukaemia virus reverse transcriptase (Invitrogen). Quantitative real-time PCR was run on a DNA Engine Opticon I system (Bio-Rad) with the following settings: 94 °C for 15 min, 94 °C for 10 s, 66 °C for 10 s, 72 °C for 10 s, and plate reading. The denaturation, annealing, elongation and reading steps were repeated for 39 cycles. A melting curve from 50 to 94 °C was generated at the end of the reaction. The 20 µl reaction mixture consisted of 10 µl Absolute QPCR SYBR green mix

(Westburg; cat. no. AB-1158), 0.2 μ M forward primer, 0.2 μ M reverse primer, and cDNA (20, 2, 0.2 or 0.02 ng per PCR). The primers used are listed in Table 1. The cycle threshold value was calculated with the Opticon Monitor software version 1.08 (Bio-Rad). Each PCR was carried out in triplicate. The relative expression of *GALI*, *GAL2*, *GAL7*, *GAL10* and *PGM2* was quantified by comparing ($2^{-\Delta\Delta C_t}$) with *ACT1* as the reference gene. The results for the steady state and each time point after the perturbation (5, 10, 15, 30 and 120 min) were derived from two independent replicate cultures.

Enzyme assays. Cell extracts were prepared with a FastPrep according to Canelas *et al.* (2008). Enzyme activities were assayed with freshly prepared cell extracts in spectrophotometric enzyme-linked assays, using a Tecan Genios microtitre plate reader. All determinations were performed at 30 °C and 340 nm ($\epsilon_{\text{NAD(P)H}}$ at 340 nm = 6.33 mM⁻¹). Samples were prepared manually in microtitre plates (transparent flat-bottom 96-well plates; Corning) in a 12-assay run with a total volume of 300 μ l per well. All assays were performed with two concentrations of cell extracts; data are presented as U (mg protein)⁻¹ (1 U = 1 μ mol min⁻¹). Protein concentrations in cell-free extracts were determined by the Lowry method. Dried BSA (fatty-acid-free, Sigma) was used as a standard.

Galactokinase (EC 2.7.1.6) was assayed according to Platt *et al.* (2000) with minor modifications. The assay mixture contained 100 mM potassium phosphate buffer (pH 7.5), 100 mM NaCl, 5 mM MgCl₂, 0.5 mM phosphoenolpyruvate, 1 mM DTT, 0.15 mM NADH, 5 mM ATP, 2.2 units pyruvate kinase, 3.0 units lactate dehydrogenase (PK/LDH; Sigma) and cell extract. The reaction was started with 5 mM D-galactose.

Galactose-1-phosphate uridylyltransferase (EC 2.7.7.12) was assayed according to Colowick & Kaplan (1967) with minor modifications. The assay mixture contained 100 mM glycine/KOH (pH 8.5), 10 mM L-cysteine, 5 mM MgCl₂, 0.4 mM NADP⁺, 40 mM galactose 1-phosphate, 0.01 mM glucose 1,6-biphosphate, 1.6 units phosphoglucosyltransferase, 1.8 units glucose-6-phosphate dehydrogenase and cell extract. The reaction was started with 2 mM UDP-D-glucose.

UDP-galactose 4-epimerase (EC 5.1.3.2) was assayed according to Majumdar *et al.* (2004) with minor modifications. The assay mixture contained 100 mM glycylglycine (pH 8.0), 0.40 mM NAD, 0.16 units UDP-glucose-6-dehydrogenase and cell extract. The reaction was started with 0.4 mM UDP-D-galactose.

Phosphoglucosyltransferase (EC 5.4.2.2) was assayed according to Masuda *et al.* (2001) with minor modifications. The assay mixture contained

50 mM Tris/HCl (pH 7.5), 5 mM MgCl₂, 1 mM NADP⁺, 0.01 mM glucose 1,6-biphosphate, 3.6 units glucose-6-dehydrogenase and cell extract. The reaction was started with 4 mM glucose 1-phosphate.

Proteomics. Samples were taken 180 min after the galactose pulse from two independent cultures under aerobic and anaerobic conditions. An equal volume of 20 % trichloroacetic acid was added to 4 ml culture sample (10–14 mg cells). After 30 min incubation on ice, the samples were centrifuged at 4 °C and washed with –20 °C acetone. The samples were centrifuged for 10 min (5 min at 4600 g, 4 °C) and the remaining acetone was evaporated. The washed pellets were suspended in 500 μ l 100 mM ammonium bicarbonate pH 7.8 and 20 μ l 250 μ g ml⁻¹ TPCK-treated trypsin in 0.025 % HCl (pH 3). Proteins were digested overnight by incubation at 37 °C in a Thermomixer. Then 6 μ l 100 mM 1,4-DTT was added to the digests and incubated for 30 min at room temperature. Samples were acidified with 5 % formic acid (FA) and any insolubles were removed by centrifugation (5 min at 4600 g, 4 °C). The supernatant was analysed by LC-MS/MS.

Peptides were separated and analysed using an Accela high-performance liquid chromatograph coupled to an LTQ linear iontrap mass spectrometer. LC-MS/MS was performed using a gradient of buffer A (LC-MS-grade water containing 0.1 % FA) and buffer B (LC-MS-grade acetonitrile containing 0.1 % FA). A 5 μ l sample of the peptide solution was trapped on an NS-MP-10-Robust pre-column (OD/ID 360/100 mm, Poros 10R2 and C₁₈ 5 μ m particles 120 Å pores; NanoSeparations) at 5 % B with a flow rate of 15 μ l min⁻¹ for 5 min. Peptides were eluted from the pre-column onto an analytical column (OD/ID 360/75 μ m, Biosphere C₁₈ 3 μ m particles 120 Å pores, length 23 cm; NanoSeparations) and to the MS using a switching valve (Meiring *et al.*, 2002). The gradient was run at 200 nl min⁻¹ from (i) 5.01 min 15 % B to 60 min 25 % B, (ii) 60 min 25 % B to 155 min 35 % B.

An Nth-order triple-play MS-method was used in the LTQ. This method consisted of three scan events: (i) an MS scan from *m/z* 300 to 2000, (ii) data-dependent zoom scans on the top three peaks from the first scan event, (iii) data-dependent MS/MS scans on the top three peaks from the first scan event. The acquired data were searched against the *Saccharomyces* Genome Database protein database (<http://www.yeastgenome.org/>), using Sequest in the Bioworks software (version 3.3.1; Thermo Fisher Scientific). Identified peptides were filtered for SF final score >0.85 and Peptide Probability <0.001. Peptide and protein areas were calculated using PepQuan in the Bioworks software.

Table 1. Primers used for quantitative real-time PCR

Gene name	Systematic name	Sequence (5'–3')	Fragment size (nt)
<i>ACT1</i>	YFL039C	FW GGCTTCTTTGACTACCTTCCA RV AGAAACACTTGTGGTGAACGA	88
<i>GALI</i>	YBR020W	FW ATCAGAGGGCTAAGCATGTG RV GCAGTAAAGCTCGCTGTAGTC	81
<i>GAL2</i>	YLR081W	FW AGCCAGCCATCTTCTAAAGGT RV CCCAGGTTGTGGCATAACAG	81
<i>GAL7</i>	YBR018C	FW TCTGGCCATTGTGAGACCTTG RV AGGTCCTCCTTCACCATTTG	81
<i>GAL10</i>	YBR019C	FW TGAGGGTACGGAGATTATGGT RV TCGACGATATTACCCGTAGGA	80
<i>PGM2</i>	YMR105C	FW CATAGACCGTGTTCACCAAGG RV TAGCGTCGAACAAAGCACAG	85

Adenosine phosphates. Intracellular ATP was determined using the ATP Bioluminescence Assay kit CLS II (Roche Diagnostics; cat. no. 1699 695), according to the manufacturer's instructions, in black Costar 96-well microtitre plates. Luminescence was read on a Mediators PhL plate reader (Mediators Diagnostics). Intracellular ADP and AMP were determined enzymically according to Mashego *et al.* (2005) based on myokinase, pyruvate kinase and lactate dehydrogenase (Bergmeyer *et al.*, 1985).

The energy charge (EC) (Atkinson, 1968) was calculated as follows:

$$EC = \frac{([ATP] + 0.5[ADP])}{([ATP] + [ADP] + [AMP])}$$

Trehalose and glycogen. Trehalose and glycogen concentration measurements were performed as described by Parrou & Francois (1997) in duplicate measurements on two independent replicate cultures. Glucose was determined using the UV method based on Roche kit no. 0716251.

RESULTS AND DISCUSSION

S. cerevisiae cannot consume galactose after a sudden switch from glucose to galactose in the absence of oxygen

Experiments on substrate changes are typically performed in shake flasks. In such experiments, biomass grown on a specific substrate is harvested and washed to remove this substrate prior to addition of a new substrate (Ashe *et al.*, 2000; Martinez-Pastor & Estruch, 1996; Nilsson *et al.*, 2001). This processing, during which biomass is temporarily starved and potentially deprived of (or exposed to) oxygen, may significantly affect experimental reproducibility and responses of the cells to substrate transitions. In the present study, *S. cerevisiae* CEN.PK113-7D was grown in glucose-limited chemostat cultures. Subsequently, excess galactose (200 mM) was injected into the cultures while concomitantly stopping the glucose feed. This set-up is excellently suited for studying substrate transitions, because a different carbon source can be added directly into the fermenter without prior processing of the biomass. Another advantage of this set-up is the alleviation of

glucose repression. Glucose exerts a strong catabolite repression on the utilization of alternative carbon sources (e.g. maltose, ethanol and galactose) (Gancedo, 1998). During shifts starting with glucose excess the transcriptional repression of the genes involved in alternative carbon source utilization first has to be relieved. In glucose-limited chemostats, the low residual glucose concentration (below 0.17 mM at $D=0.10\text{ h}^{-1}$ in aerobic cultures) causes glucose repression to be alleviated already at the onset of the experiment.

When *S. cerevisiae* was grown under glucose limitation in the presence of oxygen, galactose metabolism was induced as expected and was detectable 4 h after galactose addition (Fig. 1). The length of this adaptation phase was consistent with previously reported data for aerobic batch cultures grown on glucose/galactose mixtures (Ostergaard *et al.*, 2001b; Raamsdonk *et al.*, 2001). The high initial galactose concentration made it difficult to measure the exact time point at which galactose consumption set in. Measurements of intracellular intermediates of the Leloir pathway demonstrated that galactose metabolism started much earlier than the 4 h suggested by the extracellular galactose measurements. Concentrations of galactose 1-phosphate, glucose 1-phosphate and UDP-galactose were already increased 60 min after galactose addition (Fig. 2). Conversely, intracellular concentrations of glucose 6-phosphate and UDP-glucose rapidly decreased within the first 15 min after the shift from glucose limitation to galactose excess. The decrease of glucose 6-phosphate and UDP-glucose concentration may reflect the role of UDP-glucose in storage carbohydrate metabolism and that of glucose 6-phosphate is as an intermediate of the glycolytic pathway, which was temporarily interrupted after the shift. After this initial decrease, the intracellular glucose 6-phosphate and UDP-glucose concentrations increased and followed the increase in flux through the Leloir pathway.

When the same experiment was performed in the absence of oxygen (during both the glucose limitation and galactose excess phases) no galactose consumption at all was detected

Fig. 1. Concentrations of galactose (■) and ethanol (□) after a galactose pulse to aerobic (left panel) and anaerobic (right panel) glucose-limited chemostat cultures of *S. cerevisiae*. The anaerobic cultures were shifted to aerobically 10 h after the galactose pulse. Data points represent the average and mean-deviation of values from at least two independent cultures.

Fig. 2. Intracellular metabolite concentrations involved in galactose metabolism in aerobic (■) and anaerobic (□) glucose-to-galactose shift experiments (see Fig. 1). Data points represent the average and mean-deviation of values from two independent experiments. All metabolite concentrations are given in $\mu\text{mol (g dry weight)}^{-1}$.

(Fig. 1), even several days after the shift (data not shown). The inability of *S. cerevisiae* to consume galactose under these conditions was also observed with other laboratory strains (e.g. CBS8066), confirming that the phenomenon was not strain-specific (data not shown). To investigate whether low rates of galactose consumption might have gone unnoticed due to the relative insensitivity of the extracellular galactose measurements, intracellular concentrations of the intermediates of the Leloir pathway were measured (Fig. 2). The intracellular concentrations of UDP-glucose and glucose 6-phosphate, metabolites already present when glucose is the carbon source, rapidly decreased after glucose exhaustion and were undetectable 2 h after the shift from glucose to galactose. Glucose 1-phosphate concentration did not show significant changes and remained close to the detection limit. Although the noise of the enzymic assays was largely due to the low concentrations, galactose 1-phosphate concentration initially increased, possibly reflecting non-specific kinase activity. UDP-galactose, one of the two galactose-specific intermediates, could not be detected after galactose addition, suggesting that this metabolite was not synthesized under anaerobiosis. The apparent inability to induce galactose metabolism in the anaerobic experiments was fully reversible, since sparging the cultures with air resulted

in galactose consumption (Fig. 1). Galactose consumption upon oxygenation of the anaerobic cultures was presumably enabled by the ATP generated via ethanol consumption.

The absence of galactose consumption under anaerobiosis does not result from impaired transcription of the *GAL* genes

To investigate whether the absence of galactose consumption under anaerobic conditions could result from a deficiency in galactose signalling and induction of the *GAL* genes (Ostergaard *et al.*, 2000), transcript levels of the five structural steps of the Leloir pathway were monitored by quantitative PCR (Fig. 3). In both the aerobic and the anaerobic glucose–galactose transitions, transcription of the four genes of the *GAL* regulon (*GAL1*, *GAL2*, *GAL7* and *GAL10*) was immediately induced after the addition of galactose and increased in the 15 min following galactose addition before stabilizing. Under both conditions, the transcript levels of the *GAL* genes upon galactose induction were high (Fig. 3). However, they were about tenfold lower under anaerobiosis than under aerobiosis. Phosphoglucosyltransferase, encoded by *PGM2* (also called *GAL5*), catalyses the last step in the Leloir pathway, but

Fig. 3. Transcript levels of the Leloir-pathway genes after galactose pulses to glucose-limited chemostat cultures of *S. cerevisiae*, grown under aerobic and anaerobic conditions. The Leloir transcript levels are represented in relative units to *ACT1*, as measured by quantitative PCR (see Methods). The transcripts *GAL1* (■), *GAL2* (○), *GAL7* (□) and *GAL10* (●) are shown in the top panels and *PGM2* (■) in the lower panels. All data represent the average and mean-deviation of values from two independent experiments.

is also involved in other processes unrelated to galactose metabolism (Francois & Parrou, 2001). As a result, *PGM2* is not under the control of the *GAL* regulon (Bevan & Douglas, 1969) and, accordingly, its transcript profiles differed from those of the other *GAL* genes.

We conclude that a deficiency in the transcription of the genes involved in the Leloir pathway was not the cause for the impaired galactose utilization under anaerobic conditions.

The enzymes involved in galactose utilization are not synthesized upon a sudden shift from glucose to galactose in the absence of oxygen

The presence of the Leloir-pathway transcripts does not necessarily imply the presence of a functional pathway. Therefore, the $V_{\max}$ (maximum enzyme capacity) of the Leloir-pathway enzymes was assayed *in vitro* (Fig. 4). In good correlation with its corresponding transcript, the

Fig. 4. Enzyme activities of the Leloir pathway after galactose pulses to glucose-limited chemostat cultures of *S. cerevisiae*, grown under aerobic and anaerobic conditions. The Leloir enzyme activities are represented in $\mu\text{mol min}^{-1} \text{mg}_{\text{protein}}^{-1}$, as measured by *in vitro* assays (see Methods). The enzymes Gal1 (■), Gal7 (□) and Gal10 (●) are shown in the top panels and Pgm2 (■) in the lower panels. Data represent the average and mean-deviation of values from two independent cultivations.

enzyme activity of Pgm2 was already present during the glucose-limited cultivations and remained throughout the aerobic and anaerobic glucose–galactose transitions. Aerobically, *in vitro* enzyme activities for all Leloir-pathway enzymes under control of the *GAL* system (Gal1, Gal7 and Gal10) were undetectable during glucose-limited growth and slowly increased after the galactose pulse. Conversely, in the absence of oxygen the enzyme activities for Gal1, Gal7 and Gal10 remained undetectable throughout the experiment.

Because *in vitro* assays only reflect the absence of active enzyme and not necessarily of protein, the involvement of post-translational modifications had to be considered. The presence of the Leloir-pathway proteins was therefore investigated by LC-MS. At 180 min after galactose addition to aerobic cultures, all Leloir-pathway proteins were clearly identified among the 750 most abundant proteins. Conversely, besides Pgm2, none of the Leloir-pathway proteins were detected in the anaerobic samples. (The complete lists of proteins for aerobic and anaerobic conditions are given in Supplementary Tables 1 and 2, respectively, available with the online version of this paper.) These findings were confirmed by performing directed LC-MS/MS experiments on pre-selected peptide *m/z*'s of the identified Leloir proteins. This indicates that the absence of galactose consumption correlated with a lack of synthesis of Leloir-pathway proteins.

Lack of *GAL* protein does not result from active translation inhibition

This absence of protein synthesis following shifts of growing cells from glucose to alternative carbon sources has previously been ascribed to an active, though temporary, inhibition of translation (Ashe *et al.*, 2000; Kuhn *et al.*, 2001; Martinez-Pastor & Estruch, 1996; Uesono *et al.*, 2004). The resistance to translational inhibition upon glucose depletion of *reg1Δ*, *hxx2Δ* and *snf3Δrgt2Δ* mutants showed that several glucose signalling pathways were involved (Ashe *et al.*, 2000). Our experimental set-up differed significantly from that used in these previous studies. Although glucose was the initial carbon source, its concentration in our study was too low to exert significant carbon catabolite repression. Furthermore, previous reports of glucose–raffinose switches involving translational inhibition showed very long lag phases of 20 h or more before *S. cerevisiae* started to consume raffinose (Martinez-Pastor & Estruch, 1996). Our intracellular metabolite measurements revealed that in aerobic cultures, galactose utilization started after 15 min of exposure to galactose-excess conditions. This time frame was consistent with reported transcription and translation rates and suggested that translation inhibition was not involved. Moreover, galactose consumption did not occur faster in experiments where the glucose feed was not switched off upon galactose addition (data not shown). These results suggested that translation inhibition did not occur in our

experimental set-up when oxygen was supplied. In glucose-limited chemostat cultures, the residual glucose concentration, although very small, is typically slightly higher in the absence than in the presence of oxygen [0.17 mM aerobically versus 0.43 mM anaerobically (van Hoek *et al.*, 2000)], which might result in a somewhat stronger catabolite repression anaerobically. Since, therefore, occurrence of active translational inhibition under anaerobic conditions could not be ruled out, *reg1Δ* and *hxx2Δ* mutants were grown in an anaerobic glucose-limited chemostat and then exposed to galactose excess. Neither *reg1Δ* nor *hxx2Δ* grew after the switch from glucose to galactose, arguing against involvement of active translational inhibition by glucose depletion.

The inability of *S. cerevisiae* to switch from glucose to galactose utilization anaerobically is caused by ATP shortage

The mode of sugar dissimilation in *S. cerevisiae* and, consequently, its ATP yield is strongly affected by the availability of oxygen. In the presence of oxygen, ATP can be formed via both substrate-level and oxidative phosphorylation, leading to the production of up to 16 moles of ATP per mole of hexose sugar [with a P/O ratio of ~1.0 (Verduyn *et al.*, 1991)]. In the absence of oxygen, substrate-level phosphorylation is the only mode of ATP generation, resulting in only 2 ATP per sugar molecule. Compared to aerobic respiration, the anaerobic fermentative metabolism therefore has a considerably lower energy yield per sugar molecule. To assess the energetic state of the cells the intracellular concentrations of adenosine phosphates were measured (Fig. 5). In the aerobic experiments, the ATP concentration initially decreased by ~30% (from 7.0 to 5.0 $\mu\text{mol g}_{\text{dry weight}}^{-1}$) and then stabilized. Under anaerobic conditions, the ATP concentration continued to decrease and reached undetectable levels 1 h after the glucose-to-galactose switch. The concentrations of ADP and AMP slightly increased in the aerobic experiments while, under anaerobic conditions, the concentration of ADP transiently increased and the AMP concentration increased threefold after the glucose-to-galactose switch. Consistently, the energy charge did not change significantly during the aerobic switch experiment but decreased dramatically in the anaerobic experiment.

These results identify low ATP yield and energy charge as primary causes of the inability of *S. cerevisiae* to induce a functional galactose pathway in the anaerobic substrate-switch experiment. Protein synthesis is one of the most demanding cellular processes in terms of free energy requirement, with up to 50% of the cell's free energy budget being devoted to amino acid synthesis and polymerization (Verduyn *et al.*, 1991).

The mode of glucose dissimilation is not the only difference between aerobically and anaerobically grown cells. For instance, it is well documented that lipid metabolism, membrane and cell wall composition differ

Fig. 5. Intracellular ATP, ADP and AMP, sum of adenosine concentrations (AXP) and the energy charge of aerobic (left panels) and anaerobic (right panels) cultures after the shift from glucose-limited growth to galactose-excess conditions. The top panels show the concentrations of ATP (■), ADP (△) and AMP (□) in $\mu\text{mol per g dry weight (g}_{\text{DW}}^{-1})$. The lower graphs show the AXP concentrations in $\mu\text{mol g}_{\text{DW}}^{-1}$ (■) and the energy charge (calculated as described in Methods, □). Error bars of the adenine nucleotide concentrations represent average deviation of the mean of duplicate analyses on at least two independent culture samples. The energy charge values are average and mean-deviation of measurements from two independent cultivations.

between aerobic and anaerobic cultures (Snoek & Steensma, 2006). To assess the importance of energetic status and other oxygen-related effects, a respiration-deficient strain was constructed and grown in the same experimental set-up as the congenic reference strain. *rip1Δ* strains, which carry a deletion in the structural gene for the Rieske Fe-S protein of the mitochondrial cytochrome *bc₁* complex, lack a functional respiratory chain (Beckmann *et al.*, 1987). Even in the presence of oxygen, *rip1Δ* mutants rely solely on substrate-level phosphorylation for ATP synthesis, thus mimicking sugar dissimilation in anaerobically grown wild-type strains. The *rip1Δ* strain indeed failed to grow with non-fermentative carbon sources and displayed a marginal oxygen consumption rate with glucose as carbon source (details of the general physiology of the *rip1Δ* mutant are available as supplementary data with the online version of this paper). The *rip1Δ* strain was incapable of consuming galactose in aerobic substrate-switch experiments (Fig. 6). Moreover, the intracellular ATP concentration in the *rip1Δ* strain drastically decreased after the aerobic glucose-to-galactose switch (Fig. 6). These observations strongly support the notion that a shortage of ATP is the primary cause of the inability of *S. cerevisiae* to switch on galactose metabolism in the anaerobic experiments.

Reserve carbohydrates are not involved in maintaining the energy charge after a glucose-to-galactose switch

In the aerobic glucose-to-galactose switch experiments, intracellular ATP concentrations stabilized after 5 min, even though galactose dissimilation had not started at that point. Mobilization of the storage carbohydrates glycogen and/or trehalose might contribute to buffering of the energy charge. A difference in carbohydrate mobilization in

the anaerobic and aerobic experiments might also contribute to the different responses of the adenine nucleotide pools under aerobic and anaerobic conditions. To investigate a possible role of storage carbohydrate mobilization, cellular glycogen and trehalose contents were analysed. Intracellular concentrations of glycogen and trehalose were higher in aerobic glucose-limited chemostat cultures (Fig. 7). Especially the difference in trehalose concentration was striking: $30 \text{ mg g}_{\text{dry weight}}^{-1}$ under aerobiosis and $1 \text{ mg g}_{\text{dry weight}}^{-1}$ under anaerobiosis. However, neither glycogen nor trehalose was mobilized after the glucose-to-galactose switch under either aerobic or anaerobic conditions (Fig. 7). This observation indicates that other mechanisms play a role in maintaining the ATP level after the glucose-to-galactose switch under aerobic conditions. For example, consumption of minute amounts of galactose may be enough to synthesize a few Gal proteins

Fig. 6. Galactose concentration (■) and intracellular ATP concentration (□) after a galactose pulse to glucose-limited chemostat cultures of *S. cerevisiae rip1Δ* mutant under aerobic conditions. Data represent the average and mean-deviation of values from two independent cultivations.

Fig. 7. Intracellular concentrations of trehalose (■) and glycogen (□) after a galactose pulse to glucose-limited chemostat cultures of *S. cerevisiae* under aerobic and anaerobic conditions. The trehalose and glycogen concentrations are represented in mg glucose equivalents ($\text{mg}_{\text{glucose eq}} \text{g}_{\text{DW}}^{-1}$) per g dry weight (g_{DW}). All data points represent the average and mean-deviation of values from two independent cultures.

and thereby start up galactose consumption. The absence of reserve carbohydrate consumption after the anaerobic glucose-to-galactose switch demonstrates that *S. cerevisiae* is ill equipped to handle sudden switches in carbon sources under anaerobic, fermentative conditions.

Conclusions

Anaerobically grown *S. cerevisiae* cannot adapt to a new carbon substrate upon a sudden shift from carbon-limited growth on glucose, its preferred substrate, to the alternative carbon source galactose. The failure to induce a functional Leloir pathway for galactose utilization is caused by the absence of Leloir proteins, but not by the absence of the corresponding transcripts. Our results show that this absence of protein synthesis coincides with a fast depletion of ATP and a dramatic decrease of the energy charge. The amount of ATP needed for transcription is estimated to be 20-fold lower than that required for translation (Verduyn *et al.*, 1990) and the characteristic time of transcription is much shorter than that of translation (Stephanopoulos *et al.*, 1998). Transcript levels of the *GAL* genes reached upon full induction were, however, 10-fold lower in the anaerobic substrate-switch experiments than in the aerobic experiments. These observations indicate that the collapse of the energy charge in the anaerobic cultures did not allow the same transcription rates as in the anaerobic cultures and completely prevented translation of the *GAL* mRNAs.

Catabolism of the storage carbohydrates present in the anaerobic, glucose-limited cultures could, in principle, yield ATP to energize the synthesis of Leloir-pathway enzymes. However, no such mobilization of glycogen occurred during the glucose–galactose transition. Apparently, the well-established mobilization of storage carbohydrates during the start-up of glycolysis (Thomsson *et al.*, 2005; van den Brink *et al.*, 2008) cannot be extrapolated to the activation of the Leloir pathway. The fact that, through evolution, *S. cerevisiae* did not acquire the ability to switch from anaerobic, glucose-limited growth to galactose utilization suggests that it seldom encounters this type of condition in its natural environments.

This study identifies the intracellular energy status of *S. cerevisiae* as a key factor in its metabolic flexibility, i.e. its

ability to rapidly and functionally express novel pathways required for the utilization of alternative substrates upon the depletion of favoured substrates. In nature, energy shortage may occur not only as a result of a sudden depletion of the growth-limiting preferred substrate, as demonstrated in the chemostat set-up used in this study, but also by the presence of uncoupling agents under substrate-excess conditions. For example, it can be envisaged that weak organic acids have a similar negative impact on substrate transitions, since these uncouple the plasma membrane pH gradient of bakers' yeast and thereby negatively affect cellular energetics (Verduyn *et al.*, 1991). This would be of direct relevance to the application of *S. cerevisiae* for the production of fuels and chemicals from lignocellulosic hydrolysates. These feedstocks contain complex mixtures of sugars (including galactose) but also high concentrations of acetic acid and other weak organic acids (Klinke *et al.*, 2002; Palmqvist *et al.*, 1999; Rabelo *et al.*, 2008; Sun & Cheng, 2002). Research into the energetic aspects of metabolic flexibility and strategies to buffer cellular energy status [e.g. via engineering of ATP-buffering systems (Sauer & Schlattner, 2004)] is therefore not only of fundamental but also of applied significance.

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