Correlation between TCA cycle flux and glucose uptake rate during respiro-fermentative growth of *Saccharomyces cerevisiae*

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Glucose repression of the tricarboxylic acid (TCA) cycle in *Saccharomyces cerevisiae* was investigated under different environmental conditions using ^{13}C -tracer experiments. Real-time quantification of the volatile metabolites ethanol and CO_2 allowed accurate carbon balancing. In all experiments with the wild-type, a strong correlation between the rates of growth and glucose uptake was observed, indicating a constant yield of biomass. In contrast, glycerol and acetate production rates were less dependent on the rate of glucose uptake, but were affected by environmental conditions. The glycerol production rate was highest during growth in high-osmolarity medium ($2.9 \text{ mmol g}^{-1} \text{ h}^{-1}$), while the highest acetate production rate of $2.1 \text{ mmol g}^{-1} \text{ h}^{-1}$ was observed in alkaline medium of pH 6.9. Under standard growth conditions ($25 \text{ g glucose l}^{-1}$, pH 5.0, 30°C) *S. cerevisiae* had low fluxes through the pentose phosphate pathway and the TCA cycle. A significant increase in TCA cycle activity from $0.03 \text{ mmol g}^{-1} \text{ h}^{-1}$ to about $1.7 \text{ mmol g}^{-1} \text{ h}^{-1}$ was observed when *S. cerevisiae* grew more slowly as a result of environmental perturbations, including unfavourable pH values and sodium chloride stress. Compared to experiments with high glucose uptake rates, the ratio of CO_2 to ethanol increased more than 50 %, indicating an increase in flux through the TCA cycle. Although glycolysis and the ethanol production pathway still exhibited the highest fluxes, the net flux through the TCA cycle increased significantly with decreasing glucose uptake rates. Results from experiments with single gene deletion mutants partially impaired in glucose repression (*hxx2*, *grr1*) indicated that the rate of glucose uptake correlates with this increase in TCA cycle flux. These findings are discussed in the context of regulation of glucose repression.

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INTRODUCTION

The impact of changing growth environments on metabolism of the yeast *Saccharomyces cerevisiae* has been studied intensively in such diverse research fields as functional genomics (Giaever *et al.*, 2002) and medicine (Perocchi *et al.*, 2008). On the transcript level more than 1000 genes have been found to be differentially expressed as a result of altering medium osmolarity, cultivation temperature or pH (Causton *et al.*, 2001; Rep *et al.*, 1999). The impact of environmental and genetic changes on growth rates, glucose uptake rates and the flux through the tricarboxylic acid (TCA) cycle of *S. cerevisiae* has been analysed using ^{13}C -tracer experiments with compartmentalized models of yeast (Blank & Sauer, 2004; Blank *et al.*, 2005a; Maaheimo *et al.*, 2001; Raghevendran *et al.*, 2004; Szyperski, 1998; Velagapudi *et al.*, 2007).

To quantify net fluxes, glucose consumption and extracellular production rates of metabolites, inferred from changes in extracellular metabolite concentrations, must be accurately measured. During aerobic growth of *S. cerevi-*

siae, glucose concentrations above 0.1 g l^{-1} cause transcriptional repression of TCA cycle genes and genes involved in respiration (Yin *et al.*, 2003). The repression of genes involved in respiratory metabolism leads to respiro-fermentative growth, with ethanol as the major fermentation product. This regulatory phenomenon is also referred to as the Crabtree effect (De Deken, 1966). However, quantification of ethanol is difficult under conditions of aeration as it is highly volatile (vapour pressure 78.47 mmHg), potentially resulting in low data quality. Although the use of different types of culture vessels may minimize evaporation of volatile metabolites, quantification of all extracellular compounds, including ethanol and CO_2 , is important and necessary for the calculation of intracellular flux distributions.

To generate high-quality physiological data, quantitative physiology studies have been performed mainly in chemostats under glucose-limited conditions and below a critical dilution rate at which *S. cerevisiae* does not produce ethanol (Fiaux *et al.*, 2003; Nissen *et al.*, 1997; van Winden

et al., 2005). In batch cultures, ethanol production rates have been determined based on changes in medium concentrations without considering ethanol evaporation (Blank *et al.*, 2005a; Gombert *et al.*, 2001), or using a computational model that takes ethanol evaporation into account (Velagapudi *et al.*, 2006, 2007).

In this study, the major physiological limitations of shaking flask cultures and the problem of reliable determination of the carbon balance were overcome by integrating infrared (IR) sensors for online CO₂ and ethanol quantification. This new experimental set-up allowed for quantitative physiology experiments, which provide evidence that the glucose uptake rate, but to a lesser extent the growth rate, is inversely correlated with the flux through the TCA cycle during respiro-fermentative growth of *S. cerevisiae*.

METHODS

Strains and growth conditions. The haploid *S. cerevisiae* strain CEN.PK 113-7D (van Dijken *et al.*, 2000) was used throughout this study. CEN.PK 113-7D mutants *hxx2* (Blank & Sauer, 2004), *grr1* (courtesy of Peter Kötter, Frankfurt, Germany) and *hap2* (this study) were used for investigation of TCA cycle activity during glucose derepression and repression. Batch cultures were carried out in 1400 ml sealed Erlenmeyer flasks (100 ml growth medium) in a rotary shaker at 30 °C and 200 r.p.m., ensuring fully aerated conditions. Minimal medium (Verduyn *et al.*, 1992) was used, containing per litre, 25 g glucose, 5 g (NH₄)₂SO₄, 3 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 4.5 mg ZnSO₄·7H₂O, 0.3 mg CoCl₂·6H₂O, 1.0 mg MnCl₂·4H₂O, 0.3 mg CuSO₄·5H₂O, 4.5 mg CaCl₂·2H₂O, 3.0 mg FeSO₄·7H₂O, 0.4 mg NaMoO₄·2H₂O, 1.0 mg H₃BO₃, 0.1 g KI, 15 mg EDTA, 0.05 mg biotin, 1.0 mg calcium pantothenate, 1.0 mg nicotinic acid, 25 mg inositol, 1.0 mg pyridoxine, 0.2 mg *p*-aminobenzoic acid and 1.0 mg thiamine. To avoid pH shifts due to ammonia uptake and acetate production, the medium was supplemented with 50 mM potassium hydrogen phthalate. The reference pH value was set to 5.0. Changes in the growth rate were achieved by using different pH values (3.2, 6.5, 6.9 and 7.5) and applying sodium chloride stress (addition of 0.3 and 0.5 M NaCl at pH 5.0).

Analytical procedures. Cell growth was monitored by measuring the optical density of cultures at a wavelength of 600 nm (OD₆₀₀) using a spectrophotometer (Libra S11; BioChrom). Glucose, glycerol, acetate and ethanol were analysed by isocratic UV-RI-HPLC (LaChrom Elite; Hitachi). Analytes were separated on an Aminex HPLC-87-H column (Bio-Rad) with 2.5 mM H₂SO₄ as eluant (0.6 ml min⁻¹) at 60 °C. CO₂ and ethanol were measured online with BCpreFerm infrared off-gas sensors (BlueSens gas sensor GmbH). The pressure increase due to CO₂ evolution was measured to adjust for pressure-related analyte concentration changes. Taking the pressure inside the shake flask into account, the monitored volume percentages were transformed into molarities. The CO₂ concentration in the gas phase can be calculated using equation (1),

$$c_{g,CO_2} = \frac{n}{V_g} = \frac{(H - H_0) \cdot V_g}{V_R} \cdot \frac{1}{V_g} \text{ (mM)} \quad (1)$$

in which n is the amount of CO₂ (in mol), V_g is the gas volume (here 1.3 l), H_0 the volume percentage at the beginning of the experiment, H the volume percentage at the time of measurement, and V_R the real molar volume.

The ethanol concentration in the gas phase can be calculated using equation (2):

$$c_{g,ethanol} = \frac{n}{V_g} = \frac{H \cdot V_g}{V_R} \cdot \frac{1}{V_g} \text{ (mM)} \quad (2)$$

The real molar volume can be determined with equation (3):

$$V_R = \frac{V_m \cdot 273.15 \cdot p}{T \cdot 1.013} \text{ (l mol}^{-1}\text{)} \quad (3)$$

in which V_m is the volume of 1 mol of ideal gas (22.4 l mol⁻¹), and 273.15 and 1.013 are the values for standard temperature and pressure, in units of K and bar respectively.

Gas chromatography and mass spectrometry (GC-MS) analysis was carried out using a GC 3800, combined with an MS/MS 1200 unit (Varian Deutschland). Fifteen detectable amino acids were separated on a FactorFour VF-5ms column (Varian Deutschland) at a constant flow rate of 1 ml helium min⁻¹. The split ratio was 1:20, the injection volume was 1 µl, and the injector temperature was 250 °C. The temperature of the GC oven was kept constant for 2 min at 150 °C and afterwards increased to 250 °C with a gradient of 3 °C min⁻¹. The temperatures of the transfer line and the source were 280 °C and 250 °C, respectively. Ionization was performed by electron impact (EI) ionization at -70 eV. For enhanced detection, a selected ion monitoring (SIM) frame was defined for every amino acid (Wittmann, 2007). GC-MS raw data were analysed using the Workstation MS Data Review (Varian Deutschland) to check for detector overload.

Shake flasks and off-gas sensors set-up. The cultivations were performed in specially designed 1.4 litre sealed shake flasks equipped with two screw-fitted BCpreFerm off-gas sensors (BlueSens gas sensor GmbH) for online monitoring of CO₂ and ethanol. The sensors were placed in the head space of the shake flasks and were heated to avoid water condensation. In addition, a pressure detector was integrated in the CO₂ sensor to detect pressure variation in the gas phase. Growth medium was withdrawn through a septum using a plastic syringe connected to a metal needle. To minimize changes in volume, and consequently pressure, only 200 µl medium per sample was withdrawn. Gas leakage during sampling was minimized by submerging the needle. To avoid gas leakage during cultivation, all experiments were carried out at pressures below 100 mbar by limiting biomass concentrations to below 1.3 g_{CDW} l⁻¹ (CDW, cell dry weight). The total equipment consisted of the specially designed shake flasks, the sensors for CO₂, ethanol and pressure detection, a multiplexer BaCCom (BlueSens gas sensor GmbH) for connecting the sensors through one interface, and the FermVis software package (BlueSens gas sensor GmbH) for continuous data acquisition.

Determination of extracellular fluxes. Substrate uptake, product secretion and ethanol/CO₂ production rates of *S. cerevisiae* were determined during the exponential growth phase. The concentrations of glucose, glycerol and acetate were measured in the liquid phase, and CO₂ concentrations were measured in the gas phase. The ethanol production rate was calculated from concentration measurements in the liquid and gas phases. Substrate uptake and product secretion rates and their respective errors were determined using a simultaneous nonlinear fit of the concentration data represented over time. This analysis was completed using an exponential growth model in Sigma Plot (Systat Software, V.10.0.0.54).

The growth model consisted of six equations as listed below, where μ is the specific growth rate:

$$\mu = \ln(x_0 + x_t) / \Delta t \text{ (h}^{-1}\text{)} \quad (4)$$

The glucose concentration was estimated by:

$$c_{\text{glucose}} = s_0 - Y_{\text{SX}} \cdot (x_0 \cdot e^{\mu \cdot t} - x_0) \text{ (mM)} \quad (5)$$

in which s_0 is the starting concentration of glucose, Y_{SX} is the biomass yield on glucose, and x_0 is the biomass at the beginning of the experiment.

Product evolution was calculated by:

$$c_{\text{glycerol,acetate,ethanol,CO}_2} = p_0 + Y_{\text{XP}} \cdot (x_0 \cdot e^{\mu \cdot t} - x_0) \text{ (mM)} \quad (6)$$

in which p_0 is the starting concentration of the product and Y_{XP} is the yield of product on biomass. The specific rates are reported in mmol per g_{CDW} per hour and were calculated using equations (7) and (8):

$$r_{\text{glucose}} = Y_{\text{SX}} \cdot \mu \text{ (mmol g}^{-1} \text{ h}^{-1}) \quad (7)$$

$$r_{\text{glycerol,acetate,ethanol,CO}_2} = Y_{\text{XP}} \cdot \mu \text{ (mmol g}^{-1} \text{ h}^{-1}) \quad (8)$$

Data consistency was investigated using a simple black box model of respiro-fermentative growing yeast (Stephanopoulos *et al.*, 1998).

¹³C-labelling experiments. ¹³C-tracer experiments were performed under pseudo-steady-state (exponential growth) conditions. Glucose was used at a starting concentration of 25 g l⁻¹ to guarantee respiro-fermentative conditions. The glucose used was a mixture of 20 % (mol %) uniformly labelled [U-¹³C]glucose (EURISO-TOP) and 80 % (mol %) naturally labelled glucose. To determine the labelling patterns of the amino acids, the cells were hydrolysed at 105 °C for 12 h with 200 µl 6 M HCl. The dried hydrolysates were derivatized for GC-MS analysis using 50 µl *N,N*-dimethylformamide and 50 µl *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (Blank *et al.*, 2005a). The IR sensors for CO₂ and ethanol respond differently to ¹²C and ¹³C carbon and thus cannot be used for quantification during labelling experiments. The comparability of yeast physiology in labelled and unlabelled growth experiments was monitored by UV-RI-HPLC. In these experiments, rates of glucose consumption and ethanol, glycerol and acetate production differed by maximally 2 %.

¹³C-constrained metabolic flux analysis. The stoichiometric model for ¹³C-constrained metabolic flux analysis comprises the major pathways of yeast central carbon metabolism (Blank *et al.*, 2005a). This model contains 34 unknown fluxes and 30 metabolites. To calculate intracellular fluxes, the stoichiometric model was constrained with five extracellular fluxes (growth rate, evolution rates of ethanol, glycerol and acetate, and glucose uptake rate) and eight intracellular flux ratios (fraction of cytosolic oxaloacetate originating from cytosolic pyruvate, fraction of mitochondrial oxaloacetate derived through anaplerosis, fraction of phosphoenolpyruvate originating from cytosolic oxaloacetate, fraction of serine derived through glycolysis, upper and lower bounds of mitochondrial pyruvate derived through malic enzyme, contribution of glycine to serine biosynthesis, and contribution of serine to glycine biosynthesis). For estimation of the mitochondrial NADH regeneration we assumed an NADH-dependent isocitrate dehydrogenase encoded by *IDH1* and *IDH2* (Kornberg & Pricer, 1951) and not the NADPH-dependent *Isp* isoenzymes. Error minimization for the flux calculations in the determined network was carried out as described by Fischer *et al.* (2004). Neither CO₂ measurements nor NADPH production and consumption were added as constraints. The measured CO₂ evolution rates were about 99 % (± 4 %) of the calculated rates, showing that the flux calculations and the experimental data were highly consistent under the chosen experimental conditions. In contrast, the estimated NADPH production was higher than the consumption for biosynthesis (difference of 11 % up to 63 % of the total NADPH produced). The

highest deviation was found under alkaline conditions (pH 6.9 and 7.5: 56 % and 63 %, respectively), which might be an indication of an NAD⁺-dependent acetaldehyde dehydrogenase activity (e.g. by Ald2p or Ald3p), partly replacing the NADP⁺-dependent Ald5p assumed previously (Blank *et al.*, 2005a). Smaller contributions might be an overestimation of the flux towards cytosolic acetyl-CoA and flux through the malic enzyme.

RESULTS

Online monitoring of gaseous metabolites for quantitative physiology analysis of *S. cerevisiae*

To establish an experimental set-up for reliably determining baker's yeast physiology in a parallel cultivation system, online sensors for measuring gaseous metabolic products (ethanol and CO₂) were integrated into shaking flasks. First, ethanol recovery was characterized in non-modified shaking flasks during abiotic experiments. Half of the ethanol evaporated in 10 h (experimental conditions: 50 ml medium in a 250 ml shaking flask; data not shown), indicating that this set-up is not suitable for the determination of ethanol production rates. Next, ethanol recovery in newly assembled shake flasks was determined by summing the amounts of ethanol present in the aqueous and gaseous phases. The recovery of ethanol was about 99 % in these experiments after 16 h. The new set-up is therefore suitable for high-quality physiological experiments including quantification of the volatile metabolite ethanol.

The new shaking flask set-up with integrated online sensors for CO₂ and ethanol was used for quantitative physiological analysis of *S. cerevisiae* during respiro-fermentative growth (aerobic conditions and 25 g glucose l⁻¹) under reference conditions (30 °C and pH 5.0) (Fig. 1). These experimental conditions allow for exponential growth at least until an OD₆₀₀ of 9.5, which is in late exponential phase (Fig. 1a). An approximate calculation with an initial amount of oxygen in the headspace (1.3 l) of 12 mmol suggests that after glucose exhaustion (25 g l⁻¹), 1 mmol or more oxygen is still

Table 1. Growth parameters of *S. cerevisiae* during respiro-fermentative growth under reference conditions (30 °C, 25 g glucose l⁻¹ and pH 5.0)

Parameter	Value*
r_{glucose} (mmol g ⁻¹ h ⁻¹)	20.2 ± 0.6
r_{glycerol} (mmol g ⁻¹ h ⁻¹)	1.9 ± 0.3
r_{acetate} (mmol g ⁻¹ h ⁻¹)	1.0 ± 0.1
r_{ethanol} (mmol g ⁻¹ h ⁻¹)	30.0 ± 0.7
r_{CO_2} (mmol g ⁻¹ h ⁻¹)	34.4 ± 0.7
μ (h ⁻¹)	0.4 ± 0.0
C-recovery (%)	97.6 ± 0.8

*Relative errors were estimated using an exponential growth model that was implemented in the Sigma Plot statistic module.

Fig. 1. Fermentation course of *S. cerevisiae* during respiro-fermentative growth. (a) CO₂ and gaseous ethanol concentrations were monitored in the gas phase using IR sensors. (b) Biomass plotted vs CO₂ and gaseous ethanol concentrations. (c) Growth medium concentrations of glucose, ethanol, glycerol and acetate, quantified by UV-RI-HPLC. (d) Biomass plotted vs concentrations of glucose, ethanol, glycerol and acetate. Lines in (a) and (c) represent a best fit of all experimental data during exponential growth until 10 h using an exponential growth model. Lines represent individual linear fit for data up to an OD₆₀₀ value of 6 in (b) and for data up to an OD₆₀₀ value of 9 in (d). Data fitting was performed using the statistic module implemented in Sigma Plot.

present. Hence, aerobic conditions are ensured throughout the experiment. However, the data indicate that the shake flasks are leaky at pressures above 100 mbar, which correlated in this experiment with biomass concentrations above an OD₆₀₀ of 7.5 (1.3 g_{CDW} l⁻¹) and higher (data not shown). We therefore used biomass concentrations below an OD₆₀₀ value of 7.5, to ensure that neither oxygen limitation occurred nor were volatile metabolites lost due to overpressure. A carbon recovery of 97 % in the first 9 h indicated that the new cultivation system allowed for the acquisition of high-quality physiological data (Table 1). The yeast cells consumed glucose with an uptake rate of about 20 mmol g⁻¹ h⁻¹. The glucose was mainly converted into biomass, ethanol and CO₂, with glycerol and acetate as minor by-products. No pyruvate and succinate were detected in the growth medium

(detection limits of the UV-RI-HPLC used are 0.1 and 0.2 mM for pyruvate and succinate, respectively).

Quantitative physiological analysis during environmentally perturbed growth

To investigate the influence of glucose uptake and growth rates on the net flux through the TCA cycle, growth experiments under different environmental conditions were performed. Since previous studies reported a strong reduction in the rate of growth under altered pH and NaCl stress conditions (Blank & Sauer, 2004; Gustafsson *et al.*, 1993), in the experiments presented here, the growth rate was influenced by applying different pH values (pH 3.2, 5.0, 6.5, 6.9 and 7.5) or NaCl stress (0.3 and 0.5 M). The

physiological parameters (Table 2) obtained under reference conditions (pH 5.0) agreed well with previous studies (Blank & Sauer, 2004; Duarte *et al.*, 2004; Merico *et al.*, 2007; van Dijken *et al.*, 2000). *S. cerevisiae* grew fast and had a low biomass yield of $0.11 \text{ g}_{\text{CDW}} (\text{g glucose})^{-1}$ and large amounts of ethanol were produced. Growth of *S. cerevisiae* under all other conditions was significantly slower. Under the conditions tested here, the glucose uptake rate correlated linearly with the growth rate (Fig. 2e), indicating minimal changes in biomass yield. The constant biomass yield was also supported by the high correlation coefficient between the specific glucose uptake rate and the production rates of ethanol and CO_2 , with $R^2=0.978$ and $R^2=0.984$, respectively (Fig. 2a, b).

In contrast, glycerol and acetate production (Fig. 2c, d) seem to be less dependent on the glucose uptake rate, but were rather influenced directly by environmental conditions (Blank & Sauer, 2004; Hohmann, 2002). The highest rates for acetate production were measured at pH 7.5 ($1.7 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1}$) and 6.9 ($2.1 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1}$), whereas the lowest rate was observed at pH 3.2 ($0.6 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1}$). These data suggest that acetic acid production can be altered by *S. cerevisiae* in dependence on the pH of the growth medium to actively change the value to more favourable conditions. However, as the data presented are purely descriptive, a detailed investigation would be needed to clarify the regulation of acetate formation. Theoretically, one might assume that the extracellular pH is directly regulating acetic acid production. Other possible explanations include an imbalance in intracellular acetate or changes in the activity of the acetyl-CoA synthase isoenzymes Acs1p and Acs2p, which convert acetate to acetyl-CoA. Indeed it was previously reported that ACS1 expression is dependent on the glucose concentration under aerobic growth conditions (van den Berg *et al.*, 1996). However, no pH-dependent regulation of ACS1 or ACS2 expression was observed in alkaline or acid stress experiments in the time frame of 100 min (Causton *et al.*, 2001).

Glycerol is the sink for excess NADH (Vemuri *et al.*, 2007) and is the main osmo-protectant of *S. cerevisiae* (Hohmann, 2002). We observed the highest glycerol production rate ($2.9 \text{ mmol g}_{\text{CDW}}^{-1} \text{ h}^{-1}$) under high-osmolarity conditions (0.3 M NaCl), but significant

amounts were also produced during growth in all other conditions. The reason for such high production of glycerol is most likely the need to balance the cytosolic NADH produced and consumed, despite the presence of oxygen as the external electron acceptor.

The results indicate that the metabolic network initiates separate responses to environmental conditions such as the secretion of acetate or glycerol. The increase of the CO_2 /ethanol ratio with decreasing glucose uptake rate (Fig. 2f) suggests that the TCA cycle activity is influenced by the growth or glucose uptake rate.

Flux through the TCA cycle is inversely correlated with the glucose uptake rate

To clarify the impact of different growth and glucose uptake rates on the flux distribution in central carbon metabolism of baker's yeast, we carried out a physiological examination using ^{13}C tracer-based metabolic flux analysis. This analysis used a compartmented metabolic model (Blank *et al.*, 2005a) implemented in FiatFlux (Zamboni *et al.*, 2005).

As expected, glycolysis and the ethanol production pathway demonstrated the highest fluxes. In contrast, the pentose phosphate pathway sustained mainly biomass precursor formation, with only a minor contribution to glucose catabolism (Fig. 3 and 4). As NADPH for biomass synthesis is regenerated to a significant extent by this pathway, a low flux suggests low biomass yield, which was shown previously for several hemiascomycetous yeast species (Blank *et al.*, 2005b). Significant flux catalysed by the malic enzyme could be observed at lower growth rates. Phosphoenolpyruvate (PEP) carboxykinase, which catalyses the conversion of oxaloacetate to PEP, was active in all experiments, but no correlation with environmental conditions was observed.

Under reference conditions, the TCA cycle operated as a bifurcated pathway to sustain biomass precursor synthesis (Blank & Sauer, 2004; Maaheimo *et al.*, 2001). However, irrespective of the high glucose concentration of the medium, the normalized flux through the TCA cycle increased with decreasing glucose uptake rates (Fig. 3 and 4). Since relative fluxes can mask absolute changes in flux, the absolute fluxes through the TCA cycle under all tested

Table 2. Growth parameters and carbon recovery of *S. cerevisiae* under different growth conditions

Cultivation condition	$\mu \text{ (h}^{-1}\text{)}$	$r_{\text{glucose}} \text{ (mmol g}^{-1} \text{ h}^{-1}\text{)}$	$r_{\text{glycerol}} \text{ (mmol g}^{-1} \text{ h}^{-1}\text{)}$	$r_{\text{acetate}} \text{ (mmol g}^{-1} \text{ h}^{-1}\text{)}$	$r_{\text{ethanol}} \text{ (mmol g}^{-1} \text{ h}^{-1}\text{)}$	$r_{\text{CO}_2} \text{ (mmol g}^{-1} \text{ h}^{-1}\text{)}$	C-recovery (%)
pH 3.2	0.16 ± 0.01	7.2 ± 0.5	0.6 ± 0.1	0.7 ± 0.1	9.0 ± 0.6	15.4 ± 1.3	100 ± 3
pH 5.0	0.40 ± 0.01	19.9 ± 0.6	1.7 ± 0.1	1.3 ± 0.1	29.6 ± 1.0	33.8 ± 2.5	97 ± 1
pH 6.5	0.36 ± 0.01	18.4 ± 1.2	1.5 ± 0.1	1.3 ± 0.1	28.2 ± 1.8	32.0 ± 2.4	100 ± 2
pH 6.9	0.21 ± 0.01	12.3 ± 0.7	2.6 ± 0.1	2.1 ± 0.1	15.2 ± 1.0	24.0 ± 2.9	102 ± 2
pH 7.5	0.17 ± 0.02	10.2 ± 0.5	2.4 ± 0.1	1.7 ± 0.1	11.2 ± 0.6	20.6 ± 1.7	99 ± 1
0.3 M NaCl	0.33 ± 0.01	15.1 ± 1.1	2.9 ± 0.1	1.0 ± 0.1	20.1 ± 0.9	25.5 ± 2.5	99 ± 2
0.5 M NaCl	0.23 ± 0.02	12.2 ± 1.0	2.8 ± 0.2	0.9 ± 0.1	15.6 ± 1.5	23.1 ± 3.2	101 ± 4

Fig. 2. Absolute metabolic fluxes (a–d), growth rate (e), and CO₂/ethanol ratio (f) of *S. cerevisiae* during growth under seven different environmental conditions as a function of the specific glucose uptake rate. Respective errors were determined using a simultaneous nonlinear fit of concentration data of biomass, substrate and products using an exponential growth model in Sigma Plot and the Gaussian law of error propagation. All experiments were done in duplicate.

environmental conditions were plotted against the glucose uptake rate (Fig. 5a). The maximum TCA cycle flux of 1.69 mmol g⁻¹ h⁻¹ at a glucose uptake rate of 10.23 mmol g⁻¹ h⁻¹ is in the range of the highest reported flux through the TCA cycle in *S. cerevisiae*. As a comparison, during fully

derepressed growth in a glucose-limited chemostat at a similar growth rate, the flux through the TCA cycle was only 0.83 mmol g⁻¹ h⁻¹ (Gombert *et al.*, 2001), while a TCA cycle flux of about 2.5 mmol g⁻¹ h⁻¹ was reported during growth in the presence of respiratory carbon source ethanol

Fig. 3. Relative distribution of absolute carbon fluxes in *S. cerevisiae*. Cells were cultivated in minimal medium at pH 5.0 (reference conditions), pH 6.5, in the presence of 0.3 M NaCl, and at pH 6.9. All fluxes are normalized to the specific glucose uptake rate, which is shown in the top inset, and are given in the same order in each box. The relative NADH dehydrogenase fluxes are the net reduction rates of cytosolic and mitochondrial NADH, assumed to be catalysed by Nde1/2p and Ndi1p, respectively. The 95 % confidence intervals for the major fluxes were between 5 and 10 %, with the exception of the malic enzyme and PEP carboxykinase, for which larger confidence intervals were estimated. The growth substrate and the secreted products are written in capital letters. C1, one-carbon unit from C₁ metabolism.

(Daran-Lapujade *et al.*, 2004). According to the TCA cycle activity, the increase in the respiration rate coincided with a decreased glucose uptake rate and was found to be the highest, $6.2 \text{ mmol g}^{-1} \text{ h}^{-1}$, at pH 7.5. This is interestingly higher than during fully derepressed growth in a glucose-limited chemostat at a dilution rate of 0.1 with a respiration rate of $2.74 \text{ mmol g}^{-1} \text{ h}^{-1}$ but a considerably lower glucose uptake rate of $1.15 \text{ mmol g}^{-1} \text{ h}^{-1}$ (Daran-Lapujade *et al.*, 2004).

To determine whether the increase of TCA cycle activity is a direct response to the glucose uptake rate or to the

growth rate, we aimed to decouple growth rate from the rate of glucose uptake using three well-characterized single gene deletion mutants. The two mutants reported to be partially glucose repression negative (hexokinase 2, *hxx2*; and F-box protein component of the SCF ubiquitin-ligase complex, *grr1*) are central components in the major regulatory pathway of glucose repression (Westergaard *et al.*, 2007), while the *hap2* mutant, which is unable to derepress, lacks an essential subunit of the Hap2p/3p/4p/5p CCAAT-binding complex, which in the wild-type activates transcription of TCA cycle genes and genes involved in

respiration (Pinkham & Guarente, 1985). Comparison of the TCA cycle activity of the wild-type strain in dependence on the growth rate (Fig. 5a) and glucose uptake rate (Fig. 5b) shows a high correlation of the TCA cycle with both, which corresponds well with the linear correlation between glucose uptake and growth rate (Fig. 2e). In the mutants only the glucose uptake rate correlated strongly with the TCA cycle activity [i.e. *hxx2* (pH 5.0 and 6.9) and *grr1* (pH 5.0 and 6.9)], while the growth rate differed considerably (Fig. 5a). The increased flux through the TCA cycle in these mutants was also evidenced in the

biomass yield: 0.21 and 0.16 g_{CDW} (g glucose)⁻¹ for the *hxxk2* and *grr1* mutant, respectively, in pH 5.0 medium, and 0.18 and 0.13 g_{CDW} (g glucose)⁻¹ for the *hxxk2* and *grr1* mutant, respectively, in pH 6.9 medium. Interestingly, the *hap2* mutant was also able to increase the flux through the TCA cycle at slow growth (in pH 6.9 medium), despite a very high glucose uptake rate, which indicates fully fermentative metabolism in this mutant at pH 5.0, also supported by a biomass yield of 0.07 g_{CDW} (g glucose)⁻¹. As the *HAP2* gene product is essential for growth on respiratory carbon sources (Steinmetz *et al.*, 2002), the

Fig. 5. Correlation between absolute TCA cycle flux and (a) the growth rate and (b) the glucose uptake rate of the CEN.PK 113-7D wild-type during growth under seven different environmental conditions, and of the CEN.PK 113-7D knockout mutants (*hxx2*, *grr1* and *hap2*) during growth under reference conditions (pH 5.0) and at pH 6.9. Respective errors were determined via a simultaneous nonlinear fit of concentration data of biomass, substrate and products using an exponential growth model in Sigma Plot and the Gaussian law of error propagation. All experiments were done in duplicate.

results suggest that under the conditions tested here an alternative regulatory mechanism exists that guaranteed some minor activity in the electron transport chain. In summary, the findings indicate that the glucose uptake rate rather than the growth rate correlates with the TCA cycle activity. However, the way in which the glucose uptake rate is converted into a signal that is relevant for derepressing the TCA cycle remains unclear.

DISCUSSION

A previous study suggested that the respiratory activity of the TCA cycle may increase under conditions of excess

glucose and unlimited growth conditions in response to environmentally altered glucose uptake rates (Blank & Sauer, 2004). However, only indirect evidence from labelling experiments has hitherto been presented to support this hypothesis. In the present study, we provide additional evidence for increased TCA cycle activity under the same circumstances. We identified an increase in the ratio of CO_2 to ethanol and a decrease in the metabolic flux ratio mitochondrial oxaloacetate derived through anaplerosis in cells growing at lower rates. This evidence was supported by highly accurate quantification of the net flux through the TCA cycle. Our results suggest that the net TCA cycle flux is inversely correlated with the growth and/or glucose uptake rate and is not only influenced by the glucose concentration of the medium. This evidence for alternative TCA cycle regulation was obtainable due to the development of a simple shake flask set-up that allowed for the quantification of the volatile by-products CO_2 and ethanol.

To our knowledge, the only yeast gene of which expression was suggested to be growth rate dependent is *HXT5*, encoding a moderate-affinity glucose transporter, as higher expression was observed at lower growth rates (Verwaal *et al.*, 2002). Elbing *et al.* (2004) reported the glucose uptake rate as the determinant of respiratory metabolism in *S. cerevisiae*. The use of chimeric hexose transporters allowed for the reduction of the maximal glucose uptake rate, leading to the absence of ethanol production at low uptake rates (below $5 \text{ mmol g}^{-1} \text{h}^{-1}$). Interestingly, in a recent study, the same group claimed that the role of the glucose uptake rate was overestimated, despite a significant inverse correlation between glucose uptake and ethanol production rates (Bosch *et al.*, 2008).

Previous reports for the *hxx2* mutant support the idea that the glucose repression signal is related to the glucose uptake rate rather than to the growth rate (Blank & Sauer, 2004; Bisson & Kunathigan, 2003). Indeed, performing high-quality physiological experiments with the *hxx2*, but also with the *grr1* mutant, indicated that the glucose uptake rate, and not the growth rate, is inversely correlated with the TCA cycle flux (Fig. 5). While recent reports have elucidated the non-metabolic role of Hxx2p in glucose repression, as inhibitor of Mig1p phosphorylation by the kinase Snf1p (Gancedo, 2008), the primary signal that triggers Hxx2p phosphorylation and therefore relocation into the nucleus is not known. A possible candidate is cAMP, as many transcriptional changes due to the presence of glucose can be imitated in the absence of glucose by increasing the level of cAMP (Wang *et al.*, 2004). Interestingly, the primary cAMP-dependent regulatory proteins, the kinases Tpk1p, Tpk2p and Tpk3p, are not required for the cAMP-related change in transcription in the absence of glucose, suggesting an alternative regulatory mechanism, which might involve another metabolite (Gancedo, 2008).

A potential candidate that may influence the glucose uptake rate is the NADH/NAD ratio. Vemuri *et al.* (2007)

constructed NADH oxidase mutants that had high respiratory activity in the presence of glucose. Overexpression of an alternative oxidase gene in the mitochondria caused derepression of several TCA cycle genes. Also, a significant reduction in the ethanol production rate was shown during growth in glucose batch culture. Although the growth rate of this mutant was identical to the wild-type, the biomass yield was affected [$0.09 \text{ g}_{\text{CDW}} (\text{g glucose})^{-1}$ instead of the $0.11 \text{ g}_{\text{CDW}} (\text{g glucose})^{-1}$ of the control strain]. This difference indicates that biomass synthesis during respiration-fermentative growth is not limited by availability of energy. In another study, indirect evidence for an increase in TCA cycle activity was reported as a response to weak acids such as benzoate (Kresnowati *et al.*, 2008). During growth in the presence of benzoate, the dual mechanism of an increased extrusion rate of protons via the ATP-driven H^+ -ATPase and the export of benzoate potentially reduces the level of NADH. Other metabolic signals of glucose repression include intracellular glucose concentration, glucose 6-phosphate concentration and ATP availability, but as previously mentioned, the primary signal is unclear.

The methodology described here for high-quality metabolic flux analysis in shaking flasks with integrated online sensors for measuring volatile metabolites is robust, can be run in parallel, and generates data with high information content. Most of the reported fluxes are scaled with the glucose uptake rate. This indicates minimal flux redistribution in yeast, although the selected growth conditions had a significant impact on the rate of growth. Besides rerouting towards glycerol and acetate under specific environmental conditions, the sole exception was the respiratory TCA cycle flux. Considering the general robustness of yeast metabolism under different environmental conditions, it can be speculated that flexibility is required in certain pathways to fulfil special functions. These functions include osmo-control (glycerol synthesis pathway), extracellular pH control (acetate synthesis pathway) and energy generation (TCA cycle). This combination of robustness and flexibility may allow *S. cerevisiae* to adapt rapidly to quickly changing environmental conditions.

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