TCA cycle activity in *Saccharomyces cerevisiae* is a function of the environmentally determined specific growth and glucose uptake rates

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Metabolic responses of *Saccharomyces cerevisiae* to different physical and chemical environmental conditions were investigated in glucose batch culture by GC-MS-detected mass isotopomer distributions in proteinogenic amino acids from $^{13}$C-labelling experiments. For this purpose, GC-MS-based metabolic flux ratio analysis was extended from bacteria to the compartmentalized metabolism of *S. cerevisiae*. Generally, *S. cerevisiae* was shown to have low catabolic fluxes through the pentose phosphate pathway and the tricarboxylic acid (TCA) cycle. Notably, respiratory TCA cycle fluxes exhibited a strong correlation with the maximum specific growth rate that was attained under different environmental conditions, including a wide range of pH, osmolarity, decoupler and salt concentrations, but not temperature. At pH values of 4-6 with near-maximum growth rates, the TCA cycle operated as a bifurcated pathway to fulfil exclusively biosynthetic functions. Increasing or decreasing the pH beyond this physiologically optimal range, however, reduced growth and glucose uptake rates but increased the 'cyclic' respiratory mode of TCA cycle operation for catabolism. Thus, the results indicate that glucose repression of the TCA cycle is regulated by the rates of growth or glucose uptake, or signals derived from these. While sensing of extracellular glucose concentrations has a general influence on the *in vivo* TCA cycle activity, the growth-rate-dependent increase in respiratory TCA cycle activity was independent of glucose sensing.

INTRODUCTION

Genome-wide mRNA responses of the baker’s yeast *Saccharomyces cerevisiae* to changes in pH, osmolarity or temperature revealed differential expression of more than 1000 transcripts during adaptation (Causton et al., 2001; Gasch et al., 2000). Since transcriptome or proteome changes do not directly reveal cellular phenotypes, one would like to connect these inventory data with the apparent cellular physiology (Bailey, 1999). One such approach is metabolic flux analysis, which estimates material flow through biochemical reaction networks, and thus provides a direct link to the physiological phenotype (Hellerstein, 2003).

Different approaches for metabolic flux analysis based on $^{13}$C-labelling experiments have been developed, allowing precise quantification of central carbon metabolism (Sauer, 2004; Wiechert, 2001). Recent applications include the bacteria *Bacillus subtilis* (Daumer et al., 2001; Zamboni & Sauer, 2003), *Corynebacterium glutamicum* (Klapa et al., 2003; Petersen et al., 2000; Wittmann & Heinzle, 2002) and *Escherichia coli* (Emmerling et al., 2002; Fischer & Sauer, 2003b; Jiao et al., 2003; Sauer et al., 2004). Although conceptually more difficult, flux analysis has also been applied successfully to compartmentalized microbes such as *S. cerevisiae* (Christensen et al., 2002; Dos Santos et al., 2003), *Saccharomyces kluveri* (Möller et al., 2002), *Kluyveromyces marxianus* (Wittmann et al., 2002b) and *Penicillium chrysogenum* (Van Winden et al., 2003). Often, metabolic flux analysis combines $^{13}$C-labelling data with quantitative physiology data to obtain a best-fit flux solution. A somewhat different methodology is metabolic flux ratio (METAFoR) analysis, which quantifies the relative contribution of converging pathways or reactions to a given intracellular metabolite (Fischer & Sauer, 2003a). Without data fitting, this biochemical approach relies exclusively on $^{13}$C data and has been used successfully with NMR data in the yeasts *S. cerevisiae* (Maaheimo et al., 2001) and *Pichia stipitis* (Fiaux et al., 2003).

Here, we extend METAFoR analysis by GC-MS from *E. coli* (Fischer & Sauer, 2003a) to the compartmentalized *S. cerevisiae* metabolism. The particular focus of this study was to investigate the impact of different environmental conditions such as pH, osmolarity and temperature on the central carbon metabolism of *S. cerevisiae* during growth on glucose.
METHODS

Yeasts strains and growth conditions. The haploid S. cerevisiae strains used in this study are listed in Table 1. The his2Δ, rgt2Δ and snf3Δ mutants were constructed by using existing kanMX4 cassettes (Winzeler et al., 1999). The cassettes were amplified using primers located ~500 bp upstream and downstream of the start and stop codon of the corresponding gene, respectively. Batch cultures of 20 ml were performed in 500 ml baffled Erlenmeyer flasks on a rotary shaker at 30 °C and 225 r.p.m., ensuring fully aerated conditions, in yeast minimal medium (Verduyn et al., 1992) containing (per litre) 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 K₂HPO₄, 0–1 mg 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 changes due to ammonia uptake and acetate production the medium was supplemented with 100 mM potassium hydrogen phthalate. For all experiments at pH 5 or higher, 100 mM MOPS was used as the buffering reagent and the amino acids were performed as described previously (Dos Santos et al., 2003). The labelling pattern of phosphoenolpyruvate (PEP) derived from tyrosine and phenylalanine was different from the labelling pattern of mitochondrial pyruvate revealed from value. The labelling patterns of alanine and valine, however, were highly similar, suggesting that alanine is indeed synthesized from mitochondrial pyruvate in the experimental conditions used in this study.

Analytical procedures and physiological parameters. Cell growth was followed by monitoring the OD₆₀₀. Samples for extracellular metabolite determination were centrifuged at 14 000 r.p.m. in an Eppendorf tabletop centrifuge to remove cells. Glucose and glycerol concentrations in the supernatant were determined with commercial enzymic kits [Triglyceride 337-40A, Sigma; D-Glucose Test (E0716251), R-Biopharm AG]. The physiological parameters maximum specific growth rate, biomass yield on glucose, and specific glucose consumption rate were calculated during the exponential growth phase (Sauer et al., 1999).

¹³C-labelling experiments. All labelling experiments were done in batch cultures assuming pseudo-steady-state conditions during the exponential growth phase (Fischer & Sauer, 2003a; Sauer et al., 1999; Wittmann & Heinze, 2001). ¹³C-labelling of proteinogenic amino acids was achieved by growth on 5 g glucose l⁻¹ as a mixture of 80% (w/w) unlabelled and 20% (w/w) uniformly labelled [U-¹³C]glucose (¹³C-glucose, 98%; Isotech). Cells from an overnight minimal medium culture were washed and used for inoculation below an OD₆₀₀ of 0.05. ¹³C-labelled biomass aliquots were harvested during the mid-exponential growth phase at an OD₆₀₀ of ≤1. At this point the residual glucose concentration was below 1 g l⁻¹, thus clearly above the reported 0.5 g l⁻¹ threshold of inverte repressin in S. cerevisiae strain CEN.PK 113-7D (Herwig et al., 2001). The cells were harvested by centrifugation, washed once with sterile water, and hydrolysed in 500 μl 6 M HCl at 105 °C for 24 h. The hydrolysate was dried in a heating block at 80 °C under a constant airflow. The free amino acids were derivatized at 85 °C for 1 h using 25 μl dimethylformamide and 25 μl N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (Dauner & Sauer, 2000; Wittmann et al., 2002a).

GC-MS analysis was carried out as reported recently (Fischer & Sauer, 2003a) using a previously described biochemical reaction network (Maaheimo et al., 2001). The recently described cytosolic alanine synthesis in glucose/acetate co-metabolism experiments was not seen in our glucose experiments (Dos Santos et al., 2003). The labelling pattern of phosphoenolpyruvate (PEP) derived from tyrosine and phenylalanine was different from the labelling pattern of mitochondrial pyruvate revealed from value. The labelling patterns of alanine and valine, however, were highly similar, suggesting that alanine is indeed synthesized from mitochondrial pyruvate in the experimental conditions used in this study.

META FoR analysis using amino acids mass isotopomer data. The GC-MS data represent sets of ion clusters, each showing the distribution of mass isotopomers of a given amino acid fragment. For each fragment z, one mass isotopomer distribution vector (MDV) was assigned,

\[
MDV_z = \begin{pmatrix} m_0 \\ m_1 \\ \vdots \\ m_z \end{pmatrix} \text{ with } m_i = 1
\]

with \(m_0\) being the fractional abundance of the lowest mass and \(m_i\), the abundances of molecules with higher masses. To obtain the exclusive mass isotope distribution of the carbon skeleton, corrections for naturally occurring isotopes in the derivatization reagent and the amino acids were performed as described previously (Fischer & Sauer, 2003a), followed by the calculations of the mass distribution vectors for amino acids (MDVₐₐ) and metabolites (MDVₘₘ). Metabolic flux ratios were calculated from the MDVₘₘ as described for E. coli (Fischer & Sauer, 2003a) with the following exceptions, which account for the compartmentalized biochemical reaction network of S. cerevisiae. Since the mitochondrial aspartate aminotransferase is probably inactive under the conditions used (Maaheimo et al., 2001), the mass distribution of mitochondrial oxaloacetate was not directly accessible (Fig. 1). Instead, the MDV of the four-carbon C-1-C-2-C-3-C-4 fragment of mitochondrial oxaloacetate (OAA₁₄) (see Fig. 1 for abbreviations) was accessed by assuming that only anaerobiosis and the TCA cycle contribute to its labelling pattern. Thus, the MDVₘ of OAA₁₄ can be calculated.

Table 1. Yeast strains used in this work

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tr>
<td>CEN.PK113-7D</td>
<td>MATa MAL2-8Δ SUC2</td>
<td>Eurosacraft®</td>
</tr>
<tr>
<td>LMB45</td>
<td>CEN.PK 113-7D his2Δ::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>LMB179</td>
<td>CEN.PK 113-7D snf3Δ::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>LMB243</td>
<td>CEN.PK 113-7D rgt2Δ::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>CEN.PK513-3A</td>
<td>CEN.PK 113-7D grr1Δ::loxP-kanMX4-loxP</td>
<td>P. Kötter, pers. comm.</td>
</tr>
</tbody>
</table>
| MMB4      | W303-1A MATa ade1Δ can1Δ-100 his3Δ-11.15 leu2Δ-3,112 trp1Δ-1
urn3Δ-1 snf3Δ::HIS3 rgt2Δ::leu2Δ mtl1Δ::trp1Δ grr1Δ::kanMX4 | J. M. Gancedo, pers. comm. |

*European Saccharomyces cerevisiae Archive for Functional Analysis (euroscarf@em.uni-frankfurt.de).
Central carbon metabolism of *S. cerevisiae* during aerobic growth on glucose. Arrows with solid heads indicate the reaction reversibilities considered. Arrows with open heads highlight the biosynthesis pathways of amino acids that allow for direct conclusions on the $^{13}$C-labelling pattern of their precursor molecules. The superscripts „c yr“ and „m it“ refer to the cytosolic and mitochondrial species of metabolites that are considered in METAFoR analysis. Extracellular substrates and products are given in capitals. Abbreviations: C1, one-carbon unit from C1 metabolism; E4P, erythrose 4-phosphate; OAA, oxaloacetate; OGA, 2-oxoglutarate; PEP, phosphoenolpyruvate; P5P, pentose 5-phosphates; S7P, sedoheptulose 7-phosphate.

**Fig. 1.** Central carbon metabolism of *S. cerevisiae* during aerobic growth on glucose.

The following equations are used to calculate the fraction of OAA that is derived from malate via the TCA cycle.

\[ \text{OAA}_{14}^{\text{mit}} = \left( 1 - \frac{\text{OAA}_{\text{mit}}^{\text{through TCA cycle}}}{\text{OAA}_{\text{mit}}^{\text{through TCA cycle}}} \right)(\text{PEP}13 \times \text{MDV}(\text{CO}_2)) + \frac{\text{OAA}_{\text{mit}}^{\text{through TCA cycle}}}{\text{OAA}_{\text{mit}}^{\text{through TCA cycle}}} \times \text{OGA}_{25} \]  

(2)

The fraction of OAA derived through the TCA cycle is obtained from:

\[ \text{OAA}_{\text{mit}}^{\text{through TCA cycle}} = \frac{\text{OGA}_{25} - \text{GLU}_{1U} \times \text{GLU}_{1U} \times \text{GLU}_{1U}}{\text{GLU}_{2U} \times \text{GLU}_{2U} - \text{GLU}_{2U} \times \text{GLU}_{1U} \times \text{GLU}_{1U}} \]  

(3)

where GLU$_{1U}$ and GLU$_{2U}$ are uniformly $^{13}$C-labelled 1- and 2-carbon glucose fragments, respectively. This approach assumes that the bond between carbon atoms 2 and 3 of every oxaloacetate is broken per round through the TCA cycle.

A second independent equation for calculating the fraction of OAA through the TCA cycle can be formulated by assuming that OGA15 corresponds to OAA24$^{\text{mit}}$ + acetyl-CoA12. Thus, OGA25 equals OAA23$^{\text{mit}}$ + acetyl-CoA12 and OGA12 equals OAA34$^{\text{mit}}$. The relative contribution of the TCA cycle to OAA synthesis can then also be quantified by:

\[ \text{OAA}_{\text{mit}}^{\text{through TCA cycle}} = 1 - \frac{\text{OAA}_{\text{mit}}^{\text{through TCA cycle}} - (\text{GLU}_{1U} \times \text{GLU}_{1U})}{\text{PEP}23 \times (\text{GLU}_{1U} \times \text{GLU}_{1U})} \]  

(4)

Since the independent equations 3 and 4 gave very similar results in all analyses described here, only those obtained from equation 4 are reported in the following.

The relative contribution of different synthesis pathways to mitochondrial and cytosolic acetyl-CoA could not be quantified, because the MDV$_{\text{M}}$ of LEU12 was not accessible with the derivatizing agent used and LYS12 was not accessible with the procedure employed. Consequently, the MDV$_{\text{M}}$ of cytosolic acetyl-CoA was not available for comparison with the calculated mitochondrial acetyl-CoA MDV$_{\text{M}}$ from OGA.

From the MDVs of oxaloacetate one can calculate the cytosolic OAA$^{\text{c yr}}$ derived from cytosolic pyruvate:

\[ \text{OAA}_{\text{c yr}}^{\text{from pyruvate}} = \frac{\text{OAA}_{24}^{\text{c yr}} - \text{OAA}_{24}^{\text{mit}}}{\text{PEP}13 - \text{OAA}_{24}^{\text{mit}}} \]

(5)

Furthermore one can determine the activities of the reversible exchange fluxes between oxaloacetate and fumarate or succinate:

\[ \frac{\text{OAA}_{\text{mit}}^{\text{rev}} \text{ from fumarate}^{\text{mit}}}{\text{OAA}_{24}^{\text{mit}} - \text{pyruvate}^{\text{mit}} \times \text{MDV}(\text{CO}_2)} \]

(6)

\[ \text{OAA}_{\text{c yr}}^{\text{rev}} \text{ from fumarate}^{\text{c yr}} = \frac{\text{OAA}_{24}^{\text{c yr}} - \text{PEP}23 \times \text{MDV}(\text{CO}_2)}{0.5 \times \text{PEP}13 + 0.5 \times \text{PEP}23 \times \text{MDV}(\text{CO}_2)} - \text{PEP}23 \times \text{MDV}(\text{CO}_2) \]

(7)

Since the malic enzyme is located in the mitochondria (Boles *et al*., 1998), the mitochondrial pools of pyruvate and oxaloacetate are used to calculate lower and upper bounds of malic enzyme activity:

\[ \text{Pyruvate}^{\text{mit}} \text{from malate (lower bound)} = \frac{\text{pyruvate}^{\text{mit}} - \text{PEP}23}{(\text{GLU}_{1U} \times \text{GLU}_{1U}) - \text{PEP}23} \]

(8)

\[ \text{Pyruvate}^{\text{mit}} \text{from malate (upper bound)} = \frac{\text{pyruvate}^{\text{mit}} - \text{PEP}23}{(\text{GLU}_{1U} \times \text{GLU}_{1U}) - \text{PEP}23} \]

(9)

The contribution of the non-oxidative pentose phosphate (PP) pathway via transketolase to the synthesis of the triose pool can be estimated from the MDV of PEP12, revealing the fraction of trioses rearranged between carbon C-1 and C-2 by the action of the transketolase. The remaining PEP12 molecules originate from an unbroken C2 unit of glucose derived through glycolysis.

\[ \text{PEP} \text{through transketolase} = \frac{\text{PEP}12 - \text{GLU}_{2U}}{\text{GLU}_{1U} \times \text{GLU}_{1U} - \text{GLU}_{2U}} \]

(10)

An upper bound for the contribution of the PP pathway to trioses synthesis can than be calculated by assuming that five trioses are produced from three pentoses and that at least two of these trioses are rearranged by a transketolase:

\[ \text{PEP} \text{through PP pathway} = \frac{\text{PEP}12 - \text{GLU}_{2U}}{5/2 \times \text{PEP} \text{through transketolase}} \]

(11)

Since the contribution of the PP pathway to PEP synthesis is an upper bound, we present the results from equation 11 by including the error interval.
RESULTS

METAFlOr analysis by GC-MS of *S. cerevisiae* in glucose batch culture

To establish a rapid and robust methodology for flux analysis, we extended METAFlOr analysis by GC-MS from bacterial metabolism (Fischer & Sauer, 2003a; Fischer et al., 2004; Zamboni & Sauer, 2003) to the compartmentalized metabolism of *S. cerevisiae*. Firstly, we found the mass isotopomer distributions in the GC-MS-analysed proteinogenic amino acids to be generally consistent with previous network analysis (Christensen et al., 2002; Maheimo et al., 2001; Van Winden, 2002) of amino acid biosynthesis in *S. cerevisiae* (data not shown). Next, we compared GC-MS-based METAFlOr analysis of a shake-flask-grown *S. cerevisiae* batch culture on glucose at 30°C and pH 5-5 to NMR-based METAFlOr analysis under almost identical conditions (Maheimo et al., 2001). Indeed, very similar results such as low PP pathway and TCA cycle activities were found (Fig. 2). Since the interpretation of 13C-labelling pattern in amino acids by MS and NMR analyses is only connected by the assumed biochemical network, this consistency provides evidence for faithful and reliable quantification of metabolic flux ratios by either method. Minor differences in flux ratios may be attributed to the different minimal medium composition and to the somewhat lower growth rate of the NMR-analysed culture (Maheimo et al., 2001).

Impact of pH and temperature on the metabolic flux profile

To investigate the influence of physical and chemical environmental parameters on central carbon metabolism under aerobic fermentation conditions, we grew *S. cerevisiae* at 25, 30 and 37°C and at pH values of 3-5, 5-0 and 6-0. The 16 determined metabolic flux ratios were surprisingly stable under all conditions, and the relative fluxes through the TCA cycle and PP pathway remained rather low (Fig. 3). Generally, low TCA cycle activity and concomitant ethanol production were expected for the respiro-fermentative *S. cerevisiae* (Alexander & Jeffries, 1990). Previous estimates for the relative catabolic PP pathway flux to trioses vary between 0 and 4% (Fiaux et al., 2003; Maheimo et al., 2001) and 14% (Gombert et al., 2001). In the experiments described here, we observe an upper bound of 7% on the fraction of PEP derived through the PP pathway from uniformly labelled glucose experiments (Fig. 3). Thus, we conclude that the catabolic PP pathway activity of *S. cerevisiae* in batch cultures is indeed lower than seen for example in *E. coli* (Fischer & Sauer, 2003a).

The growth rate of *S. cerevisiae* correlates with the relative respiratory TCA cycle flux in batch culture

The increased fraction of OAA\textsuperscript{\textsubscript{m}} derived through the TCA cycle in the pH 3-5 culture indicates significantly increased respiratory TCA cycle activity, when compared to the biosynthetic TCA cycle activity (Fig. 3b). Since this low pH also reduced the maximum specific growth rate by 10%, we could not distinguish between a growth rate and an environmental condition effect on the TCA cycle activity. To quantify the influence of environmental conditions on the maximal specific growth rate and intracellular flux response, we grew batch cultures under aerobic fermentation conditions, with extensive ethanol formation at different pH values, osmolarities, temperatures, salt and decoupler concentrations. As shown in Fig. 4, the contribution of the TCA cycle to the synthesis of OAA\textsuperscript{\textsubscript{m}} generally increased with decreasing growth rate. This observation appeared to be independent of the wide variety of environmental conditions that were used to reduce the growth rate. The sole exception was temperature, which had only a modest effect on TCA cycle fluxes (Figs 3 and 4), although the growth rate was significantly reduced. Since the specific glucose uptake rate is closely correlated with the relative TCA cycle flux (data not shown). While we cannot exclude that the TCA cycle activity was directly influenced by the environmental conditions chosen, the wide range of conditions makes it rather unlikely that all of them have a similar and specific effect on the TCA cycle flux. Moreover, it was shown recently that the TCA cycle activity of *C. glutamicum* does not change with the osmolarity of the medium (Varela et al., 2003).

The OAA\textsuperscript{\textsubscript{m}} pool that is required for anaplerosis of the TCA cycle and biosynthesis of amino acids of the aspartate family was synthesized exclusively via pyruvate carboxylase (OAA\textsuperscript{\textsubscript{m}} from pyruvate\textsuperscript{\textsubscript{m}}) in most experiments. Only at

![Fig. 2. Origin of metabolic intermediates in *S. cerevisiae* during growth in batch cultures at 30°C and pH 5-5 determined by NMR (grey bars) and GC-MS (white bars). The standard deviations were estimated from redundant mass distributions. ND, Not determined.](image-url)
growth rates below 0·2 h\textsuperscript{-1} may a significant fraction of 7–22\% originate from the OAA\textsuperscript{mit} pool, probably catalysed by the action of the mitochondrial OAA transporter Oac1p, since transport was shown to be bidirectional (Palmieri \textit{et al.}, 1999). This flux ratio change was also independent of the environmental condition that reduced the growth rate.

In contrast to the above two flux ratios, however, none of the 10 other detected flux ratios exhibited a distinct correlation with the growth rate (data not shown). The malic enzyme for example was more active at acidic pH, but was inactive at pH 7\textsuperscript{−}5, although the growth rate was higher (0·19 h\textsuperscript{-1}) than at pH 3\textsuperscript{−}0 (0·07 h\textsuperscript{-1}). The reverse \textit{in vivo} activity pattern was determined for the gluconeogenic reaction catalysed by PEP carboxykinase, which was only detected at pH values 7\textsuperscript{−}0 and 7\textsuperscript{−}5.

To elucidate whether the growth rate was also correlated with any other physiological property, we quantified the growth physiology over a wide range of pH values (Fig. 5). For standard conditions (high glucose concentration, 30 °C, aerated batch culture, pH 5–6), these parameters (Fig. 5) were in good agreement with previous reports for \textit{S. cerevisiae} CEN.PK strains (Smits \textit{et al.}, 2000; van Dijken \textit{et al.}, 2000; van Maris \textit{et al.}, 2001). Akin to the maximum growth rate (Fig. 5a), the specific glucose uptake rate decreased significantly outside the optimal pH range of 4·0–6·0 (Fig. 5b). The biomass yield, in contrast, was rather constant and exhibited no correlation with the growth rate (Fig. 5c). The specific glycerol production rate showed a generally positive correlation with the culture pH, i.e. resulted in the highest production rate at pH 7·5 (Fig. 5d). This positive correlation may reflect a similar response as was reported for high osmolarity, where production of the main osmolyte glycerol was increased in yeast (Hohmann, 1999).
It should be noted that significant ethanol production was not reported because the high evaporation rate in the shake flasks compromised a thorough quantitative analysis.

**Glucose sensing is not required for the increase of relative TCA cycle flux during slow growth**

Although the initial glucose concentrations were identical in all the above experiments, we cannot exclude that glucose sensing was relevant for modulating the relative TCA cycle flux. We therefore constructed isogenic mutants of the two glucose sensors Snf3p and Rgt2p (Ozcan et al., 1996). If extracellular glucose sensing played a significant role in modulating the TCA cycle flux, one would expect no increase in flux upon an environmentally reduced growth rate in both mutants. However, the TCA cycle activity increased in both mutants at lower growth rates (Fig. 6). To further exclude the possibility of a partial functional overlap of the high and low glucose concentration sensors, Rgt2p and Snf3p, respectively, we used two further mutants. Strain MMB4 cannot sense extracellular glucose at all due to deletions in SNF3, RGT2, and the plasma membrane G-protein coupled sensor GPR1. To enable growth on glucose, this strain also carries the MTH1 deletion. The second strain was deleted in GRR1, which is essential for glucose sensing from the Snf3p and Rgt2p signal transduction pathway. Although both strains already exhibit high respiratory TCA cycle flux under standard conditions at growth rates of 0.39 and 0.26 h⁻¹ for MMB4 and the grr1 mutant, respectively, they still increased the relative TCA cycle flux upon an environmental modulation of the specific growth rate with high osmolarity (Fig. 6). These results strongly suggest that extracellular glucose sensing is not involved in the relative TCA cycle flux increase at slow growth rates in batch cultures. Nevertheless, the higher TCA cycle flux in the grr1 and MMB4 mutants, when

**Fig. 5.** Influence of extracellular pH on maximum specific growth rate (a), specific glucose uptake rate (b), biomass yield (c) and specific glycerol production rate (d) in batch cultures of *S. cerevisiae*. Standard deviations were estimated using SigmaPlot 8.0 (SPSS) and the Gaussian law of error propagation.

**Fig. 6.** The fraction of OAA\textsuperscript{mt} derived through the TCA cycle in *S. cerevisiae* mutants impaired in glucose sensing. The values were determined under standard batch conditions (white bars) and at 0.75 M NaCl (grey bars), which reduced the maximal specific growth rate by 40–60%. The experimental error of the TCA cycle activity was estimated from redundant mass distributions.
compared to the wild-type at the same growth rate, shows that glucose sensing has an additional repressive effect.

**Is the TCA cycle flux correlated with the rate of growth or glucose uptake?**

The specific rates of growth and glucose uptake were coupled in the batch experiments performed; thus we cannot distinguish if one or both rates were correlated with the relative TCA cycle flux. To address this question, we used a hexokinase II-deficient mutant that exhibits alleviated glucose repression in glucose batch cultures (Diderich et al., 2001). When compared to the wild-type, the specific growth rate of the hxx2 mutant was only modestly lower, but the specific glucose uptake rate was about 45% lower. The relative TCA cycle flux in this mutant exhibits no correlation with the growth rate but a much better, albeit weak, correlation with the glucose uptake rate (Fig. 7). This would suggest that it is the specific glucose uptake rate rather than the specific growth rate that correlates with the relative TCA cycle flux in wild-type *S. cerevisiae*.

**DISCUSSION**

Using the newly developed GC-MS-based METAfor analysis, we quantified intracellular flux responses of *S. cerevisiae* to a wide range of environmental conditions in batch culture. In contrast to all other monitored flux ratios, the relative respiratory activity of the TCA cycle increased with decreasing growth rate and/or glucose uptake rate at extracellular glucose concentrations between 1 and 5 g l\(^{-1}\). This correlation was independent of the four different chemical parameters that were used to modulate the growth rate. As the sole exception, temperature-induced growth rate changes gave a much less pronounced TCA cycle response.

The correlation observed here between the rates of growth and/or glucose uptake and relative respiratory TCA cycle flux contrasts with the generally held view of a catabolite-repressed TCA cycle in glucose-excess batch cultures of *S. cerevisiae* (Gancedo, 1998; Rolland et al., 2002). Under standard batch conditions, the TCA cycle operates as a bifurcated pathway to sustain biomass precursor requirements (Gombert et al., 2001). Although expression of the majority of the TCA cycle genes is subject to glucose repression (DeRisi et al., 1997) at extracellular glucose concentrations that may be as low 0.1 g l\(^{-1}\) (Yin et al., 2003), we show here that the relative in vivo respiratory activity of the TCA cycle may increase even at high glucose concentrations, provided the growth rate or the glucose uptake rate are impaired by other environmental parameters.

While the major regulation pathways of glucose repression are known (Rolland et al., 2002), the molecular mechanisms that initiate repression are still elusive and several metabolism-derived triggers have been discussed (Carlson, 1999; Gancedo, 1998; Rolland et al., 2002). Our glucose sensor mutant results exclude that glucose repression of the TCA cycle is exclusively mediated by sensing of extracellular glucose concentrations, which is known to repress several *HXT* genes (Rolland et al., 2002). The relative TCA cycle flux increase in four different mutants impaired in glucose sensing strongly suggests that the metabolic trigger for TCA cycle repression must be an intracellular, metabolism-derived signal. This view is consistent with increasing oxygen consumption rates upon genetic reduction of growth rate and glucose uptake rate (Ye et al., 1999). In addition, there is the concentration-dependent repression because mutants completely devoid of glucose sensing (e.g. *grr1* and MMB4) exhibited higher TCA cycle fluxes than would be expected from their growth rates.

Generally, one would expect the repression signal to be related to the glucose uptake rate rather than to the growth rate, but the two were coupled in all environmental modulations. In the *hxx2* mutant, however, the two parameters were decoupled and the relative TCA-cycle flux correlated better with uptake than with growth rate, thus providing some evidence for a flux-related signal of glucose repression of the TCA cycle. The imperfect uptake–TCA cycle correlation in the *hxx2* mutant, with a weaker repression of the TCA cycle than expected from pH experiments with similar growth rates, could be related to the regulatory role of Hxk2p in glucose repression, which would also influence the flux (Carlson, 1999; Gancedo, 1997).
1998; Rolland et al., 2002). Apparently, glucose repression of the TCA cycle exhibits a different pattern and probably also uses different signals than the paradigm glucose repression gene SUC2 (Meijer et al., 1998; Rolland et al., 2002). The general dependence of relative TCA cycle fluxes on the exact environmental conditions may also explain minor differences in TCA cycle activity obtained from previous 13C-labelling experiments (Christensen et al., 2002; Fiaux et al., 2003; Gombert et al., 2001; Maaheimo et al., 2001).

The methodology described for metabolic flux profiling based on GC-MS data of proteinogenic amino acids in yeast is robust, rapid, and also applicable to mini-cultivation systems. Most of the reported flux ratios have varied only within a rather narrow range. Importantly, the fluxes through the PP pathway, the malic enzyme and the gluconeogenic reaction catalysed by PEP carboxykinase are low and change little when considering the severe physiological impacts of the environmental conditions used. The sole exceptions were the respiratory TCA cycle flux and the mitochondrial exchange flux between oxaloacetate and fumarate. This suggests a general robustness of intracellular metabolism to the environmental conditions on a given substrate. While the rate at which glucose enters the cell may vary over a wide range, the relative distribution of carbon fluxes within the cell remains rather stable.

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REFERENCES


