By-product formation during exposure of respiring *Saccharomyces cerevisiae* cultures to excess glucose is not caused by a limited capacity of pyruvate carboxylase

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Abstract

Upon exposure to excess glucose, respiring cultures of *Saccharomyces cerevisiae* produce substantial amounts of ethanol and acetate. A possible role of a limited anaplerotic capacity in this process was investigated by overexpressing pyruvate carboxylase and by replacing it with a heterologous enzyme (*Escherichia coli* phosphoenolpyruvate carboxylase). Compared to the wild-type, neither the pyruvate carboxylase (Pyc)-overexpressing nor the transgenic strain exhibited reduced by-product formation after glucose pulses to aerobic glucose-limited chemostat cultures. An increased intracellular malate concentration was observed in the two engineered strains. It is concluded that by-product formation in *S. cerevisiae* is not caused by a limited anaplerotic capacity. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Production of low-molecular mass metabolites (ethanol, acetaldehyde, acetate) is an important constraint in biomass- or protein-directed applications of the yeast *Saccharomyces cerevisiae* (e.g. production of bakers yeast and heterologous proteins). These by-products are produced in aerobic cultures whenever the specific growth rate exceeds a critical value [1]. In industry, this problem is addressed by growing *S. cerevisiae* at sub-maximum specific growth rates in aerobic, sugar-limited fed-batch cultures. However, even in slowly growing, respiring cultures, by-product formation occurs instantaneously when cells are exposed to excess glucose. This situation may occur as a result of imperfect
mixing in large-scale fed-batch reactors fed with highly concentrated sugar solutions.

Pyruvate metabolism plays a key role in by-product formation, being situated at the interface between glycolysis and the TCA cycle [2]. In *S. cerevisiae*, pyruvate may be metabolized through three major reactions. (i) Oxidative decarboxylation to produce acetyl-CoA, catalyzed by the mitochondrial pyruvate-dehydrogenase complex. (ii) Decarboxylation to acetaldehyde, catalyzed by cytosolic pyruvate decarboxylase. (iii) Carboxylation of pyruvate to oxaloacetate, catalyzed by cytosolic pyruvate carboxylases (Pycs), an anaplerotic reaction replenishing the TCA cycle intermediates upon withdrawal of metabolites for biosynthetic reactions.

Pyruvate carboxylase (Pyc) (EC 6.4.1.1) is located in the cytosol [3] and catalyzes the magnesium- and ATP-dependent carboxylation of pyruvate to oxaloacetate. Its activity is highly regulated by different effectors, including acetyl-CoA, palmitoyl-CoA and aspartate [4]. *S. cerevisiae* contains two structural genes (*PYC1* and *PYC2*) encoding separate Pyc isoenzymes [5].

In theory, a low capacity of conversion of pyruvate into C4 compounds could prevent cells from rapidly increasing the rate of biomass formation and respiration upon transfer from glucose limitation to glucose excess. Consequently, such an anaplerotic limitation might explain the occurrence of overflow metabolism at the level of pyruvate. Indeed, it has been demonstrated that overproduction of the anaplerotic enzyme PEP carboxylase (Ppc) in *Escherichia coli* results in a strong reduction of the production of acetate by aerobic, glucose-grown cultures [6]. It was recently shown that *E. coli* Ppc can complement the growth defect of *S. cerevisiae pyc1 pyc2* double deletion mutants. In glucose-grown batch cultures, the respiration rate of the resulting strain, which exhibited a high Ppc activity in cell extracts, was ca. 2-fold higher than that of wild-type cells, suggesting that a high anaplerotic capacity stimulated TCA cycle activity under these conditions [7].

The aim of the present work was to investigate whether by-product formation after exposure of respiring *S. cerevisiae* cultures to excess glucose is a consequence of a limited capacity of the anaplerotic Pyc reaction. To address this question, by-product formation was studied in chemostat cultures of wild-type *S. cerevisiae* and in isogenic engineered strains overexpressing native *PYC2*-encoded Pyc or expressing *E. coli* Ppc. Physiological effects of the increased levels of anaplerotic enzymes in the engineered strains were evaluated by measuring intracellular metabolite concentrations after glucose pulses to aerobic, glucose-limited chemostat cultures.

2. Materials and methods

2.1. Yeast strains and plasmids

Strains CEN.PK113-7D (MATα MAL2-8ΔC SUC2) and CEN.PK113-5D (MATα ura3-52 MAL2-8ΔC SUC2) were obtained from the EUROSCARF strain collection (Frankfurt, Germany). Strain CEN.JB26-13B (MATα ura3-52 Δpyc1::kanMX Δpyc2::ILV2SMR) was constructed by mating strains CEN.JB10-4B (MATα Δpyc1::kanMX Δpyc2::ILV2SMR) [8] and CEN.PK113-5D with subsequent tetrad dissection and selection of a haploid uracil auxotrophic segregant carrying both deletions. CEN.JB27 was obtained by transformation of strain CEN.JB26-13B with plasmid pAN10-ppc. Strain CEN.JB28 was obtained by transformation of strain CEN.PK113-5D with plasmid pADH1-PYC2.

Plasmid pAN10-ppc is described by Flores and Gancedo [7]. For construction of plasmid pADH-PYC2, a fragment containing the coding region of the *PYC2* gene was amplified by PCR, using genomic DNA of strain CEN.PK113-7D as template. HindIII sites were introduced to allow for cloning into pAN10, a yeast-E. coli shuttle plasmid carrying a yeast 2-μm fragment and the *URA3* marker [9]. The 3.6-kbp fragment obtained was digested with HindIII and cloned into the HindIII site of pAN10.

To select for G-418 resistance after yeast transformations, YPD plates were supplemented with 200 mg l⁻¹ geneticin (G-418 sulfate from Gibco BRL, Germany). Selection for sulfometuron methyl resistance was performed by supplementation of 30 mg l⁻¹ sulfometuron methyl (kindly provided by Dupont, USA) to synthetic minimal medium containing 0.67% YNB (w/o amino acids, Difco) and 2% glucose as carbon source.
2.2. Chemostat cultivation

Carbon-limited chemostat cultivation at 30°C was performed in 5-l bioreactors (New MBR, Switzerland). A culture volume of 2.5 l was maintained via a weight controller. The dilution rate was maintained at 0.1 h⁻¹ and a stirrer speed of 500 rpm and airflow of 1.5 l min⁻¹ were maintained (Bronkhorst Mass flow controller, Ruurlo, The Netherlands) to keep the dissolved oxygen above 40% of air saturation. The culture pH was automatically controlled at 5.0 by addition of KOH (200 g l⁻¹). The mineral medium was prepared as described by Verduyn et al. [10], but with a modified glucose concentration (12.5 g l⁻¹).

2.3. Glucose pulse experiments

At steady-state, glucose pulses were made by rapid injection with a syringe at time \( t = 0 \) to give an initial concentration of 5, 20 or 50 mmol l⁻¹ glucose. During sampling, the change in reactor volume was compensated by controlling the steady-state via gravimetrically controlled reduction of feed- and outflow rate to keep the dilution rate at a constant level.

2.4. Sampling

Samples for determination of extracellular metabolite concentrations were rapidly filtered using nitrocellulose filters of 0.45-μm pore size (Millipore, USA) and supernatant was immediately frozen at −80°C until measurements. For determination of intracellular metabolites, 15-ml samples were quenched directly with 60 ml 60% methanol at −40°C under vigorous mixing. The suspension was then centrifuged at 3000 × g for 10 min at −10°C and the cell pellet frozen at −80°C until extraction.

2.5. Extraction of intracellular metabolites

Extraction of intracellular metabolites was performed by addition of 2.5 ml 0.05 mol l⁻¹ Tris in 3% perchloric acid at 4°C to the frozen cells followed by three freeze-thaw cycles in liquid nitrogen and ice, adapted from the method described by Gonzalez et al. [11]. The pH of the extract was adjusted to pH 6 by adding ice-cold 1 mol l⁻¹ KOH with thorough mixing. After incubation for 15 min on ice, the precipitated KClO₄ was removed by centrifugation (10 min, 5000×g at 4°C). A factor of 2.38 ml of intracellular volume per g dry weight was used to calculate the intracellular concentrations of metabolites [12].

2.6. Determination of culture dry weight

Dry weights of culture samples (20 ml) were determined using nitrocellulose filters (pore size 0.45 μm, Millipore, USA). After removal of the medium by filtration, the filters were washed with demineralized water and dried in a microwave oven for 30 min at 800 W.

2.7. Metabolite analysis

Metabolite measurements were performed enzymatically by reactions coupled to detection of NADH or NADPH and measured at 340 nm. Acetate, ethanol, glucose and malate were determined with test kits supplied by Boehringer Mannheim. Pyruvate was measured with a pyruvate test from Sigma. Oxaloacetate was determined as described by Rej [13].

2.8. Determination of enzyme activities

Crude extracts were prepared with glass beads [14] and the protein concentration was determined by the microbiuret method [15]. Pyc activity was determined at 30°C as described by Young et al. [16]. Activity of Ppc was measured at 30°C and pH 8.0 as described by Flores and Gancedo [7]. All enzyme activities are expressed as μmol of substrate converted per min and mg protein.

3. Results

3.1. Growth and enzyme levels in steady-state chemostat cultures

The wild-type \textit{S. cerevisiae} strain CEN.PK113-7D and the engineered strains CEN.JB27 (\textit{pyc1Δ pyc2Δ}, expressing \textit{E. coli ppc}) and CEN.JB28 (\textit{PYC1 PYC2}, carrying a multi-copy plasmid with \textit{PYC2} under con-
control of the ADH1 promoter) were grown in aerobic, glucose-limited chemostat cultures at a dilution rate of 0.10 h\(^{-1}\). At this dilution rate, growth of all three strains was completely respiratory, as evident from a biomass yield of 0.49 ± 0.01 g dry biomass g\(^{-1}\) glucose and the absence of ethanol, acetate and other fermentation products in culture supernatants. Glucose carbon in the reservoir media was quantitatively recovered (100 ± 3%) as biomass and carbon dioxide.

Cell extracts prepared from the wild-type strain exhibited a Pyc activity of 0.025 U mg\(^{-1}\) protein. A 4-fold higher activity (0.10 U mg\(^{-1}\) protein) was observed in the multi-copy PYC2 strain CEN.JB28. No Pyc activity was detectable in cell extracts of strain CEN.JB27, in which the PYC1 and PYC2 genes were absent. Cell extracts of the latter strain exhibited a high activity (0.39 U mg\(^{-1}\) protein) of Ppc, an enzyme activity that was not detectable in cell extracts of the other two strains.

To investigate whether overexpression of anaplerotic enzymes affected the critical dilution rate at which aerobic by-product formation set in, chemostat cultures of the three strains were run at various dilution rates. The observed critical dilution rate of the wild-type strain CEN.PK113-7D (0.28 h\(^{-1}\) < \(D_{\text{crit}}\) < 0.30 h\(^{-1}\)) was consistent with earlier work [17]. Overproduction of Pyc in strain CEN.JB28 did not result in an increased critical dilution rate as compared to the wild-type (0.25 < \(D_{\text{crit}}\) < 0.30 h\(^{-1}\)). A significantly lower critical dilution rate (0.23 h\(^{-1}\) < \(D_{\text{crit}}\) < 0.25 h\(^{-1}\)) was observed in the Ppc-expressing strain CEN.JB27. This was not due to a reduced Ppc production at higher dilution rates: at \(D = 0.23\) h\(^{-1}\), Ppc activity in cell extracts was ca. 3-fold higher than at \(D = 0.10\) h\(^{-1}\).

3.2. External metabolite accumulation under glucose pulse

The influence of overexpression of PYC2 or ppc on the accumulation of ethanol and acetate was investigated after glucose pulses of 5, 10 and 50 mmol l\(^{-1}\), respectively, to steady-state glucose-limited chemostat cultures. In agreement with previous experiments [18], exposure of wild-type cultures to excess glucose led to an immediate accumulation of...
external ethanol and acetate (Fig. 1). This overflow of metabolites at the level of pyruvate cannot be due to oxygen limitation, as the oxygen concentration remained above 40% saturation during the entire experiment.

In general, no reduction of the metabolite flux towards ethanol and acetate was observed for PYC2- or ppc-overexpressing strains as compared to the wild-type, irrespective of the glucose pulse concentration. The cumulative concentrations of ethanol and acetate reached around 9 mmol l\(^{-1}\) and 60 mmol l\(^{-1}\) for both transformant strains as for the wild-type control after pulses of 5 mmol l\(^{-1}\) and 50 mmol l\(^{-1}\) glucose, respectively, to the chemostat cultures (Fig. 1). A pulse of 20 mmol l\(^{-1}\) glucose led to a cumulative concentration of about 23 mmol l\(^{-1}\) ethanol and acetate for the wild-type and the ppc-overexpressing strain, whereas in the PYC2-overexpressing strain, this amounted to about 35 mmol l\(^{-1}\). The reason for this different behavior of the PYC2-overexpressing strain is not known at present, but one can see that the flux distribution was changed at the acetaldehyde branch-point as indicated by the significantly higher acetate concentration in this strain upon glucose pulses.

3.3. Internal metabolite accumulation under glucose pulse

As most metabolites at the pyruvate branch-point are not or only partially secreted, the intracellular metabolites derived from the Pyc and Ppc activities were also quantified. Thus, we investigated whether the overproduction of those enzymes led to changes in the concentrations of pyruvate, oxaloacetate, malate, citrate and aspartate. In this experiment, only 5-mmol l\(^{-1}\) glucose pulses were applied. The PYC2-overexpressing strain, and more clearly the ppc-overexpressing strain, showed lower pyruvate concentrations than the wild-type (Fig. 2A). However, no increase in the concentration of oxaloacetate over the wild-type level could be observed in both transformant strains (Fig. 2B).

Intracellular malate and aspartate concentrations were determined to investigate the cytosolic pathway of pyruvate metabolism further downstream. No increased concentrations of aspartate could be observed for the PYC2- nor the ppc-overexpressing strain (Fig. 2C). However, in both strains overproducing Pyc or Ppc, higher malate concentrations were measured than in the wild-type strain (Fig. 2D). Whereas in the wild-type, malate concentrations never exceeded 0.5 mmol l\(^{-1}\), in both transformant strains, concentrations were at least 2-fold higher,
reaching up to about 1.5 mmol l$^{-1}$ for the ppc-overexpressing strain and about 1.0 mmol l$^{-1}$ for the PYC2-overexpressing strain.

The flux into the mitochondria seemed not to be affected, as no increase in the citrate concentration was observed (data not shown).

4. Discussion

Biomass-directed applications of *S. cerevisiae* are hindered by the strong tendency to produce by-products ethanol and acetate in response to an excess sugar, which can be interpreted as a short-term overflow effect. In addition, long-term exposure to glucose leads to catabolite repression of respiratory enzymes, a phenomenon described as ‘glucose’ or ‘Crabtree effect’ [19]. Different attempts have been made in the past to reduce the formation of by-products via metabolic engineering of the pyruvate metabolism in *S. cerevisiae*. This ranged from total [20] or partial inactivation [21] of pyruvate decarboxylase to overexpression of the *ACS1*- and *ACS2*-encoded acetyl-CoA synthetases [19]. While various effects on metabolite accumulation could be observed, none of these attempts led to a solution of the problem.

The approach focused on overexpression of the anaplerotic enzymes Pyc and heterologous Ppc at high levels in aerobic, glucose-limited chemostat cultures. Despite its high Ppc activity in cell extracts, CEN.JB27 exhibited a reduced critical dilution rate. This suggests that, in the yeast context, Ppc cannot compete efficiently for phosphoenolpyruvate with the dissimilatory enzyme pyruvate kinase. This phenomenon may well be related to the complex regulation of Ppc activity by several effectors, including fructose-1,6-bisphosphate and aspartate [4].

During transient exposure to glucose excess, no effect of an increased anaplerotic capacity on the flux distribution at the pyruvate branch-point was observed. Neither a 4-fold overexpression of the Pyc-encoding *PYC2* gene nor a high expression of the *E. coli ppc* gene, encoding Ppc, resulted in a decreased external production of ethanol and acetate after glucose pulses relative to the wild-type reference strain. A simple calculation shows that this cannot be expected, however. With a culture dry weight of approximately 6 g l$^{-1}$ used in this study and the observed difference between wild-type and the Pyc-, respectively, Ppc-overexpressing mutants of the intracellular malate pool of about 0.5 mmol l$^{-1}$ (Fig. 2), a maximal difference of the malate concentration in the overall culture of 0.0075 mM could be expected. Clearly, this change will by itself not significantly affect the extracellular concentrations of either ethanol or acetate which were in the mM range (Fig. 1). In addition to this, no apparent increase in flux into the TCA cycle could be observed as the citrate concentration in transformant strains was not increased and also the oxygen consumption rate upon glucose pulse was not higher than in the reference strain (data not shown). However, the flux towards malate seemed to be higher as evident from significantly increased intracellular malate concentrations for both engineered strains upon a glucose pulse (Fig. 2D). This could indicate that anaplerotic metabolism does not control TCA cycle activity under these conditions. Alternatively, malate transport into the mitochondria may be limiting [22]. However, knowledge of malate transport in mitochondria of *S. cerevisiae* is limited. A malate-aspartate shuttle has been proposed to be functional in *S. cerevisiae*, but no detailed information is available on this.

Although subtle changes in by-product formation were observed in the two engineered strains, no reduction of by-product formation was achieved by increasing the levels of anaplerotic enzymes at the pyruvate branch-point. Together with earlier metabolic engineering attempts aimed at other key enzymes at this metabolic node [19–21], this study demonstrates that by-product formation in *S. cerevisiae* cannot simply be interpreted in terms of a competition of respiration and fermentation for pyruvate. In addition to pyruvate, these two processes compete for the NADH that is formed in the glycolytic glyceraldehyde-3P dehydrogenase. It is therefore clear that, in order to control by-product formation, also the metabolism of NADH by yeast mitochondria will have to be taken into account.

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References


