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RESEARCH ARTICLE

Anaplerotic reactions active during growth of *Saccharomyces cerevisiae* on glycerol

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One sentence summary: In comparison to glucose or ethanol as carbon sources, the yeast *S. cerevisiae* shows a higher metabolic flexibility with regard to the active anaplerotic reactions during growth on glycerol.

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ABSTRACT

Anaplerotic reactions replenish TCA cycle intermediates during growth. In *Saccharomyces cerevisiae,* pyruvate carboxylase and the glyoxylate cycle have been experimentally identified to be the main anaplerotic routes during growth on glucose (C6) and ethanol (C2), respectively. The current study investigates the importance of the two isoenzymes of pyruvate carboxylase (*PYC1* and *PYC2*) and one of the key enzymes of the glyoxylate cycle (*ICL1*) for growth on glycerol (C3) as a sole carbon source. As the wild-type strains of the CEN.PK family are unable to grow in pure synthetic glycerol medium, a reverse engineered derivative showing a maximum specific growth rate of 0.14 h^{-1} was used as the reference strain. While the deletion of *PYC1* reduced the maximum specific growth rate by about 38%, the deletion of *PYC2* had no significant impact, neither in the reference strain nor in the *pyc1*Δ mutant. The deletion of *ICL1* only marginally reduced growth of the reference strain but further decreased the growth rate of the *pyc1* deletion strain by 20%. Interestingly, the triple deletion (*pyc1*Δ *pyc2*Δ *icl1*Δ) did not show any growth. Therefore, both the pyruvate carboxylase and the glyoxylate cycle are involved in anaplerosis during growth on glycerol.

Keywords: glycerol; anaplerotic reactions; pyruvate carboxylase; glyoxylate cycle; *Saccharomyces cerevisiae*

INTRODUCTION

During the last decades, the yeast *Saccharomyces cerevisiae* has become a well-established model organism for understanding fundamental cellular processes relevant to higher eukaryotes (Galao *et al.* 2007). In addition, it is a favorite microbial cell factory in industrial biotechnology. In fact, this organism has been extensively engineered to produce a wide range of products including bulk chemicals (e.g. succinic acid), biofuels (e.g. isobutanol) as well as nutraceuticals and pharmaceuticals (Nevoigt 2008; Borodina and Nielsen 2014; Chen and Nielsen 2016).

Sugars are the preferred carbon source of *S. cerevisiae*. As a Crabtree-positive yeast, *S. cerevisiae* undergoes alcoholic fermentation when glucose in the environment exceeds a certain threshold concentration even in the presence of oxygen (Crabtree 1929; De Deken 1966). Only after glucose depletion and diauxic shift, the fermentation products, ethanol and glycerol, can also be used for the generation of energy and cellular biomass. In contrast to glucose, the latter two carbon sources are metabolized in a completely respiratory manner by wild-type *S. cerevisiae* (Schüller 2003; Turcotte *et al.* 2010).

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Table 1 *Saccharomyces cerevisiae* strains used in this study.

Strain	Genome modifications	Source or reference
CEN.PK113–1A *UBR2*$_{CBS}$ *GUT1*$_{JL1}$	*ubr2*::*UBR2*$_{CBS\ 6412-13A}$; *gut1*::*GUT1*$_{JL1}$	Ho *et al.* (2017)
CEN.PK113–1A *UBR2*$_{CBS}$ *GUT1*$_{JL1}$ *pyc1*Δ	*ubr2*::*UBR2*$_{CBS\ 6412-13A}$; *gut1*::*GUT1*$_{JL1}$ *pyc1*::*loxP-ble-loxP*	This study
CEN.PK113–1A *UBR2*$_{CBS}$ *GUT1*$_{JL1}$ *pyc2*Δ	*ubr2*::*UBR2*$_{CBS\ 6412-13A}$; *gut1*::*GUT1*$_{JL1}$ *pyc2*::*loxP-ble-loxP*	This study
CEN.PK113–1A *UBR2*$_{CBS}$ *GUT1*$_{JL1}$ *pyc1*Δ *pyc2*Δ	*ubr2*::*UBR2*$_{CBS\ 6412-13A}$ *gut1*::*GUT1*$_{JL1}$ *pyc1*::*loxP pyc2*::*loxP-ble-loxP*	This study
CEN.PK113–1A *UBR2*$_{CBS}$ *GUT1*$_{JL1}$ *icl1*Δ	*ubr2*::*UBR2*$_{CBS\ 6412-13A}$ *gut1*::*GUT1*$_{JL1}$ *icl1*::*loxP*	This study
CEN.PK113–1A *UBR2*$_{CBS}$ *GUT1*$_{JL1}$ *icl1*Δ *pyc1*Δ	*ubr2*::*UBR2*$_{CBS\ 6412-13A}$ *gut1*::*GUT1*$_{JL1}$ *icl1*::*loxP pyc1*::*loxP-ble-loxP*	This study
CEN.PK113–1A *UBR2*$_{CBS}$ *GUT1*$_{JL1}$ *icl1*Δ *pyc2*Δ	*ubr2*::*UBR2*$_{CBS\ 6412-13A}$ *gut1*::*GUT1*$_{JL1}$ *icl1*::*loxP pyc2*::*loxP-ble-loxP*	This study
CEN.PK113–1A *UBR2*$_{CBS}$ *GUT1*$_{JL1}$ *icl1*Δ *pyc1*Δ *pyc2*Δ	*ubr2*::*UBR2*$_{CBS\ 6412-13A}$; *gut1*::*GUT1*$_{JL1}$ *icl1*::*loxP pyc1*::*loxP-ble-loxP* *pyc2*::*loxP-nat-loxP*	This study

Anaplerotic reactions are pivotal in central carbon metabolism since they are responsible for the replenishment of TCA cycle intermediates during growth. In industrial biotechnology, anaplerotic reactions are of utmost importance when re-routing fluxes of the central carbon metabolism towards valuable fermentation products because the operating route strongly influences ATP and co-factor balance of the cell (Zelle *et al.* 2010; Zelle *et al.* 2011). The major anaplerotic routes in *S. cerevisiae* have been characterized when cells were grown on glucose or ethanol. On glucose, pyruvate carboxylase, which is encoded by the two isogenes *PYC1* and *PYC2* in this species, is the main anaplerotic enzyme (Morris, Lim and Wallace 1987; Stucka *et al.* 1991; Walker *et al.* 1991). The enzyme catalyzes the ATP-dependent carboxylation of pyruvate to oxaloacetate (Gailiusis, Rinne and Benedict 1964) and a *pyc1*Δ *pyc2*Δ double deletion mutant is not able to grow in medium containing glucose as the sole carbon source unless supplemented with aspartate (Stucka *et al.* 1991; Brewster *et al.* 1994; Blazquez, Gamo and Gancedo 1995). The deamination of aspartate yields oxaloacetate, thus bypassing the need for an anaplerotic conversion of C3 to C4 compounds (De Jong-Gubbels *et al.* 1998). During growth on ethanol (and other C2 carbon sources), C4 carbon units for the replenishment of the TCA cycle (at the level of succinate) are synthesized from acetyl-CoA as the central intermediate via the activity of the glyoxylate cycle. This has been confirmed by Schöler and Schüller (1993) who showed that a mutant strain deleted for isocitrate lyase (encoded by *ICL1*), which is one of the key enzymes for the glyoxylate cycle, cannot grow on ethanol. Knowledge about the anaplerotic reactions occurring when *S. cerevisiae* grows on glycerol is fairly fragmentary. Although glycerol has been considered a non-fermentable carbon source in *S. cerevisiae* similar to ethanol (Schüller 2003; Turcotte *et al.* 2010), the different numbers of carbon atoms (C3 vs. C2) of these two compounds might require substantially different metabolic fluxes (including anaplerotic reactions) during their metabolism as discussed by Xiberras, Klein and Nevoigt (2019). Therefore, it is questionable whether the glyoxylate cycle is indispensable during growth on glycerol.

Notably, glycerol is an attractive substrate for biotechnological processes because it is an inevitable by-product of the biodiesel industry, which generates ∼10% (w/v) glycerol (Yazdani and Gonzalez 2007). Apart from being abundant, glycerol also has a higher degree of reduction (4.7) compared to sugars such as glucose and xylose (4.0). This feature can generally lead to higher maximum theoretical product yields of reduced target molecules (Yazdani and Gonzalez 2007; Mattam *et al.* 2013; Zhang *et al.* 2013). The fact that it does not exert the above-mentioned Crabtree effect caused by glucose and other sugars can be another advantage of the 'non-fermentable' carbon source glycerol for the production of biomass and biomass-related products (Fowler and Dunlop 1989; Delvigne *et al.* 2006; Ochoa-Estopier *et al.* 2011).

The goal of the current study was to investigate the main anaplerotic routes active in *S. cerevisiae* growing in synthetic glycerol medium and particularly clarify the role of the glyoxylate cycle. A prerequisite for such a study is a strain able to grow in pure synthetic glycerol medium since growth-supporting supplements, such as amino acids and nucleic bases can interfere with the anaplerotic reactions. However, it is well-known that wild-type strains of the CEN.PK family, which are popular for physiological studies in *S. cerevisiae*, are not able to grow in synthetic glycerol medium (Swinnen *et al.* 2013). Therefore, a reverse engineered derivative of CEN.PK113–1A constructed by Ho *et al.* (2017) (CEN.PK113–1A *UBR2*$_{CBS}$ *GUT1*$_{JL1}$), which grows with a maximum rate (μ_{max}) of ∼0.14 h^{-1} in synthetic glycerol medium, was used as a reference strain in the current study. This strain carries allele replacements for both *UBR2* (encoding a cytoplasmic ubiquitin-protein ligase (E3)) and *GUT1* (encoding the glycerol kinase involved in the glycerol catabolic pathway). The implemented *UBR2* allele originated from the natural glycerol-consuming *S. cerevisiae* isolate CBS 6412–13A (Swinnen *et al.* 2016) while the used *GUT1* allele was obtained from the CEN.PK113–7D derivative generated for improved glycerol utilization by adaptive laboratory evolution (Ho *et al.* 2017).

MATERIALS AND METHODS

Strains, plasmids, medium composition and general cultivation conditions

The strains used in this study are listed in Table 1. Yeast cells were routinely grown on either solid YPD (*icl1*Δ, *icl1*Δ *pyc1*Δ and *icl1*Δ *pyc2*Δ mutants), YPD with 0.5% aspartate (*icl1*Δ *pyc1*Δ *pyc2*Δ mutant) or YPE medium (*pyc1*Δ, *pyc2*Δ and *pyc1*Δ *pyc2*Δ mutants) containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose or 30 mL/L ethanol and 15 g/L agar. Agar plates were cultivated in a static incubator at 30°C. Media were supplemented with phleomycin (20 mg/L) or nourseothricin (100 mg/L) for selection purposes when needed.

For qualitative and quantitative growth assessment, cells were grown in synthetic medium containing either 20 g/L glucose, 30 mL/L ethanol or 60 mL/L glycerol and ammonium sulfate as nitrogen source, respectively. The synthetic medium was prepared according to Verduyn *et al.* (1992), including 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 15 mg/L EDTA, 4.5 mg/L $ZnSO_4{\cdot}7H_2O$, 0.84 mg/L $MnCl_2{\cdot}2H_2O$, 0.3 mg/L $CoCl_2{\cdot}6H_2O$, 0.3 mg/L $CuSO_4{\cdot}5H_2O$, 0.4 mg/L $NaMoO_4{\cdot}2H_2O$, 4.5 mg/L $CaCl_2{\cdot}2H_2O$, 3 mg/L $FeSO_4{\cdot}7H_2O$, 1 mg/L H_3BO_3 and 0.1 mg/L KI. After heat sterilization of the medium, filter sterilized vitamins were added. Final vitamin concentrations were 0.05

Table 2. Primers used in this study.

Purpose	Primer number	Sequence (5′–3′)
Deletion of *PYC1*	775	AGATAACAAAAGGAAAATCTCAGCCTCTCCCCTTCCTCTTAGACACCAGCTGAAGCTTCGTACGC
	776	AGATTCGGGTATATTATACATTATAAATGAGAACTAACCGGTCGCATAGGCCACTAGTGGATCTG
Deletion of *PYC2*	828	GGCACAGCCAGCTCTTCTGTGATTGGCAGAGAGGGGTCCTTCCAGCTGAAGCTTCGTACGC
	829	CATTAAGGAAGAAACACAGTTAGCACGTATTTCATCGAAAGCATAGGCCACTAGTGGATCTG
Deletion of *ICL1*	1189	AACAATTGAGAGAAAACTCTTAGCATAACATAACAAAAAGTCAACGAAAACCAGCTGAAGCTTCGTACGC
	1190	ATATACTTGTCAGGAAATGCCGGCAGTTCTAATGGTTAATCCTTGTCCGCATAGGCCACTAGTGGATCTG

mg/L D-(+)-biotin, 1 mg/L D-pantothenic acid hemicalcium salt, 1 mg/L nicotinic acid, 25 mg/L myo-inositol, 1 mg/L thiamine chloride hydrochloride, 1 mg/L pyridoxine hydrochloride and 0.2 mg/L 4-aminobenzoic acid. In case aspartate was used as a supplement, 0.5% was added to the medium prior autoclaving. The pH of the synthetic glucose and synthetic ethanol medium was adjusted to 6.5 with 4 M KOH, while that of synthetic glycerol medium was adjusted to 4.0 with 2 M H_3PO_4.

Escherichia coli DH5α was used for plasmid construction and isolation, and cells were routinely grown in lysogeny broth (LB) containing 10 g/L NaCl, 5 g/L yeast extract and 10 g/L peptone, and adjusted to a pH of 7.5 with 2 M NaOH (Bertani 1951). For selection and maintenance of plasmid containing cells, 100 mg/L ampicillin was added.

General molecular biology techniques

Preparative PCRs for cloning as well as for sequence determination of deletion cassettes integrated into the genome were performed using Phusion® High-Fidelity DNA Polymerase (New England BioLabs, Frankfurt am Main, Germany). PCR conditions were adapted to the guidelines of the respective manufacturer. PCR products were purified by using the GeneJET PCR Purification Kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA).

Genetic modifications of *S. cerevisiae*

Deletions of either *PYC1*, *PYC2* or *ICL1* were obtained using a disruption cassette containing either a phleomycin (*ble*^{r}) or nourseothricin (clonNAT, *nat*^{r}) resistance marker. The deletion cassettes were amplified from plasmid pUG66 and pUG74, respectively, (Gueldener *et al.* 2002) using primers listed in Table 2. The primers contained at their 5′ terminal end, a 60-bp sequence, complementary to the region immediately upstream or downstream of the start or stop codon of the gene to be deleted. Transformation of *S. cerevisiae* with the deletion cassettes was performed according to the lithium acetate method described by Gietz *et al.* (1995). Double and/or triple deletions were achieved either by consecutive transformations of the respective deletion cassettes or else by first removing the existing marker according to Sauer and Henderson (1988) and then transforming *S. cerevisiae* with the respective deletion cassette.

Qualitative and quantitative analysis of growth on glycerol

The qualitative and quantitative analyses of *S. cerevisiae* growth were conducted following the procedure described by Swinnen *et al.* (2013) with slight modifications. The main difference between the qualitative and the quantitative growth analysis was the use of the carbon source for pre- and intermediate cultures. For qualitative analysis, pre- and intermediate cultures were performed using glucose (for *icl1*Δ, *icl1*Δ *pyc1*Δ and *icl1*Δ *pyc2*Δ mutants), glucose plus 0.5% aspartate (for the *icl1*Δ *pyc1*Δ *pyc2*Δ mutant) or ethanol (for *pyc1*Δ, *pyc2*Δ and *pyc1*Δ *pyc2*Δ mutants). For quantitative analysis, the respective cultivations were all performed using glycerol as the carbon source. In general, cells from a single colony were used to inoculate 3 mL of the synthetic medium in a 10 mL glass tube with the respective carbon source and this pre-culture was incubated at orbital shaking of 200 rpm and 30°C for 24 hours. The pre-culture was then used to inoculate 4 mL of the same medium in a 10 mL glass tube adjusting to an OD_{600} of 0.2. This culture, referred to as intermediate culture, was cultivated at the same conditions for 48 hours. For qualitative analysis, the appropriate volume from the intermediate culture necessary to adjust an OD_{600} of 0.2 was centrifuged at 800 *g* for 5 minutes and the supernatant discarded. The cell pellet was then washed once by re-suspending the cells in synthetic glycerol medium, re-centrifuged and re-suspended in 4 mL of the same medium in a 10 mL glass tube. For quantitative analysis, the appropriate culture volume to adjust an OD_{600} of 0.2 was directly resuspended in 4 mL of the main culture medium in a 10 mL glass tube. The main cultures for qualitative growth analysis were incubated at orbital shaking of 200 rpm and 30°C and growth relative to the reference strain was assessed at regular time intervals. For quantitative analysis, two aliquots (750 μL each) of the main culture were immediately transferred from the 10 mL glass tube to separate wells of a white Krystal TM 24-well clear bottom microplate (Porvair Sciences, Leatherhead, United Kingdom) and cultivated in the Growth Profiler 1152 (Enzyscreen, Haarlem, The Netherlands) at 30°C and orbital shaking at 200 rpm. The growth profiler took a scan of the plate every 40 minutes. These scans were used to calculate the density of the cultures expressed as green values (G-values). Subsequently, the G-values were expressed as OD_{600} values (referred to as OD_{600} equivalents) using the following calibration curve: OD_{600} equivalent $= 6.1761\ 10^{-8} * (\text{G-value})^{3.4784}$.

RESULTS AND DISCUSSION

To identify the anaplerotic reactions active on glycerol, we first focused on those routes that were known to be important on glucose and ethanol, i.e. pyruvate carboxylase and the glyoxylate cycle as mentioned in the introduction. To study the role of PYC (Fig. 1), we deleted *PYC1* and *PYC2* individually and in combination in our chosen reference strain. Growth of all constructed strains was first qualitatively assessed in liquid synthetic glycerol medium as described in Materials and Methods. As *pyc1/2* double deletion mutants are incapable of growth on glucose as the sole carbon source (Stucka *et al.* 1991; Brewster

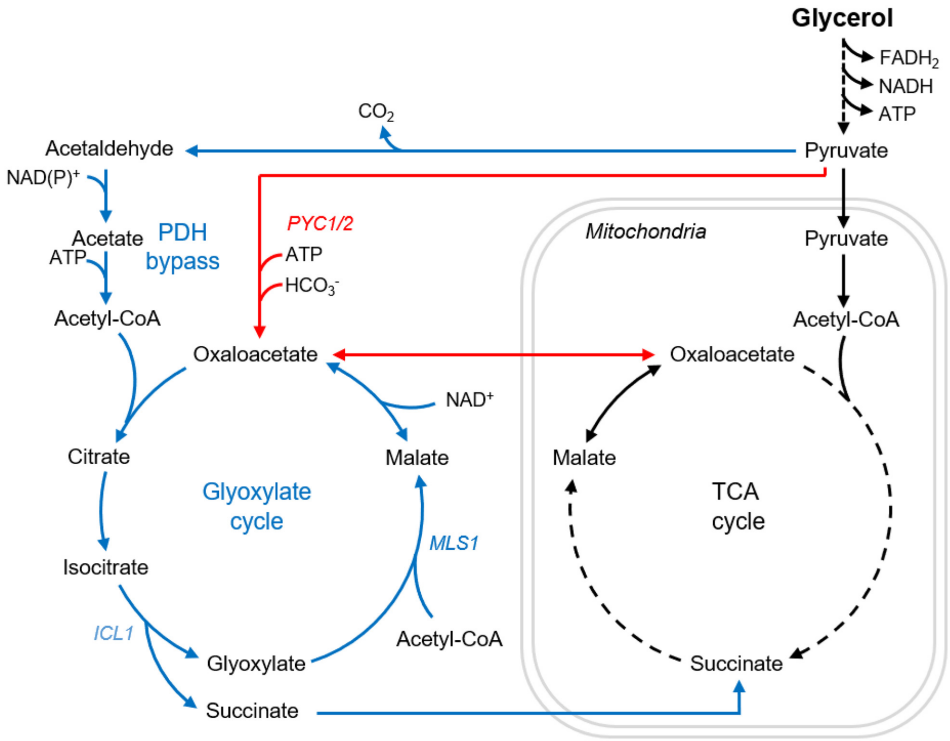

Figure 1. The two major anaplerotic routes in *S. cerevisiae* during growth on pure synthetic glycerol medium, pyruvate carboxylase shown in red and the glyoxylate cycle in combination with the pyruvate dehydrogenase (PDH) bypass shown in blue. Abbreviations: *PYC1/PYC2*, pyruvate carboxylase; *ICL1*, isocitrate lyase; *MLS1*, malate synthase.

Table 3. Growth assessment of CEN.PK113–1A $UBR2_{CBS}$ $GUT1_{JL1}$ derivatives carrying individual and combined deletions in the genes *PYC1*, *PYC2* and *ICL1*. *PYC1* and *PYC2* code for isoforms of PYC, while *ICL1* encodes isocitrate lyase, a key enzyme of the glyoxylate cycle.

Deletion(s)	Growth in synthetic glycerol medium*
—	++
*pyc1*Δ[1]	+
*pyc2*Δ[1]	++
*pyc1*Δ *pyc2*Δ[1]	+
*icl1*Δ[2]	++
*pyc1*Δ *icl1*Δ[2]	+
*pyc2*Δ *icl1*Δ[2]	++
*pyc1*Δ *pyc2*Δ *icl1*Δ[3]	-

*The experiments were carried out in synthetic medium containing 6% (v/v) glycerol as the sole carbon source. As the deletion mutants have specific growth deficits on different carbon sources, different media were used for the pre-cultivations.
[1]Pre-cultivations were conducted in synthetic ethanol medium.
[2]Pre-cultivations were conducted in synthetic glucose medium.
[3]Pre-cultivations were conducted in synthetic glucose medium supplemented with aspartate.
++Growth comparable to parent strain.
+Significantly reduced growth.
-No growth.

et al. 1994; Blazquez, Gamo and Gancedo 1995), pre-cultivations were performed in synthetic medium with ethanol. The reference strain as well as the *pyc1*Δ and *pyc2*Δ mutants were treated in the same way for a reliable comparison. The data (Table 3) show that both the *pyc1*Δ and *pyc2*Δ single and the *pyc1*Δ *pyc2*Δ double deletion strain were all able to grow in synthetic glycerol medium. Still, there was a difference with regard to the two *PYC* isogenes: mutants carrying a deletion of *PYC1* (either individually or in combination with the *PYC2* deletion) were negatively affected in their growth, while the *pyc2* single deletion strain did not show any reduction in growth compared to the reference strain. In order to quantify the strains' maximum growth rates, all mutants were pre-cultivated in synthetic glycerol medium. Main cultures were then adjusted for the same start optical density (600 nm) and the growth rates of all strains were afterwards determined as described in Materials and Methods. The results show that the deletion of *PYC2* (alone and in combination) indeed did not significantly affect growth on glycerol (Fig. 2A). In contrast, the deletion of *PYC1* remarkably reduced μ_{max} in all strains confirming the major importance of this enzyme for anaplerosis of the TCA cycle under these conditions (Fig. 2A). Our results match a previous finding showing that *PYC1* expression is highly upregulated on glycerol (Menéndez and Gancedo 1998). The strong impact of the *PYC1* deletion on growth is very different from the situation on glucose. In fact, neither the individual deletion of *PYC1* nor of *PYC2* resulted in significant phenotypic changes on glucose (Stucka *et al.* 1991). In contrast to glycerol, it seems that the pyruvate carboxylase isoenzyme encoded by *PYC2* is able to effectively compensate a lack of Pyc1 activity when cells grow on glucose.

The fact that the *pyc1*Δ *pyc2*Δ double mutant strain still grows fairly well on glycerol clearly suggests that a metabolic route other than PYC significantly contributes to the replenishment of the TCA cycle intermediates on glycerol. The next hypothetical route tested was the glyoxylate cycle. This cycle, which is essential for growth on C2 compounds, could also serve as an anaplerotic route if carbon from pyruvate was channeled via the pyruvate dehydrogenase (PDH) bypass to form cytosolic acetyl-CoA (Fig. 1). We speculated that the metabolic

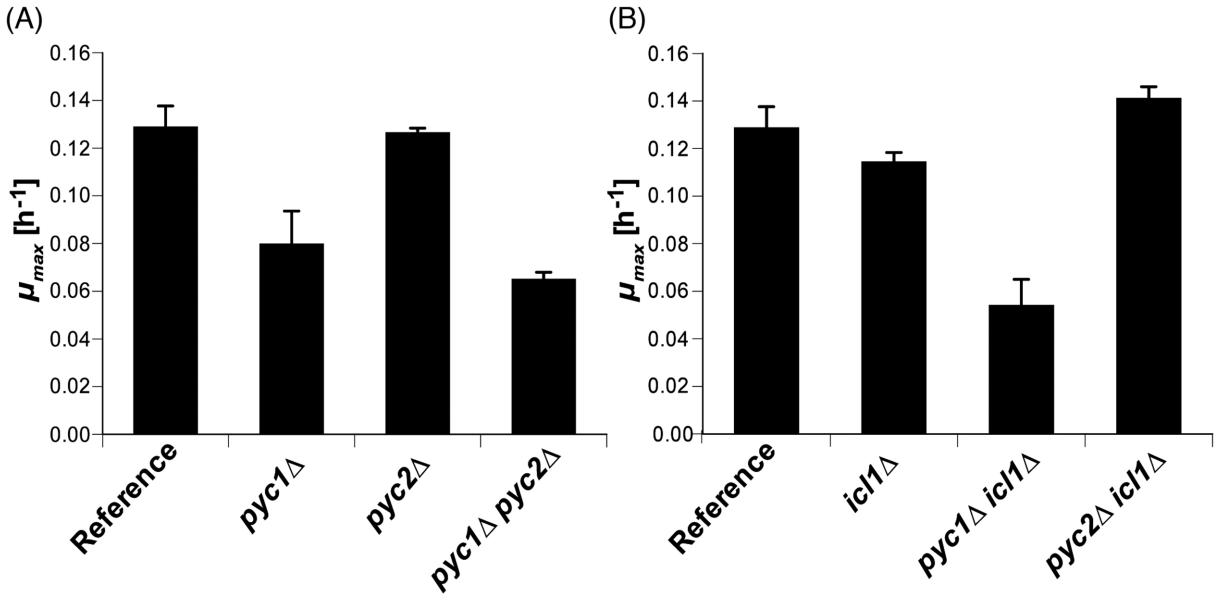

Figure 2. μ_{max} of *S. cerevisiae* strain CEN.PK113–1A $UBR2_{CBS}$ $GUT1_{JL1}$ and its derivatives carrying individual or combined deletions in the isogenes encoding for pyruvate carboxylase (*PYC1* and *PYC2*) **(A)** as well as in the gene *ICL1* (encoding isocitrate lyase, a key enzyme of the glyoxylate cycle) alone or in combination with a deletion of *PYC1* or *PYC2* **(B)**. Pre-cultures, intermediate and main cultures were performed in synthetic medium containing 6% (v/v) glycerol as the sole carbon source (SMG). Mean values and standard deviations were determined from at least three biological replicates.

flux via the pyruvate dehydrogenase bypass might be relatively strong in glycerol. This idea is supported by Kito *et al.* (2016) and Dueñas-Sánchez *et al.* (2012) who reported in their studies a higher expression of genes encoding the enzymes contributing to the pyruvate dehydrogenase bypass such as Ald2, Ald3 and Acs1. The authors analyzed the respective expression levels in cells grown in complex medium with glycerol in comparison to glucose as the carbon source either at the level of mRNA (Dueñas-Sánchez *et al.* 2012) or protein (Kito *et al.* 2016). To verify this hypothesis, we abolished the activity of the glyoxylate cycle by deleting *ICL1* individually as well as in combination with the aforementioned *PYC1/2* deletions. The pre-cultivations to assess the qualitative growth of the mutant strains on glycerol could not be performed in synthetic ethanol medium because an active glyoxylate cycle is indispensable for growth on C2 carbon sources such as ethanol and acetate. Instead, synthetic glucose medium was used for the pre-cultivation of the *icl1*Δ, *pyc1*Δ *icl1*Δ and *pyc2*Δ *icl1*Δ mutants and, for the *pyc1*Δ *pyc2*Δ *icl1*Δ mutant, the pre-cultivation medium was supplemented with aspartate. The data in Table 3 shows that all strains except the *pyc1*Δ *pyc2*Δ *icl1*Δ triple deletion mutant grew in synthetic glycerol medium. Further quantitative analysis of the first three strains on glycerol (with pre-cultivations on glycerol) showed that the deletion of *ICL1* only marginally reduced the growth rate of the reference strain, but it did not significantly reduce growth in the *pyc2*Δ deletion mutant (Fig. 2B). Interestingly, the same *ICL1* deletion in a *pyc1*Δ background significantly reduced the growth rate by additional 20% (Fig. 2B). The first conclusion drawn from these results is that the glyoxylate cycle is not crucial during growth on synthetic glycerol medium. This is based on the fact that the impact on growth of the sole deletion of *ICL1* was only marginal and in clear contrast to growth on ethanol. The second conclusion is that both Pyc2 and the glyoxylate cycle seem to be able to replenish the TCA cycle when Pyc1 is absent (Fig. 2). This assumption was based on the findings that (i) the triple deletion mutant (*pyc1*Δ *pyc2*Δ *icl1*Δ strain) did not grow at all on glycerol, and (ii) the deletion of neither *ICL1* nor *PYC2* in a *pyc1*Δ mutant strain led to growth abolishment. Another conclusion that can be drawn from our result with the triple deletion mutant (*pyc1*Δ *pyc2*Δ *icl1*Δ strain) is that there seems to be no anaplerotic route of major importance other than PYC and the glyoxylate cycle when *S. cerevisiae* is growing on glycerol, and both can partly take over the function of being the main anaplerotic reaction in the absence of the other route. The latter result also implies that phosphoenolpyruvate carboxykinase (*PCK1*), which reversibly converts phosphoenolpyruvate to oxaloacetate, does not seem to be crucial as an anaplerotic route on glycerol. This finding is in line with previous reports where PEPCK is generally considered a decarboxylating enzyme with a function in gluconeogenesis (De Torrontegui, Palacián and Losada 1966).

In the light of our results, it is interesting to note that there is a previous report about a *pyc1*Δ *pyc2*Δ double deletion mutant that was able to grow on glucose after mutagenesis. It turned out that the respective mutant showed a derepression of the glyoxylate cycle which allowed to overcome the necessity of supplementing the medium with C4 compounds (Blazquez, Gamo and Gancedo 1995). This metabolic situation resembles the one we assume for our strain growing on glycerol, a carbon source that does not show catabolite repression of the glyoxylate cycle. Similarly, the *pyc1*Δ *pyc2*Δ double deletion mutant was also able to grow on pyruvate (C3) but it did not grow on fructose (C6) and galactose (C6) as sole carbon source (data not shown).

As reviewed by Klein *et al.* (2017), *S. cerevisiae* naturally catabolizes glycerol via the so-called L-G3P pathway. We previously replaced the endogenous L-G3P pathway by an artificial NAD^+-dependent pathway referred to as 'DHA pathway' (Klein *et al.* 2016). This modification was meant to allow the entrapment of all electrons originating from glycerol in the form of soluble cytosolic NADH (instead of enzyme-bound $FADH_2$). We also tested some of the above-mentioned deletions in a DHA pathway strain background in order to investigate whether the same routes are responsible for anaplerosis as in the strain CEN.PK113–1A $UBR2_{CBS}$ $GUT1_{JL1}$. At least, we have been able to

show so far that individual deletions of the genes *PYC1*, *PYC2* and *ICL1* led to a similar growth reduction compared to the respective control strain (data not shown).

Glycerol is an attractive carbon source in industrial biotechnology due to its relatively high degree of reduction and its liquid nature. Moreover, it is a major by-product during biodiesel production. Our current study demonstrates that *S. cerevisiae* shows a higher metabolic flexibility with regard to the use of anaplerotic routes on glycerol. This is different from glucose and ethanol where only one route is used and the abolishment of the respective route leads to a growth defect. Although Pyc1 seems to be the most important anaplerotic enzyme, alternative routes, i.e. Pyc2 and the glyoxylate cycle (in combination with the pyruvate dehydrogenase bypass) contribute to this function.

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Conflict of interest. The authors declare no competing interest.

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