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Research review paper

Glycerol as a substrate for *Saccharomyces cerevisiae* based bioprocesses – Knowledge gaps regarding the central carbon catabolism of this 'non-fermentable' carbon source

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ABSTRACT

Glycerol is an interesting alternative carbon source in industrial bioprocesses due to its higher degree of reduction per carbon atom compared to sugars. During the last few years, significant progress has been made in improving the well-known industrial platform organism *Saccharomyces cerevisiae* with regard to its glycerol utilization capability, particularly in synthetic medium. This provided a basis for future metabolic engineering focusing on the production of valuable chemicals from glycerol. However, profound knowledge about the central carbon catabolism in synthetic glycerol medium is a prerequisite for such incentives. As a matter of fact, the current assumptions about the actual *in vivo* fluxes active on glycerol as the sole carbon source have mainly been based on *omics* data collected in complex media or were even deduced from studies with other non-fermentable carbon sources, such as ethanol or acetate. A number of uncertainties have been identified which particularly regard the role of the glyoxylate cycle, the subcellular localization of the respective enzymes, the contributions of mitochondrial transporters and the active anaplerotic reactions under these conditions. The review scrutinizes the current knowledge, highlights the necessity to collect novel experimental data using cells growing in synthetic glycerol medium and summarizes the current state of the art with regard to the production of valuable fermentation products from a carbon source that has been considered so far as 'non-fermentable' for the yeast *S. cerevisiae*.

1. Introduction

The yeast *Saccharomyces cerevisiae* has become a popular production organism in industrial biotechnology for several obvious reasons. First of all, it is a simple, fast growing and single-cell eukaryotic model organism, already having a relatively long history in large-scale industrial use. Moreover, *S. cerevisiae* is robust with regard to low pH values and high alcohol concentrations and many industrial applications profit from these characteristics. In contrast to microbial fermentations using bacteria, yeast cultures are not vulnerable to bacteriophage infection. However, the property that particularly boosted the popularity of *S. cerevisiae* in industrial biotechnology in the last decades is its highly efficient homologous recombination, which considerably facilitates the stable genomic incorporation of multiple expression cassettes as often required for extensive metabolic engineering endeavors.

As virtually every natural microorganism, wild-type *S. cerevisiae* also has its weaknesses when considered as a platform organism for industrial biotechnology. Its relatively narrow substrate spectrum is limiting in the view of using renewable feedstocks, which contain a broad spectrum of potential carbon sources. Apart from hexose sugars, wild-type *S. cerevisiae* cannot use many other constituents of polymers present in 'energy crops' or agricultural waste streams, such as pentose sugars or sugar acids. For this reason, huge efforts have been undertaken to extend the substrate spectrum of baker's yeast by metabolic engineering. Particularly *S. cerevisiae* strains carrying pathways for pentose sugar utilization have been developed with great success (Kim et al., 2013; Kwak and Jin, 2017; Lane et al., 2018). Another carbon source that can be obtained from renewable resources, especially from raw materials rich in oils and fats, is glycerol. This valuable substrate has been neglected as a carbon source for *S. cerevisiae*-based bioprocesses so far, which can be attributed to its rather inefficient utilization by most commonly used wild-type strains of this yeast species.

A major advantage of using glycerol as a carbon source in industrial bioprocesses is its higher degree of reduction per carbon compared to sugars (4.7 versus 4.0), which is accompanied by higher maximum theoretical yields of reduced target molecules (Clomburg and Gonzalez,

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2013). Furthermore, the additional reducing power of glycerol can support the (co-)fermentation of compounds more oxidized than glucose, such as acetic acid (de Bont et al., 2013). Currently, the major part of the available glycerol results from biodiesel production, where it is generated as an inevitable by-product. In fact, the remarkable growth of the biodiesel industry about 15 years ago initiated a huge interest in glycerol valorization in order to improve the economics of the biodiesel production process (Yazdani and Gonzalez, 2007). Several biotechnological processes (including commercial ones) based on glycerol as raw material and microorganisms other than *S. cerevisiae* have already been developed (Chen and Liu, 2016).

The fact that *S. cerevisiae* barely grows on glycerol as a carbon source holds for media in which growth-supporting supplements, such as amino acids and nucleic bases, are omitted (Swinnen et al., 2013). Indeed, many relevant industrial and laboratory strains do not grow at all under these conditions. However, when considering the industrial conversion of glycerol into products of relatively low market price, the addition of complex supplements is not economically viable. Therefore, our group has scrutinized the growth of 52 *S. cerevisiae* strains with regard to growth in synthetic glycerol medium without any supplements. Interestingly, a few wild-type isolates were identified that are able to grow up to a maximum specific growth rate of $\sim 0.15\,h^{-1}$ (Swinnen et al., 2013). A haploid meiotic segregant of one strain showing superior growth on glycerol was characterized in more detail (Swinnen et al., 2013). This segregant referred to as CBS 6412-13A has been used as a major baseline strain in our metabolic engineering endeavors for exploiting glycerol's reducing power for the production of valuable chemicals (see section 6). Apart from using natural glycerol-utilizing wild-type isolates, it has been relatively straightforward to apply adaptive laboratory evolution (ALE) for the establishment of glycerol utilization in strains unable to grow in synthetic glycerol medium by nature. Indeed, several virtually non-growing *S. cerevisiae* strains have been evolved for this phenotype so far (Ho et al., 2017; Merico et al., 2011; Ochoa-Estopier et al., 2011; Strucko et al., 2018). The identification of the genetic determinants underlying natural and artificial diversity with respect to the glycerol growth phenotype within the species *S. cerevisiae* has also provided the basis for reverse engineering approaches. In this methodology, the crucial genetic determinants identified in one strain with a superior phenotype are transferred to another strain showing an inferior phenotype as comprehensively reviewed by Oud et al. (2012). Reverse engineering was successfully applied for establishing growth on glycerol in the popular (non-growing) laboratory strain CEN.PK via different strategies (Ho et al., 2017; Strucko et al., 2018; Swinnen et al., 2016).

As reviewed by Klein et al. (2017), *S. cerevisiae* naturally catabolizes glycerol via the so-called L-G3P pathway. This pathway is composed of three proteins: one transporter (a glycerol/H^+ symporter) encoded by *STL1*, a glycerol kinase encoded by *GUT1* and a L-glycerol-3-phosphate dehydrogenase encoded by *GUT2* (Ferreira et al., 2005; Klein et al., 2016a; Ronnow and Kielland-Brandt, 1993; Sprague and Cronan, 1977; Swinnen et al., 2013). Gut2 is located at the outer face of the inner mitochondrial membrane. By the action of this FAD-dependent enzyme, the electrons originating from glycerol oxidation end up in the ubiquinone pool of the respiratory chain and are finally transferred to the inorganic electron acceptor oxygen. The product of this reaction is dihydroxyacetone phosphate (DHAP) that is further channeled into the central carbon metabolism (glycolysis and gluconeogenesis) as depicted in Fig. 1.

For any metabolic engineering endeavor aiming at small molecule production from glycerol, a profound knowledge about the actual metabolic fluxes active in synthetic glycerol medium and their regulation is crucial. As discussed later, glycerol belongs to the carbon sources that have been considered non-fermentable for *S. cerevisiae*. Comprehensive reviews about the central carbon catabolism during the utilization of non-fermentable carbon sources by *S. cerevisiae* have been provided by Schüller (2003) and Turcotte et al. (2010). However, a closer look reveals that the majority of our assumptions about the fluxes results from transcriptome studies, a fact that has to be considered with caution. Moreover, published experimental data are rare when it comes to the utilization of true synthetic glycerol media (i.e. media without amino acid or nucleic base supplements). Even studies that have focused on complex glycerol media are relatively low in number compared to other non-fermentable carbon sources such as ethanol, acetate or oleate. Several fundamental studies have used mixtures of ethanol and glycerol, and a few crucial experimental data have not been collected at all on glycerol as the sole carbon source. The many uncertainties we came across when searching for details regarding the central carbon catabolism of glycerol in *S. cerevisiae* motivated us to write this critical review. We would like to stimulate scientific discussion as well as basic research to clarify the open questions.

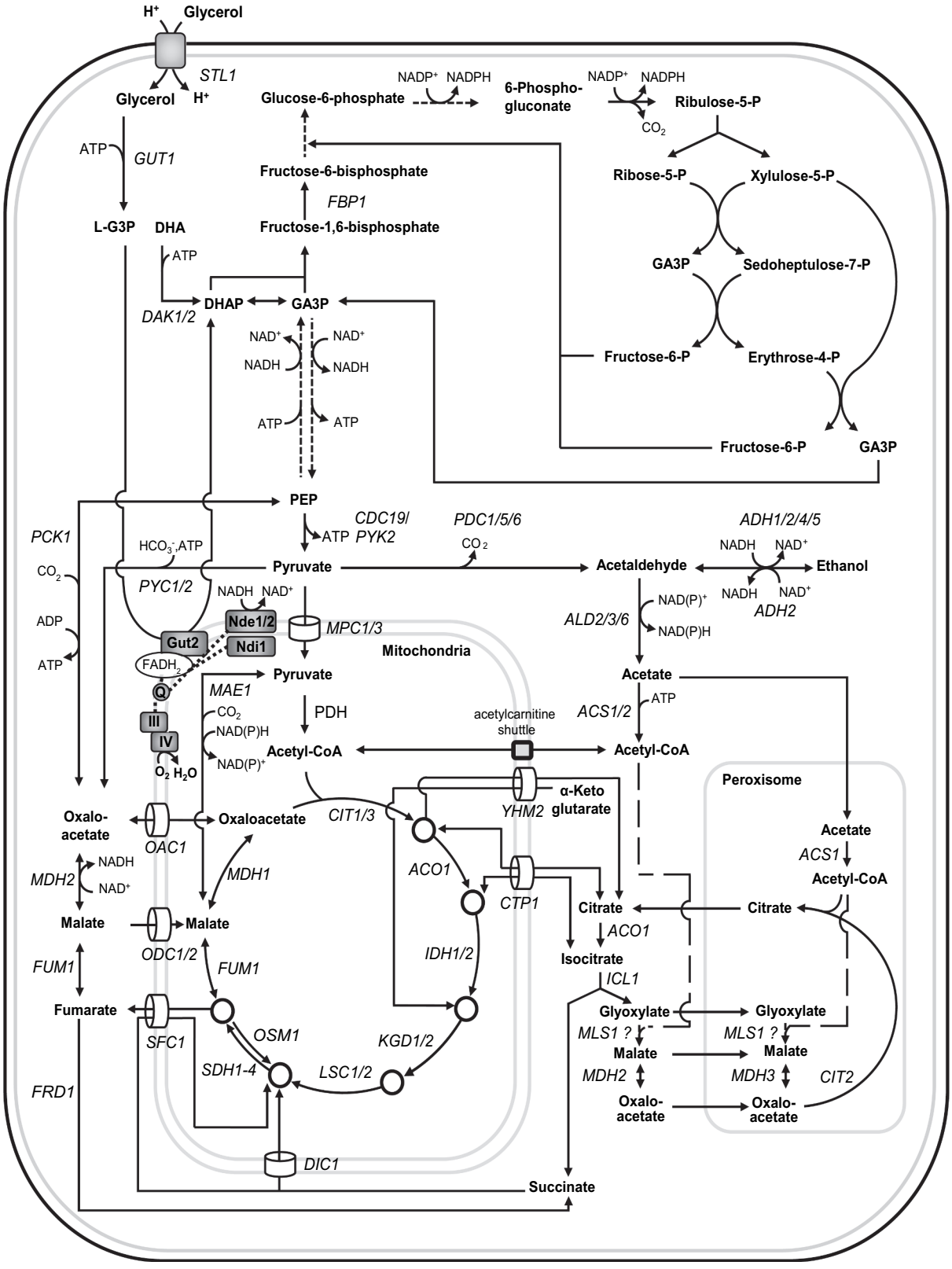
We first critically discuss the general assumptions about the central carbon catabolism on non-fermentable carbon sources (including glycerol) which are mainly based on transcriptome and proteome studies. Afterwards, we particularly scrutinize the role of the glyoxylate cycle during growth on glycerol and what is known about the localization of several enzymes contributing to the activity of this pathway. Moreover, we address intriguing experimental results regarding mitochondrial transporters and anaplerotic reactions active on glycerol. The last section is devoted to the potential biotechnological exploitation of glycerol's reducing power for the production of small molecules including promising results obtained during our own recent research activities.

2. Assumptions regarding the central carbon fluxes on non-fermentable carbon sources including glycerol

In lack of any experimental data with regard to a ^{13}C flux analysis and comprehensive *in vitro* enzyme activity measurements for *S. cerevisiae* cells exponentially growing on glycerol as the sole source of carbon, to date any assumption about the active metabolic fluxes on glycerol can only be considered hypothetical. According to previous reviews regarding the metabolism of *S. cerevisiae* on non-fermentable carbon sources, there has been a consensus that the TCA cycle, oxidative phosphorylation, the glyoxylate cycle and gluconeogenesis represent those central metabolic pathways which seem to be activated during growth on non-fermentable carbon sources but downregulated during growth on excess glucose (Carlson, 1999; Schüller, 2003; Turcotte et al., 2010). It has, however, to be recognized that most of these well-accepted assumptions have been mainly based on experimental data obtained from comparing transcript and protein abundances from cells growing on non-fermentable carbon sources and comparing them to those obtained from cells growing on glucose. We have summarized the most important of these studies in Table 1. First of all, it has to be emphasized that it is not correct to directly translate mRNA or protein abundances into metabolic fluxes since such an approach would ignore post-transcriptional modifications and/or metabolic regulation (e.g. allosteric regulation). Both levels of regulation can have a significant impact on the actual *in vivo* fluxes. Moreover, one has to realize that most of the respective *omics* studies have been conducted on non-fermentable carbon sources other than glycerol. Although all carbon sources tested in these studies have been considered to be catabolized in a fully respiratory manner, glycerol is a C3 carbon source while ethanol and acetate are C2 carbon sources whose metabolism require different metabolic fluxes. In the following, we want to scrutinize the available experimental data in more detail. We also want to alert the reader about the fact that it is still somewhat unclear how much of the global picture obtained from experiments in ethanol/glycerol mixtures or ethanol can actually be transferred to the situation when glycerol is the only carbon source, particularly if synthetic medium is considered.

In most transcriptome studies focusing on non-fermentable carbon sources, the respective transcript levels have been compared to the transcript levels detected in cells growing in excess glucose. Working

with glucose excess as a reference condition inherently leads to difficulties when making interpretations about the metabolic pathways/enzymes active or inactive on the non-fermentable carbon source under investigation. The reasons have been comprehensively summarized by Daran-Lapujade et al. (2004). First, glucose excess leads to glucose repression of genes involved in the utilization of alternate carbon sources, gluconeogenesis, respiration and peroxisomal functions (Carlson, 1999). Therefore, it is impossible to dissect the transcriptional changes that have been caused by i) the relief from glucose repression and ii) the presence of the non-fermentable carbon source. Another



(caption on next page)

Fig. 1. Central metabolic pathways in *S. cerevisiae* during growth on glycerol. The two possible localizations of Mls1 are shown with long dash lines and question mark. Abbreviations: L-G3P: L-glycerol-3-phosphate, DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, GA3P: glyceraldehyde-3-phosphate, PEP: phosphoenolpyruvate, P: phosphate, *STL1*: glycerol/H^+ symporter, *GUT1*: glycerol kinase, Gut2: FAD-dependent glycerol-3-phosphate dehydrogenase, *DAK1/2*: dihydroxyacetone kinase, *FBP1*: fructose-1,6-bisphosphatase, *CDC19/PYK2*: pyruvate kinase, *PCK1*: phosphoenolpyruvate carboxykinase, *PYC1/2*: pyruvate carboxylase, *MDH1/MDH2/MDH3*: malate dehydrogenase, *FUM1*: fumarase, PDH: pyruvate dehydrogenase complex, *MAE1*: malic enzyme, *CIT1/2/3*: citrate synthase, *ACO1*: aconitase, *IDH1/2*: isocitrate dehydrogenase, *KGD1/2:* α-ketoglutarate dehydrogenase, *LSC1/2*: succinyl-CoA ligase, *SDH1/2/3/4*: succinate dehydrogenase, *ICL1*: isocitrate lyase, *MLS1*: malate synthase, *ACS1/2*: acetyl-coA synthetase, *PDC1/5/6*: pyruvate decarboxylase, *ADH1/3/4/5*: alcohol dehydrogenase, *ADH2*: alcohol dehydrogenase, *ALD2/3/6*: aldehyde dehydrogenase, *MPC1/3*: pyruvate carrier, *YHM2*: citrate and α-ketoglutarate transporter, *CTP1*: citrate transporter, *DIC1*: dicarboxylate transporter, *SFC1*: succinate-fumarate transporter, *ODC1/2*: oxodicarboxylate carriers, *OAC1*: oxaloacetate and sulfate transporter, Nde1/2: mitochondrial external NADH dehydrogenase, Ndi1: NADH:ubiquinone oxidoreductase, Q: ubiquinone, III: complex III, IV: complex IV.

difficulty arises from the fact that toxic compounds, such as ethanol and acetic acid, are formed in batch cultivations with excess glucose, causing a stress-dependent transcriptional regulation. Moreover, excess glucose is associated with a much higher maximum specific growth rate compared to those achieved with non-fermentable carbon sources. This leads to growth-rate specific differences in global transcription interfering with the real differences solely resulting from metabolizing different carbon sources. These issues should be kept in mind when discussing the transcriptome data obtained from experiments such as those provided by DeRisi et al. (1997) and Gasch et al. (2000). The first authors performed microarray-based studies with cells harvested from batch cultivations after the diauxic shift, i.e. when excess glucose was consumed and the cells grew on a mixture of ethanol and glycerol. Gasch et al. (2000) harvested the cells from ethanol-grown batch cultures and compared the transcript levels to those obtained from exponential growth in excess glucose. DeRisi et al. (1997) detected more than 400 transcripts that were found to change by a factor of 2, while Gasch et al. (2000) identified more than 600. These two benchmarking studies shown in Table 1 have been central with regard to our understanding of metabolism when *S. cerevisiae* grows on non-fermentable carbon sources.

Nevertheless, uncertainties result from the fact that assumptions made about active metabolic fluxes which are solely based on transcriptome data inherently disregard post-transcriptional regulation. It has been well known that post-transcriptional regulation contributes to the shift from excess glucose to non-fermentable carbon sources (Schüller, 2003; Yin et al., 2000). Tripodi et al. (2015) and Chen and Nielsen (2016) summarized numerous examples for this type of

Table 1
Overview about the most relevant available transcriptome and proteome studies focusing on differentially expressed genes when *S. cerevisiae* grows in ethanol/glycerol mixtures (diauxic shift), ethanol or glycerol. Cells harvested during growth on glucose were used as a reference in all studies. Notably, the experiments were conducted in different media and cultivation conditions. Thus, conclusions have to be considered with caution. Upregulated gene clusters (represented by a bold ´ + ´) as well as up- and downregulated genes encoding key enzymes in the respective cluster (represented by ´ + ´ and ´-´) are listed according to what has been specified by the authors of the reference publication. In all cases where a gene cluster or gene was not mentioned at all in the publication, we added a question mark (´?´). If the mRNA/protein level was not significantly affected by the carbon source, an equality sign (´ = ´) was used.

	Carbon source(s) used	**Ethanol and glycerol (cells harvested after diauxic shift when grown in glucose)**		**Ethanol**		**Glycerol**	
	Reference	DeRisi et al. (1997)	Gasch et al. (2000)	Daran-Lapujade et al. (2004)	Kolkman et al. (2005)	Ohlmeier et al. (2004)	Roberts & Hudson (2006)
	Medium used	Complex	Complex	Synthetic	Synthetic	Complex	Complex
	Cultivation conditions	Batch	Batch	Chemostat	Chemostat	Batch	Batch
	Type of *omics* study	Transcriptome	Transcriptome	Transcriptome	Proteome	Transcriptome	Transcriptome
Gene cluster and/or genes encoding key enzymes	**Glycerol catabolism**						**+**
	GUT1	?	?	?	?	?	+
	GUT2	?	?	?	?	?	+
	STL1	+	?	+	?	?	+
	Ethanol catabolism						
	ADH2	?	?	=	=	+	?
	ALD2	+	?	=	?	?	?
	ALD4	?	?	=	-	+	?
	Gluconeogenesis						**+**
	PCK1	+	?	+	+	?	?
	FBP1	+	?	+	not detectable	?	?
	Glyoxylate cycle		**+**				**+**
	ICL1	+	?	+	+	?	?
	MLS1	+	?	+	+	?	?
	TCA cycle		**+**				**+**
	CIT1	+	?	=	+	+	?
	ACO1	+	?	=	+	+	?
	IDH1/2	+	?	=	*IDH1* +	-	?
	KGD1/2	+	?	=	*KGD2* +	+	?
	LSC1/2	+	?	=	*LSC2* +	+	?
	SDH1/2/3/4	+	?	*SDH1/3* +	*SDH1* +	*SDH1/2/4* +	?
	FUM1	+	?	+	+	?	?
	MDH1	+	?	=	=	+	?
	Oxidative phosphorylation	**+**	**+**	**+**	**+**	**+**	**+**

regulation with regard to selected enzymes of yeast central carbon metabolism. Various studies have shown that e.g. phosphorylation of metabolic enzymes is very widespread, with two-thirds of the respective *S. cerevisiae* metabolic proteins being targets of kinase and phosphatase signalling networks (Bodenmiller et al., 2010; Breitkreutz et al., 2010; Oliveira et al., 2012; Ptacek et al., 2005). For example, it has been shown that the E1 α subunit of the pyruvate dehydrogenase complex, the glycerol-3-phosphate dehydrogenase (Gpd1) and the Pfk2 β subunit of the phosphofructose-1-kinase complex are regulated by phosphorylation (Oliveira et al., 2012).

An important indication for a significant contribution of post-transcriptional regulation between growth on glucose and non-fermentable carbon sources at the whole-genome level has been delivered by the work of Daran-Lapujade et al. (2004), who used aerobic carbon-limited chemostat cultures in order to compare both the metabolic fluxes (based on metabolic flux balancing using a compartmented stoichiometric model) and the transcriptomes in cells grown on four different carbon sources. The authors used two fermentable carbon sources (glucose and maltose) and two non-fermentable ones (the C2 compounds ethanol and acetate). Thereby, they avoided excess glucose and adjusted the growth rates to exactly the same level in all carbon sources. Notably, hexose sugars are fully respired during this mode of cultivation and any regulation caused by excess glucose is obsolete. Of course, one has to consider that continuous cultures are barely used in industrial practice. However, the study has been very useful to better understand the regulation contributing to the use of different carbon sources independent from carbon catabolite repression. The genes which were transcriptionally upregulated during growth on C2 compounds are included in Table 1. A first, very interesting result obtained in the study of Daran-Lapujade et al. (2004) has been that only 117 genes were differentially expressed at the level of transcription in a comparison between sugars and C2 compounds. This number is significantly lower than those obtained in the studies from DeRisi et al. (1997) and Gasch et al. (2000) analyzing cells after the diauxic shift. This huge discrepancy reflects the above-mentioned differences in transcriptional regulation caused by using glucose excess and batch cultivations instead of applying chemostats. Second, the study revealed that the fluxes predicted by the stoichiometric model were only partly supported by the experimental data obtained by the transcriptome analysis. In fact, only the differences in transcript levels for the enzymes involved in the glyoxylate cycle and gluconeogenesis showed a good correlation with the predicted *in vivo* fluxes when a C6 carbon source was compared with a C2 carbon source. The latter two pathways are indeed not required during growth on glucose. As expected, the flux through acetyl-coenzyme A synthetase was also low in glucose-grown cultures but high for C2 compounds. In contrast, the model-predicted fluxes for growth on C2 carbon sources, such as a remarkably higher flux through the TCA cycle and a reduced flux through the pentose phosphate pathway, were not accordingly reflected by the transcript levels corresponding to the respective enzymes involved in these pathways. Provided that the used metabolic model was sufficiently accurate and indeed reflected the actual fluxes, the results strongly indicated that the *in vivo* fluxes in the central carbon metabolism are controlled to a large part via post-transcriptional mechanisms.

A follow-up study of Kolkman et al. (2005) on the proteome used the same strain and tested it under exactly the same conditions used in the study by Daran-Lapujade et al. (2004). In comparison to the above-mentioned 117 transcriptional differences on ethanol versus glucose (Daran-Lapujade et al., 2004), Kolkman et al. (2005) only identified 15 protein spots whose abundance significantly changed with the carbon source in their proteome study. A closer look at the results show that the key enzymes involved in the glyoxylate cycle (i.e. Mls1 and Icl1) and gluconeogenesis (Pck1), as well as most enzymes involved in the TCA cycle are regulated at the transcriptional level (Table 1). However, most glycolytic enzymes, except for Hxk1 appear to significantly rely on post-transcriptional regulation. The TCA cycle enzymes seem to be worth a particular discussion since the published results regarding the protein levels are not consistent. While Kolkman et al. (2005) identified several enzymes to be upregulated at the protein level, a parallel study of Ohlmeier et al. (2004), who solely focused on the mitochondrial proteome, only found the expression of succinate dehydrogenase to be significantly upregulated. The latter authors even suggested that the TCA cycle enzyme abundance between glucose and non-fermentable carbon sources might be similar due to the anabolic function of this cycle in addition to its role in catabolism. It is worth mentioning in this context that the study of Kolkman et al. (2005) was carried out in defined mineral medium with ethanol in a chemostat while Ohlmeier et al. (2004) conducted their experiments on complex medium with glycerol in batch cultivations. The amino acids present in complex media (Ohlmeier et al., 2004) can provide TCA cycle intermediates for anabolic reactions and might have reduced the need to generate them from the carbon source.

Except the study from Ohlmeier et al. (2004) exclusively analyzing mitochondrial proteins, none of the *omics* studies discussed here so far provided information about the specific situation on glycerol. In contrast to the utilization of glucose and C2 non-fermentable carbon sources, glycerol specifically requires activity of the initial catabolic steps (catalyzed by Stl1, Gut1 and Gut2) in order to be channeled into the central carbon catabolism (glycolysis/gluconeogenesis) via DHAP. Interestingly, the study of Daran-Lapujade et al. (2004) conducted on ethanol and acetate showed that the expression of *STL1*, the gene that has later been demonstrated to encode the active transporter for the uptake of glycerol (Ferreira et al., 2005), is also upregulated on C2 carbon sources.

Due to the fact that glycerol is a C3 carbon source, the central metabolism on glycerol should differ from ethanol or acetate. In theory, the catabolism of glycerol neither requires the glyoxylate cycle (also see section 3) for the replenishment of TCA cycle intermediates nor the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (encoded by *PCK1*), which converts oxaloacetate to phosphoenolpyruvate (PEP). In order to compare the theoretical considerations with experimental data, results from studies on glycerol as the sole carbon source are required. In this context, Roberts and Hudson (2006) provided a transcriptome study on glycerol even though it was conducted in complex medium, i.e. YPG vs. YPD (Table 1). The study used excess glucose as a reference, and the experiments were conducted in shake flask cultivations. At the level of transcriptome, the corresponding results matched to a large part with what had been reported in the transcriptome studies during the diauxic shift conducted by DeRisi et al. (1997) and Gasch et al. (2000). Notably, the study of Roberts and Hudson (2006) conducted a parallel experiment on ethanol. The comparison of the respective data obtained with glycerol surprisingly revealed that the majority of genes was shown to be regulated in the same way on both non-fermentable carbon sources. The gene clusters encoding enzymes involved in respiration (oxidative phosphorylation and TCA cycle), the glyoxylate cycle, and gluconeogenesis were highly upregulated. Additionally, clusters encoding for proteins related to mitochondrial function, energy generation and stress responses were upregulated. Downregulated genes in the study of Roberts and Hudson (2006) clustered in functional categories such as ribosome biogenesis, transcription of RNA polymerase I and III promoters, as well as membrane lipid and sphingolipid metabolism reflecting the slower growth rate of *S. cerevisiae* on non-fermentable carbon sources compared to glucose. A closer look at the genes which are particularly required for channeling glycerol or ethanol into the central carbon metabolism revealed that *STL1*, *GUT1* and *GUT2* were strongly upregulated on glycerol but also on ethanol even though the fold changes were slightly lower on ethanol. The upregulation of *STL1* on ethanol is consistent with the respective finding of Daran-Lapujade et al. (2004). Roberts and Hudson (2006) also identified a few differences between ethanol and glycerol. In fact, genes found in clusters covering carbohydrate and fatty acid metabolism were less upregulated on ethanol in comparison to glycerol. A

rationale behind this finding was not provided.

Although the study of Ohlmeier et al. (2004) solely addressed the mitochondrial proteins on glycerol, the authors also conducted a genome-wide transcriptome analysis in parallel to just confirm that the cells had indeed undergone the metabolic shift (Table 1). The authors identified 18 mitochondrial proteins whose abundance significantly changed in glycerol but detected 4000 differentially expressed genes at the transcriptome level. The authors concluded that the discrepancy between transcript and protein levels is caused by changes in translational efficiency and protein turnover. Nevertheless, the study confirmed at the proteome level that the proteins Adh2 and Ald4 are also strongly upregulated on glycerol. Both proteins are key enzymes involved in the utilization of C2 carbon sources but are obviously also abundant during growth on glycerol. Similar results were obtained by the proteome analysis conducted by Kito et al. (2016). The obvious fact that the regulation of the metabolism seems to be strikingly similar when cells are shifted from excess glucose to either ethanol or glycerol might be attributed to the circumstance that glycerol often co-exists with ethanol in natural environments of *S. cerevisiae*. In fact, ethanol is the major fermentation product resulting from catabolism of excess glucose by *S. cerevisiae* and some glycerol is formed as a by-product for cytosolic redox balancing (Bakker et al., 2001).

A number of regulatory networks are involved in the coordinated biosynthesis of enzymes necessary for the utilization of non-fermentable carbon sources as soon as glucose repression is abolished. As already mentioned, it is generally difficult to dissect the regulatory mechanisms involved in glucose repression from those that are related to a particular non-fermentable carbon source. We will not go into detail about the regulation of glucose repression as several comprehensive reviews have been provided in this context (Hedbacker and Carlson, 2008; Kayikci and Nielsen, 2015). Here, we only want to scrutinize whether the currently available information about transcription factors important for the regulation of genes during growth on ethanol and glycerol support the idea of common regulatory networks on both carbon sources. Table 2 summarizes a number of transcription factors for which studies in complex glycerol medium are available. The majority of tested transcription factors shows the same deletion phenotype when grown in ethanol or glycerol. However, there are two suspicious proteins, Rsf1 and Rsf2, whose mutant phenotypes indicate that they play a more specific role only on glycerol but are probably dispensable on ethanol. Indeed, the respective two haploid mutant strains showed severe growth defects on glycerol but not on ethanol (Lu et al., 2003; Lu et al., 2005). The same authors demonstrated that the expression of both *RSF1* and *RSF2* was strongly induced on glycerol, and the induction was lower on ethanol. The growth defect on glycerol of the two haploid mutants can be explained by the strong decrease in *GUT1* and *GUT2* transcript levels, the products of both genes are essential for glycerol utilization. *GUT1* and *GUT2* are not required for growth on ethanol which could explain the fact that the deletion of neither Rsf1 nor Rsf2 has an effect on ethanol-based growth. It has also been shown that the transcript levels of genes encoding products with a function in stress response were increased in the *rsf1Δ* mutant during exponential growth on glycerol but not on ethanol-based medium (Roberts and Hudson, 2009). Furthermore, loss of Rsf1 resulted in a decrease of the transcript levels of genes encoding proteins involved in electron transport and glycerol anabolism during growth on glycerol-based, but not on ethanol-based medium.

In summary, many aspects with respect to the regulation of the transcriptional changes that are crucial for the utilization of glycerol as the sole carbon source in synthetic medium are still not understood. Apart from basic research, a more detailed knowledge about regulatory proteins could be an advantage for metabolic engineering endeavors. Several studies have demonstrated that, for example, the deletion and/or the overexpression of selected transcriptional regulators can improve industrially important strain characteristics in *S. cerevisiae* (Chen et al., 2016; Lin et al., 2014). Moreover, the random mutagenesis of regulon-specific transcriptional factors is an interesting approach to reprogram expression of genes relevant for a particular phenotype (Swinnen et al., 2017). Such approaches could be applied for strain optimization in the context of glycerol utilization as well provided that a better understanding of the crucial transcription factors is available.

3. Physiological role of the glyoxylate cycle on glycerol and subcellular localization of the respective enzymes

In *S. cerevisiae,* the glyoxylate cycle consists of five reactions catalyzed by isocitrate lyase, malate synthase, malate dehydrogenase, citrate synthase, and aconitase. Several individual reactions of this cycle are known to take place in the cytosol while others have been localized in the peroxisomes (Fig. 1). During the utilization of C2 carbon compounds and oleate, the physiological function of the glyoxylate cycle is to convert acetyl-CoA into C4 metabolites, which can then be diverted to anabolic reactions e.g. gluconeogenesis and amino acid biosynthesis (Hiltunen et al., 2003; Kunze et al., 2006). As already mentioned in section 2, the key enzymes of the glyoxylate cycle, i.e. isocitrate lyase and malate synthase, have been shown to be highly upregulated on C2 compounds as well as on oleate (Kal et al., 1999). Interestingly, the respective upregulation was also recognized in all studies available so far on glycerol at both transcriptome and proteome level (see section 2), even though the glyoxylate cycle is, at least in theory, dispensable on C3 carbon sources. It could therefore be that the upregulation of the glyoxylate cycle enzymes is simply caused by the assumed co-regulation of *S. cerevisiae*'s metabolism for the co-utilization of glycerol and ethanol. In the following, we will scrutinize this aspect by having a closer look at the experimental data obtained by studying mutants defective in glyoxylate cycle (iso)enzymes. Out of the five enzymes contributing to the cycle, only isocitrate lyase and malate synthase have a unique function in this pathway, while the other three enzyme activities can also be found in the TCA cycle. Therefore, it is inherently difficult to draw conclusions about the role of the latter three enzymes for the glyoxylate cycle. Still, we want to provide the information regarding all isoenzymes known in *S. cerevisiae* and, if available, the phenotypes of the respective deletion mutants particularly on glycerol.

S. cerevisiae contains two actively transcribed aconitase isoenzymes called Aco1 and Aco2 (Gangloff et al., 1990; Przybyla-Zawislak et al., 1999). Deletion of *ACO1* resulted in an inability of the respective mutant to grow on glycerol (Gangloff et al., 1990). This result indicates that Aco1p is the major citric acid cycle aconitase in *S. cerevisiae.* Aco2 seems to exclusively contribute to lysine biosynthesis (Fazius et al., 2012).

Citrate synthase has three isoenzymes in *S. cerevisiae*, but only Cit2 (localized in peroxisomes) seems to be involved in the glyoxylate cycle, whereas Cit1 and Cit3 function as mitochondrial isoforms within the TCA cycle. In contrast to Cit1 and Cit2, Cit3 has been identified as a dual specificity mitochondrial citrate and methylcitrate synthase (Graybill et al., 2007). Kispal et al. (1988) have shown that individual deletions of *CIT1*, *CIT2* and *CIT3* resulted in strains that grew well on glycerol, while a double mutant strain *cit1Δ cit2Δ* is not viable on glycerol, glucose, and galactose. Jia et al. (1997) also tested the individual deletions of *CIT1* and *CIT3* on the same medium as Kispal et al. (1988). In contrast to Kispal et al. (1988), Jia et al. (1997) observed a small reduction in growth for the individual deletion of *CIT1*. Furthermore, a double mutant strain *cit1Δ cit3Δ* led to a very strong reduction in growth on glycerol. However, results regarding the growth of the double deletion strain *cit2Δ cit3Δ* on glycerol are still missing in order to allow a final conclusion about the requirement of the different isoenzymes for growth on glycerol.

The third enzyme with a function in both TCA cycle and glyoxylate cycle is malate dehydrogenase. Again, three isoenzymes have been identified, all of which can reversibly convert malate to oxaloacetate. Depending on the carbon sources, different isoenzymes of malate dehydrogenase seem to be essential. For instance, cells lacking *MDH2* are

Table 2
List of selected transcription factors (TF) potentially important during growth on glycerol as the sole carbon source. The selection has been based on the following criteria: (i) the DNA-binding target(s) of the respective transcriptional factor were experimentally characterized using glycerol as the sole carbon source (Hap2/3/4/5, Ino2/Ino4, Oaf1, Opi1, Pip2, Rds2, Rsf1, Rsf2) and/or (ii) the respective deletion mutant showed a clear growth impairment on glycerol (Adr1, Cat8, Rds2, Rsf1, Rsf2, and Znf1). Ert1 has been shown to bind to genes whose products are involved in gluconeogenesis on ethanol as the sole carbon source and a function in glycerol medium cannot be ruled out due to the lack of experimental data on glycerol. Notably, Roberts and Hudson (2006) identified a number of additional transcriptional factors upregulated during growth on glycerol. Some of them have not been included in the table since no experimental data have been available about their DNA-binding or deletion mutant phenotypes.

	DNA-binding studies			Phenotype of the deletion mutant lacking the respective transcriptional factor
TF	Target gene(s) for which DNA binding to the respective promoter(s) was demonstrated	Experimental condition for which DNA binding was shown	Method(s) and reference(s) for the DNA binding assay(s) and target gene determination[1]	
Adr1	Utilization of ethanol (*ADH2*, *ALD4*, *ALD5*, *ALD6*, *ACS1*) Utilization of glycerol (*GUT1*, *GUT2*) Utilization of lactate (e.g. *CYB2*, *JEN1, DLD3*) Mitochondrial inner membrane proteins (*CTP1*, *OAC1*, *DIC1*) *β*-oxidation (*OAF1*) Peroxisome biogenesis (*CTA1*)	Diauxic shift	ChIP-chip (Young et al., 2003) ChIP-chip, qRT-PCR (Tachibana et al., 2005)	Glycerol: no growth (Young et al., 2003) Ethanol: poor growth (Young et al., 2003)
Cat8	Gluconeogenesis (*PCK1, FBP1*) Glyoxylate shunt (*ACS1, ICL1, MDH2, MLS1*) Transporters (*PUT4*, *SFC1*, *JEN1*) Transcriptional factors *SIP4* and *HAP4* Utilization of ethanol (*ADH2*) Isocitrate dehydrogenase (*IDP2*)	Diauxic shift	ChIP-chip, qRT-PCR (Tachibana et al., 2005)	Glycerol: no growth (Akache et al., 2001; Hedges et al., 1995) Ethanol: no growth (Gasmi et al., 2014)
Ert1	Gluconeogenesis (*PCK1*, *FBP1, MAE1*) Transporters (e.g. *SFC1, FMP43*) Respiration (*HAP4*) TCA cycle (e.g. *LSC2*) Mitochondrial proteins (e.g. *PET9*)	Ethanol	ChIP-chip, qRT-PCR, gene reporter assay (Gasmi et al., 2014)	Glycerol:? Ethanol: growth (Gasmi et al., 2014)
Hap2/3/4/5	Utilization of glycerol (*GUT2*)	Glycerol	Gene reporter assay (Grauslund and Ronnow, 2000)	Glycerol: no growth (Buschlen et al., 2003) Ethanol: no growth (Buschlen et al., 2003)
Ino2/Ino4	Utilization of glycerol (*GUT1*)	Glycerol	Gene reporter assay – (Grauslund et al., 1999)	Glycerol:? Ethanol:?
Oaf1	Fatty acids utilization (e.g. *POX1*, *FOX1*) Peroxisome biogenesis (e.g. *PEX5*, *PEX11*) Transcriptional factor *PIP2*	Glycerol	ChIP-chip, qRT-PCR (Karpichev et al., 2008)	Glycerol: growth (Rottensteiner et al., 1997) Ethanol: growth (Rottensteiner et al., 1997)
Opi1	Utilization of glycerol (*GUT1, GUT2*)	Glycerol	Gene reporter assay (Grauslund et al., 1999; Grauslund and Ronnow, 2000)	Glycerol: no growth (Grauslund et al., 1999) Ethanol: no growth (Grauslund et al., 1999)

(*continued on next page*)

Table 2 (*continued*)

	DNA-binding studies			Phenotype of the deletion mutant lacking the respective transcriptional factor
TF	Target gene(s) for which DNA binding to the respective promoter(s) was demonstrated	Experimental condition for which DNA binding was shown	Method(s) and reference(s) for the DNA binding assay(s) and target gene determination[1]	
Pip2	Fatty acids utilization (e.g. *POX1*, *FOX1*) Peroxisome biogenesis (e.g. *PEX5*, *PEX11*)	Glycerol	ChIP-chip, qRT-PCR (Karpichev et al., 2008)	Glycerol: growth (Rottensteiner et al., 1997) Ethanol: growth (Rottensteiner et al., 1997)
Rds2	Gluconeogenesis (*PCK1, FBP1*) Glyoxylate shunt (*MLS1*, *MDH2*) TCA cycle (*CIT1*, *KGD2*, *SDH4*, *LSC2*) Respiration (*COX6*, *CYC1*) Transporter (*SFC1*) Transcriptional factors *HAP4* and *SIP4*	Ethanol	ChIP-chip (Soontorngun et al., 2007)	Glycerol: no growth (Akache et al., 2001; Soontorngun et al., 2012) Ethanol: no growth (Soontorngun et al., 2012)
	Gluconeogenesis (*PCK1, FBP1*) Glyoxylate shunt (*ACS1, MLS1*) TCA cycle (*LSC2*) Respiration (*COX4*) Transporters (*SFC1*) Transcriptional factors *HAP4*, *ADR1* and *SIP4*	Glycerol	ChIP-chip, qRT-PCR (Soontorngun et al., 2012)	
Rsf1	Utilization of glycerol (*GUT1, GUT2*) Respiration (*OLI1*)	Glycerol, ethanol	Y1H, qRT-PCR (Lu et al., 2003)	Glycerol: severe growth defect (Lu et al., 2003) Ethanol: growth (Lu et al., 2003)
Rsf2	Utilization of glycerol (*GUT1*, *GUT2*) Mitochondrial genes (*OLI1*) Respiration (*COX4*)	Glycerol, ethanol	qRT-PCR, DNA microarray (Lu et al., 2005)	Glycerol: growth defect (Lu et al., 2005) Ethanol: growth (Lu et al., 2005)
Znf1	Gluconeogenesis (*FBP1, PCK1*) Glyoxylate shunt (*ICL1, MDH2, MLS1*) TCA cycle (*ACO1*) Transporters (*ADY2*, *SFC1*)	Ethanol	ChIP-chip, qRT-PCR (Tangsombatvichit et al., 2015)	Glycerol: no growth (Akache et al., 2001; Tangsombatvichit et al., 2015) Ethanol: no growth (Akache et al., 2001; Tangsombatvichit et al., 2015)

[1] Target genes have been characterized by chromatin immunoprecipitation (ChIP-chip), by DNA microarray, by gene reporter assay, by yeast one-hybrid analysis (Y1H) and/or by quantitative RT-PCR (qRT-PCR).

Table 3
Localization of enzymatic activities required for the glyoxylate cycle in *S. cerevisiae*. The respective studies have been conducted in media containing C2 compounds (ethanol or acetate) or oleate as the sole carbon source.

Enzyme	Gene	ORF	C2 compounds (ethanol/acetate)		Oleate	
			Localization	Reference	Localization	Reference
Aconitase	*ACO1*	YLR304C	Cytosol (less than 5%) and mitochondria	Regev-Rudzki et al. (2005)	Cytosol (less than 5%) and mitochondria	Regev-Rudzki et al. (2005)
Citrate synthase	*CIT2*	YCR005C	Peroxisome	Lee et al. (2011)	Peroxisome	Lewin et al. (1990)
Isocitrate lyase	*ICL1*	YER065C	Cytosol	Chaves et al. (1997)	Cytosol	Chaves et al. (1997) Taylor et al. (1996)
Malate dehydrogenase	*MDH2*	YOL126C	Cytosol	Minard and McAlister-Henn (1991)	Cytosol	McCammon et al. (1990)
	MDH3	YDL078C	Peroxisome	Steffan and McAlister-Henn (1992)	Peroxisome	van Roermund et al. (1995)
Malate synthase	*MLS1*	YNL117W	Cytosol	Kunze et al. (2002)	Peroxisome	Kunze et al. (2002)

unable to utilize ethanol as the sole carbon source (Minard and McAlister-Henn, 1991) while cells lacking *MDH3* cannot grow on oleate (van Roermund et al., 1995). With regard to the role of malate dehydrogenase isoenzymes active on glycerol, the published results do not allow clear conclusions because all these studies have used either complex media or minimal media supplemented with yeast extract (McAlister-Henn and Thompson, 1987; Minard and McAlister-Henn, 1991; Przybyla-Zawislak et al., 1999; Steffan and McAlister-Henn, 1992). One might argue that the anabolic function of the TCA cycle was unnecessary under the latter conditions.

The two key enzymes in the glyoxylate cycle, malate synthase (Mls1) and isocitrate lyase (Icl1), are both encoded by a single gene in *S. cerevisiae*. If the glyoxylate cycle was indeed dispensable for glycerol utilization, the growth of the respective single deletion mutants should not be negatively affected on this carbon source. Based on spot tests, Chen et al. (2012) concluded that the *mls1*Δ and *icl1*Δ mutant strains exhibited a growth defect on glycerol as the sole carbon source. It must, however, be noted that the wild-type control only formed a faint spot. This is an inherent problem when working with strains not able to grow on synthetic glycerol medium, but show weak growth as soon as the necessary amino acid(s) or nucleic base(s) is/are added in order to supplement the strain's auxotrophy/auxotrophies. It is well known that the use of auxotrophic strains together with the respective supplements can lead to misinterpretations of phenotypes (Pronk, 2002). Therefore, we strongly recommend repeating the investigation of the *mls1*Δ and *icl1*Δ mutant strains by using a prototrophic strain able to grow in liquid glycerol medium. This holds of course for many other mutant strains as well.

The localization of the (iso)enzymes contributing to the glyoxylate cycle has experimentally been carried out using cells growing on C2 compounds and oleate. According to the results, which have been summarized in Table 3, Aco1 is mainly localized in the mitochondria and a minor fraction was detected in the cytosol. Icl1 and Mdh2 were detected in the cytosol, while Cit2p and Mdh3p are localized in the peroxisomes (Fig. 1). With regard to Mls1, the subcellular localization seems to depend on the carbon source (Kunze et al., 2002). While the enzyme seems to be located in the cytosol when cells grow on ethanol, it was detected in the peroxisomes during growth on oleate. Due to this uncertainty, we allocated Mls1 to both compartments in Fig. 1 and added a question mark. The different targeting of Mls1 on ethanol and oleate has been demonstrated to depend on the peroxisomal membrane signal receptor (encoded by *PEX9*), that is involved in peroxisomal import of a subset of matrix proteins (Effelsberg et al., 2016). Pex9 is not expressed in ethanol-grown cells, but its expression is strongly induced on oleate. Therefore, in *S. cerevisiae* cells growing on oleate, Pex9 acts as a cytosolic and membrane-bound peroxisome import receptor for Mls1 (Effelsberg et al., 2016). This example demonstrates that enzyme and pathway localization in a cell is not static but may vary according to the employed conditions. Apart from the aforementioned examples, one has to note that there is currently no available information about the localization of Mls1 on glycerol.

In general, the knowledge about the subcellular localisztion of a particular enzyme may be required for metabolic engineering approaches. Particularly if compartmentalization of metabolic pathways is used to increase product formation, this type of information is crucial. In fact, targeting enzyme localization has been of growing interest for yeast metabolic engineering (Avalos et al., 2013; DeLoache et al., 2016; Sheng et al., 2016). However, metabolic engineers should be aware that the localization of a particular enzyme can even change as a side effect of certain genetic modifications. For example, in the context of profiling cytosolic and peroxisomal acetyl-CoA metabolism in *S. cerevisiae* on ethanol, Chen et al. (2012) have found that, upon deletion of *ACS1* (a gene encoding one of the isoenzymes of acetyl-CoA synthetase), Mls1 unexpectedly relocated from the cytosol to the peroxisome. Such an unintended relocation may have a dramatic impact on the outcome of the experiments and it might not be trivial to determine the underlying reason.

4. Mitochondrial transporters active on glycerol

In eukaryotic cells such as *S. cerevisiae,* membranes often separate different metabolic pathways, which partially or completely take place in different cellular compartments. For example, the TCA cycle occurs in the mitochondrial matrix while the enzymes of the glyoxylate cycle are either located in the cytosol or in the peroxisome (see section 3). The spatial separation of the two pathways allows their differential metabolic regulation, even though both pathways partially share the same enzymatic activities. In order to translocate specific organic solutes and inorganic ions across the intracellular membranes, specific transporters are required. Knowledge about these transport mechanisms is crucial for metabolic engineering of the central carbon metabolism, especially when it relates to the target product itself or one of the necessary pathway intermediates. Here, we want to focus on known mitochondrial *S. cerevisiae* transporters that translocate main intermediates of the central carbon metabolism i.e. pyruvate, acetyl-CoA, citrate, isocitrate, succinate, fumarate, malate and oxaloacetate (Fig. 1), and scrutinize their role when cells grow on glycerol. Particularly with regard to the glyoxylate cycle intermediates, it would be of great interest to also obtain knowledge about the respective peroxisomal transport mechanisms. However, genetic screens performed in *S. cerevisiae* were not able to identify any peroxisomal transporter responsible for the transport of the relevant intermediates (Kunze and Hartig, 2013; Kunze et al., 2006). Although we still cannot completely exclude the existence of such specific peroxisomal transporters, it has been suggested that the transfer of the glyoxylate cycle intermediates might be fulfilled by peroxisomal channels, as reviewed by Antonenkov and Hiltunen (2012).

When cells grow on glycerol, they form pyruvate from DHAP similar

to cells growing on glucose. One could expect that the mitochondrial pyruvate carrier complex is required for the transport of pyruvate from the cytosol into the mitochondria (Bricker et al., 2012). Two alternative carrier complexes exist that contain either Mpc1 and Mpc3 or Mpc1 and Mpc2. The former complex is required during respiratory growth on glycerol (Fig. 1), while the latter is necessary under fermentative conditions such as growth on excess glucose (Bender et al., 2015). On glycerol, a strain in which all three homologous genes (*MPC1*, *MPC2*, and *MPC3*) are deleted is still viable, even though a clearly reduced maximum specific growth rate was observed (Timon-Gomez et al., 2013). The ability of the triple mutant to still grow on glycerol may be explained by the existence of the pyruvate dehydrogenase bypass pathway (Pronk et al., 1996) (Fig. 1). In this pathway, pyruvate is first decarboxylated to acetaldehyde in the cytosol by one of the pyruvate decarboxylases (encoded by the structural genes Pdc1/5/6) (Hohmann, 1991; Hohmann and Cederberg, 1990). Afterwards, the acetaldehyde is converted to acetate by the aldehyde dehydrogenase (Ald2/3/6) and further to acetyl-CoA by acetyl-CoA synthetase (Acs1/2) in the cytosol or acetate enters the peroxisome, where it is converted to acetyl-CoA by Acs1. The cytosolic acetyl-CoA can enter the glyoxylate cycle and replenish the TCA cycle via succinic acid that is translocated into the mitochondria (Fig. 1). In principle, cytosolic acetyl-CoA can also directly enter the mitochondria via a carnitine-dependent route (Fig. 1) (van Roermund et al., 1995). However, the latter route can only be used by *S. cerevisiae* if carnitine is added to the growth medium since it is not capable of de novo synthesis of carnitine (van Roermund et al., 1999).

Notably, Timon-Gomez et al. (2013) tested their above-mentioned triple *mpc* mutant strain in synthetic glycerol medium that contained 50 mM succinic acid as a buffering agent. Succinic acid is an intermediate of the TCA cycle and as such may have falsified the obtained result. Anyway, an *mpc1*Δ *mpc2*Δ *mpc3*Δ mutant of a glycerol-utilizing strain should be tested in pure glycerol medium to verify whether its growth phenotype can be confirmed. In theory, there could also be another route how pyruvate indirectly enters the mitochondria. Pyruvate could be converted to oxaloacetate (or malate) by pyruvate carboxylase (PYC) and malate dehydrogenase in the cytosol. These compounds can enter the mitochondria via respective transporters (Fig. 1). Malate can be finally converted to pyruvate by means of malic enzyme (Mae1) as it can be deduced from Fig. 1. To prove that such route indeed exists, one should first investigate whether an *mpc1*Δ *mpc2*Δ *mpc3*Δ mutant is able to grow on pure glycerol medium (without addition of carnitine). The question whether malic enzyme is active on glycerol is also discussed in section 5.

The dicarboxylate carrier, encoded by *DIC1*, transports dicarboxylates, such as succinate and malate, into mitochondria in exchange for internal phosphate (Palmieri et al., 1999b). In Fig. 1, only the transport of succinate is exemplarily shown. A mutant strain lacking this dicarboxylate carrier was not able to grow on ethanol or acetate, but it was viable on other non-fermentable carbon sources including glycerol, pyruvate and lactate, which are all C3 carbon sources (Palmieri et al., 1999b). As growth of the *dic1*Δ mutant strain on acetate or ethanol was only restored by addition of compounds such as oxaloacetate able to generate TCA cycle intermediates, the suggested function of this transporter is to transport cytoplasmic dicarboxylates into the mitochondrial matrix, fulfilling an essential role for the replenishment of TCA cycle intermediates (Palmieri et al., 1999b). The fact that Dic1 is not essential on C3 carbon sources could support the hypothesis that the glyoxylate cycle is not essential as an anaplerotic route when cells grow on glycerol (see section 'Anaplerotic reactions').

The mitochondrial membrane of *S. cerevisiae* has been shown to also carry a succinate/fumarate transporter encoded by *SFC1*. This carrier exports fumarate to the cytosol in exchange with succinate. In the cytosol, fumarate is channeled into the gluconeogenic pathway (via cytosolic Fum1, Mdh2 and Pck1), i.e. a route which is indispensable for *S. cerevisiae* growth on ethanol or acetate (Palmieri et al., 1997; Palmieri et al., 2000). Consequently, an *sfc1*Δ mutant strain was neither capable to grow on ethanol nor on acetate, but was still viable on pyruvate (Fernandez et al., 1994). Although the *sfc1*Δ mutant strain was not tested in glycerol medium, we expect that this mutant is still able to grow, comparable to the situation on pyruvate. On C3 substrates, oxaloacetate can be produced from pyruvate by PYC (encoded by *PYC1/2*) in the cytosol and transported into the mitochondria via Oac1 as described in the following paragraph. In order to verify this assumption, it might be worth investigating whether a *dic1*Δ *sfc1*Δ double deletion strain can still grow in synthetic glycerol medium.

The yeast mitochondrial transporter for oxaloacetate encoded by *OAC1*, uses the proton-motive force to take up oxaloacetate produced by cytoplasmic PYC (an anaplerotic reaction) into the mitochondria as shown in Fig. 1 (Palmieri et al., 1999a). The single deletion mutant *oac1*Δ was still able to grow on rich medium containing glycerol (Colleaux et al., 1992). In line with this, it has been reported that Oac1 is not required for growth of *S. cerevisiae* on glucose, galactose, ethanol, acetate and lactate, because in the absence of Oac1, oxaloacetate can be converted to malate in the cytoplasm by Mdh2, and malate enters the mitochondrion via Dic1. Malate can subsequently be converted to oxaloacetate in the mitochondria as shown in Fig. 1 (Palmieri et al., 1999a). Indeed, an *oac1*Δ *dic1*Δ double deletion strain did neither grow on glycerol nor ethanol (Palmieri et al., 1999a). However, it has to be emphasized that the exact medium composition used to test the phenotype for the *oac1*Δ *dic1*Δ double deletion strain has not been specified. The authors only mention rich medium and synthetic complete medium containing the aforementioned carbon sources. If it was possible to confirm this phenotype on pure synthetic glycerol medium, this would prove that Dic1 plays an anaplerotic role on glycerol in the absence of the mitochondrial carrier Oac1. There have been two isoforms of another mitochondrial transporter (encoded by *ODC1* and *ODC2*) which besides transporting oxoadipate and oxoglutarate (α-ketoglutarate) with high efficiency by a counter-exchange mechanism, also transports to a lower extent malate (Palmieri et al., 2001). However, this transporter does not seem to be capable to transport sufficient malate into the mitochondria in the absence of Oac1 and Dic1. In Fig. 1, only the transport of malate by Odc1 and Odc2 is shown.

In *S. cerevisiae*, two mitochondrial citrate transporters have been identified. The first one is the mitochondrial transporter Ctp1, which transports citric acid or isocitrate through the mitochondrial inner membrane (Kaplan et al., 1995), while the second carrier Yhm2 transports citrate out of the mitochondria in exchange of α-ketoglutarate (Castegna et al., 2010) (Fig. 1). Additionally, Yhm2 was also demonstrated to transport oxaloacetate, succinate and fumarate to a lesser extent, but not malate or isocitrate. In higher eukaryotes, the function of citric acid transport from mitochondria to the cytosol is to deliver cytosolic acetyl-CoA (via cytosolic citrate lyase) to fuel both fatty acid and sterol biosynthesis (Endemann et al., 1982; Watson and Lowenstein, 1970). In *S. cerevisiae*, the actual physiological function(s) of the two citrate transporters has/have remained unclear. In fact, *ctp1*Δ and *yhm2*Δ mutant strains were shown to be capable to grow on ethanol (Kaplan et al., 1995), acetate (Castegna et al., 2010) and glucose (Kaplan et al., 1996; Scarcia et al., 2017). These results do not seem to be surprising. On ethanol and acetate cytosolic acetyl-CoA is directly produced from the substrates as shown in Fig. 1 and does not have to be transported out of the mitochondria. On glucose, *S. cerevisiae* synthesizes cytosolic acetyl-CoA via pyruvate decarboxylase (Pdc1/5/6), aldehyde dehydrogenase (Ald2/3/6) and acetyl-CoA synthetase (Acs2). No data have been available on glycerol, but one might speculate that described transporters are also not required for synthesis of acetyl-CoA in the cytosol and instead, *S. cerevisiae* synthesizes cytosolic acetyl-CoA via the same route as on glucose. The question of whether the two transporters have an actual physiological function remains unsolved. Nevertheless, one interesting hypothesis has been provided for the mitochondrial citrate carrier Ymh2 which transports citrate out of the mitochondria in exchange of α-ketoglutarate. In studies conducted on acetate, it has been demonstrated that upon deletion of *YHM2*, citrate

accumulates in the mitochondria of *S. cerevisiae*. In the cytosol, citrate can be converted to isocitrate by aconitase (encoded by *ACO1*), and consequently, the latter is used to produce α-ketoglutarate and NADPH by the action of an $NADP^+$-dependent cytosolic isocitrate dehydrogenase (encoded by *IDP2*). Thus, Yhm2 could function in a shuttle mechanism which indirectly transports NADPH from the mitochondria to the cytosol (Castegna et al., 2010). In fact, production of cytosolic free fatty acids from glucose, which require NADPH for their biosynthesis, was slightly increased by overexpression of *YHM2* (Yu et al., 2018).

5. Anaplerotic reactions active during growth on glycerol

Anaplerotic reactions replenish intermediates of the TCA cycle, which is necessary to dynamically balance the reactions that steer TCA intermediates, such as α-ketoglutarate, into biosynthetic pathways. This balancing, for which evolution has developed different metabolic routes (Nelson et al., 2013), results in an almost constant concentration of citric acid cycle intermediates. In nature, there are four known anaplerotic enzymes including PYC, PEP carboxykinase, PEP carboxylase, and malic enzyme, all of which provide the C4 compounds oxaloacetate or malate from the C3 compounds pyruvate or PEP. A fifth possibility to replenish the TCA cycle (at the level of the C4 compound succinate) is the glyoxylate cycle (Fig. 1). As mentioned in section 3, this pathway is the only anaplerotic route when an organism grows on C2 carbon sources or fatty acids. From a metabolic engineer's point of view, anaplerotic reactions are of utmost importance for re-routing the central carbon metabolism towards valuable products. Obviously, they are particularly relevant if the target product is a TCA cycle intermediate (e.g. succinic acid, fumaric acid or malic acid) or whose production pathway starts from such an intermediate (e.g. glutamate and derivatives).

The actual use of the different anaplerotic routes varies between organisms and growth conditions (carbon source). Except PEP carboxylase, *S. cerevisiae* commands all enzymes mentioned that have potential anaplerotic functions. *S. cerevisiae* also possesses the glyoxylate cycle as an anaplerotic route, which has been discussed in detail in the previous sections. Similar to many of the reactions discussed in the previous sections, it is currently not completely understood which anaplerotic route(s) are the predominant ones when *S. cerevisiae* cells grow on glycerol. Therefore, we first discuss the relevance of PYC which is the major anaplerotic reaction on glucose in *S. cerevisiae* and the glyoxylate cycle which takes over this role during growth on ethanol. Afterwards, the two remaining potentially anaplerotic reactions are discussed.

PYC consists of two cytosolic isoenzymes, encoded by the genes *PYC1* and *PYC2* (Morris et al., 1987; Stucka et al., 1991; Walker et al., 1991) (Fig. 1). Both isoenzymes catalyse the ATP-dependent carboxylation of pyruvate to oxaloacetate (Gailiusis et al., 1964). The localization of this enzyme in *S. cerevisiae* is exclusively cytosolic which is in contrast to many higher organisms where PYC is a mitochondrial enzyme (Haarasilta and Taskinen, 1977; Rohde et al., 1991; van Urk et al., 1989; Walker et al., 1991). As mentioned, PYC seems to represent the major anaplerotic reaction in *S. cerevisiae* growing in glucose-containing media. Single deletion mutants (deleted in either *PYC1* or *PYC2*) can sustain growth on glucose, but the double deletion mutant (*pyc1*Δ *pyc2*Δ) cannot grow unless the medium is supplemented with aspartate (Blazquez et al., 1995; Brewster et al., 1994; Stucka et al., 1991). The latter is able to deliver the necessary oxaloacetate for TCA cycle replenishment. The result obtained with the double deletion strain suggests that other anaplerotic routes are not sufficiently active to take over the role of PYC on glucose. As expected, PYC is dispensable during growth on C2 compounds; a *pyc1/2* double deletion mutant was able to grow on ethanol (de Jong-Gubbels et al., 1998). Regarding the situation on glycerol, one has to consider that it is a C3 carbon source, and thereby the anaplerotic role of PYC might be similar to that on glucose. However, a *pyc1/2* double deletion mutant was able to grow on glycerol even without the requirement of aspartate (Blazquez et al., 1995). Although this study did not provide growth rates, the result suggests that PYC is not the major anaplerotic enzyme during growth on glycerol or that its function can easily be replaced by another mechanism such as the glyoxylate cycle.

During growth on ethanol (C2), the replenishment of oxaloacetate is supposed to occur via the glyoxylate cycle at the level of succinate. This has been confirmed by Schöler and Schüller (1993) who showed that an *icl1* mutant strain cannot grow on ethanol. In order to analyze the impact of deleting *ICL1* on growth in synthetic glycerol medium, we used a CEN.PK derivative that has been previously obtained by reverse engineering (see introduction) (Ho et al., 2017). The *icl1* deletion mutant of this strain was able to grow on glycerol but not on ethanol (Xiberras et al., manuscript in preparation). This result suggests that the glyoxylate cycle is also not the major anaplerotic route on glycerol. Another explanation might be that the cell is very flexible in using PYC and/or the glyoxylate cycle under these conditions and one route can easily be replaced by the other. It will therefore be very interesting to check whether a *pyc1*Δ *pyc2*Δ *icl1*Δ triple deletion mutant is viable in synthetic glycerol medium.

In principle, it still cannot be completely excluded that either PEP carboxykinase (*PCK1*) and/or the malic enzyme (*MAE1*) are also active as anaplerotic enzymes on glycerol (Fig. 1). Pck1 is generally considered as a decarboxylating enzyme with a function in gluconeogenesis in *S. cerevisiae* (de Torrontegui et al., 1966). However, Pck1 is the main enzyme catalysing the C3 carboxylating reaction (PEP to oxaloacetate) in *Actinobacillus succinogenes* (McKinlay et al., 2007). We have recently deleted *PCK1* in a *S. cerevisiae pyc1/2* double deletion mutant and this triple deletion mutant was still able to grow on synthetic glycerol medium (Xiberras et al., manuscript in preparation). This result supports the assumption that Pck1 does not play an important role as an anaplerotic enzyme in *S. cerevisiae*, at least under these conditions. Malic enzyme catalyses the reversible oxidative decarboxylation of malate to pyruvate and CO_2 (Fig. 1). However, the inability of pyruvate carboxylase negative *S. cerevisiae* strains to grow on glucose showed that malic enzyme cannot act as a pyruvate carboxylating anaplerotic enzyme (Zelle et al., 2011). Interestingly, this enzyme provides the pyruvate required for biosynthesis in pyruvate kinase-negative strains growing on ethanol (Boles et al., 1998). Experimental data regarding a potential function of malic enzyme on glycerol are underway.

A remarkable aspect with regard to the anaplerotic reactions and its implication in biotechnological applications is the fact that the operating route strongly influences ATP and co-factor balance of the cell. Replacing a native anaplerotic reaction by an alternative one may therefore help to improve product yields (Zelle et al., 2011; Zelle et al., 2010). This approach would also be interesting for fermentative production of compounds whose synthesis require an increased ATP yield, e.g. target products whose export from the cells require cellular energy.

6. Fermentation of the 'non-fermentable' carbon source glycerol for chemical production

It is obvious that the exploitation of glycerol's reducing power for the production of small molecules requires that fermentation is the predominant route of carbon and electrons rather than respiration. Certain wild-type bacteria are able to anaerobically ferment glycerol and naturally produce reduced fermentation products such as 1,3-propanediol (Celinska, 2010). However, glycerol has always been considered a 'non-fermentable' (or respiratory) carbon source in *S. cerevisiae* (Schüller, 2003; Turcotte et al., 2010). This is also reflected by the fact that glycerol-based medium has generally been used to select strains for a respiration-deficient phenotype (Hampsey, 1997). A fermentative metabolism of glycerol (i.e. ethanol production) has even not been observed in an evolved *S. cerevisiae* strain which showed a significantly improved glycerol utilization in synthetic medium i.e. a

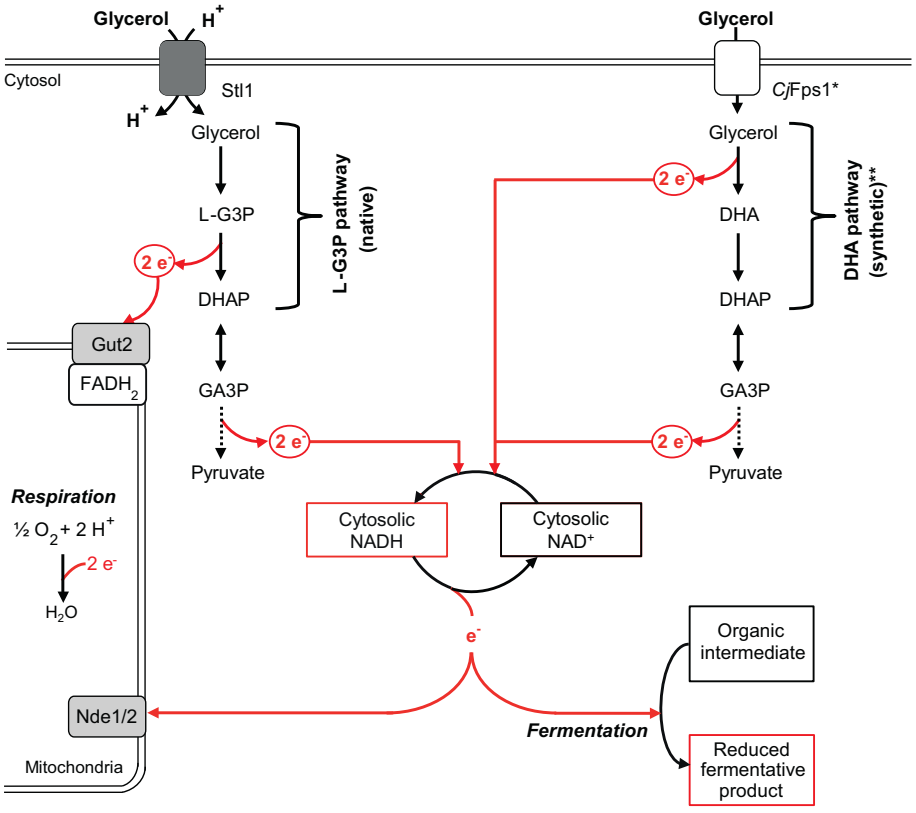


Fig. 2. Fate of electrons originating from glycerol if i) the native L-G3P pathway or ii) the engineered DHA pathway is used for glycerol catabolism. The DHA pathway allows the capturing of more electrons from glycerol in the form of cytosolic NADH. In theory, electrons from cytosolic NADH can subsequently either enter the respiratory chain (e.g. via Nde1/2) resulting in the formation of H_2O or be transferred to metabolic intermediates resulting in the formation of reduced fermentation products. *In contrast to overexpression of *S. cerevisiae*'s endogenous *FPS1* gene, the expression of a heterologous Fps1 homologue such as the one from *Cyberlindnera jadinii* (*Cj*Fps1) has been demonstrated to improve glycerol utilization in the CBS 6412-13A background (Klein et al., 2016b). **The synthetic DHA pathway consists of an NAD^+-dependent glycerol dehydrogenase from *Ogataea parapolymorpha* and the overexpression of the endogenous *DAK1* gene. The respective 'DHA pathway' strains also carry a deletion of the endogenous *GUT1* gene to abolish the native L-G3P pathway (Klein et al., 2016a). Abbreviations: L-G3P: L-glycerol 3-phosphate, DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, GA3P: glyceraldehyde 3-phosphate, Gut2: FAD-dependent glycerol 3-phosphate dehydrogenase, Stl1: glycerol/H^+ symporter, Nde1/2: mitochondrial external NADH dehydrogenase.

maximum specific growth rate of up to 0.2 h^{-1} (Ochoa-Estopier et al., 2011).

A closer look at the native pathway for glycerol catabolism in *S. cerevisiae* (L-G3P pathway) reveals that it is oxygen-dependent since two electrons from glycerol are directly transferred to the mitochondrial respiratory chain via the FAD-dependent Gut2 enzyme (Fig. 2). This respiratory route of the electrons is counterproductive in the view of generating valuable fermentation products from glycerol. We therefore recently replaced the endogenous L-G3P pathway by an artificial NAD^+-dependent pathway (the so-called DHA pathway) allowing the entrapment of the respective electrons in the form of cytosolic NADH (Klein et al., 2016a), and thus facilitating the potential formation of fermentation products (Fig. 2). Employing a CRISPR/Cas9 based strategy, the pathway swapping was achieved in various *S. cerevisiae* strains via a single transformation step. To our surprise, the DHA pathway even allowed growth on glycerol of wild-type strains originally unable to use glycerol in synthetic medium including a laboratory strain of the popular CEN.PK family (Klein et al., 2016a).

The first fermentation product that has been generated from synthetic glycerol medium by an engineered *S. cerevisiae* strain is 1,2-propanediol (1,2-PDO). A recent study of Islam et al. (2017) reported the conversion of ca. 45 g L^{-1} glycerol to 3.2 g L^{-1} 1,2-PDO. A significant amount of the natural fermentation product ethanol was also produced (8 g L^{-1}). The same study also demonstrated that the replacement of the L-G3P pathway for glycerol catabolism by the DHA pathway was a major prerequisite for the generation of 1,2-PDO from glycerol. With regard to directing the carbon and the electrons from glycerol towards reduced small molecules (Fig. 2), there are still a number of challenges to be overcome, particularly controlling the flux distribution between respiration and fermentation. In fact, electrons captured in the form of cytosolic NADH can enter the mitochondrial respiratory chain as long as oxygen is available. *S. cerevisiae* exhibits an external NADH dehydrogenase encoded by the genes *NDE1* and *NDE2* (Fig. 2). Particularly the isoenzyme Nde1 seems to have an important function in the regeneration of cytosolic NAD^+ when *S. cerevisiae* grows in synthetic glycerol medium in shake flask cultures (Asskamp et al., 2019).

One has to note, that our previous studies included the heterologous expression of an aquaglyceroporin contributing to glycerol uptake (Islam et al., 2017; Klein et al., 2016b). The presence of this type of channel protein such as Fps1 from *C. jadinii* (Fig. 2) increased the maximum specific growth rate on glycerol of strain CBS 6412-13A from 0.13 to 0.18 h^{-1} (Klein et al., 2016b). In contrast, no improvement was visible in CBS 6412-13A after overexpression of *S. cerevisiae FPS1*. These results match the previous finding of Liu et al. (2013) that only an Fps1 homologue from another yeast species can complement a deletion of *STL1*. It is possible that the increased rate of glycerol utilization (and glycolytic flux) caused by an improved influx of glycerol via the heterologous *Cj*Fps1 was crucial for the fact that glycerol is not fully respired anymore in a strain that exclusively uses the DHA pathway (Fig. 2).

Although we see the major industrial attractiveness of glycerol in its fermentative conversion to reduced small molecules, there is also an advantage of glycerol when used by a fully respiratory metabolism (i.e. in wild-type cells). Glycerol does not exert the so-called Crabtree effect as known from glucose (Crabtree, 1929). This favors the production of biomass and biomass-related products as discussed by Ochoa-Estopier et al. (2011). Baker's yeast strains able to efficiently utilize glycerol in a fully respiratory manner might therefore also be of great interest for industry.

When discussing glycerol as a carbon source for industrial processes, one cannot ignore the fact that the current and future biodiesel industry has to face a number of uncertainties due to changing crude oil prices, politics and governmental priorities, as recently reviewed by Naylor and Higgins (2017). It would be advisable to explore novel routes for the efficient direct or indirect generation of glycerol from carbon dioxide in order to provide a sustainable supply of this valuable carbon source.

7. Concluding remarks

As illustrated here by numerous examples, there have been a number of uncertainties with regard to the central carbon fluxes active during growth on glycerol as the sole source of carbon. They mainly result from the fact that the laboratory strains commonly used in fundamental research are unable to utilize glycerol in synthetic medium and useful strains for such experiments have not been available until recently. In fact, the previously isolated wild-type as well as evolved and reverse engineered *S. cerevisiae* strains able to grow in synthetic glycerol medium will facilitate more fundamental studies in synthetic glycerol medium in order to fill the knowledge gaps with regard to the central carbon catabolism of glycerol. Particularly, a ^{13}C metabolic flux analysis might help to identify the actual metabolic fluxes active under the relevant conditions. This information together with a profound knowledge about the active anaplerotic reactions, the localization of (iso)enzymes and the transporters active in synthetic glycerol medium will facilitate the efficient engineering of *S. cerevisiae* for the production of useful chemicals from glycerol. Importantly, glycerol is not per se a 'non-fermentable' carbon source for *S. cerevisiae.* As demonstrated for 1,2-propandiol, fermentation of glycerol into useful small molecules via fermentative routes seems to be possible as soon as the native L-G3P pathway for glycerol catabolism is replaced by the DHA pathway. The results are very encouraging with regard to the exploitation of glycerol's higher reducing power in baker's yeast-based fermentative processes. Therefore, it will be important to also include DHA-pathway strains into fundamental studies regarding the central metabolic fluxes active on glycerol.

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