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Replacement of the *Saccharomyces cerevisiae* acetyl-CoA synthetases by alternative pathways for cytosolic acetyl-CoA synthesis ☆

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ABSTRACT

Cytosolic acetyl-coenzyme A is a precursor for many biotechnologically relevant compounds produced by *Saccharomyces cerevisiae*. In this yeast, cytosolic acetyl-CoA synthesis and growth strictly depend on expression of either the Acs1 or Acs2 isoenzyme of acetyl-CoA synthetase (ACS). Since hydrolysis of ATP to AMP and pyrophosphate in the ACS reaction constrains maximum yields of acetyl-CoA-derived products, this study explores replacement of ACS by two ATP-independent pathways for acetyl-CoA synthesis. After evaluating expression of different bacterial genes encoding acetylating acetaldehyde dehydrogenase (A-ALD) and pyruvate-formate lyase (PFL), *acs1Δ acs2Δ S. cerevisiae* strains were constructed in which A-ALD or PFL successfully replaced ACS. In A-ALD-dependent strains, aerobic growth rates of up to 0.27 h^{-1} were observed, while anaerobic growth rates of PFL-dependent *S. cerevisiae* (0.20 h^{-1}) were stoichiometrically coupled to formate production. In glucose-limited chemostat cultures, intracellular metabolite analysis did not reveal major differences between A-ALD-dependent and reference strains. However, biomass yields on glucose of A-ALD- and PFL-dependent strains were lower than those of the reference strain. Transcriptome analysis suggested that reduced biomass yields were caused by acetaldehyde and formate in A-ALD- and PFL-dependent strains, respectively. Transcript profiles also indicated that a previously proposed role of Acs2 in histone acetylation is probably linked to cytosolic acetyl-CoA levels rather than to direct involvement of Acs2 in histone acetylation. While demonstrating that yeast ACS can be fully replaced, this study demonstrates that further modifications are needed to achieve optimal *in vivo* performance of the alternative reactions for supply of cytosolic acetyl-CoA as a product precursor.

1. Introduction

The robustness of *Saccharomyces cerevisiae* in industrial fermentation processes, combined with fast developments in yeast synthetic biology and systems biology, have made this microorganism a popular platform for metabolic engineering (Hong and Nielsen, 2012). Many natural and heterologous compounds whose production from sugars is under investigation or already implemented in industry require acetyl-coenzyme A (acetyl-CoA) as a key precursor. Examples of such products include *n*-butanol, isoprenoids, lipids and flavonoids (Dyer et al., 2002; Koopman et al., 2012; Shiba et al., 2007; Steen et al., 2008; Veen and Lang, 2004).

Acetyl-CoA metabolism in *S. cerevisiae* is compartmented (Pronk et al., 1996; van Roermund et al., 1995). During respiratory growth on sugars, a substantial flux through acetyl-CoA occurs via the mitochondrial pyruvate dehydrogenase complex (Pronk et al., 1994). However, mutant analysis has shown that mitochondrial acetyl-CoA cannot meet the extramitochondrial requirement for acetyl-CoA in the yeast cytosol, which includes, for example, its use as a precursor for synthesis of lipids and lysine (Flikweert et al., 1999; van den Berg and Steensma, 1995). In this respect, it is relevant to note that *S. cerevisiae* does not contain ATP-citrate

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lyase, an enzyme that plays a major role in translocation of acetyl-CoA across the mitochondrial membrane in mammalian cells and in several non-*Saccharomyces* yeasts (Boulton and Ratledge, 1981). When sugars are used as the carbon source, cytosolic acetyl-CoA synthesis in *S. cerevisiae* occurs via the concerted action of pyruvate decarboxylase (Pdc1, 5 and 6), acetaldehyde dehydrogenase (Ald2, 3, 4, 5 and 6) and acetyl-CoA synthetase (Acs1 and 2) (Pronk et al., 1996). Heterologous, acetyl-CoA-dependent product pathways expressed in the *S. cerevisiae* cytosol exclusively depend on this 'pyruvate dehydrogenase bypass' for provision of acetyl-CoA. Indeed, overexpression of acetyl-CoA synthetase (ACS) from *Salmonella enterica* has been shown to lead to increased productivities of the isoprenoid amorphadiene by engineered *S. cerevisiae* strains (Shiba et al., 2007).

The ACS reaction involves the hydrolysis of ATP to AMP and pyrophosphate (PP_i):

$$\text{Acetate} + \text{ATP} + \text{CoA} \rightarrow \text{Acetyl} - \text{CoA} + \text{AMP} + \text{PP}_i$$

Together with the subsequent hydrolysis of PP_i to inorganic phosphate (P_i), this ATP consumption is equivalent to the hydrolysis of 2 ATP to 2 ADP and $2P_i$. The resulting ATP expenditure for acetate activation can have a huge impact on the maximum theoretical yields of acetyl-CoA derived products. For example, the production of a C16 lipid from sugars requires 8 acetyl-CoA, whose synthesis via ACS requires 16 ATP. At an effective P/O ratio of respiration in *S. cerevisiae* of 1 (Verduyn, 1991), this ATP requirement for acetyl-CoA synthesis corresponds to 1 mol of glucose that needs to be respired for the synthesis of 1 mol of product.

In addition to the pyruvate-dehydrogenase complex, other reactions have been described in nature that enable the ATP-independent conversion of pyruvate into acetyl-CoA (Powlowski et al., 1993; Rudolph et al., 1968; Smith and Kaplan, 1980). Many prokaryotes contain an acetylating acetaldehyde dehydrogenase (A-ALD; EC 1.2.1.10) which catalyses the reversible reaction:

$$\text{Acetaldehyde} + \text{NAD}^+ + \text{CoA} \leftrightarrow \text{Acetyl} - \text{CoA} + \text{NADH} + \text{H}^+.$$

Although functional expression of bacterial genes encoding A-ALD in *S. cerevisiae* has been described in the literature, these studies focused on reductive conversion of acetyl-CoA to ethanol as part of a phosphoketolase pathway for pentose fermentation (Sonderegger et al., 2004) or as part of a metabolic engineering strategy to convert acetic acid to ethanol (Guadalupe Medina et al., 2010). Complete replacement of the native acetaldehyde dehydrogenases and/or ACS of *S. cerevisiae* by A-ALD, thereby bypassing ATP hydrolysis in the ACS reaction, has not been demonstrated.

In many anaerobic bacteria and some eukaryotes (Stairs et al., 2011), pyruvate can be converted into acetyl-CoA and formate in the non-oxidative, ATP-independent reaction catalysed by pyruvate-formate lyase (PFL; EC 2.3.1.54):

$$\text{Pyruvate} + \text{CoA} \leftrightarrow \text{Acetyl} - \text{CoA} + \text{Formate}.$$

PFL and PFL-activating enzyme (PFL-AE; EC 1.97.1.4) from *Escherichia coli* have previously been expressed in *S. cerevisiae* (Waks and Silver, 2009). Although formate production by this oxygen-sensitive enzyme system was demonstrated in anaerobic yeast cultures, its impact on cytosolic acetyl-CoA metabolism has not been investigated.

To gain the full potential benefit of ATP-independent cytosolic acetyl-CoA synthesis, the implemented heterologous pathways expressed in *S. cerevisiae* should, ideally, completely replace the ACS reaction. In wild-type strain backgrounds, deletion of both *ACS1* and *ACS2* is lethal (van den Berg and Steensma, 1995) and, during batch cultivation on glucose, presence of a functional *ACS2* gene is essential (van den Berg and Steensma, 1995) because *ACS1* is subject to glucose repression (van den Berg et al., 1996) and its product is inactivated in the presence of glucose (de Jong-Gubbels et al., 1997). Moreover, it has been proposed that Acs2, which was demonstrated to be partially localized in the yeast nucleus, is involved in histone acetylation (Takahashi et al., 2006). Involvement of Acs isoenzymes in the acetylation of histones and/or other proteins might present an additional challenge in replacing them with heterologous reactions, if this includes another mechanism than merely the provision of extramitochondrial acetyl-CoA.

The goal of this study is to investigate whether the heterologous ATP-independent A-ALD and PFL pathways can support the growth of *acs1 acs2* mutants of *S. cerevisiae* by providing extramitochondrial acetyl-CoA and to study the impact of such an intervention on growth, energetics and cellular regulation. To this end, several heterologous genes encoding A-ALD and PFL were screened by expression in appropriate yeast genetic backgrounds, followed by detailed analysis of *S. cerevisiae* strains in which both *ACS1* and *ACS2* were replaced by either of the alternative reactions. The resulting strains were studied in batch and chemostat cultures. Furthermore, genome-wide transcriptional responses to these modifications were studied by chemostat-based transcriptome analysis of engineered and reference strains.

2. Methods

2.1. Strains and maintenance

The *S. cerevisiae* strains used in this study (Table 1) share the CEN.PK genetic background (Entian and Kotter, 2007; Nijkamp et al., 2012). Stock cultures were grown aerobically in synthetic medium (Verduyn et al., 1992). When required, auxotrophic requirements were complemented with synthetic yeast drop-out medium supplements (Sigma-Aldrich, St. Louis, MO, USA) or by growth in YP medium (demineralized water, 10 g L^{-1} Bacto yeast extract, 20 g L^{-1} Bacto peptone). Carbon sources were either 20 g L^{-1} glucose, 2% (v/v) ethanol and/or 11.3 g L^{-1} sodium acetate trihydrate. Stock cultures of *S. cerevisiae* strains IMZ383 and IMZ384 were grown anaerobically and supplemented with Tween-80 (420 mg L^{-1}) and ergosterol (10 mg L^{-1}) added to the medium. Frozen stocks of *S. cerevisiae* and *E. coli* were prepared by the addition of glycerol (30% v/v) to the growing shake-flask cultures and aseptically storing 1 mL aliquots at −80 °C.

2.2. Plasmid construction

Coding sequences of *Staphylococcus aureus adhE* (NP_370672.1), *Escherichia coli eutE* (YP_001459232.1) and *Listeria innocua lin1129* (NP_470466) were codon-optimized for *S. cerevisiae* with the JCat algorithm (Grote et al., 2005). Custom-synthesized coding sequences cloned in the pMA vector (Table 2) were provided by GeneArt GmbH (Regensburg, Germany). Gateway Cloning technology (Invitrogen, Carlsbad, A) was used to insert these coding sequences in the intermediate vector pDONR221 (BP reaction) and subsequently into pAG426-pGPD (Alberti et al., 2007) (LR reaction). The resulting plasmids pUDE150 to pUDE152 were transformed into *E.* coli and their sequences checked by Sanger sequencing (BaseClear BV, Leiden, The Netherlands).

PFL- and PFL-AE-encoding sequences from *Thalassiosira pseudonana* (Genbank accession no. XM_002296598.1 and XM_002296597.1), *Chlamydomonas reinhardtii* (AJ620191.1 and AY831434.1), *E. coli* (X08035.1), *Lactobacillus plantarum* (YP_003064242.1 and YP_003064243.1) and *Neocallimastix frontalis* (AY500825.1 and AY500826.1) were codon-optimized for expression in *S. cerevisiae* and signal sequences, predicted by TargetP, were removed (Emanuelsson et al., 2007). Expression cassettes in which these coding sequences were flanked by the *TPI1* promoter