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RESEARCH ARTICLE

Ach1 is involved in shuttling mitochondrial acetyl units for cytosolic C2 provision in Saccharomyces cerevisiae lacking pyruvate decarboxylase

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One sentence summary: Saccharomyces cerevisiae lacking pyruvate decarboxylase uses acetate instead of citrate to transfer acetyl units from the mitochondria to the cytosol.

Editor: Hyun Ah Kang

ABSTRACT

Acetyl-coenzyme A (acetyl-CoA) is not only an essential intermediate in central carbon metabolism, but also an important precursor metabolite for native or engineered pathways that can produce many products of commercial interest such as pharmaceuticals, chemicals or biofuels. In the yeast Saccharomyces cerevisiae, acetyl-CoA is compartmentalized in the cytosol, mitochondrion, peroxisome and nucleus, and cannot be directly transported between these compartments. With the acetyl-carnitine or glyoxylate shuttle, acetyl-CoA produced in peroxisomes or the cytoplasm can be transported into the cytoplasm or the mitochondria. However, whether acetyl-CoA generated in the mitochondria can be exported to the cytoplasm is still unclear. Here, we investigated whether the transfer of acetyl-CoA from the mitochondria to the cytoplasm can occur using a pyruvate decarboxylase negative, non-fermentative yeast strain. We found that mitochondrial Ach1 can convert acetyl-CoA in this compartment into acetate, which crosses the mitochondrial membrane before being converted into acetyl-CoA in the cytosol. Based on our finding we propose a model in which acetate can be used to exchange acetyl units between mitochondria and the cytosol. These results will increase our fundamental understanding of intracellular transport of acetyl units, and also help to develop microbial cell factories for many kinds of acetyl-CoA derived products.

Keywords: acetyl-CoA; central carbon metabolism; yeast; mitochondria

INTRODUCTION

Acetyl-CoA is recognized as one of the most central molecules in the metabolism of most known organisms. It functions as a link between anabolism and catabolism and is involved in various acetyl transfer reactions. In addition to essential roles in carbon and energy metabolism, it plays important regulatory roles in eukaryotic cells as the acetyl group donor in post-translational protein acetylation, e.g. of histones (Takahashi et al. 2006). Besides native cellular functions, the availability of nucleocytoplasmic acetyl-CoA has been shown to affect autophagy during yeast aging (Eisenberg et al. 2014). Also, acetyl-CoA metabolism and transport was reported to play an important role for full virulence in the fungal pathogen Candida albicans (Carman, Vylkova and Lorenz 2008). Furthermore, acetyl-CoA is a key precursor metabolite for a wide range of industrially important chemical products (Chen et al. 2013; Nielsen 2014), and there is much...
interest in engineering acetyl-CoA metabolism in connection with metabolic engineering and synthetic biology of yeast (David and Siewers 2015; Jensen and Keasling 2015; Redden, Morse and Alper 2015).

Of special interest, the acetyl-CoA metabolism in the yeast Saccharomyces cerevisiae has been well studied for both basic and applied interests (Krivoruchko et al. 2014; Nielsen 2014). In this fungus, acetyl-CoA is present in at least four different cellular compartments (cytosol, mitochondria, peroxisomes and nucleus) where it is either synthesized or transferred to specific shuttle systems (Fig. 1) (Chen, Siewers and Nielsen 2012). To date, it is well acknowledged that S. cerevisiae exhibits two routes for the transport of acetyl units (van Roermund et al. 1995). In one route, acetyl units in the form of acetyl-CoA enter the glyoxylic cycle to synthesize C4 dicarboxylic acids, which can be transported from the peroxisomes to the cytosol or from the cytosol to the mitochondria where they serve as substrate for the TCA cycle or gluconeogenesis, respectively. The other route is a carnitine-dependent route in which acetyl units in a form of acetyl-carnitine, can be transported to the cytosol and/or the mitochondria. S. cerevisiae is not capable of de novo synthesis of carnitine (van Roermund et al. 1999) and can therefore only use the carnitine-dependent route if carnitine is provided to the growth medium.

Yeast has been widely used as a model organism to study intracellular transport of acetyl units in eukaryotes (Strijbis and Distel 2010). However, it is not clear whether acetyl group equivalents can be transported from the mitochondrial matrix to the cytosol across the acetyl-CoA-impermeable mitochondrial inner membrane (van Roermund et al. 1995). It is widely accepted that almost all eukaryotes use the citrate/malate shuttle and/or citrate transporter to transfer acetyl units from the peroxisomes to the cytosol. In this shuttle, intramitochondrial acetyl-CoA is converted to citrate through reaction with oxaloacetate, which can be exported to the cytosol and then converted back to acetyl-CoA by cytosolic ATP-citrate lyase. However, ATP-citrate lyase that is widely present in fungi, is absent in S. cerevisiae (Hynes and Murray 2010). There is no experimental data that confirm either of the shuttle systems can be used to transfer acetyl units from the mitochondria to the cytosol. Therefore, the question remains whether acetyl-CoA formed in the mitochondrial matrix can be exported to the cytoplasm.

In yeast, acetyl-CoA in the cytosol is formed via the pyruvate dehydrogenase bypass route (Fig. 1), where pyruvate is decarboxylated (by pyruvate decarboxylase, Pdc) to acetaldehyde that is further converted to acetate. Since the availability of acetyl units in the cytosol is essential for the biosynthesis of lipids and certain amino acids (Prönk, Steensma and van Dijken 1996), Pdc negative strains require C2 compounds such as ethanol or acetate for growth (Flikweert et al. 1999). Furthermore, these strains are sensitive to high glucose concentrations. These limitations were partially solved by evolving a Pdc negative strain, resulting in a glucose-tolerant mutant having unknown mechanisms for provision of cytosolic acetyl-CoA (van Maris et al. 2004). In a later study, the mechanisms for growth in excess glucose were identified to be related to an in-frame internal deletion of MTH1, which is involved in transcriptional regulation of glucose sensing (Oud et al. 2012). The mutation in MTH1 results in reduced glucose uptake through attenuating the expression of genes encoding hexose transporters and hereby reducing the glucose uptake rate. However, it was not identified which mechanism ensured provision of cytosolic acetyl-CoA for lipid biosynthesis.

Here, we aimed at identifying the mechanism responsible for the exchange of acetyl units between the mitochondrial matrix and the cytoplasm in S. cerevisiae. Our results identified a route relying on ACh1 that could transfer acetyl units from the mitochondria to the cytoplasm. Based on our results, we propose a new model in which acetate can be used to exchange acetyl units between the mitochondria and cytosolic compartments.

**MATERIALS AND METHODS**

**Materials and strain handling**

All PCRs were performed with Phusion or DreamTaq DNA Polymerase from Thermo-Scientific (Waltham, MA, USA), or PrimeSTAR HS DNA Polymerase from Takara Bio Europe (Segeltorp, Sweden). Oligonucleotides were custom synthesized from Sigma-Aldrich (Stockholm, Sweden). Escherichia coli plasmid extraction, PCR product purification and DNA gel extraction were performed with kits from Thermo-Scientific (Waltham, MA, USA). Saccharomyces cerevisiae plasmid extraction was performed with a Zymoprep Yeast Plasmid Miniprep II from Zymo Research (Nordic Biolabs, Täby, Sweden). All restriction enzymes were purchased from Thermo-Scientific (St. Leon-Rot, Germany).

Escherichia coli DH5α was used for routine-cloning procedures and selection and maintenance of E. coli recombinant strains was performed in LB medium containing 10 g L⁻¹ of peptone, 10 g L⁻¹ of NaCl and 5 g L⁻¹ of yeast extract and supplemented with 100 µg mL⁻¹ of ampicillin sodium salt. Solid version of this medium also included 16 g L⁻¹ of agar. Recombinant yeast strains were selected for Ura⁺ phenotypes on synthetic dextrose (SD) medium containing 6.7 g L⁻¹ of yeast nitrogen base without amino acids (ForMedium, Hunstanton, UK), 0.77 g L⁻¹ of complete supplement mixture (lacking uracil) (ForMedium, Hunstanton, UK) and 20 g L⁻¹ of glucose. For KanMX marker selection the cells were cultivated in YPD medium, containing 10 g L⁻¹ yeast extract, 20 g L⁻¹ of peptone, 20 g L⁻¹ of glucose and supplemented with 200 mg L⁻¹ G-418 disulfate (ForMedium, Hunstanton, UK).
Strains were preserved for long-term storage after overnight cultivation in selective media by adding glycerol to a final concentration of 20% and stored at −80°C.

**Plasmid and strain construction**

All plasmids and strains used and constructed in this study are summarized in Table 1, as well as their main features and source. All primers used in this study are listed in Table 2.

The plasmid pSP-GM1 (Chen et al. 2012) was amplified from CEN.PK113–5D (Entian and Kötter 2007) genomic DNA using primers OEA˙UP/OEA˙DW (Table 2) and cloned into pSP-GM1 under the promoter TEF1, resulting in the plasmid pACH1 (Table 1). tACH1 (coding for the truncated version of Ach1) was amplified from CEN.PK113–5D genomic DNA using primers OEA˙tUP/OEA˙DW and cloned into pSP-GM1 using the same restriction sites BamHI/XhoI, forming the plasmid ptACH1.

Transformation of the plasmid pSP-GM1 into IMI076 resulted in the strain YACH00.

To delete the gene ACH1 in strain IMI076 (Oud et al. 2012), the coding region of this gene was replaced by the functional URA3 cassette. This was achieved by amplification of the upstream and downstream regions of ACH1 from the yeast genome using primer pairs AHF/UAUR and UADF/AHR, and the URA3 cassette from pSP-GM1 by primers AUF/AUR. All PCR products were fused together by primers AHF/AHR via fusion PCR, and then the linear DNA fragment was transformed into strain IMI076. Transformants were selected on SD plates and single-colony isolates were confirmed to have the correct chromosomal replacement by PCR using primers DPAF/DPAR. The resulting strain was named YACH01.

Complementation of the ACH1 gene deletion in strain YACH01 was done through integration of the ACH1 gene under control of the TEF1 promoter together with the KanMX marker gene into chromosome XI site No.3 (XI-3) (Mikkelsen et al. 2012). The upstream and downstream regions of the genome were amplified using primers AchIntUF/AchIntUR and AchIntDF/AchIntDR. The ACH1 cassette was amplified from the plasmid pACH1 using primers AchIntF/AchIntR and the KanMX cassette was cloned from the vector pUG6 (Guldener et al. 1996) by using primers AchIntKanF/KanInR, KanInF/AchIntKanR. The PCR products were fused together into two fragments by using primers AchIntF/KanInR and AchIntKanF/AchIntDR.

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<th>Table 1. Plasmids and strains used in this work.</th>
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two overlapping cassettes were transformed into strain YACH01 and the transformants were selected on YPD supplemented with 200 mg L$^{-1}$ G418. Single-colony isolates were confirmed to have the correct insert by PCR, and the resulting strain was named YACH02. Similarly, the truncated version of ACH1 gene was also introduced into strain YACH01 with the slight difference of amplifying the fragment from the plasmid pTACH1 instead of pACH1, but using the same primers. The obtained strain was named as YACH03.

**Cultivation procedures**

Pre-cultivations were performed in defined minimal medium with 2% (v/v) of ethanol. The minimal medium consisted of 7.5 g L$^{-1}$ of (NH$_4$)$_2$SO$_4$, 14.4 g L$^{-1}$ of KH$_2$PO$_4$, 0.5 g L$^{-1}$ of MgSO$_4$.7H$_2$O, 1 mL of a vitamin solution and 1 mL of a trace metal solution. The pH was adjusted to 6.5 with NaOH before autoclaving, as described by Verduyn et al. (1992). Cells for culture inoculation were harvested by centrifugation during exponential growth phase, and washed twice using sterile water.

Cultivations in 100 mL shake flasks were performed in triplicates with an initial OD$_{600}$ of 0.05 using minimal medium with 20 g L$^{-1}$ of glucose. Cultivations in Bioscreen C (Oy Growth Curves Ab Ltd, Finland) were performed in octuplicates at 30°C in a microplate (10 x 10 wells) with 200 μL minimal glucose medium per well. A total of 20 mg L$^{-1}$ uracil were added when required. For supplementation of the minimal medium with succinate, filter sterilized succinic acid (Merck, Hohenbrunn, Germany) solution was added to a final concentration of 0.5 g L$^{-1}$ at the beginning of the cultivations, resulting in a pH drop from 6.35 to 6.19. When the minimal medium was supplemented with UK-5099, UK-5099 was first dissolved in dimethyl sulfoxide (DMSO), and then added to a final concentration of 200 mM during the exponential growth phase, while the same amount of DMSO was added to the control.

Spot assays were performed as described before (Chen et al. 2012). Cells were grown in SD-ura medium with 2% (v/v) ethanol at 30°C. The cells were harvested and washed twice with sterile water. After measuring the OD$_{600}$ serial dilutions with final cell concentrations of 1 x 10$^6$, 1 x 10$^5$, 1 x 10$^4$, 1 x 10$^3$ cells per mL were prepared. From each dilution, 4 μL was spotted on synthetic medium agarose plates with 20 g L$^{-1}$ glucose or 20 g L$^{-1}$ glucose supplemented with 0.3% (v/v) ethanol.

**Analytical methods**

Biomass was determined by measuring optical density at 600 nm (OD$_{600}$) with a GENESYS 20 Visible Spectrophotometer (Thermo Electron Scientific, Madison, USA). Samples from liquid cultures were taken and filtered using syringe nylon filters with a pore size of 0.45 μm into HPLC vials and stored at −20°C until being analyzed. HPLC analysis was performed in a Dionex Ultimate 3000 Systems using a refractive index detector (Shodex RI-101; Showa Denko, Tokyo, Japan) and a UV detector set at 210 nm (Ultimate 3000 VWD from Dionex, Sunnyvale, CA USA). The samples were analyzed using a Bio-Rad HPX 87H Column (Hercules, USA), which was kept at 45°C, 5 mM H$_2$SO$_4$ was used as the mobile phase with a flow rate of 0.6 mL min$^{-1}$. Quantitative analysis of glucose, pyruvate, glycerol, acetate and ethanol was performed by injecting a mixture of standards with known concentrations of each metabolite. Calibration curves were calculated using the peak areas of the RI detector for glucose, glycerol and ethanol and of the UV detector for pyruvate and acetate.

**RESULTS**

**Possible alternative routes for acetyl-CoA synthesis**

In yeast, the production of acetyl-CoA in the cytosol is vital for the synthesis of important cellular components such as lipids and certain amino acids. A Pdc negative strain, which lacks the cytosolic route to acetyl-CoA via acetaldehyde (Fig. 1) when cells grow on glucose as the sole carbon source, is therefore a good platform strain to study alternative routes for acetyl-CoA synthesis in the cytosol. First, we searched the latest genome scale metabolic model of S. cerevisiae (Osterlund et al. 2013). In this model, 34 reactions are directly related with acetyl-CoA metabolism and transport. As C$_2$ compounds such as ethanol, acetaldehyde and acetate are also associated with acetyl-CoA production, 23 additional reactions involved in C$_2$ compound metabolism were also included in this initial analysis.

When analyzing the possible role of these S7 reactions (Table S1, Supporting Information) in providing cytosolic acetyl-CoA in a Pdc negative strain two possible routes were identified for provision of C$_2$ compounds/acetyl-CoA in the absence of carnitine. One possible route is the catabolism of threonine via threonine aldolase Gly1 to release acetaldehyde, which can be converted to acetyl-CoA via acetate in the cytosol. Although it has been shown that overexpression of GLY1 can circumvent the essential biosynthetic role of Pdc in glucose-limited chemostat cultures of S. cerevisiae (van Maris et al. 2003), Gly1 as a possible source for cytosolic acetyl-CoA production was excluded due to its low affinity for threonine and the relatively low intracellular threonine concentration when yeast is grown at excess glucose (van Maris et al. 2004).

The other potential route is conversion of acetyl-CoA to acetate in the mitochondria, followed by transport of acetate across the mitochondrial inner membrane to the cytosol where it can be converted into acetyl-CoA by cytosolic acetyl-CoA synthetase (ACS). One gene product, encoded by ACH1, is associated with acetyl-CoA and acetate in the mitochondria, although the actual function of Ach1 is not conclusive. It was originally proposed to be an acetyl-CoA hydrolase to catalyze the scission of acetyl-CoA into acetate and CoA or to be involved in acetate utilization (Lee, Lin and Smith 1989, 1990, 1996; Buu, Chen and Lee 2003). Later, it was however characterized as a CoA transferase and as being involved in mitochondrial acetate detoxification (Fleck and Brock 2009). In this study, Ach1 showed the highest specific activity for the CoA transfer from succinyl-CoA to acetate in vitro, but the substrate promiscuity of this enzyme did not exclude CoA transferase activity on additional substrates. We therefore proposed that this enzyme can transfer the CoA from acetyl-CoA to succinate with the formation of acetate and succinyl-CoA in a Pdc negative strain with the above mentioned MTH1 internal deletion.

**Addition of succinate improves the growth of MTH1-ΔT strain IMI076**

To test our assumption, we firstly evaluated the impact of adding succinate to the medium of the Pdc negative strain IMI076 with an internal deletion of MTH1 (MTH1-ΔT, see Oud et al. 2012) and the control strain CEN.PK113–5D. The cultivations were tested in a microplate incubator/reader. By addition of 0.5 g L$^{-1}$ succinate to the medium, there was no obvious difference in the growth of the control strain (Fig. 2A), but it was clear that external succinate supplementation decreased the length of the lag phase for the IMI076 strain. This was further shown by the cultivation of strain IMI076 in shake flasks supplemented with succinate. As
Figure 2. Addition of succinate improves the growth of strain IMI076 (Pdc− MTH1− ΔT) in microplate (A) and shake flask (B) cultivations. Cells were grown in defined minimal medium with 20 g L−1 glucose. Supplementation with 0.5 g L−1 of succinate was performed before inoculation. The control strain in (A) was CEN.PK113–5D. Data points are from one representative experiment of at least three replicates. Specific growth rates for the other replicates were similar when cultivated with (0.074 ± 0.002 h−1) or without (0.066 ± 0.001 h−1) addition of succinate.

Figure 3. The growth of strain IMI076 (Pdc− MTH1− ΔT) relies on Ach1. (A) Growth assays on solid synthetic medium with 20 g L−1 glucose or 20 g L−1 glucose plus 0.3% (v/v) ethanol. The plates were incubated at 30°C and recorded photographically 4 days after inoculation. (B) Growth assays in defined liquid minimal media with 20 g L−1 glucose as the sole carbon source. The strains used were: IMI076 (Pdc− MTH1− ΔT), YACH00 (IMI076, empty plasmid pSP-GM1), YACH01 (IMI076 ach1::URA3). Data for liquid cultures are from one representative experiment of three replicates. For strain IMI076, all replicates had identical specific growth rates of 0.066 ± 0.001 h−1.

shown in Fig. 2B, the lag phase was substantially shortened, and the maximum specific growth rate increased by 13%. This was consistent with observed increased rates of glucose consumption and pyruvate accumulation (data not shown).

The growth of MTH1− ΔT strain IMI076 relies on Ach1

Next, in order to test the hypothesis that Ach1 is a key player in channeling acetyl units from the mitochondrial matrix to the cytoplasm the Ach1 gene was deleted by replacing it with a functional URA3 cassette in strain IMI076 (Pdc− MTH1− ΔT ura3, Oud et al. 2012). As control, the parental Pdc negative strain was transformed with empty plasmid pSP-GM1 (Chen et al. 2012) containing the same URA3 cassette, resulting in the strain YACH00.

As previously reported, the unevolved Pdc negative strain carrying the MTH1− ΔT allele (strain IMI076) grew on synthetic medium agarose plates with glucose as sole carbon source supplemented with uracil (Fig. 3A). There was no major difference in growth between this parental strain and the strain expressing URA3 (strain YACH00). However, the growth of the Ach1 deletion strain YACH01 was slightly impaired as shown in Fig. 3A. All strains grew well on glucose supplemented with ethanol, and there was no difference between strains with or without Ach1 deletion (Fig. 3A).

The growth in absence of Ach1 on plates could be an effect of the medium used for the solid growth assays, i.e. this could contain threonine or there could be C2 contamination as speculated by Oud et al. (2012). To exclude any potential C2 contamination in the medium growth was also investigated in liquid cultures using defined minimal medium (Verduyn et al. 1992). Cells of IMI076 and the ach1 mutant YACH01 were washed twice after pregrowth in synthetic ethanol media, and then transferred to a minimal glucose media. Strain IMI076 grew as described before (Oud et al. 2012), with a specific growth rate of 0.066 ± 0.001 h−1, whereas the ach1 mutant YACH01 could not grow on glucose
as the sole carbon source (Fig. 3B), clearly showing that ACH1 is essential for growth of the IMI076 strain.

**Complementation of ACH1 but not a truncated version restores growth of the ach1 mutant**

The inability of growth on glucose as sole carbon source in absence of Ach1 (Fig. 3B) points to that this enzyme being responsible for transferring acetyl units from the mitochondria to the cytosol. To further confirm this hypothesis, we performed complementation of the ach1 deletion strain with both the wild-type gene and a truncated version of ACH1, where the N-terminus of Ach1 was removed which is reported to result in redirection of this protein to the cytoplasm (Buu, Chen and Lee 2003). ACH1 with its N-terminal region being deleted or the entire protein coding gene was reintroduced into the ach1 mutant by chromosomal integration. Growth assays of the resulting strains showed that complementation with the complete ACH1 gene could restore growth of the deletion mutant, whereas complementation with the truncated version could not restore growth of the ach1 mutant (Fig. 4). The maximum specific growth rate of the ACH1 complemented strain (YACH02) was increased by 51% compared with the control strain IMI076, which may be ascribed to increased expression in the complementation strain, where ACH1 was expressed under control of the strong TEF1 promoter.

**Inhibition of the mitochondrial pyruvate carrier retards the growth of the IMI076 strain**

The compound UK-5099, an analogue of o-cyanocinnamate, is known as a specific and potent inhibitor of the mitochondrial pyruvate carrier (Halestrap 1975; Bricker et al. 2012). Uptake of pyruvate into the mitochondria in yeast is reduced by about 70% in presence of 0.2 mM UK-5099 compared to no presence of the inhibitor (Bricker et al. 2012). The sole source of acetyl-CoA in the mitochondria is through the oxidative decarboxylation of pyruvate, catalyzed by the pyruvate dehydrogenase complex. Reduction of mitochondrial pyruvate uptake will therefore limit the availability of acetyl units in this compartment. This will further restrict the supply of acetyl-CoA to the cytoplasm and may therefore affect cell growth. To further validate if the cytosolic acetyl-CoA in IMI076 is likely coming from mitochondrial acetyl-CoA, we therefore cultivated this strain in absence or presence of UK-5099. When the cells entered the exponential growth phase 0.2 mM UK-5099 was added to the culture (Fig. 5). This resulted in a significant decrease in growth compared with the control experiment where no inhibitor was added. While there is no significant effect on the growth of the control strain (CEN.PK 113–5D) when supplemented with 0.2 mM UK-5099 (data not shown), the maximum specific growth rate of strain IMI076 decreased in presence of UK-5099, from 0.066 ± 0.001 to 0.018 ± 0.002 h⁻¹. These observations clearly indicate that the flux of mitochondrial pyruvate uptake is limiting cell growth, which again supports the hypothesis that cytosolic acetyl-CoA is derived from mitochondrial acetyl-CoA.

**DISCUSSION**

A fundamental feature of most eukaryotes is the existence of different compartments and the flow of carbon metabolites between subcellular compartments. One of the key intermediates in cellular metabolism is acetyl-CoA, as it plays an essential role in carbon and energy metabolism. While acetyl-CoA metabolism has been extensively studied in the budding yeast S. cerevisiae, it is still not known whether acetyl-CoA produced in the mitochondria can be transported to the cytosol. To address this fundamental question, we used a recently reported yeast strain IMI076 with a disruption of Pdc genes together with an internal deletion of MTH1, which enables cell growth in conditions of excess glucose (Oud et al. 2012). Using this Pdc-deficient strain (IMI076), that lacks the first step of the pyruvate dehydrogenase bypass pathway involving Pdc, acetaldehyde dehydrogenase and ACS, and hereby exclude the normal route for acetyl-CoA biosynthesis.

Through analysis of a recent genome scale metabolic model, we identified a putative route for acetyl-CoA biosynthesis, namely formation of acetate in the mitochondrial matrix followed by transport of acetate to the cytosol where ACS can convert it to acetyl-CoA. In this route, Ach1 forms acetate from acetyl-CoA in the mitochondria. Ach1 has originally been recognized as a hydrolase catalyzing the hydrolysis of acetyl-CoA.
to acetate and CoA (Lee, Lin and Smith 1989), like many other acetyl-CoA hydrolases found in mammalian tissues (Gergely, Hele and Ramakrishnan 1952; Knowles et al. 1974). The exact catalytic role of this enzyme was later questioned by the observations of its role in acetate but not ethanol utilization (Lee, Lin and Smith 1996). It was also proposed that this enzyme may have a novel function concerning acetyl-CoA metabolism, but it was not until recently, when Fleck and Brock characterized Ach1 as a CoA transferase not just wasting energy by hydrolyzing acetyl-CoA (Fleck and Brock 2009) that new insight was provided on this enzyme.

Inspired by the function as a CoA transferase and the promiscuity of this enzyme on its substrates, we hypothesized that Ach1 could transfer CoA from acetyl-CoA to succinate. From the supplementation results, we observed that addition of succinate improves growth of the strain IMI076, but has no impact on the growth of the control strain (Fig. 2). Next, by disruption of ACH1 in strain IMI076, the growth on glucose was abolished (Fig. 3B), which clearly indicated that Ach1 plays an essential role in the Pdc-deficient strain for growth on glucose. Finally, complementation with the ACH1 gene under control of the strong TEF1 promoter, not only rescued growth on glucose but resulted in slightly faster growth than observed for the IMI076 strain (Fig. 4). Further confirming that Ach1 is involved in using mitochondrial acetyl-CoA was our finding that a truncated version of Ach1 that is targeted to the cytosol could not rescue growth of an ach1 deletion in IMI076 and that inhibition of the mitochondrial pyruvate carrier reduced growth of IMI076.

Based on these results, we propose the model of acetyl-CoA metabolism for the Pdc-deficient strain shown in Fig. 6. When cells grow on glucose acetyl-CoA produced in the mitochondria can be converted to acetate potentially through acetate:succinyl-CoA transferase Ach1. The acetate formed in the mitochondria crosses the membrane to the cytosol, where it is to be converted into acetyl-CoA for ensuring the provision of cytosolic acetyl-CoA for the production of lipids and amino acids. Therefore result in decreased repression of ACH1 expression. As shown in Fig. 6, Ach1 has to be localized in the mitochondria to enable the channeling of acetyl units from the mitochondria to the cytosol under growth on glucose. This is consistent with a previous study that showed that most Ach1 was distributed in the mitochondria by immunofluorescence microscopy (Buu, Chen and Lee 2003).

In conclusion, we here show that a Pdc negative S. cerevisiae strain under glucose derepressed conditions uses acetate instead of citrate to transfer acetyl units from the mitochondria to the cytosol. This alternative shuttle route has been reported in other eukaryotes, e.g. in the procyclic stage of Trypanosoma brucei (Riviere et al. 2009). In this transfer system acetate is produced in the mitochondrial matrix from acetyl-CoA by Ach1. The alternative function of Ach1 in exporting acetyl units from the mitochondria does not contradict its possible function in acetate detoxification as suggested earlier (Lee, Lin and Smith 1996; Fleck and Brock 2009) as the CoA transfer is likely a reversible reaction. The transferase activity of Ach1 could be potentially useful for designing yeast as a microbial cell factory to produce acetyl-CoA derived products. For instance, the function of Ach1 in shuffling mitochondrial acetyl units for provision of cytosolic acetate should be considered when trying to establish heterologous pathways for cytosolic acetyl-CoA supply.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Conflict of interest. None declared.

REFERENCES


Van Roermund CWT, Elgersma Y, Singh N, et al. The membrane of peroxisomes in Saccharomyces cerevisiae is impermeable to NAD(H) and acetyl-CoA under in vivo conditions. EMBO J 1995;14:3480–6.