Process Biochemistry 46 (2011) 1436–1441

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

# Oleaginous yeast *Yarrowia lipolytica* mutants with a disrupted fatty acyl-CoA synthetase gene accumulate saturated fatty acid

Jinjing Wang[a,b], Borun Zhang[a], Shulin Chen[b,*]

[a] The Laboratory of Molecular Genetics and Breeding of Yeasts, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PR China
[b] BBEL, Department of Biological Systems Engineering, Washington State University, Pullman, WA 99163, USA

ARTICLE INFO

*Article history:*
Received 14 January 2011
Received in revised form 8 March 2011
Accepted 21 March 2011

*Keywords:*
Acyl-CoA synthetase
Fatty acid
Oleaginous yeast
*Yarrowia lipolytica*

ABSTRACT

Fatty acyl-CoA synthetases are critical enzymes involved in lipid metabolism. The oleaginous yeast *Yarrowia lipolytica* is currently generating interest in biofuel research due to its ability to convert raw materials into value-added end products. In this study, the putative acyl-CoA synthetase gene *YAL1* in *Y. lipolytica* was interrupted with the copper resistance (*CRF1*) gene to allow selection without antibiotics to facilitate industrial applications. Deletion of *YAL1* led to reduced acyl-CoA synthetase activity. Furthermore, the fatty acid profile and lipid content of the mutant were different from the wild-type strain. The ratio of saturated to unsaturated fatty acids increased 6-fold, and the total lipid production of the mutant strain increased to 1.47-fold of the wild-type strain. The results indicate that *YAL1* in *Y. lipolytica* is involved in fatty acid elongation and desaturation, whereas the homologous, highly conserved *FAA1* gene from *Saccharomyces cerevisiae* was shown to be responsible for fatty acid activation. The increased ratio of saturated to unsaturated fatty acids would result in a higher combustion value and better oxidative stability for biofuel products obtained from the fatty acids from the engineered *Y. lipolytica* mutant.


## 1. Introduction

Biofuels as alternatives for petroleum fuel have generated great interest in recent years. As alternative lipid sources, oleaginous microbes are being used as feedstock of biofuel [10,11,26]. Various oleaginous yeast species have a high lipid content (40–70% of dry biomass) [22]. Among them, *Yarrowia lipolytica* is one of the most extensively studied "unconventional" microbes. Metabolism in *Y. lipolytica* is directed toward lipid accumulation. Moreover, the unique ability of this yeast to efficiently use hydrophobic substrates (HS) makes this microorganism a prime candidate for use in the production of bio-oils [1]. Genetic and metabolic engineering have been used to modify the strains for the production of a higher amount of lipids and alternative novel chemicals and fuels [1]. Several enzymes including acyl-CoA oxidase (Aox), glycerol-3-phosphate dehydrogenase (G3PDH), diacylglycerol acyltransferase, and sterol acyltransferase were shown to be involved in lipid accumulation pathways [1]. It was reported that deletion of the *POX1-6* genes coding for six Aox-related enzymes in β-oxidation pathways [18] and eliminating the *GUT2* gene coding for G3PDH of the glycerol pathway triggered lipid accumulation [2]. It has been concluded that genes involved in every step of fatty acid metabolism, including carbon flux toward triglycerides (TAG) and fatty acid β-oxidation, could affect or control lipid accumulation [1,2,18].

Fatty acyl-CoA synthetases (E.C. 6.2.1.3) are present in most organisms and are involved in the utilization of fatty acids. Fatty acids are channeled through fatty acyl-CoA derivatives into the β-oxidation pathway for degradation (Fig. 1); the activation of the CoA molecule is catalyzed by the enzyme fatty acyl-CoA synthetase. Several genes encoding acyl-CoA synthetases in *Saccharomyces cerevisiae* were identified, including *FAA1*, *FAA2*, *FAA3*, *FAA4,* and *FAT1*. Studies have shown that these enzymes are involved in fatty acid trafficking, recycling, and intracellular utilization. Michinaka et al. [17] and Scharnewski et al. [23] also demonstrated that *S. cerevisiae* deficient in acyl-CoA synthetase could secrete fatty acids. Interestingly, a number of studies in adipocytes suggest that different isoforms of acyl-CoA synthetase are involved in lipid storage and possibly channel fatty acids into TAG synthesis rather than into β-oxidation and energy production [21,25]. However, the function of the fatty acyl-CoA synthetase of the oleaginous yeast *Y. lipolytica*, which is comprised of 15 different genes located on several chromosomes, remains largely unstudied in terms of its role in lipid metabolism. Located on chromosome D, the *YAL1* gene was reported to have similar functions as the *FAA1* gene encoding the acyl-CoA synthetase in *S. cerevisiae* [5]. Thus, this study was designed to understand the role of acyl-CoA synthetase in fatty acid metabolism in the oleaginous yeast *Y. lipolytica*. The *YAL1* gene was deleted via homologous recombination to understand how it

* Corresponding author. Tel.: +1 509 335 3743; fax: +1 509 335 2722.
*E-mail addresses:* chens@wsu.edu, jj851014@hotmail.com (S. Chen).

1359-5113/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2011.03.011

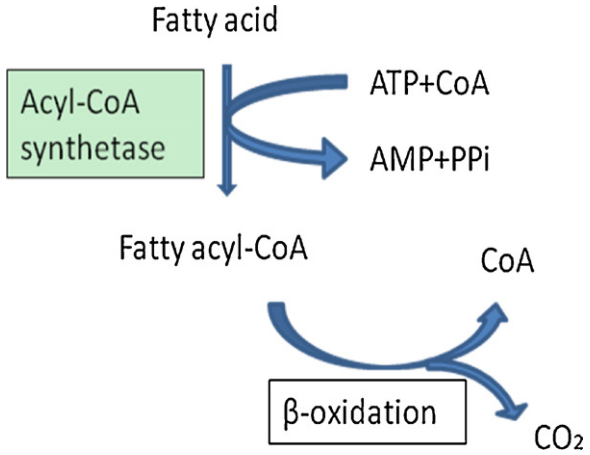


**Fig. 1.** Fatty acid present in the cytoplasm first reacts with CoA catalyzed by acyl-CoA synthetase and then enters the β-oxidation pathway for fatty acid degradation. Adapted from Beopoulos et al. [1].

affects lipid content and fatty acid composition, and its potential to produce energy-efficient biofuel products.

## 2. Materials and methods

### 2.1. Strains, media, and growth conditions

The yeast strains used are shown in Table 1. Yeasts were grown at 30 °C on YPD medium [2% (w/v) glucose, 2% (w/v) peptone, and 1% (w/v) yeast extract]. Recombinant strains were selected on YPD plates containing various amounts of $CuSO_4$. *Escherichia coli* cells were grown at 37 °C on LB medium [1% (w/v) tryptone, 1% (w/v) NaCl, and 0.5% (w/v) yeast extract] containing 50 μg/ml ampicillin when necessary. 1.5% agar was added to produce solid media. Cells were harvested at specified time points by centrifugation and the cell pellets were resuspended in deionized water to achieve desired cell densities ($OD_{600}$ in the range of 1.2–1.5).

### 2.2. Construction of plasmids

Yeast genomic DNA was prepared as described by Burke et al. [4]. The *YAL1* gene, whose sequence was used for homologous recombination, was amplified from W29 genomic DNA by PCR with the primers YAL1-F (CGAATTCGCATCCACAATCTTCTTTCGG (*EcoR*I))/YAL1-R (AAGGATCCACACATTTACGCCCAGACCT (*Bam*HI)) (Invitrogen). The PCR product was subsequently sub-cloned into a pGEM-T Easy vector to generate the plasmid pTA1. The *CRF1* gene conferring copper resistance was amplified from W29 genomic DNA by PCR with the primers CRF1-F (CGAGATCTGATGTGAGCCGTTATTCG (*Bgl*II))/CRF1-R (CCCTCGAGAAGAAACGCACATCTGTAATCC (*Xho*I)) and sub-cloned into a pGEM-T Easy vector to generate the plasmid pTCRF. Plasmid pTA1 was digested by *Bgl*II and *Xho*I, and the 2.9-kb fragment containing the *CRF1* gene from pTCRF coding for copper resistance was ligated into the *Bgl*II/*Xho*I site of pTA1. The resulting plasmid bearing the expression cassette YC (*yal1Δ::CRF1*) was designated as pTAC (Fig. 2a).

### 2.3. Yeast transformation and confirmation of recombinant strains

Plasmid pTAC was digested by *Sca*I and the linearized fragment containing cassette YC was transformed into *Y. lipolytica* W29 by electroporation using the Gene Pulser Xcell™ Electroporation System (Biorad). Recombinant strains bearing the deleted genes were selected on YPD plates with 31 mM $CuSO_4$ as a selection marker. Genomic DNA was extracted using a yeast DNA extraction kit (Thermo) and PCR was performed to confirm the gene disruption, which results from the homologous recombination between the introduced linearized DNA and the homologous regions of the genome. The 50 μl assay volume contained 25 μl *Taq* master mix (NEB) and 0.2 μM primers; the PCR cycling parameters were 30 s at 94 °C, 45 s at 48–55 °C, and 3 min at 72 °C for a total of 30 cycles, followed by a final extension for 15 min at 72 °C. PCR products were sent for sequencing to confirm the disruption of *YAL1* gene.

### 2.4. Acyl-CoA synthetase assay

The acyl-CoA synthetase extraction protocol was modified from the approach described by Fægeman et al. [8]. Once the cultures had reached the logarithmic phase, they were centrifuged for 4 min at 3000 × *g* and 4 °C. The pellet, which contained the yeast cells, was washed with cold distilled water and re-suspended in a lysis buffer composed of 200 mM Tris–HCl (pH 8.0), 5 mM β-mercaptoethanol, 4 mM EDTA, 10% glycerol, 0.01% Triton X-100, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed by a vigorous vortex of the cell suspension containing glass beads with five 1-min cycles at 4 °C. After centrifugation for 5 min at 1500 × *g* and 4 °C, the supernatants were used for the acyl-CoA synthetase activity assay. The amount of protein was determined by the Coomassie Brilliant Blue method [3]. The acyl-CoA synthetase activity was determined as described by Ichihara et al. [24]. Briefly, reactions (0.2 ml) contained 0.15 M MOPS-NaOH (pH 7.7), 1 mM DTT, 0.25 mM CoA $Na_3$, 4.5 mM ATP $Na_2$, 10 mM $MgCl_2$, 1% methanol, 1.5 U acyl-CoA oxidase, 2 KU catalase, 0.2 mM potassium salt of fatty acid, 0.55 mM Triton X-100, and fractions prepared from *Y. lipolytica* (up to 100 μg protein). The reaction was performed at 30 °C for 30 min. During the reaction, synthesized acyl-CoA was converted into *trans*-enoyl-CoA and $H_2O_2$ by the action of acyl-CoA oxidase *in situ*, and then formaldehyde was produced from the $H_2O_2$ and methanol by catalase. The sequential reactions were terminated by adding 0.2 ml of 2 M KOH at 0 °C. The color-producing reagent for aldehydes, 0.2 ml of 0.6% 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole in 0.5 M HCl, was then added to the chilled alkaline solution. The mixture was incubated at 37 °C for 10 min, and then 0.5 ml of 1% $NaIO_4$ was added. The molar absorption coefficient of the resulting purple dye was 29,200/M/cm at 550 nm. One unit of acyl-CoA synthetase was calculated as the amount of enzyme required to form 1.0 nmol of acyl-CoA per minute at 30 °C.

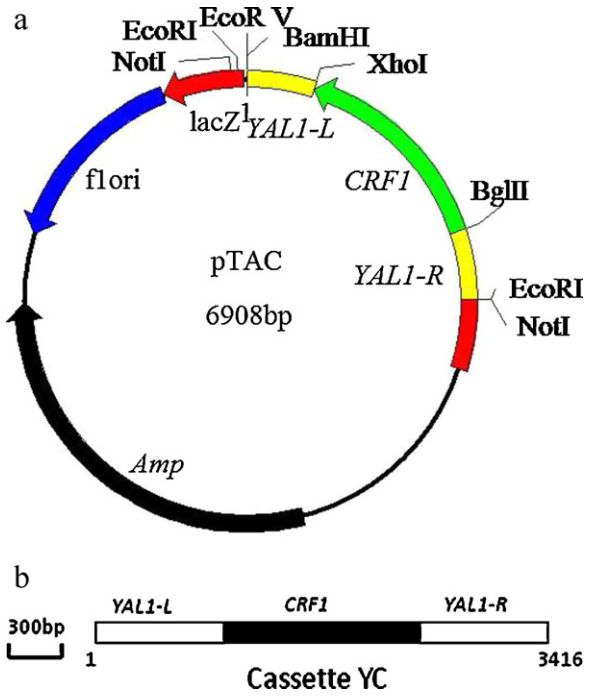


**Fig. 2.** (a) Construction of plasmid pTAC and (b) cassette YC used for homologous recombination.

### 2.5. Preparation of yeast cells and fatty acid analysis

Yeast cells and supernatant were separated by centrifugation at 8000 × *g* for 5 min. The cell pellet was washed in 1 ml of distilled water and centrifuged at 8000 × *g* for 3 min. The fatty acid concentrations of cell pellet and supernatant were measured using the gas chromatography (GC) method described by O'Fallon et al. [19]. For biomass determination, cells were washed with distilled water and dried at 80 °C until a constant weight was reached (approximately 24 h).

## 3. Results and discussion

### 3.1. Genetic manipulation of Y. lipolytica

Plasmid pTAC (Fig. 2a) was successfully constructed and confirmed by enzyme digestion and PCR. The linearized *Sca*I fragment from pTAC was transformed into *Y. lipolytica* W29. Recombinant strains were selected on 31 mM $CuSO_4$, as the host strain could only grow on less than 22 mM $CuSO_4$. Seven different transformants were selected for resistance to higher copper concentrations (31 mM $CuSO_4$) and sequentially named using the nomenclature TY1 up to TY7. The copper-resistant gene served as a selection