Elongation of C16:0 to C18:0 fatty acids in methylotrophic yeast *Hansenula polymorpha* CBS 1976 and fatty acid auxotrophic mutants

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Abstract

Fatty acid elongation defective mutant was isolated from the ethyl methanesulfonate treated *Hansenula polymorpha* based on the growth ability. Using biochemical and genetic approaches, the mutant was characterized. When compared with the fatty acid phenotype of the parental strain, the differences in profile and content of fatty acids in V1 mutant were found. In this V1 mutant, polyunsaturated fatty acids, linoleic and α-linolenic acids, could not be detected with a corresponding increase in the content of mono-unsaturated fatty acids. The ratio of C16/C18 fatty acids revealed that the accumulation of C16 fatty acids was increased significantly. The experiments on fatty acid supplementation indicated that the mutant required C18:0 for the proper growth. The results of genetic complementation with the elongase genes of *Saccharomyces cerevisiae* confirmed that the lesion was occurred at least in the extension of C16:0 to C18:0 of V1. The *H. polymorpha* mutant obtained in this work will be used as a useful tool for unraveling the pathway of fatty acid synthesis and the role of fatty acids on biological processes.

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1. Introduction

The yeast *Hansenula polymorpha*, a thermotolerant methylotrophic yeast, has many characteristics that are desirable for biotechnological implication [1–4]. This yeast has become a favorable model organism for studying certain metabolic pathways and cellular processes including methanol utilization, peroxisome biogenesis and fatty acid metabolism [5,6]. In contrast to *Saccharomyces cerevisiae*, *H. polymorpha* can produce polyunsaturated fatty acids such as linoleic acid (C18:2) and α-linolenic acid (C18:3) [7]. These advantages make *H. polymorpha* as an alternative model for study of genes and regulatory mechanisms controlling fatty acids biosynthesis in higher eukaryotes.

Fatty acids are well known to be vital compounds for cellular organisms. The bulk of cellular fatty acids that serve structural and biological functions contain acyl chains of 14–18 carbon atoms in length. Long-chain saturated fatty acids of 16–18 carbon atoms are the product of de novo biosynthesis by the fatty acid elongase [8,9]. Besides these long-chain fatty acids, a small proportion (1–5%) of very-long-chain fatty acids (VLCFAs) with lengths of 20–30 carbon atoms is usually found in eukaryotic membrane lipid [10]. Like long-chain fatty acid synthesis, the biosynthesis of VLCFAs requires...
malonyl-CoA as the chain-extending substrate and elongase system [11]. Fatty acid elongase is supposed to be functionally analogous to fatty acid synthase (FAS) but uses longer-chain acyl-CoAs rather than acetyl-CoA as a substrate. Several different strategies have been combined to study elongase multienzyme systems by molecular or biochemical means. Gathering data from previous studies, three separate fatty acid elongation systems with different chain length specificity have been reported. Based on the approaches of mutant screening and homology search, three genes encoding for fatty acid elongases, ELO1, ELO2 and ELO3 were identified from S. cerevisiae. The S. cerevisiae ELO1 gene is responsible for the elongation of C14- to C16-fatty acids [11,12]. The ELO2 and ELO3 are apparently related in VLCFAs biosynthetic machinery. The synthesis of saturated and monounsaturated fatty acids of up to 24 carbon atoms are the result of ELO2 activity while ELO3 is essential for the conversion of C24:0 to C26:0 [13].

Although enzymatic function of fatty acid elongase has been investigated in a variety of organisms by employing several approaches, a conventional method such as screening and isolation of mutants based on the fatty acid phenotype offers the potential for generating a novel strain useful for studying the fatty acid biosynthesis pathway as well as the physiological and biological roles of cellular fatty acid. In H. polymorpha, a precise biochemical pathway of fatty acid synthesis, and detailed structural and biochemical characterization of the elongase system are still lacking. In order to achieve a useful tool for providing valuable information in fatty acid elongation pathway of H. polymorpha, we isolated and characterized mutants requiring a specific saturated fatty acid for growth. The growth characteristic and fatty acid composition of the mutant were determined.

2. Materials and methods

2.1. Organisms, plasmids, and DNAs

The auxotrophic strains leu1-1 and ura3-1 of H. polymorpha were derived from CBS 1976 (NCYC 495) [14,15], donated by Prof. J.A.K.W. Kiel (University of Groningen, The Netherlands) and Prof. M.A. Gleeson (University of Sheffield, UK). The leu1-1 strain was used as parental strain for generating and screening mutant strains. The ura3-1 strain was employed for crossing the phenotype of interest. Plasmids YCpELO1.MOD and YCpGALELO2(U) carrying the S. cerevisiae ELO1 and ELO2, respectively, were kindly provided by Prof. C. Martin (Rutgers University, Piscataway, NJ). These plasmids contain galactose-inducible GAL promoter and the URA3 gene as a selectable marker.

2.2. Media and cultivations

Standard yeast genetics methods were subjected for mating, sporulation and tetrads analysis [16,17]. The yeast cells were grown either in complete medium (yeast extract-peptone-dextrose, YEPD) containing 1% yeast extract (Difco Laboratories, Detroit, MI), 2% Bacto peptone (Difco) and 2% glucose or in minimal medium (synthetic dextrose) containing 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose [14]. To screen the mutants defective in elongation of medium- to long-chain fatty acids, saturated fatty acids C16:0 and C18:0 emulsifying in 1% Triton X-100 were supplemented in the media at the concentration of 1 or 2 mM. All fatty acids were obtained from Aldrich (Aldrich, Inc., Milwaukee, WI.). The LB medium for Escherichia coli cultivation was described previously [18].

2.3. Mutagenic treatment of H. polymorpha and selection of the mutants

Mutagenesis of H. polymorpha was performed by treatment with ethyl methanesulfonate (EMS) as previously described [6]. Mutagenized cells were plated onto YEPD supplemented with a mixture of 1 mM each of C14:0, C16:0 and C18:0 (14/16/18 YEPD) and were incubated for 2–3 days at 37 °C until colonies appeared. The master plates were replica-plated onto YEPD plates for screening the mutants with a phenotype of interest. Determination of mutant growth on the media containing the individual fatty acids was further done to characterize the prospective elongation-deficient mutants.

2.4. Determination of growth ability of the mutants

The mutant cells were suspended at the concentration of 2×10⁶ cells in 5 ml YEPD supplemented with different saturated fatty acids or a mixture of C16:0 and C18:0 (1 mM each) and then were cultivated to mid logarithmic phase on a reciprocalshaker (120 rpm). Cell density was determined by spectrophotometry with absorbance at 660 nm. In the case of solid media, cultures were streaked onto 16/18 YEPD plates and replicated after 3–5 days growth onto YEPD and YEPD supplemented with different saturated fatty acids.

2.5. Complementation of the H. polymorpha fatty acid auxotrophic mutant

The plasmids YCpELO1.MOD and YCpGALELO2(U) were transformed into H. polymorpha fatty acid auxotrophic mutants using the lithium acetate method [18]. In ducible GAL promoter was described previously [13]. The yeast transformants were cultivated to exponential phase and screened prospective transformants
2.6. Lipid extraction and fatty acid analyses

Lipids were extracted from cell homogenates as previously described [6]. Fatty acids were converted to methyl esters by BF$_3$-catalyzed methanolysis [19], separated by gas chromatography using a Hewlett-Packard HP 6890 Series GC, equipped with an HP-5 column (30 m × 0.32 mm × 0.25 μm in film thickness), with a temperature gradient (20 min at 200 °C, 10 °C/min to 250 °C, and 10 min at 250 °C) and analyzed on a 3 mm glass column of 15% diethylene glycol succinate (DEGS) on 60/80 mesh Chromasorb W AW at 180 °C using Shimadzu 15A Gas Chromatograph (Shimadzu Ltd., Kyoto). Fatty acids were identified by comparison their retention time to those of commercially available methyl ester standards (NuCheck, Inc., Elysian, MN). The fatty acid compositions were expressed as percentages in total fatty acids [20].

3. Results and discussion

3.1. Isolation and characterization of elongation defective mutants

Cells of a parental strain (leu1-1) of H. polymorpha were subjected to EMS mutagenesis. To identify mutants with incapability to synthesize C18:0, the mutagen-treated cells were plated on to the growth medium YEPD agar containing a mixture of C14:0, C16:0 and C18:0. Colonies were then replicated on the YEPD medium without fatty acids supplementation. Therefore, cells defective in fatty acid synthesis or elongation would fail to grow on medium without fatty acids supplementation. After replication on the selective plates, all the mutants which were unable to grow on YEPD were further characterized. From comparison of 95,000 colonies between the master and replica plates based on the growth ability, one group possessed a phenotype of interest was identified and designated as V1 (leu1-1, fae1-1). The V1 group including 6 presumptive mutants demonstrated their inability to proliferate on solid YEPD media without addition of exogenous fatty acids. In our analysis of the defective steps in fatty acid biosynthetic pathway of V1 strain, we determined their growth on solid media supplemented with individual saturated fatty acids, C16:0 and C18:0, at the concentrations of 1–2 mM (Fig. 1). It was found that these mutants did not grow on media supplemented with 1 mM of C16:0 (data not shown). However, 2 mM of C16:0 and 1 and 2 mM of C18:0 could support the growth of the mutants as shown in Figs. 1 and 2. Ability of the V1 mutant to grow in the presence of high concentration of C16:0 (2 mM) can be explained from the viewpoint by assuming either weak activity of the partially damaged elongase enzyme in the step of C16–C18 extension or the presence
of other enzymes involved in fatty acid biosynthesis. Nevertheless, the growth of V1 mutant on media supplemented with C16:0 was also less than the growth of parental strain and V1 supplemented with C18:0 (Figs. 1 and 2). This result showed that C18 fatty acid was required for proper growth of *H. polymorpha*. Actually, most fatty acids in *H. polymorpha* are predominantly 16 and 18 carbons in length revealing that these fatty acids are critical for the normal development and cellular function.

The fatty acid profiles and compositions of V1 mutant cultivated in YEPD broth to late logarithmic stage (24 h) at 37 °C were further analysed by gas chromatography and compared with those of the parental strain (Fig. 3). The fatty acid profiles of the representative mutant from the V1 group were different from those of the parental strain. The fatty acid analysis showed that linoleic (18:2, cis-D9,12) and linolenic (18:3, cis-D9,12,15) could not be detected while mono-unsaturated fatty acids (C16:1 and C18:1) were accumulated at high relative level in V1 grown at 37 °C (Table 1 and Fig. 3). Especially, C16:1 which was normally found in minor amounts in total fatty acids of parental strain was increased significantly in V1 strain. The absence of the poly-unsaturated fatty acids and the increase of mono-unsaturated fatty acids in V1 seem to be the result of defect in desaturation processes. It was noted that C16:0 level was reduced and C18:0 was increased in V1 strain. However, when consider on the ratio of fatty acids of C16 and C18 in length, the C16/C18 ratio of V1 was increased about 3-folds when compared with those of the wild-type. The increased accumulation of C16 fatty acids (C16:0+C16:1) in V1 might be explained by the lesion in elongation of C16:0 to C18:0. Taken together, the alteration of fatty acid phenotype in V1 might be caused from the defects in either elongation or desaturation processes. To further define the defective step in fatty acid biosynthesis of V1 mutant, in addition to the growth performance, fatty acid phenotype of V1 cultivated in the media containing C18:0 was determined as shown in Table 1. The result showed that the C18 saturated fatty acid was incorporated into the cells and subsequently converted to C18:1 and C18:2 by D9- and D12-desaturases, respectively. Although the low amount of C18:2 was detected, the biotransformation of C18 fatty acids found in V1 indicated the presence of desaturation system. Moreover, the uptake of the exogenous C18:0 fatty acid resulted in the decrease of C16/C18 ratio in V1 cells. The C16/C18 ratio of V1 grown in the media with the addition of C18:0 was similar to the ratio of wild-type. This finding provides the good explanation on the effect of C18 supplementation for promoting the growth of V1 as mentioned above and previous studies [21–23]. However, the triene fatty acid was not observed in the V1 culture. This might be the partial defective in desaturation or low concentration of fatty acid substrate (C18:2) accumulated in the mutant cells.

### 3.2. Complementation study of *H. polymorpha* fatty acid auxotrophic mutant

The marked change in fatty acid composition of V1 particularly in C16/C18 ratio exhibits the possibility that the defect was occurred at the elongation of C16:0 to C18:0. The result of fatty acid supplementation could not clearly clarify the genetic lesion in V1 mutant of *H. polymorpha*. In earlier studies, at least three different yeast elongases have been defined. Elongase I extends C12–C16 fatty acyl-CoAs to C16–C18 fatty acids [12,24]. Elongase II elongates palmitoyl-CoA and stearoyl-CoA up to C22 fatty acids and elongase III synthesizes 20–26 carbon fatty acids from C18-CoA primers [13,25,26]. Thus, *ELO1* and *ELO2* are apparently involved in the elongation of C16. In order to investigate...
the defect in the elongation step, the V1 mutant was transformed with S. cerevisiae ELO1 and ELO2 genes. From crosses of the V1 mutant (leu1-1, fae1-1) with an auxotrophic strain (ura3-1), all diploid hybrids were selected on minimal medium containing a mixture of C14:0, C16:0 and C18:0 saturated fatty acids (14/16/18 MIN) without uracil and minimal medium with uracil. The segregant (ura3-1, fae1-1) was chosen as a recipient strain for complementation test. Using the plasmids YCpELO1.MOD and YCpGALELO2(U) harboring the coding sequences of S. cerevisiae ELO1 and ELO2, respectively, the ura3-1, fae1-1 strains of H. polymorpha were transformed using the lithium acetate method [18], and then plated onto the minimal medium supplemented with the fatty acids. Transformation efficiency was determined by plating aliquots of transformed cells on the medium. It was found that only the ELO2 transformants could grow on the medium containing galactose without fatty acid supplementation. Therefore, fifteen of ~150 transformant cells carrying ELO2 were randomly chosen. Each single colony was used for phenotypic testing comparative with the recipient strain (ura3-1). Phenotypic analysis demonstrated that the growth abilities of the representative transformant designated as V1 (URA3, FAE1) was similar to that of the ura3-1 strain (Fig. 2). On the contrary of the V1 mutant, the V1 strain could grow on both the media with or without exogenous fatty acids. We further analysed the growth characteristic of VT1 transformant in liquid media containing different saturated fatty acids revealed the similar results as found in solid media in which the growth of VT1 could reach closely to that of the WT strain under any conditions (data not shown). Interestingly, comparison of chromatograms of fatty acid methyl esters from the parental strain and VT1 mutant noted that their fatty acids were similar in content and profile (Fig. 3 and Table 1) that is coincided with the characteristics of growth. This result demonstrated that the ELO2 gene of S. cerevisiae could gain function in H. polymorpha V1. Taken together, our finding indicated a clear picture of the lesion in fatty acid biosynthesis in V1 mutant that the partial defect at least in the elongation step of C16–C18 which is involved in ELO2 enzyme was found. The defect of this elongation system of the H. polymorpha mutant might be occurred in either transcription or translation levels.

In conclusion, selection and characterization of the mutant deficient in the specific step of C16–C18 elongation in this study was based on their inability to grow in the absence of exogenous fatty acids. This is the first report on the study of elongation of fatty acids in H. polymorpha. The mutant strain gained in this work will be used as a valuable tool for unraveling the metabolic pathway of fatty acid synthesis and the role of fatty acids in H. polymorpha as well as in higher eukaryotes.

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References


Table 1

<table>
<thead>
<tr>
<th>Culture</th>
<th>Fatty acid composition (%) of total fatty acids in cells</th>
<th>C16/C18 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14:0</td>
<td>16:0</td>
</tr>
<tr>
<td>WT</td>
<td>1.0±0.2</td>
<td>22.0±0.1</td>
</tr>
<tr>
<td>V1</td>
<td>0.5±0.4</td>
<td>5.8±0.7</td>
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<tr>
<td>V1, 1 mM</td>
<td>0.6±0.1</td>
<td>27.4±0.6</td>
</tr>
<tr>
<td>WT</td>
<td>1.0±0.2</td>
<td>22.0±0.1</td>
</tr>
</tbody>
</table>

Stearic acid (C18:0) at the concentration of 1 mM was supplemented in V1 culture. ±SD of at least three independent experiments. The fatty acid composition of each sample was analyzed twice.


