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The growth analysis of *ylsnf1* gene deletion mutant of *Yarrowia lipolytica* on hydrophobic substrate media

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Abstract

Yarrowia lipolytica is able to utilize hydrophobic substrates, including *n*-alkanes and fatty acids, as sole carbon sources. However, their control mechanisms have still not completely understood in *Y. lipolytica*. Thus, we aim to further investigate on a new factor involved in hydrophobic substrate utilization by focusing on YlSnf1p, known as a regulator in lipid accumulation in this yeast. The deletion mutant of *YlSNF1* was successfully generated by a gene replacement at *YlSNF1* locus with *ADE1* gene (Δ*snf1*::*ADE1)*. First, the growth of Δ*snf1*::*ADE1* was determined in different hydrophobic substrate media. In contrast to glycerol medium, Δ*snf1*::*ADE1* was unable to grow on media containing *n*-decane and *n*-hexadecane as sole carbon sources. Furthermore, it exhibited a partly defective growth in oleic acid medium. In addition, both *n*-alkanes and fatty acids were also utilized as carbon sources *via* β-oxidation pathway. Thus, the involvement of *YlSNF1* to the control of β-oxidation pathway was investigated by analyzing the expression of *lacZ* gene under the control of promoter of *PAT1*, a gene encoding peroxisomal acetoacetyl-CoA thiolase. The blue colony of Δ*snf1*::*ADE* was paler than that of the wild-type. These results suggested that YlSnf1p is associated with a normal growth in the hydrophobic substrate media and is likely to also positively regulate the *PAT1* expression.

Introduction

Yarrowia lipolytica can utilize hydrophobic substrate as a sole carbon source[1]. Hydrophobic substrate utilization involves several metabolic pathways residing in different subcellular compartments. When *n*-alkanes are uptaken by a cell, *n*-alkanes are converted to fatty acid through sequential terminal oxidation occurred in the endoplasmic reticulum or peroxisomes. The first step involves a terminal hydroxylation of *n*-alkanes to fatty alcohol by cytochrome P450Alks (*ALKs1-12*)[1,2,3,4]. Second, fatty alcohol is converted to fatty aldehydes by fatty alcohol dehydrogenase or fatty alcohol oxidase[1,5]. The third step is the oxidation of the fatty aldehydes to fatty acids by fatty aldehyde dehydrogenase (*HFD1-4*)[6]. Next, fatty acids are activated to fatty-acyl CoA by acyl-CoA synthetase I and II and enter a β-oxidation cycle in peroxisomes[1]. One cycle of β-oxidation consists of four reactions that will shorten two carbons of the fatty acyl-CoA but is catalyzed by three enzyme groups that consists of acyl-CoA oxidase (*POX1-6*), a multifunctional enzyme containing hydratase and dehydrogenase activities (*MEF2*) and thiolases (*POT1* and *PAT1*)[1,7,8]. Finally, endogenous synthesized acetyl-CoA is used as energy source for growth.

In *Y. lipolytica*, the control of hydrophobic substrate utilization has not completely revealed. Thus far, there are only two steps that are known at a transcriptional regulation level.

First, *ALKs* expression is controlled by Yas1-Yas2-Yas3 system[4,9,10]. Second, the expression of β-oxidation gene is controlled by Por1p[10]. To entirely understand the control of hydrophobic substrate utilization, we further investigate on YlSnf1 that is another regulator in lipid accumulation of *Y. lipolytica.*

Methodology

Yeast strains and media

Y. lipolytica strain CXAUI (*ura3, ade1*), CXAU/AI (*ura3, ade1::ADEI*) were kindly obtained from Prof. Akinori Ohta, (Laboratory of Cellular Genetics, The University of Tokyo). CXAU/AI was used as a wild-type strain in growth analysis[9]. *Y. lipolytica* was grown in YNB medium (0.17% yeast nitrogen base without amino acid and ammonium sulfate, and 0.5% ammonium sulfate) containing 2% glycerol (SG) or 2% glucose (SD). For solid media, 0.1% fatty acids were dispersed by adding 0.05% Triton X-100. *n*-Alkanes were supplied by vapor to YNB solid media with a piece of filter paper soaked with *n*-alkanes on a lid of petri dish which was sealed and placed upside down. Uracil (24 mg/liter) was added, if necessary.

Construction of deletion cassette for YlSNF1 gene

The deletion cassettes were constructed by using approximately 1000 bp flanking regions upstream and downstream of *YlSNF1 (Yal0ID02101g*) for homologous recombination. These regions were amplified by using specific primers (Table 1). The amplified 5'- and 3'-flanking regions were successfully cloned into pBluescript (pBS) between *Xba*I and *Xho*I sites. *ADE1* fragment was then inserted at *Bam*HI site located in the cloned 5'- and 3'- flanking regions of pBS. Next, the deletion cassette (101A) was amplified from the recombinant plasmid by using primers (YALI0D02101-5F and YALI0D02101-3R). PCR products were purified by using HiYield™ Gel/PCR DNA extraction kit (RBC Bioscience, Taiwan).

Table 1. List of primers for construction of deletion cassette of *YlSNF1*

Name	Sequence
YALI0D02101-5F	5'- GC***TCTAGA***GGGTGAAGCGGGAAATCAAG-3'
YALI0D02101-5R	5'- CG***GGATCC***TTGTGAGGTGGTGGAAGGAG-3'
YALI0D02101-3F	5'- CG***GGATCC***GCACTTGTAGAGCACACTAG-3'
YALI0D02101-3R	5'- CCG***CTCGAG***CGAATTGCACCAGTCGTTC-3'

* Underlines indicate the site of restriction enzymes

Generation of deletion mutant of YlSNF1

The deletion cassette (101A) was introduced into CXAUI strain by electroporation[2] Colonies were picked and streaked to isolate yeast cells on minimum media without adenine twice. Deletion mutant of *YlSNF1* was verified by Southern blot analysis.

Verification of deletion mutant by Southern blot analysis

Genomic DNA was extracted by Dr.GenTLE™ (from yeast) high recovery kit (Takara Inc, JAPAN) and was completely digested with *Pst*I or *Sph*I. The digested products were separated on agarose gel electrophoresis. DNA fragments were transferred to HyBond-N^+ membrane by standard alkaline transfer method. The membrane was subjected to hybridization by AlkPhos direct nucleic acid labeling kit and detection system (GE Healthcare, USA). The 5'-flanking region of *YlSNF1* was used as DNA probe.

Growth analysis in hydrophobic substrate media

Wild-type and deletion mutant of *YlSNF1* were streaked on YNB agar containing various carbon sources and incubated at 30 ^{o}C.

Blue colony assay on hydrophobic substrate media

The plasmid, pPpro-*LacZ*[11], was introduced in wild-type and deletion mutant of *YlSNF1* by electroporation[2]. Transformants were isolated twice on SG media. After that both strains were grown in SG medium at 30 ^{o}C for 2 days. Cell density was adjusted to 1 OD at 600 nm and made a serial dilution from 1:10 to 1:10^4 with YNB medium. 5 µL of each dilution was spotted on agar medium containing X-gal and hydrophobic substrate (oleic acid or *n*-decane). Plates were incubated at 30 ^{o}C for 3-4 days.

Results and Discussion

Generation of deletion mutant of YlSNF1

A deletion cassette for *YlSNF1* was constructed and introduced into CXAUI strain. The deletion of *YlSNF1* was verified with Southern blot analysis as shown in Figure 1. The restriction map and probe sites were shown in Figure 1A. Genomic DNA of parents and the deletion mutant strain were completely digested with *Pst*I or *Sph*I. The result of *Sph*I- digestion showed the bands at 3.9 kb and 2.0 kb in the wild-type strain and deletion mutant of *YlSNF1*, respectively. For *Pst*1 digestion, the bands of both strains were also observed as expected at 4.0 and 3.3 kb (Fig.1B). From Southern blot analysis, the results demonstrated that *ADE1* was successfully replaced at the *YlSNF1* locus. This mutant strain was designated as Δ*snf1*::*ADE1*. In addition, the lipid droplet within Δ*snf1*::*ADE1* cells was stained with Nile Red dye and visualized by confocal microscopic technique. The result showed an accumulation of Nile Red fluorescent intensity inside Δ*snf1*::*ADE1* more than the wild-type strain, thereby suggesting an increase in lipid accumulation, consistent with a previous report on an increase in lipid accumulation in the deletion mutant of *SNF1* in glucose medium[12]. Thus, the deletion mutant of *YlSNF1* was successfully generated in our laboratory.

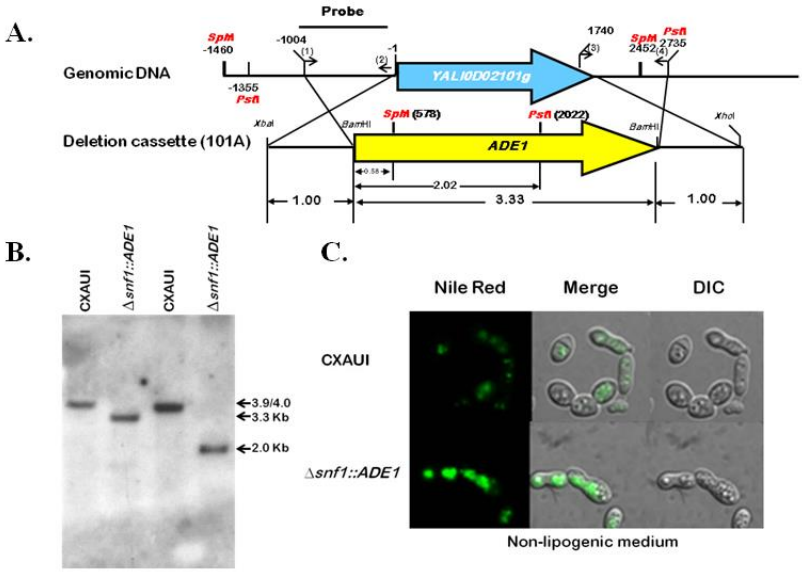

Figure 1. Generation and verification of deletion mutant of *YlSNF1*. A: Schematic representation of the deletion strategy. B: Verification of Δ*snf1::ADE1* by Southern blot analysis. C: Lipid accumulation of Δ*snf1::ADE1*; Lipid droplets were stained with Nile Red dye. Right: Nile Red fluorescence (excitation at 485-530 nm, emission at 525-605 nm). Center: Merge Right: DIC, differential interference contrast.

The growth of Δ*snf1*::*ADE1 on hydrophobic substrate*

To investigate a novel role of *YlSNF1* in *Y. lipolytica*, growth of Δ*snf1*::*ADE1* was tested on various hydrophobic substrates. Wild-type and Δ*snf1*::*ADE1* were streaked on each type of solid media and incubated at 30 ºC for 3 or 4 days depending on media types. The results revealed that Δ*snf1*::*ADE1* grew normally on glycerol and glucose. In addition, it grew with slight impairments on oleic acid, lauric acid and myristic acid. However, Δ*snf1*::*ADE1* was unable to grow on palmitic acid and all *n*-alkanes (Fig. 2). These results indicated that *YlSNF1* was mainly involved in alkane utilization, but partly implicated in fatty acid utilization.

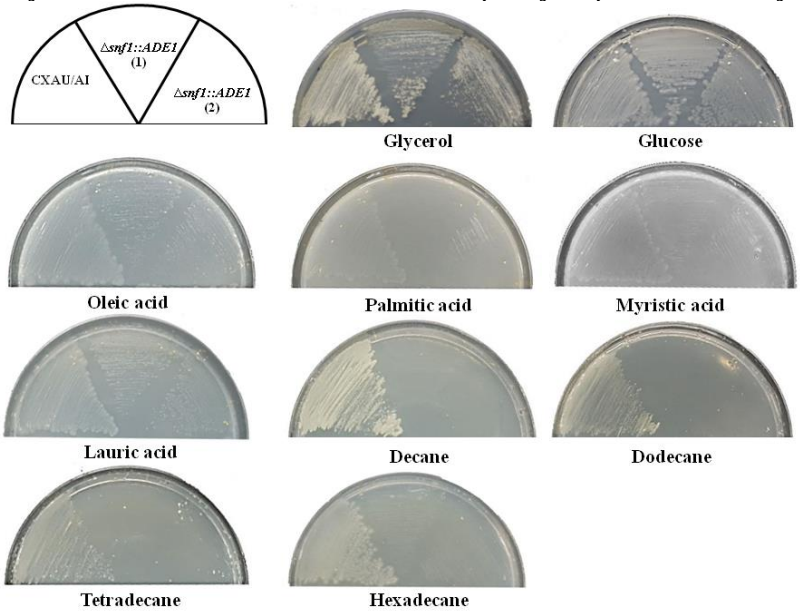

Figure 2. Growth of Δ*snf1*::*ADE1* on various carbon sources. Yeast strains were grown at 30 ºC for 3 days on glycerol and glucose, or for 4 days on oleic acid, palmitic acid, myristic acid, lauric acid, *n*-decane, *n*-dodecane, *n*-tetradecane and *n*-hexadecane.

The first step in alkane utilization is the terminal hydroxylation of *n*-alkanes by a catalytic activity of cytochrome P450 *ALK* 1-12[2,3,4]. The Δ*alk1-12* strain is unable to grow on 10-16 carbon alkanes but the complementation of Δ*alk1-12* with *ALK1* restored the growth on all *n*-alkanes[6,13]. Furthermore, *ALK*1 expression is transcriptionally regulated by Yas1p-Yas2p-Yas3p system[4,9,10]. The Δ*yas1* and Δ*yas2* strains also lost their growth ability on decane and hexadecane[9,10]. From the growth analysis of Δ*snf1*::*ADE1* in this study, the phenotype of Δ*snf1*::*ADE1* was similar to that of Δ*alk1-12*, Δ*yas1* and Δ*yas2* strains. Therefore, it is possible that the deletion of *YlSNF1* directly affected the Alk1-12p activity in terminal hydroxylation of *n*-alkanes or transcriptional activation of *ALK*1-12 expression by Yas1p-Yas2p-Yas3p system. On the other hand, fatty acids are utilized as carbon source *via* acetyl-CoA through β-oxidation pathway. Deletion mutant of *POT1*, a gene encoding peroxisomal 3-oxoacyl-CoA thiolase, is not able to grow on oleic acid as a sole carbon source[1]. Moreover, expression of β-oxidation genes (*POX2*, *POT1* and *PAT1*) in oleic acid media is primarily controlled by Por1p. From the growth analysis of Δ*por1* strain, the growth exhibited severe defects on lauric acid and myristic acid but were partially impaired on oleic acid[9]. Although the phenotype of Δ*snf1*::*ADE1* was not entirely similar to that of Δ*por1*, the growth defect on fatty acids was also observed. Thus, it was postulated that YlSnf1p would involve in fatty acid utilization *via* β-oxidation pathway.

Preliminary analysis of PAT1 expression in Δsnf1::ADE1

The utilization of hydrophobic substrate also requires β-oxidation pathway for synthesis of acetyl-CoA from exogenous fatty acid or endogenous fatty acid converted from alkane. One of the key enzymes is peroxisomal acetoacetyl-CoA thiolase encoded from *PAT1* gene. To investigate the association of *YlSNF1* to the regulation of β-oxidation pathway in the transcriptional level, the expression of *lacZ* reporter gene under the control of *PAT1* promoter was analyzed by blue colony assay. pPpro-*LacZ*[11] (Fig 3A) was introduced into wild-type and Δ*snf1*::*ADE1*. Both strains were grown on oleic acid- or decane- media containing X-gal. Blue colonies of Δ*snf1*::*ADE* were paler than that of the wild-type (Fig. 3B). This preliminary result demonstrated that YlSnf1p was likely to be involved with the regulation of the *PAT1* expression.

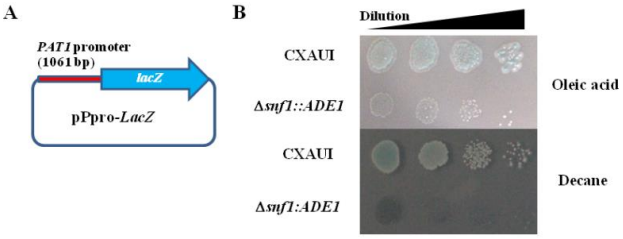

Figure 3. Effect of *SNF1* deletion on the expression of reporter gene under the control of *PAT1* promoter. A: Schematic representation of pPpro-*LacZ* plasmid. B: Blue colony assay on oleic acid and decane media at dilution 10^{-1} to 10^{-4}.

Conclusion

From this research, the deletion mutant *YlSNF1* was successfully generated by gene replacement using homologous recombination. The lack of *YlSNF1* in *Y. lipolytica* leads to a complete loss in growth on *n*-alkanes and a partial loss in growth on fatty acids. In addition, the *PAT1* expression was also affected by the deletion of *YlSNF1*. Altogether, these results shed some light on roles of YlSnf1p in controlling hydrophobic substrate utilization, especially *n*-alkane, in *Y. lipolytica*.

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