Acyl-CoA synthetases, Aal4 and Aal7, are involved in the utilization of exogenous fatty acids in *Yarrowia lipolytica*

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The yeast *Yarrowia lipolytica* assimilates hydrophobic compounds, such as *n*-aldehydes and fatty acids, as sole carbon and energy sources. It has been shown that the acyl-CoA synthetase (ACS) genes, *FAT1* and *FAA1*, are involved in the activation of fatty acids produced during the metabolism of *n*-alkanes, but the ACS genes that are involved in the metabolism of fatty acids from the culture medium remains to be identified. In this paper, we have identified the ACS genes involved in the utilization of exogenous fatty acids. RNA-seq analysis and qRT-PCR revealed that the transcript levels of the peroxisomal ACS-like protein-encoding genes *AAL4* and *AAL7* were increased in the presence of oleic acid. The single deletion mutant of *AAL4* or *AAL7* and double deletion mutant of *AAL4* and *AAL7* did not show any defects in the growth on the medium containing glucose, glycerol, *n*-alkanes, or fatty acids. In contrast, the mutant with deletion of seven genes, *FAA1*, *FAT1-FAT4*, *AAL4*, and *AAL7*, showed severe growth defects on the medium containing dodecanoic acid or oleic acid. These results suggest that Aal4p and Aal7p play important roles in the metabolism of exogenous fatty acids in collaboration with Faa1p and Fat1p-Fat4p.

Key Words: acyl-CoA synthetase; fatty acid; peroxisome; *Yarrowia lipolytica*

Abbreviations: ACS, acyl-CoA synthetase; ER, endoplasmic reticulum; 5-FOA, 5-fluoroorotic acid; qRT-PCR, quantitative real time PCR; RPKM, reads per kilobase of exon per million mapped reads; PTS1, peroxisomal targeting signal 1

Introduction

Fatty acids are important building blocks of membrane lipids, such as phospholipids and sphingolipids. They are also used in various processes, including protein acylation and synthesis of signaling molecules. In addition, they are catabolized for energy production through β-oxidation. In such important cellular processes, fatty acids are used in the form of acyl-CoAs. Acyl-CoA synthetase (ACS) is a group of enzymes that catalyze the...
thioesterification of fatty acids with coenzyme A (Black and DiRusso, 2007; Grengengo et al., 2014; Watkins and Ellis, 2012). In eukaryotic cells, there are multiple ACS isoforms, which exhibit different cellular localization and distinct substrate specificities.

Yarrowia lipolytica is an ascomycetous yeast, which efficiently utilizes hydrophobic carbon sources, such as n-

Fig. 1. Phylogenetic tree of acyl-CoA synthetases of Y. lipolytica.

The phylogenetic tree of acyl-CoA synthetases of Saccharomyces cerevisiae and Y. lipolytica was constructed using ClustalW and drawn using Njplot. The scale bar denotes 0.05 substitutions per site. The bootstrap values by 1,000 repetitions are indicated. The accession numbers of sequences from UniProtKB are as follows: Faa1p (P30624), Faa2p (P39518), Faa3p (P39002), Faa4p (P47912), Fat1p (P38225) and Fat2p (P38137) of S. cerevisiae (Sc) and Faa1p (Q6C8Q3), Fat1p (Q6C5Q8), Fat2p (Q6C634), Fat3p (Q6CFN2), Fat4p (Q6CCH6), Aal1p (Q6C670), Aal2p (Q6CH10), Aal3p (Q6C6V4), Aal4p (Q6C650), Aal5p (Q6C2M7), Aal6p (Q6C6W9), Aal7p (Q6CS77), Aal8p (Q6CFE4), Aal9p (Q6CGX7), Aal10p (Q6C8S6) of Y. lipolytica (Yl).

Table 1. Yeast strains used in this study.

| Strain | Genotype | Source of reference |
|--------|----------|---------------------|
| CXAU1  | MATA ade1 ura3 | Iida et al. (1998) |
| ∆aai1∆fat1-4 | CXAU1 ∆aai1 ∆fat1 ∆fat2 ∆fat3 ∆fat4 | Tenagy et al. (2015) |
| ∆aai4 | CXAU1 ∆aai4 | This study |
| ∆aai7 | CXAU1 ∆aai7 | This study |
| ∆aai4∆aai7-4 | CXAU1 ∆aai4 ∆aai7-4 | This study |
| ∆aai4∆aai7∆aai1-4 | CXAU1 ∆aai4 ∆aai7 ∆aai1-4 | This study |
| ∆aai7∆aai1∆aai4-4 | CXAU1 ∆aai7 ∆aai1 ∆aai4-4 | This study |

Table 2. Plasmids used in this study.

| Plasmid  | Description | Source of reference |
|----------|-------------|---------------------|
| pBURA3   | pBlueScript II SK (+) carrying URA3 | Takai et al. (2012) |
| pBUAAL4PT | A plasmid for deletion of AAL4 by pop-in/pop-out method | This study |
| pBUAAL7PT | A plasmid for deletion of AAL7 by pop-in/pop-out method | This study |
Material and Methods

Yeast strains, media and growth conditions. Strains used in this study are shown in Table 1. Yeasts were grown in YNB medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco), and 0.5% ammonium sulfate) supplemented with an appropriate carbon source as follows: 2% (w/v) glucose; 2% (w/v) glycerol; 2% (v/v) n-alkanes (n-decane and n-hexadecane), or 0.1% (w/v) dodecanoic acid. Oleic acid was added to YNB medium in a final concentration of 0.1% (v/v) for solid medium or 2% (v/v) for liquid medium. Dodecanoic acid and oleic acid were added to solid medium with 0.5% (v/v) Triton X-100. n-Alkanes were supplied in the vapor phase to YNB solid media as described previously (Endoh-Yamagami et al., 2007). Uracil (24 mg/l) and adenine (24 mg/l) were added if necessary. Yeast cells were grown at 30°C.

Construction of deletion mutants. Deletion of AAL4 and AAL7 were carried out with the pop-in/pop-out method using URA3 marker and 5-fluoroorotic acid (5-FOA) as described previously (Takai et al., 2012). Correct removal of AAL4, AAL7, URA3, and vector sequences was confirmed by PCR.

Plasmids. Plasmids used in this study are listed in Table 2. Sequences of the primers used to construct plasmids are shown in Table S1.

Transformation of Y. lipolytica. Y. lipolytica was transformed by electroporation using the Gene Pulser (BIO-RAD) as described previously (Iida et al., 1998; Tenagy et al., 2015).

RNA preparation and RNA-seq analysis. RNA was prepared from Y. lipolytica as described previously (Hirakawa et al., 2009). Sequencing samples were prepared using TruSeq RNA Sample Preparation Kit (Illumina) and sequenced by illumina GAIIx system (Illumina) according to the manufacturer’s instructions, generating 100-bp paired-end reads and 6-bp index tags. Sequence data were analyzed by CLC Genomics Workbench (QIAGEN) ver. 11.0. Quality trimmed reads were mapped to the Y. lipolytica genome (NC_002659, and NC_006067 - NC_006072) using RNA-seq tool with default parameters.

Quantitative real time PCR (qRT-PCR). Total RNA was prepared from Y. lipolytica using the hot phenol method (Collart and Oliviero, 2001). The total RNA was reverse-transcribed with ReverTra Ace® qPCR RT Master Mix with gDNA Remover (TOYOBO). Quantitative real time PCR was performed using StepOne Real Time PCR System (Applied Biosystem). The gene-specific primers are qPCR(AAL4-F) and qPCR(AAL4-R) for AAL4, qPCR(AAL7-F) and qPCR(AAL7-R) for AAL7, qPCR(Histone H3-F) and qPCR(Histone H3-R) for YAL10F25905g encoding histone H3, which was named HHT1 (Table S1).

Fig. 2. The transcript levels of AAL4 and AAL7.
A. The wild-type CXAU1 strain precultured to the logarithmic phase in the YNB liquid medium containing glucose at 30°C was seeded to the YNB liquid medium containing glucose (Glc) or oleic acid (OA) at an initial OD<sub>600</sub> = 1.0, and incubated for 1 h at 30°C. RNA was extracted and RNA-seq analysis was performed. RPKM (reads per kilobase of exon per million mapped reads) values of FAA1, FAT1-FAT4, and AAL1-AAL10 in two independent experiments (Exp. 1 and 2) are shown. The lower panel indicates the magnified view of the part of the upper panel. B. The wild-type CXAU1 strain grown in the YNB liquid medium containing glucose was seeded to the YNB liquid medium with glucose (Glc), oleic acid (OA), n-decane (C10), or n-hexadecane (C16) at an initial OD<sub>600</sub> = 1.0, and incubated for 1 h. Total RNAs were extracted from the cells and reverse-transcribed to cDNAs, which were detected using primers specific for AAL4 or AAL7. Each result represents an average of three independent experiments ± standard error (S.E.) Gene expression levels of AAL4 and AAL7 were normalized to that of histone H3 gene HHT1.
Fig. 3. Growth of the mutants deleted for ACS genes on glycerol, glucose, oleic acid, dodecanoic acid, n-decane, and n-hexadecane. The wild-type CXAU1 strain and the mutants deleted for FAA1, FAT1-FAT4, AAL4, and/or AAL7 were grown at 30°C for 2 days on YNB media containing glucose (Glc), glycerol (Gly), glucose and glycerol (Glc+Gly), glucose and dodecanoic acid (Glc+DA), glucose and oleic acid (Glc+OA), glycerol and dodecanoic acid (Gly+DA), and glycerol and oleic acid (Gly+OA), and for 5 days on oleic acid (OA), dodecanoic acid (DA), n-decane (C10), and n-hexadecane (C16).
Results and Discussion

In Y. lipolytica, transcription of the genes involved in the assimilation of n-alkanes is upregulated by n-alkanes (Endoh-Yamagami et al., 2007; Hirakawa et al., 2009; Iida et al., 1998, 2000; Kobayashi et al., 2008, 2013, 2015; Mori et al., 2013; Tenagy et al., 2015; Yamagami et al., 2004), and it has been shown that transcription of FAA1 and FAT1 was upregulated in response to n-alkanes (Tenagy et al., 2015). Similarly, transcription of the genes involved in fatty acid metabolism was upregulated by a fatty acid (oleic acid) (Poopanitpan et al., 2010; Tezaki et al., 2017). RNA-seq analysis was performed using RNA prepared from the wild-type Y. lipolytica strain cultured in a medium containing glucose or oleic acid for 1 h to investigate the transcript levels of ACS genes in Y. lipolytica (Fig. 2A) (Tezaki et al., 2017). As shown previously (Tenagy et al., 2015), the transcript level of FAT1, but not that of FAA1, was increased in the presence of oleic acid. In addition, the transcripts of FAT3 and AAL2-AAL9 were suggested to be increased in the presence of oleic acid (Fig. 2A). The transcript levels of AAL4 and AAL7 were found to be much higher than those of other AAL genes in the cells cultured in the oleic acid-containing medium. Here, we focused on the roles of AAL4 and AAL7 in the utilization of fatty acids in Y. lipolytica.

The transcript levels of AAL4 and AAL7 in the wild-type strain cultured with various carbon sources were analyzed by qRT-PCR (Fig. 2B). The wild-type strain cultured to the logarithmic phase in a glucose-containing medium was shifted to the medium containing glucose, n-decane, n-hexadecane, or oleic acid, after which it was further incubated for 1 h. The transcript levels of AAL4 and AAL7 were significantly higher in the cells grown in media containing n-alkanes or oleic acid compared to those grown in a glucose-containing medium (Fig. 2B). These results suggest that the transcription of AAL4 and AAL7 is upregulated by n-alkanes and oleic acid.

Next, we constructed single deletion mutant of AAL4 (∆aal4) or AAL7 (∆aal7), a double deletion mutant of AAL4 and AAL7 (∆aal4∆aal7), and multi-gene deletion mutants, in which AAL4 and/or AAL7 were/was deleted in the Δfaa1Δfat1-4 strain (Tenagy et al., 2015) (Δaal4Δaal7Δfaa1Δfat1-4, Δaal4Δaal7Δfaa1Δfat1-4, and Δaal7Δfaa1Δfat1-4), and analyzed their growth on various carbon sources (Fig. 3). The Δaal4, Δaal7, and Δaal4Δaal7 strains showed growth comparable to that of the wild-type strain on glucose, glycerol, fatty acids, and n-alkanes, when comparing their colony sizes. The Δaal4Δfaa1Δfat1-4 and Δaal7Δfaa1Δfat1-4 strains showed slightly slower growth on glucose but severely defective growth on n-decane or n-hexadecane, similar to the Δfaa1Δfat1-4 strain. The Δaal4Δfaa1Δfat1-4 and Δaal7Δfaa1Δfat1-4 strains were able to grow on fatty acids, dodecanoic acid and oleic acid, but the colony sizes of the Δaal7Δfaa1Δfat1-4 strain were smaller than those of the Δaal4Δfaa1Δfat1-4 strain, demonstrating that the growth of the Δaal7Δfaa1Δfat1-4 strain is weaker than that of the Δaal4Δfaa1Δfat1-4 strain on dodecanoic acid. The Δaal4Δaal7Δfaa1Δfat1-4 strain grew on glycerol similarly to the Δfaa1Δfat1-4, Δaal4Δfaa1Δfat1-4, and Δaal7Δfaa1Δfat1-4 strains, but it showed a partial growth defect on glucose. The Δaal4Δaal7Δfaa1Δfat1-4 strain exhibited severe growth defects on dodecanoic acid and oleic acid. The Δaal4Δaal7Δfaa1Δfat1-4 strain grew on fatty acids present along with glycerol, raising the possibility that the growth defects of the Δaal4Δaal7Δfaa1Δfat1-4 strain on fatty acids are not due to the toxicity of fatty acids. These results suggest that AAL4 and AAL7 are involved in the metabolism of exogenous fatty acids in collaboration with FAA1 and FAT1-FAT4.

It was suggested that the mutants of Aal4 and Aal7, in which their PTS1-like sequences were deleted, have ACS activities in the cytosol (Dulermo et al., 2016). Therefore, our results raise the possibility that Aal4 and Aal7 function as peroxisomal ACSs in fatty acid metabolism. Since the growth of the Δaal7Δfaa1Δfat1-4 strain on dodecanoic acid was weaker than that of the Δaal4Δfaa1Δfat1-4 strain, Aal7 is considered to play a more important role in the assimilation of short chain fatty acid than Aal4. The reason for the growth defect of the Δaal4Δaal7Δfaa1Δfat1-4 strain on glucose remains to be elucidated. This strain grew to a substantial extent on the medium containing both glucose and glycerol, suggesting that glucose or its metabolites are not toxic to this strain. The Δaal4Δaal7Δfaa1Δfat1-4 strain probably has a defect in the process of glucose metabolism. Transcription of FAT1, AAL4, and AAL7 was suggested to be highly upregulated by oleic acid. In Y. lipolytica, transcription of genes involved in fatty acid metabolism is upregulated by the Zn2Cys6 transcription factor Por1p (Poopanitpan et al., 2010). It is of interest to analyze the transcript levels of these genes in the deletion mutant of POR1.

Roles of other AAL genes remain unclear. Since our RNA-seq analysis suggested that transcription of AAL2-AAL9 was upregulated in the presence of oleic acid, it is possible that AAL2, AAL3, AAL5, AAL6, AAL8, and AAL9 are also involved in fatty acid utilization. Orthologs of AAL genes were searched in the genome sequences of 6 oleaginous and 22 non-oleaginous yeasts of ascomycetes and basidiomycetes, and it was reported that AAL orthologs are encoded in the genomes of Y. lipolytica and two basidiomycetous yeasts, Rhodosporidium toruloides and Rhodotorula glutinis, but not in others (Dulermo et al., 2016). Thus, orthologs of AAL genes appear to be conserved in a limited subset of yeast species. Since Aal proteins share similarities with plant or bacterial 4-coumarate-CoA ligases or insect luciferases (Tenagy et al., 2015), Aal proteins may also catalyze other reactions in addition to fatty acid activation.

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Supplementary Materials

Supplementary table is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).
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