広島大学学位請求論文

Study on long-chain alcohol utilization pathways in the glycolipid-producing yeast *Starmerella bombicola*

（糖脂質生産菌 *Starmerella bombicola* における
長鎖アルコール資化経路に関する研究）

２０１８年

広島大学大学院理学研究科
数理分子生命理学専攻

髙橋　史員

目　次

1．主論文

Study on long-chain alcohol utilization pathways in the glycolipid-producing yeast *Starmerella bombicola*

（糖脂質生産菌 *Starmerella bombicola* における長鎖アルコール資化経路に関する研究）

2．公表論文

Takahashi, F., Igarashi, K., Hagihara, H. Identification of the fatty alcohol oxidase FAO1 from *Starmerella bombicola* and improved novel glycolipids production in an FAO1 knockout mutant. ***Applied Microbiology and Biotechnology*** 100: 9519-9528 (2016)

3．参考論文

1) Takahashi, F., Sumitomo, N., Hagihara, H., Ozaki, K. Increased dipicolinic acid production with an enhanced spoVF operon in *Bacillus subtilis* and medium optimization. 2015 ***Bioscience, Biotechnology and Biochemistry*** 79: 505-511 (2015)

2) Tanaka, T., Takahashi, F., Fukui, T., Fujiwara, S., Atomi, H., Imanaka, T. Characterization of a novel glucosamine-6-phosphate deaminase from a hyperthermophilic archaeon. ***Journal of Bacteriology*** 187: 7038-7044 (2005)

主論文

Study on long-chain alcohol utilization pathways in the glycolipid-producing yeast *Starmerella bombicola*

（糖脂質生産菌 *Starmerella bombicola* における
長鎖アルコール資化経路に関する研究）

Fumikazu Takahashi

2018

Contents

General Introduction -2-

Chapter 1. Identification of the fatty alcohol oxidase FAO1 from *Starmerella bombicola* and improved novel glycolipids production in an FAO1 knockout mutant. -5-

Chapter 2. Elucidation of secondary alcohol metabolism in *Starmerella bombicola* and contribution of primary alcohol oxidase FAO1. -35-

General Conclusions -58-

Acknowledgements -62-

General Introduction

Alkyl polyglycosides (APGs) are safe and superior detergents having both high foaming properties and non-irritative to the skin, and can be blended with other detergents. Industrial production of APGs is mainly carried out by chemical synthesis using glucose and alcohol as raw materials, but requires large amounts of alcohol and suffers from glucose is denaturation during high temperature reactions. In order to develop more efficient methods, APG production at ambient temperature under atmospheric pressure using microorganisms or enzymes has been studied. For instance, *Starmerella bombicola* produces alkyl sophoroside which is a type of APGs from glucose and secondary fatty alcohols (Brakemeier et al., 1995).

S. bombicola is known as a sophorolipids-producing yeast. Our company was the first in the world to undertake industrial production of sophorolipids, and formulate them into cosmetic bases (Inoue and Ito, 1982; Kimura, 1992). In recent years, many other companies (Evonik, Ecover, Saraya, Allied Carbon Solutions and so on) have begun to commercialize sophorolipids, due to the rise in environmental awareness. However, molecular biological research on sophorolipids has lagged behind the progress in their commercial use. When I started this study, a genome analysis of *S. bombicola* had not been done and only one group had published a report on a transformation system (Van Vogaert et al., 2008).

As mentioned above, *S. bombicola* can produce APGs from secondary alcohols, however it is difficult to produce APGs from primary alcohols. There are no reports on the APG production pathway and metabolic system for fatty alcohols in *S. bombicola*. The NAD dehydrogenase pathway is generally known as the embolism pathway for

fatty alcohols. The reaction proceeds from the alcohol dehydrogenase and aldehyde dehydrogenase, and the corresponding fatty acid is produced (Iwama et al., 2015). On the other hand, the fatty alcohol oxidase (FAO) pathway was reported in some *Candida* yeasts and fungi (Cheng et al., 2005). In *S. bombicola*, intracellular FAO activity has been confirmed (Hommel and Ratledge, 1990). However, the role of FAO in alcohol assimilation, its amino acid and gene sequence of FAO remain unknown. The aims of this study are to elucidate the fatty alcohol utilization pathway in *S. bombicola* and to construct an efficient APG production method.

In Chapter 1, I describe a cDNA analysis of *S. bombicola*, construction of recombination system, and identification of primary alcohol oxidase FAO1. In addition, a high yield of APGs and a novel diol-type glycolipid production method using an FAO1-deficient strain are constructed. In Chapter 2, I clarify the contribution of FAO1 to the secondary alcohol utilization pathway, and examine for the whole fatty alcohol utilization pathway in *S. bombicola*.

References

Brakemeier, A., Lang, S., Wullbrandt, D., Merschel, L., Benninghoven, A., Buschmann, N., Wagner, F. (1995) Novel sophorose lipids from microbial conversion of 2-alkanols. ***Biotechnol. Lett.*** 17: 1183–1188.

Cheng, Q., Sanglard, D., Vanhanen, S., Huan, T.L., Bombelli, P., Smith, A., Slabas, A.R. (2005) *Candida* yeast long chain fatty alcohol oxidase is a c-type haemoprotein and plays an important role in long chain fatty acid metabolism. ***Biochim. Biophys. Acta*** 1735: 192–203.

Hommel, R., Ratledge, C. (1990) Evidence for two fatty alcohol oxidases in the biosurfactant-producing yeast *Candida* (*Torulopsis*) *bombicola*. ***FEMS Microbiol. Lett.*** 70: 183–186.

Inoue, S., Ito, S. (1982) Sophorolipids from *Torulopsis bombicola* as microbial surfactants in alkane fermentations. ***Biotechnol. Lett.*** 4: 3–8.

Iwama, R., Kobayashi, S., Ohta, A., Horiuchi, H., Fukuda, R. (2015) Alcohol dehydrogenases and an alcohol oxidase involved in the assimilation of exogenous fatty alcohols in *Yarrowia lipolytica*. ***FEMS Yeast Res.*** 15: 1–12.

Kimura, Y. (1992) Feature/Functions of metabolites produced by yeast and their applications to cosmetics and toiletries. A glycolipid surfactant and its application. ***Fragr. J.*** 20: 22-29.

Van Bogaert, I.N.A., De Maeseneire, S.L., Develter, D., Soetaert, W., Vandamme, E.J. (2008) Development of a transformation and selection system for the glycolipid-producing yeast *Candida bombicola*. ***Yeast*** 25: 273–278.

Chapter 1

Identification of the fatty alcohol oxidase FAO1 in *Starmerella bombicola* and improved novel glycolipids production in an FAO1 knockout mutant strain

Summary

Alkyl polyglucosides (APGs), which were first commercialized in the 1990s, are mild, non-ionic surfactants comprising fatty alcohols and glucose derived from recyclable starch. APGs have good properties as cleaners, foaming agents, and emulsifiers, and they do not undergo hydrolysis at an alkaline pH. In addition to their advantages over traditional synthetic surfactants, APGs are low-irritant surfactants that are nontoxic and easily degradable in the environment. Thus, APGs are considered to be environmentally friendly surfactants. *Starmerella bombicola* glycosylates long-chain ω or ω-1 hydroxy fatty acids, and it also directly glycosylates secondary alcohols. Although it is generally difficult to directly glycosylate primary alcohols, they are easily converted to the corresponding fatty acids by *S. bombicola* because of its strong alcohol oxidase activity. To redirect unconventional substrates toward APG synthesis, the long-chain alcohol oxidation pathway was blocked by knocking out the fatty alcohol oxidase gene. The complete sequence of the *S. bombicola FAO1* gene (2,046 bp) was cloned, and the obtained nucleotide sequence was used to construct a knockout cassette. An *FAO1* knockout mutant with the correct genotype and phenotype was evaluated by fermentation on 1-tetradecanol. The mutant produced tetradecyl disaccharides and tetradecanediol tetrasaccharides. The APGs and diol polyglucosides (DPGs) production

of the mutant was 27.3g/L ((APGs + DPGs) / de novo sophorolipids ratio was about 15:1), while the parent strain did not produce APG or DPG. These data indicate that the substrates had been redirected toward novel glycolipids synthesis in the mutant.

Introduction

The yeast *Starmerella bombicola* is a potent producer of economically relevant biosurfactants, which are referred to as sophorolipids (Spencer et al., 1970). Sophorolipids derived from *S. bombicola* are glycolipids that have a disaccharide sophorose linked glycosidically to the hydroxyl group at the ω or ω-1 carbon of C16 to C18 chain fatty acids. Several approaches have been used to improve sophorolipid productivity (>3.5 g L^{-1} h^{-1}) and determine the sophorolipid biosynthetic pathway (Gao et al., 2013; Van Bogaert et al., 2013). Novel sophorose lipids were successfully produced by microbial conversion using *S. bombicola* from blends of glucose and 2-alkanols as the carbon source, and 2-dodecanol and 2-tetradecanol were directly attached without further metabolization to their corresponding disaccharides (Brakemeier et al., 1995). Compared with classical sophorose lipids, most of these compounds have better water solubility and more effectively decrease the surface tension of water. The sophorolipid and alkyl polyglucoside (APG) biosynthesis pathways in *S. bombicola* are shown in Fig. 1. The sophorolipid biosynthesis system was clarified (Van Bogaert et al., 2013), and the group of enzymes that participate in hydroxylation, glycosylation, acetylation, and lactonization reactions were identified. In contrast, APGs may be synthesized in a single step by *S. bombicola*. Unfortunately, secondary alcohols are costly, and there are few commercial suppliers. However, primary alcohols are inexpensive and readily available. However, it is difficult for *S.*

bombicola to produce APGs from primary alcohols, because this strain has strong alcohol oxidase (AO) activity (Hommel and Ratledge, 1990). A better understanding of the APG synthesis pathway and subsequent genetic engineering of this yeast species could provide methods for producing higher APG yields, as well as modifications of the APG structure.

The fatty AO (FAO) pathway in *Candida tropicalis* has been characterized extensively (Cheng et al., 2005). Three FAO genes were cloned from *C. tropicalis*, one of which was designated *FAO1*, and two putative allelic genes were designated *FAO2a* and *FAO2b*. Both enzymes oxidize long-chain primary alcohols. FAO1 oxidizes ω-hydroxy fatty acids, but not 2-alkanols, whereas FAO2 oxidizes 2-alkanols, but not ω-hydroxy fatty acids. However, the FAO pathway in *S. bombicola* has not yet been determined, although it is proposed to have multiple FAO enzyme activities (Hommel and Ratledge, 1990). There is no information regarding the FAO gene sequences and whether the main FAO enzyme is expressed when *S. bombicola* is grown on a primary alcohol as the sole carbon source.

This study is the first to report the sequence of an FAO gene in *S. bombicola*, and it demonstrates that this enzyme plays a major role in the long-chain alcohol oxidation pathway. Here we describe the disruption of the FAO gene of *S. bombicola* to achieve a strain that is deficient in the long-chain alcohol oxidation pathway. This strain not only was used to increase the production of APGs, but also was able to produce novel diolpolyglucosides (DPGs) from primary alcohols.

Materials and methods

Strains, fermentation, and glycolipids production

The strains used in this study are shown in Table 1. *S. bombicola* KSM36 (an industrial strain for sophorolipid production (Inoue and Ito, 1982) was used as the parent strain. *S. bombicola* NBRC10243 (a type strain) was used for cDNA analysis. *Escherichia coli* DH5α (TaKaRa Bio Inc., Kusatsu, Japan) was used for plasmid maintenance, and *E. coli* BL21(DE3) (Funakoshi Co., Ltd., Tokyo, Japan) was used for protein expression. *S. bombicola* was cultured on yeast peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose) or on synthetic dextrose (SD) medium (0.67% yeast nitrogen base without amino acids (Sigma-Aldrich, St. Louis, MO, USA) and 2% glucose). To obtain better growth of the mutants, 0.03% uracil was added to the YPD medium. The SD medium used for the selection of auxotrophic mutants was supplemented with 0.03% uracil and 0.5% 5-fluoroorotic acid (5-FOA). Liquid yeast shake-flask cultures were incubated at 30°C with shaking at 120 rpm. *E. coli* was grown in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) supplemented with 0.01% sodium ampicillin if necessary. Liquid *E. coli* cultures were incubated at 37°C with shaking at 120 rpm. *S. bombicola* was grown on a sophorolipid production medium (10% glucose, 10% methyl palmitate, 0.5% yeast extract, and 1% urea (the pH was set to 5.0 with HCl)), or on an APG production medium comprising 15% glucose, 0.5% sodium citrate·$3H_2O$, 0.04% yeast extract, 0.0154% NH_4Cl, 0.1% KH_2PO_4, 0.07% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, 0.027% $CaCl_2 \cdot 2H_2O$, and 0.016% $K_2HPO_4 \cdot 3H_2O$. The seed culture (5 ml of YPD in a 100-ml test tube) was incubated for 48 h at 30°C with shaking (250 rpm) on a rotary shaker. An aliquot (1 ml) of this culture was added to 100 ml of production medium in a 500-ml Sakaguchi flask. After 48 h, 10 g L^{-1} of a primary alcohol was added to the microbial culture in the case of APG production medium. The pH was not adjusted, and the total incubation time was 120 h.

Washing cells from cultures

Culture samples (20 mL) were centrifuged at approximately 5,000 × *g* for 10 min at 4°C to pellet the cells. The pellet was washed by resuspension in 10 ml of 0.08% NaCl, followed by centrifugation at 5,000 × g at 4°C for 1 min. This washing procedure was repeated twice.

Preparation of cell extracts and microsomes

The washed cell pellet was resuspended in 10 ml of 1/15 M potassium buffer (pH7.4). Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM prior to breaking the cells by shaking vigorously three times at 2,700 rpm for 1 min with 0.5-mm glass beads and a Multi-beads shocker (Yasui Kikai, Osaka, Japan). Then, the broken cell suspension was centrifuged at approximately 15,000 × *g* for 60 min at 4°C. The supernatant consisted of the soluble cell extract. Microsomes were prepared from the cell extract by centrifugation at 100,000 × *g* for 1.5 h at 4°C. The supernatant was removed, and the microsomal pellet was resuspended in 200 μl of 1/15 M potassium buffer (pH7.4) containing 1 mM phenylmethylsulfonyl fluoride, and it was assayed for FAO activity. Cell extracts from *E. coli* were prepared in the same manner.

Nucleic acid extraction and cDNA synthesis

Yeast genomic DNA was isolated using the Mr. Gentle High Recovery Kit (TaKaRa Bio). Plasmid DNA was isolated using a plasmid purification kit (Roche, Basel, Switzerland). RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, except that in the cell lysis step, zymolyase-20T (Seikagaku Corporation, Tokyo, Japan) was added to a final

concentration of 100 units L^{-1} in 1 mL of buffer Y1. A full-length cDNA library was synthesized using the SMARTer cDNA Synthesis Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. *S. bombicola* 10243 cells that were fermented for 48 h in sophorolipid production medium were used for RNA extraction and cDNA sequencing (described below).

Library construction and 454 sequencing

A 454 library was constructed using the Roche GS FLX Titanium Rapid Library Preparation Kit according to the manufacturer's instructions. The library was sequenced using a GS FLX Titanium Sequencing Kit XLR70 on a 454 FLX Titanium Genome Sequencer (Roche).

Sequence assembly and functional annotation

To obtain unique transcripts, all high-quality reads were assembled *de novo* using the cDNA mode of Newbler v2.3 software (www.454.com) with default assembly parameters. The amino acid sequence of *C. tropicalis* FAO2 (accession no. AAS46880.1) was used to search for the putative AO gene in *S. bombicola* using the translated nucleotide Basic Local Alignment Search Tool (TBLASTN) (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/) with default parameters, excluding –F F (low complexity filter = OFF).

Transformation

S. bombicola cells were transformed via electroporation (Wang et al., 2001) using a Gene Pulser Xcell (Bio-Rad, Carlsbad, CA, USA) set to 25 μF, 350 Ω, and 2.5 kV.

Transformants were selected on SD plates. *E. coli* cells were transformed as described previously (Sambrook and Russell 2001).

Polymerase chain reaction (PCR) and sequencing analysis

The primers used in this study are shown in Table 2. The *URA3* genes of the selected mutants were amplified by yeast colony PCR with the primers Pura3F and ura3F. The obtained PCR fragments were cloned into pUC118/HincII(BAP) (TaKaRa Bio), and one of the resulting vectors was called pUC-*URA3*. The yeast colony PCR used KOD FX Neo (TOYOBO, Osaka, Japan). PCR amplification was performed with an initial denaturation of 94°C for 2 min, followed by 30 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 2 min, followed by a final 2-min elongation at 72°C. All DNA sequences were determined using a Big Dye ver. 3.1 (ABI) and 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Subcloning and expression of FAO1

The FAO1 F and FAO1 R primers that were used to PCR the open reading frame (ORF) of *FAO1* were designed to provide a 15-bp region that was complementary to pET21a in the final PCR product. The primers for pET21a were pET21 F and pET21 R. The PCR products were purified with a PCR Purification Kit (Roche). The *FAO1* amplicon was ligated into pET21a using the In-Fusion HD Cloning Kit (TaKaRa Bio). The *FAO1* expression vector was designated pET-*FAO1*.

Production of FAO1 in E. coli

Overnight cultures of respective *E. coli* clones were grown at 30°C in 5 ml of LB

medium containing 0.01% sodium ampicillin with shaking at 250 rpm. Fifty milliliters of LB medium containing 0.01% sodium ampicillin was placed in each of two 500-ml Sakaguchi-flasks. The flasks were inoculated with overnight cultures and grown at 37°C with shaking at 120 rpm. At an optical density at 600 nm of 0.3 to 0.4, the cultures were induced with isopropyl β-D-1-thiogalactopyranoside (0.1 mM final concentration). Then, the cultures were incubated at 16 h at 25°C and harvested by centrifugation.

FAO activity assay

The assay procedure was modified from the protocol of Kemp et al. (1988). The assay is a two-enzyme, coupled reaction. 1-Dodecanol was used as the standard for the general FAO assay. The final reaction mixture for the general AO assay comprised 100 µl of 100 mM sodium phosphate buffer (pH7.4), 50 µl of 2.8 mg ml^{-1} 2,2'-azino-di [3-ethylbenzothiazoline-(6)-sulfonic acid] solution in deionized water, 30 µl of a horseradish peroxidase (47 units mL^{-1}) solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 10 µl of microsomal protein or cell extract solutions, and 10 µl of a 5 mM substrate solution. FAO activity was measured at 30°C at 405 nm. FAO activity was reported as specific activity units per milligram of protein (1 U = 1 µmol of oxidized substrate min^{-1}).

Protein determination

The protein concentration in the extracts was determined using a Bradford protein assay kit (Bio-Rad). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970).

Creation of the knockout fragments

The strategy for knocking out the *FAO1* gene is shown in Fig. 2. The 1,000-bp upstream and 1,000-bp downstream fragments of the *FAO1* gene were amplified with the primers FAO1USinF and FAO1US-UraR, and Ura-FAO1DSF and FAO1DSinR, respectively. The primers Pura3F and ura3R were designed according to the manufacturer's guidelines and used to integrate the functional *S. bombicola URA3* sequence into *FAO1*. The primers pUC F and pUC R were used to amplify a linear DNA fragment of pUC118. pUC-Δ*FAO1* was constructed such that these fragments were ligated using the In-Fusion HD Kit (TaKaRa Bio). The primer pair FAO1USinF and FAO1DSinR was used to amplify a 1,023-bp fragment, which contained the *URA3* sequence and 1,000-bp of the *FAO1* upstream and downstream flanking sequences, from pUC-Δ*FAO1*. The fragment was used to transform the *ura3*-auxotrophic KSM-*ura3*Δ strain, which was isolated using the 5-FOA selection method (Van Bogaert et al., 2008). We used this method to select a spontaneous *ura3*-auxotrophic mutant, designated KSM-*ura3*Δ, of *S. bombicola* KSM36.

Analysis of ura3-auxotrophic mutants

The sequences of the *URA3* gene in NBRC 10243 (GenBank accession no. DQ916828), KSM36, and KSM-*ura3*Δ were analyzed. The NBRC10243 and KSM36 *URA3* genes were identical. KSM-*ura3*Δ had a single C54Y mutation in the *URA3* gene (data not shown); thus, this strain requires ura3 for growth, and it is 5-FOA resistant.

The pUC-*URA3* vector carries the functional *URA3* gene of *S. bombicola* NBRC10243, including the 789-bp coding sequence and a 234-bp 5′-untranslated region. When 1 μg of pUC-*URA3* vector was used in the transformation, over 100

colonies appeared on minimal medium plates. Insertion of the wild-type *URA3* gene into the KSM-*ura3*Δ genome was verified by PCR. The primers Pura3 and ura3R specifically amplified the *URA3* gene, while pUC118F and pUC118R were specific for the pUC118-containing fragment. Eleven randomly selected prototrophs that were obtained after the transformation with linear DNA were tested. Ten of the tested colonies resulted from a single crossover or non-homologous recombination event, that is, they contained the pUC118 fragment. For one transformant, a double-crossover event occurred in the *URA3* region. This strain was designated KSM-*ura3*Δ::*URA3*, and it was used as the comparative strain for the KS-*ura3*Δ strain. The KSM *ura3*Δ*::URA3* strain had the same ability to produce sophorolipids from oleic acid, as well as an alkyl glucoside from 2-tetradecanol, as the wild-type strain (data not shown).

High-pressure liquid chromatography/charged aerosol detection (HPLC/CAD) and mass spectroscopy (MS) analysis

Glycolipids samples were analyzed by an Chromaster HPLC system (Hitachi, Tokyo, Japan) and Corona CAD (Thermo Scientific, Waltham, MA, USA) using an Acclaim Surfactant column (4.6 x 150 mm, 5 μm (Dionex, Sunnyvale, CA, USA)) at 40°C. Parameters of CAD were set to the nitrogen gas pressure of 35 psi and the detector response range of 100 pA. A gradient of two eluents, a 0.1% formic acid (w/w) aqueous solution and acetonitrile, was used to separate the components. The gradient started at 20% acetonitrile for 3 min, and it increased linearly to 95% over 37 min and to 95% acetonitrile for 5 min. The mixture was maintained at this composition for 30 min and then brought back to 20% acetonitrile over 5 min. A flow rate of 0.5 ml min^{-1} was applied. A Prominence UFLC HPLC System (Shimadzu, Kyoto, Japan) and liquid

chromatography (LC)-MS 2020 (Shimadzu) were used for the HPLC-MS analysis. A scan analysis was performed for molecules with masses between 30 and 1,500 Da.

Sampling and analytical methods

Analytical glycolipids samples were prepared as follows. Five hundred microliters of butanol containing 0.05% 1-octadecanol as an internal standard was added to 500 µl of culture broth and shaken vigorously for 5 min. After centrifugation at 3,000 × *g* for 5 min, the upper solvent layer was removed, placed in fresh glass culture tubes (ST-13M, Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and dried using a centrifugal evaporator. For alkaline hydrolysis of ester linkages, the solid sample was refluxed in 500 µl of a 1 M NaOH solution for 4 h. After adjusting the pH to 4 with 1 M HCl, 1 ml of butanol was added and shaken vigorously for 5 min. After centrifugation at 3,000 × *g* for 5 min, the upper solvent layer was removed, placed in fresh glass culture tubes (ST-13M), and dried using a centrifugal evaporator. A 1-(trimethylsilyl)imidazole solution (500 µl, GL Sciences Inc., Tokyo, Japan) was added and incubated at 60°C for 10 min. An equal volume (500 µl) of hexane and saturated saline were added and shaken vigorously for 5 min. After centrifugation at 3,000 × *g* for 5 min, the upper solvent layer was analyzed by gas chromatography/MS (GC-MS) on 7890A (Agilent Technologies, Santa Clara, CA, USA) and 5975C Systems (Agilent Technology) using a DB-1 ms column (20 m × 0.10 mm × 0.1 µm), helium gas as the mobile phase at a flow rate 0.3 ml min^{-1}, and the following time program: 100°C for 2 min, followed by an increase of 15°C min^{-1} to 300°C (20 min). n-Dodecyl-β-D-maltoside (Calbiochem, EMD Millipore, Billerica, MA, USA) was used as a standard. Colony-forming units were determined by plating serial dilutions on YPD agar plates, which were incubated at 30°C for 3 d.

Nucleotide sequence accession number

The nucleotide sequence described in this paper was deposited in the GenBank nucleotide database under the accession number AB907775.

Results

Isolation of the *FAO1* gene sequence

In this study, a cDNA sequence analysis was performed on the type strain of *S. bombicola* NBRC10243. A putative AO gene was found that had weak homology (identity 35%) to *C. tropicalis FAO2*. The complete ORF of 2,046 bp encodes a putative FAO comprising 681 amino acids, which we named *FAO1* (Fig. 3). A single exon encoding a genomic clone contained the full-length *FAO1* coding 4688 sequence.

To isolate the complete *FAO1* gene from the *S. bombicola* KSM36 genome, the 2,787-bp *FAO1* fragment was amplified by high-fidelity PCR of freshly isolated genomic DNA. The DNA sequence of the *FAO1* gene from the KSM36 strain was the same as that of NBRC10243; thus, NBRC10243 and KSM36 are likely to be closely related. The ORF encodes a protein with an estimated molecular mass of 75.0 kDa and an estimated isoelectric point of 8.48.

Sequence homology to other FAO proteins

Analysis of the putative FAO1 protein sequence via the Conserved Domain Database available at the National Center for Biotechnology Information website (Marchler-Bauer et al., 2009) indicated that this protein (EC 1.1.3.20) belongs to the GMC oxidoreductase superfamily, which is a polyspecific family that harbors over 30 different inverting oxidases that use molecular O_2. The other FAO, *Candida cloacae*

FAO1 (CAB75352.1, Vanhanen et al., 2000), has a consensus SKL amino acid sequence motif that is a consensus peroxisome-targeting sequence (PTS, http://elm.eu.org/elms/TRG_PTS1). The corresponding FAO1 carboxyl-terminal sequence is TKL, which also may be a PTS.

Expression of FAO1 in *E. coli*, and its AO activity

FAO1 was expressed in *E. coli* BL21(DE3). FAO1 migrated as an ~75 kDa band in sodium dodecyl sulfate-polyacrylamide gels (Fig. 4a), which corresponds well to its calculated molecular mass (75 kDa). FAO1 had AO activity towards 1-dodecanol, while *E. coli* BL21(DE3) did not (Fig. 4b). The substrate specificity of FAO1 was verified (Fig. 4c). FAO1 had oxidase activity toward primary alcohols with chain lengths of 4 to

Construction of an *FAO1*-negative *S. bombicola* strain

A linear *FAO1* knockout cassette was used to transform the KSM *ura3*Δ strain, and the transformants were selected by complementation as a result of the disruption of the *FAO1* gene by the insertion of the *URA3* marker. Four days after the transformation, many transformants were obtained on selective plates, and 10 of them were subjected to colony PCR using the primers FAOUSinF and FAO1DSinR to check their genotype. Of the 10 transformants, seven yielded the expected amplicon for double-crossover events. One of the strains was designated KSM-*fao1*Δ. The other mutants probably arose from non-homologous recombination events or recombination of the URA3 marker with the KSM *ura3*Δ allele.

Growth of the KSM-*fao1*Δ strain in various long-chain alcohol substrates

S. bombicola strains were grown on a basal medium containing 1-tetradecanol as the sole carbon source. We determined the ability of the KSM-*ura3*Δ*::URA3* and KSM-*fao1*Δ strains to use several carbon sources. As shown in Table 3, both strains grew on glucose as the sole carbon source. The KSM-*ura3*Δ*::URA3* strain, but not the KSM-*fao1*Δ strain, also grew on media containing 1-tetradecanol or 1-hexadecanol. The AO activities of the KSM-*ura3*Δ*::URA3* and KSM-*fao1*Δ strains were determined. These strains were cultivated on SD medium containing 1% 1-hexadecanol for 48 h. The specific FAO activities toward 1-dodecanol in the microsome fractions of the KSM-*ura3*Δ*::URA3* and KSM-*fao1*Δ strains were 70.6 ± 16.2 and 6.9 ± 3.9 U/g-protein, respectively. These data suggest that FAO1 is the main AO for the utilization of long-chain alcohols.

Structural analysis of glycolipids produced by the KSM-*fao1*Δ strain

The ability of the strains to produce glycolipids from 1-tetradecanol was tested. Seventy-two hours after the addition of alcohol, glycolipids production by the KSM-*ura3*Δ*::URA3* and KSM-*fao1*Δ strains was determined. Tetradecanoic acid was detected in strain KSM *ura3*Δ*::URA3*, but not in strain KSM-*fao1*Δ, via GC. This indicated that alcohol oxidation was suppressed by deleting the *FAO1* gene.

The chromatograms obtained for the KSM-*fao1*Δ strain, but not the KSM-*ura3*Δ*::URA3* strain, showed a peak that corresponded to the mobility of dodecyl maltoside (18.9 min) (Fig. 5). The supernatant was then subjected to HPLC/CAD and HPLC/MS. Monoacetylated and diacetylated tetradecyl disaccharide hydronium ions had *m/z* of 598.5 and 640.5, respectively. These ions were observed at 26.7 min (peak 2 in Fig. 6b)

and 28.3 min (peak 3 in Fig. 6b) in the culture of the KSM-*fao1*Δ strain. Based on a previous study (Fleurackers et al., 2010), we estimated that the disaccharide backbone was a sophoroside. Monoacetylated 1-O-tetradecyl-(2-O-β-D-glucopyranosyl-β-D-glucopyranoside (molecular weight 580 Da) and diacetylated 1-O-tetradecyl-(2′-O-β-D-glucopyranosyl-β-D-glucopyranoside) (molecular weight 622 Da) were contained in the mixture (Fig. 7).

The main product (peak 1) ion had an *m/z* of 1,064.7 in the positive ion mode and an *m/z* of 1,045.6 in the negative ion mode. This ion corresponded to tetraacetylated tetradecandiol tetrasaccharide (molecular weight 1,046 Da). The results of acidic methanolysis of butanol extracts from the cultures and an additional GC analysis are presented in Table 4. There was a very high 1,14-tetradecandiol content in the glycolipids, which was also true to some extent for 2,13-tetradecandiol and 1-tetradecanol. Based on a previous study (Brakemeier et al., 1995), we estimated that the constituents of peak 1 were sophorolipids SL-C and SL-D (Fig. 7). The peaks from 26 to 32 min (26.4, 27.1, 29.0, and 31.1 min) are *de novo*-synthesized sophorolipids (Fig. 6c).

Production of glycolipids by the KSM-*fao1*Δ strain

To determine the novel glycolipids yield, the same sample was analyzed by HPLC/CAD. *De novo* sophorolipids production were 6.4 g/L/120 h and 1.8 g/L/120 h in the KSM-*ura3*Δ*::URA* and KSM-*fao1*Δ strains, respectively. While the KSM-*ura3*Δ*::URA3* strain did not produce APGs or DPGs from primary alcohols (Fig. 8), 27.3g L^{-1} novel glycolipids were obtained from the KSM-*fao1*Δ strain. Thus, the KSM-*fao1*Δ strain efficiently converted primary alcohols to glycolipids. The ratios of

glycolipids were 0.78, 0.16, 0.06 in alkyldisophorosides (SL-C, D), alkylsophorosides (SL-A, B), and classical sophorolipids, respectively, in the culture of the KSM-*fao1*Δ strain.

Discussion

A few studies that altered sophorolipid structures, such as their sugar or lipid components, via gene recombination in *S. bombicola* have been reported. More recently, the biosyntheses of sophorolipids and sophorolipid transporters were reported by Van Bogaert et al. (2013), and host-modified research for sophorolipid production is expected to progress rapidly (Van Bogaert et al., 2013). Moreover, Van Bogaert et al. (2009) also reported that a higher production of medium-chain length sophorolipids was achieved by blocking the beta-oxidation pathway. We attempted to determine the main enzyme responsible for alcohol oxidation in *S. bombicola*, and to convert this strain to one that produces tetradecyl disaccharide and novel diol tetrasaccharide by deleting its AO gene. Alcohol oxidation enzymes may be categorized as alcohol dehydrogenases or alcohol oxidases based on the mechanisms involved in the catalytic reactions. *S. bombicola* was suggested to have multiple alcohol oxidases (Hommel and Ratledge, 1990).

We identified *FAO1*, a novel gene encoding an AO, in the *S. bombicola* genome. The deletion of *FAO1* prevented the growth of *S. bombicola* in media containing primary alcohols as the carbon source, and AO activity in the microsomal fraction decreased by ~90% compared with that of the parent strain. Thus, FAO1 must be a dominant factor in the assimilation of primary alcohols. Deletion of *FAO1* improved the yields of tetradecanol-based sophorolipids, and tetradecanediol-based sophorolipids

from 0 to 16.9% and from 0 to 46.2%, respectively (Fig. 8). It was unexpected that tetradecanediol-based sophorolipids were major products. *S. bombicola* should have ω and ω-1 hydroxylation activity toward alcohols. These glycolipids were not detected in the parent strain because alcohols and diols could be easily oxidized to their corresponding fatty acids by FAO1.

The KSM-*fao1*Δ strain efficiently produces novel glycolipids from primary alcohols. Although sophorolipids are classified as low-foaming detergents, the culture supernatant of the KSM-*fao1*Δ strain mutant clearly exhibited increased foam formation and stability compared with the supernatant containing wild-type yeast sophorolipids. These strong foaming properties are of interest to several industries. With a yield of approximately 27g L^{-1}, the *FAO1* mutant offers an environmentally acceptable method compared to chemical synthetic procedure at a moderate temperature, standard pressure, and in a water reaction field.

Figures

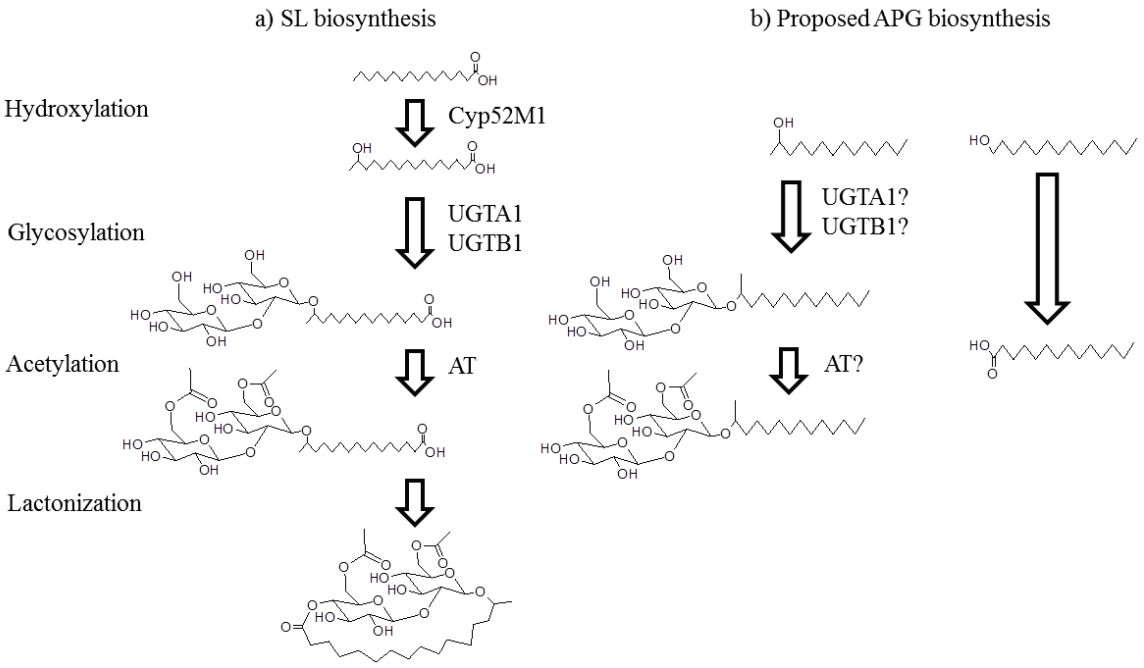

Fig. 1 Sophorolipid biosynthesis and proposed APG biosynthesis in *S. bombicola*

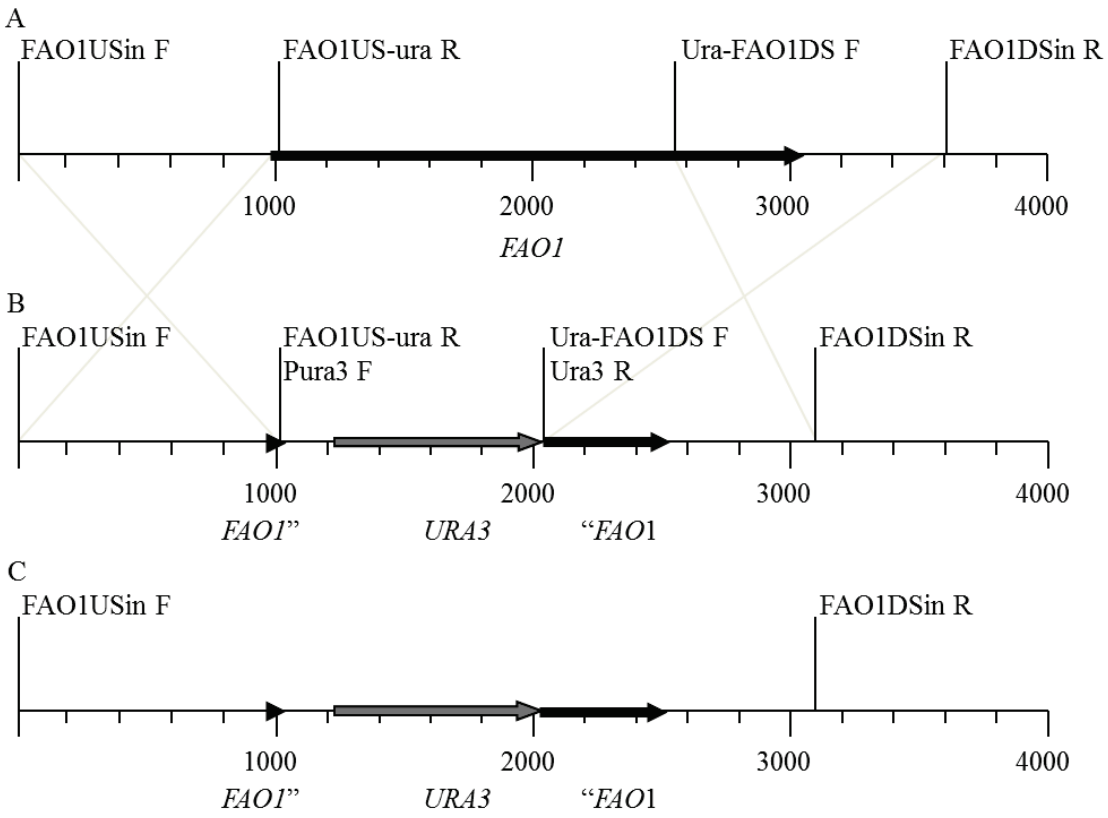

Fig. 2 *FAO1* gene knockout strategy.

A, *S. bombicola* wild-type *FAO1* allele, B, knockout cassette, C, mutant allele.

Sites of homologous recombination between the genome and the cassette are indicated, and all theprimers used to create the cassette and determine the genotype of the mutant are indicated.

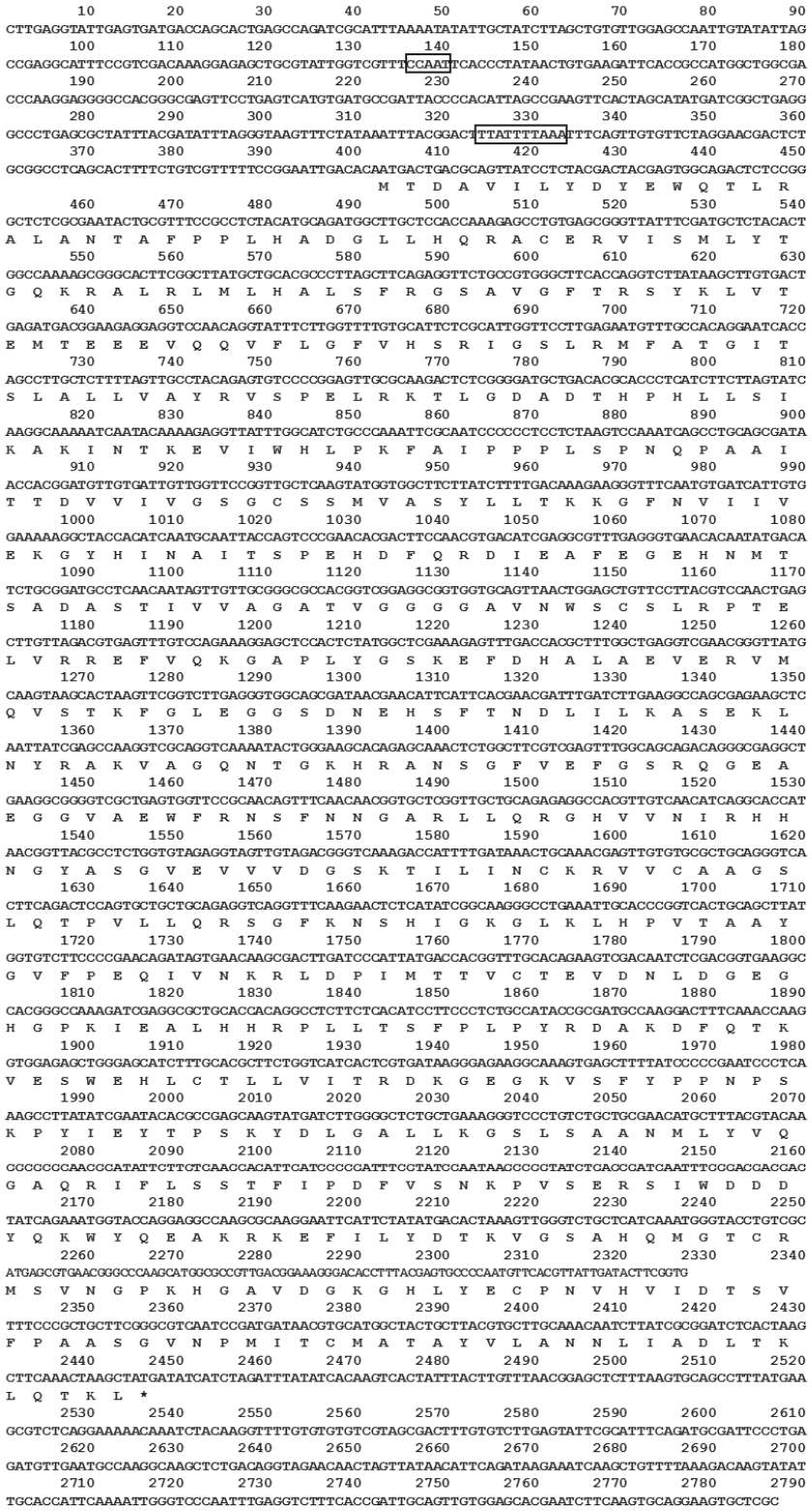

Fig. 3 Nucleotide and deduced amino acid sequence of the *FAO1* gene of *S. bombicola*. Possible promoter elements are boxed.

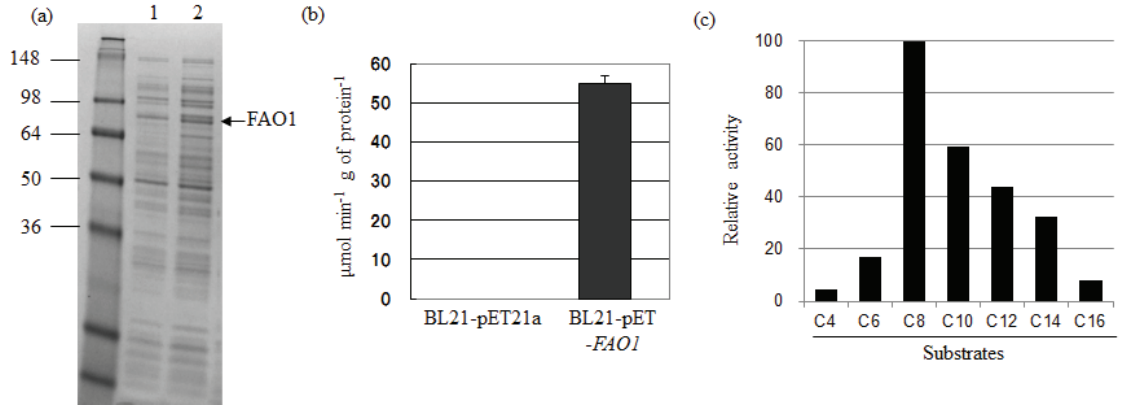

Fig. 4 (a) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of protein profiles of *E. coli* BL21(DE3)-expressed FAO1. Samples were obtained 19 h after induction. The induced protein is indicated by an arrow. Lane 1, cell-free extract of BL21-pET21a; lane 2, cell-free extract of BL21-pET-*FAO1*. (b) Specific FAO activity in cell-free extracts of *E. coli* BL21-pET21a and of BL21-pET-*FAO1*. The substrate for the enzyme assay was 1-dodecnol. (c) Substrate specificity of FAO1. C4, 1-butanol; C6, 1-hexanol; C8, 1-octanol; C10, 1-decanol; C12, 1-dodecanol; C14, 1-tetradecanol; C16, 1-hexadecanol

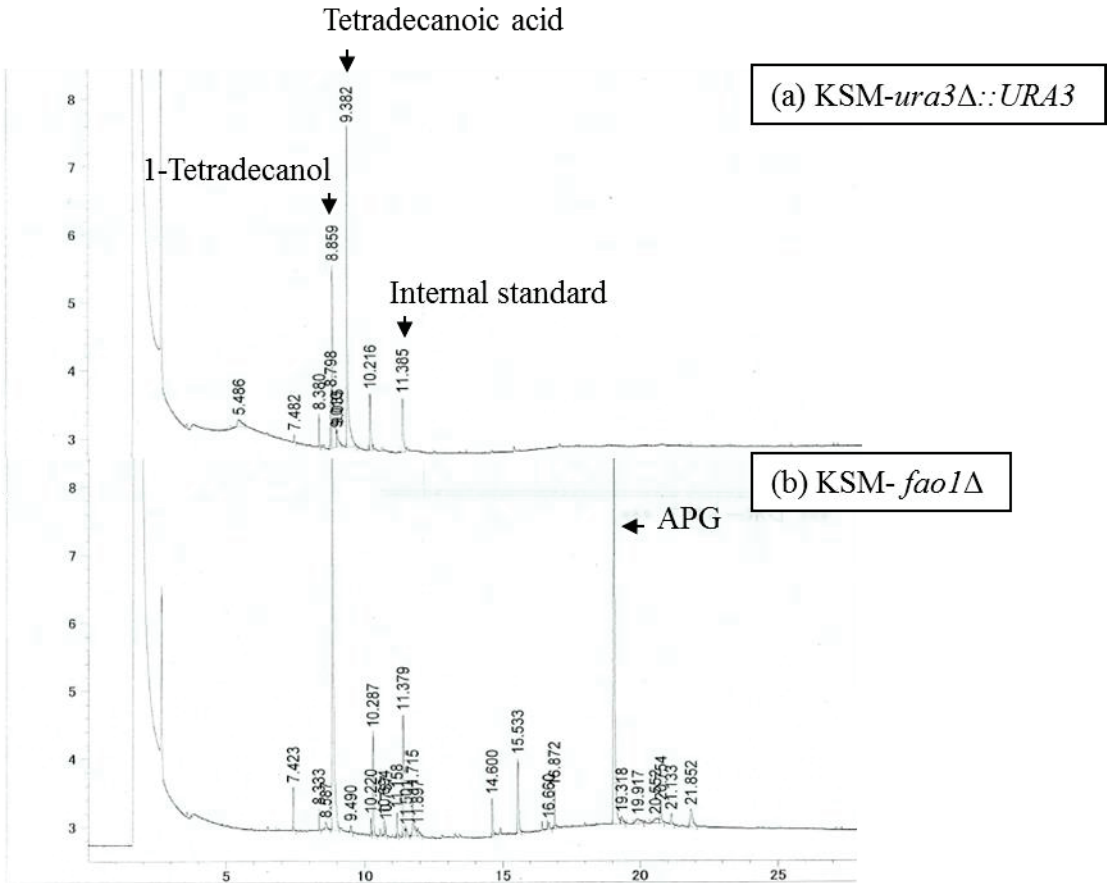

Fig. 5 Gas chromatography analysis of alkyl polyglucosides from (a) *S. bombicola* the KSM-*ura3*Δ*::URA3* and (b) KSM-*fao1*Δ strains grown on 1-tetradecanol.

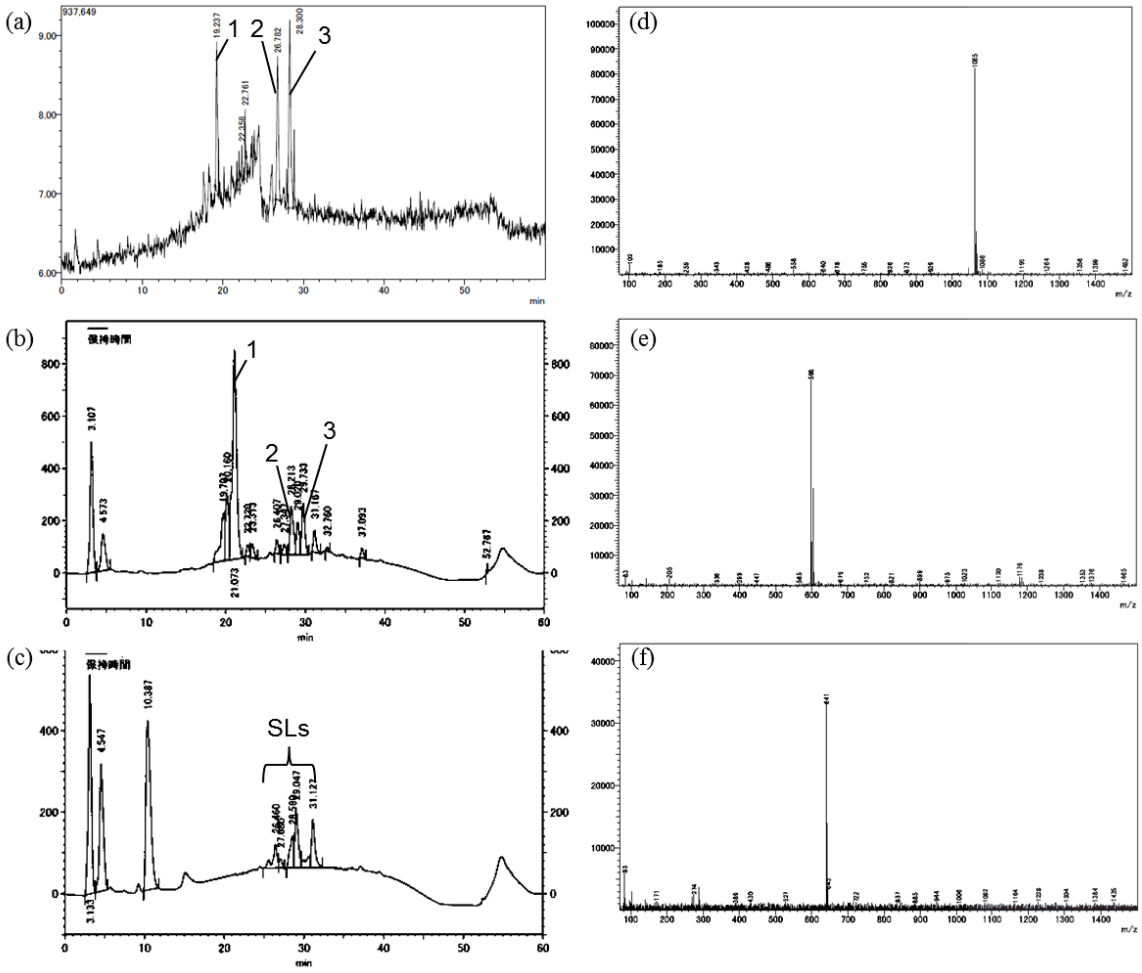

Fig. 6 HPLC analysis of glycolipids from KSM-*fao1*Δ grown on 1-tetradecanol, (a) the chromatogram of ESI MS, (b) the chromatogram of CAD. (c) HPLC/CAD analysis of glycolipids from KSM-*fao1*Δ absent 1-tetradecanol. Panel D, E, F show a detail of the positive ion mode mas spectrum of LC-ESI peak 1, 2, 3 in (a), respectively.

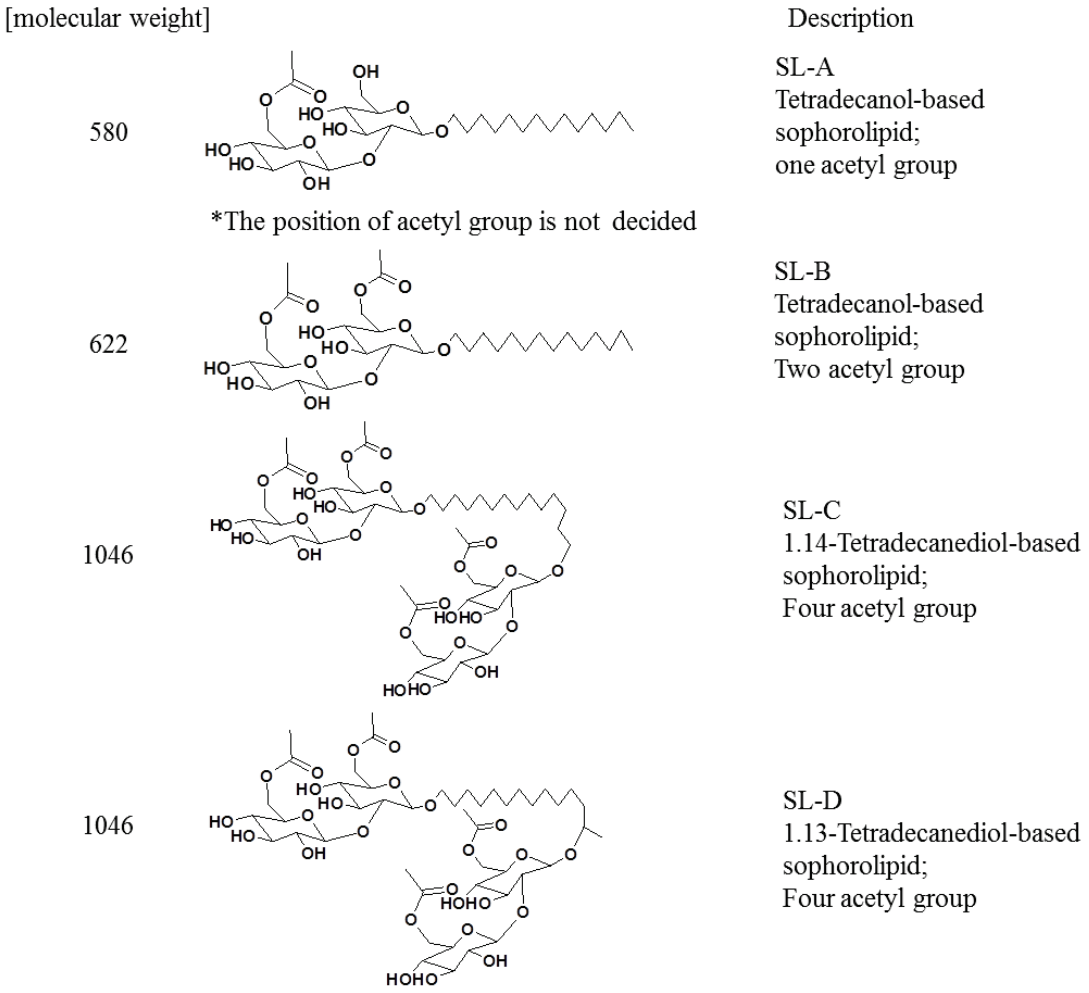

Fig. 7 Putative structure of the glycolipids from the KSM-*fao1*Δ strain grown on 1-tetradecanol. SL, sophorolipid.

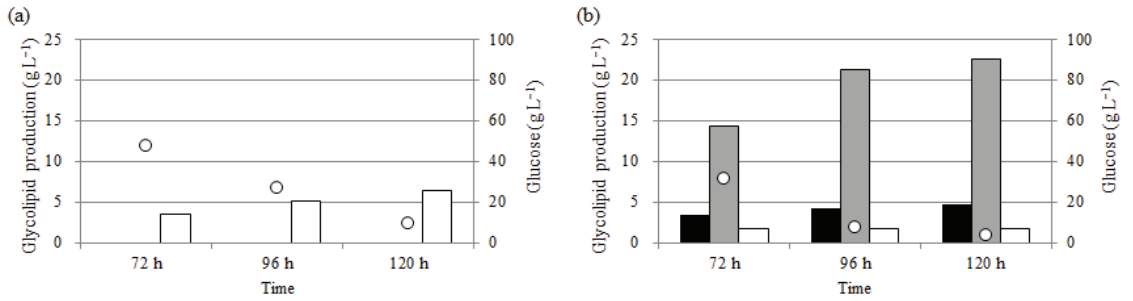

Fig. 8 Glycolipid production of the parent and the KSM-*fao1*Δ strains grown on 1-tetradecanol. (a) The KSM-*ura3*Δ*:URA3* strain, and (b) the KSM-*fao1*Δ strain. Filled bars indicate alkyl sophorosides; gray bars indicated tetradecanediol-based sophorolipids; open bars indicate *de novo*-synthesized sophorolipids; and open circles indicate the glucose concentration in the culture

Tables

Table 1 Yeast and bacterial strains used or constructed in this study

Strain	Properties	Source or reference
KSM36	*S. bombicola* KSM36	FERM-BP 799
KSM-*ura3*Δ	KSM36Δ*URA3*	This study
KSM-*ura3*Δ*::URA3*	KSMΔ*URA3::URA3*	This study
KSM- *fao1*Δ	KSM36Δ*URA3, FAO1::URA3*	This study
NBRC10243	Type strain	NITE Biological Resource Center, Japan
Escherichia coli BL21(DE3)		TaKaRa BIO

Table 2 Primers used for isolating, cloning, expression and knocking out the *S. bombicola* FAO1 gene.

Primer	Sequence (5'-3')	Purpose in this study
Pura3 F	AGTACATATTTTTCGAAACAGCTCGCAA	Cloning of *URA3*
ura3 R	CTAAGAAACTCATCTTGACTGAACTTTTC	Construction of KSM-*fao1*Δ and KSM-*ura3*Δ*::URA3*
FAO1 F	GAAGGAGATATACATATGACTGACGCAG TTATCCTC	Construction of pET-*FAO1*
FAO1 R	AGTGCGGCCGCAAGCTAGCTTAGTTTGA AGCTTAG	
pET21 F	GCTTGCGGCCGCACTCGAG	
pET21 R	ATGTATATCTCCTTCTTAAAG	
FAO1USin F	AGCTTGCATGCCTGCTTTAAATCCAGAA AGAACTG	Construction of KSM-*fao1*Δ
FAO1US-ura R	CGAAAAATATGTACTGATAACTGCGTCA GTCATTG	
Ura-FAO1DS F	AGATGAGTTTCTTAGAAGCCTTATATCG AATACAC	
FAO1DSin R	ATTCGAGCTCGGTACGACACTTCTCAGG AACCCTC	
pUC F	GTACCGAGCTCGAATTCGT	
pUC R	GCAGGCATGCAAGCTTGGC	
pUC118 F	GGCGAAAGGGGGATGTGC	Checking for illegitimate recombination of pUC118 region
pUC118 R	GCACCCCAGGCTTTACAC	

Table 3 Growth of KSM36 and mutant strains grown in primary alcohols.

Substrates/strains	KSM36	KSM-*ura3*Δ::*URA3*	KSM- *fao1*Δ
Glucose	+	+	+
1-dodecanol	−	−	−
1-tetradecanol	+	+	−
1-hexadecanol	+	+	−

'+: Growth, −: no growth.

Table 4 GC analysis of lipid constituents after the methanolysis of glycolipids after cultivation on glucose/1-tetradecanol

Lipid constituents of glycolipid	Lipid quantities in glycolipids after cultivation on 1-tetradecanol
1-tetradecanol	25.9%
1,13-tetradecanediol	22.4%
1,14-tetradecanediol	49.9%
17-hydroxyoleate	1.0%
17-hydroxystearate	0.8%

References

Brakemeier, A., Lang, S., Wullbrandt, D., Merschel, L., Benninghoven, A., Buschmann, N., Wagner, F. (1995) Novel sophorose lipids from microbial conversion of 2-alkanols. ***Biotechnol. Lett.*** 17: 1183–1188.

Cheng, Q., Sanglard, D., Vanhanen, S., Huan, T.L., Bombelli, P., Smith, A., Slabas, A.R. (2005) *Candida* yeast long chain fatty alcohol oxidase is a c-type haemoprotein and

plays an important role in long chain fatty acid metabolism. ***Biochim. Biophys. Acta*** 1735: 192–203.

Fleurackers, S.J.J., Van Bogaert, I.N.A., Develter, D. (2010) On the production and identification of medium-chained sophorolipids. ***Euro. J. Lipid Science Technol.*** 112: 655–662.

Gao, R., Falkeborg, M., Xu, X., Guo, Z. (2013) Production of sophorolipids with enhanced volumetric productivity by means of high cell density fermentation. ***Appl. Microbiol. Biotechnol.*** 97: 1103–1111.

Hommel, R., Ratledge, C. (1990) Evidence for two fatty alcohol oxidases in the biosurfactant-producing yeast *Candida* (*Torulopsis*) *bombicola*. ***FEMS Microbiol. Lett.*** 70: 183–186.

Inoue, S., Ito, S. (1982) Sophorolipids from *Torulopsis bombicola* as microbial surfactants in alkane fermentations. ***Biotechnol. Lett.*** 4: 3–8.

Kemp, G.D., Dickinson, F.M., Ratledge, C. (1988) Inducible long chain alcohol oxidase from alkane-grown *Candida tropicalis*. ***Appl. Microbiol. Biotechnol.*** 29: 370–374.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. ***Nature*** 227: 680–685.

Marchler-Bauer, A., Anderson, J.B., Chitsaz, F., Derbyshire, M.K., DeWeese-Scott, C., Fong, J.H., Geer, L.Y., Geer, R.C., Gonzales, N.R., Gwadz, M., He, S., Hurwitz, D.I., Jackson, J.D., Ke, Z., Lanczycki, C.J., Liebert, C.A., Liu, C., Lu, F., Lu, S., Marchler, G.H., Mullokandov, M., Song, J.S., Tasneem, A., Thanki, N., Yamashita, R.A., Zhang, D., Zhang, N., Bryant, S.H. (2009) CDD: specific functional annotation with the conserved domain database. ***Nucleic Acids Res.*** 37: 205–210.

Sambrook, J., Russell, D.W. (2001) Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Spencer, J.F.T., Gorin, P.A.J., Tulloch, A.P. (1970) *Torulopsis bombicola* sp. Antonie Van Leeuwenhoek 36: 129–133.

Van Bogaert INA, De Maeseneire SL, Develter D, Soetaert W, Vandamme EJ (2008) Development of a transformation and selection system for the glycolipid-producing yeast *Candida bombicola*. ***Yeast*** 25: 273–278.

Van Bogaert, I.N.A., Sabiora, J., Develter, D., Soetaert, W., Vandamme, E.J. (2009) Knocking out the MFE-2 gene of *Candida bombicola* leads to improved medium-chain sophorolipid production. ***FEMS Yeast Res.*** 9: 610–617.

Van Bogaert INA, Holvoet K, Roelants SLKW, Li B, Lin Y-C, Van de Peer Y, Soetaert W (2013) The biosynthetic gene cluster for sophorolipids: A biotechnological interesting biosurfactant produced by *Starmerella bombicola*. ***Mol. Microbiol.*** 88: 501–

509.

Vanhanen S., West M., Kroon JT, Lindner N, Casey J, Cheng Q, Elborough KM and Slabas AR (2000) A consensus sequence for long-chain fatty-acid alcohol oxidases from Candida identifies a family of genes involved in lipid ω-oxidation in yeast with homologues in plants and bacteria. ***J. Biol. Chem.*** 275: 4445–4452.

Wang T-T, Choi Y-J, Lee BH (2001) Transformation systems of non-*Saccharomyces* yeasts. ***Crit. Rev. Biotechnol.*** 21: 177–218.

Chapter 2

Elucidation of secondary alcohol metabolism in *Starmerella bombicola* and contribution of primary alcohol oxidase FAO1

Summary

The yeast *Starmerella bombicola* NBRC10243 is an excellent producer of sophorolipids, which are among the most useful biosurfactants. The primary alcoholic metabolic pathway of *S. bombicola* has been elucidated using alcohol oxidase FAO1, but the secondary alcohol metabolic pathway remains unknown. Although the FAO1 mutant was unable to grow with secondary alcohols and seemed to be involved in the secondary alcohol metabolism pathway of *S. bombicola*, it had very low activity toward secondary alcohols. By analyzing the products of secondary alcohol metabolism, alkyl polyglucosides hydroxylated at the ω position in the alkyl chain of the secondary alcohol were observed in the FAO1 mutant, but not in the wild-type yeast. In the double mutant of FAO1 and UGTA1, accumulation of 1,13-tetradecandiol and 2,13-tetradecandiol was observed. The above results indicated that hydroxylation occurred first at the ω and ω–1 positions in the secondary alcohol metabolism of *S. bombicola*, followed by primary alcohol oxidation.

Introduction

The yeast *Starmerella bombicola* is an excellent sophorolipid producer (Spencer et al., 1970). Sophorolipids derived from *S. bombicola* are glycolipids in which disaccharide sophorose is linked glycosidically to the hydroxyl group at the ω or ω-1 carbons of

C16–C18 fatty acids. Alkyl polyglucosides were also reported to be produced by *S. bombicola* using secondary alcohols as a raw material (Brakemeier et al., 1995). Compared with classical sophorolipids, sophorolipids derived from secondary alcohols have better water solubility and more effectively decrease the surface tension of water. We previously reported that *S. bombicola* fatty alcohol oxidase 1 (FAO1) is a primary alcohol oxidase essential in the primary alcohol utilization pathway of *S. bombicola* (Takahashi et al., 2016). In contrast, the secondary alcohol metabolism pathway of *S. bombicola* has not been clarified. The fatty alcohol oxidase (FAO) pathway has been characterized in *Candida tropicalis*, a yeast with high fatty alcohol and alkane utilization (Cheng et al., 2005). Three FAO genes were identified in *C. tropicalis*, namely *FAO1* and two allelic genes designated as *FAO2a* and *FAO2b*. Both enzymes oxidize long-chain primary alcohols. FAO1 oxidizes ω-hydroxy fatty acids, but not 2-alkanols, while FAO2 oxidizes 2-alkanols, but not ω-hydroxy fatty acids. In general for alkane-utilizing microbes, the *n*-alkane terminus is oxidized to produce a primary alcohol, which is further oxidized to an aldehyde by alcohol dehydrogenase or FAO, and then to a fatty acid by aldehyde dehydrogenase. Furthermore, it has been suggested that a subterminal oxidation pathway exists in some microorganisms. In the subterminal oxidation pathway, *n*-alkanes are thought to be converted into secondary alcohols, which are further oxidized to ketones by alcohol dehydrogenase or FAO and then to esters by Baeyer–Villiger monooxygenase, and then finally transformed into primary alcohols and fatty acids by esterase (Van Beilen et al., 2003).

Herein, I report the secondary alcohol metabolism pathway in *S. bombicola* for the first time, and show that *S. bombicola* FAO1 contributes to this metabolic pathway. Furthermore, we demonstrate that the FAO1 mutants are not only deficient in the

secondary alcohol oxidation pathway, but also able to produce novel sophorose lipids derived from 1,13-tetradecanediol.

Materials and methods

Strains, fermentation, and glycolipids production

The strains used in this study are shown in Table 1. *S. bombicola* KSM36, an industrial strain for sophorolipid production (Inoue and Ito, 1982), ura3-auxotrophic mutant KSM-*ura3*Δ, and FAO1-negative mutant KSM-*fao1*Δ (Takahashi et al., 2016) were used as the parent strains. *Escherichia coli* DH5α (TaKaRa Bio Inc., Kusatsu, Japan) was used for plasmid maintenance and *E. coli* BL21(DE3) (Funakoshi Co., Ltd., Tokyo, Japan) was used for protein expression. *S. bombicola* was cultured on yeast peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, and 2% glucose) or on synthetic dextrose (SD) medium (0.67% yeast nitrogen base without amino acids (Sigma-Aldrich, St. Louis, MO, USA) and 2% glucose). To obtain hygromycin resistant strains, 0.05% hygromycin B (FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to the synthetic dextrose (SD) medium (0.67% yeast nitrogen base without amino acids (Sigma-Aldrich, St. Louis, MO, USA) and 2% glucose). To obtain better mutant growth, 0.03% uracil was added to the YPD medium. Liquid yeast shake-flask cultures were incubated at 30°C with shaking at 120 rpm. *E. coli* was grown in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) supplemented with 0.01% sodium ampicillin if necessary. Liquid *E. coli* cultures were incubated at 37°C with shaking at 120 rpm. *S. bombicola* was grown on a sophorolipid production medium (10% glucose, 10% methyl palmitate, 0.5% yeast extract, and 1% urea; pH adjusted to 5.0 using HCl), or on an APG production medium comprising 15%

glucose, 0.5% sodium citrate·$3H_2O$, 0.04% yeast extract, 0.0154% NH_4Cl, 0.1% KH_2PO_4, 0.07% $MgSO_4·7H_2O$, 0.05% NaCl, 0.027% $CaCl_2·2H_2O$, and 0.016% $K_2HPO_4·3H_2O$. The seed culture (5 mL of YPD in a 100-mL test tube) was incubated for 48 h at 30°C with shaking at 250 rpm using a rotary shaker. An 1-mL aliquot of this culture was added to 100 mL of production medium in a 500-mL Sakaguchi flask. After 48 h, 10 g L^{-1} of secondary alcohol was added to the microbial culture in the case of APG production medium. The pH was not adjusted and the total incubation time was 120 h.

Washing cells from cultures

Culture samples (20 mL) were centrifuged at approximately 5,000 ×*g* for 10 min at 4°C to obtain cell pellets. The pellets were washed twice by resuspending in 0.08% NaCl (10 mL) and centrifuging at 5,000 ×g and 4°C for 1 min.

Preparation of cell extracts and microsomes

The washed cell pellet was resuspended in 1/15 M potassium buffer (10 mL, pH 7.4). Phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM prior to breaking the cells by shaking vigorously with 0.5-mm glass beads three times at 2,700 rpm for 1 min using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). The broken cell suspension was centrifuged at approximately 15,000 ×*g* for 60 min at 4°C. The supernatant consisted of soluble cell extract. Microsomes were prepared from the cell extract by centrifugation at 100,000 ×*g* for 1.5 h at 4°C. The supernatant was removed and the microsomal pellet resuspended in 1/15 M potassium buffer (200 μL, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride. This mixture was assayed for FAO

activity. Cell extracts from *E. coli* were prepared in the same manner.

Nucleic acid extraction

Yeast genomic DNA was isolated using the Mr. Gentle High Recovery Kit (TaKaRa Bio). Plasmid DNA was isolated using a plasmid purification kit (Roche, Basel, Switzerland).

Transformation

S. bombicola cells were transformed via electroporation (Wang et al., 2001) using a Gene Pulser Xcell (Bio-Rad, Carlsbad, CA, USA) set to 25 μF, 350 Ω, and 2.5 kV. Transformants were selected on SD plates. *E. coli* cells were transformed as described previously (Sambrook and Russell 2001).

Polymerase chain reaction (PCR) and sequencing analysis

The primers used in this study are shown in Table 2. PCR was performed using KOD FX Neo (TOYOBO, Osaka, Japan). PCR amplification was performed with initial denaturation at 94°C for 2 min, followed by 30 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 2 min, followed by a final 2-min elongation at 72°C. All DNA sequences were determined using a Big Dye ver. 3.1 kit (ABI) and 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

FAO activity assay

The assay procedure followed the protocol of Kemp et al. (1988) with modifications. The assay was a two-enzyme coupled reaction. 1-Dodecanol was used as the standard in

the general FAO assay. The final reaction mixture for the general AO assay comprised 100 mM sodium phosphate buffer (100 μL, pH 7.4), 2,2'-azino-di [3-ethylbenzothiazoline-(6)-sulfonic acid] solution in deionized water (50 μL, 2.8 mg mL^{-1}), horseradish peroxidase solution (30 μL, 47 units mL^{-1}; Wako Pure Chemical Industries, Ltd., Osaka, Japan), microsomal protein or cell extract solution (10 μL), and 5 mM substrate solution (10 μL). FAO activity was measured at 30°C and 405 nm, and was reported as the specific activity units per milligram of protein (1 U = 1 μmol of oxidized substrate min^{-1}).

Protein determination

The protein concentration in the extracts was determined using a Bradford protein assay kit (Bio-Rad). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method of Laemmli (1970).

Creation of knockout fragments

The strategy for knocking out the *UGTA1* and *FAO1* genes is shown in Fig. 1. The 1,000-bp upstream and 1,000-bp downstream fragments of the *UGTA1* gene were amplified using the primers UGTAUS-in F and UGTAUS-ura R, and Ura-UGTADS F and UGTADSin R, respectively. Primers Pura3F and ura3R were prepared according to manufacturer guidelines and used to integrate the functional *S. bombicola URA3* sequence into *UGTA1*. Primers pUC F and pUC R were used to amplify a linear DNA fragment of pUC118 (TaKaRa Bio). pUC-Δ*UGTA1* was constructed such that these fragments were ligated using the In-Fusion HD Kit (TaKaRa Bio). Primer pair UGTAUS-in F and UGTADSin R was used to amplify a 3,124-bp fragment, which

contained the *URA3* sequence and 1,020-bp of the *UGTA1* upstream and 1081-bp downstream flanking sequences from pUC-Δ*UGTA1*. The fragment was used to transform the *ura3*-auxotrophic KSM-*ura3*Δ strain. Primers Pura3 R and FAO1DS F were prepared and used to integrate the functional hygromycin resistance gene *HPT* sequence from pPREP4 (Thermo Fisher Scientific) into *FAO1*. Primers Pura3 R and FAO1DS F were used to amplify a linear DNA fragment of pUC-Δ*FAO1* (Takahashi et al., 2016). pUC-Δ*FAO1::HPT* was constructed such that these fragments were ligated using the In-Fusion HD Kit. The primer pair FAO1USinF and FAO1DSinR was used to amplify a 3,400-bp fragment, which contained the HPT sequence and 1,230-bp of the *FAO1* upstream and promoter sequence of *URA3*, and 1,027-bp downstream flanking sequences from pUC-Δ*FAO1::HPT*. The fragment was used to transform the KSM-*ugta*Δ strain.

High-pressure liquid chromatography (HPLC)–mass spectroscopy (MS) analysis

A Prominence UFLC HPLC System (Shimadzu, Kyoto, Japan) and liquid chromatography (LC)-MS 2020 system (Shimadzu) were used for HPLC-MS analysis. Glycolipid samples were analyzed using an Acclaim Surfactant column (4.6 × 150 mm, 5 μm; Dionex, Sunnyvale, CA, USA) at 40°C. A gradient of two eluents, namely a 0.1% formic acid (w/w) aqueous solution and acetonitrile, were used to separate the components. The gradient started at 20% acetonitrile for 3 min and then increased linearly to 95% acetonitrile over 37 min, holding at 95% acetonitrile for 5 min. The mixture was maintained at this composition for 30 min and then brought back to 20% acetonitrile over 5 min. A flow rate of 0.5 mL min^{-1} was used. Scan analysis was performed for molecules with masses between 30 and 1,500 Da.

Sampling and analytical methods

Analytical glycolipid samples were prepared as follows. 1-Butanol (500 μL) containing 0.05% 1-octadecanol as an internal standard was added to culture broth (500 μL) and shaken vigorously for 5 min. After centrifugation at 3,000 ×*g* for 5 min, the upper solvent layer was removed, placed in fresh glass culture tubes (ST-13M, Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and dried using a centrifugal evaporator. For alkaline hydrolysis of the ester linkages, the solid sample was refluxed in 1 M NaOH solution (500 μL) for 4 h. After adjusting the mixture to pH 4 with 1 M HCl, 1-butanol (1 mL) was added and shaken vigorously for 5 min. After centrifugation at 3,000 ×*g* for 5 min, the upper solvent layer was removed, placed in fresh glass culture tubes (ST-13M), and dried using a centrifugal evaporator. A 1-(trimethylsilyl)imidazole solution (500 μL; GL Sciences Inc., Tokyo, Japan) was added and incubated at 60°C for 10 min. Equal volumes (500 μL) of hexane and saturated saline were added and shaken vigorously for 5 min. After centrifugation at 3,000 × *g* for 5 min, the upper solvent layer was analyzed by gas chromatography/MS (GC-MS) on Agilent 7890A and 5975C systems (Agilent Technologies, Santa Clara, CA, USA) using a DB-1ms column (20 m × 0.10 mm × 0.1 μm) and helium gas as the mobile phase at a flow rate 0.3 mL min^{-1}. The following time program was used: 100°C for 2 min, then increased to 300°C at 15 °C min^{-1} (20 min). *n*-Dodecyl-β-D-maltoside (Calbiochem, EMD Millipore, Billerica, MA, USA) was used as a standard. Colony-forming units were determined by plating serial dilutions on YPD agar plates, which were incubated at 30°C for 3 days.

Results

Growth of the KSM-*fao1*Δ strain in secondary alcohol substrates

S. bombicola strains were grown on a basal medium containing 2-tetradecanol as the sole carbon source. We determined the ability of KSM-*ura3*Δ*::URA3* and KSM-*fao1*Δ strains to use several carbon sources. As shown in Table 3, both strains grew on glucose as the sole carbon source. The KSM-*ura3*Δ*::URA3* strain, but not the KSM-*fao1*Δ strain, also grew on media containing 2-tetradecanol or 2-hexadecanol.

Expression of FAO1 in *E. coli* and its AO activity

FAO1 was expressed in *E. coli* BL21(DE3) according to a previous report (Takahashi et al., 2016) and its substrate specificity was verified (Table 4). FAO1 had oxidase activity toward primary alcohols, but no activity was observed toward secondary alcohols.

Structural analysis of glycolipids produced by KSM-*fao1*Δ from secondary alcohols

Although FAO1 had no secondary alcohol oxidizing activity, the assimilability of secondary alcohol disappeared in the *FAO1* mutant. To estimate the secondary alcohol metabolic pathway in *S. bombicola*, we analyzed the products from 2-tetradecanol in KSM-*ura3*Δ*::URA3* and KSM-*fao1*Δ. Each strain was inoculated into the APG production medium, 2-tetradecanol was added at 20 g L^{-1}, and the culture was conducted for 2 days. The main product (peak 1) and minor product (peak 2) were detected in strains KSM-*ura3*Δ*::URA3* and KSM-*fao1*Δ using GC (Fig. 2). Based on a previous study (Brakemeier et al., 1995), we estimated that the main product was a 2-tetradecylsophoroside. In contrast, only the culture of KSM-*fao1*Δstrain showed byproducts (peaks 3–6).

To estimate the structure of byproduct peaks, the supernatant was analyzed by HPLC/MS. The molecular weights of monoacetylated and diacetylated 2-tetradecyl disaccharide were 580 Da and 622 Da, respectively. These ions were observed at 10.5 min (peak A in Fig. 3) and 11 min (peak B in Fig. 3) in the culture of the KSM-*ura3*Δ*::URA3* strain. The major (peak A) and minor (peak B) product ions appeared at *m/z* 579.5 and 621.5, respectively, in negative ion mode. Based on a previous study (Fleurackers et al., 2010), we estimated that peak A was monoacetylated 2-O-tetradecyl-(2-O-β-D-glucopyranosyl-β-D-glucopyranoside) and peak B was diacetylated 2-O-tetradecyl-(2′-O-β-D-glucopyranosyl-β-D-glucopyranoside).

In contrast, another peak was observed at 9 min (peak C) in the supernatant of KSM-*fao1*Δ with *m/z* 637.5 in negative ion mode. The molecular weight of peak C was 16 larger than that of peak B, and peak C was eluted at a higher polarity position than peak B. Therefore, peak C was attributed to a hydroxyl group added to any position of diacetylated 2-O-tetradecyl-(2′-O-β-D-glucopyranosyl-β-D-glucopyranoside).

Subsequently, the position of the additional hydroxyl group was analyzed by GC-MS. When GC-MS analysis is performed after trimethylsilylation of an alkyl compound bearing a hydroxyl group, a fragment ion on the alkyl terminal side containing a hydroxyl group is obtained. Fragment ions of *m/z* 73 and 117 were observed from peak 3, while fragment ions of *m/z* 73, 103, and 117 were observed from peak 4. The fragment ions at *m/z* 103 and 117 were derived from TMS compounds of ω-terminal and ω–1, respectively. This suggested that peaks 3 and 4 were 2,13-tetradecanediol and 1,13-tetradecanediol, respectively. Subsequently, fragment ions at *m/z* 73, 117, and 271.1, *m/z* 73 and 103, and *m/z* 73, 117 and 271.1 were detected from peaks 2, 5, and 6, respectively. Therefore, peak 5 was attributed to an alkyl

sophoroside in which the sugar was transferred to the hydroxy group at position 13 of 1,13-tetradecanediol (peak 4). In the cell of the KSM-*fao1*Δ strain, the primary alcohol oxidizing activity was greatly reduced, but the oxidase activity for the secondary alcohol was hardly changed. Therefore, compared with KSM-*ura3*Δ*::URA3*, the KSM-*fao1*Δ strain was thought to accumulate α-ω-type diols. As peak 6 was observed only in the KSM-*fao1*Δ strain, it was attributed to 1,13-tetradecanediol glycosylated on the hydroxy group at position 1. Peak 2, which is commonly observed in both strains, was thought to be attributed to glycosylation at either of the hydroxy groups in 2,13-tetradecanediol (Fig. 4).

Construction of an *FAO1–UGTA1*-double negative *S. bombicola* strain

The glycosyltransferases involved in APG production have not been reported, but UGTA 1, known as the ω or ω-1 hydroxy fatty acid glycosyltransferase for sophorolipid biosynthesis, was considered a candidate. KSM-*ugta*Δ*fao1*Δ was inoculated into the APG production medium, 2-tetradecanol was added at 20 g L^{-1}, and the culture was conducted for 2 days. Peaks 1, 2, 5, and 6 were not detected (Fig. 2(c)). Therefore, KSM-*ugta*Δ*fao1*Δ had lost the ability to glycosylate alcohols or diols. The amount of both diols reached 2.5 g L^{-1} with a yield of 11.6% (mol/mol).

Discussion

The results of this study suggested that peroxisomal fatty alcohol oxidase, FAO1, plays crucial roles in the metabolism of secondary alcohols in *S. bombicola*.

In wild-type *S. bombicola*, the production of glycolipids from primary alcohols is hardly observed, but sophorose lipids are known to be formed from secondary

alcohols (Takahashi et al., 2016; Brakemeier et al., 1995). In *FAO1* deficient strains, glycosylated products of 1,13-tetradecanediol and 2,13-tetradecanediol were observed in the cultures containing 2-tetradecanol. 2,13-Tetradecanediol was detected in the culture solution of the wild-type, while 1,13-tetradecanediol was not. FAO1 has high oxidizing activity for 1-tetradecanol, but not for 2-tetradecanol. In the wild-type strain, the primary hydroxyl group of 1,13-tetradecanediol should be rapidly oxidized by FAO1, while 2,13-tetradecanediol should accumulate in the yeast cells because FAO1 does not easily oxidize secondary alcohols, resulting in detection of the glycosylated product. Disruption of FAO1 suppressed the assimilation of primary alcohol and produced a glycosylated product derived from 1,13-tetradecanediol. Therefore, the initial step of the secondary alcohol utilization pathway in *S. bombicola* was thought to be hydroxylation at the ω-terminal.

Subsequently, *UGTA1* involved in sophorolipid biosynthesis was deleted against KSM-*fao1*Δ strain to clarify the factor for glycosylation. In the culture of KSM-*ugta*Δ*fao1*Δ strain, glycosylated products were not detected and the formation of 1,13-tetradecanediol and 2,13-tetradecanediol was observed from 2-tetradecanol. The synthesis of alkyl sophorosides in *S. bombicola* was shown to be due to UGTA1. To summarize, the utilization of secondary alcohol in *S. bombicola* is thought to proceed via the route shown in Fig. 5.

In addition, productivity improvements of long-chain diols were recognized after deletion of *UGTA1* and *FAO1*. Long-chain diols are expected to be used as fine chemicals. ω-Terminal hydroxylation of fatty alcohols is a difficult reaction to achieve in organic synthesis, and no examples of preparing (α,ω)-diols in one step from 1-decanol or 1-tetradecanol have been reported. There are also few reports of such

microbial reactions. Genetically modified *E. coli* can produce 722 mg L^{-1} of 1,8-octanediol or 15 mg L^{-1} of 1,9-nonanediol (every 4 h) from the corresponding alcohols (Fujii et al., 2006; Scheps et al., 2011). The diol production system reported herein shows higher productivity than those in the previous reports.

This study not only elucidates the metabolic pathway of secondary alcohols in *S. bombicola*, but also provides a system for producing long-chain diols such as tetradecanediol.

Figures

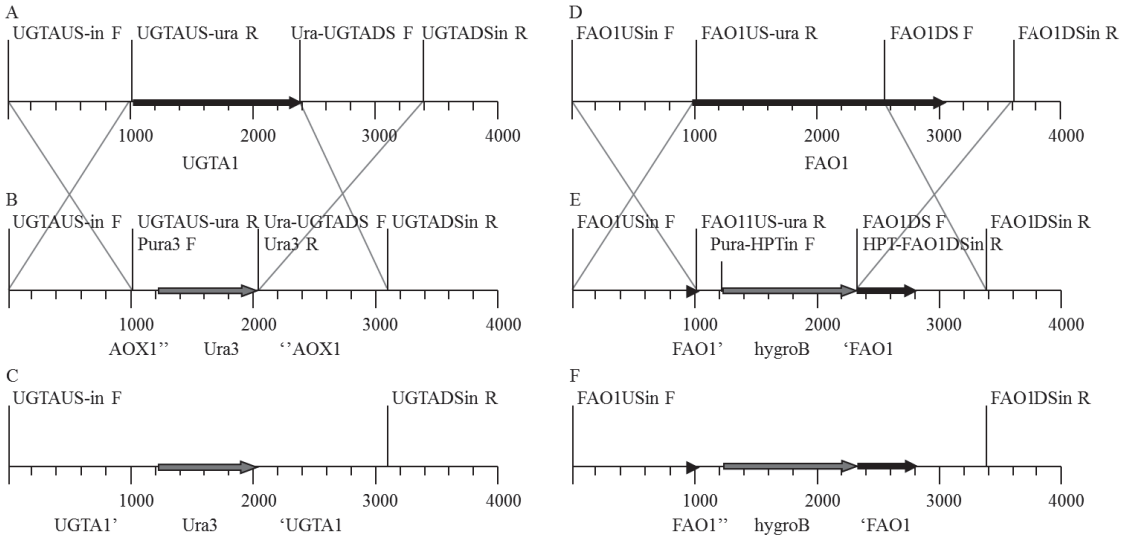

Fig. 1 Gene knockout strategy for UGTA1 and FAO1.

(A, D) *S. bombicola* wild-type UGTA1 or FAO1 allele, (B, E) knockout cassette, (C, F) mutant allele. Sites of homologous recombination between the genome and cassette are indicated and all primers used to create the cassette and control the genotype of the mutants are indicated.

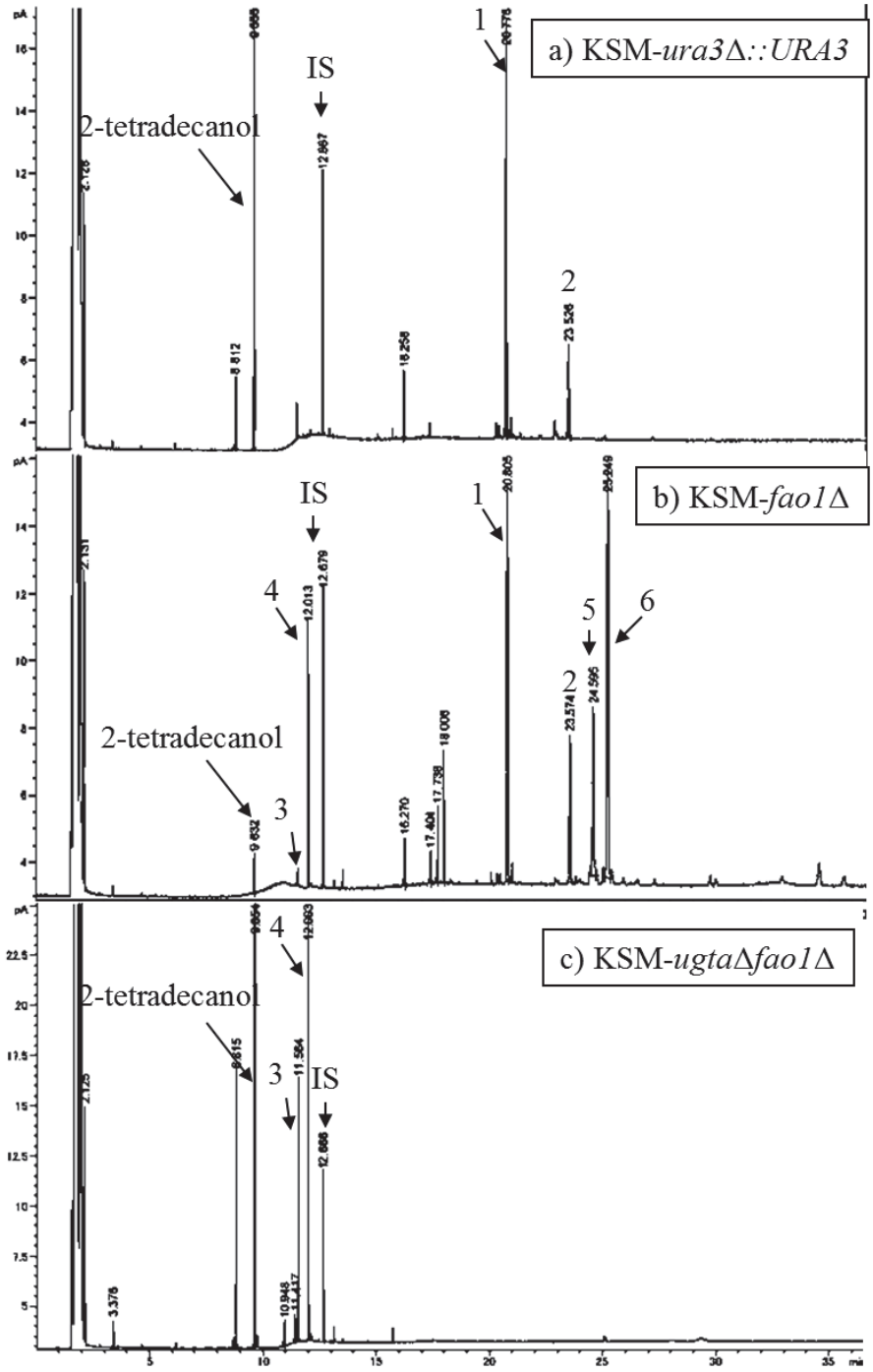

Fig. 2 Chromatogram of GC-FID analysis of glycolipids from *S. bombicola* grown on 2-tetradecanol: (a) KSM-*ura3*Δ*::URA3*, (b) KSM-*fao1*Δ, (c) KSM-*ugta*Δ*fao1*Δ. IS, internal standard (1-octadecanol).

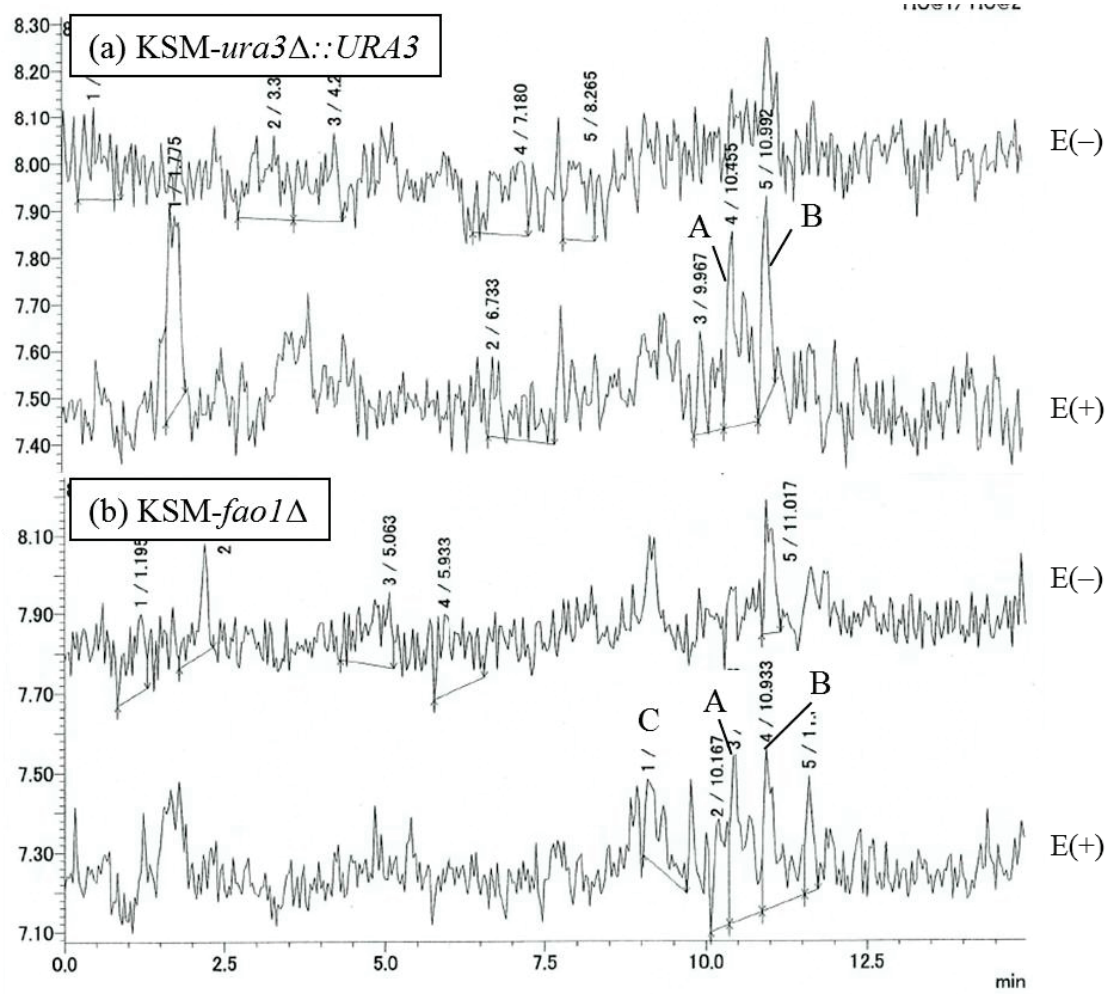

Fig. 3 HPLC-ESI MS analysis of glycolipids from *S. bombicola* grown on 2-tetradecanol: (a) KSM-*ura3*Δ*::URA3*, (b) KSM-*fao1*Δ. E(–), negative ion mode; E(+), positive ion mode.

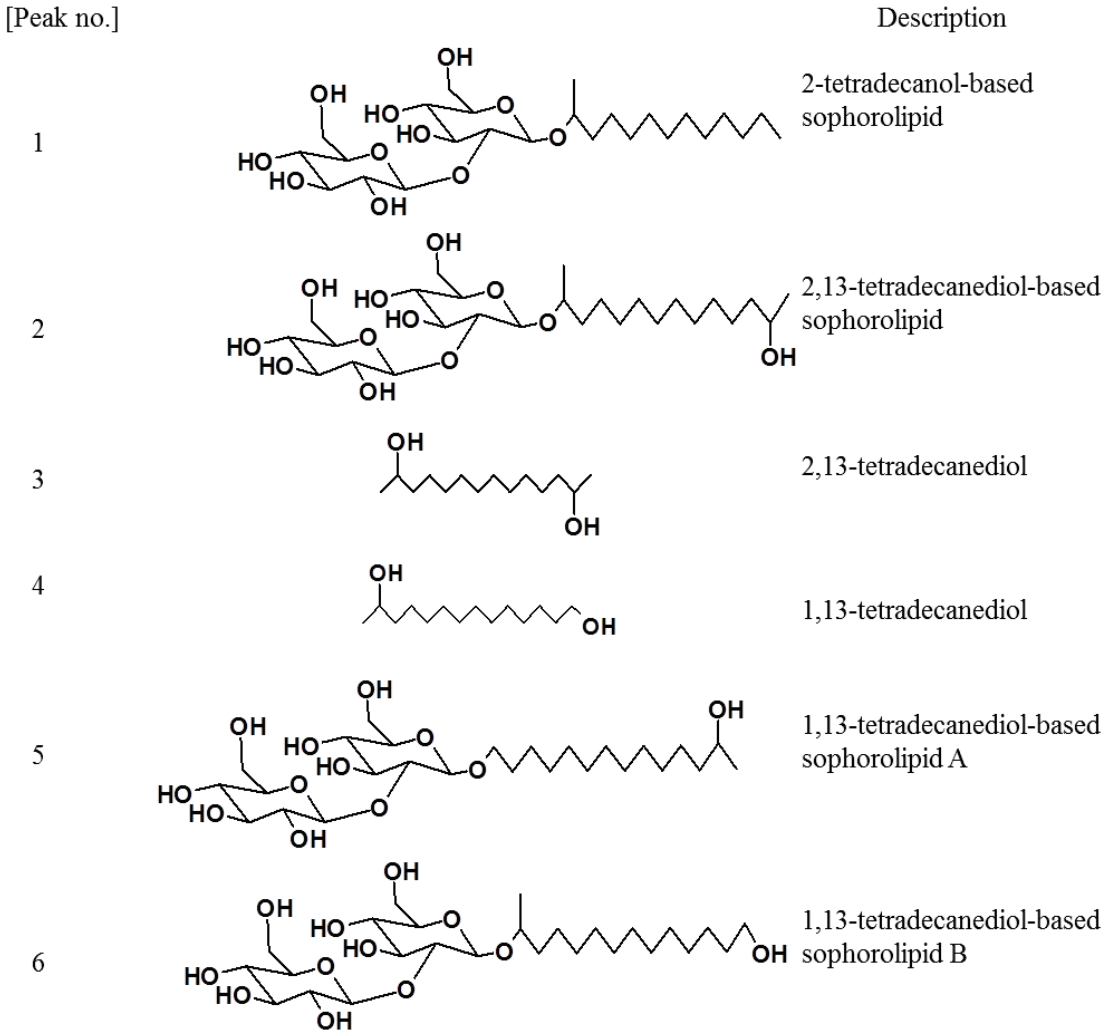

Fig. 4 Putative structure of products from the *FAO1* knockout strain grown on 2-tetradecanol.

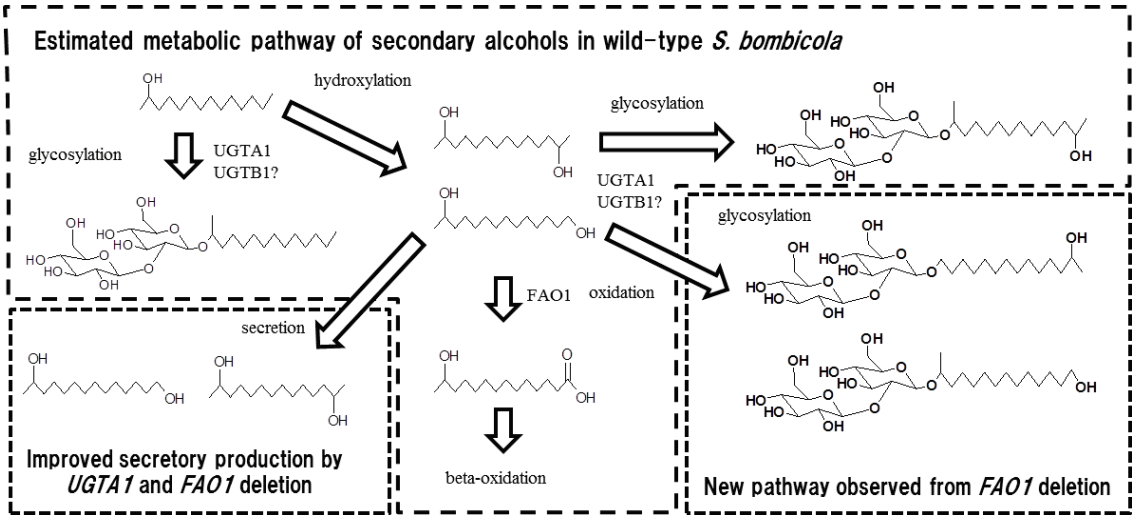

Fig. 5 Secondary alcohol metabolic pathway in *S. bombicola* and the new pathway observed after *FAO1* deletion.

Tables

Table 1 Microbial strains and plasmids used or constructed in this study.

Strain	Properties	Source or reference
KSM36	*S. bombicola* KSM36	FERM-BP 799
KSM-*ura3*Δ	KSM36Δ*URA3*	1
KSM-*ura3*Δ*::URA3*	KSMΔ*URA3::URA3*	1
KSM-*fao1*Δ	KSM36Δ*URA3, FAO1::URA3*	1
KSM-*ugta*Δ	KSM36Δ*URA3, UGTA1::URA3*	This study
KSM-*ugta*Δ*fao1*Δ	KSM36Δ*URA3, UGTA1::URA3, FAO1::HPT*	This study
NBRC10243	Type strain	NITE Biological Resource Center, Japan
Escherichia coli BL21(DE3)		TaKaRa BIO
pUC-ΔFAO1	Construction of an FAO1-negative *S. bombicola* strain	1

Table 2 Primers used for isolating, cloning, expression, and knocking out the *S. bombicola* FAO1 gene.

Primer	Sequence (5'-3')	Purpose in this study
Pura3 F	AGTACATATTTTTCGAAACAGCTCGCAA	Cloning of *URA3*
ura3 R	CTAAGAAACTCATCTTGACTGAACTTTTC	Construction of KSM-*fao1*Δ and KSM-*ura3*Δ*::URA3*
pUC F	GTACCGAGCTCGAATTCGT	
pUC R	GCAGGCATGCAAGCTTGGC	
UGTAUS-in F	AGCTTGCATGCCTGCTATTAACTCCGCAGCATGAC	Construction of KSM-*ugta*Δ
UGTAUS-ura R	CGAAAAATATGTACTGAATATTCGTAGGGAGAAGC	
Ura-UGTADS F	AGATGAGTTTCTTAGTAGAATCGTACGATCAAATC	
UGTADSin R	ATTCGAGCTCGGTACTCCTTGCCTCATTCCACCTC	
FAO1DS F	AAGCCTTATATCGAATACAC	
Pura3 R	ATTATTTCTCTACAGTAGTG	
Pura-HPT in F	CTGTAGAGAAATAATATGAAAAAGCCTGAACTCAC	Construction of KSM-*ugta*Δ*fao1*Δ
HPT-FAO1DS in R	TTCGATATAAGGCTTTTATGAACAAACGACCCAAC	
FAO1USinF	AGCTTGCATGCCTGCTTTAAATCCAGAAAGAACTG	
FAO1DSinR	ATTCGAGCTCGGTACGACACTTCTCAGGAACCCTC	
pUC118 F	GGCGAAAGGGGGATGTGC	Checkingfor illegitimate recombinationof pUC118 region
pUC118 R	GCACCCCAGGCTTTACAC	

Table 3 Growth of KSM36 and mutant strains grown in secondary alcohols.

Substrates/strains	KSM36	KSM-*ura3*Δ::*URA3*	KSM-*fao1*Δ
Glucose	+	+	+
2-tetradecanol	+	+	–
2-hexadecanol	+	+	–

+, Growth; –, no growth.

Table 4 Substrate specificity of FAO1 for primary or secondary alcohols.

Substrates	Relative activity
1-tetradecanol	100%
2-tetradecanol	n.d.

n.d., not detected.

References

Brakemeier, A., Lang, S., Wullbrandt, D., Merschel, L., Benninghoven, A., Buschmann, N., Wagner, F. (1995) Novel sophorose lipids from microbial conversion of 2-alkanols. ***Biotechnol. Lett.*** 17: 1183–1188.

Cheng, Q., Sanglard, D., Vanhanen, S., Huan, T.L., Bombelli, P., Smith, A., Slabas, A.R (2005) *Candida* yeast long chain fatty alcohol oxidase is a c-type haemoprotein and plays an important role in long chain fatty acid metabolism. ***Biochim. Biophys. Acta*** 1735: 192–203.

Fleurackers, S.J.J., Van Bogaert, I.N.A., Develter, D. (2010) On the production and identification of medium-chained sophorolipids. ***Euro. J. Lipid Sci. Technol.*** 112: 655–662.

Fujii, T., Narikawa, T., Sumisa, F., Takeda, K., Kato, J. (2006) Production of α,ω-alkanediols using *Escherichia coli* expressing a cytochrome P450 from *Acinetobacter* sp. OC4. ***Biosci. Biotech. Biochem.*** 70: 1379–1385.

Inoue, S., Ito, S. (1982) Sophorolipids from *Torulopsis bombicola* as microbial surfactants in alkane fermentations. ***Biotechnol. Lett.*** 4: 3–8.

Kemp, G.D., Dickinson, F.M., Ratledge, C. (1988) Inducible long chain alcohol oxidase from alkane-grown *Candida tropicalis*. ***Appl. Microbiol. Biotechnol.*** 29: 370–374.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. ***Nature*** 227: 680–685.

Sambrook, J., Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual, 3rd ed*. New York: Cold Spring Harbor Laboratory Press

Scheps, D., Malca, S., Hoffmann, H., Nestl, B.M., Hauer, B. (2011) Regioselective ω-hydroxylation of medium-chain n-alkanes and primary alcohols by CYP153 enzymes from *Mycobacterium marinum* and *Polaromonas* sp. strain JS666. ***Org. Biomol. Chem.*** 9: 6727–6733.

Spencer, J.F.T., Gorin, P.A.J., Tulloch, A.P. (1970) *Torulopsis bombicola* sp. ***Antonie Van Leeuwenhoek*** 36: 129–133.

Takahashi, F., Igarashi, K., Hagihara, H. (2016) Identification of the fatty alcohol oxidase FAO1 from *Starmerella bombicola* and improved novel glycolipids production in an FAO1 knockout mutant. ***Appl. Microbiol. Biotechnol.*** 100: 9519–9528.

Van Beilen, J.B., Li, Z., Duetz, W.A., Smits, T.H.M., Witholt, B. (2003) Diversity of alkane hydroxylase systems in the environment. ***Oil Gas Sci. Technol.*** 58: 427–440

Wang, T-T., Choi, Y-J., Lee, B.H. (2001) Transformation systems of non-*Saccharomyces* yeasts. ***Crit. Rev. Biotechnol.*** 21: 177–218.

General Discussion

In the thesis, I aimed to perform the basic molecular biological research and clarify the fatty alcohol metabolism pathway in *S. bombicola* to improve the glycolipid production system of this yeast. In Chapter 1, I described cDNA analysis, construction of a recombinant system, and identification of the primary alcohol oxidase in *S. bombicola* were performed. In Chapter 2, I demonstrated that primary alcohol oxidase is also involved in the secondary alcohol utilization pathway and determined the fatty alcohol utilization pathway in this yeast.

I performed cDNA and genome analyses of *S. bombicola*. The *FAO1* gene does not contain intron, similar to the case for other genes in *S. bombicola* (Van Bogaert, et al., 2009). The physiological significance of non-intron containing genes in this yeast remains to be elucidated and will be investigated in future research. Regarding the recombination system, the electroporation method achieved higher transformation efficiency than a previously reported system (Van Bogaert et al., 2008). The results of destruction of *FAO1* suggested that *S. bombicola* is a haploid yeast. *S. bombicola* appears to be easy to analyze in terms of strain improvement for industrial application and fundamental molecular biology.

In Chapter 1, I showed that FAO 1 is a fatty alcohol oxidase by expression analysis in *E. coli*, and identified it as a major fatty alcohol oxidase localized in peroxisomes by the *FAO1* disruption in *S. bombicola*. Hommel and Ratledge (1990) suggested that two alcohol oxidase activities exist in *S. bombicola*. Although we did not report the findings, we identified an oxidase FAO2 that shows weak homology to *S.*

bombicola FAO1, but the alcohol utilization was not decreased by deletion of *FAO2*. Recently, another group reported a destruction study of *FAO2* in *S. bombicola* (Van Renterghem et al., 2018). The deletion of the *FAO1* improved the productivity of bolaform sophorolipids, while deletion of the *FAO2* did not. These results supported that the notion that FAO1 is the main fatty alcohol oxidase in *S. bombicola*. The function of FAO2 remains to be elucidated.

Deletion of *FAO1* improved the yields of tetradecanol-based sophorolipids, and tetradecanediol-based sophorolipids. Bolaform sophorolipids are considered to be excellent as hypoallergenic, film-forming ability, degreasing agents, or suppression of scale formation in comparison with normal APGs (Van Renterghem et al., 2018). Through functional analyses of FAO1, we were able to construct a production system for bolaform glycolipids with excellent physical properties.

As another issue, I tried to elucidate the metabolic utilization pathway of secondary alcohols in *S. bombicola*. The *FAO1*-deficient strain was unable to assimilate secondary alcohols, suggesting that FAO1 had a role in secondary alcohol metabolism. However, FAO1 did not show oxidation activity for secondary alcohol. The initial oxidation stage in the alcohol metabolism pathway was not considered to be oxidation of the hydroxyl group on the secondary alcohol. As a result of structural analysis of the product using secondary alcohols as substrates in the wild-type and *FAO1*-deficient strain, a diol-type glycolipid having an ω-terminal hydroxyl group was only detected in the *FAO1*-deficient strain. Moreover, when both of *UGTA1*, a glycosyltransferases for sophorolipid biosynthesis, and *FAO1* were deleted, the diol-containing APGs were disappeared and accumulation of ω or ω-1 diols was observed. From these results, secondary alcohol metabolism in *S.bombicola* involves initial hydroxylation at the ω

terminal, then oxidiation by FAO1, and finally assimilation in the beta-oxidation pathway.

The above results showed that FAO1 makes a large contribution to the fatty alcohol metabolism pathway in *S. bombicola* and that fatty alcohols can be efficiently glycosylated in the *FAO1*-deficient strain. This study is considered to be useful not only for the basic results elucidating the alcohol metabolism pathway in *S. bombicola* but also for the method for synthesis of novel glycolipids.

Finally, the glycosyltransferase for alcohol and diol was unknown, and this study revealed that UGTA1 catalyzes the reaction. Multiple hydroxylases in *S. bombicola* have been identified and are involved in sophorolipid biosynthesis or alkane assimilation (Van Bogaert et al., 2009). These enzymes hydroxylate the ω or ω-1 positions of fatty acids, alcohols and alkanes. Unlike the oxidase enzymes possessed by other alkane-utilizing microorganisms, the hydroxylases in *S. bombicola* have high hydroxylation activity at the ω-1 position, necessarily producing secondary alcohols. When the glycosyltransferase is deficient, the secondary alcohols remain in the yeast body without being assimilated. As one hypothesis, it seems that the glucose addition and secretion system developed as a secondary alcohol export pathway, and then further developed into a sophorolipid biosynthesis system.

References

Hommel, R., Ratledge, C. (1990) Evidence for two fatty alcohol oxidases in the biosurfactant-producing yeast *Candida (Torulopsis) bombicola*. ***FEMS Microbiol. Lett.*** 70: 183–186.

Van Bogaert, I.N.A., De Maeseneire, S.L., Develter, D., Soetaert, W., Vandamme, E.J. (2008) Development of a transformation and selection system for the glycolipid-producing yeast *Candida bombicola*. ***Yeast*** 25: 273–278.

Van Bogaert, I.N.A., Demey, M., Develter, D., Soetaert, W., Vandamme, E.J. (2009) Importance of the cytochrome P450 monooxygenase CYP52 family for the sophorolipid‐producing yeast *Candida bombicola*. ***Yeast Res.*** 9: 87–94.

Van Renterghem, L., Roelants, S.L.K.W., Baccile, N., Uyttersprot, K., Taelman, M.C., Everaert, B., Mincke, S., Ledegen, S., Debrouwer, S., Scholtens, K., Stevens, C., Soetaert, W. (2018) From lab to market: An integrated bioprocess design approach for new-to-nature biosurfactants produced by *Starmerella bombicola*. ***Biotechnol. Bioeng.*** 115: 1195–1206.

Acknowledgments

I wish to express my sincere gratitude and appreciation to my supervisor, Prof. Takashi Yamamoto at Hiroshima University for valuable advice, supervision, and crucial contributions throughout this study, and for the excellent opportunities provided to me.

I am deeply grateful to Yoshinori Takema, Tadashi Hase and Atsushi Ouchi, from Kao Corporation who provided me with this opportunity, as well as constructive comments and warm support. I would like to give heartfelt thanks to Tadayuki Imanaka who was my professor in my Kyoto University era and provided valuable advice. Special thanks also go to Toshiaki Fukui (Tokyo Institute of Technology) and Takeshi Tanaka (National Institute of Advanced Industrial Science and Technology) who gave me invaluable comments and warm encouragement.

I am greatly indebted to Katsuya Ozaki, Hiroshi Hagihara and Yasushi Takimura from Kao Corporation, who have been responsible for helping me in this research. This work could not have been completed without their help. My special thanks go to my senior associate, Kazuaki Igarashi at Kao Corporation, who is a best collaborator for this work.

I would like to give special thanks to my colleagues at Kao Corporation; Kenji Manabe, Takuya Morimoto, Atsuko Hayase, Akihito Kawahara, Yuichi Tsuboi, Nozomu Shibata, Tatsuya Osamura, Jitsurou Kaneda, Kyoushiro Nonaka, Kousuke Ike, Mao Shaku, and Hikaru Nagai, for their warm encouragement.

Finally, special thanks to my mother, wife, daughter, and late father for their unflagging encouragement and mental and financial support throughout my study.