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RESEARCH ARTICLE

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

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One sentence summary: We clarified the long-chain secondary alcohol utilization pathway in glycolipid producing yeast *Starmerella bombicola* and clarified the contribution of FAO1, which is a primary alcohol oxidase.

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ABSTRACT

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.

Keywords: Fatty alcohol oxidase; FAO1; secondary alcohol metabolism; sophorolipids, *Starmerella bombicola*; diols production

INTRODUCTION

The yeast *Starmerella bombicola* is an excellent sophorolipid producer (Spencer, Gorin and Tulloch 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 (APG) 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. In addition, the synthesis of 2-tridecyl sophorosides having antibacterial activity against Gram-positive bacteria using 2-tridecanone as a raw material has been reported (Recke *et al.* 2013). There are no reports on enzymes and genes involved in glycosylation of alcohols in *S. bombicola*, but sophorolipids with similar structures

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have been studied well. Initially, Cyp52M1 hydroxylates ω or ω-1 position of fatty acid (Van Bogaert *et al*. 2009). Next, two distinct UDP-glucosyltransferases UGTA1 and UGTB1 conjugate two glucose units to the hydroxyl group of ω or ω-1 hydroxy fatty acid (Saerens et al. 2011a,b). Following biosynthesis steps are acetylation and lactonization of sophorolipids by acetyltransferase and lactone esterase, respectively (Saerens, Van Bogaert and Soetaert 2015, Ciesielska *et al*. 2016).

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, Igarashi and Hagihara 2016). It has been reported that *S. bombicola* has another fatty alcohol oxidase (FAO), which is a gene having homology with FAO1 (Van Renterghem *et al*. 2018). However, registered FAO2 sequence (Genbank MF431618.1) had homology with putative guanosine triphosphatase and this protein was blasted with 7.9% identity with *S. bombicola* FAO1. Therefore, FAO 2 is not an alcohol oxidase. FAO 1 was considered to be the only enzyme reported as a reliable alcohol oxidase in *S. bombicola*. In contrast to the primary alcohol utilization pathway, the secondary alcohol metabolism pathway of *S. bombicola* has not been clarified. The FAO pathway has been characterized in *Candida tropicalis* 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). On the other hand, the secondary alcohol metabolism pathway in yeast producing glycolipids has not been studied well.

Herein, we 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. *Starmerella 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, Igarashi and Hagihara 2016) were used as the parent strains. The methods for plasmid maintenance, cultivation of *S. bombicola* and preparation of cell extracts were reported earlier without hygromycin (Takahashi, Igarashi and Hagihara 2016). 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).

DNA preparation, gene sequencing and creation of knockout fragments

The methods used for DNA isolation, purification, polymerase chain reaction (PCR), sequencing and transformation were reported earlier (Takahashi, Igarashi and Hagihara 2016). The strategy for knocking out the *UGTA1* and *FAO1* genes is shown in Fig. S1 (Supporting Information). The 1000-bp upstream and 1000-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., Kusatsu, Japan). pUC-Δ*UGTA1* was constructed such that these fragments were ligated using the In-Fusion HD Kit (TaKaRa Bio., Kusatsu, Japan). Primer pair UGTAUS-in F and UGTADSin R was used to amplify a 3124-bp fragment, which contained the *URA3* sequence and 1020-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, Waltham, MA, USA) into *FAO1*. Primers Pura3 R and FAO1DS F were used to amplify a linear DNA fragment of pUC-Δ*FAO1* (Takahashi, Igarashi and Hagihara 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 3400-bp fragment, which contained the HPT sequence and 1230-bp of the *FAO1* upstream and promoter sequence of *URA3*, and 1027-bp downstream flanking sequences from pUC-Δ*FAO1*::*HPT*. The fragment was used to transform the KSM-*ugta*Δ strain. Primers used in this paper are listed in Table 2.

Protein determination and FAO activity assay

The methods for protein determination and FAO activity assay were reported earlier (Takahashi, Igarashi and Hagihara 2016). 1-tetradecanol (FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2-dodecanol (FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2-tetradecanol (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), 7-tetradecanol (Avocado Research Chemicals Limited, London, UK) and 2-hexadecanol (Acros organics, Geel, Belgium) were used for substrates.

High-pressure liquid chromatography–mass spectroscopy analysis

A Prominence UFLC high-pressure liquid chromatography (HPLC) System (Shimadzu, Kyoto, Japan) and liquid chromatography (LC)-mass spectroscopy (MS) 2020 system (Shimadzu, Kyoto, Japan) 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), 5 mM ammonium formate (FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan) 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 minutes and then returned to 20% acetonitrile over 5 minutes. A flow rate of 1

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*	Takahashi, Igarashi and Hagihara 2016
KSM-*ura3*Δ::*URA3*	KSMΔ*URA3*::*URA3*	Takahashi, Igarashi and Hagihara 2016
KSM-*fao1*Δ	KSM36Δ*URA3*, *FAO1*::*URA3*	Takahashi, Igarashi and Hagihara 2016
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	Takahashi, Igarashi and Hagihara 2016

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	Construction of KSM-*ugta*Δ
pUC R	GCAGGCATGCAAGCTTGGC	
UGTAUS-in F	AGCTTGCATGCCTGCTATTAACTCCGCAGCATGAC	
UGTAUS-ura R	CGAAAAATATGTACTGAATATTCGTAGGGAGAAGC	
Ura-UGTADS F	AGATGAGTTTCTTAGTAGAATCGTACGATCAAATC	
UGTADSin R	ATTCGAGCTCGGTACTCCTTGCCTCATTCCACCTC	
FAO1DS F	AAGCCTTATATCGAATACAC	Construction of KSM-*ugta*Δ*fao1*Δ
Pura3 R	ATTATTTCTCTACAGTAGTG	
Pura-HPT in F	CTGTAGAGAAATAATATGAAAAAGCCTGAACTCAC	
HPT-FAO1DS in R	TTCGATATAAGGCTTTTATGAACAAACGACCCAAC	
FAO1USinF	AGCTTGCATGCCTGCTTTAAATCCAGAAAGAACTG	
FAO1DSinR	ATTCGAGCTCGGTACGACACTTCTCAGGAACCCTC	
pUC118 F	GGCGAAAGGGGGATGTGC	Checking for illegitimate recombination of pUC118 region
pUC118 R	GCACCCCAGGCTTTACAC	

mL min^{-1} was used. Scan analysis was performed for molecules with masses between 50 and 1500 Da. The ESI-voltage was set at −3.5 kV and nitrogen was used both as a nebulizer gas and as drying gas (250°C). The methods used for sampling and analytical methods were reported earlier (Takahashi, Igarashi and Hagihara 2016).

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 Fig. 1, both the 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.

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

FAO1 was expressed in *E. coli* BL21(DE3) according to a previous report (Takahashi, Igarashi and Hagihara 2016) and its substrate specificity was verified (Table 3). FAO1 had oxidase activity toward primary alcohols, but no activity was observed toward secondary alcohols.

Table 3. Substrate specificity of FAO1 for primary or secondary alcohols.

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

n.d., not detected.

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

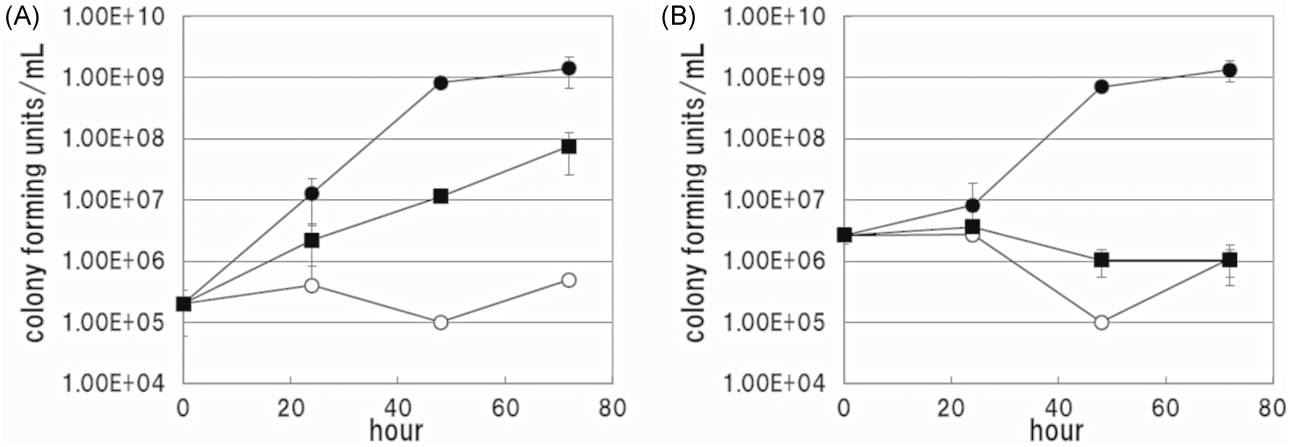

Figure 1. Growth of KSM36 and mutant strains grown in various carbon sources. Filled circles indicated glucose; opened circles indicated none carbon source and filled squares indicated 2-tetradecanol. **(A)** KSM-*ura3*Δ::*URA3*, **(B)** KSM-*fao1*Δ. Each point represents an average of the two experiments with its standard deviation.

in KSM-*ura3*Δ::*URA3* and KSM-*fao1*Δ. Each strain was inoculated into the alkyl polyglucosides 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*Δ strain showed byproducts (peaks 3–6).

To estimate the structure of byproduct peaks, the culture solution of the KSM-*fao1*Δ after alkaline hydrolysis treatment was analyzed by HPLC/MS. The molecular weights of n-dodecyl-β-D-maltoside and 2-tetradecyl disaccharide were 510 Da and 538 Da, respectively. These ions were observed as proton elimination (M-1) or the formate ion addition product (M + 45) at 16.5 min (peak 1 in Fig. 3a) and 17 min (peak 2 in Fig. 3b). The major (peak 2) product ions appeared at *m/z* 537, 583 and 651, respectively, in negative ion mode. Based on a previous study (Fleurackers, Van Bogaert and Develter 2010), we estimated that peak 2 was 2-O-tetradecyl-(2-O-β-D-glucopyranosyl-β-D-glucopyranoside). In contrast, another peak was observed at 10.5 min (peak 3 at Fig. 3b) in the supernatant of KSM-*fao1*Δ with *m/z* 553, 599 and 667 in negative ion mode. The molecular weight of peak 3 was 16 larger than that of peak 2, and peak 3 was eluted at a higher polarity position than peak 2. Therefore, peak 3 was attributed to a hydroxyl group added to any position of 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 alkyl polyglucosides 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 alkyl polyglucosides production medium. After 24 h, 2-tetradecanol was added at 10 g L^{-1}, and the total cultivation time 48 h. Peaks 1, 2, 5 and 6 were not detected (Fig. 2d). Therefore, KSM-*ugta*Δ*fao1*Δ had lost the ability to glycosylate alcohols or diols. The amount of both diols reached 2.9 g L^{-1} with a yield of 21.4% (mol/mol) (Fig. 5).

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, Igarashi and Hagihara 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 and glycosylated product were detected in the culture solution of the wild-type, while 1,13-tetradecanediol was not. These glycosylated products were identified in previous report (Brakemeier, Wullbrandt and Lang 1998). 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

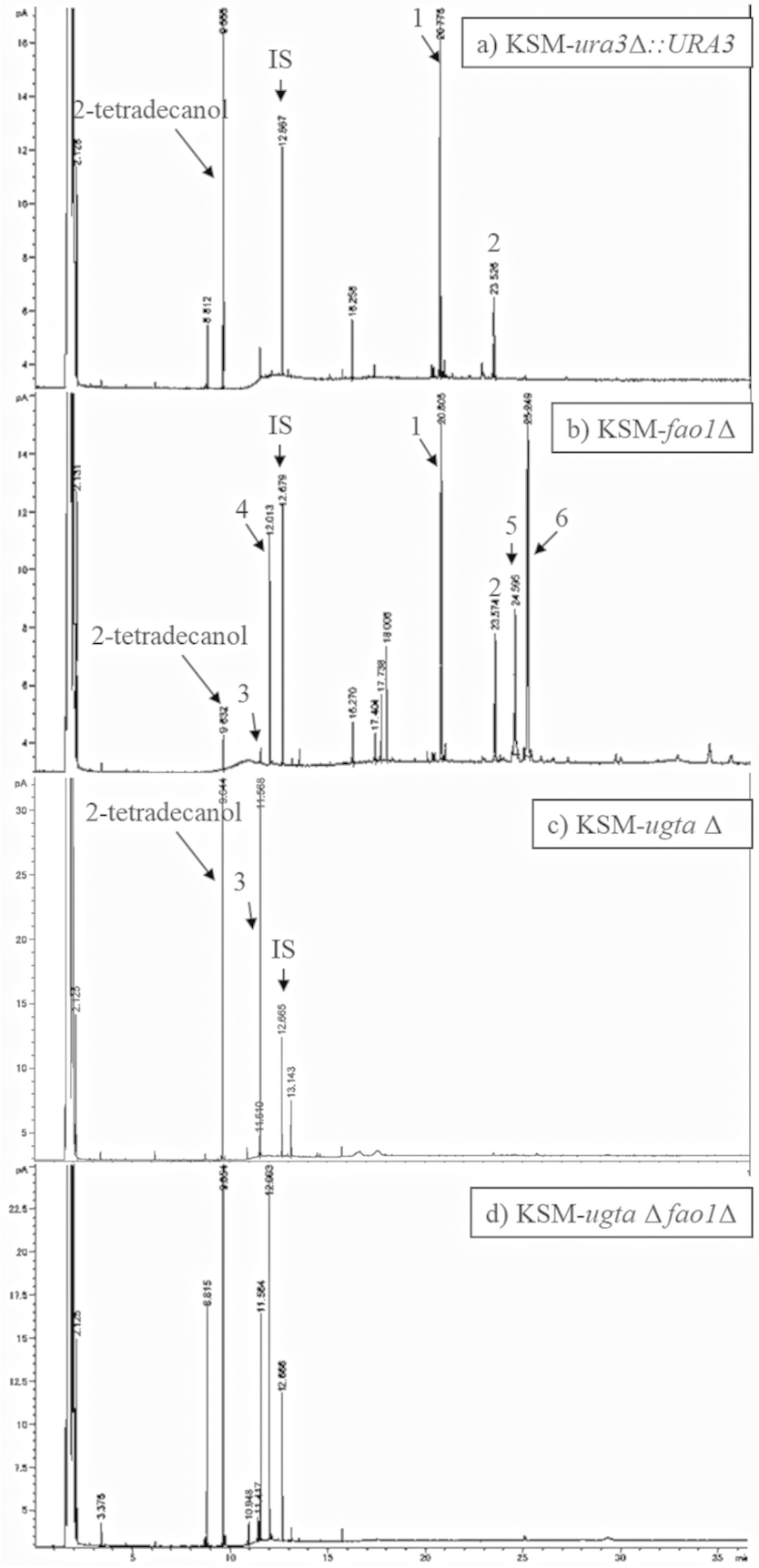

Figure 2. Chromatogram of GC-FID analysis of glycolipids from *S. bombicola* grown on 2-tetradecanol. **(a)** KSM-*ura3*Δ::*URA3*, **(b)** KSM-*fao1*Δ, **(c)** KSM-*ugta*Δ, **(d)** KSM-*ugta*Δ*fao1*Δ. IS, internal standard (1-octadecanol).

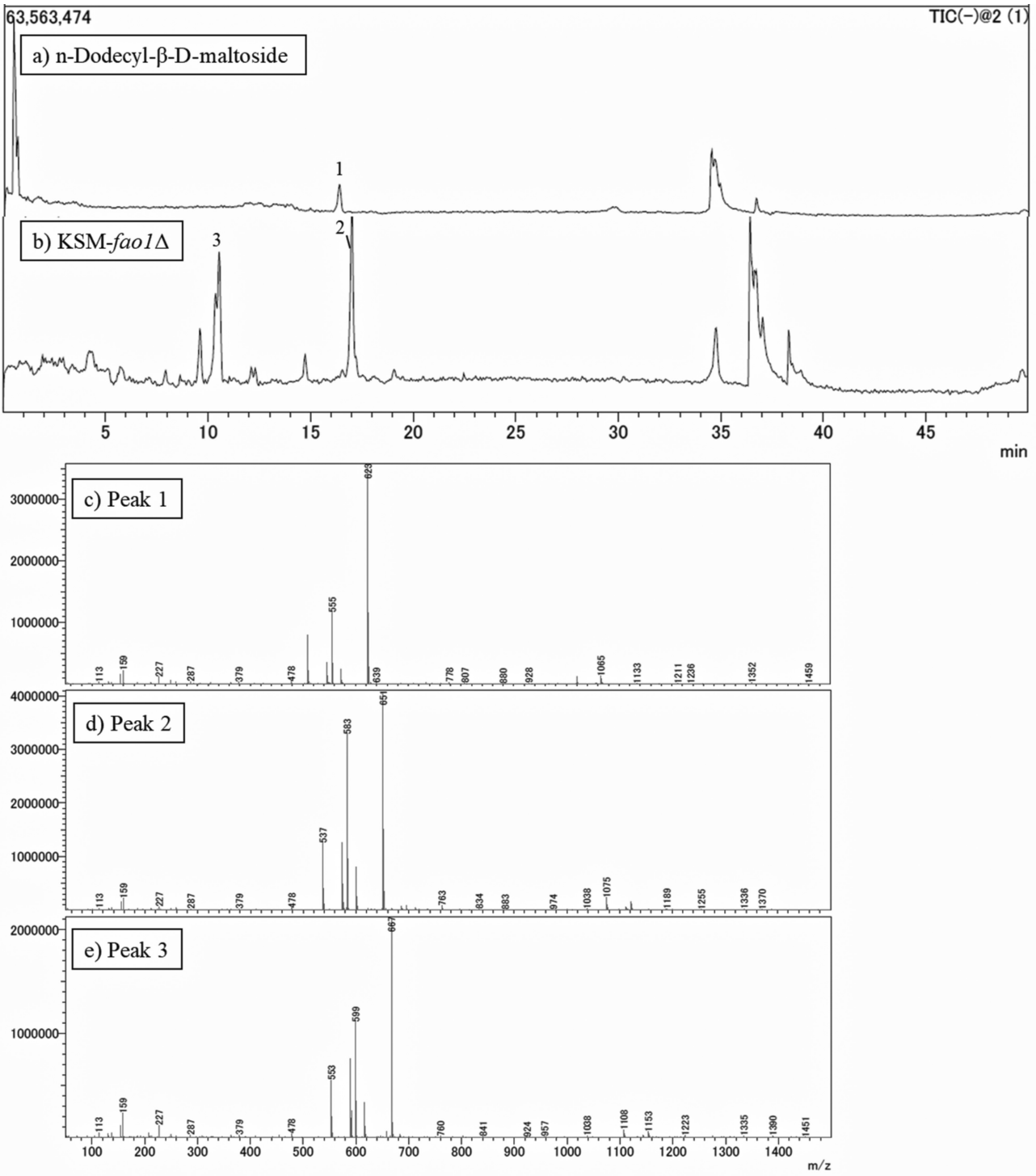

Figure 3. HPLC-ESI MS analysis of glycolipids from KSM-fao1Δ grown on 2-tetradecanol. Total ion chromatogram (TIC) by negative ion mode of **(a)** n-Dodecyl-β-D-maltoside and **(b)** glycolipids from KSM-*fao1*Δ. Panel **(c)**, **(d)**, **(e)** show a detail of the negative ion mode mass spectrum of LC-ESI peak 1 in (a), 2, 3 in (b), respectively. $*$M-1([M—H]$^-$), M + 45([M + CHOO]$^-$) and M + 113([M + CF_3COO]$^-$) ions were observed. CF_3COOH was used in the past for HPLC/MS. It was thought that CF_3COO adduct ion was detected because it remained in the detector.

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

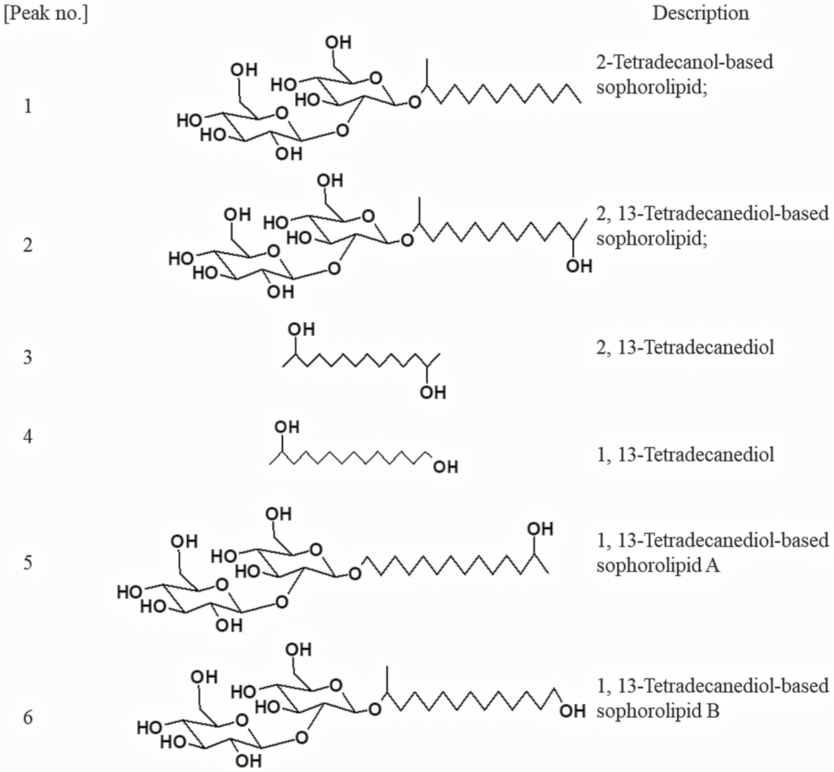

Figure 4. Putative structure of products from the *FAO1* knockout strain grown on 2-tetradecanol. Peak no. is related to Fig. 2.

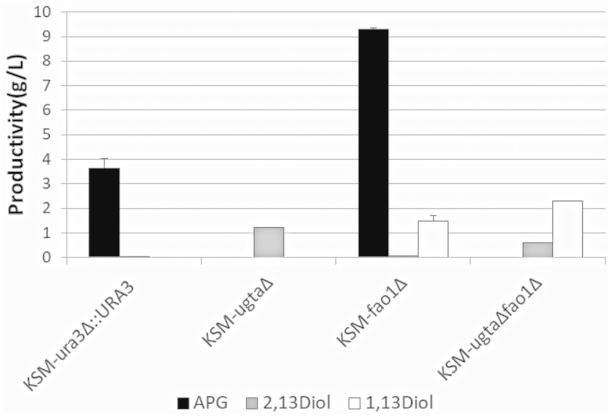

Figure 5. Glycolipids and diols production of the parent, *FAO1* or *UGTA1* knockout strain grown on 2-tetradecanol. Filled bars indicated alkyl sophorosides; gray bars indicated 2,13-tetradecanediol-based sophorolipids; open bars indicated 1,13-tetradecanediol-based sophorolipids. Each bar represents an average of the two experiments with its standard deviation.

glycosylation. In the culture of KSM-*ugtaΔfao1Δ* 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. Tetradecyl sophoroside was formed from 2-tetradecanol in previous research (Brakemeier *et al.* 1995). Together with the fact that the first glucose transfer to 2-tetradecanol was UGTA, it was speculated that second glucose transfer occurred by UGTB as like as traditional sophorolipid. To summarize, the utilization of secondary alcohol in *S. bombicola* is thought to proceed via the route shown in Fig. 6.

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

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

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None declared.

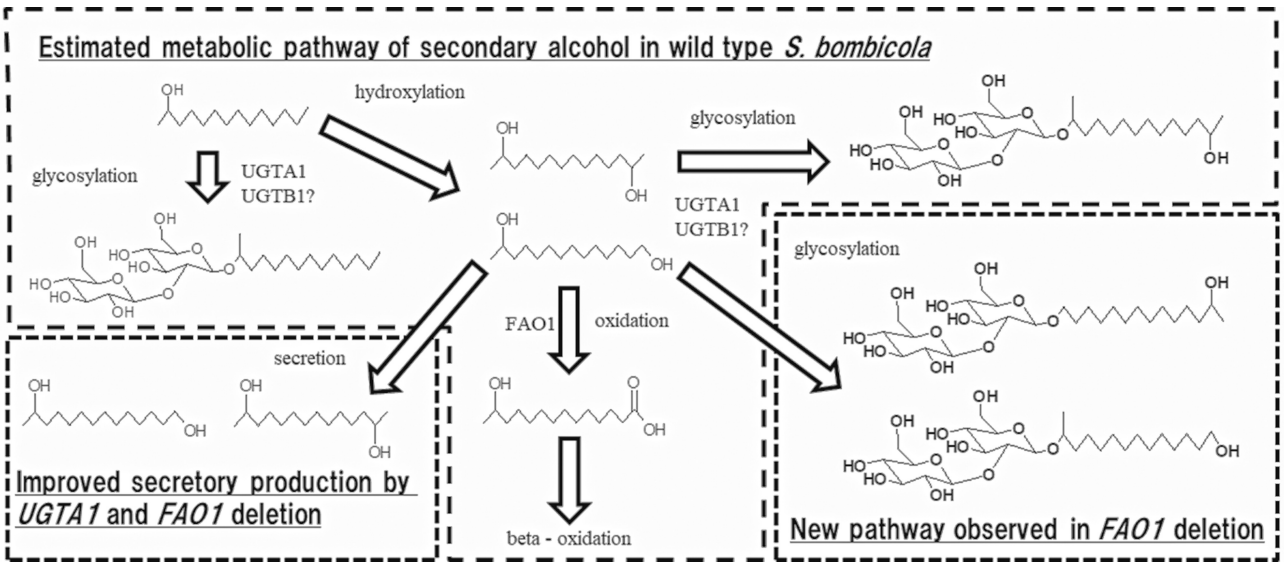

Figure 6. Secondary alcohol metabolic pathway in *S. bombicola* and the new pathway observed after *FAO1* deletion.

Ethical Approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest. None declared.

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