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...
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 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 monoxygenase, 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-ura3Δ and FAO1-negative mutant KSM-fao1Δ (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 Purα3F 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-ΔUGTA1 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-ΔUGTA1. The fragment was used to transform the ura3-auxotrophic KSM-ura3Δ strain. Primers Purα3 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 Purα3 R and FAO1DS F were used to amplify a linear DNA fragment of pUC-ΔFAO1 (Takahashi, Igarashi and Hagihara 2016). pUC-ΔFAO1::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 1320-bp of the FAO1 upstream and promoter sequence of URA3, and 1027-bp downstream flanking sequences from pUC-ΔFAO1::HPT. The fragment was used to transform the KSM-ura3Δ 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–mass spectrometry (LC-MS) 2020 system (Shimadzu, Kyoto, Japan) were used for HPLC–MS analysis. Glycolipid samples were analyzed using an Acclaim Surfactant column (4.6 x 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-ura3Δ         | KSM36ΔURA3                  | Takahashi, Igarashi and Hagihara 2016|
| KSM-ura3Δ::URA3   | KSM36ΔURA3::URA3            | Takahashi, Igarashi and Hagihara 2016|
| KSM-fao1Δ         | KSM36ΔURA3, FAO1::URA3      | Takahashi, Igarashi and Hagihara 2016|
| KSM-ugtaΔ         | KSM36ΔURA3, UGTA1::URA3     | This study                           |
| KSM-ugtaΔfao1Δ    | KSM36ΔURA3, 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           | AGTACATATTTTTTCGAAACAGCTCGCAA                        | Cloning of URA3                                         |
| Pura3 R           | ATTATTTCTCTACAGTAGTG                                  |                                                        |
| ura3 R            | CTAGAAACACTCATCTTGACTGAACTTATTTTC                    | Construction of KSM-ura3Δ and KSM-ura3Δ::URA3          |
| pUC F             | GTACCCGAGCTCGGAATTCGTT                                | Construction of KSM-ugtaΔ                                |
| pUC R             | GCAGGCATAGCAAGGCTTGGC                                |                                                        |
| UGTADS-in F       | AGCTTGGATATATCGCTCTGAC                               | Construction of KSM-ugtaΔfao1Δ                          |
| UGTADS-ura R      | CAGAAAATATGGTACTGAAATATCGTGAGGAGAGGAGC              |                                                        |
| Ura-UGTAUS ΔF     | AGATGAGTTCTTTAGAATACGGATCAATGCGAATC                 |                                                        |
| UGTADSin R        | ATTCGAGCTCGTACTCCTGGGCTCATTCACCTC                   |                                                        |
| FAO1DS F          | GAAGCGTTATATCGAATCAC                                 |                                                        |
| Pura3 R           | ATATTCTCTACAGTACTG                                  |                                                        |
| Pura-HPT in F     | CTGTAAGAGAAATAATATGAAAAAGCGCAGACCTGAC               | Checking for illegitimate recombination of pUC118 region|
| HPT-FAO1DS in R   | TTTGCAATTAGAGGCTCTTTTGTGAAACACGACCCCAAC             |                                                        |
| FAO1ISinF         | AGCTTGGATATATCGCTCTGAC                               |                                                        |
| FAO1ISinR         | ATTCGAGCTCGTACGGACACCTTCCTGCAAGAAGACCTC             |                                                        |
| pUC118 F          | GCAGAAAGGGGATGTC                                     |                                                        |
| pUC118 R          | GCACCCCCAGGCTTTACAC                                  |                                                        |

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-fao1Δ strain in secondary alcohol substrates

S. bombicola strains were grown on a basal medium containing 2-tetradeanol as the sole carbon source. We determined the ability of KSM-ura3Δ::URA3 and KSM-fao1Δ strains to use several carbon sources. As shown in Fig. 1, both the strains grew on glucose as the sole carbon source. The KSM-ura3Δ::URA3 strain, but not the KSM-fao1Δ strain, also grew on media containing 2-tetradeanol.

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-tetradeanol    | 100%              |
| 2-dodecanol      | n.d.              |
| 2-tetradeanol    | n.d.              |
| 7-tetradeanol    | n.d.              |
| 2-hexadecanol    | n.d.              |
| n.d., not detected. |

Structural analysis of glycolipids produced by KSM-fao1Δ 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-tetradeanol...
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 alcohol was hardly changed. Therefore, compared with KSM-ura3Δ::URA3, the KSM-fao1Δ strain was thought to accumulate α-ω-type diols. As peak 6 was observed only in the KSM-fao1Δ 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 polyglycosides production have not been reported, but UGTA1, known as the ω-type diols. As peak 6 was observed only in the KSM-fao1Δ strain, it was thought to accumulate α-ω-type diols. As peak 6 was observed only in the KSM-fao1Δ 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).

in KSM-ura3Δ::URA3 and KSM-fao1Δ. Each strain was inoculated into the alkyl polyglycosides 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-ura3Δ::URA3 and KSM-fao1Δ using GC (Fig. 2). Based on a previous study (Brakemeier et al. 1995), we estimated that the main product was a 2-tetradecylosphoroside. In contrast, only the culture of KSM-fao1Δ strain showed byproducts (peaks 3–6).

To estimate the structure of byproduct peaks, the culture solution of the KSM-fao1Δ strain 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+538 Da, respectively. These ions were observed as proton elimination (M-1) or the formate ion addition product (M+45). Each point represents an average of the two experiments with its standard deviation.

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-ura3Δ::URA3, (B) KSM-fao1Δ. Each point represents an average of the two experiments with its standard deviation.
Figure 2. Chromatogram of GC-FID analysis of glycolipids from S. bombicola grown on 2-tetradecanol. (a) KSM-ura3Δ::URA3, (b) KSM-fao1Δ, (c) KSM-ugtaΔ, (d) KSM-ugtaΔfao1Δ. 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-fao1Δ. 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+CHO2]-) and M+113([M+CF3COO]-) ions were observed. CF3COOH was used in the past for HPLC/MS. It was thought that CF3COO 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-fao1Δ strain to clarify the factor for
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 (ω,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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