Journal of Bioscience and Bioengineering
VOL. xx No. xx, , 2017

www.elsevier.com/locate/jbiosc

Selective production of deacetylated mannosylerythritol lipid, MEL-D, by acetyltransferase disruption mutant of *Pseudozyma hubeiensis*

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Received 8 May 2017; accepted 4 August 2017
Available online xxx

Mannosylerythritol lipids (MELs) are produced by several smut fungi of the Ustilaginaceae family; they are promising microbial biosurfactants and have excellent surface-active and self-assembling properties. *Pseudozyma hubeiensis* is a candidate for abundant MEL production and produces large amounts of 4-*O*-[(4′-mono-*O*-acetyl-2′,3′-di-*O*-alkanoyl)-β-D-mannopyranosyl]-*meso*-erythritol (MEL-C). An acetyltransferase disruption mutant of *P. hubeiensis*, SY62-MM36, was obtained to selectively produce deacetylated 4-*O*-[(2′,3′-di-*O*-alkanoyl)-β-D-mannopyranosyl]-*meso*-erythritol (MEL-D), and the structures of the products were determined. Lower mobility of major spots of the mutant on silica gel thin-layer chromatography verified its more hydrophilic nature than that of wild-type MEL-A, B, and C. Structural analyses confirmed the product to be MEL-D, which comprises acyl chains of caproic acid (C6:0), capric acid (C10:0), and lauric acid (C12:0). The critical micelle concentration (CMC) and the surface tension (γCMC) of the MEL-D were 2.0×10^{-5} M and 29.7 mN/m, respectively. SY62-MM36 also produced a minor product that was estimated as triacylated MEL-D. The triacylated MEL-D had a CMC of 3.5×10^{-5} M and a γCMC of 29.6 mN/m. In water, MEL-D formed a lamella liquid crystal phase over a broad range of concentrations. By fed-batch cultivation, the mutant produced 91.6 ± 6.3 g/L of MEL-D for 7 days.

[**Key words:** Acetyltransferase; Biosurfactant; Mannosylerythritol lipids; *Pseudozyama*; Yeast]

Mannosylerythritol lipids (MELs) are produced by several smut fungi of the Ustilaginaceae family; they are promising microbial biosurfactants (1–4). MELs have low surface tension at low critical micelle concentrations (3). They have excellent self-assembling properties and form lyotropic liquid crystals in aqueous phase; they self-assemble into a monolayer on surface plasmon resonance sensor chips with alkanethiol groups (5–7) and into a bilayer on mica (6). Immunoglobulins and lectins bind to such biosurfactant layers (5–7). MELs enhance melanoma cell apoptosis activity (8), thereby causing the outgrowth of neurites and partial cellular differentiation in rat pheochromocytoma PC12 cells (9). Furthermore, they have moisturizing capability that can help in the recovery of damaged cultured human skin cells (10) and repair damaged hair (11). Therefore, MELs have great potential for use in cosmetics, pharmaceuticals, and other industrial applications.

MEL molecules comprise two acyl chains bound to saccharides composed of mannose and erythritol. The number of acetyl groups and the length of the acyl carbon chains produced during metabolism depend on the species of fungus (1). Hewald et al. (12) first reported the gene cluster for MEL biosynthesis in *Ustilago maydis*. The gene cluster for MEL biosynthesis was also observed in other MEL-producing fungi: *Pseudozyma antarctica* (13) and *Pseudozyma hubeiensis* (14). The gene cluster encodes four responsible enzymes: Emt1, erythritol/mannose transferase; Mac1 and Mac2, acyltransferases; and Mat1, acetyltransferase (Fig. 1). When vegetable oils and saccharides are used as carbon sources, the overall MEL biosynthesis can be summarized by the following steps: (i) oils are hydrolyzed by lipase, and the carbons of the resulting fatty acids are trimmed through the beta-oxidation pathway; (ii) glycerol is partially hydrolyzed, and the supplied saccharides are converted to *meso*-erythritol and uridine triphosphate (UDP)-mannose; (iii) mannosylerythritol (ME) is synthesized from *meso*-erythritol and UDP-mannose by Emt1; (iv) two acyl portions derived from acyl-CoA in beta-oxidation pathway are bound to hydroxyl groups at the 2′- and 3′-positions of the mannose in ME by Mac1 and Mac2, and deacetylated MEL (MEL-D) is synthesized; and (v) acetyl moieties derived from acetyl-CoA are bound to the 4′- and 6′-positions in MEL by Mat1 to form acetylated MELs including MEL-A, B, and C. The acetyl groups of MELs are considered to play an important role in the physical, physiological, and biological activities of the materials, including immunoglobulin and lectin binding (5–7), apoptosis (8), and cell differentiation activity (9). A detailed characterization of all the derivatives is essential to reveal their structure–function relationship. Furthermore, the acetyl groups are potentially hydrolyzed to free acetate. The acetate can decrease the pH and promote further MEL decomposition. To the best of our knowledge, wild-type MEL-producing strains produce acetylated MELs including MEL-A, B, C, and—to a lesser extent—D. To investigate the aqueous phase behavior of MEL-D, Fukuoka et al. (15) synthesized MEL-D by the lipase-catalyzed hydrolysis of acetyl groups in MEL-B derived from *Pseudozyma tsukubaensis*. However, an efficient one-step process is necessary for the further detailed

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1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2017.08.003

Please cite this article in press as: Konishi, M., and Makino, M., Selective production of deacetylated mannosylerythritol lipid, MEL-D, by acetyltransferase disruption mutant of *Pseudozyma hubeiensis*, J. Biosci. Bioeng., (2017), http://dx.doi.org/10.1016/j.jbiosc.2017.08.003

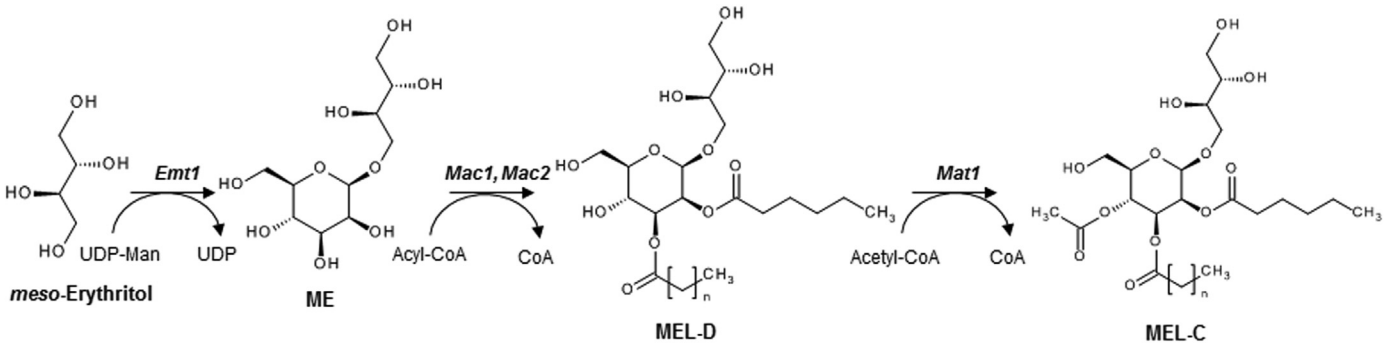

FIG. 1. *Pseudozyma hubeiensis* SY62 mannosylerythritol lipid synthesis pathway. Emt1, glycosyltransferase, Mac1, Mac2, acyltransferase, Mat1, and acetyltransferase; n = 6 or 8. ME, mannosylerythritol; MEL-D, deacetylated mannosylerythritol lipid, MEL-C, 4′-acetylated mannosylerythritol lipid.

investigation of the physical and physiological properties of MEL-D. This would facilitate the development of applications using MEL-D.

P. hubeiensis SY62, which was isolated from a deep-sea clam, is a candidate for abundant MEL production (16). Volumetric productivity and yield have been reported to reach 18.4 g/L/day and 129 g/L over 7 days, respectively (17). However, SY62 mainly produces MEL-C and only small amounts of MEL-A and B. A MEL synthesis gene cluster was identified by draft genome analysis (14), and an optimal gene transformation method was developed (18). In this study, we constructed a *mat1*-knockout mutant of SY62 to selectively produce MEL-D and subsequently investigated the structure and function of the lipid. Furthermore, a novel type of MEL derivative was found. This manuscript describes a strategy for tailor-made production of MEL derivatives using a gene knock-out mutant.

MATERIALS AND METHODS

Strains and plasmids *P. hubeiensis* SY62 was isolated from a deep-sea clam (16). *Escherichia coli* NEB was purchased from New England Bio Labs Japan (Tokyo, Japan). Plasmid pUC19 was purchased from Takara Bio Ltd. (Shiga, Japan). Plasmid pUXV1, a *Ustilago-Escherichia* shuttle vector, was purchased from the American Type Culture Collection. The plasmid was transferred into DH5α-competent cells (Nippon Gene Co., Ltd., Tokyo, Japan). Plasmids were extracted from the Luria-Bertani (LB) culture medium using an alkaline Miniprep Kit (NucleoSpin Plasmid EasyPure, Takara Bio).

Preparation of genomic DNA To prepare genomic DNA, *P. hubeiensis* SY62 was cultured at 28°C for 2 days in yeast mold (YM) broth comprising 3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, and 10 g/L of glucose. The cells were harvested from 1 mL of the culture by centrifugation (2500 ×*g*, 5 min) and resuspended in 5 mL of Westase solution (Takara Bio). The treated cell pellet was collected by centrifugation at 5000 ×*g* and 4°C for 1 min and resuspended in 530 μL of Tris-ethylenediaminetetraacetic acid (TE) buffer containing 10 μL of 10 mg/mL proteinase K (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 60 μL of 10% (w/v) sodium dodecyl sulfate. The samples were incubated at room temperature for 1 h. After incubation, phenol–chloroform extraction was performed twice. The resulting genomic DNA was recovered by ethanol precipitation. The precipitate was resuspended in TE buffer (pH 8.0).

Plasmid construction A plasmid containing a *mat1*-knockout cassette was constructed by cloning three fragments into the *Bam*HI site of pUC19 using a Gibson Assembly Kit (New England BioLab). DNA fragments of mat1L and mat1R were prepared from genomic DNA by polymerase chain reaction (PCR) amplification using the following primers: mat1_F1 (5′-CGGTACCCGGGGATCTTCACTTTGCATCGCTGCC-3′), mat1_R1 (5′-TCGCTGTCAAGGACATCTTC-3′), mat1_F2 (5′-CCTCTACCGACACC ACTCGTACATGCGTTGGTGC-3′), and mat1_R2 (5′-CGACTCTAGAGGATCTGCAG GGTCCAGTCTTAGC-3′). The PCR reaction mixture (100 μL) comprised 0.5 U/μL of TaKaRa LA-taq DNA polymerase (Takara Bio), 1 × LA-PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTP mixture, and 0.4 μM primers. Thermal cycling was performed using a T-100 thermocycler (Bio-Rad, Hercules, CA, USA) as follows: a preliminary denaturation at 94°C for 4 min; followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min; and a final elongation at 72°C for 7 min. The products were purified using a Wizard SV Gel and a PCR Clean-Up System (Promega, Madison, WI, USA). A hygromycin B-resistant gene cassette was amplified from pUXV1 using hsp-L (5′-TGTCCTTGACAGCGAGAACGTGGTAACTACCAGCGAGTTC-3′) and TT_R (5′-TGGTGTCGGTAGAGGCGTACAG-3′) primers and the same PCR regimen described above. The obtained DNA fragments were cloned into the *Bam*HI site of pUC19 using a Gibson Assembly Kit to produce a pUC19Δmat1 plasmid. The cloning procedures were performed according to the user's manual. The plasmid construction was confirmed by PCR amplification using the primers described above.

Construction of *mat1*-disruption mutant Gene disruption construct was amplified by PCR using primers mat1_F1 and mat1_R2 from pUC19Δmat1. The PCR product was transferred into *P. hubeiensis* SY62 by an electroporation method reported previously (18). To select the *mat1*-disruption mutant, we performed PCR amplification of the genomic DNA, which was extracted from each transformant, using primers mat1_F1 and mat1_R2. Here, fragments of approximately 2 kbp indicate wild-type *mat1*, and fragments of approximately 4 kbp indicate the inserted fragments, which resulted in homologous recombination.

MEL production using a 5-L table-top fermentor Jar fermentor cultivation was performed to investigate the glycolipid (GL) productivity of the *mat1*-disrupted mutant. The recombinant was cultivated in 20 mL of YM broth comprising 100 mg/L of hygromycin B in a 200-mL baffled Erlenmeyer flask at 25°C for 2 days for seed culture. The seed culture was transferred to 2 L of production medium comprising 100 g/L of glucose, 100 g/L of olive oil, 3 g/L of $NaNO_3$, 0.5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, and 100 mg/L of hygromycin B prepared in a 5-L jar fermentor (Bioneer 500-5L, B.R. Marubishi Co., Tokyo, Japan). The temperature was set at 25°C, the dissolved oxygen was maintained at above 50% saturation, the pH was not controlled, and 100 g/L olive oil was supplied on the third and fifth days.

The GLs were extracted with an equal volume of ethyl acetate. The ethyl acetate extracts were analyzed by thin-layer chromatography (TLC) on silica plates (silica gel 60F; Merck) with a solvent system comprising chloroform–methanol–7 N ammonium hydroxide (65:15:2, v/v) or chloroform–methanol–water (65:15:2, v/v). The compounds on the plates were visualized by charring at 110°C for 5 min and then spraying the plates with anthrone reagent. To quantify GL MEL-D, high-performance liquid chromatography (HPLC) was performed using a Waters e2695 separation module equipped with a silica gel column (Inertsil SIL-100A, 5 μm, 4.6 × 250 mm, GL Science, Tokyo, Japan) and a 2424 ELS detector. A mobile phase comprising chloroform–methanol was used with a liner gradient flow of 1 mL/min from 100:0 to 0:100 for 20 min.

Dry cell weight (DCW) was determined according to a previously reported method (17).

Structure determination The major structural components of the purified GL were characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy using a JASCO ECA600 instrument (600 MHz, JASCO, Tokyo, Japan) at 30°C in $CDCl_3$ or $CDCl_3/CD_3OD$ (50:50) solution. The fatty acid profiles of the purified GLs were determined using a previously reported method (16). Methyl ester derivatives of fatty acids were prepared by mixing the purified GLs mentioned above (approximately 10 mg) with 1 mL of 5% HCl–methanol reagent (Wako Pure Chemical Industries). After heating at 80°C for 30 min, the reaction was quenched with water (1 mL). The methyl ester derivatives were extracted with n-hexane (2 mL) and analyzed by gas chromatography–mass spectrometry (GC–MS) (Agilent 7890B and 5977A) using a TC-WAX column (0.25 mm × 30 m, 0.25 μm, GL-Science, Tokyo). The oven temperature was held at 80°C for 4 min and then increased by 10°C/min to 250°C. The molecular weight of the purified GL was measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS, Bruker Ultraflex) with an α-cyano-4-hydroxycinnamic acid matrix.

Determination of surface tension The surface tension values of the purified GLs was determined using the Wilhelmy-type automatic tension meter (CBVP-Z, Kyowa Interface Science Co., Ltd., Tokyo, Japan) at room temperature (25°C), as reported previously (16).

Water penetration scan The water penetration scan technique was used to examine the lyotropic liquid crystalline phase behavior of the GL, as reported

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