A Gene Cluster for Biosynthesis of Mannosylerythritol Lipids Consisted of 4-O-β-D-Mannopyranosyl-(2R,3S)-Erythritol as the Sugar Moiety in a Basidiomycetous Yeast *Pseudozyma tsukubaensis*

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Abstract

Mannosylerythritol lipids (MELs) belong to the glycolipid biosurfactants and are produced by various fungi. The basidiomycetous yeast *Pseudozyma tsukubaensis* produces diastereomer type of MEL-B, which contains 4-O-β-D-mannopyranosyl-(2R,3S)-erythritol (R-form) as the sugar moiety. In this respect it differs from conventional type of MELs, which contain 4-O-β-D-mannopyranosyl-(2S,3R)-erythritol (S-form) as the sugar moiety. While the biosynthetic gene cluster for conventional type of MELs has been previously identified in *Ustilago maydis* and *Pseudozyma antarctica*, the genetic basis for MEL biosynthesis in *P. tsukubaensis* is unknown. Here, we identified a gene cluster involved in MEL biosynthesis in *P. tsukubaensis*. Among these genes, *PtEMT1*, which encodes erythritol/mannose transferase, had greater than 69% identity with homologs from strains in the genera *Ustilago*, *Melanopsischiu*, *Sporisorium* and *Pseudozyma*. However, phylogenetic analysis placed *PtEMT1p* in a separate clade from the other proteins. To investigate the function of *PtEMT1*, we introduced the gene into a *P. antarctica* mutant strain, ΔPaEMT1, which lacks MEL biosynthesis ability owing to the deletion of PaEMT1. Using NMR spectroscopy, we identified the biosynthetic product as MEL-A with altered sugar conformation. These results indicate that *PtEMT1p* catalyzes the sugar conformation of MELs. This is the first report of a gene cluster for the biosynthesis of diastereomer type of MEL.
Introduction

Mannosylerythritol lipids (MELs) belong to the glycolipid biosurfactants which consist of mannosylerythritol (ME) as the hydrophilic moiety, and fatty acids as the hydrophobic moiety. MELs distinguish by conformation of ME. MELs which consist of 4-O-β-D-mannopyranosyl-(2S,3R)-erythritol (S-form) is termed conventional type of MELs, and consist of 4-O-β-D-mannopyranosyl-(2R,3S)-erythritol (R-form) is termed diastereomer type of MEL (Fig 1). MELs have received a great deal of industrial attention, owing to factors such as their biodegradability, biocompatibility, and favorable interfacial and self-assembling properties [1–3]. MELs have also been used as an ingredient in cosmetics because of their beneficial role in damaged hair and skin repair, cell activation and anti-oxidation [4–7].

MELs are produced from feedstocks by various fungi such as the genera *Ustilago* and *Pseudozyma*. *Ustilago maydis*, a fungal plant pathogen, has been identified as a MEL producer [8, 9] and its complete genome has been sequenced and published [10]. *P. antarctica* T-34 (formerly *Candida antarctica* T-34) has also been isolated and found to be an excellent MEL producer, producing more than 100 g L⁻¹ of MELs [11]. Similarly, several other *Pseudozyma* species have been established as MEL producers [12, 13]. Recently, genome sequences of species from the genus *Pseudozyma* have been reported. These species include *P. antarctica* T-34 and JCM10317[14, 15], *P. aphidis* DSM70725 [16] and *P. hubeiensis* SY62 [17]. Gene expression vectors and transformation methods have also been developed for this genus [18–20]. Owing to their high productivity and the availability of gene manipulation techniques [21], these *Pseudozyma* species are promising candidates for the commercial production of MELs.

Of the yeast species in the genus *Pseudozyma*, *P. antarctica*, *P. parantarctica*, *P. aphidis* and *P. rugulosa* produce mainly MEL-A (more than 70% of all MELs produced) [11, 12, 22–26]. *P. graminicola*, *P. hubeiensis*, *P. siamensis* and *P. shanxiensis* primarily produce MEL-C [27–31], and various strains of *P. tsukubaensis* produce the diastereomer type of MEL-B (Fig 1B) in large quantities [32]. The physicochemical properties of these molecules depend on their chemical structure, including the acetylation pattern, the conformation of fatty acids and the sugar moiety. The hydrophilicity of MEL-A (di-acetylated MEL) is lower than those of MEL-B and MEL-C (mono-acetylated MELs). Also, the water-holding property of R-form ME in diastereomer type of MELs is higher than that of S-form ME [33]. Our previous reports showed that diastereomer type of MEL-B has self-assembling properties across a wide range of

Fig 1. Chemical structure of MELs. (A) Conventional type of MELs. (B) Diastereomer type of MEL-B.

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concentrations and temperatures [34] and shows higher hydration ability than conventional type of MELs [35]. Therefore, diastereomer type of MEL-B produced by *P. tsukubaensis* may facilitate the use of MELs in aqueous solutions.

Over the past decade, a gene cluster involved in the biosynthesis of various glycolipids (such as MELs, cellobiose lipids and sophorolipids) has been identified [36–40]. In *U. maydis*, the MEL biosynthetic pathway consists of five proteins: an erythritol/mannose transferase (Emt1p), two acyl transferases (Mac1p and Mac2p), an acetyltransferase (Mat1p) [36, 37] and a putative transporter (Mmf1p) (Fig 2). While the products of *P. antarctica* are similar to those of *U. maydis*, the sugar conformation of MEL produced by *P. tsukubaensis* differs from those of the other *Ustilago* and *Pseudozyma* species. Furthermore, the degree of acetylation of the mannose moiety in *P. tsukubaensis* differs from that of *P. antarctica*, because the main product of *P. tsukubaensis* is a monoacetylated MEL (MEL-B). Therefore, the reaction of MEL biosynthesis in *P. tsukubaensis* differs from the reactions in *P. antarctica* and *U. maydis*, particularly with respect to sugar conformation and acetylation. We therefore focused the current study on the gene cluster for MEL biosynthesis in *P. tsukubaensis*.

We identified the gene cluster responsible for biosynthesis of the diastereomer type of MEL-B in *P. tsukubaensis* NBRC1940 based on amino acid sequence analysis. The putative amino acid sequence encoded by the gene PtEMT1 exhibited high identity to that of *P. antarctica*, but had an independent position on the phylogenetic tree. We altered the sugar conformation of MELs in *P. antarctica* from *S*-form to *R*-form by introducing PtEMT1 from *P. tsukubaensis* into a gene-disrupted mutant of *P. antarctica* lacking MEL biosynthesis activity.

**Materials and Methods**

**Pseudozyma** strains and plasmid

*Pseudozyma tsukubaensis* NBRC1940 was purchased from NITE Biological Resource Center (NBRC; Tokyo, Japan). A gene-disrupted mutant of *P. antarctica* T-34, ΔPaEMT1, is our laboratory stock [21]. A PtEMT1 expression vector, pUXV1_neo::PtEMT1, and PaEMT1 expression vector, PUXV1_neo::PaEMT1 were introduced to a host strain, ΔPaEMT1, by electroporation [19], resulting in a strain complemented the lacking of MEL biosynthesis ability.

**Sequence analysis**

The draft genome sequence of *P. tsukubaensis* NBRC1940 was performed (reported elsewhere). The BLAST program was used for sequence similarity searching in a database available on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were displayed using the ClustalW program. Phylogenetic analysis was performed using the neighbor-joining method [41] with the program MEGA6 [42] and bootstrap analysis based on 1,000 replicates [43].

**Plasmid construction**

The plasmid pUXV1_neo::PaEMT1 (Fig 3A) was constructed in Morita et al. (2013) [44]. The plasmid pUXV1_neo::PtEMT1 (Fig 3B) was constructed as follows. A PtEMT1 fragment was amplified with BamHI site by PCR using the complementary DNA of *P. tsukubaensis* NBRC1940 as template, and a set of oligonucleotide primers: 5'-GTTTGGATCCATGAAAGTGGCACTGCTTTC-3' (forward), and 5'-CGGGATCCCATGAGGGAACTGATGTGCG-3' (reverse). The 1.8-kb PtEMT1 fragment was digested by BamHI, and then inserted at the corresponding site in pUXV1_neo, yielding the plasmid pUXV1_neo::PtEMT1.
Fig 2. The biosynthetic pathway of MELs. Emt1p: erythritol/mannose transferase. Mac1p and Mac2p: acyl transferases. Mat1p: acetyl transferase. Mmf1p: putative transporter.

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Transformation

The plasmid pUXV1_neo::PtEMT1 and pUXV1_neo::PaEMT1 were introduced into ΔPaEMT1 by electroporation according to Morita et al. [19] with suitable modification. ΔPaEMT1 was grown in 3 mL MEL production medium (1 g L⁻¹ of yeast extract, 3 g L⁻¹ of NaNO₃, 0.3 g L⁻¹ of KH₂PO₄ and 0.3 g L⁻¹ of MgSO₄·7H₂O) containing 10% (w/v) glycerol at 25°C for 3 days as a seed culture. One milliliter of seed culture was inoculated into 50 mL of MEL production medium containing 10% (w/v) glycerol and cultivated at 25°C for 15 h with 250 stroke min⁻¹. The cells were harvested by centrifugation at 5,000 rpm for 5 min and washed twice with chilled 1 M sorbitol. 0.1 mL of cell suspension containing about 3 μg of plasmid was pulsed by electroporation using the Bio-Rad Gene Pulser II with Pulse Controller Plus (Bio-Rad, Tokyo, Japan). The cells were pulsed twice with a square-wave electroporation pulse of 1000 V and a pulse length of 1.0 ms at a pulse interval of 5 s. The electroporated cells were immediately diluted in 0.9 mL of chilled 1 M sorbitol and 3 mL of YM medium and incubated at 25°C for 1 h. After incubation, an aliquot (0.2 mL) was spread on the YM medium plate containing 500 μg L⁻¹ G418, and the resulting colonies were grown at 25°C for 4 days.

MEL production

ΔPaEMT1 harboring pUVX1_neo::PtEMT1 or pUXV1_neo::PaEMT1 were cultivated in 2 mL YM medium (3 g L⁻¹ of yeast extract, 3 g L⁻¹ of malt extract, 5 g L⁻¹ peptone and 10 g L⁻¹ glucose) containing 200 μg mL⁻¹ G418 at 25°C for 3 days as a seed culture. In a 300-mL flask, the seed culture was inoculated with 30 mL MEL production medium containing 10% (w/v) glucose as a carbon source and incubated at 25°C for 7 days with 200 stroke min⁻¹. 200 μg mL⁻¹ of G418 was supplied in the culture medium to maintain the plasmid. The produced MELs were detected using TLC [19]. The MELs were extracted from the cell culture with an equal volume of ethyl acetate, and 50 μL of ethyl acetate extracts were analyzed by TLC using chloroform, methanol and NH₄OH in a 65:15:2 (v:v:v) ratio as an eluent. The MELs were detected on the TLC plate by spraying with 2% anthrone-sulfate reagent and heating at 90°C for 5 min. A mixture of purified MEL-A, MEL-B and MEL-C was used as a reference.

MEL purification

The ethyl acetate fractions containing MELs were evaporated. The concentrated MELs were dissolved in chloroform and purified using silica gel (Wako-gel C-200) column
chromatography with a gradient elution of chloroform/acetone (10:0 to 0:10, v/v) mixtures as solvent systems [23]. The purified MEL-A was used in the following experiments.

Structural analysis
The structure of the purified MEL-A was characterized by $^1$H and $^{13}$C nuclear magnetic resonance spectroscopy (NMR) with a Bruker AVANCE 400 (400 MHz) at 30°C in a CDCl$_3$ solution. Tetramethylsilane [(CH$_3$)$_4$Si] was used as an internal chemical shift standard and the purified MEL-A was used as a reference for NMR analysis.

Results
Identification of the gene cluster of MEL biosynthesis in P. tsukubaensis NBRC1940
Based on BLASTP analyses, the genes involved in MEL biosynthesis (emt1, mac1, mac2, mmf1 and mat1 for U. maydis; PaEMT1, PaMAC1, PaMAC2, PaMMF1 and PaMAT1 for P. antarctica) were found in the draft genome of P. tsukubaensis NBRC1940 (PtEMT1, PtMAC1, PtMAC2, PtMMF1 and PtMAT1, respectively) (Fig 4). The gene arrangement in the MEL biosynthesis cluster of P. tsukubaensis NBRC1940 is more similar to U. maydis than P. antarctica strains JCM10317 and T-34 (Fig 4). The PtEMT1 and PtMAC2 of P. tsukubaensis NBRC1940 are rearranged, as well as U. maydis, compared with P. antarctica JCM10317 and T-34.

The amino acid sequence homology of five proteins involved in the biosynthesis of MELs (PtEMT1p, PtMAC1p, PtMAC2p, PtMMF1p and PtMAT1p) was compared to those from U. maydis and P. antarctica strains JCM10317 and T-34 (Table 1). According to BLASTP analysis, PtEMT1p, PtMAC1p, PtMAC2p and PtMMF1p have high (over 50%) homology to the corresponding proteins in U. maydis and P. antarctica.

![Gene clusters of MEL biosynthesis](image-url)
Amino acid sequence analysis of PtEMT1 from *P. tsukubaensis* NBRC1940

The amino acid sequence of PtEMT1p is similar to those of the corresponding proteins in *U. maydis* and *P. antarctica*. However, the sugar conformation of MEL produced by *P. tsukubaensis* differed from those of other MEL producers. We thus performed further amino acid sequence analysis (Table 2). PtEMT1p consists of 612 amino acids, and the corresponding proteins from *U. maydis* UM521, *P. antarctica* JCM10317 and *P. antarctica* T-34 consist of 615, 617 and 617 amino acids, respectively. Surprisingly, only nine proteins, from strains in the genera *Ustilago*, *Melanopsichium*, *Sporisorium* and *Pseudozyma*, showed high sequence identities with PtEMT1p (69–72%) (Table 2). All of these strains, except for those in the genus

Table 2. The homologous proteins of PtEMT1p by BLASTP analysis.

| # | Accession a | Description a | Strains a | e-value b | Identity (%) b |
|---|-------------|---------------|-----------|-----------|---------------|
| 1 | CDR99457.1 | hypothetical protein | Sporisorium scitamineum | 0 | 72 |
| 2 | CCF52717 | uncharacterized protein | Ustilago hordei | 0 | 70 |
| 3 | CDI53946 | conserved hypothetical protein | Melanopsichium pensylvanicum | 0 | 70 |
| 4 | ETS61959 | mannosyltransferase | Pseudozyma aphidis DSM 70725 | 0 | 69 |
| 5 | GAC96558 | glycosyltransferase | Pseudozyma hubeiensis SY62 | 0 | 70 |
| 6 | GAK68006 | glycosyltransferase | Pseudozyma antarctica | 0 | 69 |
| 7 | GAC75887 | hypothetical protein | Pseudozyma antarctica T-34 | 0 | 70 |
| 8 | CBQ73522 | conserved hypothetical protein | Sporisorium reilianum SRZ2 | 0 | 70 |
| 9 | XP_011389468 | erythritol-mannosyl-transferase involved in MEL production | Ustilago maydis 521 | 0 | 69 |
| 10 | BA177915 | mannosyltransferase | Pseudozyma antarctica | 0 | 68 |
| 11 | XP_006666634 | putative glycosyltransferase | Cordyceps militaris CM01 | 5E-101 | 37 |
| 12 | XP_681404 | hypothetical protein | Aspergillus nidulans FGSC A4 | 4.62E-94 | 35 |
| 13 | XP_008597660 | mannosyltransferase-like | Beauveria bassiana ARSEF 2860 | 9.3E-92 | 35 |
| 14 | KH93740 | UDP-glucuronosyl/UDP-glucosyltransferase | Metarhizium album ARSEF 1941 | 1.13E-86 | 39 |
| 15 | KGQ12602 | putative UDP-glucuronosyltransferase | Beauveria bassiana D1-5 | 3.8E-65 | 38 |
| 16 | KIM30075 | glycosyltransferase family 1 protein | Serendipita vermifera MAFF 305830 | 6.33E-23 | 24 |
| 17 | CCP99492 | unnamed protein | Malassezia sympodialis ATCC 42132 | 4.33E-19 | 37 |
| 18 | XP_001730757 | hypothetical protein | Malassezia globosa CBS 7966 | 3.22E-18 | 35 |
| 19 | XP_001728828 | hypothetical protein | Malassezia globosa CBS 7966 | 1.47E-17 | 36 |
| 20 | KIM75119 | glycosyltransferase family 1 protein | Piloderma croceum F 1598 | 1.4E-15 | 47 |

a Referred from NCBI database.
b Analyzed by BLASTP.

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Pseudozyma, have been reported as plant pathogens [9, 45, 46]. Table 3 shows Emt1p sequence identities among *P. tsukubaensis* NBRC1940 and nine other strains. While PtEMT1p has approximately 70% identity with homologous proteins, other pairs of species (e.g. *P. aphidis* and *P. antarctica* or *S. scitamineum* and *S. reilianum*) share more than 90% sequence identity.

We aligned nine proteins that exhibited high identity to PtEMT1p using ClustalW. While sequence identity was greater than 69%, we found three regions with relatively low identity (Fig 5). The three regions in *P. tsukubaensis* NBRC1940 each consisted of about 30 amino acids. The amino acid position of regions I, II and III were from 272 to 300, 372 to 397, and 569 to 597, respectively (Fig 6). Putative sugar binding domain was not found. Phylogenetic analysis based on the amino acid sequences of Emt1p indicated that PtEMT1p diverged significantly from the other nine proteins (Fig 7). Thus, amino acid sequence analysis suggests that PtEMT1p is a novel structure that differs substantially from homologous proteins.

MEL production by *P. antarctica* ΔPaEMT1 harboring pUXV1_neo::PtEMT1 from *P. tsukubaensis* NBRC1940

Because ΔPaEMT1 derived from *P. antarctica* T-34 had its PaEMT1 function disrupted, it provides a useful host to investigate the function of PtEMT1 in MEL production. The plasmid harboring PtEMT1 from *P. tsukubaensis* NBRC1940 was introduced into ΔPaEMT1. The empty vector pUXV1_neo was used as a negative control, and pUXV1_neo::PaEMT1 which harboring PaEMT1 from *P. antarctica* T-34 was used as a positive control.

The ΔPaEMT1 harboring pUXV1_neo::PtEMT1 was cultivated in MEL production medium containing 10% (w/v) glucose for 7 days at 25°C. The produced MELs were extracted by ethyl acetate and detected by thin-layer chromatography (TLC) analysis (Fig 8). ΔPaEMT1 harboring pUXV1_neo::PtEMT1 and pUXV1_neo::PaEMT1 produced MELs from glucose, whereas ΔPaEMT1 harboring empty vector PUVX1_neo failed to produce MELs, as expected. Thus, the gene PtEMT1 from *P. tsukubaensis* NBRC1940 restored MEL production in ΔPaEMT1.

![Table 3. Comparison of amino acid sequence homology of PtEMT1p and homologous proteins.](image)

| Subject sequence | Identity (%)a |
|------------------|--------------|
|                  | Pt. tsu | S. sci | U. hor | M. pen | P. aph | P. hub | P. ant | P. ant T-34 | S. rei | U. may |
| P. tsu           | -      | 72     | 70     | 70     | 69     | 70     | 69     | 70          | 71     | 69     |
| S. sci           | 72     | -      | 80     | 82     | 77     | 81     | 78     | 77          | 93     | 82     |
| U. hor           | 70     | 80     | -      | 78     | 75     | 77     | 76     | 76          | 79     | 78     |
| M. pen           | 70     | 82     | 79     | -      | 78     | 80     | 78     | 77          | 82     | 79     |
| P. aph           | 69     | 77     | 76     | 78     | -      | 77     | 97     | 94          | 78     | 75     |
| P. hub           | 70     | 81     | 77     | 80     | 77     | -      | 78     | 77          | 80     | 82     |
| P. ant           | 69     | 78     | 77     | 78     | 97     | 78     | -      | 95          | 78     | 76     |
| P. ant T-34      | 70     | 77     | 77     | 77     | 94     | 77     | 95     | -           | 77     | 75     |
| S. rei           | 70     | 92     | 77     | 81     | 77     | 80     | 78     | 77          | -      | 80     |
| U. may           | 69     | 82     | 78     | 79     | 75     | 82     | 76     | 75          | 81     | -      |

a Analyzed by BLASTP.

| Subject sequence | Identity (%)a |
|------------------|--------------|
|                  | Pt. tsu | S. sci | U. hor | M. pen | P. aph | P. hub | P. ant | P. ant T-34 | S. rei | U. may |
| P. tsu           | -      | 72     | 70     | 70     | 69     | 70     | 69     | 70          | 71     | 69     |
| S. sci           | 72     | -      | 80     | 82     | 77     | 81     | 78     | 77          | 93     | 82     |
| U. hor           | 70     | 80     | -      | 78     | 75     | 77     | 76     | 76          | 79     | 78     |
| M. pen           | 70     | 82     | 79     | -      | 78     | 80     | 78     | 77          | 82     | 79     |
| P. aph           | 69     | 77     | 76     | 78     | -      | 77     | 97     | 94          | 78     | 75     |
| P. hub           | 70     | 81     | 77     | 80     | 77     | -      | 78     | 77          | 80     | 82     |
| P. ant           | 69     | 78     | 77     | 78     | 97     | 78     | -      | 95          | 78     | 76     |
| P. ant T-34      | 70     | 77     | 77     | 77     | 94     | 77     | 95     | -           | 77     | 75     |
| S. rei           | 70     | 92     | 77     | 81     | 77     | 80     | 78     | 77          | -      | 80     |
| U. may           | 69     | 82     | 78     | 79     | 75     | 82     | 76     | 75          | 81     | -      |

P. tsu, *Pseudozyma tsukubaensis* NBRC1940; S. sci, CDR99457.1,_Sporisorium scitamineum_; U. hor, CCF52717,_Ustilago hordei_; M. pen, CDI53946,_Melanopsichium pennsylvanicum_ 4; P. aph, ETS61999,_Pseudozyma aphidis_ DSM70255; P. hub, GAC96558,_Pseudozyma hubeiensis_ SY62; P. ant, GAK68006,_Pseudozyma antarctica_; P. ant, GAC75887,_Pseudozyma antarctica_ T-34; S. rei, CDR99457.1,_Sporisorium reilianum_ SRZ2; U. may, XP_011389468,_Ustilago maydis_ 521.

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Fig 5. Amino acid sequence alignment of the PtEMT1p from *P. tsukubaensis* NBRC1940 and nine homologous proteins. (I), (II) and (III) show low-identity regions I, II, and III, respectively. Identical residues are shown on a black background. GAC96558_P.hub: *Pseudozyma hubeiensis* SY62. XP_011389468_U.may: *Ustilago maydis* 521. CDR94457.1_S.sci: *Sporisorium scitamineum*. CBQ73522_S.rei: *Sporisorium reilianum* SRZ2. CDI53946_M.pen: *Melanopsichium pennsylvanicum* 4. CCF52717_U.hor: *Ustilago hordei*. ETS61959_P.aph: *Pseudozyma aphidis*. MEL Biosynthesis Gene Cluster from *P. tsukubaensis* PLOS ONE | DOI:10.1371/journal.pone.0157858 June 21, 2016 9/1 6
Structural analysis of diastereomer type of MEL-A biosynthesized by ΔPaEMT1 harboring pUXV1_neo::PtEMT1

To determine the structure of MEL-A produced by ΔPaEMT1 expressing PtEMT1, we subjected purified MEL-A to NMR analysis and compared the signal pattern to that of conventional type of MEL-A produced by ΔPaEMT1 expressing PaEMT1 (Fig 9A and 9B). Each signal was assigned as previously described [47–49]. The diastereomer and conventional type of MELs showed very similar 1H NMR spectra (Fig 9A). However, two resonances arising from H-4a and H-4b in the erythritol moiety were significantly different. In conventional type of MEL the H-4 signals were widely separated (H-4b: 3.98–4.01 ppm; H-4a: 3.80–3.83 ppm), while the signals observed from diastereomer type of MEL overlapped with one another (H-4a and H-4b: 3.87–3.97 ppm). Moreover, the H-1 signal from the mannose anomeric hydrogen was shifted to a lower field in MEL-A produced by recombinant strain ΔPaEMT1 harboring pUXV1_neo::PtEMT1 (from 4.73 ppm to 4.74 ppm). In 13C NMR analyses (Fig 9B), we observed a...
Fig 7. The phylogenetic relationships among *P. tsukubaensis* NBRC1940 and homologous strains, based on the Emt1p amino acid sequence.

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Fig 8. TLC analysis of MEL production by Δ*PaEMT1* harboring pUXV1_neo::PtEMT1. STD: standard MELs containing MEL-A, MEL-B and MEL-C. Δ*PaEMT1*::neo: Δ*PaEMT1* harboring pUXV1_neo. Δ*PaEMT1*::neo_PaEMT1: Δ*PaEMT1* harboring pUXV1_neo::PaEMT1. Δ*PaEMT1*::neo_PtEMT1: Δ*PaEMT1* harboring pUXV1_neo::PtEMT1. Each strain was cultivated in MEL production medium containing 10% (w/v) glucose for 7 days at 25°C. The spots were visualized using anthrone reagent.

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characteristic chemical shift in the C-2 and C-3 signals of the erythritol moiety (from 72.0 ppm to 71.8 ppm and 71.3 ppm to 71.5 ppm, respectively), which corresponded to the C-2 and C-3 signals in diastereomer type of MEL-A [47–49]. Based on these observations, we concluded that ΔPaEMT1 harboring pUXV1_neo::PtEMT1 produced the diastereomer type of MEL-A.

**Discussion**

In this study, we obtained for the first time the gene cluster involved in the diastereomer type of MEL-B biosynthesis in *P. tsukubaensis*. The gene PtEMT1, which plays a crucial role in determining the sugar conformation of ME, was introduced into ΔPaEMT1. PtEMT1 restored MEL production in ΔPaEMT1 and the product was determined to be diastereomer type of MEL-A.

MEL biosynthesis clusters have been previously reported in conventional type of MEL producers such as *U. maydis, P. antarctica, P. aphidis* and *P. hubeiensis* [14–17, 36, 37]. Earlier
studies assessed the function of Emt1 using gene disruption methods and found that Emt1 is essential for MEL biosynthesis [21, 36]. Therefore, we strongly expected Emt1 to contribute to the chirality of MEL-B in P. tsukubaensis. To investigate this hypothesis, we introduced the plasmid pUXV1_neo::PtEMT1 into ΔPaEMT1, which lacks MEL production. NMR analyses revealed that the MEL produced was diastereomer type of MEL-A (Fig 9). We therefore concluded that PtEMT1p plays a critical role in the formation of the sugar moiety in MELs.

Morita et al. [50] reported that P. tsukubaensis JCM16987 synthesizes mannosyl-L-arabitol lipid-B from L-arabitol as a substrate but does not utilize D-arabitol. In contrast, P. parantarc-tica, a conventional type of MEL-A producer, biosynthesized only mannosyl-D-mannitol lipid from D-arabitol. This suggests that substrate specificity in P. tsukubaensis differs from that of conventional type of MEL producers [50]. Based on Emt1p amino acid sequence alignment (Fig 5), we observed three low-identity regions in the C-terminal half of P. tsukubaensis NBRC1940. We predict that these regions may be related to the substrate specificity of sugar alcohols in P. tsukubaensis. Important avenues for future research, include chimeric enzyme construction, crystal structure analysis, and determination of the active site of PtEMT1p, will provide us with greater insight into the function of PtEMT1p.

To our knowledge, P. tsukubaensis and P. crassa are the only species that biosynthesize diastereomer type of MEL [48, 49]. P. crassa biosynthesizes a mixture of diastereomer type of MELs containing MEL-A, MEL-B and MEL-C; however, the genomics of this species has not yet been studied. Therefore, genetic and structural analysis of Emt1p in P. crassa will help to elucidate the catalytic mechanism of sugar conformation.

Various homologs of conventional type of MELs have been reported to date, such as MEL-A, MEL-B, MEL-C and MEL-D. Other MEL homologs, containing D-arabitol, D-mannitol and ribitol instead of erythritol, have also been biosynthesized [51, 52]. In previous studies, diastereomer type of MEL-B and MEL-D exhibited higher hydrophilicity and water-holding properties than conventional type of MELs [35]. Based on these findings, it was determined that the sugar conformation of ME affects the function of MELs. Hitherto, MEL-B is the only diastereomer type of MEL capable of commercial-scale production. Diastereomer type of MEL-A and MEL-C are biosynthesized by P. crassa; however, this species produces only small amounts of MELs. While diastereomer type of MEL-D can be obtained in vitro by enzymatic reaction, there is no previously reported means of producing this glycolipid through microbial biosynthesis. In the present study, we demonstrated that diastereomer type of MEL-A can be produced by a conventional type of MEL-A producer modified to express PtEMT1. Further investigation of the PtEMT1p enzyme will facilitate the expansion of structural and functional varieties of MELs through gene engineering methods.

In conclusion, we identified for the first time the gene cluster involved in diastereomer type of MEL-B biosynthesis and demonstrated that PtEMT1p plays a crucial role in sugar conformation. Further elucidation of the MEL biosynthesis gene cluster will provide opportunities for metabolic engineering using this biosynthetic pathway.

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**Author Contributions**

Conceived and designed the experiments: AS TM. Performed the experiments: AS HK TF SY TK TM. Analyzed the data: AS HK TF SY TK TM. Wrote the paper: AS TM.
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