Deficiency of biodegradable plastic-degrading enzyme production in a gene-deletion mutant of phyllosphere yeast, *Pseudozyma antarctica* defective in mannosylerythritol lipid biosynthesis

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**Abstract**

The basidiomycetous yeast *Pseudozyma antarctica* (currently designated *Moesziomyces antarcticus*) produces extracellular enzymes and glycolipids, including mannosylerythritol lipids (MELs), which are biosurfactants. Strain GB-4(0) of this species was previously isolated from rice husks and produces biodegradable plastic-degrading enzyme (*Pseudozyma antarctica* esterase; PaE). In this study, we generated a MEL biosynthesis-deficient strain (∆PaEMT1) by deleting the gene PaEMT1, which is essential to MEL biosynthesis in strain GB-4(0). The resulting ∆PaEMT1 strain showed deficient PaE activity, and the corresponding signal was hardly detected in its culture supernatant through western blotting analysis using rabbit anti-PaE serum. On the other hand, the relative expression of the gene PaCLE1, encoding PaE, was identical between GB-4(0) and ∆PaEMT1 based on quantitative real-time PCR. When strain ∆PaEMT1 was grown in culture media supplemented with various surfactants, i.e., Tween20, BRIJ35 and TritonX-100, and MELs, PaE activity and secretion recovered. We also attempted to detect intracellular PaE using cell-free extract, but observed no signal in the soluble or insoluble fractions of ∆PaEMT1. This result suggested that the PaCLE1 gene was not translated to PaE, or that expressed PaE was degraded immediately in ∆PaEMT1. Based on these results, MEL biosynthesis is an important contributor to PaE production.

**Keywords:** *Pseudozyma antarctica*, Esterase, Biodegradable plastic, Mannosylerythritol lipid, Glycosyltransferase, Gene deletion

**Introduction**

*Pseudozyma antarctica* (currently designated *Moesziomyces antarcticus*) has the ability to produce some materials, including enzymes and glycolipids (Boekhout and Fell 1998). In recent years, we have focused on an esterase (*Pseudozyma antarctica* esterase; PaE) that degrades biodegradable plastics (BP), including poly(butylene succinate) (PBS), poly(butylene succinate-co-adipate) (PBSA), poly(ɛ-caprolactone) (PCL), and poly(lactide) (PLA) (Kitamoto et al. 2011; Shinozaki et al. 2013). *P. antarctica* strains isolated from rice husks secrete relatively high amounts of PaE into the culture supernatant, and PaE production is strongly enhanced when these strains are cultivated with xylose (Watanabe et al. 2014). PBSA, PBS and commercially available BP mulch films submerged in the culture supernatant of *P. antarctica* are rapidly degraded (Watanabe et al. 2014). To accelerate PaE utilization, we constructed a recombinant strain that is able to produce large amounts of PaE (13.4-fold higher amount than that of the wild-type strain) (Watanabe et al. 2016). Additionally, a targeted gene manipulation method has been developed recently (Yarimizu et al. 2017).
that allows more efficient modification of the PaE production of this strain.

*Pseudozyma antarctica* is well known to produce glycolipid-type biosurfactants known as mannosylerythritol lipids (MELs), which are composed of mannose, erythritol and fatty acids. MELs show excellent interfacial properties, as well as moisturizing activity toward cultured human skin cells; therefore, they have been commercially in skincare products and cosmetics (Yamamoto et al. 2012). MEL production by *P. antarctica* was first reported in strain T-34, isolated from the exudate of a tree on Mt. Tsukuba in Japan (Kitamoto et al. 1990). The genomic sequence of *P. antarctica* T-34 has been reported, and five genes (PaEMTI, PaMAC1, PaMAC2, PaMAT1 and PaMMF1) involved in MEL biosynthesis have been identified (Morita et al. 2013a).

Recently, we found that the BP film degradation activity of PaE was inhibited by MELs through in vitro analysis using surface plasmon resonance (Fukuoka et al. 2016). The hydrophobic lipid domain of MEL interacted with the BP film, and the hydrophilic sugar domains of MEL were regularly oriented on the MEL-coated film surface. The hydrophobic portion of PaE attaches to hydrophobic substrates such as BPs, as interaction between the enzyme and substrate is crucial for the degradation activity (Shinozaki et al. 2013). The negative effects of synthetic surfactants on production of esterases by yeasts have been studied in several previous papers. Namely, production of lipases, a subclass of esterases, by the yeasts *Candida viswanathii* and *Yarrowia lipolytica* was suppressed with the addition of either Tween80 or TritonX-100 (Almeida et al. 2013; Dominguez et al. 2003). The natMX4 cassette with the nourseothricin acetyltransferase gene (*nat1*) controlled by *Ashbya gossypii* TEF promoter and terminator (1.2-kb) was amplified via PCR with the primer set (PaEMTI_up_F1, R1 and PaEMTI_down_F1, R1) listed in Table 1, and with the genomic DNA of strain GB-4(0) used as a template. The natMX4 cassette with the nourseothricin acetyltransferase gene (*nat1*) controlled by *Ashbya gossypii* TEF promoter and terminator (1.2-kb) was amplified via PCR with the primer set (Nat_F1, R1) listed in Table 1, using plasmid pAG25 (Goldstein and McCusker 1999) as a template. The gene fragment natMX4, flanked by the sequences of the PaEMTI locus, was amplified by overlap PCR using three fragments that share 40 base-pair end-terminal homology as templates, yielding fragment PaEMTI::natMX4 (Additional file 1: Figure S1A), checked using gel electrophoresis, and purified with the MagExtractor-PCR & Gel Clean-up kit (TOYOBO Co., Ltd., Osaka, Japan). Approximately 10 μg of this DNA fragment was introduced into strain GB-4(0) using the lithium acetate method (Yarimizu et al. 2017). The transformed cells were grown on YPD medium agar plates containing 100 μg/mL nourseothricin (clonNAT; Jena Bioscience, Jena, Germany), and PaEMTI deletion was confirmed using colony PCR.

**PaE production**

Seed cultures of the strains were grown in 2 mL YM medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 10 g/L glucose) in test tubes at 30 °C for 3 days with shaking at 250 strokes/min (reciprocal). Then, 300 μL of seed culture was inoculated into a 300-mL
flask with 30 mL modified(m)-3 × FMM (fungal mini-
mum medium; 3 g/L yeast extract, 2 g/L NaNO₃, 0.6 g/L
KH₂PO₄ and 0.6 g/L MgSO₄·7H₂O) containing 8 (w/v)%
xylose that modified from previous report (Watanabe
et al. 2014). The strains were cultivated at 30 °C for 4 days
with 200 strokes/min shaking (rotary). After cultivation,
the culture was centrifuged at 20,400 g, and its superna-
tant was used for the PaE activity assay. To evaluate the
functional complementation of ΔPaEMT1, PaEMT1 was introduced using a plasmid that carries a bacterial
neomycin resistance gene (neo), namely pUXV1_neo-
PaEMT1, via the lithium acetate method, and allowing
selection on YM medium containing 500 μg/mL gene-
 ticin (G418). 200 μg/mL G418 was added to the cul-
ture medium to maintain the plasmid. Cell growth was
determined by measuring the optical density of samples
at 600 nm (OD₆₀₀). All cultivations were performed in
triplicate.

**PaE detection through SDS-PAGE and western blotting**

PaE production in the culture supernatant and cell-free
extract were analyzed through sodium dodecyl sul-
fate polyacrylamide gel electrophoresis (SDS-PAGE)
according to the method of Laemmli (1970), using a 14%
polyacrylamide slab gel (culture supernatant) or Any kD
Mini-Protein TGX gel (cell-free extract; Bio-Rad, Rich-
mond, CA, USA). Proteins were visualized with Coomas-
sie brilliant blue (CBB) staining using Quick-CBB (culture
supernatant; FUJIFILM Wako Pure Chemical Corpora-
tion, Osaka, Japan) or SimplyBlue™ SafeStain (cell-free
extract; Invitrogen, Carlsbad, CA, USA) kits. The pro-
teins separated through SDS-PAGE were transferred to
PVDF membranes using Trans-Blot SD Semi-Dry Trans-
fer Cell (Bio-Rad) at 60 mA for 60 min. Detection of PaE
through western blot analysis was performed using rabbit
anti-PaE serum (1/3.5 × 10⁴) (Kitamoto et al. 2011) and
HRP-linked donkey anti-rabbit polyclonal antibody (GE
Healthcare, Little Chalfont, UK) as the primary and sec-
tary antibodies, respectively. The PaE-antibody con-
jugate was detected with FluorChem and AlphaEase FC
Software (Alpha Innotech, San Jose, CA, USA) using ECL
western blotting detection reagent (GE Healthcare). Puri-
ﬁed PaE was used as a control, as described previously
(Suzuki et al. 2013).

**Preparation of cell-free extract**

Preparation of the cell-free extract is summarized in
Additional file 1: Figure S2. To prepare cell-free extract,
cells were harvested from 1 mL culture broth through centrifugation at 9100g for 2 min. The cells were resuspended in 0.5 mL 20 mM Tris–HCl (pH 6.8) containing 1 mM PMSE, and then disrupted with two 5-mm and twenty 2-mm zirconia beads at 3200 rpm for 5 min (three times) using the Beads Crusher μT-12 (TAITEC, Saitama, Japan). After disruption, 0.5 mL 20 mM Tris–HCl (pH 6.8) was added, and 0.8 mL of sample was transferred to a 1.5-mL tube. The cell debris was removed through centrifugation at 9100g for 2 min and re-suspended in 0.8 mL 20 mM Tris–HCl (pH 6.8) as the insoluble fraction. TritonX-100 was added to the insoluble fraction (final concentration: 0.01%) to treat the insoluble fraction. TritonX-100 was added to the sample with an equal volume of ethyl acetate, and 10 μL of this emulsion was measured at 660 nm (OD660). One unit (U) of PBSA degradation activity was defined as the activity that decrease OD660 by 1 in 1 mL reaction solution in 1 min. All analyses were performed in triplicate.

Production and detection of MELS
Cells were cultivated in MEL production medium (1 g/L yeast extract, 3 g/L NaNO₃, 0.3 g/L KH₂PO₄ and 0.3 g/L MgSO₄·7H₂O) containing 2 (w/v)% soybean oil at 30 °C for 4 days. The MELS produced were detected using thin-layer chromatography (TLC), as described previously (Kitamoto et al. 2011). Briefly, a reaction mixture containing 20 mM Tris–HCl (pH 6.8), 0.045 (w/v)% emulsified PBSA and supernatant of the yeast culture or purified MELS was incubated at 30 °C for 15 min with 180 strokes/min shaking. After addition of the culture supernatant, the decrease in absorbance of the PBSA emulsion was measured at 660 nm (OD₆₆₀). One unit (U) of PBSA degradation activity was defined as the activity that decrease OD₆₆₀ by 1 in 1 mL reaction solution in 1 min. All analyses were performed in triplicate.

The effects of surfactant addition on PaE production by strain ΔPaEMT1
ΔPaEMT1 was cultivated in m-3×FMM containing 8 (w/v)% xylose supplemented with 0.01 (w/v)% of MELS, Tween20, BRIJ35, TritonX-100, SDS, sodium laurate (C12Na) or sodium stearate (C18Na). After 4 days of cultivation, the PaE activity in the culture supernatant was analyzed.}

Analysis of PaCLE1 expression levels through quantitative real-time PCR
The gene expression levels of PaCLE1, encoding PaE, were analyzed through quantitative real-time PCR (qRT-PCR). P. antarctica GB-4(0) and strain ΔPaEMT1 were cultivated in m-3×FMM medium containing 8 (w/v)% xylose with or without 0.01 (w/v)% TritonX-100. After 3 days of cultivation, cells were harvested from 30 mL of culture by centrifugation at 800g for 3 min and washed with distilled water twice. Total RNA was isolated from each sample with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions, and the total concentration of purified RNA was determined using a Nanodrop fluorometer (Nano Drop Technologies, Wilmington, DE, USA). Then, mRNA was purified with the Oligotex-dT30<Super>mRNA Purification Kit (Takara Bio, Shiga, Japan). cDNA was synthesized from a template of 50 ng mRNA using the SuperScript VILO cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. Primers for qRT-PCR were designed using Primer 3 (http://bioinfo.ut.ee/prime r3-0.4.0/prime r3/) (Table 1). The actin gene was used as a housekeeping gene. qRT-PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and visualized with LightCycler 2.0 with LightCycler software (ver. 5.0; Roche Diagnostics, Basel, Switzerland). Primer specificity was examined via melting curve analysis. Gene expression levels were quantified using the following equation: gene expression level = 2^−[Ct(PaCLE1)−Ct(Actinant)]. The accession numbers of the actin gene and PaCLE1 are LC466625 and LC276896, respectively.

Results
Deletion of PaEMT1 in strain GB-4(0)
To obtain the MEL biosynthesis-deficient strain of GB-4(0), PaEMT1 was deleted and replaced with natMX4 through homologous recombination (Additional file 1: Figure S1A). The transformants generated on YPD plates with clonNAT were picked and analyzed using colony PCR to confirm PaEMT1 deletion (Additional file 1: Figure S1B). The inner region (1.0 kb) of PaEMT1 was amplified via PCR with primers PaEMT1_inner_F1
4 days cultivation) (Fig. 2b). In SDS-PAGE and western blot analyses, the signal corresponding to PaE from ΔPaEMT1 was weaker than that from the wild-type (Fig. 2a). Contrary to our expectation, PaE activity in the culture supernatant of ΔPaEMT1 (0.2 ± 0.2 U/mL, 4 days cultivation) was clearly lower than that of the wild-type (1.0 ± 0.2 U/mL, 4 days cultivation) (Fig. 2b). In SDS-PAGE and western blot analyses, the signal corresponding to PaE from ΔPaEMT1 was weaker than that from the wild-type (Fig. 2c). This result suggested that PaE expression and/or secretion decreased with defective MEL production. On the other hand, strain GB-4(0) secretes large amounts of 33-kDa endo-β-xylanase in the presence of xylose (Watanabe et al. 2015). We observed similar signal intensity corresponding to xylanase in the culture supernatants of ΔPaEMT1 and wild-type cells (Fig. 2c). In addition, weak signals corresponding to other unknown proteins from ΔPaEMT1 were the same as those from the wild-type.

PaE activity of strain ΔPaEMT1
To investigate the effect of PaEMT1 deletion on PaE production, strains GB-4(0) and PaEMT1-deletion strain PaEMT1Δ::NAT (ΔPaEMT1) were cultivated in m-3×EMM-8% xylose. Cell growth of ΔPaEMT1 was the same as that of the wild-type (Fig. 2a). Contrary to our expectation, PaE activity in the culture supernatant of ΔPaEMT1 (0.2 ± 0.2 U/mL, 4 days cultivation) was clearly lower than that of the wild-type (1.0 ± 0.2 U/mL, 4 days cultivation) (Fig. 2b). In SDS-PAGE and western blot analyses, the signal corresponding to PaE from ΔPaEMT1 was weaker than that from the wild-type (Fig. 2c). This result suggested that PaE expression and/or secretion decreased with defective MEL production. On the other hand, strain GB-4(0) secretes large amounts of 33-kDa endo-β-xylanase in the presence of xylose (Watanabe et al. 2015). We observed similar signal intensity corresponding to xylanase in the culture supernatants of ΔPaEMT1 and wild-type cells (Fig. 2c). In addition, weak signals corresponding to other unknown proteins from ΔPaEMT1 were the same as those from the wild-type.

Based on these results, MEL biosynthesis is likely to contribute strongly to PaE production.

Complementation of PaEMT1 deletion
To confirm the phenotypic changes due to loss of MEL production described above, PaEMT1 deletion was complemented through introduction of PaEMT1 with the plasmid pUXV1.neo-PaEMT1, and pUXV1.neo was used as a control. The recombinant strains ΔPaEMT1 harboring pUXV1.neo-PaEMT1 or pUXV1.neo were cultivated in MEL production medium containing 2 (w/v)% soybean oil and 200 μg/mL G418 for 4 days at 30°C. In TLC analysis, MEL production was recovered in ΔPaEMT1 harboring pUXV1.neo-PaEMT1 (Fig. 3a). Cell growth of both strains was identical (Fig. 3b). PaE activity and secretion in the culture supernatant were recovered in ΔPaEMT1 harboring pUXV1.neo-PaEMT1 (Fig. 3b, c) in addition to MEL production. These results strongly support MEL biosynthesis being necessary for PaE secretion or production in strain GB-4(0).

The effects of surfactant addition on PaE production in strain ΔPaEMT1
We then investigated the effects of 0.01% MELs and various synthetic surfactants on PaE production in ΔPaEMT1. While SDS and C12Na affected cell growth, the other additions (MELs, Tween20, BRIJ35, TritonX-100, C18Na) generally maintained normal growth of ΔPaEMT1 (Fig. 4a). The PaE activities in the cultures supplemented with Tween20, BRIJ35 and TritonX-100 were markedly elevated compared to the control (Fig. 4b). A signal corresponding to PaE was detected in all cultures supplemented with Tween20, BRIJ35 and TritonX-100 in SDS-PAGE and western blotting analyses (Fig. 4c). However, PaE activity and the PaE signal did not recover with 0.01% MEL addition, even though MEL is the native surfactant of strain GB-4(0).

The effect of varying the concentration of MELs was tested. A higher concentration of MELs did not affect cell growth (Fig. 5a). Recovery of PaE secretion was observed in the presence of 0.1 and 0.5% MELs based on PaE activity and PaE detection through western blotting (Fig. 5a, b). Based on this result, a higher concentration of MEL was required to recover PaE productivity compared to other surfactants.

Relative expression level of PaCLE1 in ΔPaEMT1 strain
Although the protein secretion/degradation pathway is unknown in P. antarctica, we analyzed the expression of PaCLE1, encoding PaE, through qRT-PCR to determine whether PaE production deficiency depends on transcriptional regulation or an unknown protein degradation pathway. The expression level of the gene was the
same in ∆PaEMT1 (2.26 ± 0.22), wild-type (2.12 ± 0.25) and ∆PaEMT1 grown in the medium supplemented with 0.01% TritonX-100 (2.10 ± 0.51). This result indicates that PaCLE1 is transcribed normally in ∆PaEMT1, but that the gene product secretion system was defective.

Detection of PaE in cell-free extract
As mentioned above, PaE was not secreted into the culture supernatant of ∆PaEMT1, although the PaCLE1 gene was expressed at the same level as in the wild-type. From these results, we speculated that PaE secretion failed due to the loss of MEL production.

To test this hypothesis, cell-free extract was prepared and analysis of intracellular PaE was carried out using SDS-PAGE and western blotting. The detailed preparation method for cell-free extract is summarized in Additional file 1: Figure S2. According to Fig. 6, PaE was detected only in the culture supernatant of strain GB-4(0) (Fig. 6b, Lane 1), and showed no signals in the soluble or insoluble fractions of either strain GB-4(0) (Fig. 6b, Lane 2 and 3) or ∆PaEMT1 (Fig. 6b, Lane 7 and 8). Additionally, precipitate of the insoluble fraction was washed using 0.01% TritonX-100 for 1 h at 30 °C (Additional file 1: Figure S2). Because PaE
productivity recovered with the addition of 0.01% TritonX-100 (Fig. 4), we expected that PaE would be eluted after 0.01% TritonX-100 treatment if PaE was insolubilized. However, no signal was present in the fraction obtained after TritonX-100 treatment (Fig. 6b, Lane 9 and 10). These results suggest that the gene PaCLE1 was not translated to PaE or that expressed PaE was degraded immediately in ∆PaEMT1.
Fig. 4  PaE production by strain ΔPaEMT1 in m-3×FMM medium supplemented with 8% xylose and 0.01% surfactants.  

a  Cell growth. b  PaE activity.  

SDS-PAGE and PaE western blot analysis of culture supernatant. M, marker; P, purified PaE; Lane 1, control; Lane 2, MEL; Lane 3, Tween20; Lane 4, BRIJ35; Lane 5, TritonX-100; Lane 6, SDS; Lane 7, sodium dodecanoate (C12Na); Lane 8, sodium octadecanoate (C18Na). The amount of each culture supernatant loaded in the gel was 10 μL for CBB staining and western blotting. The results of the cell growth and PaE activity assays are shown as the average of three different experiments. Error bars show standard deviations.

Fig. 5  PaE production by strain ΔPaEMT1 in m-3×FMM medium supplemented with 8% xylose and various concentrations of MEL.  

a  Cell growth (white) and PaE activity (gray). b  SDS-PAGE and western blot analysis of culture supernatant. M, marker; P, purified PaE. The amount of each culture supernatant loaded in the gel was 10 μL for CBB staining and western blotting. The results of the cell growth and PaE activity assays are shown as the average of three different experiments. Error bars show standard deviations.
Discussion

In this study, we found that the biosynthesis of extracellular glycolipids known as MELs contributes to production of an extracellular esterase, PaE, in *P. antarctica* GB-4(0). Furthermore, we demonstrated that addition of various surfactants including MEL complement this deficiency; thus, MEL biosynthesis is required for PaE production in strain GB-4(0).

A gene cluster consisting of five genes responsible for MEL biosynthesis has been reported in *U. maydis* UM521 and *P. antarctica* T-34, *P. antarctica* JCM10317T, *P. aphidis* DSM70725 and *P. tsukubaensis* NBRC1940 (Hewald et al. 2006; Lorenz et al. 2014; Morita et al. 2013a; Saika et al. 2014, 2016). Of these genes, a gene encoding glycosyltransferase (corresponding to Emt1 of *U. maydis* and PaEmt1p of *P. antarctica*) is essential for
MEL production in *U. maydis* UM521 and *P. antarctica* T-34, as reported previously (Hewald et al. 2005; Morita et al. 2010). PaEMT1 from strain GB-4(0) shares high amino acid sequence identity with *U. maydis* UM521, *P. antarctica* T-34 and *P. antarctica* JCM10317; 75, 100 and 95%, respectively. The PaEMT1-deletion strain exhibits MEL biosynthesis deficiency, and the phenotype was complemented by transformation with PaEMT1. Thus, PaEMT1 is essential for MEL production in GB-4(0), in accordance with results from strain T-34 (Morita et al. 2010).

PaE activity recovered with the addition of synthetized 0.01% Tween20, BRIJ35, or TritonX-100, while the other surfactants, including 0.01% MEL, did not lead to such activity (Fig. 4). The critical micelle concentration (CMC) of each surfactant was as follows: MEL: 0.0027 mM (1.8 × 10⁻⁴%), Tween20: 0.06 mM (0.0074%), BRIJ35: 0.09 mM (0.011%), TritonX-100: 0.24 mM (0.015%), SDS: 8 mM (0.23%), C12Na: 27.5 mM (0.61%), and C18Na: 0.5 mM (0.015%). A concentration of 0.01% is lower than the CMC for BRIJ35, Triton-X-100, SDS, C12Na and C18Na; therefore, CMC is likely unrelated to the recovery of PaE production. In addition, we estimated the effect of the initial content of Triton-X-100 on PaE production with addition of 0, 0.2, 0.5, 1, and 5 CMC. As shown in Additional file 1: Figure S3, recovery of PaE production was observed at 0.2–5 CMC, supporting the hypothesis described above. SDS, C12Na and C18Na, which did not have an effect on recovery of PaE, are categorized as anionic surfactants, while MEL, Tween20, BRIJ35 and TritonX-100 are categorized as non-ionic surfactant. From this result, the type of surfactant might affect the ability to recover PaE production. According to Fig. 5, a higher concentration of MEL was needed to recover PaE production. *P. antarctica* is known to produce large amounts of MEL (over 14% in culture medium) (Kitamoto et al. 2001), which could be related to the requirement for a high concentration of MEL, but further investigation is needed to understand the mechanism of PaE secretion.

Fukuoka et al. (2016) showed that PaE activity decreased with the addition of MELs at final concentrations of 5 and 50 mg/L (0.0005 and 0.005%, respectively). While the MEL concentrations shown in Fig. 5 were 1000 times higher than those in previous reports, PaE activities were not inhibited. The culture supernatant used for the PaE activity assay shown in Fig. 5 was analyzed through TLC (Additional file 1: Figure S4). According to the results, the added MEL was not detected in the supernatant of every culture (Additional file 1: Figure S4A). On the other hand, added MEL was detected in the precipitate that included cells, but the amount of MEL was lower than the MEL standard (Additional file 1: Figure S4B). Based on these results, addition of MEL did not inhibit PaE activity because the MEL was precipitated during centrifugation, and some of the added MEL may have degraded during cultivation.

As the PaCLE1 expression level of strain ∆PaEMT1 was the same as that of the parent strain, recovery of deficient PaE production with surfactants may be linked to post-translational events, such as protein trafficking and degradation pathways. Because these amphiphilic compounds will interact with the cell membrane, various intracellular pathways related to cell integrity, e.g., biosynthesis of components of the cell membrane and cell wall and the protein kinase C pathway, respond to treatment with detergents. Moreover, the absence of MELs may induce deficiency of protein trafficking in transport vesicles, because the composition of intracellular membranes affects their function (Phillips et al. 2009; Alexander et al. 2011; Iwamoto and Oiki 2013). After incorporation into the intracellular membrane via endocytosis, the amphiphilic compounds may complement the absence of MELs in the intracellular membrane, although the function of MELs in protein sorting is unknown. Further study of the molecular cell biology of *P. antarctica*, including protein trafficking, degradation, secretion, and the localization and transportation pathways of MELs, will clarify the mechanisms of PaE production.

Additional file

**Additional file 1:** Figure S1. PCR analysis of the parental strain GB-4(0) and strain ∆PaEMT1. The primer sets used in PCR amplification to assess PaEMT1 deletion (A). Agarose gel electrophoresis of amplified DNA fragments to confirm gene disruption. The gene fragments were amplified using PaEMT1_inner_F1 (Primer A) and PaEMT1_inner_R1 (Primer B), or PaEMT1_up_F2 (Primer C) and NATfragment_R1 (Primer D) (B). Figure S2. Schematic diagram of the procedure used to obtain cell-free extract. Figure S3. PaE production by strain ∆PaEMT1 in m-3×FMM medium supplemented with 8% xylose and various concentrations of TritonX-100. Cell growth (white) and PaE activity (gray) (A) and SDS-PAGE and western blot analyses of culture supernatant (B). CMC, critical micelle concentration; M, marker; P, purified PaE. The amount of each culture supernatant loaded in the gel was 10 µl for CBB staining and western blotting. The results of the cell growth and PaE activity assays are shown as the average of three different experiments. Error bars show standard deviations. Figure S4. TLC analysis of culture supernatants used for the PaE activity assay. Culture supernatants (A) and precipitates including cells (B).

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Authors’ contributions
AS designed the study, carried out most of the biological studies and drafted the manuscript. HK (H. Koike) carried out gene analysis and evaluated and discussed the results. TY and TW helped in the generation of ∆PaEMT1 and evaluated and discussed the results. HK (H. Kitamoto) and TM conceived and designed the study, and drafted the manuscript. All authors read and approved the final manuscript.
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Ethics approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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