Metabolomic Evaluation of the Central Metabolic Pathways of Mannosylerythritol Lipid Biosynthesis in *Moesziomyces antarcticus* T-34

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Abstract: *Moesziomyces antarcticus* is a basidiomycetous yeast that produces mannosylerythritol lipids (MELs), which have potential applications as bio-based functional materials in various oleochemical industries, the cosmetics, toiletry, agriculture, and pharmaceutical industries. To better understand the MEL producer, we characterized the central metabolic pathways of *M. antarcticus* strain T-34 grown on glucose or olive oil via metabolomics. The relative fatty acid content was higher in the cells cultured in olive oil compared to glucose, while the acetyl-CoA content was lower in cells cultured in olive oil. The levels of the tricarboxylic acid cycle metabolites citrate/isocitrate, α-ketoglutarate, and succinate were lower in olive oil compared to glucose, while fumarate and malate levels exhibited the opposite pattern. Pyruvate was not detected in olive oil compared to glucose culture. The levels of glycerol, as well as trehalose, myo-inositol, threitol/erythritol, and mannitol/sorbitol, were higher in olive oil compared to glucose cultures. The ATP level was lower in olive oil compared to glucose culture, although the assimilation of fatty acids produced by digestion of olive oil should promote large amounts of ATP production. The possibility that ATP regeneration by respiratory chain complex promote oil utilization and MEL production in *M. antarcticus* T-34 was found based on the results of this metabolomic analysis.

Key words: *Moesziomyces antarcticus*, mannosylerythritol lipids, metabolomics, central metabolic pathway

1 Introduction

*Moesziomyces antarcticus* T-34 (renamed from *Pseudozyma antarctica* T-34) produces large quantities of glycolipid-type biosurfactants, mannosylerythritol lipids (MELs), when vegetable oil is used as its carbon source. MELs are composed of hydrophilic 4-O-β-D-mannopyranosylmeso-erythritol and hydrophobic fatty acids. MELs exhibit high productivity and excellent surface activity, among other unique properties including self-assembly, anti-tumor activity, promotion of cell differentiation, and moisturizing and hair-repairing properties. Studies aimed at improving the efficiency of MEL production, as well as biological, nanotechnological and environmental applications of MELs, have been conducted.

The production quantity of MEL by *M. antarcticus* T-34 reached to 40 g/L when soybean oil is used as the carbon source. *P. aphidis* is another efficient producer of MELs, with yields of 165 g/L when vegetable oil is used as the carbon source in a fed-batch culture system. Furthermore, genetically modified *P. tsukubaensis* expressing an extracellular lipase can produce 23.3 g/L of a MEL-B diastereomer, corresponding to a production yield of 0.29 g/g substrate. MEL-producing fungi are currently being developed using genetic engineering techniques; such genetically modified strains show great potential for large-scale industrial production of MELs.

Recently, we performed a transcriptomic analysis of *M. antarcticus* T-34, which highlighted key feature of the central metabolic pathways. We compared the transcriptomes of *M. antarcticus* T-34 grown with glucose versus soybean oil as the carbon source, and revealed that the central metabolism of *M. antarcticus* T-34 contributes

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to its excellent oil utilization and MEL production. The genes encoding enzymes in the tricarboxylic acid (TCA) cycle (citrate synthase, aconitase, succinate dehydrogenase, fumarase, and malate dehydrogenase), and those involved in glyoxylate shunt (isocitrate lyase and malate synthase) and anaplerotic reactions (malic enzyme and phosphoenolpyruvate carboxykinase), were highly expressed in *M. antarcticus* T-34 when cultured in soybean oil. However, the information of gene functions and expression levels is insufficient to explain the influence of genotype and environment change on biological phenotype through the intracellular crosstalk between different molecular layers.

In this study, we focused on the central metabolic pathway and evaluated the levels of metabolites in *M. antarcticus* T-34 grown in olive oil or glucose using metabolomics which is a powerful technique for the analysis of phenotype. Through the measurement of intermediates in the metabolic reactions, the metabolic state during MEL production was clarified. Furthermore, by integrating the results of the metabolomic analysis with the results of previously reported transcriptomic analysis, it was possible to interpret a combination of genotype and phenotype. These results improve our understanding of the molecular mechanisms underlying oil utilization and MEL production in basidiomycetous yeast.

### 2 Materials and Methods

#### 2.1 Culture conditions

*M. antarcticus* T-34 was pre-cultured in yeast mold medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L glucose) for 48 h at 25°C with 250 rpm agitation. Pre-cultures were washed twice with 9 g/L sodium phosphate, 3 g/L yeast extract, 3 g/L sodium nitrate, 0.3 g/L monopotassium phosphate, and 0.3 g/L magnesium sulfate, to an initial optical density at 600 nm of 0.5. Glucose or olive oil was added as a sole carbon source. Cells grown in olive oil were washed once with hexane to remove oily compounds before the OD_{600} measurements. For MEL production, cells were cultivated in 500-mL Erlenmeyer flasks (50 mL volume) for 96 h at 25°C, with 250 rpm agitation. Dry cell weight (DCW) was measured after filtering 10 mL of culture, washing cells with deionized water, and then drying them at 60°C for 5 days; a standard curve was generated to calculate the conversion coefficient used to interpolate DCW values from OD_{600}.

#### 2.2 Measurement of extracellular metabolites

Culture broth was collected every 24 h and the concentrations of extracellular metabolites (glucose, olive oil, and MELs) were measured. The glucose concentration of the culture broth supernatant was measured by high-performance liquid chromatography (HPLC), as described previously. MELs and residual olive oil were extracted and measured as outlined in a previous study.

#### 2.3 Collection of intracellular metabolites

First, 5 mL of culture was filtrated at 72 h after cultivation using 1.0 μm pore size Omnipore filter disks (Merck KGaA, Darmstadt, Germany). Harvested cells were immediately (within 30 s) immersed in pre-chilled methanol containing 50 μM (+)-10-camphorsulfonate and 50 μM ribitol to quench metabolic activity. The mixture was stored in −80°C until extraction. Intracellular metabolites were extracted from the cell mixture using chloroform and Milli-Q water. Supernatants containing intracellular metabolites were concentrated using a CC-105 centrifugal concentrator (Tony, Tokyo, Japan).

#### 2.4 Gas chromatography/mass spectrometry (GC/MS) measurement of intracellular metabolites

The dried samples were resuspended in 40 g/L methoxyamine dissolved in pyridine, and shaken at 30°C for 1.5 h. Then, N-methyl-N-trimethylsilyl trifluoroacetamide with 1% trimethylchlorosilane was added to the mixture and shaken at 37°C for 0.5 h. The resulting mixture was analyzed by a GC-MS using 7890B gas instrument coupled to a 5977B mass spectrometer (Agilent Technology, Santa Clara, CA, USA), under previously described analytical conditions. The data were analyzed for statistical significance by Student’s *t*-test. Differences were assessed by the two-sided test with *α* = 0.05.

#### 2.5 Liquid chromatography/mass spectrometry (LC/MS) measurement of intracellular metabolites

The dried samples were resuspended in Milli-Q water and analyzed with a Dionex Ultimate 3000 HPLC machine coupled to an Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a PE capped CERI L-column 2 ODS (150 mm × 2.1 mm, 2.5 μm particle size; Chemicals Evaluation and Research Institute, Tokyo, Japan). The operating conditions were as previously described. The data analysis method was the same as for GC-MS.

### 3 Results

#### 3.1 MEL production profiles of *M. antarcticus* T-34

First, we characterized the MEL production profile of *M. antarcticus* T-34 grown using olive oil as the carbon source. As a control, *M. antarcticus* T-34 was grown in glucose, the most commonly used carbon source. *M. ant-
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M. antarcticus T-34 was cultured for 96 h with either glucose or olive oil as the sole carbon source (Fig. 1). The DCW conversion coefficient was 0.3 gDCW/L·OD_{600} for M. antarcticus T-34 grown in both conditions. MEL production was the highest after 96 h cultivation; 27.8 ± 3.8 g/L of MELs were produced from 60.3 ± 2.0 g/L of olive oil (initial oil concentration: 73.6 ± 1.8 g/L), for a MEL production yield of 0.46 ± 0.06 g/g substrate. These results are similar to previous findings. The amount of biomass was reached to 29.1 ± 1.2 gDCW/L, indicating that 94% of the olive oil consumed was converted into biomass and MELs. In the glucose condition, 7.5 ± 1.2 g/L of MELs were obtained from 47.8 ± 2.1 g/L of glucose (initial glucose concentration: 73.3 ± 1.8 g/L), corresponding to a MEL production yield 0.16 ± 0.02 g/g substrate. The amount of biomass was reached to 20.1 ± 3.4 gDCW/L, indicating that 59% of the glucose consumed was converted into biomass and MELs.

The metabolic profiles of M. antarcticus T-34 under both carbon sources are summarized in Table 1. Since continuous substrate consumption and MEL production were observed in 48–96 h cultivation under both carbon source conditions, cells were harvested after 72 h cultivation for metabolomic analysis.

### 3.2 Metabolomic analysis of M. antarcticus T-34

The central metabolic pathway metabolites of M. antarcticus T-34 cells cultivated with olive oil for 72 h were analyzed by GC-MS and LC-MS, using cells grown in glucose as a control.

#### 3.2.1 Substrate-related metabolites and energies

M. antarcticus T-34 digested the olive oil using an endogenous lipase to produce fatty acid and glycerol, which were used as carbon sources. The relative amounts of palmitate, oleate, linoleate, and stearate were 11-, 82-, 6-, and 4-fold higher in olive oil compared to glucose culture, respectively (Fig. 2A). Glycerol was only detected in olive oil, indicating that olive oil was assimilated by M. antarcticus T-34 cells in the form of fatty acids and glycerol following lipase digestion. The relative glucose concentration was 229-fold in glucose than olive oil. Ingested olive oil and glucose are converted to the key compound, acetyl-CoA, through β-oxidation pathway and glycolysis, respectively. The level of acetyl-CoA was 95% lower in olive oil compared to glucose (Fig. 2A). Central metabolic pathways are used to convert ingested carbon sources not only for MEL production, but also for energy production to maintain the cellular activities. ATP, ADP, and AMP, which are used as intracellular common currencies, were observed (Fig. 2B). The levels of ATP and ADP, which are energy-carrying molecules, were 45% and 93% lower in olive oil compared to glucose, respectively, while the AMP levels were 4-fold higher in olive oil compared to glucose.

#### 3.2.2 TCA cycle and anaplerotic reactions

As the metabolites in TCA cycle, citrate/isocitrate, α-ketoglutarate, succinate, fumarate, malate were observed, notaconitate, succinyl-CoA, oxaloacetate, and gly-

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**Fig. 1** Culture profiles of M. antarcticus T-34 under (A) glucose and (B) olive oil culture conditions. Symbols: circle, biomass; square, substrates (glucose or olive oil); diamonds, mannosylerythritol lipids (MELs); arrow, sampling point for metabolomic analysis. Data are expressed as mean ± standard deviation (N = 3).

**Table 1** Summary of culture profiles.

| Conditions | Substrates | MELs | Consumed substrates | Biomass | MELs |
|------------|------------|------|---------------------|---------|------|
| Glucose    | Substrates | MELs | 30.6 ± 1.7          | 6.2 ± 1.4| 36.2 ± 1.3 | 15.9 ± 0.1 | 4.6 ± 0.3 |
| Olive oil  | Substrates | MELs | 20.1 ± 3.4          | 12.5 ± 3.5| 48.1 ± 6.8 | 19.9 ± 0.5 | 24.9 ± 5.5 |

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oxylate (Fig. 2C). The level of citrate/isocitrate, α-ketoglutarate, and succinate, which are metabolites in a former step of TCA cycle, were 78%, 85%, and 58% lower in olive oil compared to glucose, respectively. On the other hand, the levels of fumarate and malate, which are metabolites in a latter step of TCA cycle, were both 1.1-fold higher in olive oil than glucose. Malate and oxaloacetate are converted to pyruvate and phosphoenolpyruvate, respectively, by anaplerotic reaction (malic enzyme: PANT_7c00096, malate + NAD[P]⁺ → pyruvate + CO₂ + NAD[P]H; phosphoenolpyruvate carboxykinase: PANT_2d00021, oxaloacetate + ATP → phosphoenolpyruvate + CO₂ + ADP) to supply sugar intermediates and their phosphates. Unfortunately, oxaloacetate and phosphoenolpyruvate were not detected and pyruvate was observed in glucose only, while 3-phosphoglycerate, which is synthesized from phosphoenolpyruvate through 2-phosphoglycerate (enolase: PANT_14d00015, phosphoenolpyruvate → 2-phosphoglycerate; phosphoglycerate mutase: PANT_15d00053, 2-phosphoglycerate → 3-phosphoglycerate), was observed at 1.8-fold higher level in olive oil than in glucose (Fig. 2C).

3.2.3 Sugar synthesis

In olive oil, sugar intermediates required for growth and MEL production are supplied from 3-phosphoglycerate via glycolysis/gluconeogenesis. The levels of detected metabolites were shown in Fig. 2D. Glucose 6-phosphate/fructose 6-phosphate, fructose 1,6-bisphosphate, and 6-phosphogluconate, which are intermediates in glycolysis/gluconeogenesis and pentose phosphate pathway, were 32%, 17%, and 13% lower in olive oil compared to glucose, respectively. Regarding the precursors of the sugar backbone of MEL, mannitol/sorbitol was 1.4-fold higher in olive oil compared to glucose, while threitol/erythritol was 28% lower in olive oil compared to glucose. Regarding the by-products during MEL production, myo-inositol and trehalose were 3.0- and 1.8-fold higher in olive oil than glucose, respectively.

4 Discussion

In present study, we performed metabolomic analysis focused on central metabolic pathways of M. antarcticus T-34 in glucose or olive oil as carbon source. As results of metabolomic analysis using GC-MS and LC-MS, 24 metabolites were observed (Fig. 2). Fatty acids and glycerol, which are able to supply directly by digestion of olive oil, were higher in olive oil than that in glucose. Metabolites in the former TCA cycle were lower in olive oil than in glucose,
while metabolites in the latter TCA cycle exhibited the opposite pattern. Sugar intermediates in glycolysis/glucogenesis and pentose phosphate pathway were lower in olive oil than in glucose, while by-product and the precursor of MEL component exhibited the opposite pattern. In order to analyze metabolic state during MEL production in more detail, the results of the metabolomic analysis in the present study were integrated with the previous transcriptomic analysis. The M- and A-value described below were defined in previous study as the logarithmic induction ratio and the average of the logarithmic signal intensities, respectively.

Olive oil was digested into fatty acids and glycerol by the reaction of lipase secreted by M. antarcticus T-34 (lipase: PANT2c00005, triacylglycerol + 3 H2O→3 fatty acids + glycerol), and then these were assimilated into the cells. Since the main component of fatty acids contained in olive oil is oleate, its abundance ratio was much higher than other kind of fatty acids. The amount of oleate was 82-fold higher in olive oil than in glucose. Besides, the ATP level was 45% lower in olive oil than in glucose, although the assimilation of fatty acids produced by digestion of olive oil should promote large amounts of ATP production. These results suggest that assimilation of fatty acids by β-oxidation pathway is rate-limiting step. However, carnitine acyltransferase (PANT9c00423), the generally known as rate-limiting enzyme in β-oxidation pathway, was highly expressed under vegetable oil conditions (M-value = 0.71, A-value = 14.06). Therefore, it is considered that there are other rate-limiting reactions in β-oxidation pathway.

In general, assimilated fatty acids are converted acyl-AMP (acyl-CoA synthetase: PANT11c00027, fatty acid + ATP→acyl-AMP + pyrophosphate), and then replaces AMP with CoA, resulting acyl-CoA. Besides, the AMP level was 4.0-fold higher in olive oil than in glucose and our previous transcriptomic analysis revealed that acyl-CoA synthetase was highly expressed under vegetable oil conditions (M-value = 0.71, A-value = 14.06). Therefore, it is suggested that the ATP regeneration may be rate-limiting step. ATP was regenerated by respiratory chain complex involving NADH-ubiquinone reductase forming proton motive force via NADH oxidation (PANT8c000413, M-value = 0.00, A-value = 14.81) and ATP synthase forming ATP using proton motive force (PANT14c00109, M-value = 0.05, A-value = 15.89). These are similar under the both glucose and vegetable oil conditions. Consequently, ATP regeneration may be triggered to promote oil utilization and MEL production in M. antarcticus T-34, although further studies are required to elucidate the molecular mechanisms underlying this phenomenon.

Acetyl-CoA is generated from fatty acids via β-oxidation pathway in case of olive oil culture condition and from glucose via glycolysis in case of glucose culture condition. The level of acetyl-CoA was 95% lower in olive oil than in glucose. Our previous transcriptomic analysis revealed that citrate synthase expression (PANT22c00009, acetyl-CoA + oxaloacetate→citrate, M-value = 0.23, A-value = 15.68) remained relatively high under both glucose and olive oil culture conditions, indicating that acetyl-CoA was equivalently converted to citrate under both carbon sources. Together, it is suggested that acetyl-CoA concentration is dependent on flux through pathways supplying acetyl-CoA from glucose or olive oil. Moreover, the level of metabolites in a former step of TCA cycle (citrate/isocitrate, α-ketoglutarate, and succinate) were lower in olive oil than in glucose, while level of metabolites in a latter step of TCA cycle (fumarate and malate) exhibited opposite pattern. Previous transcriptomic analysis revealed that the A-value of genes related to glyoxylate shunt (isocitrate lyase: PANT15d00022, isocitrate→succinate + glyoxylate, A-value = 15.78; malate synthase: PANT14d00059, glyoxylate + acetyl-CoA→malate, A-value = 15.00) were higher than those of genes related to the TCA cycle (isocitrate dehydrogenase: PANT12d00048, isocitrate + NAD [P] →α-ketoglutarate + NAD[P]H + CO2, A-value = 14.80; α-ketoglutarate dehydrogenase: PANT11c00079, α-ketoglutarate + NAD2 + CoA→succinyl-CoA + NADH + CO2, A-value = 14.27), which compete for utilization of isocitrate as common substrate. Together, these results of metabolomic and transcriptomic analysis suggest that fumarate and malate were generated as a result of the TCA cycle being bypassed by activation of glyoxylate shunt in M. antarcticus T-34 under olive oil culture conditions.

Metabolites in TCA cycle were converted to sugar intermediates via anaplerotic reactions (malic enzyme: PANT7c00006, malate + NAD[P] →pyruvate + NAD[P]H + CO2, M-value = 0.43, A-value = 14.80; phosphoenolpyruvate carboxykinase: PANT24d00021, oxaloacetate + ATP→phosphoenolpyruvate + ADP + CO2, A-value = 1.06, A-value = 15.34). Although both genes highly expressed in olive oil conditions, the reaction by malic enzyme should not supply sugar intermediates because no phosphoenolpyruvate synthase (pyruvate + ATP→phosphoenolpyruvate + AMP + pyrophosphate) is annotated in M. antarcticus T-34 genome. Therefore, mainly used anaplerotic reaction must be phosphoenolpyruvate carboxykinase. On the evidence to the activity of anaplerotic reaction, 3-phosphoglycerate, intermediate in glycolysis/glucogenesis, was 1.8-fold higher in olive oil than in glucose. Moreover, M. antarcticus T-34 in olive oil produced by-products as trehalose for 1.8-fold in glucose during MEL production. The intracellular trehalose level in M. antarcticus T-34 was estimated to be 50 mg/gDCW based on that in model yeast Saccharomyces cerevisiae. Since the amount of biomass generated in olive oil at 96 h cultivation was 29.1 gDCW/L, the total trehalose was equivalent to 1.5 g/L, indicating it was quite low compared...
to MEL production and biomass. Moreover, other sugar intermediates such as mannitol/sorbitol and myo-inositol, was also 1.4- and 3.0-fold higher in olive oil than in glucose. These phenomena may be involved with the assimilation of glycerol produced by digestion of olive oil; however, this requires further verification. Together, these findings suggest that the sugar intermediates are synthesized efficiently from olive oil via central metabolic pathway including β-oxidation pathway, TCA cycle, glyoxylate shunt, anaplerotic reaction, glycolysis/gluconeogenesis, and pentose-phosphate pathway.

5 Conclusion

We performed a metabolomic analysis to explore the central metabolic pathways in *M. antarctica* T-34 cultured in olive oil. Previously, we identified the most important metabolic pathways involved in oil assimilation and MEL biosynthesis in *M. antarctica* T-34 via transcriptomics; to complement this, we conducted the present study to analyze intracellular metabolism in more detail. This metabolomic analysis provided insight into the various metabolites in central metabolic pathways and energy generation mechanisms during MEL biosynthesis. Combining -omics data, such as transcriptomics and metabolomics, will facilitate the development of genetically modified strains for more efficient MEL production and expand our knowledge regarding the characteristics of oleaginous yeast strains.

Author Contribution

Conceptualization; KW, TM. Methodology; KW, AS. Validation; KW. Formal analysis; KW, AS. Investigation; KW. Resource; KW. Data curation; KW. Original draft preparation; KW, AS, TM. Review and editing; KW, AS, SU, SS, TF, TM. Visualization; KW. Supervision; TM.

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Conflict of Interest

The authors declare there are no conflicts of interest.

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