Engineering *Ustilago maydis* for production of tailor-made mannosylerythritol lipids

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**ABSTRACT**

Mannosylerythritol lipids (MELs) are surface active glycolipids secreted by various fungi. MELs can be used as biosurfactants and are a biodegradable resource for the production of detergents or pharmaceuticals. Different fungal species synthesize a unique mixture of MELs differing in acetyl- and acyl-groups attached to the sugar moiety. Here, we report the construction of a toolbox for production of glycolipids with predictable fatty acid side chains in the basidiomycete *Ustilago maydis*. Genes coding for acyl-transferases involved in MEL production (Mac1 and Mac2) from different fungal species were combined to obtain altered MEL variants with distinct physical properties and altered antimicrobial activity. We also demonstrate that a *U. maydis* paralog of the acyltransferase Mac2 with a different substrate specificity can be employed for the biosynthesis of modified MEL variants. In summary, our data showcase how the fungal repertoire of Mac enzymes can be used to engineer tailor-made MELs according to specific biotechnological or pharmaceutical requirements.

1. Introduction

Many basidiomycetous fungi produce Mannosylerythritol lipids (MELs) (Fig. 1A; Arutchelvi et al., 2008). The biosynthetic pathway has been characterized in *Ustilago maydis* (Hewald et al., 2005, 2006). Enzymes for MEL production are encoded in a gene cluster and include the glycosyl-transferase Emt1, the acetyl-transferase Mat1 and the two acyl-transferases Mac1 and Mac2. Acyl-transferases are required to decorate the carbohydrate backbone with acyl groups of different length. In *U. maydis*, Mac1 attaches the short side chain at position C2, while Mac2 catalyzes the transfer of the long side chain to the carbohydrate moiety at position C3 (Fig. 1A; Deinzer et al., 2019). Acylation of MELs is coupled to β-oxidation and occurs inside peroxisomes (Freitag et al., 2014; Kitamoto et al., 1998). Mat1 catalyzes acetylation at two residues of the carbohydrate backbone leading to the production of four variants termed MEL-A, -B, -C and -D that differ in their degree of acetylation and as a consequence in their hydrophobicity (Hewald et al., 2006; Kitamoto et al., 1990).

While the natural function of MELs is yet elusive, their potential for biotechnology, cosmetics and pharmaceutical industry is well documented (Arutchelvi et al., 2008; Bölker et al., 2008; Coelho et al., 2020; Morita et al., 2013a, 2015). MELs exhibit antimicrobial and antioxidant activity, can aid gene delivery into mammalian cells and have been suggested to counteract hair damage or help to protect the skin (Kitamoto et al., 2002; Morita et al., 2013a; Onwosi et al., 2020; Takahashi et al., 2012). One major challenge for industrial use of MELs is the customized biosynthesis of desired MEL molecules (Saika et al., 2018). The production of MEL species can be modified by feeding different carbon sources or by changing the environmental conditions but the produced spectrum of MELs is mostly species specific (Beck et al., 2019; Deinzer et al., 2019; Morita et al., 2013b; Saika et al., 2018).

We recently demonstrated functional complementation of *U. maydis* mac1 and mac2 genes by orthologs from *Ustilagohordei*, which resulted in a changed acylation pattern of MELs (Deinzer et al., 2019). *U. maydis* is an attractive model-system, for which multiple genetic tools have been established (Kämper et al., 2006; Schuster et al., 2016). The fungus has been successfully used as a platform for unconventional protein secretion and is a producer of the bioplastic precursor itaconic acid (Geiser et al., 2016a, 2016b; Stock et al., 2012; Terfrüchte et al., 2017). Here, we describe a system for production of customized and predictable MELs.

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species with novel properties.

2. Materials and methods

2.1. Strains and growth conditions

Fungal strains were grown in liquid YEPSL (1% yeast extract, 0.4% peptone, 0.4% sucrose) or on solid potato dextrose broth containing 1.5% Bacto agar at 28°C and 23°C, respectively. The U. maydis strains used and generated in this study are listed in Supplementary Table S1 and are derivatives of the U. maydis strain MB215 (Hewald et al., 2005). U. hordei (Uh4857-4) and Sporisorium scitamineum (RK109) were kind gifts from Regine Kahmann (MPI Marburg, Germany). Moesziomyces aphidis (formerly Pseudozyma aphidis) (DSM70725) was received from Michael Günther (Fraunhofer Institute Stuttgart, Germany). To induce glycolipid production U. maydis strains were grown to stationary phase in YEPSL and then transferred to nitrogen starvation medium (OD = 0.1) containing 0.17% YNB (yeast nitrogen base without ammonia) and 1% glucose as carbon source. Glycolipid production was then allowed for 6 days at 23°C under rotation. 

2.2. Molecular cloning and nucleic acid procedures

Standard procedures were followed for generation of pTB1 and its derivatives (Sambrook et al., 1989). Plasmids are based on the plasmid petGFP-Ala6-MMXN (Böhmer et al., 2008; Spellig et al., 1996). Genomic DNAs of U. maydis, U. hordei, S. scitamineum and M. aphidis cells were prepared according to an established protocol (Hoffman and Winston, 1987). Primer sequences are listed in Supplementary Table S2 and plasmids are listed in Supplementary Table S3. Plasmids were verified by sequencing.

2.3. Strain construction

Transformation of U. maydis was conducted as described (Schulz et al., 1990). Strain MB215 Δmac1 Δmac2 Δmat1 was created by the deletion of the mat1 gene in the previously described MB215 Δmac1 Δmac2 strain (Freitag et al., 2014) with help of the CRISPR/Cas9 system (Schuster et al., 2016; Wege et al., manuscript in preparation). Briefly, mat1 (UMAG_03114) was deleted using 100 ng pCas9-sgmat1 and 5 pmol of donor-DNA containing 40 nt upstream and 40 nt downstream sequence of the open reading frame of mat1. For transformation plasmids were linearized with SspI. Integration of plasmids into the ip-locus of U. maydis can produce carboxin resistant strains (Broomfield and Hargreaves, 1992). Integration of plasmids was verified by Southern Blot analysis (Sambrook et al., 1989).
2.4. Analysis of glycolipids

Extracellular glycolipids were extracted as previously described (Hewald et al., 2005). Glycolipids (MELs and CLs) were separated by thin-layer chromatography (TLC) on silica plates first with a solvent system consisting of chloroform-methanol-water (65:25:4, v/v/v) for 5 min followed by a second solvent system consisting of chloroform-methanol (9:1, v/v; 2x 18 min) (Kurz et al., 2003). Alternatively, MELs were separated with a solvent system consisting of chloroform-methanol (9:1, v/v) for 3x 18 min. The plates were dried, and sugar containing compounds were visualized by application of a mixture of ethanol: sulphuric acid: p-anisaldehyde (18:1:1, v/v) followed by heating at 150 °C for 2 min (Frautz et al., 1986).

2.5. Mass spectrometry

2.5.1. HPLC

High-performance liquid chromatography (HPLC) separation of the extracted MELs (50 μl) was performed with a 1100-HPLC system (Agilent) equipped with a EC 125/2 Nucleodur 100-3 C8 ec column (Merck-Nagel). The gradient applied at a flow rate of 0.2 ml/min and a column temperature of 45 °C was as follows (buffer A is water with 0.05% formic acid; buffer B is methanol with 0.045% formic acid): linear gradient from 60% buffer B to 95% buffer B within 30 min and then holding 95% buffer B for 10 min.

2.5.2. Electrospray ionization

Online electrospray ionization MS and MSⁿ of the HPLC-separated compounds was done with a Finnigan LTQ-FT Ultra ion cyclotron resonance (FT-ICR) mass spectrometer (Thermo Scientific). Electrospray ionization parameters were adapted to the flow rate and mass range. Accurate masses (accuracy, 2 ppm), allowing the determination of the chemical formulas of the eluting compounds, were obtained by using the FT mass analyzer at a resolution of 100,000. Meanwhile, fragment ions were acquired and analyzed in the LTQ mass analyser. Alternatively, data-dependent fragmentation or fixed m/z fragmentation was used; the latter resulted in better sensitivity. The accurate FT masses in combination with MS² experiments were sufficient to identify the acylation pattern of the compounds. Data were analyzed using the software XcaliburTM.

2.6. Surface tension

Surface tension was determined by measuring the diameter of a liquid droplet spotted on a petri dish. A larger area indicates a higher reduction of surface tension. Water was stained with 0.01% Xylenecyanol. 45 μl water were mixed with 5 μl MEL (2 μg/μl in MetOH). 5 μl were spotted on the lid of a Petri dish and allowed to dry. Pictures were taken with a CoolPix 4500 camera (Nikon). The areas of the circles were measured with ImageJ. Experiments were at least repeated three times with different MEL preparations.

2.7. Hemolysis

Sheep blood agar plates were obtained from MERCK. 4 μl MELs in solution (2 μg/μl) were spotted on plates and incubated at 30 °C for 2 days. Pictures were taken with a CoolPix 4500 camera (Nikon).

2.8. Emulsification

The potential of MEL-D to emulsify oil and fatty acids was analyzed according to the protocol of Fukuoka et al. (2007). Briefly, 4 ml distilled water were mixed thoroughly for 1 min with 25 μl of MEL-D (2 μg/μl Methanol) and either 500 μl corn oil (Tegut, Germany) or octanoic acid (caprylic acid) (Sigma-Aldrich, Germany). The mixture was incubated for 18 h at room temperature and the turbidity was measured photometrically at 620 nm. Methanol was used as control.

2.9. Antibacterial activity

The antibacterial effects of MEL-D variants were measured in growth assays using a micro plate reader (Synergy Mix, BioTek). 100 μl of bacterial cells of OD = 0.1 were grown in LB medium (10 g/l yeast extract; 10 g/l Trypton; 5 g/l NaCl) in the presence of MEL-D. Growth was recorded every 30 min for 13 h.

2.10. Statistical analysis

Superplots and Student’s t-tests were computed using RStudio 1.2.1335 with R 3.6.0 (Lord et al., 2020; Strehlik et al., 2020). Plots are structured as follows: center line, mean; error bars, standard error of the mean; big circles, mean of experiments. P-values were calculated using an unpaired, two-sided Student’s t-test (Table S4).

2.11. Accession numbers

Accession numbers from National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov): U. hordei: Mac1 (UHOR_04874; CCF52716), Mac2 (UHOR_04878; CCF52718). U. maydis: Mat1 (UMAG_03114; XP_011389465), Mac1 (UMAG_03116; XP_011389467), Mac2 (UMAG_10636; XP_011389530), Mac3 (UMAG_01438; XP_011387307). M. aphidis: Mac1 (PaG_03509; ETS61961), Mac2 (PaG_03508; ETS61960). S. scitamineum: Mac1 (SPSC_06354; CDU26160), Mac2 (SPSC_06352; CDU26158).

3. Results

3.1. A Toolbox-vector for MEL biosynthesis

We have previously demonstrated that MEL biosynthesis of an U. maydis Δmac1 Δmac2 double mutant can be successfully restored by genomic integration of mac1 and mac2 open reading frames (ORFs) from U. maydis but also from U. hordei (Deinzer et al., 2019; Freitag et al., 2014). In this study we examined the production of novel MEL species with altered acylation patterns. To this end, we engineered a vector (pTB1) enabling expression of fluorophore-tagged Mac1 and Mac2 proteins under control of promoters induced in the absence of a nitrogen source (GFP and mCherry: Shaner et al., 2004; Tsien, 1998). pTB1 also contains the sdh2 locus of U. maydis, which allows for site-specific integration of the vector into the genome (Broomfield and Hargreaves, 1992) (Figs. 1B and S1A). U. maydis cells transformed with pTB1 showed cytosolic GFP and mCherry fluorescence when grown in medium without nitrogen source (Fig. S1B). To further characterize this test system, we inserted the ORFs of mac1 and mac2 into pTB1 and transformed an U. maydis MB215 strain (Hewald et al., 2005) deleted for mat1, mac1 and mac2 (MB4059). Employing the mat1 mutant (MB3115) simplifies the analysis of chromatographic and mass spectrometric data as it can only produce the non-acylated MEL-D variant (Hewald et al., 2006). Both fusion proteins localized inside of peroxisomes and MEL production from this strain was indistinguishable from the Δmat1 strain (MB3115) thin-layer chromatography (TLC) (Figs. 1C and 1D; Freitag et al., 2014). We further examined MELs by mass-spectrometry. Total ion-count spectra of MELs from MB3115 and the complemented triple mutant (MB4059U.mac1UmMac2) were highly similar and MS² fragmentation analysis revealed comparable patterns of attached acyl groups (Figs. 1E and 1F; Table S5). Together, the data qualify pTB1 as a suitable tool to
study biosynthesis of different MELs.

### 3.2. MEL biosynthesis via acyltransferases Mac1 and Mac2 from different species

We have recently demonstrated that the specific acylation patterns of MELs are determined by acyl-transferases Mac1 and Mac2 (Deinzer et al., 2019). *U. hordei* mac1 and mac2 genes introduced into an *U. maydis* Δmac1 Δmac2 double mutant restored MEL production but the resulting MELs resembled those produced by *U. hordei* (Deinzer et al., 2019). To test whether this is a general principle, we engineered plasmids based on pTB1 containing the mac genes from *U. hordei* or the mac genes from the basidiomycete *M. aphidis* (Fig. 2A; Uh1Uh2 and Ma1Ma2, respectively). We used *M. aphidis* mac1 and mac2 genes as it was recently observed that *M. aphidis* produces MEL species with an acylation pattern quite different from that of *U. hordei* (Beck et al., 2019). While the *U. hordei* MEL-D contains a long (C12-C16) and a short (C2-C6) acyl group, in *M. aphidis* two acyl groups of medium length (C8-C10) are linked to the carbohydrate backbone. MELs produced by strains containing the described constructs were investigated by TLC and mass spectrometry (Figs. 2B-2D). TLC analysis showed that the amount of MELs were comparable in all strains. Total ion count spectra revealed the expected differences in MEL species produced by the different strains (Fig. 2C). This was confirmed by MS² analysis. Acylation patterns of MELs produced by *U. maydis* expressing both Mac1 and Mac2 from either *U. hordei* or *M. aphidis* are highly similar to those produced by the respective donor organism (Fig. 2D and Fig. S2; Table S5). Thus, specificity for fatty acids of a certain length appears to be a property of the Mac enzymes and thus the composition of MELs is predictable if the specificity of acyl-transferases has been investigated.

### 3.3. Generation of novel MEL species

To test this hypothesis, we created strains containing combinations of Mac1 and Mac2 from different species (Uh1Ma2 and Ma1Uh2, respectively; Fig. 2A). We found that MEL-D variants produced from these combinations displayed novel migration patterns in a TLC experiment and also showed altered total ion count spectra (Figs. 2B and 2C) compared to WT, Uh1Uh2 and Ma1Ma2. By MS² measurements we could verify that fatty acid preferences of Mac1 and Mac2 proteins from different organisms remained unchanged when combinations of these enzymes, which do not occur in nature, were expressed in *U. maydis* (Fig. 2D). The Uh1Ma2 expressing strain produced MEL-D molecules of unusually low mass containing relatively short fatty acids attached to C2 and C3 (Figs. 2C and 2D; Table S5). On the contrary, expression of Ma1Uh2 lead to the production of MELs with exceptionally long fatty acid side chains (Fig. 2D). Together, these data suggest that a desired fatty acid composition of MELs can likely be realized by employing Mac enzymes of the required specificity. Moreover, we show that these enzymes can be freely combined without perturbing their functionality or specificity. To ascertain that our results can be generalized, we created a comparable set of plasmids containing Mac1 and Mac2 combinations from *M. aphidis* and *S. scitamineum* (Figs. S2 and S3). Performing the analyses described above, we obtained similar results. The tested combinations resulted in MEL species with distinct acylation patterns reflecting the substrate specificity of tested enzyme combinations (Figs. S2 and S3). This strengthens our idea that Mac enzymes can be freely combined to synthesize MELs with a defined acylation pattern.
3.4. Mac3 as an alternative acyltransferase for MEL production

We have hitherto identified in *U. maydis* a paralog of Mac2 (Fig. S4), whose gene is neither localized within the gene cluster for MEL biosynthesis nor co-regulated with the cluster genes (UMAG_01438; Hewald et al., 2006, 2005; Lanver et al., 2018). We tested the potential activity of this enzyme for MEL biosynthesis by transforming *U. maydis* Δmac2 cells with a construct containing umag_01438 under control of the constitutive oef promoter (Spellig et al., 1996). TLC and MS experiments revealed that expression of UMAG_01438 (from now on: Mac3) restores MEL biosynthesis in a Δmac2 strain. However, acyl groups attached to C3 were exceptionally short (Figs. 3A and 3B; Table S5). To confirm that the substrate specificity remains unchanged if combined with a different Mac1 we inserted Mac3 into pTB1 also containing mac1 from *U. hordei*. Fatty acids linked to C3 were very similar independent of the Mac1 source used in combination with Mac3 (Fig. 3C and 3D). We could identify putative mac3 genes in several other fungi indicating that testing these in *U. maydis* may again enlarge the repertoire of different MELs.

3.5. Characteristics of the novel molecules

MELs have been shown to exhibit a variety of characteristics useful for biotechnological and pharmaceutical purposes. A prominent quality is the reduction of surface tension (Morita et al., 2015). To get an idea of possible changes in their ability to lower surface tension, we spotted MEL solutions derived from engineered strains on petri dishes and measured the diameter of the resulting droplet. All MEL variants tested behaved very similar in these assays (Figs. S5A and S6A), but may well be different in more accurate and quantitative assays for surface tension. We also measured the efficiency of MELs to emulsify octanoic acid and corn oil, respectively, and found that MELs produced by Ma1Uh2 harboring very long side chains were most efficient in the emulsification of octanoic acid (Figs. 4A and 4B and Figs. S7A and S7B). On the contrary, these MELs...
were less or similar efficient emulsifiers of corn oil compared to MELs derived from the natural combinations (Figs. 4C and S7C). In addition, novel MEL species derived from combinations of different organisms exhibited a significant reduction in hemolysis (Figs. S5B and S6B). Compared to naturally occurring MELs these molecules lead to an increased LD50 value, when they were incubated with gram positive Bacillus subtilis bacteria (Fig. 5A and 5B; Table S6). Thus, our approach led to the generation of MELs with substantially different features.

4. Conclusion

Here, we have generated a principle workflow for the construction of novel MEL molecules. Acylation of the sugar moiety fulfills two major criteria, which are a premise for our approach. First, the substrate specificity (Beck et al., 2019; Deinzer et al., 2019) of both, Mac1 and Mac2, did not change if the enzymes were expressed in a different species. Second, all combinations of Mac enzymes tested in this study lead to the synthesis of predictable MELs. Preferences towards fatty acids of specific length were largely independent of the partner enzyme used. Thus, a large number of different MELs with a predefined composition can theoretically be produced.

The variety of molecules could be increased even more by the use of genes from outside of the original gene cluster such as mac3. Recently, it was reported that a strain lacking the gene for the putative transporter MmF1, produces mono-acylated MELs (Saika et al., 2020), which again enlarges the number of possible compounds. During our previous investigation of MEL biosynthesis inside of the peroxisome we could show that acyl-transferases for MEL production can also use hydroxylated fatty acids when Mac enzymes are targeted to the cytosol (Freitag et al., 2014). Hydroxylated acyl-CoA are used for production of the glycolipid u-stiglic acid in this compartment (Freitag et al., 2014; Teichmann et al., 2007). Engineering strains favoring production of MEL containing these unusual acyl groups could be one next step to expand the chemical variability of MELs. In addition, these data suggest that providing unusual fatty acid substrates inside peroxisomes (e.g. by peroxisomal targeting of fatty acid modifying enzymes) may an alternative strategy to direct the synthesis of modified MELs. Also forced production of tri-acylated MELs, which have been observed previously (Fukuoka et al., 2007), may be possible.

Other attempts to engineer biosurfactants e.g. sophorolipids with novel properties have been described that focused on isolating mutants that produce or accumulate intermediates of the final product (e.g. by deleting the gene for the multifunctional enzyme for peroxisomal β-oxidation) (Jezierska et al., 2018). One of these attempts even lead to the synthesis of balsaamphiphilic molecules with very useful features (Van Bogaert et al., 2016). For the analysis of tailor-made MEL production, we focused so far on nat1 mutants to simplify downstream analysis. Introducing the engineered combinations of Mac enzymes in a wildtype background should allow for the production of acylated variants of novel MEL molecules. Our study therefore might be a framework for the generation of a large library of small amphiphilic glycolipids.

The importance of biodegradable and renewable resources is emerging. Hence, glycolipids are expected to gain greater economic importance in the future. Today, only a small fraction of surfactants is produced fully bio-based, while the majority is still a product of petrochemical industry (Beck and Zibek, 2020). Having a repertoire of MEL molecules with very different characteristics may contribute to a more regular economic use of this renewable resource.

Declaration of competing interest

All authors have seen and approved the manuscript. All authors have contributed significantly to the work, and the manuscript has not been published and is not being considered for publication elsewhere. All authors declare that they have no competing financial interests.

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Appendix A. Supplementary data

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