An *Ustilago maydis* chassis for itaconic acid production without by-products

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**Summary**

*Ustilago maydis* is a promising yeast for the production of a range of valuable metabolites, including itaconate, malate, glycolipids and triacylglycerols. However, wild-type strains generally produce a potpourri of all of these metabolites, which hinders efficient production of single target chemicals. In this study, the diverse by-product spectrum of *U. maydis* was reduced through strain engineering using CRISPR/Cas9 and FLP/FRT, greatly increasing the metabolic flux into the targeted itaconate biosynthesis pathway. With this strategy, a marker-free chassis strain could be engineered, which produces itaconate from glucose with significantly enhanced titre, rate and yield. The lack of by-product formation not only benefited itaconate production, it also increases the efficiency of downstream processing improving cell handling and product purity.

**Introduction**

*Ustilago maydis*, a member of the Ustilaginaceae family, is a well-established model organism for the investigation of fungal mating, DNA recombination, RNA biology, cell signalling and plant-pathogen interactions (León-Ramírez et al., 2014). It also has large biotechnological potential due to features such as natural production of a wide range of value-added molecules, insensitivity to medium impurities and hydromechanic stress, and unicellular growth (Klement et al., 2012; Klement and Büchs, 2013; Maassen et al., 2014). Main products include organic acids (malate, succinate, itaconate, itatartarate and (S)-2-hydroxypropionic acid), polyols (erythritol and mannitol), glycolipids (mannosylerythritol lipids and ustilagic acid), intracellular triacylglycerols (Guevarra and Tabuchi, 1990; Bölker et al., 2008; Moon et al., 2010; Feldbrügge et al., 2013; Aguilà et al., 2017) and proteins (Kämper et al., 2006; Mueller et al., 2008; Geiser et al., 2013). Culture conditions influence the composition of the product spectrum. For instance, nitrogen starvation is an important activator for the synthesis of organic acids and glycolipids (Hewald et al., 2005, 2006; Teichmann et al., 2007). A decrease in pH however leads to a switch from the production of organic acids to polyols and glycolipids as preferred metabolite classes (Geiser et al., 2014).

Itaconate is an established bio-based product with high potential as platform chemical. This chemical and its derivatives already have a widespread application spectrum, for example in the industrial production of fibres, plastics, rubbers and surfactants, but also as bioactive compound in the agriculture, pharmacy or medical sectors (Willke and Vorlop, 2001; Okabe et al., 2009; Betancourt et al., 2010; Bera et al., 2015; De Carvalho et al., 2018; Kuenz and Krull, 2018). The market for itaconic acid is expected to expand in the next years (Weastra, 2012). So far, this chemical is commercially produced in fermentation processes using the filamentous fungus *Aspergillus terreus*. Other natural producers like *U. maydis*, *Candida* sp. or *Rhodotorula* sp. are to date not competitive with the production parameters of *A. terreus* that is able to reach itaconate titres of up to 160 g l⁻¹, yields of 0.63 gITA Glu⁻¹ and rates of 1.53 g l⁻¹ h⁻¹ (Steiger et al., 2013; Krull et al., 2017).

Nevertheless, *A. terreus* has a high sensitivity to fermentation conditions such as medium impurities, hydro-mechanical stress, viscosity of fermentation broth or oxygen...
supply (Klement et al., 2012; Kuenz et al., 2012). Morphology control is a major challenge which hinders reproducibility of cultivations, even when they are performed with the same strains (Willke and Vorlop, 2001; Kuenz et al., 2012).

Therefore, research increasingly focuses on Ustilaginaceae as alternative unicellular biocatalysts. *U. maydis* represents a promising organism for application in industrial itaconate production. Recent efforts have achieved considerable improvements in itaconate production with *Ustilago*, including characterization and upregulation of the itaconate gene cluster and associated pathway (Geiser et al., 2016b,c, 2018), engineering of transporters involved in itaconate production (Hosseinpour Tehrani et al., 2019b), and morphological and metabolic engineering of the acid-tolerant *U. cynodontis* (Hosseinpour Tehrani et al., 2019c). However, the potpourri of metabolites naturally synthesized by *U. maydis* results in suboptimal specificity, productivity and yield of itaconate as a product. These impurities hinder downstream purification of itaconate as the main product by for example co-precipitation and co-crystallization and can also impede prior biomass separation unit operations such as settling or centrifugation (Regestein et al., 2018). This issue can be addressed by metabolic engineering, which is enabled by a strong basis of genomic tools and data, including a well-annotated genome sequence (Kämper et al., 2006) as well as shotgun sequences of related itaconate producing Ustilaginaceae (Geiser et al., 2016a, 2018). Engineered promoters enable strong heterologous gene expression under a range of conditions (Botting et al., 1996; Brachmann et al., 2001; Flor-Parra et al., 2006; Zarnack et al., 2006; Sarkari et al., 2014; Zambanini et al., 2017), and in vivo and ex vivo sensors enable high-resolution monitoring of a range of cellular factors (Büchs, 2001; John et al., 2003; Anderlei et al., 2004; Hartmann et al., 2018). For efficient gene deletion, gene insertion, and promoter exchange, different genetic tools are available, including CRISPR/Cas9 genome editing (Schuster et al., 2016), FLP/FRT system with marker recycling (Khruyk et al., 2010) or Golden Gate Cloning (Terfrüchte et al., 2014).

Previous studies already achieved promising results in characterizing and engineering product formation pathways. Regarding itaconate production, a combination of deleting cyp3, which encodes an itaconate oxidase that converts itaconate into 2-hydroxyparaconate, and overexpressing ria1, which encodes the itaconate gene cluster regulator, increased itaconate titres by nearly 4-fold (Geiser et al., 2016b). This also led to a strong decrease in malate production due to upregulation of mtt1 encoding a cis-aconitate/malate antiporter (Geiser et al., 2016b; Scarcia et al., 2019). MEL biosynthesis is mediated by a gene cluster comprising five genes, and single mutant strains *U. maydis* MB215 Δemt1, Δmac1 and Δmac2 completely lost their ability to produce MELs (Hewald et al., 2005, 2006). The gene cluster enabling UA biosynthesis is formed by 12 open reading frames including the cytochrome P450 monoxygenase encoded by cyp1 (Hewald et al., 2005; Teichmann et al., 2007, 2010). Intracellular triacylglycerol (TAG) formation has not been studied in detail in *Ustilago*, but in other oleaginous species, diacylglycerol acyltransferases (DGAT) represent main contributors by mediating the final acylation of diacylglycerols to TAGs (Guo et al., 2009; Aguilar et al., 2017).

Based on this detailed methodical and biochemical knowledge, this study reduces the product spectrum of *U. maydis* through marker-free gene cluster deletion and promoter replacement (Fig. 1). This successfully focused most of the carbon flux into itaconate as main product, while simultaneously generating a chassis that is more manageable in a process context.

**Fig. 1.** Overview of known *U. maydis* MB215 metabolites. The targets addressed by metabolic engineering are labelled in red, the main product itaconic acid in blue. Grey arrows indicate metabolites that were not detected under the conditions tested in this study.

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Results and discussion

Insights into the genome sequence of an U. maydis deletion strain engineered for an improved itaconate production

To generate an itaconate producing U. maydis chassis with reduced by-product formation, multiple knockouts were successively performed in one strain. Due to a limited number of selection markers suitable for U. maydis, and considering the benefit of marker-free strains in an industrial biotechnology setting, two cloning techniques were used to generate a marker-free strain: CRISPR/Cas9 (Schuster et al., 2016) and marker insertion by homologous recombination with subsequent FRT-FLP-mediated marker removal (Khrunyk et al., 2010). When using the Cas9-based system, repair templates were used for all knockouts in order to achieve full gene deletions through homologous recombination. These templates were transformed along with the Cas9 plasmid which included the guide RNA of the target gene. Primers for construction of all associated sequences and the corresponding plasmids are listed in Tables S2 and S3.

The conversion of itaconate into (S)-2-hydroxyparacconate was abolished by the deletion of cyp3 (Geiser et al., 2016b). To stop glycolipid production, repair templates were designed to remove the whole gene clusters for MEL (Hewald et al., 2006) and UA (Teichmann et al., 2007) biosynthesis. The production of TAGs, forming intracellular lipid droplets as main energy storage in U. maydis (Aguilar et al., 2017), was reduced by single gene deletion of UMAG_03937. This gene, subsequently named dgat, encodes a putative diacylglycerol acyltransferase based on sequence alignment of DGAT2 from Saccharomyces cerevisiae (Liu et al., 2011). As last modification, ria1, encoding the itaconate cluster regulator, was overexpressed by a promoter exchange to improve itaconate and decrease malate production (Geiser et al., 2016b). This was achieved by the inclusion of the Petefria1 promoter into the repair template yielding a marker-free insertion (Hosseinpour Tehrani et al., 2019a).

For the generation of Δcyp3, ΔMEL, ΔUA and ΔPria1::Petef, the CRISPR/Cas9 system was used, while Δdgat was achieved using the FRT/FLP-mediated approach. All modifications were performed consecutively in the order Δcyp3, ΔMEL, ΔUA, Δdgat and ΔPria1::Petef, ultimately yielding the final chassis strain U. maydis MB215 Δcyp3 ΔMEL ΔUA Δdgat ΔPria1::Petef (hence called U. maydis MB215 ITA chassis). While Δcyp3, Δdgat, and ΔPria1::Petef could successfully be verified by PCR analysis, the confirmation of the deletion of larger genomic fragments, such as the 18.5 kb MEL cluster (Hewald et al., 2006) and the 58 kb UA cluster (Teichmann et al., 2007), could not be conclusively confirmed this way. Resequencing of the chassis strain genome and comparison to the reference genome of U. maydis 521 wild type (Kämper et al., 2006) confirmed the successful deletion of cyp3 and dgat, but also exposed several unexpected phenomena with the other modifications (Fig. 2). As expected, no reads mapped against the native ria1 promoter region, indicating successful deletion of the 1334 bp fragment. The coverage of reads mapped against the native Petef promoter was almost twice as high as the baseline (not shown), indicating that the stronger Petef promoter was successfully inserted. However, the coverage of the two 1000 bp regions flanking the promoter, precisely matching the homology arms of the repair template, is approximately twice the mean value. Further single read analysis and PCR verification indicated that the entire pJET1.2-vector carrying the repair template had been integrated into the genome (Fig. S1). Thereby, both flanks exist as duplicates, in the native and in the synthetically generated form. This led to a duplication of Petef ria1, with one truncated 985 bp ria1 and one full 1231 bp gene. Although it is unclear if this truncated version is active, it is unlikely that further overexpression of Ria1 increases itaconate production, given that separate experiments aimed at increasing Ria1 expression through multicopy insertion of a Petefria1 construct did not increase production compared to the single copy insertion strain reported by Geiser et al. (2016c) (Bator, 2017, unpublished). The duplication of the ria1 flanks comes with a slight risk of genetic instability, although this would equally be the case for the aforementioned insertion of Petefria1 into the CBX locus. The chance of duplication can be avoided in the future through the use of a linear PCR-generated repair template.

The aspired deletion of the 18.5 kb MEL gene cluster identified a disadvantage of the Cas9 system. The system is based on a Cas9 nuclelease cleaving within a specific target gene, in this case emt1. The formed double strand break was supposed to be repaired via homologous recombination using the repair template specifically designed for the deletion of the MEL cluster. However, the sequencing shows only the successful deletion of the complete mac2 gene and of the first 43% of the emt1 sequence, while the other cluster genes are still present. The adjacent UMAG_03120 gene might also be partially deleted, although coverage was generally poor in this region. Even though the full cluster was not deleted as intended, the deletion of mac2 and the disruption of emt1 are sufficient to abolish MEL synthesis in U. maydis (Hewald et al., 2005, 2006). A similar effect occurred with the deletion of the 58 kb UA biosynthesis gene cluster. Besides the targeted elimination of all UA cluster genes, additional genes upstream of the cluster were also deleted. UMAG_06454, UMAG_06455 and UMAG_06456 encode hypothetical proteins with
unknown function. This event of co-elimination of adjacent sequences also occurred for a part of gene UMAG_03120 flanking the MEL cluster and encoding an uncharacterized protein. No significant sequence similarities between the repair template and sequences surrounding the affected regions were found. A lack of reads in the end of chromosome 23 where the UA cluster is encoded was also noticeable. However, this is likely a mapping artefact, as the coverage of the telomeric regions of other chromosomes exhibited an overall downgraded coverage quality in these highly repetitive sequences.

Summarized, all intended genomic modifications were achieved but in three out of four cases where the Cas9 system was used unintended side-effects occurred. These unintentional effects indicate that homologous recombination often fails to resolve along the designed repair templates, even with long 1 kb flanks. Although it is currently unclear what causes this, it could possibly be avoided in the future by using higher concentrations of linear repair template with longer flanks for recombination. In the case of deletion of longer genomic stretches, the unpredictability of the chosen method is exacerbated by the fact that correct deletions are harder to detect by PCR methods due to the lack of a suitable positive control. This highlights the usefulness of genome resequencing, especially in this case of multiple iterative deletions. Although unexpected deletions did occur, these had no observable negative effect on the fitness of the strain, as described below.

**Fig. 2.** Genome analysis of the ITA chassis strain *U. maydis* MB215 Δcyp3 ΔMEL ΔUA Δdgat ΔP_int::P_etr by whole-genome sequencing. The coverage of Illumina sequencing reads mapped against the *U. maydis* 521 wild-type sequence visualizes the different deletion loci. Targeted genes are marked in red, verified deletions in dark red and unexpectedly non-performed deletions in light red. Further unexpected gene deletions are marked in black.

**Detailed analysis of lipid production in U. maydis ITA chassis and its progenitors**

The lipid spectra of the deletion strains were quantitatively characterized by GC-FID analysis of fatty acid
methyl esters (FAMES). Lipidic extracts were obtained from the whole culture broths after 72 h of cultivation (screening medium, 50 g l\(^{-1}\) glucose, pH 6.5 buffered with 100 mM MES, System Duetz\(^{®}\)) in order to quantify both intra- and extracellular lipids. The ITA chassis and all engineered precursor strains were analysed along with the wild type in order to identify the impact of each single mutation (Fig. 3A).

The molecular structures of the three investigated lipid classes (MELs, UA and TAGs) feature different fatty acid chain length profiles: two short- to medium-chain (C\(_2\) to C\(_{14}\)) fatty acids for MELs (Hewald et al., 2006; Huang et al., 2011), one short-chain (C\(_6\) or C\(_8\)) and a C\(_{16}\) fatty acid for UA (Teichmann et al., 2007, 2011), and three long-chain fatty acids, predominantly C\(_{16}\) and C\(_{18}\), for TAGs (Aguilar et al., 2017). As expected from the molecular structures, the deletion of glycolipid production, especially ΔMEL, led to a drastic reduction or even complete absence of C\(_6\), C\(_{12}\) and C\(_{14}\) fatty acids (Fig. 3A). Consequently, a general shift in the fatty acid production spectrum was observed, represented by an increased production of long-chain fatty acids, indicating a fostered TAG synthesis by glycolipid elimination. The subsequent deletion of dgat resulted in a significant reduction or complete loss of these long-chain fatty acids. The production of C\(_{18:0}\) as well as the whole spectrum of C\(_{20}\) to C\(_{24}\) fatty acids was completely eliminated, while C\(_{16}\) and C\(_{18:1}\) fatty acids were reduced by 38, respectively, 60% in comparison to the values of the precursor triple mutant. This confirms that the putative dgat (UMAG_03937) plays a major role in TAG production. With the final overexpression of ria1 included, the resulting U. maydis MB215 ΔUA (Fig. S3). This peak is assumed to be caused by MELs, since it is absent in all strains, which only have the deletion of the MEL cluster in common. The HPLC analysis further confirmed the absence of (S)-2-hydroxyxipaconate (rRT of 0.75) in the cyp3 deletion mutant. The deletion of all by-product genes in U. maydis MB215 Δcyp3 ΔMEL ΔUA Δdgat, resulted in a 1.1-fold increase in itaconate production compared to the wild type. With the additional overexpression of ria1 in the final ITA chassis, this increase was 10.2-fold. The differences in the degree of enhancement between FAMES and HPLC analyses are likely due to C\(_{10}\)/ITA overlapping in FAMES analysis. Simultaneously to the increase in ITA synthesis, malate (rRT of 0.70) secretion was drastically reduced by 84% due to the upregulation of the mitochondrial antiporter mtt1 as consequence of the ria1 overexpression (Jaklitsch et al., 1991; Geiser et al., 2016b).

In the ITA chassis strain, the production of virtually all detectable by-products could be abolished, while the metabolic flux towards the target product itaconate was drastically increased compared to the wild type. The deletion of lipidic by-product formation was turned out to be a major advantage in handling and efficiency of unit operations such as settling or centrifugation. Within minutes of centrifugation, cells of the ITA chassis strain formed a pellet with clear supernatant (Fig. 3D). With the wild type, this effect was not achievable within 60 min. Microscopy images

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identified strong extracellular lipid production of the wild type (Fig. S2) likely resulting in the formation of a cloudy supernatant of suspended glycolipids during centrifugation. It is also expected that TAG formation affects the density of the cells making a subpopulation neutrally buoyant. Both effects are avoided in the ITA chassis, facilitating downstream processing with this strain.
Influence of the reduced by-product spectrum on itaconate production

To evaluate the effect of the elimination of by-product formation on itaconate production in detail, the performance of the *U. maydis* MB215 ITA chassis (Δcyp3 ΔMEL ΔUA Δdgat ΔPria1::Petef) and its precursor strain *U. maydis* MB215 Δcyp3 ΔMEL ΔUA Δdgat were investigated over time (Fig. 4, Table 1). *U. maydis* MB215 wild type and *U. maydis* MB215 Δcyp3 ΔMEL ΔUA Δdgat ΔPria1::Petef, a strain equivalent to the already engineered and published itaconate hyper-producing strain *U. maydis* MB215 Δcyp3 Petefria1 (Geiser et al., 2016b), were included as references. The strains were cultivated in 24-well System Duetz® plates in screening medium containing an initial concentration of 50 g l\(^{-1}\) glucose as sole carbon source buffered with 100 mM MES at a pH of 6.5.

The strain with all four by-product-forming genes/ gene clusters deleted, but without ria1 overexpression (*U. maydis* MB215 Δcyp3 ΔMEL ΔUA Δdgat) produced 2.1 ± 0.4 g l\(^{-1}\) itaconate, which is not significantly different from the wild type. However, *U. maydis* MB215 Δcyp3 ΔMEL ΔUA Δdgat consumed 25% less glucose than the wild type resulting in a 51% higher product to substrate yield (y\(_{ps}\)) for itaconate from glucose. This confirms the previous observations which suggest that this strain lacks sufficient metabolic ‘outlet’, thereby reducing its substrate uptake rate, when growth as predominant C-sink is no longer possible under the applied nitrogen limitation.

The itaconate producing strain *U. maydis* MB215 Δcyp3 ΔPria1::Petef achieved a maximum titre of 15.3 ± 0.4 g l\(^{-1}\) itaconate, which is 8.1-fold higher than that of the wild type. With the ITA chassis, the itaconate titre was further increased to 19.4 ± 0.3 g l\(^{-1}\) constituting a 10.2-fold increase compared to the wild type and accordingly a 1.3-fold increase compared to *U. maydis* MB215 Δcyp3 ΔPria1::Petef. The OD\(_{600}\) of the ITA chassis dropped significantly towards the end of the culture, likely caused by a change in morphology (Hosseinipour Tehrani et al., 2019c). The maximum volumetric production rate (q\(_{p,max}\)) was increased by 13%, while the overall rate (q\(_{p}\)) was increased by 19%, and the y\(_{ps}\) by 29% compared to the latter strain. These results show that the four deletions reduce the metabolic flux of glucose into these by-product pathways, reflected in a decreased

![Fig. 4. System Duetz® cultivation of various *U. maydis* MB215 strains in screening medium with 50 g l\(^{-1}\) glucose and 100 mM MES, incubated in 24-well plates with a filling volume of 1.5 ml (shaking diameter = 50 mm, n = 300 rpm, T = 30 °C and Φ = 80%). Concentrations of itaconate (A), malate (B), (S)-2-hydroxyparaconate (C) and glucose (D) are shown, as well as OD\(_{600}\) (E) and pH (F) of cultures of *U. maydis* MB215 wild type (orange, inverted triangle), Δcyp3 ΔPria1::Petef (green, triangle), Δcyp3 ΔMEL ΔUA Δdgat (light blue, circle) and Δcyp3 ΔMEL ΔUA Δdgat ΔPria1::Petef (dark blue, square). Error bars indicate the standard error of the mean (n = 3).](image-url)

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was changed from MES to CaCO₃ and the substrate eliminate this potential pH inhibition, the buffer system.

In the previous experiment, the pH decreased from 6.5 along with a drastic drop of the pH value in the medium. The much higher buffer capacity of 66 g l⁻¹ CaCO₃ solved the problem, the ITA chassis reached a titre of 53.5 ± 5.0 g l⁻¹ accomplishing an yₚ/s of 0.47 ± 0.03 gITA gGlu⁻¹ and qₚ of 0.28 ± 0.00 g l⁻¹ h⁻¹. These parameters confirm that the three by-product deletions ∆MEL, ∆UA and ∆dgat resulted in a more efficient conversion of glucose into itaconate and therefore in a 21 – 27% improvement of itaconate yield, titre and rate (Table 1). The same trend was observed with an initial glucose concentration of 150 g l⁻¹ (Fig. S4; Table S1), but with slightly lower yields and production rates likely due to higher osmotic stress.

Conclusions

This study focused on the reduction of the U. maydis metabolite spectrum to improve its itaconate production. The elimination of by-product formation significantly enhanced itaconate titre, rate, and yield. In addition, the lack by-products, and better handling of the strain, will contribute to a more efficient downstream processing. This is particularly crucial for itaconate applications in the polymer industry, where high purities are generally required. With its optimized performance, the designed strain lays the basis for further process optimizations such as fed-batch fermentations and cell retention. In addition, upon removal of the itaconate gene cluster, it’s clean product background and marker-free genome makes the U. maydis MB215 ITA chassis an ideal platform for the synthesis of a variety of other industrially relevant products such as malate or erythritol.

Experimental procedures

Media and culture conditions

Escherichia coli strains were grown in medium containing 10 g l⁻¹ peptone, 5 g l⁻¹ sodium chloride, 5 g l⁻¹ yeast extract and 5 g l⁻¹ glucose. U. maydis strains
were grown in YEPS medium containing 10 g l\(^{-1}\) yeast extract, 10 g l\(^{-1}\) peptone and 10 g l\(^{-1}\) sucrose. As screening medium for production experiments, \(U. \) \textit{maydis} was cultivated in modified Tabuchi medium (MTM) according to Geiser \textit{et al.} (2014). Besides varying glucose and buffer (2-(N-morpholino) ethanesulfonic acid (MES; pH adjusted to 6.5 with NaOH) or CaCO\(_3\)) concentrations, this medium contained 0.8 g \(l^{-1}\) \(\text{NH}_4\)Cl, 0.2 g \(l^{-1}\) MgSO\(_4\)\(\cdot\)7H\(_2\)O, 0.01 g \(l^{-1}\) FeSO\(_4\)\(\cdot\)7H\(_2\)O, 0.5 g \(l^{-1}\) KH\(_2\)PO\(_4\), 1 ml \(l^{-1}\) vitamin solution and 1 ml \(l^{-1}\) trace element solution. The vitamin solution contained (per litre) 0.05 g D-biotin, 1 g D-calcium pantothenate, 1 g nicotinic acid, 25 g myo-inositol, 1 g thiamine hydrochloride, 1 g pyridoxal hydrochloride and 0.2 g para-aminobenzoic acid. The trace element solution contained (per litre) 1.5 g EDTA, 0.45 g ZnSO\(_4\)\(\cdot\)7H\(_2\)O, 0.10 g MnCl\(_2\)\(\cdot\)4H\(_2\)O, 0.03 g CoCl\(_2\)\(\cdot\)6H\(_2\)O, 0.03 g CuSO\(_4\)\(\cdot\)5H\(_2\)O, 0.04 g Na\(_2\)MoO\(_4\)\(\cdot\)2H\(_2\)O, 0.45 g CaCl\(_2\)\(\cdot\)2H\(_2\)O, 0.3 g FeSO\(_4\)\(\cdot\)7H\(_2\)O, 0.10 g H\(_2\)BO\(_3\) and 0.01 g KI. When using CaCO\(_3\), medium components were added relative to the total volume of solids + liquid, leading to a higher aqueous concentration of soluble components. Shaking cultures of \(U. \) \textit{maydis} were performed in 24-well System Duetz\textsuperscript{®} plates with a filling volume of 1.5 ml (shaking diameter = 50 mm, \(n = 300 \text{ rpm}, T = 30 \degree \text{C and } \Phi = 80\%\)) (Duetz \textit{et al.}, 2000) or in 500 ml shaking flasks with a filling volume of 50 ml (shaking diameter = 25 mm, \(n = 200 \text{ rpm}, T = 30 \degree \text{C and } \Phi = 80\%\)). When using System Duetz\textsuperscript{®}, cultures were inoculated in parallel into multiple plates in order to ensure continuous oxygenation by taking a complete plate as sacrificial sample for each sample point (Table 2).

### Table 2. \textit{Ustilago maydis} MB215 strains used in this study.

| Strain designation | Resistance | Reference |
|--------------------|------------|-----------|
| \textit{U. maydis} MB215 |           | Hewald \textit{et al.} (2005) |
| \textit{U. maydis} MB215 \(\Delta\)cyP3 \(\Delta\)P\(_{\text{ria}}\):\(\text{P}_{\text{ela}}\) | hyg\(^R\) | Hewald \textit{et al.} (2005) |
| \textit{U. maydis} MB215 \(\Delta\)cyP1 \(\Delta\)emt1 | hyg\(^R\) | Hewald \textit{et al.} (2005) |
| \textit{U. maydis} MB215 \(\Delta\)cyP3 | This study |
| \textit{U. maydis} MB215 \(\Delta\)emt1 | This study |
| \textit{U. maydis} MB215 \(\Delta\)UA | This study |
| \textit{U. maydis} MB215 \(\Delta\)MEL | This study |
| \textit{U. maydis} MB215 \(\Delta\)cyP3 \(\Delta\)MEL | This study |
| \textit{U. maydis} MB215 \(\Delta\)P\(_{\text{ria}}\):\(\text{P}_{\text{ela}}\) | \(\Delta\)UA |
| \textit{U. maydis} MB215 \(\Delta\)cyP3 \(\Delta\)MEL \(\Delta\)UA | This study |
| \textit{U. maydis} MB215 \(\Delta\)cyP3 \(\Delta\)MEL \(\Delta\)UA \(\Delta\)dgat | This study |
| \textit{U. maydis} MB215 \(\Delta\)cyP3 \(\Delta\)MEL | This study |
| \textit{U. maydis} MB215 \(\Delta\)P\(_{\text{ria}}\):\(\text{P}_{\text{ela}}\) \(\Phi_{\text{TIA}}\) | This study |

\(\Phi_{\text{TIA}}\) = 80\%. Itaconate production was shown to be in phase with a complete plate as sacrificial sample for each sample point (Table 2).

**Analytical methods**

All values are the arithmetic mean of at least three biological replicates. Error bars or ± values indicate the standard error of the mean.

When using CaCO\(_3\) as buffer, 1 ml culture broth was taken for OD\(_{600}\) and HPLC analysis. The CaCO\(_3\) was dissolved 1:1 with HCl prior to further measurement as described in (Zambanini \textit{et al.}, 2016). Cell densities were measured by determining the absorption at 600 nm with an Ultraspec 10 Cell Density Meter (Amer sham Biosciences, Chalfont Saint Giles, UK).

For thin-layer chromatography (TLC) analysis of mannosylerythritol lipids and ustilagic acid, the \textit{U. maydis} strains were cultivated for 72 h in yeast nitrogen base/5% glucose medium. 0.3 ml culture was mixed with 0.6 ml ethyl acetate for 20 min. After centrifugation (5 min, 14 000 g), the ethyl acetate phase was dried overnight at room temperature (RT). The residue was resuspended in 50 µl methanol, whereof 6 µl were spotted on a TLC Silica gel 60 aluminium sheet (20 x 20 cm, Merck KGaA, Darmstadt, Germany) and dried for 5 min at RT. As running buffer, a mixture of chloroform, methanol and H\(_2\)O (70:26:4, v/v) was used. The plate...
was dried for 10 min at RT, before glycolipids were visualized by staining with a mixture of acetic acid, p-anisaldehyde and sulphuric acid (97:1:2, v/v) followed by heating at 120 °C for 3–5 min.

For fatty acid methyl esters (FAMES) analysis, lipid extracts were obtained from 9 ml culture broth after 72 h of cultivation in MTM (50 g l⁻¹ glucose, pH 6.5 buffered with 100 mM MES, System Duetz®). Lipid composition was analysed by lipid hydrolysis, esterification to fatty acid methyl esters using methanolic H₂SO₄ as transmethyllating reagent and hexane extraction. GC-FID separation was performed in a Shimadzu GC-MS QP 2010 using an OPTIMA® 225 column (30 m, 0.25 mm, 0.25 µm; Macherey-Nagel, Germany).

For high-performance liquid chromatography (HPLC) analysis, all samples were filtered with Rotilabo® (CA, 0.2 µm, Ø 15 mm) or Acrodisc® Syringe Filters (GHP, 0.2 µm, Ø 13 mm) and diluted 1:10 with 5 mM H₂SO₄. Products in the supernatant were analysed in a DIONEX UltiMate 3000 HPLC System (Thermo Scientific, Langen, Germany) with an ISERA Metab-AAC column 300 x 7.8 mm column (ISERA, Germany). As solvent, 5 mM H₂SO₄ with a constant flow rate of 0.6 ml min⁻¹ and a temperature of 40 °C was used. For detection, a SHODEX RI-101 detector (Showa Denko Europe GmbH, Munich, Germany) and a DIONEX UltiMate 3000 Variable Wavelength Detector set to 210 nm were used. Analytes were identified via retention time and UV/RI quotient compared to corresponding standards. For optimal comparison of HPLC chromatograms, retention times were specified relative to the retention time of itaconate. With this normalization, small analytical variations are minimized, enabling a more accurate comparison of overlapping peaks in the start of the chromatogram.

For genome re-sequencing, genomic DNA was isolated by phenol-chloroform-isooamyl alcohol extraction. Whole-genome sequencing was performed by GATC Biotech AG (Konstanz, Germany) using Genome Sequencer Illumina HiSeq 2500 technology (sequence mode: 2 x 150 bp read length) and the HiSeq Control Software v2.0.12.0 (Illumina Inc., San Diego, CA, USA). Variance analysis in terms of SNP and InDel detection was conducted by mapping the re-sequencing data onto the U. maydis 521 reference genome (Kämper et al., 2006; Refseq assembly GCF_000328475.2). Mapping and variant calling results were visualized with Integrative Genomics Viewer (IGV) software (Thorvaldsdottir et al., 2013). The sequencing data are available through the NCBI Sequence Read Archive (PRJNA592070).

**Plasmid cloning and strain engineering**

Plasmids were constructed by Gibson assembly (Gibson et al., 2009) using the NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs (NEB), Ipswich, MA, USA). Primers were ordered as DNA oligonucleotides from Eurofins Genomics (Ebersberg, Germany). As polymerase, Q5® High-Fidelity DNA Polymerase (NEB) was used. Detailed information about utilized primers and plasmids are listed in Tables S2 and S3. Competent *E. coli* DH5α were used for standard cloning and plasmid maintenance according to Sambrook and Russell (2001). Plasmids were confirmed by PCR or sequencing. Generation of protoplasts and transformation of *U. maydis* were performed according to (Brachmann et al. (2004). Genomic DNA of *U. maydis* was isolated according to Hoffman and Winston (1987). For the deletion of *cyp3*, MEL and UA gene cluster, and for the exchange of the native *ria1* promoter with the strong constitutive *P*_eth, the CRISPR/Cas9 system (Schuster et al., 2016) in combination with repair templates was used. The repair templates consisted of two 1000 bp long fragments corresponding to the flanking regions upstream and downstream of the sequence to be eliminated. For the deletion of *dgap*, homologous recombination with 1000 bp flanking regions including FRT-sites and a hygromycin resistance cassette were used (Khrunkyt et al., 2010). Successful integration and deletion were verified by PCR and sequencing.

**Acknowledgements**

We thank Dr. Mariana Schuster and Prof. Dr. Regine Kahmann (Max Planck Institute for Terrestrial Microbiology, Department of Organismic Interactions, Marburg, Germany) for providing the plasmid pCas9_sgRNA_0 and Dr. Kerstin Schipper and Prof. Dr. Michael Feldbrügge (Institute for Microbiology, Heinrich Heine University Düsseldorf, Germany) for providing the plasmids pstorI_1rh_WT (pUMa1522) and pFLPexPC. We thank Dr. Emad Albaroukhi, Prof. Dr. Jan Schirawski and Dr. Tino Polen for their help with the interpretation of genome resequencing data.

**Conflict of interest**

None declared.

**Author contributions**

All authors contributed significantly to the work. NW conceived the study. JL designed and performed experiments and analysed results with the help of NW, HHT and LMB. MG and JM performed FAMES lipid analysis. JL wrote the manuscript with help of NW and LMB.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Genomic sequence after exchange of the native *ria1* promoter by the constitutive *etef* promoter encoding gene.

**Fig. S2.** Identification of mannosylerythritol lipid (white arrow) and ustilagic acid (black arrow) production in *U. maydis* MB215 wildtype (left) and *U. maydis* MB215 ITA chassis (right) by microscopy.

**Fig. S3.** FAMES and HPLC diagrams of different *U. maydis* MB215 mutant strains.

**Fig. S4.** Shake flask cultivation of two *U. maydis* MB215 mutant strains in screening medium.

**Table S1.** Production parameters of two engineered *U. maydis* MB215 strains resulting from a cultivation in screening medium.

**Table S2.** Plasmids used in this study.

**Table S3.** Oligonucleotides used for deletion and overexpression constructs.