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Modulation of the central carbon metabolism of *Corynebacterium glutamicum* improves malonyl-CoA availability and increases plant polyphenol synthesis

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Abstract

In recent years microorganisms have been engineered towards synthesizing interesting plant polyphenols such as flavonoids and stilbenes from glucose. Currently, the low endogenous supply of malonyl-CoA, indispensable for plant polyphenol synthesis, impedes high product titers. Usually, limited malonyl-CoA availability during plant polyphenol production is avoided by supplementing fatty acid synthesis-inhibiting antibiotics such as cerulenin, which are known to increase the intracellular malonyl-CoA pool as a side effect. Motivated by the goal of microbial polyphenol synthesis being independent of such expensive additives, we used rational metabolic engineering approaches to modulate regulation of fatty acid synthesis and flux into the tricarboxylic acid cycle (TCA cycle) in *Corynebacterium glutamicum* strains capable of flavonoid and stilbene synthesis. Initial experiments showed that sole overexpression of genes coding for the native malonyl-CoA-forming acetyl-CoA carboxylase is not sufficient for increasing polyphenol production in *C. glutamicum*. Hence, the intracellular acetyl-CoA availability was also increased by reducing the flux into the TCA cycle through reduction of citrate synthase activity. In defined cultivation medium, the constructed *C. glutamicum* strains accumulated 24 mg·L⁻¹ (0.088 mM) naringenin or 112 mg·L⁻¹ (0.49 mM) resveratrol from glucose without supplementation of phenylpropanoid precursor molecules or any inhibitors of fatty acid synthesis.

KEYWORDS

citrate synthase, *Corynebacterium glutamicum*, malonyl-CoA, naringenin, polyphenols, resveratrol

1 | INTRODUCTION

Across all domains of life, malonyl-CoA serves as building block for chain elongation steps during fatty acid biosynthesis. Beyond that, mostly in bacteria, fungi, and plants, malonyl-CoA is also required for

the synthesis of polyketides, a large class of secondary metabolites with a multitude of functions (Han et al., 2000; van Summeren-Wesenhagen & Marienhagen, 2015; Zabala, Braña, Salas, & Méndez, 2015). In plants, polyphenols such as stilbenes and chalcones are produced from a phenylpropanoyl-CoA starter molecule (derived from the aromatic amino acids L-phenylalanine or L-tyrosine) and three molecules of malonyl-CoA (Figure 1). In this context, plant-derived

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FIGURE 1 Metabolic pathways involved in the synthesis of the polyphenol precursors *p*-coumaroyl-CoA and malonyl-CoA. *p*-Coumaroyl-CoA and malonyl-CoA are key precursor molecules for the synthesis of the plant polyphenols naringenin and resveratrol. *p*-Coumaroyl-CoA is the CoA thioester of *p*-coumaric acid, which in turn is obtained from L-tyrosine by nonoxidative deamination. Malonyl-CoA is synthesized by carboxylation of acetyl-CoA. Metabolic pathways involved in the synthesis or consumption of the mentioned polyphenol precursors include the pentose phosphate pathway (supply of the shikimate pathways substrate erythrose 4-phosphate), the shikimate pathway (synthesis of L-tyrosine), glycolysis (pyruvate synthesis), and the TCA cycle (oxidation of acetyl-CoA). In the course of this study, several genetic modifications were introduced in to *C. glutamicum* and evaluated for their effect on polyphenol synthesis. 4CL: 4-coumarate: CoA ligase; ACC: acetyl-CoA carboxylase; CHI: chalcone isomerase; CHS: chalcone synthase; CS: citrate synthase; DAHP: 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthase; E4P: erythrose 4-phosphate; FAS: fatty acid synthase; FASR: repressor of *accBC*-, *accD1*-, *fas-IA*- and *fas-IB*-expression; PPP: pentose phosphate pathway; SDH: succinate dehydrogenase; STS: stilbene synthase; TAL: tyrosine ammonia lyase. Abolished enzymes/transcriptional regulators: crossed out; increased enzyme activity: green arrow upwards, reduced enzyme activity: red arrow downwards [Color figure can be viewed at wileyonlinelibrary.com]

Type III polyketide synthases, either stilbene synthases (STS) or chalcone synthases (CHS), catalyze the malonyl-CoA-dependent chain elongation reaction of the phenylpropanoyl-CoA starter unit and the subsequent formation of a second aromatic ring (Ferrer, Jez, Bowman, Dixon, & Noel, 1999). Stilbenes act as phytoalexins in plants, whereas chalcones are precursor molecules of more than 5,000 flavonoids known today (Ross & Kasum, 2002). In general, polyphenols are of commercial interest because of the manifold of health-promoting effects including antioxidative, antibiotic, anti-inflammatory, antiallergic, and anticancer activities (Daglia, 2012).

Unfortunately, plants produce polyphenols only at very low rates and the overall polyphenol content is always subject to incalculable seasonal and climatic variations. Microorganisms, genetically engineered for plant polyphenol production, represent a promising alternative for the production of these valuable compounds (Milke, Aschenbrenner, Marienhagen, & Kallscheuer, 2018). However, tight regulation of the endogenous malonyl-CoA synthesis with the aim to maintain only low levels of this fatty acid precursor in microorganisms, turned out to be the decisive bottleneck during microbial polyphenol production utilizing *Escherichia coli* and *Saccharomyces cerevisiae* (Kim & Ahn, 2014; Leonard, Lim, Saw, & Koffas, 2007; M. Li, Schneider,

Kristensen, Borodina, & Nielsen, 2016; Lim, Fowler, Hueller, Schaffer, & Koffas, 2011; Yang, Lin, Li, Linhardt, & Yan, 2015). Hitherto, metabolic engineering of the microbial central carbon metabolism focused on increasing the metabolic flux towards malonyl-CoA formation (Zha, Rubin-Pitel, Shao, & Zhao, 2009). The ATP-dependent carboxylation of acetyl-CoA, catalyzed by acetyl-CoA carboxylases (ACC) comprised up to four different subunits is the sole natural source of malonyl-CoA (S.-J. Li & Cronan, 1992). To this end, genes coding for ACC subunits from various sources were heterologously expressed for increasing the intracellular malonyl-CoA availability during plant polyphenol production; a strategy, which was not always successful (Katsuyama, Funa, Miyahisa, & Horinouchi, 2007; Miyahisa et al., 2005; van Summeren-Wesenhagen & Marienhagen, 2015; Zha et al., 2009). Interestingly, in the context of these efforts, the ACC of *Corynebacterium glutamicum* has often been exploited in various species as this enzyme consists only of two instead of four different subunits (Gande et al., 2007). Expression of heterologous *acc* genes was often combined with deletion or downregulation of native genes coding for enzymes involved in competing pathways, for example, for acetate or ethanol formation from acetyl-CoA (Zha et al., 2009). However, primary fate of acetyl-CoA is its oxidation in the tricarboxylic acid cycle (TCA cycle).

Here, deletion of the *sdhCDAB* operon coding for the succinate dehydrogenase complex of the TCA cycle of *E. coli* resulted in increased production of chalcone-derived (2S)-flavanones with this organism (Fowler, Gikandi, & Koffas, 2009). A substantial increase in polyphenol titers in engineered microbial production strains can also be achieved by the addition of the antibiotic cerulenin (Leonard et al., 2008). Cerulenin acts as a nonselective, irreversibly-inhibiting agent on fatty acid synthases by covalent binding to the active site's cysteine and occupation of the β -ketoacyl-ACP synthase domain (Johansson et al., 2008; Price et al., 2000). This blocks fatty acid synthesis as the major "competing" pathway for malonyl-CoA consumption and thus increases the microbial production of polyphenols (Lim et al., 2011). Unfortunately, fatty acid biosynthesis-inhibiting agents such as cerulenin are very expensive and usually inhibit microbial growth as a result of rapid fatty acid depletion. Therefore, use of such antibiotics is not an option for larger-scale microbial polyphenol production.

C. glutamicum is already used for decades for the multimillion ton-scale biotechnological production of amino acids and has been recently engineered towards producing plant polyphenols such as stilbenes, (2S)-flavanones, flavonols, and flavanonols, either from supplemented precursors or directly from glucose using natural or nonnatural pathways (Kallscheuer, Vogt, Bott, & Marienhagen, 2017; Kallscheuer, Vogt, & Marienhagen, 2016; Kallscheuer, Vogt, Stenzel et al., 2016). Similar to other microorganisms engineered for polyphenol production, low availability of malonyl-CoA also prohibits synthesis of higher polyphenol concentrations in *C. glutamicum* (Kallscheuer, Vogt, Stenzel et al., 2016).

We here addressed this challenge and followed several rational metabolic engineering strategies to modulate the central carbon metabolism of *C. glutamicum* with the aim to increase the intracellular malonyl-CoA availability for microbial plant polyphenol synthesis.

2 | MATERIAL AND METHODS

2.1 | Bacterial strains, plasmids, media, and growth conditions

All bacterial strains and plasmids used in this study along with their respective relevant characteristics are listed in Table 1. *C. glutamicum* strains were routinely cultivated aerobically at 30°C in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, MI) or defined CGXII medium with glucose as sole carbon and energy source (Keilhauer, Eggeling, & Sahm, 1993). *E. coli* DH5 α was used for plasmid constructions and was cultivated in LB medium (Bertani, 1951) at 37°C. Where appropriate, kanamycin (50 $\mu\text{g}\cdot\text{ml}^{-1}$ for *E. coli* or 25 $\mu\text{g}\cdot\text{ml}^{-1}$ for *C. glutamicum*) or spectinomycin (100 $\mu\text{g}\cdot\text{ml}^{-1}$ for *E. coli* and *C. glutamicum*) was added to the medium. Bacterial growth was followed by measuring the optical density at 600 nm (OD_{600}).

For cultivation of *C. glutamicum*, a single colony was picked from an agar plate and grown for six–eight hours in a test tube with 5 ml BHI medium on a rotary shaker at 170 rpm (first preculture). This culture was used to inoculate 50 ml defined CGXII medium with 4% glucose in 500 ml baffled Erlenmeyer flasks (second preculture). The

cell suspensions were cultivated overnight on a rotary shaker at 130 rpm. The main culture was inoculated to an OD_{600} of 5 in CGXII medium with 4% glucose. For optimizing the production of the (2S)-flavanone naringenin and the stilbene resveratrol, *p*-coumaric acid (solved in dimethyl sulfoxide) was added to a final concentration of 5 mM. Heterologous gene expression was induced one hour after inoculation of the main culture using 1 mM isopropyl β -D-thiogalactopyranoside (IPTG). Where indicated, 25 μM cerulenin was added to the main culture at an OD_{600} of 18–20. 1 ml of the culture broth was collected at defined time points and stored at -20°C until extraction.

2.2 | Plasmid and strain construction

Standard protocols of molecular cloning, such as polymerase chain reaction (PCR), DNA restriction, and ligation were carried out for recombinant DNA work (Sambrook & Russell, 2001). Techniques specific for *C. glutamicum*, for example, electroporation for the transformation of strains, were done as described previously (Eggeling & Bott, 2005). All enzymes were obtained from Thermo Fisher Scientific (Schwerte, Germany). Codon-optimized synthetic genes for *C. glutamicum* ATCC13032 were obtained from Life Technologies (Darmstadt, Germany). Genes were amplified by PCR from genomic DNA or synthetic genes as a template using primers containing unique restriction sites (Table 2). PCR products were used for cloning of genes into plasmid vectors using the introduced restrictions site. For plasmids containing more than one insert, genes were cloned sequentially. In-frame gene deletions or integration of DNA into the genome of *C. glutamicum* were performed using the pK19*mobsacB* system (Schäfer et al., 1994) by a two-step homologous recombination method described previously (Niebisch & Bott, 2001). All constructed plasmids were finally verified by DNA sequencing at Eurofins MWG Operon (Ebersberg, Germany).

2.3 | Bioreactor cultivations of polyphenol-producing *C. glutamicum* strains

All bioreactor experiments were performed in a 2 L bioreactor (Eppendorf, Hamburg, Germany) with 1 L of working volume. CGXII medium supplemented with 4% glucose but without urea and without 3-morpholino propanesulfonic acid (MOPS) was used in all bioreactor experiments. The pH was maintained at 7.0 by automatic addition of either HCl (3 M) or NaOH (2 M). Dissolved oxygen was kept above 30% saturation by feedback control of the stirring speed from 650 rpm until a maximum of 1,200 rpm and an air-flow rate of 1 vvm (volume air per volume medium and minute). The foam was disrupted by addition of antifoaming agent Antifoam 204 (Sigma-Aldrich, Schnelldorf, Germany). Bacterial growth was followed by measuring the OD_{600} (Synergy HT Multi-Detection Microplate Reader; BioTek Instruments, Winooski, VT) and obtained values were converted to biomass dry weight using a calibration curve. Heterologous gene expression was induced at biomass concentrations ranging from 0.9 to 1.4 $\text{g}\cdot\text{L}^{-1}$ using 1 mM IPTG. One milliliter of the culture broth was collected at defined time points and stored at -20°C until further processing.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
<i>C. glutamicum</i>		
DelAro ⁴ -4cl _{PC}	<i>C. glutamicum</i> DelAro ⁴ derivative with a chromosomally encoded 4cl _{PC} gene coding for 4-coumarate: CoA ligase under control of the T7 promoter (Δcg0344–47::P _{T7} -4cl _{PC})	Kallscheuer, Vogt, Stenzel et al. (2016)
Nar1	DelAro ⁴ -4cl _{PC} strain harboring pMKEx2_chs _{ph} _chi _{ph}	This study
Nar1 ΔfasR	Nar1 derivative with in-frame deletion of <i>fasR</i> (cg2737)	This study
Nar1 ΔsdhCAB	Nar1 derivative with in-frame deletion of <i>sdhCAB</i> (cg0445–47)	This study
Nar1 ΔfasR ΔsdhCAB	Nar1 derivative with an in-frame deletion of <i>fasR</i> and <i>sdhCAB</i>	This study
Nar1_A16	Nar1 derivative with the replacement of the native <i>gltA</i> promoter with the <i>dapA</i> promoter variant A16	This study
Nar1_L1	Nar1 derivative with the replacement of the native <i>gltA</i> promoter with the <i>dapA</i> promoter variant L1	This study
Nar1_C7	Nar1 derivative with the replacement of the native <i>gltA</i> promoter with the <i>dapA</i> promoter variant C7	This study
Nar1_C7 ΔfasR	Nar1_C7 derivative with an in-frame deletion of <i>fasR</i>	This study
Res1_C7	DelAro ⁴ -4cl _{PC} strain harboring pMKEx2_sts _{Ah} -4cl _{PC} and replacement of the native <i>gltA</i> promoter with the <i>dapA</i> promoter variant C7	This study
<i>E. coli</i>		
DH5α	F [−] Φ80lacZΔM15 Δ(lacZYA-argF) U169 <i>recA1 endA1 hsdR17</i> (r _k [−] , m _k ⁺) <i>phoA supE44 thi-1 gyrA96 relA1 λ</i> [−]	Invitrogen (Karlsruhe, Germany)
Plasmids		
pK19mobsacB	<i>kan</i> ^r ; vector for allelic exchange in <i>C. glutamicum</i> (pK18, oriV _{Ec} , <i>sacB</i> , <i>lacZα</i>)	Schäfer et al. (1994)
pK19mobsacBΔsdhCAB	Vector for in-frame deletion of <i>sdhCAB</i>	This study
pK19mobsacBΔfasR	Vector for in-frame deletion of <i>fasR</i>	Nickel, Irzik, Van Ooyen, and Eggeling (2010)
pK19mobsacB_gltA_A16	Vector for exchanging the native promoter of <i>gltA</i> against the <i>dapA</i> promoter variant A16	van Ooyen et al. (2012)
pK19mobsacB_gltA_L1	Vector for exchanging the native promoter of <i>gltA</i> against the <i>dapA</i> promoter variant L1	van Ooyen et al. (2012)
pK19mobsacB_gltA_C7	Vector for exchanging the native promoter of <i>gltA</i> against the <i>dapA</i> promoter variant C7	van Ooyen et al. (2012)
pMKEx2	<i>kan</i> ^r ; <i>E. coli</i> - <i>C. glutamicum</i> shuttle vector (<i>lacI</i> , P _{T7} , <i>lacO1</i> , pHM1519 ori _{CG} , pACYC177 ori _{Ec})	Kortmann, Kuhl, Klaffl, and Bott (2015)
pMKEx2_chs _{ph} _chi _{ph}	<i>kan</i> ^r ; pMKEx2 derivative with genes coding for chalcone synthase and chalcone isomerase from <i>Petunia x hybrida</i> (codon-optimized)	Kallscheuer, Vogt, Stenzel et al. (2016)
pMKEx2_sts _{Ah} -4cl _{PC}	<i>kan</i> ^r ; pMKEx2 derivative with genes coding for stilbene synthase from <i>Arachis hypogaea</i> and 4-coumarate:CoA ligase from <i>Petroselinum crispum</i> (codon-optimized)	Kallscheuer, Vogt, Stenzel et al. (2016)
pEKEx3	<i>spec</i> ^r ; <i>E. coli</i> - <i>C. glutamicum</i> shuttle vector (<i>lacI</i> , P _{tac} , <i>lacO1</i> , pBL1 ori _{CG} , pUC ori _{Ec})	Gande et al. (2007)
pEKEx3_accBC_accD1	<i>spec</i> ^r ; pEKEx3 derivative containing <i>accBC</i> and <i>accD1</i> genes from <i>C. glutamicum</i>	This study
pEKEx3_aroH _{Ec} _tal _{Fj}	<i>spec</i> ^r ; pEKEx3 derivative containing native <i>aroH</i> from <i>Escherichia coli</i> and a codon-optimized gene encoding tyrosine ammonia lyase from <i>Flavobacterium johnsoniae</i>	Kallscheuer, Vogt, Stenzel et al. (2016)

kan^r: kanamycin resistance, *spec*^r: spectinomycin resistance

TABLE 2 Oligonucleotides used in this study

Primer name	Sequence (5'→3')	Relevant site
Sbfl-accBC-s	ACACCTGCAGGAAGGAGGACTGCTATGTCTAGTCGAGACTAGGAAGATCACCAAGGTTCTTGTCGCTAAC	<i>Sbfl</i>
KpnI-accBC-as	TTCGGTACCTTACTTGTATCTCGAGGAGAACAACGCCCTTG	<i>KpnI</i>
KpnI-accD1-s	ATAGGTACCTCTAAGGAGGCGTTTCGATGACCATTCTCTACCTTTGATTGACGTC	<i>KpnI</i>
BamHI-accD1-as	CGTGATCCTTACAGTGGCATGTTGCCGTGCTTG	<i>BamHI</i>
chk-pEEx3-s	GCAAATATTCTGAAATGAGCTGTTGACAATTAATCATC	–
chk-pEEx3-as	CGTTCTGATTTAATCTGTATCAGGCTGAAAATCTTCTC	–
chk-sdhCAB-s	GATTTACATAACTTGCAGATAAGCC	–
chk-sdhCAB-as	CGAGGTCAGAGGTCACAATCTC	–
chk-fasR-s	GCGTGCCACTGCGCTACTCCTG	–
chk-fasR-as	CCGTCCCGGACAACATGTACGGCC	–
chk-gltA-s	ATCGTTAACGATCTGACCCAACAA	–
chk-gltA-as	CGTAAGCAGCCTCTGGCGGAA	–

Note. Restriction sites relevant for cloning are underlined.

2.4 | Polyphenol extraction and LC-MS quantification

Polyphenols (naringenin or resveratrol) were extracted from cultivation broth for subsequent LC-MS analysis by mixing 1 ml of the culture broth with 1 ml ethyl acetate and vigorous shaking (1,400 rpm, 10 min, 20°C) in a Thermomixer (Eppendorf). The suspension was centrifuged for 5 min at 13,000 rpm and the ethyl acetate layer (800 µl) was transferred to an organic solvent resistant deep-well plate (Eppendorf). After evaporation of the ethyl acetate overnight, dried extracts were resuspended in the same volume of acetonitrile and directly used for LC-MS analysis. Polyphenols were quantified using an Agilent ultrahigh-performance LC (uHPLC) 1290 Infinity System coupled to a 6130 Quadrupole LC-MS System (Agilent Technologies, Waldbronn, Germany). LC separation was carried out with a Kinetex 1.7 µm C₁₈ 100 Å pore size column (2.1×50 mm; Phenomenex, Torrance, CA) at 50°C. For elution, 0.1% acetic acid (solvent A) and acetonitrile supplemented with 0.1% acetic acid (solvent B) were applied as the mobile phases at a flow rate of 0.5 ml·min^{−1}. A gradient was used, where the amount of solvent B was increased stepwise: minute 0–6: 10–30%, minute 6–7: 30–50%, minute 7–8: 50–100%, and minute 8–8.5: 100–10%. The mass spectrometer was operated in the negative electrospray ionization (ESI) mode, and data acquisition was performed in selected ion monitoring mode. Authentic naringenin and resveratrol standards were purchased from Sigma-Aldrich. Area values for [M-H][−] mass signals were linear up to metabolite concentrations of at least 250 mg·L^{−1}. Benzoic acid (final concentration 100 mg·L^{−1}) was used as internal standard. Calibration curves were calculated based on analyte/internal standard ratios for the obtained area values.

2.5 | Extraction of malonyl-CoA as free acid malonate and quantification using LC-MS/MS

Quenching, metabolite extraction, and sampling for intracellular quantification of malonyl-CoA in form of the free acid malonate were

performed as described previously for phenylpropanoid CoA-thioesters (Kallscheuer, Vogt, Kappelmann et al., 2016). Briefly, 5 ml culture broth of each biological replicate were quenched in 15 ml ice-cold 60% MeOH in ddH₂O (v/v) in triplicates. After centrifugation of the quenched biomass, one part of the quenching supernatant was passed through a 0.2 µm cellulose acetate filter and subsequently stored at −20°C until analysis of malonate concentration. The residual quenching supernatant was carefully discarded from the pelleted biomass. The cell pellet was stored at −80°C until preparation of the cell extract samples. In addition, one part of the culture broth was filtrated as described above of which 250 µl were diluted with 750 µl 60% MeOH. This culture supernatant sample was used to quantify extracellular malonate, which was intentionally allowed to lyse from CoA. Cell extracts, culture supernatant and quenching supernatant samples were separated on an Agilent 1260 Infinity HPLC system (Agilent Technologies) equipped with a Sequant ZIC-pHILIC column (2.1×150 mm) with 5 µm particle size and an appropriate 20 mm guard column (internal diameter 2.1 mm; Merck, Darmstadt, Germany) which were both maintained at 40°C. For LC separation, 10 mM ammonium acetate buffer (pH 9.2, buffer A) and acetonitrile (buffer B) were used. Before each injection, the column was equilibrated for 15 min at 90% B. After 5 µl of each sample was injected, the following gradient was applied: 0 min: 90% B, 1 min: 90% B, 10 min: 70% B, 25 min: 65% B, 35 min: 10% B, 45 min: 10% B, 55 min: 10% B. The LC-eluent was coupled to an ESI-QqTOF MS (TripleTOF 6600; AB Sciex, Darmstadt, Germany) equipped with an IonDrive ion source. Data acquisition was performed using Analyst TF 1.7 (AB Sciex, Concord, ON, Canada).

For quantification, a fully ¹³C-labeled cell extract of *E. coli* was spiked with [Malonyl-¹³C₃]-malonyl-CoA to attain a concentration of approximately 12.5 µM (based on the molar weight of the free acid). The latter contained [U-¹³C₃]-malonate as contamination (data not shown). This compound most probably originates from the spontaneous thioester lysis of [Malonyl-¹³C₃]-malonyl-CoA and served as the internal standard for quantification of malonate.

The internal standard solution was spiked to an equal volume of each sample and an external standard series spanning malonate concentrations from 0.01 to 100 μM in 50% MeOH/ddH₂O was used. A separate external standard series for malonyl-CoA was prepared analogously from the tetra lithium salt of malonyl-CoA acquired from Sigma-Aldrich.

The optimal collision energies for the most intense transitions of malonyl-CoA (852.1 > 79) as well as malonate (103 > 59) were -130 and -11 eV, respectively. These energies were determined from a direct infusion of authentic standards. During the elution, these transitions as well as those of their internal standards (855.1 > 79 and 106 > 61, respectively) were targeted in MS/MS High Sensitivity Mode using the optimal collision energy.

For both metabolites the ^{12}C - ^{13}C isotope ratio was used for quantification. The obtained ratios for malonate were below the linear dynamic range of the calibration curve and no intracellular malonate concentrations could be calculated. Nevertheless, the intensity of the malonate signals with regard to the overall area allowed for a direct comparison of the different strains analyzed. Obtained areas for intracellular malonate were calculated as mean values with standard deviation from six samples in total per strain (two biological replicates with technical triplicates each).

3 | RESULTS

3.1 | Modulation of the endogenous ACC activity alone does not increase polyphenol synthesis

The parental strain *C. glutamicum* DelAro⁴-4cl_{P_C} pMKEx2_chs_{P_P}_chi_{P_P} (designated *C. glutamicum* Nar1) is devoid of catabolic pathways for phenylpropanoids and benzoic acids and was previously engineered to produce naringenin from supplemented *p*-coumaric acid in presence of the fatty acid synthesis inhibitor cerulenin (Kallscheuer, Vogt, Stenzel et al., 2016). For this purpose, genes for the 4-coumarate: CoA ligase (4CL) from parsley (*Petroselinum crispum*) and for the CHS and the chalcone isomerase (CHI) from *Petunia x hybrida* are heterologously expressed. First efforts towards increasing the intracellular malonyl-CoA availability focused on increasing acetyl-CoA carboxylation yielding malonyl-CoA. The native ACC of *C. glutamicum* is a heterodimer consisting of the two subunits AccBC (α -subunit) and AccD1 (β -subunit; Gande et al., 2007). The respective genes *accBC* (cg0802) and *accD1* (*dtsR1*, cg0812) were separately amplified from genomic DNA of *C. glutamicum* and cloned as synthetic operon under control of the *tac* promoter in the expression plasmid pKEEx3 yielding pKEEx3_accB-C_accD1. The strain *C. glutamicum* Nar1 pKEEx3_accBC_accD1 was cultivated in comparison to the initial strain *C. glutamicum* Nar1. However, in the absence of cerulenin, no difference to the control strain with regard to naringenin titers was observable when *accBC* and *accD1* were overexpressed. Both strains accumulated 2 mg·L⁻¹ (0.007 mM) naringenin (data not shown). However, in the presence of 25 μM cerulenin, a naringenin concentration of 26 mg·L⁻¹ (0.095 mM) could be determined for *C. glutamicum* Nar1 pKEEx3_accBC_accD1, whereas the control strain *C. glutamicum* Nar1 accumulated 22 mg·L⁻¹

(0.081 mM) (data not shown). Noteworthy, because the presence of cerulenin inhibits fatty acid synthesis, cell growth stopped one hour after addition of this compound to the culture broth.

In *C. glutamicum*, the transcriptional repressor FasR controls the expression of genes coding for enzymes of the fatty acid synthesis machinery, namely the two fatty acid synthases Fas-IA (cg2743) and Fas-IB (cg0957) as well as AccBC and AccD1 (Nickel et al., 2010). In presence of increased long-chain acyl-CoA concentrations, expression of the FasR target genes is inhibited (Irzik et al., 2014). Hence, it was assumed that deletion of *fasR* (cg2737) would increase *accBC* and *accD1* expression levels and thus also increases the intracellular malonyl-CoA pool. Compared with the attempted plasmid-based overexpression of *accBC* and *accD1*, this could be more beneficial with regard to a balanced expression level of both genes. Interestingly, when cultivated in CGXII medium with 4% glucose, the resulting strain *C. glutamicum* Nar1 Δ *fasR* showed a 35% reduced growth rate (0.22 hr⁻¹) in comparison to the reference strain *C. glutamicum* Nar1 (0.34 hr⁻¹). However, both strain strains reached the same final biomass as determined by the optical density of the cultures (Table 3). When comparing the polyphenol concentrations, differences between both strains were less clear as deletion of *fasR* increased naringenin production from 2 to 4 mg·L⁻¹ (0.007–0.015 mM).

3.2 | Reduced acetyl-CoA consumption in the central carbon metabolism increases malonyl-CoA availability

At this stage, we speculated that an increased ACC activity would have a more pronounced effect on the malonyl-CoA pool, and ultimately on polyphenol production, when the availability of ACC substrate acetyl-CoA would be also increased. This can be achieved by decreasing the overall activity of the TCA cycle in *C. glutamicum* in which acetyl-CoA is oxidized. The citrate synthase (CS) as the pace-making enzyme of the TCA cycle, condensing acetyl-CoA and oxaloacetate to yield citrate, represents a promising target. In contrast to the closely related mycobacteria, *C. glutamicum* has only a single CS, which is encoded by the essential gene *gltA* (cg0949; van Ooyen, Noack, Bott, Reth, & Eggeling, 2012). Hence, deletion of this gene is not a feasible strategy for reducing TCA cycle activity. Instead, expression of *gltA* can be downregulated by exchanging its native promoter to weaker promoter variants. This strategy was already followed previously when *gltA*-promoter engineering in *C. glutamicum* improved oxaloacetate supply, ultimately leading to increased L-lysine titers (van Ooyen et al., 2012). In this particular study, eight synthetic promoter variants of the dihydrodipicolinate synthase gene *dapA* were constructed (Vašicová, Pátek, Nešvera, Sahn, & Eikmanns, 1999). Three of these promoter variants, A16, L1, and C7 reducing CS activity to 32%, 16%, and 10% compared to wild-type CS activity, respectively, were individually introduced into *C. glutamicum* Nar1. The resulting strains *C. glutamicum* Nar1_A16, *C. glutamicum* Nar1_L1 and *C. glutamicum* Nar1_C7 were further characterized with regard to growth behavior and naringenin production. *C. glutamicum* Nar1_A16 with 32% residual CS activity reached the same growth rate and final biomass as the reference strain

TABLE 3 Growth properties and naringenin titers of *C. glutamicum* strains engineered for naringenin production

Strain	Growth rate hr ⁻¹	Final biomass OD ₆₀₀	Naringenin titer mg·L ⁻¹	Normalized naringenin titer mg·L ⁻¹ ·OD ₆₀₀ ⁻¹
<i>C. glutamicum</i> Nar1	0.32 ± 0.01	55.2 ± 1.7	2.1 ± 0.5	0.04
<i>C. glutamicum</i> Nar1 Δ fasR	0.22 ± 0.02	49.6 ± 1.5	3.3 ± 0.2	0.07
<i>C. glutamicum</i> Nar1 Δ sdhCAB	0.27 ± 0.02	28.1 ± 0.7	3.8 ± 0.2	0.13
<i>C. glutamicum</i> Nar1 Δ fasR Δ sdhCAB	0.14 ± 0.02	7.6 ± 0.6	1.9 ± 0.9	0.25
<i>C. glutamicum</i> Nar1_A16	0.32 ± 0.01	51.2 ± 2.1	4.5 ± 1.2	0.09
<i>C. glutamicum</i> Nar1_L1	0.29 ± 0.01	50.8 ± 1.8	10.3 ± 2.4	0.20
<i>C. glutamicum</i> Nar1_C7	0.24 ± 0.02	48.0 ± 0.1	18.5 ± 2.5	0.39
<i>C. glutamicum</i> Nar1_C7 Δ fasR	0.19 ± 0.02	45.2 ± 1.8	12.4 ± 1.7	0.27

The strains were cultivated in 50 ml CGXII medium with 4% glucose and in the presence of 5 mM *p*-coumaric acid. Heterologous gene expression was induced with 1 mM IPTG. All cultivations were performed in baffled shaking flasks, and all data represent average values and standard deviations from three biological replicates.

C. glutamicum Nar1 (Table 3), but showed only a small increase of the overall polyphenol titer (5 mg·L⁻¹ [0.018 mM] naringenin compared with 2 mg·L⁻¹ [0.007 mM] obtained with *C. glutamicum* Nar1; Table 3). Further reduction of CS activity directly correlated with an increased naringenin production. *C. glutamicum* Nar1_L1 (16% residual CS activity) and *C. glutamicum* Nar1_C7 (10% residual activity) were capable of producing 12 mg·L⁻¹ (0.044 mM) and 19 mg·L⁻¹ (0.07 mM) naringenin, respectively (Table 3). All strains with altered *gltA* expression levels reached the same final biomass (OD₆₀₀ of 48–52). *C. glutamicum* Nar1_C7 with 10% residual CS activity was characterized by a growth rate of 0.24 hr⁻¹, which is 70% of the growth rate of the reference strain, while production of naringenin was increased 10-fold to 19 mg·L⁻¹ (0.07 mM). The strain also showed the highest naringenin titer of 0.39 mg·L⁻¹·OD₆₀₀⁻¹ when normalized to biomass (Table 3).

Alternatively, we followed the strategy of deleting the *sdhCAB* (cg0445-47) operon encoding the succinate dehydrogenase complex (SDH) in *C. glutamicum*. This enzyme is not essential for growth of *C. glutamicum* and deletion of this operon was also predicted to have a positive impact on polyphenol synthesis in a recent bioinformatics study using a genome-scale model of this bacterium (Hartmann et al., 2017). As a result of the deletion of *sdhCAB*, growth rate and final biomass of *C. glutamicum* Nar1 Δ sdhCAB were reduced by 20% and 50%, respectively, in comparison to the reference strain (Table 3). Although reaching only half of the maximal biomass, *C. glutamicum* Nar1 Δ sdhCAB accumulated twice as much naringenin (*C. glutamicum* Nar1: 2 mg·L⁻¹ [0.007 mM]; *C. glutamicum* Nar1 Δ sdhCAB: 4 mg·L⁻¹ [0.015 mM]). However, additional plasmid-based expression of *accBC* and *accD1* in the engineered strain *C. glutamicum* Nar1 Δ sdhCAB pEKEx3_accBC_accD1 only moderately increased the naringenin titer to 5 mg·L⁻¹ (0.018 mM) after 48 hr of cultivation.

3.3 | Combination of different metabolic engineering strategies

With the aim of ascertaining whether a combination of individual strategies is suitable to further increase the production of naringenin,

several genetic modifications were combined in one strain. To this end, the double deletion strain *C. glutamicum* Nar1 Δ fasR Δ sdhCAB was constructed to check if simultaneous deregulation of the ACC-encoding genes and reduced activity of the TCA cycle show a synergistic effect on naringenin production. Unfortunately, growth rate, final OD₆₀₀ and naringenin titer of *C. glutamicum* Δ fasR Δ sdhCAB were drastically reduced, while the normalized naringenin titer of 0.25 mg·L⁻¹·OD₆₀₀⁻¹ was higher compared with the parental strain *C. glutamicum* Nar1 (Table 3). The gene *fasR* was also deleted in the best-performing strain *C. glutamicum* Nar1_C7, resulting in a growth rate reduction from 0.24 (*C. glutamicum* Nar1_C7) to 0.19 hr⁻¹ (*C. glutamicum* Nar1_C7 Δ fasR). Unfortunately, also in this strain the naringenin production was negatively affected. *C. glutamicum* Nar1_C7 Δ fasR accumulated 40% less naringenin (12 mg·L⁻¹ [0.044 mM]) compared with *C. glutamicum* Nar1_C7, whereas final biomass concentrations (OD₆₀₀ of 45–48) were similar (Table 3). In contrast, plasmid-based expression of the ACC-encoding genes in the constructed strain *C. glutamicum* Nar1_C7 pEKEx3_accBC_accD1 increased the naringenin production by 40% to 26 mg·L⁻¹ (0.095 mM) compared with *C. glutamicum* Nar1_C7.

3.4 | Quantification of the intracellular malonyl-CoA pool by LC-MS/MS analysis

Hitherto, changes in the intracellular malonyl-CoA levels in the constructed strains could be only evaluated indirectly by determination of the naringenin titer. To confirm that the constructed strains indeed generate higher amounts of malonyl-CoA, we evaluated several reported methods for intracellular malonyl-CoA extraction and quantification. However, in case of *C. glutamicum* these did either not work at all or delivered unreliable results. As a consequence, we established a method for extraction and LC-MS/MS quantification of the intracellular malonyl-CoA pools in the engineered *C. glutamicum* variants, which is based on a method we used earlier for quantifying phenylpropanoid-CoA thioesters (Kallscheuer, Vogt, Kappelmann et al., 2016). On the basis of our calibration curve for quantification, we estimate the limit of detection to be 0.1 μ M. Given this sensitivity, we

FIGURE 2 LC-MS/MS analysis of intracellular malonate concentrations. Areas for malonate acquired by LC-MS/MS analysis of cytoplasmic extracts are plotted against the determined naringenin concentration during cultivation of the respective strains. The plotted naringenin concentrations represent mean values with standard deviations from biological triplicates, whereas the obtained malonate areas represent mean values with standard deviations from biological duplicates with three technical replicates each (six samples in total). Asterisks represent the level of significance of an unpaired two-tailed *t* test ($p < 0.05$) [Color figure can be viewed at wileyonlinelibrary.com]

could not detect any malonyl-CoA directly in cell extracts. Interestingly, larger amounts of malonate were detectable. This acid was also present in authentic malonyl-CoA standards, due to spontaneous degradation. To the best of our knowledge, *C. glutamicum* is not capable of synthesizing or degrading malonate. Therefore, we expect that all malonate detectable in the analyzed samples, solely originates from such spontaneous hydrolysis of malonyl-CoA, and we also assume that higher malonate concentrations are a reflection of intracellular malonyl-CoA levels.

For all experiments, strains without the plasmid pMKEx2_chs_{Ph}_chi_{Ph} were used as any CHS activity would consume malonyl-CoA, thereby distorting the metabolite quantification (Figure 1). Due to the laborious extraction method of intracellular metabolites, only *C. glutamicum* strains L1 and C7 with reduced CS activity, in which corresponding naringenin titers indicated the biggest improvement in malonyl-CoA supply were compared with the reference strain *C. glutamicum* DelAro⁴-4cl_{Pc}. Because the obtained ¹²C-¹³C isotope ratios for the *C. glutamicum* DelAro⁴-4cl_{Pc} and L1 strain were below the limit of quantification, no intracellular malonate concentration could be calculated. Subsequently, the integrated areas of the ¹²C-malonate signal of the extracted samples were adduced to allow for the comparison of all strains. The integrated areas of the malonate signals were plotted against the naringenin titers of the tested *C. glutamicum* strains (Figure 2). For the parental strain *C. glutamicum* DelAro⁴-4cl_{Pc} the measured area was 22,566 ± 6,966, whereas malonate signals for the engineered *C. glutamicum* strains L1 and C7 were determined to be 35,426 ± 6,337 (1.6-fold increase) and 43,065 ± 757 (1.9-fold increase), respectively. A strong linear relationship between naringenin titers and malonate areas could be observed, indicating that the developed methods for extraction and LC-MS/MS analysis of the cytoplasmic malonyl-CoA and malonate indeed reflect the

intracellular concentrations of these important polyphenol precursor molecules.

3.5 | Polyphenol production from glucose and upscaling

The strain *C. glutamicum* Nar1_C7 was chosen for bioreactor cultivations as this strain accumulated the highest naringenin concentrations during shake flask cultivations. During strain engineering for improved malonyl-CoA availability, the naringenin precursor *p*-coumaric acid was always supplemented to exclude any possible limitation of the CHS substrate *p*-coumaroyl-CoA. However, for large-scale fermentations, microbial naringenin production with *C. glutamicum* would be much more economical when starting from cheap glucose. We demonstrated earlier that the plasmid-borne heterologous expression of two additional genes is sufficient for enabling naringenin synthesis from glucose in *C. glutamicum* (Kallscheuer, Vogt, Stenzel et al., 2016). These genes include *aroH* from *E. coli* and *tal* from *Flavobacterium johnsoniae* coding for 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase (the first enzyme of the shikimate pathway) and tyrosine ammonia lyase (TAL), respectively. Expression of *aroH* allows for the production of moderate amounts of L-tyrosine, as AroH of *E. coli* is not feedback-inhibited by this aromatic amino acid, whereas the heterologous TAL catalyzes the subsequent nonoxidative deamination of L-tyrosine yielding *p*-coumaric acid (Kallscheuer, Vogt, Stenzel et al., 2016).

C. glutamicum Nar1_C7 pEKEx3_aroH_{Ec}_tal_{Fj} was cultivated in a bioreactor without supplementation of *p*-coumaric acid. Naringenin production started 3 hr after induction of heterologous gene expression. Until 28 hr of cultivation, the product titer increased only slowly and remained below 5 mg·L⁻¹ (0.018 mM; Figure 3a). During the transition from the late exponential growth phase to the stationary phase (hours 28–35), the naringenin titer increased rapidly from 4 to 20 mg·L⁻¹ (0.015–0.073 mM). The final titer of 24 mg·L⁻¹ (0.088 mM) was reached at the end of the cultivation, 52 hr after inoculation (Figure 3a). In shaking flasks with the same defined medium (only MOPS-buffered), a maximum growth rate of only 0.08 hr⁻¹ and a naringenin titer of 1.7 mg·L⁻¹ (0.006 mM) could be determined (data not shown).

In principle, flavonoids and stilbenes share the same metabolic route, which is also reflected in the requirement of three mol malonyl-CoA/mol product for both compound classes (Figure 1). With the aim to demonstrate that the improvements with regard to intracellular malonyl-CoA availability are transferable from flavonoid synthesis to stilbene synthesis, additional bioreactor cultivations were performed. In this context, *C. glutamicum* Res1_C7 pEKEx3_aroH_{Ec}_tal_{Fj} as the engineered variant with the largest intracellular malonyl-CoA pool was constructed using the plasmid pMKEx2_st-SA_H-4cl_{Pc} conferring the capability to produce the stilbene resveratrol (Kallscheuer, Vogt, Stenzel et al., 2016). In performed bioreactor cultivations the strain reached a final biomass concentration of 8.7 g·L⁻¹ and exhibited a specific growth rate of 0.20 hr⁻¹ (Figure 3b). Resveratrol synthesis set in 3 hr after induction of heterologous gene

FIGURE 3 Microbial production of naringenin and resveratrol from glucose during bioreactor cultivations with engineered *C. glutamicum* strains. (a) Naringenin production from glucose: concentration of glucose and biomass and naringenin titers during batch cultivation of *C. glutamicum* Nar1_C7 pEKEx3_aroH_{Ec}_talF_j. (b) Resveratrol production from glucose: concentration of glucose and biomass and resveratrol titers during batch cultivation of *C. glutamicum* Res1_C7 pEKEx3_aroH_{Ec}_talF_j. The data represent average values and standard deviations from three independent experiments [Color figure can be viewed at wileyonlinelibrary.com]

expression and a final maximum resveratrol concentration of 112 mg·L⁻¹ (0.491 mM) could be determined (Figure 3b). These experiments show that the improvements achieved with regard to the intracellular malonyl-CoA availability are beneficial for flavonoid- as well as stilbene production with *C. glutamicum*, emphasizing the versatility of this platform organism for plant polyphenol synthesis.

4 | DISCUSSION

Currently, microbial synthesis of polyphenols with *C. glutamicum*, as well as with other microorganisms engineered towards producing these compounds, relies on the supplementation of cerulenin, which acts as an inhibitor of fatty acid synthesis (Kallscheuer, Vogt, Stenzel et al., 2016). Although this strategy is often successfully applied in the context of microbial synthesis of various stilbenes, (2S)-flavanones, flavones, and flavonols at lab scale, it was early recognized that any large-scale microbial polyphenol production using cerulenin would be uneconomical due to the high costs of this compound (Milke et al., 2018).

The expression of genes coding for ACCs is a reasonable strategy to increase the endogenous pool of malonyl-CoA, which is the rate-limiting metabolite for polyketide-derived compounds in engineered bacteria. *Streptomyces venezuelae*, an actinobacterium related to *C. glutamicum*, is a natural producer of the antibiotic jadomycin, which requires altogether nine molecules of malonyl-CoA per mol product (Jakeman et al., 2009). Interestingly, in the gene cluster essential for jadomycin synthesis, the gene *jadJ* codes for an ACC α -subunit (Han et al., 2000). Deletion of this gene in *S. venezuelae* severely affected jadomycin production, which underlines the significance of high ACC activity for sufficient malonyl-CoA levels. In the context of engineering microorganisms for polyphenol production, the heterodimeric ACC from *C. glutamicum* was used several times to overcome the tight regulation of endogenous malonyl-CoA synthesis in *E. coli* (Miyahisa et al., 2005; van Summeren-Wesenhausen & Marienhagen, 2015; Zha

et al., 2009; Zhao, Wu, Liu, Qiao, & Zhao, 2018). In *C. glutamicum*, the presence of long-chain acyl-CoA molecules or transition from the exponential phase to the stationary phase leads to downregulation of *accBC* and *accD1* expression (Irzik et al., 2014; Larisch, Nakunst, Hüser, Tauch, & Kalinowski, 2007). In our study, both genes were put under control of the IPTG-inducible *tac* promoter and expressed as a bicistronic transcript with the aim to circumvent this regulation. Alternatively, the gene coding for the fatty acid repressor FasR was deleted, a strategy, which proved to be successful in the past when *C. glutamicum* was engineered for the overproduction of fatty acids (Takeno et al., 2013). Unfortunately, both approaches resulted only in a moderate increase of naringenin biosynthesis. The limited effect of the deletion of *fasR* on naringenin synthesis could be explained by the previous finding that absence of the transcriptional repressor *fasR* in *C. glutamicum* also relieves repression of both genes encoding for the fatty acid synthases Fas-IA and Fas-IB, which consume malonyl-CoA (Nickel et al., 2010). Presumably, this leads to increased fatty acid synthesis and does not necessarily provide more malonyl-CoA for plant polyphenol synthesis.

From the data obtained to this point, we concluded that increased expression of the ACC-encoding genes only leads to an increase in malonyl-CoA synthesis when the acetyl-CoA-level is also increased. In *E. coli*, deletion of the *sdh*-operon was performed to increase acetyl-CoA availability for polyphenol synthesis (Fowler et al., 2009). As a result, naringenin titers increased 1.5-fold. In case of *C. glutamicum*, it has already been described that loss of SDH activity interrupting the TCA cycle results in reduced growth and accumulation of succinate and acetate up to titers of 40 and 125 mM, respectively (Litsanov, Kabus, Brocker, & Bott, 2012). Nonetheless, an SDH-deficient *C. glutamicum* variant was constructed to find out how the loss of SDH activity impacts polyphenol synthesis. Interestingly, naringenin production increased 1.8-fold, but growth rate and overall biomass formation were drastically reduced, rendering this strain unsuitable for further engineering towards microbial polyphenol synthesis. In contrast, reduction of the endogenous CS activity turned out to be

more beneficial as the growth of *C. glutamicum* was less negatively affected and polyphenol concentrations were increased. Reduction of the CS activity already proved to be successful during metabolic engineering of a *C. glutamicum* strain towards microbial L-lysine production (van Ooyen et al., 2012). In this study, reduction of the CS activity increased L-lysine concentration in the culture supernatant from 7.51 to 13.19 g·L⁻¹. The same *C. glutamicum* strain, only differing in the genetic constitution related to product synthesis, was found to produce different naringenin (24 mg·L⁻¹) and resveratrol (112 mg·L⁻¹) concentrations from glucose, although both molecules are derived from the same precursor molecules. This observation is in consistency with a preceding study from our lab, in which synthesis of resveratrol seemed to be more efficient compared with the synthesis of naringenin (Kallscheuer, Vogt, Stenzel et al., 2016).

When comparing microbial resveratrol and naringenin production in *C. glutamicum*, *E. coli* or *S. cerevisiae*, either from supplemented precursors or directly from glucose, resveratrol titers generally seem to exceed the ones of naringenin independent from the utilized host or the heterologous genes used (Chouhan, Sharma, Zha, Guleria, & Koffas, 2017; Jeandet et al., 2018; Milke et al., 2018). Similar observations have also been made for two very similar *E. coli* strains also engineered towards naringenin and resveratrol synthesis (Yang et al., 2015). This suggests that the difference in product concentrations observed might be due to the chemical reactions catalyzed by STS and CHS/CHI, respectively. We calculated the change of the Gibbs free energy for the final reaction of resveratrol and naringenin biosynthesis using the BioCYC database (Caspi et al., 2007; Caspi et al., 2015) to obtain the free energy for all metabolites (substrates and products) involved. Indeed, we found that the final step of the resveratrol biosynthesis appears to be thermodynamically more favorable compared to the last step of naringenin biosynthesis ($\Delta G^{\circ} = -70$ kJ·mol⁻¹ for resveratrol compared with $\Delta G^{\circ} = +46$ kJ·mol⁻¹ for naringenin). This difference is due to an additional molecule of CO₂ released during resveratrol synthesis and might help to explain this interesting observation.

Taken together, by reducing the CS activity in the parental strain *C. glutamicum* DelAro⁴-4cl_{PC}, dependency on the costly antibiotic cerulenin for efficient polyphenol synthesis could be overcome. As demonstrated by the applicability of the best performing *C. glutamicum* DelAro⁴-4cl_{PC}-C7 strain for both, flavonoid and resveratrol synthesis, it can be excluded that the observed improvements regarding malonyl-CoA availability are for any reason limited to naringenin synthesis only. Thus, the *C. glutamicum* DelAro⁴-4cl_{PC}-C7 strain constructed in this study represents a promising starting point for further tailoring this bacterium towards increased microbial plant polyphenol synthesis.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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