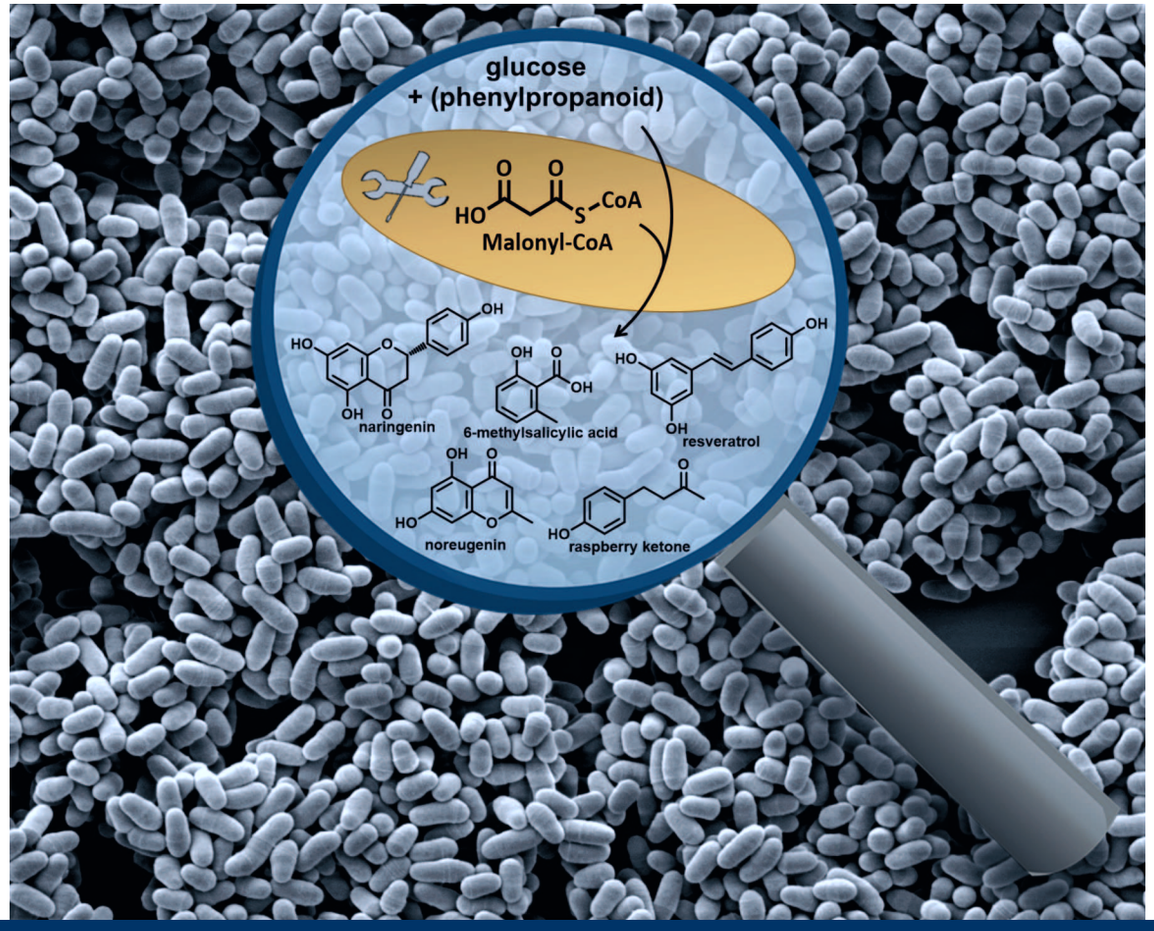

Engineering of *Corynebacterium glutamicum* towards increased malonyl-CoA availability for polyketide synthesis

Lars Milke

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Abstract

Polyketides are a structurally highly diverse group of natural products with interesting health-promoting effects on humans. Despite all structural differences, polyketides are synthesized from simple CoA-activated carboxylic acid derivatives, such as acetyl-CoA or malonyl-CoA following a mechanism closely related to fatty acid biosynthesis. Unfortunately, polyketides are only synthesized in small quantities by the respective native organism. In contrast, microbial polyketide synthesis using engineered bacteria is a promising approach to get access to the desired products. Against this background, *Corynebacterium glutamicum* strains for the production of different plant polyketides such as stilbenes and flavonoids have been constructed recently. However, it soon became evident that the intracellular availability of malonyl-CoA is limiting the overall product formation in these strains. Hence, the main goal of this thesis was to optimize the intracellular malonyl-CoA availability in *C. glutamicum* by metabolic engineering. Additionally, the tailored strains should be used for establishing synthesis of biotechnological interesting polyketides. The following results were obtained:

1) Reduction of the citrate synthase activity to 5.5 % compared to the *C. glutamicum* wild type by exchanging the promotor of the encoding *gltA* gene, reduced acetyl-CoA consumption via the tricarboxylic acid cycle, which in turn improved malonyl-CoA availability. Upon transcriptional deregulation of *accBC* and *accD1* encoding the two subunits of acetyl-CoA carboxylase, malonyl-CoA synthesis from acetyl-CoA was drastically improved allowing for the synthesis of 65 mg/L (0.24 mM) naringenin und 450 mg/L (1.97 mM) resveratrol. Furthermore, improving the glucose uptake and elimination of anaplerotic pyruvate carboxylation reaction further contributed to an improved intracellular malonyl-CoA availability in the ultimately constructed strain *C. glutamicum* M-CoA.

2) Through episomal expression of genes encoding heterologous type III polyketide synthases from various plant species in the constructed strain *C. glutamicum* M-CoA, microbial synthesis of a pentaketide (noreugenin) but also phenylbutanoids (raspberry ketone, zingerone, benzylacetone) with a *ldhA*-deficient variant could be established. The respective strains allowed for the synthesis of up to 53.3 mg/L (0.28 mM) noreugenin, 100 mg/L (0.61 mM) raspberry ketone, 70 mg/L (0.36 mM) zingerone and 10.5 mg/L (0.07 mM) benzylacetone from simple precursor molecules, respectively.

3) Hitherto, only type III polyketides can be synthesized by engineered *C. glutamicum* strains. In the context of this study, functional expression of a codon-optimized gene variant encoding the type I polyketide synthase 6-methylsalicylic acid synthase ChlB1 from *Streptomyces antibioticus* of 1,756 amino acids size was achieved. This allowed for the synthesis of up to 41 mg/L (0.27 mM) 6-methylsalicylic acid. It was found that *C. glutamicum* has an endogenous phosphopantetheinyltransferase activity, which can post-translationally activate ChlB1. This makes *C. glutamicum* a promising host for the production of other interesting type I polyketides.

Zusammenfassung

Polyketide sind eine Gruppe strukturell vielfältiger Naturprodukte mit interessanten gesundheitsfördernden Eigenschaften für den Menschen. Trotz aller strukturellen Unterschiede werden Polyketide aus einfachen CoA-aktivierten Carbonsäurederivaten wie Acetyl-CoA oder Malonyl-CoA nach einem mit der Fettsäurebiosynthese eng verwandten Mechanismus synthetisiert. Leider werden Polyketide im jeweiligen nativen Organismus nur in geringen Mengen produziert. Im Gegensatz dazu ist die heterologe mikrobielle Synthese mit entwickelten Bakterienstämmen ein vielversprechender Ansatz, um Zugang zu den gewünschten Produkten zu erhalten. In diesem Zusammenhang wurden kürzlich *Corynebacterium glutamicum* Stämme zur Synthese verschiedener pflanzlicher Polyketide wie Stilbene und Flavonoide konstruiert. Es zeigte sich jedoch bald, dass die intrazelluläre Verfügbarkeit von Malonyl-CoA die gesamte Produktbildung in diesen Stämmen limitiert. Daher war das Hauptziel dieser Arbeit die Optimierung der intrazellulären Malonyl-CoA Verfügbarkeit in *C. glutamicum* durch *metabolic engineering*. Darüber hinaus sollten die maßgeschneiderten Stämme für die Etablierung der Synthese biotechnologisch interessanter Polyketide verwendet werden. Folgende Ergebnisse wurden erzielt:

1) Die Verringerung der Citrat-Synthase Aktivität auf 5,5 % im Vergleich zum *C. glutamicum* Wildtyp durch Promotoraustausch des kodierenden *gltA*-Gens, reduzierte den Verbrauch von Acetyl-CoA durch den Tricarbonsäurezyklus und verbesserte damit die Malonyl-CoA Verfügbarkeit. Durch die transkriptionelle Deregulierung von *accBC* und *accD1*, die für die zwei Untereinheiten der Acetyl-CoA Carboxylase kodieren, wurde die Malonyl-CoA-Synthese ausgehend von Acetyl-CoA drastisch verbessert, sodass letztendlich 65 mg/L (0,24 mM) Naringenin und 450 mg/L (1,97 mM) Resveratrol synthetisiert werden konnten. Darüber hinaus trugen die Verbesserung der Glukoseaufnahme und die Beseitigung der anaplerotischen Pyruvatcarboxylierungsreaktion weiterhin zu einer verbesserten Malonyl-CoA Verfügbarkeit im konstruierten Stamm *C. glutamicum* M-CoA bei.

2) Durch episomale Expression von Genen, die für heterologe Typ III Polyketid Synthasen aus verschiedenen Pflanzenarten im konstruierten Stamm *C. glutamicum* M-CoA, konnte die mikrobielle Synthese eines Pentaketids (Noreugenin) aber auch von Phenylbutanoiden (Himbeerketon, Zingeron, Benzylaceton) mit einer *ldhA*-defizienten Variante nachgewiesen werden. Die jeweiligen Stämme erlaubten die Synthese von bis zu 53,32 mg/L (0,28 mM) Noreugenin, 100 mg/L (0,61 mM) Himbeerketon, 70,03 mg/L (0,36 mM) Zingeron und 10,5 mg/L (0,07 mM) Benzylaceton aus einfachen Vorläufermolekülen.

3) Bisher können nur Typ III Polyketide mit *C. glutamicum*-Stämmen synthetisiert werden. Im Rahmen dieser Studie wurde die funktionelle Expression einer codon-optimierten Genvariante erreicht, die für die Typ I Polyketid Synthase 6-Methylsalicylsäure Synthase ChlB1 aus *Streptomyces antibioticus* von 1756 Aminosäuren Größe kodiert. Dies ermöglichte die Synthese von 41 mg/L (0,27 mM) 6-Methylsalicylsäure. Weiterhin wurde festgestellt, dass *C. glutamicum* eine endogene Phosphopantetheinyltransferase besitzt, die ChlB1 post-translational aktivieren kann. Dies macht *C. glutamicum* zu einem vielversprechenden mikrobiellen Wirt für die Synthese weiterer interessanter Typ I Polyketide.

Abbreviations

4CL	4-coumarate: CoA ligase
6-MSA	6-methylsalicylic acid
aa	Amino acids
ACC	Acetyl-CoA carboxylase
ACP	Acyl carrier protein
asRNA	Antisense RNA
AT	Acyl transferase
BAR	Benzalacetone reductase
BAS	Benzalacetone synthase
BC	Biotin carboxylase
BCCP	Biotin carboxyl carrier protein
BHI	Brain heart infusion
C4H	Cinnamic acid-4-hydroxylase
CHI	Chalcone isomerase
CHS	Chalcone synthase
CoA	Coenzyme A
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRi	CRISPR interference
CS	Citrate synthase
CT	Carboxyltransferase
DAHP	3-deoxy-D-arabinoheptulosonate 7-phosphate
DH	Dehydratase
DMSO	Dimethyl sulfoxide
DS	DAHP synthase
ER	Enoyl reductase
ESI	Electrospray ionization
FACS	Fluorescence activated cell sorting
FAS	Fatty acid synthase
GAPDH	NAD^+-dependent glyceraldehyde 3-phophate dehydrogenase
HPLC	High-performance liquid chromatography
IC_{50}	half maximal inhibitory concentration of a specific compound
IPTG	Isopropyl-β-D-thiogalactopyranosid
kDa	Kilodalton
KR	Keto reductase
KS	β-keto synthase
LB	Lysogeny broth
LC-MS	Liquid chromatography with mass spectrometry coupling
LC-MS/MS	Liquid chromatography with tandem mass spectrometry coupling
m/z	Mass-to-charge ratio
MDa	Megadalton
MOPS	3-morpholino propanesulfonic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

NMR	Nuclear magnetic resonance
NRPS	Nonribosomal peptide synthetases
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PAL	Phenylalanine ammonia lyase
*p*CA	*p*-coumaric acid
PCS	Pentaketide chromone synthase
PEP	Phosphoenolpyruvate
PEPC	PEP carboxylase
*p*HBA	*p*-hydroxybenzalacetone
Phe	L-phenylalanine
PKS	Polyketide synthase
PPTase	Phosphopantetheinyltransferase
PYC	Pyruvate carboxylase
RK	Raspberry ketone
RPM	Rounds per minute
RZS1	Raspberry ketone/zingerone synthase
SAM	*S*-adenosyl methionine
SDH	Succinate dehydrogenase
SIM	Selected ion monitoring
sRNA	Small regulatory RNA
STS	Stilbene synthase
TAL	Tyrosine ammonia lyase
TCA	Tricarboxylic acid
TPBD	1-(2,4,6-trihydroxyphenyl)butane-1,3-dione
Tyr	L-tyrosine
XAL	Aromatic amino acid ammonia lyase

1 Scientific context and key results of this thesis

1.1 Polyketides – a highly diverse group of natural products with pharmacological relevance

Even though more than 200,000 plant natural products are currently known – and probably many more are to be identified in the future – the group of polyketides is outstanding with regard to their structural diversity and the number of their clinical applications (Hopwood, 2009; Osbourn & Lanzotti, 2009; Robertsen & Musiol-Kroll, 2019; Wink, 2010). Polyketides comprise antibiotics (erythromycin A, azithromycin), anticancer drugs (enediynes), drugs for cardiovascular diseases (statins) and immunosuppressants (rapamycin) (Figure 1). Amongst known polyketides, more than 20 have been marketed, resembling a success rate of 0.3 % compared to standard pharmaceutical screenings (<0.001 %). In total, polyketide-derived pharmaceuticals make up 20 % of the top-selling drugs, generating a worldwide revenue of over 14 billion €, annually (Weissman & Leadlay, 2005).

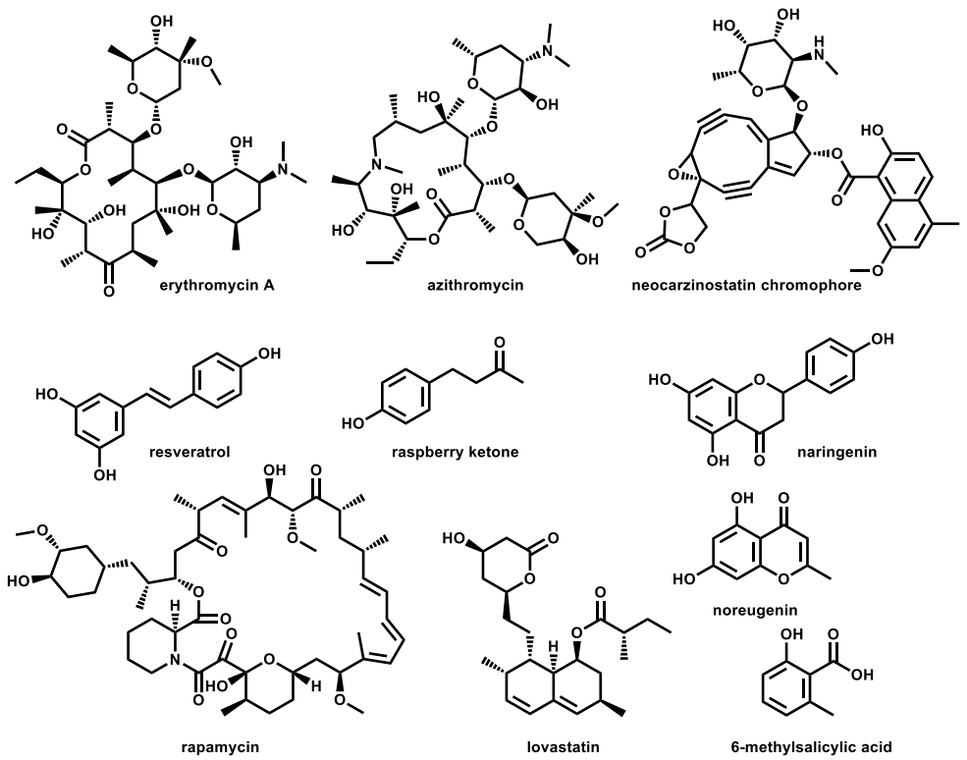

Figure 1: Chemical structure of various polyketides.

Despite their broad structural variety, all polyketides are synthesized by a mechanism closely related to fatty acid biosynthesis using simple CoA-activated carboxylic acid derivatives. Although first predictions on the synthesis mechanism of polyphenols, a major subgroup of polyketides, by the chemist James Collie around 1900 were accurate, Arthur Birch confirmed Collie's predictions not before the 1950s (Birch *et al.*, 1955; Birch & Donovan, 1953; Staunton & Weissman, 2001). By using the at that time novel technique of radionuclear labelling, he could demonstrate that 6-methylsalicylic acid (6-MSA) synthesis in *Penicillium patulum* originates from *the head-to-tail linkage of acetic acid units*. With the advent of NMR technology, scientist gained detailed insights into polyketide biosynthesis so that currently over 10,000 structures have been elucidated.

Soon it became clear, that the formed β-ketoacyl chain must be folded *in vivo* under enzymatic control to prevent premature cyclization at early stages of chain elongation (Harris & Harris, 1977; Harris & Wittek, 1975). In fact, with regard to the overall enzyme architecture, there are remarkable homologies between polyketides synthases (PKSs) and fatty acid synthases (FASs) as involved enzymes. Thus, proceedings in one field often had immediate effects on the other (Smith & Tsai, 2007).

Due to the similarities to fatty acid synthesis, the same nomenclature was adopted to classify PKSs (Austin & Noel, 2003). Based on the architecture and respective assembly lines, PKSs are classified into type I, II and III families. Mutuality of all PKSs is a β-keto synthase (KS) activity that catalyzes the sequential head-to-tail incorporation of two-carbon acetate units into a growing polyketide chain. Whereas the type I family comprises giant multifunctional polypeptides with multiple domains catalyzing the individual reaction steps, type II PKSs are enzyme complexes of dissociable, usually monofunctional enzymes (Hertweck, 2009). In accordance to FAS classification, type I PKSs predominantly occur in fungi and animals, whereas type II PKSs are commonly found in prokaryotes, especially actinomycetes.

The general reaction scheme of type I and II PKSs involves a priming substrate, usually an acetyl or propionyl moiety derived from the respective CoA-thioester. The acyl group is transferred from the CoA moiety to the prosthetic phosphopantetheine group of the acyl carrier protein (ACP) domain by an acyl transferase (AT) domain (Figure 2). The essential post-translational phosphopantetheinylation of the ACP domain is catalyzed by a phosphopantetheinyl transferase (PPTase), also required for the activation of fatty acid synthases (Beld *et al.*, 2014). This phosphopantetheinyl residue serves as a flexible arm that moves the substrates and intermediates between the different the individual active sites of the PKS (Staunton & Weissman, 2001).

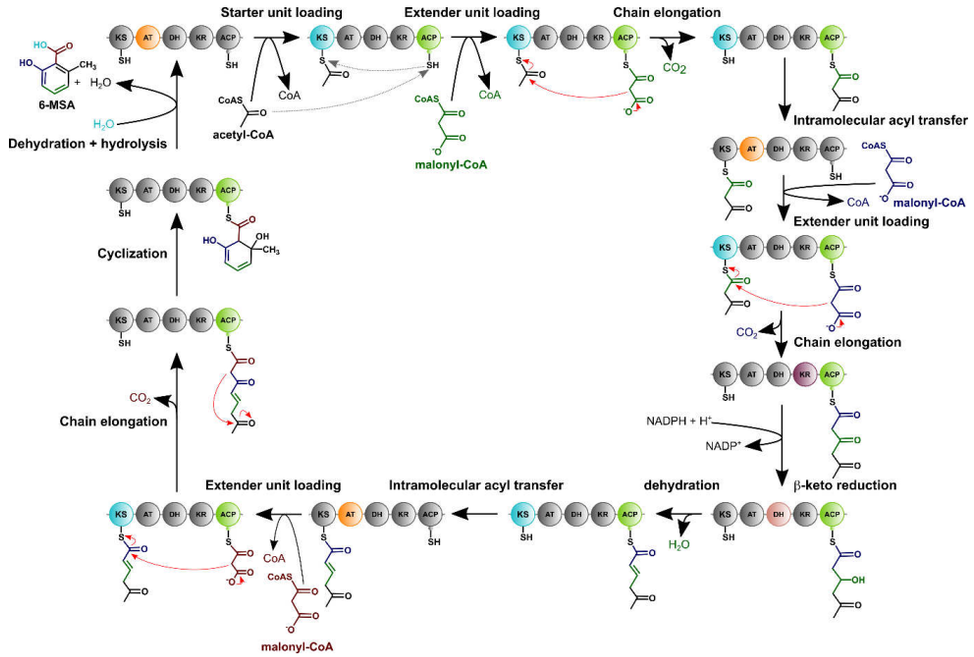

Figure 2: Schematic representation of the individual reaction steps of a typical type I polyketide synthase, here the 6-methylsalicylate synthase ChlB1 from *Streptomyces antibioticus*. ACP: acyl carrier protein, AT: acyl transferase, DH: dehydratase, KR: ketoreductase, KS: β-keto synthase. The smaller green sphere attached to the ACP domain represents the phosphopantetheinyl residue.

Subsequently, the ACP-bound starter unit is transferred to the active site cysteine of the KS domain by the inherent transferase function of this domain leaving a free phosphopantetheinyl residue of the ACP domain. This starter substrate is subsequently elongated by repetitive decarboxylative Claisen condensations of malonyl or methylmalonyl extender units, creating a growing β-ketoacyl chain. In detail, the AT transfers a malonyl extender moiety from the thioester onto the free ACP. The KS domain catalyzes the Claisen condensation of the loaded starter and the delivered extender unit, driven by decarboxylation of the latter, resulting in a β-ketoacyl product bound to the ACP. Subsequently, optional processing of the β-carbon by ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) domains generates a saturated β-ketoacyl backbone incorporating complexity into the growing polyketide chain (Hertweck, 2009). After the first round of elongation is completed, these steps are repeated until a defined β-ketoacyl chain length is reached. Finally, the β-ketoacyl chain is released by a thioesterase (TE) domain from the enzyme complex and may be further modified.

In contrast to large and multi-domain type I and II PKSs, homodimeric type III PKSs manage to define starter unit selection, chain elongation and cyclization patterns – despite their structural simplicity – solely by enzyme cavities and the architecture of a single KS-like active

site (Austin & Noel, 2003; Hopwood, 2009). Interestingly, type III PKSs show a much broader acceptance of CoA-activated starter units, for instance phenylpropanoid thioesters such as *p*-coumaroyl-CoA, allowing for the generation of considerably great structural diversity. Type III PKSs are usually found in plants, but also bacterial and fungal enzymes have been identified in the past decade (Moore & Hopke, 2001; Pfeifer *et al.*, 2001; Seshime *et al.*, 2005).

As this dissertation focusses on the application of type I and III PKSs, relevant examples and peculiarities are described in more detail in the following chapters.

1.2 Polyketide synthases

1.2.1 Type I polyketide synthases are large multi-domain enzymes

The family of type I PKSs is subdivided into iterative and non-iterative or modular PKSs. In iterative PKSs, the KS domain catalyzes more than one round of chain elongation. Depending on the presence of β-ketoacyl processing domains, type I PKSs can be further classified into non-reducing, partially reducing and highly reducing subfamilies. Although typically found in fungi, there are examples of bacterial iterative type I PKSs such as the 6-methylsalicylic acid synthase ChlB1 from *Streptomyces antibioticus* harboring characteristic KS, AT, DH, KR and ACP domains in a single polypeptide chain of 1,756 amino acids (Shao *et al.*, 2006). It has been shown that this enzyme synthesizes 6-MSA from one molecule of acetyl-CoA and three malonyl-CoA extender units, also partially reducing the nascent polyketide chain (Figure 2).

In contrast, non-iterative so called megasynthases of bacterial origin consist of several individual elongation modules, each harboring KS, AT and ACP and optional β-keto processing domains (Hertweck, 2009). According to the *principle of colinearity*, one module catalyzes only a single elongation step, allowing for deducing the polyketide structure from the enzyme architecture and vice versa. These class of PKSs is notorious for harboring gigantic enzyme complexes such as the erythromycin PKS from *Saccharopolyspora erythraea*, which consists of three individual multienzyme polypeptides each of ~350 kDa size (Staunton & Weissman, 2001). Even this PKS is easily surpassed by the size of the PKS machinery involved in the biosynthesis of the immunosuppressant rapamycin in *Streptomyces hygroscopicus* (Schwecke *et al.*, 1995). In total, 70 active sites divided into 14 modules with a total size of 2.63 MDa make this PKS one of the largest enzyme complexes known to date.

1.2.2 Small but versatile – type III polyketide synthases

Type III PKSs, also known as the chalcone synthase (CHS) / stilbene synthase (STS) superfamily, resemble a class of ubiquitous enzymes found in higher plants. CHSs catalyze the initial step in flavonoid biosynthesis, resembling a major subfamiliy of polyphenols besides stilbenoids, which are synthesized by STSs (Austin & Noel, 2003).

Derived from the aromatic amino acid metabolism, L-phenylalanine or L-tyrosine are initially non-oxidatively deaminated *in planta* (Figure 3). Catalyzed by phenylalanine ammonia lyases (PAL) or tyrosine ammonia lyases (TAL), the phenylpropanoids cinnamic acid or *p*-coumaric acid are yielded, respectively. In plants however, this reaction is often catalyzed by a bifunctional enzyme, where PAL activity dominates (Barros *et al.*, 2016). Following deamination of L-phenylalanine, cinnamic acid is hydroxylated by a cytochrome P450-dependent cinnamic acid-4-hydroxylase (C4H) generating *p*-coumaric acid (Rodrigues *et al.*, 2015; Watts *et al.*, 2004). Subsequently, phenylpropanoids are CoA-activated in an ATP-dependent reaction by a 4-coumarate: CoA ligase (4CL). Finally, by iterative decarboxylation and condensation of three malonyl-CoA extender units with a starting *p*-coumaroyl-CoA, the bicyclic naringenin chalcone is formed by CHS, which is subsequently cyclized to the tricyclic (2*S*)-flavanone naringenin by a chalcone isomerase (CHI) (Jez & Noel, 2002).

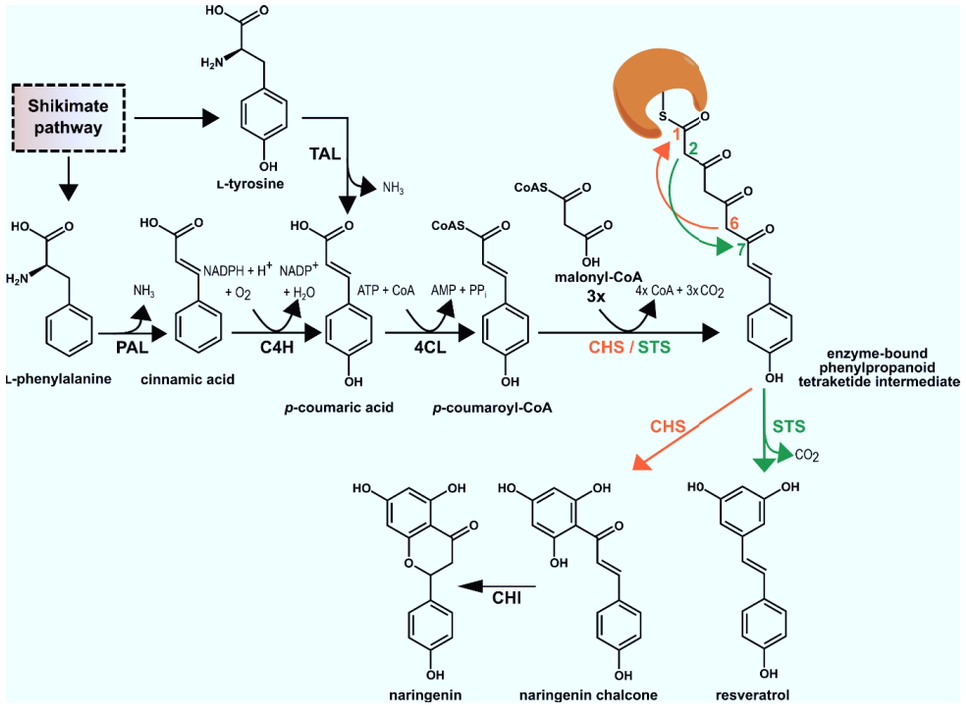

Figure 3: Synthesis of *p*-coumaric acid from aromatic amino acids L-tyrosine and L-phenylalanine and subsequent synthesis of stilbenes and chalcones catalyzed by type III PKSs. The intramolecular C6-C1 Claisen condensation catalyzed by CHS is indicated by an orange arrow; the intramolecular C2-C7 Aldol condensation catalyzed by an STS is indicated by a green arrow. 4CL: 4-coumarate: CoA ligase, C4H: cinnamic acid 4-hydroxylase (cytochrome P450 monooxygenase), CHI: chalcone isomerase, CHS: chalcone synthase, PAL: phenylalanine ammonia lyase, STS: stilbene synthase, TAL: tyrosine ammonia lyase.

From here, flavonoids branch into the different subgroups, which are utilized by plants for the purpose of antimicrobial defense, protection against UV radiation, petal pigmentation and also induction of symbiotic root nodulation with *Rhizobium* bacteria (Austin & Noel, 2003). Due to their antioxidant characteristics, polyphenols in general demonstrate a plethora of beneficial effects on human health including anti-inflammatory or anti-cancerous traits, prevention or treatment of cardiovascular and neurodegenerative diseases, diabetes but also obesity (Khurana *et al.*, 2013; Pandey & Rizvi, 2009). In this context, naringenin in particular is a promising therapeutic agent as demonstrated by *in vitro* and *in vivo* animal studies but also first clinical trials (Salehi *et al.*, 2019; Zaidun *et al.*, 2018).

Type III PKSs strictly differ from their large type I and II counterparts, not only by their unique architecture, but also by the absence of phosphopantetheinylation required for enzymatic activity or even ACP domains (Abe & Morita, 2010; Austin & Noel, 2003). It is assumed that the CHS/STS superfamily originates from a gain of function mutation from a homodimeric KS domain of modular type II FAS, originally catalyzing the initial chain elongation generating C_4 intermediates (Austin & Noel, 2003; Jaworski *et al.*, 1989; Walsh *et al.*, 1990). Besides the catalytic cysteine, an asparagine and a histidine residue are involved in the characteristic formation of a catalytic C-N-H triad (Ferrer *et al.*, 1999). The reaction sequence is initiated by binding of the *p*-coumaroyl moiety to the nucleophilic cysteine. Subsequently, the asparagine and histidine residues facilitate correct orientation and decarboxylation of malonyl-CoA providing the prerequisite for following chain elongation. This reaction is repeated until a tetraketide intermediate is formed, which is subsequently cyclized by intramolecular C6-C1 Claisen condensation yielding naringenin chalcone (Figure 3) (Ferrer *et al.*, 1999; Jez *et al.*, 2000).

Despite producing the same tetraketide intermediate from *p*-coumaroyl-CoA and three molecules of malonyl-CoA, STSs generate structurally clearly different molecules than CHSs. In contrast to CHSs, STSs catalyze an intramolecular C2-C7 aldol cyclization yielding bicyclic stilbenes such as resveratrol (Figure 3) (Austin *et al.*, 2004; Shomura *et al.*, 2005). Unlike predicted to be under steric control, aldol cyclization was demonstrated to be rather electronically mediated. Whereas C6-C1 cyclization automatically results in the cleavage of the C1 thioester bond, C2-C7 cyclization requires not only a thioesterase-like hydrolysis step to cleave the C1 thioester linkage from the catalytically active cysteine but also an additional decarboxylation. In STSs, a hydrogen bonding network generating a hydroxide anion – termed as aldol switch – was shown to have a thioesterase-like activity promoting the hydrolytic cleavage of the C1 thioester bond.

Resveratrol is a good example for the pharmacological potential of polyphenols. Besides mentioned anti-cancerous and antidiabetic characteristics, antioxidant properties of resveratrol

have been used to explain the ‘French paradox’, which links the moderate consumption of red wine, usually characterized by a high resveratrol content (1 – 5 mg/L), to low mortality caused by cardiovascular diseases of the South European population (Frankel *et al.*, 1993; Renaud & De Lorgeril, 1992; Vestergaard & Ingmer, 2019). However, subsequent research queried this explanation as the consumed dose of resveratrol seems to be insufficient to explain this paradox. In general, it is doubtful whether the French paradox exists or if it is actually a statistical illusion. (Goldberg *et al.*, 2003; Law & Wald, 1999; Rimm *et al.*, 1996) Nonetheless, this led to a successful marketing of resveratrol as a dietary supplement (Catalgol *et al.*, 2012).

Besides STSs, additional functionally divergent type III PKSs emerged from CHSs during evolution (Abe & Morita, 2010). Not only the number of iterative elongation cycles, but also the range of accepted CoA-thioester starter units has evolved. Among these emerged type III PKSs are the benzalacetone synthase (BAS) from Chinese rhubarb (*Rheum palmatum*) as well as the pentaketide chromone synthase (PCS) from the medicinal plant *Aloe arborescens*. The following examples illustrate the importance of the active site cavity architecture for starter unit selection, chain elongation and cyclization patterns.

BAS from *R. palmatum* was demonstrated to catalyze the condensation of *p*-coumaroyl-CoA and one molecule of malonyl-CoA generating the diketide *p*-hydroxybenzalacetone (*p*HBA), which is the precursor for phenylbutanoids such as raspberry ketone (Figure 1) (Abe *et al.*, 2001; Borejsza-Wysocki & Hrazdina, 1996, 1994). Raspberry ketone has not only gained commercial relevance as a natural flavoring agent providing the typical scent and flavor of ripe raspberries, but also as a dietary and cosmetic supplement (Beekwilder *et al.*, 2007; Kim *et al.*, 2016; Morimoto *et al.*, 2005). Low abundance in raspberries and legal regulations are the reason for the high costs of natural raspberry ketone creating a potential market value of 6 to 10 million € (European Council, 2008; Häkkinen *et al.*, 2015; Vandamme & Soetaert, 2002). Opposed to all known CHSs, a gatekeeping phenylalanine residue probably involved in *p*-coumaroyl-CoA orientation and facilitating decarboxylation is uniquely replaced by leucine (F208L) in BAS from *R. palmatum* (T. Abe *et al.*, 2007). This impedes proper locking of *p*-coumaroyl-CoA in the traditional binding pocket. Instead, a novel active site pocket locks the aromatic moiety. Mutagenesis studies of this particular phenylalanine residue demonstrated restoration of CHS activity, suggesting to be involved in the termination of chain elongation at the diketide stage (T. Abe *et al.*, 2007; Shimokawa *et al.*, 2010).

Yet another interesting example for the evolutionary divergence of type III PKS is the PCS from *A. arborescens*, catalyzing the iterative decarboxylative condensation of five malonyl-CoA molecules yielding one molecule of the pentaketide noreugenin, which serves as precursor for the synthesis of the antiasthmatic furochromones khellin and visnagin (Figure 1) (Abe *et al.*, 2005; Dewick, 2002). Although the catalytic C-N-H triad is conserved in PCS and

p-coumaroyl-CoA is accepted *in vitro* as CoA-thioester starter unit, PCS only produces triketide and tetraketide α–pyrones bisnoryangionin and *p*-coumaroyl triacetic acid lactone in contrast to naringenin chalcone synthesized by CHSs (Abe *et al.*, 2005). Extensive elucidation of the PCS active site cavity not only demonstrated a three-fold smaller cavity volume compared to the CHS from *Medicago sativa*, but also a glycine to leucine amino acid substitution (G266L) appearing to be specific for utilizing acetyl- or malonyl-CoA starter units (Morita *et al.*, 2007). Moreover, amino acid substitutions drastically expanding the active site cavity volume were demonstrated to allow for the synthesis octaketides and a nonaketide (I. Abe *et al.*, 2007). These findings clearly support the idea that enzyme cavities and architectures of type III PKSs, often drastically influenced by single amino acid substitutions, are of utter importance for starter unit selection, chain elongation and cyclization.

1.3 Access to polyketides

Common limitation for further pharmacological investigations and/or marketing of type I - and III polyketides is often the extraction of the respective compound from its natural sources such as plants (Chemler & Koffas, 2008). Polyketides tend to accumulate to low quantities, which are further subject to seasonal and regional changes. A further complication is that the desired polyketide must be separated from a large number of structurally similar molecules. On the other hand, total chemical synthesis routes exist, but are typically commercially unfeasible considering the increasing complexity of polyketide structures (Marienhagen & Bott, 2013).

A promising third route to obtain polyketides of interest, is the synthesis with engineered microbial hosts. Microbial synthesis is not only based on inexpensive renewable feedstocks, it furthermore profits from rapid microbial growth, which drastically reduces the overall process time. In addition, microbial synthesis is usually more environmentally friendly opposed to chemical synthesis, as it avoids using organic solvents, heavy metal catalysts and strong acids or bases (Marienhagen & Bott, 2013).

1.4 Current status of microbial polyketide synthesis

Despite having numerous advantages over traditional extraction from plant material and chemical synthesis, microbial polyketide production features its own challenges. These are either specific for certain PKS subfamilies, but can also be limiting for polyketide synthesis in general. This includes the construction of biosynthetic pathways in the microbial host as well as tailoring the metabolism of this host towards desired needs, such as improved precursor availability (Lussier *et al.*, 2012).

Type III PKS such as CHS, STS and BAS, rely on phenylpropanoid-derived CoA-thioester starter units. In plants, *p*-coumaric acid is mainly derived from deamination and hydroxylation

of L-phenylalanine by PAL and C4H. Currently, no C4H enzyme has been functionally expressed in prokaryotic organisms (Rodrigues *et al.*, 2015; Watts *et al.*, 2004). Hence, heterologous TAL activity for the formation of *p*-coumaric acid by deaminating L-tyrosine has been established in microorganisms (Kallscheuer *et al.*, 2016b; Leonard *et al.*, 2008; Wu *et al.*, 2014b; Xu *et al.*, 2011). Here, prerequisite for enabling efficient *p*-coumaric acid supply by a TAL activity, is to ensure availability of free L-tyrosine. Similar to other amino acid biosynthetic pathways, the shikimate pathway generating aromatic amino acids is mostly feedback-regulated by inhibition of the 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, which catalyzes the initial step of the shikimate pathway (Herrmann & Weaver, 1999; Ikeda, 2006). This regulation is usually circumvented by the application of feedback-resistant isoenzymes, which allow for high fluxes through the desired pathways (Kallscheuer *et al.*, 2016b; Luttik *et al.*, 2008; Ray *et al.*, 1988). Unfortunately, even upon L-tyrosine supplementation, low enzymatic activity of both heterologous PALs and TALs is regarded as a bottleneck in the production of phenylpropanoid-derived polyketides (Eudes *et al.*, 2013; Kallscheuer *et al.*, 2016b; Lin & Yan, 2012; Zhou *et al.*, 2016). To overcome this, directed evolution of an aromatic amino acid ammonia lyase (XAL) by combination of transcriptional biosensors and fluorescence activated cell sorting (FACS) was performed to isolate PAL variants with improved activity in *Escherichia coli* and *in vitro* (Flachbart *et al.*, 2019). The advent of such techniques enables fast identification of muteins with desired characteristics. To date, these two bottlenecks are usually circumvented by the supplementation of *p*-coumaric acid.

In general, metabolization of pathway intermediates by the host organism is detrimental for product synthesis. In this context, the *phd* gene cluster, enabling utilization of phenylpropanoids such as *p*-coumaric acid or ferulic acid as sole carbon and energy source in *C. glutamicum*, was identified during metabolic engineering towards polyphenolic polyketide synthesis (Kallscheuer *et al.*, 2016a). Deletion of this catabolic pathway was prerequisite for enabling polyphenolic polyketide synthesis using this bacterium (Kallscheuer *et al.*, 2016b). In *E. coli* the transport of phenylpropanoid precursors is considered to be subject to catabolic repression (Watts *et al.*, 2006).

In contrast to type III PKSs, type I PKSs feature a distinct ACP domain, that requires to be post-translationally phosphopantetheinylated by a PPTase for activity (Pfeifer & Khosla, 2001). Although PPTases are present in all domains of life, perhaps with exception of archaea, the individual enzymes have different specific configurations or act on a varying range of polypeptides (Beld *et al.*, 2014). Whereas the PPTase from *E. coli* has a relatively tight selectivity, PPTase Sfp from *Bacillus subtilis* can activate a broad spectrum of PKSs as well as nonribosomal peptide synthetases (Lambalot *et al.*, 1996; Pfeifer & Khosla, 2001).

Therefore, choice of the PPTase is often crucial for heterologous PKS activity. In addition, the size of type I PKSs appears to be challenging for the respective host's gene expression machinery as often only insoluble protein aggregates are formed.

Despite significant differences in enzyme architecture and resulting characteristics of different PKSs types, the scope of extender units is rather small with malonyl-CoA, whose synthesis resembles the first committed step of fatty acid synthesis, being the most prominent (Chan *et al.*, 2009). Studies have shown that a low intracellular malonyl-CoA availability is the decisive bottleneck for the efficient synthesis of polyketides (Marienhagen & Bott, 2013; Palmer & Alper, 2018; Pandey *et al.*, 2016; van Summeren-Wesenhagen & Marienhagen, 2015). In bacteria and yeast, malonyl-CoA is almost exclusively used for fatty acid synthesis and therefore its intracellular availability is strictly regulated (Brownsey *et al.*, 2006; Cronan & Thomas, 2009; Tehlivets *et al.*, 2007). In general, metabolic engineering strategies to increase malonyl-CoA availability either aim for improving its formation or at decreasing its consumption in fatty acid biosynthesis.

In order to reduce undesired malonyl-CoA consumption, supplementation of the potent FAS inhibitor cerulenin can be used, which allows for an increased synthesis of polyphenolic polyketides in *E. coli* and *C. glutamicum* (Kallscheuer *et al.*, 2016b; Leonard *et al.*, 2008; Santos *et al.*, 2011; van Summeren-Wesenhagen & Marienhagen, 2015). Although efficiently improving malonyl-CoA availability, the high costs (23 € per mg) as well as a pronounced growth-inhibiting effect of cerulenin due to rapid depletion of fatty acids render this strategy unsuitable for large-scale applications. Alternatively, post-transcriptional downregulation of genes involved in fatty acid synthesis in *E. coli* using antisense RNAs, small regulatory RNAs or CRISPR interference were demonstrated to improve naringenin, resveratrol and pinosylvin synthesis (Cress *et al.*, 2015; Liang *et al.*, 2016; Wu *et al.*, 2015, 2014a; Yang *et al.*, 2018, 2015).

In most organisms, malonyl-CoA is exclusively synthesized by the carboxylation of acetyl-CoA catalyzed by the acetyl-CoA carboxylase (ACC). Hence, increasing both, the substrate availability by tailoring the central carbon metabolism as well as ACC activity, contributed to increased synthesis of malonyl-CoA-dependent products. Increasing flux through the glycolytic pathway by genes encoding the phosphoglycerate kinase and pyruvate dehydrogenase added to the almost 6-fold increased final volumetric production of 474 mg/L (1.74 mM) naringenin using *E. coli* (Xu *et al.*, 2011). With the advent of computational metabolic models, various gene knock-out combinations were predicted and verified to increase availability of acetyl-CoA and ultimately malonyl-CoA. In both, *E. coli* and *C. glutamicum*, interruption of the tricarboxylic acid (TCA) cycle by deletion of succinate dehydrogenase complex encoding genes was shown to reduce the consumption of acetyl-CoA allowing for the 2-fold increased naringenin synthesis

in both organisms (Fowler *et al.*, 2009; Hartmann *et al.*, 2017). Combinatorial deletion of predicted target genes *adhE*, *brnQ* and *citE* in the particular *sdhA*-deficient *E. coli* strain led to an overall 3-fold increased synthesis of 215 mg/L (0.79 mM) naringenin over the parental strain.

Increasing ACC activity is mandatory to unleash synergistic effects of increased acetyl-CoA availability for improved malonyl-CoA supply. In this context, expression of ACC encoding genes of various origin was demonstrated to contribute to an increased intracellular malonyl-CoA availability (Leonard *et al.*, 2007; Miyahisa *et al.*, 2005; Shi *et al.*, 2014; Shin *et al.*, 2012). Here, streamlining the carbon flux in *E. coli* towards acetyl-CoA by abolishing formation of by-products acetate and ethanol in combination with improving ACC activity was demonstrated to contribute to the 15-fold increased malonyl-CoA availability, ultimately allowing for a 2.5-fold increase in phloroglucinol synthesis (Zha *et al.*, 2009). Alternatively, the ACC independent formation of malonyl-CoA by exploiting a malonate assimilation pathway from *Rhizobium trifolii* increased microbial synthesis of the polyphenolic polyketide pinocembrin up to 15-fold (final titer 480 mg/L, 1.88 mM) using *E. coli* (An & Kim, 1998; Leonard *et al.*, 2008; Wu *et al.*, 2013).

One successful example for the relevance of microbial synthesis for the supply of polyketides is the commercialization of resveratrol obtained from engineered yeast (Evolva, 2019). Furthermore, cultivation of *Streptomyces natalensis* for the synthesis of the antifungal agent natamycin, which is used as food preservative for cheese and sausages (Farid *et al.*, 2000). The blood cholesterol lowering lovastatin is mainly obtained by submerged cultivation of filamentous fungi including *Aspergillus terreus*, *Monascus ruber* and various *Penicillium* species (Manzoni & Rollini, 2002).

1.5 *Corynebacterium glutamicum* – a promising host for polyketide synthesis?

1.5.1 Industrial relevance of *C. glutamicum*

C. glutamicum was isolated 1956 from soil samples in a research project aiming for the identification of glutamic acid-secreting microorganisms and was subsequently applied in an industrial setting for amino acid production by Kyowa Hakko Kogyo Ltd (Kinoshita *et al.*, 1957; Yukawa & Inui, 2013). The Gram-positive, non-motile and biotin-auxotrophic actinobacterium, is a non-pathogenic representative of *Corynebacterineae* serving as model organism for studying closely related pathogenic species *Mycobacterium tuberculosis*, *Mycobacterium leprae* or *Corynebacterium diphtheriae* (Stackebrandt *et al.*, 1997). Characteristic property of this actinobacterial suborder is its unique cell wall structure. In addition to *N*-acetylmuraminic acid and *N*-acetylglucosamine, the cell wall consists of

arabinogalactan and mycolic acids, forming a lipid monolayer similar to the outer membrane of Gram-negative bacteria (Brennan & Nikaido, 1995; McNeil *et al.*, 1990).

Several features, such as comparatively high growth rates or the possibility to reach high cell densities in defined media helped to make *C. glutamicum* a relevant industrial microbial host of white biotechnology since its isolation (Eggeling & Bott, 2005; Kalinowski *et al.*, 2003; Yukawa & Inui, 2013). Genetic accessibility, availability of plasmids and strong promoters allowed for tailoring this microorganism towards industrial scale synthesis of commercial products. Additionally, *C. glutamicum* does not produce endotoxins, thus fermentation products are *generally recognized as safe* (GRAS status) (Baritugo *et al.*, 2018). Today, *C. glutamicum* has become a workhorse for the production of several proteinogenic amino acids (Yukawa & Inui, 2013). Currently, optimized strains are used for the production of up to 3.1 million tons L-glutamate, 2.4 million tons L-lysine and 1 million tons D,L-methionine (Lee & Wendisch, 2017). Extensive metabolic engineering of *C. glutamicum* led to the availability of additional strains for the synthesis of L-leucine, L-valine, L-arginine but also D,L-serine (Hirasawa & Shimizu, 2016; Stäbler *et al.*, 2011; Vogt *et al.*, 2014).

Beyond that, the product portfolio of *C. glutamicum* has been expanded in the past decade to alcohols (ethanol, butanol, isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol) and organic acids (xylonate, α-ketoglutarate, succinate, hydroxybenzoic acids) (Blombach *et al.*, 2011; Kallscheuer & Marienhagen, 2018; Litsanov *et al.*, 2012; Tenhaef *et al.*, 2018; Vogt *et al.*, 2016; Yukawa & Inui, 2013). To date, approximately 70 natural and non-natural compounds can be synthesized with engineered *C. glutamicum* cell factories (Becker *et al.*, 2018).

1.5.2 Malonyl-CoA-dependent synthesis of polyphenolic polyketides with *C. glutamicum*

Naturally, *C. glutamicum* shows high resistance to a variety of aromatics, partly due to the activity of a catabolic network for such compounds (Palmer & Alper, 2018; Shen *et al.*, 2012). Despite of being devoid of said aromatic degradation pathways, the constructed platform strain *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ still tolerates the supplementation of at least 5 mM *p*-coumaric acid in shaking flask cultivations during the synthesis of polyphenolic polyketides such as naringenin, resveratrol, pterostilbene and kaempferol (Kallscheuer *et al.*, 2017, 2016a, 2016b). This is in the range of supplemented *p*-coumaric acid concentrations reported for other microbial hosts such as *Streptomyces venezulae* (1.2 mM), *E. coli* (2.6 mM) and *Saccharomyces cerevisiae* (5 mM) (Beekwilder *et al.*, 2006; Park *et al.*, 2009; Xu *et al.*, 2011). Expression of plant-derived codon-optimized genes encoding 4CL and STS enabled the synthesis of 12 mg/L resveratrol (Kallscheuer *et al.*, 2016b). As observed for other microbial hosts, synthesis of polyketides was limited by the intracellular availability of malonyl-CoA. To

improve malonyl-CoA accessibility, its consumption via fatty acid synthesis was inhibited by addition of 25 µM cerulenin. As a result, up to 158 mg/L resveratrol accumulated during cultivations of the engineered *C. glutamicum* production strain. Utilizing the same strategy for the synthesis of naringenin, up to 35 mg/L naringenin were produced upon heterologous expression of genes encoding 4CL-, CHS- and CHI- activities.

Under physiological conditions, the sole fate of malonyl-CoA in the metabolism of *C. glutamicum* is the incorporation as extender unit for *de novo* fatty acid synthesis (Gago *et al.*, 2011). Opposed to bacteria in general and interestingly also opposed to the closely related *Mycobacteria* and *Streptomyces*, all *Corynebacteria* lack functional type II FAS systems. Instead, they exclusively feature functional type I fatty acid synthases (Gago *et al.*, 2011; Radmacher *et al.*, 2005). *C. glutamicum* harbors an essential (FasA) and a supplementary (FasB) FAS that are both required to generate the characteristic lipid environment. Here, absence of FasA was shown to result in oleate auxotrophy, whereas FasB deficiency reduces synthesis of palmitate and causes the absence of thereof derived mycolic acids (Radmacher *et al.*, 2005).

The initial step of fatty acid synthesis is the carboxylation of acetyl-CoA yielding malonyl-CoA. Here, the key enzyme is the ACC comprised of three different domains catalyzing two distinct reaction steps (Cronan & Waldrop, 2002). An ATP-dependent biotin carboxylase (BC) domain catalyzes the transfer of a carboxyl group to biotin, which is covalently attached to the biotin carboxyl carrier protein (BCCP) domain over a lysine residue, forming carboxybiotin and finishing the first reaction (Figure 4). The flexible arm of biotin transports CO_2 between the BC domain and the carboxyltransferase (CT) domain, which catalyzes the second half reaction where the carboxygroup is transferred to acetyl-CoA eventually forming malonyl-CoA.

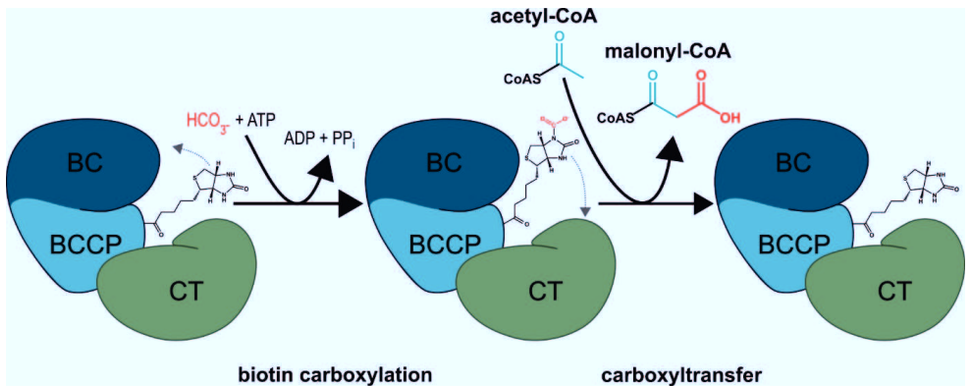

Figure 4: Schematic representation of the two-step mechanism catalyzed by the acetyl-CoA carboxylase from *C. glutamicum*. BC: biotin carboxylase, BCCP: biotin carboxyl carrier protein, CT: carboxyltransferase.

Similar to the enzymatic architecture of FAS, mammalian, fungal and plant cytosolic ACCs are single polypeptide chains possessing the three domains. In contrast, bacterial and plant chloroplastic ACCs are organized as complexes of distinct dissociable polypeptides (Cronan & Waldrop, 2002). The ACC complex of *E. coli* consists of four separate polypeptides AccC (BC domain), AccB (BCCP domain), AccA and AccD (CT-α and CT-β domain) providing the three domains (Guchhait *et al.*, 1974; Li *et al.*, 1992; Li & Cronan, 1992a, 1992b). Not only in *Mycobacteria* and *Streptomyces*, but also in *C. glutamicum* BC and BCCP domains are organized as a single polypeptide (α-subunit), a separate CT domain polypeptide (β-subunit) and a small ε-peptide (Gande *et al.*, 2007). In *C. glutamicum*, *accBC* (cg0802), *accD1* (cg0812) and *accE* (cg0810) encode the α-, β- and ε-subunit, respectively (Nickel *et al.*, 2010). Interestingly, *accE* is not essential for ACC activity in this bacterium (Miyahisa *et al.*, 2005).

In addition to *accD1*, *C. glutamicum* harbors three additional genes (*accD2-4*) encoding β-subunits with CT activity (Gande *et al.*, 2007, 2004). In combination with the same AccBC and AccE of the ACC complex, AccD2 and AccD3 constitute an acyl carboxylase essential for the corynomycolic acid synthesis. Although cell extract assays point towards AccD4 having a carboxylase activity, the absence of a phenotype of an *accD4* mutant led to the suggestion that AccD4 is not directly involved in lipid biosynthesis (Gande *et al.*, 2004).

As in other organisms, fatty acid synthesis is transcriptionally regulated in *C. glutamicum* (Nickel *et al.*, 2010; Schujman *et al.*, 2006). The TetR-type transcriptional repressor FasR binds to a highly conserved *fasO* motif upstream of *accBC*, *accD1*, *fasA* and *fasB*, thus preventing transcription of fatty acid synthesis key genes. Here, acyl-CoA thioesters (oleoyl-CoA and palmitoyl-CoA) are regarded as effectors interacting with FasR (Irzik *et al.*, 2014). A FasR-S20N mutant, characterized by increased transcript levels of *accD1*, *fasA* and *fasB* was shown to drastically improve fatty acid production, mainly oleic acid (Takeno *et al.*, 2013). The amino acid substitution was postulated to either interfere with formation of the FasR-acyl-CoA complex or binding of the repressor-effector complex to the *fasO* motifs, both resulting in the derepression of controlled genes. Furthermore, extensive mutational studies of the *fasO* motif revealed essential nucleotides for the interaction with FasR (Nickel *et al.*, 2010).

In *S. cerevisiae*, phosphorylation of the single polypeptide ACC was demonstrated to post-translationally regulate enzyme activity (Shi *et al.*, 2014). Interestingly, generation of a *C. glutamicum* phosphoproteome map revealed phosphorylation of the α-subunit AccBC (Bendt *et al.*, 2003). However, there is no experimental data whether this modification affects ACC activity.

1.6 Aims of this thesis

The major objective of this thesis is to unleash the potential of *C. glutamicum* platform strains for the microbial synthesis of polyketides by increasing the intracellular malonyl-CoA availability. By following strategies of rational metabolic engineering, including deletion, overexpression, de- and downregulation of genes encoding key enzymes in the central carbon and fatty acid metabolism, *C. glutamicum* should be tailored towards the cerulenin-independent increased supply of malonyl-CoA. Furthermore, the product portfolio synthesized with the engineered *C. glutamicum* cell factory should be expanded to additional biotechnologically interesting type III polyketides such as phenylbutanoids and chromonones, but also to yet untapped type I polyketides.

1.7 Key results on engineering *C. glutamicum* for increased malonyl-CoA availability for polyketide synthesis

1.7.1 Increasing acetyl-CoA supply is essential for improving intracellular malonyl-CoA availability

(Milke et al. 2019a, cf. chapter 2.2; Milke et al. 2019b, cf. chapter 2.3)

Starting point for this thesis was the previously constructed *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ platform strain, harboring a chromosomally integrated codon-optimized gene variant encoding 4CL from *Petroselinum crispum* under control of the inducible T7 promotor (P_{T7}-*4cl*$_{PcCg}$). In order to improve the established synthesis of plant polyphenolic polyketides resveratrol and naringenin, efforts aimed for increasing malonyl-CoA availability by rational modulation of the central carbon metabolism. To evaluate a modifications impact on malonyl-CoA availability, naringenin as a malonyl-CoA dependent polyphenolic polyketide was chosen as a reporter molecule. Therefore, engineered strains were transformed using the existing plasmid pMKEx2-*chs*$_{PhCg}$-*chi*$_{PhCg}$ providing codon-optimized genes encoding CHS and CHI from *Petunia x hybrida*, organized as a synthetic operon under control of the inducible T7 promotor (*chs*$_{PhCg}$-*chi*$_{PhCg}$). Subsequently, constructed strains were cultivated in defined CGXII medium with 4 % glucose supplemented with 5 mM *p*-coumaric acid as well as 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Naringenin was extracted from the cultivation broth using ethyl acetate and subsequently analyzed by LC-MS. When the titer was increased compared to the control strain, a beneficial impact on malonyl-CoA supply was attributed to a particular modification.

Initially, the *sdhCAB* operon encoding the subunits of the succinate dehydrogenase complex was deleted, which had only a neglectable positive effect on naringenin synthesis. However, since both, growth rate and in particular biomass formation were reduced upon loss of this

operon, this deletion can be regarded as rather disadvantageous. Nevertheless, these results indicated that the TCA cycle indeed represents a promising target to increase acetyl-CoA and ultimately malonyl-CoA availability.

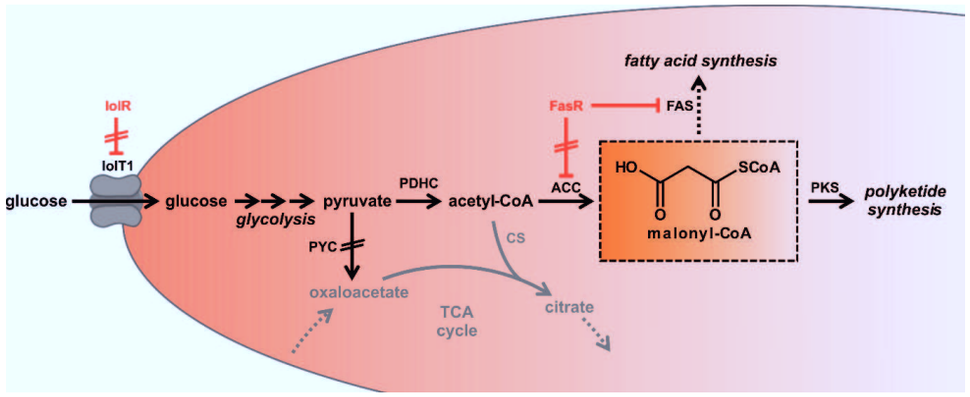

Figure 5: Metabolic engineering of the central carbon metabolism of *C. glutamicum* towards increased malonyl-CoA availability for polyketide synthesis. ACC: acetyl-CoA carboxylase; CS: citrate synthase; FAS: fatty acid synthase; FasR: transcriptional repressor of *accBC*, *accD1*, *fasIA* and *fasIB*, all involved in fatty acid synthesis; IolR: transcriptional repressor of genes involved in *myo*-inositol catabolism; IolT1: glucose/*myo*-inositol permease; PDHC: pyruvate dehydrogenase complex; PKS: polyketide synthase; PYC: pyruvate carboxylase; TCA: tricarboxylic acid, Eliminated enzymatic reactions (black) or regulatory circuits (red): crossed out; reduced enzyme/pathway activity: grey.

In contrast to closely related *Mycobacteria*, *C. glutamicum* harbors only a single citrate synthase (CS) as the pace-making enzyme of the TCA cycle, which is encoded by the essential *gltA* gene (cg0949). CS catalyzes the condensation of acetyl-CoA and oxaloacetate yielding citrate (Figure 5). Therefore, a reduced CS activity could be beneficial to enhance acetyl-CoA availability to ultimately also improve malonyl-CoA biosynthesis. Previously, it was demonstrated that exchanging the promotor of the *gltA* gene with three candidates from a set of synthetic promotor variants of the dihydrodipicinolate synthase gene *dapA* (P_{dapA}) decreased CS activity and in turn increased acetyl-CoA supply. (van Ooyen *et al.*, 2012). Thus, exchanging the promotor of the *gltA* gene might also improve malonyl-CoA availability and ultimately polyphenolic polyketide synthesis.

Individual exchange of the native *gltA* promotor P_{gltA} against the three variants P_{dapA}-A16, P_{dapA}-L1 and P_{dapA}-C7 reduced CS activity to 32 %, 16 % and 10 % compared to *C. glutamicum* wild-type CS activity, respectively. Further characterization with regard to growth rate and biomass formation demonstrated that *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ reacts quite robust to reduced CS activities. Biomass formation in all constructed strains was barely distinguishable from the parental strain (OD_{600} = 55.2 ± 1.7). Even the strain with only 10 % residual CS activity (*C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C7) still maintained a growth rate of

0.24 ± 0.02 h^{-1} compared to the parental strain's growth rate (μ = 0.32 ± 0.01 h^{-1}). Whereas the parental strain produced only 2 mg/L (0.007 mM) naringenin, the engineered strains with 32 % and 16 % residual CS activity already produced 5 mg/L (0.018 mM) and 12 mg/L (0.044 mM) naringenin. Using the constructed strain *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C7 with 10 % residual CS activity, naringenin synthesis was improved almost ten-fold to 19 mg/L (0.07 mM). Summarizing, LC-MS analysis showed antiproportional correlation of residual CS activities and naringenin titers.

However, since the assessment of malonyl-CoA availability by the synthesis of reporter molecules such as naringenin is a well-established but only an indirect method, an LC-MS/MS-based method for direct quantification of the intracellular malonyl-CoA pool was established. As malonyl-CoA spontaneously hydrolyses under the experimental conditions yielding malonate, the free acid was used to determine malonyl-CoA availability. In consideration of current knowledge, *C. glutamicum* is not capable of synthesizing or degrading malonate, suggesting that detected malonate solely originates from spontaneous decay of malonyl-CoA as a consequence of the sample treatment during LC-MS/MS analysis. Indeed, the engineered strains either harboring the P_{dapA}-L1 or P_{dapA}-C7 promotor variant controlling *gltA* expression showed 1.6- and 1.9-fold increased integrated malonate signals compared the parental strain (Figure 6).

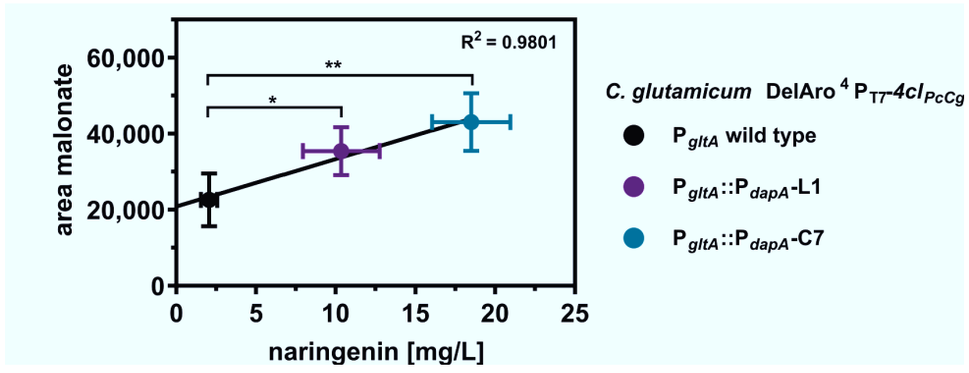

Figure 6: LC-MS/MS analysis of intracellular malonate concentrations. Areas for malonate acquired by LC-MS/MS analysis of cytoplasmatic extracts are plotted against the determined naringenin concentration during cultivation of the respective strains. The plotted naringenin concentrations represent mean 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 ($p < 0.05$).

Furthermore, 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 intracellular concentrations.

For large-scale fermentations, microbial naringenin production with *C. glutamicum* would be much more economical when starting from cheap glucose. Therefore, *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C7 not only harboring pMKEx2-*chs*$_{PhCg}$-*chi*$_{PhCg}$ but also pEKEx3-*aroH*$_{Ec}$-*tal*$_{FjCg}$ was constructed. The latter plasmid contains genes encoding DAHP synthase from *E. coli* and TAL from *Flavobacterium johnsoniae*, whose episomal heterologous expression was demonstrated previously to be sufficient for the synthesis of naringenin from glucose (Kallscheuer *et al.*, 2016b). Cultivation of the constructed *C. glutamicum* strain in a bioreactor generated up to 24 mg/L (0.09 mM) naringenin exclusively from glucose.

To emphasize the versatility of the constructed *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C7 strain, the naringenin-specific production plasmid was replaced by the plasmid pMKEx2-*sts*$_{AhCg}$-*4cl*$_{PcCg}$ for enabling resveratrol synthesis. Ultimately, the constructed strain harboring pMKEx2-*sts*$_{AhCg}$-*4cl*$_{PcCg}$ and pEKEx3-*aroH*$_{Ec}$-*tal*$_{FjCg}$ produced up to 112 mg/L (0.49 mM) resveratrol directly from glucose, which is a ten-fold increase to the parental strain (Braga *et al.*, 2018). Moreover, dependency on the costly fatty acid synthesis inhibitor cerulenin has been abolished.

This particular strain was tailored towards increased ACC activity to further improve malonyl-CoA availability (*C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C7 mu*fasO*$_{BCD1}$, cf. chapter 1.7.2). Building on this variant, the integrated P_{dapA}-C7 promotor was replaced by the P_{dapA}-C5 variant, further reducing the CS activity to 5.5 %. This allowed to improve the newly established synthesis of the plant pentaketide noreugenin, which is produced by condensation of five molecules of malonyl-CoA and was therefore used as a sensor molecule for this very purpose, more than 2-fold from 1.98 mg/L (0.01 mM) to 4.13 mg/L (0.22 mM) (cf. chapter 1.7.2 and 1.7.3).

In addition to improving acetyl-CoA availability by reducing its consumption via the TCA cycle, strategies to increase its biosynthesis were also followed. Usually, acetyl-CoA is generated by oxidative decarboxylation of pyruvate. Streamlining the cellular glucose metabolism towards pyruvate should therefore be advantageous to ultimately improve acetyl-CoA and malonyl-CoA availability for polyketide synthesis (Figure 5).

Recently, deregulated expression of *iolT1* (cg0223) encoding the glucose permease/*myo*-inositol transporter IolT1 was demonstrated to promote D-xylose utilization by engineered *C. glutamicum* (Brüsseler *et al.*, 2018). As glucose uptake should also benefit from increased abundance of IolT1, the respective nucleotide substitutions were introduced into the promotor sequence of *iolT1* resulting in the strain *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C5 mu*fasO*$_{BCD1}$ P_{O6}-*iolT1*. When characterized

with regard to noreugenin synthesis, only minor positive effects on the absolute product titer could be observed during shake flask cultivations (4.4 mg/L, 0.02 mM noreugenin). Nevertheless, growth rate, negatively influenced by downregulated CS activity, was partly restored. Thus, bearing important parameters of large-scale applications such as space-time yield in mind, this modification was regarded as beneficial.

Beyond increasing glucose uptake, withdrawal of phosphoenolpyruvate and pyruvate from glycolysis by the anaplerotic reactions generating oxaloacetate was targeted. In contrast to most microorganisms, *C. glutamicum* is able to catalyze the carboxylation of both phosphoenolpyruvate and pyruvate. Previously, it was demonstrated that only one of the two enzymes needs to be present to enable growth on glucose. In fact, 90 % of total oxaloacetate synthesis is ascribed to the activity of pyruvate carboxylase (PYC) encoded by *pyc* (cg0791). Construction of *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C5 mu*fasO*$_{BCD1}$ P_{O6}-*iolT1* Δ*pyc* (later termed *C. glutamicum* M-CoA, cf. chapter 2.5) and subsequent cultivation for the synthesis of noreugenin demonstrated the accumulation of 5.63 mg/L (0.03 mM) noreugenin within 72 hours. Thus, *pyc* deficiency was regarded to promote malonyl-CoA availability in the constructed strain.

It can be concluded that increasing acetyl-CoA availability is prerequisite for improving malonyl-CoA availability in *C. glutamicum*. The metabolic strategies successfully implemented in the context of this study can possibly be also transferred to other microbial hosts.

1.7.2 Increasing ACC activity through modulation of FasR-mediated gene regulation improves malonyl-CoA availability

(Milke et al. 2019a, cf. chapter 2.2; Milke et al. 2019b, cf. chapter 2.3)

As malonyl-CoA is solely derived from the carboxylation of acetyl-CoA in *C. glutamicum*, increasing ACC activity with the aim to improve malonyl-CoA availability is a promising approach. Here, a widely used strategy is the episomal expression of genes encoding the ACC from *C. glutamicum* as the protein synthesis of only two subunits needs to be coordinated to form a functional enzyme. Whereas this strategy was applied multiple times in the context of engineering *E. coli* for polyphenolic polyketide synthesis, no effects on naringenin formation in *C. glutamicum* itself were discernible. It turned out that an increasing supply of acetyl-CoA, being the substrate of ACC, is mandatory in order to increase intracellular malonyl-CoA availability for efficient naringenin synthesis. Episomal expression of *accBC* and *accD1* increased naringenin production by 40 % to 26 mg/L (0.095 mM) compared to the reference strain *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C7. This demonstrated the general benefits of enhanced ACC activity on malonyl-CoA supply in *C. glutamicum*.

In general, use of multiple plasmids might increase the metabolic burden for the cells as well as overall process costs during production due to the required supplementation of additional antibiotics (Glick, 1995; Wu *et al.*, 2016). Thus, increasing the genomic expression of *accBC* and *accD1* was targeted to increase overall ACC activity. With the aim to improve the synthesis of malonyl-CoA-derived fatty acids with *C. glutamicum*, *fasR,* encoding the transcriptional repressor of fatty acid synthesis, was previously deleted (Takeno *et al.*, 2013). Application of this strategy in *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ hardly improved naringenin synthesis, but drastically decreased the growth rate from 0.32 ± 0.01 h^{-1} to 0.22 ± 0.02 h^{-1}. Deletion of *fasR* in *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C7 further reduced the growth rate to 0.19 ± 0.02 h^{-1} and also reduced naringenin synthesis by 40 % (12 mg/L, 0.044 mM), rendering this strategy unsuitable for the synthesis of polyketides with *C. glutamicum*. Presumably, absence of the transcriptional repressor FasR not only relieves repression of *accBC* and *accD1* but also of the genes *fasA* (cg0957) and *fasB* (cg2743) encoding the malonyl-CoA consuming fatty acid synthases FasA and FasB. Thus, this modification does not necessarily promote accumulation of malonyl-CoA for polyketide synthesis and was therefore discarded.

Mutational studies of the operator regions of *accBC* and *accD1* revealed essential nucleotides in the *fasO* sites for binding of FasR (Nickel *et al.*, 2010). Based on these findings, the *fasO* motifs upstream of the open reading frames (ORFs) of *accBC* and *accD1* were mutated in the *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C7 strain background, both individually (mu*fasO*$_{BC}$, mu*fasO*$_{D1}$) and in combination (mu*fasO*$_{BCD1}$). Important to note in this context, since the FasR binding site of *accD1* overlaps with the ORF of this gene, only nucleotide substitutions were introduced that do not alter the amino acid sequence of AccD1 (Figure 7).

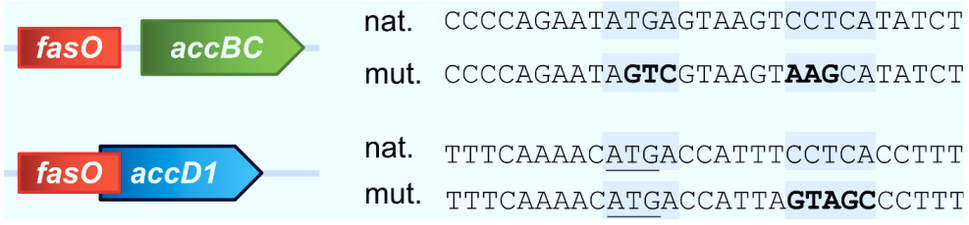

Figure 7: Schematic representation and relative postion of the *fasO* sites of *accBC* and *accD1*. In both cases, the mutated (mut.) *fasO* sites are aligned with the respective native (nat.) *fasO* sites. Grey-backed nucleotides are conserved in all known *C. glutamicum fasO*-sites essential for FasR binding. Mutated nucleotides are highlighted by bold letters. The start codon of *accD1* is underlined.

The engineered strains were transformed using the plasmid pMKEx2-*pcs*$_{AaCg}$-short providing a codon-optimized gene variant encoding a N-terminally truncated PCS from *A. arborescens* under control of the inducible T7 promotor (*pcs*$_{AaCg}$-short) to allow for the synthesis of

noreugenin (cf. chapter 1.7.3). Subsequently, the constructed strains were cultivated in defined CGXII medium with 4 % glucose and 1 mM IPTG. Noreugenin was extracted from the cultivation broth using ethyl acetate for LC-MS analysis. Again, an increased product titer over the control strain was regarded to reflect increased malonyl-CoA availability.

Whereas *C. glutamicum* strains with the individually mutated *fasO* sites even showed reduced noreugenin synthesis, the constructed strain *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C7 mu*fasO*$_{BCD1}$, harboring both mutated *fasO* sites, carrying pMKEx2-*pcs*$_{AaCg}$-short generated 1.98 mg/L (0.01 mM) noreugenin within 72 hours indicating an increased malonyl-CoA supply. In comparison, the parental strain with wild-type FasR-mediated regulation accumulated only 0.8 mg/L (0.004 mM) noreugenin. To confirm the assumption of increased malonyl-CoA availability upon mutation of both *fasO* sites, the intracellular malonyl-CoA pool was quantified in form of its free acid malonate by LC-MS/MS. The obtained malonate signals showed a 2.8-fold increase upon deregulated expression of both, *accBC* and *accD1*, in comparison to the parental strain. Using a calibration curve, an intracellular malonate concentration of 1.8 mM was determined, the isotope ratio of the parental strain was below the linear range of the calibration curve.

This novel approach to specifically increase intracellular ACC activity by repealing FasR-mediated regulation of genome-encoded ACC genes in *C. glutamicum*, turned out to be the least intrusive but most effective, allowing to efficiently increase malonyl-CoA availability. When equipped with the respective plasmids, *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C7 mu*fasO*$_{BCD1}$ accumulated up to 65 mg/L (0.24 mM) naringenin and 450 mg/L (1.97 mM) resveratrol from 5 mM supplemented *p*-coumaric acid using standard cultivation conditions (unpublished data). In general, this strategy should be applicable to all microbial hosts, in which the ACC activity is controlled on the transcriptional level.

1.7.3 *C. glutamicum* as a microbial host for the synthesis of the pentaketide noreugenin

(Milke et al. 2019b, cf. chapter 2.3)

In close interdependence with the projects to increase intracellular malonyl-CoA availability, the establishment of further relevant product classes was also aimed at. One of these novel compounds is the plant pentaketide noreugenin from the medicinal plant *A. arborescens*. Noreugenin belongs to the class of chromones, which are gaining attention due to their ascribed health-promoting effects (Gaspar *et al.*, 2014). Beyond that, noreugenin resembles a very suitable reporter molecule for intracellular malonyl-CoA availability, as it is exclusively

produced from just this metabolite (Yang *et al.*, 2018). Opposed to naringenin or resveratrol synthesis, the number of enzymatic reaction steps is reduced to a single enzyme (Figure 8).

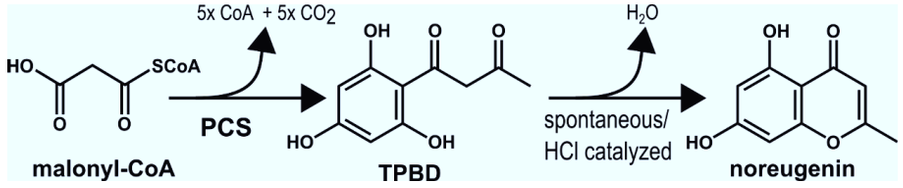

Figure 8: Biosynthetic noreugenin pathway. PCS: pentaketide chromone synthase, TPBD: 1-(2,4,6-tri-hydroxyphenyl)butane-1,3-dione

In order to establish noreugenin synthesis in *C. glutamicum*, a codon-optimized variant of the *pcs* gene originating from *A. arborescens* (pcs_{AaCg}) encoding a pentaketide chromone synthase was subcloned into the pMKEx2 plasmid allowing for the IPTG inducible gene expression under control of the T7 promotor. Initial cultivation experiments in CGXII medium with 4 % glucose and 1 mM IPTG yielded only traces of noreugenin close to the LC-MS detection limit.

Amino acid sequence alignments to similar type III PKS enzymes, which were already functionally implemented into *C. glutamicum* for the synthesis of resveratrol (STS from *Arachis hypogea*, STS_{Ah}) or naringenin (CHS from *Petunia x hybrida*, CHS_{Ph}) revealed that the first ten amino acid residues in PCS_{Aa} have no equivalent in the other two enzymes despite their overall sequence identity of 70 - 80 %. Interestingly, residues M11 and V14 of PCS_{Aa} aligned with M1 and V4 of both, CHS_{Ph} and STS_{Ah}, suggesting a simple misannotation of the translational start of the protein sequence deposited in UniProt (ID: Q58VP7). To exclude a possible function of the ten N-terminal amino acids of PCS_{Aa} as signal sequence for protein localization in the plant cell, three algorithms to detect signal and targeting peptide sequences were consulted. None of them proposed such a function for the ten first amino acids. However, a truncated variant pcs_{AaCg}-short lacking the first 30 nucleotides was constructed and cloned into pMKEx2. Indeed, expression of the truncated gene variant significantly improved noreugenin synthesis with *C. glutamicum* yielding 0.8 mg/L (0.004 mM) after 72 hours under standard cultivation conditions.

Ensuing metabolic engineering of *C. glutamicum* and LC-MS/MS based quantification of the intracellular malonyl-CoA pool demonstrated that an increased precursor availability is reflected by the noreugenin titer (cf. chapter 1.7.2). This allows to regard noreugenin as a reliable reporter molecule to evaluate the impact of genetic modifications on the intracellular availability of malonyl-CoA in *C. glutamicum*, circumventing the laborious extraction and LC-MS/MS quantification method.

Over the course of tailoring *C. glutamicum* towards improved malonyl-CoA availability for noreugenin synthesis, all constructed strains showed continuous noreugenin synthesis over prolonged process times, even when the cultures already had reached the stationary growth phase after 24 hours. This observation is contrary to the current believe assuming a strict exponential growth phase-dependent malonyl-CoA supply in *C. glutamicum*. This means that noreugenin synthesis should have stopped upon reaching the stationary growth phase. As such a phenomenon had never been observed for the synthesis of naringenin or resveratrol, the reason for this observation was rather assumed to be connected to the product formation of noreugenin than the engineered *C. glutamicum* strain background.

In silico reconstruction of the PCS_{Aa} reaction mechanism postulated a C6-C1 cyclized pentaketide intermediate (1-(2,4,6-tri-hydroxyphenyl)butane-1,3-dione; TPBD, M = 210,05 g/mol, Figure 8), which subsequently undergoes spontaneous isomerization and dehydration forming a pyrone moiety eventually yielding noreugenin. In fact, a corresponding TPBD signal was detected in extracted cultivation samples during LC-MS analysis that decreases over time, whereas the noreugenin signal gains abundance (Figure 9A+B). Further analysis revealed that the malonyl-CoA dependent synthesis of the TPBD intermediate is indeed coupled to the exponential growth phase, whereas the seemingly continuous noreugenin synthesis was resolved to arise solely from the slow spontaneous formation of the pyrone moiety (Figure 9C).

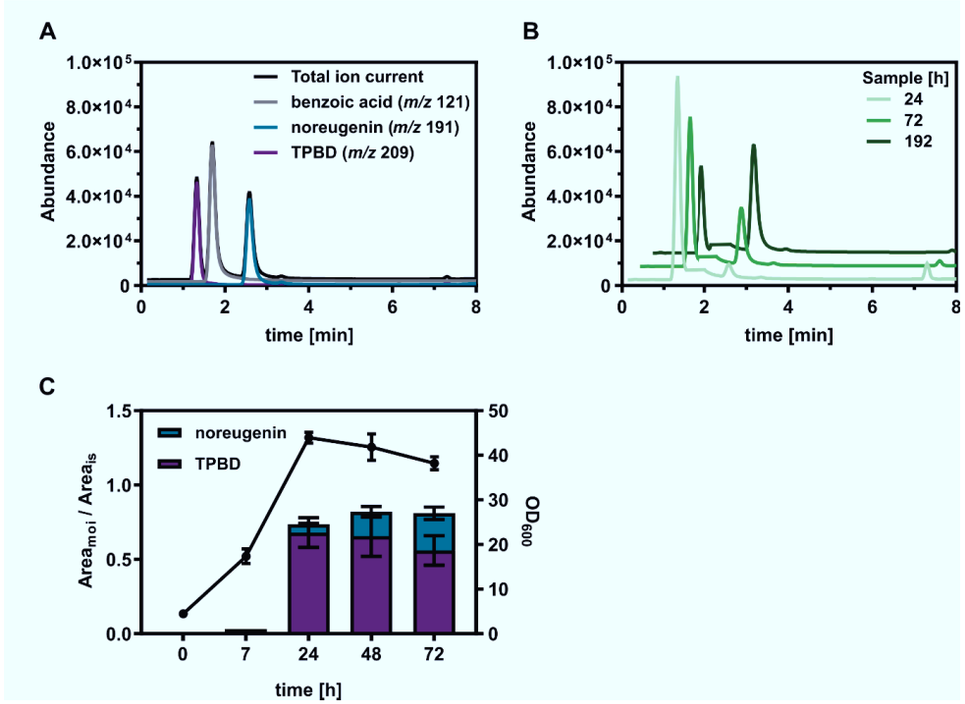

Figure 9: Detection of the noreugenin synthesis intermediate 1-(2,4,6-trihydroxyphenyl)butane-1,3-dione (TPBD) in extracted cultivation samples. (A) Exemplary chromatogramm of an extracted cultivation sample both as total ion current and the individual *m/z* ratios. (B) Total ion current chromatograms of extracted samples resembling the spontaneous cyclization of TPBD towards noreugenin over the cultivation time. For better visualization, the benzoic acid signal was removed from the chromatogram. (C) Growth curve and product abundance for the strain *C. glutamicum* M-CoA pMKEx2-pcs_{AaCg}-short. Unavailability of an authentic TPBD standard prevented quantification of this particular molecule. To evaluate product formation over time, the normalized signal areas of the molecules of interest ($Area_{moi}$) TPBD and noreugenin were added up. Signal area of the internal standard ($Area_{is}$) benzoic acid was used for normalization. The calculated ratios are depicted on the primary Y-axis. At the given sampling time points, OD_{600} of the cultures was also determined (filled circles, shown on secondary Y-axis). The obtained data represent mean values with standard deviations from biological triplicates.

1.7.4 *C. glutamicum* as a microbial host for the synthesis of the phenylbutanoids raspberry ketone, zingerone and benzylacetone

(Milke et al. 2020, cf. chapter 2.5)

The phenylbutanoid raspberry ketone, characteristic for the scent and flavor of ripe raspberries, is used as a natural flavoring agent in food and beverages. Unfortunately, abundance of raspberry ketone in raspberries is low, rendering the extraction of this molecule from raspberries very costly. Although chemical routes allow for the economical synthesis, raspberry ketone produced by such processes cannot be marketed as *natural flavoring*

substance in accordance with food regulations (European Council, 2008; Smith, 1996; Vandamme & Soetaert, 2002). Contrary to this, RK obtained from microbial synthesis is regarded as *natural*. As the biosynthetic pathway resembles naringenin and resveratrol synthesis and thus requires 4CL and type III PKS activity with inherent malonyl-CoA dependency, the already available platform strain *C. glutamicum* M-CoA was selected as a promising host for raspberry ketone synthesis (Figure 10). The phenylpropanoid precursor *p*-coumaric acid is CoA-activated by a 4CL and subsequently condensed with one molecule of malonyl-CoA, catalyzed by a BAS, yielding *p*-hydroxybenzalacetone. Ultimately, the biosynthetic pathway features a dedicated reduction of the diketide intermediate *p*-hydroxybenzalacetone catalyzed by BAR yielding raspberry ketone.

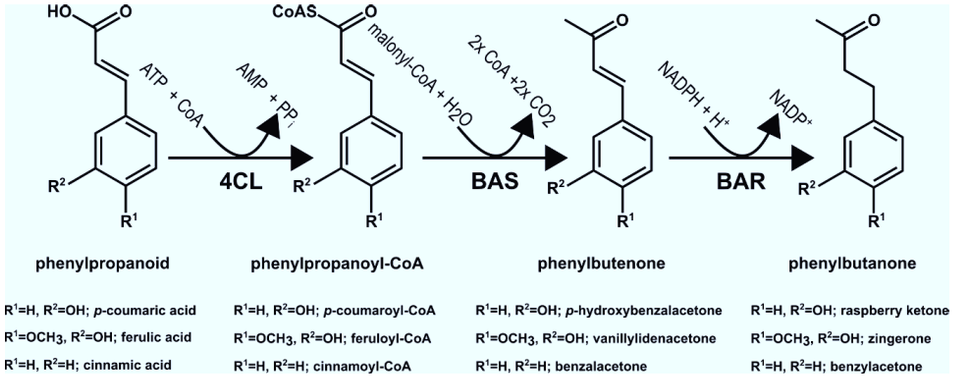

Figure 10: Biosynthetic pathway for phenylbutanoids from phenylpropanoids. 4CL: 4-coumarate: CoA ligase, BAR: benzalacetone reductase, BAS: benzalacetone synthase.

In preceding experiments to evaluate cytotoxicity of the pathway intermediates and raspberry ketone itself, *C. glutamicum* was cultivated in the presence of *p*-hydroxybenzalacetone and raspberry ketone. Here, *C. glutamicum* showed a superior resistance against both compounds in comparison to *E. coli* and *S. cerevisiae*, which are already used for microbial raspberry ketone synthesis. Furthermore, it was found that *C. glutamicum* possesses a hitherto unknown endogenous BAR activity allowing for the reduction of *p*-hydroxybenzalacetone yielding raspberry ketone.

To further increase intracellular BAR activity, codon-optimized gene variants encoding the NADPH-dependent raspberry ketone/zingerone reductase RZS1 from *Rubus idaeus* were tested for the reduction of 500 mg/L (3.09 mM) *p*-hydroxybenzalacetone. Unfortunately, both variants failed to increase BAR activity in *C. glutamicum*. Additionally, the NADPH-dependent curcumin/dihydrocurcumin reductase CurA from *E. coli* ($CurA_{Ec}$) was investigated for BAR activity, as the close structural resemblance of curcumin and *p*-hydroxybenzalacetone suggests a potential activity of $CurA_{Ec}$ with the latter compound. Indeed, heterologous

expression of *curA*$_{Ec}$ from *E. coli* MG1655 increased BAR activity, which could be further improved upon expression of the codon-optimized variant *curA*$_{EcCg}$ yielding 68.67 mg/L (0.42 mM) raspberry ketone.

To further improve intracellular BAR activity, efforts were made to improve the availability of the cofactor NADPH. In this context, heterologous expression of a codon-optimized gene encoding the cytosolic transhydrogenase UdhA from *E. coli* (*udhA*$_{EcCg}$) in a lactate dehydrogenase deficient strain (*C. glutamicum* M-CoA Δ*ldhA*) further improved raspberry ketone synthesis from supplemented *p*-hydroxybenzalacetone by 25 %.

With the aim of enabling the *p*-coumaric acid-derived synthesis of raspberry ketone, codon-optimized genes of different plant origin encoding type III PKSs with BAS activity were tested for the formation of *p*-hydroxybenzalacetone from *p*-coumaric acid or more precisely, *p*-coumaroyl-CoA. The bifunctional chalcone synthases PKS1 and PKS4 from raspberry turned out unsuitable for *p*-hydroxybenzalacetone and ultimately raspberry ketone synthesis. However, a monofunctional BAS from *R. palmatum* enabled synthesis of 14.03 mg/L (0.09 mM) raspberry ketone and 9.43 mg/L (0.06 mM) *p*-hydroxybenzalacetone from supplemented *p*-coumaric acid (5 mM) in the absence of *udhA*$_{EcCg}$. As expression of *udhA*$_{EcCg}$ surprisingly reduced the cumulated synthesis of *p*-hydroxybenzalacetone and raspberry ketone, the strain *C. glutamicum* M-CoA Δ*ldhA* harboring pMKEx2-*bas*$_{RpCg}$-*curA*$_{EcCg}$ and pEKEx3-*udhA*$_{EcCg}$ was regarded as the most suitable strain for the synthesis of raspberry ketone from supplemented *p*-hydroxybenzalacetone, whereas additional *udhA*$_{EcCg}$ expression was not suitable for synthesis from *p*-coumaric acid.

In addition to raspberry ketone, other phenylbutanoids such as the ferulic acid-derived zingerone or the cinnamic acid-derived benzylacetone are of commercial interest drawn by their flavoring traits (Figure 10). Zingerone provides the flavor of cooked ginger, whereas benzylacetone has a strawberry and jasmine flavor. Motivated by the discovered enzyme promiscuity of CurA$_{Ec}$ also accepting *p*-hydroxybenzalcetone, the enzymes of the established pathway for raspberry ketone synthesis were challenged with the other precursors / intermediates to see whether the constructed *C. glutamicum* strain could also be used for the synthesis of other phenylbutanoids. Resembling the strategy for establishing raspberry ketone synthesis, a potential reductase activity with the respective intermediates vanillylidenacetone (zingerone) and benzalacetone (benzylacetone) was elucidated first. The findings indicated that both intermediates are accepted by the endogenous reductase of *C. glutamicum*, whereas only vanillylidenacetone is also accepted by CurA$_{Ec}$, which is resembled by an increased accumulation of zingerone upon expression of *curA*$_{EcCg}$. Investigation of 4CL and BAS activity with the alternative phenylpropanoid substrates ferulic acid and caffeic acid, the synthesis of both intermediates, vanillylidenacetone and

benzalacetone, was verified by HPLC analysis. Probably due to the lower ability to reduce benzalacetone, no benzylacetone could be detected during cultivations with supplemented caffeic acid, although all individual reaction steps in general are feasible. Contrary, the established pathway allowed for the synthesis of 14.14 mg/L (0.07 mM) zingerone from 5 mM ferulic acid.

1.7.5 *C. glutamicum* as a microbial host for the synthesis of the type I polyketide 6-methylsalicylic acid

(Kallscheuer et al., 2019, cf. chapter 2.4)

Establishing novel type III PKS dependent biosynthetic pathways for the microbial production of pentaketides and phenylbutanoids in engineered *C. glutamicum* strains demonstrated the versatility of the constructed cell factory. To explore the limitations of the engineered platform strain, the synthesis of type I polyketides, e.g. 6-MSA, was examined. In case of type I PKSs, proper folding of the large polypeptide chains and the required post-translational phosphopantetheinylation by a dedicated PTTase are challenging in the respective microbial host system.

As expected, the strain *C. glutamicum* DelAro^4 P_{T7}-*4cl*$_{PcCg}$ P_{gltA}::P_{dapA}-C5 mu*fasO*$_{BCD1}$ was neither able to catabolize nor modify supplemented 6-MSA due to the deletion of 21 genes involved in the degradation of multiple aromatic compounds. Instead, toxicity experiments demonstrated *C. glutamicum* and *S. cerevisiae* to be more a suitable host than *E. coli* for 6-MSA production. Furthermore, the provoked improved availability of acetyl-CoA and malonyl-CoA, which are required as substrates for the synthesis of 6-MSA, made this *C. glutamicum* strain a promising starting point for this particular project. Unfortunately, no accumulation of detectable amounts of 6-MSA upon co-expression of codon-optimized genes encoding the 6-MSA synthase ChlB1 from *Streptomyces antibioticus* (ChlB1$_{Sa}$) and the broad-spectrum PPTase Svp from *Streptomyces verticillus* (Svp$_{Sv}$) could be determined. Having the difficulties with regard to proper folding of type I PKSs in mind, a translational fusion of ChlB1$_{Sa}$ to the C-terminus of the maltose binding protein from *E. coli* (MalE$_{Ec}$) was constructed. Despite being even larger, the fusion protein MalE$_{Ec}$-ChlB1$_{Sa}$ allowed for the synthesis of up to 6 mg/L (0.039 mM) 6-MSA in defined CGXII medium with 4 % glucose and 1 mM IPTG.

Heterologous expression of *svp*$_{Sv}$ was assumed to cause rapid depletion of Mg^{2+} ions, which are required for PPTase activity, which would explain the observed limited biomass formation. Hence, standard CGXII medium only containing 1 mM $MgSO_4$ as sole source of Mg^{2+} ions was supplemented with different Mg^{2+} ion concentrations ranging from 15 to 200 mM. Although all tested concentrations restored the reduced growth phenotype, 50 mM $MgSO_4$ turned out to be optimal for 6-MSA synthesis (20 mg/L, 0.13 mM).

In order to improve 6-MSA synthesis, additional PPTases were screened to find a better suited enzyme for the required ChlB1$_{Sa}$ activation. Unexpectedly, the constructed strain *C. glutamicum* DelAro^4 P$_{T7}$-*4cl*$_{PcCg}$ P$_{gltA}$::P$_{dapA}$-C5 mu*fasO*$_{BCD1}$ pMKEx2-*malE*$_{Ec}$-*chlB1*$_{Sa}$ without any heterologous PPTase activity turned out to accumulate the highest 6-MSA concentrations (41 mg/L, 0.27 mM), indicating that *C. glutamicum* provides endogenous PPTase activity enabling ChlB1$_{Sa}$ activation. Genome analysis revealed two genes encoding the known PPTases in *C. glutamicum*, *pptA*$_{Cg}$ (cg2171) and *acpS*$_{Cg}$ (cg2738) (Ikeda & Nakagawa, 2003; Kalinowski *et al.*, 2003). Amino acid sequence alignments with Svp$_{Sv}$ suggested that PptA$_{Cg}$ is more likely to activate ChlB1$_{Sa}$ than AcpS$_{Cg}$ as both, Svp$_{Sv}$ and PptA$_{Cg}$, belong to the EntD superfamily of PPTases. The deletion of *pptA*$_{Cg}$ made little sense since a serious growth phenotype is known that would prevent a reliable assessment of the influence on 6-MSA production (Chalut *et al.*, 2006). Here, the putative *in vivo* function of PptA$_{Cg}$ is the activation of the sole type I PKS13, which is involved in the synthesis of corynomycolic acids in *C. glutamicum*. Instead, an overexpression of *pptA*$_{Cg}$ was performed to evaluate whether this PPTase contributes to 6-MSA synthesis. Similarly to the episomal expression of heterologous PPTases, overexpression of *pptA*$_{Cg}$ reduced the final 6-MSA titer (18 mg/L, 0.12 mM). Eventually, the reduced titers upon episomal expression of PPTase-encoding genes were demonstrated to be due to an increased metabolic burden. It was concluded that the native expression level of the genomically enconded *pptA*$_{Cg}$ gene is most suitable for ChlB1$_{Sa}$ activation.

In addition to type I PKSs, nonribosomal peptide synthases (NRPSs) also require post-translational phosphopantetheinylation for catalytic activity. In *E. coli*, the NRPSs EntB and EntF, both activated by the PPTase EntD, are involved in the synthesis of enterobactin. To characterize the spectrum of PKSs and NRPS activated by PptA$_{Cg}$, it was investigated whether PptA$_{Cg}$ from *C. glutamicum* could compensate for the loss of EntD in *E. coli*. Indeed, enterobactin synthesis was restored upon expression of *pptA*$_{Cg}$. This suggests that PptA$_{Cg}$ is a broad spectrum PPTase capable of activating both PKSs in *C. glutamicum* and NRPSs in *E. coli*.

1.8 Conclusion and outlook

In the course of this thesis, *C. glutamicum* was tailored towards improved malonyl-CoA availability by rational metabolic engineering. In this context, modulation of the central carbon metabolism and transcriptional deregulation of *accBC* and *accD1* encoding the two subunits of ACC were demonstrated to be promising strategies for efficiently increasing malonyl-CoA availability without relying on the addition of cerulenin. This allowed for increasing the microbial synthesis of polyphenolic polyketides naringenin (65 mg/L, 0.24 mM) and resveratrol (450 mg/L; 1.97 mM) even beyond reported product titers obtained when supplementing

cerulenin during the production phase (Figure 11). Considering of this performance, *C. glutamicum* M-CoA was established as the third host for the microbial synthesis of these compounds alongside *E. coli* and *S. cerevisiae*.

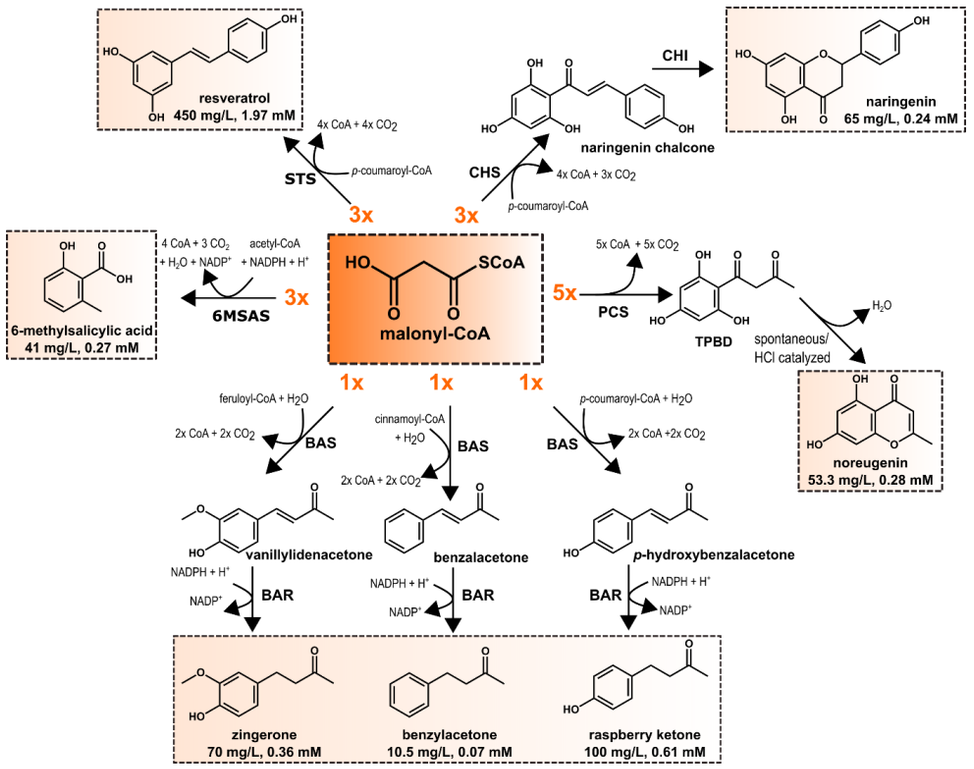

Figure 11: Overview of the different malonyl-CoA dependent polyketides microbially synthesized with *C. glutamicum* in this thesis. 6MSAS: 6-methylsalicylic acid synthase, BAR: benzalacetone reductase, BAS: benzalacetone synthase, CHI: chalcone isomerase, CHS: chalcone synthase, PCS: pentaketide chromone synthase, STS: stilbene synthase, TPBD: 1-(2,4,6-tri-hydroxyphenyl)butane-1,3-dione.

Additionally, the type III PKS catalyzed biosynthesis of a pentaketide and phenylbutanoids was established in this microorganism using the extensively engineered variant *C. glutamicum* M-CoA. Upon expression of a codon-optimized gene variant encoding a truncated PCS from *A. arborescens*, 53.3 mg/L (0.28 mM) noreugenin accumulated in defined CGXII medium supplemented with casamino acids within 24 hours. Furthermore, 100 mg/L (0.61 mM) raspberry ketone, 70 mg/L (0.36 mM) zingerone and 10.5 mg/L (0.07 mM) benzylacetone were synthesized using a *ldhA*-deficient variant of *C. glutamicum* M-CoA. In the context of this study, a codon-optimized gene variant encoding BAS from *R. palmatum* was functionally expressed. Not only an endogenous BAR activity of *C. glutamicum* was demonstrated, but also a yet unknown substrate promiscuity of CurA from *E. coli*.

Establishing the synthesis of the type I polyketide 6-MSA in *C. glutamicum* is of particular significance as the functional expression of large genes encoding for the required type I PKS is generally regarded to be very challenging. Furthermore, an endogenous PPTase of *C. glutamicum* indicated activity with a broad range of substrates. This could significantly accelerate the functional expression of future type I PKS genes compared to other host organisms. Often, these genes originate from the genus of *Streptomyces*, characterized by a high GC-content. As *C. glutamicum* belongs to the common order of *Actinomycetales*, a general compatibility with *Streptomyces* genes can be expected. Moreover, the close structural similarity between type I FAS of *C. glutamicum* and type I PKSs suggests that the expression machinery of *C. glutamicum* in general is capable of synthesizing such large multi-domain polypeptides, which could prove to be advantageous. Therefore, the constructed strain *C. glutamicum* M-CoA represents a promising platform for future studies aiming at the microbial synthesis of type I PKS-derived polyketides.

2 Peer-reviewed publications

2.1 Limitations for the microbial synthesis of plant polyphenolic polyketides

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MINI-REVIEW

Production of plant-derived polyphenols in microorganisms: current state and perspectives

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Abstract
Plants synthesize several thousand different polyphenols of which many have the potential to aid in preventing or treating cancer, cardiovascular, and neurodegenerative diseases. However, plants usually contain complex polyphenol mixtures impeding access to individual compounds in larger quantities. In contrast, functional integration of biosynthetic plant polyphenol pathways into microorganisms allows for the production of individual polyphenols as chemically distinct compounds, which can be synthesized in large amounts and can be more easily isolated. Over the last decade, microbial synthesis of many plant polyphenols could be achieved, and along the way, many decisive bottlenecks in the endogenous microbial host metabolism as well as in the heterologous plant pathways could be identified. In this review, we present recent advancements in metabolic engineering of microorganisms for the production of plant polyphenols and discuss how current challenges could be addressed in the future.

Keywords Polyphenols · Metabolic engineering · Phenylpropanoids · Resveratrol · Naringenin · Malonyl-CoA

Introduction

In addition to alkaloids and isoprenoids, polyphenols constitute the third class of plant secondary metabolites (Bourgaud et al. 2001). By definition, polyphenols are characterized by two or more aromatic rings and at least two phenolic hydroxy groups, but a few hydroxylated one-ring aromatics such as gallic acid or pyrogallol have been also added to this group (Badhani et al. 2015; Tinh et al. 2016). Typically, polyphenolic compounds are not directly involved in plant growth and propagation. Instead, they counteract microbial infections or are involved in protecting the plant against UV radiation, i.e., by neutralizing reactive oxygen species in light-exposed plant tissues (Bennett and Wallsgrove 1994; Kootstra 1994). Furthermore, polyphenols can also confer coloration, attract pollinators, or provide protection from herbivores (Crozier et al. 2006; Holton and Cornish 1995).

Due to their natural function as antioxidants and radical-scavenging agents, some polyphenols demonstrate health-promoting effects in humans and thus have the potential to help in preventing or treating of certain types of cancer, cardiovascular and neurodegenerative diseases, diabetes, and obesity (Khurana et al. 2013; Landete 2012; Pandey and Rizvi 2009). It is assumed that the described positive effects strongly depend on the bioavailability and the consumed amount of polyphenols (Saura-Calixto et al. 2007). In this context, access to individual polyphenolic compounds is of great interest for studying the presumed health-promoting effects in more detail. Unfortunately, only low amounts of individual polyphenols accumulate in plants and not all of these compounds are produced at all times as their biosynthesis often requires environmental triggers. Polyphenol compositions in plants strongly vary among different plant species and plant tissues, and are also subjected to seasonal and geographical variation. Thus, polyphenol extraction from the native plant is in most cases economically not feasible. Only in rare cases plant-extracted polyphenols made it to commercialization as dietary supplements, e.g., in case of the stilbene resveratrol or the flavonol quercetin. In most cases, chemical polyphenol synthesis is also not profitable as it involves complex reaction cascades, utilization or accumulation of toxic chemicals, and laborious purification (Quideau et al. 2011). Alternatively, functional introduction of plant-derived

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pathways in genetically engineered microbial host strains represents a promising strategy towards a more environmentally friendly polyphenol production from renewable and inexpensive resources.

In plants, the two major classes of polyphenols, stilbenoids and flavonoids, are derived from the ubiquitous aromatic amino acids L-phenylalanine (Phe) or L-tyrosine (Tyr). Both amino acids are initially converted to phenylpropanoids by non-oxidative deamination. In this reaction, phenylalanine ammonia lyases (PAL) and tyrosine ammonia lyases (TAL) convert Phe and Tyr to the phenylpropanoid cinnamic acid and *p*-coumaric acid, respectively (MacDonald and D'Cunha 2007; Rosler et al. 1997) (Fig. 1). Both phenylpropanoids can be further modified by hydroxylation- or *O*-methylation reactions, which gives rise to additional phenylpropanoids such as caffeic acid, ferulic acid, hydroxyferulic acid, and sinapic acid (Hahlbrock and Scheel 1989) (Fig. 1). For the synthesis of stilbenoids and flavonoids, phenylpropanoids as direct precursor molecules are first converted to their respective coenzyme A (CoA) thioesters. These ATP-dependent CoA ligation reactions are catalyzed by 4-coumarate: CoA ligases (4CL). Subsequently, two different type III polyketide synthases (stilbene synthases, STS or chalcone synthases, CHS) consume the phenylpropanoid CoA thioesters as starter units and catalyze three malonyl-CoA-dependent chain elongation steps yielding identical tetraketide intermediates, which are subsequently converted either into a stilbene (STS-catalyzed reaction) or into a chalcone (CHS-catalyzed reaction) (Tropf et al. 1994) (Fig. 1). Chalcone isomerases catalyze the subsequent isomerization of chalcones to (2*S*)-flavanones. The (2*S*)-flavanone naringenin is the first compound in the flavonoid pathway constituting the "flavonoid core", and represents the most important precursor molecule for almost all flavonoids. Naringenin can be further converted to dihydroflavonols, flavonols, isoflavones, and anthocyanidins (all belonging to the class of flavonoids) by

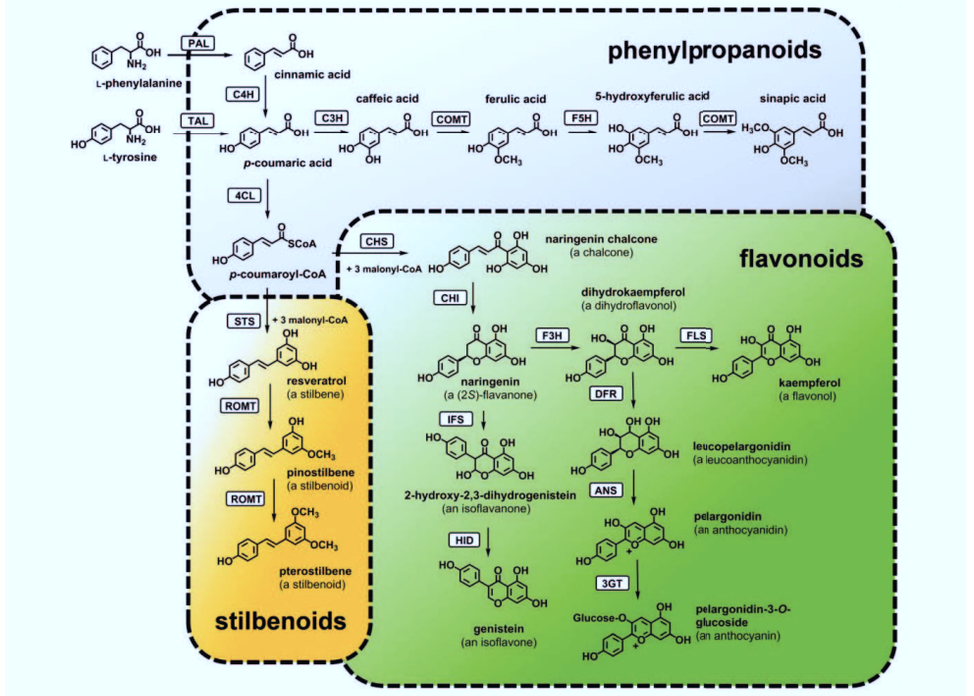

Fig. 1 Pathways for phenylpropanoid, stilbenoid, and flavonoid synthesis starting from aromatic amino acids. For the sake of simplicity, only the stilbenoids and flavonoids derived from *p*-coumaric acid are depicted. PAL phenylalanine ammonia lyase, C4H cinnamate 4-hydroxylase, TAL tyrosine ammonia lyase, C3H coumarate 3-hydroxylase, COMT caffeate *O*-methyltransferase, F5H ferulate 5-hydroxylase, 4CL 4-coumarate: CoA ligase, STS stilbene synthase, ROMT resveratrol-di-*O*-methyltransferase, CHS chalcone synthase, CHI chalcone isomerase, IFS 2-hydroxyisoflavanone synthase, HID 2-hydroxyisoflavanone dehydratase, F3H flavanone 3-hydroxylase, FLS flavonol synthase, DFR dihydroflavonol reductase, ANS anthocyanidin synthase, 3GT anthocyanidin 3-glycosyltransferase

2-oxoglutarate dioxygenases and reductases (Fig. 1). Flavonoids as well as stilbenes can be decorated with glycosyl, methyl, acetyl or other acyl groups forming a large set of different stilbenoids and flavonoids with different chemical properties (Ibrahim et al. 1998; Vogt and Jones 2000). Polyphenol decoration is typically associated with an increase in water solubility, bioavailability, or molecule stability (Xiao and Högger 2015).

Plants and microorganisms share a similar primary metabolism, which means that the microbial metabolism can in principle also provide aromatic amino acids and malonyl-CoA as polyphenol precursor molecules. This also applies for relevant cosubstrates used for decoration, e.g., the methyl donor *S*-adenosyl methionine (SAM) and the glycosyl donor UDP-glucose. Hence, microorganisms represent an attractive alternative production platform for polyphenols when the respective plant pathways consuming these precursor molecules can be functionally introduced.

In this review article, we summarize key advancements in engineering microorganisms towards polyphenol production of the last 15 years. During these years, researchers focused on the functional introduction of plant-derived enzymes into the microbial hosts and on the optimal connection of the respective microbial carbon metabolism to the heterologous polyphenol pathways. In some organisms also competing pathways, consuming polyphenol precursor metabolites had to be identified and abolished.

Functional introduction of plant-derived pathways enables polyphenol production in microorganisms

The first plant-derived polyphenols produced in a microorganism were the (2*S*)-flavanones naringenin and pinocembrin, which are derived from the phenylpropanoids *p*-coumaric acid and cinnamic acid, respectively. In one of the first studies, expression of heterologous genes coding for TAL from the yeast *Rhodotorula rubra*, 4CL from *Streptomyces coelicolor* and CHS from *Glycyrrhiza echinata* in *Escherichia coli* allowed for the accumulation of 0.5 mg/L naringenin and 0.8 mg/L pinocembrin from supplemented Tyr and Phe, respectively (Hwang et al. 2003). The product titer could be considerably increased in 2004, when genes coding for a TAL from *Rhodobacter sphaeroides* and for 4CL and CHS from *Arabidopsis thaliana* were expressed in *E. coli*. The obtained strain produced 21 mg/L naringenin starting from Tyr (Watts et al. 2004). The same group also reported production of 105 mg/L resveratrol from *p*-coumaric acid in an *E. coli* strain expressing an STS-encoding gene from peanut (*Arachis hypogaea*) (Watts et al. 2006). In 2003, it could be shown that *Saccharomyces cerevisiae* can also serve as host organism for polyphenol production. By expressing a *4cl* gene from *Populus sp*. in combination with an *sts* gene from grape (*Vitis vinifera*) 0.001 mg/L resveratrol could be produced (Becker et al. 2003). Starting from 2005, other compound classes of the large flavonoid family were tapped by demonstrating production of flavones and flavonols in *E. coli* and *S. cerevisiae* for the first time (Leonard et al. 2005; Leonard et al. 2006a; Leonard et al. 2006b).

Since 2009, other microbial host organisms such as *Corynebacterium glutamicum*, *Lactococcus lactis*, and *Streptomyces venezuelae* were successfully introduced as alternative hosts for the microbial production of polyphenols (Donnez et al. 2009; Kallscheuer et al. 2016c; Park et al. 2009). In case of *C. glutamicum*, the natural competence for degrading phenylpropanoid precursor molecules needed to be eliminated before the organism could be successfully employed for polyphenol synthesis (Kallscheuer et al. 2016a; Kallscheuer et al. 2016c).

Increasing the supply of malonyl-CoA for improved polyphenol production

In these first studies, it became obvious that supplemented aromatic amino acids or phenylpropanoids were never completely converted to the corresponding polyphenols. This finding was attributed to the low intracellular availability of malonyl-CoA, which was identified as major bottleneck during polyphenol production in engineered microorganisms (Miyahisa et al. 2005). Malonyl-CoA, produced from acetyl-CoA by an acetyl-CoA carboxylase (ACC), is for the most part endogenously consumed during chain elongation reactions in fatty acid biosynthesis. Hence, the intracellular level of malonyl-CoA is strictly regulated at the level of ACC activity to prevent any fatty acid overproduction (Brownsey et al. 2006; Tehlivets et al. 2007). Metabolic engineering of polyphenol-producing microorganisms aimed at enhancing the malonyl-CoA supply by increasing its biosynthesis and by decreasing its conversion during fatty acid biosynthesis.

A considerable increase in the obtained polyphenol titers could be observed in the presence of fatty acid biosynthesis inhibitors. In most studies, the natural fatty acid synthase-inhibiting antibiotic cerulenin was supplemented, which increased the production of polyphenols in engineered *E. coli* 3- to 20-fold (Leonard et al. 2008; Santos et al. 2011; van Summeren-Wesenhagen and Marienhagen 2015). A constructed *E. coli* strain expressing heterologous genes coding for a 4CL from *A. thaliana* and for an STS from *V. vinifera* produced 2.3 g/L of the stilbene resveratrol by a two-step biotransformation from *p*-coumaric acid in presence of cerulenin (Lim et al. 2011). This is the highest reported resveratrol titer obtained from the precursor *p*-coumaric acid in a microorganism to date. In *C. glutamicum*, the production of resveratrol increased 13-fold (final titer 158 mg/L) in presence of cerulenin (Kallscheuer et al. 2016c). Cerulenin is very expensive (20 € per mg), and thus, cannot be used in large-scale fermentations. Alternatively, fatty acid synthesis was down-regulated by posttranscriptional gene

silencing using antisense RNA (asRNA). Expression of an asRNA targeting the mRNA of the malonyl-CoA-acyl carrier protein (ACP) transacylase gene *fadD* resulted in a 4.5-fold increase of the intracellular malonyl-CoA concentration in *E. coli* (Yang et al. 2015). This in turn allowed for a 1.5-fold increase of the naringenin titer (91 mg/L) and a 1.7-fold increase of the resveratrol titer (268 mg/L). In a similar study, an increase of the naringenin titer from 91 to 391 mg/L (more than fourfold increase) was observed when the expression of the genes *fabH* and *fapB* was downregulated by posttranscriptional gene silencing (Wu et al. 2014a). FabH and FabB are 3-oxoacyl-ACP synthases, which catalyze the initial malonyl-CoA-dependent elongation reaction of the iterative fatty acid synthesis (Tsay et al. 1992). More recently, expression of genes involved in fatty acid biosynthesis was down-regulated by using CRISPRi, which increased the obtained polyphenol titer of naringenin sevenfold (final titer 422 mg/L) and of the stilbene pinosylvin twofold (final titer 47 mg/L) (Cress et al. 2015; Liang et al. 2016; Wu et al. 2015).

Improved supply of malonyl-CoA typically relies on increasing the ACC activity for enhancing the conversion of acetyl-CoA to malonyl-CoA. Alternatively, a malonate assimilation pathway from *Rhizobium trifolii* was used, in which malonate is initially converted to malonyl-CoA by CoA-ligation (An and Kim 1998). Two of the involved genes coding for a malonate carrier protein (*matC*) and a malonyl-CoA synthetase (*matB*) were exploited for increased polyphenol production from supplemented malonate (Fig. 2). By following this strategy in *E. coli*, the production of the (2*S*)-flavanone pinocembrin was increased up to 15-fold (final titer 480 mg/L) (Leonard et al. 2008; Wu et al. 2013). This approach requires the supplementation of malonate as precursor

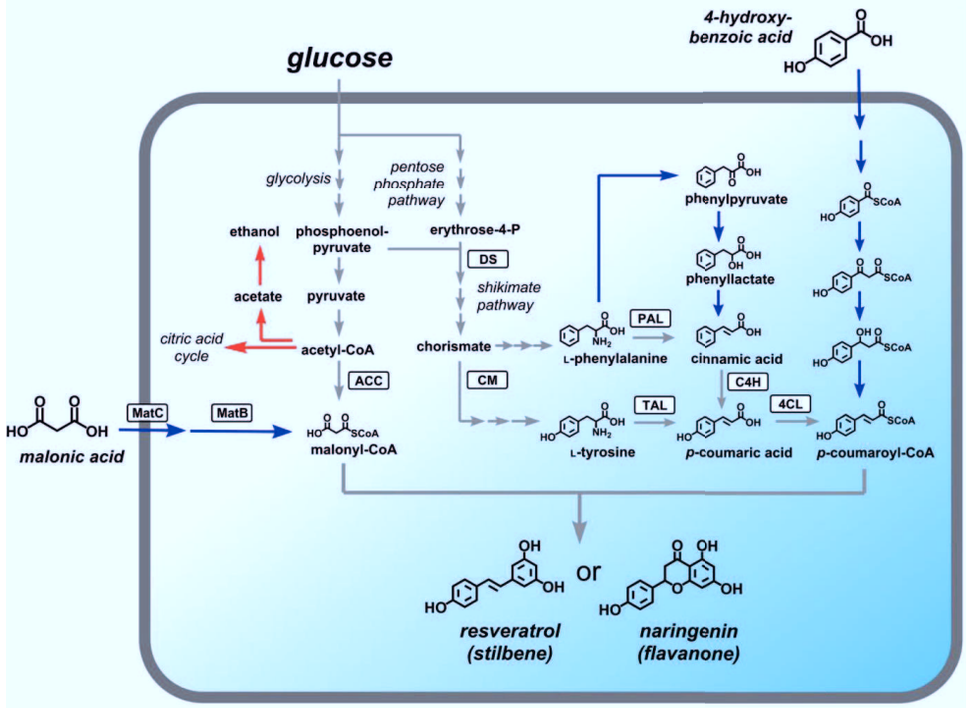

Fig. 2 Overview of engineered natural and non-natural pathways supplying *p*-coumaroyl-CoA and malonyl-CoA as polyphenol precursor molecules. The relevant natural pathways for the synthesis of aromatic amino acids (shikimate pathway), phenylpropanoids (ammonia-lyase reaction), and malonyl-CoA (carboxylation of acetyl-CoA) are indicated by gray arrows. Red arrows indicate competing reactions consuming acetyl-CoA, which are either abolished or downregulated in engineered microbial producer strains. The orthogonal pathway for malonyl-CoA (malonic acid assimilation pathway from *Rhizobium trifolii)* and the functional non-natural pathways leading to cinnamic acid or *p*-coumaroyl-CoA are indicated by blue arrows. ACC acetyl-CoA carboxylase, MatC malonate carrier protein, MatB malonyl-CoA synthetase, DS 3-Deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase, CM chorismate mutase, PAL phenylalanine ammonia lyase, TAL tyrosine ammonia lyase, C4H cinnamate 4-hydroxylase, 4CL 4-coumarate: CoA ligase

and was also functionally implemented in other organisms such as *S. venezuelae* (Park et al. 2011).

Alternatively, for an increased ACC activity, four genes encoding a heterotetrameric ACC along with a gene for a necessary biotin ligase from *Photorhabdus luminescens* were heterologously expressed in *E. coli* (Leonard et al. 2007). This strategy allowed for a sevenfold increase of the microbial pinocembrin production from supplemented cinnamic acid with a maximal product titer of 196 mg/L (Leonard et al. 2007). Alternatively, the heterodimeric enzyme AccBC-AccD1 of *C. glutamicum* was employed as a functional ACC in *E. coli* as it only requires expression of two genes (Gande et al. 2007; Miyahisa et al. 2005). This strategy boosted the intracellular malonyl-CoA concentration in *E. coli* 15-fold and allowed for the accumulation of 1.3 g/L phloroglucinol (Zha et al. 2009). In contrast, the monomeric ACC of *S. cerevisiae* is a multi-domain protein encoded by the gene *acc1*. Overexpression of *acc1* improved the resveratrol production in *S. cerevisiae* twofold to 6 mg/L (Shin et al. 2012). ACC1 activity was found to be repressed by protein kinase-dependent phosphorylation of two serine side chains. Substitution of serine for alanine at both positions abolished the posttranslational regulation and led to increased levels of malonyl-CoA (Shi et al. 2014).

The central carbon metabolism of polyphenol-producing *E. coli* strains was additionally engineered towards increased acetyl-CoA supply. The elimination of pathways for acetate and ethanol formation, interruption of the citric acid cycle, and an increased flux from glucose to pyruvate proved to be suitable strategies for increasing acetyl-CoA supply (Fowler et al. 2009; Xu et al. 2011; Zha et al. 2009) (Fig. 2). Combination of genetic modifications ensuring increased ACC activity and higher intracellular acetyl-CoA availability yielded an *E. coli* strain capable of producing 470 mg/L naringenin from supplemented *p*-coumaric acid (Xu et al. 2011).

Engineering of the microbial aromatic amino acid metabolism enables phenylpropanoid and polyphenol synthesis from glucose

In initial studies, engineered microorganisms typically produced the desired plant polyphenols from supplemented phenylpropanoids or amino acids as precursor molecules (Lussier et al. 2012). However, microbial production from cheap substrates such as glucose or ethanol would be more economical. Metabolic engineering into this direction included two major goals: (i) increasing the carbon flux into the shikimate pathway for the biosynthesis of the aromatic amino acids Phe or Tyr and (ii) the functional introduction of ammonia lyases for the efficient conversion of aromatic amino acids to the respective phenylpropanoids (Fig. 2). Similar to other amino acid biosynthetic pathways, the shikimate pathway is mainly regulated by feedback inhibition of allosterically controlled key enzymes (Herrmann and Weaver 1999; Ikeda 2006). In most microorganisms, the aromatic amino acids inhibit the activity of the 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase (DS), which catalyzes the initial committed step of the pathway (Fig. 2). In *E. coli*, an increased flux into the shikimate pathway was achieved by employing feedback-resistant DS variants (Ger et al. 1994; Jossek et al. 2001; Ray et al. 1988). Introduction of a feedback-resistant DS in combination with a feedback-resistant chorismate mutase/prephenate dehydrogenase (catalyzing the reaction at the metabolic branch leading to Tyr) and a TAL enabled coupling of the endogenous primary metabolism to the heterologous polyphenol pathway. In *E. coli*, naringenin was produced from glucose with maximal titers of 80–100 mg/L (Santos et al. 2011; Wu et al. 2014b). By following the same strategy for elevated intracellular Phe availability, production of 40 mg/L pinocembrin from glucose could be established (Wu et al. 2013). The production of sakuranetin (7-*O*-methylnaringenin) and ponciretin (4′-*O*-methylnaringenin) as more complex flavonoids from glucose in *E. coli* required heterologous expression of altogether eight genes (Kim et al. 2013). In this study, synthesis of 40 mg/L sakuranetin and 43 mg/L ponciretin could be observed. In *C. glutamicum*, deregulation of the shikimate pathway and introduction of a heterologous TAL from *Flavobacterium johnsoniae* enabled the production of 60 mg/L resveratrol and 32 mg/L naringenin from glucose (Kallscheuer et al. 2016c). In *S. cerevisiae*, the same strategy as in bacteria could be successfully adopted for overproducing aromatic amino acids as polyphenol precursor molecules (Rodriguez et al. 2015). In addition to these genetic modifications, the Ehrlich pathway competing for aromatic amino acids was abolished. The resulting yeast strain proved to be a suitable platform for the production of phenylpropanoid-derived compounds from glucose as accumulation of 2 g/L *p*-coumaric acid could be observed. In a follow-up study, *S. cerevisiae* was further engineered for the production of resveratrol by introducing genes for 4CL and STS enzyme activities and by increasing the malonyl-CoA supply (Li et al. 2016). The best-performing strain accumulated 800 mg/L resveratrol, which is the highest reported polyphenol titer obtained from glucose in an engineered microorganism to date.

Especially in bacterial production strains, low activity of the heterologously introduced PALs or TALs was found to be another bottleneck limiting overall polyphenol production from glucose (Eudes et al. 2013; Kallscheuer et al. 2016c; Lin and Yan 2012). This is most probably related to the enzyme-catalyzed reaction mechanism, which includes elimination of the non-acidic proton at the C3 carbon and was proposed to be associated with a dearomatization of the aromatic ring of the substrate (MacDonald and D'Cunha 2007). In a comparative in vivo study, ammonia lyases from various donor organisms were evaluated in the three microbial platform organisms *E. coli*, *S. cerevisiae*, and *L. lactis*, and several

enzymes exhibiting high activity and substrate specificity could be identified (Jendresen et al. 2015). In plants, no ammonia lyase-independent route to phenylpropanoids is known. More recently, bio-inspired, synthetic pathways leading to these polyphenol precursor molecules were designed in silico and successfully implemented into microbial production strains (Fig. 2). In *C. glutamicum*, the use of a CoA-dependent, β-oxidative phenylpropanoid catabolic pathway from *Azoarcus sp.* EbN1 was exploited in the non-natural anabolic direction for conversion of supplemented 4-hydroxybenzoic acid to *p*-coumaroyl-CoA, which was subsequently used for the microbial production of resveratrol (Kallscheuer et al. 2016b). In *E. coli*, a PAL-independent α-reductive pathway starting from phenylpyruvate yielded cinnamic acid via the pathway intermediate phenyllactate (Masuo et al. 2016) (Fig. 2).

Decoration of polyphenols in microbial production strains

Plants synthesize a large diversity of several thousand polyphenols by decoration of only a few stilbenoid and flavonoid backbone molecules. Decoration of polyphenols results from hydroxylation and subsequent *O*-methylation, *O*-acylation, or glycosylation reactions, which give rise to polyphenols with altered chemical properties influencing, e.g., water solubility and overall molecule stability (Tsao 2010). Initially, metabolic engineering of microorganisms for polyphenol production focused on the synthesis of stilbenoid and flavonoid backbone molecules, but scientists also started to tap the large natural polyphenol diversity by implementing downstream reactions for polyphenol decoration in the microbial production strains. Depending on the pathway and the hydroxy group to be decorated, required reactions can either be included at the earlier stage of phenylpropanoid precursor synthesis or at a later stage when the polyphenol core structure is available.

At the stage of phenylpropanoids, engineering efforts focused on the synthesis of *O*-methylated compounds such as ferulic acid and sinapic acid (Fig. 1) as these phenylpropanoids also serve as precursors for other compounds related to polyphenols (e.g., lignans, coumarins, and curcumins) (Marienhagen and Bott 2013). For this purpose, the phenylpropanoid *p*-coumaric acid was hydroxylated to caffeic acid in *E. coli* (Furuya and Kino 2014; Huang et al. 2013). Depending on the amount of supplemented *p*-coumaric acid, up to 10.2 g/L caffeic acid could be produced, whereas the highest product titer obtained from glucose was 0.8 g/L. With an additional caffeate *O*-methyltransferase, 0.2 g/L ferulic acid could be produced from glucose (Kang et al. 2012), while sinapic acid synthesis could not be achieved in microorganisms yet.

Hydroxylation of polyphenol core structures in plants is mediated by membrane-bound cytochrome P450-dependent monooxygenases, which are difficult to functionally introduce into microorganisms (Rodrigues et al. 2015). In *E. coli*, translational fusion of a flavonoid 3′,5′-hydroxylase with a P450 reductase enabled production of flavonols with different hydroxylation patterns (Leonard et al. 2006b). The same group also published the functional introduction of a P450-dependent isoflavone synthase in *E. coli* and *S. cerevisiae* and demonstrated the production of the isoflavone genistein in both organisms (Leonard and Koffas 2007). Furthermore, the pathway for the flavonol fisetin, in which the intermediate resokaempferol is hydroxylated to fisetin in the last step, was reconstructed in *E. coli* (Stahlhut et al. 2015). In this study, the translational fusion of a truncated version of a flavonoid 3′-monooxygenase from *A. thaliana* with a truncated version of a cytochrome P450 reductase from *Catharanthus roseus* allowed for a fisetin titer of 1.2 mg/L. *C. glutamicum* was found to be a suitable microbial host organism for the production of flavonols. The functional introduction of two 2-oxoglutarate-dependent dioxygenases (flavanone 3-hydroxylase and flavonol synthase) in a (2*S*)-flavanone-producing *C. glutamicum* strain was key for developing a strain capable of accumulating the highest reported flavonol concentrations of 23 mg/L kaempferol and 10 mg/L quercetin from supplemented *p*-coumaric acid and caffeic acid, respectively, in the culture medium (Kallscheuer et al. 2017).

O-methylation of the stilbene resveratrol was achieved in *E. coli* and *C. glutamicum* by functional introduction of different *O*-methyltransferases. This allowed for the production of the mono-, di-, and tri-*O*-methylated resveratrol derivatives with maximal titers of 50 mg/L (Heo et al. 2017; Jeong et al. 2014; Kallscheuer et al. 2017; Kang et al. 2014; Wang et al. 2015b). In a similar manner, the introduction of glycosyltransferases from various donor organisms enabled production of glycosylated resveratrol derivatives comprising the 4′-*O*-glucoside and the 3-*O*-glucoside in *E. coli* (Choi et al. 2014; Ozaki et al. 2012). Anthocyanins, the most abundant flavonoids in fruits and vegetables, are glycosylated as this decoration improves the molecule stability compared to the very unstable anthocyanin agylcones referred to as anthocyanidins (Vogt and Jones 2000). For the production of anthocyanins in *E. coli*, genes coding for a UDP-glucose: flavonoid 3-*O*-glucosyltransferase from *Petunia hybrida* and *A. thaliana* were expressed and cultivations were performed under acidic conditions as anthocyanins show higher stability at a lower pH (Yan et al. 2008). Under optimized conditions, anthocyanin titers of 70–80 mg/L could be achieved. Very recently, more complex anthocyanins such as peonidin 3-*O*-glucoside were produced (Cress et al. 2017), while production of 10 mg/L pelargonidin-3-*O*-glucoside from glucose was demonstrated

using a four-strain *E. coli* polyculture collectively expressing 15 heterologous genes (Jones et al. 2017).

E. coli was used as a glycosylation platform supplying different UDP- or TDP-activated sugars. By using this strategy, several glycosylated flavonol derivatives were produced when the respective aglycone was supplemented (De Bruyn et al. 2015; Pandey et al. 2013; Simkhada et al. 2010; Yang et al. 2014). For the glycosylation of various flavonoids also bacterial glycosyltransferases from *Bacillus cereus* or *Xanthomonas campestris* were successfully employed, e.g., for the synthesis of kaempferol 7-*O*-glucoside, quercetin 3-*O*-glucoside, and luteolin-3′-*O*-glucoside (Hyung Ko et al. 2006; Kim et al. 2007). In a recent study, the glycosyl donor metabolite dTDP-glucose was modified by an additional dehydratase and an aminotransferase, which enabled synthesis of the non-natural compound fisetin 3-*O*-4-amino-4,6-dideoxy-galactoside (Pandey et al. 2016). This recent study demonstrates that manipulation of pathways for the respective sugar donors can be used to carry out tailor-made decorations, which eventually lead to synthetic polyphenol glycosides. In parallel, supplementation of different precursor metabolites enabled synthesis of nearly 100 different polyphenol aglycones and half of these represent non-natural compounds (Chemler et al. 2007; Katsuyama et al. 2007). Taken together, the great natural diversity of polyphenols can even be surpassed by combining non-natural polyphenols with modified sugar residues. When considering that polyphenols can carry several decorations, one can imagine that a very large variety of compounds with different properties can be made accessible by this approach.

Table 1 Production of phenylpropanoids, stilbenoids and flavonoids in engineered microorganisms

Product	Product titer		Precursor	Precursor titer		Organism	Special conditions	Reference
	[mg/L]	[mM]		[mg/L]	[mM]			
Phenylpropanoids								
p-Coumaric acid	1930	11.8	None, from glucose	–	–	*S. cerevisiae*		Rodriguez et al. 2015
Caffeic acid	767	4.2	None, from glucose	–	–	*E. coli*		Huang et al. 2013
Caffeic acid	10,200	56.6	*p*-Coumaric acid	16,400	100	*E. coli*		Furuya and Kino 2014
Ferulic acid	196	1.0	None, from glucose	–	–	*E. coli*		Kang et al. 2012
Stilbenoids								
Pinosylvin	91	0.43	Phenylalanine	496	3	*E. coli*	Addition of cerulenin	van Summeren-Wesenhagen and Marienhagen 2015
Pinosylvin	121	0.57	Cinnamic acid	741	5	*C. glutamicum*	Addition of cerulenin	Kallscheuer et al. 2016c
Resveratrol	2340	10.3	*p*-Coumaric acid	2462	15	*E. coli*	Addition of cerulenin	Lim et al. 2011
Resveratrol	812	3.6	None, from glucose	–	–	*S. cerevisiae*		Li et al. 2016
Piceatannol	65	0.27	Resveratrol	100	0.44	*E. coli*		Wang et al. 2015a
Piceatannol	56	0.23	Caffeic acid	900	5	*C. glutamicum*	Addition of cerulenin	Kallscheuer et al. 2016c
Pinostilbene	34	0.14	Resveratrol	228	1	*E. coli*		Jeong et al. 2014
Pterostilbene	34	0.13	None, from glucose	–	–	*E. coli*		Heo et al. 2017
Pterostilbene	170	0.66	Resveratrol	228	1	*E. coli*		Wang et al. 2015b
(2S)-Flavanones								
Pinocembrin	710	2.8	Cinnamic acid	296	2	*E. coli*	Addition of cerulenin	Leonard et al. 2008
Pinocembrin	432	1.7	None, from glucose	–	–	*E. coli*	Malonate assimilation	Wu et al. 2016
Naringenin	474	1.7	*p*-Coumaric acid	426	2.6	*E. coli*		Xu et al. 2011
Naringenin	391	1.4	L-Tyrosine	543	3	*E. coli*	Malonate assimilation	Wu et al. 2014a
Naringenin	84	0.31	None, from glucose	–	–	*E. coli*	Addition of cerulenin	Santos et al. 2011
Eriodictyol	107	0.37	L-Tyrosine	181	1	*E. coli*		Zhu et al. 2014
Flavones								
Chrysin	9	0.04	L-Phenylalanine	543	3	*E. coli*		Miyahisa et al. 2006
Chrysin	31	0.12	Cinnamic acid	178	1.2	*S. venezuelae*	Malonate assimilation	Park et al. 2011
Apigenin	30	0.11	*p*-Coumaric acid	49	0.3	*E. coli*		Lee et al. 2015
Apigenin	15	0.06	*p*-Coumaric acid	197	1.2	*S. venezuelae*	Malonate assimilation	Park et al. 2011
Flavonols								
Kaempferol	66	0.23	*p*-Coumaric acid	164	1	*S. cerevisiae*	Fed-batch cultivation	Duan et al. 2017
Kaempferol	23	0.08	*p*-Coumaric acid	820	5	*C. glutamicum*	Addition of cerulenin	Kallscheuer et al. 2017
Quercetin	10	0.03	Caffeic acid	900	5	*C. glutamicum*	Addition of cerulenin	Kallscheuer et al. 2017
Isoflavones								
Genistein	8	0.03	Naringenin	136	0.5	*S. cerevisiae*		Trantas et al. 2009
Anthocyanins								
Pelargonidin-3-*O*-glucoside	79	0.18	(+)-Afzelechin	206	0.75	*E. coli*		Yan et al. 2008
Cyanidin-3-*O*-glucoside	350	0.78	(+)-Catechin	1016	3.5	*E. coli*		Lim et al. 2015

Conclusion and outlook

Major breakthroughs in this field allowed for the construction of microbial cell factories for a broad range of different plant-derived polyphenols in the last 15 years. However, whereas flavonoid and stilbene core structures can now be produced at (almost) gram-scale from cheap glucose (Table 1), microbial synthesis of more complex polyphenol structures remains challenging. In such cases, accumulation of only low product concentrations usually impedes any commercial production. At least a tenfold increase in the current polyphenol titers would be necessary to render microbial production of these compounds economically feasible. Major limitations are (still) insufficient supply of precursor molecules by the microbial metabolism and low activity of plant-derived enzymes in heterologous hosts. However, recent technological innovations such as the development of biosensor-driven directed evolution approaches could enable rapid engineering of microbial host strains for providing more precursor molecules to increase polyphenol synthesis (Schallmey et al. 2014; Siedler et al. 2017). New molecular techniques for the rapid assembly of biosynthetic pathways and balancing of (heterologous) gene expression in the metabolic context of the microbial host could improve rerouting of carbon fluxes to and through polyphenol-providing pathways of interest (van Summeren-Wesenhagen et al. 2015). Similarly, approaches and concepts from synthetic biology allowing for dynamic pathway regulation and metabolic control could be also employed to harmonize endogenous and heterologous pathways for maximizing product titers (Xu et al. 2014). First examples of new pathways for polyphenol synthesis, not found in nature, have been presented and more functional synthetic routes from the scientist's drawing board are expected to become available in the future (Kallscheuer et al. 2016b). Furthermore, protein engineering strategies might help to improve the performance of plant enzymes in the respective microbial host and chemical diversification of precursor molecules or the final polyphenol products might give rise to compounds with new properties. No less important than the genetic design of the microbial cell factories is the aspect of process engineering as optimization and balancing of microbial growth and product formation are decisive for success. In addition, in situ product removal for preventing undesired oxidation of the target compounds and for minimizing potentially toxic effects of the polyphenols on the microbial host will be of increasing importance as the obtained product titers also increase (Braga et al. 2017).

Future developments require joint efforts of multiple disciplines such as biology, biochemistry, chemistry, process engineering, and other disciplines (Dudnik et al. 2017). For this reason, collaborative projects in which scientists with different backgrounds and ideas work on the goal of an economically feasible microbial polyphenol production are of great importance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

An JH, Kim YS (1998) A gene cluster encoding malonyl-CoA decarboxylase (MatA), malonyl-CoA synthetase (MatB) and a putative dicarboxylate carrier protein (MatC) in *Rhizobium trifolii*. FEBS J 257(2):395–402

Badhani B, Sharma N, Kakkar R (2015) Gallic acid: a versatile antioxidant with promising therapeutic and industrial applications. RSC Adv 5(35):27540–27557. https://doi.org/10.1039/C5RA01911G

Becker JV, Armstrong GO, van der Merwe MJ, Lambrechts MG, Vivier MA, Pretorius IS (2003) Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. FEMS Yeast Res 4(1):79–85. https://doi.org/10.1016/S1567-1356(03)00157-0

Bennett RN, Wallsgrove RM (1994) Secondary metabolites in plant defence mechanisms. New Phytol 127(4):617–633. https://doi.org/10.1111/j.1469-8137.1994.tb02968.x

Bourgaud F, Gravot A, Milesi S, Gontier E (2001) Production of plant secondary metabolites: a historical perspective. Plant Sci 161(5):839–851. https://doi.org/10.1016/S0168-9452(01)00490-3

Braga A, Oliveira J, Silva R, Ferreira P, Rocha I, Kallscheuer N, Marienhagen J, Faria N (2017) Impact of the cultivation strategy on resveratrol production from glucose in engineered *Corynebacterium glutamicum*. J Biotechnol 265:70–75

Brownsey R, Boone A, Elliott J, Kulpa J, Lee W (2006) Regulation of acetyl-CoA carboxylase. Biochem Soc Trans 34(Pt 2):223–227. https://doi.org/10.1042/BST0340223

Chemler JA, Yan Y, Leonard E, Koffas MA (2007) Combinatorial mutasynthesis of flavonoid analogues from acrylic acids in microorganisms. Org Lett 9(10):1855–1858. https://doi.org/10.1021/ol0703736

Choi O, Lee JK, Kang S-Y, Pandey RP, Sohng J-K, Ahn JS, Hong Y-S (2014) Construction of artificial biosynthetic pathways for resveratrol glucoside derivatives. J Microbiol Biotechnol 24(5):614–618. https://doi.org/10.4014/jmb.1401.01031

Cress BF, Leitz QD, Kim DC, Amore TD, Suzuki JY, Linhardt RJ, Koffas MA (2017) CRISPRi-mediated metabolic engineering of *E. coli* for *O*-methylated anthocyanin production. Microb Cell Fact 16(1):10. https://doi.org/10.1186/s12934-016-0623-3

Cress BF, Toparlak OD, Guleria S, Lebovich M, Stieglitz JT, Englaender JA, Jones JA, Linhardt RJ, Koffas MA (2015) CRISPathBrick: modular combinatorial assembly of type II-A CRISPR arrays for dCas9-mediated multiplex transcriptional repression in *E. coli*. ACS Synth Biol 4(9):987–1000. https://doi.org/10.1021/acssynbio.5b00012

Crozier A, Jaganath IB, Clifford MN (2006) Chapter 1: phenols, polyphenols and tannins: an overview. In: Plant secondary metabolites: occurrence, structure and role in the human diet. Blackwell Publishing Ltd. https://doi.org/10.1002/9780470988558.ch1

De Bruyn F, Van Brempt M, Maertens J, Van Bellegem W, Duchi D, De Mey M (2015) Metabolic engineering of *Escherichia coli* into a versatile glycosylation platform: production of bio-active quercetin glycosides. Microb Cell Factories 14(1):138. https://doi.org/10.1186/s12934-015-0326-1

Donnez D, Jeandet P, Clément C, Courot E (2009) Bioproduction of resveratrol and stilbene derivatives by plant cells and microorganisms. Trends Biotechnol 27(12):706–713. https://doi.org/10.1016/j.tibtech.2009.09.005

Duan L, Ding W, Liu X, Cheng X, Cai J, Hua E, Jiang H (2017) Biosynthesis and engineering of kaempferol in *Saccharomyces cerevisiae*. Microb Cell Factories 16(1):165. https://doi.org/10.1186/s12934-017-0774-x

Dudnik A, Almeida AF, Andrade R, Avila B, Bañados P, Barbay D, Bassard J-E, Benkoulouche M, Bott M, Braga A, Breitel D, Brennan R, Bulteau L, Chanforan C, Costa I, Costa RS, Doostmohammadi M, Faria N, Feng C, Fernandes A, Ferreira P, Ferro R, Foito A, Freitag S, Garcia G, Gaspar P, Godinho-Pereira J, Hamberger B, Hartmann A, Heider H, Jardim C, Julien-Laferriere A, Kallscheuer N, Kerbe W, Kuipers OP, Li S, Love N, Marchetti-Spaccamela A, Marienhagen J, Martin C, Mary A, Mazurek V, Meinhart C, Sevillano DM, Menezes R, Naesby M, Nørholm MHH, Okkels FT, Oliveira J, Ottens M, Parrot D, Pei L, Rocha I, Rosado-Ramos R, Rousseau C, Sagot M-F, dos Santos CN, Schmidt M, Shelenga T, Shepherd L, Silva AR, da Silva MH, Simon O, Stahlhut SG, Solopova A, Sorokin A, Stewart D, Stougie L, Su S, Thole V, Tikhonova O, Trick M, Vain P, Veríssimo A, Vila-Santa A, Vinga S, Vogt M, Wang L, Wang L, Wei W, Youssef S, Neves AR, Forster J (2017) BacHBerry: BACterial Hosts for production of Bioactive phenolics from bERRY fruits. Phytochem Rev. https://doi.org/10.1007/s11101-017-9532-2

Eudes A, Juminaga D, Baidoo EE, Collins FW, Keasling JD, Loqué D (2013) Production of hydroxycinnamoyl anthranilates from glucose in *Escherichia coli*. Microb Cell Factories 12(1):62. https://doi.org/10.1186/1475-2859-12-62

Fowler ZL, Gikandi WW, Koffas MA (2009) Increased malonyl coenzyme A biosynthesis by tuning the *Escherichia coli* metabolic network and its application to flavanone production. Appl Environ Microbiol 75(18):5831–5839. https://doi.org/10.1128/AEM.00270-09

Furuya T, Kino K (2014) Catalytic activity of the two-component flavin-dependent monooxygenase from *Pseudomonas aeruginosa* toward cinnamic acid derivatives. Appl Microbiol Biotechnol 98(3):1145–1154. https://doi.org/10.1007/s00253-013-4958-y

Gande R, Dover LG, Krumbach K, Besra GS, Sahm H, Oikawa T, Eggeling L (2007) The two carboxylases of *Corynebacterium glutamicum* essential for fatty acid and mycolic acid synthesis. J Bacteriol 189(14):5257–5264. https://doi.org/10.1128/JB.00254-07

Ger Y-M, Chen S-L, Chiang H-J, Shiuan D (1994) A single Ser-180 mutation desensitizes feedback inhibition of the phenylalanine-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthetase in *Escherichia coli*. J Biochem 116(5):986–990. https://doi.org/10.1093/oxfordjournals.jbchem.a124657

Hahlbrock K, Scheel D (1989) Physiology and molecular biology of phenylpropanoid metabolism. Annu Rev Plant Biol 40(1):347–369. https://doi.org/10.1146/annurev.pp.40.060189.002023

Heo KT, Kang S-Y, Hong Y-S (2017) *De novo* biosynthesis of pterostilbene in an *Escherichia coli* strain using a new resveratrol *O*-methyltransferase from *Arabidopsis*. Microb Cell Factories 16(1):30. https://doi.org/10.1186/s12934-017-0644-6

Herrmann KM, Weaver LM (1999) The shikimate pathway. Annu Rev Plant Biol 50(1):473–503. https://doi.org/10.1146/annurev.arplant.50.1.473

Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7(7):1071–1083. https://doi.org/10.1105/tpc.7.7.1071

Huang Q, Lin Y, Yan Y (2013) Caffeic acid production enhancement by engineering a phenylalanine over-producing *Escherichia coli* strain. Biotechnol Bioeng 110(12):3188–3196. https://doi.org/10.1002/bit.24988

Hwang EI, Kaneko M, Ohnishi Y, Horinouchi S (2003) Production of plant-specific flavanones by *Escherichia coli* containing an artificial gene cluster. Appl Environ Microbiol 69(5):2699–2706. https://doi.org/10.1128/AEM.69.5.2699-2706.2003

Hyung Ko J, Gyu Kim B, Joong-Hoon A (2006) Glycosylation of flavonoids with a glycosyltransferase from *Bacillus cereus*. FEMS Microbiol Lett 258(2):263–268. https://doi.org/10.1111/j.1574-6968.2006.00226.x

Ibrahim RK, Bruneau A, Bantignies B (1998) Plant *O*-methyltransferases: molecular analysis, common signature and classification. Plant Mol Biol 36(1):1–10. https://doi.org/10.1023/A:1005939803300

Ikeda M (2006) Towards bacterial strains overproducing L-tryptophan and other aromatics by metabolic engineering. Appl Microbiol Biotechnol 69(6):615–626. https://doi.org/10.1007/s00253-005-0252-y

Jendresen CB, Stahlhut SG, Li M, Gaspar P, Siedler S, Förster J, Maury J, Borodina I, Nielsen AT (2015) Novel highly active and specific tyrosine ammonia-lyases from diverse origins enable enhanced production of aromatic compounds in bacteria and yeast. Appl Environ Microbiol 81(13):4458–4476. https://doi.org/10.1128/AEM.00405-15

Jeong YJ, An CH, Woo SG, Jeong HJ, Kim Y-M, Park S-J, Yoon BD, Kim CY (2014) Production of pinostilbene compounds by the expression of resveratrol *O*-methyltransferase genes in *Escherichia coli*. Enzym Microb Technol 54:8–14. https://doi.org/10.1016/j.enzmictec.2013.09.005

Jones JA, Vernacchio VR, Collins SM, Shirke AN, Xiu Y, Englaender JA, Cress BF, McCutcheon CC, Linhardt RJ, Gross RA, Koffas MAG (2017) Complete biosynthesis of anthocyanins using *E. coli* polycultures. *mBio* 8:e00621-17. https://doi.org/10.1128/mBio.00621-17

Jossek R, Bongaerts J, Sprenger GA (2001) Characterization of a new feedback-resistant 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase AroF of *Escherichia coli*. FEMS Microbiol Lett 202(1):145–148

Kallscheuer N, Vogt M, Bott M, Marienhagen J (2017) Functional expression of plant-derived *O*-methyltransferase, flavanone 3-hydroxylase, and flavonol synthase in *Corynebacterium glutamicum* for production of pterostilbene, kaempferol, and quercetin. J Biotechnol 258:190–196. https://doi.org/10.1016/j.jbiotec.2017.01.006

Kallscheuer N, Vogt M, Kappelmann J, Krumbach K, Noack S, Bott M, Marienhagen J (2016a) Identification of the *phd* gene cluster responsible for phenylpropanoid utilization in *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 100(4):1871–1881. https://doi.org/10.1007/s00253-015-7165-1

Kallscheuer N, Vogt M, Marienhagen J (2016b) A novel synthetic pathway enables microbial production of polyphenols independent from the endogenous aromatic amino acid metabolism. ACS Synth Biol 6(3):410–415. https://doi.org/10.1021/acssynbio.6b00291

Kallscheuer N, Vogt M, Stenzel A, Gätgens J, Bott M, Marienhagen J (2016c) Construction of a *Corynebacterium glutamicum* platform strain for the production of stilbenes and (2*S*)-flavanones. Metab Eng 38:47–55. https://doi.org/10.1016/j.ymben.2016.06.003

Kang S-Y, Choi O, Lee JK, Hwang BY, Uhm T-B, Hong Y-S (2012) Artificial biosynthesis of phenylpropanoic acids in a tyrosine overproducing *Escherichia coli* strain. Microb Cell Factories 11(1):153. https://doi.org/10.1186/1475-2859-11-153

Kang S-Y, Lee JK, Choi O, Kim CY, Jang J-H, Hwang BY, Hong Y-S (2014) Biosynthesis of methylated resveratrol analogs through the

construction of an artificial biosynthetic pathway in *E. coli*. BMC Biotechnol 14(1):67. https://doi.org/10.1186/1472-6750-14-67

Katsuyama Y, Funa N, Miyahisa I, Horinouchi S (2007) Synthesis of unnatural flavonoids and stilbenes by exploiting the plant biosynthetic pathway in *Escherichia coli*. Chem Biol 14(6):613–621. https://doi.org/10.1016/j.chembiol.2007.05.004

Khurana S, Venkataraman K, Hollingsworth A, Piche M, Tai T (2013) Polyphenols: benefits to the cardiovascular system in health and in aging. Nutrients 5(10):3779–3827. https://doi.org/10.3390/nu5103779

Kim JH, Kim BG, Kim JA, Park Y, Lee YJ, Lim Y, Ahn J (2007) Glycosylation of flavonoids with *E. coli* expressing glycosyltransferase from *Xanthomonas campestris*. J Microbiol Biotechnol 17(3):539–542

Kim M-J, Kim B-G, Ahn J-H (2013) Biosynthesis of bioactive *O*-methylated flavonoids in *Escherichia coli*. Appl Microbiol Biotechnol 97(16):7195–7204. https://doi.org/10.1007/s00253-013-5020-9

Kootstra A (1994) Protection from UV-B-induced DNA damage by flavonoids. Plant Mol Biol 26(2):771–774. https://doi.org/10.1007/BF00013762

Landete J (2012) Updated knowledge about polyphenols: functions, bioavailability, metabolism, and health. Crit Rev Food Sci Nutr 52(10):936–948. https://doi.org/10.1080/10408398.2010.513779

Lee HJ, Kim BG, Kim M, Ahn J-H (2015) Biosynthesis of two flavones, apigenin and genkwanin in *Escherichia coli*. J Microbiol Biotechnol 25(9):1442–1448. https://doi.org/10.4014/jmb.1503.03011

Leonard E, Chemler J, Lim KH, Koffas MA (2006a) Expression of a soluble flavone synthase allows the biosynthesis of phytoestrogen derivatives in *Escherichia coli*. Appl Microbiol Biotechnol 70(1):85–91. https://doi.org/10.1007/s00253-005-0059-x

Leonard E, Koffas MA (2007) Engineering of artificial plant cytochrome P450 enzymes for synthesis of isoflavones by *Escherichia coli*. Appl Environ Microbiol 73(22):7246–7251. https://doi.org/10.1128/AEM.01411-07

Leonard E, Lim K-H, Saw P-N, Koffas MA (2007) Engineering central metabolic pathways for high-level flavonoid production in *Escherichia coli*. Appl Environ Microbiol 73(12):3877–3886. https://doi.org/10.1128/AEM.00200-07

Leonard E, Yan Y, Fowler ZL, Li Z, Lim C-G, Lim K-H, Koffas MA (2008) Strain improvement of recombinant *Escherichia coli* for efficient production of plant flavonoids. Mol Pharm 5(2):257–265. https://doi.org/10.1021/mp7001472

Leonard E, Yan Y, Koffas MA (2006b) Functional expression of a P450 flavonoid hydroxylase for the biosynthesis of plant-specific hydroxylated flavonols in *Escherichia coli*. Metab Eng 8(2):172–181. https://doi.org/10.1016/j.ymben.2005.11.001

Leonard E, Yan Y, Lim KH, Koffas MA (2005) Investigation of two distinct flavone synthases for plant-specific flavone biosynthesis in *Saccharomyces cerevisiae*. Appl Environ Microbiol 71(12):8241–8248. https://doi.org/10.1128/AEM.71.12.8241-8248.2005

Li M, Schneider K, Kristensen M, Borodina I, Nielsen J (2016) Engineering yeast for high-level production of stilbenoid antioxidants. Sci Rep 6:Article number 36827

Liang J-l, Guo L-Q, Lin J-F, He Z-Q, Cai F-J, Chen J-F (2016) A novel process for obtaining pinosylvin using combinatorial bioengineering in *Escherichia coli*. World J Microbiol Biotechnol 32(6):102. https://doi.org/10.1007/s11274-016-2062-z

Lim CG, Fowler ZL, Hueller T, Schaffer S, Koffas MA (2011) High-yield resveratrol production in engineered *Escherichia coli*. Appl Environ Microbiol 77(10):3451–3460. https://doi.org/10.1128/AEM.02186-10

Lim CG, Wong L, Bhan N, Dvora H, Xu P, Venkiteswaran S, Koffas MA (2015) Development of a recombinant *Escherichia coli* strain for overproduction of the plant pigment anthocyanin. Appl Environ Microbiol 81(18):6276–6284. https://doi.org/10.1128/AEM.01448-15

Lin Y, Yan Y (2012) Biosynthesis of caffeic acid in *Escherichia coli* using its endogenous hydroxylase complex. Microb Cell Factories 11(1):42. https://doi.org/10.1186/1475-2859-11-42

Lussier F-X, Colatriano D, Wiltshire Z, Page JE, Martin VJ (2012) Engineering microbes for plant polyketide biosynthesis. Comput Struct Biotechnol J 3(4):1–11

MacDonald MJ, D'Cunha GB (2007) A modern view of phenylalanine ammonia lyase. Biochem Cell Biol 85(3):273–282. https://doi.org/10.1139/O07-018

Marienhagen J, Bott M (2013) Metabolic engineering of microorganisms for the synthesis of plant natural products. J Biotechnol 163(2):166–178. https://doi.org/10.1016/j.jbiotec.2012.06.001

Masuo S, Kobayashi Y, Oinuma K-I, Takaya N (2016) Alternative fermentation pathway of cinnamic acid production via phenyllactic acid. Appl Microbiol Biotechnol 100(20):8701–8709. https://doi.org/10.1007/s00253-016-7623-4

Miyahisa I, Funa N, Ohnishi Y, Martens S, Moriguchi T, Horinouchi S (2006) Combinatorial biosynthesis of flavones and flavonols in *Escherichia coli*. Appl Microbiol Biotechnol 71(1):53–58. https://doi.org/10.1007/s00253-005-0116-5

Miyahisa I, Kaneko M, Funa N, Kawasaki H, Kojima H, Ohnishi Y, Horinouchi S (2005) Efficient production of (2*S*)-flavanones by *Escherichia coli* containing an artificial biosynthetic gene cluster. Appl Microbiol Biotechnol 68(4):498–504. https://doi.org/10.1007/s00253-005-1916-3

Ozaki S-i, Imai H, Iwakiri T, Sato T, Shimoda K, Nakayama T, Hamada H (2012) Regioselective glucosidation of *trans*-resveratrol in *Escherichia coli* expressing glucosyltransferase from *Phytolacca americana*. Biotechnol Lett 34(3):475–481. https://doi.org/10.1007/s10529-011-0784-4

Pandey KB, Rizvi SI (2009) Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Med Cell Longev 2(5):270–278. https://doi.org/10.4161/oxim.2.5.9498

Pandey RP, Malla S, Simkhada D, Kim B-G, Sohng JK (2013) Production of 3-*O*-xylosyl quercetin in *Escherichia coli*. Appl Microbiol Biotechnol 97(5):1889–1901. https://doi.org/10.1007/s00253-012-4438-9

Pandey RP, Parajuli P, Chu LL, Kim S-Y, Sohng JK (2016) Biosynthesis of a novel fisetin glycoside from engineered *Escherichia coli*. J Ind Eng Chem 43:13–19. https://doi.org/10.1016/j.jiec.2016.07.054

Park SR, Ahn MS, Han AR, Park JW, Yoon YJ (2011) Enhanced flavonoid production in *Streptomyces venezuelae* via metabolic engineering. J Microbiol Biotechnol 21(11):1143–1146. https://doi.org/10.4014/jmb.1108.08012

Park SR, Yoon JA, Paik JH, Park JW, Jung WS, Ban Y-H, Kim EJ, Yoo YJ, Han AR, Yoon YJ (2009) Engineering of plant-specific phenylpropanoids biosynthesis in *Streptomyces venezuelae*. J Biotechnol 141(3):181–188. https://doi.org/10.1016/j.jbiotec.2009.03.013

Quideau S, Deffieux D, Douat-Casassus C, Pouysegu L (2011) Plant polyphenols: chemical properties, biological activities, and synthesis. Angew Chem Int Ed 50(3):586–621. https://doi.org/10.1002/anie.201000044

Ray JM, Yanofsky C, Bauerle R (1988) Mutational analysis of the catalytic and feedback sites of the tryptophan-sensitive 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase of *Escherichia coli*. J Bacteriol 170(12):5500–5506. https://doi.org/10.1128/jb.170.12.5500-5506.1988

Rodrigues JL, Araújo R, Prather KL, Kluskens L, Rodrigues L (2015) Heterologous production of caffeic acid from tyrosine in *Escherichia coli*. Enzym Microb Technol 71:36–44. https://doi.org/10.1016/j.enzmictec.2015.01.001

Rodriguez A, Kildegaard KR, Li M, Borodina I, Nielsen J (2015) Establishment of a yeast platform strain for production of *p*-coumaric acid through metabolic engineering of aromatic amino

acid biosynthesis. Metab Eng 31:181–188. https://doi.org/10.1016/j.ymben.2015.08.003
Rosler J, Krekel F, Amrhein N, Schmid J (1997) Maize phenylalanine ammonia-lyase has tyrosine ammonia-lyase activity. Plant Physiol 113(1):175–179. https://doi.org/10.1104/pp.113.1.175
Santos CNS, Koffas M, Stephanopoulos G (2011) Optimization of a heterologous pathway for the production of flavonoids from glucose. Metab Eng 13(4):392–400. https://doi.org/10.1016/j.ymben.2011.02.002
Saura-Calixto F, Serrano J, Goñi I (2007) Intake and bioaccessibility of total polyphenols in a whole diet. Food Chem 101(2):492–501. https://doi.org/10.1016/j.foodchem.2006.02.006
Schallmey M, Frunzke J, Eggeling L, Marienhagen J (2014) Looking for the pick of the bunch: high-throughput screening of producing microorganisms with biosensors. Curr Opin Biotechnol 26:148–154. https://doi.org/10.1016/j.copbio.2014.01.005
Shi S, Chen Y, Siewers V, Nielsen J (2014) Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. MBio 5(3):e01130–e01114. https://doi.org/10.1128/mBio.01130-14
Shin S-Y, Jung S-M, Kim M-D, Han NS, Seo J-H (2012) Production of resveratrol from tyrosine in metabolically engineered *Saccharomyces cerevisiae*. Enzym Microb Technol 51(4):211–216. https://doi.org/10.1016/j.enzmictec.2012.06.005
Siedler S, Khatri NK, Zsohár A, Kjærbølling I, Vogt M, Hammar P, Nielsen CF, Marienhagen J, Sommer MOA, Joensson HN (2017) Development of a Bacterial Biosensor for Rapid Screening of Yeast p-Coumaric Acid Production. ACS Synth Biol. 2017 Oct 20;6(10):1860–1869. https://doi.org/10.1021/acssynbio.7b00009. Epub 2017 Jun 21
Simkhada D, Lee HC, Sohng JK (2010) Genetic engineering approach for the production of rhamnosyl and allosyl flavonoids from *Escherichia coli*. Biotechnol Bioeng 107(1):154–162. https://doi.org/10.1002/bit.22782
Stahlhut SG, Siedler S, Malla S, Harrison SJ, Maury J, Neves AR, Forster J (2015) Assembly of a novel biosynthetic pathway for production of the plant flavonoid fisetin in *Escherichia coli*. Metab Eng 31:84–93. https://doi.org/10.1016/j.ymben.2015.07.002
Tehlivets O, Scheuringer K, Kohlwein SD (2007) Fatty acid synthesis and elongation in yeast. Biochim Biophys Acta Mol Cell Biol Lipids 1771(3):255–270. https://doi.org/10.1016/j.bbalip.2006.07.004
Tinh TH, Nuidate T, Vuddhakul V, Rodkhum C (2016) Antibacterial activity of pyrogallol, a polyphenol compound against *Vibrio parahaemolyticus* isolated from the central region of Thailand. Procedia Chem 18:162–168. https://doi.org/10.1016/j.proche.2016.01.025
Trantas E, Panopoulos N, Ververidis F (2009) Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in *Saccharomyces cerevisiae*. Metab Eng 11(6):355–366. https://doi.org/10.1016/j.ymben.2009.07.004
Tropf S, Lanz T, Rensing S, Schröder J, Schröder G (1994) Evidence that stilbene synthases have developed from chalcone synthases several times in the course of evolution. J Mol Evol 38(6):610–618
Tsao R (2010) Chemistry and biochemistry of dietary polyphenols. Nutrients 2(12):1231–1246. https://doi.org/10.3390/nu2121231
Tsay J-T, Oh W, Larson T, Jackowski S, Rock C (1992) Isolation and characterization of the beta-ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. J Biol Chem 267(10):6807–6814
van Summeren-Wesenhagen PV, Marienhagen J (2015) Metabolic engineering of *Escherichia coli* for the synthesis of the plant polyphenol pinosylvin. Appl Environ Microbiol 81(3):840–849. https://doi.org/10.1128/AEM.02966-14
van Summeren-Wesenhagen PV, Voges R, Dennig A, Sokolowsky S, Noack S, Schwaneberg U, Marienhagen J (2015) Combinatorial optimization of synthetic operons for the microbial production of *p*-coumaryl alcohol with *Escherichia coli*. Microb Cell Factories 14(1):79. https://doi.org/10.1186/s12934-015-0274-9
Vogt T, Jones P (2000) Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. Trends Plant Sci 5(9):380–386. https://doi.org/10.1016/S1360-1385(00)01720-9
Wang S, Zhang S, Xiao A, Rasmussen M, Skidmore C, Zhan J (2015a) Metabolic engineering of *Escherichia coli* for the biosynthesis of various phenylpropanoid derivatives. Metab Eng 29:153–159. https://doi.org/10.1016/j.ymben.2015.03.011
Wang Y, Bhuiya MW, Zhou R, Yu O (2015b) Pterostilbene production by microorganisms expressing resveratrol *O*-methyltransferase. Ann Microbiol 65(2):817–826. https://doi.org/10.1007/s13213-014-0922-z
Watts KT, Lee PC, Schmidt-Dannert C (2006) Biosynthesis of plant-specific stilbene polyketides in metabolically engineered *Escherichia coli*. BMC Biotechnol 6(1):22. https://doi.org/10.1186/1472-6750-6-22
Watts KT, Lee PC, Schmidt-Dannert C (2004) Exploring recombinant flavonoid biosynthesis in metabolically engineered *Escherichia coli*. Chembiochem 5(4):500–507. https://doi.org/10.1002/cbic.200300783
Wu J, Du G, Chen J, Zhou J (2015) Enhancing flavonoid production by systematically tuning the central metabolic pathways based on a CRISPR interference system in *Escherichia coli*. Sci Rep 5:article number 13477
Wu J, Du G, Zhou J, Chen J (2013) Metabolic engineering of *Escherichia coli* for (2*S*)-pinocembrin production from glucose by a modular metabolic strategy. Metab Eng 16:48–55. https://doi.org/10.1016/j.ymben.2012.11.009
Wu J, Yu O, Du G, Zhou J, Chen J (2014a) Fine-tuning of the fatty acid pathway by synthetic antisense RNA for enhanced (2*S*)-naringenin production from L-tyrosine in *Escherichia coli*. Appl Environ Microbiol 80(23):7283–7292. https://doi.org/10.1128/AEM.02411-14
Wu J, Zhang X, Dong M, Zhou J (2016) Stepwise modular pathway engineering of *Escherichia coli* for efficient one-step production of (2*S*)-pinocembrin. J Biotechnol 231:183–192. https://doi.org/10.1016/j.jbiotec.2016.06.007
Wu J, Zhou T, Du G, Zhou J, Chen J (2014b) Modular optimization of heterologous pathways for *de novo* synthesis of (2*S*)-naringenin in *Escherichia coli*. PLoS One 9(7):e101492. https://doi.org/10.1371/journal.pone.0101492
Xiao J, Högger P (2015) Stability of dietary polyphenols under the cell culture conditions: avoiding erroneous conclusions. J Agr Food Chem 63(5):1547–1557. https://doi.org/10.1021/jf505514d
Xu P, Li L, Zhang F, Stephanopoulos G, Koffas M (2014) Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. Proc Natl Acad Sci 111(31):11299–11304. https://doi.org/10.1073/pnas.1406401111
Xu P, Ranganathan S, Fowler ZL, Maranas CD, Koffas MA (2011) Genome-scale metabolic network modeling results in minimal interventions that cooperatively force carbon flux towards malonyl-CoA. Metab Eng 13(5):578–587. https://doi.org/10.1016/j.ymben.2011.06.008
Yan Y, Li Z, Koffas MA (2008) High-yield anthocyanin biosynthesis in engineered *Escherichia coli*. Biotechnol Bioeng 100(1):126–140. https://doi.org/10.1002/bit.21721
Yang S-M, Han SH, Kim B-G, Ahn J-H (2014) Production of kaempferol 3-*O*-rhamnoside from glucose using engineered *Escherichia coli*. J Ind Microbiol Biotechnol 41(8):1311–1318. https://doi.org/10.1007/s10295-014-1465-9
Yang Y, Lin Y, Li L, Linhardt RJ, Yan Y (2015) Regulating malonyl-CoA metabolism via synthetic antisense RNAs for enhanced biosynthesis of natural products. Metab Eng 29:217–226. https://doi.org/10.1016/j.ymben.2015.03.018
Zha W, Rubin-Pitel SB, Shao Z, Zhao H (2009) Improving cellular malonyl-CoA level in *Escherichia coli* via metabolic engineering. Metab Eng 11(3):192–198. https://doi.org/10.1016/j.ymben.2009.01.005
Zhu S, Wu J, Du G, Zhou J, Chen J (2014) Efficient synthesis of eriodictyol from L-tyrosine in *Escherichia coli*. Appl Environ Microbiol 80(10):3072–3080. https://doi.org/10.1128/AEM.03986-13

2.2 Engineering *C. glutamicum* towards increased malonyl-CoA availability for plant polyphenol synthesis

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ARTICLE

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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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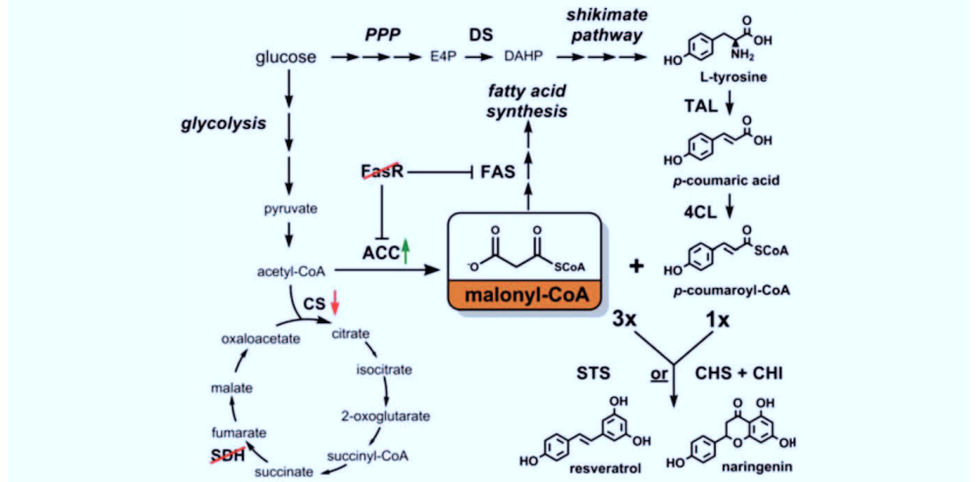

FIGURE 1 Metabolic pathways involved in the synthesis of the polyphenol precursors *p*-coumaroyl-CoA and malonyl-CoA. *p*-Coumaroyl-CoA and malonyl-CoA are key precursor molecules for the synthesis of the plant polyphenols naringenin and resveratrol. *p*-Coumaroyl-CoA is the CoA thioester of *p*-coumaric 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; DS: 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) compromised 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 (2*S*)-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, (2*S*)-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* DH5α was used for plasmid constructions and was cultivated in LB medium (Bertani, 1951) at 37°C. Where appropriate, kanamycin (50 μg·ml^{-1} for *E. coli* or 25 μg·ml^{-1} for *C. glutamicum*) or spectinomycin (100 μg·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 (2*S*)-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 g·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		
C. glutamicum		
DelAro^4-*4cl*$_{Pc}$	*C. glutamicum* 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 Δ*fasR*	Nar1 derivative with in-frame deletion of *fasR* (cg2737)	This study
Nar1 Δ*sdhCAB*	Nar1 derivative with in-frame deletion of *sdhCAB* (cg0445–47)	This study
Nar1 Δ*fasR* Δ*sdhCAB*	Nar1 derivative with an in-frame deletion of *fasR* and *sdhCAB*	This study
Nar1_A16	Nar1 derivative with the replacement of the native *gltA* promotor with the *dapA* promotor variant A16	This study
Nar1_L1	Nar1 derivative with the replacement of the native *gltA* promotor with the *dapA* promotor variant L1	This study
Nar1_C7	Nar1 derivative with the replacement of the native *gltA* promotor with the *dapA* promotor variant C7	This study
Nar1_C7 Δ*fasR*	Nar1_C7 derivative with an in-frame deletion of *fasR*	This study
Res1_C7	DelAro^4-*4cl*$_{Pc}$ strain harboring pMKEx2_*sts*$_{Ah}$_*4cl*$_{Pc}$ and replacement of the native *gltA* promotor with the *dapA* promotor variant C7	This study
E. coli		
DH5α	F$^-$ Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA*1 *endA*1 *hsdR*17 (r_k^-, m_k^+) *phoA supE*44 *thi*-1 *gyrA*96 *relA*1 λ$^-$	Invitrogen (Karlsruhe, Germany)
Plasmids		
pK19*mobsacB*	*kan*^{r}; vector for allelic exchange in *C. glutamicum* (pK18, oriV$_{Ec}$, *sacB*, *lacZα*)	Schäfer et al. (1994)
pK19*mobsacB*Δ*sdhCAB*	Vector for in-frame deletion of *sdhCAB*	This study
pK19*mobsacB*Δ*fasR*	Vector for in-frame deletion of *fasR*	Nickel, Irzik, Van Ooyen, and Eggeling (2010)
pK19*mobsacB_gltA*_A16	Vector for exchanging the native promoter of *gltA* against the *dapA* promotor variant A16	van Ooyen et al. (2012)
pK19*mobsacB_gltA*_L1	Vector for exchanging the native promoter of *gltA* against the *dapA* promotor variant L1	van Ooyen et al. (2012)
pK19*mobsacB_gltA*_C7	Vector for exchanging the native promotor of *gltA* against the *dapA* promotor variant C7	van Ooyen et al. (2012)
pMKEx2	*kan*^{r}; *E. coli-C. glutamicum* shuttle vector (*lacI*, P$_{T7}$, *lacO*1, pHM1519 ori$_{Cg}$; pACYC177 ori$_{Ec}$)	Kortmann, Kuhl, Klaffl, and Bott (2015)
pMKEx2_*chs*$_{Ph}$_*chi*$_{Ph}$	*kan*^{r}; pMKEx2 derivative with genes coding for chalcone synthase and chalcone isomerase from *Petunia x hybrida* (codon-optimized)	Kallscheuer, Vogt, Stenzel et al. (2016)
pMKEx2_*sts*$_{Ah}$_*4cl*$_{Pc}$	*kan*^{r}; pMKEx2 derivative with genes coding for stilbene synthase from *Arachis hypogaea* and 4-coumarate:CoA ligase from *Petroselinum crispum* (codon-optimized)	Kallscheuer, Vogt, Stenzel et al. (2016)
pEKEx3	*spec*^{r}; *E. coli-C. glutamicum* shuttle vector (*lacI*, P$_{tac}$, *lacO*1, pBL1 ori$_{Cg}$; pUC ori$_{Ec}$)	Gande et al. (2007)
pEKEx3_*accBC_accD*1	*spec*^{r}; pEKEx3 derivative containing *accBC* and *accD*1 genes from *C. glutamicum*	This study
pEKEx3_*aroH*$_{Ec}$_*tal*$_{Fj}$	*spec*^{r}; pEKEx3 derivative containing native *aroH* from *Escherichia coli* and a codon-optimized gene encoding tyrosine ammonia lyase from *Flavobacterium johnsoniae*	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
SbfI-accBC-s	ACACCTGCAGGAAGGAGGACTGCTATGTCAGTCGAGACTAGGAAGATCACCAAGGTTCTTGTCGCTAAC	*Sbf*I
*Kpn*I-accBC-as	TTCGGTACCTTACTTGATCTCGAGGAGAACAACGCCCTTG	*Kpn*I
*Kpn*I-accD1-s	ATAGGTACCTCTAAGGAGGCGTTCGATGACCATTTCCTCACCTTTGATTGACGTC	*Kpn*I
*Bam*HI-accD1-as	CGTGGATCCTTACAGTGGCATGTTGCCGTGCTTG	*Bam*HI
chk-pEKEx3-s	GCAAATATTCTGAAATGAGCTGTTGACAATTAATCATC	–
chk-pEKEx3-as	CGTTCTGATTTAATCTGTATCAGGCTGAAAATCTTCTC	–
chk-sdhCAB-s	GATTTACATAACTTGCAGATAAGCC	–
chk-sdhCAB-as	CGAGGTCAGAGGTCACAATCTC	–
chk-fasR-s	GCGTGCCACTGCGCTACTCCTG	–
chk-fasR-as	CCGTCCCCGACAACATGTACGGCC	–
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 C_{18} 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 $[M\text{-}H]^-$ 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 ddH_2O (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 ^{13}C-labeled cell extract of *E. coli* was spiked with [*Malonyl*-^{13}C3]-malonyl-CoA to attain a concentration of approximately 12.5 µM (based on the molar weight of the free acid). The latter contained [U-^{13}C3]-malonate as contamination (data not shown). This compound most probably originates from the spontaneous thioester lysis of [*Malonyl*-^{13}C3]-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/ddH_2O 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* DelAro^4-*4cl*$_{Pc}$ pMKEx2_*chs*$_{Ph}$_*chi*$_{Ph}$ (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 pEKEx3 yielding pEKEx3_*accB-C_accD1*. The strain *C. glutamicum* Nar1 pEKEx3_*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 pEKEx3_*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, Sahm, & 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 $mg \cdot L^{-1}$	Normalized naringenin titer $mg \cdot L^{-1} \cdot OD_{600}^{-1}$
C. glutamicum Nar1	0.32 ± 0.01	55.2 ± 1.7	2.1 ± 0.5	0.04
C. glutamicum Nar1 Δ*fasR*	0.22 ± 0.02	49.6 ± 1.5	3.3 ± 0.2	0.07
C. glutamicum Nar1 Δ*sdhCAB*	0.27 ± 0.02	28.1 ± 0.7	3.8 ± 0.2	0.13
C. glutamicum Nar1 Δ*fasR* Δ*sdhCAB*	0.14 ± 0.02	7.6 ± 0.6	1.9 ± 0.9	0.25
C. glutamicum Nar1_A16	0.32 ± 0.01	51.2 ± 2.1	4.5 ± 1.2	0.09
C. glutamicum Nar1_L1	0.29 ± 0.01	50.8 ± 1.8	10.3 ± 2.4	0.20
C. glutamicum Nar1_C7	0.24 ± 0.02	48.0 ± 0.1	18.5 ± 2.5	0.39
C. glutamicum 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 $mg \cdot L^{-1}$ [0.018 mM] naringenin compared with 2 $mg \cdot L^{-1}$ [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 $mg \cdot L^{-1}$ (0.044 mM) and 19 $mg \cdot L^{-1}$ (0.07 mM) naringenin, respectively (Table 3). All strains with altered *gltA* expression levels reached the same final biomass (OD_{600} 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 $mg \cdot L^{-1}$ (0.07 mM). The strain also showed the highest naringenin titer of 0.39 $mg \cdot L^{-1} \cdot OD_{600}^{-1}$ 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 $mg \cdot L^{-1}$ [0.007 mM]; *C. glutamicum* Nar1 Δ*sdhCAB*: 4 $mg \cdot L^{-1}$ [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 $mg \cdot L^{-1}$ (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 OD_{600} and naringenin titer of *C. glutamicum* Δ*fasR*Δ*sdhCAB* were drastically reduced, while the normalized naringenin titer of 0.25 $mg \cdot L^{-1} \cdot OD_{600}^{-1}$ 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 $mg \cdot L^{-1}$ [0.044 mM]) compared with *C. glutamicum* Nar1_C7, whereas final biomass concentrations (OD_{600} 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 $mg \cdot L^{-1}$ (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 μM. Given this sensitivity, we

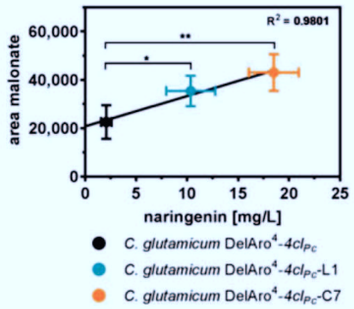

FIGURE 2 LC-MS/MS analysis of intracellular malonate concentrations. Areas for malonate acquired by LC-MS/MS analysis of cytoplasmatic extracts are plotted against the determined naringenin concentration during cultivation of the respective strains. The plotted naringenin concentrations represent mean 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* DelAro^4-$4cl_{Pc}$. Because the obtained ^{12}C–^{13}C isotope ratios for the *C. glutamicum* DelAro^4-$4cl_{Pc}$ and L1 strain were below the limit of quantification, no intracellular malonate concentration could be calculated. Subsequently, the integrated areas of the ^{12}C-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* DelAro^4-$4cl_{Pc}$ 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_sts_{Ah}_$4cl_{Pc}$ 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

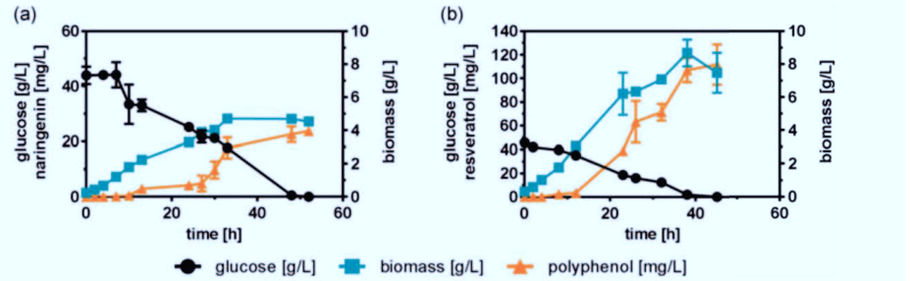

FIGURE 3 Microbial production of naringenin and resveratrol from glucose during bioreactor cultivations with engineered *C. glutamicum* strains. (a) Naringenin production from glucose: concentration of glucose and biomass and naringenin titers during batch cultivation of *C. glutamicum* Nar1_C7 pEKEx3_$aroH_{Ec}$_tal_{Fj}. (b) Resveratrol production from glucose: concentration of glucose and biomass and resveratrol titers during batch cultivation of *C. glutamicum* Res1_C7 pEKEx3_$aroH_{Ec}$_tal_{Fj}. 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 $mg \cdot L^{-1}$ (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, (2*S*)-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-Wesenhagen & 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 $g \cdot L^{-1}$. The same *C. glutamicum* strain, only differing in the genetic constitution related to product synthesis, was found to produce different naringenin (24 $mg \cdot L^{-1}$) and resveratrol (112 $mg \cdot L^{-1}$) 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 ($\Delta G^{0\prime} = -70$ $kJ \cdot mol^{-1}$ for resveratrol compared with $\Delta G^{0\prime} = +46$ $kJ \cdot mol^{-1}$ for naringenin). This difference is due to an additional molecule of CO_2 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* DelAro^4-*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* DelAro^4-*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* DelAro^4-*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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REFERENCES

Bertani, G. (1951). Studies on lysogenesis I: The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology*, *62*(3), 293–300.

Caspi, R., Foerster, H., Fulcher, C. A., Kaipa, P., Krummenacker, M., Latendresse, M., … Karp, P. D. (2007). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Research*, *36*, D623–D631. https://doi.org/10.1093/nar/gkm900

Caspi, R., Billington, R., Ferrer, L., Foerster, H., Fulcher, C. A., Keseler, I. M., … Karp, P. D. (2015). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Research*, *44*(D1), D471–D480. https://doi.org/10.1093/nar/gkv1164

Chouhan, S., Sharma, K., Zha, J., Guleria, S., & Koffas, M. A. G. (2017). Recent advances in the recombinant biosynthesis of polyphenols. *Frontiers in Microbiology*, *8*, 2259. https://doi.org/10.3389/fmicb.2017.02259

Daglia, M. (2012). Polyphenols as antimicrobial agents. *Current Opinion in Biotechnology*, *23*(2), 174–181. https://doi.org/10.1016/j.copbio.2011.08.007

Eggeling, L. , & Bott, M. (2005). *Handbook of Corynebacterium glutamicum* (537–564). Boca Raton, FL: CRC press. https://doi.org/10.1201/9781420039696.

Ferrer, J. -L., Jez, J. M., Bowman, M. E., Dixon, R. A., & Noel, J. P. (1999). Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. *Nature Structural Biology*, *6*(8), 775–784. https://doi.org/10.1038/11553

Fowler, Z. L., Gikandi, W. W., & Koffas, M. A. G. (2009). Increased malonyl coenzyme A biosynthesis by tuning the *Escherichia coli* metabolic network and its application to flavanone production. *Applied and Environmental Microbiology*, *75*(18), 5831–5839. https://doi.org/10.1128/AEM.00270-09

Gande, R., Dover, L. G., Krumbach, K., Besra, G. S., Sahm, H., Oikawa, T., & Eggeling, L. (2007). The two carboxylases of *Corynebacterium glutamicum* essential for fatty acid and mycolic acid synthesis. *Journal of Bacteriology*, *189*(14), 5257–5264. https://doi.org/10.1128/JB.00254-07

Hartmann, A., Vila-Santa, A., Kallscheuer, N., Vogt, M., Julien-Laferrière, A., Sagot, M. -F., … Vinga, S. (2017). OptPipe—A pipeline for optimizing metabolic engineering targets. *BMC Systems Biology*, *11*(1), 143. https://doi.org/10.1186/s12918-017-0515-0

Irzik, K., van Ooyen, J., Gätgens, J., Krumbach, K., Bott, M., & Eggeling, L. (2014). Acyl-CoA sensing by FasR to adjust fatty acid synthesis in *Corynebacterium glutamicum*. *Journal of Biotechnology*, *192*, 96–101. https://doi.org/10.1016/j.jbiotec.2014.10.031

Jakeman, D. L., Bandi, S., Graham, C. L., Reid, T. R., Wentzell, J. R., & Douglas, S. E. (2009). Antimicrobial activities of jadomycin B and

structurally related analogues. *Antimicrobial Agents and Chemotherapy*, *53*(3), 1245–1247. https://doi.org/10.1128/AAC.00801-08

Jeandet, P., Sobarzo-Sánchez, E., Clément, C., Nabavi, S. F., Habtemariam, S., Nabavi, S. M., & Cordelier, S. (2018). Engineering stilbene metabolic pathways in microbial cells. *Biotechnology Advances*, *36*, 2264–2283. https://doi.org/10.1016/j.biotechadv.2018.11.002

Johansson, P., Wiltschi, B., Kumari, P., Kessler, B., Vonrhein, C., Vonck, J., … Grininger, M. (2008). Inhibition of the fungal fatty acid synthase type I multienzyme complex. *Proceedings of the National Academy of Sciences*, *105*(35), 12803–12808. https://doi.org/10.1073/pnas.0805827105

Kallscheuer, N., Vogt, M., Bott, M., & Marienhagen, J. (2017). Functional expression of plant-derived *O*-methyltransferase, flavanone 3-hydroxylase, and flavonol synthase in *Corynebacterium glutamicum* for production of pterostilbene, kaempferol, and quercetin. *Journal of Biotechnology*, *258*, 190–196. https://doi.org/10.1016/j.jbiotec.2017.01.006

Kallscheuer, N., Vogt, M., Kappelmann, J., Krumbach, K., Noack, S., Bott, M., & Marienhagen, J. (2016). Identification of the *phd* gene cluster responsible for phenylpropanoid utilization in *Corynebacterium glutamicum*. *Applied Microbiology and Biotechnology*, *100*(4), 1871–1881. https://doi.org/10.1007/s00253-015-7165-1

Kallscheuer, N., Vogt, M., & Marienhagen, J. (2016). A novel synthetic pathway enables microbial production of polyphenols independent from the endogenous aromatic amino acid metabolism. *ACS Synthetic Biology*, *6*(3), 410–415. https://doi.org/10.1021/acssynbio.6b00291

Kallscheuer, N., Vogt, M., Stenzel, A., Gätgens, J., Bott, M., & Marienhagen, J. (2016). Construction of a *Corynebacterium glutamicum* platform strain for the production of stilbenes and (2*S*)-flavanones. *Metabolic Engineering*, *38*, 47–55. https://doi.org/10.1016/j.ymben.2016.06.003

Katsuyama, Y., Funa, N., Miyahisa, I., & Horinouchi, S. (2007). Synthesis of unnatural flavonoids and stilbenes by exploiting the plant biosynthetic pathway in *Escherichia coli*. *Chemistry & Biology*, *14*(6), 613–621. https://doi.org/10.1016/j.chembiol.2007.05.004

Keilhauer, C., Eggeling, L., & Sahm, H. (1993). Isoleucine synthesis in *Corynebacterium glutamicum*: Molecular analysis of the *ilvB-ilvN-ilvC* operon. *Journal of Bacteriology*, *175*(17), 5595–5603. https://doi.org/10.1128/jb.175.17.5595-5603.1993

Kim, B. G., Lee, H., & Ahn, J. H. (2014). Biosynthesis of pinocembrin from glucose using engineered *Escherichia coli*. *Journal of Microbiology and Biotechnology*, *24*(11), 1536–1541. https://doi.org/10.4014/jmb.1406.06011

Kortmann, M., Kuhl, V., Klaffl, S., & Bott, M. (2015). A chromosomally encoded T7 RNA polymerase-dependent gene expression system for *Corynebacterium glutamicum*: Construction and comparative evaluation at the single-cell level. *Microbial Biotechnology*, *8*(2), 253–265. https://doi.org/10.1111/1751-7915.12236

Han, L., Yang, K., Kulowski, K., Wendt-Pienkowski, E., Hutchinson, C. R., & Vining, L. C. (2000). An acyl-coenzyme A carboxylase encoding gene associated with jadomycin biosynthesis in *Streptomyces venezuelae* ISP5230. *Microbiology*, *146*(4), 903–910.

Larisch, C., Nakunst, D., Hüser, A. T., Tauch, A., & Kalinowski, J. (2007). The alternative sigma factor SigB of *Corynebacterium glutamicum* modulates global gene expression during transition from exponential growth to stationary phase. *BMC Genomics*, *8*(1), 1. https://doi.org/10.1186/1471-2164-8-4

Leonard, E., Lim, K. -H., Saw, P. -N., & Koffas, M. A. G. (2007). Engineering central metabolic pathways for high-level flavonoid production in *Escherichia coli*. *Applied and Environmental Microbiology*, *73*(12), 3877–3886. https://doi.org/10.1128/AEM.00200-0

Leonard, E., Yan, Y., Fowler, Z. L., Li, Z., Lim, C.-G., Lim, K.-H., & Koffas, M. A. G. (2008). Strain improvement of recombinant *Escherichia coli* for efficient production of plant flavonoids. *Molecular Pharmaceutics*, *5*(2), 257–265. https://doi.org/10.1021/mp7001472

Li, M., Schneider, K., Kristensen, M., Borodina, I., & Nielsen, J. (2016). Engineering yeast for high-level production of stilbenoid antioxidants. *Scientific Reports*, *6*, 36827. https://doi.org/10.1038/srep36827

Li, S.-J., & Cronan, J. E. (1992). The genes encoding the two carboxyltransferase subunits of *Escherichia coli* acetyl-CoA carboxylase. *Journal of Biological Chemistry*, *267*(24), 16841–16847.

Lim, C. G., Fowler, Z. L., Hueller. T., Schaffer, S., & Koffas, M. A. G. (2011). High-yield resveratrol production in engineered *Escherichia coli*. *Applied and Environmental Microbiology*, *77*(10), 3451–3460. https://doi.org/10.1128/AEM.02186-10

Litsanov, B., Kabus, A., Brocker, M., & Bott, M. (2012). Efficient aerobic succinate production from glucose in minimal medium with *Corynebacterium glutamicum*. *Microbial Biotechnology*, *5*(1), 116–128. https://doi.org/10.1111/j.1751-7915.2011.00310.x

Milke, L., Aschenbrenner, J., Marienhagen, J., & Kallscheuer, N. (2018). Production of plant-derived polyphenols in microorganisms: Current state and perspectives. *Applied Microbiology and Biotechnology*, *102*(4), 1575–1585. https://doi.org/10.1007/s00253-018-8747-5

Miyahisa, I., Kaneko, M., Funa, N., Kawasaki, H., Kojima, H., Ohnishi, Y., & Horinouchi, S. (2005). Efficient production of (2*S*)-flavanones by *Escherichia coli* containing an artificial biosynthetic gene cluster. *Applied Microbiology and Biotechnology*, *68*(4), 498–504. https://doi.org/10.1007/s00253-005-1916-3

Nickel, J., Irzik, K., van Ooyen, J., & Eggeling, L. (2010). The TetR-type transcriptional regulator FasR of *Corynebacterium glutamicum* controls genes of lipid synthesis during growth on acetate. *Molecular Microbiology*, *78*(1), 253–265. https://doi.org/10.1111/j.1365-2958.2010.07337.x

Niebisch, A., & Bott, M. (2001). Molecular analysis of the cytochrome bc_1-aa_3 branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome c_1. *Archives of Microbiology*, *175*(4), 282–294. https://doi.org/10.1007/s002030100262

Price, Allen C., Choi, Keum-Hwa, Heath, Richard J., Li, Zhenmei, White, Stephen W., & Rock, Charles O. (2000). Inhibition of β-Ketoacyl-Acyl Carrier Protein Synthases by Thiolactomycin and Cerulenin. *Journal of Biological Chemistry*, *276*(9), 6551–6559. https://doi.org/10.1074/jbc.m007101200

Ross, J. A., & Kasum, C. M. (2002). Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annual Review of Nutrition*, *22*(1), 19–34. https://doi.org/10.1146/annurev.nutr.22.111401.144957

Sambrook, J., & Russell, D. (2001). *Molecular cloning*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., & Pühler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: Selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene*, *145*(1), 69–73. https://doi.org/10.1016/0378-1119(94)90324-7

Takeno, S., Takasaki, M., Urabayashi, A., Mimura, A., Muramatsu, T., Mitsuhashi, S., & Ikeda, M. (2013). Development of fatty acid-producing *Corynebacterium glutamicum* strains. *Applied and Environmental Microbiology*, *79*(21), 6776–6783. https://doi.org/10.1128/AEM.02003-13

van Ooyen, J., Noack, S., Bott, M., Reth, A., & Eggeling, L. (2012). Improved L-lysine production with *Corynebacterium glutamicum* and systemic insight into citrate synthase flux and activity. *Biotechnology and Bioengineering*, *109*(8), 2070–2081. https://doi.org/10.1002/bit.24486

van Summeren-Wesenhagen, P. V., & Marienhagen, J. (2015). Metabolic engineering of *Escherichia coli* for the synthesis of the plant polyphenol pinosylvin. *Applied and Environmental Microbiology*, *81*(3), 840–849. https://doi.org/10.1128/AEM.02966-14

Vasicová, P., Pátek, M., Nesvera, J., Sahm, H., & Eikmanns, B. (1999). Analysis of the *Corynebacterium glutamicum dapA* promoter. *Journal of Bacteriology*, *181*(19), 6188–6191.

Yang, Y., Lin, Y., Li, L., Linhardt, R. J., & Yan, Y. (2015). Regulating malonyl-CoA metabolism via synthetic antisense RNAs for enhanced biosynthesis of natural products. *Metabolic Engineering*, *29*, 217–226. https://doi.org/10.1016/j.ymben.2015.03.018

Zabala, D., Braña, A. F., Salas, J. A., & Méndez, C. (2015). Increasing antibiotic production yields by favoring the biosynthesis of precursor metabolites glucose-1-phosphate and/or malonyl-CoA in *Streptomyces* producer strains. *The Journal of Antibiotics*, *69*, 179–182. https://doi.org/10.1038/ja.2015.104

Zha, W., Rubin-Pitel, S. B., Shao, Z., & Zhao, H. (2009). Improving cellular malonyl-CoA level in *Escherichia coli* via metabolic engineering. *Metabolic Engineering*, *11*(3), 192–198. https://doi.org/10.1016/j.ymben.2009.01.005

Zhao, Y., Wu, B. -H., Liu, Z. -N., Qiao, J., & Zhao, G. -R. (2018). Combinatorial optimization of resveratrol production in engineered *E. coli*. *Journal of Agricultural and Food Chemistry*, *66*, 13444–13453. https://doi.org/10.1021/acs.jafc.8b05014

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2.3 Increasing malonyl-CoA availability for improved noreugenin synthesis

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Microbial Cell Factories

RESEARCH Open Access

Tailoring *Corynebacterium glutamicum* towards increased malonyl-CoA availability for efficient synthesis of the plant pentaketide noreugenin

Lars Milke[1], Nicolai Kallscheuer[1,2], Jannick Kappelmann[1] and Jan Marienhagen[1,2,3*]

Abstract

Background: In the last years, different biotechnologically relevant microorganisms have been engineered for the synthesis of plant polyphenols such as flavonoids and stilbenes. However, low intracellular availability of malonyl-CoA as essential precursor for most plant polyphenols of interest is regarded as the decisive bottleneck preventing high product titers.

Results: In this study, *Corynebacterium glutamicum*, which emerged as promising cell factory for plant polyphenol production, was tailored by rational metabolic engineering towards providing significantly more malonyl-CoA for product synthesis. This was achieved by improving carbon source uptake, transcriptional deregulation of *accBC and accD1* encoding the two subunits of the acetyl-CoA carboxylase (ACC), reduced flux into the tricarboxylic acid (TCA) cycle, and elimination of anaplerotic carboxylation of pyruvate. The constructed strains were used for the synthesis of the pharmacologically interesting plant pentaketide noreugenin, which is produced by plants such as *Aloe arborescens* from five molecules of malonyl-CoA. In this context, accumulation of the C_1/C_6 cyclized intermediate 1-(2,4,6-trihydroxyphenyl)butane-1,3-dione (TPBD) was observed, which could be fully cyclized to the bicyclic product noreugenin by acidification.

Conclusion: The best strain *C. glutamicum* Nor2 C5 mu*fasO*$_{BCD1}$ P_{O6}-*iolT1* Δ*pyc* allowed for synthesis of 53.32 mg/L (0.278 mM) noreugenin in CGXII medium supplemented with casamino acids within 24 h.

Keywords: Malonyl-CoA, *Corynebacterium glutamicum*, Noreugenin, Metabolic engineering, Acetyl-CoA carboxylase

Background

Besides alkaloids and isoprenoids, polyphenols constitute the third large group of plant secondary metabolites [1]. In plants, polyphenols such as flavonoids, stilbenes or lignans do not contribute directly to growth or propagation, they rather protect the plant from UV radiation, aid in the defense against pathogens or herbivores, and color petals and fruits to attract animals [2, 3]. Due to anti-oxidative, anti-depressive, anti-hepatotoxic, anti-cancerous as well anti-inflammatory effects described for many polyphenols, these compounds received a lot of attention over the last years [4–8].

Recently, the Gram-positive soil bacterium *Corynebacterium glutamicum* was engineered towards plant polyphenols synthesis [9, 10]. Important prerequisite for establishing *C. glutamicum* as polyphenol producing cell factory was the identification and abolishment of a catabolic phenylpropanoid pathway. To date, several stilbenes (e.g. resveratrol and its *O*-methylated derivatives) as well as flavonoids (e.g. naringenin, kaempferol and quercetin) could be produced with *C. glutamicum* from supplemented phenylpropanoid precursors or directly from glucose [10, 11]. In the context of these studies, low

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intracellular availability of malonyl-CoA provided by the central metabolism could be identified as major bottleneck impeding higher product titers [2]. When considering the stoichiometry of flavonoid and stilbene synthesis, however, the importance of larger amounts of malonyl-CoA for plant polyphenol synthesis is not surprising as three moles of malonyl-CoA are consumed by chalcone synthases (CHS) and stilbene synthases (STS) during condensation with a CoA-activated phenylpropanoid thioester yielding chalcones (as flavonoid precursors) and stilbenes, respectively [12]. Similar to engineered polyphenol production in other microorganisms, the observed limitation at the stage of malonyl-CoA could be overcome in *C. glutamicum* at lab-scale by supplementing the antibiotic cerulenin, which selectively inhibits fatty acid synthesis as main malonyl-CoA sink in the microbial carbon metabolism [10, 13, 14]. Very recently, dependency on cerulenin for plant polyphenol synthesis could be repealed by rationally engineering the central metabolism yielding the *C. glutamicum* C7 strain, which enabled the accumulation of 24 mg/L (0.088 mM) naringenin or 112 mg/L (0.49 mM) resveratrol from glucose [15]. Key to success was a reduction of the flux into the tricarboxylic acid (TCA) cycle, whereas episomal overexpression of *accBC* (cg0802) and *accD1* (cg0812) encoding the two subunits of the acetyl-CoA carboxylase complex (ACC) hardly increased product titers. In *C. glutamicum*, transcription of *accBC* and *accD1* is controlled by the transcriptional repressor FasR [16]. Hence, *fasR* (cg2737) was also deleted to deregulate expression of *accBC* and *accD1*, but the observed positive effect on malonyl-CoA availability and accumulation of the flavonoid naringenin was very limited [15]. This was explained by the previous finding that FasR also represses expression of *fas*-IA (cg2743) and *fas*-IB (cg0957) coding for the two fatty acid synthases of *C. glutamicum*, which consume malonyl-CoA provided by the ACC for fatty acid biosynthesis [16].

About a decade ago, the pentaketide chromone synthase (PCS_{Aa}, EC 2.3.1.216, UniProt ID: Q58VP7) was identified in the medicinal plant *Aloe arborescens* [17]. Like CHS- and STS-enzymes, PCS_{Aa} is a type III polyketide synthase (PKS), but instead of catalyzing the condensation of three malonyl-CoA extender units with a CoA-activated phenylpropanoid thioester, this enzyme catalyzes the iterative decarboxylation and condensation of five malonyl-CoA molecules yielding the chromone noreugenin (Fig. 1). Plant-derived chromones such as noreugenin are gaining attention drawn by their beneficial impacts on human health including anti-inflammatory, anti-cancerous, anti-diabetic but also antimicrobial traits [18]. Thus, the chromone scaffold is considered to be a promising lead structure for medicinal chemistry. Noteworthy, the furochromones khellin and visnagin, both regarded to have anti-asthmatic effects, are derived from the pentaketide noreugenin [19]. Chemical routes for the synthesis of noreugenin include acid- or base-catalyzed reactions, microwave irradiation and solid-support catalysts [20, 21]. In addition to chemical synthesis, noreugenin could also be obtained by extraction from the producing plant material. However, typically, chemical synthesis of more complex secondary plant metabolites is economically not feasible and product concentrations in the naturally producing plants are usually quite low. In this context, microbial production of secondary plant metabolites has emerged as true alternative [22]. In case of noreugenin, synthesis of this compound has been first described in in vitro enzyme assays with purified PCS_{Aa} [17]. Recently, microbial noreugenin synthesis in *Escherichia coli* using PCS_{Aa} has been reported for the first time as proof of principle, but no product titer was determined [23].

Here, we present rational engineering of the central carbon metabolism and of fatty acid synthesis in *C. glutamicum* towards further increasing malonyl-CoA availability. Furthermore, we demonstrate the functional integration of PCS_{Aa} into these engineered *C. glutamicum* strains and the microbial synthesis of noreugenin.

Results

Establishing a heterologous pathway for the synthesis of noreugenin

For establishing noreugenin synthesis in *C. glutamicum*, a codon-optimized, synthetic variant of the *pcs* gene (pcs_{AaCg}) originating from *A. arborescens* was cloned into the vector pMKEx2 yielding pMKEx2-pcs_{AaCg}, which allows for isopropyl β-D-thiogalactopyranoside (IPTG) inducible gene expression of pcs_{AaCg} from the strong T7 promoter [24]. After transformation of *C. glutamicum* C7 selected as production strain due to its improved capability to supply malonyl-CoA, the resulting strain *C. glutamicum* C7 pMKEx2-pcs_{AaCg} (designated *C. glutamicum* Nor1 C7) was cultivated in defined CGXII minimal medium with 4% glucose and 1 mM IPTG. However, these initial experiments yielded only traces of noreugenin close to the detection limit of our LC–MS system (Fig. 2).

Detailed comparison of the amino acid sequence of PCS_{Aa} to similar type III PKS such as the STS from *Arachis hypogea* (STS_{Ah}) and CHS from *Petunia x hybrida* (CHS_{Ph}) revealed that the first ten amino acids of PCS_{Aa} have no equivalent sequence in any of the two other enzymes, despite their otherwise high sequence identity of 70–80% over the whole amino acid sequence (Additional file 1: Figure S1). Surprisingly, residues M11 and V14 of PCS_{Aa} align with the starting methionine M1 and V4 of both enzymes, CHS_{Ph} and STS_{Ah}. This pointed

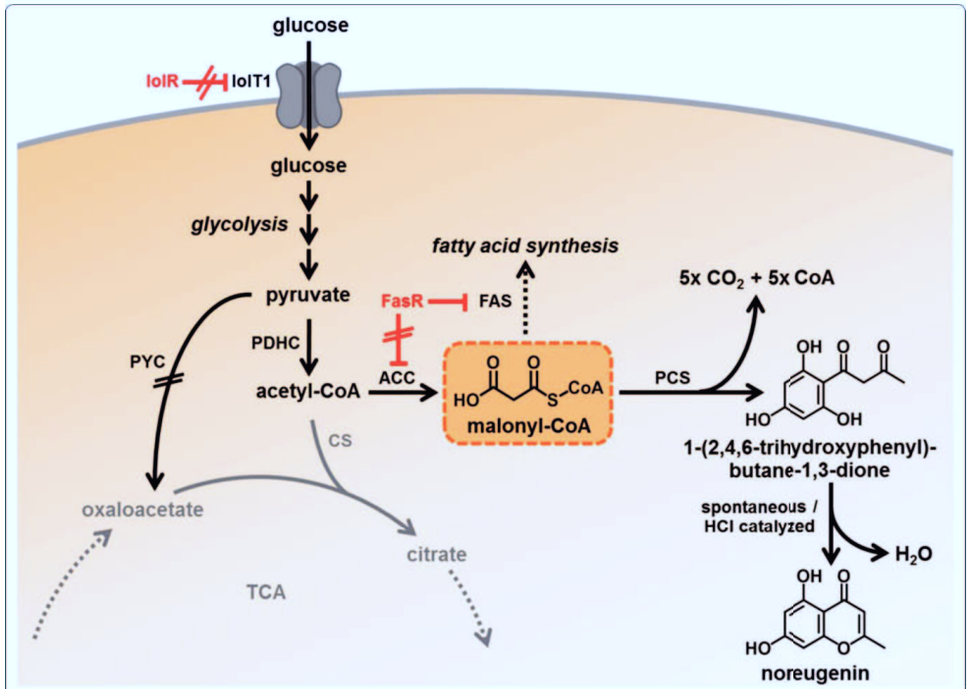

Fig. 1 Metabolic engineering of the central carbon metabolism of *C. glutamicum* for increased malonyl-CoA availability and noreugenin synthesis. ACC: acetyl-CoA carboxylase; CS: citrate synthase; FAS: fatty acid synthase; FasR: transcriptional repressor of *accBC*, *accD1*, *fasIA* and *fasIB*, all involved in fatty acid synthesis; IolR: transcriptional repressor of genes involved in *myo*-inositol catabolism; IolT1: glucose/*myo*-inositol permease; PCS: pentaketide chromone synthase; PDHC: pyruvate dehydrogenase complex; PYC: pyruvate carboxylase; TCA: tricarboxylic acid cycle, Eliminated enzymatic reactions (black) or regulatory circuits (red): crossed out; reduced enzyme/pathway activity: grey

either to a simple misannotation of the translational start or to the ten N-terminal amino acids of PCS_{Aa} functioning as signal sequence for protein localization in the plant cell. Three algorithms (SignalP [25], TargetP [26] and WoLF PSORT [27]) developed to detect signal- and targeting peptide sequences were used to interpret this sequence, but none proposed a possible function for this short amino acid stretch. However, a pMKEx2 plasmid harboring a truncated pcs_{AaCg} variant (pcs_{AaCg}-short, pMKEx2-pcs_{AaCg}-short) without the first 30 nucleotides encoding the ten N-terminal amino acids in question was constructed. Expression of the truncated pcs_{AaCg} gene in the strain *C. glutamicum* C7 (designated *C. glutamicum* Nor2 C7) under aforementioned conditions, significantly improved noreugenin synthesis leading to a product titer of 0.8 mg/L (0.004 mM) after 72 h (Fig. 2). Hence, most likely the pcs_{Aa} was simply not annotated correctly, likely reducing the activity of the enzyme or preventing the formation of the active PCS_{Aa} dimer under the described conditions.

Deregulation of genes involved in fatty acid synthesis increases malonyl-CoA availability

In order to further improve noreugenin synthesis, fatty acid metabolism of *C. glutamicum* Nor2 C7 was engineered aiming for increased malonyl-CoA supply. The fatty acid regulator protein FasR acts as transcriptional repressor of the genes *accBC*, *accD1*, *fasIA* and *fasIB*, all involved in fatty acid biosynthesis [16]. Deletion of *fasR* in *C. glutamicum* C7 was shown to negatively affect flavonoid synthesis, presumably due to an also increased fatty acid synthase (FAS) activity consuming malonyl-CoA again [15]. In contrast, episomal overexpression of ACC subunit genes *accBC* and *accD1* increased the polyphenol

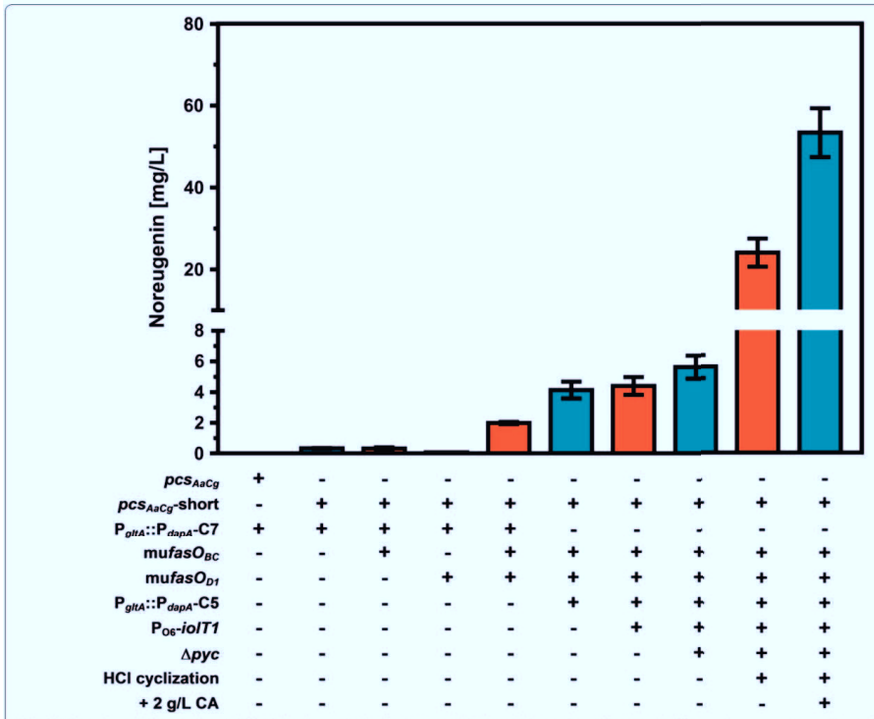

Fig. 2 Overview of the constructed *C. glutamicum* strains for improved intracellular malonyl-CoA availability and noreugenin synthesis. Given noreugenin concentrations were determined after 72 h of cultivation and represent mean values with standard deviations from biological triplicates. For more clarity, + and - represent presence or absence of the depicted genetic modifications or plasmids. For details the reader is referred to the main text

production by 40%, which shows that acetyl-CoA carboxylation is indeed a promising target for further metabolic engineering. Therefore, fatty acid metabolism of *C. glutamicum* should be tailored towards increased endogenous ACC activity yielding more malonyl-CoA without simultaneously increasing FAS activity consuming this noreugenin precursor. Mutational analysis of the operator regions of both *acc* genes identified the *fasO* motifs essential for FasR binding [16]. Based on these findings, the *fasO* motifs upstream of the *accBC*- and *accD1*-open reading frames were mutated in the *C. glutamicum* C7 strain background, both individually (mu*fasO*$_{BC}$, mu*fasO*$_{D1}$) and in combination (mu*fasO*$_{BCD1}$). Important to note in this context, since the FasR binding site of *accD1* overlaps with the open reading frame of this gene, only nucleotide substitutions were introduced that do not alter the amino acid sequence of AccD1 (Fig. 3). Subsequently, the resulting strains *C. glutamicum* Nor2 C7 mu*fasO*$_{BC}$, *C. glutamicum* Nor2 C7 mu*fasO*$_{D1}$ and *C. glutamicum* Nor2 C7 mu*fasO*$_{BCD1}$ were cultivated to compare their ability to produce noreugenin (Fig. 2). Whereas the strains with individually mutated *fasO* sites accumulated 0.31 mg/L (0.002 mM) and 0.06 mg/L (0.0003 mM), respectively, did *C. glutamicum* Nor2 C7 mu*fasO*$_{BCD1}$ generate 1.98 mg/L (0.010 mM) noreugenin after 72 h of cultivation in CGXII medium with 4% glucose.

With the aim of verifying that *C. glutamicum* C7 mu*fasO*$_{BCD1}$ indeed provides more malonyl-CoA compared to *C. glutamicum* C7, intracellular malonyl-CoA was quantified in form of its free acid malonate by LC–MS/MS as previously described [15]. As the expression of *pcs*$_{AaCg}$-short would lead to malonyl-CoA consumption and thus distort intracellular metabolite concentrations, the strains without the pMKEx2-*pcs*$_{AaCg}$-short plasmid were examined. Since the obtained isotope ratio for the parental *C. glutamicum* C7 strain was

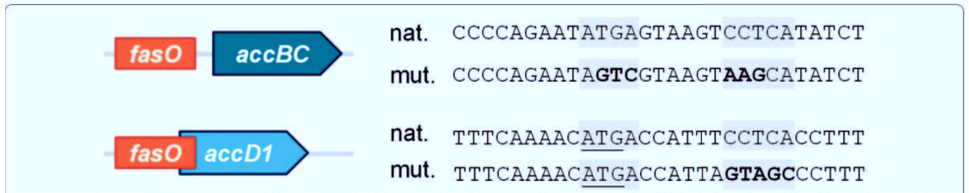

Fig. 3 Schematic representation and relative postion of the *fasO* sites of *accBC* and *accD1*. In both cases, the mutated (mut.) *fasO* sites are aligned with the respective native (nat.) *fasO* sites. Grey-backed nucleotides are conserved in all known *C. glutamicum fasO*-sites essential for FasR binding. Mutated nucleotides are highlighted by bold letters. The start codon of *accD1* is underlined

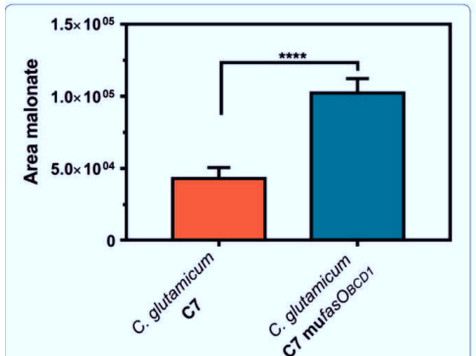

Fig. 4 LC–MS/MS analysis of intracellular malonate concentrations representing intracellular malonyl-CoA availability. Areas for malonate acquired by LC-MS/MS analysis of cytoplasmatic extracts are plotted for the indicated strains. The obtained malonate areas represent mean values with standard deviations from biological duplicates with three technical replicates each (six samples in total). The four asterisks represent the level of significance of an unpaired two-tailed *t* test ($p < 0.0001$)

below the limit of quantification, obtained signal areas themselves were used for comparison (Fig. 4). The malonate area for the parental strain *C. glutamicum* C7 was determined to be 43,065 ± 3090, whereas the malonate signal for *C. glutamicum* C7 mu*fasO*$_{BCD1}$ was increased 2.8-fold (102,304 ± 4495). As the isotope ratio corresponding to the latter area was within the linear range of the calibration curve, an intracellular malonate concentration of 1.8 mM for *C. glutamicum* C7 mu*fasO*$_{BCD1}$ could be calculated. These findings show that noreugenin synthesis can be used to quickly report differences in intracellular malonyl-CoA pools of constructed *C. glutamicum* strains, instead of always using the laborious LC–MS/MS-based method. This allows considering noreugenin as a reliable reporter molecule to evaluate the impact of metabolic engineering on intracellular malonyl-CoA availability in addition to the relevance of the product itself.

Increased glucose uptake and reduced flux into the tricarboxylic acid cycle increase the malonyl-CoA-pool

Previously, reduction of the activity of citrate synthase (CS, encoded by the gene *gltA*, cg0949) to 10% compared to wild-type CS activity by promoter replacement enabled increased plant polyphenol production with *C. glutamicum* due to a reduced flux of acetyl-CoA in the TCA cycle and thus increased malonyl-CoA availability [15]. Further reduction of CS activity to 5.5% was achieved by exchanging the P$_{dapA}$-C7 promoter upstream of *gltA* by the even weaker P$_{dapA}$-C5 variant [15, 28]. The constructed strain *C. glutamicum* Nor2 C5 mu*fasO*$_{BCD1}$ showed reduced growth, but accumulated two times more noreugenin after 72 h of cultivation (4.13 mg/L, 0.022 mM) compared to *C. glutamicum* Nor2 C7 mu*fasO*$_{BCD1}$ (1.98 mg/L, 0.010 mM, Fig. 2).

The dependency of noreugenin synthesis on the availability of the glucose-derived metabolite malonyl-CoA suggests that an improved glucose uptake of *C. glutamicum* might further increase the intracellular malonyl-CoA pool. Recently, we reported that relieve of IolR-mediated repression of the *iolT1* gene encoding the glucose/*myo*-inositol permease IolT1 by promoter engineering increases D-xylose and D-glucose uptake in *C. glutamicum* [29, 30]. Hence, two point mutations in the *iolT1* promoter (P$_{O6}$-*iolT1*) were also introduced into *C. glutamicum* Nor2 C5 mu*fasO*$_{BCD1}$ yielding *C. glutamicum* Nor2 C5 mu*fasO*$_{BCD1}$ P$_{O6}$-*iolT1*. Interestingly, this modification appears to have only a minor positive effect on noreugenin synthesis (4.40 mg/L, 0023 mM, Fig. 2), but growth, negatively influenced by downregulation of *gltA* expression, was partly improved.

To further increase acetyl-CoA availability for malonyl-CoA synthesis, anaplerotic reactions withdrawing

phosphoenolpyruvate (PEP) and pyruvate as glycolytic acetyl-CoA precursors were targeted. Interestingly, *C. glutamicum* is capable of catalyzing the carboxylation of both PEP and pyruvate during growth on glucose, which is rather uncommon for most microorganisms [31]. It was demonstrated, that PEP carboxylase (PEPC) and pyruvate carboxylase (PYC) could replace each other as anaplerotic enzymes when glucose is used as sole carbon and energy source [32]. This means that at least one of the two enzymes needs to be present for enabling growth on glucose. In fact, 90% of total oxaloacetate synthesis is ascribed to the activity of PYC encoded by *pyc* (cg0791) in *C. glutamicum* [33]. Therefore, we decided to delete *pyc*. The strain *C. glutamicum* Nor2 C5 mu*fasO*$_{BCD1}$ P$_{O6}$-*iolT1* Δ*pyc* was constructed, cultivated and characterized with regard to noreugenin synthesis. Deletion of the *pyc* gene in this strain increased noreugenin synthesis and enabled accumulation of 5.63 mg/L (0.029 mM) noreugenin within 72 h (Fig. 2).

Identification and acid-catalyzed cyclization of the intermediate 1-(2,4,6-trihydroxyphenyl) butane-1,3-dione

To our surprise, synthesis of noreugenin continued over the whole process time in all *C. glutamicum* strains constructed in this study, although the cells reached the stationary growth phase already after 24 h. To the best of our knowledge, malonyl-CoA supply in *C. glutamicum* is strictly coupled to the exponential growth phase, implying that noreugenin synthesis should cease when the cultures reach the stationary growth phase. In preceding studies focusing on the synthesis of malonyl-CoA-dependent polyphenols naringenin and resveratrol with *C. glutamicum* C7-based strains, continuous production of the respective polyphenols was never observed [15]. Even when prolonging the cultivation times from 72 to 192 h, noreugenin formation continued and did not reach a plateau (Additional file 1: Figure S2). We assumed that this phenomenon was unlikely to be due to the engineered *C. glutamicum* strain background, instead we concluded that the reason for this finding must be connected to the product noreugenin itself. As noreugenin synthesis from malonyl-CoA is catalyzed by a single enzyme, further investigations focused on PCS$_{Aa}$ and its reaction mechanism. PCS$_{Aa}$ is described to catalyze a C_1/C_6 Claisen-type cyclization of the enzyme-bound pentaketide followed by spontaneous pyrone ring formation yielding noreugenin [34]. In silico reconstruction of the PCS$_{Aa}$ reaction mechanism suggested 1-(2,4,6-trihydroxyphenyl)butane-1,3-dione (TPBD, M = 210,05 g/mol) to be the C_1/C_6 cyclized pentaketide intermediate that subsequently undergoes spontaneous isomerization and dehydration (Fig. 1). When the mass spectrometer was operated in negative electrospray ionization (ESI) mode and data acquisition was performed in selected ion monitoring (SIM) mode, an additional mass signal at *m/z* 209 (retention time = 1.33 min) was detected in all prepared samples representing the $[M-H]^-$ mass signal for the presumed intermediate TPBD (Fig. 5a). Over the cultivation time, a decreasing TPBD signal could be observed whereas the noreugenin signal increased (Fig. 5b). Unfortunately, unavailability of an authentic TPBD standard rendered quantification of this intermediate impossible. To evaluate product formation over time, we added up the signal areas of TPBD and noreugenin, both normalized to the area of the internal standard benzoic acid $((Area_{Noreugenin}/Area_{Berzoic\ acid}) + (Area_{TPBD}/Area_{Benzoic\ acid}))$ (Fig. 5c). Here, no net change of summed ratios beyond 24 h of cultivation could be observed. We concluded, that the synthesized TPBD circularizes spontaneously after this time point, forming the pyrone moiety yielding noreugenin. This would explain the observed decreasing TPBD concentration and an increasing noreugenin concentration over time. However, since the summed ratio of TPBD and noreugenin does not change after 24 h, no more TPBD is synthesized when the cells have reached the stationary growth phase and malonyl-CoA supply stopped after 24 h when glucose is depleted. Therefore, the observed continuous noreugenin synthesis can solely be ascribed to slow, spontaneous TPBD conversion and is not due to any unlikely growth-decoupled supply of malonyl-CoA.

As the slow, spontaneous cyclization of the pyrone moiety limits the absolute noreugenin titer and needlessly prolongs the overall process time, speeding up TPBD conversion was targeted to fully convert TPBD to noreugenin. For this purpose, acetonitrile extracts prepared for LC–MS analysis were acidified with methanolic HCl at various concentrations. After evaporation to dryness and subsequent resuspension in acetonitrile, conversion of TPBD to noreugenin was quantified using LC–MS analysis. These experiments showed, that a concentration of at least 142 mM HCl was required to achieve full conversion of TPBD to noreugenin (data not shown). Here, we proposed an acid-catalyzed mechanism for the cyclization of TPBD (Additional file 1: Figure S3). Application of this method for samples obtained from standard cultivation of the best performing strain *C. glutamicum* Nor2 C5 mu*fasO*$_{BCD1}$ P$_{O6}$-*iolT1* Δ*pyc* increased the total noreugenin titer to 23.99 mg/L (0.125 mM, Fig. 2).

Supplementation of defined CGXII medium with casamino acids

Recently, standard CGXII medium was described to be insufficient to support heterologous expression of the plant-derived genes *ans* and *3gt* encoding an

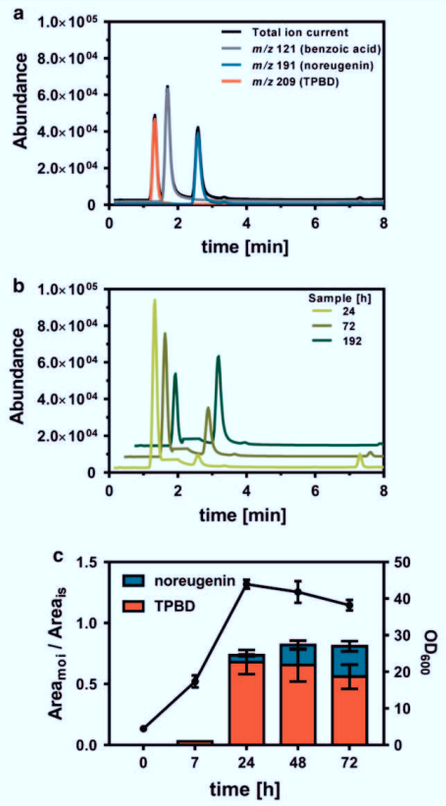

Fig. 5 Detection of the noreugenin synthesis intermediate 1-(2,4,6-trihydroxyphenyl)butane-1,3-dione (TPBD) in extracted cultivation samples. **a** Exemplary chromatogramm of an extracted cultivation sample both as total ion current and the individual *m/z* ratios. **b** Total ion current chromatogramms of extracted samples resembling the spontaneous cyclization of TPBD towards noreugenin over the cultivation time. For better visualization, the benzoic acid signal was removed from the chromatogramm. **c** Growth curve and product abundance for the strain *C. glutamicum* C5 $fasO_{BCD1}$ P_{O6}-*iolT1* Δ*pyc* pMKEx2-pcs_{AaCg}-short. Unavailability of an authentic TPBD standard prevented quantification of this particular molecule. To evaluate product formation over time, the normalized signal areas of the molecules of interest ($Area_{moi}$) TPBD and noreugenin were added up. Signal area of the internal standard ($Area_{is}$) benzoic acid was used for normalization. The calculated ratios are depicted on the primary Y-axis. At the given sampling time points, OD_{600} of the cultures was also determined (filled circles, shown on secondary Y-axis). The obtained data represent mean values with standard deviations from biological triplicates

anthocyanidin synthase and an anthocyanidin 3-*O*-glucosyltransferase required for anthocyanin biosynthesis in *C. glutamicum* [35]. In this context, utilization of Andrew's magic medium (AMM), described as a rich medium for *E. coli* already containing 2 g/L casamino acids, was tested [36]. Additional supplementation of 2 g/L casamino acids for improving the functional expression of these genes allowed for higher anthocyanin titers with *C. glutamicum* from supplemented catechin. With the aim to exclude that microbial noreugenin synthesis with *C. glutamicum* Nor2 C5 mu$fasO_{BCD1}$ P_{O6}-*iolT1* Δ*pyc* suffers from poor expression of pcs_{AaCg}-short due to an unfavorable medium composition, standard CGXII medium with 4% glucose was additionally supplemented with 2 g/L casamino acids. By doing so, noreugenin synthesis could be doubled yielding 53.32 mg/L (0.278 mM) after 72 h of cultivation and subsequent HCl-based cyclization of the TPBD intermediate (Fig. 2).

Discussion

In this study, we describe engineering of *C. glutamicum* for increased malonyl-CoA availability and microbial synthesis of the plant pentaketide noreugenin originally found in the medicinal plant *A. arborescens*. Key to success was the N-terminal truncation of the type III PKS enabling noreugenin synthesis. Originally, the ten N-terminal residues of PCS_{Aa} were believed to contribute to the formation of an expanded surface-exposed loop [34]. However, since this short amino acid stretch is not present in closely related CHS- and STS-enzymes, which could be already functionally implemented in *C. glutamicum*, we deleted the first 30 nucleotides of the pcs_{Aa} gene. Eventually, this modification drastically improved noreugenin synthesis.

A common approach in the context of improving malonyl-CoA availability for product synthesis aims at increasing ACC activity in the respective microbial workhorse [2]. Previously, by abolishing Snf1-dependent posttranslational regulation of Acc1, malonyl-CoA availability in *Saccharomyces cerevisiae* could be increased for the synthesis of fatty acid ethyl esters and 3-hydroxypropionic acid [37]. Another strategy for increasing ACC activity is overexpression of genes encoding for the respective subunits of this enzyme complex. For example, episomal overexpression of four genes coding for a heterotetrameric ACC from *Photorhabdus luminescens* in *E. coli* increased the titer of the malonyl-CoA-derived polyphenol pinocembrin sevenfold, yielding 196 mg/L of this product [38]. Furthermore, the active AccBC-AccD1 heterodimer of *C. glutamicum* could be functionally introduced into *E. coli* [39]. By following this strategy, the intracellular malonyl-CoA availability in *E. coli* was increased 15-fold allowing for the synthesis of 1.3 g/L phloroglucinol [40].

In this study, we developed a different strategy aiming for an increased expression of the genomically encoded ACC genes in *C. glutamicum*, instead of following the more traditional approach of episomal overexpression of (heterologous) genes. By mutation of the *fasO*-sites upstream of *accBC* and *accD1*, repression of gene expression mediated by the transcription regulator FasR could be repealed, which almost tripled intracellular malonyl-CoA availability and thus enabled increased product formation. Important to note, mutation of both FasR binding sites was required to increase ACC activity, probably because a functional ACC requires both subunits, AccBC and AccD1, in equimolar amounts [39, 41]. Furthermore, the intracellular malonyl-CoA availability was positively influenced by increasing glucose uptake through deregulation of the gene for the glucose/*myo*-inositol permease IolT1. However, this had only limited beneficial effects on noreugenin synthesis at shaking flask scale, but might be beneficial for large-scale applications when considering important process criteria such as space–time yield. Efficacy of this strain modification was already demonstrated for the de novo synthesis of hydroxybenzoic acids in *C. glutamicum* from glucose [30]. Potential other strategies for increasing the intracellular malonyl-CoA availability include establishing an ACC-independent pathway for malonyl-CoA synthesis from supplied malonate through heterologous expression of genes for malonate uptake and CoA-activation of malonate originating from the malonate assimilation pathway in *Rhizobium trifolii* [42]. This particular approach enabled a 15-fold increase of pinocembrin production using *E. coli* [13, 43]. Furthermore, state-of-the-art techniques for gene silencing or downregulation such as CRISPR interference (CRISPRi) or methods employing synthetic small regulatory RNA (sRNA) libraries or synthetic antisense RNA (asRNA) could be used to specifically knock down genes encoding for malonyl-CoA consuming enzymes [23, 44, 45].

Moreover, we predicted and detected the TPBD intermediate as the actual product of PCS_{Aa} and could show that formation of TPBD is strictly limited to the exponential growth phase in *C. glutamicum* in which malonyl-CoA is exclusively supplied. This is in line with our observations regarding flavonoid- and stilbene synthesis with *C. glutamicum* [10, 15]. Through HCl acidification of acetonitrile extracts we achieved full conversion of TPBD to noreugenin within 3 h allowing to significantly reduce the overall cultivation time.

Very recently, it could be demonstrated that composition of the defined CGXII medium might not be optimal for the expression of plant-derived genes involved in anthocyanin synthesis in *C. glutamicum* [35]. In this particular study, supplementation of the defined AMM medium with 2 g/L casamino acids significantly improved heterologous gene expression and thus anthocyanin synthesis. Casamino acids, obtained through acid hydrolysis of casein, represent a valuable source of all proteinogenic amino acids except tryptophan [46, 47]. Although it could be shown here that supplementation of casamino acids also promotes noreugenin synthesis, we prefer the simplicity of defined CGXII medium, especially as the composition of casamino acids varies from supplier to supplier. Nevertheless, this supplementation strategy is helpful to meet possible future challenges connected to heterologous gene expression. Alternatively, translational fusions of the target protein with the maltose-binding protein MalE from *E. coli* can be generated. This strategy already proved to be beneficial for the functional expression of a plant-derived *O*-methyltransferase gene from *Vitis vinifera* in *C. glutamicum* [11].

Conclusion

In the present work, we applied the knowledge of the well-characterized central carbon metabolism to tailor *C. glutamicum* towards increased malonyl-CoA availability by rational metabolic engineering. Additionally, our work contributes to a better understanding of the PCS_{Aa} reaction mechanism as we could detect the intermediate TPBD, which spontaneously cyclizes to noreugenin. Acidification accelerated TPBD conversion to the product allowing for a titer of 53.32 mg/L (0.278 mM) noreugenin. Taken together, the constructed strain *C. glutamicum* C5 mu$fasO_{BCD1}$ P_{O6}-*iolT1* Δ*pyc* represents a promising strain for the microbial production of noreugenin and other malonyl-CoA derived products.

Materials and methods

Bacterial strains, plasmids, media and growth conditions

All bacterial strains and plasmids with their respective characteristics used in this study are listed in Table 1. *C. glutamicum* strains were routinely cultivated aerobically at 30 °C in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, USA) or defined CGXII medium with glucose as sole carbon and energy source [48]. When indicated, 2 g/L casamino acids were supplemented (Becton–Dickinson, Franklin Lakes, USA). *E. coli* DH5α was used for plasmid constructions and was cultivated in LB medium [49] at 37 °C. Where appropriate, kanamycin (*E. coli* 50 µg/mL, *C. glutamicum* 25 µg/mL) was added to the medium. Bacterial growth was followed by measuring the optical density at 600 nm (OD_{600}).

To cultivate *C. glutamicum*, a test tube with 5 mL BHI medium was inoculated with a single colony from an agar plate and grown for 6–8 h on a rotary shaker at 170 rpm (first preculture). This first preculture was used to inoculate 50 mL of defined CGXII medium with 4% glucose in

Table 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source
C. glutamicum strains		
DelAro^4-*4cl*$_{Pc}$ C7	*C. glutamicum* derivative with in-frame deletions of cg0344-47, cg0502, cg1226 and cg2625-40, harboring a chromosomally encoded codon-optimized *4cl*$_{Pc}$ gene coding for 4-coumarate:CoA ligase from *Petroselinum crispum* under control of the T7 promoter (Δcg0344-47::P$_{T7}$-*4cl*$_{Pc}$) and replacement of the native *gltA* promoter with the *dapA* promoter variant C7 (P$_{gltA}$::P$_{dapA}$-C7)	[15]
Nor1 C7	DelAro^4-*4cl*$_{Pc}$ C7 strain harboring pMKEx2-*pcs*$_{AaCg}$	This work
Nor2 C7	DelAro^4-*4cl*$_{Pc}$ C7 strain harboring pMKEx2-*pcs*$_{AaCg}$-short	This work
Nor2 C7 mu*fasO*$_{BC}$	Nor2 C7 derivative with mutated *fasO* binding site upstream of *accBC*	This work
Nor2 C7 mu*fasO*$_{D1}$	Nor2 C7 derivative with mutated *fasO* binding site upstream of *accD1*	This work
Nor2 C7 mu*fasO*$_{BCD1}$	Nor2 C7 derivative with mutated *fasO* binding site upstream of *accBC* and *accD1*	This work
Nor2 C5 mu*fasO*$_{BCD1}$	Nor2 C7 mu*fasO*$_{BCD1}$ derivative with replacement of the *dapA* promoter variant C7 with the *dapA* promoter variant C5	This work
Nor2 C5 mu*fasO*$_{BCD1}$ P$_{O6}$-*iolT1*	Nor2 C5 mu*fasO*$_{BCD1}$ derivative with two nucleotide exchanges in the *iolT1* promoter at position -113 (A→G) and -112 (C→G) relative to the start codon	This work
Nor2 C5 mu*fasO*$_{BCD1}$ P$_{O6}$-*iolT1* Δ*pyc*	Nor2 C5 mu*fasO*$_{BCD1}$ P$_{O6}$-*iolT1* derivative with in-frame deletion of *pyc*	This work
E. coli strains		
DH5α	F−Φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (rK−,mK+)*phoA supE44*λ−*thi-1 gyrA96relA1*	Invitrogen (Karlsruhe, Germany)
Plasmids		
pK19*mobsacB*	*kan*^{r}; vector for allelic exchange in *C. glutamicum* (pK18, oriV_{Ec}, *sacB*, *lacZ*α)	[52]
pK19*mobsacB*-mu*fasO*$_{BC}$	Vector for mutation of the *fasO* binding site upstream of *accBC*	This work
pK19*mobsacB*-mu*fasO*$_{D1}$	Vector for mutation of the *fasO* binding site upstream of *accD1*	This work
pK19*mobsacB*-P$_{gltA}$::P$_{dapA}$-C5	Vector for exchanging the P$_{dapA}$-C7 variant upstream of *gltA* against the P$_{dapA}$-C5 variant	[28]
pK19*mobsacB*-P$_{O6}$-*iolT1*	Vector for mutation of the *iolT1* promoter	[29]
pK19*mobsacB*-Δ*pyc*	Vector for in-frame deletion of *pyc*	This work
pMKEx2	*kan*^{r}; *E. coli*-*C. glutamicum* shuttle vector (*lacI*, P$_{T7}$, *lacO1*, pHM1519 ori$_{Cg}$; pACYC177 ori$_{Ec}$)	[24]
pMKEx2-*pcs*$_{AaCg}$	pMKEx2 derivative with gene coding for pentaketide chromone synthase from *Aloe arborescens* (codon-optimized)	This work
pMKEx2-*pcs*$_{AaCg}$-short	pMKEx2 derivative with truncated gene coding for pentaketide chromone synthase from *Aloe arborescens* (codon-optimized)	This work

a 500 mL baffled Erlenmeyer flask (second preculture). The second preculture was cultivated overnight on a rotary shaker at 130 rpm. The main culture was subsequently inoculated from the second preculture to an OD_{600} of 5 in defined CGXII medium with 4% glucose. For synthesis of noreugenin, heterologous gene expression was induced 90 min after inoculation of the main culture using 1 mM IPTG. 1 mL of the culture broth was sampled at defined time points and stored at −20 °C until ethyl acetate extraction and LC–MS analysis.

Plasmid and strain construction

Standard protocols of molecular cloning, such as PCR, restriction and ligation of DNA were carried out for recombinant DNA work [50]. 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 and chromosomal fragments were amplified by PCR from synthetic genes or genomic *C. glutamicum* DNA as template using the listed oligonucleotides (Table 2). PCR products were subsequently used for cloning of genes and genomic fragments into plasmid vectors using Gibson Assembly [51]. In-frame gene deletions and introduction of genomic mutations in *C. glutamicum* were performed using the pK19mobsacB system [52] by a two-step homologous recombination method described previously [53]. Integrity of all constructed plasmids was verified by colony PCR, restriction analysis, and DNA sequencing at Eurofins MWG Operon (Ebersberg, Germany) Techniques specific for *C. glutamicum*, e.g. electroporation of cells, were performed as described previously [54].

Table 2 Oligonucleotides used in this study

Primer	Sequence (5′–> 3′)
PCS-s	ACTTTAAGAAGGAGATATACCATGGTAAGGAGGACAGCTATGTCCTCCTTGTCCAAC
PCS-as	CCAGGACTAGTTTCCAGAGTACTATTACATGAGTGGCAGGGAG
PCS-short-s	ACTTTAAGAAGGAGATATACCATGGTAAGGAGGACAGCTATGGAAGATGTGCAGGGC
mu-accBC-up-s	ATCCCCGGGTACCGAGCTCGAACCAGCGCGCGTTCGTG
mu-accBC-up-as	TTACGACTATTCTGGGGGAATTCTTCTGTTTTAGGCAGGAAATATGGCTTATG
mu-accBC-down-s	AGAAGAATTCCCCCAGAATAGTCGTAAGTAAGCATATCTGGTTGAGTTCTTCGGGGTTG
mu-accBC-down-as	TTGTAAAACGACGGCCAGTGGCCTTGGCGGTATCTGCG
chk-accBC-s	GTTCGGCCACTCCGATGTCCGCCTG
chk-accBC-as	GCCTTGATGGCGATTGGGAGACC
mu-accD1-up-s	ATCCCCGGGTACCGAGCTCGTCATTCAACGCATCCATGACAGC
mu-accD1-up-as	CTAATGGTCATGTTTTGAAATCGTAGCGGTAGGCGGGG
mu-accD1-down-s	ACCGCTACGATTTCAAAACATGACCATTAGTAGCCCTTTGATTGACGTCGCCAACCTTC
mu-accD1-down-as	TTGTAAAACGACGGCCAGTGCGCCAGAAGCCTGAATGTTTTG
chk-accD1-s	GGCTGATATTAGTGCCCCAACCGATGAC
chk-accD1-as	GATCACGTCTGGGCCGGTAACGAAC
chk-gltA-s	ATCGTTAACGATCTGACCCAACAA
chk-gltA-as	CGTAAGCAGCCTCTGGCGGAA
chk-P_{O6}-iolT1-s	TACGAATGCCCACTTCGCACCCTT
chk-P_{O6}-iolT1-as	CAACTCATTACGGCCAGCCAGTGAGC
pyc-up-s	ATCCCCGGGTACCGAGCTCGAATTCCTGATACCTTCGCGGTGTAC
pyc-up-as	CACCTTCCACAGATGTGTGAGTCGACAC
pyc-down-s	TCACACATCTGTGGAAGGTGGCGACTTG
pyc-down-as	TTGTAAAACGACGGCCAGTGAATTCCCTGAAAGTGCAGAATGTCTTTTTC
chk-pyc-s	GCCGTAACTCTGGCCTGATC
chk-pyc-as	CTGGCAACCACATCTGCACTGCG
chk-pMKEx2-s	CCCTCAAGACCCGTTTAGAGGC
chk-pMKEx2-as	TTAATACGACTCACTATAGGGGAATTGTGAGC
rsp	CACAGGAAACAGCTATGACCATG
univ	CGCCAGGGTTTTCCCAGTCACGAC

Noreugenin extraction and LC–MS quantification

Noreugenin extraction was performed by mixing 1 mL of the culture broth with 1 mL ethyl acetate and subsequent vigorous shaking (1400 rpm, 10 min, 20 °C) in a thermomixer (Eppendorf, Hamburg, Germany). The suspension was centrifuged for 5 min at 13,300 rpm and the upper, organic layer (800 µL) was transferred to an organic solvent resistant deep-well plate (Eppendorf, Hamburg, Germany). After evaporation of the ethyl acetate overnight, the same volume of acetonitrile was used to resuspend the dried extracts for LC–MS analysis. Noreugenin was quantified using an Agilent ultra-high-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 C_{18} 100 Å pore size column (2.1 * 50 mm; Phenomenex, Torrance, CA, USA) 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. A gradient elution was used, where the amount of solvent B was increased stepwise: minute 0 to 6: 10% to 30%, minute 6 to 7: 30% to 50%, minute 7 to 8: 50% to 100% and minute 8 to 8.5: 100% to 10%. The mass spectrometer was operated in the negative electrospray ionization (ESI) mode, and data were acquired using the selected ion monitoring (SIM) mode. An authentic noreugenin standard was purchased from Carbosynth (Compton, Newbury, United Kingdom). Area values for $[M-H]^-$ mass signals were linear up to metabolite concentrations of at least 50 mg/L. Benzoic acid (final concentration 100 mg/L) was used as internal standard. A calibration curve was calculated based on analyte/internal standard ratios for the obtained area values.

Acid-catalyzed cyclization of 1-(2,4,6-trihydroxyphenyl) butane-1,3-dione

For the cyclization of 1-(2,4,6-trihydroxyphenyl)butane-1,3-dione, a 250 µL aliquot of the ethyl acetate-extracted acetonitrile samples prepared for LC–MS was acidified by addition of 100 µL 0.5 M HCl in MeOH (final concentration 0.15 M). The samples were incubated (700 rpm, 3 h, 50 °C) in a thermomixer (Eppendorf, Hamburg, Germany), evaporated to dryness and subsequently resuspended in 250 µL acetonitrile. LC–MS analysis of obtained samples was performed as described above.

Additional file

Additional file 1. Additional information containing detailed alignment of amino acid sequences, further results and a proposed mechanism for the acid-catalyzed cyclization of TPBD.

Authors' contributions

LM and NK conceived the design of this study. LM performed the construction of plasmids and strains, cultivation, sample preparation and analysis. JK performed the experiments for the determination of the intracellular malonyl-CoA availability. LM, NK and JM wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Additional files.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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References

1. Bourgaud F, Gravot A, Milesi S, Gontier E. Production of plant secondary metabolites: a historical perspective. Plant Sci. 2001;161:839–51. https://doi.org/10.1016/S0168-9452(01)00490-3.
2. Milke L, Aschenbrenner J, Marienhagen J, Kallscheuer N. Production of plant-derived polyphenols in microorganisms: current state and perspectives. Appl Microbiol Biotechnol. 2018;102:1575–85. https://doi.org/10.1007/s00253-018-8747-5.
3. Wink M. Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. Theor Appl Genet. 1988;75:225–33. https://doi.org/10.1007/BF00303957.
4. Erlund I. Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. Nutr Res. 2004;24:851–74. https://doi.org/10.1016/j.nutres.2004.07.005.
5. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev. 2009;2:270–8. https://doi.org/10.4161/oxim.2.5.9498.
6. Nile SH, Park SW. Edible berries: bioactive components and their effect on human health. Nutrition. 2014;30:134–44. https://doi.org/10.1016/j.nut.2013.04.007.
7. Rodriguez-Mateos A, Heiss C, Borges G, Crozier A. Berry (poly)phenols and cardiovascular health. J Agric Food Chem. 2014;62:3842–51. https://doi.org/10.1021/jf403757g.
8. Bensalem J, Dal-Pan A, Gillard E, Calon F, Pallet V. Protective effects of berry polyphenols against age-related cognitive impairment. Nutr Aging. 2016;3:89–106. https://doi.org/10.3233/nua-150051.
9. Kallscheuer N, Vogt M, Kappelmann J, Krumbach K, Noack S, Bott M, et al. Identification of the *phd* gene cluster responsible for phenylpropanoid utilization in *Corynebacterium glutamicum*. Appl Microbiol Biotechnol. 2016;100:1871–81. https://doi.org/10.1007/s00253-015-7165-1.
10. Kallscheuer N, Vogt M, Stenzel A, Gätgens J, Bott M, Marienhagen J. Construction of a *Corynebacterium glutamicum* platform strain for the production of stilbenes and (2S)-flavanones. Metab Eng. 2016;38:47–55. https://doi.org/10.1016/j.ymben.2016.06.003.
11. Kallscheuer N, Vogt M, Bott M, Marienhagen J. Functional expression of plant-derived *O*-methyltransferase, flavanone 3-hydroxylase, and flavonol synthase in *Corynebacterium glutamicum* for production of pterostilbene, kaempferol, and quercetin. J Biotechnol. 2017;258:190–6. https://doi.org/10.1016/j.jbiotec.2017.01.006.
12. van Summeren-Wesenhagen PV, Marienhagen J. Putting bugs to the blush—metabolic engineering for phenylpropanoid-derived products in microorganisms. Bioengineered. 2013;4:355–62. https://doi.org/10.4161/bioe.23885.
13. Leonard E, Yan Y, Fowler ZL, Li Z, Lim CG, Lim KH, et al. Strain improvement of recombinant *Escherichia coli* for efficient production of plant flavonoids. Mol Pharm. 2008;5:257–65. https://doi.org/10.1021/mp700472.
14. Lim CG, Fowler ZL, Hueller T, Schaffer S, Koffas MAG. High-yield resveratrol production in engineered *Escherichia coli*. Appl Environ Microbiol. 2011;77:3451–60. https://doi.org/10.1128/AEM.02186-10.
15. Milke L, Ferreira P, Kallscheuer N, Braga A, Vogt M, Kappelmann J, et al. Modulation of the central carbon metabolism of *Corynebacterium glutamicum* improves malonyl-CoA availability and increases plant polyphenol synthesis. Biotechnol Bioeng. 2019. https://doi.org/10.1002/bit.26939 **(Epub ahead of print)**.
16. Nickel J, Irzik K, Van Ooyen J, Eggeling L. The TetR-type transcriptional regulator FasR of *Corynebacterium glutamicum* controls genes of lipid synthesis during growth on acetate. Mol Microbiol. 2010;78:253–65. https://doi.org/10.1111/j.1365-2958.2010.07337.x.

17. Abe I, Utsumi Y, Oguro S, Morita H, Sano Y, Noguchi H. A plant type III polyketide synthase that produces pentaketide chromone. J Am Chem Soc. 2005;338:1362–3. https://doi.org/10.1021/ja0431206.
18. Gaspar A, Matos MJ, Garrido J, Uriarte E, Borges F. Chromone: a valid scaffold in medicinal chemistry. Chem Rev. 2014;114:4960–92. https://doi.org/10.1021/cr400265z.
19. Dewick PM. Medicinal natural products: a biosynthetic approach. 2nd ed. Chichester: Wiley; 2002. https://doi.org/10.1016/j.jbiosc.2010.01.005.
20. Kabalka GW, Mereddy AR. Microwave-assisted synthesis of functionalized flavones and chromones. Tetrahedron Lett. 2005;46:6315–7. https://doi.org/10.1016/j.tetlet.2005.07.038.
21. Tawfik HA, Ewies EF, El-Hamouly WS. Synthesis of chromones and their applications during the last ten years. Int J Res Pharm Chem. 2014;4:1046–85.
22. Marienhagen J, Bott M. Metabolic engineering of microorganisms for the synthesis of plant natural products. J Biotechnol. 2013;163:166–78. https://doi.org/10.1016/j.jbiotec.2012.06.001.
23. Yang D, Kim WJ, Yoo SM, Choi JH, Ha SH, Lee MH, et al. Repurposing type III polyketide synthase as a malonyl-CoA biosensor for metabolic engineering in bacteria. PNAS. 2018;115:9835–44. https://doi.org/10.1073/pnas.1808567115.
24. Kortmann M, Kuhl V, Klaffl S, Bott M. A chromosomally encoded T7 RNA polymerase-dependent gene expression system for *Corynebacterium glutamicum*: construction and comparative evaluation at the single-cell level. Microb Biotechnol. 2015;8:253–65. https://doi.org/10.1111/1751-7915.12236.
25. Emanuelsson O, Brunak S, von Heijne G, Nielsen H. Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc. 2007;2:953–71. https://doi.org/10.1038/nprot.2007.131.
26. Horton P, Park KJ, Obayashi T, Fujita N, Harada H, Adams-Collier CJ, et al. WoLF PSORT: protein localization predictor. Nucleic Acids Res. 2007;35:585–7. https://doi.org/10.1093/nar/gkm259.
27. Nielsen H. Protein function prediction. In: Kihara D, editor. Protein function prediction, vol. 1611. West Lafayette: Humana Press; 2017. p. 59–73. https://doi.org/10.1007/978-1-4939-7015-5.
28. van Ooyen J, Noack S, Bott M, Reth A, Eggeling L. Improved L-lysine production with *Corynebacterium glutamicum* and systemic insight into citrate synthase flux and activity. Biotechnol Bioeng. 2012;109:2070–81. https://doi.org/10.1002/bit.24486.
29. Brüsseler C, Radek A, Tenhaef N, Krumbach K, Noack S, Marienhagen J. The *myo*- inositol/proton symporter IolT1 contributes to D-xylose uptake in *Corynebacterium glutamicum*. Bioresour Technol. 2018;249:953–61. https://doi.org/10.1016/j.biortech.2017.10.098.
30. Kallscheuer N, Marienhagen J. *Corynebacterium glutamicum* as platform for the production of hydroxybenzoic acids. Microb Cell Fact. 2018. https://doi.org/10.1186/s12934-018-0923-x.
31. Sauer U, Eikmanns BJ. The PEP-pyruvate-oxaloacetate node as the switch point for carbon flux distribution in bacteria. FEMS Microbiol Rev. 2005;29:765–94. https://doi.org/10.1016/j.femsre.2004.11.002.
32. Peters-Wendisch PG, Kreutzer C, Kalinowski J, Pátek M, Sahm H, Eikmanns BJ. Pyruvate carboxylase from *Corynebacterium glutamicum*: characterization, expression and inactivation of the *pyc* gene. Microbiology. 1998;144:915–27. https://doi.org/10.1099/00221287-144-4-915.
33. Petersen S, De Graaf AA, Eggeling L, Mollney M, Wiechert W, Sahm H. In vivo quantification of parallel and bidirectional fluxes in the anaplerosis of *Corynebacterium glutamicum*. J Biol Chem. 2000;275:35932–41. https://doi.org/10.1074/jbc.M908728199.
34. Morita H, Kondo S, Oguro S, Noguchi H, Sugio S. Structural insight into chain-length control and product specificity of pentaketide chromone synthase from *Aloe arborescens*. Chem Biol. 2007;14:359–69. https://doi.org/10.1016/j.chembiol.2007.02.003.
35. Zha J, Zang Y, Mattozzi M, Plassmeier J, Gupta M, Wu X, et al. Metabolic engineering of *Corynebacterium glutamicum* for anthocyanin production. Microb Cell Fact. 2018;17:366–75. https://doi.org/10.1016/j.jbiotec.2013.12.020.
36. He W, Li G, Fu L, Jones JA, Linhardt RJ, Koffas M. Production of chondroitin in metabolically engineered *E. coli*. Metab Eng. 2015;27:92–100. https://doi.org/10.1016/j.ymben.2014.11.003.
37. Shi S, Chen Y, Siewers V, Nielsen J. Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1. MBio. 2014;5:e01130-14. https://doi.org/10.1128/mBio.01130-14.
38. Leonard E, Lim K-H, Saw P-N, Koffas MAG. Engineering central metabolic pathways for high-level flavonoid production in *Escherichia coli*. Appl Environ Microbiol. 2007;73:3877–86. https://doi.org/10.1128/AEM.00200-07.
39. Miyahisa I, Kaneko M, Funa N, Kawasaki H, Kojima H, Ohnishi Y, et al. Efficient production of (2*S*)-flavanones by *Escherichia coli* containing an artificial biosynthetic gene cluster. Appl Microbiol Biotechnol. 2005;68:498–504. https://doi.org/10.1007/s00253-005-1916-3.
40. Zha W, Rubin-Pitel SB, Shao Z, Zhao H. Improving cellular malonyl-CoA level in *Escherichia coli* via metabolic engineering. Metab Eng. 2009;11:192–8. https://doi.org/10.1016/j.ymben.2009.01.005.
41. Gande R, Dover LG, Krumbach K, Besra GS, Sahm H, Oikawa T, et al. The two carboxylases of *Corynebacterium glutamicum* essential for fatty acid and mycolic acid synthesis. J Bacteriol. 2007;189:5257–64. https://doi.org/10.1128/JB.00254-07.
42. An JH, Kim YS. A gene cluster encoding malonyl-CoA decarboxylase (MatA), malonyl-CoA synthetase (MatB) and a putative dicarboxylate carrier protein (MatC) in *Rhizobium trifolii*–cloning, sequencing, and expression of the enzymes in *Escherichia coli*. Eur J Biochem. 1998;257:395–402. https://doi.org/10.1046/j.1432-1327.1998.2570395.x.
43. Wu J, Du G, Zhou J, Chen J. Metabolic engineering of *Escherichia coli* for (2*S*)-pinocembrin production from glucose by a modular metabolic strategy. Metab Eng. 2013;16:48–55. https://doi.org/10.1016/j.ymben.2012.11.009.
44. Yang Y, Lin Y, Li L, Linhardt RJ, Yan Y. Regulating malonyl-CoA metabolism via synthetic antisense RNAs for enhanced biosynthesis of natural products. Metab Eng. 2015;29:217–26. https://doi.org/10.1016/j.ymben.2015.03.018.
45. Wu J, Du G, Chen J, Zhou J. Enhancing flavonoid production by systematically tuning the central metabolic pathways based on a CRISPR interference system in *Escherichia coli*. Sci Rep. 2015;5:1–14. https://doi.org/10.1038/srep13477.
46. Biosciences BD. BD Bionutrients ™ technical manual. 4th ed. BD Biosciences: Franklin Lakes; 2006.
47. Chung YJ, Lee JA, Jung MY, Lee SM, Kim TY, Choe YK, et al. Optimization of diphtheria toxin production by *Corynebacterium diphtheriae* using a casein-based medium in a fermenter. Biotechnol Bioprocess Eng. 2016;21:537–43. https://doi.org/10.1007/s12257-016-0360-9.
48. Keilhauer C, Eggeling L, Sahm H. Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. J Bacteriol. 1993;175:5595–603. https://doi.org/10.1128/jb.175.17.5595-5603.1993.
49. Bertani G. Studies on lysogenesis.I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol. 1951;62:293–300.
50. Green MR, Sambrook J. Molecular cloning: a laboratory manual. 4th ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 2012.
51. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2009;6:343–5. https://doi.org/10.1038/nmeth.1318.
52. Schäfer A, Tauch A, Jaeger W, Kalinowski J, Thierbach G, Pühler A. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene. 1994;145:69–73. https://doi.org/10.1016/0378-1119(94)90324-7.
53. Niebisch A, Bott M. Molecular analysis of the cytochrome bc_1-aa_3 branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome c_1. Arch Microbiol. 2001;175:282–94. https://doi.org/10.1007/s002030100262.
54. Eggeling L, Bott M. Handbook of *Corynebacterium glutamicum*. Boca Raton: CRC Press; 2005.

2.3.1 Supplementary Material

Additional file 1

Tailoring *Corynebacterium glutamicum* towards increased malonyl-CoA availability for efficient synthesis of the plant pentaketide noreugenin

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Keywords: malonyl-CoA, *Corynebacterium glutamicum*, noreugenin, metabolic engineering, acetyl-CoA carboxylase

Additional file 1

```
PCS_Aa (Q58VP7)   MSSLSNSLPLMEDVQGIRKAQKADGTATVMAIGTAHPPHIFPQDTYADVYFRATNSEHKV 60
STS_Ah (K7XD27)   ----------MVSVSGIRNVERAEGPATVLAIGTANPSNCVDQSTYADYYFRVTNSEHMT 50
CHS_Ph (Q43040)   ----------MVTVEEYRKAQRCEGPATVMAIGTATPTNCVDQSTYPDYYFRITNSEHKT 50
                            *  *.  *:.::.:* ***:***** * : . *.** * *** ***** .

PCS_Aa (Q58VP7)   ELKKKFDHICKKTMIGKRYFNYDEEFLKKYPNITSYDEPSLNDRQDICVPGVPALGTEAA 120
STS_Ah (K7XD27)   DLKKKFQRICERTQIKNRHMYLTEEILKENPNICAYKAPSLDAREDMMIREVPRVGKEAA 110
CHS_Ph (Q43040)   DLKEKFKRMCEKSMIKKRYMHLTEEILKENPSMCEYMAPSLDARQDIVVVEVPKLGKEAA 110
                  :**:**.::*::: * :*::   **:**: *.:  *  ***: *:*: :  ** :*.***

PCS_Aa (Q58VP7)   VKAIEEWGRPKSEITHLVFCTSCGVDMPSADFQCAKLLGLHANVNKYCIYMQGCYAGGTV 180
STS_Ah (K7XD27)   TKAIKEWGQPMSKITHLIFCTTSGVALPGVDYELIVLLGLDPCVKRYMMYHQGCFAGGTV 170
CHS_Ph (Q43040)   QKAIKEWGQPKSKITHLVFCTTSGVDMPGCDYQLTKLLGLRPSVKRLMMYQQGCFAGGTV 170
                   ***:***:* *:****:***:.** :*. *::   ****   *::  :* ***:*****

PCS_Aa (Q58VP7)   MRYAKDLAENNRGARVLVVCAELTIMMLRAPNETHLDNAIGISLFGDGAAALIIGSDPII 240
STS_Ah (K7XD27)   LRLAKDLAENNKDARVLIVCSENTAVTFRGPSETDMDSLVGQALFADGAAAIIIGSDPVP 230
CHS_Ph (Q43040)   LRLRKDLAENNKGARVLVVCSEITAVTFRGPNDTHLDSLVGQALFGDGAGAIIIGSDPIP 230
                  :*  *******:.****:**:* * : :*.*.:*.:*. :* :**.***.*:******:

PCS_Aa (Q58VP7)   GVEKPMFEIVCTKQTVIPNTEDVIHLHLRETGMMFYLSKGSPMTISNNVEACLIDVFKSV 300
STS_Ah (K7XD27)   EVEKPLFEIVSTDQKLVPGSHGAIGGLLREVGLTFYLNKSVPDIISQNINDALSKAFDPL 290
CHS_Ph (Q43040)   GVERPLFELVSAAQTLLPDSHGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLEEAFKPL 290
                   **:*:**:*.: *.::*.:...*   ***.*: *:* *. *  **:*:: .* ..*. :

PCS_Aa (Q58VP7)   GITPPEDWNSLFWIPHPGGRAILDQVEAKLKLRPEKFRAARTVLWDYGNMVSASVGYILD 360
STS_Ah (K7XD27)   GI---SDYNSIFWIAHPGGRAILDQVEQKVNLKPEKMKATRDVLSNYGNMSSACVFFIMD 347
CHS_Ph (Q43040)   GI---SDWNSLFWIAHPGGPAILDQVEIKLGLKPEKLKATRNVLSDYGNMSSACVLFILD 347
                  **   .*:**:*** **** ******* *: *:***::*:* ** :**** **.* :*:*

PCS_Aa (Q58VP7)   EMRRKSAAKGLETYGEGLEWGVLLGFGPGITVETILLHSLPLM 403
STS_Ah (K7XD27)   LMRKKSLEEGLKTTGEGLDWGVLFGFGPGLTIETVVLRSVAI- 389
CHS_Ph (Q43040)   EMRKASAKEGLGTTGEGLEWGVLFGFGPGLTVETVVLHSVAT- 389
                   **: *  :** * ****:****:*****:*:**::*:*:
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Figure S1: Alignment of the amino acid sequences of the pentaketide chromone synthase from *Aloe arborescens* (PCS_{Aa}, UniProt ID Q58VP7), the stilbene synthase from *Arachis hypogea* (STS_{Ah}, UniProt ID K7XD27) and the chalcone synthase from *Petunia x hybrida* (CHS_{Ph}, UniProt ID Q43040). The ten N-terminal amino acids of PCS_{Aa} which were deleted in the course of this study are highlighted by the orange box.

Additional file 1

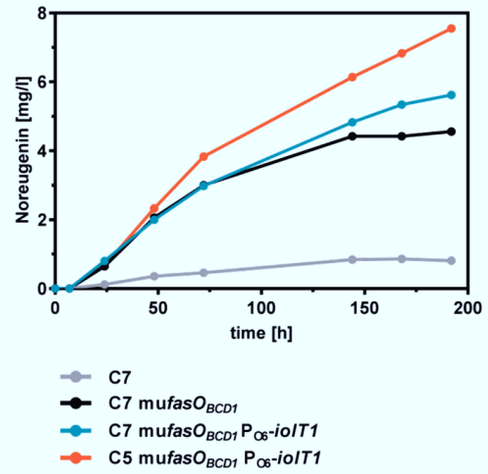

Figure S2: Microbial production of noreugenin from glucose during shake flask cultivations with engineered *C. glutamicum* strains harboring pMKEx2-*pcs*$_{AaCg}$-short during extended cultivation times. The data represent values from single replicate experiments.

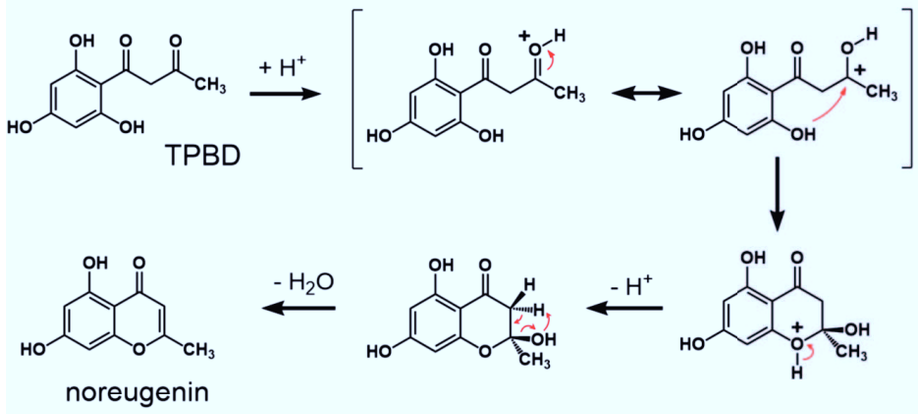

Figure S3: Proposed mechanism for the HCl-catalyzed cyclization of the intermediate 1-(2,4,6-trihydroxyphenyl)butane-1,3-dione (TPBD) yielding noreugenin.

2.4 *C. glutamicum* is a promising host for the synthesis of type I polyketides

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Microbial synthesis of the type I polyketide 6-methylsalicylate with *Corynebacterium glutamicum*

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Abstract
Type I polyketide synthases (PKSs) are large multi-domain proteins converting simple acyl-CoA thioesters such as acetyl-CoA and malonyl-CoA to a large diversity of biotechnologically interesting molecules. Such multi-step reaction cascades are of particular interest for applications in engineered microbial cell factories, as the introduction of a single protein with many enzymatic activities does not require balancing of several individual enzymatic activities. However, functional introduction of type I PKSs into heterologous hosts is very challenging as the large polypeptide chains often do not fold properly. In addition, PKS usually require post-translational activation by dedicated 4′-phosphopantetheinyl transferases (PPTases). Here, we introduce an engineered *Corynebacterium glutamicum* strain as a novel microbial cell factory for type I PKS-derived products. Suitability of *C. glutamicum* for polyketide synthesis could be demonstrated by the functional introduction of the 6-methylsalicylic acid synthase ChlB1 from *Streptomyces antibioticus*. Challenges related to protein folding could be overcome by translation fusion of $ChlB1_{Sa}$ to the C-terminus of the maltose-binding protein MalE from *Escherichia coli*. Surprisingly, $ChlB1_{Sa}$ was also active in the absence of a heterologous PPTase, which finally led to the discovery that the endogenous PPTase $PptA_{Cg}$ of *C. glutamicum* can also activate $ChlB1_{Sa}$. The best strain, engineered to provide increased levels of acetyl-CoA and malonyl-CoA, accumulated up to 41 mg/L (0.27 mM) 6-methylsalicylic acid within 48 h of cultivation. Further experiments showed that $PptA_{Cg}$ of *C. glutamicum* can also activate nonribosomal peptide synthetases (NRPSs), rendering *C. glutamicum* a promising microbial cell factory for the production of several fine chemicals and medicinal drugs.

Keywords Polyketide · 6-methylsalicylate · Type I polyketide synthase · Phosphopantetheinyl transferase · *Corynebacterium glutamicum* · Malonyl-CoA

Introduction

In microorganisms and plants, polyketide synthases (PKSs) synthesize a broad range of chemically diverse secondary metabolites, including aromatics, macrolides, polyenes, and polyethers (Shen 2003). Due to their potent bioactivities, e.g., as antibiotics or antioxidants, polyketide-derived products are of interest for the development of pharmaceuticals or nutraceuticals (Kallscheuer et al. 2019; Shi et al. 2018; Yin et al. 2015). In bacteria, PKSs are typically involved in the synthesis of antibiotics or secondary lipids, whereas in plants, PKSs are essential for the production of polyphenols such as stilbenes and flavonoids (Schröder 1997).

As a common feature, PKSs catalyze the condensation of coenzyme A (CoA)-activated starter units and, in most cases, consume 3–12 malonyl-CoA molecules as building blocks during repetitive chain elongation reactions (Abe and Morita 2010; Hertweck 2009; Ray and Moore 2016; Shen 2003). This assembly process is often accompanied by defined β-keto processing reactions. The resulting intermediate is subsequently converted to the final product by an intrinsic cyclase activity of the PKS or, alternatively, subject to hydrolysis or

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lactonization. Once released from the PKS, the polyketide can be further modified by decorating enzymes such as methyltransferases or glycosyltransferases or it serves as a starter molecule for the synthesis of more complex compounds (Schmidlin et al. 2008; Wang et al. 2014). The architecture of PKSs is reflected by the presence of different catalytically active domains responsible for substrate selection, chain elongation, reductive processing, and product release. Of particular interest are iterative type I PKSs, in which the catalytically active domains are combined in a single polypeptide chain. These domains bear acyltransferase (AT), ketosynthase (KS), ketoreductase (KR), dehydratase (DH), enoylreductase (ER), and thioesterase (TE) activities (Cox 2007; Kage et al. 2015). During polyketide synthesis, the growing carbon chain is bound to an acyl carrier protein (ACP) domain, which must have undergone a post-translational modification to provide an active thiol group for the required thioesterification (Hertweck 2009). This activation step is catalyzed by discrete 4′-phosphopantetheinyl transferases (PPTases), which transfer a 4′-phosphopantetheine residue from CoA to a conserved serine residue in the ACP domain, thereby converting the inactive *apo* form of the PKS to the active *holo*-PKS (Lambalot et al. 1996).

In recent years, an increasing interest in using PKSs for the production of high value compounds in heterologous microorganisms arose. However, functional expression of genes coding for type I PKSs in such hosts is challenging (Yuzawa et al. 2012). In particular, correct folding of PKS enzymes characterized by a typical length ranging from 1500 to 4000 amino acids and post-translational phosphopantetheinylation were identified as key issues. Nevertheless, functional introduction of several PKSs was achieved, e.g., in *Escherichia coli* and *Streptomyces* spp., by using strategies for improved protein folding and by co-expression of PPTase-encoding genes (Baltz 2010; Liu et al. 2015; Ugai et al. 2015).

Since decades, *Corynebacterium glutamicum* is used at industrial scale for the production of amino acids, in particular of L-glutamate and L-lysine (Eggeling and Bott 2015), but this bacterium was also engineered for the synthesis of other biotechnologically interesting compounds, e.g., alcohols, diamines, dicarboxylic acids, aromatic compounds, and secondary metabolites (Heider et al. 2014; Kallscheuer and Marienhagen 2018; Kallscheuer et al. 2016a; Litsanov et al. 2012; Nguyen et al. 2015; Vogt et al. 2016). In previous studies, we already established the functional introduction of plant-derived type III PKSs with a typically length of 300–400 amino acids into *C. glutamicum*, which enabled plant polyphenol synthesis with this bacterium (Kallscheuer et al. 2016b; Milke et al. 2019b).

In this study, we introduce *C. glutamicum* as a promising microbial host for polyketide production as we could functionally introduce the type I PKS 6-methylsalicylic acid synthase ChlB1 from *Streptomyces antibioticus* ($ChlB1_{Sa}$, 1756 aa, 186 kDa, UniProt ID Q0R4P8) into this bacterium (Jia et al. 2006; Shao et al. 2006). $ChlB1_{Sa}$ catalyzes the conversion of the starter unit acetyl-CoA and three molecules of malonyl-CoA into the aromatic compound 6-methylsalicylic acid (6-MSA) (Fig. 1) (Parascandolo et al. 2016). The latter is an important building block in the biosynthesis of several antibiotics, including chlorothricin, maduropeptin, pactamycin, and polyketomycin (Daum et al. 2009; Ito et al. 2009; Jia et al. 2006; Van Lanen et al. 2007).

Material and methods

Bacterial strains, plasmids, media, and growth conditions

All bacterial strains and plasmids used in this study and their relevant characteristics are listed in Table 1. *C. glutamicum* was routinely cultivated aerobically at 30 °C in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, USA) or in defined CGXII medium with 4% (w/v) glucose as sole carbon and energy source (Keilhauer et al. 1993). *E. coli* DH5α used for plasmid constructions was cultivated in LB medium (Bertani 1951) at 37 °C. For maintenance of plasmids, kanamycin (50 μg/mL for *E. coli* or 25 μg/mL for *C. glutamicum*), chloramphenicol (25 μg/mL for *E. coli*), or spectinomycin (100 μg/mL 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}). *C. glutamicum* was grown for 6–8 h in test tubes with 5 mL BHI medium on a rotary shaker at 170 rpm (first preculture) and was subsequently inoculated into 50 mL defined CGXII medium with 4% (w/v) glucose in 500-mL baffled Erlenmeyer flasks (second preculture). Cell suspensions were cultivated overnight on a rotary shaker at 130 rpm. The main culture was inoculated to an OD_{600} of 5 in defined CGXII medium with 4% (w/v) glucose, and heterologous gene expression was induced 1 h after inoculation using the indicated amount of isopropyl β-D-1-thiogalactopyranoside (IPTG).

For comparison of different heterologously expressed inactive PPTase gene variants, microbioreactor cultivations were performed. Multiple 48-well Flowerplates containing 1000 μL CGXII medium with 4% (w/v) glucose, 50 mM $MgSO_4$, and 1 mM IPTG were inoculated from a preculture to an OD_{600} of 5 and cultivated at 30 °C, 1200 rpm, and a

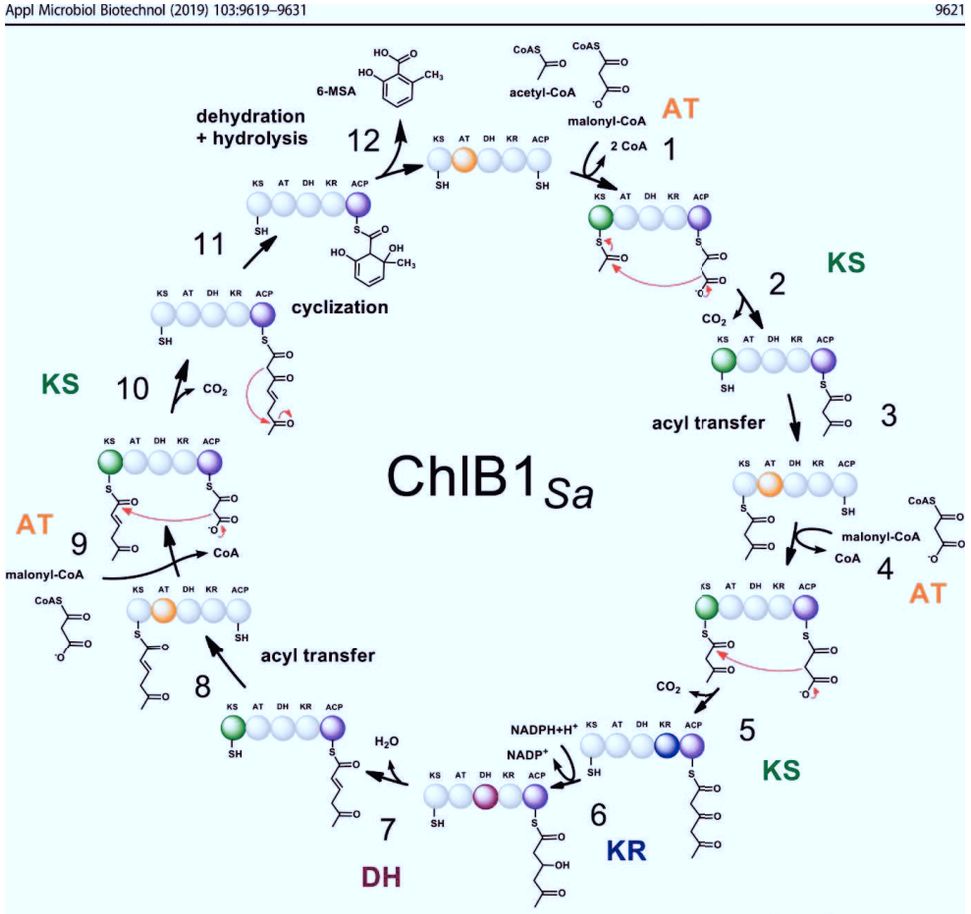

Fig. 1 6-Methylsalicylic acid formation by the type I PKS ChlB1$_{Sa}$. The depicted steps follow a previously established reaction sequence. 6-Methylsalicylic acid is produced from acetyl-CoA and three molecules of malonyl-CoA. Acetyl-CoA as starter unit is consumed in step 1, whereas three malonyl-CoA molecules are consumed in steps 1, 4, and 9, respectively. The required domains involved in the respective reaction steps are highlighted by different colors. ACP: acyl carrier protein domain, AT acyltransferase domain, KS ketosynthase domain, KR ketoreductase domain, DH dehydratase domain, 6-MSA 6-methylsalicylic acid

humidity of 85% in a BioLector microbioreactor (m2p-labs GmbH, Baesweiler, Germany). At indicated time points, cells of individual wells were harvested for determination of cell growth and 6-MSA analysis by LC-MS.

Construction of plasmids and strains

Plasmids and strains used in this study are listed in Table 1. Standard protocols of molecular cloning, such as PCR, DNA restriction, and ligation (Sambrook and Russell 2001), were carried out for recombinant DNA work. Techniques specific for *C. glutamicum*, e.g., electroporation for transformation of strains, were performed as described previously (Eggeling and Bott 2005). Synthetic genes were obtained from Thermo Fisher Scientific, formerly GeneArt (Darmstadt, Germany). Genes were amplified by PCR using oligonucleotides with unique restriction sites for cloning (Table 2). All constructed plasmids were finally verified by DNA sequencing at Eurofins Genomics (Ebersberg, Germany). The PPTase gene *entD* in *E. coli* BW25113 was inactivated by λ/Red recombineering (Datsenko and Wanner 2000). For this purpose, the ampicillin resistance gene from pUC19 was furnished with *entD*

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli strains		
DH5α	F– Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* (rK–, mK+) *phoA supE44* λ– *thi-1 gyrA96 relA1*	Invitrogen (Karlsruhe, Germany)
BW25113	Δ(*araD-araB*)*567* Δ(*rhaD-rhaB*)*568* Δ*lacZ4787* (::rrnB-3) *hsdR514 rph-1*	Grenier et al. (2014)
BW25113 *entD*::*amp*^{r}	*entD* insertional mutant of BW25113	This study
BL21(DE3)	F$^-$ *ompT hsdS*B(r_B^- m_B^-) *gal dcm* (DE3)	Merck KGaA (Darmstadt, Germany)
C. glutamicum strains		
DelAro^4 C5 mu*fasO*$_{BCD1}$	derivative of prophage-free *C. glutamicum* MB001(DE3) with the following modifications: Δcg0344-cg0347, Δcg0502, Δcg1226, Δcg2625–40 (DelAro^4); exchange of the native promoter of the citrate synthase gene *gltA* by the *dapA* promoter variant C5; elimination of the fatty acid biosynthesis regulator (FasR) operator sites in *accBC* and *accD1* encoding the heterodimeric acetyl-CoA carboxylase (mu*fasO*$_{BCD1}$)	Milke et al. (2019b)
S. cerevisiae strains		
CEN.PK 2-1C	*MATa leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2–8*^{c} *SUC2*	Entian and Kötter (2007)
Plasmids		
pMKEx2	*kan*^{r}; *E. coli-C. glutamicum* shuttle vector (*lacI*, P_{T7}, lacO1, pIIM1519 ori$_{Cg}$; pACYC177 ori$_{Ec}$)	Kortmann et al. (2015)
pMKEx2_ *malE*$_{Ec}$-*chlB1*$_{Sa}$	pMKEx2 derivative for expression of a codon-optimized gene coding for the 6-methylsalicylate synthase ChlB1 from *S. antibioticus* (GenBank: MN342166)	This study
pMKEx2_ *malE*$_{Ec}$-*chlB1*$_{Sa}$	pMKEx2 derivative harboring the genes *malE* from *E. coli* (native gene) and *chlB1*$_{Sa}$ for obtaining fusion of the maltose-binding protein MalE to the N-terminus of ChlB1$_{Sa}$ (GenBank: MN342167)	This study
pEKEx3	*spec*^{r}; *E. coli-C. glutamicum* shuttle vector (*lacI*, P_{tac}, lacO1, pBL1ori$_{Cg}$; pUCori$_{Ec}$)	Gande et al. (2007)
pEKEx3_*svp*$_{Sv}$	pEKEx3 derivative for expression of the PPTase gene *svp* from *S. verticillus* (GenBank: AF210311)	This study
pEKEx3_*sfp*$_{Bs}$	pEKEx3 derivative for expression of the PPTase gene *sfp* from *B. subtilis* (GenBank: X63158)	This study
pEKEx3_ *sfp*$_{Bs}$-D107N	pEKEx3 derivative for expression of the gene variant *sfp*-D107N encoding the PPTase from *B. subtilis*	This study
pEKEx3_ *npgA*$_{An}$	pEKEx3 derivative for expression of the PPTase gene *npgA* from *A. nidulans* (GenBank: AF198117)	This study
pEKEx3_ *npgA*$_{An}$--D148N	pEKEx3 derivative for expression of the gene variant *npgA*-D148N encoding the PPTase from *A. nidulans*	This study
pEKEx3_ *pptA*$_{Cg}$	pEKEx3 derivative for overexpression of the endogenous PPTase gene *pptA* (GenBank: BAB99373)	This study
pIJ790	*cm*^{r}, Red recombination vector (*gam, bet, exo*, *araC*, pSC101^{ts} ori$_{Ec}$)	Gust et al. (2003)

kan^r kanamycin resistance, *spec*^{r} spectinomycin resistance, *cm*^{r} chloramphenicol resistance

homologous arms by PCR using oligonucleotides *entD*-s and *entD*-as. The 1.3-kb sized amplicon was introduced into *E. coli* BW25113/pIJ790 by electroporation after induction of λ *red* genes following a previously reported procedure (Kreutzer et al. 2011). The resulting transformants were incubated overnight at 37 °C on LB agar containing ampicillin (50 μg/mL). To identify recombinants with the desired mutation, PCRs using

Table 2 Oligonucleotides used in this study

Oligonucleotide	Sequence (5′-3′)	Restriction site
chlB1Sa-s	TTCGCTCTTCAAAGCTGCTTAAGGAGGCTATCTA TGCAGTCGCACGACGTGG	*Sap*I
chlB1Sa-as	TACGCTCTTCTTCGTTAGGCGGTGGCGGCCGGCT CGGCC	*Sap*I
entD-s	TAAGCGTAGCGCATCAGGCAGTTTTGCGTTTGTCATCA GTCTCGAATATGCATTCAAATATGTATCCGCTC	–
entD-as	TGCGGGCGAATCGTACCAGATGTTGTCAATTAATCGTG TTGGCACAGCGTAGAGTTGGTAGCTCTTGATC	–
entDSQ-s	CGGCCTACAAAAATCTTGCC	–
entDSQ-as	AGGCGTCGCATTCTTCAGAT	–
malEEc-s	TTCGCTCTTCAAAGTATACCATGGTAAGGAGGTT CAGCATGAAAACTGAAGAAGGTAAACTGGTAATCTG GATTAACGGCGATAAAGGCTATAAC	*Sap*I
malEEc-as	TACGCTCTTCTTCGGAGCCGGAACCGGAAGAGGA AGTCTG	*Sap*I
svpSv-s	CTCCTGCAGAAGGAGGCTGTCTATGATCGCCGCCC TCCTGC	*Pst*I
svpSv-as	CACGGATCCTTACGGGACGGCGGTCCGGT	*Bam*HI
sfpBs-s	CTCGGATCCAAGGAGGCTGTCTATGAAGATTTACG GAATTTATATGGACCGCCCGCTTTC	*Bam*HI
sfpBs-as	CACGAATTCTTATAAAAGCTCTTCGTACGAGACCA TTGTGATATCCTC	*Eco*RI
sfpBs-D107N-up-s	CCTGCAGGTCGACTCTAGAGGATCCAAGG AGGCTGTCTATGAAGATTTAC	*Bam*HI
sfpBs-D107N-up-as	TTTTTTCGATGTTTATGCCGATCGGCTGTGAATC	–
sfpBs-D107N-down-s	CGGCATAAACATCGAAAAAACGAAACCG	–
sfpBs-D107N-down-as	CTGTAAAACGACGGCCAGTGAATTCTTATAAAAGCTCTT CGTACG	*Eco*RI
npgAAn-s	CCTGCAGGTCGACTCTAGAGAAGGAGGCTGTCTATGGT GCAAGACACATCAAG	–
npgAAn-as	ATTCGAGCTCGGTACCCGGGTTAGGATAGGCAATTACA CAC	–
npgAAn-D148N-up-s	GCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGAA GGAG	*Sbf*I
npgAAn-D148N-up-as	ACGTAATGTTAATTCCGACTTCGGGTTTG	–
npgAAn-D148N-down-s	AGTCGGAATTAACATTACGTGCGTAAACGAG	–
npgAAn-D148N-down-as	AGTGAATTCGAGCTCGGTACCCGGGTTAGGATAGGCAAT TACAC	*Xma*I
pptACg-s	CTCGGATCCAAGGAGGCTGTCTATGCTGGATGAGT CTTTGTTTCCAAATTCGGCAAAG	*Bam*HI
pptACg-as	CACGAATTCTCAAGTCACTGCAGTCGCAGCTATGA CATAACCATC	*Eco*RI

Relevant restriction sites are underlined; *Sap*I cuts outside of its recognition site; the obtained 5′-overhangs after *Sap*I cleavage used for Electra Cloning are shown in bold. Due to the presence of internal restriction sites, the $npgA_{An}$ gene was cloned using Gibson assembly. Mutant variants $npgA_{An}$-D148N and sfp_{Bs}-D107N were also constructed using Gibson assembly

oligonucleotides *entD*SQ-s and *entD*SQ-as were conducted. The mutation in *E. coli* BW25113 *entD*::*amp*^{r} was confirmed by DNA sequencing.

LC-MS analysis for quantification of 6-MSA in culture supernatants

6-MSA was quantified in culture supernatants by LC-MS using an Agilent ultra-high-performance LC 1290 Infinity System coupled to a 6130 Quadrupole LC-MS System (Waldbronn, Germany). LC separation was carried out using a Kinetex 1.7 µm C_{18} 100-Å pore size column (50 mm by 2.1 mm (internal diameter)) (Phenomenex, Torrance, CA, USA) at 50 °C. For elution, 0.1% (v/v) acetic acid (solvent A) and acetonitrile supplemented with 0.1% (v/v) acetic acid (solvent B) were applied as the mobile phases at a flow rate of 0.3 mL/min. A gradient was used, where the amount of solvent B was increased stepwise: minutes 0 to 6, 5 to 30%;

minutes 6 to 7, 30 to 50%; minutes 7 to 8, 50 to 100%; and minutes 8 to 8.5, 100 to 5%. The mass spectrometer was operated in the negative electrospray ionization (ESI) mode, and data acquisition was performed in selected-ion-monitoring (SIM) mode. Authentic metabolite standards were purchased from Thermo Fisher Scientific, formerly Acros Chemicals (Geel, Belgium). Area values for $[M\text{-}H]^-$ mass signals were linear up to metabolite concentrations of at least 250 mg/L. Benzoic acid (final concentration 100 mg/L) was used as internal standard. Calibration curves were calculated based on analyte/internal standard ratios for the obtained area values.

PPTase complementation in an *entD*-deficient *E. coli* variant

The promiscuity of $PptA_{Cg}$ was tested through the restoration of enterobactin biosynthesis by complementing the inactivated *entD* gene. For this, *E. coli* BW25113 *entD*::*amp*^{r} was transformed either with the empty vector pEKEx3 or with the vector pEKEx3_$pptA_{Cg}$ harboring the $pptA_{Cg}$ gene from *C. glutamicum*. For analysis of enterobactin production, the two resulting *E. coli* strains were cultivated in 50 mL M9 mineral medium without iron supplementation at 37 °C to an OD_{600} of 0.6. At this time, expression of the $pptA_{Cg}$ gene was induced with 1 mM IPTG. Afterwards, the incubation was continued overnight at 37 °C. To verify the production of enterobactin, the cultures were acidified with hydrochloric acid (pH 2) and exhaustively extracted with ethyl acetate. After removal of the organic solvent, the crude extracts were dissolved in methanol. The extracts were analyzed by HPLC-MS using an Agilent 1260 Infinity System (Agilent, Waldbronn, Germany) coupled to a Bruker Compact ESI-Q-TOF mass spectrometer (Bruker, Bremen, Germany) equipped with a Nucleoshell RP-C_{18} column (100 mm × 2 mm, 2.7 μm, Macherey Nagel, Düren, Germany). A linear gradient of acetonitrile in water with 0.1% (v/v) formic acid (from 5% to 98% acetonitrile within 10 min; flow rate, 0.4 mL/min) was used for metabolic profiling. UV chromatograms were recorded at 316 nm.

6-MSA toxicity tests

With the aim to analyze cytotoxic effects of 6-MSA on growth of *C. glutamicum* and *E. coli*, both organisms were cultivated in the presence of increasing 6-MSA concentrations (0, 5, 10, 20, 30, 40, 50 mM), which was dissolved in DMSO. Forty-eight-well Flowerplates (m2p-labs GmbH, Baesweiler, Germany) containing 1000 μl BHI medium were inoculated from an overnight preculture to an OD_{600} of 1 and cultivated at 30 °C (*C. glutamicum*) or 37 °C (*E. coli*), 1200 rpm, and a humidity of 85% in a BioLector microbioreactor (m2p-labs GmbH, Baesweiler, Germany). Formation of biomass was followed by measuring the backscattered light intensity at a wavelength of 620 nm (signal gain factor 15).

For 6-MSA toxicity tests with yeast, *Saccharomyces cerevisiae* CEN.PK 2-1C was grown overnight in 50 mL HPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L D-glucose) in 250-mL shake flasks at 180 rpm and 30 °C. This preculture was used to inoculate 20 mL HPD + 5% (v/v) ethanol in 100-mL shake flasks with baffles, supplemented with different concentrations of 6-MSA (0, 5, 10, 20, 30, 40, 50 mM). The 6-MSA cultures were incubated at 180 rpm and 30 °C for 24 h. All experiments were carried out in two biological replicates. The maximum growth rate was determined during the exponential growth phase for all experiments. For an easier comparison, growth rates, relative to cultivations in the absence of 6-MSA, were calculated for each organism.

Results

Functional introduction of $chlB1_{Sa}$ into *C. glutamicum* enables 6-MSA synthesis

In the course of developing a *C. glutamicum* platform strain for plant polyphenol synthesis, the central carbon metabolism was engineered towards increased availability of the PKS substrates acetyl-CoA and malonyl-CoA, and the entire catabolic network for aromatic compounds was eliminated to avoid any product degradation (Kallscheuer et al. 2016b; Milke et al. 2019a; Milke et al. 2019b). Against this background, *C. glutamicum* DelAro^4 C5 mu$fasO_{BCD1}$, characterized by (I) deletion of 21 genes involved in the degradation of various aromatic compounds, (II) reduction of the citrate synthase activity and (III) deregulation of genes encoding the acetyl-CoA carboxylase, was selected as parent strain for further experiments (Fig. 2). Initially, it was tested if *C. glutamicum* DelAro^4 C5 mu$fasO_{BCD1}$ can metabolize supplemented 6-MSA. However, cultivations in the presence of 200 mg/L (1.3 mM) 6-MSA and analysis of culture supernatants by LC-MS confirmed that *C. glutamicum* DelAro^4 C5 mu$fasO_{BCD1}$ is neither capable of catabolizing nor unspecifically modifying this compound (data not shown).

For heterologous expression of the 6-MSA synthase gene from *S. antibioticus*, the codon-optimized $chlB1_{Sa}$ gene was cloned into the expression plasmid pMKEx2, which allows for an IPTG-inducible gene expression under the control of the strong T7 promoter (Kortmann et al. 2015). In addition, a gene coding for the broad-spectrum PPTase Svp from *Streptomyces verticillus* (Svp_{Sv}, UniProt ID Q9F0Q6) was co-expressed from the plasmid pEKEx3_svp_{Sv} for phosphopantetheinylation of $ChlB1_{Sa}$ (Sánchez et al. 2001). Unfortunately, the constructed strain *C. glutamicum* DelAro^4

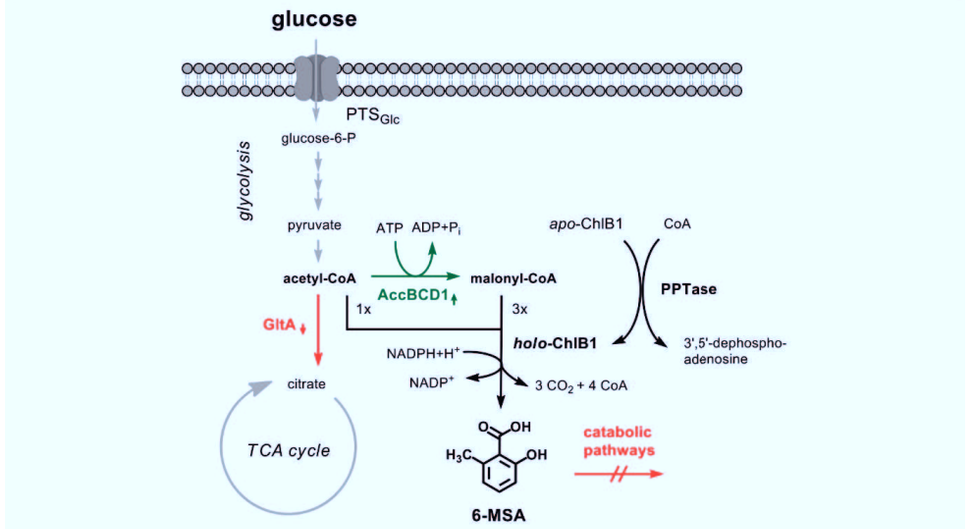

Fig. 2 Metabolic engineering of *C. glutamicum* for 6-MSA production. The engineered central carbon metabolism of *C. glutamicum* and the ChlB1 and PPTase-catalyzed reactions are shown. During the construction of *C. glutamicum* DelAro^4 C5 mu*fasO*$_{BCD1}$ used for 6-MSA production, the following genomic modifications were introduced: (I) Replacement of the native *gltA* promoter by the constitutive *dapA* promoter variant C5 for reducing the overall citrate synthase (GltA) activity to 5% (compared to the GltA activity in the *C. glutamicum* wild type); (II) mutation of regulator binding sites for the transcriptional repressor FasR upstream of the open reading frames of *accBC* and *accD1* (coding for the heterodimeric acetyl-CoA carboxylase AccBCD1) to abolish gene repression; (III) deletion of genes coding for enzymes involved in the degradation of aromatic compounds to avoid any potential 6-MSA consumption by *C. glutamicum*. PTS_{Glc} glucose-specific phosphotransferase system, PPTase 4′-phosphopantetheinyl transferase, TCA cycle tricarboxylic acid cycle

C5 mu*fasO*$_{BCD1}$ pMKEx2_*chlB1*$_{Sa}$ pEKEx3_*svp*$_{Sv}$ failed to produce any LC-MS-detectable amounts of 6-MSA when cultivated in defined CGXII medium with 4% (w/v) glucose and two different IPTG concentrations (20 μM or 1 mM). However, problems related to folding of "challenging" proteins such as ChlB1$_{Sa}$ were not unexpected when considering its protein size of 186 kDa. Thus, we decided to test translational fusion of ChlB1$_{Sa}$ to the C-terminus of the maltose binding protein MalE of *E. coli*. Although being even larger than ChlB1$_{Sa}$ (1756 aa, 186 kDa), the MalE$_{Ec}$-ChlB1$_{Sa}$ fusion protein (2139 aa, 228 kDa) appeared to be active in *C. glutamicum* as accumulation of 2 mg/L (0.013 mM) and 6 mg/L 6-MSA (0.039 mM) after induction with 20 μM or 1 mM IPTG, respectively, could be detected (Fig. 3a, b). Neither under non-inducing conditions (no IPTG) nor in case of cultivation of a control strain harboring only the two empty plasmids, 6-MSA synthesis could be observed.

Formation of 6-MSA suggested that ChlB1$_{Sa}$ can be successfully activated by the PPTase Svp$_{Sv}$. However, the *svp*$_{Sv}$-expressing *C. glutamicum* strain only reached a drastically reduced final biomass (OD_{600} = 30–33) during the cultivation and 6-MSA production experiments. Considering that standard CGXII medium contains only 1 mM $MgSO_4$ as sole source of Mg^{2+}-ions required as metal cofactor for PPTase activity, we assumed that heterologous expression of *svp*$_{Sv}$ leads to rapid depletion of Mg^{2+} ions, which in turn would limit overall cell growth. Hence, different $MgSO_4$ concentrations ranging from 15 to 200 mM were tested in cultivations of the engineered *C. glutamicum* strain (Fig. 4). These experiments revealed that limited Mg^{2+} availability was indeed the reason for the observed reduced growth phenotype since all cultures with $MgSO_4$ concentrations > 1 mM reached a final OD_{600} between 50 and 55, typical for shake flask cultivations of *C. glutamicum*. In this context, it was not surprising that low Mg^{2+} concentrations also limited 6-MSA synthesis as cultivations with 15 mM $MgSO_4$ doubled product titers, reaching 14 mg/L (0.09 mM) 6-MSA. The highest product titer of 20 mg/L (0.13 mM) at this stage was determined in cultivations with supplementation of 50 mM $MgSO_4$, whereas supplementation of 100 mM $MgSO_4$ and above had a negative effect on product formation (Fig. 4). All subsequent cultivations for 6-MSA production were thus performed in defined CGXII medium containing 50 mM $MgSO_4$.

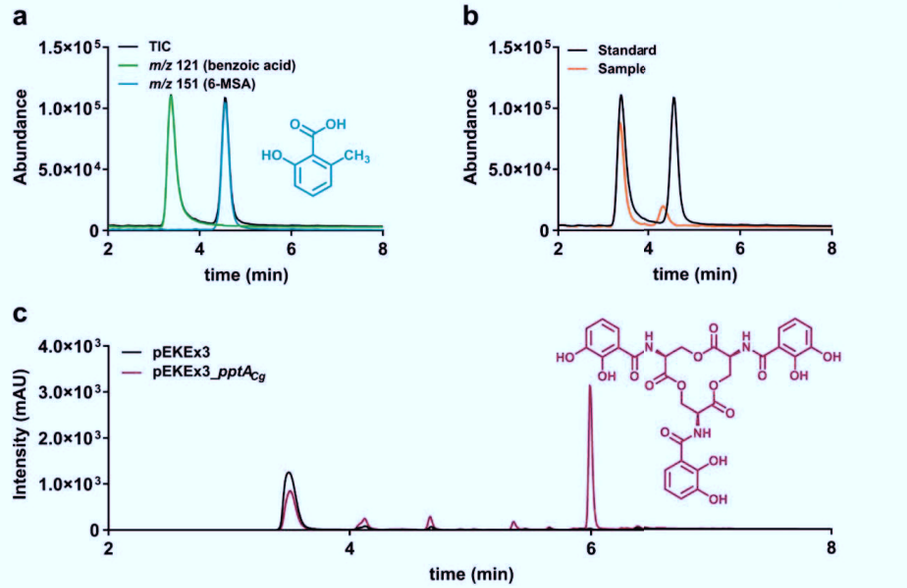

Fig. 3 Analysis of 6-MSA production with *C. glutamicum* and enterobactin synthesis in *E. coli*. **a** Chromatogram of an authentic 6-MSA/benzoic acid standard, both as total ion current and as individual *m/z* ratios for 6-MSA and benzoic acid, respectively. **b** Total ion current chromatograms of a typical sample obtained from the cultivation of *C. glutamicum* DelAro^4 C5 mu*fasO*$_{BCD1}$ pMKEx2_*malE*$_{Ec}$-*chlB1*$_{Sa}$ pEKEx3_*svp*$_{Sv}$ producing 6-MSA and an authentic 6-MSA standard for comparison. A slight shift in the retention time of the 6-MSA peak in culture supernatants of *C. glutamicum* in comparison to the metabolite standard was observed. Integrity of the shifted 6-MSA peak was verified by addition of 6-MSA to the culture supernatant (spiking). **c** UV-chromatograms of culture supernatants from *E. coli* BW25113 *entD*::*amp*^{r} harboring either an empty plasmid (pEKEx3) or a plasmid featuring *pptA*$_{Cg}$ from *C. glutamicum* (pEKEx3_*pptA*$_{Cg}$). Both chromatograms were recorded at 316 nm. The enterobactin peak at a retention time of 6 min was identified by ESI-MS analysis

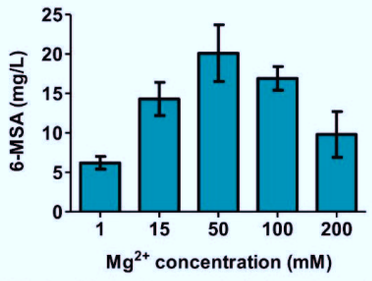

Fig. 4 Variation of the Mg^{2+} concentration in the culture medium for optimizing PPTase activity in *C. glutamicum*. $MgSO_4$ concentrations ranging from 1 to 200 mM were tested during the microbial production of 6-MSA in defined CGXII medium with 4% glucose and 1 mM IPTG using *C. glutamicum* DelAro^4 C5 mu*fasO*$_{BCD1}$ pMKEx2_*chlB1*$_{Sa}$ pEKEx3_*svp*$_{Sv}$. Notably, the standard $MgSO_4$ concentration in defined CGXII medium is 1 mM (first column). Data represent average values and standard deviations of three biological replicates

An endogenous PPTase of *C. glutamicum* can activate ChlB1$_{Sa}$

In microbial host systems engineered for polyketide synthesis involving type I PKSs, PPTase-mediated PKS activation is essential for product formation, but not every PPTase is capable of activating every PKS (Cox et al. 1997; Shen et al. 1992). The PPTase Svp$_{Sv}$ from *S. verticillus* was initially selected due to its well-described ability to activate a broad range of different type I PKSs and the close relationship of *S. verticillus* to *S. antibioticus*, from which ChlB1$_{Sa}$ originates (Sánchez et al. 2001). With the aim to identify an even more suitable PPTase for ChlB1$_{Sa}$ activation in *C. glutamicum*, we evaluated the performance of other broad-spectrum PPTases such as Sfp$_{Bs}$ (UniProt ID P39135) from *Bacillus subtilis* and NpgA$_{An}$ (UniProt ID G5EB87) from *Aspergillus nidulans* (Oberegger et al. 2003; Quadri et al. 1998). Interestingly, *C. glutamicum* DelAro^4 C5 mu*fasO*$_{BCD1}$ pMKEx2_*malE*$_{Ec}$-*chlB1*$_{Sa}$ pEKEx3_*npgA*$_{An}$

expressing the gene for the PPTase from *A. nidulans* did not grow at all and thus also failed to produce 6-MSA. At this stage, we could only speculate that the $npgA_{An}$ gene product is toxic for *C. glutamicum*. In contrast, coexpression of sfp_{Bs} enabled accumulation of 28 mg/L (0.18 mM) 6-MSA. To our surprise, the highest product titer was obtained in cultivations of a constructed *C. glutamicum* DelAro^4 C5 mu$fasO_{BCD1}$ variant without any heterologous expression of a PPTase-encoding gene, which was originally supposed to serve as negative control. This strain, *C. glutamicum* DelAro^4 C5 mu$fasO_{BCD1}$ pMKEx2_$malE_{Ec}$-$chlB1_{Sa}$, accumulated 41 mg/L (0.27 mM) 6-MSA, indicating that *C. glutamicum* harbors an endogenous enzyme capable of activating $ChlB1_{Sa}$. At this point, we speculated that this endogenous PPTase activity is present in all constructed strains and that lower 6-MSA concentrations determined in cultivations of *C. glutamicum* strains expressing heterologous PPTase genes are due to the metabolic burden of the plasmid-based expression of additional genes from a second plasmid also requiring supplementation of an additional antibiotic. To elucidate this, gene variants of sfpBs and npgAAn, both encoding for inactive PPTases, were designed and constructed by Gibson assembly (Quadri et al. 1998; Vickery et al. 2014). Both genes carry an asparagine instead of a highly conserved aspartate residue close to the essential Mg2+ binding site. The resulting strains expressing either npgAAn-D148N or sfpBs-D107N as well as strains expressing their wild-type counterparts and the best 6-MSA producer strain only carrying the empty pEKEx3 vector, were cultivated using the BioLector microbioreactor. Over the course of cultivation, cell growth and 6-MSA synthesis were followed (Supp. Fig. 2). In general, 6-MSA titers are reduced in microbioreactor cultivations compared to baffled flasks, but ratios of titers between the respective strains are similar and the control strain only harboring the empty pEKEx3 vector still accumulated the most 6-MSA. As expected from previous experiments, the strain expressing $npgA_{An}$ neither grew in the BioLector cultivation nor produced any 6-MSA. Interestingly, inactivation of $NpgA_{An}$ restored both microbial growth and 6-MSA synthesis supporting the speculation that the gene product of $npgA_{An}$ is toxic for *C. glutamicum*. In direct comparison to the wild-type enzyme, expression of the sfp_{Bs}-D107N did not significantly affect 6-MSA synthesis. These findings support the presumption that the metabolic burden of episomal gene expression from a second plasmid is detrimental for the microbial synthesis of 6-MSA in *C. glutamicum*.

Analysis of the genome sequence of *C. glutamicum* ATCC 13032 identified two genes encoding PPTases, namely *pptA* (cg2171) and *acpS* (cg2738). $AcpS_{Cg}$ (UniProt ID Q8NMS4) was shown to be crucial for the activation of the fatty acid synthases FasI-A and FasI-B in this bacterium (Chalut et al. 2006). The putative function of $PptA_{Cg}$ (UniProt ID Q8NP45) is the activation of the sole type I PKS13 in *C. glutamicum* (Cg3178, 1610 aa, 172 kDa), which is required for the synthesis of corynomycolic acids as important building blocks for cell wall synthesis in coryneform bacteria (Gande et al. 2004).

Motivated by the finding that an endogenous PPTase of *C. glutamicum* is capable of activating $ChlB1_{Sa}$, we compared the sequence of the two endogenous PPTase candidates $PptA_{Cg}$ and $AcpS_{Cg}$ to Svp_{Sv}. $PptA_{Cg}$ and Svp_{Sv} share a sequence identity of 34% (sequence similarity 58%), whereas $AcpS_{Cg}$ appears to be unrelated to Svp_{Sv} (sequence identity 8%; sequence similarity 19%) (Fig. S1). Hence, it appears to be more likely that $PptA_{Cg}$ rather than $AcpS_{Cg}$ can activate $ChlB1_{Sa}$ in *C. glutamicum*. This notion is also supported by the domain architecture as both enzymes, $PptA_{Cg}$ and Svp_{Sv}, belong to the EntD superfamily of PPTases (NCBI domain cl27525), whereas $AcpS_{Cg}$ is a PPTase of the ACPS superfamily (NCBI domain cl00500). In order to confirm the relevance of $PptA_{Cg}$ for $ChlB1_{Sa}$ activation, deletion of the respective gene *pptA* was planned. However, a *pptA* deletion mutant was previously shown to exhibit a severe growth phenotype reflected in cell aggregation during cultivation in liquid medium (Chalut et al. 2006). For this reason, the idea of deleting *pptA* was abandoned as discrimination between lacking 6-MSA production due to missing $PptA_{Cg}$-mediated $ChlB1_{Sa}$ activation or poor growth is not possible. Instead, it was tested whether overexpression of *pptA* could further improve 6-MSA production with *C. glutamicum*. The strain *C. glutamicum* DelAro^4 C5 mu$fasO_{BCD1}$ pMKEx2_$malE_{Ec}$-$chlB1_{Sa}$ pEKEx3_$pptA_{Cg}$ accumulated only 18 mg/L (0.12 mM) 6-MSA, which further supports the notion that reduced 6-MSA titers in the strains additionally expressing PPTase-encoding genes are due to an increased metabolic burden and that the native expression level of $pptA_{Cg}$ expression is already sufficient for $ChlB1_{Sa}$ activation.

To judge the suitability of *C. glutamicum* as host for 6-MSA synthesis in comparison to the other well-established biotechnological workhorses *E. coli* and *S. cerevisiae*, toxicity experiments were performed. In this context, *C. glutamicum* DelAro^4 C5 mu$fasO_{BCD1}$, *E. coli* DH5α, and *S. cerevisiae* CEN.PK 2-1C were cultivated in the presence of different 6-MSA concentrations (0–50 mM). Maximum growth rates were determined during exponential growth and subsequently normalized for comparability of results (Supp. Fig. S3). For a better comparison, the growth rate determined in the absence of 6-MSA (0 mM) was set to 100% for each organism. Whereas *E. coli* did not grow in the presence of 10 mM 6-MSA, the relative growth rate of *C. glutamicum* was higher than 50%. In contrast, the presence of 10 mM 6-MSA in the growth medium did

not affect growth of *S. cerevisiae* as 85% of the growth rate was retained. However, at concentrations of 20 mM 6-MSA or above, the relative growth of *C. glutamicum* and *S. cerevisiae* could not be distinguished, which makes both microorganisms equally good 6-MSA production hosts.

PptA of *C. glutamicum* is a broad-spectrum PPTase

To interrogate a possible broad-spectrum PPTase activity of $PptA_{Cg}$, further experiments were performed in *E. coli*. This bacterium is in general capable of producing the catecholate siderophore enterobactin under iron-limited conditions (Grass 2006). Biosynthesis of enterobactin involves six proteins of which two, EntB and EntF, feature carrier protein domains (Gehring et al. 1998). Analogous to PKS systems, both EntB and EntF are phosphopantetheinylated by a PPTase named EntD (UniProt ID P19925) encoded in the enterobactin locus (Gehring et al. 1997; Lambalot et al. 1996). While inactivation of *entD* abolishes production of enterobactin in *E. coli* (Cox et al. 1970), this phenotype can be rescued by complementation with an exogenous PPTase (Barekzi et al. 2004). To test whether $PptA_{Cg}$ is similarly capable to act as a substitute for the enterobactin PPTase, an *entD* mutation was introduced into the *E. coli* strain BW25113, which was previously reported as enterobactin producer (Ma and Payne 2012). Comparative LC-MS analyses with the wild type confirmed the loss of enterobactin in the *E. coli* BW25113 *entD*::*amp*^{r} strain (data not shown). Hence, it was evident that *E. coli* BW25113 does not possess another PPTase, which could compensate for the functional loss of EntD. Following the expression of $pptA_{Cg}$ in *E. coli* BW25113 *entD*::*amp*^{r}, the mutant resumed the production of enterobactin, which strongly suggests that $PptA_{Cg}$ is capable to phosphopantetheinylate the two carrier protein domains in EntB and EntF (Fig. 3c). This experiment indicates that $PptA_{Cg}$ of *C. glutamicum* can indeed activate various carrier protein domains and can thus be regarded as broad-spectrum PPTase with many possible applications in microbial natural product synthesis.

Discussion

Iterative type I PKS biocatalysts are particularly appealing for the production of high-value molecules from simple CoA-activated precursors derived from the central carbon metabolism. Once an iterative PKS is activated, all reaction steps are catalyzed by individual domains; in that sense, the PKS itself represents a biochemical "assembly line" within a microbial cell factory. This avoids to a large degree any engineered deregulation of natural biosynthetic pathways and thereby circumvents the accumulation of undesired side-products. In consequence, iterative PKSs can be considered as ideal targets for the production of chemical building blocks as well as medicinal drugs in engineered microbial cell factories.

Here, we functionally integrated the 6-MSA synthase ChlB1 from *S. antibioticus* into *C. glutamicum* and demonstrated its suitability as a cell factory for 6-MSA synthesis. Production of related hydroxybenzoic acids in engineered microorganism such as salicylic acid (a derivative of 6-MSA lacking the methyl group) requires deregulation and engineering of the shikimate pathway and can lead to metabolic imbalances, auxotrophic strains, or undesired accumulation of side products (Kallscheuer and Marienhagen 2018; Lin et al. 2014). In contrast, after introduction of $ChlB1_{Sa}$ in *C. glutamicum*, no significant accumulation of side products could be observed.

Microbial production of 6-MSA using the 6-MSA synthase from *Penicillium patulum* was already demonstrated earlier using *E. coli* and *Saccharomyces cerevisiae* as production hosts (Kealey et al. 1998; Wattanachaisaereekul et al. 2007; Wattanachaisaereekul et al. 2008). For the essential PPTase-mediated activation of the heterologous PKS, co-expression of a broad spectrum PPTase gene, such as *sfp* from *B. subtilis*, was mandatory in both hosts. In the direct comparison, *S. cerevisiae* was more suitable for 6-MSA production as titers from 0.2 to 1.7 g/L (1.3–11.2 mM) 6-MSA were reported, whereas *E. coli* accumulated only 75 mg/L (0.49 mM) (Kealey et al. 1998; Wattanachaisaereekul et al. 2008). The determined product concentrations in *E. coli* are comparable to 6-MSA titers obtained using engineered *Streptomyces coelicolor* strains (Bedford et al. 1995) and with *C. glutamicum* in this study. The performance of the different production hosts, however, is difficult to compare as we decided to use ChlB1 from *S. antibioticus* in this study instead of the enzyme from *P. patulum*.

In both organisms, *C. glutamicum* and *E. coli*, challenges related to protein insolubility are needed to be addressed. In our study, improved folding was achieved by translation fusion of the PKS to the maltose-binding protein MalE, whereas co-expression of chaperone-encoding genes from *S. coelicolor* improved folding and solubility of different type I PKSs in *E. coli* (Betancor et al. 2008). In case of the essential PPTase-mediated activation of PKSs, we could show that *C. glutamicum* harbors a broad-spectrum PPTase ($PptA_{Cg}$), which is not only capable of activating $ChlB1_{Sa}$, but also activates the enterobactin biosynthesis enzymes EntB and EntF in *E. coli*. It is thus likely that expression of a heterologous PPTase gene can be omitted in future PKS- and NRPS-related applications using *C. glutamicum*, which will significantly simplify and shorten strain development times as only the core biosynthesis gene needs to be functionally expressed.

This important property of *C. glutamicum* cannot be valued highly enough as phosphopantetheinylation in heterologous hosts often fails due to the unavailability of a suitable PPTase (Crosby et al. 1995; Li and Neubauer 2014; Roberts et al. 1993; Shen et al. 1992).

Taken together, we consider *C. glutamicum* as a promising host organism for microbial polyketide production using type I PKSs. *C. glutamicum* is an actinomycete and thus closely related to Streptomycetes, which are a valuable source of many interesting type I PKSs (including $ChlB1_{Sa}$). Access to *C. glutamicum* variants with increased acetyl-CoA and malonyl-CoA availability and easy-to-handle production plasmids enabling the expression of soluble MalE-fusion proteins further underlines the potential of this microorganism for polyketide synthesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human or animal participants performed by any of the authors.

References

Abe I, Morita H (2010) Structure and function of the chalcone synthase superfamily of plant type III polyketide synthases. Nat Prod Rep 27: 809–838

Baltz R (2010) *Streptomyces* and *Saccharopolyspora* hosts for heterologous expression of secondary metabolite gene clusters. J Ind Microbiol Biotechnol 37:759–772

Barekzi N, Joshi S, Irwin S, Ontl T, Schweizer HP (2004) Genetic characterization of *pcpS*, encoding the multifunctional phosphopantetheinyl transferase of *Pseudomonas aeruginosa*. Microbiology 150:795–803

Bedford DJ, Schweizer E, Hopwood DA, Khosla C (1995) Expression of a functional fungal polyketide synthase in the bacterium *Streptomyces coelicolor* A3 (2). J Bacteriol 177:4544–4548

Bertani G (1951) Studies on lysogenesis I: the mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol 62:293

Betancor L, Fernández MJ, Weissman KJ, Leadlay PF (2008) Improved catalytic activity of a purified multienzyme from a modular polyketide synthase after coexpression with *Streptomyces* chaperonins in *Escherichia coli*. ChemBioChem 9:2962–2966

Chalut C, Botella L, de Sousa-D'Auria C, Houssin C, Guilhot C (2006) The nonredundant roles of two 4′-phosphopantetheinyl transferases in vital processes of Mycobacteria. Proc Natl Acad Sci U S A 103: 8511–8516

Cox RJ (2007) Polyketides, proteins and genes in fungi: programmed nano-machines begin to reveal their secrets. Org Biomol Chem 5: 2010–2026

Cox G, Gibson F, Luke R, Newton N, O'Brien I, Rosenberg H (1970) Mutations affecting iron transport in *Escherichia coli*. J Bacteriol 104:219–226

Cox RJ, Hitchman TS, Byrom KJ, Findlow ISC, Tanner JA, Crosby J, Simpson TJ (1997) Post-translational modification of heterologously expressed *Streptomyces* type II polyketide synthase acyl carrier proteins. FEBS Lett 405:267–272

Crosby J, Sherman DH, Bibb MJ, Revill WP, Hopwood DA, Simpson TJ (1995) Polyketide synthase acyl carrier proteins from *Streptomyces*: expression in *Escherichia coli*, purification and partial characterisation. Biochim Biophys Acta -Protein Structure and Molecular Enzymology 1251:32–42

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640–6645

Daum M, Peintner I, Linnenbrink A, Frerich A, Weber M, Paululat T, Bechthold A (2009) Organisation of the biosynthetic gene cluster and tailoring enzymes in the biosynthesis of the tetracyclic quinone glycoside antibiotic polyketomycin. ChemBioChem 10:1073–1083

Eggeling L, Bott M (2005) Handbook of *Corynebacterium glutamicum*. CRC press, Boca Raton

Eggeling L, Bott M (2015) A giant market and a powerful metabolism: L-lysine provided by *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 99:3387–3394

Entian KD, Kötter P (2007) Yeast genetic strain and plasmid collections. Methods Microbiol 36:629–666

Gande R, Gibson KJ, Brown AK, Krumbach K, Dover LG, Sahm H, Shioyama S, Oikawa T, Besra GS, Eggeling L (2004) Acyl-CoA carboxylases (*accD2* and *accD3*), together with a unique polyketide synthase (*Cg-pks*), are key to mycolic acid biosynthesis in *Corynebacterianeae* such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*. J Biol Chem 279:44847–44857

Gande R, Dover LG, Krumbach K, Besra GS, Sahm H, Oikawa T, Eggeling L (2007) The two carboxylases of *Corynebacterium glutamicum* essential for fatty acid and mycolic acid synthesis. J Bacteriol 189:5257–5264

Gehring AM, Bradley KA, Walsh CT (1997) Enterobactin biosynthesis in *Escherichia coli*: isochorismate lyase (EntB) is a bifunctional enzyme that is phosphopantetheinylated by EntD and then acylated by EntE using ATP and 2,3-dihydroxybenzoate. Biochemistry 36: 8495–8503

Gehring AM, Mori I, Walsh CT (1998) Reconstitution and characterization of the *Escherichia coli* enterobactin synthetase from EntB, EntE, and EntF. Biochemistry 37:2648–2659

Grass G (2006) Iron transport in *Escherichia coli*: all has not been said and done. BioMetals 19:159–172

Grenier F, Matteau D, Baby V, Rodrigue S (2014) Complete genome sequence of *Escherichia coli* BW25113. Genome Announc 2: e01038–e01014

Gust B, Challis GL, Fowler K, Kieser T, Chater KF (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc Natl Acad Sci U S A 100:1541–1546

Heider SA, Wolf N, Hofemeier A, Peters-Wendisch P, Wendisch VF (2014) Optimization of the IPP precursor supply for the production of lycopene, decaprenoxanthin and astaxanthin by *Corynebacterium glutamicum*. Front Bioeng Biotechnol 2:28

Hertweck C (2009) The biosynthetic logic of polyketide diversity. Angew Chem Int Ed Engl 48:4688–4716

Ito T, Roongsawang N, Shirasaka N, Lu W, Flatt PM, Kasanah N, Miranda C, Mahmud T (2009) Deciphering pactamycin biosynthesis and engineered production of new pactamycin analogues. ChemBioChem 10:2253–2265

Jia XY, Tian ZH, Shao L, Qu XD, Zhao QF, Tang J, Tang GL, Liu W (2006) Genetic characterization of the chlorothricin gene cluster as a model for spirotetronate antibiotic biosynthesis. Chem Biol 13:575–585

Kage H, Riva E, Parascandolo JS, Kreutzer MF, Tosin M, Nett M (2015) Chemical chain termination resolves the timing of ketoreduction in a

partially reducing iterative type I polyketide synthase. Org Biomol Chem 13:11414–11417

Kallscheuer N, Marienhagen J (2018) *Corynebacterium glutamicum* as platform for the production of hydroxybenzoic acids. Microb Cell Factories 17:70

Kallscheuer N, Vogt M, Marienhagen J (2016a) A novel synthetic pathway enables microbial production of polyphenols independent from the endogenous aromatic amino acid metabolism. ACS Synth Biol 6:410–415

Kallscheuer N, Vogt M, Stenzel A, Gätgens J, Bott M, Marienhagen J (2016b) Construction of a *Corynebacterium glutamicum* platform strain for the production of stilbenes and (2*S*)-flavanones. Metab Eng 38:47–55

Kallscheuer N, Classen T, Drepper T, Marienhagen J (2019) Production of plant metabolites with applications in the food industry using engineered microorganisms. Curr Opin Biotechnol 56:7–17

Kealey JT, Liu L, Santi DV, Betlach MC, Barr PJ (1998) Production of a polyketide natural product in nonpolyketide-producing prokaryotic and eukaryotic hosts. Proc Natl Acad Sci U S A 95:505–509

Keilhauer C, Eggeling L, Sahm H (1993) Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. J Bacteriol 175:5595–5603

Kortmann M, Kuhl V, Klaffl S, Bott M (2015) A chromosomally encoded T7 RNA polymerase-dependent gene expression system for *Corynebacterium glutamicum*: construction and comparative evaluation at the single-cell level. Microb Biotechnol 8:253–265

Kreutzer MF, Kage H, Gebhardt P, Wackler B, Saluz HP, Hoffmeister D, Nett M (2011) Biosynthesis of a complex yersiniabactin-like natural product via the *mic* locus in phytopathogen *Ralstonia solanacearum*. Appl Environ Microbiol 77:6117–6124

Lambalot RH, Gehring AM, Flugel RS, Zuber P, LaCelle M, Marahiel MA, Reid R, Khosla C, Walsh CT (1996) A new enzyme superfamily—the phosphopantetheinyl transferases. Chem Biol 3:923–936

Li J, Neubauer P (2014) *Escherichia coli* as a cell factory for heterologous production of nonribosomal peptides and polyketides. New Biotechnol 31:579–585

Lin Y, Sun X, Yuan Q, Yan Y (2014) Extending shikimate pathway for the production of muconic acid and its precursor salicylic acid in *Escherichia coli*. Metab Eng 23:62–69

Litsanov B, Kabus A, Brocker M, Bott M (2012) Efficient aerobic succinate production from glucose in minimal medium with *Corynebacterium glutamicum*. Microb Biotechnol 5:116–128

Liu Q, Wu K, Cheng Y, Lu L, Xiao E, Zhang Y, Deng Z, Liu T (2015) Engineering an iterative polyketide pathway in *Escherichia coli* results in single-form alkene and alkane overproduction. Metab Eng 28:82–90

Ma L, Payne SM (2012) AhpC is required for optimal production of enterobactin by *Escherichia coli*. J Bacteriol 194:6748–6757

Milke L, Ferreira P, Kallscheuer N, Braga A, Vogt M, Kappelmann J, Oliveira J, Silva AR, Rocha I, Bott M, Noack S, Faria N, Marienhagen J (2019a) Modulation of the central carbon metabolism of *Corynebacterium glutamicum* improves malonyl-CoA availability and increases plant polyphenol synthesis. Biotechnol Bioeng 116:1380–1391

Milke L, Kallscheuer N, Kappelmann J, Marienhagen J (2019b) Tailoring *Corynebacterium glutamicum* towards increased malonyl-CoA availability for efficient synthesis of the plant pentaketide noreugenin. Microb Cell Fact:1871

Nguyen AQ, Schneider J, Wendisch VF (2015) Elimination of polyamine *N*-acetylation and regulatory engineering improved putrescine production by *Corynebacterium glutamicum*. J Biotechnol 201:75–85

Oberegger H, Eisendle M, Schrettl M, Graessle S, Haas H (2003) 4′-Phosphopantetheinyl transferase-encoding *npgA* is essential for siderophore biosynthesis in *Aspergillus nidulans*. Curr Genet 44:211–215

Parascandolo JS, Havemann J, Potter HK, Huang F, Riva E, Connolly J, Wilkening I, Song L, Leadlay PF, Tosin M (2016) Insights into 6-methylsalicylic acid bio-assembly by using chemical probes. Angew Chem Int Ed Engl 55:3463–3467

Quadri LE, Weinreb PH, Lei M, Nakano MM, Zuber P, Walsh CT (1998) Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases. Biochemistry 37:1585–1595

Ray L, Moore BS (2016) Recent advances in the biosynthesis of unusual polyketide synthase substrates. Nat Prod Rep 33:150–161

Roberts GA, Staunton J, Leadlay PF (1993) Heterologous expression in *Escherichia coli* of an intact multienzyme component of the erythromycin-producing polyketide synthase. Eur J Biochem 214:305–311

Sambrook J, Russell D (2001) Molecular cloning, vol 1-3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sánchez C, Du L, Edwards DJ, Toney MD, Shen B (2001) Cloning and characterization of a phosphopantetheinyl transferase from *Streptomyces verticillus* ATCC15003, the producer of the hybrid peptide–polyketide antitumor drug bleomycin. Chem Biol 8:725–738

Schmidlin L, Poutaraud A, Claudel P, Mestre P, Prado E, Santos-Rosa M, Wiedemann-Merdinoglu S, Karst F, Merdinoglu D, Hugueney P (2008) A stress-inducible resveratrol *O*-methyltransferase involved in the biosynthesis of pterostilbene in grapevine. Plant Physiol 148:1630–1639

Schröder J (1997) A family of plant-specific polyketide synthases: facts and predictions. Trends Plant Sci 2:373–378

Shao L, Qu XD, Jia XY, Zhao QF, Tian ZH, Wang M, Tang GL, Liu W (2006) Cloning and characterization of a bacterial iterative type I polyketide synthase gene encoding the 6-methylsalicyclic acid synthase. Biochem Biophys Res Commun 345:133–139

Shen B (2003) Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms. Curr Opin Chem Biol 7:285–295

Shen B, Summers R, Gramajo H, Bibb M, Hutchinson C (1992) Purification and characterization of the acyl carrier protein of the *Streptomyces glaucescens* tetracenomycin C polyketide synthase. J Bacteriol 174:3818–3821

Shi YM, Richter C, Challinor VL, Grün P, Girela del Rio A, Kaiser M, Schüffler A, Piepenbring M, Schwalbe H, Bode HB (2018) Georatusin, a specific antiparasitic polyketide–peptide hybrid from the fungus *Geomyces auratus*. Org Lett 20:1563–1567

Ugai T, Minami A, Fujii R, Tanaka M, Oguri H, Gomi K, Oikawa H (2015) Heterologous expression of highly reducing polyketide synthase involved in betaenone biosynthesis. Chem Commun (Camb) 51:1878–1881

Van Lanen SG, Oh TJ, Liu W, Wendt-Pienkowski E, Shen B (2007) Characterization of the maduropeptin biosynthetic gene cluster from *Actinomadura madurae* ATCC 39144 supporting a unifying paradigm for enediyne biosynthesis. J Am Chem Soc 129:13082–13094

Vickery CR, Kosa NM, Casavant EP, Duan S, Noel JP, Burkart MD (2014) Structure, biochemistry, and inhibition of essential 4′-phosphopantetheinyl transferases from two species of *Mycobacteria*. ACS Chem Biol 9:1939–1944

Vogt M, Brüsseler C, van Ooyen J, Bott M, Marienhagen J (2016) Production of 2-methyl-1-butanol and 3-methyl-1-butanol in engineered *Corynebacterium glutamicum*. Metab Eng 38:436–445

Wang YY, Ran XX, Chen WB, Liu SP, Zhang XS, Guo YY, Jiang XH, Jiang H, Li YQ (2014) Characterization of type II thioesterases involved in natamycin biosynthesis in *Streptomyces chattanoogensis* L10. FEBS Lett 588:3259–3264

Wattanachaisaereekul S, Lantz AE, Nielsen ML, Andrésson ÓS, Nielsen J (2007) Optimization of heterologous production of the polyketide 6-MSA in *Saccharomyces cerevisiae*. Biotechnol Bioeng 97:893–900

Wattanachaisaereekul S, Lantz AE, Nielsen ML, Nielsen J (2008) Production of the polyketide 6-MSA in yeast engineered for increased malonyl-CoA supply. Metab Eng 10:246–254

Yin J, Hoffmann M, Bian X, Tu Q, Yan F, Xia L, Ding X, Stewart AF, Müller R, Fu J (2015) Direct cloning and heterologous expression of the salinomycin biosynthetic gene cluster from *Streptomyces albus* DSM41398 in *Streptomyces coelicolor* A3 (2). Sci Rep 5:15081

Yuzawa S, Kim W, Katz L, Keasling JD (2012) Heterologous production of polyketides by modular type I polyketide synthases in *Escherichia coli*. Curr Opin Biotechnol 23:727–735

2.4.1 Supplementary material

Supplementary Material

Applied Microbiology and Biotechnology

Microbial synthesis of the type I polyketide 6-methylsalicylate with *Corynebacterium glutamicum*

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Running Title: 6-methylsalicylate production in *C. glutamicum*

Keywords: polyketide, 6-methylsalicylate, type I polyketide synthase, phosphopantetheinyl transferase, *Corynebacterium glutamicum*, malonyl-CoA

Supplementary Material

a

```
PptA(Cg)    1   MLDESLFPNSAK--FSFIKTGDAVNLDHFHQLHPLEKALVAHSVDIRKAEFGDARWCAHQ   58
Svp(Sv)     1   -MIAALLPSWAVTEHAFTD----APDDPVSLLFPEEAAHVARAVPKRLHEFATVRVCARA   55
                 :  :*:*. *   .:* .    .  * .  *.* * * **::*  *  **. .* **:

PptA(Cg)   59   ALQALGRDSGDPILRGERGMPLWPSSVSGSLTHTDGFRAAVVAPRLLVRSMGLDAEPAEP   118
Svp(Sv)    56   ALGRLGLPP-GPLLPGRRGAPSWPDGVVGSMTHCQGFRGAAVARAADAASLGIDAEPNGP   114
                ** **    .*:* *.** * **..* **:** :***.*.**    . *:*:**** *

PptA(Cg)  119   LPKDVLGSIARVGEIPQLKRLEEQ-GVHCADRLLFCAKEATYKAWFPLTHRWLGFEQAEI   177
Svp(Sv)   115   LPDGVLAMVSLPSEREWLAGLAARRPDVHWDRLLFSAKESVFKAWYPLTGLELDFDEAEL   174
                **..**. ::  .*   *  *  :      *****.***:.:***:***   *.*::**:

PptA(Cg)  178   DLRDDG-TFVSYLLVRPTP------VPFISGKWVLRDGYVIAATAVT-------------   217
Svp(Sv)   175   AVDPDAGTFTARLL-VPGPVVGGRRLDGFEGRWAAGEGLVVTAIAVAAPAGTAEESAEGA   233
                 :  *. **.: **  * *      :  :.*:*.  :* *::* **:

PptA(Cg)  218   -------------                                               217
Svp(Sv)   234   GKEATADDRTAVP                                               246
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b

```
AcpS(Cg)    1   ------------------------------------------------------------   0
Svp(Sv)     1   MIAALLPSWAVTEHAFTDAPDDPVSLLFPEEAAHVARAVPKRLHEFATVRVCARAALGRL   60

AcpS(Cg)    1   ------------------------------------------------------------   0
Svp(Sv)    61   GLPPGPLLPGRRGAPSWPDGVVGSMTHCQGFRGAAVARAADAASLGIDAEPNGPLPDGVL  120

AcpS(Cg)    1   -MISIGTDLVHI---------------------------------------------   11
Svp(Sv)   121   AMVSLPSEREWLAGLAARRPDVHWDRLLFSAKESVFKAWYPLTGLELDFDEAELAVDPDA  180
                 *:*: ::   :

AcpS(Cg)   12   SAFAEQLAQPGSSFMEVFSAGERRKANERQASRYAEHLAGRWAAKESFIKAWSQAIYGQP   71
Svp(Sv)   181   GTFTARLLVPGP---------------VVGGRRLDGFEGRWAAGEGLVVTAIAV--AAP  222
                .:*: :*  **                 ..*  : : ***** *.:: :   .  . *

AcpS(Cg)   72   PVIAEEAVVWRDIEVRADAWGRVAIELAPELAAVVRESIGEFSSSLSISHDGDYAVATCV   131
Svp(Sv)   223   AGTAEESAEGAGKEATADD--RT-----------------------------AVP---  246
                  ***:.   . *. **   *.                             **

AcpS(Cg)  132   LTIQ                                                        135
Svp(Sv)   247   ----                                                        246
```

Figure S1. Sequence alignment of different PPTases evaluated for an application in *C. glutamicum*. (a) Sequence comparison of the native PPTase PptA of *C. glutamicum* and Svp from *Streptomyces verticillus*; **(b)** Sequence comparison of the native PPTase AcpS of *C. glutamicum* and Svp from *Streptomyces verticillus*. Alignments were performed at the UniProt database website using Clusal Omega (Sievers *et al.* 2011).

Supplementary Material

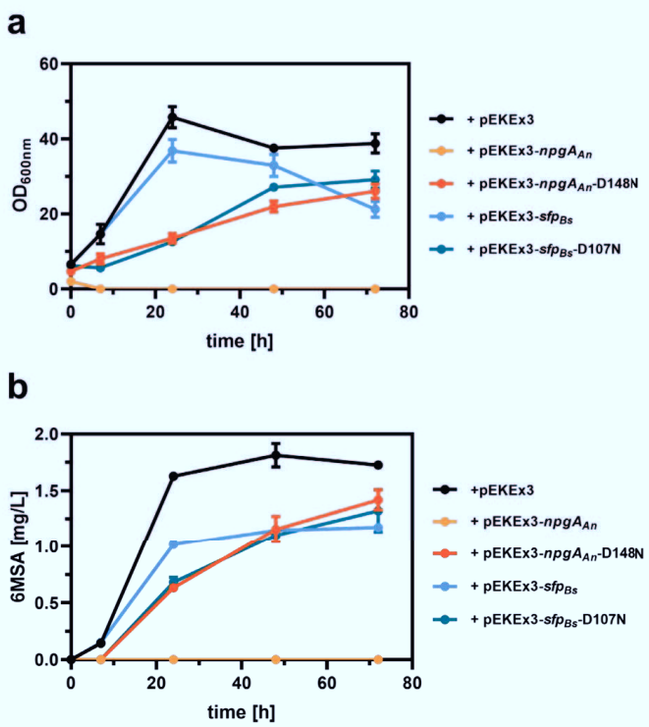

Figure S2. Microbial synthesis of 6-MSA with *C. glutamicum* DelAro^4 C5 mu*fasO*$_{BCD1}$ pMKEx2_*malE*$_{Ec}$-*chlB1*$_{Sa}$ expressing different gene variants of *npgA*$_{An}$ and *sfb*$_{Bs}$ encoding inactive PPTases using the BioLector microbioreactor system. At indicated time points, samples were taken to determine cell growth and the 6-MSA titer by LC-MS. **(a**) Microbial growth of indicated strains **(b)** 6-MSA titers. In general, product titers are lower compared to 6-MSA concentrations obtained during flask cultivations, but ratios between different strains are similar. Depicted data represents mean values and standard deviations from biological triplicates

Supplementary Material

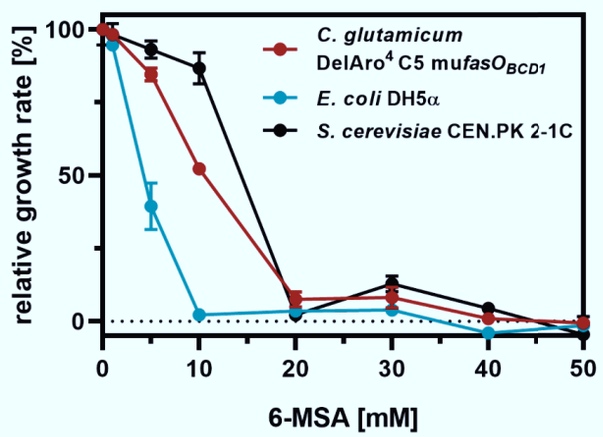

Figure S3. Determination of 6-MSA toxicity for *C. glutamicum*, *E. coli* and *S. cerevisiae*. All three different organisms were cultivated in the presence of different 6-MSA concentrations ranging from 0 - 50 mM 6-MSA. Growth rates were determined during exponential growth phase and subsequently normalized. Here, the growth rate determined in the absence of 6-MSA (0 mM) was set to 100 %. Data for *C. glutamicum* and *E. coli* represents mean values and standard deviations from biological triplicates, whereas data for *S. cerevisiae* is calculated on the basis of biological duplicates.

References

Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular systems biology 7(1):539

2.5 Synthesis of flavoring phenylbutanoids with *C. glutamicum*

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Microbial Cell Factories

RESEARCH Open Access

Synthesis of the character impact compound raspberry ketone and additional flavoring phenylbutanoids of biotechnological interest with *Corynebacterium glutamicum*

Lars Milke[1], Mario Mutz[1] and Jan Marienhagen[1,2,3*]

Abstract

Background: The phenylbutanoid 4-(4-hydroxyphenyl)butan-2-one, commonly known as raspberry ketone, is responsible for the typical scent and flavor of ripe raspberries. Chemical production of nature-identical raspberry ketone is well established as this compound is frequently used to flavor food, beverages and perfumes. However, high demand for natural raspberry ketone, but low natural abundance in raspberries, render raspberry ketone one of the most expensive natural flavoring components.

Results: In this study, *Corynebacterium glutamicum* was engineered for the microbial synthesis of the character impact compound raspberry ketone from supplemented *p*-coumaric acid. In this context, the NADPH-dependent curcumin/dihydrocurcumin reductase CurA from *Escherichia coli* was employed to catalyze the final step of raspberry ketone synthesis as it provides a hitherto unknown benzalacetone reductase activity. In combination with a 4-coumarate: CoA ligase from parsley (*Petroselinum crispum*) and a monofunctional benzalacetone synthase from Chinese rhubarb (*Rheum palmatum*), CurA constitutes the synthetic pathway for raspberry ketone synthesis in *C. glutamicum*. The resulting strain accumulated up to 99.8 mg/L (0.61 mM) raspberry ketone. In addition, supplementation of other phenylpropanoids allowed for the synthesis of two other naturally-occurring and flavoring phenylbutanoids, zingerone (70 mg/L, 0.36 mM) and benzylacetone (10.5 mg/L, 0.07 mM).

Conclusion: The aromatic product portfolio of *C. glutamicum* was extended towards the synthesis of the flavoring phenylbutanoids raspberry ketone, zingerone and benzylacetone. Key to success was the identification of CurA from *E. coli* having a benzalacetone reductase activity. We believe, that the constructed *C. glutamicum* strain represents a versatile platform for the production of natural flavoring phenylbutanoids at larger scale.

Keywords: NADPH-dependent curcumin reductase, Benzalacetone reductase, *Corynebacterium glutamicum*, Raspberry ketone, Metabolic engineering, Character impact compound

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Introduction

The phenylbutanoid character impact compound raspberry ketone (4-(4-hydroxyphenyl)butan-2-one, RK) defines the typical scent and taste of raspberries. Thus, it is utilized by food and beverage industries to flavor beverages and foods, e.g. pudding, yogurt or sweets [1, 2]. In addition, its presumed activity as an anti-obesity or skin-whitening agent, drew consumers interest, although a

potential toxicity of this compound for humans has not yet been clarified [2–6].

Different strategies can be followed to obtain RK, e.g. extraction from natural plant material or chemical synthesis. Adversely, the natural concentration of RK in raspberries is not only very low (1–4 mg/kg), but also subject to seasonal and regional fluctuations, leading to high product costs of 3000–20,000 US$ per kg of natural, extracted RK [1, 7, 8]. Alternatively, RK can be chemically synthesized, but any RK produced by such processes is only considered as a nature-identical flavoring substance according to EU and US regulations, which no longer meets customers' demands [9–11]. Contrary to this, RK obtained from microbial synthesis is regarded as natural. Thus, microbial synthesis represents a promising approach for the sustainable production of natural RK. Prerequisite for establishing a microbial RK production process is the functional introduction of the natural biosynthesis pathway from the plant into a heterologous host.

In raspberry plants, RK synthesis starts from L-phenylalanine, which is provided by the shikimate pathway [12]. From there, L-phenylalanine is non-oxidatively deaminated by a phenylalanine ammonia lyase (PAL), yielding the phenylpropanoid cinnamic acid, which is subsequently hydroxylated towards *p*-coumaric acid (*p*CA). This compound in turn undergoes CoA-activation catalyzed by a 4-coumarate: CoA ligase (4CL, Fig. 1). The activated thioester is then condensed with one molecule of malonyl-CoA by a benzalacetone synthase (BAS), a type III polyketide synthase (PKS), yielding the diketide intermediate *p*-hydroxybenzalacetone (*p*HBA). Finally, a NADPH-dependent benzalacetone reductase (BAR) reduces *p*HBA to RK.

First studies on microbial RK production from supplemented *p*CA using BAS from Chinese rhubarb (*Rheum palmatum*) and CHS from raspberry (*Rubus idaeus*), respectively, reported product titers below 10 mg/L (0.06 mM) when using *Escherichia coli* or *Saccharomyces cerevisiae* as host strains [1, 13]. Interestingly, both studies relied on endogenous BAR activities by unknown endogenous reductase(s) in the respective host, rendering heterologous expression of a BAR-encoding gene unnecessary. Only recently, synthesis of up to 91 mg/L (0.55 mM) RK was demonstrated using *E. coli* BL21(DE3), which was developed for the expression of *bas* from *R. palmatum* and *rzs1* from raspberry [8, 14]. The latter gene codes for the raspberry ketone/zingerone synthase RZS1 ($RZS1_{Ri}$, UniProt ID: G1FCG0), which provides the required BAR activity.

Since various type III PKS-encoding genes of plant origin (encoding for stilbene synthases, chalcone synthases and a pentaketide chromone synthase) have been functionally expressed in *Corynebacterium glutamicum* previously, it is reasonable to assume that this is also true for a type III PKS gene providing BAS activity [15, 16].

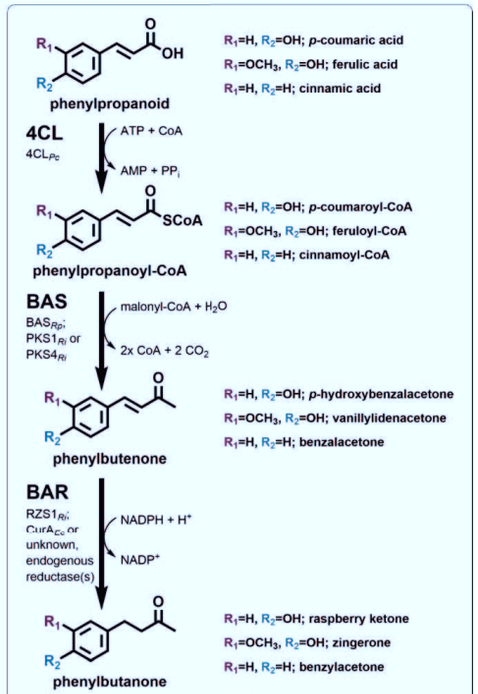

Fig. 1 Synthesis of different phenylbutanoids from phenylpropanoids. 4CL: 4-coumarate: CoA ligase, BAS: benzalacetone synthase, BAR: benzalacetone reductase, CoA: coenzyme A. Particular enzymes used in this study are assigned below the respective general enzyme activities. For further information on the individual enzymes used, the reader is referred to the text

In this context, *C. glutamicum* strains have been tailored towards increased malonyl-CoA supply for efficient synthesis of plant polyphenols and polyketides [16–18]. This was necessary, as typically only low intracellular concentrations of the unstable fatty acid precursor malonyl-CoA are maintained in microorganisms as its synthesis is strictly regulated, limiting overall product formation [19]. Although only one molecule of malonyl-CoA is required for the synthesis of one RK molecule, a *C. glutamicum* strain with increased malonyl-CoA availability is predestined for also establishing a heterologous pathway for the synthesis of RK.

In this study, we present the construction of a microbial *C. glutamicum* cell factory for the synthesis of the flavoring phenylbutanoids RK, zingerone and benzylacetone. Additionally, we identified a hitherto unknown BAR activity of the NADPH-dependent curcumin/dihydrocurcumin reductase CurA from *E. coli* allowing for the reduction of diketide intermediates.

Results

Cytotoxicity of *p*-hydroxybenzalacetone and raspberry ketone

In preparation of establishing a heterologous RK biosynthesis pathway from supplemented *p*CA, intermediate (*p*HBA) and product (RK) cytotoxicity on the designated host *C. glutamicum* was investigated. For this purpose, the strain *C. glutamicum* M-CoA, previously constructed for providing increased malonyl-CoA levels [16], was cultivated in CGXII medium with 4% glucose supplemented with different concentrations ranging from 0 to 1 g/L (6.17 mM) *p*HBA and RK (6.1 mM) using the BioLector microbioreactor system (Fig. 2). Concentrations ≥ 125 mg/L (0.77 mM) *p*HBA negatively affected microbial growth up to complete growth inhibition in the presence of 1 g/L (6.17 mM) *p*HBA. Bearing the designated supplementation of 5 mM *p*CA as precursor for RK synthesis in mind, resembling the standard production conditions for the synthesis of *p*CA-derived plant polyphenols using *C. glutamicum*, such toxic concentrations cannot be reached [18]. In contrast, no significant negative impact on growth could be observed upon supplementation of up to 1 g/L (6.1 mM) RK.

Previous studies utilizing either *E. coli* or *S. cerevisiae* for microbial RK synthesis demonstrated that both hosts provide an endogenous BAR activity [1, 13]. Hence, supernatants from the *C. glutamicum* microbioreactor cultivations performed in the context of the *p*HBA cytotoxicity experiments, were analyzed by HPLC for the presence of potentially accumulating RK. Indeed, RK was detected in all samples from cultivations supplemented with ≥ 125 mg/L (0.77 mM) *p*HBA reaching a maximum of 15.4 mg/L (0.094 mM) RK when 500 mg/L (3.09 mM) *p*HBA was present in the microbioreactor cultivations. Interestingly, even though the *C. glutamicum* cells did not grow in the presence of 1 g/L (6.17 mM) *p*HBA, up to 9.3 mg/L (0.057 mM) RK were formed. This particular experiment was repeated without cells to verify that *p*HBA reduction yielding RK is due to the presence of the *C. glutamicum* cells conferring an endogenous BAR activity and not the result of a spontaneous reduction under the selected cultivation conditions in CGXII medium. This control experiment showed that RK formation was only detectable in the presence of *C. glutamicum* cells. Therefore, a yet unknown endogenous BAR activity can also be ascribed to *C. glutamicum*.

The curcumin reductase CurA from *E. coli* improves the BAR activity in *C. glutamicum*

With the aim to increase the endogenous BAR activity and to establish the full RK pathway in *C. glutamicum*, heterologous genes coding for BAS and BAR enzymes were episomally introduced into this bacterium. For this purpose, a codon-optimized gene variant encoding BAS

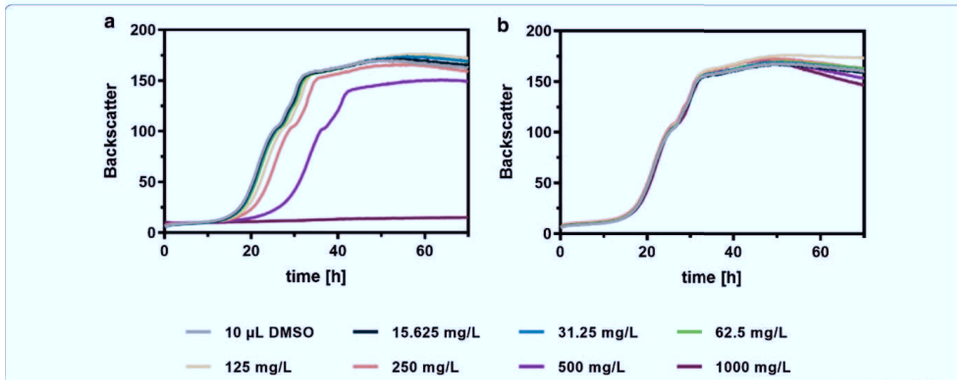

Fig. 2 Cytotoxic effects of **a** *p*-hydroxybenzalacetone and **b** raspberry ketone on growth of *C. glutamicum*. *C. glutamicum* M-CoA was cultivated in CGXII medium with 4% glucose supplemented with increasing concentrations of either *p*-hydroxybenzalacetone or raspberry ketone dissolved in DMSO using a BioLector microbioreactor. Biomass formation was followed by measuring the backscattered light intensity (gain 10) at a wavelength of 620 nm. The depicted data represent mean values from biological triplicates

from *Rheum palmatum* (bas_{RpCg}, UniProt ID: Q94FV7) was combined with a gene for different BAR variants. A codon-optimized gene variant of $RZS1_{Ri}$ ($rzs1_{RiCg}$, UniProt ID: G1FCG0) was used as this particular enzyme has already been successfully applied for the microbial synthesis of RK in *E. coli* [8]. Previously, cofactor specificity in a $RZS1_{Ri}$-G191D mutant was reported to be relaxed resulting in the acceptance of NADH as reducing agent [14]. Based on this observation, the same amino acid substitution was also introduced into $rzs1_{RiCg}$ ($rzs1_{RiCg}$-G191D). Additionally, available scientific data was analyzed to identify endogenous reductases involved in *p*HBA reduction in *E. coli* and *C. glutamicum*. In case of *E. coli*, the NADPH-dependent curcumin/dihydrocurcumin reductase CurA involved in the degradation of this polyphenol ($CurA_{Ec}$, UniProt ID: P76113) was identified as a promising candidate. Its natural substrate curcumin is a dimer of *p*HBA and thus the enzyme might also be active on the monomers (Additional file 1: Figure S1) [20]. Therefore, the native *curA* gene was amplified from the genome of *E. coli* MG1655 ($curA_{Ec}$) but also ordered as codon-optimized variant ($curA_{EcCg}$) for a possible application in *C. glutamicum*. To enable IPTG-inducible heterologous gene expression from the strong T7 promotor, the plasmid pMKEx2 was selected [21]. The constructed plasmids were used for the transformation of *C. glutamicum* M-CoA. For evaluation of reductase activity, the generated strains were cultivated for 72 h in 50 mL defined CGXII medium with 4% glucose and 1 mM IPTG supplemented with 500 mg/L (3.09 mM) *p*HBA. Taken samples were extracted with ethyl acetate and analyzed for the synthesis of RK by HPLC (Fig. 3).

Surprisingly, both strains harboring an episomally encoded $rzs1_{RiCg}$ gene variant did not synthesize more RK from supplemented *p*HBA compared to *C. glutamicum* M-CoA harboring the empty vector (12.9 mg/L, 0.08 mM). This indicates that both $rzs1_{RiCg}$ variants are not functionally expressed in *C. glutamicum.* However, in presence of the $curA_{Ec}$, 49.4 mg/L (0.3 mM) RK were synthesized, indicating not only its functional expression in *C. glutamicum* but also the capability of $CurA_{Ec}$ to reduce *p*HBA. When utilizing the codon-optimized $curA_{EcCg}$ gene, RK synthesis was increased further to 68.7 mg/L (0.42 mM). Hence, the pMKEx2-bas_{RpCg}-$curA_{EcCg}$ plasmid was selected for all subsequent experiments. Noteworthy, at least one additional reductase conferring *p*HBA reducing abilities must be present in *E. coli*, since *E. coli* BL21 strains, previously also utilized for microbial RK synthesis, do not have the *curA* gene [1].

Increased NADPH availability improves *p*HBA reduction

The observed incomplete conversion of *p*HBA to RK suggested intracellular NADPH supply to be a limiting factor during phenylbutanoid synthesis. With the aim to improve *p*HBA reduction, previously described strategies for increasing NADPH availability were followed [22]. In particular, elimination of the endogenous lactate dehydrogenase activity, especially in combination with the heterologous expression of the transhydrogenase genes *pntAB* from *E. coli*, was shown to contribute to increased NADPH availability in *C. glutamicum*. The constructed strain *C. glutamicum* M-CoA Δ*ldhA* was transformed using the plasmid pMKEx2-bas_{RpCg}-$curA_{EcCg}$. To evaluate, if the deletion of *ldhA* also increases NADPH availability and thus improves *p*HBA reduction of *C. glutamicum*, strains were cultivated both, absence or presence of pMKEx2-bas_{RpCg}-$curA_{EcCg}$. Strain cultivation and analysis of taken samples were

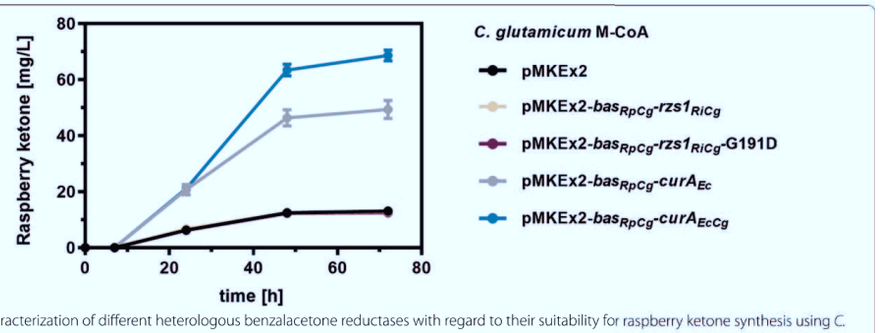

Fig. 3 Characterization of different heterologous benzalacetone reductases with regard to their suitability for raspberry ketone synthesis using *C. glutamicum*. Different *C. glutamicum* M-CoA variants harboring one of the indicated expression plasmids were cultivated in 50 mL CGXII medium with 4% glucose and 500 mg/L supplemented *p*-hydroxybenzalacetone in baffled flasks at 30 °C and 130 rpm for 72 h. The depicted data represent mean values with standard deviations from biological triplicates

conducted as described above (Additional file 1: Figure S2). Under standard cultivation conditions, sole *ldhA* deletion did not affect growth, but did also not improve RK synthesis. However, the approach of deleting *ldhA* was not abandoned although no positive effect on absolute RK titers was observed at this stage. Obviously, limited effects on NADPH-dependent *p*HBA reduction generating RK are not surprising when taking into consideration that the abolished lactate-forming reaction is NADH-dependent, consequently increasing NADH availability. For increasing NADPH supply from NADH ($NADH + NADP^+ \rightarrow NAD^+ + NADPH$), the membrane-bound transhydrogenase PntAB from *E. coli* (UniProt IDs: P07001 and P0AB67) described earlier was tested [22]. *E. coli* harbors two transhydrogenase isoforms. Whereas the energy-dependent PntAB enzyme catalyzes the transfer of a hydride ion from NADH to $NADP^+$ under physiological conditions, the energy-independent cytoplasmatic variant UdhA (UniProt ID: P27306) operates in the reverse direction, when an excess of NADPH is present in the cell [23, 24]. However, in principal, both enzymes are capable of catalyzing both reactions.

The expression plasmids pEKEx3-$pntAB_{Ec}$ and pEKEx3-$udhA_{EcCg}$, either harboring the native *pntAB* genes from *E. coli* ($pntAB_{Ec}$) or a codon-optimized *udhA* variant ($udhA_{EcCg}$), each under control of the *tac* promoter, were constructed. Subsequently, these plasmids were used for transformation of *C. glutamicum* M-CoA Δ*ldhA* harboring pMKEx2-bas_{RpCg}-$curA_{EcCg}$. The resulting strains were cultivated under the same conditions as described before. As heterologous expression of genes encoding for integral membrane proteins often cause growth defects, multiple IPTG concentrations ranging from 10 to 1000 µM were tested for the heterologous expression of $pntAB_{Ec}$. These experiments showed that an increasing IPTG concentration was always associated with an increasing growth defect up to a complete arrest of growth (Additional file 1: Figure S3). HPLC analysis indicated a drastically impaired RK synthesis for all IPTG concentrations compared to the reference strain with 1 mM IPTG, rendering the heterologous expression of *pntAB* unsuitable for RK synthesis in *C. glutamicum*.

Contrary, episomal expression of $udhA_{EcCg}$ barely affected microbial growth but increased RK titers up to 25% (Additional file 1: Figure S4). This indicates that the functional expression of $udhA_{EcCg}$ in *C. glutamicum* allows for the hydride ion transfer from NADH to $NADP^+$. Interestingly, functional expression of *udhA* from *E. coli* in *C. glutamicum* has already been demonstrated earlier, though utilized for the opposite hydride ion transfer [25]. Thus, *C. glutamicum* M-CoA Δ*ldhA* harboring the two expression plasmids pMKEx2-bas_{RpCg}-$curA_{EcCg}$ and pEKEx3-$udhA_{EcCg}$ provides the highest BAR activity, resembling a promising candidate for establishing (4CL and) BAS activity to complete the heterologous pathway for RK synthesis from supplemented *p*CA.

Raspberry ketone synthesis from *p*-coumaric acid

So far, RK synthesis in *C. glutamicum* was only achieved by supplementation of the diketide intermediate *p*HBA. As *C. glutamicum* M-CoA provides increased amounts of malonyl-CoA, synthesis of *p*HBA from *p*CA should be also possible. In addition to BAS from Chinese rhubarb (*R. palmatum*), which was shown to feature a novel catalytic mechanism allowing for the sole synthesis of *p*HBA, the bifunctional chalcone synthases PKS1 (UniProt ID: Q9AU11) and PKS4 (UniProt ID: B0LDU5) from raspberry (*R. idaeus*) were tested for *p*HBA synthesis from supplemented *p*CA in *C. glutamicum* [26–29]. Both enzymes were described to have a BAS side activity in addition to their CHS activity. Codon-optimized gene variants $pks1_{RiCg}$ and $pks4_{RiCg}$ were used to construct pMKEx2-$pks1_{RiCg}$-$curA_{EcCg}$ and pMKEx2-$pks4_{RiCg}$-$curA_{EcCg}$. For evaluation of (4CL and) BAS activity, the constructed strains were cultivated and analyzed as described above with supplementation of 5 mM *p*CA instead of 3.09 mM *p*HBA. Stacked concentrations of *p*HBA and RK were used to assess the (4CL and) BAS activity (Fig. 4).

Utilization of the already applied *bas* gene from *R. palmatum* enabled RK synthesis from *p*CA in *C. glutamicum*. After 72 h of cultivation in the absence of $udhA_{EcCg}$, 14 mg/L (0.09 mM) RK and 9.4 mg/L (0.06 mM) *p*HBA were detected by HPLC. In contrast to the previous experiments, $udhA_{EcCg}$ expression reduced *p*HBA- and RK synthesis. In addition, neither expression of *pks1* nor *pks4* in combination with *curA* enabled RK synthesis, suggesting that both genes were not functionally expressed in *C. glutamicum*. Since both enzymes are primarily chalcone synthases, it was also tested whether naringenin chalcone, or more precisely, naringenin was formed. However, also no detectable amounts of naringenin were synthesized, indicating that PKS1 and PKS4 might be incorrectly folded in *C. glutamicum*. Previously, N-terminal translational fusion with the maltose binding protein from *E. coli* ($MalE_{Ec}$) was demonstrated to efficiently increase functional expression of heterologous plant genes in *C. glutamicum* [30]. To test whether functional expression of *pks* genes could be achieved by mimicking this strategy, *C. glutamicum* strains harboring the plasmids pMKEx2-$malE_{Ec}$-$pks1_{RiCg}$-$curA_{EcCg}$ and pMKEx2-$malE_{Ec}$-$pks4_{RiCg}$-$curA_{EcCg}$ were constructed and cultivated. Although, general applicability of this strategy was indicated by the formation of RK when expressing

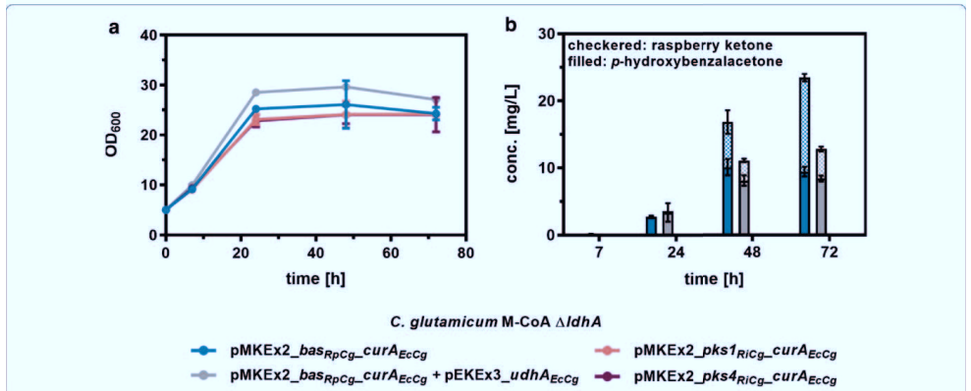

Fig. 4 Evaluation of different benzalacetone synthases for raspberry ketone production from *p*-coumaric acid using *C. glutamicum*. *C. glutamicum* M-CoA Δ*ldhA* harboring the indicated expression plasmids was cultivated in 50 mL CGXII medium with 4% glucose and 5 mM *p*-coumaric acid in baffled flasks at 30 °C and 130 rpm for 72 h. **a** Growth and **b** *p*-hydroxybenzalacetone- and raspberry ketone synthesis was followed over time. Determined concentrations for *p*-hydroxybenzalacetone and raspberry ketone in cell extracts were stacked for a better visualization. The depicted data represent mean values with standard deviations from biological triplicates

$malE_{Ec}$-$pks1_{RiCg}$ and $malE_{Ec}$-$pks4_{RiCg}$, significantly less RK was formed in comparison to BAS_{Rp}.

Taken together, *C. glutamicum* M-CoA Δ*ldhA* harboring pMKEx2-bas_{RpCg}-$curA_{EcCg}$ and pEKEx3-$udhA_{EcCg}$ is regarded as the most suitable strain for the synthesis of RK from supplemented diketide intermediate *p*HBA, whereas additional $udhA_{EcCg}$ expression was not beneficial for the synthesis from *p*CA.

Microbial synthesis of zingerone and benzylacetone

Besides RK, other phenylbutanoids such as the ferulic acid-derived zingerone or the cinnamic acid-derived benzylacetone are of commercial interest as well. Zingerone is regarded as the molecule providing the characteristic flavor of cooked ginger, whereas benzylacetone is described to contribute to the characteristic taste of strawberries and jasmine [31–34]. The respective molecules differ from RK only in their hydroxylation/methoxylation pattern of the aromatic ring (Fig. 1). Thus, it is reasonable to assume that the enzymes of the RK pathway also accept ferulic acid and cinnamic acid (and their derivatives) as substrates, which would offer the opportunity for a combinatorial biosynthesis of zingerone or benzylacetone using the very same *C. glutamicum* strain (Fig. 1). First, confirmation of reductase activity with the respective diketide intermediates of zingerone and benzylacetone synthesis was addressed as formation of the diketide *p*HBA from *p*CA by $4CL_{Pc}$ and BAS_{Rp} was rather inefficient and might be even more challenging with alternative phenylpropanoids as substrates. To this end, CGXII medium was supplemented with the respective diketide precursors (3.09 mM) during cultivations of *C. glutamicum* M-CoA Δ*ldhA*, optionally harboring pMKEx2-bas_{RpCg}-$curA_{EcCg}$. HPLC analysis of extracted samples demonstrated synthesis of 40.2 mg/L (0.21 mM) zingerone upon $curA_{EcCg}$ expression whereas the synthesis of benzylacetone was unaffected (0.6 mg/L, 0.01 mM) indicating that $CurA_{EcCg}$ cannot reduce benzalacetone (Additional file 1: Figure S5). Therefore, benzalacetone appears to be solely reduced by the unknown endogenous reductase activity of *C. glutamicum* yielding benzylacetone. Interestingly, less zingerone (70 mg/L, 0.36 mM) compared to RK (99.8 mg/L, 0.61 mM) was produced from the respective diketide intermediate despite an even higher similarity to the curcumin structure (Fig. 5b).

Nevertheless, reductase activity of the constructed strain *C. glutamicum* M-CoA Δ*ldhA* carrying pMKEx2-bas_{RpCg}-$curA_{EcCg}$ and pEKEx3-$udhA_{EcCg}$ was verified for all tested substitution patterns of the aromatic ring.

To evaluate substrate promiscuity of the diketide forming enzymes $4CL_{Pc}$ and BAS_{Rp}, *C. glutamicum* M-CoA Δ*ldhA* harboring pMKEx2-bas_{RpCg}-$curA_{EcCg}$ and pEKEx3-$udhA_{EcCg}$ was cultivated using standard conditions with supplementation of the respective phenylpropanoids (5 mM). Extracted samples were analyzed by HPLC for the presence of respective diketides and ketones (Fig. 5d). After 72 h of cultivation, 7.9 mg/L (0.05 mM) *p*HBA and 4.7 mg/L (0.05 mM) RK

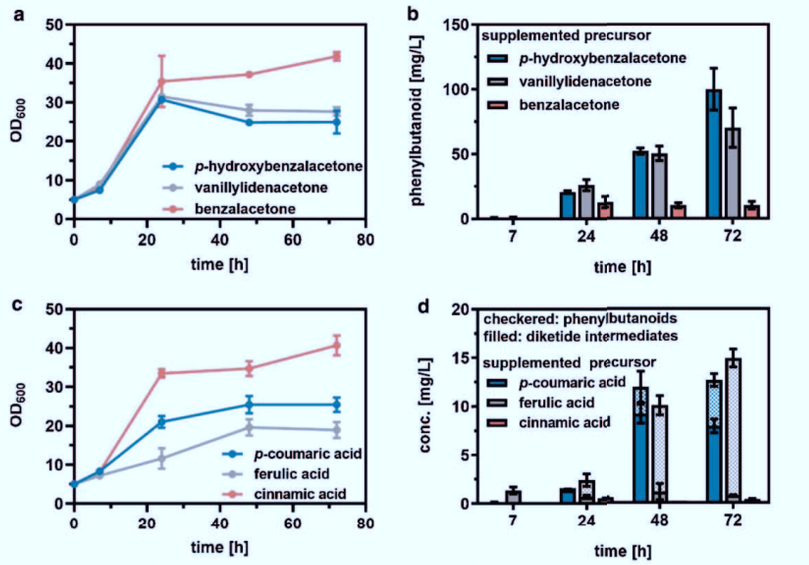

Fig. 5 Combinatorial biosynthesis of phenylbutanoids zingerone and benzylacetone from supplemented phenylpropanoid- and diketide precursors using *C. glutamicum* M-CoA Δ*ldhA* pMKEx2-bas_{RpCg}-$curA_{EcCg}$ pEKEx3-$udhA_{EcCg}$. Cultivations were performed in 50 mL CGXII medium with 4% glucose and either 3.09 mM diketide or 5 mM phenylpropanoid precursors in baffled flasks at 30 °C and 130 rpm for 72 h. Here, supplementation of *p*-coumaric acid and *p*-hydroxybenzalacetone for the synthesis of RK served as a positive control. **a** Microbial growth and **b** phenylbutanoid synthesis from supplemented diketides. **c** Microbial growth and **d** phenylbutanoid synthesis from supplemented phenylpropanoids. Where appropriate, determined concentrations for diketides (filled bars) and phenylbutanoids (checkered bars) were stacked for a better data visualization. Depicted data represent mean values with standard deviations from biological triplicates

were formed from *p*CA. When supplementing either ferulic acid or cinnamic acid, 0.8 mg/L (0.01 mM) vanillylidenacetone and 14.1 mg/L (0.07 mM) zingerone or 0.4 mg/L (0.01 mM) benzalacetone but no detectable benzylacetone was formed, respectively.

In principle, the precursors and intermediates of zingerone and benzylacetone synthesis can be converted also by the heterologous pathway for RK synthesis established in *C. glutamicum*. Nevertheless, benzylacetone could not be produced from cinnamic acid, probably due to the insufficient synthesis of the diketide intermediate benzalacetone. Contrary to previous results obtained from cultivations with supplemented diketide intermediates, the reduction of vanillylidenacetone appears to be more efficient compared to *p*HBA reduction, as almost all vanillylidenacetone synthesized was converted to zingerone.

Discussion

In this study, we constructed a *C. glutamicum* variant for the microbial synthesis of the flavoring phenylbutanoids RK, zingerone and benzylacetone. Initial cytotoxicity experiments of *p*HBA and RK suggested *C. glutamicum* to be more resistant to these compounds compared to *E. coli* and *S. cerevisiae*. For the latter two microorganisms, half maximal inhibitory concentrations (IC_{50}) have been calculated for both *S. cerevisiae* and *E. coli* [1]. Here, concentrations of 100 mg/L or 300 mg/L *p*HBA and 500 mg/L or 900 mg/L were determined for *S. cerevisiae* and *E. coli* to reduce biomass formation by 50%, respectively. Since the calculation of IC_{50} values for *C. glutamicum* would be imprecise due to insufficient data for higher concentrations of both molecules, we cannot provide exact concentrations. Nevertheless, the cytotoxicity experiments allow to consider *C. glutamicum* to be more resistant to both *p*HBA and RK as the IC_{50} concentrations have to be > 500 mg/L and > 1000 mg/L, respectively. More importantly, the constructed strain *C. glutamicum* M-CoA Δ*ldhA* pMKEx2-bas_{RpCg}-$curA_{EcCg}$ accumulates up to 14 mg/L (0.09 mM) RK from supplemented *p*CA, which is comparable to the product titer determined for a *S. cerevisiae* strain (7.5 mg/L RK (0.05 mM)) [13].

However, synthesis of 91 mg/L (0.55 mM) RK from *p*CA using an engineered *E. coli* BL21(DE3) variant was recently reported [8].

Moreover, a yet unknown substrate promiscuity of the NADPH-dependent curcumin/dihydrocurcumin reductase CurA from *E. coli* MG1655 allowing for the efficient reduction of *p*HBA and vanillylidenacetone, respectively, was identified. Although *E. coli* BL21 has been previously reported to possess an endogenous BAR activity, this activity presumably cannot be traced back to CurA as this particular gene is not present in the utilized strain background [1]. Thus, it is likely that at least one additional reductase also features BAR activity in *E. coli* BL21. We presumed a possible enzymatic activity of CurA with *p*HBA due to structural similarities with its natural substrate curcumin (Additional file 1: Figure S1). As the additional methoxy group of vanillylidenacetone increases structural similarity to curcumin even more, a more efficient conversion to the corresponding ketone compared to the RK branch was expected prior to the conducted experiments. Indeed, when producing flavoring phenylbutanoids from supplemented phenylpropanoids, an almost complete conversion of vanillylidenacetone to zingerone was observed, whereas *p*HBA reduction was less efficient.

Furthermore, a hitherto unknown endogenous BAR activity must be also present in *C. glutamicum* as demonstrated by the reduction of the three tested diketide intermediates in the absence of $curA_{EcCg}$. Apart from *E. coli* and *C. glutamicum*, such an activity has already been described for *S. cerevisiae* [13].

To increase NADPH supply for the efficient reduction of diketide intermediates, heterologous expression of genes encoding the membrane-bound as well as the cytoplasmatic transhydrogenases from *E. coli* was evaluated. Despite already being used to increase NADPH availability in an isobutanol producing *C. glutamicum* variant, the membrane-bound transhydrogenase PntAB turned out to be unsuitable for RK synthesis with the same bacterium [22]. With increasing induction strength, a severe growth defect in *C. glutamicum* was observed upon *pntAB* expression. This might indicate cytotoxic effects of the transhydrogenase itself, but also the absence of chaperones supporting folding or an altered membrane composition could be the cause [35]. Contrary, the cytoplasmatic transhydrogenase UdhA was beneficial for RK synthesis resembling a promising alternative to the membrane-bound PntAB. Despite being rather involved in the energy-independent hydride ion transfer from NADPH to NAD^+ in vivo, UdhA can still catalyze the transfer in the reverse direction [23]. Nevertheless, the equilibrium of the transhydrogenation reaction could be shifted towards NADPH, when NADPH is constantly withdrawn by the reduction of *p*HBA to RK. Furthermore, deletion of *ldhA* increases NADH availability, which in turn also shifts the equilibrium of the transhydrogenase reaction towards NADPH. Further strategies for increased NADPH supply in *C. glutamicum* involve altering the coenzyme specificity of the NAD^+-dependent glyceraldehyde 3-phophate dehydrogenase (GAPDH) to $NADP^+$, which was done in the context of L-lysine production with *C. glutamicum* [36]. It should be noted, that an imbalanced NADH/NADPH distribution could perturb the cellular metabolism and might even inhibit cellular growth or glucose consumption [36].

Interestingly, expression of $udhA_{EcCg}$ appears to be disadvantageous for the synthesis of *p*HBA and RK from *p*CA. NADPH availability could not be limiting for the small amounts of *p*HBA produced from *p*CA, so that the described positive effect of additional NADPH supply only become significant at higher *p*HBA concentrations. The reduced cumulated titer of *p*HBA and RK might be due to the increased metabolic burden of the cell due to maintenance of the pEKEx3-$udhA_{EcCg}$ plasmid (requiring supplementation of a second antibiotic and expression of an additional antibiotic resistance gene) [37, 38].

Conclusion

In the present work, we extended the product portfolio of *C. glutamicum* towards flavoring phenylbutanoids. We identified an endogenous BAR activity of *C. glutamicum* and a yet unknown substrate promiscuity of CurA from *E. coli* that turned out to be a promising BAR. Moreover, the cytoplasmatic transhydrogenase UdhA from *E. coli* allowed for increased NADPH supply and ultimately improved RK synthesis. Taken together, the constructed strain *C. glutamicum* M-CoA Δ*ldhA* harboring pMKEx2-bas_{RpCg}-$curA_{EcCg}$ and pEKEx3-$udhA_{EcCg}$ represents a versatile host for the synthesis of up to 99.8 mg/L (0.61 mM) RK, 70 mg/L (0.36 mM) zingerone and 10.5 mg/L (0.07 mM) benzylacetone.

Materials and methods

Bacterial strains, plasmids, media and growth conditions

All bacterial strains and plasmids with their respective characteristics used in this study are listed in Table 1. *C. glutamicum* strains were routinely cultivated aerobically at 30 °C in brain heart infusion (BHI) medium (Difco Laboratories, Detroit, USA) or defined CGXII medium with 4% (w/v) glucose as sole carbon and energy source [39]. *E. coli* DH5α, used solely for plasmid constructions, was cultivated in LB medium at 37 °C [40]. Where appropriate, kanamycin (*E. coli* 50 µg/mL, *C. glutamicum* 25 µg/mL) and/or spectinomycin (100 µg/mL for *E. coli* and *C. glutamicum*) was added to the respective medium.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source
C. glutamicum strains		
DelAro^4-*4cl*$_{Pc}$ C5 mu*fasO*$_{BCD1}$ P$_{O6}$-*iolT1* Δ*pyc* (M-CoA)	*C. glutamicum* derivative with in-frame deletions of cg0344-47, cg0503 cg2625-40 and cg1226; harboring a chromosomally encoded codon-optimized *4cl*$_{Pc}$ gene coding for 4-coumarate:CoA ligase from *P. crispum* under control of the T7 promoter and replacement of the native *gltA* promotor with the *dapA* promotor variant C5, mutated *fasO* binding sites upstream of *accBC* and *accD1*, two nucleotide exchanges in the *iolT1* promotor and in-frame deletion of *pyc*	[16]
M-CoA Δ*ldhA*	*C. glutamicum* M-CoA derivative with in-frame deletion of *ldhA*	This work
E. coli strains		
DH5α	F$^-$Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR*17 (r_K^-,m_K^+) *phoA supE*44 λ$^-$ *thi-1 gyrA*96 *relA1*	Invitrogen (Karlsruhe, Germany)
Plasmids		
pK19*mobsacB*-Δ*ldhA*	Vector for in-frame deletion of *ldhA*	[46]
pMKEx2	*kan*^{r}; *E. coli*-*C. glutamicum* shuttle vector (*lacI*, P_{T7}, *lacO1*, pHM1519 ori$_{Cg}$; pACYC177 ori$_{Ec}$)	[21]
pMKEx2-*bas*$_{RpCg}$-*rzs1*$_{RiCg}$	*kan*^{r}; pMKEx2 derivative containing codon-optimized genes encoding benzalacetone synthase from *R. palmatum* (*bas*$_{RpCg}$) and NADPH-dependent raspberry ketone/zingerone reductase from *R. idaeus* (*rzs1*$_{RiCg}$)	This work
pMKEx2-*bas*$_{RpCg}$-*rzs1*$_{RiCg}$-G191D	*kan*^{r}; pMKEx2-*bas*$_{RpCg}$-*rzs1*$_{RiCg}$ derivative with mutations in the rzs1$_{RiCg}$ nucleotide sequence leading to amino acid substitution G191D in RZS1$_{Ri}$.	This work
pMKEx2-*bas*$_{RpCg}$-*curA*$_{Ec}$	*kan*^{r}; pMKEx2 derivative containing a codon-optimized gene encoding benzalacetone synthase from *R. palmatum* (*bas*$_{RpCg}$) and the native gene encoding NADPH-dependent curcumin/dihydrocurcumin reductase CurA from *E. coli* (*curA*$_{Ec}$)	This work
pMKEx2-*bas*$_{RpCg}$-*curA*$_{EcCg}$	*kan*^{r}; pMKEx2 derivative containing codon-optimized genes encoding benzalacetone synthase from *R. palmatum* (*bas*$_{RpCg}$) and NADPH-dependent curcumin/dihydrocurcumin reductase CurA from *E. coli* (*curA*$_{EcCg}$)	This work
pMKEx2-*pks1*$_{RiCg}$-*curA*$_{EcCg}$	*kan*^{r}; pMKEx2 derivative containing codon-optimized genes encoding PKS1 from *R. idaeus* (*pks1*$_{RiCg}$) and NADPH-dependent curcumin/dihydrocurcumin reductase CurA from *E. coli* (*curA*$_{EcCg}$)	This work
pMKEx2-*pks4*$_{RiCg}$-*curA*$_{EcCg}$	*kan*^{r}; pMKEx2 derivative containing codon-optimized genes encoding PKS4 from *R. idaeus* (*pks4*$_{RiCg}$) and NADPH-dependent curcumin/dihydrocurcumin reductase CurA from *E. coli* (*curA*$_{EcCg}$)	This work
pMKEx2-*malE*$_{Ec}$-*pks1*$_{RiCg}$-*curA*$_{EcCg}$	*kan*^{r}; pMKEx2 derivative containing the native *malE* gene from *E. coli* fused to the codon-optimized gene encoding PKS1 from *R. idaeus* (*malE*$_{Ec}$-*pks1*$_{RiCg}$) and a codon-optimized gene encoding NADPH-dependent curcumin/dihydrocurcumin reductase CurA from *E. coli* (*curA*$_{EcCg}$)	This work
pMKEx2-*malE*$_{Ec}$-*pks4*$_{RiCg}$-*curA*$_{EcCg}$	*kan*^{r}; pMKEx2 derivative containing the native *malE* gene from *E. coli* fused to the codon-optimized gene encoding PKS4 from *R. idaeus* (*malE*$_{Ec}$-*pks4*$_{RiCg}$) and a codon-optimized gene encoding NADPH-dependent curcumin/dihydrocurcumin reductase CurA from *E. coli* (*curA*$_{EcCg}$)	This work
pEKEx3	*spec*^{r}; *E. coli*-*C. glutamicum* shuttle vector (*lacI*,P_{tac}, *lacO1*, pBL1ori$_{Cg}$; pUCori$_{Ec}$)	[47]
pEKEx3-*pntAB*$_{Ec}$	*spec*^{r}; pEKEx3 derivative containing native *pntAB* genes from *E. coli* (*pntAB*$_{Ec}$) encoding a membrane-bound transhydrogenase	This work
pEKEx3-*udhA*$_{EcCg}$	*spec*^{r}; pEKEx3 derivative containing codon-optimized *udhA* gene variant from *E. coli* (*udhA*$_{EcCg}$) encoding a cytoplasmatic transhydrogenase	This work
pEKEx3-*malE*$_{Ec}$-*omt*$_{Vv}$	*spec*^{r}; pEKEx3 derivative containing *malE* gene from *E. coli* (*malE*$_{Ec}$) fused to the codon-optimized gene coding for resveratrol-di-*O*-methyltransferase from *V. vinifera* (*omt*$_{Vv}$)	[30]

Bacterial growth was followed by measuring the optical density at 600 nm (OD_{600}).

To cultivate *C. glutamicum*, a test tube with 5 mL BHI medium was inoculated with a single colony from an agar plate and grown for 6–8 h on a rotary shaker at 170 rpm (first preculture). This first preculture was used to inoculate 50 mL of defined CGXII medium with 4% (w/v) glucose in a 500 mL baffled Erlenmeyer flask (second preculture). The second preculture was cultivated overnight on a rotary shaker at 130 rpm. The main culture was subsequently inoculated from the second preculture to the indicated OD_{600} in defined CGXII medium with 4% (w/v) glucose. For microbial synthesis of phenylbutanoids, the main culture was inoculated to an OD_{600} of 5 in defined CGXII medium with 4% glucose and heterologous gene expression was induced 90 min after inoculation using 1 mM IPTG. 1 mL of the culture broth was sampled at

defined time points and stored at − 20 °C until ethyl acetate extraction and HPLC analysis.

For evaluating cytotoxicity of *p*HBA and RK, *C. glutamicum* M-CoA was cultivated at 30 °C, 900 rpm and a humidity of 85% in 48-well Flowerplates containing 800 µL CGXII medium with 4% (w/v) glucose inoculated to an OD_{600} of 1, using the BioLector microbioreactor (m2p-labs, Baesweiler, Germany). Increasing concentrations of both *p*HBA and RK (final concentrations 0, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 mg/L), dissolved in 10 µL DMSO were added. Online measurement of the backscattered light intensity (620 nm, gain 10) was used for evaluation of cellular growth. To estimate IC_{50} values, obtained backscattered light intensities after 72 h were plotted against the respective *p*HBA- and RK concentrations and subsequently analyzed using the GraphPad Prism 8.1.2 software (San Diego, CA, USA). The nonlinear regression *[inhibitor] vs. response − Variable slope (four parameters)* with the following specifications was used: top = 165, bottom = 15, as well as $IC_{50} > 0$. The values for top and bottom correspond to the mean values for the determined backscatter values after 72 h in the absence of *p*HBA or RK, or the average value for the backscatter after 72 h in the presence of 1000 mg/L (6.17 mM) *p*HBA.

Plasmid and strain construction

Standard protocols of molecular cloning, such as PCR, restriction and ligation of DNA were carried out for recombinant DNA work [41]. All enzymes were obtained from Thermo Fisher Scientific (Schwerte, Germany). Codon-optimized synthetic genes for *C. glutamicum* ATCC13032 were obtained from Thermo Fisher Scientific (formerly GeneArt, Darmstadt, Germany). Genes and chromosomal fragments were amplified by PCR from synthetic genes or genomic *E. coli* DNA as template using the listed oligonucleotides (Additional file 1: Table S1). PCR products were subsequently used to clone genes and genomic fragments into plasmid vectors using Gibson assembly [42]. In-frame gene deletions in the *C. glutamicum* genome were performed using the pK19*mobsacB* system by a two-step homologous recombination method described previously [43, 44]. Integrity of all constructed plasmids was verified by colony PCR, restriction analysis, and DNA sequencing at Eurofins MWG Operon (Ebersberg, Germany) Techniques specific for *C. glutamicum*, e.g. electroporation of cells, were performed as described previously [45].

Ethyl acetate extraction and HPLC quantification

Phenylbutanoids and pathway intermediates were extracted from cultivation broth for subsequent HPLC analysis by mixing 1 mL of the culture broth with 1 mL ethyl acetate and vigorous shaking (1400 rpm, 10 min, 20 °C) in a Thermomixer (Eppendorf, Hamburg, Germany). The suspension was centrifuged for 5 min at 13,000 rpm and the upper ethyl acetate layer (800 µL) was transferred to an organic solvent resistant deep-well plate (Eppendorf, Hamburg, Germany). After evaporation to dryness, extracts were resuspended in the same volume of acetonitrile and subsequently used for HPLC analysis.

Metabolites were quantified using an Agilent high-performance liquid chromatography (HPLC) 1260 Infinity System equipped with a 1260 Infinity DAD (Agilent Technologies, Waldbronn, Germany). Authentic standards of benzalacetone, benzylacetone, cinnamic acid, ferulic acid, *p*-coumaric acid, vanillylidenacetone and zingerone were purchased from Sigma-Aldrich (Taufkirchen, Germany), *p*-hydroxybenzalacetone was obtained from Alfa Aesar (Kandel, Germany) and raspberry ketone from Acros Organics (Geel, Belgium). LC separation was carried out with an InfinityLab Poroshell 120 2.7 µm EC-C_{18} column (3.0 × 150 mm; Agilent Technologies, Waldbronn, Germany) 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 0.7 mL/min. Depending on the analyte, a different elution gradient was used, where the amount of solvent B was increased stepwise. *Raspberry ketone*: minute 0–10: 10%, minute 10–11: 10–90%, minute 11–13: 90%, minute 13–15: 90–10% and minute 15–17: 10%. Absorption was determined at 275 nm (raspberry ketone), 310 nm (*p*-coumaric acid) and 320 nm (*p*-hydroxybenzalacetone). *Zingerone*: minute 0–15: 10%, minute 15–16: 10–90%, minute 16–18: 90%, minute 18–20: 90–10% and minute 20–22: 10%. Absorption was determined at 275 nm (zingerone) and 320 nm (ferulic acid and vanillylidenacetone). *Benzylacetone*: minute 0–13: 10–50%, minute 13–15: 50%, minute 15–17: 50–10% and minute 17–19: 10%. Absorption was determined at 260 nm (benzylacetone) and 320 nm (cinnamic acid and benzalacetone). Area values of integrated signals were linear up to metabolite concentrations of at least 83.3 mg/L.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12934-020-01351-y.

Additional file 1. Additional information containing a list of oligonucleotides used in this study, the chemical structure of curcumin with highlighted *p*HBA structure and additional cultivation results.

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Authors' contributions
LM and MM conceived the design of this study. LM and MM performed the experimental work. LM and JM wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional files.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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

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References

1. Beekwilder J, Van der Meer IM, Sibbbesen O, Broekgaarden M, Qvist I, Mikkelsen JD, et al. Microbial production of natural raspberry ketone. Biotechnol J. 2007;2:1270–9. https://doi.org/10.1002/biot.200700076.
2. Bredsdorff L, Wedebye EB, Nikolov NG, Hallas-Møller T, Pilegaard K. Raspberry ketone in food supplements—high intake, few toxicity data—a cause for safety concern? Regul Toxicol Pharmacol. 2015;73:196–200. https://doi.org/10.1016/j.yrtph.2015.06.022.
3. Kim M, Baek HS, Lee M, Park H, Shin SS, Choi DW, et al. Rhododenol and raspberry ketone impair the normal proliferation of melanocytes through reactive oxygen species-dependent activation of GADD45. Toxicol In Vitro. 2016;32:339–46. https://doi.org/10.1016/j.tiv.2016.02.003.
4. Lee J. Further research on the biological activities and the safety of raspberry ketone is needed. NFS J. 2016;2:15–8. https://doi.org/10.1016/j.nfs.2015.12.001.
5. Morimoto C, Satoh Y, Hara M, Inoue S, Tsujita T, Okuda H. Anti-obese action of raspberry ketone. Life Sci. 2005;77:194–204. https://doi.org/10.1016/j.lfs.2004.12.029.
6. Wang L, Meng X, Zhang F. Raspberry ketone protects rats fed high-fat diets against nonalcoholic steatohepatitis. J Med Food. 2012;15:495–503. https://doi.org/10.1089/jmf.2011.1717.
7. Larsen M, Poll L, Callesen O, Lewis M. Relations between the content of aroma compounds and the sensory evaluation of 10 raspberry varieties (*Rubus idaeus* L). Acta Agric Scard. 1991;41:447–54. https://doi.org/10.1080/00015129109439927.
8. Wang C, Zheng P, Chen P. Construction of synthetic pathways for raspberry ketone production in engineered *Escherichia coli*. Appl Microbiol Biotechnol. 2019;103:3715–25. https://doi.org/10.1007/s00253-019-09748-5.
9. Vandamme EJ, Soetaert W. Bioflavours and fragrances via fermentation and biocatalysis. J Chem Technol Biotechnol. 2002;77:1323–32. https://doi.org/10.1002/jctb.722.
10. Smith LR. Rheosmin ("raspberry ketone") and zingerone, and their preparation by crossed aldol-catalytic hydrogenation sequences. Chem Educ. 1996;1:1–18. https://doi.org/10.1007/s00397960034a.
11. European Council. Regulation (EC) No 1334/2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. 2008.
12. Borejsza-Wysocki W, Hrazdina G. Biosynthesis of *p*-hydroxyphenylbutan-2-one in raspberry fruits and tissue cultures. Phytochemistry. 1994;35:623–8. https://doi.org/10.1016/S0031-9422(00)90575-2.
13. Lee D, Lloyd NDR, Pretorius IS, Borneman AR. Heterologous production of raspberry ketone in the wine yeast *Saccharomyces cerevisiae* via pathway engineering and synthetic enzyme fusion. Microb Cell Fact. 2016;15:1–7. https://doi.org/10.1186/s12934-016-0446-2.
14. Koeduka T, Watanabe B, Suzuki S, Hiratake J, Mano J, Yazaki K. Characterization of raspberry ketone/zingerone synthase, catalyzing the alpha, beta-hydrogenation of phenylbutenones in raspberry fruits. Biochem Biophys Res Commun. 2011;412:104–8. https://doi.org/10.1016/j.bbrc.2011.07.052.
15. Kallscheuer N, Vogt M, Stenzel A, Gätgens J, Bott M, Marienhagen J. Construction of a *Corynebacterium glutamicum* platform strain for the production of stilbenes and (2*S*)-flavanones. Metab Eng. 2016;38:47–55. https://doi.org/10.1016/j.ymben.2016.06.003.
16. Milke L, Kallscheuer N, Kappelmann J, Marienhagen J. Tailoring *Corynebacterium glutamicum* towards increased malonyl-CoA availability for efficient synthesis of the plant pentaketide noreugenin. Microb Cell Fact. 2019;18:71. https://doi.org/10.1186/s12934-019-1117-x.
17. Kallscheuer N, Kage H, Milke L, Nett M, Marienhagen J. Microbial synthesis of the type I polyketide 6-methylsalicylate with *Corynebacterium glutamicum*. Appl Microbiol Biotechnol. 2019;103:9619–31. https://doi.org/10.1007/s00253-019-10121-9.
18. Milke L, Ferreira P, Kallscheuer N, Braga A, Vogt M, Kappelmann J, et al. Modulation of the central carbon metabolism of *Corynebacterium glutamicum* improves malonyl-CoA availability and increases plant polyphenol synthesis. Biotechnol Bioeng. 2019;116:1380–91. https://doi.org/10.1002/bit.26939.
19. Milke L, Aschenbrenner J, Marienhagen J, Kallscheuer N. Production of plant-derived polyphenols in microorganisms: current state and perspectives. Appl Microbiol Biotechnol. 2018;102:1575–85. https://doi.org/10.1007/s00253-018-8747-5.
20. Hassaninasab A, Hashimoto Y, Tomita-Yokotani K, Kobayashi M. Discovery of the curcumin metabolic pathway involving a unique enzyme in an intestinal microorganism. Proc Natl Acad Sci. 2011;108:6615–20. https://doi.org/10.1073/pnas.1016217108.
21. Kortmann M, Kuhl V, Klaffl S, Bott M. A chromosomally encoded T7 RNA polymerase-dependent gene expression system for *Corynebacterium glutamicum*: construction and comparative evaluation at the single-cell level. Microb Biotechnol. 2015;8:253–65. https://doi.org/10.1111/1751-7915.12236.
22. Blombach B, Riester T, Wieschalka S, Ziert C, Youn JW, Wendisch VF, et al. *Corynebacterium glutamicum* tailored for efficient isobutanol production. Appl Environ Microbiol. 2011;77:3300–10. https://doi.org/10.1128/AEM.02972-10.
23. Sauer U, Canonaco F, Heri S, Perrenoud A, Fischer E. The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*. J Biol Chem. 2004;279:6613–9. https://doi.org/10.1074/jbc.M311657200.

24. Jackson JB. Proton translocation by transhydrogenase. FEBS Lett. 2003;545:18–24. https://doi.org/10.1016/S0014-5793(03)00388-0.
25. Reddy GK, Lindner SN, Wendisch VF. Metabolic engineering of an ATP-neutral Embden–Meyerhof–Parnas pathway in *Corynebacterium glutamicum*: growth restoration by an adaptive point mutation in NADH dehydrogenase. Appl Environ Microbiol. 2015;81:1996–2005. https://doi.org/10.1128/AEM.03116-14.
26. Abe I, Takahashi Y, Morita H, Noguchi H. Benzalacetone synthase. A novel polyketide synthase that plays a crucial role in the biosynthesis of phenylbutanones in *Rheum palmatum*. Eur J Biochem. 2001;268:3354–9. https://doi.org/10.1046/j.1432-1327.2001.02255.x.
27. Shimokawa Y, Morita H, Abe I. Benzalacetone synthase. Front Plant Sci. 2012;3:57. https://doi.org/10.3389/fpls.2012.00057.
28. Zheng D, Schröder G, Schröder J, Hrazdina G. Molecular and biochemical characterization of three aromatic polyketide synthase genes from *Rubus idaeus*. Plant Mol Biol. 2001;46:1–15. https://doi.org/10.1023/A:1010642517738.
29. Zheng D, Hrazdina G. Molecular and biochemical characterization of benzalacetone synthase and chalcone synthase genes and their proteins from raspberry (*Rubus idaeus* L.). Arch Biochem Biophys. 2008;470:139–45. https://doi.org/10.1016/j.abb.2007.11.013.
30. Kallscheuer N, Vogt M, Bott M, Marienhagen J. Functional expression of plant-derived *O*-methyltransferase, flavanone 3-hydroxylase, and flavonol synthase in *Corynebacterium glutamicum* for production of pterostilbene, kaempferol, and quercetin. J Biotechnol. 2017;258:190–6. https://doi.org/10.1016/j.jbiotec.2017.01.006.
31. Kuhn W, Funk H-U, Senft G. Process for the preparation of benzylacetone. US 6,441,242 B1, 2002.
32. Parthasarathy VA, Chempakam B, Zachariah TJ. Chemistry of spices. vol. 32. 2008. https://doi.org/10.4327/jsnfs1949.32.267.
33. Rajan I, Narayanan N, Rabindran R, Jayasree PR, Manish Kumar PR. Zingerone protects against stannous chloride-induced and hydrogen peroxide-induced oxidative DNA damage in vitro. Biol Trace Elem Res. 2013;155:455–9. https://doi.org/10.1007/s12011-013-9801-x.
34. Svetaz LA, Di Liberto MG, Zanardi MM, Suárez AG, Zacchino SA. Efficient production of the flavoring agent zingerone and of both (*R*)- and (*S*)-zingerols via green fungal biocatalysis. Comparative antifungal activities between enantiomers. Int J Mol Sci. 2014;15:22042–58. https://doi.org/10.3390/ijms151222042.
35. Wagner S, Bader ML, Drew D, de Gier JW. Rationalizing membrane protein overexpression. Trends Biotechnol. 2006;24:364–71. https://doi.org/10.1016/j.tibtech.2006.06.008.
36. Bommareddy RR, Chen Z, Rappert S, Zeng A. A de novo NADPH generation pathway for improving lysine production of *Corynebacterium glutamicum* by rational design of the coenzyme specificity of glyceraldehyde 3-phosphate dehydrogenase. Metab Eng. 2014;25:30–7. https://doi.org/10.1016/j.ymben.2014.06.005.
37. Glick BR. Metabolic load and heterologous gene expression. Biotechnol Adv. 1995;13:247–61. https://doi.org/10.1016/0734-9750(95)00004-a.
38. Wu G, Yan Q, Jones JA, Tang YJ, Fong SS, Koffas MAG. Metabolic Burden: cornerstones in synthetic biology and metabolic engineering applications. Trends Biotechnol. 2016;34:652–64. https://doi.org/10.1016/j.tibtech.2016.02.010.
39. Keilhauer C, Eggeling L, Sahm H. Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. J Bacteriol. 1993;175:5595–603. https://doi.org/10.1128/jb.175.17.5595-5603.1993.
40. Bertani G. Studies on lysogenesis. I. The mode of phage liberation by lysogenis *Escherichia coli*. J Bacteriol. 1951;62:293–300.
41. Green MR, Sambrook J. Molecular cloning: a laboratory manual. 4th ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 2012.
42. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods. 2009;6:343–5. https://doi.org/10.1038/nmeth.1318.
43. Niebisch A, Bott M. Molecular analysis of the cytochrome bc_1-aa_3 branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome c_1. Arch Microbiol. 2001;175:282–94. https://doi.org/10.1007/s002030100262.
44. Schäfer A, Tauch A, Jaeger W, Kalinowski J, Thierbach G, Pühler A. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene. 1994;145:69–73. https://doi.org/10.1016/0378-1119(94)90324-7.
45. Eggeling L, Bott M. Handbook of *Corynebacterium glutamicum*. Boca Raton: CRC Press; 2005. https://doi.org/10.1201/9781420039696.
46. Litsanov B, Brocker M, Bott M. Toward homosuccinate fermentation: metabolic engineering of *Corynebacterium glutamicum* for anaerobic production of succinate from glucose and formate. Appl Environ Microbiol. 2012;78:3325–37. https://doi.org/10.1128/AEM.07790-11.
47. Gande R, Dover LG, Krumbach K, Besra GS, Sahm H, Oikawa T, et al. The two carboxylases of *Corynebacterium glutamicum* essential for fatty acid and mycolic acid synthesis. J Bacteriol. 2007;189:5257–64. https://doi.org/10.1128/JB.00254-07.

Publisher's Note

2.5.1 Supplementary material

Additional file

Synthesis of the character impact compound raspberry ketone and additional flavoring phenylbutanoids of biotechnological interest with *Corynebacterium glutamicum*

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Keywords: NADPH-dependent curcumin reductase, benzalacetone reductase, *Corynebacterium glutamicum*, raspberry ketone, metabolic engineering, character impact compound.

Additional file

Table S1: Oligonucleotides used in this study.

Primer	Sequence (5'→3')
bas_{RpCg}-s	ATTGTGAGCGGATAACAATTCCCCTCTAGA**AAGGAGG**TCGAAGATGGCA ACCGAAGAAATG
bas_{RpCg}-as	CTCCTTTAGCACCATGGTTAGGAGATCACTGGCAC
$rzs1_{RiCg}$-s	AGTGATCTCCTAACCATGGTGCTA**AAGGAGG**TCGAAGATGGCATCCGGT GGCGAA
$rzs1_{RiCg}$-as	TCTGCGGCCGCGTCGACTTGTACAGGATCCTTATTCACGGGACACCACC AC
$rzs1_{RiCg}$-G191D-s	CCTTTTCTTTGGAATCTGCGGAGCCCACCACGTA
$rzs1_{RiCg}$-G191D-as	GGTGGGCTCCGCAGATTCCAAAGAAAAGGTGGATC
$curA_{Ec}$-s	CGTGCCAGTGATCTCCTAACCATGGTGCTA**AAGGAGG**TCGAAGATGGGG CAACAAAAGCAG
$curA_{Ec}$-as	GGCCGCGTCGACTTGTACAGGATCCTTAATCATCACCCGCCAC
$curA_{EcCg}$-s	GATCTCCTAACCATGGTGCTA**AAGGAGG**TCGAAGATGGGCCAGCAGAAG CAG
$curA_{EcCg}$-as	GGCCGCGTCGACTTGTACAGGATCCTTAATCGTCGCCAGCCAC
$pntA_{Ec}$-s	CCTGCAGGTCGACTCTAGAGGATCC**AAGGAGG**TCATATCATGGAAGGGA ATATCATGC
$pntA_{Fc}$-as	ACGATTCCTCCTTGTACATTCACGGCCAGATTTAATTTTTGCGGAACATTT TC
$pntB_{Ec}$-s	ATCTGGCCGTGAATGTAC**AAGGAGG**AATCGTATGTCTGGAGGATTAGTTA CAGCT
$pntB_{Ec}$-as	CTGTAAAACGACGGCCAGTGAATTCTTACAGAGCTTTCAGGATTG
$udhA_{EcCg}$-s	CCTGCAGGTCGACTCTAGAGGATCC**AAGGAGG**TCATATCATGGGCCTGG TGAAGCAG
$udhA_{EcCg}$-as	CTGTAAAACGACGGCCAGTGAATTCTTAGATATCTTCGATCAGGTGTGC
$pks1_{RiCg}$-s	GAGCGGATAACAATTCCCCTCTAGA**AAGGAGG**TCGAAGATGGTGACCGT GGATGAAG
$pks1_{RiCg}$-as	CTTCGACCTCCTTTAGCACCATGGTTAGGTGGATGCTGCCAC
$pks4_{RiCg}$-s	GAGCGGATAACAATTCCCCTCTAGA**AAGGAGG**TCGAAGATGGTGACCGT GGAAGAAG
$pks4_{RiCg}$-as	CTTCGACCTCCTTTAGCACCATGGTTACACCAGGGAGAACAG
$malE_{Ec}$-s	GAGCGGATAACAATTCCCCTCTAGA**AAGGAGG**TCGAAGATGAAAACTGA AGAAGGTAAACTGGTAATCTG
$malE_{Ec}$-as	CCACGGTCACGCCGGAACCGGAAGAGGA
$malE_{Ec}$-$pks1_{RiCg}$-s	CGGTTCCGGCGTGACCGTGGAAGAAGTG
$malE_{Ec}$-$pks4_{RiCg}$-s	CGGTTCCGGCGTGACCGTGGATGAAGTG
chk-pMKEx2-s	CCCTCAAGACCCGTTTAGAGGC
chk-pMKEx2-as	TTAATACGACTCACTATAGGGGAATTGTGAGC
chk-ldhA-s	GTGCGATGCCTGATCAATCCCACAACCG
chk-ldhA-as	GGTTTCATCGTCGTGTGCACAGTTG

C. glutamicum ribosome binding sites are highlighted in bold, relevant restriction sites are underlined.

Additional file

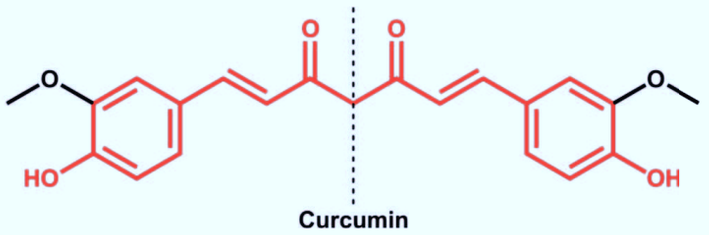

Figure S1: Chemical structure of curcumin. The structure of *p*-hydroxybenzalacetone can be found twice in the curcumin structure as highlighted by the red coloration and dashed line.

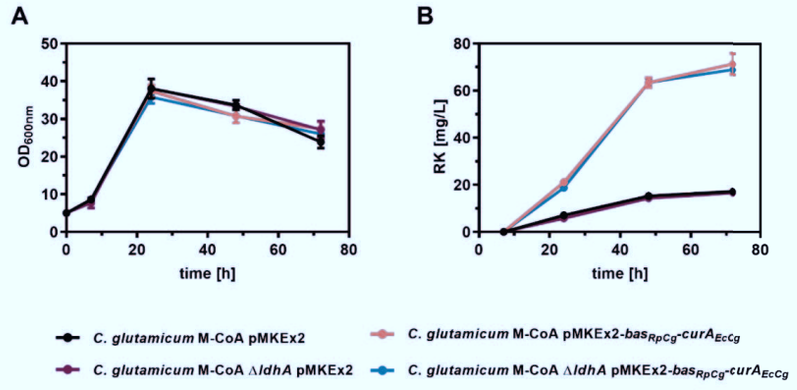

Figure S2: Effect of *ldhA* deletion on microbial (A) growth of *C. glutamicum* and (B) benzalacetone reductase activity. Indicated strains were cultivated for 72 hours in 50 ml defined CGXII medium with 4 % glucose and 1 mM IPTG supplemented with 500 mg/L (3.09 mM) *p*HBA. Taken samples were extracted with ethyl acetate and analyzed for the synthesis of RK by HPLC. All data presented are mean values including standard deviations from biological triplicates.

Additional file

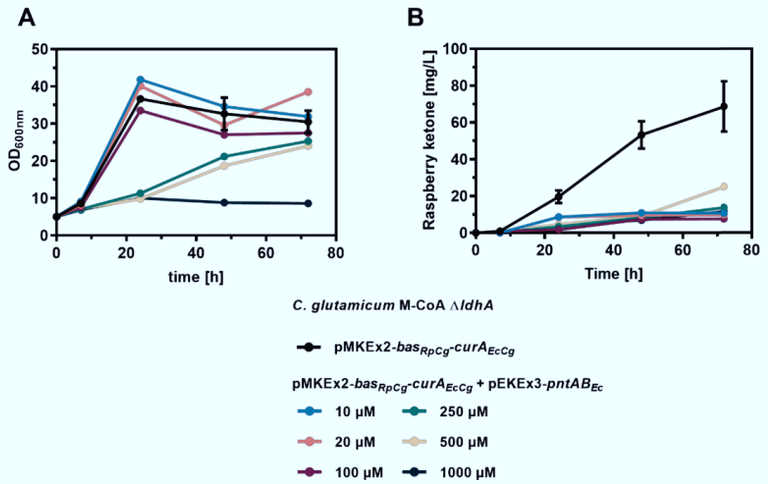

Figure S3: Effect of heterologous expression of *pntAB*$_{EC}$ encoding the membrane bound transhydrogenase from *E. coli* on (A) growth of *C. glutamicum* and (B) raspberry ketone synthesis. *C. glutamicum* M-CoA Δ*ldhA* harboring the indicated expression plasmids was cultivated in 50 mL CGXII medium with 4 % glucose and 500 mg/L supplemented *p*-hydroxybenzalacetone in baffled flasks at 30 °C and 130 rpm for 72 h. The depicted data for the strain solely harboring pMKEx2-*bas*$_{RpCg}$-*curA*$_{EcCg}$ (black) represents mean values with standard deviations from biological triplicates. The coloured data points indicating the varying IPTG concentrations (10 - 1000 µM) were obtained from single cultivations.

Additional file

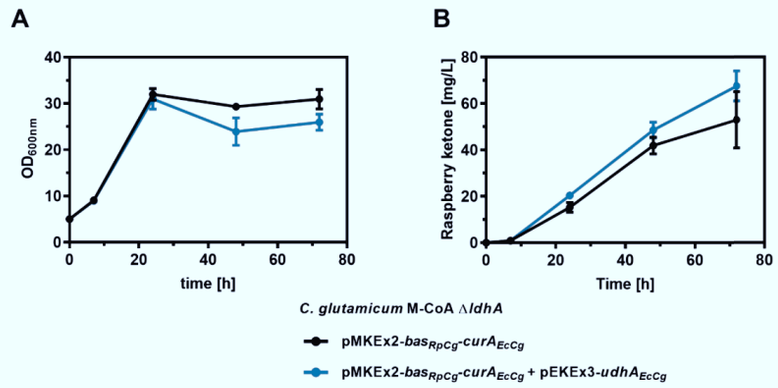

Figure S4: Effect of heterologous expression of a codon-optimized *udhA*$_{EcCg}$ gene variant encoding the cytosolic transhydrogenase from *E. coli* on (A) growth of *C. glutamicum* and (B) raspberry ketone synthesis. *C. glutamicum* M-CoA Δ*ldhA* harboring the indicated expression plasmids was cultivated in 50 mL CGXII medium with 4 % glucose and 500 mg/L supplemented *p*-hydroxybenzalacetone in baffled flasks at 30 °C and 130 rpm for 72 h. The depicted data represent mean values with standard deviations from biological triplicates.

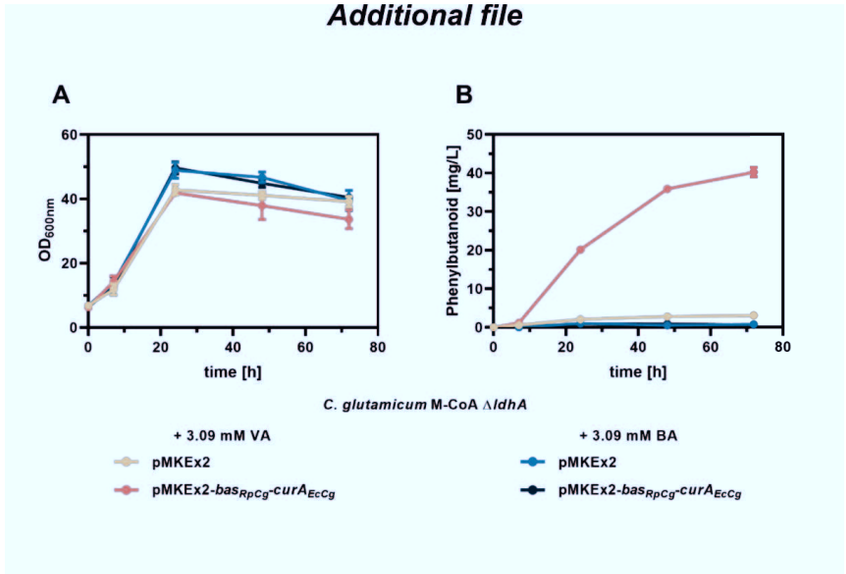

Figure S5: Effect of *curA*$_{EcCg}$ expression on the reduction of diketide intermediates in *C. glutamicum*. *C. glutamicum* M-CoA Δ*ldhA* optionally harboring one of the indicated expression plasmids was cultivated in 50 mL CGXII medium with 4 % glucose and 3.09 mM supplemented diketides vanillylidenacetone (VA) and benzalacetone (BA) in baffled flasks at 30 °C and 130 rpm for 72 h. Microbial growth (A) and phenylbutanoid synthesis (B) were followed over the process time. The depicted data represent mean values with standard deviations from biological triplicates.

3 References

Abe, I. ; Morita, H., 2010. “Structure and function of the chalcone synthase superfamily of plant type III polyketide synthases.” *Natural Product Reports*, 27 (6), 809–838. https://doi.org/10.1039/b909988n

Abe, I. ; Morita, H. ; Oguro, S. ; Noma, H. ; Wanibuchi, K. ; Kawahara, N. ; Goda, Y. ; Noguchi, H. ; Kohno, T., 2007. “Structure-Based Engineering of a Plant Type III Polyketide Synthase : Formation of an Unnatural Nonaketide Naphthopyrone.” *Journal of the American Chemical Society*, 129 , 5976–5980. https://doi.org/10.1021/ja070375l

Abe, I. ; Takahashi, Y. ; Morita, H. ; Noguchi, H., 2001. “Benzalacetone synthase. A novel polyketide synthase that plays a crucial role in the biosynthesis of phenylbutanones in *Rheum palmatum*.” *European Journal of Biochemistry*, 268 (11), 3354–3359. https://doi.org/10.1046/j.1432-1327.2001.02255.x

Abe, I. ; Utsumi, Y. ; Oguro, S. ; Morita, H. ; Sano, Y. ; Noguchi, H., 2005. “A Plant Type III Polyketide Synthase that Produces Pentaketide Chromone.” *Journal of the American Chemical Society*, 338 , 1362–1363. https://doi.org/10.1021/ja0431206

Abe, T. ; Morita, H. ; Noma, H. ; Kohno, T. ; Noguchi, H. ; Abe, I., 2007. “Structure function analysis of benzalacetone synthase from *Rheum palmatum*.” *Bioorganic and Medicinal Chemistry Letters*, 17 (11), 3161–3166. https://doi.org/10.1016/j.bmcl.2007.03.029

An, J.H. ; Kim, Y.S., 1998. “A gene cluster encoding malonyl-CoA decarboxylase (MatA), malonyl-CoA synthetase (MatB) and a putative dicarboxylate carrier protein (MatC) in *Rhizobium trifolii* - cloning, sequencing, and expression of the enzymes in *Escherichia coli*.” *European Journal of Biochemistry*, 257 (2), 395–402. https://doi.org/10.1046/j.1432-1327.1998.2570395.x

Austin, M.B. ; Bowman, M.E. ; Ferrer, J.-L. ; Schröder, J. ; Noel, J.P., 2004. “An Aldol Switch Discovered in Stilbene Synthases Mediates Cyclization Specificity of Type III Polyketide Synthases.” *Chemistry & Biology*, 11 , 1179–1194. https://doi.org/10.1016/j.chembiol .2004.05.024

Austin, M.B. ; Noel, J.P., 2003. “The chalcone synthase superfamily of type III polyketide synthases.” *Natural Product Reports*, 20 (1), 79–110. https://doi.org/10.1039/b100917f

Baritugo, K.-A. ; Kim, H.T. ; David, Y. ; Choi, J. ; Hong, S.H. ; Jeong, K.J. ; Choi, J.H. ; Joo, J.C. ; Park, S.J., 2018. “Metabolic engineering of *Corynebacterium glutamicum* for fermentative production of chemicals in biorefinery.” *Applied Microbiology and Biotechnology*, 102 , 3915–3937. https://doi.org/10.1007/s00253-018-8896-6

Barros, J. ; Serrani-Yarce, J.C. ; Chen, F. ; Baxter, D. ; Venables, B.J. ; Dixon, R.A., 2016. “Role of bifunctional ammonia-lyase in grass cell wall biosynthesis.” *Nature Plants*, 2 (6), 1–9. https://doi.org/10.1038/NPLANTS.2016.50

Becker, J. ; Rohles, C.M. ; Wittmann, C., 2018. “Metabolically engineered *Corynebacterium glutamicum* for bio-based production of chemicals, fuels, materials, and healthcare products.” *Metabolic Engineering*, 50 , 122–141. https://doi.org/10.1016/j.ymben.2018.07.008

Beekwilder, J. ; Van der Meer, I.M. ; Sibbbesen, O. ; Broekgaarden, M. ; Qvist, I. ; Mikkelsen, J.D. ; Hall, R.D., 2007. “Microbial production of natural raspberry ketone.” *Biotechnology Journal*, 2 (10), 1270–1279. https://doi.org/10.1002/biot.200700076

Beekwilder, J. ; Wolswinkel, R. ; Jonker, H. ; Hall, R. ; De Rie Vos, C.H. ; Bovy, A., 2006. “Production of resveratrol in recombinant microorganisms.” *Applied and Environmental Microbiology*, 72 (8), 5670–5672. https://doi.org/10.1128/AEM.00609-06

Beld, J. ; Sonnenschein, E.C. ; Vickery, C.R. ; Noel, J.P. ; Burkart, M.D., 2014. “The Phosphopantetheinyl Transferases: Catalysis of a Posttranslational Modification Crucial for Life.” *Natural Product Reports*, 31 (1), 61–108. https://doi.org/10.1371/journal.pone.0178059

Bendt, A.K. ; Burkovski, A. ; Schaffer, S. ; Bott, M. ; Farwick, M. ; Hermann, T., 2003. “Towards a phosphoproteome map of *Corynebacterium glutamicum*.” *Proteomics*, 3 (8), 1637–1646. https://doi.org/10.1002/pmic.200300494

Birch, A.J. ; Donovan, F.W., 1953. “Studies In Relation To Biosynthesis. I. Some possible derivatives of orcinol and phloroglucinol.” *Australian Journal of Chemistry*, 6 (4), 360–368.

Birch, A.J. ; Massy-Westropp, R.A. ; Moye, C.J., 1955. “Studies in relation to biosynthesis. VII. 2-hydroxy-6-methylbenzoic acid in *Penicillium Griseofulvum Dierckx*.” *Australian Journal of Chemistry*, 8 (4), 539–544. https://doi.org/10.1071/CH9550539

Blombach, B. ; Riester, T. ; Wieschalka, S. ; Ziert, C. ; Youn, J.W. ; Wendisch, V.F. ; Eikmanns, B.J., 2011. “*Corynebacterium glutamicum* tailored for efficient isobutanol production.” *Applied and Environmental Microbiology*, 77 (10), 3300–3310. https://doi.org/10.1128/AEM.02972-10

Borejsza-Wysocki, W. ; Hrazdina, G., 1996. “Purification, Characterization, and Antibody Development to Benzalacetone Synthase from Raspberry Fruits.” *Plant Physiology*, 110 , 791–799. https://doi.org/10.1104/pp.110.3.791

Borejsza-Wysocki, W. ; Hrazdina, G., 1994. “Biosynthesis of *p*-hydroxyphenylbutan-2-one in raspberry fruits and tissue cultures.” *Phytochemistry*, 35 (3), 623–628. https://doi.org/10.1016/S0031-9422(00)90575-2

Braga, A. ; Oliveira, J. ; Silva, R. ; Ferreira, P. ; Rocha, I. ; Kallscheuer, N. ; Marienhagen, J. ; Faria, N., 2018. “Impact of the cultivation strategy on resveratrol production from glucose in engineered *Corynebacterium glutamicum*.” *Journal of Biotechnology*, 265 , 70–75. https://doi.org/10.1016/J.JBIOTEC.2017.11.006

Brennan, P.J. ; Nikaido, H., 1995. “The Envelope of *Mycobacteria*.” *Annual Review of Biochemistry*, 64 , 29–63. https://doi.org/10.1017/CBO9781107415324.004

Brownsey, R.W. ; Boone, A.N. ; Elliott, J.E. ; Kulpa, J.E. ; Lee, W.M., 2006. “Regulation of acetyl-CoA carboxylase.” *Biochemical Society Transactions*, 34 , 223–227. https://doi.org/10.1042/BST20060223

Brüsseler, C. ; Radek, A. ; Tenhaef, N. ; Krumbach, K. ; Noack, S. ; Marienhagen, J., 2018. “The *myo*-inositol/proton symporter IolT1 contributes to D-xylose uptake in *Corynebacterium glutamicum*.” *Bioresource Technology*, 249 , 953–961. https://doi.org/10.1016/j.biortech.2017.10.098

Catalgol, B. ; Batirel, S. ; Taga, Y. ; Ozer, N.K., 2012. “Resveratrol: French paradox revisited.” *Frontiers in Pharmacology*, 3 , 1–18. https://doi.org/10.3389/fphar.2012.00141

Chalut, C. ; Botella, L. ; De Sousa-D’Auria, C. ; Houssin, C. ; Guilhot, C., 2006. “The nonredundant roles of two 4’-phosphopantetheinyl transferases in vital processes of *Mycobacteria*.” *Proceedings of the National Academy of Sciences of the United States of America*, 103 (22), 8511–8516.

Chan, Y.A. ; Podevels, A.M. ; Kevany, B.M. ; Thomas, M.G., 2009. “Biosynthesis of polyketide synthase extender units.” *Natural Product Reports*, 26 , 90–114. https://doi.org/10.1039/b801658p

Chemler, J.A. ; Koffas, M.A., 2008. “Metabolic engineering for plant natural product biosynthesis in microbes.” *Current Opinion in Biotechnology*, 19 (6), 597–605. https://doi.org/10.1016/j.copbio.2008.10.011

Cress, B.F. ; Toparlak, O.D. ; Guleria, S. ; Lebovich, M. ; Stieglitz, J.T. ; Englaender, J.A. ; Jones, J.A. ; Linhardt, R.J. ; Koffas, M.A.G., 2015. “CRISPathBrick: Modular Combinatorial Assembly of Type II-A CRISPR Arrays for dCas9-Mediated Multiplex Transcriptional Repression in *E. coli*.” *ACS Synthetic Biology*, 4 (9), 987–1000. https://doi.org/10.1021/acssynbio.5b00012

Cronan, J.E. ; Thomas, J., 2009. “Bacterial Fatty Acid Synthesis and its Relationships with Polyketide Synthetic Pathways.” *Methods in Enzymology*, 459 , 395–433. https://doi.org/10.1016/S0076-6879(09)04617-5

Cronan, J.E. ; Waldrop, G.L., 2002. “Multi-subunit acetyl-CoA carboxylases.” *Progress in Lipid Research*, 41 (5), 407–35. https://doi.org/10.1016/s0163-7827(02)00007-3

Dewick, P.M., 2002. Medicinal Natural Products: A Biosynthetic Approach, 2nd ed. John Wiley & Sons, Ltd, Chichester, West Sussex, England. https://doi.org/10.1016/j.jbiosc.2010.01.005

Eggeling, L. ; Bott, M., 2005. Handbook of *Corynebacterium glutamicum*. CRC Press, Boca Raton, FL, USA. https://doi.org/10.1201/9781420039696

Eudes, A. ; Juminaga, D. ; Baidoo, E.E.K. ; Collins, F.W. ; Keasling, J.D. ; Loqué, D., 2013. “Production of hydroxycinnamoyl anthranilates from glucose in *Escherichia coli*.” *Microbial Cell Factories*, 12 (62), 1–10. https://doi.org/10.1186/1475-2859-12-62

European Council, 2008. Regulation (EC) No 1334/2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC.

Evolva, 2019. Veri-te Resveratrol [WWW Document]. URL https://veriteresveratrol.com/quality/ (accessed 10.19.19).

Farid, M.A. ; El-Enshasy, H.A. ; El-Diwany, A.I. ; El-Sayed, E.S.A., 2000. “Optimization of the cultivation medium for natamycin production by *Streptomyces natalensis*.” *Journal of Basic Microbiology*, 40 (3), 157–166. https://doi.org/10.1002/1521-4028(200007)40:3<157::AID-JOBM157>3.0.CO;2-1

Ferrer, J.-L. ; Jez, J.M. ; Bowman, M.E. ; Dixon, R.A. ; Noel, J.P., 1999. “Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis.” *Nature Structural & Molecular Biology*, 6 , 775–784. https://doi.org/10.1038/11553

Flachbart, L.K. ; Sokolowsky, S. ; Marienhagen, J., 2019. “Displaced by Deceivers: Prevention of Biosensor Cross-Talk Is Pivotal for Successful Biosensor-Based High-Throughput Screening Campaigns.” *ACS Synthetic Biology*, 8 (8), 1847–1857. https://doi.org/10.1021/acssynbio.9b00149

Fowler, Z.L. ; Gikandi, W.W. ; Koffas, M.A.G., 2009. “Increased malonyl coenzyme A biosynthesis by tuning the *Escherichia coli* metabolic network and its application to flavanone production.” *Applied and Environmental Microbiology*, 75 , 5831–5839. https://doi.org/10.1128/AEM.00270-09

Frankel, E.N. ; Waterhouse, A.L. ; Kinsella, J.E., 1993. “Inhibition of human LDL oxidation by resveratrol.” *The Lancet*, 341 (8852), 1103–1104. https://doi.org/10.1016/0140-6736(93)92472-6

Gago, G. ; Diacovich, L. ; Arabolaza, A. ; Tsai, S.-C. ; Gramajo, H., 2011. “Fatty acid biosynthesis in actinomycetes.” *FEMS Microbiology Reviews*, 35 , 475–497. https://doi.org/10.1111/j.1574-6976.2010.00259.x

Gande, R. ; Dover, L.G. ; Krumbach, K. ; Besra, G.S. ; Sahm, H. ; Oikawa, T. ; Eggeling, L., 2007. “The two carboxylases of *Corynebacterium glutamicum* essential for fatty acid and mycolic acid synthesis.” *Journal of Bacteriology*, 189 (14), 5257–5264. https://doi.org/10.1128/JB.00254-07

Gande, R. ; Gibson, K.J.C. ; Brown, A.K. ; Krumbach, K. ; Dover, L.G. ; Sahm, H. ; Shioyama, S. ; Oikawa, T. ; Besra, G.S. ; Eggeling, L., 2004. “Acyl-CoA carboxylases (*accD2* and *accD3*), together with a unique polyketide synthase (*Cg-pks*), are key to mycolic acid biosynthesis in *Corynebacterianeae* such as *Corynebacterium glutamicum* and *Mycobacterium tuberculosis*.” *The Journal of Biological Chemistry*, 279 (43), 44847–44857. https://doi.org/10.1074/jbc.M408648200

Gaspar, A. ; Matos, M.J. ; Garrido, J. ; Uriarte, E. ; Borges, F., 2014. “Chromone: A valid scaffold in medicinal chemistry.” *Chemical Reviews*, 114 (9), 4960–4992. https://doi.org/10.1021/cr400265z

Glick, B.R., 1995. “Metabolic load and heterologous gene expression.” *Biotechnology Advances*, 13 (2), 247–261. https://doi.org/10.1016/0734-9750(95)00004-a

Goldberg, D.M. ; Yan, J. ; Soleas, G.J., 2003. “Absorption of three wine-related polyphenols in three different matrices by healthy subjects.” *Clinical Biochemistry*, 36 (1), 79–87. https://doi.org/10.1016/S0009-9120(02)00397-1

Guchhait, R.B. ; Polakis, S.E. ; Dimroth, P. ; Stoll, E. ; Moss, J. ; Lane, M.D., 1974. “Acetyl Coenzyme A Carboxylase System of *Escherichia coli*.” *The Journal of Biological Chemistry*, 249 (20), 6633–6645.

Häkkinen, S.T. ; Seppänen-Laakso, T. ; Oksman-Caldentey, K.-M. ; Rischer, H., 2015. “Bioconversion to Raspberry Ketone is Achieved by Several Non-related Plant Cell Cultures.” *Frontiers in Plant Science*, 6 (November), 1–9. https://doi.org/10.3389/fpls.2015.01035

Harris, T. ; Harris, M., 1977. “Synthesis of polyketide-type aromatic natural products by biogenetically modeled routes.” *Tetrahedron*, (36), 2159–2185. https://doi.org/10.1016/0040-4020(77)80001-X

Harris, T.M. ; Wittek, P.J., 1975. “Biogenetic-Type Syntheses of Polycyclic Polyketide Metabolites using Partially Protected *β*-Hexa- and *β*-Heptaketones. 6-Hydroxymusizin, Barakol, Emodin, and Eleutherinol.” *Journal of the American Chemical Society*, 97 (11), 3270–3271. https://doi.org/10.1021/ja00844a085

Hartmann, A. ; Vila-Santa, A. ; Kallscheuer, N. ; Vogt, M. ; Julien-Laferrière, A. ; Sagot, M.-F. ; Marienhagen, J. ; Vinga, S., 2017. “OptPipe - a pipeline for optimizing metabolic engineering targets.” *BMC Systems Biology*, 11 , 1–9. https://doi.org/10.1186/s12918-017-0515-0

Herrmann, K.M. ; Weaver, L.M., 1999. “The Shikimate Pathway.” *Annual Review of Plant Physiology and Plant Molecular Biology*, 50 (1), 473–503. https://doi.org/10.1146/annurev.arplant.50.1.473

Hertweck, C., 2009. “The biosynthetic logic of polyketide diversity.” *Angewandte Chemie - International Edition*, 48 (26), 4688–4716. https://doi.org/10.1002/anie.200806121

Hirasawa, T. ; Shimizu, H., 2016. “Recent advances in amino acid production by microbial cells.” *Current Opinion in Biotechnology*, 42 , 133–146. https://doi.org/10.1016/j.copbio.2016.04.017

Hopwood, D.A. (Ed.), 2009. Complex Enzymes in Microbial Natural Product Biosynthesis, Part B: Polyketides, Aminocoumarins and Carbohydrates, First. ed, Methods in Enzymology. Academic Press. https://doi.org/10.1016/S0076-6879(09)04622-9

Ikeda, M., 2006. “Towards bacterial strains overproducing L-tryptophan and other aromatics by metabolic engineering.” *Applied Microbiology and Biotechnology*, 69 (6), 615–626. https://doi.org/10.1007/s00253-005-0252-y

Ikeda, M. ; Nakagawa, S., 2003. “The *Corynebacterium glutamicum* genome: Features and impacts on biotechnological processes.” *Applied Microbiology and Biotechnology*, 62 (2–3), 99–109. https://doi.org/10.1007/s00253-003-1328-1

Irzik, K. ; van Ooyen, J. ; Gätgens, J. ; Krumbach, K. ; Bott, M. ; Eggeling, L., 2014. “Acyl-CoA sensing by FasR to adjust fatty acid synthesis in *Corynebacterium glutamicum*.” *Journal of Biotechnology*, 192 , 96–101. https://doi.org/10.1016/j.jbiotec.2014.10.031

Jaworski, J.G. ; Clough, R.C. ; Barnum, S.R., 1989. “ A Cerulenin Insensitive Short Chain 3-Ketoacyl-Acyl Carrier Protein Synthase in *Spinacia oleracea* Leaves .” *Plant Physiology*, 90 (1), 41–44. https://doi.org/10.1104/pp.90.1.41

Jez, J.M. ; Ferrer, J.L. ; Bowman, M.E. ; Dixon, R.A. ; Noel, J.P., 2000. “Dissection of malonyl-coenzyme A decarboxylation from polyketide formation in the reaction mechanism of a plant polyketide synthase.” *Biochemistry*, 39 (5), 890–902. https://doi.org/10.1021/bi991489f

Jez, J.M. ; Noel, J.P., 2002. “Reaction mechanism of chalcone isomerase: pH dependence, diffusion control, and product binding differences.” *Journal of Biological Chemistry*, 277 (2), 1361–1369. https://doi.org/10.1074/jbc.M109224200

Kalinowski, J. ; Bathe, B. ; Bartels, D. ; Bischoff, N. ; Bott, M. ; Burkovski, A. ; Dusch, N. ; Eggeling, L. ; Eikmanns, B.J. ; Gaigalat, L. ; Goesmann, A. ; Hartmann, M. ; Huthmacher, K. ; Krämer, R. ; Linke, B. ; McHardy, A.C. ; Meyer, F. ; Möckel, B. ; Pfefferle, W. ; Pühler, A. ; Rey, D.A. ; Rückert, C. ; Rupp, O. ; Sahm, H. ; Wendisch, V.F. ; Wiegräbe, I. ; Tauch, A., 2003. "The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins." *Journal of Biotechnology*, 104 (1–3), 5–25. https://doi.org/10.1016/S0168-1656(03)00154-8

Kallscheuer, N. ; Marienhagen, J., 2018. "*Corynebacterium glutamicum* as platform for the production of hydroxybenzoic acids." *Microbial Cell Factories*, 17 (70). https://doi.org/10.1186/s12934-018-0923-x

Kallscheuer, N. ; Vogt, M. ; Bott, M. ; Marienhagen, J., 2017. "Functional expression of plant-derived *O*-methyltransferase, flavanone 3-hydroxylase, and flavonol synthase in *Corynebacterium glutamicum* for production of pterostilbene, kaempferol, and quercetin." *Journal of Biotechnology*, 258 , 190–196. https://doi.org/10.1016/j.jbiotec.2017.01.006

Kallscheuer, N. ; Vogt, M. ; Kappelmann, J. ; Krumbach, K. ; Noack, S. ; Bott, M. ; Marienhagen, J., 2016a. "Identification of the *phd* gene cluster responsible for phenylpropanoid utilization in *Corynebacterium glutamicum*." *Applied Microbiology and Biotechnology*, 100 , 1871–1881. https://doi.org/10.1007/s00253-015-7165-1

Kallscheuer, N. ; Vogt, M. ; Stenzel, A. ; Gätgens, J. ; Bott, M. ; Marienhagen, J., 2016b. "Construction of a *Corynebacterium glutamicum* platform strain for the production of stilbenes and (2*S*)-flavanones." *Metabolic Engineering*, 38 , 47–55. https://doi.org/10.1016/j.ymben.2016.06.003

Khurana, S. ; Venkataraman, K. ; Hollingsworth, A. ; Piche, M. ; Tai, T.C., 2013. "Polyphenols: Benefits to the cardiovascular system in health and in aging." *Nutrients*, 5 (10), 3779–3827. https://doi.org/10.3390/nu5103779

Kim, M. ; Baek, H.S. ; Lee, M. ; Park, H. ; Shin, S.S. ; Choi, D.W. ; Lim, K.M., 2016. "Rhododenol and raspberry ketone impair the normal proliferation of melanocytes through reactive oxygen species-dependent activation of GADD45." *Toxicology in Vitro*, 32 , 339–346. https://doi.org/10.1016/j.tiv.2016.02.003

Kinoshita, S. ; Udaka, S. ; Shimono, M., 1957. "Studies On the Amino Acid Fermentation." *The Journal of General and Applied Microbiology*, 3 (3), 193–205. https://doi.org/10.2323/jgam.3.193

Lambalot, R.H. ; Gehring, A.M. ; Fluge, R.S. ; Zuber, P. ; LaCelle, M. ; Marahiel, M.A. ; Reid, R. ; Khosia, C. ; Walsh, C.T., 1996. "A new enzyme transferases superfamily - the phosphopantetheinyl transferases." *Chemistry & Biology*, 3 , 923–936. https://doi.org/10.1016/S1074-5521(96)90181-7

Law, M. ; Wald, N., 1999. "Education and debate the time lag explanation." *British Medical Journal*, 318 , 1471–1476. https://doi.org/10.1136/bmj.318.7196.1471

Lee, J.H. ; Wendisch, V.F., 2017. "Production of amino acids – Genetic and metabolic engineering approaches." *Bioresource Technology*, 245 , 1575–1587. https://doi.org/10.1016/j.biortech.2017.05.065

Leonard, E. ; Lim, K.-H. ; Saw, P.-N. ; Koffas, M.A.G., 2007. "Engineering central metabolic pathways for high-level flavonoid production in *Escherichia coli*." *Applied and Environmental Microbiology*, 73 , 3877–3886.

Leonard, E. ; Yan, Y. ; Fowler, Z.L. ; Li, Z. ; Lim, C.G. ; Lim, K.H. ; Koffas, M., 2008. "Strain improvement of recombinant *Escherichia coli* for efficient production of plant flavonoids." *Molecular Pharmaceutics*, 5 (2), 257–265. https://doi.org/10.1021/mp7001472

Li, S.-J. ; Cronan, J.E., 1992a. "The Gene Encoding the Biotin Carboxylase Subunit of *Escherichia coli* Acetyl-CoA Carboxylase." *The Journal of Biological Chemistry*, 267 (2), 855–863.

Li, S.-J. ; Cronan, J.E., 1992b. “The Genes Encoding the Two Carboxyltransferase Subunits of *Escherichia coli* Acetyl-coA Carboxylase.” *The Journal of Biological Chemistry*, 267 (24), 16841–16847.

Li, S.-J. ; Rock, C.O. ; Cronan, J.E., 1992. “The *dedB* (*usg*) open reading frame of *Escherichia coli* encodes a subunit of acetyl-coenzyme A carboxylase.” *Journal of Bacteriology*, 174 (17), 5755–5757. https://doi.org/10.1128/jb.174.17.5755-5757.1992

Liang, J. long ; Guo, L. qiong ; Lin, J. fang ; He, Z. qi ; Cai, F. ji ; Chen, J. fei, 2016. “A novel process for obtaining pinosylvin using combinatorial bioengineering in *Escherichia coli*.” *World Journal of Microbiology and Biotechnology*, 32 (6). https://doi.org/10.1007/s11274-016-2062-z

Lin, Y. ; Yan, Y., 2012. “Biosynthesis of caffeic acid in *Escherichia coli* using its endogenous hydroxylase complex.” *Microbial Cell Factories*, 11 (42), 1–9. https://doi.org/10.1186/1475-2859-11-42

Litsanov, B. ; Brocker, M. ; Bott, M., 2012. “Toward homosuccinate fermentation: Metabolic engineering of *Corynebacterium glutamicum* for anaerobic production of succinate from glucose and formate.” *Applied and Environmental Microbiology*, 78 (9), 3325–3337. https://doi.org/10.1128/AEM.07790-11

Lussier, F.-X. ; Colatriano, D. ; Wiltshire, Z. ; Page, J.E. ; Martin, V.J.J., 2012. “Engineering Microbes for Plant Polyketide Biosynthesis Polyketides are synthesized fr.” *Computational And Structural Biotechnology Journal*, 3 (4).

Luttik, M.A.H. ; Vuralhan, Z. ; Suir, E. ; Braus, G.H. ; Pronk, J.T. ; Daran, J.M., 2008. “Alleviation of feedback inhibition in *Saccharomyces cerevisiae* aromatic amino acid biosynthesis : Quantification of metabolic impact.” *Metabolic Engineering*, 10 , 141–153. https://doi.org/10.1016/j.ymben.2008.02.002

Manzoni, M. ; Rollini, M., 2002. “Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs.” *Applied Microbiology and Biotechnology*, 58 (5), 555–564. https://doi.org/10.1007/s00253-002-0932-9

Marienhagen, J. ; Bott, M., 2013. “Metabolic engineering of microorganisms for the synthesis of plant natural products.” *Journal of Biotechnology*, 163 , 166–178. https://doi.org/10.1016/j.jbiotec.2012.06.001

McNeil, M. ; Daffe, M. ; Brennan, P.J., 1990. “Evidence for the nature of the link between the arabinogalactan and peptidoglycan of mycobacterial cell walls.” *Journal of Biological Chemistry*, 265 (30), 18200–18206.

Miyahisa, I. ; Kaneko, M. ; Funa, N. ; Kawasaki, H. ; Kojima, H. ; Ohnishi, Y. ; Horinouchi, S., 2005. “Efficient production of (2*S*)-flavanones by *Escherichia coli* containing an artificial biosynthetic gene cluster.” *Applied Microbiology and Biotechnology*, 68 (4), 498–504. https://doi.org/10.1007/s00253-005-1916-3

Moore, B.S. ; Hopke, J.N., 2001. “Discovery of a new bacterial polyketide biosynthetic pathway.” *ChemBioChem*, 2 (1), 35–38. https://doi.org/10.1002/1439-7633(20010105)2:1<35::AID-CBIC35>3.0.CO;2-1

Morimoto, C. ; Satoh, Y. ; Hara, M. ; Inoue, S. ; Tsujita, T. ; Okuda, H., 2005. “Anti-obese action of raspberry ketone.” *Life Sciences*, 77 (2), 194–204. https://doi.org/10.1016/j.lfs.2004.12.029

Morita, H. ; Kondo, S. ; Oguro, S. ; Noguchi, H. ; Sugio, S., 2007. “Structural Insight into Chain-Length Control and Product Specificity of Pentaketide Chromone Synthase from *Aloe arborescens*.” *Chemistry & Biology*, 14 , 359–369. https://doi.org/10.1016/j.chembiol.2007.02.003

Nickel, J. ; Irzik, K. ; Van Ooyen, J. ; Eggeling, L., 2010. “The TetR-type transcriptional regulator FasR of *Corynebacterium glutamicum* controls genes of lipid synthesis during growth on acetate.” *Molecular Microbiology*, 78 (1), 253–265. https://doi.org/10.1111/j.1365-2958.2010.07337.x

Osbourn, A.E. ; Lanzotti, V., 2009. Plant-derived Natural Products - Synthesis, Function, and Application. Springer New York, New York, NY. https://doi.org/10.1017/CBO9781107415324.004

Palmer, C.M. ; Alper, H.S., 2018. “Expanding the Chemical Palette of Industrial Microbes: Metabolic Engineering for Type III PKS-Derived Polyketides.” *Biotechnology Journal*, 1700463 , 1–14. https://doi.org/10.1002/biot.201700463

Pandey, K.B. ; Rizvi, S.I., 2009. “Plant polyphenols as dietary antioxidants in human health and disease.” *Oxidative Medicine and Cellular Longevity*, 2 (5), 270–278. https://doi.org/10.4161/oxim.2.5.9498

Pandey, R.P. ; Parajuli, P. ; Koffas, M.A.G. ; Sohng, J.K., 2016. “Microbial production of natural and non-natural flavonoids: Pathway engineering, directed evolution and systems/synthetic biology.” *Biotechnology Advances*, 34 (5), 634–662. https://doi.org/10.1016/j.biotechadv.2016.02.012

Park, S.R. ; Yoon, J.A. ; Paik, J.H. ; Park, J.W. ; Jung, W.S. ; Ban, Y.H. ; Kim, E.J. ; Yoo, Y.J. ; Han, A.R. ; Yoon, Y.J., 2009. “Engineering of plant-specific phenylpropanoids biosynthesis in *Streptomyces venezuelae*.” *Journal of Biotechnology*, 141 (3–4), 181–188. https://doi.org/10.1016/j.jbiotec.2009.03.013

Pfeifer, B.A. ; Khosla, C., 2001. “Biosynthesis of Polyketides in Heterologous Hosts.” *Microbiology and Molecular Biology Reviews*, 65 (1), 106–118. https://doi.org/10.1128/mmbr.65.1.106-118.2001

Pfeifer, V. ; Nicholson, G.J. ; Ries, J. ; Recktenwald, J. ; Schefer, A.B. ; Shawky, R.M. ; Schröder, J. ; Wohlleben, W. ; Pelzer, S., 2001. “A polyketide synthase in glycopeptide biosynthesis. The biosynthesis of the non-proteinogenic amino acid (*S*)-3,5-dihydroxyphenylglycine.” *Journal of Biological Chemistry*, 276 (42), 38370–38377. https://doi.org/10.1074/jbc.M106580200

Radmacher, E. ; Alderwick, L.J. ; Besra, G.S. ; Brown, A.K. ; Gibson, K.J.C. ; Sahm, H. ; Eggeling, L., 2005. “Two functional FAS-I type fatty acid synthases in *Corynebacterium glutamicum*.” *Microbiology*, 151 (7), 2421–2427. https://doi.org/10.1099/mic.0.28012-0

Ray, J.M. ; Yanofsky, C. ; Bauerlel, R., 1988. “Mutational Analysis of the Catalytic and Feedback Sites of the Synthase of *Escherichia coli*.” *Journal of Bacteriology*, 170 (12), 5500–5506. https://doi.org/10.1128/jb.170.12.5500-5506.1988

Renaud, S. ; De Lorgeril, M., 1992. “Wine, alcohol, platelets and the French paradox for coronary heart disease.” *The Lancet*, 339 , 1523–1526. https://doi.org/10.1016/0140-6736(92)91277-F

Rimm, E.B. ; Klatsky, A. ; Grobbee, D. ; Stampfer, M.J., 1996. “Review of moderate alcohol consumption and reduced risk of coronary heart disease: Is the effect due to beer, wine, or spirits?” *British Medical Journal*, 312 , 731–736. https://doi.org/10.1136/bmj.312.7033.731

Robertsen, H.L. ; Musiol-Kroll, E.M., 2019. “Actinomycete-Derived Polyketides as a Source of Antibiotics and Lead Structures for the Development of New Antimicrobial Drugs.” *Antibiotics*, 8 (4). https://doi.org/10.3390/antibiotics8040157

Rodrigues, J.L. ; Araújo, R.G. ; Prather, K.L.J. ; Kluskens, L.D. ; Rodrigues, L.R., 2015. “Heterologous production of caffeic acid from tyrosine in *Escherichia coli*.” *Enzyme and Microbial Technology*, 71 , 36–44. https://doi.org/10.1016/j.enzmictec.2015.01.001

Salehi, B. ; Fokou, P.V.T. ; Sharifi-Rad, M. ; Zucca, P. ; Pezzani, R. ; Martins, N. ; Sharifi-Rad, J., 2019. “The therapeutic potential of naringenin: A review of clinical trials.” *Pharmaceuticals*, 12 (1), 1–18. https://doi.org/10.3390/ph12010011

Santos, C.N.S. ; Koffas, M. ; Stephanopoulos, G., 2011. “Optimization of a heterologous pathway for the production of flavonoids from glucose.” *Metabolic Engineering*, 13 (4), 392–400. https://doi.org/10.1016/j.ymben.2011.02.002

Schujman, G.E. ; Guerin, M. ; Buschiazzo, A. ; Schaeffer, F. ; Llarrull, L.I. ; Reh, G. ; Vila, A.J. ; Alzari, P.M. ; de Mendoza, D., 2006. “Structural basis of lipid biosynthesis regulation in Gram-positive bacteria.” *The EMBO Journal*, 25 (17), 4074–83. https://doi.org/10.1038/sj.emboj.7601284

Schwecke, T. ; Aparicio, J.F. ; Molnár, I. ; König, A. ; Khaw, L.E. ; Haydock, S.F. ; Oliynyk, M. ; Caffrey, P. ; Cortés, J. ; Lester, J.B. ; Böhm, G.A. ; Staunton, J. ; Leadlay, P.F., 1995. “The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin.” *Proceedings of the National Academy of Sciences of the United States of America*, 92 (17), 7839–7843. https://doi.org/10.1073/pnas.92.17.7839

Seshime, Y. ; Juvvadi, P.R. ; Fujii, I. ; Kitamoto, K., 2005. “Discovery of a novel superfamily of type III polyketide synthases in *Aspergillus oryzae*.” *Biochemical and Biophysical Research Communications*, 331 (1), 253–260. https://doi.org/10.1016/j.bbrc.2005.03.160

Shao, L. ; Qu, X.D. ; Jia, X.Y. ; Zhao, Q.F. ; Tian, Z.H. ; Wang, M. ; Tang, G.L. ; Liu, W., 2006. “Cloning and characterization of a bacterial iterative type I polyketide synthase gene encoding the 6-methylsalicyclic acid synthase.” *Biochemical and Biophysical Research Communications*, 345 (1), 133–139. https://doi.org/10.1016/j.bbrc.2006.04.069

Shen, X.H. ; Zhou, N.Y. ; Liu, S.J., 2012. “Degradation and assimilation of aromatic compounds by *Corynebacterium glutamicum*: Another potential for applications for this bacterium?” *Applied Microbiology and Biotechnology*, 95 (1), 77–89. https://doi.org/10.1007/s00253-012-4139-4

Shi, S. ; Chen, Y. ; Siewers, V. ; Nielsen, J., 2014. “Improving production of malonyl coenzyme A-derived metabolites by abolishing Snf1-dependent regulation of Acc1.” *mBio*, 5 (3), E01130-14. https://doi.org/10.1128/mBio.01130-14

Shimokawa, Y. ; Morita, H. ; Abe, I., 2010. “Structure-based engineering of benzalacetone synthase.” *Bioorganic and Medicinal Chemistry Letters*, 20 (17), 5099–5103. https://doi.org/10.1016/j.bmcl.2010.07.022

Shin, S.Y. ; Jung, S.M. ; Kim, M.D. ; Han, N.S. ; Seo, J.H., 2012. “Production of resveratrol from tyrosine in metabolically engineered *Saccharomyces cerevisiae*.” *Enzyme and Microbial Technology*, 51 (4), 211–216. https://doi.org/10.1016/j.enzmictec.2012.06.005

Shomura, Y. ; Torayama, I. ; Suh, D.Y. ; Xiang, T. ; Kita, A. ; Sankawa, U. ; Miki, K., 2005. “Crystal structure of stilbene synthase from *Arachis hypogaea*.” *Proteins: Structure, Function and Genetics*, 60 (4), 803–806. https://doi.org/10.1002/prot.20584

Smith, L.R., 1996. “Rheosmin (‘Raspberry Ketone’) and Zingerone, and Their Preparation by Crossed Aldol-Catalytic Hydrogenation Sequences.” *The Chemical Educator*, 1 (3), 1–18. https://doi.org/10.1007/s00897960034a

Smith, S. ; Tsai, S.C., 2007. “The type I fatty acid and polyketide synthases: A tale of two megasynthases.” *Natural Product Reports*, 24 (5), 1041–1072. https://doi.org/10.1039/b603600g

Stäbler, N. ; Oikawa, T. ; Bott, M. ; Eggeling, L., 2011. “*Corynebacterium glutamicum* as a host for synthesis and export of D-amino acids.” *Journal of Bacteriology*, 193 (7), 1702–1709. https://doi.org/10.1128/JB.01295-10

Stackebrandt, E. ; Rainey, F.A. ; Ward-Rainey, N.L., 1997. “Proposal for a New Hierarchic Classification System, *Actinobacteria* classis nov.” *International Journal of Systemic Bacteriology*, 47 (2), 479–491. https://doi.org/10.1017/CBO9781107415324.004

Staunton, J. ; Weissman, K.J., 2001. “Polyketide biosynthesis: A millennium review.” *Natural Product Reports*, 18 (4), 380–416. https://doi.org/10.1039/a909079g

Takeno, S. ; Takasaki, M. ; Urabayashi, A. ; Mimura, A. ; Muramatsu, T. ; Mitsuhashi, S. ; Ikeda, M., 2013. “Development of Fatty Acid-Producing *Corynebacterium glutamicum* Strains.” *Applied and Environmental Microbiology*, 79 (21), 6776–6783. https://doi.org/10.1128/AEM.02003-13

Tehlivets, O. ; Scheuringer, K. ; Kohlwein, S.D., 2007. “Fatty acid synthesis and elongation in yeast.” *Biochimica et Biophysica Acta*, 1771 , 255–270. https://doi.org/10.1016/j.bbalip.2006.07.004

Tenhaef, N. ; Brüsseler, C. ; Radek, A. ; Hilmes, R. ; Unrean, P. ; Marienhagen, J. ; Noack, S., 2018. “Production of D-xylonic acid using a non-recombinant *Corynebacterium glutamicum* strain.” *Bioresource Technology*, 268 , 332–339. https://doi.org/10.1016/j.biortech.2018.07.127

van Ooyen, J. ; Noack, S. ; Bott, M. ; Reth, A. ; Eggeling, L., 2012. “Improved L-lysine production with *Corynebacterium glutamicum* and systemic insight into citrate synthase flux and activity.” *Biotechnology and Bioengineering*, 109 (8), 2070–2081. https://doi.org/10.1002/bit.24486

van Summeren-Wesenhagen, P.V. ; Marienhagen, J., 2015. “Metabolic engineering of *Escherichia coli* for the synthesis of the plant polyphenol pinosylvin.” *Applied and Environmental Microbiology*, 81 (3), 840–849. https://doi.org/10.1128/AEM.02966-14

Vandamme, E.J. ; Soetaert, W., 2002. “Bioflavours and fragrances via fermentation and biocatalysis.” *Journal of Chemical Technology & Biotechnology*, 77 (12), 1323–1332. https://doi.org/10.1002/jctb.722

Vestergaard, M. ; Ingmer, H., 2019. “Antibacterial and antifungal properties of resveratrol.” *International Journal of Antimicrobial Agents*, 53 (6), 716–723. https://doi.org/10.1016/j.ijantimicag.2019.02.015

Vogt, M. ; Brüsseler, C. ; van Ooyen, J. ; Bott, M. ; Marienhagen, J., 2016. “Production of 2-methyl-1-butanol and 3-methyl-1-butanol in engineered *Corynebacterium glutamicum*.” *Metabolic Engineering*, 38 , 436–445. https://doi.org/10.1016/j.ymben.2016.10.007

Vogt, M. ; Haas, S. ; Klaffl, S. ; Polen, T. ; Eggeling, L. ; van Ooyen, J. ; Bott, M., 2014. “Pushing product formation to its limit: Metabolic engineering of *Corynebacterium glutamicum* for L-leucine overproduction.” *Metabolic Engineering*, 22 , 40–52. https://doi.org/10.1016/j.ymben.2013.12.001

Walsh, M.C. ; Klopfenstein, W.E. ; Harwood, J.L., 1990. “The short chain condensing enzyme has a widespread occurrence in the fatty acid synthetases from higher plants.” *Phytochemistry*, 29 (12), 3797–3799. https://doi.org/10.1016/0031-9422(90)85334-C

Watts, K.T. ; Lee, P.C. ; Schmidt-Dannert, C., 2006. “Biosynthesis of plant-specific stilbene polyketides in metabolically engineered *Escherichia coli*.” *BMC Biotechnology*, 6 , 1–12. https://doi.org/10.1186/1472-6750-6-22

Watts, K.T. ; Lee, P.C. ; Schmidt-Dannert, C., 2004. “Exploring recombinant flavonoid biosynthesis in metabolically engineered *Escherichia coli*.” *ChemBioChem*, 5 (4), 500–507. https://doi.org/10.1002/cbic.200300783

Weissman, K.J. ; Leadlay, P.F., 2005. “Combinatorial biosynthesis of reduced polyketides.” *Nature Reviews Microbiology*, 3 (12), 925–936. https://doi.org/10.1038/nrmicro1287

Wink, M., 2010. Functions and Biotechnology of Plant Secondary Metabolites, Second. ed, Annual Plant Reviews. Wiley-Blackwell. https://doi.org/10.1002/9781444318876

Wu, G. ; Yan, Q. ; Jones, J.A. ; Tang, Y.J. ; Fong, S.S. ; Koffas, M.A.G., 2016. “Metabolic Burden: Cornerstones in Synthetic Biology and Metabolic Engineering Applications.” *Trends in Biotechnology*, 34 (8), 652–664. https://doi.org/10.1016/j.tibtech.2016.02.010

Wu, J. ; Du, G. ; Chen, J. ; Zhou, J., 2015. “Enhancing flavonoid production by systematically tuning the central metabolic pathways based on a CRISPR interference system in *Escherichia coli*.” *Scientific Reports*, 5 , 1–14. https://doi.org/10.1038/srep13477

Wu, J. ; Du, G. ; Zhou, J. ; Chen, J., 2013. “Metabolic engineering of *Escherichia coli* for (2*S*)-pinocembrin production from glucose by a modular metabolic strategy.” *Metabolic Engineering*, 16 (1), 48–55. https://doi.org/10.1016/j.ymben.2012.11.009

Wu, J. ; Yu, O. ; Du, G. ; Zhou, J. ; Chen, J., 2014a. “Fine-tuning of the fatty acid pathway by synthetic antisense RNA for enhanced (2*S*)-naringenin production from L-tyrosine in *Escherichia coli*.” *Applied and Environmental Microbiology*, 80 (23), 7283–7292. https://doi.org/10.1128/AEM.02411-14

Wu, J. ; Zhou, T. ; Du, G. ; Zhou, J. ; Chen, J., 2014b. “Modular optimization of heterologous pathways for De Novo synthesis of (2*S*)-Naringenin in *Escherichia coli*.” *PLoS ONE*, 9 (7), 1–9. https://doi.org/10.1371/journal.pone.0101492

Xu, P. ; Ranganathan, S. ; Fowler, Z.L. ; Maranas, C.D. ; Koffas, M.A.G., 2011. “Genome-scale metabolic network modeling results in minimal interventions that cooperatively force carbon flux towards malonyl-CoA.” *Metabolic Engineering*, 13 (5), 578–587. https://doi.org/10.1016/j.ymben.2011.06.008

Yang, D. ; Kim, W.J. ; Yoo, S.M. ; Choi, J.H. ; Ha, S.H. ; Lee, M.H. ; Lee, S.Y., 2018. “Repurposing type III polyketide synthase as a malonyl-CoA biosensor for metabolic engineering in bacteria.” *Proceedings of the National Academy of Sciences of the United States of America*, 115 (40), 9835–9844. https://doi.org/10.1073/pnas.1808567115

Yang, Y. ; Lin, Y. ; Li, L. ; Linhardt, R.J. ; Yan, Y., 2015. “Regulating malonyl-CoA metabolism via synthetic antisense RNAs for enhanced biosynthesis of natural products.” *Metabolic Engineering*, 29 , 217–226. https://doi.org/10.1016/j.ymben.2015.03.018

Yukawa, H. ; Inui, M. (Eds.), 2013. Microbiology Monographs Vol. 23, 1st ed, *Corynebacterium glutamicum* Biology and Biotechnology. Springer-Verlag Berlin Heidelberg. https://doi.org/10.1007/978-3-642-29857-8

Zaidun, N.H. ; Thent, Z.C. ; Latiff, A.A., 2018. “Combating oxidative stress disorders with citrus flavonoid: Naringenin.” *Life Sciences*, 208 , 111–122. https://doi.org/10.1016/j.lfs.2018.07.017

Zha, W. ; Rubin-Pitel, S.B. ; Shao, Z. ; Zhao, H., 2009. “Improving cellular malonyl-CoA level in *Escherichia coli* via metabolic engineering.” *Metabolic Engineering*, 11 (3), 192–198. https://doi.org/10.1016/j.ymben.2009.01.005

Zhou, S. ; Liu, P. ; Chen, J. ; Du, G. ; Li, H., 2016. “Characterization of mutants of a tyrosine ammonia-lyase from *Rhodotorula glutinis*.” *Applied Microbiology and Biotechnology*, 100 (24), 10443–10452. https://doi.org/10.1007/s00253-016-7672-8

4 Appendix

4.1 Author Contributions

Milke, L. *; Aschenbrenner, J. *; Marienhagen, J. ; Kallscheuer, N., 2018. “Production of plant-derived polyphenols in microorganisms: current state and perspectives.” *Applied Microbiology and Biotechnology*, 102 (4), 1575–1585. https://doi.org/10.1007/s00253-018-8747-5

* shared first authorship

LM, JA and NK performed literature and data base research, collected and compiled the material. LM, JA, JM and NK discussed the structure of the manuscript, wrote and revised it.

Milke, L. *; Ferreira, P. *; Kallscheuer, N. ; Braga, A. ; Vogt, M. ; Kappelmann, J. ; Oliveira, J. ; Silva, A.R. ; Rocha, I. ; Bott, M. ; Noack, S. ; Faria, N. ; Marienhagen, J., 2019a. “Modulation of the central carbon metabolism of *Corynebacterium glutamicum* improves malonyl-CoA availability and increases plant polyphenol synthesis.” *Biotechnology and Bioengineering*, 116 (6), 1380–1391. https://doi.org/10.1002/bit.26939

* shared first authorship

LM constructed the plasmids and strains, performed shake flask cultivation experiments, metabolite extraction, LC-MS analysis and contributed to intracellular malonyl-CoA extraction and wrote the respective parts of the manuscript. PF performed the bioreactor experiments and wrote the respective part of the manuscript. JK developed and perfomed the LC-MS/MS measurement for malonyl-CoA quantification. NK, AB, MV, ARS and IR contributed to manuscript preparation. MB, SN, NF and JM revised the manuscript.

Milke, L. ; Kallscheuer, N. ; Kappelmann, J. ; Marienhagen, J., 2019b. “Tailoring *Corynebacterium glutamicum* towards increased malonyl-CoA availability for efficient synthesis of the plant pentaketide noreugenin.” *Microbial Cell Factories*, 18, 71. https://doi.org/10.1186/s12934-019-1117-x

LM conceived the design of the study, constructed plasmids and strains, performed the cultivation experiments, noreugenin extraction, cyclization and LC-MS analysis and wrote the manuscript. JK performed the LC-MS/MS measurements for intracellular malonyl-CoA quantification. NK and JM revised the manuscript.

Kallscheuer, N. ; Kage, H. ; Milke, L. ; Nett, M. ; Marienhagen, J., 2019. “Microbial synthesis of the type I polyketide 6-methylsalicylate with *Corynebacterium glutamicum*.” *Applied Microbiology and Biotechnology*, 103 (23-24), 9619–9631, https://doi.org/10.1007/s00253-019-10121-9

LM performed toxicity experiments with *C. glutamicum* and *E. coli*, plasmid construction, cultivation and LC-MS analysis of inactive PPTase variants and contributed to the manuscript. NK conceived the design of the study, performed all other *C. glutamicum* experiments and wrote the manuscript. HK performed the enterobactin experiments with *E. coli* and the toxicity experiment with *S. cerevisiae*. MN contributed to the manuscript. JM revised the manuscript.

Milke, L. ; Mutz, M. ; Marienhagen, J., 2020. “Synthesis of the character impact compound raspberry ketone and other flavoring phenylbutanoids of biotechnological interest with *Corynebacterium glutamicum*.” *Microbial Cell Factories*, 19, 92. https://doi.org/10.1186/s12934-020-01351-y

LM conceived the experimental design of the study, searched for enzyme candidates and wrote the manuscript. MM performed the experimental works as master’s student under guidance of LM. LM and JM wrote the manuscript.

4.2 Patent application

Kallscheuer, N. ; Milke, L. ; Bott, M. ; Marienhagen, J., 2018. Patentanmeldung PT 1.2829 “Bereitstellung von Malonyl-CoA in coryneformen Bakterien sowie Verfahren zur Herstellung von Polyphenolen und Polyketiden mit coryneformen Bakterien“

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6 Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in der vorgelegten oder ähnlichen Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Jülich, den 17.12.2019

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Lars Milke

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