# A *Pseudomonas taiwanensis* malonyl-CoA platform strain for polyketide synthesis

Tobias Schwanemanna, Maike Ottoa, Benedikt Wynandsa, Jan Marienhagena,b and Nick Wierckxa‡

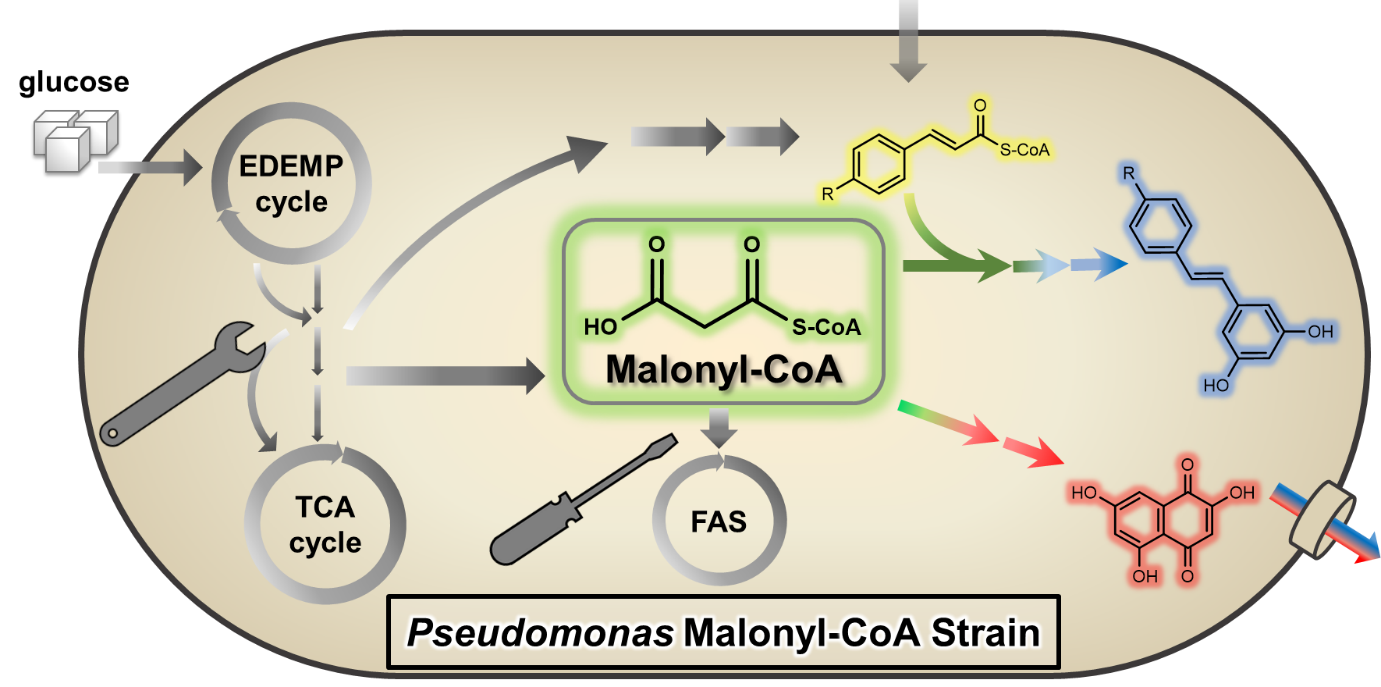
a Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich GmbH, 52425 Jülich, Germany

b Institute of Biotechnology, RWTH Aachen University, Worringer Weg 3, D-52074 Aachen, Germany

‡ correspondingauthor**:**

Nick Wierckx, Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich, Wilhelm-Johnen-Straße, 52425 Jülich, Germany. e-mail: n.wierckx@fz-juelich.de

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# Abstract:

Malonyl-CoA is a central precursor for biosynthesis of a wide range of complex secondary metabolites, in native fatty acid biosynthesis and thus of its products. The development of platform strains with increased malonyl‑CoA supply can contribute to the efficient production of secondary metabolites, especially if such strains exhibit high tolerance towards these chemicals. In this study, *Pseudomonas* *taiwanensis* VLB120 was engineered for increased malonyl-CoA availability to produce bacterial and plant-derived polyketides. A multi-target metabolic engineering strategy focusing on decreasing the malonyl-CoA drain and increasing malonyl-CoA precursor availability, led to an increased production of various malonyl-CoA-derived products, including pinosylvin, resveratrol and flaviolin. The production of flaviolin, a molecule deriving from five malonyl-CoA molecules, was doubled compared to the parental strain by this malonyl-CoA increasing strategy. Additionally, the engineered platform strain enabled production of up to 84 mg L-1 resveratrol from supplemented *p*‑coumarate. One key finding of this study was that acetyl-CoA carboxylase overexpression majorly contributed to an increased malonyl-CoA availability for polyketide production in dependence on the used strain-background and whether downstream fatty acid synthesis was impaired, reflecting its complexity in metabolism. Hence, malonyl-CoA availability is primarily determined by competition of the production pathway with downstream fatty acid synthesis, while supply reactions are of secondary importance for compounds that derive directly from malonyl-CoA in *Pseudomonas*.

**Keywords:** Polyketide; Malonyl-CoA; *Pseudomonas*; Stilbene; Flaviolin; fatty acid biosynthesis

# Introduction:

Chemical synthesis of plant polyketides is often laborious or unfeasible for very complex products. Currently, the main source of such chemicals are often the natural producers, which accumulate only low amounts of product embedded in a complex biomass matrix. Their production thus requires extensive and costly extraction methods. This can be avoided by the transfer of the natural biosynthesis pathway to industrially established microbial hosts (Braga & Faria, 2022; Wolf et al., 2021; Yang et al., 2020, 2022; Palmer & Alper, 2019), which is often essential for the development of a cost-efficient production process (Liu et al., 2017). Such microbial processes can increase product concentrations, enable better scale-up of production, and facilitate downstream processing (Tharmasothirajan et al., 2021; Wang et al., 2016).

When designing a novel biotechnological production process, it is important to choose a host whose properties best match the expected conditions encountered in the envisioned process applications (Blombach et al., 2021). In this respect, bacteria of the genus *Pseudomonas* are well-known for their resistance towards xenobiotics and solvent tolerance (Bitzenhofer et al., 2021; Ramos et al., 2015). This tolerance is of interest for polyketide products displaying antimicrobial properties. Some Pseudomonads are even natural producers of polyketide antibiotics, such as 2,4-diacetylphloroglucinol (Yang & Cao, 2012) or mupirocin (Gurney & Thomas, 2011). Pseudomonads may also facilitate functional expression of secondary metabolite gene clusters including e.g. polyketide synthases (PKS) and polyketide modifying enzymes from organisms with varying GC-content due to agnostic acceptance of AT-rich regions (Ackermann et al., 2021) whilst having a naturally high GC-content (Gross et al., 2006). Additionally they possess a native phosphopantetheinyl transferase with broad substrate spectrum (Beld et al., 2014; Owen et al., 2011). These traits, *inter alia*, make Pseudomonadsvery suitable hosts for heterologous pathway implementations (Blombach et al., 2021; Loeschcke & Thies, 2015; Nikel & de Lorenzo, 2018). However, *Pseudomonas* is so far rarely used for heterologous polyketide synthesis approaches (Incha et al., 2020). Albeit, they allow fermentation process designs that are not feasible for other organisms such as two-phase cultivations with toxic solvents (e.g. toluene (Ramos-González et al., 2003), decanol, methyl decanoate and more (Demling et al., 2020)) for *in situ* product extraction (Heipieper et al., 2007). Hence, development of a *Pseudomonas* platform for secondary metabolite production, especially polyketides, is highly desirable.

Aromatics production via the shikimate pathway is well established in Pseudomonads and many different molecules derived from aromatic amino acids can be synthesized efficiently such as phenol (Wynands et al., 2018), *trans*-cinnamate (Otto et al., 2019), or *cis*,*cis*-muconate (Kuatsjah et al., 2022; Ling et al., 2022) among many more (Schwanemann et al., 2020). Alternatively, aromatics are produced from condensation of coenzyme A (CoA) esters by type III PKS (Bisht et al., 2021; Morita et al., 2010). High-value polyketides like stilbenoids are synthesized by stilbene synthases (STS) with CoA-bound phenylpropanoids as starter units, which are extended by three malonyl-CoA (also called MaCoA) molecules to form a tetraketide intermediate, followed by a C2→C7 aldol condensation reaction for aromatic ring formation. Pinosylvin (*trans*-3,5-dihydroxystilbene) and resveratrol (*trans*-3,5,4’-trihydroxystilbene) are made from cinnamoyl-CoA or *p*-coumaroyl-CoA as starter molecules, respectively (Jeandet et al., 2021). Other polyketides are exclusively synthesized from malonyl-CoA like the colorant flaviolin which is made by 1,3,6,8‑tetrahydroxynaphthalene synthase (THNS) and subsequent spontaneous oxidation (Funa et al., 1999; D. Yang et al., 2018). Synthesis of these secondary metabolites is often limited by product toxicity and intracellular malonyl-CoA content of the host (Milke et al., 2018; van Summeren-Wesenhagen & Marienhagen, 2015). The limiting malonyl-CoA supply is likely especially relevant for *Pseudomonas* due to the lower native CoA ester content compared to other species (Gläser et al., 2020).

Malonyl-CoA is a central metabolite for fatty acid *de novo* synthesis (FAS) and it serves as precursor for a plethora of secondary metabolites in diverse organisms (Cronan & Thomas, 2009). Especially in polyketide biosynthesis, malonyl-CoA is a frequent extender unit. Due to its universal use as precursor in different biosynthetic pathways, several microbial hosts have been engineered for increased availability of malonyl-CoA for heterologous secondary metabolite production (J. Liu et al., 2022; Milke & Marienhagen, 2020; Palmer & Alper, 2019). These include *Escherichia coli* (Yang et al., 2018), *Corynebacterium glutamicum* (Milke, Ferreira, et al., 2019; Milke, Kallscheuer, et al., 2019)*, Streptomyces* spp.(Liao et al., 2022), *Saccharomyces cerevisiae* (S. Li et al., 2021),and *Yarrowia lipolytica* (Sáez-Sáez et al., 2020).

In this work, a robust genome-reduced *Pseudomonas* *taiwanensis* VLB120 is engineered as a platform strain for the synthesis of polyketides by increasing intracellular malonyl-CoA availability and reducing the drain into FAS (Fig. 1).



Fig. 1 Schematic representation of heterologous stilbene and flaviolin synthesis from malonyl-CoA. Target of inhibition by cerulenin and the respective heterologous enzymes for product synthesis are indicated. R represents H or OH group. Abbreviations: EDEMP cycle, Entner-Doudoroff Embden-Meyerhof-Parnas cycle (Nikel et al., 2015); TCA cycle, tricarboxylic acid cycle; FAS, fatty acid biosynthesis; PAL, phenylalanine ammonia-lyase; TAL, tyrosine ammonia-lyase; 4CL, 4-coumaroyl:CoA ligase; STS, stilbene synthase; RppA, 1,3,6,8‑tetrahydroxynaphthalene synthase

# Materials and Methods:

## Bacterial Strains, Plasmids, and Cultivation Conditions

In this study, *Escherichia coli* and *Pseudomonas taiwanensis* VLB120 strains were cultured in LB medium at 37°C and 30°C, respectively, with antibiotics if required (50 mg L-1 kanamycin sulfate; 20 mg L-1 gentamycin disulfate G418; 100 mg L‑1 ampicillin sodium salt; 50 mg L-1 apramycin sulfate; tetracycline hydrochlorid 30 mg L-1). LB medium contained 10 g L−1 peptone, 5 g L−1 sodium chloride, and 5 g L−1 yeast extract. Solid agar plates contained 1.5% (w/v) agar. For selection of *P. taiwanensis* after conjugational matings, cetrimide agar (Sigma-Aldrich) with 10 mL L-1 glycerol or LB with 25 mg L‑1 irgasan (triclosan) were used. Strains and plasmids used or generated in this study are shown in Supplementary Table S1 and Supplementary Table S2, respectively. The strains are available upon request.

Growth and production experiments started with a liquid LB seed culture inoculated from cryo‑conserved stock, followed by an adaptation and main culture in mineral salt medium (MSM) adapted from Hartmans *et al.* (1989) at pH 7 with varying carbon sources and antibiotics if necessary. The medium buffer components (22.3 mM K2HPO4 and 13.6 mM NaH2PO4) were added 1- to 3-fold. Cerulenin (Sigma-Aldrich) (freshly dissolved in ethanol or methanol when indicated) was added to a final concentration of 180 µM in indicated experiments. Glucose was applied at 30 mM in production cultures and 20 mM in adaptation precultures. The nitrogen concentration of 2 g L-1 (NH4)2SO4 (≈30 mM NH4+) was reduced for nitrogen-limiting conditions to 0.333 g L-1 (≈5.05 mM NH4+), which corresponds to a C/N ratio of 6:1 under normal conditions and 36:1 when 30 mM glucose was used. MSM adaptation cultures were inoculated from LB seed cultures to an initial OD600 of 0.2. In biotransformation experiments a higher initial OD600 was used in main cultures as indicated for the respective experiment. Production experiments were performed in 100 mL Erlenmeyer flasks with 10 mL filling volume or in 24 square well System Duetz plates (EnzyScreen, Leiden, Netherlands) with 1.5 mL filling volume. Cultures were shaken at a frequency of 200 rpm (shake flasks) or 300 rpm (System Duetz well plates) in rotary shakers with a throw of 50 mm. Growth and toxicity experiments were performed in MSM with 20 mM glucose in the Growth Profiler (EnzyScreen, Leiden, Netherlands) in 96 square well microtiter plates with 200 µL filling volume at 30°C, 225 rpm and a throw of 50 mm.

## Plasmid construction and genetic modifications – DNA Techniques

Plasmids were cloned applying the Gibson assembly methodology using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, New Ipswich, USA). DNA oligonucleotides for PCR and sequencing were purchased from Eurofins Genomics (Ebersberg, Germany) (Supplementary Table S3). DNA fragments used for cloning were amplified using the Q5 High-Fidelity Polymerase Master Mix or purchase synthetically (Supplementary Table S4). Colony PCRs and other diagnostic PCRs were performed with OneTaq 2x Master Mix (New England Biolabs, New Ipswich, USA) after pre-lysis with alkaline PEG 200 pH 12.75 (Chomczynski & Rymaszewski, 2006). The deletion procedure is based on two successive homologous recombination events. The second homologous recombination is selected through the induction of DNA double strand breaks introduced through I-SceI at its recognition sites introduced by pEMG (Martínez-García & de Lorenzo, 2011), pGNW2 (Wirth et al., 2020) or pSNW2 plasmids (Volke et al., 2020). In short, 400-800 bp of flanking regions of the target gene were amplified from the genome and cloned into a suicide plasmid which integrates into the genome by homologous recombination (HR). Sequences between the flanking sequences result in exchanges instead of full deletions. Subsequent, I-SceI expression induces double strand break and HR results in either wild-type or modified mutant. Lastly, positive strains were cured from the I-SceI expression plasmid (Wynands et al., 2018). Genes for pinosylvin synthesis or flaviolin synthesis were integrated at the Tn7-site and expressed using synthetic constitutive promoters (Zobel et al., 2015). For the recycling of antibiotic resistance marker, constructs with FRT sites flanking the antibiotic resistance gene were used and followed by transformation with pBBFLP for marker excision (Ackermann et al., 2021).

The genes *AtPAL2 and Sc4CLA294G* were codon-optimized for *P. taiwanensis* VLB120 and obtained from a previous study by Otto *et al*. (2019; 2020). The sequence for AhSTS was equipped with a N-terminal his6-tag and additionally a codon-optimized version (his.AhSTSopt) for *P. taiwanensis* VLB120, using the online tool OPTIMIZER (Puigbò et al., 2007) with manual curation of restriction sites according to the SEVA standard, was purchased (Damalas et al., 2020; Martínez-García et al., 2022). Genes were ordered as synthetic DNA fragments from Thermo Fisher Scientific *GeneArt* (Regensburg, Germany). Stilbene synthases AhSTS, PsSTS, and HisPsSTST248A were provided by van Summeren-Wesenhagen and Marienhagen (2015). Template for acetyl-CoA carboxylase from *C. glutamicum* (CgACC) was pEKEx3\_*accBC\_accD1* (Milke, Ferreira, et al., 2019); SgRppA was codon-optimized as described before for his.AhSTSopt. Sequences of ordered synthetic DNA fragments can be seen in Table S4.

## Sampling and analysis:

Manual measurements of the optical density were performed at 600 nm with GE Healthcare UltrospecTM 10 device from Fischer Scientific GmbH (Schwerte, Germany).

The online analysis of culture growth by the Growth Profiler device is based on read out of the green pixels of a taken photo. Measured green values by Growth Profiler from photographs’ pixels were converted into OD600 equivalent values based on a calibration with *P. taiwanensis* VLB120 wild-type strain (Supplementary S5).

Compounds of interest were analyzed by high performance liquid chromatography (HPLC). Samples of stilbenoids were made from 1 mL culture broth mixed with an equal amount of ethyl acetate for extraction. The mixture was shaken for at least 15 minutes at 1500 rpm in an IKA VIBRAX VXR basic at room temperature. This was followed by a 10-15 minutes centrifugation step in Centrifuge 5425 from Eppendorf AG (Wesseling-Berzdorf/Germany). Eight hundred microliter of the top layer were transferred into an amber glass vial for evaporation at room temperature. After full evaporation, acetonitrile was added to reach a one- to eight-fold concentration of extracted compounds being in the linear correlation of HPLC analysis. HPLC vials were closed with a solvent resistant PTFE lined cap. Cinnamate was not quantified from extracts but from filtered aqueous supernatants of a culture. Authentic reference solutions of pinosylvin were made in acetonitrile. Cinnamate stock solutions were dissolved in water titrated with NaOH. HPLC analysis was performed in a 1260 Infinity II HPLC equipped with a 1260 DAD WR (Agilent Technologies) using an ISAspher 100-5 C18 BDS column (Isera, Düren, Germany) at a temperature of 40°C and a flow of 0.8 mL min-1. Quantification was done with a DAD detector at 245 nm for cinnamate and 300 nm for pinosylvin. The injection volume was 10 µL and the flow profile of the solvents for the first 2 minutes was initially 10% acetonitrile (ACN) and 90% H2O with 0.1% trifluoroacetic acid (TFA). Afterwards a gradient from 2-6 min from 10% to 100% ACN was performed. From 6-8 min the conditions were hold at 100% ACN. From 8-10 min ACN decreased back to 10% and 90% H2O with 0.1% TFA. Finally, from 10-12 min the conditions were hold at these conditions. Samples were stored at 14°C during analysis. Retention time of cinnamate is at 7.75 min (245 nm) and pinosylvin elutes after 8.01 min (300 nm).

Flaviolin was measured from culture supernatant when cell pellets appeared colorless, indicating full secretion of flaviolin. HPLC method for flaviolin used the same equipment and 0.8 mL min-1 flow rate but at 30°C for the column and an injection volume of 5 µL. Initial flow was 5% ACN and 95% water with 0.1% TFA for 1 min, then from 1-2 min to 30% ACN, 2-11 min gradient to 60% ACN, 11-13 min up to 95% ACN. ACN 95% was hold for 2 min then from 15-17 min ACN decreased back to 5% and hold for 2 min. Detection of flaviolin was done at 310 nm after 8.93 min, after 10.76 another unknown compound eluted in flaviolin supernatants. Flaviolin was identified by reported UV spectrum from Gross et al. (2006) (Supplementary Fig. S6 and Fig. S7). Using the same acquisition method, *p*-coumarate was detected at 280 nm after 7.13 min, resveratrol after 9.08 min at 310 nm, cinnamate at 245 nm after 11.54 min and pinosylvin after 14.35 min at 300 nm.

Significance analysis was performed by determination of the standard deviation or standard error of the mean when indicated, followed by an ordinary one-way or two-way ANOVA using the software GraphPad Prism 9 with assumed Gaussian distribution, minimum p<0.05.

# Results and Discussion:

## *Pseudomonas* as tolerant host towards the stilbenoid pinosylvin

Besides specific cytotoxic properties of stilbenoids, their physical properties also likely pose a general stress on the cell due to their hydrophobicity that can be described as the logarithmic distribution of a compound in an *n*-octanol-water two-phase system (log PO/W). In this work, the log PO/W of pinosylvin was determined to be 3.65 ± 0.19 (initial concentration of 35 mg L-1, pH=6.2). The log PO/W of a hydrophobic chemical correlates linearly with its partitioning between bacterial membranes and an aqueous buffer (Sikkema et al., 1994) and is therefore an indicator for a compounds toxicity. Solvent-tolerant Pseudomonadsare considered especially resistant to chemicals with a log PO/W value between 2.5 and 4 (Rojas et al., 2004; Sardessai & Bhosle, 2004) making them promising hosts for stilbene synthesis. *P. taiwanensis* VLB120 was chosen over other Pseudomonads due to its solvent-tolerance and classification as biosafety level 1 organism in Germany. Furthermore, there are genome-reduced chassis strains available for this species with greatly improved bioprocess features (Wynands et al., 2019). To assess the tolerance of *P. taiwanensis* VLB120 wild-type, genome-reduced *chassis* strains GRC1, GRC2, GRC3 (Wynands et al., 2019) and *E. coli* BL21 (DE3), these strains (Supplementary Table S1) were cultured in mineral salt medium (MSM) with 0, 50, 100, and 150 mg L-1 pinosylvin (Fig. 2). The growth of *E. coli* BL21 (DE3) was greatly reduced, reaching only 63% of the final biomass in the presence of 50 mg L-1 (0.24 mM) pinosylvin compared to the unstressed control and completely inhibited by 100 and 150 mg L-1 (0.47 mM and 0.71 mM). In contrast, all *P. taiwanensis* strains were able to grow at all applied concentrations with a reduction of final biomass by about 72% for the highest pinosylvin concentration. With increasing pinosylvin concentration the biomass density decreased, indicating an energy-demanding tolerance mechanism (Isken et al., 1999). Compared to wild-type *P. taiwanensis* VLB120,the genome-reduced *chassis* strains all grew better in the presence of pinosylvin. Strains GRC1 and GRC3 reached the highest final biomass, while GRC2 which constitutively expresses the TtgGHI solvent efflux pump performed slightly worse, indicating that this efflux pump does not significantly contribute to pinosylvin tolerance. However, this efflux pump might be beneficial for later bioprocess development employing biphasic cultivations, and therefore GRC3 with an inducible solvent efflux pump was used as a base strain for polyketide synthesis in this study.

Pinosylvin concentrations decreased during the toxicity assessment experiment with only approximately 30-70% remaining after four days in the sterile medium control and in culture supernatants (Supplementary Fig. S8). This abiotic loss of pinosylvin is consistent with previous reports of resveratrol instability under aerobic conditions (Braga et al., 2018). It is strongly affected by a variety of experimental parameters, thus highlighting the importance of reference cultivations in each experiment in the following sections because varying cultivation times and applied biomasses will affect the final pinosylvin concentration. Prospectively, stabilization of the product by e.g. *in situ* extraction might be an option.



Fig. 2 Growth of *E. coli* BL21 (DE3) (A) and *P. taiwanensis* VLB120 (B) in presence of increasing concentrations of pinosylvin; and genome-reduced *chassis* strains GRC1, GRC2 and GRC3 in presence of 100 mg L-1 (C) and 150 mg L-1 pinosylvin (D) in MSM with 20 mM glucose in 96-square half deepwell plates in the Growth Profiler. The corresponding cultures of *E. coli* BL21, *P. taiwanensis* VLB120 wild-type are included in C and D for comparison. Error bars indicate the standard deviation (n=3).

## Conversion of a phenylalanine platform strain into a pinosylvin producer

We previously engineered a *P. taiwanensis* phenylalanine platform strain (GRC3 Δ8Δ*pykA*-tap; here called GRC3 PHE) with multiple modifications of the shikimate pathway, producing 2.6 mM phenylalanine from 20 mM glucose or 3.3 ± 0.07 mM cinnamate (22.8% Cmol Cmol−1) when equipped with phenylalanine ammonia-lyase from *Arabidopsis thaliana* (AtPAL2) (Otto et al., 2019). Pinosylvin synthesis from phenylalanine requires deamination to cinnamate and subsequent CoA activation. Finally, an STS converts the resulting cinnamoyl-CoA and three malonyl-CoA into pinosylvin and four CO2 (Fig. 1). Genomic integration of pinosylvin synthesis module *AhSTS-Sc4CLA294G-AtPAL2*, consisting of his-tagged stilbene synthase AhSTS from *Arachis hypogaea* (Schöppner & Kindl, 1984), cinnamate:coenzyme A ligase mutant Sc4CLA294G from *Streptomyces coelicolor* A3 (Kaneko et al., 2003) and AtPAL2 (Cochrane et al., 2004) were made to enable *de novo* cinnamate and pinosylvin synthesis. High initial biomass and addition of 180 µM of FAS inhibiting cerulenin was used to elevate malonyl-CoA availability (Supplementary S9). Cerulenin addition resulted in strong growth inhibition of the host, leading to only one further doubling of the OD600 after its addition. It should be noted that the applied cerulenin concentration was very high compared to concentrations typically used in other hosts such as *E. coli* (≤100 µM (Hu et al., 2022; Yang et al., 2018)) or *C. glutamicum* (25 µM (Kallscheuer et al., 2016)) or pathogenic *Mycobacterium avium* (22 µM (McCarthy, 1988)), likely reflecting the high tolerance of *P. taiwanensis* to chemical stress. Different carbon sources in presence of cerulenin were tested since these can greatly affect product yields due to the entry point into the central carbon metabolism and consequential metabolic rearrangements (Otto et al., 2019). Indeed, final pinosylvin titers were highly dependent on the used carbon source (Fig. 3A). The use of glycolytic substrates like glucose and glycerol enabled higher pinosylvin titers of 43.8 ± 0.4 mg L-1 (0.21 mM) and 34.4 ± 1.6 mg L-1 (0.16 mM), respectively, compared to gluconeogenic substrates like succinate (13.2 ± 0.3 mg L-1, 0.06 mM) and xylose (14.1 ± 0.3 mg L-1, 0.07 mM) (Fig. 3) which enter central metabolism in the TCA cycle (Köhler et al., 2015). Glucose enabled higher pinosylvin titers than glycerol under the tested conditions. Interestingly, not just pinosylvin, but also cinnamate titers were lower on glycerol than on glucose in the cerulenin-inhibited bioconversion tested here (Fig. 3B). This is contrary to cinnamate yields of the parent strain, which were higher on glycerol (Otto et al., 2019). Titers reached on succinate and xylose differ in their cinnamate formation but not in pinosylvin production. An excess of cinnamate was produced under all tested conditions, indicating that malonyl-CoA availability, rather than aromatics production, is the main limiting factor. Addition of formic acid as an auxiliary energy-yielding substrate to glucose increased the titer significantly by 22% although only 5.6% additional carbon was added as formic acid. The formic acid alters the NADH supply (Zobel et al., 2017), which influences enzyme kinetics of central metabolism (Chittori et al., 2011; Ebert et al., 2011), likely reducing flux into the TCA cycle and thus making more acetyl-CoA available as direct precursor of malonyl-CoA. Octanoate is known to counteract cerulenin inhibition (McCarthy, 1988) thereby explaining the low titer of approximately 4 mg L-1 (0.02 mM) with this carbon source.

In summary, the tested glycolytic substrates enabled the highest pinosylvin yields, cinnamate accumulated in all cases, and without the effect of cerulenin, pinosylvin titers were low as can be seen for octanoate. The results of this proof-of-principle experiment indicate that malonyl-CoA availability is the main limitation in pinosylvin production and that FAS inhibition with cerulenin increases this availability by enabling STS to compete with native metabolism.



Fig. 3 Pinosylvin (A) and cinnamate (B) titers from high biomass *de novo* synthesis bioconversions using *P. taiwanensis* GRC3 PHE *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* with addition of 180 µM cerulenin. Different carbon sources were applied in equal amounts of carbon (180 mM carbon atoms) or plus 10 mM formate. Cultivation in MSM with 1x buffer in 1.5 mL square-well System Duetz with inoculation to initial OD600 of 2 (n=2), sampled after 21.3 h. Error bars represent the standard error of the mean. Significance (p<0.05) is indicated by \* (\*\*, p≤0.01; \*\*\*, p≤0.001; \*\*\*\*, p≤0.0001) from one-way ANOVA. Abbreviation: ns, not significant.

## Genomic deletions to increase pinosylvin titers

The addition of cerulenin is often more costly than the actual product of interest, and the addition of a toxic antibiotic complicates downstream processing of products meant for human use. Also the reached titers with growth-inhibiting cerulenin are highly influenced by the applied initial biomass and this increases complexity due to separate biomass and production formation. Because of this, the use of cerulenin needs to be avoided. To this end, rational targeted gene deletions were performed to increase the supply of malonyl-CoA. Natively, a significant proportion of glucose is oxidized in the periplasm to gluconate by pyrroloquinoline quinone (PQQ)‑dependent glucose dehydrogenase (Gcd). Deletion of the encoding gene (*gcd*; PVLB\_05240) was reported to have a positive impact on polyhydroxyalkanoate (PHA) production (Poblete-Castro et al., 2013). It also slightly elevated intracellular malonyl-CoA in *P. putida* while simultaneously reducing the level of free CoA (Gläser et al., 2020).

Deletion of *gcd* in the *P. taiwanensis* phenylalanine platform strain GRC3 PHE had a positive effect on pinosylvin titers (Fig. 4). In contrast, the deletion of PHA synthesis cluster *phaCZC2* (PVLB\_02155-02165) and a type II thioesterase (encoded by *tesB*; PVLB\_03305) had no beneficial effect or were even detrimental in the GRC3 PHE strain background, even though these were key modifications for increasing the formation of methyl ketones deriving from full procession of FAS in *P. taiwanensis* VLB120 (Nies et al., 2020).

The deletion of *gcd* was shown to have an influence on primary metabolic fluxes and the regulation of various genes of the central metabolism, thereby positively affecting PHA synthesis in *P. putida* (Poblete-Castro et al., 2013). Here, the deletion nearly doubled the pinosylvin titer compared to the parental strain revealing that products using malonyl-CoA as direct precursor also benefit from the deletion of *gcd* and not only peripheral products like PHA, which gain their precursors from full procession of FAS which in turn consumes malonyl-CoA.



Fig. 4 Pinosylvin titers from *de novo* synthesis based on *P. taiwanensis* GRC3 PHE *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* with deletions of PHA production cluster *phaCZC2*, glucose dehydrogenase *gcd* or type II thioesterase *tesB*. Cultivation in MSM 30 mM glucose with 1x buffer in 1.5 mL square-well System Duetz plate, initial OD600 was 2. Error bars represent the standard deviation (n=3) and significance (p<0.05) is indicated by \* (\*\*\*\*, p≤0.0001) from two-way ANOVA. Abbreviation: ns, not significant.

## Engineering the acetyl-CoA node by modulation of citrate synthase activity, ACC expression and deletion of the pyruvate shunt

In previous studies, downregulation of the citrate synthase, the pace-making enzyme of the TCA cycle, increased acetyl-CoA availability in *P. putida* (Kozaeva et al., 2021). It also increased malonyl-CoA availability in *C. glutamicum*, if combined with deregulated expression of the gene for acetyl‑CoA carboxylase (ACC) (Milke, Ferreira, et al., 2019; Milke, Kallscheuer, et al., 2019). The fact that formate supplementation increased pinosylvin production in our *P. taiwanensis* GRC3 PHE platform also indicates that a reduced TCA cycle flux increases malonyl-CoA availability. Thus, we sought to decrease the flux into the TCA cycle by rational strain engineering in the phenylalanine platform.

Attempts to delete the *gltA* gene encoding citrate synthase (PVLB\_16320) were unsuccessful in our hands, likely because this gene is considered essential for growth (Molina-Henares *et al.* 2010). We therefore sought to reduce GltA activity with two complementary approaches.

The promoter region upstream of *gltA* (*PgltA*) was predicted by BPROM (Solovyev & Salamov, 2011) and exchanged by the weak synthetic promoter *P14a* together with a bicistronic design element (BCD2) (Mutalik et al., 2013; Zobel et al., 2015) (Fig. 5). Analysis of the promoter exchange variant with SAPPHIRE (Coppens & Lavigne, 2020) revealed a potential -35 region with appropriate distance upstream of the *P14a* promoter (Supplementary Fig. S10). Originally *P14a* has a truncated spacer between its -10 and -35 region (Zobel et al., 2015). Due to the potential -35 region upstream of *P14a* expression might be altered. Therefore, the inserted promoter fused to the potential -35 box is referred to as *P14a*\* (Supplementary Fig. S10).

Besides this transcriptional modulation by promoter exchange, an enzymatic modulation was achieved by replacing *gltA* (PVLB\_16320) with *prpC* (PVLB\_08385), in the native locus of *gltA*. PrpC is a methylcitrate synthase used in propionate metabolism (Dolan et al., 2022; Ewering et al., 2006). The putative PrpC of *P. taiwanensis* VLB120 is uncharacterized, but for PrpC from *P.* *aeruginosa* (87.5% aa identity) there are indications that it has a citrate synthase side activity (Dolan et al., 2022; Mitchell et al., 1995; Watson et al., 1983). This has also been shown for other methylcitrate synthases, which have a side activity for acetyl-CoA with higher Km (Chittori et al., 2011). Thus, a replacement of *gltA* with the PrpC-encoding gene likely reduces citrate synthase activity. To achieve this, *prpC* was first deleted, followed by replacement of *gltA* by *prpC* through homologous recombination, leaving the first 99 bp of *gltA* intact followed by a stop codon and 24 bp upstream sequence of the native *prpC* to avoid major changes in regulation and ribosome binding. This gene replacement strategy ensures expression of *prpC* in the absence of its native inducer propionate (Dolan et al., 2022; Watson et al., 1983). The successful generation of a strain with replacement of *gltA* by *prpC* showed that citrate synthase function can also be attributed to PrpC. This revealed citrate synthase exchangeability with the native methylcitrate synthase in *P. taiwanensis* VLB120, confirming published results obtained from *P. aeruginosa* (Dolan et al., 2022). Conditional essentiality of *gltA* in *P. taiwanensis* VLB120 must therefore result from *prpC* expression profile which might vary from that of *P. aeruginosa* or other pseudomonads during the used deletion procedure.

In addition, the two strategies were combined by replacing the native promoter of *gltA* with *P14a\** in the Δ*gltA*::*prpC* strain, resulting in a Δ*PgltA*::*P14a\** Δ*gltA*::*prpC* genotype.

Lastly, in order to shift carbon fluxes further towards acetyl-CoA, the pyruvate shunt into the TCA cycle was blocked by deletion of the pyruvate carboxylase-encoding *pycAB*.

Beside the accumulation of the central intermediate acetyl-CoA by reducing the activity of acetyl-CoA-consuming reactions or metabolic bypasses, the conversion of acetyl-CoA to malonyl-CoA by ACC is a known bottleneck in polyketide synthesis (Leonard et al., 2007). In *E. coli*, a significant improvement of malonyl-CoA availability was achieved using balanced expression of the four-subunit ACC from *Photorhabdus* *luminescens* (Leonard et al., 2007). The ACC of *P. taiwanensis* VLB120 also consists of four subunits *accA*, *accB*, *accC*, and *accD*, coding for carboxyltransferase α, biotin carboxyl carrier protein (BCCP), biotin carboxylase, and carboxyltransferase β, respectively. In *C. glutamicum*, the overexpression of ACC alone had no effect on malonyl-CoA availability, but it was beneficial in combination with further modifications causing increased acetyl-CoA supply (Milke, Ferreira, et al., 2019). To test the effect of an additional heterologous ACC on malonyl-CoA availability in *P. taiwanensis*, the dimeric ACC from *C. glutamicum* (*Cg\_accBC-accD1*; CgACC) was episomally expressed using plasmid pBT’T in pinosylvin-producing strains with modified acetyl-CoA node to identify possible synergistic effects.

Pinosylvin production with *P. taiwanensis* GRC3 PHEstrains modified around their acetyl-CoA node resulted in varying growth (Supplementary Fig. S11) and titers when compared to the respective control (Fig. 5). Without the heterologous expression of CgACC, none of the modifications resulted in increased titers compared to the GRC3 PHE control (Fig. 5A). Sometimes contrary tendencies in titers were observed between those strains with and without pBT’T-*CgACC*, as for the strain with *PgltA* promoter exchange (Fig. 5A, B). This is likely related to the complex (and partly unknown) regulation of acetyl-CoA and malonyl-CoA homeostasis in primary metabolism of *P. taiwanensis*. We hypothesize that it might also be influenced by the drain of erytrose-4-phosphate and phosphoenolpyruvate into the shikimate pathway in the used phenylalanine platform strain in these experiments. Here, availability of malonyl-CoA, and not cinnamate, is the major bottleneck.



Fig. 5 Pinosylvin titers of *P. taiwanensis* GRC3 PHE *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* with modifications concerning the acetyl-CoA node (A) and with additional plasmid pBT’T-*CgACC* (B). Cultivation in MSM 30 mM glucose with 1x buffer in 1.5 mL square-well System Duetz plate, initial OD600 was 2 in A and 0.4 in B. Error bars represent the standard error of the mean (n=2) and significance (p<0.05) is indicated by \* (\*\*, p≤0.01; \*\*\*\*, p≤0.0001) from two-way ANOVA of t2. Abbreviation: ns, not significant; ND, not determined.

The main improvements in pinosylvin production were achieved by plasmid-based overexpression of ACC from *C. glutamicum* as it was the case in previous studies (Miyahisa et al., 2005; Zha et al., 2009; Zhao et al., 2018). Overexpression of CgACC increased pinosylvin formation compared to the control strain *P. taiwanensis* GRC3PHE *chassis* without the pBT’T-*CgACC* plasmid. The combination of a *PgtlA* promoter exchange with CgACC overexpression led to a further increase of the pinosylvin titer to 14 mg L-1 (0.07 mM), which was more than a three-fold increase compared to the *P. taiwanensis* GRC3 PHE starting strain under similar conditions. We reasoned that the modifications and gained knowledge should be applied in a metabolic context lacking an increased flux into the shikimate pathway.

## Transfer to a platform strain without enhanced aromatics production

Many polyketides do not necessarily require an aromatic CoA ester precursor. Instead, they can be formed from malonyl-CoA exclusively, like the colorant flaviolin. Hence, we aimed to have a strain purely engineered for malonyl-CoA availability without interference of cinnamate formation. Deletion of *gcd* and altered citrate synthase expression in combination with heterologous CgACC expression enabled higher pinosylvin titers in a phenylalanine platform strain. Yet, cinnamate *de novo* supply was higher than its conversion to pinosylvin in these strains which were based on an aromatic production strain and so the shikimate pathway competes with carbon flux towards malonyl‑CoA. Therefore, to reduce complexity of the production system and enable a better evaluation of engineering strategies around malonyl-CoA, beneficial modifications were transferred to a strain without an enhanced flux into the shikimate pathway and thus limited aromatics production. The chosen strain, GRC3Δ6 (∆*pobA*, ∆*hpd*, ∆*quiC*, ∆*quiC1*, ∆*quiC2*, ∆*benABCD*), is a GRC3 derivative lacking several degradation pathways of aromatics to prevent precursor depletion. A codon-optimized version of *SgRppA* from *Streptomyces* *griseus*, encoding THNS, was integrated into the genome of this strain at the Tn7 attachment site (*attTn7*) followed by kanamycin resistance marker recycling (Ackermann et al., 2021) to yield a constitutive flaviolin producer. Thereby, a product formed exclusively from 5 units of malonyl-CoA serves as reporter for malonyl-CoA availability to reduce probable additional complexity from precursor supplementation. Experiments were conducted in 3-fold buffered MSM to avoid pH shifts and ensure flaviolin secretion during cultivation because if buffered insufficiently, cell pellets appeared dark and flaviolin accumulation in the supernatant was distinctly reduced (Supplementary Fig. S12).

Flaviolin was quantified from culture supernatant by comparing absorbance peak area in HPLC due to the unavailability of an authentic standard (Fig. 6 A). HPLC analysis confirmed the previously identified positive effect of Δ*gcd* on polyketide production from glucose with an increased flaviolin titer by about 25% compared to the parental GRC3Δ6 flaviolin producer. Addition of formate also significantly increased the flaviolin titer, but this effect was abolished upon deletion of *gcd*. Notably, both, deletion of Gcd and supplementation of formate changes formation of reducing equivalents (NADPH and NADH), either by forcing the use of glucose-6-phosphate dehydrogenase (Volke et al., 2021) or through formate dehydrogenases (Zobel et al., 2017). This could be a reason why these two approaches do not cumulatively increase flaviolin titers.

The promoter exchange of *PgltA*::*P14a\** caused a significant additional increase of the flaviolin titer by 7% compared to the Gcd deletion, while constitutive genomic expression from *P14f* of CgACC at integration site PVLB\_23545-40 had no additional positive effect here (Fig. 6 A). 3-oxoacyl-ACP synthase III (FabH) is required for initiation of fatty acid biosynthesis (McNaught et al., 2023). Deletion of a FabH homologue-encoding PVLB\_18090 (84.7% aa identity to fabH\_1 (PFL\_1532) from *P. protegens* Pf-5; 74.6% aa identity to fabH1 (PA3286) from *P. aeruginosa* PAO1) (Kondakova et al., 2015) had no effect on the flaviolin titer either. FabH2 (PVLB\_17265) (85.9% aa identity to fabH\_2 (PFL\_1626) from *P. protegens* Pf-5; 84.9% aa identity to fabH (Pp\_4379) from *P. putida* KT2440) seems to be essential for FAS initiation because multiple deletion attempts failed. A malonyl-CoA platform strain of the first generation (GRC3Δ6MC-I) was made by removal of the *attTn7*-based flaviolin production module though pEMG-mediated repair of the Tn7-site to wild-type sequence (Fig. 6 A). When using ethanol as sole carbon and energy source or nitrogen limitation, none of the implemented modifications of GRC3Δ6MC‑I-based flaviolin producer showed a positive effect on production (Supplementary Fig. S13), but GRC3Δ6MC‑I may be beneficial for synthesis of products further downstream deriving from FAS. Deletion of the pyruvate carboxylase (PycAB) resulted in decreased titers in all tested approaches as observed previously for the GRC3 PHE. This anaplerotic bypass of acetyl-CoA through the pyruvate shunt into the TCA cycle highlights that malonyl-CoA supply requires more than just streamlining central carbon metabolism flux to acetyl-CoA in *Pseudomonas*.

While the previously described modifications were made with the intention to increase the formation of malonyl-CoA, the drain of malonyl-CoA into FAS is known to heavily influence malonyl-CoA availability and should thus be addressed. Expression of FabF-2, a FabF (3-ketoacyl-ACP synthase II) homologue identified in *P*. *putida* F1, allowed deletion of 3-ketoacyl-ACP synthases in *E. coli*, the essential and pace-making reaction of FAS (Dong et al., 2021). Here, the FabF-2 homologue from *P. putida* KT2440 (PP\_3303) was chromosomally integrated at PVLB\_02480/85 and expressed by the constitutive promoter *PEM7* in the flaviolin producer GRC3Δ6Δ*gcd*Δ*PgltA*::*P14a\**. Deletion of natively essential FabF (PVLB\_07185) and exchange by a unique barcoding sequence was subsequently achieved (Fig. 6 B). However, 55 bp of *fabF* remained after deletion due to promoter-like regions within *fabF* coding sequence and partly overlapping coding sequence of 4-amino-4-deoxychorismate lyase gene (Supplementary Table S2). This new flaviolin producer reached the highest flaviolin titers, exceeding previous production by 2‑fold compared to its predecessor GRC3Δ6Δ*gcd*Δ*PgltA*::*P14a\**. The second-generation platform strain GRC3Δ6MC‑II was made from this FAS-modified flaviolin producer. Integration of FabF-2 alone reduced flaviolin titers by about 10% indicating a slightly increased precursor drain into FAS, which might be of interest for products deriving from acyl-ACPs. Modifications that did not lead to increased polyketide product titers were listed in Supplementary S14. Based on homologies (Kondakova et al., 2015; McNaught et al., 2023; Whaley et al., 2021) and deletions, we depicted the fatty acid biosynthesis pathway in *P. taiwanensis* (Supplementary Fig. S15).

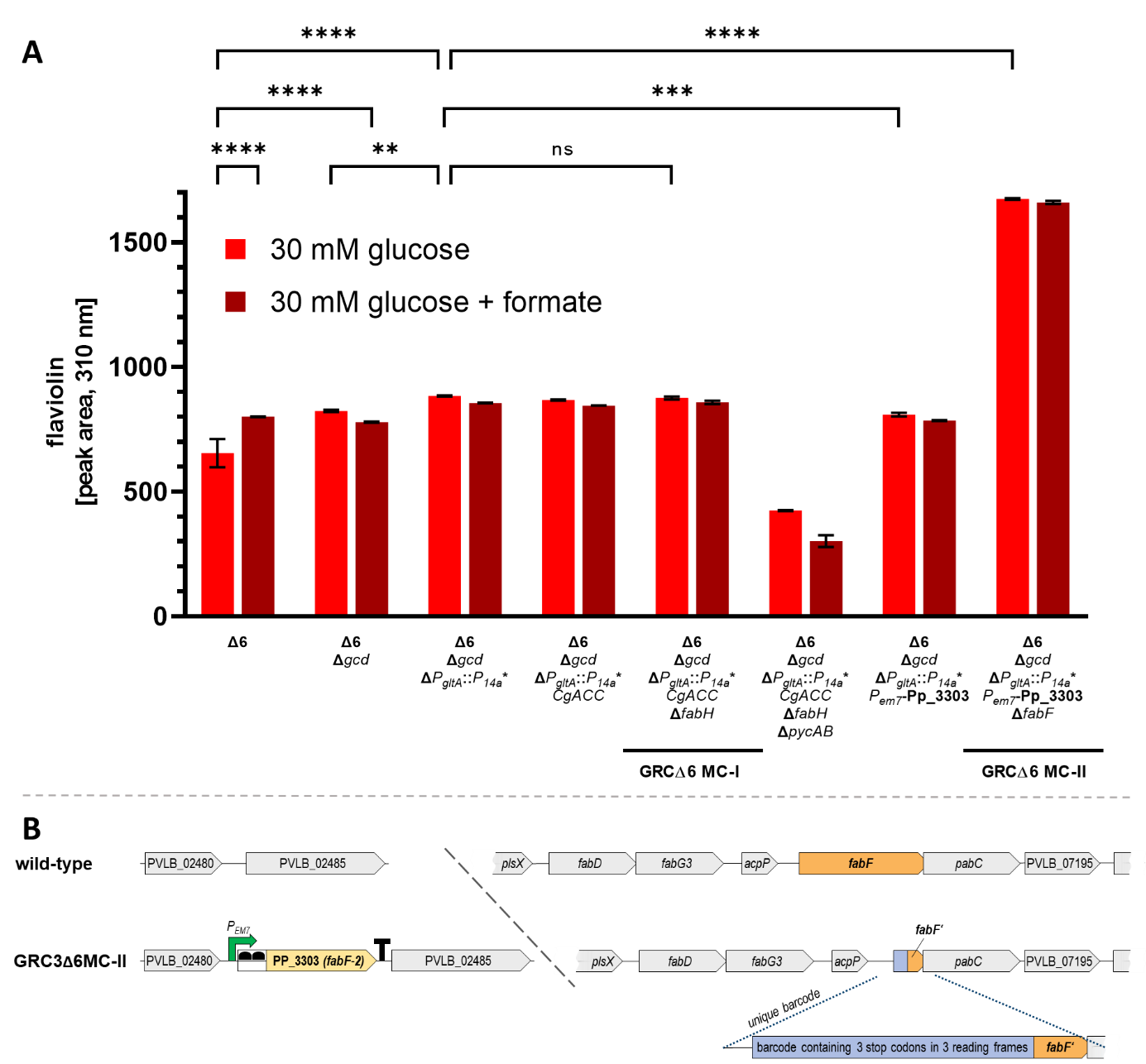


Fig. 6 Flaviolin titers in supernatants of different flaviolin producer strains based on aromatics catabolism deficient GRC3Δ6 with additional indicated modifications (A). Cultivation in MSM with 3x buffer, 30 mM glucose or with additional 10 mM formate in 1.5 mL square-well System Duetz plate, inoculation with 1% of adaption culture. Samples were taken after 91 h and 92.5 h, respectively to ensure full growth and THN to flaviolin oxidation. Error bars represent the standard deviation (n=3) and significance (p<0.05) is indicated by \* (\*\*, p≤0.01; \*\*\*, p≤0.001; \*\*\*\*, p≤0.0001) from two-way ANOVA. Abbreviation: ns, not significant; GRC3Δ6 MC-I, genotype of malonyl-CoA platform strain 1; GRC3Δ6 MC-II, genotype of malonyl-CoA platform strain 2. An illustration of the genetic design of *fabF-2* integration and deletion of native *fabF* in the genomic context of the wild-type sequence and GRC3Δ6MC-II is shown in (B). PVLB\_02480, hypothetical protein; PVLB\_02485, carbamoyltransferase; *plsX*, phosphate acyltransferase; *fabD*, malonyl CoA-acyl carrier protein transacylase; *fabG3*, 3-ketoacyl-(acyl-carrier-protein) reductase; *acpP*, acyl carrier protein; *fabF*, 3-oxoacyl-(acyl carrier protein) synthase II; *pabC*, 4-amino-4-deoxychorismate lyase; PVLB\_07195, hypothetical protein

## Overexpression of CgACC enhances stilbenoid production

After increasing flaviolin titers by reducing FAS, additional expression of CgACC did not further increase flaviolin titers (Fig. 6, Supplementary S12). The modifications likely increased the net flux towards malonyl-CoA, but they also likely increased the intracellular malonyl-CoA concentration, affecting enzyme kinetics. This effect is not apparent with RppA due to its low Km (Supplementary S12), but other synthases for more complex molecules often have a much higher Km, thus highlighting a limitation of flaviolin as reporter.

To test this hypothesis, and to determine whether the overexpression of acetyl-CoA synthaseprovides a benefit for other polyketide synthases, the pinosylvin production module was inserted into *P. taiwanensis* strains GRC3Δ6MC‑II with and without genomic *CgACC* expression.The resulting strains were tested using high initial biomass concentrations for a biotransformation with phenylalanine, cerulenin, or both as supplements. Under all of the tested conditions, overexpression of *CgACC* significantly increased pinosylvin titers (Fig. 7, Supplementary Fig. S16). The effect was most prominent with phenylalanine supplementation without cerulenin, in which case the strain with *CgACC* produced 3.5-fold more pinosylvin than the control. With phenylalanine and cerulenin, a titer of 71 ± 1 mg L-1 (0.35 mM) was reached by GRC3Δ6MC‑II *CgACC*. Hence, GRC3Δ6MC‑II *CgACC* without any production module was named GRC3Δ6MC‑III in following experiments due to its superior performance compared to GRC3Δ6MC‑II in all plant polyketide synthesis approaches with supplements. Interestingly, the newly obtained GRC3Δ6MC‑III-based strains with Δ*fabF* and additional ACC acidify batch culture medium if not buffered sufficiently (Supplementary Fig. S12). Under cerulenin inhibition, biotransformations of the GRC3 PHE strain (Fig. 3) produce only slightly less pinosylvin titers compared to GRC3Δ6MC-III with cerulenin (Fig. 7), although conditions vary somewhat making a direct comparison difficult. In contrast, the impact of the malonyl-CoA modifications becomes apparent in the absence of cerulenin, where MC-III produces 3-fold more than the PHE chassis in the absence of phenylalanine supplementation, and nearly 8-fold more when phenylalanine was supplemented to MC-III (comp. Fig 4 & 7).

Titers from supplemented phenylalanine were about 33.3 mg L-1 (0.16 mM) for the new GRC3Δ6MC-III from only 30 mM (5.4 g L-1) glucose as source for biomass and thus for malonyl-CoA. In other studies with a pinosylvin forming *E. coli* titers of 53 mg L-1 (0.25 mM) were reached from LB medium with additional 10 g L-1 glycerol and supplemented cinnamate (Salas-Navarrete et al., 2018). Pinosylvin titers of *C. glutamicum* DelAro3 from supplemented cinnamate, 40 g L-1 glucose and 25 µM cerulenin were 121 ± 2 mg L-1 (0.57 mM) and thus about twice the titer obtained by GRC3Δ6MC-III in biotransformation with all supplements while DelAro3 used approximately 7-fold glucose concentration (Kallscheuer et al., 2016).



Fig. 7 Biotransformation approach of GRC3Δ6MC‑II and GRC3Δ6MC‑II *CgACC* with pinosylvin production module for pinosylvin synthesis. Cultivation in MSM 30 mM glucose with 3x buffer and different supplements (2 mM phenylalanine, 180 µM cerulenin) in 1.5 mL square-well System Duetz plate, initial OD600 was 3.4 and 2.7 (Supplementary S16), sampled after 21 h. Error bars represent the standard deviation (n=3) and significance (p<0.05) is indicated by \* (\*\*\*\*, p≤0.0001) from two-way ANOVA.

## Evaluation of platform strain GRC3Δ6MC-III by stilbenoid synthesis

In order to elucidate probable effects by more than one reporter molecule deriving from phenylalanine, resveratrol as a product of tyrosine and *p*‑coumarate conversion (Feng et al., 2022) was produced (Fig. 8). The inclusion of this additional product has two main advantages: (1) it broadens the applicable product-spectrum of the new platform strain GRC3Δ6MC-III, and (2) it makes optimal use of the CoA-substrate preference of AhSTS which may reflect in malonyl-CoA consumption. The respective strains were equipped with the pinosylvin production module (Supplementary S17), or with a resveratrol production module (Fig. 8) in which phenylalanine-specific AtPAL2 was replaced by tyrosine-specific ammonia-lyase StsTAL (Cui et al., 2020). In order to assess the possibility of *de novo* synthesis of phenylpropanoid precursors with a lower metabolic burden, a point mutation was introduced in *aroF-1* (P148L) (Wynands et al., 2018) leading to a more moderate increase of metabolic flux into the shikimate pathway compared to the GRC3 PHE strain used in section 3.3 (Fig. 8). However, when supplemented with *p*-coumarate, resveratrol concentrations were 84 ± 2.2 mg L-1 (0.37 mM) and 62.5 ± 2.6 mg L-1 (0.27 mM) for GRC3Δ6MC-III and GRC3Δ6MC-III aroF-1P148L, respectively (Fig. 8). These results indicate that even a moderate increase in aromatics production caused a negative effect likely due to the metabolic burden of aromatics production (Fig. S17, Fig. 8). Approximately 72% of the supplemented *p*-coumarate (0.5 mM) was converted to the product resveratrol with 30 mM glucose in medium for growth and CoA-esters supply (Supplementary Fig. S18). In previous studies using *C. glutamicum* about 169 ± 11.8 mg L−1 (0.8 mM) resveratrol were reached in shake flasks from 220 mM glucose and 5 mM *p*-coumarate which corresponds to a 7-fold higher glucose supply but only 2-fold higher product titer than in this study. However, 1.71 g L-1 (7.5 mM) were reached in biphasic fed-batch cultivation with that strain (Tharmasothirajan et al., 2021). Highest titers so far were reached by yeast *Y. lipolytica* production systems reaching 12.4 g L-1 (54.4 mM) (Sáez-Sáez et al., 2020) and up to 22.5 g L-1 (98.7 mM) resveratrol (Liu et al., 2022).



Fig. 8 Titers of resveratrol and *p*-coumarate in *de novo* production experiments and with supplementation of tyrosine (0.5 mM) or *p*-coumarate (0.5 mM). Cultivation was performed in MSM 30 mM glucose with 3x buffer in 1.5 mL square-well System Duetz plate, initial OD600 was 0.2, sampled after 24 h. Triangular symbols indicate individual resveratrol titers of the replicates. Error bars represent the standard deviation (n=3) and \* indicates p<0.05 confidence interval (\*\*\*, p≤0.001; \*\*\*\*, p≤0.0001) of two-way ANOVA analysis. Abbreviation: ns, not significant, MSM, mineral salt medium; tyr, tyrosine; coum, *p*-coumarate.

Overall, this experiment highlights three aspects. First, even a relatively moderate shift of carbon into the shikimate pathway by introduction of AroF-1P148L results in reduced stilbenoid production. Second, using non-preferred CoA substrates by the used PKS III influences overall achievable product titers (Supplementary Fig. S17). Given the difference in price and availability of the phenylpropanoids precursors and the stilbenes, a biotransformation approach is therefore likely the most economic option. Third, the new malonyl-CoA platform strain GRC3Δ6MC-III can easily be equipped with different production modules to produce different polyketide products (Supplementary S19).

# Conclusion

In this study, a genome-reduced *P. taiwanensis* VLB120 strain with high tolerance towards pinosylvin was engineered for stilbenoid synthesis. Elimination of the metabolic burden of *de novo* synthesis of aromatic precursors was beneficial to achieving efficient production. Malonyl-CoA platform strains were developed using flaviolin as reporter and stilbenes as demonstrator products. Replacement of 3-ketoacyl-ACP synthase II (FabF) in native fatty acid metabolism by the heterologous isoenzyme FabF-2 from *P. putida* KT2440 significantly increased flaviolin titers up to the point where malonyl-CoA availability was no longer limiting for flaviolin synthesis, likely by reducing the drain on malonyl-CoA imposed by fatty acid biosynthesis. The benefit of additional CgACC expression only became apparent for pinosylvin production, likely due to the higher Km value of AhSTS compared to RppA. In general, this study confirms that the malonyl-CoA node is highly complex, and that engineering of increased malonyl-CoA availably requires a cumulative, multi-factorial approach. This was achieved in the engineered *P. taiwanensis* GRC3Δ6MC-III malonyl-CoA platform, which enabled efficient synthesis of both pinosylvin and resveratrol. The strategies for increasing malonyl-CoA supply in *Pseudomonas* will be valuable for future metabolic engineering approaches in related species, and the *P. taiwanensis* GRC3Δ6MC-III platform has a broad applicability for production of malonyl-CoA-derived secondary metabolites.

# CRediT authorship contribution statement:

**Tobias Schwanemann**: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization, Funding acquisition

**Maike Otto**: Methodology, Formal analysis, Investigation

**Benedikt Wynands**: Validation, Writing - Review & Editing, Supervision

**Jan Marienhagen**: Resources, Writing - review & Editing, Supervision

**Nick Wierckx**: Conceptualization, Validation, Resources, Writing - Review & Editing, Supervision, Funding acquisition, Project administration

# Declaration of competing interest

The authors declare no competing interest.

# Funding

This work was supported by the German Federal Environmental Foundation (DBU) [PhD Scholarship 20019/638-32], and the German Federal Ministry of Education and Research (BMBF) with the project NO-STRESS [FKZ 031B0852A].

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# Supporting information to article “A *Pseudomonas taiwanensis* malonyl-CoA platform strain for polyketide synthesis”

Tobias Schwanemanna, Maike Ottoa, Benedikt Wynandsa, Jan Marienhagena,b and Nick Wierckxa‡

aInstitute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich GmbH, 52425 Jülich, Germany

bInstitute of Biotechnology, RWTH Aachen University, Worringer Weg 3, D-52074 Aachen, Germany

‡ correspondingauthor**:**

Nick Wierckx, Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich, Wilhelm-Johnen-Straße, 52425 Jülich, Germany. e-mail: n.wierckx@fz-juelich.de

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## **Table S1:** Bacterial strains used in this study

|  |  |  |
| --- | --- | --- |
| **Strains** | **Relevant characteristics** | **Reference** |
| ***Escherichia coli*** |  |  |
| HB101 pRK2013 | HB101 with pRK2013 | Ditta et al. (1980) |
| PIR2 | *F−*∆*lac169* *rpoS(Am)* *robA1 creC510 hsdR514 endA* *recA1 uidA(*∆*MluI)*::*pir*; host for *oriV(R6K)* plasmids | Thermo Fischer Scientific |
| DH5α | F− *Φ80 lac*ZΔM15 Δ(*lac*ZYA-*arg*F)*U169* *recA1 endA1* *hsdR17*(rk−, mk+) *phoA* *supE44 thi-1 gyrA96* *relA1* λ− | Thermo Fischer Scientific |
| DH5α λ*pir* | λ*pir* lysogen of DH5α; host for *oriV(R6K)* plasmids | Víctor de Lorenzo lab |
| DH5α λ*pir* pTNS1 | DH5α λ*pir* with pTNS1 | Choi et al. (2005) |
| DH5α pSW-2 | DH5α with pSW-2 | Martínez-García & de Lorenzo (2011) |
| BL21 (DE3) | F– *omp*T *hsdSB* (rB–, mB–) *gal dcm* (DE3) | Thermo Fischer Scientific |
| ***Pseudomonas taiwanensis*** |  |  |
| VLB120 | Wild-type | Panke et al. (1998); MiKat#1 |
| GRC1 | Genome-reduced-chassis strain (deleted megaplasmid pSTY, four prophages, flagella apparatus and major biofilm-formation) | Wynands et al. (2019); MiKat#3 |
| GRC2 | Genome-reduced-chassis strain; Δprophage1/2::*ttgGHI* | Wynands et al. (2019); MiKat#4 |
| GRC3 | Genome-reduced-chassis strain; Δprophage1/2::*ttgVWGHI* | Wynands et al. (2019); MiKat#5 |
| GRC3 Δ8pykA-tap (=GRC3 PHE) | Phenylalanine platform strain; GRC3 Δ*pobA*, Δ*hpd*, Δ*quiC*, Δ*quiC1*, Δ*quiC2*, Δ*phhAB*, Δ*katG*, ΔPVLB\_10925, Δ*pykA*, *trpE*P290S, *aroF‑1*P148L, *pheA*T310I | Otto et al. (2019); MiKat#74 |
| GRC3 PHE *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | GRC3 PHE; with pinosylvin production module | This study; MiKat#182 |
| GRC3 PHE ∆*phaCZC2* | Phenylalanine platform strain; deleted PHA cluster (PVLB\_02155-65) | This study; MiKat#338 |
| GRC3 PHE ∆*phaCZC2* *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | GRC3 PHE ∆*phaCZC2*; with pinosylvin production module | This study; MiKat#365 |
| GRC3 PHE ∆*gcd* | Phenylalanine platform strain; deleted glucose dehydrogenase (PVLB\_05240) | This study; MiKat#339 |
| GRC3 PHE ∆*gcd* *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | GRC3 PHE ∆*gcd*; with pinosylvin production module | This study; MiKat#366 |
| GRC3 PHE ∆*tesB* | Phenylalanine platform strain; deleted type II thioesterase (PVLB\_03305) | This study; MiKat#383 |
| GRC3 PHE ∆*tesB* *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | GRC3 PHE ∆*tesB*; with pinosylvin production module | This study; MiKat#388 |
| GRC3 PHE ∆PVLB\_02920 | Phenylalanine platform strain; deleted putative regulator PVLB\_02920 | This study; MiKat#271 |
| GRC3 PHE ∆PVLB\_02920 *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | GRC3 PHE ∆PVLB\_02920; with pinosylvin production module | This study; MiKat#299 |
| GRC3 PHE ∆PVLB\_01730-01815 | Phenylalanine platform strain; deleted putative secondary metabolite cluster | This study; MiKat#262 |
| GRC3 PHE ∆PVLB\_01730-01815 *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | GRC3 PHE ∆PVLB\_01730-01815; with pinosylvin production module | This study; MiKat#265 |
| GRC3 PHE ∆*PgltA*::*P14a*\* | Phenylalanine platform strain; exchanged promoter of citrate synthase | This study; MiKat#263 |
| GRC3 PHE ∆*PgltA*::*P14a\** *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | Phenylalanine platform strain; exchanged promoter of citrate synthase; with pinosylvin production module | This study; MiKat#266 |
| GRC3 PHE ∆*prpC* | Phenylalanine platform strain; deleted methyl citrate synthase | This study; MiKat#314 |
| GRC3 PHE ∆*prpC* *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | Phenylalanine platform strain; deleted methyl citrate synthase; with pinosylvin production module | This study; MiKat#364 |
| GRC3 PHE ∆*prpC* ∆*gltA*::*prpC* | Phenylalanine platform strain; deleted methyl citrate synthase in native locus and replaced citrate synthase | This study; MiKat#361 |
| GRC3 PHE ∆*prpC* ∆*gltA*::*prpC* *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | Phenylalanine platform strain; deleted methyl citrate synthase in native locus and replaced citrate synthase; with pinosylvin production module | This study; MiKat#367 |
| GRC3 PHE ∆*prpC* ∆*PgltA*::*P14a*\* ∆*gltA*::*prpC* | Phenylalanine platform strain; exchanged promoter of citrate synthase; deleted methyl citrate synthase in native locus and replaced citrate synthase | This study; MiKat#518 |
| GRC3 PHE ∆*prpC* ∆*PgltA*::*P14a*\* ∆*gltA*::*prpC* *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | Phenylalanine platform strain; exchanged promoter of citrate synthase; deleted methyl citrate synthase in native locus and replaced citrate synthase; with pinosylvin production module | This study; MiKat#527 |
| GRC3 PHE ∆*pycAB* | Phenylalanine platform strain; deleted pyruvate decarboxylase (PVLB\_25325-30) | This study; MiKat#368 |
| GRC3 PHE ∆*pycAB* *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | Phenylalanine platform strain; deleted pyruvate decarboxylase (PVLB\_25325-30); with pinosylvin production module | This study; MiKat#385 |
| GRC3 PHE ∆*prpC* ∆*gltA*::*prpC* ∆*pycAB* | Phenylalanine platform strain; deleted methyl citrate synthase in native locus and replaced citrate synthase, deleted pyruvate decarboxylase (PVLB\_25325-30) | This study; MiKat#370 |
| GRC3 PHE ∆*prpC* ∆*gltA*::*prpC* ∆*pycAB attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | Phenylalanine platform strain; deleted methyl citrate synthase in native locus and replaced citrate synthase, deleted pyruvate decarboxylase (PVLB\_25325-30); with pinosylvin production module | This study; MiKat#387 |
| GRC3 PHE ∆*prpC* ∆*PgltA*::*P14a*\* ∆*gltA*::*prpC* ∆*pycAB* | Phenylalanine platform strain; exchanged promoter of citrate synthase; deleted methyl citrate synthase in native locus and replaced citrate synthase, deleted pyruvate decarboxylase (PVLB\_25325-30) | This study; MiKat#519 |
| GRC3 PHE ∆*prpC* ∆*PgltA*::*P14a*\* ∆*gltA*::*prpC* ∆*pycAB* *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | Phenylalanine platform strain; exchanged promoter of citrate synthase; deleted methyl citrate synthase in native locus and replaced citrate synthase, deleted pyruvate decarboxylase (PVLB\_25325-30); with pinosylvin production module | This study; MiKat#528 |
| GRC3 Δ5 | GRC3 derivative incapable of growing on 4-hydroxybenzoate, tyrosine and quinate (∆*pobA*∆*hpd*∆*quiC*∆*quiC1*∆*quiC2*) | This study; MiKat#376 |
| GRC3 Δ6 | deleted *benABCD* in GRC3 Δ5 | This study; MiKat#382 |
| GRC3 Δ6 *attTn7*::*FRT-P14f-SgRppA* | with 1,3,6,8-tetrahydroxynaphthalene synthase SgRppA for flaviolin synthesis without resistance marker | This study; MiKat#546 |
| GRC3 Δ6 Δ*gcd* *attTn7*::*FRT-P14f-SgRppA* | Deleted gcd in flaviolin producer | This study; MiKat#668 |
| GRC3 Δ6 Δ*gcd* ∆*PgltA*::*P14a*\* *attTn7*::*FRT-P14f-SgRppA* | Exchanged promoter of citrate synthase in flaviolin producer | This study; MiKat#691 |
| GRC3 Δ6 Δ*gcd* ∆*PgltA*::*P14a\** PVLB\_23545-40::*P14f*-*Cg\_accBC-Cg\_accD1* *attTn7*::*FRT-P14f-SgRppA* | Integration of ACC from *C. glutamicum* in flaviolin producer | This study; MiKat#801 |
| GRC3 Δ6 Δ*gcd* ∆*PgltA*::*P14a*\* PVLB\_23545-40::*P14f*-*Cg\_accBC-Cg\_accD1* ΔPVLB\_18090 *attTn7*::*FRT-P14f-SgRppA* | Deleted putative 3-oxoacyl-ACP synthase III in a flaviolin producer | This study; MiKat#813 |
| GRC3 Δ6 Δ*gcd* ∆*PgltA*::*P14a*\* PVLB\_23545-40::P14f-*Cg\_accBC-Cg\_accD1* ΔPVLB\_18090 (=GRC3Δ6 MC-I) | Malonyl-CoA platform strain No.1 (GRC3Δ6MC I) | This study; MiKat#822 |
| GRC3 Δ6 Δ*gcd* ∆*PgltA*::P14a\* PVLB\_23545-40::*P14f*-*Cg\_accBC-Cg\_accD1* ΔPVLB\_18090 Δ*pycAB* *attTn7*::*FRT-P14f-SgRppA* | deleted pyruvate decarboxylase (PVLB\_25325-30) in a flaviolin producer | This study; MiKat#852 |
| GRC3 Δ6 Δ*gcd* ∆*PgltA*::*P14a*\* PVLB\_02480-85::*PEM7*\_PP3303 (*fabF-2*) *attTn7*::*FRT-P14f-SgRppA* | Integrated cryptic long-chain 3-oxoacyl-ACP synthase II (FabF-2, PP\_3303) from *P. putida* KT2440 in a flaviolin producer | This study; MiKat#912 |
| GRC3 Δ6 Δ*gcd* ∆*PgltA*::*P14a*\* PVLB\_02480-85::*PEM7*\_PP3303 (*fabF-2*) Δ*fabF* (PVLB\_07185)(=GRC3Δ6 MC-II) *attTn7*::*FRT-P14f-SgRppA* | Deleted *fabF* (PVLB\_07185) in a flaviolin producer with *fabF-2;* Platform strain No.2 flaviolin producer | This study; MiKat#912 |
| GRC3 Δ6 Δ*gcd* ∆*PgltA*::*P14a\** PVLB\_02480-85::*PEM7*\_PP3303 (*fabF-2*) Δ*fabF* (PVLB\_07185)(=GRC3Δ6 MC-II) | Malonyl-CoA platform strain No.2 (GRC3Δ6MC II) | This study; MiKat#975 |
| GRC3 Δ6 Δ*gcd* ∆*PgltA*::*P14a\** PVLB\_02480-85::*PEM7*\_PP3303 (*fabF-2*) Δ*fabF* (PVLB\_07185)PVLB\_23545-40::*P14f*-*Cg\_accBC-Cg\_accD1*  (=GRC3Δ6 MC-III) | Malonyl-CoA platform strain No.2 with ACC from *C. glutamicum* (=> No.3) (GRC3Δ6MC III) | This study; MiKat#1058 |
| GRC3 Δ6MC-II PVLB\_23545-40::*P14f-Cg\_accBC-Cg\_accD1* (=GRC3Δ6 MC-III) *attTn7*::*FRT-P14f-SgRppA* | Malonyl-CoA platform strain No.3 flaviolin producer | This study; MiKat#1058 |
| GRC3 Δ6MC-II *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | Malonyl-CoA platform strain No.2 with pinosylvin production module | This study; MiKat#1003 |
| GRC3 Δ6MC-III *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | Malonyl-CoA platform strain No.3 with pinosylvin production module | This study; MiKat#1024 |
| GRC3 Δ6MC-III *attTn7::P14g-his.AhSTS-Sc4CLA294G-StsTAL* | Malonyl-CoA platform strain No.3 with resveratrol production module | This study; MiKat#1127 |
| GRC3 Δ6MC-III *aroF-1P148L* | Malonyl-CoA platform strain No.3 aroF-1 mutant | This study; MiKat#1151 |
| GRC3 Δ6MC-III *aroF-1P148L* *attTn7::P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | Malonyl-CoA platform strain No.3 aroF-1 mutant; with pinosylvin production module | This study; MiKat#1628 |
| GRC3 Δ6MC-III *aroF-1P148L* *attTn7::P14g-his.AhSTS-Sc4CLA294G-StsTAL* | Malonyl-CoA platform strain No.3 aroF-1 mutant with resveratrol production module | This study; MiKat#1629 |

## **Table S2:** Plasmids used in this study

|  |  |  |  |
| --- | --- | --- | --- |
| **Plasmid** | **Relevant characteristics** | **HiFi assembly note** | **Reference & No.** |
| pTNS1 | AmpR, *oriV(R6K)*, TnSABC+D operon | - | Choi et al. (2005) |
| pBBFLP | plasmid for antibiotic markers excision in *P. putida* strains; TcR, *oriV*(pBBR1) *oriT*(RK2) mob+ λPR::FLP λ(cI857) *sacB* *tet* | - | De Las Heras et al. (2008) |
| pEMG | KmR, *oriV(R6K)*, *oriT*, *traJ*, *lacZα*-MCS flanked by two I-SceI restriction sites | - | Martínez-García & de Lorenzo (2011) |
| pGNW2 | Derivative of vector pEMG carrying *P14g*→*msfGFP* | - | Wirth et al. (2020) plasmid #19 |
| pSNW2 | Derivative of vector pEMG carrying *P14g*-BCD2→*msfGFP* | - | Volke et al. (2020, 2021)  plasmid #142 |
| pSW-2 | GmR, *oriV(RK2), oriT, xylS, Pm→* *I-SceI* | - | Martínez-García & de Lorenzo (2011) |
| pSEVA6213S | GmR, *oriV(RK2)*, *PEM7*→*I-SceI*; | - | Wirth et al. (2020) |
| pGNW2-fabD(Ts) W258Q | Genomic exchange of TGG772-774 -> CAG (W258Q) (temperature sensitive) in *fabD* (PVLB\_07170) | Fragment BW682/BW683 from pGNW2; Fragment TS029/TS030 and TS031/TS032 from VLB120 genome | This study  Plasmid #59, #84 |
| pEMG-Ex-PgltA-P14a | Exchange native promoter region of *gltA* (PVLB\_16320) *PgltA* by *P14a*-BCD2 (*P14a*\*) | Cut pEMG with EcoRI and XbaI; Fragment TS042/TS043 and TS046/TS047 from VLB120 genome, Fragment TS044/TS045 from pBG14a | This study  Plasmid #80 |
| pGNW2-KO-prpC | Deletion vector for *prpC* (PVLB\_08385) | Fragment BW682/BW683 from pGNW2; Fragment TS091/TS092 and TS093/TS094 from VLB120 genome | This study  Plasmid #90 |
| pEMG-KO-gltA | Deletion vector for *gltA* (PVLB\_16320) | Cut pEMG with EcoRI and XbaI; Fragment TS042/TS097 and TS098/TS099 from VLB120 genome | This study  Plasmid #91 |
| pSNW2-KO-gltA-apraR | Deletion vector for *gltA* (leave 99bp) and exchange with apramycin resistance | Cut pSNW2 with EcoRI and XbaI; Fragment TS042/TS097 and TS189/TS099 from VLB120 genome, Fragment TS187/TS188 from pQT8 | This study  Plasmid #190 |
| pEMG-Ex-gltA-prpC | Exchange of *gltA* (leave 99bp) with *prpC* | Cut pEMG with EcoRI and XbaI; Fragment TS042/TS097 and TS101/TS099 and TS102/TS103 from VLB120 genome | This study  Plasmid #92 |
| pEMG-Ex-gltA-prpC with *P14a* | Exchange of *PgltA* by *P14a*\* and exchange of *gltA* (leave 99bp) with prpC | Cut pEMG with EcoRI and XbaI; Fragment TS101/TS099 and TS102/TS103 from VLB120 genome, TS042/TS097 from VLB-genome with PGltA::P14a\* | This study  Plasmid #93 |
| pEMG-KO-phaCZCII | Deletion vector for *phaCZC2* (PVLB\_02155-65) |  | Nies et al. (2020) |
| pEMG-KO-tesB | Deletion vector for *tesB* (PVLB\_03305) |  | Nies et al. (2020) |
| pEMGg-aroF-1P148L | Genomic exchange of CCG442 -> CTG (P148L) in *aroF-1* (PVLB\_08330) |  | Wynands et al. (2018) |
| pGNW-KO-pycAB | Deletion vector for *pycAB* (PVLB\_25325-25330) | Fragment BW682/BW683 from pGNW2; Fragment TS135/TS136 and TS137/TS138 from VLB120 genome | This study  Plasmid #135 |
| pGNW-KO\_FabF | Deletion vector for aa residues 1-386 of *fabF* (PVLB\_07185) with insertion of unique barcode with stop codon in all reading frames | Fragment BW682/BW683 from pGNW2; Fragment TS163/TS164 and TS165/TS166 from VLB120 genome | This study  Plasmid #141 |
| pSNW2-KO\_PVLB\_16225 | Deletion vector for *fabB* (PVLB\_16225) | Fragment BW682/BW683 from pSNW2; Fragment TS254/TS255 and TS256/TS257 from GRC3 genome | This study  Plasmid #289 |
| pSNW2-KO-oprF | Deletion vector for *oprF* (PVLB\_07655) | Cut pSNW2 with EcoRI and XbaI; Fragment TS204/TS205 and TS206/TS207 from VLB120 genome | This study  Plasmid #193 |
| pSNW2-KO-PVLB17265 (fabH2) | Deletion vector for PVLB\_17265 (*fabH2*) | Cut pSNW2 with EcoRI and XbaI; Fragment TS232/TS217 and TS218/TS235 from GRC3 genome | This study  Plasmid #196 |
| pSNW2-KO\_PVLB18090 (fabH) | Deletion vector for PVLB\_18090 (*fabH*) | Cut pSNW2 with EcoRI and XbaI; Fragment TS210/TS211 and TS212/TS213 from VLB120 genome | This study  Plasmid #195 |
| pSNW2-attTn7recycling VLB120 | Deletion of marker-free insert at Tn7-site for wt sequence | Cut pSNW2 with EcoRI and XbaI; Fragment TS239/TS240 from VLB120 genome | This study  Plasmid #228 |
| pSEVA412S-benABCD | Deletion vector for PVLB\_12215-12230 (*benABCD*) |  | Otto et al. (2020) |
| pEMG-PVLB\_02480/85-*Pem7*-msfgfp | Integration of *msfgfp* at landing pad PVLB\_02480/85 with *PEM7* |  | Lechtenberg et al., manuscript in preparation |
| pEMG-PVLB\_02480/85-*Pem7*-fabF2 | Integration of *fabF2* (PP\_3303) from *P. putida* KT2440 at landing pad PVLB\_02480/85 with *PEM7* | Fragment TS-019/BW463 from plasmid pEMG-PVLB\_02480/85-Pem7-msfgfp and TS251/TS252 from *P. putida* KT2440 | This study  Plasmid #277 |
| pEMG-PVLB\_23545-40-*P14f*- tyrAfbr | Integration at landing pad PVLB\_23545/40 (homologue to PP\_0340-PP\_0341) with *P14f* |  | Lechtenberg, Wynands; personal communication |
| pEMG-PVLB\_23545-40-P14f-CgaccBC-accD1 | Integration of *accBC-accD1* genes from *C. glutamicum* at landing pad PVLB\_23545-23540 with *P14f* | Fragment BW-463/TS-019 from pEMG-PVLB\_23545-P14f-tyrA(fbr), Fragment TS036/TS250 from pEKEx3\_accBC\_accD1 | This study  Plasmid #249 |
| pBT’Tmcs | Derivative of pBT’mcs, KmR, Ori/IHF, expression vector, constitutive *Ptac* promoter, with RBS, no terminator |  | Koopman et al. (2010) |
| pBNTmcs(t) | KmR, oriV(pBBR1) expression vector containing the salicylate-inducible *nagR/pNagAa* promotor |  | Verhoef et al. (2010) |
| pEKEx3\_accBC\_accD1 | specR; pEKEx3 derivative containing *accBC* and *accD1* genes from *C. glutamicum* |  | Milke et al. (2019) |
| pBT'T-CgaccBC-accD1 | Expression vector for *accBC-D1* from *C. glutamicum* | Cut pBT’Tmcs’ with EcoRI, Fragment TS034/TS035 from pEKEx3\_accBC\_accD1 | This study  Plasmid #78 |
| pBNT-FabD | Salicylate inducible expression vector for *fabD* (PVLB\_07170) | Cut pBNT’Tmcs’ with EcoRI, Fragment TS224/TS225 from VLB120 genome | This study  Plasmid #203 |
| pBNT-FabD W258Q | Salicylate inducible expression vector for *fabDW258Q* (PVLB\_07170) | Cut pBNT’Tmcs’ with EcoRI, Fragment TS224/TS225 from pGNW-fabD(Ts) W258Q | This study  Plasmid #204 |
| pBNT-sigX | Salicylate inducible expression vector for *sigX* (PVLB\_07650) | Cut pBNT’Tmcs’ with EcoRI, Fragment TS222/TS223 from VLB120 genome | This study  Plasmid #205 |
| pBG14g | Tn7 delivery vector; KmR GmR, oriV(R6K), Tn7L, and Tn7R flanks, BCD2-*msfgfp* fusion, synthetic promoter variants |  | Zobel et al. (2015) |
| pBG14g-PstrSTS-Sc4CLA294G-AtPAL2 | Pinosylvin synthesis module with STS from *P. strobus* |  | Otto (2019)  Plasmid #90 |
| pBG14g-PstrSTS\*-Sc4CLA294G-AtPAL2 | Pinosylvin synthesis module with STS from *P. strobus* (codon optimized for *E. coli*) |  | Otto (2019)  Plasmid #89 |
| pBG14g-AhSTS-Sc4CLA294G-AtPAL2 | Pinosylvin synthesis module with STS from *A. hypogaea* (UniProt: Q9SLV5; GeneBank: AXN70034.1; UniProt: P45724) |  | Otto (2019)  Plasmid #54 |
| pBG14g-his.AhSTS-Sc4CLA294G-AtPAL2 | Pinosylvin synthesis module with his-tag | Fragment TS015/TS001 and TS016/TS004 from pBG14g-ahSTS-4CL-atPAL | This study  Plasmid #52 |
| pBG14g-his.AhSTS.opt-Sc4CLA294G-AtPAL2 | Pinosylvin synthesis module with his-tag (codon optimized) | Fragment TS001/TS002 and TS003/TS004 from pBG14g-ahSTS-4CL-atPAL; gBlock opt.AhSTS from synthesis | This study  Plasmid #53 |
| pBG14f\_Kan\_FRT\_StsTAL | Plasmid containing tyrosine ammonia lyase from *Streptomyces sp*. NRRL F-4489 (UniProt: A0A0X3WEK2) |  | Wynands et al., manuscript in preparation, Plasmid #279 |
| pBG14g-his.AhSTS-Sc4CLA294G-stsTAL | Resveratrol synthesis module with his-tag | Fragment TS-275/TS-276 from pBG14g-his.AhSTS-Sc4CLA294G-AtPAL2 (plasmid #52), TS277/TS278 from pBG14f\_Kan\_FRT\_StsTAL (Wynands, manuscript in preparation) | This study  Plasmid #343 |
| pBG14f\_FRT\_Kan | KmR flanked by FRT sites, *oriV(R6K)*, *oriT*, mini-Tn7 transposon delivery vector, *P14f(BCD2)*→*msfgfp* |  | Ackermann et al. (2021) |
| pBG14f\_Km\_FRT\_SgRppA.opt | Recyclable flaviolin production module (1,3,6,8-tetrahydroxynaphthalene synthase) (UniProt: Q54240) | Fragment TS-106/TS-019 from pBG14f-Kan-FRT, gBlock SgRppA.opt from synthesis | This study  Plasmid #207 |
| pBG14f\_Km\_FRT\_his.AhSTS-Sc4CLA294G-AtPAL2 | Pinosylvin synthesis module with recyclable KmR | Fragment TS-106/TS-019 from pBG14f-Kan-FRT, Fragment TS020/TS238 from pBG14g-his.ahSTS-4CL-atPAL (plasmid #52) | This study  Plasmid #229 |

## **Table S3:** Oligonucleotides used in this study

Shown are their name, sequence, and description. Oligonucleotides used for diagnostic PCRs and sequencings are not included.

|  |  |  |
| --- | --- | --- |
| Primer No. | Description | Sequence |
| BW\_463 | pBG42 amplification fwd primer | gaattcgagctcggtaccc |
| BW\_682 | pGNW or pEMG BB amplification fwd | tctagagtcgacctgcag |
| BW\_683 | pGNW or pEMG BB amplification rev | gaattcagattaccctgttatcc |
| TS-001 | BB pBG14ffg\_fwd with overhang atPAL | tatctgctaaagaattcgagctcggtac |
| TS-002 | BB pBG14ffg\_rev with overhang opt.His-tag | tgatgatgcgagctgcccattagaaaacctccttagcatg |
| TS-015 | BB pBG14ffg\_rev with overhang His-tag | tgatggtgatggctgctgcccattagaaaacctccttagcatg |
| TS-016 | AhSTS fwd with His6tag overhang | atgggcagcagccatcaccatcatcaccacagccaggatccaatggtgtccgtgtccggcatc |
| TS-019 | rev on BCD2 for BB amplification | attagaaaacctccttagcatg |
| TS-020 | fwd on BCD2 for Insert amplification | atgctaaggaggttttctaatg |
| TS-029 | fwd TS1 fabD W258Q | taacagggtaatctgaattcgaaggacgccgttcgcct |
| TS-030 | rev TS1 fabD W258Q | cgcactcgacctggcgtaccggctggtacaac |
| TS-031 | fwd TS2 fabD W258Q | ggtacgccaggtcgagtgcgtgcagac |
| TS-032 | rev TS2 fabD W258Q | gcctgcaggtcgactctagacgttgttgaccaagatagccg |
| TS-034 | fwd Cg accBCD1 in pBT | acaggaaacaggaggtaccgaatatgtcagtcgagactagg |
| TS-035 | rev Cg accBCD2 in pBT | atgctcctctagactcgaggttattacagtggcatgttgcc |
| TS-036 | fwd Cg accBCD1 in pMO\_RiboJ-bcd | tgctaaggaggttttctaatgtcagtcgagactagg |
| TS-037 | rev Cg accBCD1 in pMO\_RiboJ-bcd | gcctgcaggtcgactctagaggcttacagtggcatgttgcc |
| TS-042 | fwd TS1 GltA promoter exchange | agtatagggataacagggtaatctggcgccatccagtcatagag |
| TS-043 | rev TS1 GltA promoter exchange | gtcaacctagttagctaccccgtcacgttgtc |
| TS-044 | fwd Insert promoter 14a | tgacggggtagctaactaggttgacatggatataatg |
| TS-045 | rev Insert promoter 14a | ttttttgtcagccattagaaaacctccttagc |
| TS-046 | fwd TS2 GltA promoter exchange | aggttttctaatggctgacaaaaaagcgcag |
| TS-047 | rev TS2 GltA promoter exchange | aagcttgcatgcctgcaggtcgacttcatgtgcaggaagttttc |
| TS-050 | fwd TS1 mega-operon | taacagggtaatctgaattccgtcgaactgagcgaagcaggac |
| TS-051 | rev TS1 mega operon | caacactatcccaagcgccggcgatccg |
| TS-052 | fwd TS2 mega operon | cggcgcttgggatagtgttgactttccgccc |
| TS-053 | rev TS2 mega operon | gcctgcaggtcgactctagagcgcgagaaactgtcgcaatc |
| TS-067 | fwd TS1 PVLB\_02920 - TetR | taacagggtaatctgaattcaaccagttatccacagcatcgcgcg |
| TS-068 | rev TS1 PVLB\_02920 - TetR | agtgagctgatcggctggctgcagcggc |
| TS-069 | fwd TS2 PVLB\_02920 - TetR | agccagccgatcagctcactcggctgag |
| TS-070 | rev TS2 PVLB\_02920 - TetR | gcctgcaggtcgactctagatcagctttccgtcatctcc |
| TS-091 | fwd TS1 PVLB\_08385 - PrpC | taacagggtaatctgaattcctggatgacgtgttgacc |
| TS-092 | rev TS1 PVLB\_08385 - PrpC | tcttccacgaggtttttctcctttcttgaaattg |
| TS-093 | fwd TS2 PVLB\_08385 - PrpC | gagaaaaacctcgtggaagacgccgggg |
| TS-094 | rev TS2 PVLB\_08385 - PrpC | gcctgcaggtcgactctagacgacgatctccggcaggc |
| TS-097 | rev TS1 GltA | gcggacgtcgattacatcag |
| TS-098 | fwd TS2 GltA | ctgatgtaatcgacgtccgctaagcccctggccgaacg |
| TS-099 | rev TS2 GltA | tgcatgcctgcaggtcgactggtatgtgggcagagtcgtg |
| TS-101 | fwd TS2 GltA-PrpC exchange | gaccagcgctgataagcccctggccgaacg |
| TS-102 | fwd PrpC Insert PVLB\_08385 with native RBS | tggtcctgatgtaatcgacgtccgctaacaatttcaagaaaggagaaaaaccatggc |
| TS-103 | rev PrpC Insert PVLB\_08385 | ggccaggggcttatcagcgctggtcgatcgg |
| TS-106 | fwd BB pBGxx amplification | taaagaattcgagctcggtaccc |
| TS-109 | rev on pBGxx BB to insert | tgcccgtcgtattaaagagg |
| TS-135 | fwd TS1 pycAB (PVLB\_25325-25330) | taacagggtaatctgaattccttacggacccttcaccg |
| TS-136 | rev TS1 pycAB (PVLB\_25325-25330) | gaagactccagatacgccctcatctacaag |
| TS-137 | fwd TS2 pycAB (PVLB\_25325-25330) | agggcgtatctggagtcttcccaaagccgtag |
| TS-138 | rev TS2 pycAB (PVLB\_25325-25330) | gcctgcaggtcgactctagactgggcgcggttgaccac |
| TS-163 | fwd TS1 fabF (PVLB\_07185) | taacagggtaatctgaattccgctactgtgccggtgaac |
| TS-164 | rev TS1 fabF (PVLB\_07185) | cgttagcccaatggcaggattaagtactctccttttctaataacagagtttcttg |
| TS-165 | fwd TS2 fabF (PVLB\_07185) with barcode | taatcctgccattgggctaacgaaatggcgaataactaactgaattcatgaatatcgacgttgtactgtccaactc |
| TS-166 | rev TS2 fabF (PVLB\_07185) | gcctgcaggtcgactctagatgcaccgggattgccagc |
| TS-169 | CsR counter selection for KO and Ex GltA (PVLB\_16320) | gcgcgggtgatcttcgactcgcacg |
| TS-170 | CsR counter selection for KO and Ex GltA (PVLB\_16320) | aaaccgtgcgagtcgaagatcaccc |
| TS-171 | CsR counter selection for KO FabF (PVLB\_07185) | gcgcgtggcgacagcatacccatac |
| TS-172 | CsR counter selection for KO FabF (PVLB\_07185) | aaacgtatgggtatgctgtcgccac |
| TS-173 | CsR counter selection for Point mutation FabD W258Q (PVLB\_07170) | gcgcgacgcactcgacccagcgtac |
| TS-174 | CsR counter selection for Point mutation FabD W258Q (PVLB\_07170) | aaacgtacgctgggtcgagtgcgtc |
| TS-187 | fwd Ins Apra in GltA KO | ctgatgtaatcgacgtccgctaatttacactttatgcttccggctc |
| TS-188 | rev Ins Apra in GltA KO | ttcggccaggggcttatcagccaatcgactggcg |
| TS-189 | fwd TS2 KO GltA-Apra | ctgataagcccctggccgaacg |
| TS-204 | fwd TS1 OprF (PVLB\_07655) | agggataacagggtaatctgaattcgtgatgctgaaggtgctg |
| TS-205 | rev TS1 OprF (PVLB\_07655) | aaaccaattaccgttaaatccccatctg |
| TS-206 | fwd TS2 OprF (PVLB\_07655) | gatttaacggtaattggtttgacgtttcatg |
| TS-207 | rev TS2 OprF (PVLB\_07655) | tgcatgcctgcaggtcgactctagaggtagtcaacggcatcac |
| TS-210 | fwd TS1 PVLB\_18090 (FabH1) | agggataacagggtaatctgcagatcgactttaccggc |
| TS-211 | rev TS1 PVLB\_18090 (FabH1) | aggaacgacctgagtcttgggctgacgg |
| TS-212 | fwd TS2 PVLB\_18090 (FabH1) | ccaagactcaggtcgttcctctggtcaaag |
| TS-213 | rev TS2 PVLB\_18090 (FabH1) | tgcatgcctgcaggtcgactcaccgggagtctggttcaag |
| TS-216 | fwd TS1 PVLB\_17265 (FabH2) | agggataacagggtaatctggcaagcgcgggtcagccg |
| TS-217 | rev TS1 PVLB\_17265 (FabH2) | ttgggaagcctaagcgctgccagtaggttactcttcg |
| TS-218 | fwd TS2 PVLB\_17265 (FabH2) | gcagcgcttaggcttcccaataaataacagctcaataccaccgtc |
| TS-219 | rev TS2 PVLB\_17265 (FabH2) | tgcatgcctgcaggtcgactccgtgccgacggcggtgt |
| TS-222 | fwd sigX in pBNT | acaggaaacaggaggtaccgaattcatgcgttatgacccccgc |
| TS-223 | rev sigX in pBNT | atgctcctctagactcgaggctaagtttcactcaacccggc |
| TS-224 | fwd fabD (W258Q) in pBNT | acaggaaacaggaggtaccgaattcatgtctgcatccctcgcattcgtctttcc |
| TS-225 | rev fabD (W258Q) in pBNT | atgctcctctagactcgaggtcaggccagcgccgcacg |
| TS-235 | rev new TS2 PVLB\_17265 (FabH2) | tgcatgcctgcaggtcgactagaagcactttaccctcg |
| TS-238 | rev on pBGxx BB to insert | ccgggtaccgagctcgaattc |
| TS-250 | rev CgaccBC-D1 cloning in landing pad PVLB\_23545 | cgggtaccgagctcgaattcttacagtggcatgttgcc |
| TS-251 | fwd fabF2 from KT2440 (PP\_3303) | tgctaaggaggttttctaatgactcacaacgttaatcaaaagcg |
| TS-252 | rev fabF2 from KT2440 (PP\_3303) | cgggtaccgagctcgaattctcatacgttggcctcccag |
| TS-254 | fwd TS1 PVLB\_16225 (FabB) | agggataacagggtaatctggaagacctgctgcgctgc |
| TS-255 | rev TS1 PVLB\_16225 (FabB) | cagcgtcttagcgaataacccttagaaattgtcagtg |
| TS-256 | fwd TS2 PVLB\_16225 (FabB) | ggttattcgctaagacgctgatgcggtaattg |
| TS-257 | rev TS2 PVLB\_16225 (FabB) | atccccgggtaccgagctcggccattgcgcaatcatcc |
| TS-275 | rev on gene spacer in pBG | cctcctttcggtacccgcatag |
| TS-276 | fwd on mcs of pBG | agaattcgagctcggtacc |
| TS-277 | fwd StsTAL with overhangs | atgcgggtaccgaaaggaggtctatatgccgagcctggactcc |
| TS-278 | rev StsTAL with overhangs | gggtaccgagctcgaattctttaggccgcacccgtcaa |

## **Table S4:** Synthetic DNA fragments.

Overhangs for cloning are indicated by small letters, capital letters represent coding sequences.

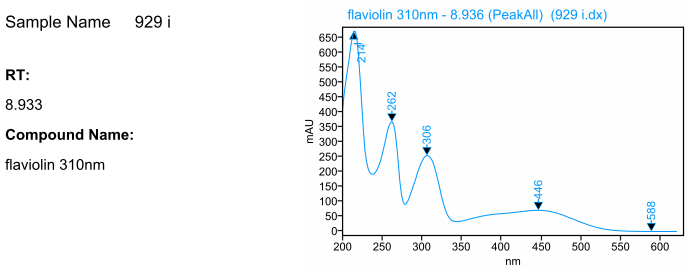
|  |  |  |
| --- | --- | --- |
| **Name** | **Sequence (5’ → 3’)** | **Note** |
| *AhSTS.opt* | ATGGGCAGCTCGCATCATCACCACCATCACAGCCAAGACCCGATGGTGTCTGTCAGCGGTATCCGTAAAGTCCAGCGTGCGGAGGGTCCGGCAACCGTGCTCGCCATCGGCACCGCGAACCCACCGAACTGCGTGGACCAGAGCACTTACGCTGACTACTACTTCCGCGTCACTAACAGCGAACATATGACCGATCTGAAGAAGAAGTTCCAGCGCATCTGCGAGCGCACCCAGATCAAGAACCGCCACATGTATCTGACCGAAGAGATCCTGAAAGAGAACCCGAACATGTGCGCTTACAAGGCCCCATCCCTCGACGCCCGCGAAGATATGATGATCCGTGAGGTCCCGCGTGTTGGCAAGGAAGCCGCTACCAAGGCCATTAAAGAGTGGGGCCAGCCTATGTCGAAAATCACCCACCTGATCTTCTGCACCACCAGTGGCGTGGCACTGCCGGGTGTGGACTACGAACTGATTGTACTGCTGGGTCTGGACCCAAGCGTGAAGCGTTACATGATGTATCACCAGGGCTGCTTCGCCGGCGGTACCGTTCTGCGCCTCGCCAAGGATCTGGCAGAGAACAACAAAGACGCCCGTGTGCTGATCGTCTGCTCGGAGAACACGAGCGTTACCTTCCGTGGTCCTTCCGAGACCGACATGGACTCCCTGGTCGGCCAGGCCCTGTTTGCCGATGGCGCTGCTGCTATCATCATCGGCTCGGACCCGGTCCCAGAAGTCGAAAACCCGCTGTTCGAGATCGTCTCGACCGACCAGCAGCTGGTTCCGAACTCCCACGGTGCCATCGGTGGTCTGCTGCGGGAAGTCGGTTTGACCTTCTACCTGAACAAGTCCGTGCCTGACATCATCTCCCAGAACATCAACGATGCACTGAGCAAGGCGTTCGACCCACTGGGTATCAGCGACTACAATAGCATCTTCTGGATCGCGCATCCGGGTGGCCGTGCAATCCTGGACCAGGTCGAGGAAAAAGTGAACCTGAAGCCTGAAAAGATGAAGGCTACGCGTGATGTGCTGTCCAACTACGGTAATATGTCCAGCGCCTGCGTCTTCTTCATCATGGACCTGATGCGTAAGAAATCGCTGGAAGCCGGCCTGAAGACCACGGGCGAAGGCCTGGATTGGGGCGTACTGTTCGGTTTCGGTCCTGGTCTGACCATCGAAACTGTTGTGCTGCGCTCCATGGCCATCTAA | codon-optimized and his-tagged stilbene synthase *AhSTS* (UniProt: Q9SLV5)  from *Arachis hypogaea* |
| *StsTAL* | ATGCCGAGCCTGGACTCCATCGTTGAGGCCGCGAGCTGGACTGCCAAGTTGGGCCCCCTCACTGACGCGGACGTCGCTCGCATGGATCGCTCGGGGGCCACCGTTGATGCCTACCTGGCTGAGGGTCGTCCTGTATATGGTCTGACGCAGGGCTTCGGCCCGCTGGTTACCTATAGCGCTACCTCGGAGATGGAGCAAGGCGCGAGCCTGATCAGCCATCTGGGCACTGCGCAGGGGCGTCCTATCGACCCCGATGCGTCGCGCCTGGTCTTCTGGCTGCGCCTCAACAGTATGCGTAAGGGCTTCAGCGCAGTCTCGACCGAGTTTTGGCAACGTCTGGCTGACCTGTGGAACGCCGGCTTTACTCCTGTAATCCCCCGCGACGGCACTGTGAGTGCAAGCGGTGACTTGCAGCCCTTGGCTCACGTGGCGCTGGCCTGCGCCGGTCATGGCGAAGCCTGGGTGCGCGATGAACAGGATCGTTGGACCCGTCGCCCAGCAGCTGAAGCACTGGCTGGTCTGGGTGCTGAACCGCTGGTGTGGCCCGTCCGCGAGGCGCTGGCATTCGTAAACGGCACCGGTGTAGGCTTGGCCGTCGCCATCTTGAACCAGCGCTCCGCTGTGCGTCTGGTGCGTGCTGTGGCGACTCTGACCGCACGTTTGACCGACCTGTTGGGCGGCAATGCCGAACACTACGATGAAGGTGTGGGTCAAGCCCGTAATCAGCTGGGCCAGTTGGAAGTAGCGCGCTGGATCCGCGCCGAAATCCCTGCCGGTCATCGGCGTGATGAGCGTCGGCCCCTGCAAGAGCCGTATAGTCTGCGCTGCGCCCCGCAGGTACTGGGCGCAGTCCTGGACCAACTGACCACTGCCGGTGAGATCCTCCTGCGCGAGGCCAACGGTTGTACCGACAATCCCTTGACCTACGAGGACCGCGTTCTCCACGCGGGTAACTTCCATGCCATGCCCGTTGGCTTCGCGAGCGAGCAGACGGGGCTGGCCATGCACATGGCCGCGTACCTCGCCGAACGTCAGTTGGGGCTGGTGGTGAATCCGACGACCAACGGCGACCTGCCGATCATGCTGACCCCACGCGCTGGGCGTGGTTGTGGCCTGGCTGGTGTACAAATTAGCGCGACCAGCTTTATCAGTCGCATCCGCCAACTGGTGACCCCGGCCTCGCTGACCACCCTCCCGACGAACGGCTGGAACCAGGACCATGTGCCAATGGCTCTCAATGGTGCAAACGGCGTCGGCGAAGCGTTGGAGCTGGGCTGGTTGGCAGTAGGTAGTCTGGCCTTGGCGGCTGCCCAATTGGCCGTCATGACTGGCAAAGCTGAGAGTGCCACCGGTGTCTGGGCGGAGCTGGCCCGCATTAGCCCGGCACTCGACGCAGACCGCCCCATGGCTGGCGAAGTCCGTGCCGCTGCGGAACTGTTCCGCGATCACGCTGAACGCCAGTTGACGGGTGCGGCCTAA | codon-optimized tyrosine ammonia lyase *StsTAL* (Uniprot: A0A0X3WEK2)  from *Streptomyces sp*. NRRL F-4489 |
| *SgRppA* | tgctaaggaggttttctaATGGCCACGCTGTGCCGTCCTGCCATTGCCGTCCCGGAACATGTGATCACCATGCAGCAGACCCTCGATCTGGCGCGCGAAACCCACGCTGGCCACCCGCAGCGGGACCTGGTCCTGCGCCTGATCCAGAACACCGGCGTCCAGACCCGCCACTTGGTGCAACCGATCGAGAAAACCCTGGCCCATCCCGGCTTCGAAGTGCGTAACCAAGTGTACGAGGCGGAGGCCAAAACGCGTGTGCCGGAGGTTGTACGTCGGGCTTTGGCGAACGCGGAAACGGAACCGAGCGAAATCGACCTGATCGTCTACGTGTCGTGCACCGGCTTCATGATGCCGTCGCTGACCGCCTGGATCATCAATTCCATGGGCTTTCGCCCCGAGACCCGTCAGCTGCCTATCGCTCAGCTGGGCTGCGCAGCCGGTGGCGCTGCGATCAACCGCGCCCACGATTTCTGTGTCGCATATCCGGATAGCAACGTCTTGATCGTCAGCTGTGAGTTCTGCTCCCTGTGCTACCAGCCTACCGATATCGGTGTCGGCTCCCTGCTGAGTAACGGCCTGTTCGGCGACGCACTGAGCGCGGCAGTGGTGCGTGGTCAGGGCGGGACCGGTATGCGCCTGGAGCGCAATGGCAGTCACCTCGTTCCCGACACCGAGGACTGGATCTCCTACGCCGTCCGCGATACCGGGTTCCACTTCCAGCTGGACAAGCGCGTGCCCGGCACCATGGAAATGCTGGCCCCCGTCCTCCTGGATCTGGTGGATCTGCACGGTTGGAGCGTGCCGAACATGGACTTCTTCATCGTACACGCGGGCGGCCCACGCATCCTGGACGATCTGTGCCACTTCCTCGACCTGCCCCCGGAGATGTTCCGGTATAGCCGCGCCACCCTGACTGAGCGGGGCAACATCGCCTCGTCCGTGGTGTTTGATGCCCTGGCACGCCTGTTCGACGATGGCGGGGCCGCCGAGTCGGCCCAGGGCCTGATCGCTGGCTTCGGCCCTGGCATCACCGCCGAGGTAGCCGTTGGCTCCTGGGCAAAGGAGGGGCTGGGCGCAGATGTGGGTCGCGACCTGGATGAGTTGGAGCTGACCGCGGGCGTCGCCCTGTCCGGCTAAagaattcgagctcggta | codon-optimized 1,3,6,8-tetrahydroxynaphthalene synthase *SgRppA* (UniProt: Q54240)  from *Streptomyces griseus* |

## **Supplement S5:** Calibration Growth Profiler

The following function was used to convert green values into an OD600 equivalent:

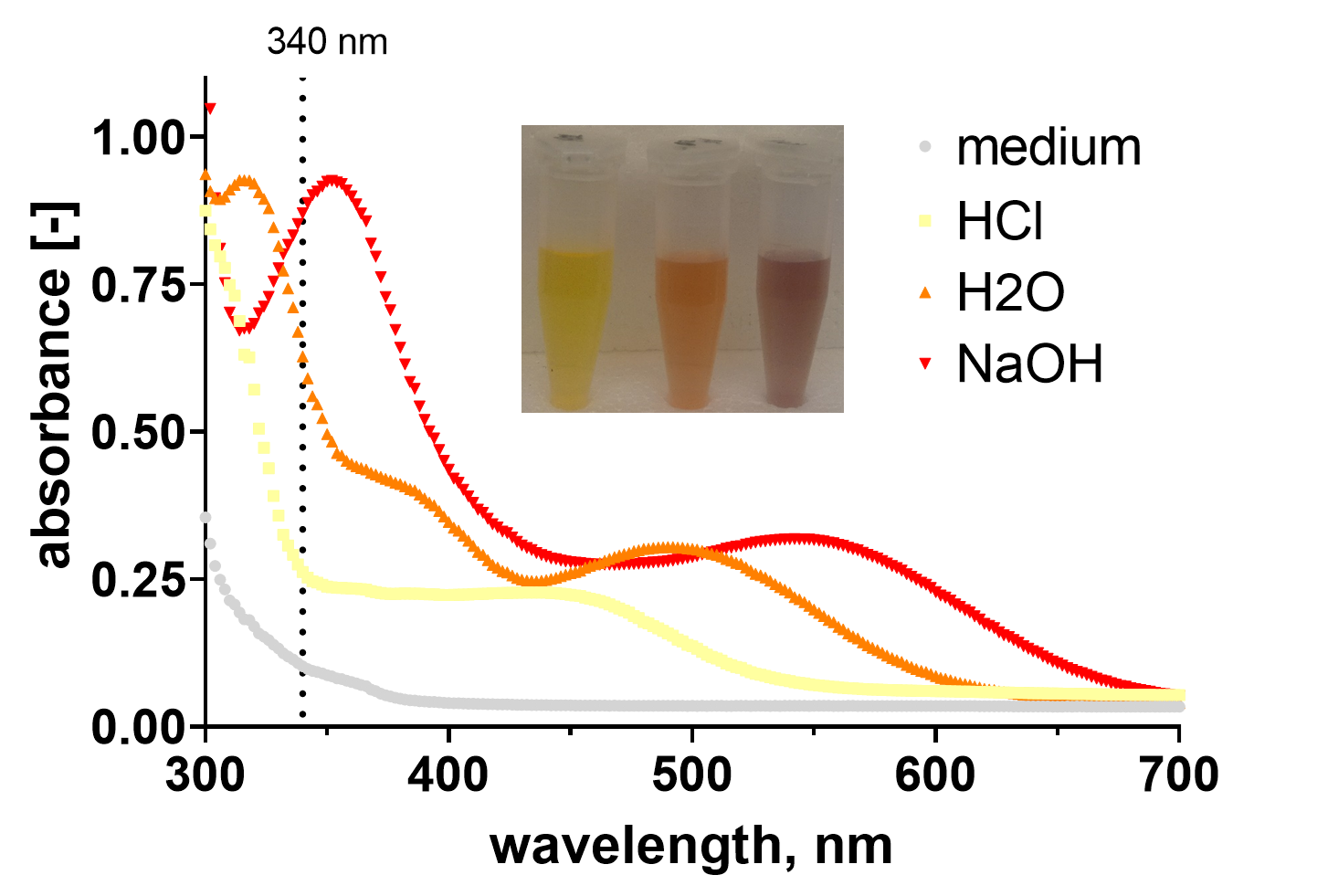
OD600 equivalent = a \* (gValue – gBlank)b + c \* (gValue – gBlank)d + e \* (gValue – gBlank)f

with a = 0.0305, b = 1, c = 8 \* 10−8, d = 3.8, e = 1.77 \* 10−13, f = 6.7 and gBlank = 18.787. Abbreviations: gValue, green value; gBlank, green value of reference medium. The calibration was performed for the *P. taiwanensis* VLB120 wild type in half-deepwell microtiter plates (CR1496d, EnzyScreen) sealed with sandwich covers (CR1296c, EnzyScreen).



## **Figure S6: Flaviolin UV spectrum.**

UV spectrum of flaviolin peak in HPLC from supernatant at retention 8.933 min. Relative quantification by peak area occurred at 310 nm. Sample derives from supernatant of strain GRC3Δ6 MC-II *attTn7*::*FRT*-*P14f*-*SgRppA* (strain #929). Reference spectrum for comparison is published by Gross et al. (2006)



## **Figure S7:**

Absorbance spectrum and picture of culture supernatant with secreted flaviolin. Addition of 50 µL 1 M HCl for acidification, 50 µL H2O for unmodified at pH 7 or 50 µL 1 M NaOH for basic conditions in 950 µL supernatant and mineral salts medium with 1-fold buffer as control. Absorption at 340 nm is indicated.



## **Figure S8:**

Remaining pinosylvin in culture supernatant after 4 days of cultivation. Initially applied pinosylvin concentrations are indicated at the x-axis. Error bars represent the standard deviation (n=3).



## **Figure S9:**

Absorbance at 340 nm of culture supernatants of flaviolin producer GRC3 Δ6 *attTn7*::*FRT-P14f-SgRppA* pSenFapRPseudoTermV1 with different concentrations of cerulenin (x-axis). Measured in plate reader in 96 well plate. Error bars represent standard deviation (n=3).



## **Figure S10: Design of promoter P14a\*.**

Intergenic sequence of *P. taiwanensis* VLB120 position 3568779 – 3568956 bp between succinate dehydrogenase (*sdh*, PVLB\_16315) and type II citrate synthase (*gltA*, PVLB\_16320) with exchanged promoter region of *gltA* with synthetic promoter *P14a\**. Sequence predictions are based on σ70 promoter prediction tool SAPPHIRE (*Psdh*, P-value 2.408E-5; *P14a*\*, P-value 5.0064E-4). Native sequence, small letters and dashed line; synthetic promoter sequence with bicistronic design 2 (BCD2), capital letters; translation start codon, red and bold; RBS, ribosome binding site; -10, Pribnow box; ‑35, consensus sequence for promoter; TSS, putative transcription start site.



## **Figure S11:**

Separate growth experiment of GRC3 PHE pinosylvin producing strains with modifications of the acetyl-CoA node in Growth Profiler in 96-square well plate at 30°C, 224rpm, 50 mm amplitude. Error bars represent the standard deviation of four replicates.



## **Figure S12:**

Flaviolin titers in supernatant and pH of LB seed culture (n=1) of GRC3Δ6 MC‑II and GRC3Δ6 MC‑II *CgACC* (MC-III) flaviolin producers without and with additional 2x buffer (left; cell pellets appeared dark of GRC3Δ6 MC-II *CgACC* in LB) and comparison of flaviolin titers from different carbon sources in 3x buffered MSM (right). Error bars represent the standard deviation (n=3). Abbreviations: glc, glucose; gly, glycerol, EtOH, ethanol



## **Figure S13**:

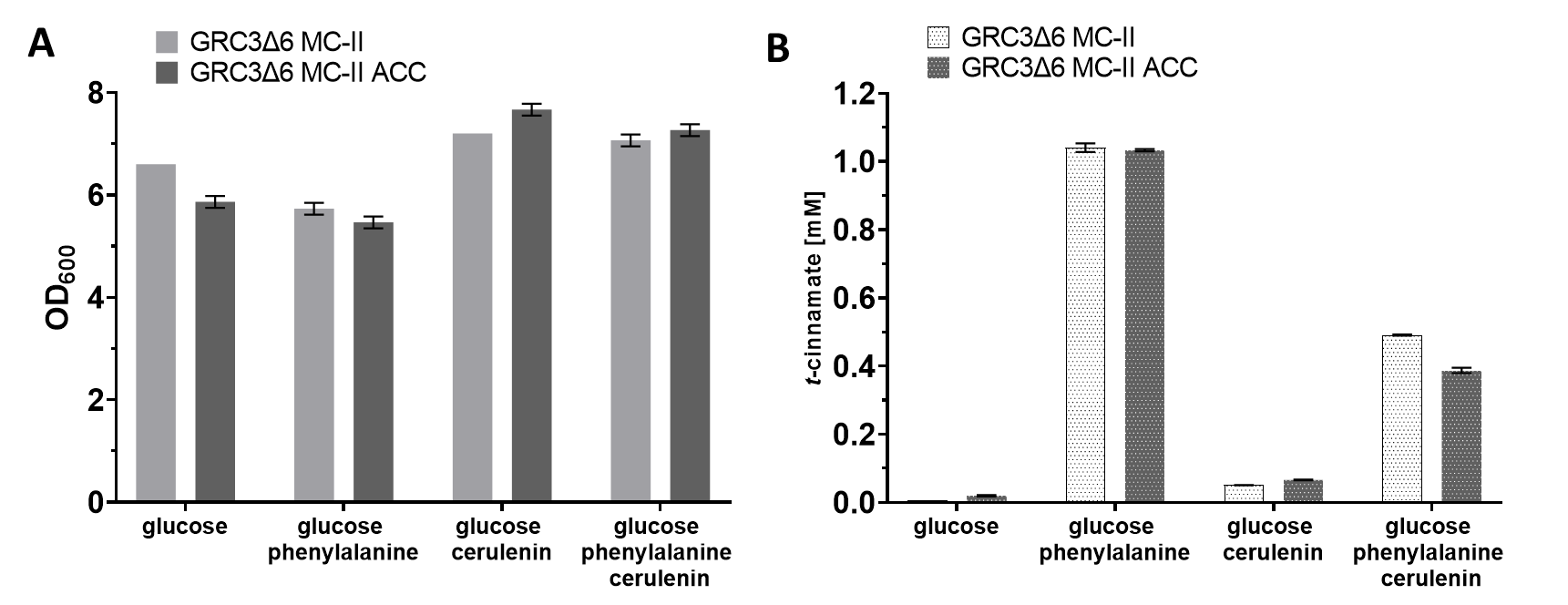
Flaviolin titers in supernatant (A) and OD600 (B) of different flaviolin producer strains based on aromatics catabolism deficient GRC3Δ6 with additional indicated modifications. Made in 3-fold buffered MSM with 30 mM glucose (glc.), or with additional 10 mM formate, or 90 mM ethanol or 1/6th of ammonium sulfate for nitrogen limiting conditions (C/N ~6) in 1.5 mL System Duetz cultures (30°C, 300 rpm, 50 mm amplitude). Samples were taken after 91 h, 92.5 h, 93h and 93.5 h, respectively to ensure full growth and THN oxidation to flaviolin. Error bars represent the standard deviation (n=3). Abbreviation: EtOH, ethanol; GRC3Δ6 MC-I, genotype of malonyl-CoA platform strain 1; GRC3Δ6 MC-II, genotype of malonyl-CoA platform strain 2.

## **Supplement S14:** Genetic modifications without positive effect on product titers

* pinosylvin production with AhSTS outperformed PstrSTS or PstrSTS\* in GRC3 PHE
* Deletion of secondary metabolite operon PVLB\_01730-PVLB\_01815 did not increase pinosylvin production in GRC3 PHE platform strain.
* The putative TetR-type regulator PVLB\_02920 shares similarities to FabR of *P. fluorescens* WH6 (73%aa identity) and some to DesT from *P. aeruginosa* PAO1(72%aa identity) which are likely involved in regulation of fatty acid biosynthesis. Deletion of fabR-like regulator encoded by PVLB\_02920 in GRC3 PHE background did not increase pinosylvin synthesis.
* Deletion of *glnB* in GRC3Δ6 MC-II flaviolin producer did not increase flaviolin production.
* Plasmid expression of *sigX*, *fabD* or *fabD*W258Q for flaviolin synthesis in strain GRC3 Δ6 Δ*gcd* Δ*PgltA*::*P14a*\*-BCD PVLB\_23545-40::*P14f*-*Cg\_accBC\_accD1* *attTn7*::FRT-*P14f-SgRppA* (MiKat#801) did not increase flaviolin production.



## **Figure S15: Proposed fatty acid biosynthesis in *P. taiwanensis* VLB120.**



## **Figure S16**:

Biomass in OD600 (A) and cinnamate titer (B) after bioconversion towards pinosylvin with supplemented phenylalanine and/or cerulenin after 21 h. Error bars represent the standard deviation (n=3); for some OD600 values the error bar is too small to be displayed.

## **Supplement S17:** Evaluation of platform strain GRC3Δ6 MC-III by stilbenoid synthesis

Biotransformation experiments with GRC3Δ6 MC-III revealed that higher pinosylvin titers can be obtained when phenylalanine is supplemented. To close the loop of the *de novo* stilbenoid synthesis and to reduce the metabolic burden, only one point mutation, namely *aroF-1*P148L (Wynands et al., 2018) was introduced into GRC3Δ6 MC-III to moderately increase the flux into the shikimate pathway.

The strains were grown in minimal medium with 30 mM glucose with and without supplementation of 0.5 mM of the respective phenylpropanoid or aromatic amino acid. Under these conditions, *de novo* synthesis of 12.3 ± 4.9 mg L-1 (0.06 mM) pinosylvin and 3.8 ± 0.8 mg L-1 (0.017 mM) resveratrol was achieved using the GRC3Δ6 MC‑III strain (Supplementary S15, Supplementary S16). *De novo* formation of pinosylvin was higher than resveratrol, but this was likely due to the different precursor supply provided by different PAL/TAL activities.

With cinnamate or phenylalanine supplementation the pinosylvin titers were not further increased but the overall high variance in pinosylvin titers may hide potential tendencies which were previously observable in high biomass biotransformation. This clonal variability remained also in a separate experiment (data not shown), indicating a genetic instability. The *p*‑coumarate titers of *aroF-1*P148L mutant were not higher than from parental GRC3Δ6 MC-III, but these titers were anyway one order of magnitude lower than that of cinnamate, likely due to a higher Km value of StsTAL (Cui et al., 2020) than AtPAL2 (Cochrane et al., 2004). The supplementation of tyrosine reduced resveratrol titers below 1 mg L-1. Here, it has to be considered that the used strain is also able to form some *de novo*-synthesized *p*‑coumarate through StsTAL activity.



**Figure S17**: Titers of pinosylvin and cinnamate in *de novo* production experiments and with supplementation of phenylalanine (0.5 mM) or cinnamate (0.5 mM). Cultivation was performed in MSM 30 mM glucose with 3x buffer in 1.5 mL square-well System Duetz plate, initial OD600 was 0.2, sampled after 24 h. Individual pinosylvin titers are indicated by a triangle. Error bars represent the standard deviation (n=3) and \* indicates p<0.05 confidence interval (\*\*\*, p≤0.001; \*\*\*\*, p≤0.0001) of two-way ANOVA analysis. Abbreviation: ns, not significant, MSM, mineral salt medium; phe, phenylalanine; cinn, cinnamate.



## **Figure S18**:

Stacked pinosylvin and cinnamate titers in mM from *de novo* production experiments and with supplementation of phenylalanine (0.5 mM) or cinnamate (0.5 mM) (A); and stacked titers of resveratrol and *p*-coumarate from *de novo* synthesis and supplemented approaches with tyrosine (0.5 mM) or *p*-coumarate (0.5 mM) (B). Experimental conditions: MSM with initial OD600 of 0.2 in 1.5 mL System Duetz cultures at 30°C, 300 rpm, 50 mm amplitude, sampling after 24 h. Error bars represent the standard deviation (n=3)

## **Table S19:** Different bioconversions in shake flasks and 24 square well plates with indicated substrate, precursor, product titers after approx. 25 h

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Strain basis** | **Tn7-site** | **Substrate** | **Supplemented precursor** | **Initial OD** | **OD** | **Remaining phenylpropanoid precursor** | **Titer [mg L-1]**  **Stilbene / phenylpropanoid** | **comment** |
| GRC3Δ6 | *P14g-his.AhSTS-Sc4CLA294G-AtPAL2* | 30 mM glc | 2 mM cinnamate | 0.2 | 4.1 | 1.38 mM | 9.9 ± 0.42 pinosylvin |  |
| GRC3Δ6MC‑I | 30 mM glc | 2 mM cinnamate | 0.2 | 3.93 | 1.49 mM | 1.4 ± 0.7 pinosylvin |  |
| GRC3Δ6MC‑II | 30 mM glc | none | 0.2 | 4.2 |  | 9.3 ± 0.13 pinosylvin  0.01 mM *t*‑cinnamate |  |
| GRC3Δ6MC‑II | 30 mM glc | 2 mM cinnamate | 0.2 | 4.1 | 1.65 mM | 8.5 ± 0.9 pinosylvin |  |
| 2 mM phenylalanine | 0.2 | 3.37 | 1.06 mM | 7.1 ± 0.8 pinosylvin |  |
| 60 mM glc | 2 mM cinnamate | 0.2 | 6.23 | 1.26 mM | 22.3 ± 0.44 pinosylvin | After 47h |
| 2 mM phenylalanine | 0.2 | 4.43 | 0.74 mM | 8.43 ± 0.5 pinosylvin | After 47h |
| 30 mM glc | 2 mM coumarate | 0.2 | 4.27 | 1.56 mM *p*‑coumarate | 65.7 ± 3.9 Resveratrol  5.4 ± 0.3 Pinosylvin  0.16 mM *t*‑cinnamate |  |
| GRC3Δ6MC‑II *Cg\_ACC* | 30 mM glc | 2 mM cinnamate | 0.2 | 3.87 | 1.73 mM | 16.2 ± 1 pinosylvin |  |
| GRC3Δ6MC‑II *trpEP290S* | 30 mM glc | none | 1 | 4 |  | 7.1 ± 0.22 pinosylvin  1.593 ± 0.004 mM cinnamate |  |
| GRC3Δ6MC‑II *trpEP290S* | 30 mM glc | none | 0.2 | 4 |  | 7.4 ± 0.3 pinosylvin  1.6 ± 0.01 mM cinnamate |  |
| 60 mM glc | none | 0.2 | 5.43 |  | 11.6 ± 0.4 pinosylvin  2.26 ± 0.03 cinnamate | After 47h |
| GRC3Δ6MC‑III | 30 mM glc | none | 1% |  |  | 12.3 ± 4.9 pinosylvin  0.035 ± 0.01 cinnamate | Figure S15 |
| GRC3Δ6MC‑III *aroF-1P148L* | 30 mM glc | none | 1% |  |  | 13.6 ± 7 pinosylvin  0.1 ± 0.05 cinnamate | Figure S15 |
| GRC3Δ6MC‑III | 30 mM glc | 0.5 mM cinnamate | 1% |  |  | 19.1 ± 9.5 pinosylvin  0.24 ± 0.02 cinnamate | Figure S15 |
| GRC3Δ6MC‑III *aroF-1P148L* | 30 mM glc | 0.5 mM cinnamate | 1% |  |  | 18.7 ± 9.5 pinosylvin  0.35 ± 0.08 cinnamate | Figure S15 |
| GRC3Δ6MC‑III | 30 mM glc | 0.5 mM phenylalanine | 1% |  |  | 15.2 ± 6.6 pinosylvin  0.156 ± 0.03 cinnamate | Figure S15 |
| GRC3Δ6MC‑III *aroF-1P148L* | 30 mM glc | 0.5 mM phenylalanine | 1% |  |  | 18.5 ± 8.6 pinosylvin  0.17 ± 0.03 cinnamate | Figure S15 |
| GRC3Δ6MC‑III | *P14g-his.AhSTS-Sc4CLA294G-StsTAL* | 30 mM glc | none | 1% |  |  | 3.8 ± 0.7 resveratrol  0.015 mM coumarate | Figure S15 |
| GRC3Δ6MC‑III *aroF-1P148L* | 30 mM glc | none | 1% |  |  | 1 resveratrol  0.015 mM coumarate | Figure S15 |
| GRC3Δ6MC‑III | 30 mM glc | 0.5 mM coumarate | 1% |  |  | 84 ± 2.2 resveratrol  0.135 ± 0.011 mM coumarate | Figure S15 |
| GRC3Δ6MC‑III *aroF-1P148L* | 30 mM glc | 0.5 mM coumarate | 1% |  |  | 62.5 ± 2.6 resveratrol  0.22 ± 0.01 mM coumarate | Figure S15 |
| GRC3Δ6MC‑III | 30 mM glc | 0.5 mM tyrosine | 1% |  |  | 0.53 ± 0.12 resveratrol  0.01 mM coumarate | Figure S15 |
| GRC3Δ6MC‑III *aroF-1P148L* | 30 mM glc | 0.5 mM tyrosine | 1% |  |  | 0.43 ± 0.07 resveratrol  0.01 mM coumarate | Figure S15 |

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