

BRIEF REPORT

Enhanced biosynthesis of poly(3-hydroxybutyrate) in engineered strains of *Pseudomonas putida* via increased malonyl-CoA availability

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Abstract

Malonyl-coenzyme A (CoA) is a key precursor for the biosynthesis of multiple value-added compounds by microbial cell factories, including polyketides, carboxylic acids, biofuels, and polyhydroxyalkanoates. Owing to its role as a metabolic hub, malonyl-CoA availability is limited by competition in several essential metabolic pathways. To address this limitation, we modified a genome-reduced *Pseudomonas putida* strain to increase acetyl-CoA carboxylation while limiting malonyl-CoA utilization. Genes involved in sugar catabolism and its regulation, the tricarboxylic acid (TCA) cycle, and fatty acid biosynthesis were knocked-out in specific combinations towards increasing the malonyl-CoA pool. An enzyme-coupled biosensor, based on the *rppA* gene, was employed to monitor malonyl-CoA levels in vivo. RppA is a type III polyketide synthase that converts malonyl-CoA into flaviolin, a red-colored polyketide. We isolated strains displaying enhanced malonyl-CoA availability via a colorimetric screening method based on the RppA-dependent red pigmentation; direct flaviolin quantification identified four engineered strains had a significant increase in malonyl-CoA levels. We further modified these strains by adding a non-canonical pathway that uses malonyl-CoA as precursor for poly(3-hydroxybutyrate) biosynthesis. These manipulations led to increased polymer accumulation in the fully engineered strains, validating our general strategy to boost the output of malonyl-CoA-dependent pathways in *P. putida*.

INTRODUCTION

Malonyl-coenzyme A (CoA) serves as a hub metabolite for the biosynthesis of lipids and acts as an attractive building block for the microbial production of biologically active polyketides and fatty acid-derived compounds, including biofuels (Li et al., 2024). In most organisms, malonyl-CoA is produced through acetyl-CoA carboxylation by acetyl-CoA carboxylase (ACC). Bacteria and plant chloroplasts possess a multi-subunit ACC enzyme,

which consists of three distinct domains responsible for catalyzing two separate reaction steps (Cronan, 2021a, 2021b; Cronan & Waldrop, 2002). The primary function of malonyl-CoA in bacterial metabolism is serving as an extender unit for fatty acids synthesis (Polyak et al., 2012), while its role in the production of native secondary metabolites appears to be relatively minor (Cronan & Thomas, 2009; McNaught et al., 2023).

Several studies have focused on engineering model microorganisms to enhance the biosynthesis

Modulating the endogenous fatty acid synthesis has been proposed as a promising approach to enhance the availability of malonyl-CoA for polyketide synthesis (Cronan & Thomas, 2009). This strategy was used in a study where synthetic antisense RNAs were

employed to reduce fatty acid biosynthesis, resulting in an enrichment of the malonyl-CoA pool in *Escherichia coli*. Such interventions led to a relatively modest increase in the production of 4-hydroxycoumarin, resveratrol, and naringenin (Yang et al., 2015). Cress et al. (2015) used a CRISPathBrick tool to modulate FadR in *E. coli*, a transcriptional regulator that represses β -oxidation and positively regulates fatty acid synthesis (Cronan, 2021a, 2021b), supporting a high malonyl-CoA turnover and improved naringenin titers. In another examples based on engineered *E. coli*, RNA interference was directed against the transcription of *fabB* and *fabF*, encoding the β -ketoacyl-acyl carrier protein (ACP) synthases (KAS) I and II (Wu et al., 2014). CRISPR interference for malonyl-CoA accumulation has also been adopted in *E. coli* to increase flavonoid production by targeting all possible malonyl-CoA-related genes, with the best effects achieved via altering *fabF* transcription (Wu et al., 2015). Other engineering approaches have been successfully applied to various microbial hosts. For instance, several transcriptional regulators of phospholipid synthesis were manipulated in *Saccharomyces cerevisiae* to increase the production of 3-hydroxypropionic acid (Chen et al., 2017), a platform chemical derived from malonyl-CoA. Another elegant example is the combined engineering of

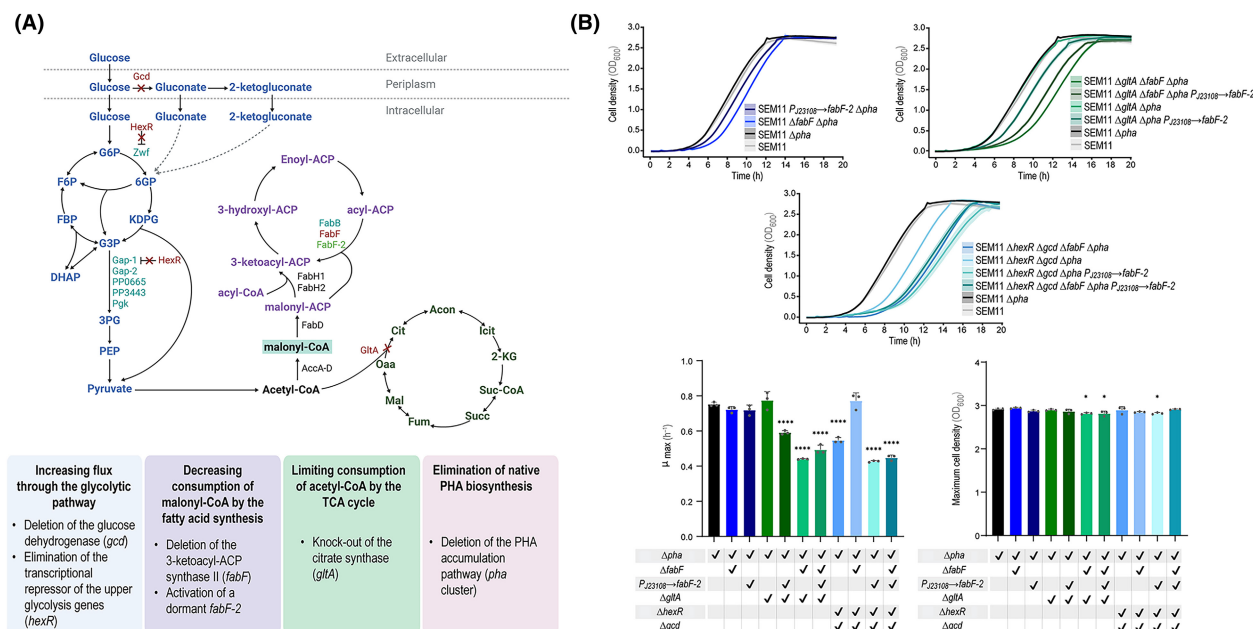


FIGURE 1 Genetic modifications to boost malonyl-CoA availability in *Pseudomonas putida*. (A) *P. putida* SEM11 was modified by knocking-out various combinations of the four genes shown in red and reactivating a dormant FabF-2 *via* promoter insertion in order to increase the malonyl-CoA pool. In addition, the native gene cluster encoding the enzymes for PHA production and degradation was deleted to eliminate endogenous biopolymer production. Abbreviations: G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; KDPG, 2-keto-3-deoxy-6-phosphogluconate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; G3P, glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; Cit, citrate; Acon, aconitate; Icit, isocitrate; 2-KG, 2-ketoglutarate; Suc-CoA, succinyl-CoA; Succ, succinate; Fum, fumarate; Mal, malate; and Oaa, oxaloacetate. (B) Growth curves of the resulting engineered strains. Cell density was estimated as the optical density measured at 600 nm (OD₆₀₀). CurvE software was used to analyze growth curves (Wirth, Funk, et al., 2023; Wirth, Rohr, et al., 2023), and the maximum specific growth rate ($\mu_{\max}$) was derived from the OD₆₀₀ measurements over time. GraphPad Prism 9 (GraphPad Software, Inc.) was used to perform all statistical analyses; the levels of significance are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. The error bars represent standard deviations; $n = 3$.

Corynebacterium glutamicum by deregulating the expression of genes encoding ACC components, reducing the acetyl-CoA entry into the tricarboxylic acid (TCA) cycle, and eliminating anaplerotic pyruvate carboxylation, which resulted in improved production of the pentaketide noreugenin (Milke et al., 2019). *E. coli* and *Pseudomonas taiwanensis* have also been engineered using similar strategies; increased production of phloroglucinol, resveratrol, and 3-hydroxypropionic acid, respectively, was achieved through these manipulations (Chen et al., 2024; Schwanemann et al., 2023; Zha et al., 2009).

Developing non-traditional microbial hosts as platform strains with enhanced malonyl-CoA supply could enable the efficient bioproduction of compounds that are challenging to produce in *E. coli* and other model species. *Pseudomonas putida* is a Gram-negative soil bacterium (Belda et al., 2016; Calero & Nikel, 2019), which, over the years, became a biotechnological chassis (Martínez-García & de Lorenzo, 2019; Schwanemann et al., 2020; Weimer et al., 2020). Owing to its robust and adaptable metabolism, *P. putida* can use a wide variety of structurally diverse molecules as carbon and energy sources (D'Arrigo et al., 2019; Fernández-Cabezón et al., 2022; Turlin et al., 2022, 2023). This bacterium can also handle the stress induced by toxic molecules or environmental conditions (Bitzenhofer et al., 2021; Nikel & de Lorenzo, 2018; Wirth et al., 2022). *P. putida* has already been successfully employed as a host for the production of natural products, e.g., rhamnolipids, terpenoids, polyketides, non-ribosomal peptides, and biopolymers—as well as other bulk and specialty chemicals (Batianis et al., 2020; de Lorenzo et al., 2024; Kozaeva et al., 2024; Prieto et al., 2016; Weimer et al., 2020; Wirth & Nikel, 2021). *Pseudomonas* species are efficient biopolymer producers (Mezzina et al., 2021), especially polyhydroxyalkanoates (PHAs). PHAs comprise a large family of natural polymers that includes poly(3-hydroxybutyrate) (PHB), the most widespread example of short-chain-length PHAs, and copolymers containing 3-hydroxyvalerate (Steinbüchel et al., 1992) and longer carbon structures (Anderson & Dawes, 1990; Suriyamongkol et al., 2007). These bio-based polymers emerged as an alternative to conventional plastics because they present many of the same characteristics and provide some benefits over petrochemical materials, e.g., a lower carbon footprint and more alternatives for waste disposal (Choi et al., 2020; Koller et al., 2017; Meng & Chen, 2018). As an intermediate derived from acetyl-CoA, malonyl-CoA directly participates in the synthesis of PHAs by supplying the key two-carbon units that are ultimately incorporated into the polymer structure (Aduhene et al., 2021; Mitra et al., 2022). Hence, some engineering strategies to enhance PHA production have focused on increasing the intracellular concentration of malonyl-CoA. For example, overexpressing the genes *fabH_{Ec}*, *fabD_{Ec}*,

and *fabD_{Ps}* in an engineered *E. coli* strain led to an increased PHA content (Taguchi et al., 1999). PHA production was also enhanced by increasing the fluxes through the CoA biosynthetic pathway, introducing a gene encoding the prokaryotic type III pantothenate kinase and supplementing pantothenate or β -alanine as CoA precursors (Kudo et al., 2023), and by overexpressing ACC components (Wang et al., 2012). Based on these examples, we reasoned that altering malonyl-CoA availability in *P. putida* could multiply the value of this host as a platform for PHA production.

In this study, we adopted a metabolic engineering approach that started by knocking-out genes associated with glycolytic pathways, the TCA cycle, and fatty acid biosynthesis to increase the levels of malonyl-CoA in a genome-reduced derivative of *P. putida* KT2440, strain SEM11 (Wirth, Funk, et al., 2023; Wirth, Rohr, et al., 2023). A colorimetric assay, based on a repurposed polyketide synthase, was used to screen for isolates with high malonyl-CoA content. These strains were then employed to assess PHB production from glucose through a non-canonical PHB biosynthesis pathway, revealing a substantial enhancement in biopolymer accumulation compared to the non-engineered, parental strain.

RESULTS AND DISCUSSION

Design, engineering, and characterization of *P. putida* strains with enhanced malonyl-CoA availability

We engineered strain SEM11, a genome-reduced variant of wild-type *P. putida* KT2440, through several gene deletions in order to increase the intracellular pool of malonyl-CoA (Figure 1A), using a well-established method for genome engineering in *Pseudomonas* species based on the I-SceI meganuclease (Martínez-García & de Lorenzo, 2011; Wirth et al., 2020). Two genes were deleted to increase the catabolic fluxes through glycolysis: (i) *hexR*, encoding the transcriptional repressor that controls the expression of several glycolytic genes, including *zwf-1* (glucose 6-phosphate dehydrogenase), the genes encoding enzymes in the Entner-Doudoroff pathway that ultimately yield glyceraldehyde-3-phosphate and pyruvate (*edd*, *eda*, and *glk*), and *gap-1*, glyceraldehyde-3-phosphate dehydrogenase (del Castillo et al., 2007, 2008; Nikel et al., 2015), and (ii) the *gcd* gene, encoding the membrane-bound glucose 2-dehydrogenase, responsible for sugar processing via the periplasmic oxidative route (Sudarsan et al., 2014; Volke et al., 2023). The deletion of *gcd* has been shown to have a positive effect on both PHA accumulation (Poblete-Castro et al., 2013) and the free CoA pool in *P. putida* (Gläser et al., 2020). This gene has been also targeted to increase the

malonyl-CoA content in *P. taiwanensis* (Schwanemann et al., 2023). In order to reduce acetyl-CoA consumption, the *gltA* gene (encoding citrate synthase) was also targeted for deletion, which would prevent acetyl-CoA from entering the TCA cycle (Figure 1A). In a previous study from our laboratory, *gltA* was identified as a promising candidate for CRISPR interference (CRISPRi) towards increasing the availability of acetyl-CoA in *P. putida* (Kozueva et al., 2021). Furthermore, deleting this gene increased malonyl-CoA levels in *P. taiwanensis* (Schwanemann et al., 2023).

Malonyl-CoA is used in the cell mainly for fatty acid biosynthesis, and we altered the flux through this pathway by activating the transcriptionally dormant *fabF-2* gene, encoding a 3-ketoacyl-ACP synthase (KAS), via promoter engineering. Specifically, the medium-strength synthetic P_{J23108} promoter was integrated upstream the *fabF-2* gene (PP_3303) via homologous recombination (Martínez-García & de Lorenzo, 2011), thereby enabling its constitutive expression. FabF-2 is expected to display a reduced activity compared to the very active variant encoded by *fabF* (Dong et al., 2021). Sequence and structure comparison between FabF and FabF-2 of *P. putida* KT2440 indicated that the two proteins share only 48.7% identity (Figure S1 in the Supporting Information). However, the relatively high structural conservation suggests that their mechanisms may still be similar. In addition to the promoter engineering approach to activate *fabF-2*, *fabF* was deleted to reduce the flux of malonyl-CoA towards fatty acid synthesis (Figure 1A). Various combinations of gene deletions and transcriptional engineering led to ten engineered strains, obtained by pairing modifications in fatty acid synthesis ($\Delta fabF$, $P_{J23108} \rightarrow fabF-2$) with deletions affecting glycolytic pathways ($\Delta hexR$ and Δgcd) or the TCA cycle ($\Delta gltA$). These strains are SEM11 $P_{J23108} \rightarrow fabF-2$, SEM11 $\Delta fabF$, SEM11 $\Delta gltA$, SEM11 $\Delta gltA \Delta fabF$, SEM11 $\Delta gltA P_{J23108} \rightarrow fabF-2$, SEM11 $\Delta gltA \Delta fabF P_{J23108} \rightarrow fabF-2$, SEM11 $\Delta hexR \Delta gcd$, SEM11 $\Delta hexR \Delta gcd \Delta fabF$, SEM11 $\Delta hexR \Delta gcd P_{J23108} \rightarrow fabF-2$, and SEM11 $\Delta hexR \Delta gcd \Delta fabF P_{J23108} \rightarrow fabF-2$. The native operon encoding the enzymes involved in PHA synthesis and depolymerization (de Eugenio et al., 2010) was knocked out in all these strains to avoid competition for precursors that support the accumulation of medium-chain-length PHAs.

We first tested whether these gene deletions were deleterious for the *P. putida* strains by growing the modified strains in mineral salt medium with glucose as a carbon source (Hartmans et al., 1989). Growth parameters, including maximum growth rate (μ_{max}), extension of the lag phase, and cell density (estimated as the optical density at 600 nm, OD_{600}), were computed with the QurvE software (Wirth, Funk, et al., 2023; Wirth, Rohr, et al., 2023). The strains containing only changes in the fatty acid synthesis and those containing a lower number of deletions had minimal variations

in the growth parameters when compared to the parental SEM11 strain, whereas strains with more gene deletions showed a decrease in μ_{max} and an increase in the extension of the lag phase (Figure 1B). The growth curves of SEM11 Δpha overlapped with that of the reference strain (SEM11, Figure 1B), suggesting that eliminating the gene cluster for PHA metabolism in *P. putida* has no substantial effect on the cell physiology under these conditions. All strains reached a maximum OD_{600} comparable to SEM11 Δpha (adopted as the reference strain henceforth, since its behavior was practically indistinguishable from that of *P. putida* SEM11); only SEM11 $\Delta gltA \Delta fabF \Delta pha$, SEM11 $\Delta gltA \Delta fabF \Delta pha P_{J23108} \rightarrow fabF-2$, and SEM11 $\Delta hexR \Delta gcd P_{J23108} \rightarrow fabF-2$ had a slight (but statistically significant) decrease in final OD_{600} compared to the reference strain (~3%). The most affected strains in terms of μ_{max} were SEM11 $\Delta gltA \Delta fabF \Delta pha$ and SEM11 $\Delta hexR \Delta gcd P_{J23108} \rightarrow fabF-2$, with a reduction of 41% and 43% compared to SEM11 Δpha , respectively.

These results align well with the expected perturbation of essential biochemical processes, i.e., the TCA cycle, fatty acid synthesis, and sugar processing. In particular, the *gltA* deletion was expected to result in several metabolic consequences that can negatively affect fitness, including reduced fluxes through the TCA cycle, an altered redox and energy metabolism, and a possible shortage of critical building-blocks needed for synthesizing amino acids, nucleotides, and other biomass constituents (Zhou et al., 2024). A previous study from our laboratory analyzed the effect of knocking-down the *gltA* gene in *P. putida* through a CRISPRi strategy (Kozueva et al., 2021). A combined metabolomic and proteomic analysis showed a substantial impact on central carbon metabolism, negatively affecting some components of the EDMP cycle (Nikel et al., 2015, 2021), the glyoxylate shunt, and enzymes involved in aromatic amino acid biosynthesis (Volke et al., 2021, 2022). The *gcd* deletion was also proven to cause a low rate of total carbon consumption and a reduced growth rate (Bentley et al., 2020), largely due to the role of Gcd in mediating sugar catabolism with energy conservation (Volke et al., 2023). Finally, deleting *fabF* in *P. putida* has been shown to cause a deficiency in unsaturated fatty acid synthesis (Dong et al., 2021), as FabF is responsible for extending palmitoleic acid ([9Z]-hexadec-9-enoic acid, C_{16}) to *cis*-vaccenic acid ([11E]-octadec-11-enoic acid, C_{18}), a key process that affects the membrane lipid composition (Do et al., 2018). Considering these observations, the metabolic effects of the gene modifications tested herein likely account for the impact on the growth of the modified *P. putida* strains. Despite the slight deleterious effect of some of the gene deletions, the resulting strains are not significantly impaired under the conditions tested. After 18 h of incubation, for instance, all strains reached a

comparable final OD₆₀₀, confirming their suitability for applications under standard growth conditions.

Semi-quantitative analysis of malonyl-CoA levels through an enzyme-coupled biosensor

We employed an enzyme-coupled biosensor, based on 1,3,6,8-tetrahydroxynaphthalene synthase (RppA), for a semi-quantitative screening of malonyl-CoA availability in the engineered strains. RppA converts malonyl-CoA to flaviolin (a red-colored compound), offering a direct colorimetric readout of malonyl-CoA levels (Figure 2A). The biosensor has been previously tested in *E. coli*, *P. putida*, and *C. glutamicum*, simplifying malonyl-CoA detection through a rapid colorimetric assay (Yang et al., 2018). We integrated the *rppA* gene in the genome of the engineered strains using a mini-Tn7 transposon system that allows for the incorporation of DNA constructs in the chromosome at the *attTn7*

attachment site that is conserved in many bacterial species (Zobel et al., 2015). The *P. putida* strains were transformed with two plasmids, one containing the mini-Tn7 transposon system itself and a helper plasmid encoding the Tn7 transposase required for transposon mobilization (Schweizer & de Lorenzo, 2004). The *rppA* gene was constitutively expressed from the strong P_{14f} promoter followed by the translational coupler BCD2 (Mutalik et al., 2013). We performed the integration in all engineered strains and in the reference strain SEM11 Δpha . After growing the cells under the same conditions indicated above, culture supernatants were visually inspected as a preliminary step prior to flaviolin quantification, and some of the samples displayed an intense red color, indicative of increased intracellular malonyl-CoA (Figure 2B).

For a more precise assessment of malonyl-CoA levels, HPLC analysis was used to directly quantify flaviolin produced by the different *P. putida* strains. The quantification of flaviolin is based on HPLC intensity as no flaviolin standard is available for direct quantification,

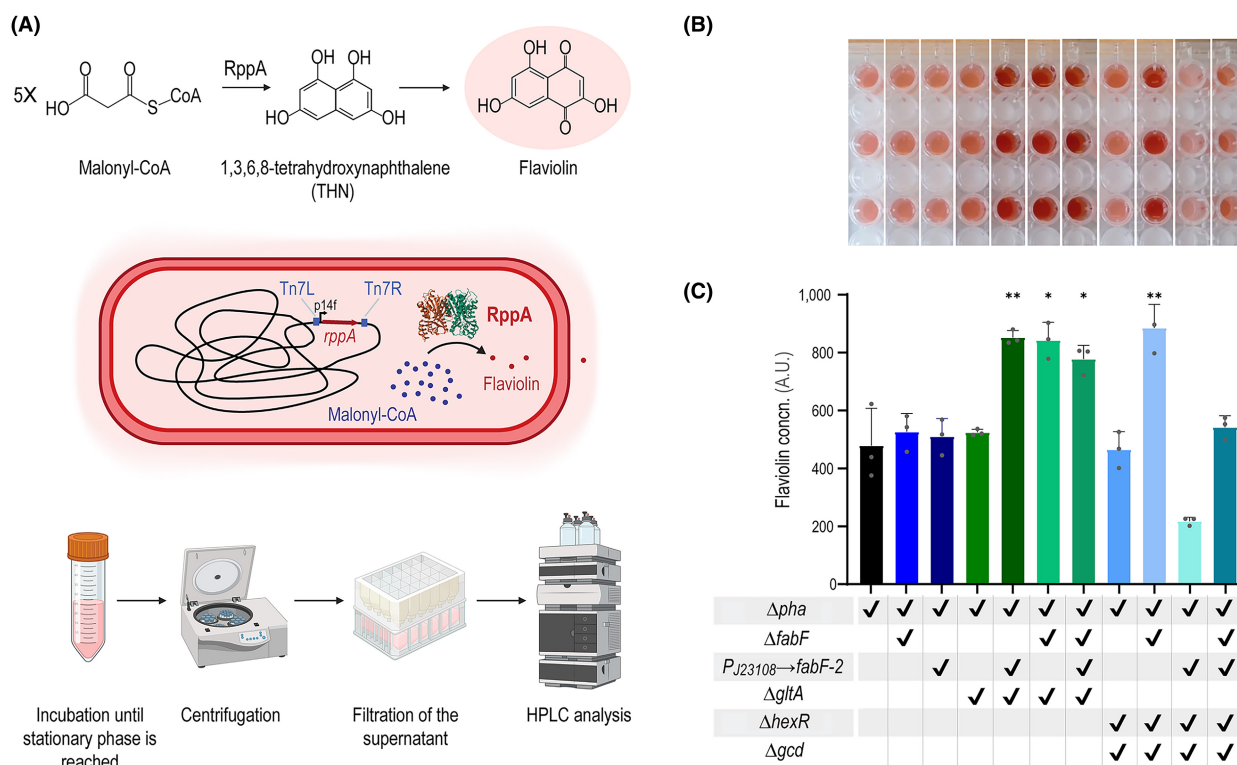


FIGURE 2 Analysis of malonyl-CoA levels with a biosensor. (A) A semi-quantitative analysis of malonyl-CoA levels in the engineered strains was carried out with an enzyme-coupled biosensor based on the *rppA* gene. This gene encodes RppA, a type III polyketide synthase that converts five molecules of malonyl-CoA into one molecule of flaviolin, which displays a red color. Malonyl-CoA is first converted to THN by RppA (THNS), and the metabolite undergoes non-enzymatic oxidation to flaviolin. In our design, the *rppA* gene was placed under the control of the constitutive P_{14f} promoter and the module was integrated in the genome at the *attTn7* site. A workflow for assessing malonyl-CoA levels in the engineered strain is shown; this illustration was created with BioRender.com. (B) Engineered *Pseudomonas putida* strains endowed with enhanced malonyl-CoA turnover were identified through a colorimetric screening method, isolating clones that displayed increased red pigmentation in the supernatant. (C) HPLC analysis was used to quantify the flaviolin produced by the different engineered strains. Arbitrary units (A.U.) indicate the intensity of the peak corresponding to flaviolin, measured at 310 nm. GraphPad Prism 9 (GraphPad Software, Inc.) was used to perform all statistical analyses; the levels of significance are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. The error bars represent standard deviations; $n = 3$.

limiting the possibility of reporting absolute yields. The analysis was performed after 24 h of growth, when the cultures had reached the same final OD₆₀₀. As shown in Figure 2C, four strains showed significantly higher flaviolin levels than the others. The strains with the highest flaviolin production were SEM11 $\Delta hexR \Delta gcd \Delta fabF \Delta pha$, SEM11 $\Delta gltA \Delta fabF \Delta pha$, SEM11 $\Delta gltA \Delta pha$ $P_{J23108} \rightarrow fabF-2$, and SEM11 $\Delta gltA \Delta fabF \Delta pha$ $P_{J23108} \rightarrow fabF-2$. These strains had an increase in the flaviolin levels of 85%, 78%, 76%, and 63%, respectively, when compared to the reference strain. These strains harbor several gene manipulations, showing that multiple pathways need to be altered to increase malonyl-CoA formation. Except for SEM11 $\Delta gltA \Delta pha$ $P_{J23108} \rightarrow fabF-2$, all strains with high flaviolin production contain the *fabF* deletion, suggesting that targeting fatty acid synthesis has a major effect on the phenotype—yet eliminating FabF alone is not sufficient. A comparable outcome was reported for a platform *P. taiwanensis* strain; in this case, enhancing malonyl-CoA availability involved a reduced demand of the thioester for fatty acid synthesis (Schwanemann et al., 2023).

Enhanced malonyl-CoA availability supports PHB accumulation through a non-canonical biosynthesis pathway

We employed a malonyl-CoA shunt-based pathway for PHB biosynthesis to evaluate the engineered *P. putida* strains for biopolymer accumulation, utilizing malonyl-CoA as a substrate. The canonical PHB biosynthesis pathway begins with de novo acetoacetyl-CoA formation, typically catalyzed by acetoacetyl-CoA thiolase via a thioester-dependent Claisen condensation between two acetyl-CoA molecules (Choi & Lee, 1999; Nikel et al., 2006). The non-canonical biosynthesis pathway used in our study differs in that its first step is catalyzed by NphT7, an acetoacetyl-CoA synthase from *Streptomyces* sp., which irreversibly condenses acetyl-CoA and malonyl-CoA to produce acetoacetyl-CoA and CoA (Okamura et al., 2010). The next two steps are mediated by PhaB and PhaC from the canonical pathway of *Cupriavidus necator* (Figure 3A). The topology of this non-canonical pathway for PHB biosynthesis offers improved control over precursor availability at the acetyl-CoA and malonyl-CoA metabolic nodes (Orsi et al., 2021, 2022). This pathway was shown to mediate PHB accumulation from glucose in engineered *P. putida* (Kozueva et al., 2021; Martínez-García, Aparicio, et al., 2014; Martínez-García, Nikel, et al., 2014; Nikel & de Lorenzo, 2013). A synthetic operon, comprising the genes encoding the three enzymes of the pathway, was expressed from plasmid pSEVA2311-PHAS, which carries the cyclohexanone-inducible *ChnR/P_{chnB}* expression system (Benedetti et al., 2016).

After all strains were transformed with plasmid pSEVA2311-PHAS, we characterized their growth patterns in the presence and absence of cyclohexanone. By analyzing the growth curves of these engineered *P. putida* strains containing the plasmid for PHB accumulation (Figure 3B), we noticed a different physiological response to the addition of the inducer, probably due to the burden caused by the production of the pathway enzymes. In particular, virtually all strains with modifications in the TCA cycle showed a higher final cell density (OD₆₀₀) in the presence of cyclohexanone. Since PHA accumulation affects light scattering and results in higher absorbance values (Martínez & Déziel, 2020), we ascribed the higher final OD₆₀₀ readings to enhanced PHB accumulation. Culturing SEM11 Δpha , the reference strain, under the same conditions with increasing cyclohexanone concentrations resulted in no substantial changes in the final OD₆₀₀ (data not shown). These results exclude a potential effect of the inducer on the absorbance. Most of the strains with gene deletions affecting glycolysis exhibited a lower maximum OD₆₀₀, suggesting that PHB accumulation may act as a stress factor in these genetic backgrounds (Ankenbauer et al., 2020).

PHB granules within the cells were stained with Nile red, a fluorescent lipophilic dye that binds hydrophobic inclusion bodies and enables their visualization by fluorescence microscopy (Martínez-García, Aparicio, et al., 2014; Martínez-García, Nikel, et al., 2014; Nikel et al., 2009; Nikel, Pettinari, Galvagno, & Méndez, 2008; Nikel, Pettinari, Ramírez, et al., 2008; Spiekermann et al., 1999). Using this protocol, we obtained semi-quantitative evidence of PHB accumulation in all engineered strains (Figure 3C). PHB granules were visualized as intensely fluorescent intracellular dots; in the negative controls, where the corresponding *P. putida* strain had been transformed with the empty pSEVA2311 vector (Martínez-García et al., 2023), only the membranes were stained.

Nile red can also be used to compare PHA accumulation levels by adding the stain to cultures upon they reach stationary phase (Nikel, Pettinari, Galvagno, & Méndez, 2008; Nikel, Pettinari, Ramírez, et al., 2008). Fluorescence and OD₆₀₀ are measured after a short incubation, providing a semi-quantitative estimation of PHB content on biomass. Almost all engineered strains had significantly higher normalized fluorescence values than SEM11 Δpha harboring the empty vector (used as a negative control), except for SEM11 Δpha $P_{J23108} \rightarrow fabF-2$, SEM11 $\Delta gltA \Delta pha$ and the reference strain SEM11 Δpha (Figure 3D). In particular, cultures of SEM11 $\Delta gltA \Delta pha$ $P_{J23108} \rightarrow fabF-2$, SEM11 $\Delta gltA \Delta fabF \Delta pha$, and SEM11 $\Delta hexR \Delta gcd \Delta fabF \Delta pha$ had an increase in the normalized fluorescence of 51%, 49%, and 141%, respectively, when compared to SEM11 Δpha . We observed that the strains that exhibited high flaviolin

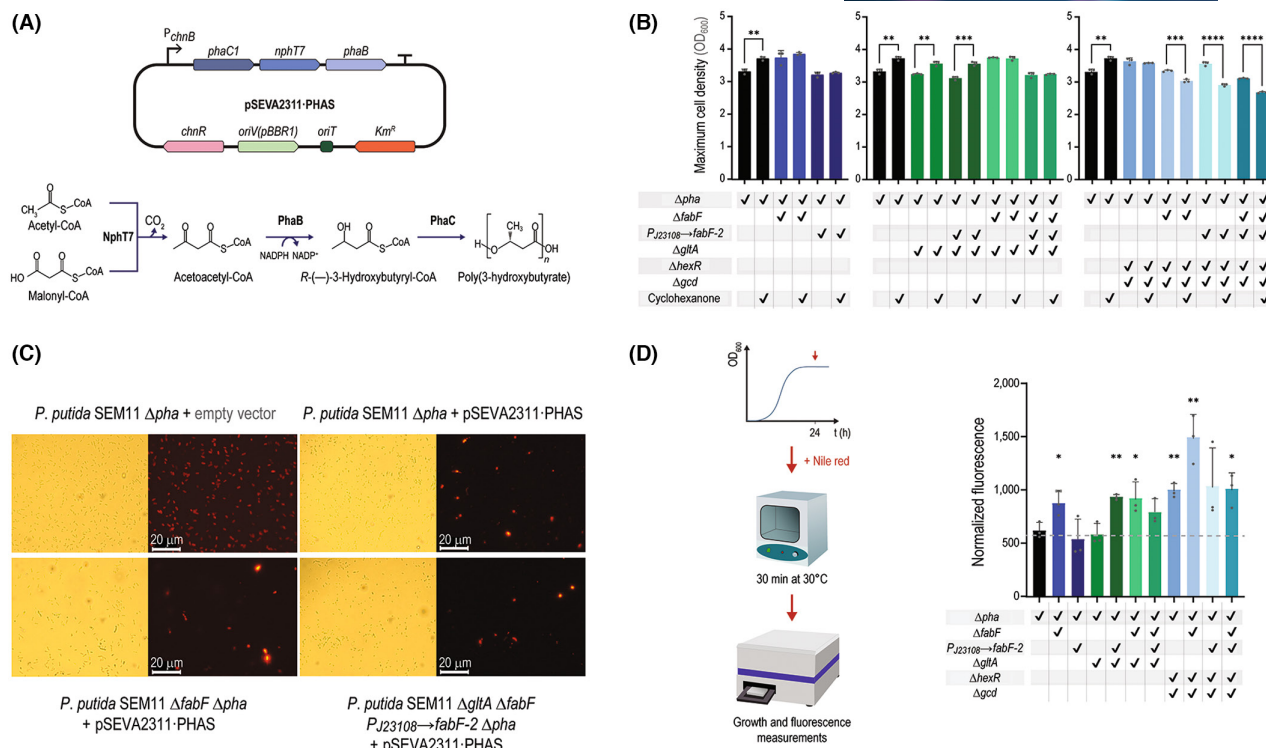


FIGURE 3 Adopting engineered *Pseudomonas putida* strains for malonyl-CoA-dependent PHA production. (A) A non-canonical PHA biosynthesis pathway, based on NphT7 (an acetoacetyl-CoA synthase from *Streptomyces* sp.), was used in this study. PhaB catalyzes the NADPH-dependent reduction of acetoacetyl-CoA, followed by polymerization by PhaC, a PHA synthase; both PhaB and PhaC are enzymes from *C. necator*. The genes encoding these three enzymes are encoded in plasmid pSEVA2311-PHAS under control of the cyclohexanone-inducible *ChnR*/*P_{chnB}* expression system. (B) Maximum cell density (estimated as the optical density at 600 nm, OD_{600}) reached by the strains transformed with plasmid pSEVA2311-PHAS, in the presence or absence of the inducer (cyclohexanone). (C) Nile Red staining for visualization of PHB granules. Nile Red is a lipophilic fluorescent dye that binds to PHB granules and can be readily detected through fluorescence microscopy, offering qualitative evidence of biopolymer accumulation. (D) Nile red staining for semi-quantitative assessment of PHB levels in engineered *P. putida*. Nile red was added to the cultures after 24 h of growth, when the cultures had reached stationary phase, and the fluorescence was read after 30 min of incubation. Nile red fluorescence values for each strain were normalized to the OD_{600} of the corresponding culture; normalized fluorescence values were compared with those of the parental strain, SEM11 Δpha . The relative fluorescence of SEM11 Δpha containing an empty pSEVA2311 vector is indicated by the dotted gray line. GraphPad Prism 9 (GraphPad Software, Inc.) was used to perform all statistical analyses; the levels of significance are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. In all cases, the error bars represent standard deviations; $n = 3$.

production also performed well in PHB accumulation experiments; in fact, the two parameters showed a strong correlation across all experimental conditions and strains (Figure S2 in the Supporting Information). In general, we concluded that deleting *gltA* in combination with an altered fatty acid synthesis seems to favor malonyl-CoA turnover and PHB accumulation. In contrast, deleting genes involved in glycolysis, coupled with fatty acid synthesis, appears to introduce a stress factor if combined with PHB accumulation—although the PHB content in these strains remained largely unaffected. Although the increase in Nile red fluorescence observed in the best-performing strains validated our engineering strategy to boost malonyl-CoA, we wanted to explore a more direct quantification method for PHB content. To this end, strain SEM11 $\Delta gltA \Delta fabF \Delta pha$ *P_{J23108}*→*fabF-2* was grown in shaken-flask cultures using glucose as the main substrate, and the biomass and PHB content was determined after a 24 h incubation by methanolysis and

detection of the 3-methyl esters of 3-hydroxybutyrate by GC-FID (Ruiz et al., 2006). Under these conditions, this engineered strain accumulated PHB to $25.1 \pm 3.9\%$ (on a cell dry weight basis). While further experiments are necessary for a detailed comparison of all engineered strains, our findings set the basis for developing improved bioprocess for PHB production via malonyl-CoA. Additionally, these engineered strains could serve as versatile platforms for other malonyl-CoA-dependent pathways and products.

CONCLUDING REMARKS

In this work, we identified and implemented gene deletions within different metabolic pathways that produce or consume malonyl-CoA, obtaining engineered strains with an increased pool of intracellular malonyl-CoA. While several metabolic pathways had to be modified and rewired simultaneously towards boosting

the thioester levels, the one that seemed most influential is fatty acids synthesis. Interestingly, the engineered *P. putida* strains generated in this study had growth features comparable to the parental strain, yet a relatively small growth compromise was observed in glucose-dependent cultivations—a consequence expected when genes involved in pathways essential for growth are deleted (Nogales et al., 2020). Yet, all strains reached comparable final OD₆₀₀ values, making them suitable for testing PHB biosynthesis. We demonstrated that these strains could support enhanced PHB production, with SEM11 $\Delta gltA \Delta fabF \Delta pha$, SEM11 $\Delta hexR \Delta gcd \Delta fabF \Delta pha$, and SEM11 $\Delta gltA \Delta fabF \Delta pha$ $P_{J23108} \rightarrow fabF-2$ as the most promising candidates for subsequent rounds of metabolic engineering to improve malonyl-CoA-dependent production.

Several strategies have been previously explored in the context of metabolic engineering for PHA production in *P. putida*. Some examples include overexpressing *phaJ* in *P. putida* KCTC1639, which enhanced medium-chain-length PHA biosynthesis from octanoate by ~9% (Vo et al., 2008). Introducing PHA synthases from various microorganisms into *P. putida* KT2442 Δpha led to the accumulation of structurally diverse PHAs (Chung et al., 2009). Other approaches exploited manipulating β -oxidation; specifically, strains with deletions in both *fadBA1* and *fadBA2*, along with a knock-out in *phaZ* (the PHA depolymerase gene), had a 20% and 100% increase in the PHA yield from *p*-coumarate and lignin, respectively, as compared to the wild-type strain (Salvachúa et al., 2020). Promoter engineering has also proven effective to boost PHA production, as demonstrated in *P. putida* KT2440 with modified transcription levels of *phaC1* and *phaC2* (the PHA synthase genes). When combined with the overexpression of genes encoding components of the pyruvate dehydrogenase complex and deletion of *Gcd*, the PHA yield on biomass was increased by 90% (Zhang et al., 2021). Unlike medium-chain-length PHAs, PHB biosynthesis in engineered *P. putida* has only been reported in a few publications (Ackermann et al., 2024; Didion et al., 2024; Kozaeva et al., 2021), and our present study indicates that malonyl-CoA could be harnessed as a precursor for biopolymer production. However, the scalability of these strains for industrial production has yet to be demonstrated, as is the feasibility of achieving high-yield biopolymer production under large-scale operational conditions. To this end, multiple factors will have to be evaluated and optimized, e.g., the robustness of the strains under changing operating conditions, potential bottlenecks in metabolic fluxes and oxygen transfer, and the overall economic viability of the bioprocess (Manikandan et al., 2021). Additionally, further studies are required to understand the systems-level impact of the genetic modifications on bacterial metabolism (Tokic et al., 2020), which could affect the productivity across extended cultivation periods and in bioreactors

of different configurations. Despite these shortcomings, this study provides a foundation for optimizing malonyl-CoA-dependent bioproduction in *P. putida*, a bacterial host that is gaining attention for industrial applications (de Lorenzo et al., 2024).

AUTHOR CONTRIBUTIONS

Giusi Favoino: Formal analysis; investigation; visualization; writing – original draft. **Nicolas Krink:** Investigation; methodology. **Tobias Schwanemann:** Investigation; writing – review and editing. **Nick Wierckx:** Conceptualization; writing – review and editing. **Pablo I. Nikel:** Conceptualization; funding acquisition; project administration; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated for this work are available within the main text or upon reasonable request to the corresponding author.

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SUPPORTING INFORMATION

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