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Metabolic engineering strategies to produce medium-chain oleochemicals via acyl-ACP:CoA transacylase activity

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ABSTRACT

Microbial lipid metabolism is an attractive route for producing aliphatic chemicals, commonly referred to as oleochemicals. The predominant metabolic engineering strategy centers on heterologous thioesterases capable of producing fatty acids of desired size. To convert acids to desired oleochemicals (e.g. fatty alcohols, ketones), metabolic engineers modify cells to block beta-oxidation, reactivate fatty acids as coenzyme-A thioesters, and redirect flux towards termination enzymes with broad substrate utilization ability. These genetic modifications narrow the substrate pool available for the termination enzyme but cost one ATP per reactivation - an expense that could be saved if the acyl-chain was directly transferred from ACP- to CoA-thioester. In this work, we demonstrate an alternative acyl-transferase strategy for producing medium-chain oleochemicals. Through bioprospecting, mutagenesis, and metabolic engineering, we developed strains of *Escherichia coli* capable of producing over 1 g/L of medium-chain free fatty acids, fatty alcohols, and methyl ketones using the transacylase strategy.

HIGHLIGHTS

- Validated PhaG as a 3-hydroxyacyl-ACP:CoA transacylase with *in vivo* characterization
- Bioprosped a higher activity PhaG homolog from *Pseudomonas koreensis*
- Isolated a PhaG Q45R G142V variant that had a 4-fold improvement in fatty acid production when compared to wild-type
- We reported production of fatty acid and fatty alcohol over 1 g/L, and methyl ketone 7.2 g/L

Keywords

Oleochemical; PhaG; 3-hydroxyacyl-ACP:CoA transacylase; fatty acid; fatty alcohol; methyl ketone; poly-hydroxyalkanoate

1. INTRODUCTION

Oleochemicals are a class of aliphatic hydrocarbons that are industrially-derived from petroleum, animal fats or oil seed crops. Depending on the terminal reductive state of the acyl-chain, oleochemicals can be classified as fatty acids, alcohols, aldehydes, ketones, esters, olefins, alkanes, etc. Oleochemicals are also categorized by size (i.e. acyl chain length). Medium-chain oleochemicals are defined as compounds with acyl moiety of 8-12 carbons and are of particular interest due to their applications as commodity and specialty chemicals ^{1,2}. For example, medium-chain fatty acids are used as herbicides, antimicrobials, and precursors for lubricant synthesis ³. Medium-chain methyl ketones are used as flavors, floral fragrances, insecticides, and renewable liquid transportation fuels ^{4,5}. Medium-chain alcohols are used as surfactants, as additives in biodiesel, as cosmetics and in consumer products. The unique molecular and chemical properties of medium-chain oleochemicals and their limited natural abundance make them attractive targets for biosynthesis.

The final enzymatic reactions in oleochemical biosynthesis use acyl-thioesters as substrates, making high-flux to desired chain length acyl-thioesters a key element to any metabolic engineering strategy. Thus far, two approaches have been applied to produce medium-chain acyl-thioesters in microbes: thiolase-driven β -reduction cycles (i.e. reversal of β -oxidation) ⁶ and thioesterase-driven diversion of fatty acid biosynthesis intermediates ^{1,7}. The reverse β -oxidation pathway incorporates a reversible, thiolase-catalyzed Claisen condensation (e.g. FadA) with acetyl-CoA as the donor to by-pass the energy consumption required for producing malonyl-CoA in fatty acid biosynthesis. The 1-ATP savings per elongation provides reversal of β -oxidation with the highest theoretical yield of all oleochemical biosynthesis routes at the cost of losing the strong driving force provided by the decarboxylative Claisen condensation in fatty acid biosynthesis. β -

reduction was used to demonstrate production of both medium-chain fatty acids and fatty alcohols^{6,8-11} in high yields, albeit with poor selectivity to specific chain length products. This is in part due to the competition between termination reactions (cleavage or reduction, respectively) and thiolase-catalyzed extension of acyl-CoA thioesters. To date, strategies to bias termination at a particular chain length have yet to be demonstrated and better results have come from finding ways to accumulate pools of desired chain-length acyl-CoAs made via fatty acid biosynthesis to avoid the competition entirely.

Plants synthesize novel oils containing medium-chain acyl groups by expressing specific acyl-ACP thioesterases in the chloroplast to generate free fatty acids of desired size⁷. Once made, the free fatty acids are transported to the cytosol for reactivation as acyl-CoA and subsequent incorporation into storage lipids¹². In microbes, plant acyl-ACP thioesterases can be leveraged to produce free fatty acids of desired chain length. Additional genetic modifications are needed to enable conversion of the free fatty acids to the desired oleochemical form (e.g. alcohol, ketone, ester). Unlike plants where biochemistries can be compartmentalized, bacteria express enzymes for both fatty acid biosynthesis and catabolism in the same locations. In order to accumulate a pool of desired acyl-CoA's β -oxidation must be blocked by eliminating all enzymes that catalyze one of the four reactions in the cycle. Depending on the desired product form, different β -oxidation steps are targeted (e.g. acyl-CoA dehydrogenase, FadE, for fatty alcohol production; thiolase, FadA/FadI, for methyl ketone production) to produce the substrate for termination enzymes (e.g. saturated acyl-CoA for acyl-CoA reductase conversion to fatty alcohol; β -ketoacyl-CoA for β -ketothioesterase conversion to methyl ketone). The last steps in the metabolic engineering strategy are expression of a specialized acyl-ACP thioesterase (to produce desired free fatty acids), expression of an acyl-CoA synthetase (to activate the free fatty acid), and expression of the desired

termination enzyme. Highly-active acyl-ACP thioesterases have been identified from natural sources and others have been engineered or evolved in the laboratory to produce octanoic acid, decanoic acid, dodecanoic acid, and tetradecanoic acid ^{13–17}. For instance, a highly active variant of a *Cuphea palustris* thioesterase FatB (referred to *C_pFatB**) was identified using a *C_pFatB* random mutagenesis library and a growth selection based on the lipoic acid requirement of *Escherichia coli*. *E. coli* strain NHL17 (MG1655 Δ *araBAD* Δ *fadD::trc-C_pfatB**) produced 1.7 g/L octanoic acid with >90% specificity from 20 g/L glycerol ¹⁴. The highly-active C8-specific *C_pFatB** enzyme was utilized to produce 1-octanol by expressing an acyl-CoA synthetase (*MtFadD6*) from *Mycobacterium tuberculosis* to reactivate octanoic acid at the cost of 1 mole ATP and expressing an acyl-CoA reductase from *Marinobacter aquaeolei* (*Ma*ACR) to convert octanoyl-CoA to 1-octanol. The resulting *E. coli* strain NHL24 produced 1.3 g/L 1-octanol ¹⁸. In a separate study, 2-heptanone was produced by converting octanoyl-CoA to β -ketooctanoyl-CoA via an acyl-CoA oxidase from *Micrococcus luteus* (Mlut_11700) and an endogenous bi-functional dehydrogenase *EcFadB*. Subsequently, β -ketooctanoyl-CoA was hydrolyzed by an β -ketoacyl-CoA thioesterase *PsFadM* from *Providencia sneebia* (*PsFadM*) and the resulting β -ketooctanoic acid was decarboxylated non-enzymatically to yield 2-heptanone. Strain *E. coli* TRS12 (MG1655 Δ *araBAD* Δ *fadD::Ptrc-C_pfatB1** Δ *fadA* Δ *fadE* Δ *fadI* Δ *fadR*) harboring pTRC99a-*MtFadD6*-*PsfadM* and pACYC-*Mlmlut_11700* plasmids produced up to 4 g/L 2-heptanone in fed-batch bioreactor experiments ⁴. While successful, this thioesterase strategy could be further improved by replacing the futile cycle of thioester cleavage and formation with a direct acyl-transfer.

In nature, some bacteria such as *Pseudomonads*, accumulate polyhydroxyalkanoate (PHA) as a means of storing carbon and energy. PHA polymerization requires a supply of (*R*)-3-hydroxyacyl-CoAs (PHA monomers), which can be derived from either fatty acid biosynthesis or

β -oxidation. Two decades ago, PhaG, an enzyme found in *Pseudomonas putida* and *Pseudomonas aeruginosa*, was identified as the enzymatic link between fatty acid biosynthesis and PHA biosynthesis^{19,20}. PhaG was hypothesized to catalyze the transfer of the (*R*)-3-hydroxyacyl moiety from the ACP thioester to CoA. *In vitro* experimental results showed a time-course of CoA release by incubating purified PhaG, (*R*)-3-hydroxydecanoyl-CoA and holo-ACP, indicating PhaG catalyzes a reversible transacylase reaction²¹. In subsequent studies, researchers overexpressed PhaG and observed increased PHA content in cells and an increased fraction of medium-chain-length 3-hydroxyalkanoate (3HA) units in the polymer^{22–25}. Given these results, we hypothesized that PhaG could provide a similar role in linking fatty acid biosynthesis with creation of tailored pools of acyl-CoAs and oleochemical products.

In the present study, we demonstrated that such a PhaG-dependent pathway is an alternative strategy to link fatty acid biosynthesis and oleochemical production with higher theoretical yields than previously demonstrated thioesterase strategies (**Figure 1 and Table S2**). We validated the ability of *P. putida* PhaG, to perform 3-hydroxyacyl-ACP:CoA transacylase reactions and proved that PhaG can direct flux towards oleochemical synthesis at rates comparable to thioesterases. Using computational bioprospecting tools, we identified seven homologs of *Ppu*PhaG and evaluated their *in vivo* activities. The PhaG variant from *Pseudomonas koreensis* produced 1.6-fold more methyl ketones than the *Ppu*PhaG variant used as a seed to the search. We constructed a random mutagenesis library of *Pk*PhaG and isolated seventeen beneficial mutations that increased octanoic acid production 3.3- to 16.3-fold above strains expressing the original *Pk*PhaG. We used these improved enzymes to construct strains capable of producing three demonstration oleochemicals - free fatty acids, fatty alcohols, and methyl ketones. Strains expressing the PhaG-dependent pathway were capable of producing 1.1 g/L C₈-C₁₄ free fatty acids,

1.5 g/L C₇-C₁₅ methyl ketones, and 1.1 g/L C₈-C₁₆ fatty alcohols depending on the tailoring enzymes co-expressed. These titers demonstrate that PhaG is a useful alternative for medium-chain length oleochemical synthesis and a promising target for future protein engineering to guide substrate selectivity.

2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids, oligonucleotides, and reagents

All bacterial strains used in this study are listed in **Supplementary Table S1**. Q5 DNA polymerase and Monarch[®] PCR and DNA Cleanup Kit was purchased from New England Biolabs (Ipswich, MA). Oligonucleotide primers and gBlocks were synthesized by Integrated DNA Technologies (IDT), Inc. (San Diego, CA). Chemicals including fatty acid, fatty alcohol and methyl ketones were purchased from Sigma-Aldrich (St. Louis, MO).

E. coli DH5 α strains were used for plasmid amplification and DNA assembly. *E. coli* RABIJ and *E. coli* CM23 were created as part of prior studies ^{11,26}. *E. coli* RAI and RADJ strains were used in those experiments presented in **Figure 2B, 2C, 2D, 3B, 5A, 5B, 6A and 6B**. *E. coli* CM23 strains were used in those experiments presented in **Figure 4B and 4C**.

2.2 Plasmid and strain construction

All plasmids used in this study are summarized in **Supplementary Table S1**. Plasmid maps are available as **Supplementary Materials**. All plasmids were constructed by Gibson assembly (New England Biolabs). The chromosomal deletion of *fadD* and *lipB* were performed using a combination of lambda red recombination and CRISPR/Cas9-mediated selection as described in prior work ^{4,11,14,18}. All cloned sequences and gene deletions were confirmed by Sanger sequencing performed by Functional Biosciences (Madison, WI). Constructs expressing FadB and PhaJ homologs were obtained from prior studies ^{4,11,26}.

2.3 Oleochemical production and quantification

All oleochemical production studies were performed by growing *E. coli* strains at 30°C in Clomburg medium containing 20 g/L glycerol, the appropriate antibiotics (Carbenicillin - 100 µg/mL; kanamycin - 50 µg/mL; chloramphenicol - 34 µg/mL), and IPTG for induction as indicated. Pre-cultures for each experiment were prepared by inoculating 5 mL LB media (+ antibiotics) with a single colony and incubating overnight at 30°C with shaking at 250 r.p.m. A 2.5 vol% inoculum was transferred into production flasks with a starting OD₆₀₀ 0.1. Fatty alcohol and methyl ketone production cultures were supplemented with 10% (v/v) dodecane to provide a product sink. Samples from each culture were extracted after 72 hours incubation at 30°C.

Fed-batch fermentation was performed using a 1-L Infors Multifors bioreactor. Overnight pre-cultures were inoculated to an initial OD₆₀₀ of 0.05 into a bioreactor containing 500 mL Clomburg medium with ~50 g/L glycerol. The bioreactor was operated at the following conditions: temperature was controlled at 30°C post-induction, air flow was 1.5 L/min, stirrer rate was varied between 250 r.p.m. and 1000 r.p.m. to control dissolved oxygen at a value of 30%, pH was maintained at 7.0 using 2 M sulfuric acid and 2 M ammonia hydroxide. When the OD₆₀₀ reached ~1.0, IPTG was added to achieve a final concentration of 1 mM and 100 mL dodecane was fed in the bioreactor. At 24 hours of post-induction, ~100 mL 5X concentrated Clomburg media containing 500 g/L glycerol was one-time bolus-fed into the bioreactor and fermentation terminated 96 hours post-induction. Measurements of methyl ketone, glycerol, optical density, and CO₂ evolution were recorded for 96 hours total.

The bioreactor outlet gas stream was directed through a chilled organic absorber to capture methyl ketone vapors stripped from the culture broth. The absorber was composed of a jacketed glass gas dryer with a ceramic sparge distributing the off gas into 100 mL of chilled dodecane. The

temperature of the absorber was maintained at 5°C by an external water cooler. Methyl ketone data was taken at 96 hours from the dodecane, and endpoint methyl ketone capture was taken from both the collected water and remaining dodecane 96-101 hours after inducing the culture. A schematic of the absorber is described in more detail in **Supplemental Figure S6**.

Methods of fatty acid, fatty alcohol and methyl ketone quantification were described in previous work ⁴. To determine the methyl ketone and fatty alcohol concentration in the distinct organic or aqueous phases, 50 mL of cell culture was centrifuged at $4500 \times g$ for 10 min and 0.5 mL samples from the dodecane layer and 2.5 mL samples from aqueous phase were collected and evaluated separately. Fatty acids were extracted from culture according to an acid-based esterification method described previously ^{14,15}. Fatty alcohols and methyl ketones were extracted from culture into *n*-hexane according to methods described previously ^{4,11}. Fatty acid and methyl ketone species were separated using an Agilent RTX-5 column and fatty alcohol species were separated using Agilent DB-Fatwax column (Santa Clara, CA). Oleochemicals were quantified by comparing GC-FID peak areas against standard curves prepared with commercial standards.

2.4 Mutagenesis of PhaG

A mutagenic PhaG library was constructed by error-prone PCR using GeneMorph II from Agilent (Santa Clara, CA) with a low mutation frequency (0 to 4.5 mutations/kb). The plasmid backbone (pBTRCK) was PCR-amplified using a high-fidelity DNA polymerase Q5 from New England Biolabs (Ipswich, MA). The library was assembled using an isothermal assembly method²⁷. Primers used in the creation of the library contained the start and stop codons in order to prevent mutation of them.

The sequence of improved PhaG variants was obtained by Sanger sequencing of colony PCR products made by high fidelity Q5 DNA polymerase. To re-introduce single point mutations, we amplified *P_k*PhaG with mutagenic primers and subcloned the fragments into pTRC99A-*'P_kphaG'-T^dTER* by an isothermal assembly method ²⁷. The resulting plasmids were transformed into *E. coli* CM23 harboring pBTRCK-*Ma_{tesB}** and pACYC-*Pa_{phaJ3}* plasmids.

2.5 Lipoic acid selection

3-Hydroxyoctanoyl-CoA producing variants of PhaG were isolated using a lipoic/octanoic acid selection described previously ¹⁴. Plasmids expressing each variant (pBTRCK-*P_kphaG**) were transformed into *E. coli* CM23-*ΔlipB* (CM23 strain, *ΔlipB*) strain harboring pTRC99a-*T^dTER-Ma_{tesB}** + pACYC-*Pa_{phaJ3}*. Transformants were plated on MOPS minimal media agarose plates containing 0.2% glucose, 20 μM IPTG, and kanamycin, chloramphenicol and carbenicillin to maintain and induce plasmids as described in previous work ¹⁴. Transformants were patched onto LB plates for archiving and secondary screening in octanoic acid production studies.

3. RESULTS AND DISCUSSION

Figure 1 Metabolic pathways for producing medium-chain oleochemicals via fatty acid biosynthesis. Oleochemical products such as methyl ketones (MK), fatty acid ethyl esters (FAEE), fatty alcohols (FAOH), and alkanes are made by enzymes that act on acyl-thioesters. Many oleochemical-producing enzymes can act on long-chain acyl-ACPs (Option 1) but fail to produce medium or short-chain products in high amounts without additional genetic modifications. The reduced activity is likely caused by lower concentrations of shorter chain length acyl-ACPs and/or reduced specific activity on these substrates, particularly when compared to enzymes in fatty acid biosynthesis that elongate the same substrates. Alternatively, if β -oxidation is blocked (often by deleting enzymes catalyzing the appropriate step in the cycle), higher concentrations of mcl-acyl-CoA's can be provided to saturate oleochemical-producing enzymes. This acyl-CoA pool can be created either by the combination of thioesterase (TE) and acyl-CoA synthetase (e.g. FadD) activity (Option 2) or by acyl-transferase activity (Option 3). Transferase activity (e.g. PhaG) saves one ATP per product relative to Option 2, leading to higher theoretical yields. Here, we have focused on transfer of 3-hydroxyacyl-thioesters that can be converted to desired substrates for oleochemical producing enzymes with additional enzymes (represented by a bold dashed arrow). Detailed pathways for Options 2 and 3 are illustrated in **Supplementary Figure S1**.

3.1 Genetic Studies Confirm PhaG is an Acyl-ACP:CoA Transacylase

Homologs of *Ppu*PhaG have been used extensively as a means of enhancing production of medium-chain length (mcl) PHA in bacteria^{19,28–32}. That said, the specific activities catalyzed by *Ppu*PhaG are less well cataloged. Prior studies connected PhaG expression with the production of hydroxy fatty acids^{32,33} and suggested that PhaG catalyzes 3-hydroxyacyl-ACP thioesterase activity. If true, the metabolic yield advantages motivating this work would be lost. Therefore, we compared the metabolic product profiles (looking for production of methyl ketones or hydroxy

fatty acids) of specifically engineered strains of *E. coli* (MG1655 $\Delta fadA$, $\Delta fadI$, $\Delta fadD$, $\Delta fadR$, pTRC99a-^{Ppu}*phaG*-^{Ec}*fadM*) to determine if heterologously expressed PhaG has thioesterase and/or transacylase activity. Strains were designed to create a 3-hydroxy fatty acid product sink to indicate potential PhaG thioesterase activity and a methyl ketone product sink for PhaG transacylase activity (**Figure 2A**). Deletion of *fadD* removes acyl-CoA synthetase activity and prevents reactivation of free fatty acids generated by either FadM or PhaG. Deletion of *fadA* and *fadI* removes known thiolase activities from *E. coli* and blocks β -oxidation from catabolizing any acyl-CoAs produced *in vivo*. Deletion of *fadR* removes repression of *fadB* expression and thereby upregulates a bi-functional enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase responsible for converting hydroxy-acyl-CoA thioesters to 3-ketoacyl-CoA thioesters. FadM^{4,34} is overexpressed to provide 3-ketoacyl-CoA thioesterase activity, resulting in conversion of any 3-hydroxyacyl-CoAs generated by PhaG to the corresponding methyl ketones. Cultures of *E. coli* RADI strain harboring pTRC99a-^{Ppu}*phaG*-^{Ec}*fadM* were grown in Clomburg media at 30°C for 48 h. Culture samples were extracted and derivatized for GC/FID and GC/MS analysis. The samples contained a total of 170 mg/L C₇-C₁₃ methyl ketones but no detectable 3-hydroxy methyl esters (**Figure 2B**) indicating that ^{Ppu}PhaG functions primarily as a 3-hydroxyacyl ACP:CoA transacylase. Strains expressing FadD (*E. coli* RAI strain pTRC99a-^{Ppu}*phaG*-^{Ec}*fadM*) produced equivalent amounts of methyl ketones, indicating that the carbon flux for methyl ketone synthesis is primarily from the activity of PhaG rather than activity of free fatty acid reactivation by FadD. Strains lacking PhaG overexpression (*E. coli* RADI strain pTRC99a-^{Ec}*fadM*) produced small amounts (<1 mg/L) of methyl ketones (**Figure 2B**) that have been previously observed in strains expressing FadM in *E. coli*^{4,34}. Strains lacking ^{Ec}FadM overexpression (*E. coli* RADI strain pTRC99a-^{Ppu}*phaG*) contained a total of 55 mg/L C₈-C₁₂ 3-hydroxy methyl esters (**Figure 2C**).

Together, this data suggests that 3-hydroxy fatty acid production observed in past studies likely comes from thioesterase activities encoded by native enzymes (e.g. YciA, FadM, TesB can potentially catalyzes (*R*)-3-hydroxyacyl-CoA to 3-hydroxy fatty acids) that are outcompeted by the methyl ketone synthesis pathway we introduced.

Figure 2 (A) Designed pathways for testing PhaG activities for methyl ketone or 3-hydroxy fatty acid synthesis. Two potential activities of PhaG is proposed, a thioesterase that converts (*R*)-3-hydroxyacyl-ACP to 3-hydroxy fatty acids or an acyl-ACP:CoA transacylase that transfers (*R*)-3-hydroxyacyl moiety from a ACP to CoA (B) - (C) Methyl ketone or 3-hydroxy fatty acid produced by *E. coli* strain RAI harboring pTRC99a-^{Ppu}phaG-^{Ec}fadM, pTRC99a-^{Ec}fadM or pTRC99a-^{Ppu}phaG. Analysis of methyl ketone production with or without chromosomal ^{Ec}FadD deletion

using *E. coli* RAI strain harboring pTRC99a-*Ppu*phaG-*Ec*fadM. (n=3). 3-hydroxy fatty acid produced by *E. coli* strain RADI harboring pTRC99a-*Ppu*phaG (n=3). (D) Sequencing similarity map of bioprospecting *Ppu*PhaG. Selected homologs are colored. Quantitative pairwise percent amino acid identity multiple alignment of each enzyme homolog can be found in **Figure S2 (E)** Mean methyl ketone titers for constructs of PhaG homologs using *E. coli* RAI harbor pTRC99a-*Pk*phaG-*Ec*fadM (n=3). Cultures were cultivated in the Clomburg medium containing 20 g/L glycerol at 30°C and shaking at 250 r.p.m. All error bars represent standard error. ** represents P value < 0.01 analyzed based on student t-test assuming unequal variances.

3.2 Bioprospecting Identifies Active PhaG Variants

Next, we sought to identify higher activity variants through bioprospecting. We conducted a homology search based on the *Pseudomonas putida* KT2440 PhaG (*Ppu*PhaG) sequence using the Basic Local Alignment Search Tool (BLAST)³⁵ to identify candidate protein sequences. BLAST hits were sorted using the Enzyme Similarity Tool³⁶. Among the homologous sequences that have similarity greater than 45%, we found >95% of sequences belong to *Pseudomonas* species, indicating that the transacylase PhaG enzyme is quite unique in *Pseudomonads* even compared to other bacteria that have reported PHA synthesis³⁷. We selected thirteen PhaG homologs which had a protein sequence similarity range of 24-88% based on a pairwise comparison of each transacylase, shown in **Figure 2D** and **Supplementary Figure S2**. We were particularly interested in homologs from *Mycobacteria* and *Corynebacteria* species because of their potential to interface with substrates linked to the ACP domain of type I fatty acid synthase (FAS) found in these species³⁸. The activity of PhaG homologs was assayed *in vivo* by monitoring methyl ketone production using *E. coli* RADI harboring a pTRC99a-*Pk*phaG-*Ec*fadM plasmid. Most PhaG variants generated similar methyl ketone titers to *Ppu*PhaG albeit with reduced levels of 2-heptanone. The PhaG variants from *Mycobacteria* and *Corynebacteria* failed to produce methyl ketones. We found that the *Pseudomonas koreensis* *Pk*PhaG showed the highest production at 350 mg/L total methyl ketone, 1.6 times higher than the *Ppu*PhaG, shown in **Figure 2D**. The methyl

ketone profile included 34 mg/L 2-heptanone, 85 mg/L 2-nonanone, 85 mg/L 2-undecanone and 145 mg/L 2-tridecanone indicating a broad activity against medium-chain 3-hydroxyacyl-ACPs.

3.3 Metabolic Engineering to Enhance Methyl Ketone Production

A central tenet of metabolic engineering states that enzyme activity must be balanced across a metabolic pathway to minimize unwanted accumulation of intermediates and maximize pathway flux. In order to assess the relative activity of PhaG to pathway flux, we varied co-expression of *Ppu*PhaG, the more-active *Pk*PhaG, and enzymes that convert 3-hydroxyacyl-CoAs to 3-ketoacyl-CoAs. In particular, we were concerned about the relative activity of ketoreductases on the two 3-hydroxyacyl-CoA stereoisomers. PhaG generates (*R*)-3-hydroxyacyl-CoA for direct polymerization into PHA. In contrast, β -oxidation passes through (*S*)-3-hydroxyacyl-CoAs. FadB, the dual-function enoyl-CoA hydratase and dehydrogenase, can isomerize (*R*)-3-hydroxy-acyl-CoA via the corresponding enoyl-acyl-CoA, likely at a reduced rate relative to its preferred substrate isomer^{39–42}. PHA producing bacteria solve this problem by expressing an alternative enoyl-CoA hydratase (PhaJ) that can generate the preferred *R*-isomer (**Figure 3A**) from β -oxidation intermediates. We selected *Ec*FadB and *Ec*FadJ, an anaerobically expressed FadB homolog, and four previously studied PhaJ variants from *P. aeruginosa*²⁶. These enzymes were important optimization points because deletion of *fadB* and *fadJ* eliminated PhaG-dependent methyl ketone production (**Figure 3B**).

A combination of *Ec*FadB, *Ec*FadB/*Ec*FadJ and four *Ppu*PhaJ homologs were cloned into an operon linked to a P_{TRC} promoter on a pACYC vector. Each of these vectors was co-expressed with pTRC99a-*Ppu*phaG-*Ec*fadM or pTRC99a-*Pk*phaG-*Ec*fadM in *E. coli* RAI. Cultures of each strain were grown at 30° for 48 hours. Methyl ketones were extracted from culture samples and

quantified by GC/FID (**Figure 3**). Strains expressing P_{pu} PhaG all produced ~160 mg/L of methyl ketones with similar distributions to prior experiments. In contrast, when P_k PhaG was expressed, methyl ketone titers increased 2-3 fold relative to the corresponding P_{pu} PhaG strains. In this series, co-expression of PhaJ1 and PhaJ3 had the biggest impact on methyl ketone titer, surpassing 0.5 g/L in cultures of the best strains. These experiments indicate that 3-hydroxyacyl-CoA isomerization can be a limiting step when PhaG transacylase activity is increased. Further, these experiments suggested that titers could be improved further with more active PhaG enzymes.

Figure 3 (A) Scheme of *in vivo* characterization of enzymes catalyzing reactions that bridge between (R)-3-hydroxyacyl-CoAs and acyl-CoA intermediates in β -oxidation pathway. (B) Evaluation of methyl ketone productions based on stepwise genetic modifications from basal strains RAI and RABII (n=3). All error bars represent standard error. *** represents P value < 0.001 analyzed based on student t-test assuming unequal variances.

3.4 Random mutagenesis PhaG and selection for lipoic acid requirement of *E. coli*

Our initial experiments indicated that PhaG has the capability of controlling the synthesis of medium-chain oleochemicals analogous to acyl-ACP thioesterases. However, unlike thioesterases, our bioprospecting study did not identify PhaG variants with dramatically different selectivities to medium-chain species. Therefore, we initiated a protein engineering study to seek variants with altered specificity and enhanced activity. We constructed an error-prone PCR library of *P_kphaG* ORFs and screened them for the ability to complement a lipoic acid auxotrophy. The lipoic acid auxotrophy was recently used to isolate C₈-specific thioesterases with enhanced $V_{\max}$ ¹⁴. *E. coli* CM23 strain¹¹, (MG1655 Δ *araBAD* Δ *fadAB*, Δ *fadIJ*, Δ *fadD*, Δ *fadR*, Δ *fadE*, Δ *ldhA*, Δ *ackApta*, Δ *adhE*, Δ *poxB*, Δ *frdABCD*, Δ *ydiO*, Δ *atoC*), which lacks all β -oxidation genes and many fermentation pathways, was chosen as the base strain as it lacks all alternative routes to lipoic acid. We constructed an *E. coli* CM23- Δ *lipB* strain to implement the lipoic acid auxotrophy. This strain requires supplementation of lipoic acid or octanoic acid in the media to enable the formation of an active pyruvate dehydrogenase and aerobic growth on glucose. To link the octanoic acid selection to PhaG activity, we added heterologous enzymes to convert the 3-hydroxyoctanoyl-CoA to octanoic acid (**Figure 4A**). In order to allow CM23- Δ *lipB* to synthesize free fatty acids, we coexpressed genes encoding *P_k*PhaG, *P_a*PhaJ3, a *Treponema denticola* reductase *T_d*TER¹¹ and a *Mycobacterium* sp. acyl-CoA thioesterase *Ma*TesB A197D (referred to as *Ma*TesB*)⁴³ (**Figure 4A**). Purified *Mycobacterium avium* *Ma*TesB* has been shown to hydrolyze octanoyl-CoA and generate octanoic acid *in vitro*⁴³. We neglected the octanoic acid synthesis activities by endogenous *E. coli* acyl-CoA thioesterase since *Ec*TesB generally has activities toward longer chain acyl-CoA (>C₁₀)⁴⁴ and *Ec*YciA has activities toward shorter chain acyl-CoA (<C₈)^{45,46}. To test the basal

octanoic acid production level, we found *E. coli* CM23- Δ *lipB* harboring pTRC99a-*P_kphaG* - *T_dTER* + pACYC-*P_aphaJ3* + pBTRCK-*Ma_{tesB}** plasmids produced ~20 mg/L octanoic acid after 48 hours, while the corresponding strain without *P_kPhaG* produced <1 mg/L octanoic acid (data not shown). In order to reduce the baseline octanoic acid titer, we subcloned *P_kphaG* onto a low copy vector (pBTRCK)¹¹ and *Ma_{tesB}** onto a high copy number vector pTRC99a. The latter was performed to ensure that octanoic acid production would be limited solely by PhaG activity. After tuning the copy number of *P_kphaG* and *Ma_{tesB}**, *E. coli* CM23- Δ *lipB* strain harboring pTRC99a-*Ma_{tesB}**-*T_dTER* + pACYC-*P_aphaJ3* + pBTRCK-'*P_kphaG*' plasmids produced ~7 mg/L octanoic acid after 48 hours. Selections of the error-prone PhaG library were performed using *E. coli* CM23- Δ *lipB* strain harboring pTRC99a-*Ma_{tesB}**-*T_dTER* + pACYC-*P_aphaJ3* + pBTRCK-'*P_kphaG*'.

In the first-round of mutagenesis, hundreds of colonies appeared on MOPS-glucose minimal media plates containing 20 μ M IPTG three days after plating. On day 4, we picked ~180 of the largest colonies and quantified the octanoic acid titer from individual liquid cultures grown in Clomburg liquid media containing 20 g/L glycerol. We found that 17 strains expressing *P_kPhaG* variants increased octanoic acid titer 3.3- to 16.3-fold over the parent *P_kPhaG* (**Figure S3**). All 17 *P_kPhaG* mutant strains increased total fatty acid production ranging 1.8- to 8.3-fold with modest changes in the overall distribution of chain-lengths.

To investigate why titers were increased in these strains, we examined PhaG abundance using a translational coupling circuit linking RFP to PhaG⁴⁷. We compared expression of five PhaG variants (WT, LM1, LM7, LM9 and LM14) by proxy using RFP fluorescence generated by the translationally coupling cassette. When YFP was cloned in place of PhaG, RFP fluorescence was proportional to YFP fluorescence over a range of IPTG (inducer) concentrations (**Supplementary Figure S4**). PhaG variants LM9, LM14, and WT demonstrated equivalent

fluorescence values across a range of IPTG concentrations. PhaG variant LM7 generated slightly more RFP fluorescence, indicating it may be present at higher concentrations. Conversely, PhaG variant LM1 produced ~60% of the RFP fluorescence of the WT. Decreased expression may be due to the occurrence of a rare codon CGG at point mutation Q45R. CGG codons have been shown in other studies to be a rate-limiting factor for translational efficiency due to its low abundance tRNA supply in *E. coli* ^{48,49}. We estimated specific PhaG activity by normalizing the sum of observed C₈-C₁₄ FAME titer to RFP fluorescence (proxy for protein abundance). These estimates indicate the LM1 and LM14 variants are 7.4- and 8.8-fold more active than wild-type PhaG (**Supplementary Figure S4**).

From the sequences of the 17 improved *P_k*PhaG variants, we identified 28 point mutations. To investigate the importance of each on *P_k*PhaG activity, we created the full library of single point mutations. We coexpressed each *P_k*PhaG variant from the high copy number pTRC99a-'*P_k*phaG'-*T_d*TER vector with pACYC-*P_a*phaJ3 + pBTRCK-*M_a*tesB* in *E. coli* CM23. Each strain was cultured for 48 hrs at 30 °C and samples were harvested for fatty acid quantification. We found that variants containing 6 of the 28 single point mutations (e.g. Q45R, R66H, H76Y, Y138F, G142V, Q277X) increased octanoic acid titer more than 2-fold over strains expressing the parent *P_k*PhaG (**Supplementary Figure S5**). Among these six single point mutations, three individual *P_k*PhaG mutants Q45R, G142V and Y138F strains had 1.9-, 1.8- and 1.5-fold higher total fatty acid titers than strains expressing wild-type *P_k*PhaG.

In order to evaluate the combinatorial effects of the three most beneficial single point mutations, we constructed all four possible combinations of Q45R, G142V and Y138F mutations in a vector pTRC99a-'*P_k*phaG'-*T_d*TER. These combinatorial mutants were co-expressed with pACYC-*P_a*phaJ3 + pBTRCK-*M_a*tesB* in *E. coli* CM23. Each strain was cultured for 48 hrs at 30°C

and samples were harvested for fatty acid quantification. Overall, the best variant, ^{Pk}PhaG Q45R G142V, strain produced the highest amount of C₈-C₁₄ free fatty acid at 1.1 g/L, a 4.0-fold increase of total C₈-C₁₄ free fatty acid compared to that of the wild-type ^{Pk}PhaG (**Figure 4B**). The fatty acid pool contained 41% tetradecanoic acid, 22% dodecanoic acid, 11% decanoic acid, and 26% octanoic acid.

3.5 Validating enzymes converting (*R*)-3-hydroxyacyl-CoA intermediates

In order to re-evaluate the effect of FadB and PhaJ activities on overall rate of the PhaG pathway with the more active ^{Pk}PhaG* (referred as to ^{Pk}PhaG Q45R G142V) variant, we cloned six FadB homologs and four PhaJ homologs in a pACYC vector, co-expressed each with pTRC99a-^{Td}TER-^{Pk}phaG* and pBTRCK-^{Ma}tesB* in strain CM23, and cultured them for fatty acid production. The strain expressing ^{Pa}PhaJ3 and ^{Pa}PhaJ1 produced the highest fatty acid titers, suggesting these enzymes have the highest activity for converting (*R*)-3-hydroxyacyl-CoA substrate to enoyl-acyl-CoA (**Figure 4C**). We found that all FadB variants produced substantially lower titers compared to the analogous strains expressing ^{Pa}PhaJ3. This suggests that FadB has lower activity on recognizing (*R*)-3-hydroxyacyl-CoAs than ^{Pa}PhaJ3. This is not surprising since in *Pseudomonas*, FadB might evolve to lower its isomerization activity to avoid crosstalk between PHA biosynthesis and beta-oxidation.

Figure 4 (A) Scheme of octanoic acid/lipoic acid selection strategies based on *E. coli* CM23-*ΔlipB* strain. *EcFadD* deletion blocks reactivation of fatty acids, *EcFadE* deletion blocks hydrogenation enoyl octanoyl-CoA to octanoyl-CoA, *EcFadBJ* deletion blocks hydration and dehydrogenation of enoyl octanoyl-CoA to β -keto-octanoyl-CoA by *EcFadBJ*, *EcFadAI* deletion blocks condensation of octanoyl-CoA and acetyl-CoA to β -ketodecanoyl-CoA, LipB deletion blocks activation of apo PDC E2 domain to octanoylated PDC E2 domain. The PhaG enzyme was designed to pull carbon flux from 3-hydroxyoctanoyl-ACP to (*R*)-3-hydroxyoctanoyl-CoA. (*R*)-3-hydroxyoctanoyl-CoA undergoes a dehydration and hydrogenation process by *PaPhaJ3* and *TdTER* to generate octanoyl-CoA. Octanoyl-CoA is cleaved by *MaTesB** to release ACP and produce octanoic acid. The produced octanoic acid is the octanoyl donor for the octanoylation process of Apo PDC E2 domain to octanoylated PDC E2 domain. When octanoic acid is produced by LipB deletion CM23 strain by the designed PhaG pathway, cells were capable of grown on minimal MOPS agarose plates. *MaTesB** and *TdTER* were cloned in a high copy vector pTRC99a, *PaPhaJ3* was cloned in a medium copy vector pACYC, *PkPhaG* was cloned in a low copy vector pBTRCK. **(B)** FAME profiles of 17 hits identified in the genetic selection strategy of lipoic acid requirement of *E. coli* using a randomly mutagenized. The strain CM23 harboring pBTRCK-'*PkphaG*' + pACYC-'*PaphaJ3*' + pTRC99a-'*TdTER*'-'*MatesB**' were cultured in test tubes containing 5 mL Clomburg 20 g/L glycerol and 1 mM IPTG at 30°C for 48 hrs. **(C)** Combinatorial effects of single point mutations on PhaG activities *in vivo* for fatty acid production (n=3). All error bars represent standard error. ** means

P value < 0.01 and *** means P value < 0.001 analyzed based on student t-test assuming unequal variances.

3.6 Validate methyl ketone and fatty alcohol production

With an enhanced 3-hydroxyacyl-ACP:CoA transacylase ^{P_k}PhaG mutant and a more active (R)-3-hydroxyacyl-CoA dehydratase ^{P_a}PhaJ3 selected, we wanted to benchmark the PhaG pathway for methyl ketone and fatty alcohol production. In order to produce methyl ketones, we cultured *E. coli* RAD1 harboring pTRC99a-^{P_k}phaG*-^{P_s}fadM + pACYC-^{P_a}phaJ3 plasmids. A β -ketoacyl-CoA thioesterase ^{P_s}FadM from *Providencia sneebia* was chosen for its high activity toward medium-chain β -ketoacyl-CoA substrates. The strain RAD1 harboring pTRC99a-^{P_k}phaG*-^{P_s}fadM + pACYC-^{P_a}phaJ3 was incubated for 72 hours at 30 °C in shake flasks containing rich glycerol media. The cultures produced 1.5 g/L total methyl ketone from 20 g/L glycerol (**Figure 5A**), representing 26% 2-heptanone, 27% 2-nonanone, 19% 2-undecanone, 14% 2-tridecanone, 14% 2-heptadecanone. To produce fatty alcohols via PhaG activity, we cultured strain *E. coli* RAD1 harboring pTRC99a-^{P_k}phaG*-^{T_d}TER + pACYC-^{P_a}phaJ3 + pBTRCK-^{M_a}ACR for 72 hrs at 30 °C in shake flasks containing rich glycerol media. The strain produced ~1.1 g/L total fatty alcohol from 20 g/L glycerol (**Figure 5B**), representing 6% 1-octanol, 6% 1-decanol, 7% 1-dodecanol, 41% 1-tetradecanol, 41% 1-hexadecanol. The titer and yield of fatty acids, methyl ketones and fatty alcohols from the PhaG-dependent strategies are comparable to values reported in literature using thioesterase strategies or reverse β -oxidation strategies, **shown in Table 1**.

Figure 5. Benchmark of methyl ketone and fatty alcohol production in batch mode shake flask cultures. (A-B) Methyl ketone production by RAD1 or RAI strain harboring pTRC99a-*P_kphaG**-*P_sfadM* + pACYC-*P_aphaJ3* plasmids. (C-D) Fatty alcohol production by RAD1 strain harboring pTRC99a-*P_kphaG**-*P_sfadM* + pACYC-*P_aphaJ3* + pBTRCK-*MaACR* and negative control corresponding strain without coexpressing PhaG*. All cultures were cultivated in 50 mL Clomburg media 20 g/L glycerol and 1 mM IPTG at 30°C for 48 hrs (n=3). All error bars represent standard error.

3.7 Methyl ketone production in bioreactors

In effort to demonstrate enhanced methyl ketone titers, we performed fed-batch cultivations in a stirred bioreactor by adding Clomburg media + glycerol pulses after cells reached high cell densities. We followed two previously developed strategies to reduce the loss of medium-chain methyl ketones in the off-gas by adding a 20% dodecane overlay to the culture ⁴. After 96 hrs of cultivation, cells consumed 90.0 g/L of glycerol, reached an OD₆₀₀ ~60, and produced 6.8 g/L total methyl ketone titer (**Figure 6A**). Additionally, we used ASPEN to evaluate and design improved gas traps to capture product leaving in the off-gas (**Supplementary Figure S6**) ⁴. We bubbled off-gas through a jacketed gas dryer filled with dodecane at 5°C and observed similar glycerol consumption, biomass formation, and methyl ketone production rates within the reactor. After 96 hrs of induced cultivation, cells reached a density of OD₆₀₀ ~60, consumed 95.2 g/L glycerol, and produced 6.7 g/L total methyl ketone titer (**Figure 6B**). At the end-point, we observed 3.4 g/L methyl ketone contained in the condensed dodecane phase, corresponding to an additional 0.51 g/L (culture volume) of total methyl ketone captured from the bioreactor (**Figure 6C**).

Figure 6. Time course of concentration of glycerol, OD₆₀₀ and total methyl ketone in fed-batch fermentation from bioreactor (n=3) (A) and bioreactor coupling a condenser (n=2) (B) using *E. coli* RAD1 strain harbors pTRC99a-*P_kphaG**-*P_sfadM* and pACYC-*P_aphaJ3* plasmids. (C) Evaluation of methyl ketone concentration from samples in dodecane layer, aqueous phase and condenser after 96 hours fermentation. All error bars represent standard error.

Table 1 Reported titers and yield of oleochemical production using established thioesterase-dependent strategies or developed PhaG-dependent strategies in this work

Strategies	Products	Titer (g/L)	Yield	Fermentation
Thioesterase				
<i>CpFatB</i> ⁴	2-heptanone, 2-nonanone, 2-undecanone	4.4 g/L, 3 g/L, 0.34 g/L	0.035 g/g consumed glycerol, 0.028 g/g consumed glycerol, 0.014 g/g consumed glycerol	fed-batch, bioreactor
<i>CpFatB</i> ¹⁸	1-octanol	1.3	0.065 g/g glycerol	batch, shake flask,
<i>CpFatB</i> ¹⁴	octanoic acid	1.7	0.085 g/g glycerol	batch, shake flask,
BTE ⁵⁰	C ₁₂ -C ₁₄ FAOH	1.6	0.13 g/g glucose	fed-batch, bioreactor
<i>EcTesA</i> ⁵¹	C ₁₁ -C ₁₇ MK	9.8	0.171 g/g glucose	fed-batch, bioreactor
r-BOX				
r-BOX ⁶	C ₆ -C ₁₆ FAOH	1.8	0.2 mol/mol consumed glucose	fed-batch, bioreactor
r-BOX ¹⁰	C ₄ -C ₁₀ FFA	4.7	0.31 g/g consumed glucose	fed-batch, bioreactor
PhaG				
<i>PkPhaG</i>	C ₈ -C ₁₄ FFA	1.2	0.06 g/g glycerol	batch, shake-flask
<i>PkPhaG</i>	C ₈ -C ₁₆ FAOH	1.1	0.055 g/g glycerol	batch, shake-flask
<i>PkPhaG</i>	C ₇ -C ₁₅ MK	7.2	0.075 g/g glycerol	fed-batch, bioreactor

4. CONCLUSION

This study demonstrated that PhaG catalyzed acyltransferase activity is capable of supporting high flux to three medium-chain oleochemical products, fatty acids, fatty alcohols, and methyl ketones. The PhaG-dependent strategy has the potential to achieve higher theoretical yields compared to the well-established thioesterase route for methyl ketone and fatty alcohol production. Here, we first validated PhaG as a 3-hydroxyacyl-ACP:CoA transacylase. We then validated methyl ketone production is driven by the activity of PhaG with a designed PhaG dependent

pathway rather than reactivation of free fatty acids by FadD. Through bioprospecting, we identified a PhaG from *Pseudomonas koreensis* that demonstrated higher activity when producing methyl ketones. After expressing a random mutagenesis library of ^{Pk}PhaG in a lipoic acid selecting strain of *E. coli*, we isolated 17 PhaG mutants that produced octanoic acid ranging from 3.3- to 16.3-fold greater than that of wild-type PhaG. Through evaluating the effect of single point mutations and their combinations, we identified a double mutant, ^{Pk}PhaG Q45R G142V, that showed 4.0-fold higher activity towards conferring total fatty acid production than the ^{Pk}PhaG. Finally, based on our optimization of the PhaG-dependent pathway enzymes, we demonstrated production of 1.1 g/L C₈-C₁₄ free fatty acids, 1.5 g/L C₇-C₁₅ methyl ketones, and 1.1 g/L C₈-C₁₆ fatty alcohols. In fed-batch fermentation, we benchmarked total methyl ketone titers, 7.2 g/L. In total, we have demonstrated that a PhaG dependent pathway is a viable alternative option for production of oleochemicals.

5. CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Qiang Yan: Conceptualization, Investigation, Data curation, Writing - original draft, Writing - review & editing. Michael A. Jindra: Investigation, Writing - review & editing, William T. Cordell: Investigation, Writing - review & editing. Dylan Cortney: Investigation, Writing - review & editing. Xuanqi Chen: Investigation, Writing - review & editing. Brian F. Pfleger: Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

6. DECLARATION OF COMPETING INTEREST

No conflict of interest was declared for this study.

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