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

Qiang Yan1,2, William T. Cordell1,2, Michael A. Jindra1,2, Dylan K. Courtney1, Madeline K. Kuckuk1, Xuanqi Chen1 & Brian F. Pfleger1,2,3✉

Microbial lipid metabolism is an attractive route for producing oleochemicals. The predominant strategy centers on heterologous thioesterases to synthesize desired chain-length fatty acids. To convert acids to oleochemicals (e.g., fatty alcohols, ketones), the narrowed fatty acid pool needs to be reactivated as coenzyme A thioesters at cost of one ATP per reactivation - an expense that could be saved if the acyl-chain was directly transferred from ACP- to CoA-thioester. Here, we demonstrate such an alternative acyl-transferase strategy by heterologous expression of PhaG, an enzyme first identified in Pseudomonads, that transfers 3-hydroxy acyl-chains between acyl-carrier protein and coenzyme A thioester forms for creating polyhydroxyalkanoate monomers. We use it to create a pool of acyl-CoA’s that can be redirected to oleochemical products. Through bioprospecting, mutagenesis, and metabolic engineering, we develop three strains of Escherichia coli capable of producing over 1 g/L of medium-chain free fatty acids, fatty alcohols, and methyl ketones.
Oleochemicals are a class of aliphatic hydrocarbons that are industrially derived from petroleum, animal fats, or oilseeds. 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 an acyl moiety of 8–12 carbons and are of particular interest due to their applications as a commodity and specialty chemicals. For example, medium-chain fatty acids are used as herbicides, antimicrobials, and precursors for lubricant synthesis. Medium-chain methyl ketones are used as flavors, floral fragrances, insecticides, and renewable liquid transportation fuels. Medium-chain alcohols are used as surfactants, as additives in biodiesel, cosmetics, and 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-thioesterases as substrates, making high-chain acyl-thioesters in microbes: thiolase-driven (i.e., reversal of the β-oxidation) and FadE-driven (β-ketoacyl-CoA thioesterase-driven) divergence of fatty acid biosynthesis intermediates. The reverse β-oxidation pathway incorporates a reversible, thiolase-catalyzed Claisen condensation (e.g., FadA) with acyl-CoA as the donor to bypass 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 the production of both medium-chain fatty acids and fatty alcohols 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 selective acyl-ACP thioesterases in the chloroplast to generate free fatty acids of the desired size. Once made, the free fatty acids are transported to the cytosol for reactivation as acyl-CoA and subsequent incorporation into storage lipids. 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 the conversion of the free fatty acids to 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/FadL, 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 an expression of a specialized acyl-ACP thioesterase (to produce desired free fatty acids), expression of 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. For instance, a highly active variant of a Cuphea palustris thioesterase FatB (referred to as ThyFatB*) was identified using a PhaG/FatB random mutagenesis library and a growth selection based on the lipoic acid requirement of Escherichia coli. E. coli strain NHL17 (MG1655 ΔaraBAD ΔfadD:FADΔfadDΔFatB*) produced 1.7 g/L octanoic acid with >90% specificity from 20 g/L glycerol. The highly active C8-specific ThyFatB enzyme was utilized to produce 1-octanol by expressing an acyl-CoA synthetase (MtFatD6) from Mycobacterium tuberculosis to reconvert octanoic acid at the cost of 1 mole ATP and expressing an acyl-CoA reductase from Marinobacter aquaeolei (MuACR) to convert octanoyl-CoA to 1-octanol. The resulting E. coli strain NHL24 produced 1.3 g/L 1-octanol. In a separate study, 2-heptanone was produced by converting octanoyl-CoA to β-ketoacyl-CoA using an acyl-CoA oxidase from Micrococcus luteus (MLut_11700) and an endogenous bi-functional dehydrogenase from E. coli FadB. Subsequently, β-ketoacyl-CoA was hydrolyzed by an β-ketoacyl-CoA thioesterase FadD from Providencia stuartii (PsFadD) and the resulting β-ketoacyclic acid was decarboxylated non-enzymatically to yield 2-heptanone. Strain E. coli TRSI2 (MG1655 ΔaraBAD ΔfadD:FADΔfadDΔFatB* Δfadb ΔfadE ΔfadF ΔfadR) harboring pTRC99a-MtFatD6, pPsFadD and pACYC-MtMlum_11700 plasmids produced up to 4 g/L 2-heptanone in fed-batch bioreactor experiments. Although 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-hydroxyacy-CoAs (PHA monomers), which can be derived from either fatty acid biosynthesis or β-oxidation. In 1998, PhaG, an enzyme found in Pseudomonas putida and Pseudomonas aeruginosa, was identified as the enzymatic link between fatty-acid biosynthesis and PHA biosynthesis. 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-hydroxydecanoy-CoA and holo-ACP, indicating PhaG catalyzes a reversible transacylase reaction. In subsequent studies, researchers overexpressed PhaG and observed increased PHA content in cells and an increased fraction of medium-chain length (mcl) 3-hydroxyalkanoate units in the polymer. Given these results, we hypothesized that PhaG could provide a similar role in linking fatty acid biosynthesis with the creation of tailored pools of acyl-CoAs and oleochemical products.

In the present study, we demonstrate a PhaG-dependent pathway as an alternative strategy to link fatty acid biosynthesis and oleochemical production. We validate the ability of P. putida PhaG to direct flux towards oleochemical synthesis at rates comparable to thioesterases. Using computational bioprospecting tools, we identify seven homologs of P. putida PhaG and evaluate their in vivo activities. The PhaG variant from Pseudomonas koreensis produces 1.6-fold more methyl ketones than the P. putida PhaG variant. We construct a random mutagenesis library of P. putida PhaG and isolate seventeen beneficial mutations that increase octanoic acid production 3.3–16.3-fold above strains expressing the original P. putida PhaG. We use 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 are capable of producing 1.1 g/L C₉–C₁₄ free fatty acids, 1.5 g/L C₁₀–C₁₅ methyl
ketones, and 1.1 g/L C₆–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. Yields remain ~50% of the theoretical limit, on par with demonstrations of many thioesterase-utilizing strategies. Continued improvement of PhaG-driven pathways will allow strains to access higher theoretical yields than current thioesterase strategies (Fig. 1).

Results and discussion

Genetic studies support PhaG has acyl-ACP:CoA transacylase activity. Homologs of PpuPhaG have been used extensively as a means of enhancing the production of mcl PHA in bacteria. This, that the specific activities catalyzed by PpuPhaG are debated. In vitro studies have confirmed the ability of PhaG to generate 3-hydroxyacyl-ACP from the corresponding CoA species and holo-ACP. This is the reverse reaction of the one desired for oleochemical production studies and no in vitro data on acyl-ACPs, the substrate in the forward direction is available. From 1998 to 2012, PhaG was generically called a 3-hydroxyacyl ACP:CoA transacylase, based on in vitro data. In 2012, Nomura and co-workers challenged the name and the ability of the enzyme to perform the transferase reaction. In this study, E. coli BL21 cells harboring a plasmid for expressing an mcl-Pha polymerase (PhaCl) were transformed with plasmids expressing PhaG and/or a CoA ligase from P. putida. Cells expressing both enzymes produced more than ten times the amount of PHA than those lacking the CoA ligase. The conclusion drawn from this study is that the ligase is needed for high-flux PHA generation and PhaG acts primarily as a thioesterase. Subsequent papers have used the name thioesterase, but have not provided further evidence to support the presence of thioesterase activity. In contrast, prior studies demonstrated that co-expression of TesB, a promiscuous CoA thioesterase enhanced the production of 3-hydroxy-fatty acids in both E. coli and P. putida. TesB was similarly used to produce 3-hydroxy fatty acids as precursors to methyl esters.

The complicated history motivated us to confirm that PhaG had substantial transferase activity. Therefore, we compared the metabolic product profiles (looking for production of methyl ketones or 3-hydroxy fatty acids) of specifically engineered strains of E. coli (MG1655 ΔfadA, ΔfadI, ΔfadD, ΔfadR, pTRC99a-PpuphaG-ΔfadM) to determine if heterologously expressed PhaG demonstrated more thioesterase 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 (Fig. 2A). Deletion of fadD removes the dominant acyl-CoA synthetase activity and prevents the reactivation of free fatty acids generated by either FadM or PhaG. Deletion of fadA and fadI removes known thiolese activities from E. coli and blocks β-oxidation from catalyzing 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 3-hydroxyacyl-CoA to 3-ketoacyl-CoA thioesters. FadM is overexpressed to provide 3-ketoacyl-CoA thioesterase activity, resulting in the conversion of any 3-hydroxyacyl-CoAs generated by PhaG to the corresponding methyl ketones. Cultures of E. coli RADI strain harboring pTRC99a-PpuphaG-ΔfadM 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 (Fig. 2B) indicating that PpuPhaG functions primarily as a 3-hydroxyacyl ACP:CoA transacylase. Strains expressing FadD (E. coli RAI strain pTRC99a-PpuphaG-ΔfadM) produced equivalent amounts of methyl ketones, indicating that the carbon flux for methyl ketone synthesis is not enhanced by FadD-catalyzed free fatty acid reactivation. Strains lacking PhaG overexpression (E. coli RADI strain pTRC99a-ΔfadM) produced small amounts (<1 mg/L) of
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 (pPhaG) sequence using the Basic Local Alignment Search Tool (BLAST) to identify candidate protein sequences. BLAST hits were sorted using the Enzyme Similarity Tool. Among the homologous sequences that have similarity >45%, we found >95% of sequences belong to *Pseudomonas* species, indicating that PhaG provides activity unique from other PHA-producing bacteria. We selected 13 PhaG homologs, which had a protein sequence similarity range of 24–88% based on a pairwise comparison, shown in Fig. 2D and Supplementary Figure 2. 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 species. The activity of PhaG homologs was assayed in vivo by monitoring methyl ketone production using *E. coli* harboring a pPhaG expression plasmid. Most PhaG variants generated similar methyl ketone titers to pPhaG albeit with reduced levels of 2-heptanone. The PhaG variants from *Mycobacteria* and *Corynebacteria* failed to produce methyl ketones. We found that the *P. koreensis* pPhaG showed the highest production at 350 mg/L total methyl ketone, 1.6 times higher than the pPhaG, shown in Fig. 2D. The methyl ketone profile included 34 mg/L 2-heptanone, 85 mg/L 2-nonenone, 85 mg/L 2-undecanone, and 145 mg/L 2-tridecanone indicating a broad activity against medium-chain 3-hydroxyacyl-ACPs. The product distribution did not vary significantly across the tested variants. Therefore, substrate preference will need to be addressed with protein engineering efforts analogous to those targeted to acyl-ACP thioesterases.

**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-overexpression of pPhaG, the more-active pPhaG, 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-hydroxyacyl-CoA via the corresponding enoyl-ACP, likely at a reduced rate relative to its regular substrate isomer. PHA-producing bacteria solve this problem by expressing an alternative enoyl-CoA hydratase (PhaI) that can generate the preferred R-isomer (Fig. 3A) from β-oxidation intermediates. We selected EcFadB and EcFadJ, an anaerobically expressed FadB homolog and four previously studied PhaI variants from *P. aeruginosa*. These methyl ketones (Fig. 2B) that have been previously observed in strains expressing FadM. Strains lacking EcFadM overexpression (*E. coli* RADI strain pTRC99a-pPhaG) contained a total of 55 mg/L C3-C12 3-hydroxy methyl esters (Fig. 2C) consistent with prior studies. Together, these data suggest 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 catalyze the cleavage of (R)-3-hydroxyacyl-CoA to 3-hydroxy fatty acids) that are outcompeted by the methyl ketone synthesis pathway we introduced.

Fig. 2 PhaG transacylase activity in vivo. A Metabolic pathways used to test for the presence of PhaG-dependent thioesterase and/or thiolase activity. B In cells lacking FadR, FadA, FadD, and FadD (E. coli RADI harboring pTRC99a-pPhaG), PhaG expression leads to the production of 50 mg/L medium-chain 3-hydroxy fatty acids (n = 3 biologically independent samples). These products could be generated by either direct PhaG thioesterase activity on medium-chain acyl-ACPs or by CoA thioesterase activity on stranded pools of 3-hydroxyacyl-CoAs made via PhaG transacylase activity. **P = 0.0005** was analyzed based on student two-tailed t test assuming unequal variances. C Co-expression of PhaG, and FadM in E. coli RAI (MG1655 ΔfadR ΔfadA ΔfadI ΔfadL harboring pTRC99a-pPhaG-pFadM) results in the production of 150 mg/L of medium-chain methyl ketones (n = 3 biologically independent samples). FadD expression did not impact methyl ketone production indicating that free fatty acid activation is not required for PhaG-dependent methyl ketone production. D A two-dimensional cluster map created with the Enzyme Similarity Tool displays the sequence similarity of PhaG variants tested in bioprospecting studies. Quantitative pairwise percent amino-acid identity of each homolog can be found in Supplementary Figure 2. Colored boxes and dots are used to indicate the sequences tested. Mean methyl ketone titers for constructs of PhaG homologs using *E. coli* RAI harbor pTRC99a-pPhaG-pFadM (n = 3 biologically independent samples). All cultures were grown in Glomberg medium containing 20 g/L glycerol at 30 °C and shaking at 250 r.p.m. **P = 0.009** was analyzed based on student two-tailed t test assuming unequal variances. All data represent the mean ± s.d. of biological triplicates. Source data underlying B-D are provided as a Source Data file.
enzymes were important optimization points because deletion of fadB and fadI eliminated PhaG-dependent methyl ketone production (Fig. 3B).

A combination of EcFadB, EcFadB/EcFadJ, and four PpphaG homologs was cloned into an operon linked to a P<sub>T</sub>TRC promoter on a pACYC vector. Each of these vectors was co-expressed with pTRC99a-P<sub>p</sub>phaG-E<sub>c</sub>fadM or pTRC99a-P<sub>p</sub>phaG-E<sub>c</sub>fadM in E. coli RAI. Cultures of each strain were grown at 30 °C for 48 h. Methyl ketones were extracted from culture samples and quantified using GC/FID (Fig. 3). Strains expressing PpphaG all produced ~20 mg/L methyl ketones with similar distributions to prior experiments. In contrast, when PkphaG was expressed, methyl ketone titers increased twofold relative to the corresponding strain without PkphaG. In this series, co-expression of PhaJ1 and PhaJ3 had the biggest impact on methyl ketone titers, surpassing ~160 mg/L of methyl ketones with similar distributions to prior experiments. We neglected the isomerization of 3-hydroxyacyl-CoA to octanoic acid (Fig. 4A). These enzymes include PkphaG, PphaJ3, a Treponema denticola trans-enoyl-CoA reductase (T<sub>D</sub>TER) and a Mycobacterium sp. acyl-CoA thioesterase M<sub>T</sub>esB A197D (referred to as M<sub>T</sub>esB<sup>B</sup>) (Fig. 4A). Purified Mycobacterium avium M<sub>T</sub>esB<sup>B</sup> has been shown to hydrolyze octanoyl-CoA and generate octanoic acid in vitro<sup>45</sup>. We neglected the octanoic acid synthesis activities by endogenous E. coli acyl-CoA thioesterase because E<sub>T</sub>esB generally has activities toward longer chain acyl-CoA (>C10)<sup>41</sup> and E<sub>Y</sub>elA has activities toward shorter chain acyl-CoA (<C<sub>8</sub>)<sup>46,47</sup>. The base strain, expressing the wild-type PkphaG, produced ~20 mg/L octanoic acids after 48 h, whereas the corresponding strain without PkphaG produced <1 mg/L octanoic acid. In order to reduce the baseline octanoic acid titer, we subcloned PkphaG onto a low-copy vector (pBTRCK)<sup>8</sup> and M<sub>T</sub>esB<sup>B</sup> 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 PkphaG and M<sub>T</sub>esB<sup>B</sup>, E. coli CM23-ΔlipB harboring pTRC99a-M<sub>T</sub>esB<sup>B</sup>-T<sub>D</sub>TER+pACYC-P<sub>p</sub>phaJ3+pBTRCK-PkphaG plasmids produced ~7 mg/L octanoic acids after 48 h. This strain was used to perform selections of the error-prone PhaG library.
In the first-round of mutagenesis, hundreds of colonies appeared three days after plating on MOPS-glucose minimal agar containing 20 μM isopropyl β-d-1-thiogalactopyranoside (IPTG). On day 4, we picked ~180 of the largest colonies and quantified the octanoic acid titer from individual liquid cultures grown in Cloburg liquid media containing 20 g/L glycerol. We found 17 PhaG variants increased octanoic acid titer (3.3–16.3-fold) and total fatty acid titer (1.8–8.3-fold) relative to the parent PhaG (Supplementary Figures 3–5 and Supplementary Method 2). The 17 improved PhaG variants contained a total of 28 point mutations. The best variant, PhaG Q45R G142V, produced 1.1 g/L 13.6-fold octanoic acid, similar to the original PhaG (Fig. 4B). The fatty-acid pool contained 41% tetradecanoic acid, 22% dodecanoic acid, 11% decanoic acid, and 26% octanoic acid, similar to the original PhaG-expressing strain. This indicates that the increased production of octanoic acid was due to a general increase in activity, not selectivity.

We next repeated the experiments described in Fig. 3 to confirm that the more-active PhaG* (referred to as PhaG Q45R G142V variant) was not exceeding the downstream FadB and PhaJ activities. Strains expressing PhaJ produced free fatty acids as model oleochemical products. To produce methyl ketones, we cultured strain PhaJ3 expressing the parent PhaG (Supplementary Method 2). The 17 improved PhaG variants contained a total of 28 point mutations. The best variant, PhaG Q45R G142V, produced 1.1 g/L 13.6-fold octanoic acid, similar to the original PhaG (Fig. 4B). The fatty-acid pool contained 41% tetradecanoic acid, 22% dodecanoic acid, 11% decanoic acid, and 26% octanoic acid, similar to the original PhaG-expressing strain. This indicates that the increased production of octanoic acid was due to a general increase in activity, not selectivity.

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Benchmarking PhaG-driven oleochemical production. Next, we benchmarked PhaG-driven production of methyl ketones and fatty alcohols as model oleochemical products. To produce methyl ketones, we cultured strain PhaJ3 expressing the parent PhaG. To produce fatty alcohols, we cultured strain PhaJ3 expressing the parent PhaG.
methyl ketone and ~1.1 g/L total fatty alcohol from 20 g/L glycerol (Fig. 5). The chain length distribution of each product was different. Methyl ketones were evenly distributed across 8–16-carbon chain lengths, whereas, fatty alcohols were dominated by 14- and 16-carbon alcohols. The titer and yield of fatty acids, methyl ketones, and fatty alcohols from the PhaG-dependent strategies are comparable to values reported in the literature using thioesterase strategies or reverse β-oxidation strategies to produce products in shake flasks (Table 1).

To further improve methyl ketone titers, we performed discontinuous fed-batch cultivations in a stirred bioreactor by adding media pulses after cells reached high cell densities. Enhanced aeration in bioreactors can lead to loss of volatile oleochemical products in the off-gas. Therefore, we added a 20% dodecane overlay to the culture to trap products in the reactor and designed a gas trap to recover products from the off-gas. Details of off-gas methyl ketone capture and ASPEN analysis can be found in the Supplementary information file (Supplementary Figure 8 and Supplementary Method 3). After 96 hrs of cultivation in reactors lacking an off-gas scrubber, cells consumed 90.0 g/L of glycerol, reached an OD600 ~60, and produced 6.8 g/L total methyl ketone (Fig. 6A). In a separate experiment, we bubbled the bioreactor off-gas through a jacketed gas dryer filled with dodecane maintained at 5 °C. After 96 hrs of induced cultivation, cells reached a density of OD600 ~60, consumed 95.2 g/L of glycerol, and produced 6.7 g/L total methyl ketone (Fig. 6B). At the endpoint of the cultivation, we observed 3.4 g/L methyl ketone contained in the condensed dodecane phase of the off-gas trap, corresponding to an additional 0.51 g/L (per L of culture volume) of total methyl ketone captured from the bioreactor (Fig. 6C).

This study demonstrated that PhaG is capable of supporting high-flux to three medium-chain oleochemical products, fatty acids, fatty alcohols, and methyl ketones. Through bioprospecting, we identified a PhaG from P. koreensis that demonstrated higher activity when producing methyl ketones. Random mutagenesis of PkPhaG produced 17 enhanced PhaG variants that were isolated by complementing lipoic acid auxotrophy. The improved activity was demonstrated by 3–16-fold more octanoic acid being produced when the PhaG variants were co-expressed in a cell designed to direct flux to octanoic acid (co-expressing TesB, PhaJ3, and Ter). Reconstitution of the individual point mutations led to the creation of a double mutant, PkPhaG Q45R G142V, that showed 4.0-fold higher activity relative to the parent enzyme. Finally, we demonstrated the production of 1.1 g/L C8–C11 free fatty acids, 1.5 g/L C7–C13 methyl ketones, and 1.1 g/L C8–C16 fatty alcohols in shake flasks and 7.2 g/L of methyl ketones in a fed-batch. These results demonstrate that PhaG is a viable alternative strategy that should be considered for oleochemical production. The PhaG-dependent strategy has the potential to achieve higher theoretical yields compared with the well-established thioesterase route. However, this impact, saving one ATP per product, is not likely to be observed until cells approach theoretical limits. Given the current state of the field, additional work is needed on both fronts. Our work motivates continued strain development as well as additional protein engineering to narrow the PhaG product profile and further increase activity.

**Methods**

**Bacterial strains, plasmids, oligonucleotides, and reagents.** All bacterial strains used in this study are listed in Supplementary Data 1. Q5 DNA polymerase and Monarch® PCR and DNA Cleanup Kit were 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).
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 | % TY* | Fermentation |
|------------|----------|-------------|--------|--------|--------------|
| Thioesterase | 2-heptanone, | 4.4 g/L, | 0.028 mol/mol consumed glycerol, | 9% | Fed-batch, bioreactor |
| PhaG | 2-nonanone, | 3 g/L, | 0.018 mol/mol consumed glycerol, | 8% | Fed-batch, bioreactor |
| PhaG | 2-decanone | 0.34 g/L | 0.0008 mol/mol consumed glycerol | 0.4% | Fed-batch, bioreactor |
| PhaG | 1-octanol | 1.3 | 0.046 mol/m glycerol | 17.4% | Batch, shake flask |
| PhaG | octanoic acid | 1.7 | 0.054 mol/m glycerol | 17.4% | Batch, shake flask |
| PhaG | C12-C14 FAOH | 1.6 | 0.109 mol/mol glucoseb | 34.8% | Fed-batch, bioreactor |
| PhaG | C8-C16 FFA | 0.5 | 0.044 mol/mol glycerol | 14% | Batch, shake flask |
| PhaG | C8-C14 FFA | 0.67 | 0.063 mol/mol glycerol | 21% | Batch, shake flask |
| PhaG | C11-C17 MK | 5.4 | 0.033 mol/mol glycerol | 11% | Batch, shake flask |
| PhaG | C4-C16 FAOH | 1.8 | 0.2 mol/mol consumed glucose | 65% | Fed-batch, bioreactor |
| PhaG | C4-C10 FFA | 4.7 | 0.25 mol/mol consumed glucose | 68% | Fed-batch, bioreactor |
| PhaG | C6-C14 FFA | 1.2 | 0.063 mol/mol glycerol | 21% | Batch, shake flask |
| PhaG | C8-C16 FAOH | 1.1 | 0.024 mol/mol glycerol | 15% | Batch, shake flask |
| PhaG | C7-C15 MK | 7.2 | 0.049 mol/mol glycerol | 31% | Fed-batch, bioreactor |

* Dodecanoic acid M.W. was used as an average M.W. of the fatty acid mixture.
* Tetradecanoic acid M.W. was used as an average M.W. of methyl ketone mixture.
* Octanoic acid M.W. was used as an average M.W. of the fatty alcohol mixture.
* Percentage of the theoretical yield.

Fig. 6 Fed-batch fermentation for methyl ketone production. Time course of glycerol consumption, OD600, and methyl ketone titer from fed-batch bioreactor cultures (n = 3 biologically independent samples). A and bioreactor coupling a condenser (n = 2 biologically independent samples) B using E. coli RADI harboring pTC99a-PphaG*-PfadM and pACYC-PphaJ3 plasmids. C Evaluation of methyl ketone concentration from samples in the aqueous phase (dark gray), dodecane layer (light gray), and condenser (shading) after 96 h fermentation. All data represent the mean ± s.d. of biological triplicates. Source data underlying A-C are provided as a Source Data file.

E. coli DH5α strains were used for plasmid amplification and DNA assembly. E. coli RABJ (MG1655 ΔarBAD ΔfadR ΔfadA ΔfadB Δfadl ΔfadD) and E. coli CM23 (MG1655 ΔarBAD ΔfadABJIDRE ΔldhA ΔackApΔ ΔilvE ΔroB ΔfadABCD ΔfadDI ΔfadC) were created as part of prior studies2,3. All cloned sequences and gene deletions were confirmed using the combination of lambda red recombination and CRISPR/Cas9-mediated selection4,14,18. All cloned sequences and gene deletions were confirmed by Sanger sequencing performed by Functional Biosciences (Madison, WI). Constructs expressing FadB8,8 and PhaJ homologs were obtained from prior studies44.
Oleochemical production and quantification. All oleochemical production studies were performed by growing E. coli strains at 30 °C in Cloburg 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 rpm. A 2.5 vol% inoculum was transferred into production flasks with a starting OD600 of 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 h incubation at 30 °C.

Fed-batch fermentation was performed using a 1-L Infors Multiforus bioreactor. Overnight pre-cultures were inoculated to an initial OD600 of 0.05 into a bioreactor containing 500 mL Cloburg medium with ~50 g/L glycerol. The bioreactor was operated at the following conditions: the temperature was controlled at 30 °C post induction, airflow was 1.5 L/min, stirrer rate was varied between 250 rpm and 1000 rpm 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 into the bioreactor. At 24 h of post induction, ~100 mL 5x concentrated Cloburg media containing 500 g/L glycerol was one-time bolus-fed into the bioreactor and fermentation terminated 96 h post induction. Measurements of methyl ketone, glycerol, optical density, and CO2 evolution were recorded for 96 h total.

Water and remaining dodecane 96 °C by an external water cooler. Methyl ketone data were taken at 96 h from the absorber and the end point methyl ketone capture was taken from both the collected water and remaining dodecane 96–101 h after inducing the culture. A schematic of the absorber is described in more detail in Supplementary Figure 7.

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 × g for 10 min and 100 μL of the dodecane layer and 2.5 mL samples from the aqueous phase were collected and evaluated separately. Fatty acids were extracted from culture according to an acid-based esterification method. Fatty alcohols and methyl ketones were extracted from culture into n-hexane. 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.

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 (0.4–4.5 mutations/kb). The plasmid backbone (pBTRCK-PhaGP) was PCR-amplified using a high-fidelity DNA polymerase Q5 from New England Biolabs (Ipswich, MA). The library was assembled using an isothermal assembly method.

Primers used in the creation of the library contained the start and stop codons in single point mutations, we amplified PhaG with mutagenic primers and subcloned the fragments into pTRC99A–PhaG–TesA by an isothermal assembly method.

The resulting plasmids were transformed into E. coli CM23 harboring pBTRCK–M6aB′ and pACYC–Pha3 plasmids.

Lipoic acid selection. 3-Hydroxyoctanoyl-CoA producing variants of PhaG were isolated using a lipoic/octanoic acid selection strategy. In brief, Gibson assembly reaction mixtures (2 μL) containing a PhaG library (pBTRCK–PhaGP) was transformed into 100 μL of electropotent E. coli CM23–Δlbp (CM23 strain, Δlbp) strain harboring pTRC99A–TesA–M6aB′ + pACYC–Pha3. 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. Cells were rescued growth and appeared on the selection plates after 3 days of incubation. Transformants were patched onto LB plates for archiving and secondary screening in octanoic acid production studies.

Statistics. We used instrument software to collect and analyze most experiments. Shimanizu Labsolutions (Long Beach, CA) was used for GC and HPLC data analysis. The fluorescence intensity of TFP and RFP was detected by the Tecan 200 Plate reader (Tecan, 391.0.0, Gröbern, Switzerland). Error bars indicate standard deviations from three biological replicates. All P values were generated from a two-tailed Student’s t test using Microsoft Excel 2016 (Microsoft Corporation, USA).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that all data supporting the findings of this study are available within the paper (and its Supplementary Information files). All strains and plasmids information are available in Supplementary Data 1. The additional reactions and metabolites used in Olen, JML1515 than JML1515 are available in Supplementary Data 2 and 3. All plasmid maps including annotations of oligonucleotide and gene sequences are available in Supplementary Data 4. Source data are provided with this paper.

Code availability

All codes for calculating theoretical yields are available at the GitHub repository [https://github.com/Pfleger-Lab/Metabolic-Modeling---Yield-Analysis-of-PhaG].

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Author contributions

Q.Y.: conceptualization, investigation, data curation, writing original draft, writing—review & editing. M.A.I.: investigation, writing—review & editing, W.T.: investigation, writing—review & editing. D.C.: investigation, writing—review & editing. X.C.: investigation, writing—review & editing. M.K.K.: investigation, writing—review & editing. B.F.P.: conceptualization, writing original draft, writing—review & editing, supervision, funding acquisition.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence

and requests for materials should be addressed to Brian F. Pfaffer.

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