Microbial production of bi-functional molecules by diversification of the fatty acid pathway

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ABSTRACT

Fatty acids that are chemically functionalized at their ω -ends are rare in nature yet offer unique chemical and physical properties with wide ranging industrial applications as feedstocks for bio-based polymers, lubricants and surfactants. Two enzymatic determinants control this ω -group functionality, the availability of an appropriate acyl-CoA substrate for initiating fatty acid biosynthesis, and a fatty acid synthase (FAS) variant that can accommodate that substrate in the initial condensation reaction of the process. In Type II FAS, 3-ketoacyl-ACP synthase III (KASIII) catalyses this initial condensation reaction. We characterized KASIIIs from diverse bacterial sources, and identified variants with novel substrate specificities towards atypical acyl-CoA substrates, including 3-hydroxybutyryl-CoA. Using *Alicyclobacillus acidocaldarius* KASIII, we demonstrate the *in vivo* diversion of FAS to produce novel ω -1 hydroxy-branched fatty acids from glucose in two bioengineered microbial hosts. This study unveils the biocatalytic potential of KASIII for synthesizing diverse ω -functionalized fatty acids.

KEYWORDS

3-Ketoacyl-ACP Synthase III; fatty acid synthesis; bio-based chemicals; omega-functionalized fatty acids; substrate diversity; microbial engineering

ABBREVIATIONS

KASIII: 3-Ketoacyl-ACP-Synthase III

HBFA: ω -1 Hydroxy Branched Fatty Acid

FAS: Fatty Acid Synthase

PHB: Polyhydroxybutyrate

INTRODUCTION

The increasing concerns with climate change and price volatility of petroleum feedstocks have prompted a growing search for sustainable sources of chemicals and fuels^{1, 2}. Fatty acids are chemically the most similar biological molecules to petroleum hydrocarbons, and are therefore the most readily reachable bioengineering targets for developing sustainable replacements of petroleum-derived fuels and chemicals^{3, 4}. The processes of fatty acid metabolism can generate diverse metabolites with different chemical functionalities, and considerable research efforts have been dedicated to identify the genetic elements, and dissect the enzymology within these metabolic pathways^{2, 5, 6}. Much of this success has been facilitated by the modular nature of the enzymatic machinery that underlies the process of fatty acid synthesis, and the more general process of polyketide biosynthesis⁷. These processes are initiated by the condensation of an acyl-CoA substrate with a 2-carbon donor (malonyl-CoA or malonyl-ACP); and this condensation process is iterated in fatty acid/polyketide biosynthesis cycle to elongate the acyl-chain. Whereas fatty acid synthesis follows each condensation reaction by a 3-reaction process (reduction-dehydration-reduction) that generates a fully reduced alkyl chain, the more general polyketide synthesis mechanism skips one or two of these latter reactions prior to the next condensation iteration, leaving a functional group in the alkyl chain⁸. Examples of such alternative products being generated by fatty acid synthase (FAS) are the mechanisms by which prokaryotes produce unsaturated fatty acids⁹, and the biosynthesis of methylketones by tomato trichomes¹⁰. Both these processes intercept the 3-reaction reduction-dehydration-reduction cycle and maintain a

chemical functional group in the alkyl-chain. The first of these is via an isomerization reaction that moves the carbon-carbon double bond, 1-bond further from the carboxylic acid end of the acyl-chain, ultimately generating an unsaturated fatty acid. In the second example, an acyl-ACP thioesterase that is specific to the 3-ketoacyl-ACP intermediate of FAS intercepts the cycle and releases a 3-ketoacid, which undergoes decarboxylation to generate a methylketone.

We evaluated the feasibility of introducing chemical functional group near the ω -end of fatty acids by exploring the substrate flexibility offered by natural variants of 3-ketoacyl-ACP synthase III (KASIII)¹¹, the enzyme that catalyses the initial condensation reaction of Type II FAS systems. Most well characterized KASIII enzymes, including the enzyme from *Escherichia coli* and most plants, use acetyl-CoA as the substrate in this reaction, and thus the ω -end of the final product is an unreactive methyl-group¹². However, KASIII from some bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus* can utilize substituted acyl-CoAs (i.e., methyl branches at the ω -1 (isobutyryl-CoA) and ω -2 (anteisovaleryl-CoA) positions of the acyl-chain, see supplementary Table 1 for chemical structures of acyl-CoA molecules)^{12, 13}. These organisms therefore produce fatty acids with methyl branches at or near the ω -ends of the molecules. Therefore, we projected that by providing chemically substituted acyl-CoA substrates to novel KASIII enzymes, one can envision biosynthesizing ω -functionalized fatty acids. Moreover, KASIII are archetypal for many Type III polyketide synthases which catalyse similar Claisen condensation reactions between an acyl-CoA and a malonyl-CoA/ACP

thioester substrate¹⁴ and also show great flexibility in being able to use a variety of acyl-CoA substrates, generating a large collection of polyketide specialized metabolites.

In this study, we specifically tested this hypothesis and demonstrated the ability to biologically produce ω -1 Hydroxy Branched Fatty Acids (HBFAs) using the novel KASIII enzyme from *Alicyclobacillus acidocaldarius* coupled with a system to generate 3-hydroxyacyl-CoA as a substrate for this novel KASIII enzyme. The utility of such bi-functional hydroxy acids is in such applications as polymers (e.g., polyesters) and specialized natural surfactants (e.g., sophorolipids)¹⁵. The system that we have developed has more general applications in that it illustrates the flexibility of Type II FAS systems. These systems can be bioengineered to accept novel initiating acyl-CoA substrates by exploiting the variant enzymes that catalyse the initial condensation reaction and define the ω -end of the final product (i.e., KASIII). A cursory examination of sequence databases¹⁶ currently identifies 14,000 potential KASIII enzymes that could be used to generate a variety of different bi-functional molecules¹⁷.

RESULTS

Computational identification of KASIIIs from diverse bacterial sources

Putative KASIII-coding genes were computationally identified by sequence homology, by searching genomic sequences from diverse bacteria that are known to produce large amounts of either terminally branched chain fatty acids or ω -cyclic fatty acids. We projected that organisms that produce large quantities of branched chain or ω -cyclic fatty acids should express novel

KASIII enzymes that are capable of utilizing branched-chain acyl-CoAs or ω -cyclic acyl-CoAs as substrates to initiate fatty acid biosynthesis. We further hypothesized that such KASIII enzymes will have relatively larger substrate binding pockets that can not only accommodate branched or ω -cyclic acyl-CoA substrates, but could also accommodate other bulky substrates, such as aromatic, hydroxylated or unsaturated acyl-CoAs.

Based on these presuppositions, we characterized two KASIII enzymes, one from the thermophile *Thermus aquaticus*, an organism that can produce 95% branched chain fatty acids¹⁸ and another from the acidothermophile *A. acidocaldarius*, which can produce large proportion of ω -alicyclic fatty acids (59%) and branched chain fatty acids (36%)¹⁹. We compared the properties of the *A. acidocaldarius* (aaKASIII) and *T. aquaticus* (taKASIII) enzymes to two functionally well-characterized KASIII, one from *E. coli* (ecKASIII, encoded by *fabH* gene), and the second from *B. subtilis* (bsKASIIIb, encoded by the *yhfB* gene). These latter two enzymes were selected because they are known to display different substrate specificity; ecKASIII is specific for acetyl-CoA and is unable to use branched chain acyl-CoA substrates^{12, 20}, whereas bsKASIIIb can utilize both acetyl-CoA and branched-chain acyl-CoA substrates¹².

Primary sequence analyses of aaKASIII and taKASIII revealed that each possesses the conserved catalytic triad residues (Cys, His and Asn; Supplementary Fig. 1) typical of KASIII enzymes, suggesting that both belong to the family of decarboxylating thiolase enzymes¹⁴. Moreover, both aaKASIII

and taKASIII contain the substrate binding residues that are conserved among well-characterized KASIII enzymes¹³ (see Supplementary Fig.1).

***In vivo* analysis of KASIII function in a *B. subtilis* $\Delta yjaX \Delta yhfB$ strain**

The bacterium *B. subtilis* primarily synthesizes branched chain fatty acids (95%)²¹, and possesses two KASIII homologs, bsKASIIIa (encoded by the *yjaX* gene) and bsKASIIIb (encoded by the *yhfB* gene); both possess high specificity for branched chain acyl-CoA substrates¹². Deletion of these two endogenous KASIII genes from *B. subtilis* results in a lethal phenotype, which can be rescued by growing the double mutant strain in the presence of branched-chain fatty acids (Jin and Nikolau, unpublished data). This *B. subtilis* $\Delta yjaX \Delta yhfB$ mutant strain was used as a vehicle to screen for KASIII enzymes that can utilize branched chain acyl-CoA substrates and can therefore synthesize branched chain fatty acids, thereby rescuing the lethal condition. The selected aaKASIII and taKASIII were assessed for their ability to support the production of branched chain fatty acids, by integrating each of them into the genome of the *B. subtilis* $\Delta yjaX \Delta yhfB$ mutant strain. In parallel, the ecKASIII that is unable to utilize branched-chain substrates¹² was also integrated into the genome of this strain. The resulting three *B. subtilis* $\Delta yjaX \Delta yhfB$ mutant strains, each expressing one of the recombinant KASIII genes, were grown in the presence and absence of exogenously supplied branched-chain fatty acids. Of the three recombinant KASIII genes that were tested, aaKASIII and taKASIII could rescue the lethal phenotype of the $\Delta yjaX \Delta yhfB$ mutant strain but as expected the ecKASIII could not, and this strain could not grow in media that does not contain branched-chain fatty acids.

Fatty acid analysis of the *B. subtilis* $\Delta yjaX \Delta yhfB$ strains harbouring either aaKASIII or taKASIII revealed that both strains could produce similar branched chain fatty acid profiles, with anteiso-branched chain fatty acids accounting for the largest portion of the fatty acids (48-52%), followed by iso-

branched chain fatty acids (27-34%) (Fig. 1). These data establish that aaKASIII and taKASIII have the ability to use both anteiso-branched and iso-branched acyl-CoA substrates for priming fatty acid biosynthesis.

***In vitro* analysis of KASIII function, and identification of KASIII enzymes with atypical substrate preferences**

We used pure enzyme systems to directly evaluate whether the active sites of aaKASIII and taKASIII could accommodate branched chain acyl-CoAs and even bulkier and more polar acyl-CoA substrates, such as hydroxylated, aromatic or diacidic acyl-CoAs (see supplementary Table 1 for chemical structures of acyl-CoAs). These *in vitro* characterizations compared the properties of the aaKASIII and taKASIII enzymes with those obtained in parallel with ecKASIII and bsKASIIIb.

Each recombinantly expressed KASIII enzyme was purified to near-homogeneity (Supplementary Fig. 2), and we initially confirmed that they each showed an appropriately folded structure by Circular Dichroism (CD) spectroscopy (Supplementary Fig. 3). All of four KASIII proteins showed similar CD spectra that indicated they are folded.

Each recombinant KASIII protein was then evaluated for its ability to bind potential acyl-CoA substrates using a fluorescence-based thermal shift assay²²⁻²⁴, which measures the thermal stability of a protein in the presence or absence of a specific ligand. A positive shift in melting temperature (T_m) of the protein in the presence of the ligand is correlated to binding, concomitant with

the stabilization of the protein, which is consistent with the substrate-induced contraction of protein structures that are often observed with enzymes. In contrast, a negative shift in T_m suggests destabilization of the protein by the ligand.

Each KASIII was assayed with ligands that are typical substrates of well-characterized KASIII enzymes, including straight, short-chain acyl-CoAs (e.g., acetyl-CoA, propionyl-CoA, butyryl-CoA, hexanoyl-CoA) and branched chain acyl-CoAs (e.g., isobutyryl-CoA, isovaleryl-CoA). In addition, we also used acyl-CoAs that are atypical of known KASIII substrates, such as diacidic (malonyl-CoA, methylmalonyl-CoA), hydroxylated (3-hydroxybutyryl-CoA), unsaturated (crotonyl-CoA), and aromatic (benzoyl-CoA and phenylacetyl-CoA) acyl-CoAs (see supplementary Table 1 for chemical structures of acyl-CoAs).

The inherent T_m s of the four KASIII enzymes in the absence of ligand are shown in Figure 2a, and these values were used as the baseline to measure the shift in T_m by addition of the potential substrate ligands. The higher T_m s of the aaKASIII and taKASIII are consistent with their origin from thermophilic bacteria. When the thermal shift assays were conducted in the presence of potential substrate ligands, the KASIIIs from *B. subtilis*, *A. acidocaldarius* and *T. aquaticus* demonstrated positive changes in T_m with a broad range of acyl-CoAs. In contrast, such a positive change in T_m for ecKASIII was obtained only with acetyl-CoA (Fig. 2b), which is consistent with the narrow substrate specificity of this enzyme¹².

Figure 2 | Effect of different acyl-CoAs on the thermal stability of different KASIIIs. **a)** The melting temperature (T_m) of each KASIII enzyme in the absence of any ligands. **b)** Change in the melting temperatures (ΔT_m) of the selected KASIIIs upon addition of different acyl-CoA ligands. Each data point represents the average of four technical replicates, and the error bars represent standard deviations.

The bsKASIIIb and aaKASIII enzymes exhibited significant increases in T_m (4-6 °C) when incubated with many different acyl-CoA ligands, including those with straight, branched, diacidic, unsaturated or aromatic acyl-chains (Fig. 2b). Specifically, aaKASIII exhibited significant positive shifts in T_m when incubated with bulky or unsaturated ligands, including 3-hydroxybutyryl-CoA, crotonyl-CoA and benzoyl-CoA. Similarly, bsKASIIIb exhibited positive shifts in T_m in the presence of 3-hydroxybutyryl-CoA, methylmalonyl-CoA, crotonyl-CoA and phenylacetyl-CoA (10 °C shift). However, some of the atypical acyl-CoAs (i.e., hexanoyl-CoA and benzoyl-CoA) destabilized the bsKASIIIb protein (i.e., decreased the T_m).

A unique KASIII that utilizes 3-hydroxybutyryl-CoA as a substrate

We focused further studies on identifying a KASIII that is most likely capable of using 3-hydroxybutyryl-CoA as a substrate in order to use such an enzyme to produce hydroxy-fatty acids. The thermal shift assays indicated that of the four KASIII enzymes tested, aaKASIII and bsKASIIIb were both thermally stabilized by 3-hydroxybutyryl-CoA, and that this stabilization was largest for the aaKASIII enzyme. This finding suggested that aaKASIII might utilize 3-hydroxybutyryl-CoA as a substrate. Using an *in vitro* spectrophotometric enzyme assay (see Methods), we tested the ability of aaKASIII to utilize 3-hydroxybutyryl-CoA and malonyl-ACP as substrates and produce 5-hydroxy-3-ketohexanoyl-ACP. The specific activity for this reaction catalysed by aaKASIII is 5-fold and 3-fold higher with isobutyryl-CoA and 3-hydroxybutyryl-CoA, respectively, than that obtained with acetyl-CoA (Table 1). Kinetic characterization of aaKASIII revealed that isobutyryl-CoA is the preferred

substrate compared to acetyl-CoA and 3-hydroxybutyryl-CoA (Table 1). The order of catalytic efficiency (i.e. k_{cat}/K_m) of aaKASIII with various substrates was: isobutyryl-CoA > 3-hydroxybutyryl-CoA > acetyl-CoA (Table 1).

Table 1. Steady state kinetic analysis of aaKASIII and comparison with ecKASIII

KASIII enzyme assay was carried out spectrophotometrically using 400 μ M ACP, 400 μ M malonyl-CoA, 10-400 μ M various acyl-CoAs, 400 μ M NADPH, 1 mM DTT, 60 μ g FabD, 30 μ g FabG and 4 μ g of aaKASIII (or 9.5 μ g of ecKASIII).

Enzyme	Substrate	K_m (μ M)	V_{max} (μ moles/s)	k_{cat} (s^{-1})	Specific constant k_{cat}/K_m (μ M $^{-1}$ s^{-1})	Specific activity (nmol/mg/min)
A. <i>acidocaldarius</i> KASIII	Acetyl-CoA	114.6 $\pm$ 14.5	1.6E-04 $\pm$ 0.2E-04	25.2E-03 $\pm$ 4.42E-03	21.8E-05 $\pm$ 1.0E-05	10.3 $\pm$ 2.6
	Isobutyryl-CoA	59.4 $\pm$ 13.6	10.2E-04 $\pm$ 2.9E-04	154.3E-03 $\pm$ 45.3E-03	255.6E-05 $\pm$ 17E-05	56.0 $\pm$ 6.3
	3-Hydroxybutyryl-CoA	249.7 $\pm$ 2.5	7.2E-04 $\pm$ 0.8E-04	109.8E-03 $\pm$ 13.6E-03	44E-05 $\pm$ 5.9E-05	33.4 $\pm$ 1.1
<i>E. coli</i> KASIII	Acetyl-CoA	96.8 $\pm$ 4.7	3.3E-04 $\pm$ 0.1E-04	21.4E-03 $\pm$ 0.93E-03	22.1E-05 $\pm$ 1.1E-06	447 $\pm$ 68
	Isobutyryl-CoA	Not a substrate				
	3-Hydroxybutyryl-CoA	Not a substrate				

Production of HBFAs by expressing aaKASIII in *Rhodospirillum rubrum*

Having identified that aaKASIII can utilize 3-hydroxybutyryl-CoA as a substrate, we expressed this enzyme in the purple phototrophic bacterium *Rhodospirillum rubrum* with the specific aim of producing HBFAs via the fatty acid synthesis pathway. *R. rubrum* was chosen for these experiments because this organism produces large quantities of the biopolymer polyhydroxybutyrate (PHB) (over 50% of dry biomass of the cells²⁵), and therefore must have the capacity to produce large quantities of the precursor, 3-hydroxybutyryl-CoA. The 3-hydroxybutyryl-CoA intermediate is synthesized via PhaA-catalyzed condensation of two acetyl-CoA molecules to yield acetoacetyl-CoA, which is then reduced by PhaB to yield 3-hydroxybutyryl-CoA. In the native host, 3-hydroxybutyryl-CoA is polymerized by PhaC to

assemble PHB (Fig. 3). The *R. rubrum* genome encodes three PhaC-encoding genes (*phaC1*, *phaC2*, and *phaC3*), and a triple *phaC* mutant strain ($\Delta phaC1 \Delta phaC2 \Delta phaC3$) is incapable of accumulating PHB, but suffers only a slight growth-penalty²⁶. We reasoned therefore that this triple mutant strain

has the capacity to generate 3-hydroxybutyryl-CoA, which could serve as a substrate for aaKASIII to produce HBFAs (Fig. 3). We tested this hypothesis by recombinantly expressing the aaKASIII in the *R. rubrum* $\Delta phaC1 \Delta phaC2 \Delta phaC3$ strain that lacks any functional phaC enzymes, and then analysed the resultant fatty acids by GC-MS.

These analyses detected a novel product in the aaKASIII-expressing strain corresponding to the silylated-derivative of the methyl-ester of 15-hydroxyhexadec-9-enoic acid, which was not produced by the control strain that did not express the recombinant aaKASIII (Fig. 4a, 4b). These results therefore confirm the aaKASIII-dependent production of a monounsaturated HBFA in the *R. rubrum* triple mutant host. This novel bi-functional fatty acid accounted for 0.19 ± 0.04 mol % of the fatty acids produced by the *R. rubrum* strain (Fig. 4c). Additionally, novel iso- and anteiso-branched chain fatty acids of varying carbon chain lengths that do not occur in the control strain were produced in the aaKASIII-expressing strain, and these accounted for approximately 1.3 mol % of the fatty acids (Fig. 4c). Taken together, our data establish that the aaKASIII is able to utilize hydroxyacyl-CoA (i.e., 3-hydroxybutyryl-CoA) (Fig. 3) or branched chain acyl-CoAs (i.e., iso- and anteiso-acyl-CoAs) *in vivo*, resulting in the respective formation of HBFAs or iso/anteiso branched chain fatty acids that are not naturally produced by the host bacterium.

Optimizing the production of HBFA by expressing aaKASIII in a bioengineered *E. coli* host. Having demonstrated the production of bi-functional fatty acids in *R. rubrum*, we attempted production of HBFAs in a bioengineered *E. coli* host. We reasoned that use of *E. coli* would open avenues to applying a wide range of molecular genetic tools to improve the titer of these novel fatty acids. Specifically, we co-expressed in *E. coli* the aaKASIII, with the *R. rubrum phaA* and *phaB* genes, thus recapitulating the initiating part of the pathway that should lead to the biosynthesis of HBFAs (Fig. 3). The *E. coli* strain (OC100) that we used for this experiment carried deletion mutations of the ecKASIII encoded by *fabH* ($\Delta fabH::kam^R$) gene and of the *fadD* ($\Delta fadD::cam^R$) gene. The rationale for incorporating these gene-disruptions in an *E. coli* production host are to 1) ensure minimal interference by the native KASIII, and 2) eliminate β -oxidation of fatty acids to facilitate the accumulation and secretion of the novel fatty acid products into the medium. We generated the bioengineered *E. coli* strain (OC101) by overexpressing aaKASIII, the *R. rubrum phaA* and *phaB* genes in OC100 strain.

After 12 hours of growth, fatty acids were extracted from the spent media used to grow strain OC101, and these were analysed by GC-MS. Figure 5a shows the presence of novel peaks corresponding to HBFAs of 14-, 16- and 18-carbon chain lengths, either without any carbon-carbon double bonds, or with one double bond at the ω -7 position of the molecules (Fig. 5a). These peaks were absent in the control *E. coli* strain OC100 that carried the $\Delta fabH$ and $\Delta fadD$ mutations, but did not carry the *phaA*, *phaB* and *aaKASIII* genes. Quantitative analysis shows that the titer of HBFA was approximately 1 mg/L,

which constituted 43% of the fatty acids in the media (Fig. 5b). 15-Hydroxyhexadec-9-enoic acid was the most abundant HBFA, followed by 15-hydroxyhexadecanoic acid (see Supplementary Fig. 4 and 5 for MS-spectral confirmation data). In summary, recapitulating the pathway for HBFA production in *E. coli* has provided a genetically tractable platform for optimizing productivity, and prior to genetic optimization it is a platform that expresses at a titer at least 1000-fold higher than that achieved in *R. rubrum*.

We attempted to increase the HBFA production levels by overexpressing an acyl-ACP thioesterase (TE) isolated from a plant source (*Cuphea* seeds), which prior studies indicated could affect a higher titer of fatty acids when

expressed in *E. coli*²⁷. Upon overexpressing this TE in strain OC101, the titers of fatty acids and HBFAs did not increase, but in fact were reduced to 20% of the levels that was produced by the OC101 strain.

Optimizing HBFA titer via manipulation of fermentation conditions

A standardized culturing system was implemented to explore the effect of different fermentation conditions on the ability of strain OC101 to produce HBFA. Unless otherwise stated, this consisted of 125 ml flasks with a working volume of 25 ml media maintained at 37 °C and agitated at 250 rpm, and induced with IPTG. In these non-optimized fermentation conditions in LB media, *E. coli* strain OC101 produced 4.6 mg/L of HBFA upon reaching stationary phase, which accounted for about 40% of the fatty acids secreted into the medium. To optimize the fermentation conditions, the carbon to nitrogen ratio (C/N) in the initial culture medium and size of the inoculum in establishing the culture were varied. The C/N ratio^{28, 29} and inoculum size^{30, 31} have been demonstrated to have significant effects on cell growth and fatty acid production by different microorganisms (supplementary Table 4). Additionally, induction temperature, induction time and concentration of the inducer, i.e., IPTG, were also varied as these are also known to be factors influencing the expression of a transgenic protein, thus may significantly affect the final fatty acid titer^{32, 33} (supplementary Table 4).

Figure 6 shows the impact of altering the five fermentation parameters described above on the HBFA and fatty acid titers when strain OC101 is cultured in M9 minimal media. HBFA and fatty acid titers increased with

increasing C/N-ratio (Fig. 6a), with the most dramatic increase occurring as the ratio was increased from 25 to 50. The highest HBFA titer occurred at a C/N ratio of 75, with the HBFA titer reaching 7.2 mg/L. Based upon these results, in the following experiments ammonium chloride was supplemented in the media at 0.41 g/L, to achieve a C/N-ratio of 75.

Altering the size of the inoculum from 1% (v/v) to 10% (v/v) resulted in increased cell growth (Supplementary Fig. 6a) and enhanced extracellular fatty acid titers (Fig. 6b). Optimal fatty acid and HBFA titers (10.1 mg/L and 9.0 mg/L at, respectively) occurred at an inoculum size of 7% (v/v).

The post-induction temperature of the culture had considerable impact on both cellular growth (Supplementary Fig. 7a) and extracellular fatty acid production (Fig. 6c). When post-induction temperature was maintained at 30 °C or 37 °C, the culture reached stationary phase by about 15 h post-induction and achieved final ODs of 2.4 at 30 °C and 1.5 at 37 °C. However, at the lower temperature (25 °C), the culture maintained logarithmic growth for about 28 h, reaching a final OD of 4.6. The highest extracellular fatty acid and HBFA titers of 14.5 mg/L and 10.8 mg/L, respectively, were achieved at 30 °C. Moreover, the post-induction temperature of the culture posed a significant effect on the proportion of HBFAs secreted by the strain (Supplementary Fig. 7b); at 20 °C or 25 °C, HBFAs accounted between 75%-80% of the extracellular fatty acids, whereas at 30 °C and 37 °C, they accounted for 85% and 62% of the fatty acids, respectively, which may be attributable to the poorer cell growth at the higher temperatures.

Finally, we also assessed the effect of inducing the culture at different stages of growth or with different concentrations of the inducer, IPTG (Fig. 6d and Supplementary Fig. 8a, 8b). Generally, the highest titers of both fatty acids

and HBFAs (13-14 mg/L) were obtained when 0.4 mM IPTG was introduced into the culture at an OD of 0.4.

In these optimization experiments, the dominant fatty acids were C16:1-, C16:0- and C18:1-HBFAs (Supplementary Fig. 6b, 7b). Overall these optimization experiments of *E. coli* strain OC101 increased titers of fatty acids and HBFAs by 3-fold from those obtained in the non-optimized culture conditions, reaching values of 25.1 mg/L and 13.8 mg/L, respectively. These optimum conditions in M9 minimal media occur at a carbon-to-nitrogen ratio of 75, inoculum size of 7% (v/v), post-induction temperature of 25 °C and with 0.4 mM IPTG added when the culture was at early log-phase at an OD 0.4 (Supplementary Fig. 9).

DISCUSSION

In this study, we demonstrated the production of HBFAs from glucose, first by eliminating the ability of *R. rubrum* to produce PHB, and diverting its precursor (i.e., 3-hydroxyacyl-CoA) into fatty acid biosynthesis with a KASIII variant that shows novel substrate specificity, aaKASIII. Subsequently, we recapitulated this metabolic pathway in a bioengineered *E. coli* host. The resultant strain produced HBFAs of 14-18 carbon-chain lengths, with or without carbon-carbon double bonds. Additional optimization by altering the fermentation conditions increased the titer of this valuable product to 13 mg/L, which was over 1000-fold greater than the titer obtained in the initial *R. rubrum* demonstration host. In this study, we made the assumption that HBFAs are recovered in the growth medium, based on many other studies that use fadD

mutation and report secretion of fatty acids in the medium^{34, 35}. However, cell lysis at stationary phase may still be the means of getting HBFAs in the growth medium.

Metabolically intercepting Type II FAS via these genetic engineering manipulations required the identification and characterization of bacterial KASIII enzyme variants isolated from *B. subtilis*, *T. aquaticus* and *A. acidocaldarius*, and their properties were compared to the *E. coli* sourced enzyme. The bsKASIIIb was known to have the property of using larger acyl-CoA substrates (i.e., branched acyl-chains) than the acetyl-CoA-specific ecKASIII. The catalytic properties of the taKASIII and aaKASIII had not previously been experimentally determined, but we projected that because they are sourced from bacteria that produce large quantities of fatty acids with bulky chemical structures at their ω -ends these enzymes may be able to utilize larger acyl-CoA substrates. Coupling the ability of the aaKASIII to utilize 3-hydroxybutyryl-CoA as a substrate with the capacity to generate this substrate *in vivo* proved successful in generating a strain that can produce HBFAs.

This achievement has wider implications, setting a precedent bioengineering strategy for diversifying the products that can be produced from the fatty acid biosynthesis pathway. Thus, one can modify the available acyl-CoA substrate pools for the KASIII enzyme in combination with KASIII variants that can make use of these acyl-CoAs. Specifically, this KASIII based technology can be used as a general platform for production of other ω -functionalized fatty

acids, such α,ω -diacids, ω -amino acids, ω -unsaturated acids, and ω -halogenated acids that have many chemical applications, but are not readily accessible by biological or chemical synthetic routes^{36, 37}. More widely, this strategy could also be applied to diversify the natural product portfolio that can be produced by the broader class of Type III iterative polyketide synthases (PKS) that are structural homologs of KASIII enzymes³⁸. Approaches to load alternative starter units to bacterial PKSs and combinatorial biosynthesis approaches have been described in the literature that could serve as alternative approaches to synthesize ω -functionalized polyketides and natural products such as macrolactam antibiotics and ω -aromatic fatty acids³⁹⁻⁴¹.

The occurrence of a double bond in the alkyl-chain of our HBFA products would alter the physical-chemical properties of the HBFAs compared to the saturated HBFAs, specifically lowering the melting point of the former molecules. Moreover, the occurrence of the double bond would allow for additional chemistries to be aimed at this functional group providing a combined biological-chemical route to additional products, such as epoxides via oxidation, diacids via metathesis, and diesters via cross-metathesis⁴². Such chemicals would have wide ranging applications in polymers, and in the manufacture of surfactants, and cleaning agents.

A wide range of possible chemical transformations of HBFAs have been experimentally described resulting in products with enhanced functionalities³⁷, thus demonstrating their potential to spur innovation in newer technological areas. Specifically, HBFAs are considered to be excellent monomers for

synthesizing polyethylene-like biobased plastics^{43, 44}, or they can be readily converted to macrocyclic lactones⁴⁵ that have applications in pharmaceutical industry⁴⁶ and flavors and fragrances⁴⁷.

HBFAs occur naturally in glycolipids, namely sophorolipids^{48, 49} and they impart superior functional properties as biosurfactants¹⁵. Sophorolipids are synthesized by fermentation of long-chain fatty acids and other long-chain compounds in certain yeasts, *Candida bombicola*⁵⁰, *Torulopsis magnoliae*⁴⁸ and *Torulopsis gropengiesser*⁵¹. These microbial HBFAs appear to be produced by the hydroxylation of a fatty acid precursor by a specific cytochrome P450 monooxygenase^{43, 52}. While this yeast-based platform for producing HBFAs require long-chain fatty acids as substrates, various chemical synthesis routes have also been proposed, and these also require expensive functionalized substrates and multi-step processes^{36, 53}. The platform described herein produces HBFAs from a sugar feedstock, which may offer an alternative metabolic process than the fatty acid-dependent process available in the sophorolipid producing yeasts^{48, 50, 51}. Thus although sugars are a less expensive feedstock than fatty acids, this new platform will require more optimization to compete with the yeast sophorolipid producing strains that yield a titer of up to 400 g/L⁵⁴.

Despite this current limitation, we did realize a nearly 1000-fold improvement in these parameters from the initial demonstration of the platform in *R. rubrum*, which offers optimism that further improvements can be achieved. The improvements to date included switching the host for expressing the

platform to an *E. coli* strain that can be more readily manipulated via genetic strategies. Targeted genetic manipulations were guided by the principle of eliminating wasteful competing reactions. For example deleting the *fabH* gene that encodes the endogenous KASIII, and eliminating processes that can potentially catabolize the HBFA, i.e., fatty acid β -oxidation pathway, by deleting the *fadD* gene.

Additional improvements in HBFA titer (by 3-fold) were achieved by optimizing the fermentation conditions, specifically optimizing the C/N ratio, inoculum size, the post-induction temperature, induction time, and concentration of the inducer of the reconstructed HBFA pathway. The theoretical yield of a 16-carbon HBFA is 37.8% based on the overall reaction: 4 Glucose $\rightarrow$ 8 Acetyl-CoA $\rightarrow$ 15-hydroxy-hexadecanoic acid. The actual yield of our process is less than 1% of the theoretical yield, and this can be possibly attributed to a number of factors including low catalytic efficiency of the aaKASIII enzyme, limited substrate availability and rate limitation by certain enzymes of the fatty acid synthesis pathway, such as acetyl CoA carboxylase. As previously demonstrated in the development of fatty acid producing strains⁵⁵⁻⁵⁸, improvements in HBFA titer and yield are possible via rational engineering of the aaKASIII enzyme to improve its catalytic efficiency, and via other standard genetic modifications of *E. coli* that increase the flux through the fatty acid biosynthetic pathway by overexpression of acyl-ACP thioesterase⁵⁹, acetyl-CoA carboxylase (*accABCD*)⁵⁸ and/or manipulation of transcription factors (e.g., *FadR*)⁵. Our initial attempts at these types of improvements with acyl-ACP thioesterase did not yield the expected improvement, which may indicate

that improvements in HBFA titers may require exploration of other mechanisms.

MATERIALS AND METHODS

Chemicals and reagents. Unless otherwise specified, all chemicals, biochemicals, solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibiotics were purchased from Fisher Scientific (Waltham, MA).

Gene synthesis and cloning. The ORF sequences coding for aaKASIII from *Alicyclobacillus acidocaldarius* subsp. *acidocaldarius* DSM 446 (Genbank accession number – ACV57087.1); and taKASIII from *Thermus aquaticus* (Genbank accession number – EED09609.1) were codon-optimized for expression in *E. coli*, chemically synthesized (with restriction sites for *Xba*I and *Hind*III at the 5' and 3' ends respectively) and cloned into the pUC57 vector (GenScript, Piscataway, NJ, USA) to generate the plasmids, pUC_aaKASIII and pUC_taKASIII. The chemically synthesized *aaKASIII* and *taKASIII* ORFs were cloned into the pDEST-17 vector with N-terminal His tag via Gateway Cloning (Invitrogen, Carlsbad, CA) to generate plasmids, pDEST_aaKASIII and pDEST_taKASIII.

The *fabH* gene (Genbank accession number - AAG55837.1) that encodes *E. coli* KASIII (ecKASIII) was PCR-amplified from *E. coli* strain MG1655 (The *E. coli* Genetic Stock Center, New Haven, CT), and cloned into pDEST17 vector using Gateway cloning (Invitrogen, Carlsbad, CA), resulting in the plasmid

pDEST_ecKASIII. The *yhfB* gene (Genbank accession number - CAB12857.1) that encodes *B. subtilis* KASIIIb (bsKASIIIb) was PCR-amplified from *B. subtilis* strain 168 (The Bacillus Genetic Stock Center, Columbus, OH) and cloned into the pDEST17 expression vector via Gateway cloning to generate plasmid pDEST_bsKASIIIb.

Four pCA24N expression vectors that harbor the *fabD*, *fabG*, *acpP* and *aas* genes were obtained from the National BioResource Project (NIG, Japan), which encode malonyl-CoA: ACP transacylase (MCAT or FabD), β -ketoacyl ACP reductase (KAR or FabG), holo-ACP and fused 2-acylglycerophosphoethanolamine acyl transferase/acyl-acyl carrier protein synthetase, respectively. The *acpP* and *aas* genes were subsequently cloned into the pETDUET vector (Novagen, Merck KGaA, Darmstadt, Germany) to generate pETDUET_ACPP_AAS vector. Each of these constructs encodes an N-terminal His-tag.

***B. subtilis* strains, plasmids, DNA manipulation and growth conditions.**

B. subtilis was routinely grown in LB medium at 37 °C. *B. subtilis* minimal medium was composed of Spizizen salts, supplemented with 0.5 % glucose and amino acids. As needed, media were supplemented with appropriate antibiotics, erythromycin (1 μ g/ml) or ampicillin (100 μ g/ml). Isopropyl- β -thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- β -galactopyranoside (X-gal) were used at concentrations of 0.4-1 mM and 40 μ g/ml respectively. As needed, media were supplemented with 10-100 μ M individual fatty acids, suspended in 0.01 % (v/v) Brij 58P detergent.

DNA manipulation techniques such as PCR amplification, plasmid preparation, restriction endonuclease digestion, agarose gel electrophoresis and genetic transformation were carried out by standard molecular biology methods. Transformation of *B. subtilis* was conducted on modified competent medium.

B. subtilis $\Delta yjaX \Delta yhfB$ deletion mutant strain was created by first deleting the *yjaX* gene, which encodes bsKASIIIa, using pMU4A plasmid and then deleting the *yhfB* gene, which encodes bsKASIIIb, by using pUCB-erm. The pMU4A plasmid was derived from pMUTIN4 plasmid⁶⁰, and it contained two *yjaX*-derived fragments, one upstream and one downstream of the *yjaX* open reading frame (ORF). The upstream portion (897 bp) spanned from 860-bp upstream of the *yjaX* ORF to 37-bp within this ORF. The downstream fragment (897 bp) spanned from 799-bp downstream to 98-bp within the 3'-end of the *yjaX* ORF. Each fragment was initially PCR amplified from *B. subtilis* strain 168 with the primer pairs, AUf-PacI and AUr-SalI, and ADf-SalI and ADr-PstI (Supplementary Table 2). The resulting fragments were cloned into the integration vector pMUTIN4, at the *PacI* and *PstI* sites, respectively, to generate fusions with the *lacZ* reporter gene. The resulting pMU4A plasmid contains an in-frame 135 bp-*yjaX* coding fragment that is missing 804 bp from the middle of the *yjaX*-ORF; the fact that this deletion allele carries an in-frame ORF avoids any polar effect on the downstream genes of the *yjaX*-containing transcription unit.

The pUCB-erm plasmid was constructed by an analogous procedure as used for pMU4A, except that this vector was derived from plasmid pUC19 and the erythromycin-resistant gene (*erm*) was inserted between the 836-bp upstream fragment and 802-bp downstream DNA fragments of the *yhfB* ORF.

The single deletion mutant strain, *B. subtilis* $\Delta yjaX$, was generated by homologous recombination via a two-step procedure using the plasmid pMU4A. Briefly, pMU4A was transformed into the wild-type strain *B. subtilis* strain 168, followed by selection for erythromycin-resistance that would be conferred by a recombination crossover event between pMU4A and the *B. subtilis* genome. The recovered integrant colonies were grown in LB liquid medium without erythromycin, and the overnight cultures were diluted 1:10⁷, and 100 μ l of the diluted culture was plated on LB medium with IPTG and X-gal. Because the pMUTIN4 plasmid harbors *lacZ*, one can identify those strains that have undergone a second recombination event resulting in the loss of β -galactosidase activity (encoded by the pMUTIN4 vector) and thus appearing as white colonies when grown on X-gal containing plates. Deletion mutants were confirmed via PCR amplification across the deleted portion of *yjaX*.

The double deletion mutant strain, $\Delta yjaX \Delta yhfB::erm$ was generated by homologous recombination via a one-step procedure using the plasmid pUCB-erm. Briefly, the *yhfB*-deletion plasmid, pUCB-erm was linearized via digestion with EcoRI and subsequently transformed into the mutant $\Delta yjaX$ strain. Resultant $\Delta yjaX \Delta yhfB$ double mutant colonies were selected on

media containing erythromycin and anteiso-C16:0 fatty acid, the inclusion of the latter was to enable rescuing of the lethal double mutant. PCR confirmation of the $\Delta yjaX$ and $\Delta yhfB::erm$ alleles were performed using the primer pairs (Supplementary Table 2), lofAf and lofAr, and lofBf and lofBr, respectively.

Genetic complementation of the *B. subtilis* $\Delta yjaX \Delta yhfB$ double mutant strain with aaKASIII, taKASIII or ecKASIII expression vectors was conducted using pUCB-erm-derived plasmids, carrying the different KASIII ORF sequences. In these vectors each of the different KASIII ORFs were under the control of the Pspac promoter, and were inserted between the downstream and upstream DNA fragments of the 135-bp ORF in the $\Delta yhfB$ allele of the $\Delta yjaX \Delta yhfB::erm$ double mutant.

***R. rubrum* strains, plasmids, DNA manipulation and growth conditions**

The aaKASIII ORF sequence was inserted into the *phaC2* locus (Rru_A2413) of the *R. rubrum* genome via a two-step recombination event. The *R. rubrum* recipient strain for this experiment was the *phaC* triple mutant ($\Delta phaC1 \Delta phaC2 \Delta phaC3$) that lacked any PhaC activity²⁶. First, the upstream 922-bp flanking sequence from the *R. rubrum phaC2* gene was cloned upstream of the aaKASIII sequence in plasmid pJQ200SK resulting in pTC_aaKASIII, and this chimeric construct (pTC_aaKASIII) was introduced into the *E. coli* strain S17-1. This construct was transferred to the *R. rubrum* $\Delta phaC1 \Delta phaC2 \Delta phaC3$ strain via transconjugation. Transconjugation was induced by co-incubating overnight the *R. rubrum phaC* triple mutant with the *E. coli* S17-1

strain harbouring pTC_aaKASIII on a 0.22 µm filter. The bacterial mixture was subsequently cultured on minimal medium containing gentamicin (25 µg/ml) for one to two weeks. The resulting colonies carry the product of the first recombination crossover event, which integrates the plasmid pTC_aaKASIII in the *R. rubrum* $\Delta phaC1 \Delta phaC2 \Delta phaC3$ strain. Following colony purification, the recovered *R. rubrum* strains were cultured in SMN rich medium for two to three days under illumination but without gentamicin selection to allow for the second-recombination event, which excises the integrated plasmid and integrates the *aaKASIII* gene at the *phaC2* gene (Rru_A2413) locus in the *R. rubrum* $\Delta phaC1 \Delta phaC2 \Delta phaC3$ strain. Products of the double-crossover events were identified via selection on supplemented malate-ammonium medium (SMN) containing 5% sucrose⁶¹. Resultant *R. rubrum* $\Delta phaC1 \Delta phaC2 \Delta phaC3$ *aaKASIII* strains were confirmed via PCR amplification of *aaKASIII* and subsequent sequencing.

The fatty acid productivity of *R. rubrum* strains was evaluated by growing cultures in RRNCO medium (but omitting ammonium chloride, hydrogen sulfide, carbon monoxide and carbon dioxide)⁶², for 5 days and the bacterial pellet was recovered for fatty acid analysis.

***E. coli* strains, plasmids, DNA manipulation and growth conditions.** Two *pha* genes, *phaA* (3-ketothiolase, *R. rubrum* locus Rru_A0274) and *phaB* (acetoacetyl-CoA reductase, *R. rubrum* locus Rru_A0273) were PCR amplified from *Rhodospirillum rubrum* ATCC 11170 using CloneAmp HiFi PCR Premix (TaKaRa, Clontech, Mountain View, CA) and cloned into a series

of pCDFDuet vectors (Merck Millipore, Darmstadt, Germany) using in-Fusion HD cloning system (Clontech, Mountain View, CA). The resulting plasmids were: a) pCDFDuet_phaA contained *phaA* at Multiple Cloning Site 1 (MCS1); b) pCDFDuet_phaB contained *phaB* at MCS2; c) pCDFDuet_phaA_phaB contained *phaA* in MCS1 and *phaB* in MCS2; d) and pCDFDuet_phaB_phaA contained the two genes in the reverse order, i.e. *phaB* in MCS1 and *phaA* in MCS2 (Supplementary Table 3).

The codon-optimized cDNA sequence of aaKASIII was PCR amplified from pUC57_aaKASIII plasmid using CloneAmp HiFi PCR Premix and cloned into a pETDuet vector to generate pETDuet_aaKASIII (Supplementary Table 3).

Mutant *E. coli* strains were derived from *E. coli* strain K-12 BW25113 (Supplementary Table 3). The JW1077 strain harboring the $\Delta fabH::kan$ allele was obtained from the Keio collection⁶³. The $\Delta fabH::kan \Delta fadD::cam$ double knockout strain (OC100) was derived from JW1077 using the one-step inactivation method of Datsenko and Wanner⁶⁴.

The *E. coli* $\Delta fabH::kan \Delta fadD::cam$ mutant was generated by starting with strains JW1077 that harbors the $\Delta fabH::kan$ allele. PCR primers containing *fadD* gene flanking sequences (fadDH1P1cam and fadDH2P2cam (Supplementary Table 2)) were used for the amplification of a *cam*^R cassette from a pKD3 plasmid template (obtained from the *E. coli* Genetic Stock Center, CGSC#: 7631, Yale, New Haven, CT). The $\Delta fabH::kan \Delta fadD::cam$ mutant cells were recovered on Tryptone Yeast Extract (TYE) agar plates

containing kanamycin and chloramphenicol. Agarose gel electrophoresis was used to confirm the replacement of the native *fadD* gene with the *cam*^R knockout cassette. PCR amplification of the native *fadD* gene was performed using gene specific primers, fadD-U, fadD-D and PCR amplification of the knockout cassette was conducted with the fadDH1P1cam, fadDH2P2cam primers (Supplementary Table 2).

The plasmids pCDFDuet_phaA_phAB and pETDuet_aaKASIII were transformed into *E. coli* $\Delta fabH::kan \Delta fadD::cam$ mutant strain (OC100) by electroporation, to generate strain OC101. Similarly, strain OC102 was obtained by transforming *E. coli* $\Delta fabH::kan \Delta fadD::cam$ mutant (OC100) with pCDFDuet_phaB_phaA and pETDuet_aaKASIII plasmids. Thioesterase (TE) sourced from *Cuphea* seeds was overexpressed in strain OC101 using plasmid pLR 506 (i.e. pETDuet vector carrying aaKASIII at MCS1 and TE at MCS2).

During strain construction, cultures were grown at 37 °C in LB medium and appropriate antibiotics were included, kanamycin (50 µg/mL), ampicillin (100 µg/mL), spectinomycin (100 µg/ml) and chloramphenicol (20 µg/mL). Overnight cultures were inoculated into fresh LB media to OD of 0.1. After 4-6 hours of incubation at 37 °C, expression of aaKASIII was induced with 0.5 mM IPTG. The cultures were cultivated at 22 °C at 250 rpm, and samples were collected 24h or 48h after induction for fatty acid analysis. All experiments were performed in triplicates.

Fatty acid extraction. Fatty acids were extracted from cells or from the growth media using a chloroform/methanol/water extraction method. In particular, the cells or media were acidified with 1 M HCl, and 4 ml chloroform-methanol (1:1 vol/vol) was added to recover the fatty acids. After vortexing for 10 min and centrifuging at 3000×g for 4 min, the lower chloroform phase was transferred to a new tube and evaporated under a stream of nitrogen gas until the samples were concentrated to ~100 µl. Samples were derivatized and 1 µl of each derivatized sample was analyzed by GC-MS. Specifically, extracted fatty acids from *B. subtilis* strains were derivatized by converting to picolinyl esters (lipidlibrary.aocs.org/ms/ms02/index.htm) or methyl esters using methanolic-HCl at 80 °C for 60 minutes. Extracted fatty acids from *R. rubrum* and *E.coli* strains were derivatized by converting to trimethylsilyl esters.

DMDS derivatization. To determine the location of the double bonds in the fatty acid chain, we used a DMDS derivatization method⁶⁵. Briefly, methylated fatty acid extracts were dissolved in hexane with DMDS iodine solution and incubated overnight at 40°C. After the addition of 5% sodium thiosulfate, fatty acid methyl esters were recovered by hexane extraction, silylated and analyzed using GC/MS.

GC-MS analysis. GC-MS analysis of derivatized fatty acids was performed on an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) equipped with an HP-5 MS fused-silica column (length 30 m, internal diameter 250 µm, film thickness 0.25 µm), coupled to an Agilent 5973 MSD

detector. The temperatures of the injector and MSD interface were both set to 250 °C. Helium (1.8 ml/min) was used as a carrier gas. The temperature gradient was from 80–220 °C at 4 °C/min, then to 320 °C at 20 °C/min, and then isothermal at 320 °C for 2 min.

Protein expression and purification. The OverExpress™ C41 (Lucigen, Middletown, WI) strain was used for expression of all KASIII, FabD, FabG and holo-ACP proteins, from their respective pDEST17, pCA24N and pETDuet constructs. The OverExpress™ C41 transformants were grown at 37 °C in 4 L LB medium, supplemented with 100 µg/ml ampicillin. Protein expression was induced by addition of IPTG to a final concentration of 0.4 mM, when the OD₆₀₀ of the culture was between 0.6-0.8. After 16-18 h of induction at 25 °C, cells were harvested by centrifugation (10,000 X g, 4 °C, 10 minutes). The cell pellet was suspended in lysis buffer (0.5 M NaCl, 5 mM imidazole, 20 mM Tris-HCl, pH 8.0, 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.1% Triton-X 100) and subjected to sonication (10 s pulses separated by 3 s intervals for a total of 3 minutes). Following centrifugation (10,000 X g, 4°C, 30 minutes), the supernatant containing the soluble protein fraction was recovered and filtered through a 0.45 µ filter (Corning, the Netherlands). The recombinant His-tagged proteins were purified using PerfectPro Ni-NTA His-bind resin (5 Prime GmbH, Gaithersburg, MD). The soluble protein extract was applied to 4 ml packed column of the resin, and after washing the unbound proteins with wash buffers (0.5 M NaCl, 20 mM Tris-HCl, pH 8.0) supplemented with 20 mM and 40 mM imidazole, the His-tagged proteins of interest were eluted from the column with the wash buffer containing 250 mM imidazole. The

purified His-tagged proteins were dialyzed against 0.1 M sodium phosphate buffer, pH 7.2, at 4 °C and concentrated using Amicon ultrafiltration centrifugal devices with 10,000 MWCO (Millipore, Billerica, MA). Protein purity was assessed by Coomassie-stained SDS-PAGE, which showed presence of near-homogenous protein preparations (greater than 98% purity). Protein concentrations were determined by Bradford's assay (BioRad, Hercules, CA). The concentrated proteins were either stored at -80°C or immediately used for enzyme activity assays and thermal shift assays.

KASIII activity assay. KASIII enzymatic activity was ascertained via spectrophotometric assay, which coupled the appearance of 3-ketoacyl-ACP to the oxidation of NADPH, using the *E. coli* FabG protein to reduce 3-ketoacyl-ACP to 3-hydroxyacyl-ACP. The assay was performed in 96-well plate-format with three technical replicates for each reaction condition. In a total volume of 100 µl for each reaction, the reaction mix containing 400 µM holo-ACP, 400 µM malonyl-CoA, 10 mM DTT, 10-400 µM acyl-CoA substrate and 400 µM NADPH in 0.1 M sodium phosphate buffer (pH 7.2). This mixture was pre-incubated with 60 µg of FabD for two minutes to initiate synthesis of malonyl-ACP from malonyl-CoA and holo-ACP. The KASIII reaction was then started by the addition of 30 µg of FabG and varying concentrations of KASIII enzyme (0.5-15 µg). As KASIII catalysed the condensation acyl-CoA with malonyl-ACP to form 3-ketoacyl-ACP, FabG reduced this 3-ketoacyl-ACP intermediate to 3-hydroxyacyl ACP in presence of NADPH. Change in absorbance at 340 nm due to the conversion of NADPH to NADP⁺, catalysed by FabG, was recorded using Synergy 2 Multi-Mode Microplate Reader

(BioTek, Winooskit, VT). This assay was used to assess the ability of different KASIII enzymes to use different acyl-CoA substrates (i.e., acetyl-CoA, isobutyryl-CoA and 3-hydroxybutyryl-CoA). Specific activity was calculated by varying the amount of KASIII and ascertaining the moles of product (NADP⁺) formed per unit time per mg of KASIII. K_m was calculated by varying the concentration of acyl-CoA substrate from 10 μ M to 400 μ M, while keeping the KASIII concentration constant.

Circular Dichroism (CD) spectroscopy. All CD spectra of purified KASIII proteins (0.1–0.25 mg/ml in 10 mM sodium phosphate buffer, pH 7.2) were collected using a Jasco J-710 Spectropolarimeter, in a 0.1 cm cell at 25 °C. Far-UV spectra were recorded with a bandwidth of 1.0 nm and a time response of 8 s with a total of two accumulations of data.

Thermal shift binding assays. Thermal shift assays were performed with a Light Cycler 480 System (Roche Applied System) using 20 μ l reactions in a 96-well plate format²². KASIII protein (2 μ M–20 μ M) was mixed with SYPRO Orange dye (Sigma-Aldrich, St. Louis, MO) (5X – 10X molar excess of protein concentration) in 0.1 M sodium phosphate buffer, pH 7.2. For each assay, an acyl-CoA ligand (coenzyme-A, acetyl-CoA, propionyl-CoA, butyryl-CoA, hexanoyl-CoA, isobutyryl-CoA, isovaleryl-CoA, 3-hydroxybutyryl-CoA, malonyl-CoA, methylmalonyl-CoA, crotonyl-CoA, benzoyl-CoA or phenylacetyl-CoA) was added in 50-fold molar excess of the KASIII protein being tested. For negative controls, water was used instead of an acyl-CoA ligand. Plates were sealed with an optical sealing tape, and then heated in the

Light Cycler 480 instrument from 20 °C to 95 °C at the rate of 1 °C/minutes. Melting temperatures of the proteins were calculated using the Light Cycler 480 Protein Melt program (Roche Applied Science, Penzberg, Germany), and the effect of different ligands on the melting temperatures of each KASIII were determined. Data from quadruplicate experiments were collected for each protein.

Strain cultivation for fermentation conditions optimization. The bioengineered *E. coli* strain OC101 strain was cultured on LB-agar plates supplemented with streptomycin (50 µg/ml), ampicillin (100 µg/ml) and kanamycin (50 µg/ml) and maintained at 37 °C. A single colony of the *E. coli* strain was inoculated into 3 mL LB liquid medium supplemented with antibiotics and cultured for 12–16 hrs at 37 °C. The inoculum was added aseptically to a 125 ml shake flask containing 25 ml M9 minimal medium supplemented with the antibiotics. M9 medium containing 20 g/L glucose as carbon source and NH₄Cl as nitrogen source as well as 0.5 g/L NaCl, Na₂HPO₄·7H₂O 12.8 g/L, KH₂PO₄ 3 g/L, MgSO₄ 0.24 g/L, CaCl₂ 0.002 g/L, FeSO₄ 0.003 g/L, 0.005 g/L thiamine HCl and 10 ml/L BME vitamins. The culture was cultivated at 37 °C and 250 rpm, and gene expression was induced by the addition of IPTG, the concentration of which varied depending on the experiment. During the cultivation, the optical density of the culture was monitored and cells were harvested for fatty acid analysis when stationary phase was reached.

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