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Engineered Production of Short-Chain Acyl-Coenzyme A Esters in *Saccharomyces cerevisiae*

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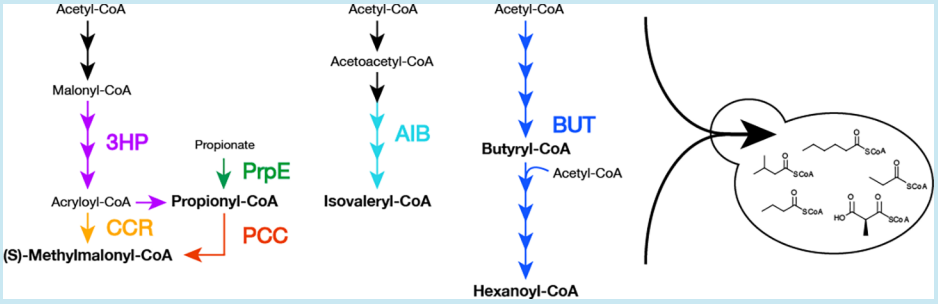
Supporting Information



ABSTRACT: Short-chain acyl-coenzyme A esters serve as intermediate compounds in fatty acid biosynthesis, and the production of polyketides, biopolymers and other value-added chemicals. *S. cerevisiae* is a model organism that has been utilized for the biosynthesis of such biologically and economically valuable compounds. However, its limited repertoire of short-chain acyl-CoAs effectively prevents its application as a production host for a plethora of natural products. Therefore, we introduced biosynthetic metabolic pathways to five different acyl-CoA esters into *S. cerevisiae*. Our engineered strains provide the following acyl-CoAs: propionyl-CoA, methylmalonyl-CoA, *n*-butyryl-CoA, isovaleryl-CoA and *n*-hexanoyl-CoA. We established a yeast-specific metabolite extraction protocol to determine the intracellular acyl-CoA concentrations in the engineered strains. Propionyl-CoA was produced at 4−9 μM; methylmalonyl-CoA at 0.5 μM; and isovaleryl-CoA, *n*-butyryl-CoA, and *n*-hexanoyl-CoA at 6 μM each. The acyl-CoAs produced in this study are common building blocks of secondary metabolites and will enable the engineered production of a variety of natural products in *S. cerevisiae*. By providing this toolbox of acyl-CoA producing strains, we have laid the foundation to explore *S. cerevisiae* as a heterologous production host for novel secondary metabolites.

KEYWORDS: *metabolic engineering, S. cerevisiae, heterologous pathway, precursor engineering, platform molecules, acyl-coenzyme A*

Saccharomyces cerevisiae, a model organism in the biotechnology industry, has been identified and established as a versatile host for the biosynthesis of several natural and engineered small molecules that comprise a number of different biochemical classes, such as flavonoids, isoprenoids, alkaloids and polyketides.[1−4] There is great potential to use *S. cerevisiae* for the production of unique secondary metabolites by introducing many of the metabolic production routes that have evolved in plants, fungi or bacteria. The first step toward such plug and play production in yeast, however, is availability of biosynthetic precursors. Despite the many advantages as a production host, *S. cerevisiae* lacks a number of short-chain acyl-CoA metabolites required in the biosynthesis of many biologically and economically important compounds.

One example are the polymers poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate (PHBV) and 2-hydroxybutyrate-containing polyhydroxyalkanoate (2HB-PHA).[5−7] Propionyl-CoA is as platform metabolite in the biosynthesis of both PHA variants, but not an intermediate of central metabolism in *S. cerevisiae*.[5−7]

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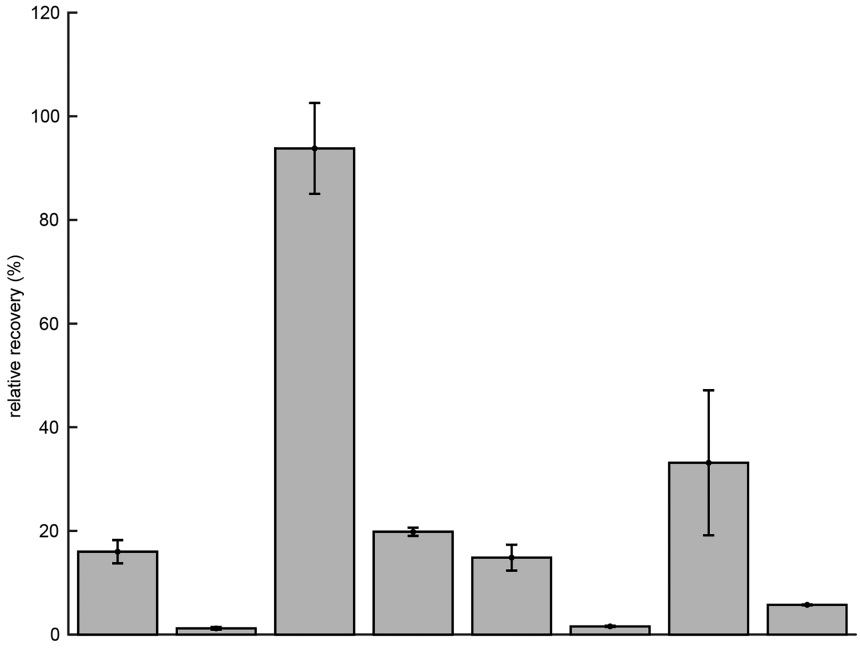

Extraction method	1	2	3	4	5	6	7	8
OD_{600} of cells	10	10	10	10	50	50	50	50
Filtration	x	x			x	x		
Centrifugation and homogenization			x	x			x	x
Lyophilization		x		x		x		x

Figure 1. Evaluation of several acyl-CoA extraction methods. Relative extraction efficiencies achieved for isovaleryl-CoA using different extraction methods (EM). For EM 1−4, a culture volume corresponding to a total OD_{600} of 10 was used; for EM 5−8, a total OD_{600} of 50 was used. For EM 1, 2, 5, and 6, cells were lysed using filtration and quenching. For EM 3, 4, 7 and 8, cells were lysed by homogenization in quenching solution. The extracted metabolite solution was additionally lyophilized in EM 2, 4, 6, and 8 and resuspended in 150 μL quenching solution. Bars show mean values of two biological replicates ± standard deviation with highest measured intracellular concentration set as 100%.

Other examples include linear, odd-chained and branched organic acids and alcohols.[8,9] These compounds are promising alternatives to petroleum-based fuels[10,11] and can be derived from fatty acid biosynthesis. Saturated straight fatty acids are synthesized from acetyl-CoA and malonyl-CoA both of which are common to yeast, while odd-chain fatty acids require propionyl-CoA[12,13] and branched-chain fatty acids are produced from short, branched-chain acyl-CoAs, such as isovaleryl-CoA.[14,15] Isovaleryl-CoA is not an intermediate in yeast metabolism, and as such limits the production of odd-chain and branched acids in *S. cerevisiae*.

Finally, many pharmaceutically relevant secondary metabolites are polyketides. Polyketides comprise a vastly diverse group of bioactive compounds made by polyketide synthases (PKSs) in bacteria, fungi, plants and animals. PKSs condense simple acyl-CoA building blocks, so-called starter and extender units, *via* successive rounds of decarboxylative Claisen-like condensations into chemically more complex products.[16] Some PKSs show an inherent substrate flexibility that could be exploited by feeding alternative carboxylates/caryboxyl-CoAs and various alkylmalonyl-CoA extender units for the production of new compounds with bioactive or special chemical properties.[17−19] Implementing an increasing variety of characterized bio- and semisynthetic routes to different acyl-CoAs in *S. cerevisiae* would allow virtually endless possibilities to create novel and non-natural chemicals in yeast.[20−29]

To expand the metabolic capabilities of yeast, we introduced biosynthetic metabolic pathways to five different acyl-CoA esters into *S. cerevisiae*. Isovaleryl-CoA was produced *via* the alternative isovaleryl biosynthetic (AIB) pathway from *Myxobacterium xanthus*, which is the first heterologous expression example of the AIB pathway to our knowledge.[30] We also report for the first time production of *n*-butyryl-CoA and *n*-hexanoyl-CoA in *S. cerevisiae via* the *n*-butanol (BUT) pathway, which has been previously established in *E. coli*.[9,31] Propionyl-CoA was produced both from glucose using the 3-hydroxypropionate biosynthetic route (3HP) and by propionate feeding using propionyl-CoA synthetase (PrpE).[32−34] Propionyl-CoA synthesis *via* the PrpE route has been reported in *S. cerevisiae* before and serves as benchmark to the performance of the 3HP pathway.[34] A biosynthetic route *via* the 3HP pathway has been established in *E. coli* previously and further improved here for production in *S. cerevisiae*.[32] Methylmalonyl-CoA was synthesized *via* propionyl-CoA using multiple pathways, including the 3HP and PrpE pathways.[19,34,35] Methylmalonyl-CoA synthesis from sugar *via* the 3HP pathway has not be shown before in *S. cerevisiae*.

With the successful implementation of these biosynthetic routes in *S. cerevisiae*, we have established yeast as a viable production host for a variety of compounds. The engineered strains can serve as a platform to implement pathways for the production of value added chemicals, ranging from alcohols, acids, to biopolymers and (un)natural fatty acids and polyketides.

RESULTS AND DISCUSSION

Pathway Design. To avoid homologous recombination, heterologous DNA fragments containing similar sequences are

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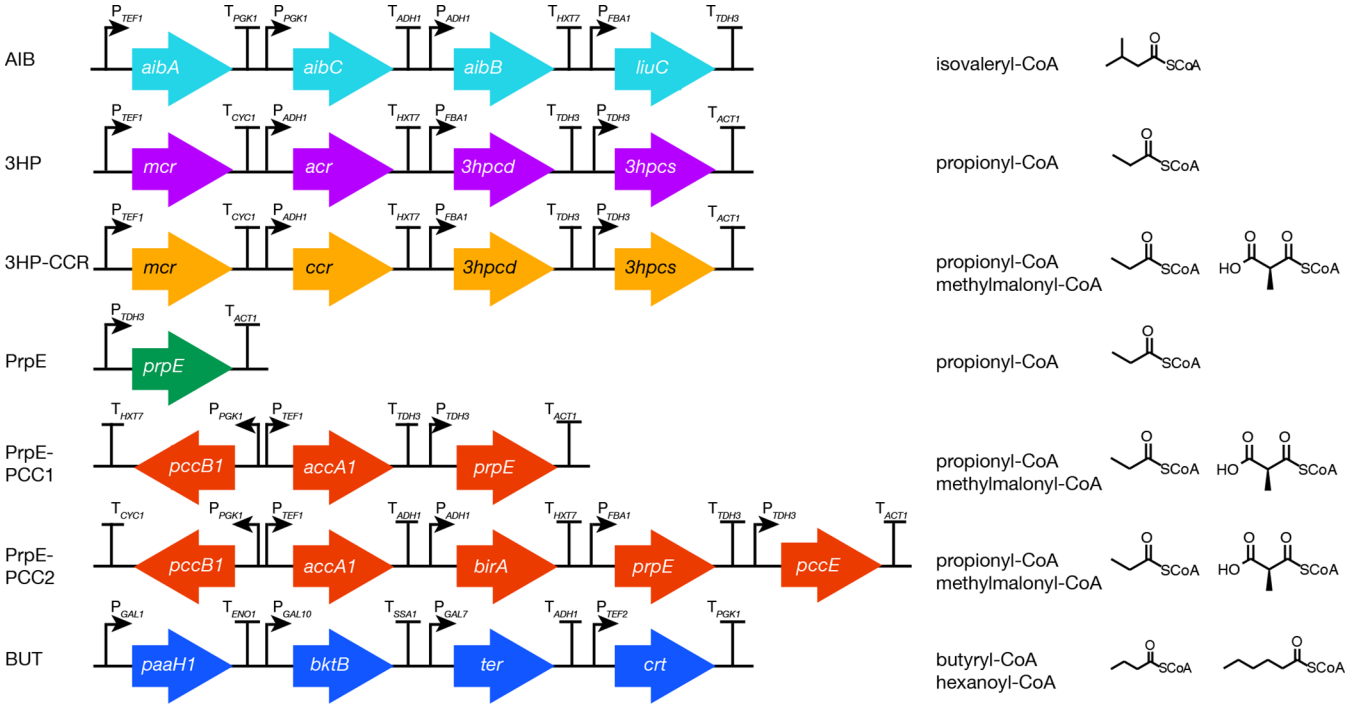

Figure 2. Scheme of gene cassettes encoding pathway enzymes and their respective acyl-CoA products. Each engineered pathway contains between one to five biosynthetic genes and was assembled with unique promoter and terminator pairs.

Table 1. Product Titers for Each Acyl-CoA Pathway in *S. cerevisiae*

pathway	description	titer in μM for acyl-CoA (product[a])	
AIB	alternative isovaleryl-CoA biosynthesis	5.5 ± 1.2 (iv-CoA)	
3HP	3-hydroxypropionate route	8.5 ± 3.7 (p-CoA)	
3HP-CCR	3-hydroxypropionate route with carboxylase/reductase	3.7 ± 2.7 (p-CoA)	0.3 ± 0.3 (mm-CoA)
PrpE	propionyl-CoA synthetase	5.3 ± 2.4 (p-CoA)	
PrpE-PCC1	propionyl-CoA synthetase with propionyl-CoA carboxylase (AccA1, PccB1)	4.6 ± 2.2 (p-CoA)	0.5 ± 0.1 (mm-CoA)
PrpE-PCC2	propionyl-CoA synthetase with propionyl-CoA carboxylase (AccA1, PccB1, PccE) and biotin ligase	6.8 ± 2.8 (p-CoA)	0.1 ± 0.1 (mm-CoA)
BUT	*n*-butanol/hexanol pathway	6 ± 1.9 (but-CoA)	5.8 ± 2 (hex-CoA)

[a]Abbreviations are p-CoA, propionyl-CoA; iv-CoA, isovaleryl-CoA; mm-CoA, methylmalonyl-CoA; but-CoA, butyryrl-CoA; hex-CoA, hexanoyl-CoA.

usually integrated into different locations of the *S. cerevisiae* genome. In this study we assembled the different biosynthetic pathway genes into cassettes. This cassette design allows for greater engineering flexibility and ultimately for rapid integration into multiple loci. To allow for fast and convenient cloning in a standardized fashion, a small cloning library (pNK series) was generated based on the yeast 2 μ plasmids pRS425 and pRS424. This library contains pairs of glycolytic promoters and terminators to be combined with any gene of interest and assembled into pathway cassettes.[36,37] The current library can be used to create pathways with up to five genes (Figure S1). We observed some variability for all intracellular acyl-CoA levels. This could be attributed to the fact that all heterologous genes were expressed from 2 μ plasmids, which are known to have copy number variability.[38] Alternatively, a part of the variability might come from acyl-CoA ester instability during metabolite extraction and analysis. All biosynthetic genes for each pathway are colocalized on one construct, therefore multiple integrations into different loci can be achieved in the future to reduce variability and at the same time increase gene copy number.

Optimization of Acyl-CoA Metabolite Extraction. Acyl-CoAs are difficult to quantify in cell extracts, because they are prone to hydrolysis and degradation. To prevent degradation we optimized the metabolite extraction protocol. We used the yeast strain expressing the AIB pathway genes as a test case for method optimization. Figure 1 summarizes the different extraction methods (EM) tested and their relative extraction efficiencies. Our results showed that using mechanical force (*i.e.*, homogenization) to disrupt the cell wall was necessary to achieve efficient extraction. Furthermore, we observed that concentration steps by lyophilization strongly reduced measurable titers up to 90% in our hands, probably due to acyl-CoA instability under tested conditions. From these results, we chose extraction method EM3 as the standard extraction protocol for our study. EM3 is based on a total cell amount of OD_{600} 10, includes a bead beating step and recovers acyl-CoAs in almost 6-fold higher concentrations compared to the original extraction method EM1. Method EM3 was applied for all metabolite extractions. We quantified the concentration of each acyl-CoA ester based on a calibration curve of synthesized standards using LC−MS/MS and calculated intracellular

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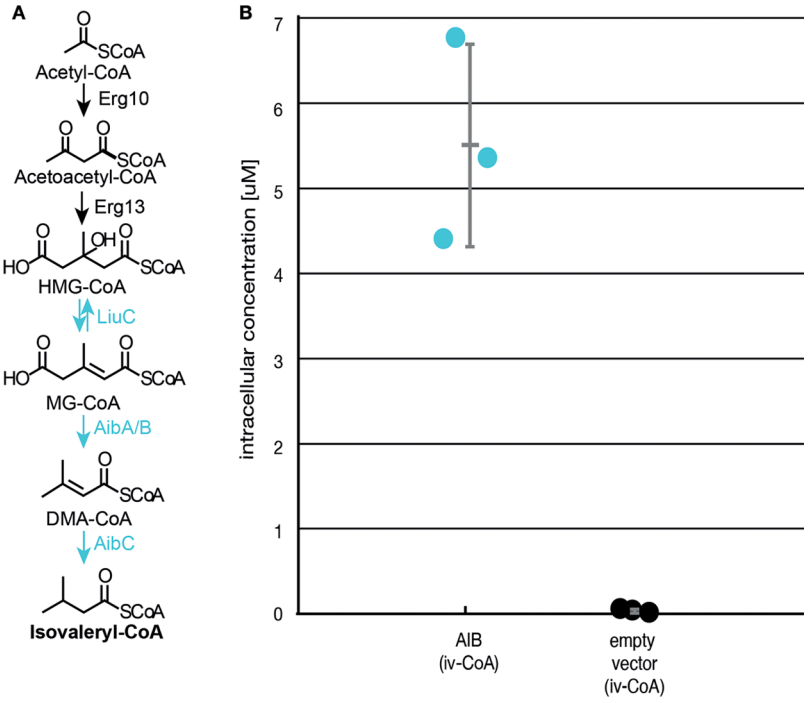

Figure 3. Engineered production of isovaleryl-CoA *via* the alternative isovaleryl-CoA pathway (AIB). (A) Biosynthetic scheme toward isovaleryl-CoA (iv-CoA) production: Proteins catalyzing the conversion of acetyl-CoA to HMG-CoA are native to yeast (black), whereas the 3-methylglutaconyl CoA hydratase LiuC, the glutaconate CoA transferase AibA/B and the dehydrogenase AibC originate from *M. xanthus* (light blue). (B) Intracellular concentrations of isovaleryl-CoA with respective standard deviations for three biological replicates of *S. cerevisiae* BJ5465 harboring pNK23 (light blue) or the empty vector control pRS424 (black).

metabolite pools taking cell concentration and extraction volume into account.

Especially for unstable metabolites, the development of a suitable and most importantly gentle extraction method is necessary. Different methods available are not specific to acyl-CoA quantification in *S. cerevisiae* or require additional equipment and could hence not be applied in our case.[39,40] Ultimately, improved extraction yields were achieved for the desired process by comparing and combining commonly used approaches.

Engineered Acyl-CoA Precursor Supply. Precursor supply is pivotal to establishing *S. cerevisiae* as a production host for various secondary metabolites. We therefore engineered several acyl-CoA pathways into *S. cerevisiae* to provide the commonly used precursors isovaleryl-CoA, butyryl-CoA, hexanoyl-CoA, propionyl-CoA and methylmalonyl-CoA (Figure 2). We based our pathway design on previously characterized biosynthetic and catabolic routes. To this end we established seven different acyl-CoA pathways in *S. cerevisiae* to create a diverse platform for secondary metabolite production. The selected pathways supply the straight-chain starter units propionyl-CoA, butyryl-CoA and hexanoyl-CoA, as well as the branched-chain starter unit isovaleryl-CoA. Additionally, multiple pathway routes were tested for the production of the extender unit methylmalonyl-CoA. The average intracellular concentration measured for each acyl-CoA is listed in Table 1.

Isovaleryl-CoA Supply. Certain bacteria and fungi produce branched-chain acyl-CoAs using various pathways.[41,42] One biosynthetic scheme is based on the bacterial route *via* the branched-chain α-keto acid dehydrogenase complex (Bkd).[43−45] We initially attempted to express this complex together with a transaminase from *Bacillus subtilis* in *S. cerevisiae* BJ5464, but did not observe any branched-chain acyl-CoA production. This is likely due to the fact that a post-translational modification of the E2 subunit is required for its activity.[46] Lipoic acid is covalently bound to a lysine residue in the lipoyl domain of the E2 subunit. However, in yeast the lipoylation enzymes are localized to the mitochondrial lumen, where they post-translationally modify the pyruvate and 2-oxoglutarate dehydrogenase complexes.[47] We additionally attempted to engineer a cytosolic lipoic acid-scavenging pathway by coexpressing the lipoyl ligase LplJ from *B. subtilis*.[48] To this end, we constructed the Bkd pathway as well as a cytosolic lipoylation route but were not able to detect any branched-chain acyl-CoAs.[42,48] As biosynthesis *via* the Bkd pathway utilizes a multisubunit complex, it is likely that no functional enzymes were formed under the conditions tested. Potential bottlenecks could be an unfavorable ratio of Bkd subunits and/or improper folding of its polypeptide chains as well as different expression dynamics preventing assembly of a functional Bkd complex.

Alternatively, isovaleryl-CoA can be produced by branching off the mevalonate pathway *via* the alternative isovaleryl-CoA biosynthetic pathway (AIB) from *M. xanthus*.[30] As in mevalonate production, biosynthesis of isovaleryl-CoA is initiated by condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA by a β-ketothiolase and conversion to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is catalyzed by the HMG-CoA synthase. The AIB pathway then diverts HMG-CoA into 3-methylglutaconyl-CoA (MG-CoA) using the hydratase LiuC. This reaction is followed by an AibA/B-dependent decarboxylation yielding 3,3-dimethylacrylyl-CoA (DMA-CoA), and the final AibC-catalyzed reduction step leads to formation of isovaleryl-CoA (Figure 3A).[49] When expressing the *aib* genes on a 2 μ plasmid in *S. cerevisiae* BJ5464, we were able to detect isovaleryl-CoA in the prepared intracellular

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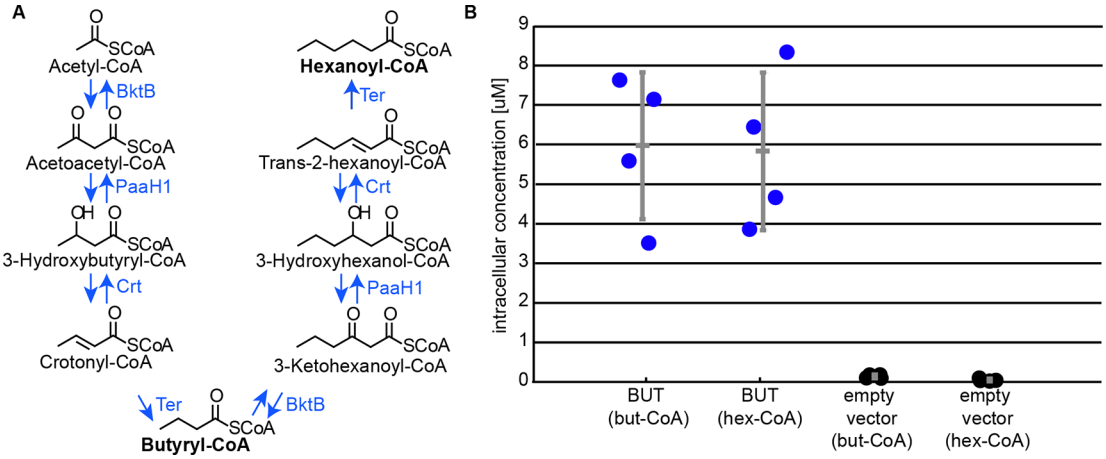

Figure 4. Engineered production of butyryl-CoA (but-CoA) and hexanoyl-CoA (hex-CoA) *via* the BUT pathway. (A) Biosynthetic scheme from acetyl-CoA *via* the beta-ketothiolase BktB from *R. eutropha*, the 3-hydroxyacyl-CoA dehydrogenase PaaH1 from *Clostridium acetobutylicum*, the crotonase Crt from *C. acetobutylicum* and the trans-enoyl-CoA reductase Ter from *Treponema denticola*. (B) Intracellular concentrations of but-CoA and hex-CoA with respective standard deviations for four biological replicates of the production strain BJ5465 harboring pNK44 (blue) or the empty vector control pRS424 (black).

metabolite extracts (Figure S2A). The intracellular concentration was measured at 5.5 ± 1.2 μM, and no isovaleryl-CoA was detected in the empty vector control extracts (Figure 3B). Previously, it was shown that bacterial fatty acid synthases (FAS) may also incorporate branched-chain acyl-CoAs, aside from acetyl and malonyl-CoA.[50] Recent advances in expanding the substrate specificity of fungal FAS lay the foundation for the exploration of synthesis of novel fatty acids by providing unnatural, short-chain acyl-CoAs.[51] For polyketides, isovaleryl-CoA and other branched-chain acyl-CoAs serve as starter units and the levels of acyl-CoAs produced within this study are consistent with the reported binding constants for various characterized PKSs.[52−54] The intracellular concentrations of acyl-CoAs, including isovaleryl-CoA, measured in this study are comparable to acyl-CoAs pool sizes of natural producer strains, suggesting that the engineered pathways can support polyketide biosynthesis in yeast.[55] As such, this engineered yeast strain can serve as a platform to convert isovaleryl-CoA into various high-value chemicals like polyketides or novel fatty acids.

Butyryl-CoA and Hexanoyl-CoA Production. Butyraldehyde and butanol production have been previously engineered into *E. coli* and *S. cerevisiae*.[9,31,56−60] To implement butyryl-CoA and hexanoyl-CoA supply in yeast, we adapted the *n*-butanol pathway previously described in *E. coli* for expression in *S. cerevisiae*.[9,31] The thiolase BktB condenses two acetyl-CoAs into acetoacetyl-CoA. The dehydrogenase/reductase PaaH1 and the crotonase Crt catalyze the reactions to 3-hydroxybutyryl-CoA and crotonyl-CoA, respectively. The reductase Ter hydrogenates the penultimate intermediate to form butyryl-CoA (Figure 4A). BktB from *Ralstonia eutropha* has been shown to have good catalytic activity on butyryl-CoA, which allows for an additional condensation toward synthesis of hexanoyl-CoA.[9,57] By expressing the *n*-butanol pathway in *S. cerevisiae*, we could detect butyryl-CoA and hexanoyl-CoA production in the engineered strain BJ5464 harboring pNK44 (Figure S2B). We determined intracellular concentrations of 6 ± 1.9 μM for butyryl-CoA and 5.8 ± 2 μM for hexanoyl-CoA (Figure 4B). Butyryl-CoA and hexanoyl-CoA are precursors to alcohols and carboxylic acids.[9] Several type I, II and III PKSs have been shown to incorporate hexanoyl-CoA into (un)-natural polyketides.[61−63] By providing a *S. cerevisiae* platform strain producing hexanoyl-CoA and additional other short- and medium-chain acyl-CoAs, the establishment of yeast as polyketide production host has been accelerated.

Propionyl-CoA Production. Multiple propionyl-CoA biosynthesis routes exist in nature. The most commonly used pathway is based on exogenous propionate supply and the expression of the propionyl-CoA synthetase PrpE from *Salmonella typhimurium*.[34] To avoid propionate feeding, we implemented a direct propionyl-CoA production route from malonyl-CoA (Figure 5A). This pathway has been previously established in *E. coli* and uses elements from the 3-hydroxypropionate (3HP) carbon assimilation cycle found in certain auxotrophic archaea and bacteria.[32,64−66] We modified the reported 3HP pathway by replacing Mcr_{St}, a malonic semialdehyde reductase from *Sulfolobus tokodaii*, with the bifunctional malonyl-CoA reductase Mcr_{Ca} from *Chloroflexus aurantiacus*.[65] A recent study showed that Mcr_{Ca} has significantly higher activity compared to Mcr_{St}.[32,67,68] The Mcr_{Ca} catalyzes two reduction steps from malonyl-CoA to 3-hydroxypropionate, which is followed by an esterification by the 3-hydroxypropionyl-CoA synthase (3Hpcs) of *Metallosphaera sedula* to 3-hydroxypropionyl-CoA. The dehydration reaction to acryloyl-CoA is catalyzed by the hydroxypropionyl-CoA dehydratase (3Hpcd) of *S. tokodaii*. The final reduction toward propionyl-CoA is catalyzed by the acryloyl-CoA reductase Acr from *M. sedula* (Figure 5A). After transforming the 3HP pathway plasmid into *S. cerevisiae* BJ5464, we determined the intracellular propionyl-CoA concentration to be 8.5 ± 3.7 μM (Figure 5B, Figure S2C). We also recapitulated the PrpE-dependent pathway for comparison and determined the propionyl-CoA level to be 5.3 ± 2.4 μM. Side-by-side comparison between both engineering approaches revealed that propionate-dependent production resulted in a smaller propionyl-CoA pool compared to the 3HP pathway (Figure 5B). Also, the PrpE dependent pathway showed a distinct growth phenotype compared to the 3HP pathway and all other acyl-CoA production pathways tested. While all strains expressing pathways for the production of acyl-CoA esters exhibited some reduced growth and flocculation, probably caused by changes in intracellular acyl-CoA (and free CoA) pools, PrpE expression compromised growth only in the

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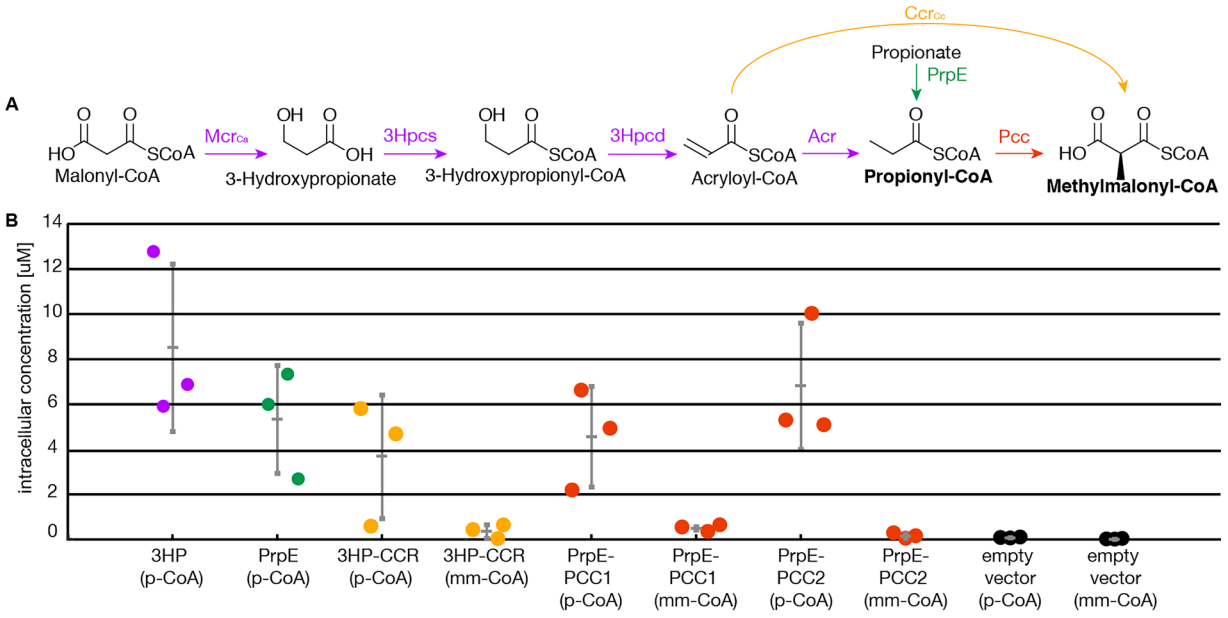

Figure 5. Propionyl-CoA (p-CoA) and methylmalonyl-CoA (mm-CoA) production *via* the various biosynthesis routes. (A) Biosynthetic schemes from malonyl-CoA or propionate using the malonyl-CoA reductase Mcr_{Ca} from *C. aurantiacus*, the 3-hydroxypropionyl-CoA synthase 3Hpcs from *M. sedula*, the 3-hydroxypropionyl-CoA dehydratase 3Hpcd from *S. tokodaii*, the acryloyl-CoA reductase Acr from *M. sedula*, the propionyl-CoA synthetase PrpE from *S. typhimurium*, the biotin-dependent propionyl-CoA carboxylase complex Pcc from *S. coelicolor*. Acryloyl-CoA can be reduced and carboxylated *via* either a two-step pathway using Acr and the Pcc complex or the crotonyl-CoA carboxylase/reductase Ccr from *C. crescentus*. (B) Intracellular propionyl-CoA concentrations in the 3HP production strain BJ5465 pNK30 (purple) and the PrpE production strain BJ5465 pNK36 (green). Intracellular concentrations of propionyl-CoA and methylmalonyl-CoA of the 3HP-CCR production strain BJ5465 pNK42 (orange), the PrpE-PCC1 and PrpE-PCC2 production strains BJ5465 pNK37 or BJ5465 pNK54 (red) and the empty vector control strain BJ5465 pRS424 (black) as well as their respective standard deviations. All concentrations were determined using three biological replica.

presence of propionate in the medium (Table S4).[89] This indicated a toxic effect of highly efficient ATP-dependent propionyl-CoA ligation in yeast.[69−72] Overall we implemented successful production of the platform molecule propionyl-CoA *via* different routes. Propionyl-CoA is a building block in polyketides, fatty acids and bioplastics. Our strains facilitate the implementation of production of these value-added chemicals.

Methylmalonyl-CoA Production. To supply the precursor methylmalonyl-CoA (mm-CoA) production we modified the 3HP biosynthetic pathway by replacing the acryloyl-CoA reductase Acr with a crotonyl-CoA carboxylase/reductase (Ccr). These enzymes are able to reductively carboxylate enoyl-CoA esters into their corresponding alkylmalonyl-CoA analogs.[73,74] We used the Ccr from *Caulobacter crescentus* (Ccr_{Cc}, Figure 5A) that was shown to convert acryloyl-CoA to methylmalonyl-CoA *in vitro*.[19] Using LC−MS/MS both propionyl-CoA and methylmalonyl-CoA were detected at intracellular concentrations of 3.7 ± 2.7 μM and 0.3 ± 0.3 μM, respectively (Figure 5B, Figure S2C). Recent investigations on the mechanism demonstrated that Ccrs are able to catalyze a single reduction step in addition to the combined reduction/carboxylation step, which explains the presence of propionyl-CoA in addition to methylmalonyl-CoA.[75,76] The ratio of the two products indicates that reduction is more efficient compared to carboxylation under the tested conditions *in vivo*.

An alternative route to mm-CoA using the PrpE-PCC pathway has been published previously.[34] Here we compared the PrpE-PCC biosynthetic route with the 3HP-CCR pathway. To this end, we established the ATP-dependent carboxylation of propionyl-CoA, catalyzed by the biotin-dependent propionyl-CoA carboxylase complex (Pcc). The Pcc complex from *Streptomyces coelicolor* consists of three subunits and requires a post-translational modification. The alpha subunit AccA1 contains two components, the biotin carboxylase and biotin-carboxyl carrier protein domain. The beta subunit PccB1 functions as carboxyl transferase carboxylating propionyl-CoA to form methylmalonyl-CoA. This mm-CoA biosynthetic pathway has been successfully implemented in the past using a multiplasmid approach.[34] In this study we combined *prpE*, *accA1* and *pccB1* on one plasmid creating the PrpE-PCC1 pathway. As this pathway produces propionyl-CoA as intermediate, we detected both propionyl-CoA and methylmalonyl-CoA. The titer was measured at 0.5 ± 0.1 μM for methylmalonyl-CoA, similar to the amount produced by the 3HP-CCR pathway. Propionyl-CoA was measured at an intracellular concentration of 4.6 ± 2.2 μM (Figure 5B).

Then, we sought to improve mm-CoA levels by engineering the PCC2 pathway comprised of AccA1, PccB1, PccE from *S. coelicolor* and BirA from *E. coli*. Expression of the epsilon subunit PccE has been found to improve complex formation and increase Pcc activity significantly.[77−79] Furthermore, the biotin ligase BirA has been shown to facilitate biotinylation of the Pcc complex.[35] The PrpE-PCC2 pathway was found to produce a higher propionyl-CoA titer at 6.8 ± 2.8 μM but a lower titer of methylmalonyl-CoA at 0.1 ± 0.1 μM compared to the PrpE-PCC1 pathway. Reasons may lie in the varied design of the genetic constructs, as *prpE* is expressed from a different promoter. It is also likely that complex formation of AccA1, PccB1 and PccE is suboptimal under the here tested conditions, forming more nonfunctional protein. To this end, we successfully implemented a number of pathways resulting in the production of methylmalonyl-CoA and quantified their intracellular pools by LC−MS/MS.

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CONCLUSION

To conclude, we metabolically engineered the budding yeast *S. cerevisiae* to produce propionyl-CoA, butyryl-CoA, isovaleryl-CoA, hexanoyl-CoA and methylmalonyl-CoA. We expressed the alternative isovaleryl-CoA pathway of *M. xanthus* in yeast. We also engineered *S. cerevisiae* for butyryl-CoA and hexanoyl-CoA production *via* the well-established *n*-butanol pathway. Finally, we evaluated multiple routes toward propionyl-CoA and methylmalonyl-CoA biosynthesis and established a feeding-free approach for the production of these acyl-CoA esters *in vivo*. In summary, this study provides a foundation for establishing *S. cerevisiae* as a general chassis for the production of secondary metabolites, where the different acyl-CoAs serve as important platform molecules. The engineered platform strains provide a metabolic engineering tool to produce diverse compounds of interest such as fatty acids and polyketides from renewable carbon sources in *S. cerevisiae*. This plug-and-play approach further provides opportunities to discover and engineer novel bioactive compounds and valuable chemicals in the future.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* strain NEB Turbo (New England Biolabs) was used for cloning and plasmid propagation. *S. cerevisiae* BJ5465 was obtained from ATCC as a protease-deficient variant of *S. cerevisiae* (Meyen ex. E.C. Hansen, ATCC 208289) (Table 2).[80] The yeast 2 μ plasmids pRS424 and pRS425 were chosen as vector backbones for acyl-CoA pathway expression.[81] The glycolytic promoters P_{TEF1}, P_{ADH1}, P_{PGK1}, P_{FBA1} and P_{TDH3} were chosen based on their expression strength and dynamic profiles using a published data set.[36] The selected promoters were previously shown to have comparable activity during mid-log phase. The terminators T_{CYC1}, T_{HXT7}, T_{TDH3}, T_{ATC1}, T_{ADH1}, T_{ENO1}, T_{SSA1} and T_{PGK1} were selected based on their efficiency to terminate transcription as characterized previously.[36,37] Promoters and terminators used in the BUT pathway were selected to investigate inducible expression of enzymes for cell growth decoupled production of acyl-CoA esters. All biosynthetic genes were codon optimized for *S. cerevisiae* using the IDT codon optimization tool, except for the 3HP pathway, which was sourced from *E. coli* codon optimized genes, as well as *birA*, which was amplified from the genome of *E. coli*.[32] The mcr_{Ca} gene was kindly provided by the Nielsen lab.[82] The complete set of genes and their source organisms are listed in Table S1.

Table 2. Strains Used in This Study

strain	genotype and description	reference
E. coli NEB Turbo	*gln V44 thi-1* Δ*(lac-proAB) galE15 galK16 R(zgb-210::*Tn10*)*Tet^S *endA1* Δ*(mcrB-hsdSM)5,* ($r_K^-m_K^-$) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15/fhuA2*]	NEB
S. cerevisiae BJ5465, ATCC 208289	*MAT*a *ura3*−*52 trp1 leu2*-Δ*1 his3*-Δ*200 pep4::HIS3 prb1*-Δ*1.6R can1 GAL*	ATCC[34,80]

We constructed the pathway plasmids in a modular approach using a small library of entry vectors, which we generated during this study and named the pNK series. The pNK plasmids carry distinct promoter-terminator pairs to construct gene expression cassettes in a modular approach. This small library was then used as template to assemble all pathway plasmids. A complete list of plasmids is provided in Table 3 and Table S2, and a complete list of primers used for plasmid assembly is available in Table S3. Each promoter, terminator, gene and backbone was amplified by PCR and each primer had an approximately 25-bp overhang to the flanking parts. Amplicons were generated by PCR using the high fidelity polymerase PrimeSTAR GXL DNA (Clontech) and *S. cerevisiae* CEN.PK as gDNA template or described plasmids. The purified PCR products were assembled using circular polymerase extension cloning (CPEC) or Gibson assembly.[83,84] Purified PCR fragments were used as input for CPEC, with 250 ng backbone and 3:1 amount of insert to vector in a 50 μL reaction volume. Five μL of the assembled plasmid was transformed into *E. coli* using electroporation. Analytical digests and subsequent sequencing identified correct plasmids. For Gibson assembly, 100 ng backbone was mixed with inserts (3:1 insert to backbone molar ratio) in a 10 μL reaction volume, of which 5 μL was added to the cells for transformation. 100 ng of the backbone amplicon was cotransformed with the insert fragments in a 1:3 ratio into competent *S. cerevisiae* BJ5465 cells using the Frozen-EZ Yeast Transformation II Kit (Zymo Research). A more detailed description of plasmid construction can be found in the Supporting Information.

Media and Cultivation. *E. coli* cells were routinely cultured in Luria−Bertani medium (LB) containing 100 μg/mL ampicillin for plasmid propagation at 37 °C. *S. cerevisiae* cells were grown at 30 °C in yeast extract-peptone-dextrose (YPD) medium consisting of 1% bacto yeast extract, 2% bacto peptone and 2% dextrose. Yeast strains carrying plasmids were grown at 30 °C on selective plates (Trp dropout or Leu dropout) and at 25 °C for liquid cultures. A modified synthetic complete medium was used for selective growth in liquid medium. Synthetic complete medium was purchased from Sunrise Scientific lacking Tryptophan (SC-TRP) or Leucine (SC-LEU). The modified synthetic complete medium contained 13.4 g/L yeast nitrogen base, 5.76 g/L SC-TRP or 5.49 g/L SC-LEU, 20 g/L glucose and 100 mM sodium phosphate buffer

Table 3. Plasmids Used in This Study[a]

plasmid	genotype and description	reference	pathway
pNK23	P_{TEF1}-*aibA*-T_{PGK1} P_{PGK1}-*aibC*-T_{ADH1} P_{ADH1}-*aibB*-T_{HXT7} P_{FBA1}-*liuC*-T_{TDH3}, 2 μ, *TRP1*, AmpR-pMB1	This study	AIB
pNK30	P_{TEF1}-*mcr*-T_{PGK1} P_{ADH1}-*acr*-T_{HXT7} P_{FBA1}-*3hpcd*- T_{TDH3} P_{TDH3}-*3hpcs*-T_{ACT1}, 2 μ, *LEU2*, AmpR-pMB1	This study	3HP
pNK36	P_{TDH3}-*prpE*-T_{ACT1}, 2 μ, *TRP1*, AmpR-pMB1	This study	PrpE
pNK37	P_{TDH3}-*prpE*-T_{ACT1} P_{TEF1}-*accA1*- T_{TDH3} P_{PGK1}-*pccB1*-T_{HXT7}, 2 μ, *TRP1*, AmpR-pMB1	This study	PrpE-PCC1
pNK42	P_{TEF1}-*mcr*-T_{CYC1} P_{ADH1}-*ccr*-T_{HXT7} P_{FBA1}-*3hpcd*-T_{TDH3} P_{TDH3}-*3hpcs*-T_{ACT1}, 2 μ, *LEU2*, AmpR-pMB1	This study	3HP-CCR
pNK44	P_{GAL1}-*paaH1*-T_{ENO1} P_{GAL10}-*bktB*-T_{SSA1} P_{GAL7}-*ter*-T_{ADH1} P_{TEF2}-*crt*-T_{PGK1}, 2 μ, *LEU2*, AmpR-pMB1	This study	BUT
pNK54	P_{TDH3}-*pccE*-T_{ACT1} P_{FBA1}-*prpE*-T_{TDH3} P_{ADH1}-*birA*-T_{HXT7} P_{TEF1} -*accA1*-T_{ADH1} P_{PGK1}-*pccB1*-T_{CYC1}, 2 μ, *TRP1*, AmpR-pMB1	This study	PrpE-PCC2

[a]All plasmids were expressed in *S. cerevisiae* BJ5465.

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pH 6.4 and was used to grow the strains containing 2 μ plasmids.

For intracellular acyl-CoA analysis, fresh single colonies were prepared. Each plasmid was transformed into competent cells of *S. cerevisiae* strain BJ5465 and plated onto selective growth agar. The plates were incubated for 3 days at 30 °C. Precultures were grown from single colonies in 5 mL selective SC medium for 3 to 4 days at 25 °C. Production cultures were then inoculated to a starting OD_{600} of 0.05 into 12 mL of selective medium in 100 mL flasks and grown at 25 °C and 200 rpm. Strains carrying *prpE* expression plasmids were supplemented with 50 mM sodium propionate to allow for propionyl-CoA production. The cells were harvested after 48 h.

To determine growth rates, cells were freshly transformed with plasmids. After growing for 3 days on plates, three colonies were picked and cultured in 5 mL media for another 3 days. Cells were then inoculated at an OD_{600} of 0.05 in 24-well plates in 1.5 mL volume with different sodium propionate concentrations and grown for 70 h at 25 °C.[89]

Metabolite Extraction. The protocol for intracellular metabolite extraction was adapted from a previous study.[32] In order to identify optimal extraction conditions for our purpose, several commonly used extraction protocols were tested and compared (Figure 1). *S. cerevisiae* transformed with plasmid pNK23 producing isovaleryl-CoA was selected for utilization in the extraction method optimization.

For the filtration step of extraction method 1, 2, 5 and 6, a culture volume corresponding to a total OD_{600} of 10 or 50 was vacuum-filtered through a 0.45-μm pore size filter. The filters that collected the cells were immediately transferred into 1 mL of quenching solution (prechilled acetonitrile/methanol/water (2:2:1)) and incubated at −20 °C for at least 1 h. Resuspended cells were transferred into 1.5 mL tubes and centrifuged at −9 °C and 17 000*g* for 30 min. The supernatant was stored at −80 °C for subsequent lyophilization or analysis using liquid chromatography coupled to triple quadrupole mass spectrometry (LC−MS/MS).

The same total OD_{600} of cells as above were harvested by centrifugation for 3 min at −9 °C at 2700*g* and supernatant was discarded for the extraction methods 3, 4, 7, and 8. Cells were taken up in 1 mL of prechilled quenching solution and transferred into 2 mL screw cap tubes, filled half with 0.5 mm glass beads. Cells were homogenized 10 times for 10 s at 4800*g* with 2 min on ice between each homogenization pulse (Precellys Evolution, Bertin Instruments). To separate the cell lysate from the beads, the lid of the tubes was removed and a hole was poked into the bottom using a hot needle. The tube containing the metabolite extract was then set into an empty 15 mL falcon, centrifuged for 2 min at 2700*g* and −9 °C, to separate the extract from the beads. After removing the empty homogenizer tube with the remaining beads, the flow-through cell suspension was transferred into prechilled 1.5 mL tubes and centrifuged for at least 20 min at 17 000*g* and −9 °C to remove any remaining cell debris. The supernatant was transferred into a new tubes and the samples were subsequently stored at −80 °C for lyophilization or LC−MS/MS analysis.

For extraction method 2, 4, 6 and 8, in which samples were lyophilized, the stored supernatants were diluted with 5 mL water and snap frozen in liquid nitrogen. Samples were lyophilized (Alpha 1−4 LCS plus, Martin Christ) at −50 °C for 15 h. Samples were taken up in 150 μL quenching solution. Of all samples, 3 μL were analyzed using LC−MS/MS.

To determine intracellular concentrations of all acyl-CoA esters from production cultures, the cells were cultivated for 48 h at 25 °C at 200 rpm and a culture volume corresponding to a total OD_{600} of 10 was harvested and extracted according to extraction method 3 (Figure 1).

LC−MS/MS Analysis of Acyl-CoA Esters. Acyl-CoA esters were measured by fast LC−MS/MS as described previously.[85] An Agilent 1290 Infinity II UHPLC system (Agilent Technologies) was used for liquid chromatography. The column was an Acquity BEH Amide 30 × 2.1 mm with 1.7 μm particle size (Waters GmbH). The temperature of the column oven was 30 °C, and the injection volume was 2 μL. LC solvent A was water with 10 mM ammonium formate and 0.1% formic acid (v/v), and LC solvent B was acetonitrile with 0.1% formic acid (v/v). The gradient was 0 min 90% B; 1.3 min 40% B; 1.5 min 40% B; 1.7 min 90% B; 2 min 90% B. The flow rate was 0.4 mL min^{-1}. An Agilent 6495 triple quadrupole mass spectrometer (Agilent Technologies) was used for mass spectrometry using ion source parameters described in Guder *et al.*[86] Parameters for multiple reaction monitoring (MRM) were adapted from Zimmermann *et al.* (Table 4).[85] Absolute concentrations of acyl-CoA esters were estimated by external calibration curves. The corresponding acyl-CoAs were synthesized according to a previous study.[20] Butyryl-CoA and propionyl-CoA were synthesized following the protocol for Symmetric Anhydride Synthesis.[20] For the hexanoyl-CoA, isobyutyryl-CoA, 2-methylbutyryl-CoA and isovaleryl-CoA synthesis, we used Carbonyldiimidazole Synthesis.[20] Methylmalonyl-CoA was synthesized using malonic acid and the malonyl-CoA synthetase MatB.[20] The purity of the products and yields were determined using HPLC−MS and Nanodrop (Nanodrop 2000, Thermo Scientific). The stocks of the compounds were stored at −80 °C and diluted into extraction solution to use as standards for LC−MS/MS. All standards were mixed at final concentrations of 2.5, 0.625, 0.156, 0.039, 0.0098 and 0.0024 μM. From these calibration curves, the absolute concentration of precursors in cell lysates were determined. Intracellular concentrations were calculated using a specific cell volume of 3.7×10^{-14} L/cell and 3×10^{7} cells/mL per OD_{600} 1.[87,88]

Table 4. Multiple Reaction Monitoring (MRM) Parameters

metabolite	formula	Q1 (m/z)	Q2 (m/z)
propionyl-CoA	$C_{24}H_{40}N_7O_{17}P_3S$	822.1	408
butyryl-CoA	$C_{25}H_{42}N_7O_{17}P_3S$	836.2	408
isovaleryl-CoA	$C_{26}H_{44}N_7O_{17}P_3S$	850.2	408
hexanoyl-CoA	$C_{27}H_{46}N_7O_{17}P_3S$	864.2	408
methylmalonyl-CoA	$C_{25}H_{40}N_7O_{19}P_3S$	866.1	822

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00466.

Tables of plasmid, strains, genes and primers (Supplementary Tables S1−S3); Text on plasmid construction (Supplementary Text); Growth rates with propionate supplementation (Supplementary Table S4); Illustration of pathway assembly (Supplementary Figure S1); LC−MS/MS chromatograms (Supplementary Figure S2) (PDF)

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Author Contributions

¶N.K.K., A.C.L., and A.L. contributed equally to this work. A.L. and N.K.K. conceived the project. N.K.K., A.C.L., A.L., and H.L. performed the experiments and data analysis. C.D. contributed to work on the BUT pathway. B.V. assisted to standard synthesis of CoA-esters and provided material. T.J.E., S.Y., T.J., L.K., M.J., V.S., and J.D.K. contributed to discussion of results. All authors provided input on the manuscript.

Notes

The authors declare the following competing financial interest(s): J.D.K. has a financial interest in Amyris, Lygos, Demetrix, Constructive Biology, and Napigen. L.K. has a financial interest in Lygos. The remaining authors declare that they have no competing interests.

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