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Engineering *Escherichia coli* for Microbial Production of Butanone

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To expand the chemical and molecular diversity of biotransformation using whole-cell biocatalysts, we genetically engineered a pathway in *Escherichia coli* for heterologous production of butanone, an important commodity ketone. First, a 1-propanol-producing *E. coli* host strain with its sleeping beauty mutase (Sbm) operon being activated was used to increase the pool of propionyl-coenzyme A (propionyl-CoA). Subsequently, molecular heterofusion of propionyl-CoA and acetyl-CoA was conducted to yield 3-ketovaleryl-CoA via a CoA-dependent elongation pathway. Lastly, 3-ketovaleryl-CoA was channeled into the clostridial acetone formation pathway for thioester hydrolysis and subsequent decarboxylation to form butanone. Biochemical, genetic, and metabolic factors affecting relative levels of ketogenesis, acidogenesis, and alcohologenesis under selected fermentative culture conditions were investigated. Using the engineered *E. coli* strain for batch cultivation with 30 g liter^{-1} glycerol as the carbon source, we achieved coproduction of 1.3 g liter^{-1} butanone and 2.9 g liter^{-1} acetone. The results suggest that approximately 42% of spent glycerol was utilized for ketone biosynthesis, and thus they demonstrate potential industrial applicability of this microbial platform.

Due to waning fossil fuel reserves, the demand for more cost-effective and environmentally conscientious bioprocesses to replace petrochemical processes has increased significantly. Whole-cell biocatalytic platforms offer several technological advantages over traditional synthetic chemical processes, such as high chemoselectivity, stereoselectivity, and regioselectivity and the ability to catalyze complex multistep reactions under ambient conditions. However, biological systems are often limited in their applicability for the production of valuable chemicals, due to the lack of natural biosynthetic pathways (1, 2). Fortunately, nature has evolved novel catalytic processes that can be manipulated and redesigned for *in vivo* chemical synthesis. Accordingly, metabolic engineering has been integrated with synthetic biology to expand the molecular capabilities and chemical diversity of living systems for scalable synthesis of a wider array of value-added chemicals and biofuels.

Herein, we report the implementation of a robust metabolic pathway in *Escherichia coli* for heterologous production of butanone (also referred as methyl ethyl ketone [MEK]), a commodity ketone. Owing to its low boiling point and high dissolution properties, butanone is used as a general solvent in trades extending from printing and textiles to domestic uses (3). Currently, butanone is manufactured exclusively using petroleum-derived feedstocks, such as 2-butanol, 2-butene, and various branched alkylbenzenes, with an annual production of 730,000 tons (A. Mueller and M. Koepke, U.S. patent application 0330809 A1). Although no natural metabolic pathways for butanone formation have been identified, a biosynthetic approach to produce butanone in engineered *E. coli* by extending the *meso*-2,3 butanediol synthetic pathway using a promiscuous vitamin B_{12}-dependent glycerol dehydratase has been proposed (4). A similar strategy was undertaken by implementing this chimeric pathway in *Saccharomyces cerevisiae* for coproduction of butanone and 2-butanol (5). However, the butanone titers and yields with these approaches are considered limited, particularly for large-scale industrial adoption.

Our proposed butanone biosynthetic pathway starts with production of the nonnative metabolite propionyl-coenzyme A (propionyl-CoA) in *E. coli*, for use as a substrate for subsequent molecular fusion. Propionyl-CoA metabolism in wild-type *E. coli* is generally confined to selected reactions associated with either the thioesterification of propionate or the dissimilation of odd-chain fatty acids and several α-amino acids (e.g., L-threonine) (6, 7). Accordingly, to increase the propionyl-CoA pool in the cell, propionate must be exogenously supplemented in the culture medium or complex genetic approaches must be used to overcome the concerted feedback inhibition exerted by the amino acid biosynthetic pathways (7–9). Recently, we reported heterologous production of 1-propanol based on genomic activation of the dormant sleeping beauty mutase (Sbm) operon in *E. coli* (10, 11). This four-gene operon (i.e., *sbm-ygfD-ygfG-ygfH*) encodes various enzymes for extended dissimilation of succinate, and its functional expression redirects carbon flux toward the propionyl-CoA node, resulting in the production of nonnative C_3-fermentative products of 1-propanol and propionate (10, 11) (Fig. 1). The presence of propionyl-CoA opens an avenue for novel microbial synthesis of several nonnative metabolites, including butanone. Genomic activation of the Sbm operon not only transforms *E. coli* to be propanologenic but also introduces an intracellular "flux competition" between the traditional C_2-fermentative pathway (forming acetate and ethanol) and the novel C_3-fermentative pathway (forming propionate and 1-propanol). As a result, further biochemical and genetic strategies were required in this study to redirect carbon flux and to increase the size of the propionyl-CoA pool.

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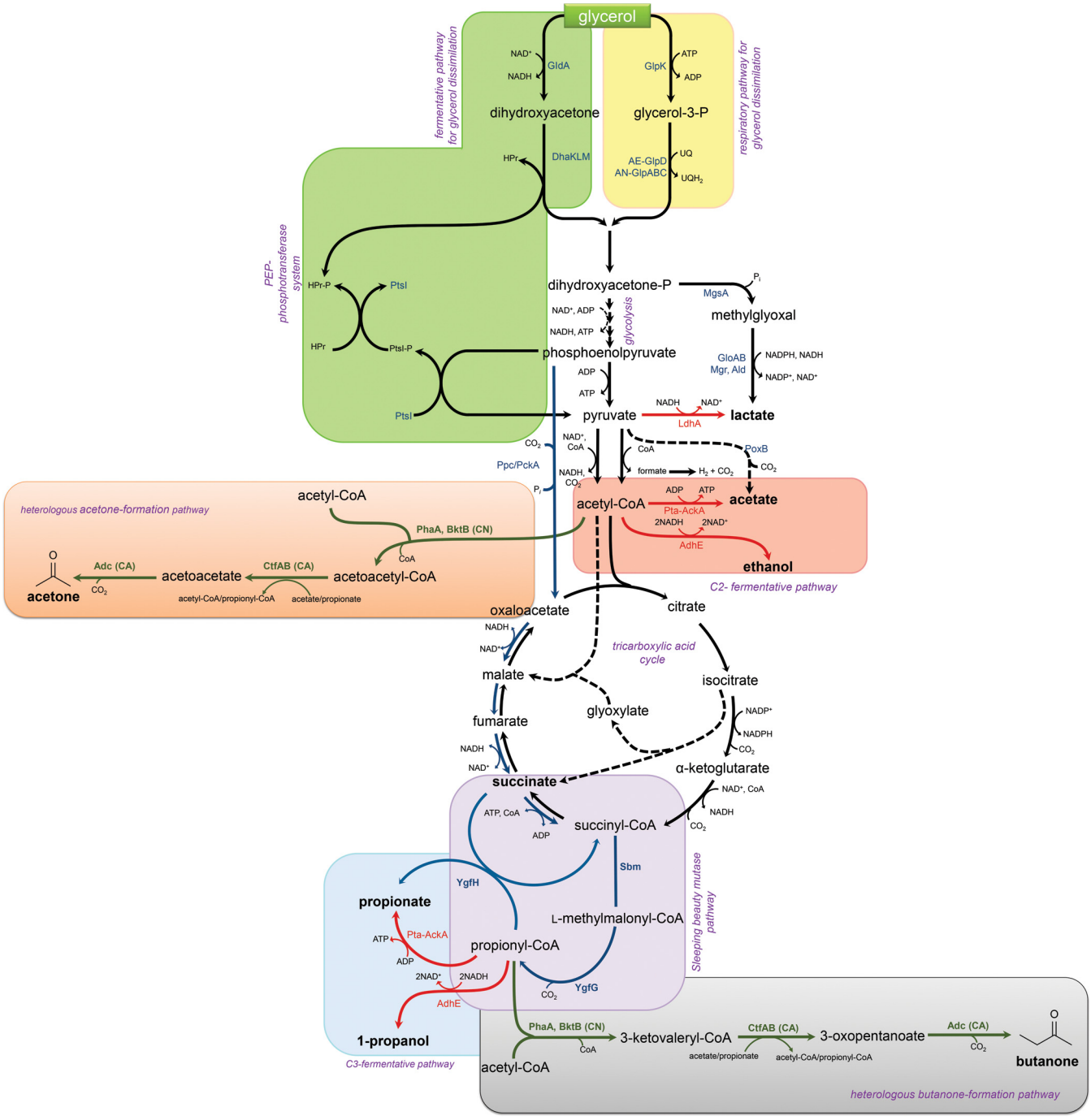

FIG 1 Schematic representation of the butanone biosynthetic pathway. Green text, heterologous enzymes from *Cupriavidus necator* (CN) and *Clostridium acetobutylicum* (CA); green box, fermentative pathway for glycerol dissimilation; yellow box, respiratory pathway for glycerol dissimilation; purple box, sleeping beauty mutase (Sbm) pathway; blue arrows, route to the C_3-fementative products; dark-orange box, C_2-fermentative pathway; blue box, C_3-fermentative pathway; light-orange box, heterologous acetone formation pathway; gray box, heterologous butanone formation pathway; blue text, relevant enzymes for production of various fermentative products as well as the enzymes of the respiratory and fermentative glycerol pathways and the Sbm pathway; red arrows, competing pathways at the pyruvate/acetyl-CoA and propionyl-CoA nodes. Abbreviations: HPr, phosphocarrier protein; P_i, inorganic phosphate; UQ, ubiquinone.

For butanone formation in the engineered *E. coli* strain, two major intracellular transformations were carried out (Fig. 1). First, propionyl-CoA was fused with acetyl-CoA to form 3-ketovaleryl-CoA (also referred to as 3-oxopentanoyl-CoA) via the CoA-dependent elongation pathway; this was implemented by expressing a set of promiscuous β-ketothiolases from *Cupriavidus necator* (formerly *Ralstonia eutropha*) (12). Next, 3-ketovaleryl-CoA was channeled into the clostridial pathway for acetone for-

TABLE 1 *E. coli* strains and plasmids used in this study

Strain or plasmid	Description and relevant genotype	Source
E. coli host strains		
HST08	F$^-$ *endA1 supE44 thi-1 recA1 relA1 gyrA96 phoA* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169* Δ(*mrr-hsdRMS-mcrBC*) Δ*mcrA* λ$^-$	TaKaRa Bio (Shiga, Japan)
BW25141	F$^-$ Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) Δ(*phoB-phoR*)*580* λ$^-$ *galU95* Δ*uidA3*::*pir*$^+$ *recA1 endA9*(del-ins)::FRT *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	Datsenko and Wanner (16)
BW25113	F$^-$ Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) λ$^-$ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	Datsenko and Wanner (16)
BW-ΔldhA	BW25113Δ*ldhA* null mutant	Srirangan et al. (10)
CPC-Sbm-Cm^r	BW-Δ*ldhA* P_{trc}::*sbm* (with FRT-Cm^r-FRT-P_{trc} cassette replacing 204 bp upstream of Sbm operon)	This study
CPC-Sbm	BW-Δ*ldhA* P_{trc}::*sbm* (with FRT-P_{trc} cassette replacing 204 bp upstream of Sbm operon)	This study
CPC-SbmΔ*adhE*	BW-Δ*ldhA* Δ*adhE* P_{trc}::*sbm* (with FRT-P_{trc} cassette replacing 204 bp upstream of Sbm operon)	This study
CPC-SbmΔ*pta*	BW-Δ*ldhA* Δ*pta* P_{trc}::*sbm* (with FRT-P_{trc} cassette replacing 204 bp upstream of Sbm operon)	This study
CPC-SbmΔ*glpD*	BW-Δ*ldhA* Δ*glpD* P_{trc}::*sbm* (with FRT-P_{trc} cassette replacing 204 bp upstream of Sbm operon)	This study
CPC-SbmΔ*dhaK*	BW-Δ*ldhA* Δ*dhaK* P_{trc}::*sbm* (with FRT-P_{trc} cassette replacing 204 bp upstream of Sbm operon)	This study
CPC-MEKCon1	CPC-Sbm/pK-PhaA and pCtfAB-Adc	This study
CPC-MEKCon2	CPC-Sbm/pK-BktB and pCtfAB-Adc	This study
CPC-MEKCon3	CPC-Sbm/pK-PhaA-BktB and pCtfAB	This study
CPC-MEKCon4	CPC-Sbm/pK-PhaA-BktB and pAdc	This study
CPC-MEK	CPC-Sbm/pK-PhaA-BktB and pCtfAB-Adc	This study
CPC-MEKΔ*adhE*	CPC-SbmΔ*adhE*/pK-PhaA-BktB and pCtfAB-Adc	This study
CPC-MEKΔ*pta*	CPC-SbmΔ*pta*/pK-PhaA-BktB and pCtfAB-Adc	This study
CPC-MEKΔ*glpD*	CPC-SbmΔ*glpD*/pK-PhaA-BktB and pCtfAB-Adc	This study
CPC-MEKΔ*dhaK*	CPC-SbmΔ*dhaK*/pK-PhaA-BktB and pCtfAB-Adc	This study
Plasmids		
pCP20	FLP$^+$ λ *cI857*$^+$ λ p_R Rep(pSC101 ori)(Ts) Ap^r Cm^r	Cherepanov and Wackernagel (18)
pKD46	RepA101(Ts) ori Ap^r *araC*-P_{araB}::*gam-bet-exo*	Datsenko and Wanner (16)
pTrc99a	ColE1 ori Ap^r P_{trc}	Amann et al. (44)
pKD3	R6K-γ ori Ap^r FRT-Cm^r-FRT	Datsenko and Wanner (16)
pK184	p15A ori Km^r P_{lac}::*lacZ*′	Jobling and Holmes (45)
pSOS95	repL ori ColE1 ori MLS^r Em^r Ap^r P_{thl}::*ctfAB-adc*	P. Soucaille and E. T. Papoutsakis, unpublished data
pK-PhaA	From pK184; P_{lac}::*phaA*	This study
pK-BktB	From pK184; P_{lac}::*bktB*	This study
pK-PhaA-BktB	From pK184; P_{lac}::*phaA-bktB*	This study
pCtfAB	From pTrc99a; P_{thl}::*ctfAB*	This study
pAdc	From pTrc99a; P_{thl}::*adc*	This study
pCtfAB-Adc	From pTrc99a; P_{thl}::*ctfAB-adc*	This study

mation; this was implemented by expressing acetoacetyl-CoA:acetate/butyrate:CoA transferase and acetoacetate decarboxylase from *Clostridium acetobutylicum*, for thioester hydrolysis and decarboxylation, respectively. It should be noted that the clostridial acetone formation pathway was previously expressed in *E. coli* for hydrolysis and decarboxylation of acetoacetyl-CoA, leading to acetone production (13). In addition to the aforementioned synthetic biology approaches, various genetic and metabolic factors and cultivation strategies to enhance the production of butanone were explored. To our knowledge, our engineered *E. coli* strains with butanone titers of up to 1.3 g liter^{-1} represent the most effective microbial platform for butanone production reported to date.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used in this study are listed in Table 1. The sequences of all oligonucleotides used for DNA cloning, verification, and homologous recombination are found in Table 2. Standard recombinant DNA technologies for molecular cloning were applied (14). *Pfu* and *Taq* DNA polymerases, T4 DNA ligase, and the large (Klenow) fragment of DNA polymerase I were obtained from New England Biolabs (Ipswich, MA, USA). All synthesized oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA). DNA sequencing was conducted by the Centre for Applied Genomics at the Hospital for Sick Children (Toronto, Canada). *E. coli* BW25141 was the parental strain for derivation of all mutant strains in this study, and *E. coli* HST08 was used for molecular cloning.

Activation of the genomic Sbm operon to form propanologenic *E. coli* CPC-Sbm was described previously (11). Briefly, the FRT-Cm^r-FRT cassette from pKD3 was PCR amplified using the c-frt primer set, whereas the *trc* promoter-operator region was PCR amplified using the c-ptrc primer set. The two DNA amplicons were fused together by splice overlap extension (SOE) PCR (15), using the forward primer of the c-frt primer set and the reverse primer of the c-ptrc primer set to generate the FRT-Cm^r-FRT-P_{trc} cassette. To generate the DNA cartridge for genomic integration, the FRT-Cm^r-FRT-P_{trc} cassette was PCR amplified using the r-frt-ptrc primer set, containing the 36-bp 5′ and 3′ homology arms. The homology arms were designed so that the FRT-Cm^r-FRT-P_{trc} cassette was inserted precisely upstream of the Sbm operon. λ-Red genomic recombineering was carried out as described by Datsenko and Wanner (16).

Gene (i.e., *adhE*, *pta*, *glpD*, and *dhaK*) knockouts were introduced into CPC-Sbm by P1 phage transduction (14), using the appropriate Keio Collection strains (Coli Genetic Stock Center, Yale University, New Haven, CT, USA) as donors (17). To eliminate the cotransduced FRT-Kn^r-FRT cassette, the transductant mutants were transformed

TABLE 2 Nucleotide sequences of the oligonucleotides used in this study

Primer set[a]	Sequences (5′ to 3′)[b]	Source
v-ldhA	GATAACGGAGATCGGGAATGATTAA; GGTTTAAAAGCGTCGATGTCCAGTA	Srirangan et al. (10)
v-adhE	ATCAGGTGTCCTGAACTGTGCG; TTGACCAGCGCAAATAACCCGATGA	This study
v-pta	GGCATGAGCGTTGACGCAATCA; CAGCTGTACGCGGTGATACTCAGG	This study
v-dhaK	CATCGAGGATAAACAGCGCA; ATCTGATAAAGCTCTTCCAGTGT	This study
v-glpD	CGTCAATGCTATAGACCACATC; TATTATTGAAGTTTGTAATATCCTTATCAC	This study
c-frt	AGATTGCAGCATTACACGTCTTGAG; CCAGCTGCATTAATGAATCGGGCCATGGTCCA TATGAATATCCTCC	This study
c-ptrc	CCGATTCATTAATGCAGCTGG; GGTCTGTTTCCTGTGTGAAATTGTTA	This study
r-frt-ptrc	**CTCGATTATGGTCACAAAGTCCTTCGTCAGGATTAA**AGATTGCAGCATTACACGTCTTGA; **GTTGGCAAGCTGTTGCCACTCCTGCACGTTAGACAT**GGTCTGTTTCCTGTGTGAAATTGT	This study
v-frt-ptrc	GCGCTCGACTATCTGTTCGTCAGCTC; TCGACAGTTTTCTCCCGACGGCTCA	This study
c-phaA1	**ACACAGGAAACAGCTATGAC**ATGACTGACGTTGTCATCGTATCC; **GAGCTCGAATTCGTAA TCAT**TTATTTGCGCTCGACTGCCA	This study
c-bktb1	**ACACAGGAAACAGCTATGAC**ATGACGCGTGAAGTGGTAGTG; **GAGCTCGAATTCGTAAT CAT**TCAGATACGCTCGAAGATGG	This study
c-pK184	GTCATAGCTGTTTCCTGTGTG; ATGATTACGAATTCGAGCTC	This study
c-phaA2	CATGATTACGAATTCGATGACTGACGTTGTCATCGTATCCGCCGCC; TACCACTTCA CGCGTCATGGTATATCTCCTTTATTTGCGCTCGACTGCCAGCGCC	This study
c-bktb2	AGGAGATATACCATGACGCGTGAAGTGGTAGTGGTGA; TACCGAGCTCGAATTCTCAGA TGCGTTCGAAGATAGCGGCAA	This study
c-ctf	CGCGTTGGTGCGGATATCGGTCGACTGTGGATGGAGTTAAGTC; TACCGAGCTCGAATTC CACACAGGAAACAGCTATGACCATG	This study
c-ctf-c3	GTTGGTGCGGATATCTTCGACTTTTTAACAAAATATATTG; TACCGAGCTCGAATTC CTAAACAGCCATGGGTCTAAG	This study
c-adc-c4A	GTTGGTGCGGATATCTTATTGAATAAAAGATATGAGAGATTTATC; **CCTTTAACA**TTTAAT CCCTCCTTTTAAATTC	This study
c-adc-c4B	**GGGATTAAA**TGTTAAAGGATGAAGTAATTAAAC; TACCGAGCTCGAATTCTTACTTAAGA TAATCATATATAACTTCAGC	This study

[a] v, verification primer; c, cloning primer; r, recombineering. Restriction recognition sequences are underlined and recombination homology arms for *in vivo* or *in vitro* recombination are in bold print.
[b] Each entry denotes a primer set (forward; reverse).

with pCP20 (18), a temperature-sensitive plasmid expressing a flippase (Flp) recombinase. Upon Flp-mediated excision of the Kn^r cassette, a single Flp recognition site (flippase recognition target [FRT] "scar site") was generated. Plasmid pCP20 was then removed by growing the cells at 42°C. The genotypes of the knockout strains derived were confirmed by colony PCR using the appropriate primer sets, as listed in Table 2.

The DNA fragment containing the three *C. acetobutylicum* ATCC 824 genes (*cftA-ctfB-adc*) under the control of the P_{thl} promoter was PCR amplified from pSOS95 (a kind gift from E. T. Papoutsakis, Delaware Biotechnology Institute, Newark, DE, USA) using the c-ctf primer set. It should be noted that this 2.3-kb PCR-amplified fragment also contained the Rho-independent transcriptional terminator from the clostridial *adc* gene. The amplified DNA fragment was then subcloned into the EcoRV and EcoRI restriction sites of pTrc99a, to generate pCtfAB-adc. Similarly, to generate control plasmid pCtfAB, *ctfA-ctfB* under the control of the P_{thl} promoter was PCR amplified from pSOS95 using the c-ctf-c3 primer set, and the amplified fragment was cloned into the EcoRV and EcoRI restriction sites of pTrc99a. To generate control plasmid pAdc, P_{thl} was PCR amplified using the c-adc-c4A primer set, whereas *adc* was PCR amplified from pSOS95 using the c-adc-c4B primer set. The two amplicons were then fused using Gibson enzymatic assembly (19) and cloned into the EcoRV and EcoRI restriction sites of pTrc99a.

The two β-ketothiolase genes were PCR amplified from the genomic DNA of the wild-type *C. necator* strain (ATCC 17699) using the c-phaA1 primer set for *phaA* and the c-bktb1 primer set for *bktB*. To generate plasmid pK-PhaA, harboring the *phaA* gene under the control of the P_{lac} promoter, the *phaA* amplicon was fused with PCR-linearized pK184 (linearized using the c-pK184 primer set) using Gibson enzymatic assembly. A clone with the correct transcriptional orientation of the *phaA* fragment with respect to the P_{lac} promoter was selected and verified by DNA sequencing. The same approach was used to generate plasmid pK-BktB, harboring the *bktB* gene under the control of the P_{lac} promoter. To generate the DNA cartridge containing the β-ketothiolases (i.e., PhaA and BktB), *phaA* and *bktB* were first individually PCR amplified from *C. necator* ATCC 17699 genomic DNA, using the c-phaA2 and c-bktb2 primer sets, respectively. The two DNA fragments were then transcriptionally fused with splice overlap extension (SOE) PCR (15), using the forward primer of c-phaA2 and the reverse primer of c-bktb2. The resulting fused fragment was cloned into the EcoRI restriction site of pK184. A clone with the correct transcriptional orientation of the *phaA-bktB* fragment with respect to the P_{lac} promoter was selected and verified by DNA sequencing, yielding pK-PhaA-BktB.

Media and cultivation conditions. All medium components were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA) except for glucose, yeast extract, and tryptone, which were obtained from BD Diagnostic Systems (Franklin Lakes, NJ, USA). Media were supplemented with antibiotics (30 μg ml^{-1} kanamycin and 12 μg ml^{-1} chloramphenicol) as required. For ketone production, the ketogenic (i.e., ketone-producing) *E. coli* strains (stored as glycerol stocks at −80°C) were streaked on LB agar plates with appropriate antibiotics and incubated at 37°C for 16 h. Single colonies were picked from LB plates to inoculate 30 ml SB medium (32 g $liter^{-1}$ tryptone, 20 g $liter^{-1}$ yeast extract, and 5 g $liter^{-1}$ NaCl) with appropriate antibiotics in 125-ml conical flasks. Overnight cultures were shaken at 37°C and 280 rpm in a rotary shaker (New Brunswick Scientific, Edison, NJ, USA) and used as seed cultures to inoculate 200 ml SB medium at 1% (vol/vol), with appropriate antibiotics, in 1-liter conical flasks. These second seed cultures were shaken at 37°C and 280 rpm for approximately 16 h. Cells were then harvested by centrifugation at 6,000 × *g* and 20°C for 15 min and were resuspended in 100 ml fresh LB

medium. The suspended culture was used to inoculate a 1-liter stirred-tank bioreactor (CelliGen 115; Eppendorf AG, Hamburg, Germany) to obtain a target initial optical density at 600 nm (OD_{600}) of ~5.5 (i.e., ~3.5 g dry cell weight [DCW] liter^{-1}), and the bioreactor was operated anaerobically, microaerobically, or semiaerobically at 30°C and 430 rpm. The production medium in the bioreactor contained 30 g liter^{-1} glycerol or 30 g liter^{-1} glucose, 0.23 g liter^{-1} K_2HPO_4, 0.51 g liter^{-1} NH_4Cl, 49.8 mg liter^{-1} $MgCl_2$, 48.1 mg liter^{-1} K_2SO_4, 1.52 mg liter^{-1} $FeSO_4$, 0.055 mg liter^{-1} $CaCl_2$, 2.93 g liter^{-1} NaCl, 0.72 g liter^{-1} tricine, 10 g liter^{-1} yeast extract, 10 mM $NaHCO_3$, 0.2 μM cyanocobalamin (vitamin B_{12}), a 1:1,000 dilution (i.e., 1 ml liter^{-1}) of Trace Metal Mix A5 [2.86 g liter^{-1} H_3BO_3, 1.81 g liter^{-1} $MnCl_2{\cdot}4H_2O$, 0.222 g liter^{-1} $ZnSO_4{\cdot}7H_2O$, 0.39 g liter^{-1} $Na_2MoO_4{\cdot}2H_2O$, 79 μg liter^{-1} $CuSO_4{\cdot}5H_2O$, 49.4 μg liter^{-1} $Co(NO_3)_2{\cdot}6H_2O$] (20), and appropriate antibiotics and was supplemented with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Anaerobic conditions were maintained by constant bubbling of nitrogen into the bulk culture at 0.1 volume of air/volume of bioreactor/min (vvm). Microaerobic conditions were maintained by purging air into the headspace at 0.1 vvm. Semiaerobic conditions were maintained by purging air into the bulk culture at 0.1 vvm. The pH of the production culture was maintained at 7.0 ± 0.1 with 30% (vol/vol) NH_4OH and 15% (vol/vol) H_3PO_4. All cultivation experiments were performed in duplicate.

Offline analyses and metabolite detection. Culture samples were appropriately diluted with saline for measurement of OD_{600} using a spectrophotometer (DU520; Beckman Coulter, Fullerton, CA). Cell-free supernatant was collected and filter sterilized for titer analysis of glycerol and various metabolites using a high-performance liquid chromatograph (HPLC) (LC-10AT; Shimadzu, Kyoto, Japan) with a refractive index detector (RID) (RID-10A; Shimadzu, Kyoto, Japan) and a chromatographic column (Aminex HPX-87H; Bio-Rad Laboratories, Hercules, CA, USA). The column temperature was maintained at 65°C, and the mobile phase was 5 mM H_2SO_4 (pH 2.0), with a flow rate of 0.6 ml min^{-1}. The RID signal was acquired and processed with a data-processing unit (Clarity Lite; DataApex, Prague, Czech Republic). While HPLC was used as the primary analytic method for quantification, the identity of all volatile metabolites (i.e., acetone, butanone, ethanol, and 1-propanol) was verified by gas chromatography (GC) with flame ionization detection (FID). The GC system was an Agilent 6890 series system (Agilent Technologies, Santa Clara, CA, USA) with a J & W Scientifics DB Wax column (30 m by 0.53 mm; film thickness, 1 μm) (Agilent Technologies). The oven program was as follows: the temperature was set initially at 80°C for 5 min, ramped to 230°C at 7.5°C min^{-1}, and then ramped to 260°C at a higher rate of 10°C min^{-1}, followed by maintenance of that temperature for analysis. The FID detector was held at 330°C. The injection volume was 1 μl, with injection at a split ratio of 15:1. Helium was used as the carrier gas.

RNA extraction, microarray hybridization, and gene expression analyses. Two aliquots of 1-ml culture samples of CPC-Sbm cells cultivated anaerobically with glycerol or glucose as the major carbon source were collected in the mid-exponential growth phase. Total RNA extraction was performed in duplicate for each sample using a High Pure RNA isolation kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions. The integrity of extracted total RNA was verified by agarose gel electrophoresis, and the RNA quality was evaluated based on the concentration and the OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios. Samples were adjusted to a final concentration of 1 μg μl^{-1} total RNA. Duplicate aliquots of 100 μl of the total RNA samples were subjected to microarray transcriptomic analysis (conducted at the Centre for Applied Genomics at the Hospital for Sick Children, Toronto, Ontario, Canada) using Affymetrix *E. coli* Genome 2.0 GeneChips (Affymetrix, Santa Clara, CA, USA). Statistical data analyses, bioinformatic annotation, and retrieval of hybridization intensity raw data from the microarray experiments were carried out using Bioconductor version 3.0 (www.bioconductor.org) and supporting R-Project Bioconductor statistical tool packages (www.cran.r-project.org) (21–23). Normalized gene expression values and expression summaries were generated for each array chip using the Bioconductor Robust Multichip Average (RMA) normalization package, with default parameters for raw data background correction, quantile normalization, and signal summation. The Bioconductor limma package was used to identify differentially expressed genes. Annotation of the probe sets was performed using the Affymetrix *E. coli* Genome 2 annotation file.

RESULTS

Implementation of synthetic pathway for ketone production in *E. coli*. A prerequisite for *in vivo* butanone biosynthesis via our proposed pathway is the intracellular presence of propionyl-CoA. This can be provided by activation of the Sbm pathway for extended dissimilation of succinyl-CoA (Fig. 1) (11). For molecular heterofusion of propionyl-CoA with acetyl-CoA to form 3-ketovaleryl-CoA, two promiscuous β-ketothiolase genes (i.e., *phaA* and *bktB* from *C. necator*) were expressed either individually (using pK-PhaA or pK-BktB) or in combination (using pK-PhaA-BktB) for CoA-dependent chain elongation. It should be noted that PhaA and BktB have enzymatic specificity toward short-chain and long-chain CoA molecules, respectively (12). However, β-ketothiolases can also mediate homofusion of two acetyl-CoA moieties into acetoacetyl-CoA. For subsequent ketone biosynthesis through thioester hydrolysis and decarboxylation of 3-ketovaleryl-CoA and acetoacetyl-CoA, we introduced the clostridial acetone formation pathway by fusing the three relevant genes in the transcriptional order *ctfA-ctfB-adc* in plasmid pCtfAB-Adc, for episomal expression under the regulation of the P_{thl} promoter. Each of the β-ketothiolase expression plasmids (i.e., pK-PhaA, pK-BktB, and pK-PhaA-BktB) was cotransferred with pCtfAB-Adc into CPC-Sbm to generate the ketogenic strains CPC-MEKCon1, CPC-MEKCon2, and CPC-MEK, respectively (Table 1 and Fig. 2).

To demonstrate ketone production, CPC-MEKCon1, CPC-MEKCon2, and CPC-MEK cells were cultivated in a bioreactor. However, culture conditions appear to critically affect ketone production. It was shown previously that glycerol, with a greater degree of reduction, outperformed glucose as the major carbon source for anaerobic cultivation of the propanologenic *E. coli* (11). Comparative transcriptomic analysis of the propanologenic strain CPC-Sbm cultivated with glucose or glycerol also indicated that most of the Sbm operon genes were upregulated when glycerol was used as the major carbon source (see Fig. S1 in the supplemental material). However, glycerol dissimilation was significantly hampered by anaerobiosis (11), and anaerobic cultivation conditions normally favor alcohologenesis. Given that ketones are less reduced than alcohols, their biosynthesis may require a more aerobic environment. Therefore, in addition to strict anaerobiosis, microaerobic and semiaerobic conditions were established by purging air at a low flow rate of 0.1 vvm into the headspace and the bulk culture, respectively, while all other cultivation parameters remained the same. These culture conditions were used to characterize CPC-MEKCon1, CPC-MEKCon2, and CPC-MEK with respect to their ketone-producing capacities, and the cultivation results are summarized in Fig. 3 (see also Fig. S2 and Table S1 in the supplemental material).

Glycerol dissimilation and cell growth were low upon anaerobic cultivation of these *E. coli* strains (data not shown) but were much improved under oxygenic conditions. While CPC-MEKCon1 and CPC-MEKCon2 were competent producers of acetone, only trace levels of butanone were detected in these strains

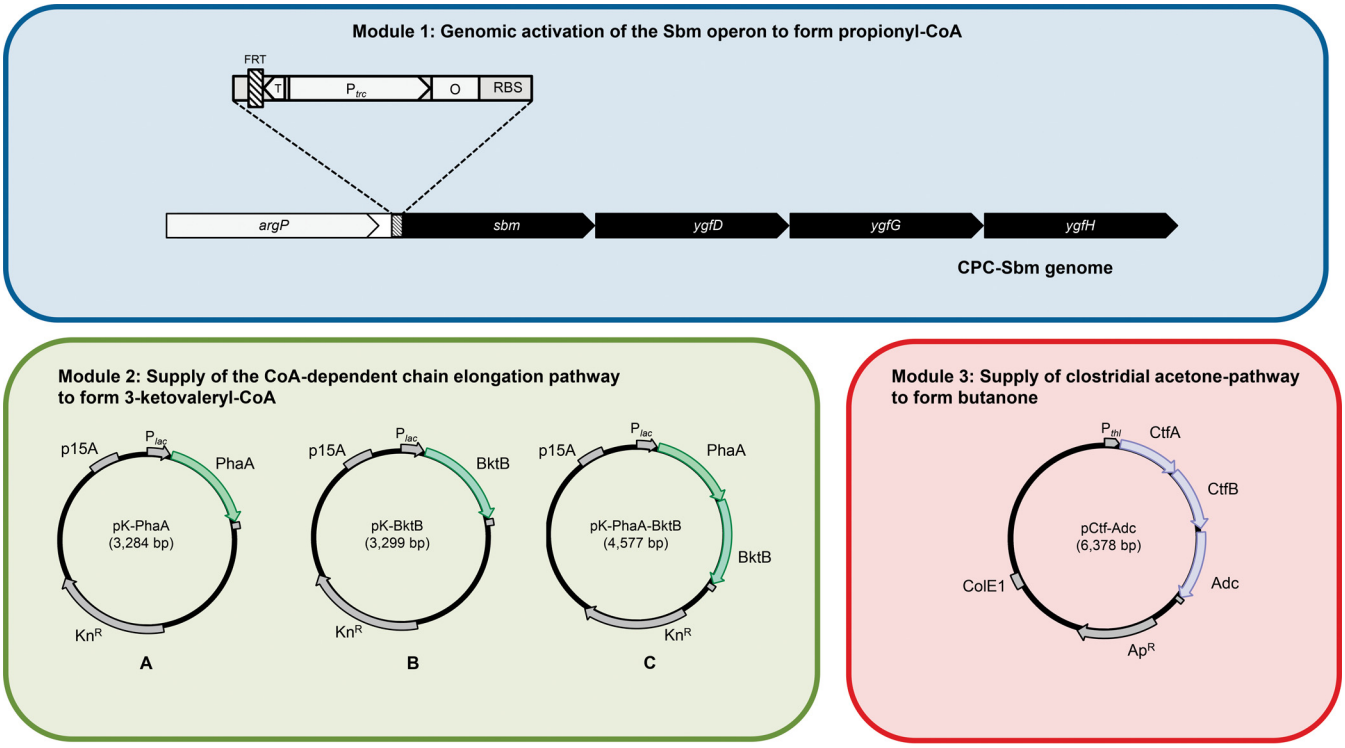

FIG 2 Synthetic biology strategies used for heterologous production of butanone in engineered *E. coli*. The ketone biosynthesis pathway consisted of three modules, as follows: module 1, the chromosomally activated Sbm pathway in CPC-Sbm for supply of propionyl-CoA; module 2, a set of promiscuous β-ketothiolases (i.e., PhaA and BktB) expressed independently or in tandem to generate the CoA-dependent chain elongation pathway for either homofusion of acetyl-CoA or heterofusion of acetyl-CoA and propionyl-CoA; module 3, the clostridial acetone formation pathway (i.e., CtfAB-Adc) for thioester hydrolysis of the fused CoA intermediate and subsequent decarboxylation. All three modules were assembled together to generate various ketone production strains, i.e., CPC-MEKCon1 contained modules 1, 2A, and 3, CPC-MEKCon2 contained modules 1, 2B, and 3, and CPC-MEK contained modules 1, 2C, and 3. The single-gene knockouts (i.e., *adhE*, *pta*, *glpD*, and *dhaK*) are all variants of the parent ketogenic strain CPC-MEK. Abbreviations: FRT, flippase recognition target; O, operator; RBS, ribosome binding site; T, terminator.

under microaerobic cultivation conditions (Fig. 3A and B; see also Fig. S2A and B and Table S1 in the supplemental material). Interestingly, heterologous expression of both β-ketothiolases, PhaA and BktB, in CPC-MEK greatly increased ketone production under microaerobic cultivation conditions, with 0.43 g liter^{-1} acetone and 0.33 g liter^{-1} butanone (Fig. 3C; see also Fig. S2C and Table S1). Semiaerobic cultivation consumed 30 g liter^{-1} glycerol in 19 h, compared to 45 h for microaerobic cultivation, and

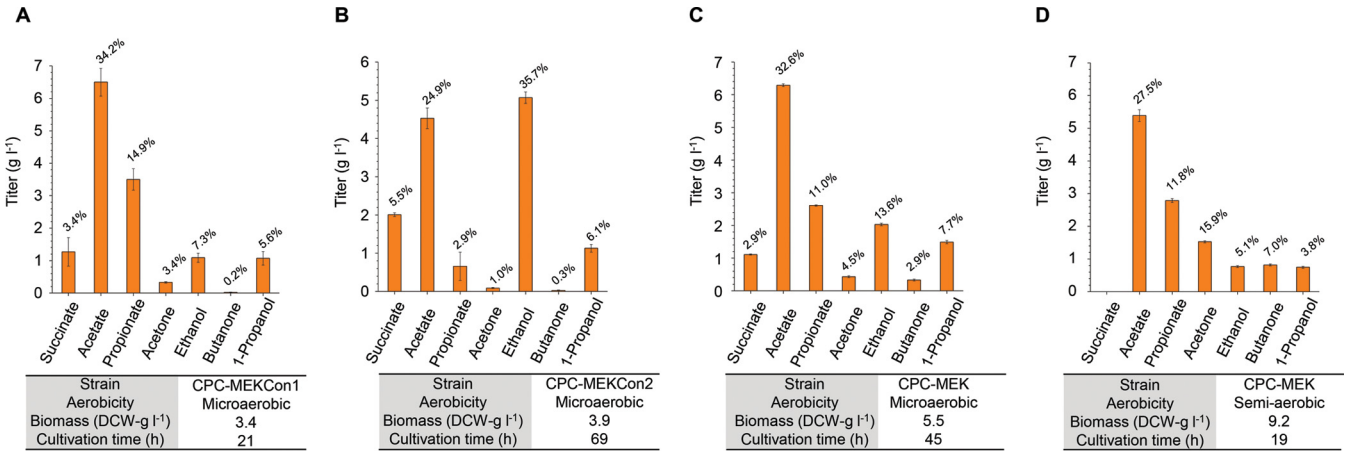

FIG 3 Construction of the biosynthetic pathway for ketone production in *E. coli*. (A and B) Final metabolite titers, including acetone and butanone, with microaerobic cultivation of the ketogenic strains CPC-MEKCon1 (A) and CPC-MEKCon2 (B), using 30 g liter^{-1} glycerol as the major carbon source. (C and D) Final metabolite titers, including acetone and butanone, with microaerobic cultivation (C) and semiaerobic cultivation (D) of the ketogenic strain CPC-MEK, using 30 g liter^{-1} glycerol as the major carbon source. All of the strains were induced at the start of the batch cultivation with 0.1 mM IPTG. Error bars, standard deviations ($n = 2$). The metabolite distribution (denoted as the percentage of spent glycerol converted to a specific metabolite) is indicated above the bars.

yielded higher ketone titers (i.e., 1.53 g liter^{-1} acetone and 0.82 g liter^{-1} butanone, respectively), with the total ketone production accounting for ~23% of the dissimilated glycerol (Fig. 3D; see also Fig. S2D and Table S1). More-oxygenic conditions produced by further increasing the air-purging rate to 1 vvm into the bulk culture, however, impaired ketone production, with most dissimilated glycerol being directed toward acidogenesis (namely, the formation of acetate and propionate) and biomass formation (data not shown). Accordingly, unless otherwise specified, subsequent cultivations were conducted under semiaerobic conditions using glycerol as the major carbon source. It should be noted that no ketones or other major metabolites were detected when glycerol was not supplemented in the medium and no ketones were detected with CPC-MEKCon3 or CPC-MEKCon4, in which *ctfAB* or *adc* was expressed independently (Table 1; see also Table S1), suggesting that all carbons among fermentative end products are derived from glycerol and that all three enzymatic components of the clostridial acetone formation pathway are required for ketone synthesis under these culture conditions. In contrast, nonpropanologenic *E. coli* BW-Δ*ldhA* harboring pK-PhaA-BktB and pCtfAB-Adc produced trace amounts of acetone only, not butanone (data not shown), implying that propionyl-CoA acts as a precursor for butanone biosynthesis. Lastly, for pK-PhaA-BktB, IPTG induction was critical for functional expression of β-ketothiolases in CPC-MEK (see Table S1). These control experiments confirmed not only functional expression of the two heterologous β-ketothiolases, PhaA and BktB, but also their synergistic effects on CoA-dependent chain elongation, particularly associated with the substrate of propionyl-CoA.

Engineering of *E. coli* to enhance ketone biosynthesis. (i) Effect of blocking alcohologenesis. While cell growth and glycerol dissimilation were improved by introducing oxygenic conditions to favor ketone biosynthesis in CPC-MEK, the formation of ethanol and that of 1-propanol may represent competing pathways, particularly under microaerobic conditions. Hence, we further manipulated CPC-MEK by disrupting the *adhE* gene encoding alcohol dehydrogenase, and the ketone-producing capacity of the resulting mutant CPC-MEKΔ*adhE* was characterized (Fig. 4A and B; see also Fig. S3A and B and Table S2 in the supplemental material). As expected, inactivation of *adhE* abolished the formation of ethanol and 1-propanol without harming cell growth and glycerol dissimilation. Under microaerobic conditions, ketone production was significantly enhanced with abolished alcohologenesis, from 0.43 g liter^{-1} acetone and 0.33 g liter^{-1} butanone for CPC-MEK to 0.84 g liter^{-1} acetone and 0.94 g liter^{-1} butanone for CPC-MEKΔ*adhE*. Such improvement was less drastic under semiaerobic conditions, with an ~30% increase in the butanone titer, i.e., from 0.82 g liter^{-1} for CPC-MEK to 1.07 g liter^{-1} for CPC-MEKΔ*adhE*.

(ii) Effect of blocking acidogenesis. While the ketone-producing capacity of CPC-MEK was enhanced by introducing oxygenic conditions, this aeration strategy also increased acidogenesis, with the sum of the acetate and propionate titers accounting for up to ~40% of dissimilated glycerol (see Table S1 in the supplemental material); this phenotype was also observed in the *adhE*-null mutant CPC-MEKΔ*adhE*. Therefore, we inactivated the phosphotransacetylase (Pta)-acetate kinase (AckA) pathway in CPC-MEK, and the ketone-producing capacity of the resulting mutant, CPC-MEKΔ*pta*, was characterized (Fig. 4C; see also Fig. S3C and Table S2 in the supplemental material). Inactivation of the Pta-AckA pathway significantly redistributed metabolites. While it was expected that knocking out *pta* would drastically reduce acidogenesis, alcohologenesis was also abolished in CPC-MEKΔ*pta*, resulting in the production of acetone (2.0 g liter^{-1}) and butanone (0.94 g liter^{-1}). However, these metabolic perturbations led to severe carbon loss, with the entire accumulated metabolites accounting for only 31% of dissimilated glycerol.

Manipulation of glycerol dissimilation to enhance ketone biosynthesis. In *E. coli*, two alternative pathways, i.e., the respiratory GlpK-GlpD and fermentative GldA-DhaK pathways (24), mediate dissimilation of glycerol prior to their merging with the glycolytic trunk. We recently observed that, upon genomic activation of the Sbm operon, the pathway utilization for glycerol dissimilation can potentially affect carbon flux competition between the C_2- and C_3-fermentative pathways (11, 25). To assess the contributions of the two glycerol dissimilation pathways to the production of the C_3-fermentative products, we first engineered CPC-Sbm by inactivating either of the two pathways, resulting in the derivation of two mutant strains, i.e., CPC-SbmΔ*glpD* and CPC-SbmΔ*dhaK*, containing a single deletion in *glpD* and *dhaK*, respectively, and their cultivation performances were evaluated (see Fig. S4A to C and Table S2 in the supplemental material). Inactivation of either pathway resulted in major changes in biomass formation, carbon flux distribution, and metabolite profiles. In comparison with the parental strain CPC-Sbm, decreased alcohologenesis and increased acidogenesis were observed for both knockout mutants, with propionate titers of 5.2 g liter^{-1} for CPC-SbmΔ*glpD* and 11.8 g liter^{-1} for CPC-SbmΔ*dhaK*. The substantial increases in the C_3/C_2 fermentative product ratio and the propionate/acetate ratio for CPC-SbmΔ*dhaK*, compared to CPC-Sbm (see Fig. S4D), suggest that inactivation of the fermentative glycerol dissimilation pathway could be used to modulate the "flux competition" between the C_2- and C_3-fermentative pathways. To potentially increase the level of propionyl-CoA, initial glycerol dissimilation should be channeled through the respiratory pathway.

To evaluate the ketone-producing capacities of these mutant strains further, CPC-SbmΔ*glpD* and CPC-SbmΔ*dhaK* were transformed with pK-PhaA-BktB-and pCtfAB-Adc to generate strains CPC-MEKΔ*glpD* and CPC-MEKΔ*dhaK*, respectively. Semiaerobic cultivations of the resulting strains were conducted, and the results are summarized in Fig. 4D and E (see also Fig. S5A and B and Table S2 in the supplemental material). For both mutants with slightly reduced glycerol dissimilation rates, compared to the parental strain CPC-MEK, alcohologenesis was completely abolished and acidogenesis was significantly reduced. The dissimilated glycerol was primarily directed toward biomass formation with limited ketone production (i.e., 0.60 g liter^{-1} acetone and 0.64 g liter^{-1} butanone) for CPC-MEKΔ*glpD*. However, higher-level ketogenesis was observed for CPC-MEKΔ*dhaK*, leading to coproduction of 2.89 g liter^{-1} acetone and 1.3 g liter^{-1} butanone. The theoretical yields of acetone and butanone based on our proposed biosynthetic pathways with glycerol as the substrate are 0.315 g/g and 0.39 g/g, respectively. As a result, our reported data for CPC-MEKΔ*dhaK* cultivation (i.e., 0.0963 g acetone/g glycerol and 0.0433 g butanone/g glycerol) suggest that approximately 42% of glycerol was utilized toward ketone biosynthesis. To our knowledge, this represents one of the highest reported butanone titers for microbial production.

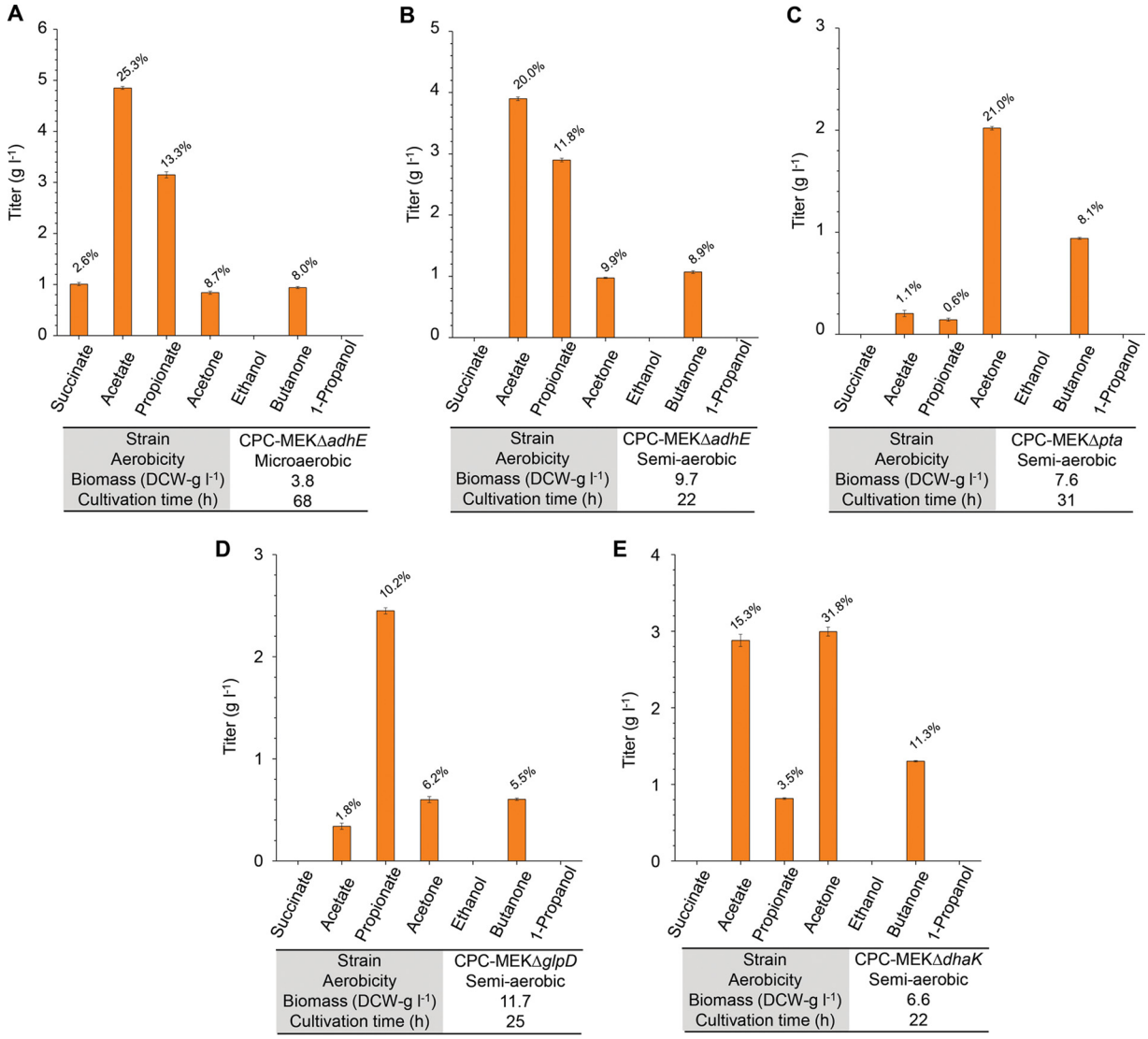

FIG 4 Enhancement of ketone production in engineered *E. coli*. Final metabolite titers with microaerobic cultivation of CPC-SbmΔ*adhE* (A), semiaerobic cultivation of CPC-SbmΔ*adhE* (B), semiaerobic cultivation of CPC-SbmΔ*pta* (C), semiaerobic cultivation of CPC-SbmΔ*glpD* (D), and semiaerobic cultivation of CPC-SbmΔ*dhaK* (E), using 30 g liter^{-1} glycerol as the major carbon source, are shown. All of the strains were treated with 0.1 mM IPTG at the start of the batch cultivation. Error bars, standard deviations ($n = 2$). The metabolite distribution (denoted as the percentage of spent glycerol converted to a specific metabolite) is indicated above the bars.

DISCUSSION

Implementing recursive pathways into genetically tractable microorganisms is particularly useful for the production of longer-chain chemicals, as the key bond-forming functional group of the substrate is regenerated in each reaction cycle (26). Here we further engineered propanologenic *E. coli* strains by episomally expressing (i) a set of promiscuous β-ketothiolases (i.e., PhaA and BktB) from *C. necator* for CoA-dependent chain elongation for either homofusion of acetyl-CoA or heterofusion of acetyl-CoA and propionyl-CoA and (ii) a clostridial acetone formation pathway for thioester hydrolysis of the fused CoA intermediate and subsequent decarboxylation. It should be noted that PhaA and BktB can synergistically enhance CoA-dependent chain elongation and, therefore, the strain's ketone-producing capacity. The genetic strategies led to the derivation of *E. coli* strains for the coproduction of acetone and butanone.

Since the CoA-dependent chain elongation reaction catalyzed by β-ketothiolases can be a major kinetic barrier (27), ensuring the abundance of key precursors of acetyl-CoA and propionyl-CoA becomes crucial. Activation of the Sbm pathway not only directed dissimilated carbon flux toward the propionyl-CoA node but also established an intracellular flux competition between the C_2-fermentative and C_3-fermentative pathways; the carbon source also appears to critically affect such competition. Glycerol was more effective than glucose as the major carbon source for cultivation of *E. coli* CPC-Sbm for 1-propanol production (11), implying a more active Sbm pathway and higher propionyl-CoA levels under such conditions. This observation was corroborated by a comparative transcriptomic analysis of CPC-Sbm cultivated with glucose or glycerol (see Fig. S1 in the supplemental material). Compared to cells cultivated in glucose, several of the Sbm operon genes and other glycolytic enzyme genes, such as *pckA* and *ppsA*,

were upregulated in cells cultivated with glycerol. Tricarboxylic acid (TCA) cycle genes, including *sdhB*, *sucC*, *sucD*, *sucA*, and *sucB*, in the pathway toward the node of succinyl-CoA (i.e., the starting precursor into the Sbm pathway) were also upregulated. Furthermore, given that ketones are less reduced than alcohols, ketogenic *E. coli* should not be cultivated under strict anaerobic conditions, which not only affect glycerol dissimilation but also favor alcohologenesis. Under slightly oxygenic (i.e., microaerobic or semiaerobic) conditions, not only were cell growth and glycerol dissimilation improved but also the cell's capacity for redox-balanced biosynthesis of metabolites through anaerobic fermentation was preserved. Our results suggest that ketone production was most effective under semiaerobic conditions. Interestingly, both CtfAB and Adc enzymes from anaerobic clostridia appear to be active under such culture conditions.

Disruption of *adhE* completely abolished alcohologenesis and increased acetone and butanone titers by >2-fold under microaerobic conditions. In addition to carbon flux redirection, the enhanced ketogenesis can be associated with potential elimination of alcohol inhibition of clostridial CoA transferase (28). Our results contrast with an earlier observation that ethanol formation is indispensable for respirofermentative utilization of glycerol (24). While such a genetic effect in enhancing ketogenesis was limited under semiaerobic conditions for acetone, the butanone titer was increased by ~30%. Also, it should be noted that the enhanced ketogenesis appeared to occur simultaneously with increased propionate titers and reduced acetate titers, suggesting that knocking out *adhE* can potentially enhance the intracellular propionyl-CoA pool by limiting 1-propanol formation.

On the other hand, knocking out *pta* significantly reduced acidogenesis while completely abolishing alcohologenesis, resulting in almost homoketogenic behavior. These results are similar to those of a previous study of semiaerobic cultivation with glycerol as the major carbon source, in which inactivation of the Pta-Ack pathway led to significant changes in the distribution of metabolites, namely, reduced ethanol production and overall increases in oxidized metabolites (24). It should be noted that acetyl phosphate, the intermediate in the Pta-AckA pathway, serves as an important metabolite for global regulation of gene expression and other fundamental processes and inactivating this pathway can potentially induce metabolic complications (29). For instance, elimination of acetyl phosphate can lead to inadequate turnover of the sigma factor RpoS and overall reductions in growth and metabolite formation (30, 31). Although this phenomenon is well known and its etiology is not fully understood, it could be associated with the depletion of free intracellular CoA moieties caused by low rates of acetyl-CoA turnover (26). Furthermore, while the residual acetate can be produced via decarboxylation of pyruvate catalyzed by pyruvate oxidase (encoded by *poxB*) (24) and the residual propionate production can be mediated by propionyl-CoA/succinyl-CoA transferase (encoded by *ygfH*), the homoketogenic behavior of this strain did not lead to higher ketone yields. Such low-level ketogenesis can potentially be associated with low levels of acid production since, for acetone-butanol-ethanol (ABE) fermentation in clostridia, acetone production is coupled with acetate and butyrate uptake via CoA transferase, for continuous regeneration of acetyl-CoA and butyryl-CoA (13). It is thus difficult to decouple acidogenesis from ketogenesis without hampering cell growth and glycerol dissimilation. An alternative approach for coping with acetate and propionate accumulation can be recycling of excreted acetate and propionate to form acetyl-CoA in an ATP-dependent manner, by overexpressing acetyl-CoA synthetase (encoded by *acs*) (32). Other strategies include establishing thioesterase-mediated ketone production platforms by relying on acetate-independent pathways (33) or fatty acid β-oxidation pathways (34).

Our results show that directing initial glycerol dissimilation through the respiratory GlpK-GlpD pathway can enhance butanone production, potentially due to increased levels of propionyl-CoA. A critical factor limiting biosynthesis based on the use of glycerol is the diversion of carbon flux at the phosphoenolpyruvate (PEP) node toward being a phosphate donor to support glycerol dissimilation via the fermentative GldA-DhaK pathway. Such a competing need for PEP as a phosphate donor is eliminated in the mutant CPC-MEKΔ*dhaK*, ostensibly increasing the PEP pool. The increased PEP pool potentially enhances propionyl-CoA production, as more carbon flux is diverted toward oxaloacetate (OAA) via the anaplerotic reactions catalyzed by the endogenous PEP carboxylase or PEP carboxykinase (encoded by *ppc* or *pckA*, respectively) (24, 35, 36). Similar to other Gram-negative bacteria that utilize glycerol (e.g., *Klebsiella pneumoniae*), inactivating the fermentative GldA-DhaK pathway in *E. coli* enhances acetate formation (24, 37). Inactivating *dhaK* is known to decrease the $NADH/NAD^+$ ratio, thus reducing the biosynthesis of reduced products such as ethanol and 1-propanol. The cell potentially compensates for the NADH deficiency by enhancing acetate formation for ATP production (24, 37). An increase in the PEP pool can also result in the decarboxylation of pyruvate to acetate catalyzed by PoxB (38). On the other hand, routing glycerol dissimilation through the fermentative GldA-DhaK pathway in the mutant strain CPC-MEKΔ*glpD* can enhance such PEP competition and divert the carbon flux away from the C_3-fermentative pathway, resulting in less butanone production. It should also be noted that GlpD plays a role in balancing the intracellular PEP and OAA pools (36, 39), and such a function appears to be critical for directing more carbon flux toward the propionyl-CoA node for butanone biosynthesis.

Engineering of *E. coli* for coproduction of acetone and butanone was demonstrated, with up to 42% of the spent carbon source of glycerol being utilized for ketone biosynthesis. While the current results appear to be promising, large-scale production will require derivation of superior ketogenic strains by targeting key steps in the ketone production pathway, eliminating latent metabolic bottlenecks and imbalances, and substantially reducing byproduct formation. For instance, 3-ketovaleryl-CoA and acetoacetyl-CoA are converted to 3-oxopentoate and acetoacetate, respectively, via the clostridial CoA transferase with the use of acetate as the major CoA acceptor, resulting in recycling of acetyl-CoA. We are exploring several higher-chain CoA transferases (e.g., 3-oxoadipate:succinyl-CoA transferase), which can potentially use propionate or succinate as the CoA acceptor and promote recycling of propionyl-CoA. It should be noted that butanone (and to a lesser extent acetone) can also be used as an intermediate for the biological conversion of other value-added products such as ethyl esters (40) and secondary alcohols such as 2-butanol (5) and isopropanol (41). Lastly, in addition to medium-chain methyl ketones, CoA-dependent elongation pathways can be applied for the biosynthesis of several other long-chain oleochemicals, such as fatty acid ethyl esters, fatty alcohols and

amines, paraffins, and olefins (42, 43), thus expanding the scope of whole-cell biocatalytic platforms.

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K.S. conceived the study, formulated the research plan, coordinated the research team, carried out experiments, performed result interpretation and data analysis, and drafted the manuscript. X.L., L.A., and M.B. participated in the design of the study, helped to conduct experiments, and performed result interpretation and data analysis. M.M.-Y. and C.P.C. conceived, planned, supervised, and managed the study and helped to draft the manuscript. All authors read and approved the final manuscript.

We declare no competing financial interests.

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REFERENCES

1. **Bond-Watts BB, Bellerose RJ, Chang MC.** 2011. Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways. Nat Chem Biol **7:**222–227. http://dx.doi.org/10.1038/nchembio.537.
2. **Lee JW, Na D, Park JM, Lee J, Choi S, Lee SY.** 2012. Systems metabolic engineering of microorganisms for natural and non-natural chemicals. Nat Chem Biol **8:**536–546. http://dx.doi.org/10.1038/nchembio.970.
3. **Hoell D, Mensing T, Roggenbuck R, Sakuth M, Sperlich E, Urban T, Neier W, Strehlke G.** 2000. 2-Butanone. Wiley-VCH Verlag, Weinheim, Germany.
4. **Yoneda H, Tantillo DJ, Atsumi S.** 2014. Biological production of 2-butanone in *Escherichia coli*. ChemSusChem **7:**92–95. http://dx.doi.org/10.1002/cssc.201300853.
5. **Ghiaci P, Norbeck J, Larsson C.** 2014. 2-Butanol and butanone production in *Saccharomyces cerevisiae* through combination of a B_{12} dependent dehydratase and a secondary alcohol dehydrogenase using a TEV-based expression system. PLoS One **9:**e102774. http://dx.doi.org/10.1371/journal.pone.0102774.
6. **Brock M, Maerker C, Schütz A, Völker U, Buckel W.** 2002. Oxidation of propionate to pyruvate in *Escherichia coli*. Eur J Biochem **269:**6184–6194. http://dx.doi.org/10.1046/j.1432-1033.2002.03336.x.
7. **Shen C, Liao J.** 2013. Synergy as design principle for metabolic engineering of 1-propanol production in *Escherichia coli*. Metab Eng **17:**12–22. http://dx.doi.org/10.1016/j.ymben.2013.01.008.
8. **Shen CR, Liao JC.** 2008. Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metab Eng **10:**312–320. http://dx.doi.org/10.1016/j.ymben.2008.08.001.
9. **Pfeifer BA, Admiraal SJ, Gramajo H, Cane DE, Khosla C.** 2001. Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*. Science **291:**1790–1792. http://dx.doi.org/10.1126/science.1058092.
10. **Srirangan K, Akawi L, Liu X, Westbrook A, Blondeel EJ, Aucoin MG, Moo-Young M, Chou CP.** 2013. Manipulating the sleeping beauty mutase operon for the production of 1-propanol in engineered *Escherichia coli*. Biotechnol Biofuels **6:**139. http://dx.doi.org/10.1186/1754-6834-6-139.
11. **Srirangan K, Liu X, Westbrook A, Akawi L, Pyne M, Moo-Young M, Chou CP.** 2014. Biochemical, genetic, and metabolic engineering strategies to enhance coproduction of 1-propanol and ethanol in engineered *Escherichia coli*. Appl Microbiol Biotechnol **98:**9499–9515. http://dx.doi.org/10.1007/s00253-014-6093-9.
12. **Slater S, Houmiel KL, Tran M, Mitsky TA, Taylor NB, Padgette SR, Gruys KJ.** 1998. Multiple β-ketothiolases mediate poly(β-hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*. J Bacteriol **180:**1979–1987.
13. **Bermejo LL, Welker NE, Papoutsakis ET.** 1998. Expression of *Clostridium acetobutylicum* ATCC 824 genes in *Escherichia coli* for acetone production and acetate detoxification. Appl Environ Microbiol **64:**1079–1085.
14. **Miller JH.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
15. **Jones ML, Barnard RT.** 2005. Chimerization of multiple antibody classes using splice overlap extension PCR. Biotechniques **38:**181–182. http://dx.doi.org/10.2144/05382BM01.
16. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
17. **Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol **2:**2006.0008.
18. **Cherepanov PP, Wackernagel W.** 1995. Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158:**9–14. http://dx.doi.org/10.1016/0378-1119(95)00193-A.
19. **Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO.** 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat Methods **6:**343–345. http://dx.doi.org/10.1038/nmeth.1318.
20. **Neidhardt FC, Bloch PL, Smith DF.** 1974. Culture medium for enterobacteria. J Bacteriol **119:**736–747.
21. **Bolstad BM, Irizarry RA, Åstrand M, Speed TP.** 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics **19:**185–193. http://dx.doi.org/10.1093/bioinformatics/19.2.185.
22. **Gautier L, Cope L, Bolstad BM, Irizarry RA.** 2004. affy—analysis of Affymetrix GeneChip data at the probe level. Bioinformatics **20:**307–315. http://dx.doi.org/10.1093/bioinformatics/btg405.
23. **Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP.** 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:**249–264. http://dx.doi.org/10.1093/biostatistics/4.2.249.
24. **Durnin G, Clomburg J, Yeates Z, Alvarez PJJ, Zygourakis K, Campbell P, Gonzalez R.** 2009. Understanding and harnessing the microaerobic metabolism of glycerol in *Escherichia coli*. Biotechnol Bioeng **103:**148–161. http://dx.doi.org/10.1002/bit.22246.
25. **Braunegg G, Sonnleitner B, Lafferty RM.** 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Appl Microbiol Biotechnol **6:**29–37. http://dx.doi.org/10.1007/BF00500854.
26. **Felnagle EA, Chaubey A, Noey EL, Houk KN, Liao JC.** 2012. Engineering synthetic recursive pathways to generate non-natural small molecules. Nat Chem Biol **8:**518–526. http://dx.doi.org/10.1038/nchembio.959.
27. **Weber AL.** 1991. Origin of fatty acid synthesis: thermodynamics and kinetics of reaction pathways. J Mol Evol **32:**93–100. http://dx.doi.org/10.1007/BF02515381.
28. **Wiesenborn DP, Rudolph F, Papoutsakis E.** 1989. Coenzyme A transferase from *Clostridium acetobutylicum* ATCC 824 and its role in the uptake of acids. Appl Environ Microbiol **55:**323–329.
29. **McCleary WR, Stock JB, Ninfa AJ.** 1993. Is acetyl phosphate a global signal in *Escherichia coli*? J Bacteriol **175:**2793–2798.
30. **Pfleger BF, Gossing M, Nielsen J.** 2015. Metabolic engineering strategies for microbial synthesis of oleochemicals. Metab Eng **29:**1–11. http://dx.doi.org/10.1016/j.ymben.2015.01.009.
31. **Marcheschi RJ, Li H, Zhang K, Noey EL, Kim S, Chaubey A, Houk K, Liao JC.** 2012. A synthetic recursive "+1" pathway for carbon chain elongation. ACS Chem Biol **7:**689–697. http://dx.doi.org/10.1021/cb200313e.
32. **Brown TDK, Jones-Mortimer MC, Kornberg HL.** 1977. The enzymic interconversion of acetate and acetyl-coenzyme A in *Escherichia coli*. J Gen Microbiol **102:**327–336. http://dx.doi.org/10.1099/00221287-102-2-327.

33. **May A, Fischer R-J, Thum SM, Schaffer S, Verseck S, Dürre P, Bahl H.** 2013. A modified pathway for the production of acetone in *Escherichia coli*. Metab Eng **15:**218–225. http://dx.doi.org/10.1016/j.ymben.2012.08.001.
34. **Mifune J, Nakamura S, Fukui T.** 2010. Engineering of *pha* operon on *Cupriavidus necator* chromosome for efficient biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from vegetable oil. Polym Degrad Stabil **95:**1305–1312. http://dx.doi.org/10.1016/j.polymdegradstab.2010.02.026.
35. **Shams Yazdani S, Gonzalez R.** 2008. Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products. Metab Eng **10:**340–351. http://dx.doi.org/10.1016/j.ymben.2008.08.005.
36. **Zhang X, Shanmugam KT, Ingram LO.** 2010. Fermentation of glycerol to succinate by metabolically engineered strains of *Escherichia coli*. Appl Environ Microbiol **76:**2397–2401. http://dx.doi.org/10.1128/AEM.02902-09.
37. **Seo M-Y, Seo J-W, Heo S-Y, Baek J-O, Rairakhwada D, Oh B-R, Seo P-S, Choi MH, Kim CH.** 2009. Elimination of by-product formation during production of 1,3-propanediol in *Klebsiella pneumoniae* by inactivation of glycerol oxidative pathway. Appl Microbiol Biotechnol **84:**527–534. http://dx.doi.org/10.1007/s00253-009-1980-1.
38. **Causey T, Shanmugam K, Yomano L, Ingram L.** 2004. Engineering *Escherichia coli* for efficient conversion of glucose to pyruvate. Proc Natl Acad Sci U S A **101:**2235–2240. http://dx.doi.org/10.1073/pnas.0308171100.
39. **Mazumdar S, Clomburg JM, Gonzalez R.** 2010. *Escherichia coli* strains engineered for homofermentative production of D-lactic acid from glycerol. Appl Environ Microbiol **76:**4327–4336. http://dx.doi.org/10.1128/AEM.00664-10.
40. **Kotani T, Yurimoto H, Kato N, Sakai Y.** 2007. Novel acetone metabolism in a propane-utilizing bacterium, *Gordonia* sp. strain TY-5. J Bacteriol **189:**886–893. http://dx.doi.org/10.1128/JB.01054-06.
41. **Hanai T, Atsumi S, Liao J.** 2007. Engineered synthetic pathway for isopropanol production in *Escherichia coli*. Appl Environ Microbiol **73:**7814–7818. http://dx.doi.org/10.1128/AEM.01140-07.
42. **Akawi L, Srirangan K, Liu X, Moo-Young M, Chou CP.** 2015. Engineering *Escherichia coli* for high-level production of propionate. J Ind Microbiol Biotechnol **42:**1057–1072. http://dx.doi.org/10.1007/s10295-015-1627-4.
43. **Yang JE, Choi YJ, Lee SJ, Kang K-H, Lee H, Oh YH, Lee SH, Park SJ, Lee SY.** 2014. Metabolic engineering of *Escherichia coli* for biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from glucose. Appl Microbiol Biotechnol **98:**95–104. http://dx.doi.org/10.1007/s00253-013-5285-z.
44. **Amann E, Ochs B, Abel K-J.** 1988. Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene **69:**301–315. http://dx.doi.org/10.1016/0378-1119(88)90440-4.
45. **Jobling MG, Holmes RK.** 1990. Construction of vectors with the pl5A replicon, kanamycin resistance, inducible *lacZ*α and pUC18 or pUC19 multiple cloning sites. Nucleic Acids Res **18:**5315–5316. http://dx.doi.org/10.1093/nar/18.17.5315.