Supporting Information for

Construction of a Part of a 3-Hydroxypropionate Cycle for Heterologous Polyketide Biosynthesis in *Escherichia coli*

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EXPERIMENTAL PROCEDURES

Chemicals

3-Hydroxypropionate was purchased from Tokyo Chemical Industry. All other chemicals were from Sigma-Aldrich. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were from invitrogen. Ni-NTA affinity resin was purchased from Qiagen. Acryloyl-CoA was synthesized from acrylate, coenzyme A (CoA), and adenosine triphosphate (ATP) by 3-hydroxypropionyl-CoA synthase (3HPCS) as previously described.[1]

Strains

Escherichia coli strain BAP1 and K207-3 were kindly provided by Kosan Biosciences.[2,3]

Synthetic Genes

The synthetic genes encoding malonyl-CoA reductase (MCR), malonic semialdehyde reductase (MSR), 3HPCS, 3-hydroxypropionyl-CoA dehydratase (3HPCD), and acryloyl-CoA reductase (ACR) with codons optimized for expression in *Escherichia coli* were ordered from DNA2.0.

Codon-optimized gene sequence of Sulfolobus tokodaii MCR

ATGAGAAGAACGTTAAAAGCAGCCATCCTCGGCGCAACCGGTCTGGTGGGTA
TTGAGTATGTTCGTATGCTGAGCAATCACCCGTACATTAAACCTGCGTATCTG
GCCGGTAAGGGTAGCGTTGGTAAGCCGTATGGCGAGGTCGTCCGTTGGCAGA
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ACCCGAAGCTGATGGACGATGTTGATATCATCTTCTCCCCGTTGCCGCAGGGT
GCGGCAGGTCCGGTCGAGGAACAATTTGCGAAAGAAGGTTTTCCAGTCATCA
GCAACTCGCCGGACCACCGCTTCGACCCAGACGTGCCGCTGCTGGTGCCGGA
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GTATACTTTGACCGCTGGGCAGGCGATATTCCGGGCATGAGCGTGGTTGTCG
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Codon-optimized gene sequence of Metallosphaera sedula MSR

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GAAGAACAACTCCCAAATTACGGAGGACGTTAACACCGTGATCAGCCGTGTC
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AAAG

Codon-optimized gene sequence of M. sedula 3HPCS
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GGTATCCCAGATCCGGTCAAAGGTGAAGCGGTCCACTTGTTTGTTGTGTTGAA
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Codon-optimized gene sequence of S. tokodaii 3HPCD
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TTAACCAACTGACCCCGGCTGAAGCATGGAAGTTTAGCAAGAAGGGTCGCGA
AATCATGGACAAAATCGAGGCGCTGTCTAAGCCGACCATTGCGATGATTAAT
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CTTGGAGAGCGTGGGCTGGGGTGTCGTTTTTAGCACGGAGGACAAGAAGGAA
GGTGTCTCCGCATTTCTGGAGAAACGTGAGCCTACCTTCAAAGGCAAA

Codon-optimized gene sequence of M. sedula ACR
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GTGCCGGACCCGAAACCGGGTAAGGATGAAGTCATTATTCGCGTTGATCGTG
CGGCACTGTGTTACCGCGACTTGCTGCAGTTGCAGGGTTACTATCCGCGTATG
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TGCGCCGGACGGTACGTGTGAGTACTGCCAGATTGGTGAAGAGGCCTACTGC
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CCCAAGAGGGCAAGATTAAGCCGGTTATTGCGGGTACTGTCTCCCTGGAGAA
CATCGACGAGGGTTATAAGATGATCAAGGACAAAAACAAGGTGGGTAAAGT
GCTGGTGAAGCCT

Plasmid Construction

Plasmids used in this study are listed in Table 1. In order to enable rapid cloning and assembly of genes, we employed the BglBrick cloning strategy using BglBrick vectors.[4]

pNC006-pNC010

Each synthetic gene was designed to have a NdeI site and a BamHI site at the 5' and 3' end, respectively, and not to include EcoRI, BglII, NdeI, BamHI, and XhoI sites. The genes were subcloned into the NdeI and BamHI sites in pBbE7a to construct pNC001-pNC005. To insert a hexahistidine tag sequence to pNC001-pNC005, we amplified AGATCTTTTAAGAAGGAGATATACCATGGGCAGCAGCCATCATCATCATCAT CACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATG sequence from pET28 and the sequence was subcloned into the N-termini using BglII and NdeI to construct pNC006-pNC010.

pNC011

The gene encoding MCR was excised from pNC001 using EcoRI and XhoI and subcloned into the EcoRI and XhoI sites in pBbE2k to construct pNC011.

pNC015

The gene encoding 3HPCS was excised from pNC003 using EcoRI and XhoI and subcloned into the EcoRI and XhoI sites in pBbA5k to construct pNC012. The gene encoding ACR was excised from pNC005 using BglII and XhoI and subcloned into the BamHI and XhoI sites in pNC012 to construct pNC013. The gene encoding MSR was excised from pNC002 using BglII and XhoI and subcloned into the BamHI and XhoI sites in pNC013 to construct pNC014. The gene encoding 3HPCD was excised from pNC004 using BglII and XhoI and subcloned into the BamHI and XhoI sites in pNC014 to construct pNC015.

pNC016

pBbE2k was cut with BglII and BamHI and then ligated to construct pNC016.

pNC017

pBbA5k was cut with BglII and BamHI and then ligated to construct pNC017.

pNC018

The segment encoding 3HPCS, ACR, MSR, and 3HPCD was excised from pNC015 using BglII and XhoI and subcloned into the BamHI and XhoI sites in pNC011 to construct pNC018.

Protein Production and Purification

E. coli BL21(DE3) strains harboring pNC006, pNC007, pNC008, pNC009, or pNC010 were grown in LB medium with appropriate antibiotics at 37°C until the OD_{600} reached

0.4-0.5. The cultures were then cooled to 18°C and induced with 250 μM isopropyl-β-D-galactopyranoside (IPTG) for 16 h. The cells were harvested by centrifugation (4000*g*, 5 min) and resuspended in lysis/wash buffer (50 mM phosphate, pH 7.6, 300 mM NaCl, 10 mM imidazole, 4°C). The cells were lysed by sonication (8 × 30 sec) and cellular debris was removed by two subsequent centrifugations (4000*g*, 30 min, 4°C). Nickel-NTA agarose resins were added directly to the supernatant (1 mL of resins/L of culture) and mixed for 1 h at 4°C. The resulting resins were poured into a fritted column, washed with 10 resin volumes of lysis/wash buffer (4°C), and eluted with 2 resin volumes of elution buffer (150 mM phosphate, pH 7.6, 50 mM NaCl, 150 mM imidazole, 4°C). The buffer was then exchanged into stock buffer (50 mM phosphate, pH 7.6, 8% glycerol, 4°C) by dialysis using an Amicon Ultra-15 Centrifugal Filter, 10K or 30K device (Millipore). Proteins were stored at -80°C.

In Vitro Reconstitution of a Part of 3-Hydroxypropionate Cycle

In vitro propionyl-CoA biosynthesis from malonyl-CoA

The Bradford protein assay was used to measure the concentrations of MCR, MSR, 3HPCS, 3HPCD, and ACR using a reaction volume of 50 μL. 250 μM of malonyl-CoA was incubated with 1 mM NADPH and 1 mM ATP in the presence of 1 μM each of MCR, MSR, 3HPCS, 3HPCD, and ACR in 100 mM phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$ and 2.5 mM DTT at 23°C. The reaction was quenched at 1 h by adding 50 μL of methanol and then filtered with an Amicon Ultra-0.5 mL Centrifugal Filter, 3K device (Millipore) at 4°C. 2 μL of the resulting solutions were analyzed by liquid chromatography-mass spectrometry (LC-MS).

In vitro acryloyl-CoA biosynthesis from 3-hydroxypropionate

The reaction was set up in a reaction volume of 50 μL. 500 μM of 3-hydroxypropinate was incubated with 150 μM of CoA and 1 mM ATP in the presence of 1 μM each of 3HPCS and 3HPCD in 100 mM phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$ and 2.5 mM DTT at 23°C. After 1 h, the reaction was quenched by adding 50 μL of methanol and then filtered with an Amicon Ultra-0.5 mL Centrifugal Filter, 3K device at 4°C. 2 μL of the resulting solutions were analyzed by LC-MS.

In vitro propionyl-CoA biosynthesis from 3-hydroxypropionate

The reaction was set up in a reaction volume of 50 μL. 500 μM of 3-hydroxypropinate was incubated with 150 μM of CoA, 1 mM ATP, and 1 mM NADPH in the presence of 1 μM each of 3HPCS, 3HPCD, and ACR in 100 mM phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$ and 2.5 mM DTT at 23°C. The reaction was quenched at 1 h by adding 50 μL of methanol and then filtered with an Amicon Ultra-0.5 mL Centrifugal Filter, 3K device at 4°C. 2 μL of the resulting solutions were analyzed by LC-MS.

In Vitro Kinetic Studies of a Part of 3-Hydroxypropionate Cycle

The substrate-dependent oxidation of NADPH was monitored at 365 nm where the extinction coefficient at 365 nm for NADPH is 3400 M^{-1} cm^{-1}. Each measurement was

set up in a reaction volume of 90 μL in 96-well plate (0.25 cm light path). 500 μM of malonyl-CoA was incubated with 2 mM NADPH and 2 mM ATP in the presence of 2 μM each of MCR, MSR, 3HPCS, 3HPCD, and ACR in 100 mM phosphate buffer, pH 7.2, containing 5 mM $MgCl_2$ and 2.5 mM DTT at 25°C or 37°C. To determine the rate-limiting enzyme in the system, each enzyme concentration was decreased by 5-fold.

Acyl-CoA Extraction from *E. coli*

E. coli strains harboring pNC011/pNC015 or pNC016/pNC017 were grown in 5 mL of LB medium with appropriate antibiotics at 37°C until the OD_{600} reached 0.4-0.5. The cultures were induced with 100 nM anhydrous tetracycline (aTc) and 250 μM IPTG for 3 h. The cells were harvested by centrifugation (4000*g*, 5 min) and resuspended in 330 μL of a mixture of acetonitrile/methanol/water (2:2:1, 4°C) with 0.1% formic acid. The cells were lysed with sonication (2 × 10 sec) and the supernatant and cellular debris were separated by centrifugation (15000*g*, 2 min, 4°C). The resulting pellets were again resuspended in 330 μL of a mixture of acetonitrile/methanol/water (2:2:1, 4°C) with 0.1% formic acid. The cells were sonicated (2 × 10 sec) and cellular debris was removed by centrifugation (15000*g*, 2 min, 4°C). The resulting supernatants (660 μL each) were neutralized by adding 5 μL of 5N ammonium hydroxide. The organic phases were evaporated under vacuum and then lyophilized. The resulting white powders were dissolved in 150 μL of methanol/water (1:1, 4°C) and then filtered with an Amicon Ultra-0.5 mL Centrifugal Filter, 3K device. 5 μL of the filtered solutions were injected for LC-MS analysis.

LC Analysis of Acyl-CoA

LC separation of acyl-CoA compounds was conducted at 50°C with an Inertsil ODS-3 reverse-phase column (250 mm length, 2.1 mm internal diameter, 3 μM particle size; GL Sciences) using a 1100 series high-performance liquid chromatography system (Agilent Technologies).

CoA, acetyl-CoA, acryloyl-CoA, propionyl-CoA, and 3-hydroxypropionyl-CoA

The mobile phase was composed of 20 mM ammonium acetate in water (solvent A) and 20 mM ammonium acetate in methanol (solvent B). CoA, acetyl-CoA, acryloyl-CoA, propionyl-CoA, and 3-hydroxypropionyl-CoA were separated with the following gradient: 10% to 22.5% B for 15 min, 22.5% to 100% B for 5 min, 100% to 10% B for 0.1 min, held at 10% B for 24.9 min. A flow rate of 0.14 mL/min was used throughout.

Succinyl-CoA and methylmalonyl-CoA

The mobile phase was composed of 20 mM ammonium acetate in water (solvent A) and 20 mM ammonium acetate in methanol (solvent B). Succinyl-CoA and methylmalonyl-CoA were separated with the following gradient: 2.5% to 25% B for 20 min, 25% to 2.5% B for 0.1 min, held at 2.5% B for 24.9 min. A flow rate of 0.14 mL/min was used throughout.

MS Analysis of Acyl-CoA

The LC system was coupled to an Agilent Technologies LC-MSD SL electrospray

ionization mass (ESI MS) spectrometer. Nitrogen gas was used as both the nebulizing and drying gas to facilitate the production of gas-phase ions. The drying and nebulizing gases were set to 10 L/min and 20 lb/in^2, respectively, and a drying gas temperature of 300°C was used throughout. ESI was conducted in the positive-ion mode with a capillary voltage of 4 kV. Mass measurements were carried out in the selected ion monitoring mode (CoA, m/z 768; acetyl-CoA, m/z 810; acryloyl-CoA, m/z 822; propionyl-CoA, m/z 824 CoA; 3-hydroxypropionyl-CoA, m/z 840) at 1.01 s/cycle with a dwell time of 1 s for the detection of $[M + H]^+$ ions. The instrument was tuned for a range of m/z 50 to 3000 *via* the Agilent ES tuning mix. Data acquisition and processing were performed by the Agilent Chemstation (Agilent technologies).

TABLES

Table 1. Plasmids

Plasmid	Promoter-*Gene*-Ori	Parent Vector	Resistance
pNC006	P_{T7}-*MCR*-ColE1	pBbE7a	Amp
pNC007	P_{T7}-*MSR*-ColE1	pBbE7a	Amp
pNC008	P_{T7}-*3HPCS*-ColE1	pBbE7a	Amp
pNC009	P_{T7}-*3HPCD*-ColE1	pBbE7a	Amp
pNC010	P_{T7}-*ACR*-ColE1	pBbE7a	Amp
pNC011	P_{Tet}-*MCR*-ColE1	pBbE2k	Kan
pNC015	P_{lacUV5}-*3HPCS-ACR-MSR-3HPCD*-p15A	pBbA5a	Amp
pNC016	P_{Tet}-*No gene*-ColE1	pBbE2k	Kan
pNC017	P_{lacUV5}-*No gene*-p15A	pBbA5a	Amp
pNC018	P_{Tet}-*MCR-3HPCS-ACR-MSR-3HPCD*-ColE1	pBbE2k	Kan

Abbreviations: Amp, ampicillin; Kan, kanamycin.

FIGURES

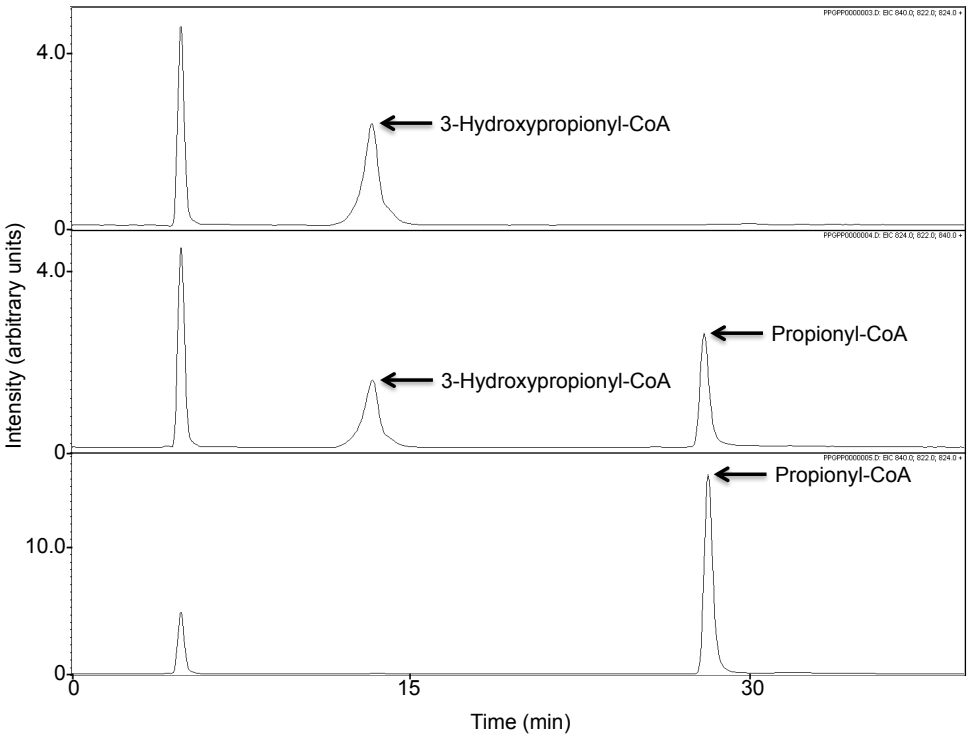


Figure 1. *In vitro* propionyl-CoA biosynthesis from 3-hydroxypropionate. 500 μM of 3-hydroxypropionate was incubated with 150 μM of CoA, 1 mM ATP in the presence of 1 μM each of 3HPCS and 3HPCD (top), in the presence of 1 mM NADPH and 1 μM each of 3HPCS and 3HPCD (middle) or in the presence of 1 mM NADPH and 1 μM each of 3HPCS and 3HPCD, and ACR (bottom) for 1 h at 23°C. The resulting reaction mixtures were analyzed by LC-MS.

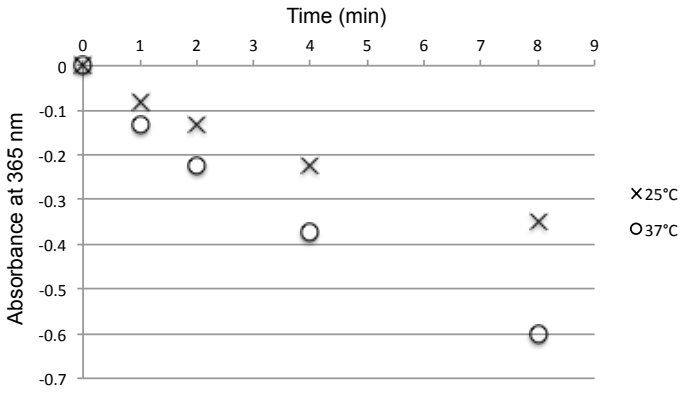
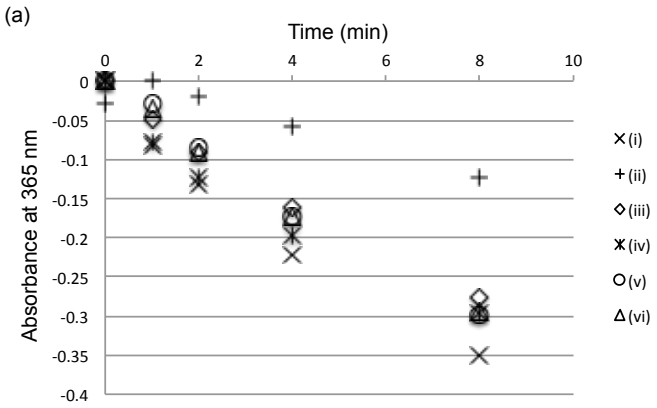
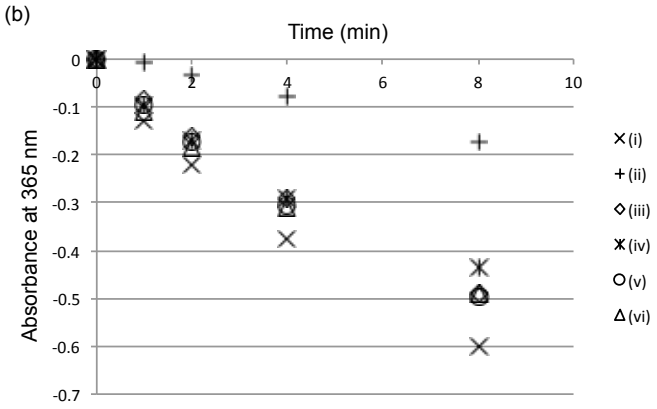


Figure 2. *In vitro* kinetic studies of propionyl-CoA biosynthesis. Substrate-dependent oxidation of NADPH was monitored at 365 nm. 500 μM of malonyl-CoA was incubated with 2 mM NADPH and 2 mM ATP in the presence of 2 μM each of MCR, MSR, 3HPCS, 3HPCD, and ACR at 25°C or 37°C.



	MCR	MSR	3HPCS	3HPCD	ACR
(i)	2 μM	2 μM	2 μM	2 μM	2 μM
(ii)	0.4 μM	2 μM	2 μM	2 μM	2 μM
(iii)	2 μM	0.4 μM	2 μM	2 μM	2 μM
(iv)	2 μM	2 μM	0.4 μM	2 μM	2 μM
(v)	2 μM	2 μM	2 μM	0.4 μM	2 μM
(vi)	2 μM	2 μM	2 μM	2 μM	0.4 μM



	MCR	MSR	3HPCS	3HPCD	ACR
(i)	2 μM	2 μM	2 μM	2 μM	2 μM
(ii)	0.4 μM	2 μM	2 μM	2 μM	2 μM
(iii)	2 μM	0.4 μM	2 μM	2 μM	2 μM
(iv)	2 μM	2 μM	0.4 μM	2 μM	2 μM
(v)	2 μM	2 μM	2 μM	0.4 μM	2 μM
(vi)	2 μM	2 μM	2 μM	2 μM	0.4 μM

Figure 3. Identification of the rate-limiting enzyme of propionyl-CoA biosynthesis from malonyl-CoA. Substrate-dependent oxidation of NADPH was monitored at 365 nm. 500 μM of malonyl-CoA was incubated with 2 mM NADPH and 2 mM ATP in the presence of 2 μM each of MCR, MSR, 3HPCS, 3HPCD, and ACR or the five enzymes where each enzyme concentration is decreased by 5-fold. The measurements were conducted at 25°C (a) and 37°C (b).

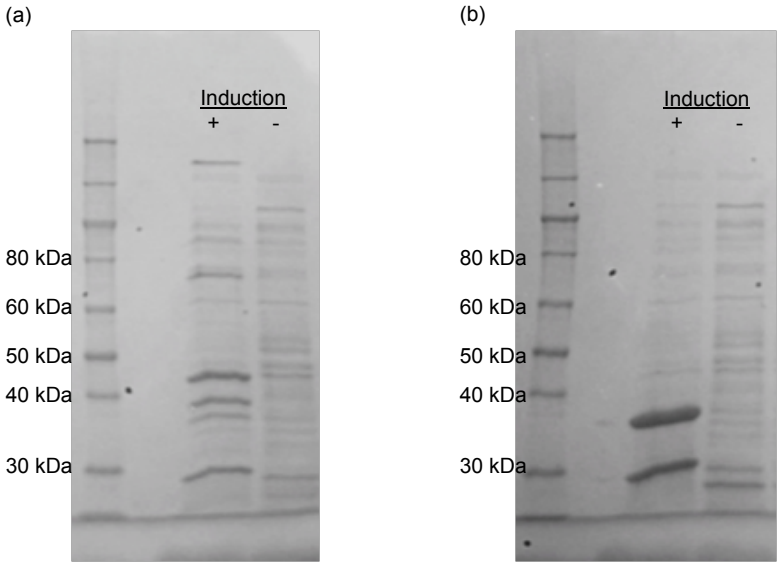


Figure 4. SDS-PAGE analysis of soluble fractions of *E. coli* BL21(DE3) carrying plasmids encoding MCR, MSR, 3HPCS, 3HPCD, and ACR (pNC011 + pNC015 or pNC018). (a) *E. coli* BL21(DE3) transformed with pNC011 + pNC015: (left) + IPTG, + aTc; (right) - IPTG, - aTc. (b) *E. coli* BL21(DE3) transformed with pNC018: (left) + aTc; (right) - aTc. Molecular weights of MCR, MSR, 3HPCS, 3HPCD, and ACR are 39 kDa, 35 kDa, 74 kDa, 28 kDa, and 36 kDa, respectively.

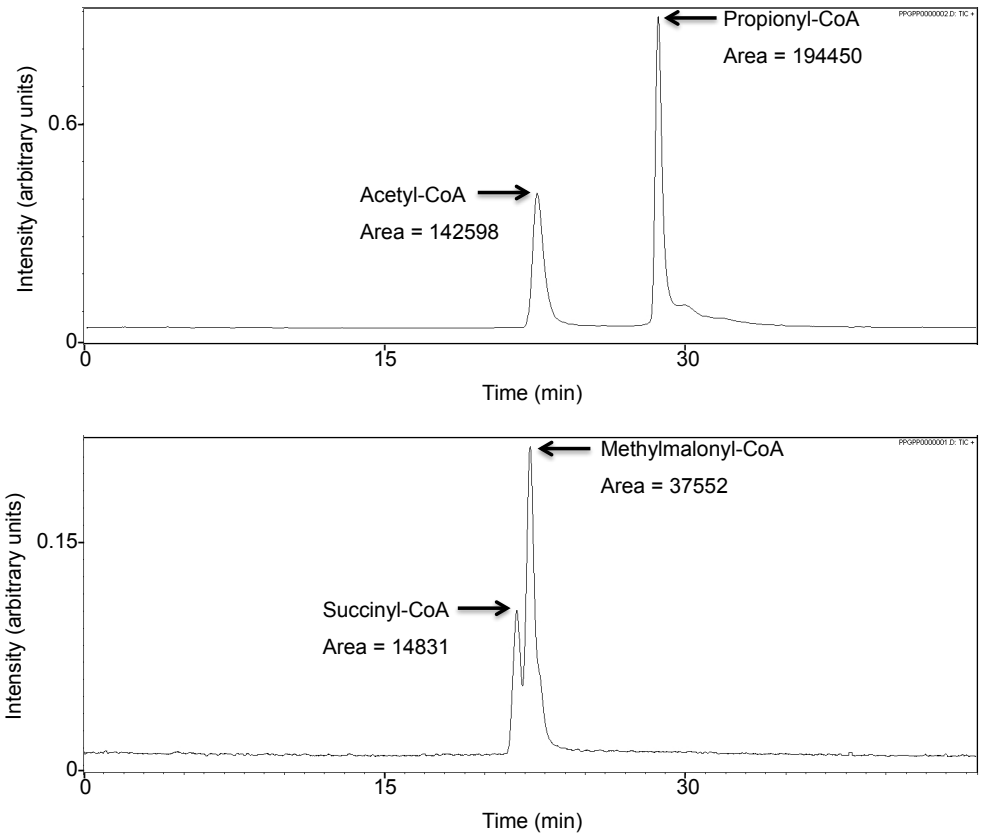


Figure 5. Standard acyl-CoA analysis. 5 μL of 1 μM standard acetyl-CoA, propionyl-CoA, succinyl-CoA, and methylamlonyl-CoA (purchased from Sigma-Aldrich) were analyzed by LC-MS. (top) Comparison of area between acetyl-CoA and propionyl-CoA. (bottom) Comparison of area between succinyl-CoA and methylmalonyl-CoA.

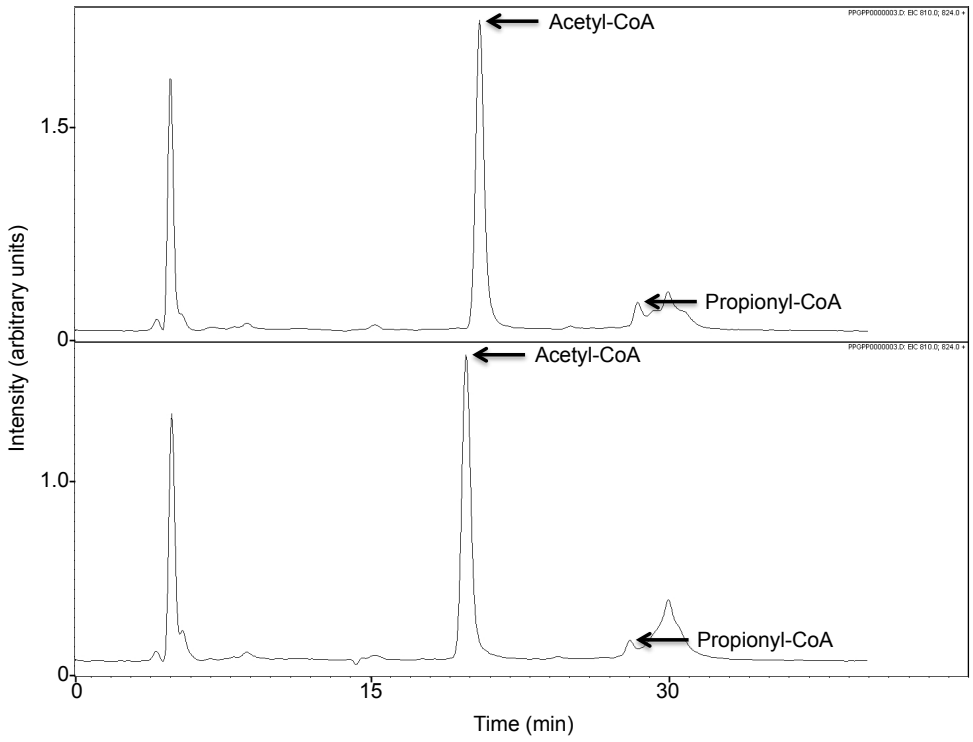


Figure 6. Propionyl-CoA biosynthesis in *E. coli*. *E. coli* BL21 (DE3) and BAP1 carrying plasmids encoding MCR, MSR, 3HPCS, 3HPCD, and ACR (pNC011 + pNC015) were extracted after induction with IPTG and aTc and analyzed by LC-MS. (top) Comparison between acetyl-CoA and propionyl-CoA in the engineered *E. coli* BL21 (DE3). (bottom) Comparison between acetyl-CoA and propionyl-CoA in the engineered *E. coli* BAP1.

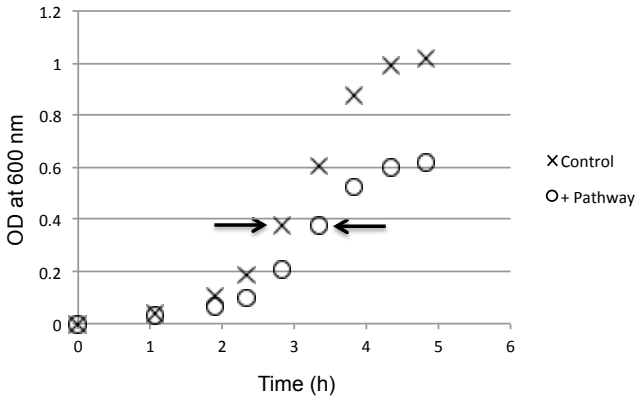


Figure 7. Comparison of growth rates in the absence and presence of the methylmalonyl-CoA biosynthetic pathway. *E. coli* K207-3 strains carrying plasmids encoding MCR, MSR, 3HPCS, 3HPCD, and ACR (pNC011 + pNC015) or the control plasmids (pNC016 + pNC017) were grown in LB medium with appropriate antibiotics at 37°C until the OD_{600} reached 0.4. The cultures were then induced with 100 nM aTc and 250 μM IPTG (indicted by arrows).

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