

Construction and modular implementation of the THETA cycle for synthetic CO₂ fixation

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Supplementary Information for

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Supplementary Methods

Production and purification of recombinant proteins

All genes were expressed in *E. coli* and purified subsequently. The plasmids (Supplementary Table 7) for *in vitro* experiments were transformed into chemical competent BL21 (DE3) cells. For Mco, Rosetta (DE3) cells were used, and for Hbs, an additional plasmid (pBB541) was used to co-express non-tagged GroEL and GroES chaperones.

The cells carrying the plasmids were grown in salt-buffered TB medium (2 L per 5 L baffled shake flask) at 37 °C and 100 rpm till OD₆₀₀ of 1.0-1.5 was reached. The cultures were cooled down to 21 °C, induced with 0.25 mM IPTG and grown overnight. For protein production of Hbd under microaerobic conditions, the cells were grown to an OD₆₀₀ of 4 and cooled down. The culture was transferred into a sterile Schott bottle, and 100 µM Fe(II)SO₄, 100 µM Fe(III)citrate and 20 mM fumarate were added before induction. After overnight expression, the cells were pelleted by centrifugation (15 min, 4 °C, 6,000 x g) and either stored at -80 °C until usage or directly resuspended in two-fold volume lysis buffer (buffer A, 500 mM NaCl, 50 mM HEPES, 10% glycerol, pH 7.8) before 5 mM MgCl₂ and 10 µg/ml DNase were added.

For lysis, the cells were disrupted using a microfluidizer (two iterations at 16,000 psi). The lysate was centrifuged at 50,000 x g for 1 h at 4 °C, and the supernatant was subsequently filtered through a 0.45 µm membrane. Lysate derived from 2 L expression culture was mixed with 2 ml preequilibrated (buffer A) Protino Ni-NTA agarose beads (Macherey-Nagel) and incubated on ice for ~30 min (70 rpm). The beads were collected in a 14 ml Protino (Macherey-Nagel) gravity column and washed with three column volumes (CVs) of lysis buffer. For removal of non-specific bound proteins, the beads were washed with three CVs of lysis buffer containing 75 mM imidazole. Elution was done with buffer B (lysis buffer containing 500 mM imidazole). The elution fractions were concentrated with an Amicon Ultra 15 ml Centrifugal Filters (Merck).

For desalting, size exclusion chromatography was done on a HiLoad 16/600 Superdex 200-pg column attached to an Äkta Pure (both GE Healthcare) using desalting/storage buffer containing 200 mM NaCl, 50 mM HEPES and 10% glycerol at pH 7.8 (buffer C). The collected fractions were pooled and concentrated again. FAD was added to Mco at a concentration equal to the protein concentration. Enzymes requiring MgCl₂ or coenzyme B₁₂ were stored in buffer C containing 2 mM of the respective cofactor. Glycerol was added to a final concentration of 20% (v/v), the proteins were flash-frozen in liquid nitrogen and stored at -80 °C.

Expression of the gox1801 gene for strep-tagged glyoxylate reductase (Gor) was done as stated above. After lysate preparation, the supernatant was loaded onto a preequilibrated (buffer C) 1-ml StrepTrap column on an Äkta Start (both GE Healthcare) and eluted using buffer

C containing 2 mM D-desthiobiotin. Concentration and storage did not differ from the steps described above.

Enzyme assays

All assays were conducted at 30 °C under aerobic conditions. The absorbance at a given wavelength was measured on a Cary 60 UV-Vis spectrophotometer (Agilent) in quartz cuvettes with a path length of 10 mm.

Activity assay of DHOD1a. The activity of DHOD1a was assayed by monitoring orotate production at various wavelengths as described previously¹. When fumarate was used as the electron acceptor, the production of orotate was measured at 300 nm ($\epsilon_{300\text{ nm}} = 2.65\text{ mM}^{-1}\text{ cm}^{-1}$). The reaction mixture (500 μl) contained 100 mM MOPS pH 7, 0.2 mM dihydroorotate (DHO), 0.2 mM fumarate, and an appropriate amount of DHOD1a (0.45 μg DHOD1a-Ef, 2.89 μg DHOD1a-LI, 1.38 μg DHOD1a-Tc, or 12.3 μg DHOD1a-Sc). When no electron acceptor was added, oxygen was used as the electron acceptor, and the production of orotate was measured at 278 nm ($\epsilon_{278\text{ nm}} = 6.70\text{ mM}^{-1}\text{ cm}^{-1}$). The reaction mixture (500 μl) contained 100 mM MOPS pH 7, 0.2 mM DHO, and an appropriate amount of DHOD1a (0.57 μg DHOD1a-Ef, 28.9 μg DHOD-LI, 13.8 μg DHOD-Tc, or 12.3 μg DHOD-Sc).

Activity assay of DHOD1b. The DHO:NAD⁺ oxidoreductase activity of DHOD1b was assayed by monitoring the decrease of absorbance at 340 nm corresponding to the consumption of NADH ($\epsilon_{340\text{ nm}} = 6.22\text{ mM}^{-1}\text{ cm}^{-1}$). The reaction mixture (500 μl) contained 100 mM MOPS pH 7, 0.2 mM orotate, 0.2 mM NADH, and an appropriate amount of DHOD1b (3.95 μg DHOD1b-LI or 2.62 μg DHOD1b-Bs). The side activity as a NADH oxidase was assayed by monitoring the decrease of absorbance at 340 nm corresponding to the consumption of NADH. The reaction mixture (500 μl) contained 100 mM MOPS pH 7, 0.2 mM NADH, and an appropriate amount of DHOD1b (39.5 μg DHOD1b-LI or 26.2 μg DHOD1b-Bs).

Activity assay of Cat2. The activity of Cat2 on crotonate was measured in a coupled assay with Ccr. Cat2 converts crotonate to crotonyl-CoA using acetyl-CoA as the CoA donor, then Ccr performs the reductive carboxylation of crotonyl-CoA using NADPH as the reducing equivalent. The oxidation of NADPH was followed spectrophotometrically at 340 nm ($\epsilon_{340\text{ nm}} = 6.22\text{ mM}^{-1}\text{ cm}^{-1}$). The reaction mixture (200 μl) contained 100 mM MOPS pH 7, 5 mM MgCl₂, 20 mM NaHCO₃, 0.2 mM NADPH, 0.5 mM crotonate, 0.1 mM acetyl-CoA, 6.05 μg Ccr, and 0.185 μg Cat2. The activity of Cat2 on 4-hydroxybutyrate (4HB) was measured in a coupled assay with Hbd and Ccr. Cat2 converts 4HB to 4HB-CoA, which is converted to crotonyl-CoA by Hbd, then Ccr performs the reductive carboxylation of crotonyl-CoA using NADPH as the reducing equivalent. The

consumption of NADPH was followed at 340 nm. The reaction mixture (200 μ l) contained 100 mM MOPS pH 7, 5 mM MgCl₂, 20 mM NaHCO₃, 0.2 mM NADPH, 0.5 mM 4HB, 0.1 mM acetyl-CoA, 6.05 μ g Ccr, 8.8 μ g Hbd, and 1.85 μ g Cat2.

Activity assay of DmdB1. The activity of DmdB1 on crotonate was measured in a coupled assay with Ccr. DmdB1 catalyses the ATP-dependent condensation of crotonate and CoA into crotonyl-CoA, then Ccr performs the reductive carboxylation of crotonyl-CoA using NADPH as the reducing equivalent. The oxidation of NADPH was followed spectrophotometrically at 340 nm. The reaction mixture (200 μ l) contained 100 mM MOPS pH 7, 5 mM MgCl₂, 20 mM NaHCO₃, 1 mM ATP, 1 mM CoA, 0.2 mM NADPH, 0.5 mM crotonate, 6.05 μ g Ccr, and 48.8 μ g DmdB1. The activity of DmdB1 on 4HB was measured in a coupled assay with Hbd and Ccr. DmdB1 converts 4HB to 4HB-CoA, which is converted to crotonyl-CoA by Hbd, then Ccr performs the reductive carboxylation of crotonyl-CoA using NADPH as the reducing equivalent. The consumption of NADPH was followed at 340 nm. The reaction mixture (200 μ l) contained 100 mM MOPS pH 7, 5 mM MgCl₂, 20 mM NaHCO₃, 1 mM ATP, 1 mM CoA, 0.2 mM NADPH, 0.5 mM 4HB, 6.05 μ g Ccr, 8.8 μ g Hbd, and 48.8 μ g DmdB1.

Activity assay of Mtk. The activity of Mtk on various substrates was measured in a coupled assay with pyruvate kinase (Pyk) and lactate dehydrogenase (Ldh). Pyk converts phosphoenolpyruvate (PEP) to pyruvate using ADP formed from the Mtk reaction, and Ldh subsequently reduces pyruvate using NADH as the reducing equivalent. The consumption of NADH was followed spectrophotometrically at 340 nm. The reaction mixture (200 μ l) contained 100 mM MOPS pH 7, 5 mM MgCl₂, 1 mM ATP, 1 mM CoA, 3 mM PEP, 0.2 mM NADH, 0.5 mM substrate (malate, succinate, methylsuccinate, mesaconate, citramalate, ethylmalonate, itaconate, or crotonate), 0.4 U Pyk, 0.4 U Ldh, and 15 μ g Mtk.

Activity assay of Sdh. The activity of Sdh on succinate or methylsuccinate was measured by monitoring the decrease of absorbance at 300 nm corresponding to the consumption of ferrocenium hexafluorophosphate (FePF₆) ($\epsilon_{300\text{ nm}} = 4.3\text{ mM}^{-1}\text{ cm}^{-1}$)², which is used as the artificial electron acceptor. The reaction mixture (200 μ l) contained 100 mM MOPS pH 7.5, 0.25 mM FePF₆, 0.5 mM succinate/methylsuccinate, and 36.3 μ g Sdh. The reaction mixture was quenched with 3% formic acid after reacting for 30 min and analyzed with LC-MS to verify the product.

Activity assays of Ccr, Epi, Ecm, Mco, Mct, Meh, Ccl, Pps, Ppc, Mdh. Activities of Ccr, Epi, Ecm, Mct, Meh, Ccl, Pps, Ppc, and Mdh were measured as described previously³. Activity of Mco was assayed as described in Schwander et al⁴.

Cell extract preparation. Single colonies of the strain transformed with the desired plasmids were cultured overnight in LB with 10 mM acetate and appropriate antibiotics. On the next day, the overnight culture was inoculated at 2% into 12 ml M9 minimal medium with 1% glucose, 10 mM acetate, and appropriate antibiotics. The cultures were grown at 37 °C to an OD₆₀₀ of 0.2 to 0.4, induced with 0.5 mM IPTG, and grown overnight at 30 °C. Then cells were harvested by centrifugation and the pellets were resuspended with 0.2 ml BugBuster HT protein extraction reagent with rLysozyme (Novagen). The cell suspension was incubated in a 30 °C shaker at 180 rpm for 15-20 min. The lysate was then centrifuged at 20,817 x g for 15 min at 4°C and the supernatant was used for subsequent enzyme assays. Total protein concentrations of the cell extracts were determined by Bradford assay.

Assay of SucCD activity in cell extracts. The activity of SucCD in cell extracts was measured in a coupled assay with Pyk and Ldh. Pyk converts PEP to pyruvate using ADP formed from the SucCD reaction, and Ldh subsequently reduces pyruvate using NADH as the reducing equivalent. The consumption of NADH was followed spectrophotometrically at 340 nm. The reaction mixture (200 µl) contained 100 mM MOPS pH 7, 5 mM MgCl₂, 1 mM ATP, 1 mM CoA, 3 mM PEP, 0.2 mM NADH, 0.5 mM succinate, 0.4 U Pyk, 0.4 U Ldh, and 2 µl cell extract.

Assay of Scr activity in cell extracts. The activity of Scr (NADH-dependent or NADPH-dependent) in cell extracts was measured in a coupled assay with SucCD. SucCD converts succinate to succinyl-CoA, which is subsequently reduced by Scr using NAD(P)H as the reducing equivalent. The consumption of NAD(P)H was followed spectrophotometrically at 340 nm. The reaction mixture (200 µl) contained 100 mM MOPS pH 7, 5 mM MgCl₂, 1 mM ATP, 1 mM CoA, 0.2 mM NAD(P)H, 0.5 mM succinate, 20.4 µg SucCD, and 2 µl cell extract.

Assay of Ssr activity in cell extracts. The activity of Ssr (NADH-dependent or NADPH-dependent) in cell extracts was measured by monitoring the decrease of absorbance at 340 nm corresponding to the consumption of NAD(P)H. The reaction mixture (200 µl) for the activity assay contained 100 mM MOPS pH 7, 5 mM MgCl₂, 0.2 mM NAD(P)H, 0.5 mM succinic semialdehyde, and 2 µl cell extract. Another reaction mixture (200 µl), which contained 100 mM MOPS pH 7, 5 mM MgCl₂, 1 mM ATP, 1 mM CoA, 0.2 mM NAD(P)H, 0.5 mM succinic semialdehyde, 8.34 µg Hbs, and 2 µl cell extract, was quenched with 3% formic acid after reacting for 30-60 min and analyzed with LC-MS to verify the product.

Assay of Cat2 activity on 4HB in cell extracts. The activity of Cat2 on 4HB in cell extracts was performed as described above. The reaction mixture (200 µl) contained 100 mM MOPS pH 7, 5 mM MgCl₂, 20 mM NaH¹³CO₃, 0.2 mM NADPH, 0.5 mM 4HB, 0.1 mM acetyl-CoA, 6.05 µg

Ccr, 8.8 µg Hbd, and 10 µl cell extract. The reaction mixture was quenched with 3% formic acid after reacting for 30 min and analyzed with LC-MS to verify the product.

Assay of Hbs activity in cell extracts. The activity of Hbs was measured in a coupled assay with Hbd and Ccr. Hbs converts 4HB to 4HB-CoA, which is converted to crotonyl-CoA by Hbd, then Ccr performs the reductive carboxylation of crotonyl-CoA using NADPH as the reducing equivalent. The consumption of NADPH was followed at 340 nm. The reaction mixture (200 µl) contained 100 mM MOPS pH 7, 5 mM MgCl₂, 20 mM NaH¹³CO₃, 1 mM ATP, 0.2 mM CoA, 0.2 mM NADPH, 0.5 mM 4HB, 3.03 µg Ccr, 8.8 µg Hbd, and 10 µl cell extra. The reaction mixture was quenched with 3% formic acid after reacting for 60 min and analyzed with LC-MS to verify the product.

Assay of Hbd activity in cell extracts. The activity of Hbd in cell extracts was measured in a coupled assay with Hbs and Ccr. Hbs converts 4HB to 4HB-CoA, which is converted to crotonyl-CoA by Hbd, then Ccr performs the reductive carboxylation of crotonyl-CoA using NADPH as the reducing equivalent. The consumption of NADPH was followed at 340 nm. The reaction mixture (200 µl) contained 100 mM MOPS pH 7, 5 mM MgCl₂, 20 mM NaH¹³CO₃, 1 mM ATP, 0.25 mM CoA, 0.2 mM NADPH, 0.5 mM 4HB, 8.34 µg Hbs, 3.03 µg Ccr, and 10 µg cell extract. The reaction mixture was quenched with 3% formic acid after reacting for 20-60 min and analyzed with LC-MS to verify the product.

High resolution LC-MS analysis of CoA Esters

For CoA esters with ¹³C-labeling, High Resolution LC-MS was used for the analysis. The chromatographic separation was performed on an Agilent Infinity II 1290 HPLC system using a Kinetex EVO C18 column (150 × 2.1 mm, 3 µm particle size, 100 Å pore size, Phenomenex) connected to a guard column of similar specificity (20 × 2.1 mm, 3 µm particle size, Phenomenex) at a constant flow rate of 0.25 ml/min with mobile phase comprised of 50 mM ammonium acetate in water at pH 8.1 (A) and 100% methanol (Honeywell) (B). The column oven was set to 25 °C and the autosampler was maintained at 4 °C. The injection volume was 5 µL. The mobile phase profile consisted of the following steps and linear gradients: 0 to 1 min constant at 2.5% B; 1 to 6 min from 2.5 to 95% B; 6 to 8 min constant at 95% B; 8 to 8.1 min from 95 to 2.5% B; 8.1 to 10 min constant at 2.5% B.

An Agilent 6550 ion funnel Q-TOF mass spectrometer was used in positive mode with an dual jet stream electrospray ionization source and the following conditions: ESI spray voltage 3500 V, nozzle voltage 500 V, sheath gas 400 °C at 12 L/min, nebulizer pressure 20 psig and drying gas 225 °C at 13 L/min. The TOF was calibrated using an ESI-L Low Concentration Tuning Mix (Agilent) before measurement (residuals less than 2 ppm for five reference ions) and

was recalibrated during a run using 922 m/z as reference mass. MS data were acquired with a scan range of 100-1200 m/z . LC-MS data were analyzed using MassHunter Qualitative Analysis software (Agilent).

LC-MS/MS analysis of acetyl-CoA, malonyl-CoA and glycolate

Acetyl-CoA, malonyl-CoA and glycolate were analyzed via LC-MS/MS and quantified using external standard curves. For glycolate and malonyl-CoA we used previously described methods (glycolate: Pandi et al.⁵, malonyl-CoA: Diehl et al.⁶). For acetyl-CoA we used the method described in the following:

The chromatographic separation was performed on an Agilent Infinity II 1290 HPLC system using a Kinetex EVO C18 column (150 × 2.1 mm, 3 μ m particle size, 100 Å pore size, Phenomenex) connected to a guard column of similar specificity (20 × 2.1 mm, 3 μ m particle size, Phenomenex) at a constant flow rate of 0.25 ml/min with mobile phase A being 50 mM ammonium acetate in water at a pH of 8.1 and phase B being 100 % methanol (Honeywell). The column oven was set to 25 °C and the autosampler was maintained at 4 °C. The injection volume was 1 μ l of a 1:20 diluted quenched sample. The mobile phase profile consisted of the following steps and linear gradients: 0 to 1 min constant at 0% B; 1 to 4.5 min from 0 to 80% B; 4.5 to 5.5 min constant at 80% B; 5.5 to 5.6 min from 80 to 0% B; 5.6 to 8 min constant at 0% B.

An Agilent 6495 mass spectrometer was used in positive mode with an electrospray ionization source and the following conditions: ESI spray voltage 4500 V, nozzle voltage 1000 V, sheath gas 400 °C at 11 L/min, nebulizer pressure 20 psig and drying gas 100 °C at 11 L/min. Compounds were identified based on their mass transition and retention time compared to standards. Chromatograms were integrated using MassHunter software (Agilent). Relative abundance was determined based on the peak area.

High resolution LC-MS analysis of amino acids

Amino acids (¹³C-labeled amino acid) were analyzed using HRES LC-MS. The chromatographic separation was performed on an Agilent Infinity II 1260 HPLC system using a ZichILIC SeQuant column (150 × 2.1 mm, 5 μ m particle size, 100 Å pore size) connected to a ZichILIC guard column (20 × 2.1 mm, 5 μ m particle size) (Merck KgAA) at a constant flow rate of 0.3 ml/min with mobile phase A being 0.1 % formic acid in 99:1 water:acetonitrile (Honeywell) and phase B being 0.1 % formic acid in 99:1 acetonitrile:water. The column oven was set to 25 °C and the autosampler was maintained at 4 °C. The injection volume was 1 μ l. The mobile phase profile consisted of the following steps and linear gradients: 0 to 12 min from 90 to 70% B;

12 to 15 min from 70 to 20% B; 15 to 17 min constant at 20% B; 17 to 17.1 min from 20 to 90% B; 17.1 to 20 min constant at 90% B.

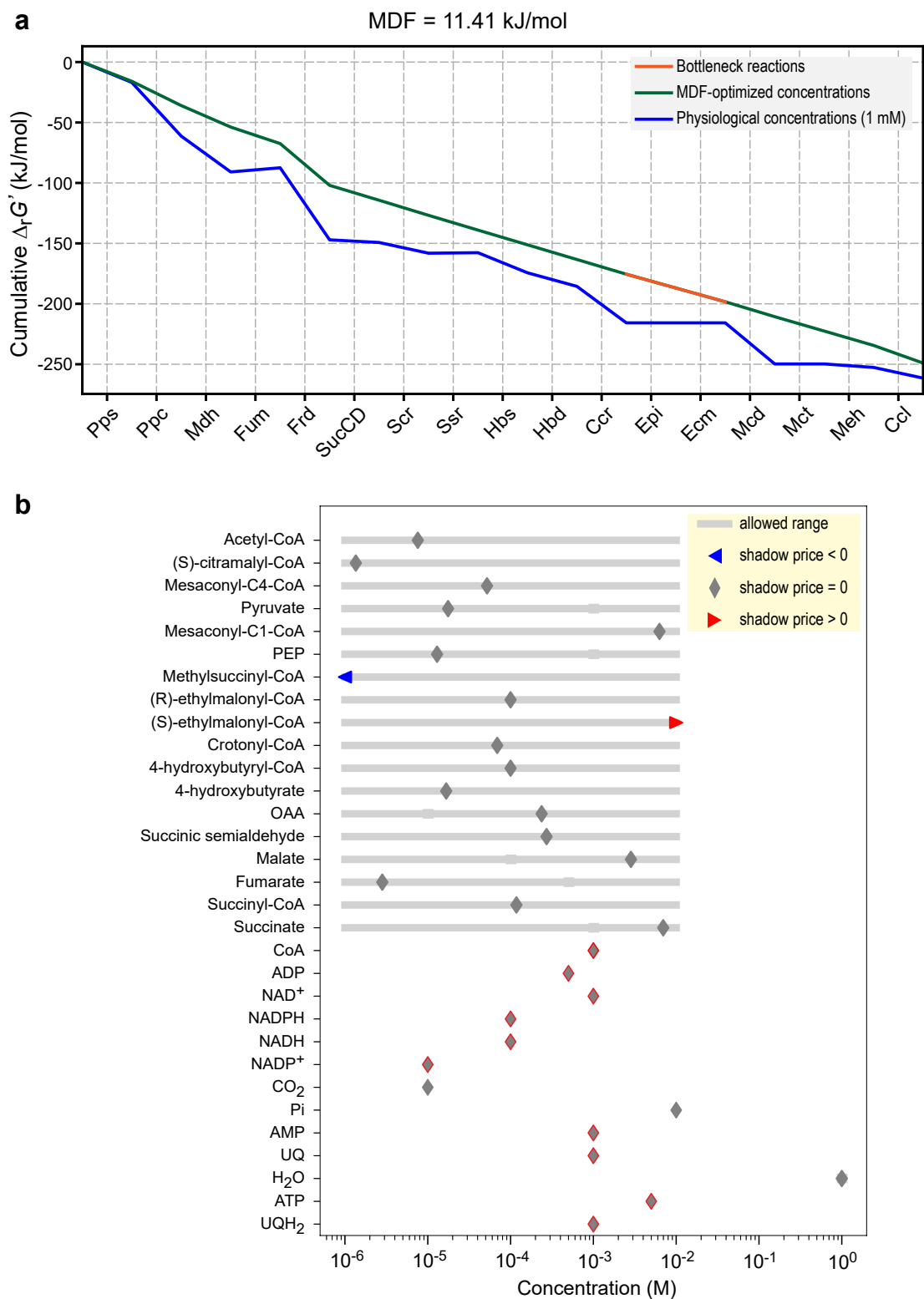
An Agilent 6550 ion funnel Q-TOF mass spectrometer was used in positive mode with a dual jet stream electrospray ionization source and the following conditions: ESI spray voltage 1000 V, nozzle voltage 900 V, sheath gas 200° C at 9 L/min, nebulizer pressure 35 psig, and drying gas 130° C at 12 L/min. The TOF was calibrated using an ESI-L Low Concentration Tuning Mix (Agilent) before measurement (residuals less than 2 ppm for five reference ions) and was recalibrated during a run using 121.050873 m/z as reference mass. MS data were acquired with a scan range of 50-250 m/z. LC-MS data were analyzed using MassHunter Qualitative Analysis software (Agilent).

LC-MS/MS analysis of organic acids

Organic acids were analyzed using LC-MS/MS. The chromatographic separation was performed on an Agilent Infinity II 1290 HPLC system using a Kinetex EVO C18 column (150 × 2.1 mm, 3 µm particle size, 100 Å pore size, Phenomenex) connected to a guard column of similar specificity (20 × 2.1 mm, 3 µm particle size, Phenomenex) at a constant flow rate of 0.2 ml/min with mobile phase A being 0.1 % formic acid in water and phase B being 0.1 % formic acid methanol (Honeywell). The column oven was set to 25 °C and the autosampler was maintained at 4 °C. The injection volume was 1 µl. The mobile phase profile consisted of the following steps and linear gradients: 0 to 4 min constant at 0% B; 4 to 4.5 min from 0 to 20 % B; 4.5 to 9.5 min from 20 to 46% B; 9.5 to 10 min from 46 to 100% B; 10 to 12 min constant at 100% B; 12 to 12.1 min from 100 to 0% B; 12.1 to 15 min constant at 0% B.

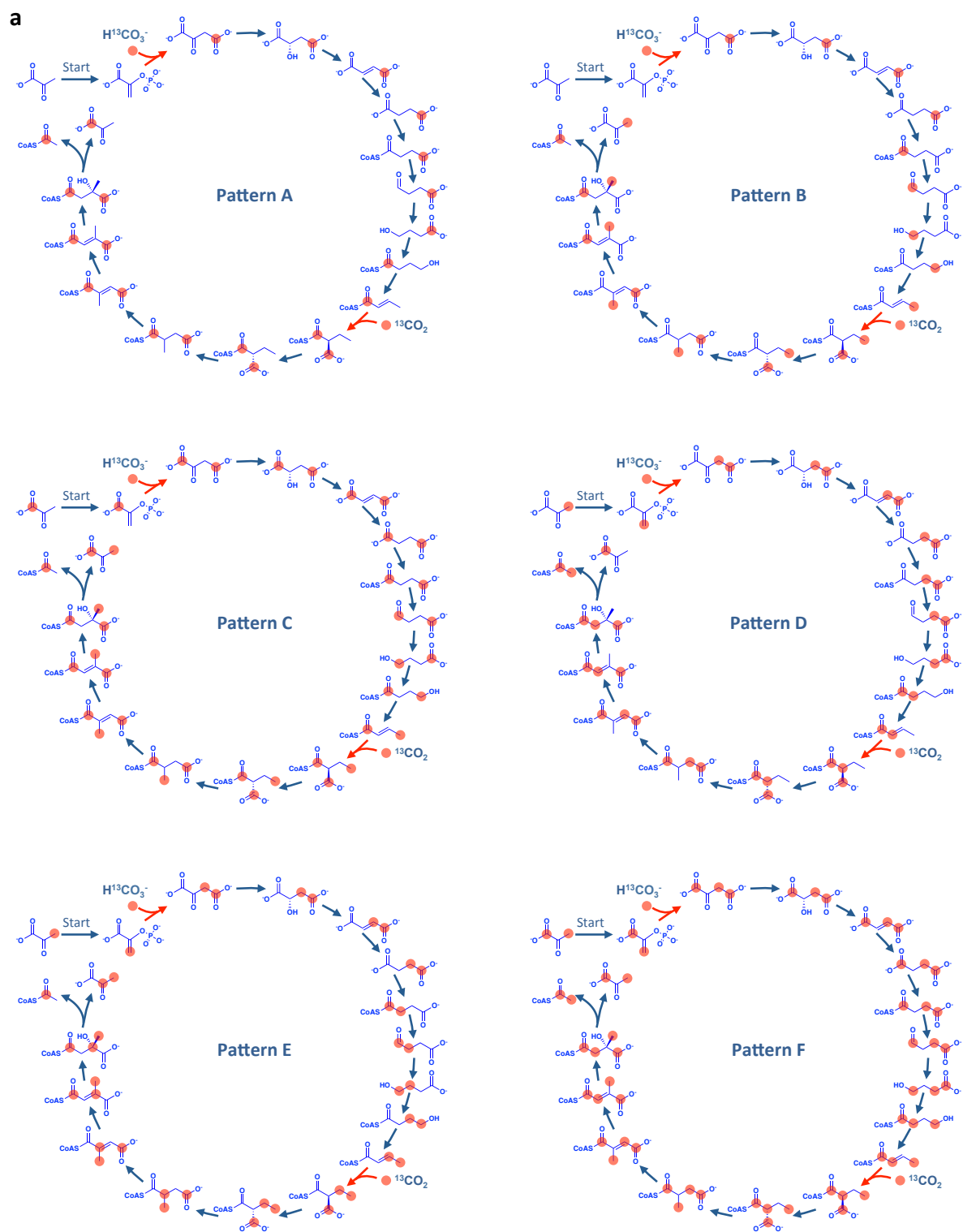
An Agilent 6495 ion funnel mass spectrometer was used in negative mode with an electrospray ionization source and the following conditions: ESI spray voltage 2000 V, nozzle voltage 500 V, sheath gas 260° C at 10 L/min, nebulizer pressure 35 psig, and drying gas 100° C at 13 L/min. For measuring non-labeled organic acids, cell accelerator voltage, dwell time, and fragmentor voltage were set to 5 V, 60 ms, and 380 V respectively. Mass transitions and optimized collision energy used were as follows: Citramalate (147 m/z → 87.1 m/z, 14 V, 147 m/z → 57.1 m/z, 19 V); Malate (133.1 m/z → 115.1 m/z, 8 V, 133.1 m/z → 71.2 m/z, 12 V); Ethylmalonate/Methylsuccinate (131 m/z → 131 m/z, 0 V, 131 m/z → 87.2 m/z, 10 V); Itaconate/Mesaconate (129 m/z → 129 m/z, 0 V, 129 m/z → 85.2 m/z, 0 V, 129 m/z → 41.2 m/z, 15 V); Succinate (117.2 m/z → 73.2 m/z, 9 V, 117.2 m/z → 55.1 m/z, 15 V); Fumarate (115.1 m/z → 71.2 m/z, 4 V, 115.1 m/z → 27.3 m/z, 9 V); and Pyruvate (87.1 m/z → 87.1 m/z, 0 V, 87.1 m/z → 43.1 m/z, 4 V). For measuring ¹³C-labeled succinate, cell accelerator voltage, dwell

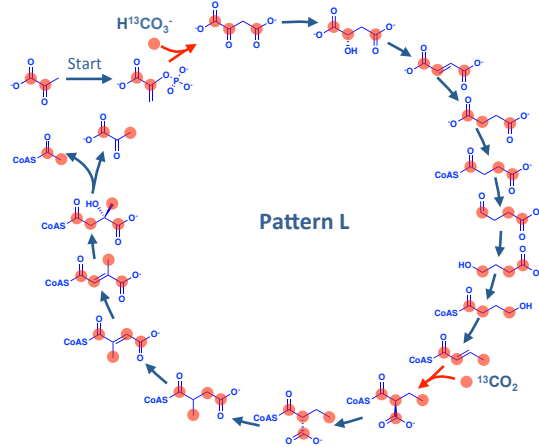
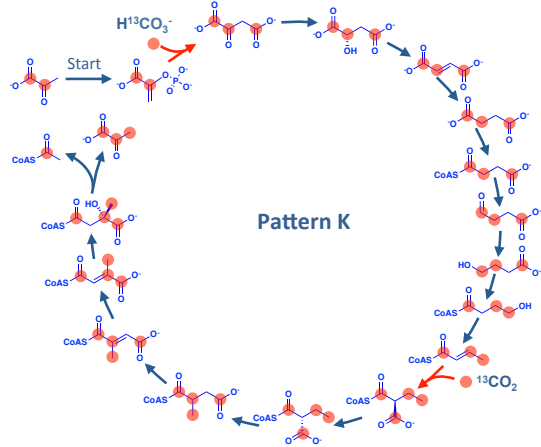
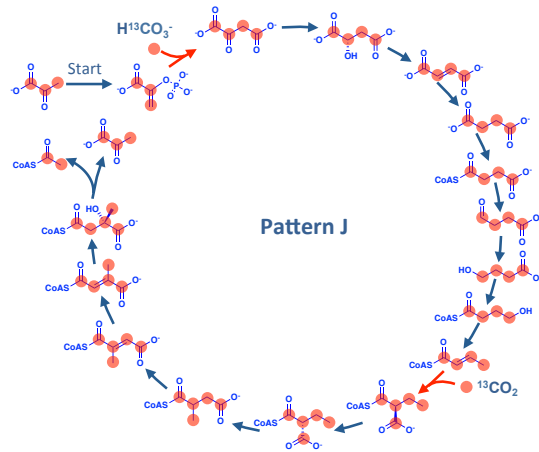
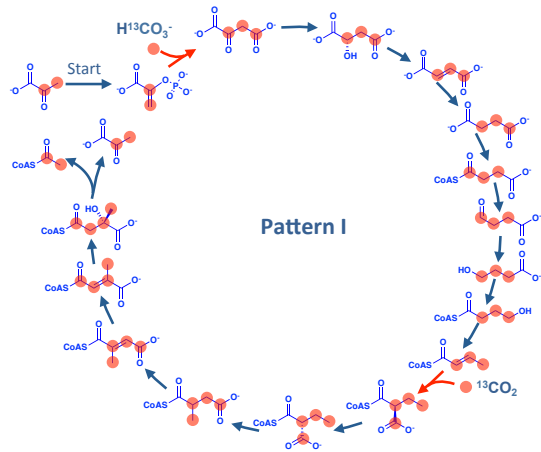
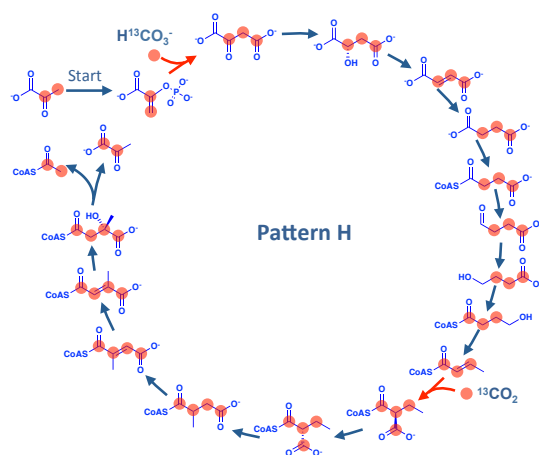
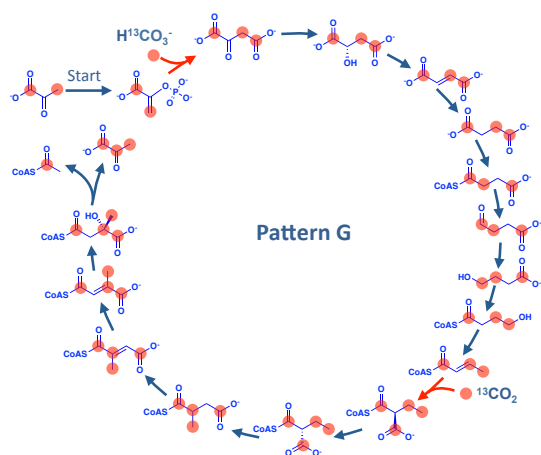
time, fragmentor voltage, optimized collision energy were set to 5 V, 30 ms, 380 V, and 9V respectively. Mass transitions used for ^{13}C -labeled succinate were as follows: Succinate M+0 (117.2 m/z $\rightarrow$ 73.2 m/z); Succinate M+1 (118.2 m/z $\rightarrow$ 73.2 m/z, 118.2 m/z $\rightarrow$ 74.2 m/z); Succinate M+2 (119.2 m/z $\rightarrow$ 74.2 m/z, 119.2 m/z $\rightarrow$ 75.2 m/z); Succinate M+3 (120.2 m/z $\rightarrow$ 75.2 m/z, 120.2 m/z $\rightarrow$ 76.2 m/z); and Succinate M+4 (121.2 m/z $\rightarrow$ 76.2 m/z). LC-MS data were analyzed and quantified using MassHunter Qualitative Navigator and QQQ Quantitative Analysis software (Agilent). Absolute concentrations were calculated based on an external calibration curve prepared in sample matrix.



Supplementary Fig. 1. Thermodynamic profiles and Max-min Driving Force (MDF) analysis of the THETA cycle. (a) Thermodynamic profiles of the THETA cycle. Blue lines correspond to $\Delta_r G'^m$ values of pathway reactions under physiological conditions (at 25 °C, 1 bar

pressure, pH = 7.0, pMg = 3, Ionic strength = 0.25 M, and 1 mM for all reactants). Green lines correspond to $\Delta_r G'$ values of pathway reactions after MDF optimization. The orange lines represent the predicted bottleneck reactions. (b) Metabolite concentrations after MDF optimization. Cofactors, marked with a red frame, have fixed concentrations. Compounds with concentrations limiting the driving force, as determined by MDF analysis, are represented by triangles. See the Methods section for calculation details. PEP, phosphoenolpyruvate; OAA, oxaloacetate; UQ, ubiquinone; UQH₂, ubiquinol.

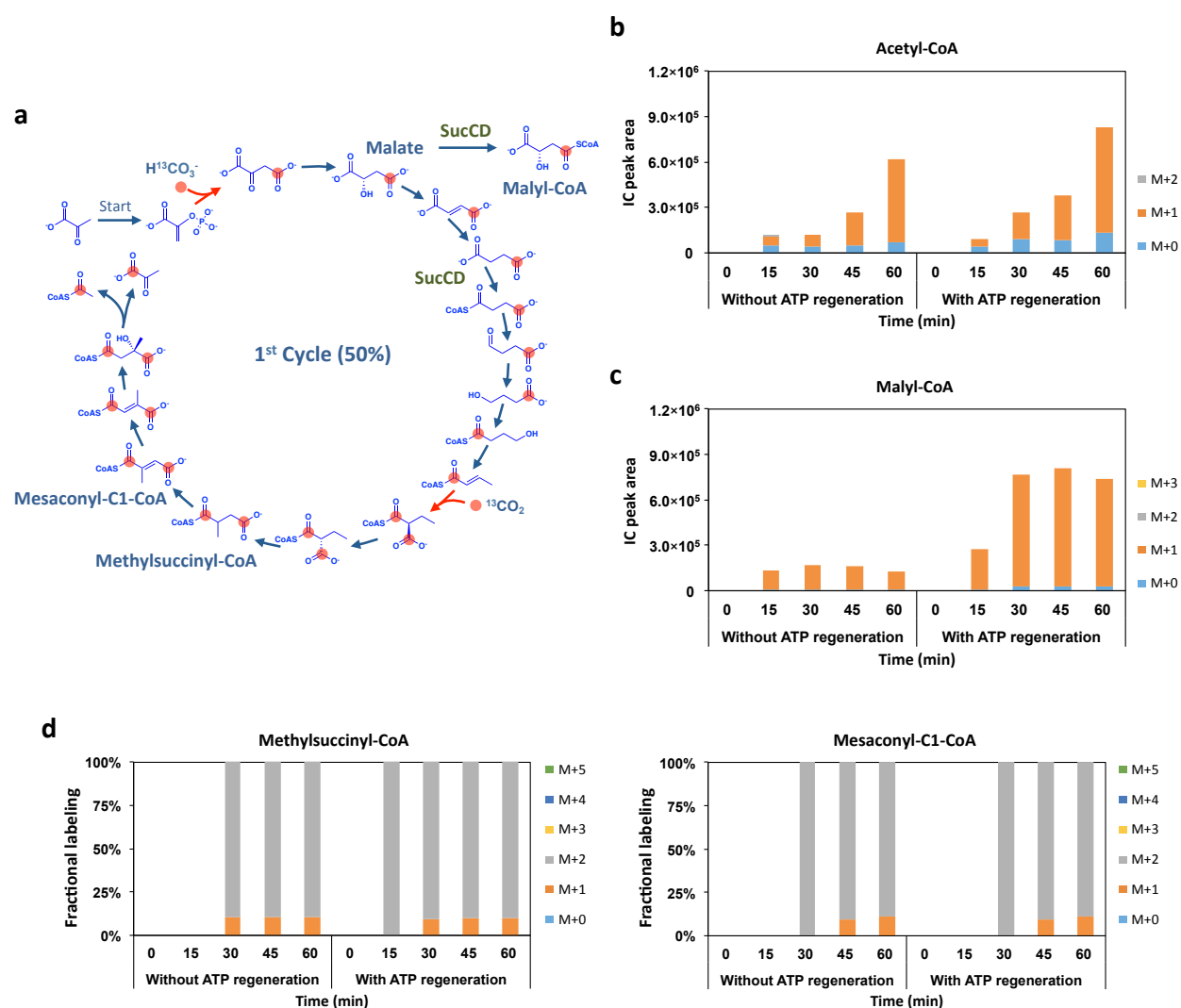




b

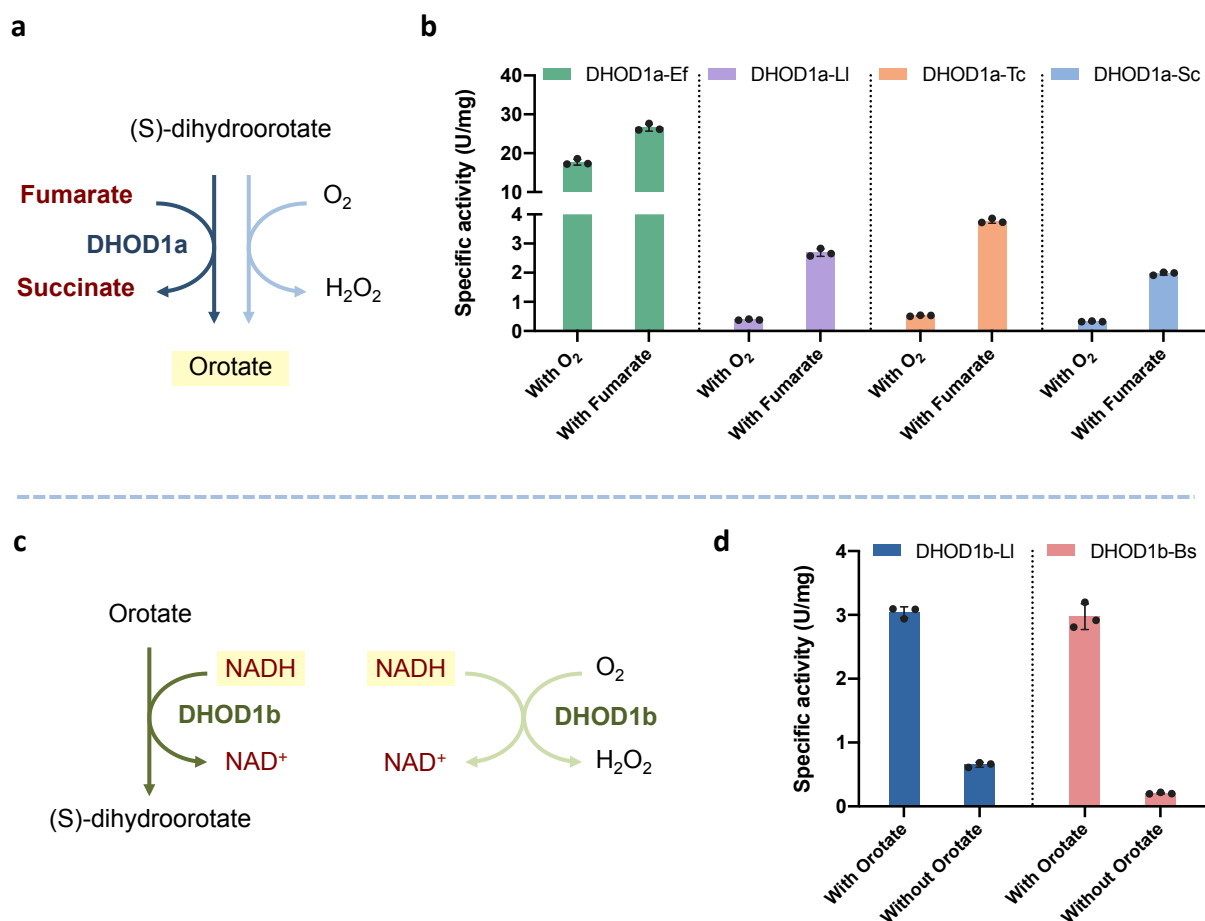
Cycle	1 st	2 nd	3 rd	4 th	5 th	n th (n ≥ 4)
A	50%					
B	50%					
C		50%	25%			
D		25%				
E		25%				
F			25%	25%	15.625%	$\frac{n}{2^n}$
G			25%	25%	15.625%	$\frac{n}{2^n}$
H			12.5%	6.25%	3.125%	$\frac{1}{2^n}$
I			12.5%	6.25%	3.125%	$\frac{1}{2^n}$
J				25%	56.25%	$1 - \frac{2+n}{2^{n-1}}$
K				6.25%	3.125%	$\frac{1}{2^n}$
L				6.25%	3.125%	$\frac{1}{2^n}$
Output						
M+1 Acetyl-CoA	100%	75%	50%	31.25%	18.75%	$\frac{1+n}{2^n}$
M+2 Acetyl-CoA		25%	50%	68.75%	81.25%	$1 - \frac{1+n}{2^n}$

Supplementary Fig. 2. Expected ¹³C-labeling patterns of the THETA cycle with ¹³CO₂ and H¹³CO₃⁻. Two carbon fixation steps are featured in red. Due to the symmetry of succinate, each turn of the THETA cycle has multiple possible expected labeling patterns. **(a)** Details of 12 possible labeling patterns (pattern A to L) of the THETA cycle starting from pyruvate. **(b)** Summary of percentages of possible labeling patterns and single or double labeled acetyl-CoA as the output molecule for the first 5 turns and the nth turn of the THETA cycle. All ¹³C-labeled carbons are shown in red dots.

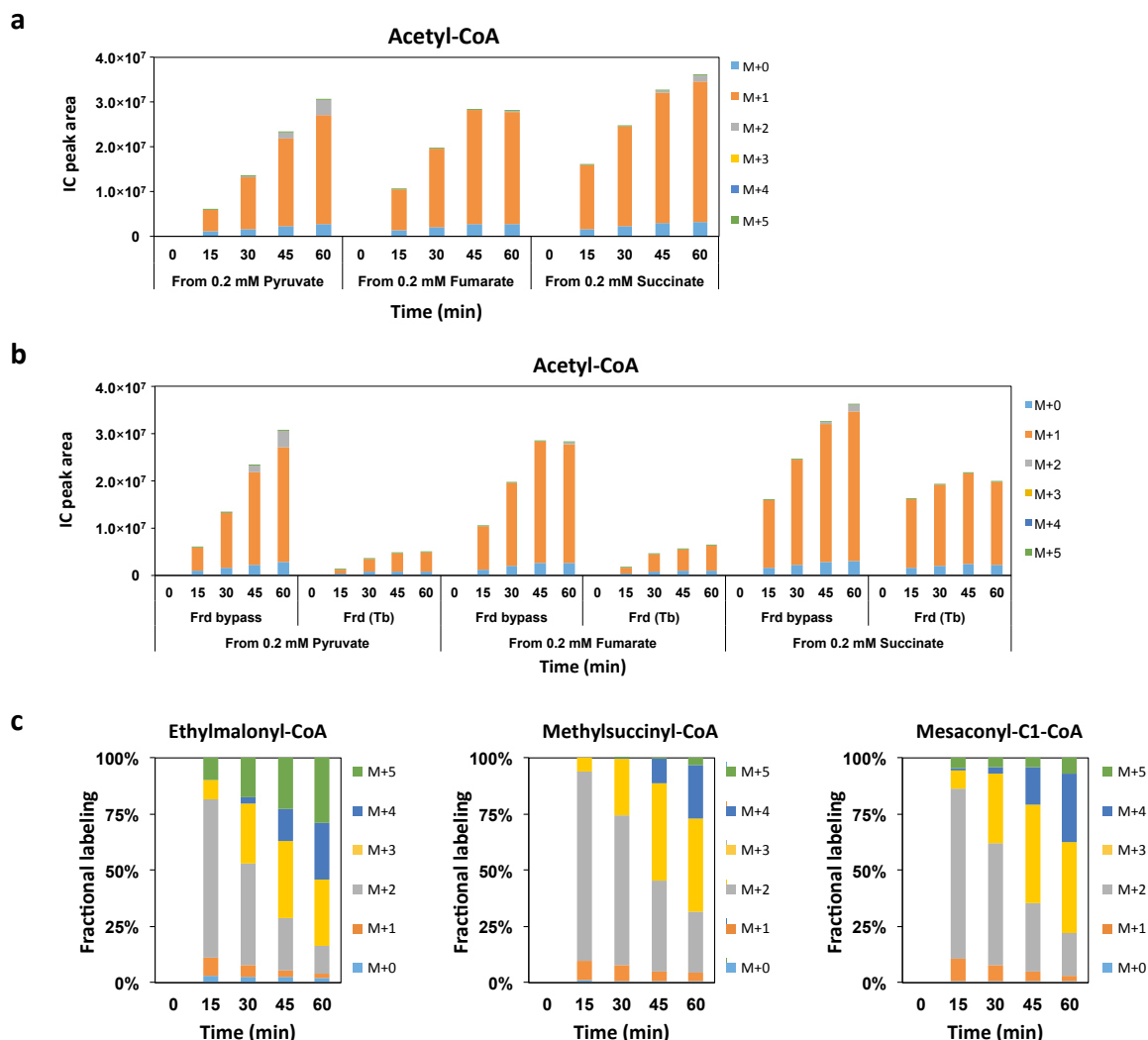


Supplementary Fig. 3. THETA 1.0 (malyl-CoA build-up results from the promiscuous activity of SucCD on malate). (a) Scheme of THETA 1.0 with labeling patterns (50% of the 1st cycle). THETA 1.1 is THETA 1.0 with ATP regeneration through creatine phosphate and creatine phosphokinase. (b) Levels (ion counts) and labeling patterns of acetyl-CoA with and without ATP regeneration over 60 minutes. The increase of labeled acetyl-CoA with time showed THETA 1.0 was functional *in vitro*. (c) Levels (ion counts) and labeling patterns of malyl-CoA with and without ATP regeneration over 60 minutes. Malyl-CoA was produced from the promiscuous activity of SucCD on malate and its build-up was more significant with ATP regeneration. (d) Labeling patterns of major detectable CoA intermediates with and without ATP regeneration over 60 minutes. The cycle was started with 0.2 mM pyruvate. All labeled species originated from naturally occurring labeled species, due to natural isotopic distribution, were subtracted from the results.

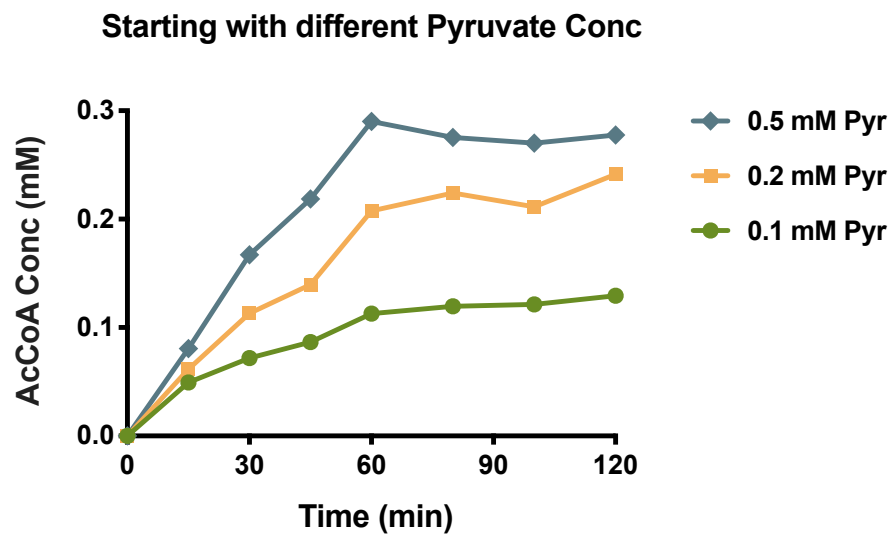
(50% of the 1st cycle). ATP was regenerated using creatine phosphate (CP) and creatine phosphokinase (Cpk). NAD(P)H was regenerated using glucose-6-phosphate (G6P) and glucose-6-phosphate dehydrogenase (G6PDH). (b) Levels (ion counts) and labeling patterns of CoA esters at time 0 (0 min), 1 (15 min), 2 (30 min), 3 (45 min), 4 (60 min), showed acetyl-CoA was the predominate product and there was no significant accumulation of CoA intermediates or side products. (c) Levels (ion counts) and labeling patterns of acetyl-CoA from different starting substrate (0.2 mM of pyruvate, fumarate, or Succinate) over 60 minutes, showed the Frd step was the bottleneck in THETA 2.0. (d) Labeling patterns of major detectable CoA intermediates over 60 minutes. The cycle was started with 0.2 mM pyruvate in (b) and (d). All labeled species originated from naturally occurring labeled species, due to natural isotopic distribution, were subtracted from the results. 6PG, 6-phosphogluconolactone; 3HB-CoA, 3-hydroxybutyryl-CoA; 4HB-CoA, 4-hydroxybutyryl-CoA.



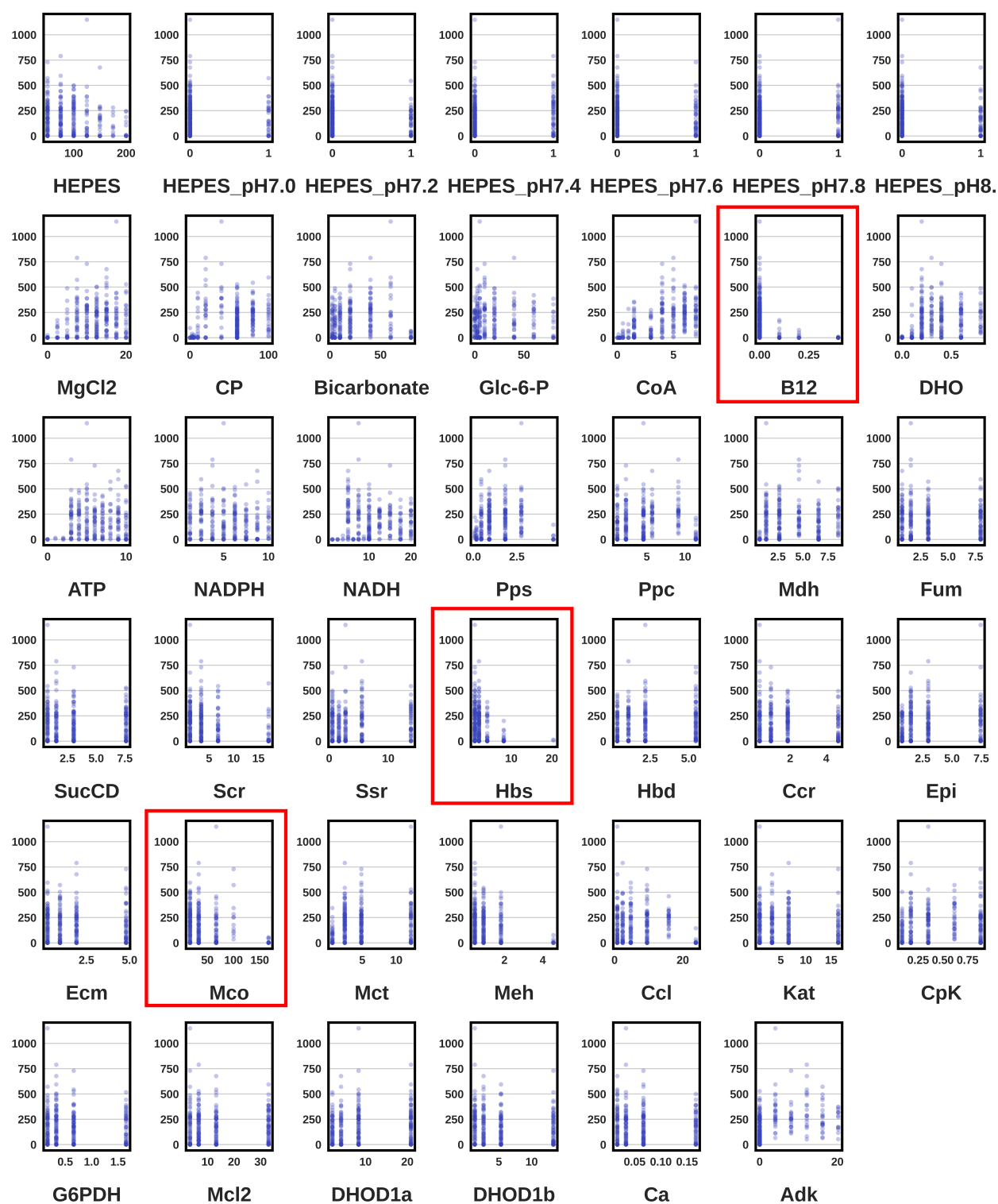
Supplementary Fig. 5. Specific activities of four DHOD1a and two DHOD1b for building the dihydroorotate-based Frd bypass. (a) Kinetic assay scheme of DHOD1a. DHOD1a catalyses the conversion of dihydroorotate to orotate using either fumarate or O₂ as electron acceptor. **(b)** Specific activities of four DHOD1a with fumarate or O₂ as electron acceptor. DHOD1a-Ef got the highest activity for fumarate-dependent dihydroorotate oxidation. However its activity for O₂-dependent dihydroorotate oxidation was also quite high, which combined with DHOD1b could form a very active futile cycle of NADH oxidation in the absence of fumarate. Thus DHOD1a-Tc was chosen. **(c)** Kinetic assay scheme of DHOD1b. DHOD1b catalyses the conversion of orotate to dihydroorotate using NADH as reducing equivalent. It can also oxidize NADH in the absence of orotate. **(d)** Specific activities of two DHOD1b for NADH-dependent orotate reduction or NADH oxidation. DHOD1b-Bs was chosen for its lower NADH oxidation activity. The data represent the mean $\pm$ s.d. (standard deviation) determined from n = 3 independent measurements. Ef, *Enterococcus faecalis*; LI, *Lactococcus lactis*; Tc, *Trypanosoma cruzi*; Sc, *Saccharomyces cerevisiae*; Bs, *Bacillus subtilis*.



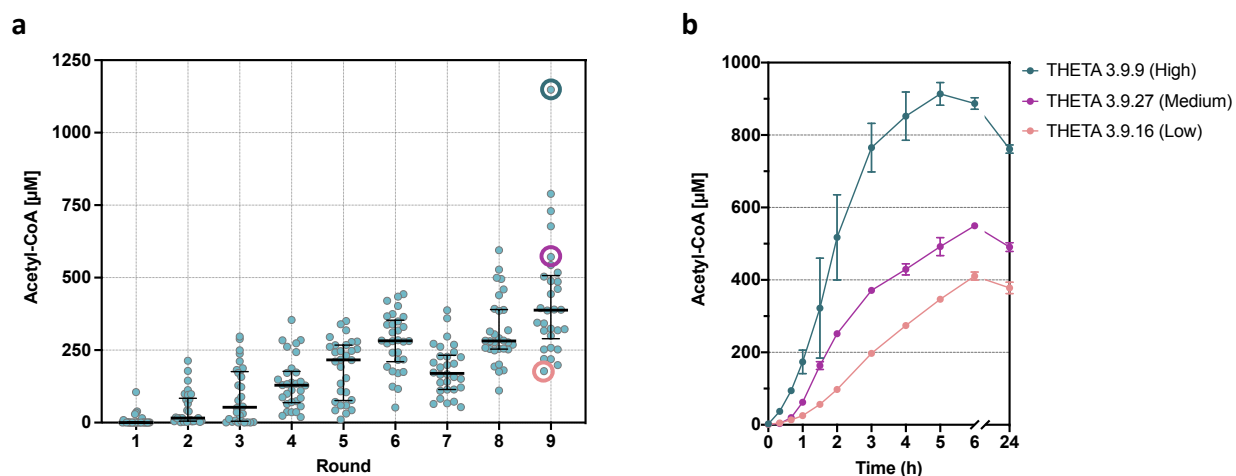
Supplementary Fig. 6. THETA 3.0 (replacing Frd-Tb with the dihydroorotate-based Frd bypass (DHOD1a-Tc and DHDA1b-Bs)). (a) Levels (ion counts) and labeling patterns of acetyl-CoA from different starting substrate (0.2 mM of pyruvate, fumarate, or succinate) over 60 minutes, showed the Frd step was no longer the bottleneck in THETA 3.0. (b) Comparison of the acetyl-CoA production between THETA 2.0 (using Frd-Tb) and THETA 3.0 (using the dihydroorotate-based Frd bypass). (c) Labeling patterns of major detectable CoA intermediates over 60 minutes. The cycle was started with 0.2 mM pyruvate in (c). All labeled species originated from naturally occurring labeled species, due to natural isotopic distribution, were subtracted from the results. Tb, *Trypanosoma brucei*.



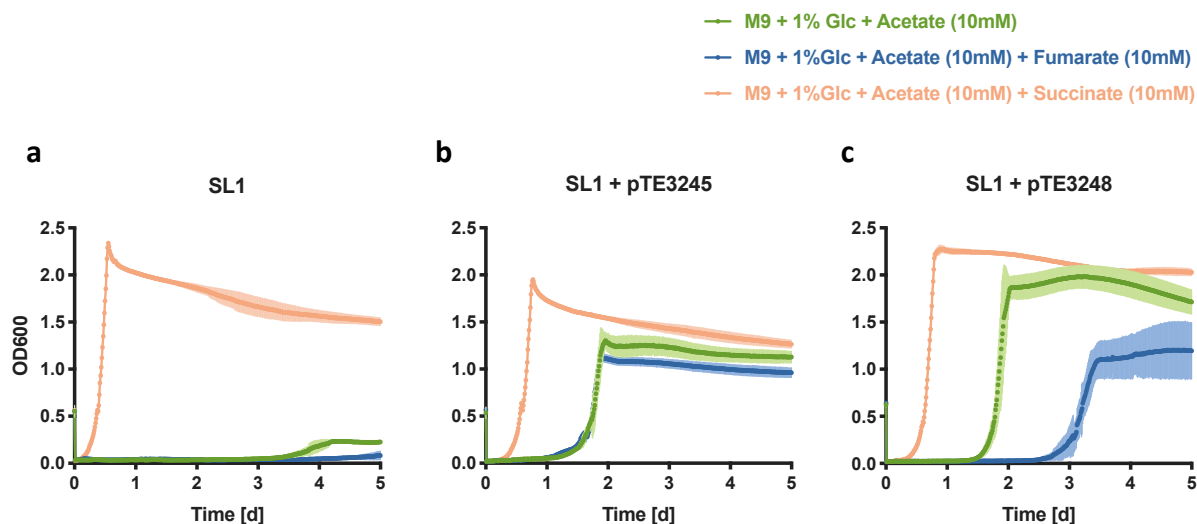
Supplementary Fig. 7. The acetyl-CoA production of THETA 3.0 starting with different concentrations of pyruvate over 2 hours. The cycle stopped around 1 hour and the acetyl-CoA concentration could not reach higher than 0.3 mM. Pyr, pyruvate.



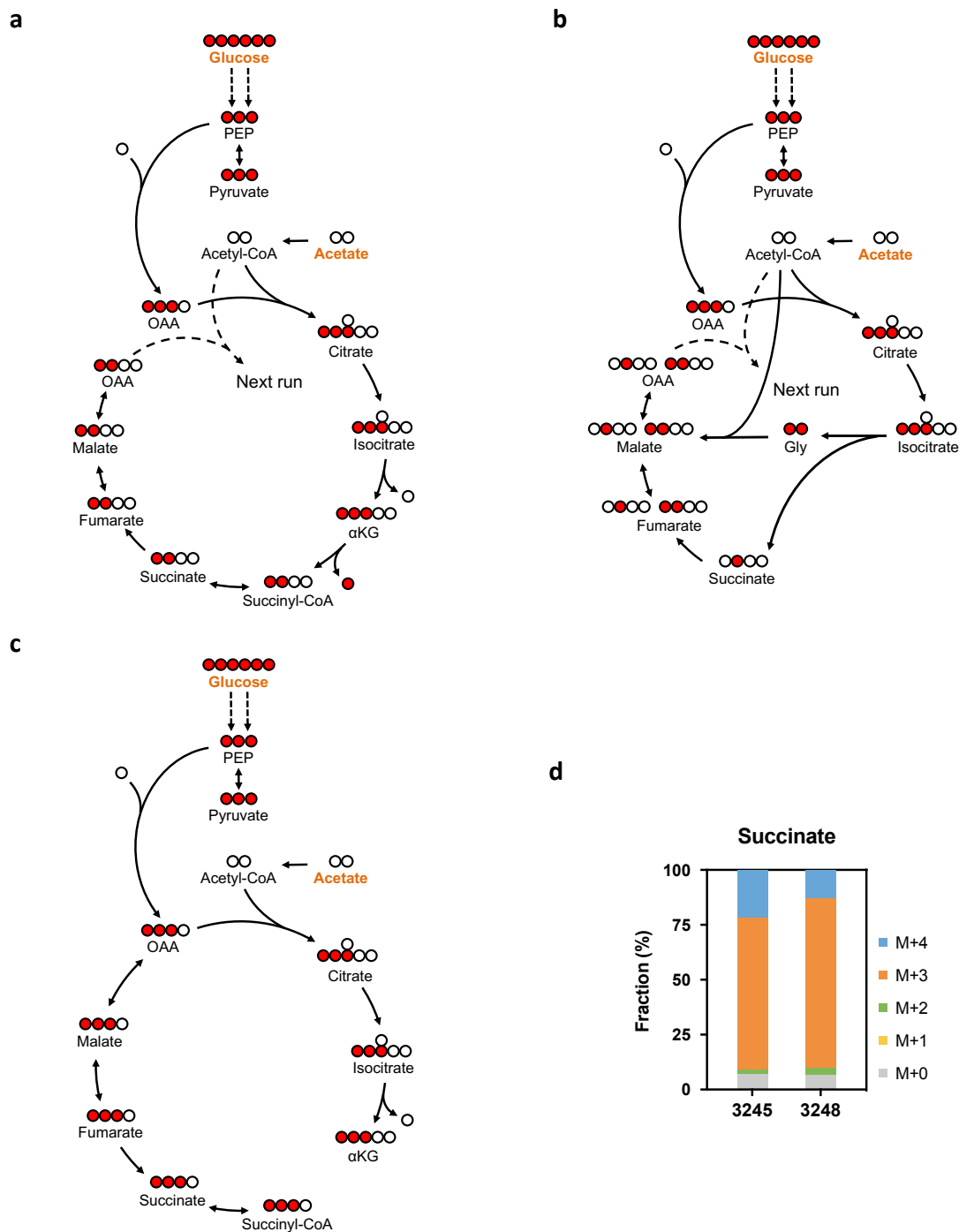
Supplementary Fig. 8. Distribution of the acetyl-CoA yield at different concentrations of assay components. All yields (y-axis, μM) from nine rounds of optimization ($x30 = 270$) are plotted against the different concentrations (x-axis, μM for enzymes, mM for others) used within the respective conditions. Components mentioned in the main text are highlighted in red.



Supplementary Fig. 9. Verification of the scalability of optimized conditions. To dismiss any severe effects on the assay performance by using the automated procedure conducted in 10 μl volumes⁵, we repeated different conditions by manually pipetting the same compositions in a final volume of 120 μl . The differences between the assay procedures are the pipetting method (Echo[®] liquid handler vs. manual pipette), the aforementioned pipetting volume (10 vs 120 μl) and reaction vessel (96-well plate vs. 1.5 ml Eppendorf tube). Those differences lead to different surface to volume ratios, evaporation rates and assay preparation times. The methodical details are described in the Methods section. **(a)** Three conditions (in circles) after machine learning-guided optimization were picked for scalability test. Shown are acetyl-CoA production of nine rounds of machine learning-guided optimization of the THETA cycle *in vitro*. Each round tested 30 different conditions in triplicates. Assays (10 μl) were pipetted by an Echo[®] liquid handler, started with 200 μM pyruvate, and stopped after 3 h. Formed acetyl-CoA was quantified by LC-MS. **(b)** Manually repeated timepoint resolved experiments (120 μl) for THETA 3.9.9, THETA 3.9.27, and THETA 3.9.16, representing high, medium, and low acetyl-CoA production condition, respectively. Assays were started with 200 μM pyruvate, samples were withdrawn at different timepoints and quantified by LC-MS. Data in (a) represent the median with interquartile range derived from $n=30$ different testing conditions, with each individual data point representing the mean obtained in $n=3$ independent experiments. Data in (b) is color-coded according to (a) and represent the mean $\pm$ s.d. obtained in $n=3$ independent experiments.

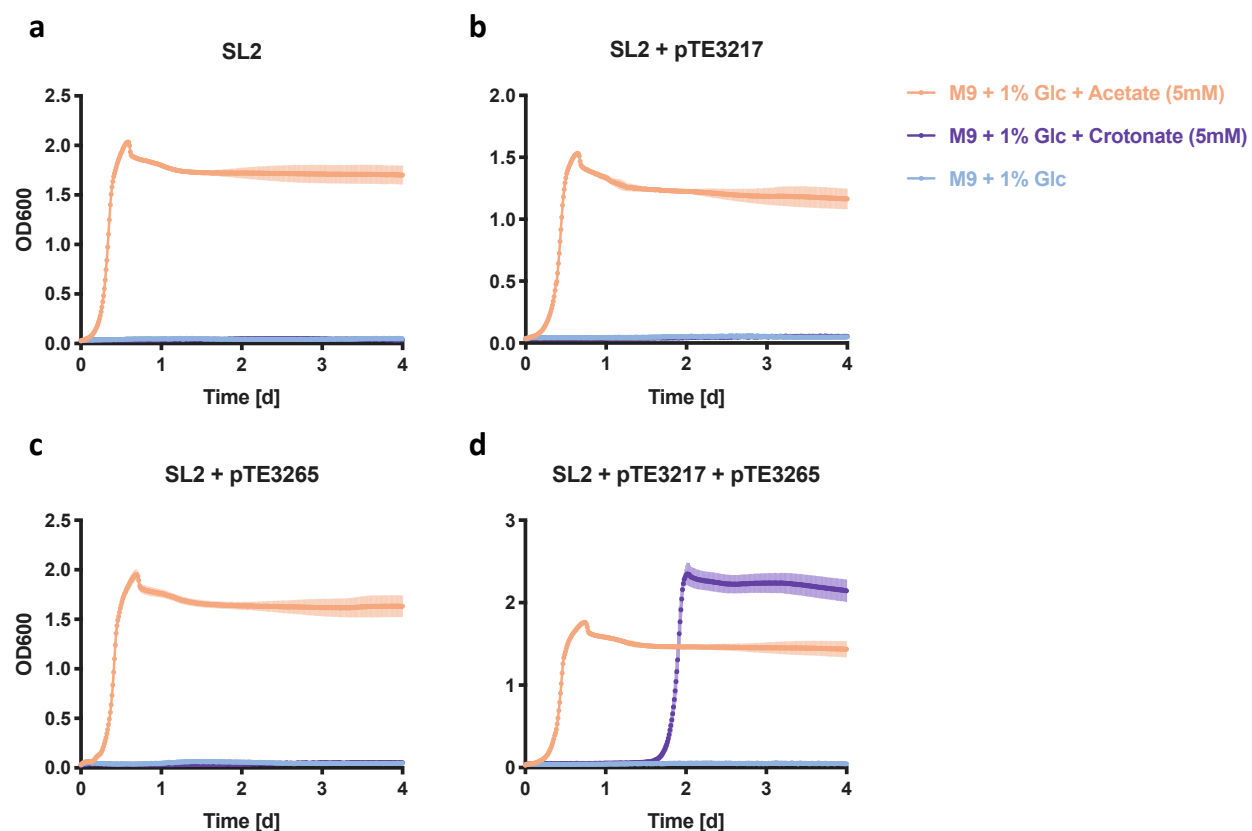


Supplementary Fig. 10. Growth profiles of (a) strain SL1, (b) strain SL1 carrying plasmid pTE3245, and (c) strain SL1 carrying plasmid pTE3248 in three minimal media. Media used in this test: M9 minimal medium with 1% glucose and 10 mM acetate (green line); M9 minimal medium with 1% glucose, 10 mM acetate, and 10 mM fumarate (blue line); M9 minimal medium with 1% glucose, 10 mM acetate, and 10 mM succinate (orange line and as the positive control). The growth data represent the mean $\pm$ s.d. obtained in $n=3$ independent experiments.

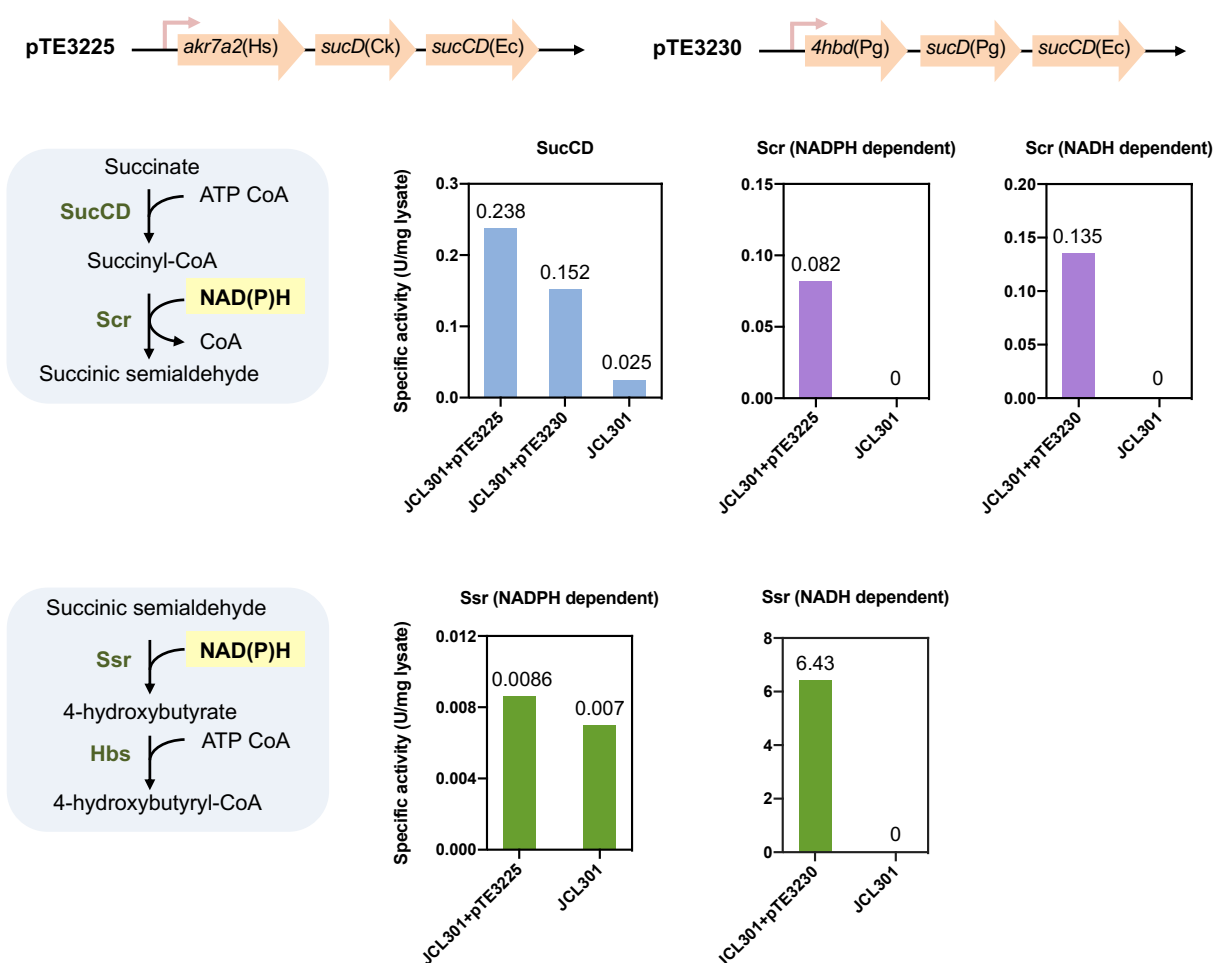


Supplementary Fig 11. Verification of module 1 of THETA *in vivo* with ¹³C-labeling. Expected ¹³C-labeling patterns when the selection strain SL1 (Ac-CoA Suc-CoA auxotroph) operates (a) oxidative TCA cycle, (b) glyoxylate shunt, or (c) module 1 of THETA. The labeling is determined with [U-¹³C]glucose, non-labeled acetate, and non-labeled CO₂. (d) Detected ¹³C-labeling of extracellular succinate after cultivating the strains (3245: SL1 containing pTE3245, 3248: SL2 containing pTE3248) in M9 medium with 1% [U-¹³C]glucose and 10 mM acetate under 5% CO₂ in the gas phase. The detected labeling pattern aligns well with the expected

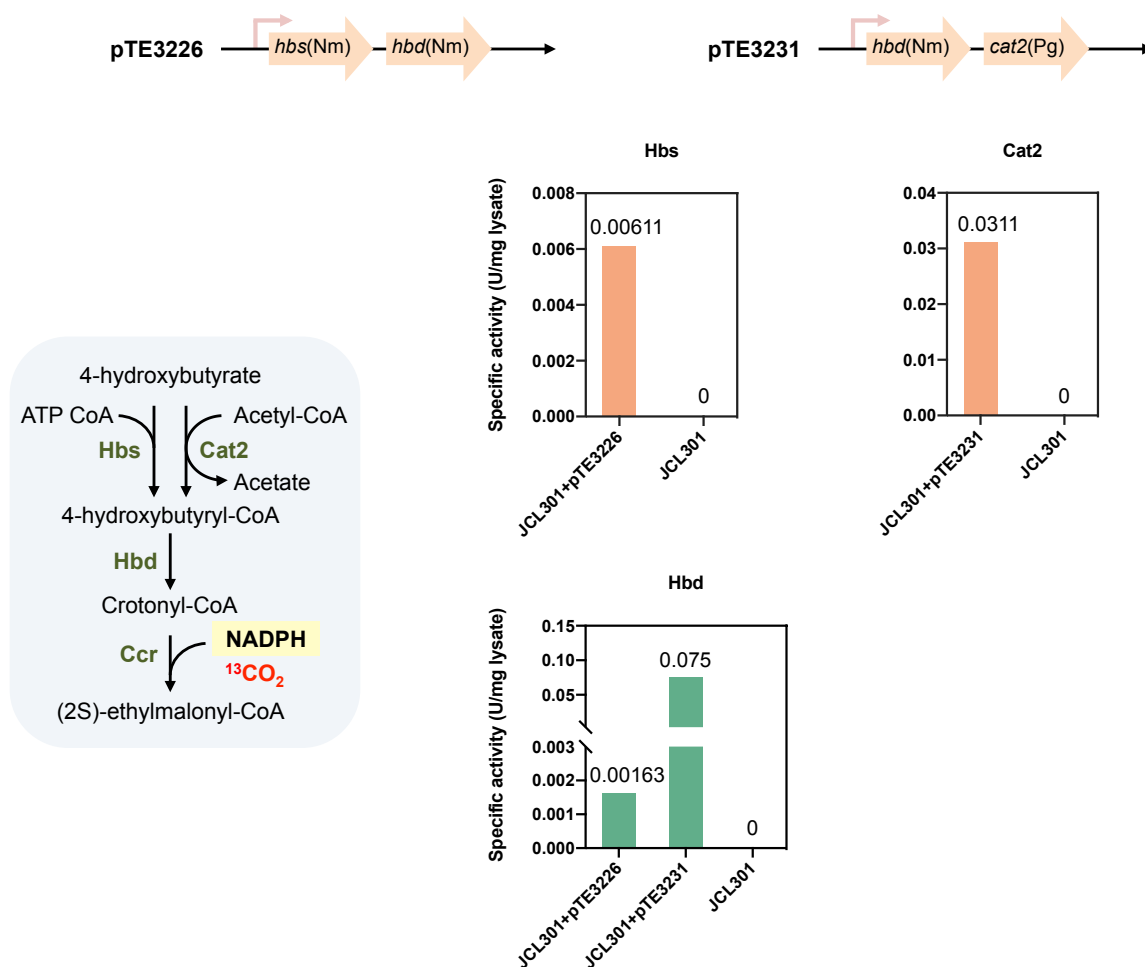
pattern of module 1 operation, predominantly producing M+3 labeled succinate. M+4 labeled succinate was detected, indicating the incorporation of intracellularly generated $^{13}\text{CO}_2$ from [U- ^{13}C]glucose. ^{13}C -labeled carbons are depicted as red dots, while non-labeled carbons are represented as empty dots. The labeling data represent the mean values obtained in n=3 independent experiments.



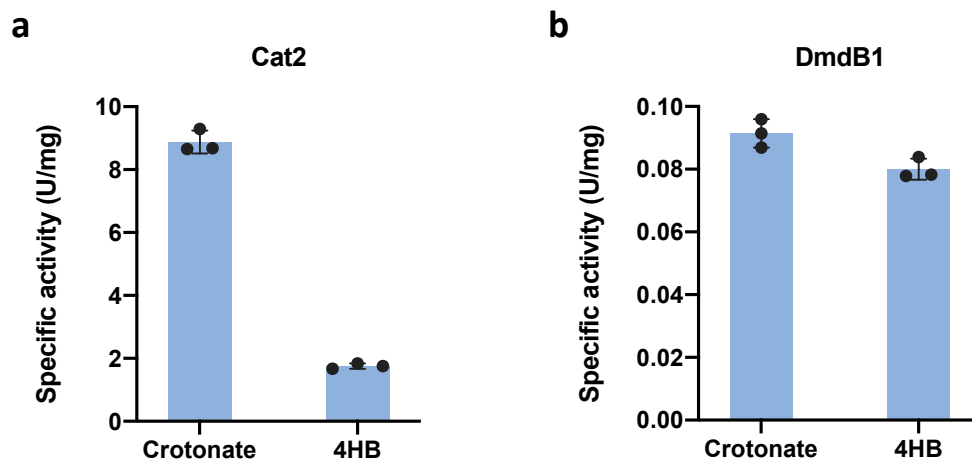
Supplementary Fig. 12. Growth profiles of (a) strain SL2, (b) strain SL2 carrying plasmid pTE3217, (c) strain SL2 carrying plasmid pTE3265, and (d) strain SL2 carrying plasmids pTE3217 and pTE3265 in three minimal media. Media used in this test: M9 minimal medium with 1% glucose and 5 mM acetate (orange line and as the positive control); M9 minimal medium with 1% glucose and 5 mM crotonate (purple line); M9 minimal medium with 1% glucose (light blue line and as the negative control). The growth data represent the mean $\pm$ s.d. obtained in n=3 independent experiments.



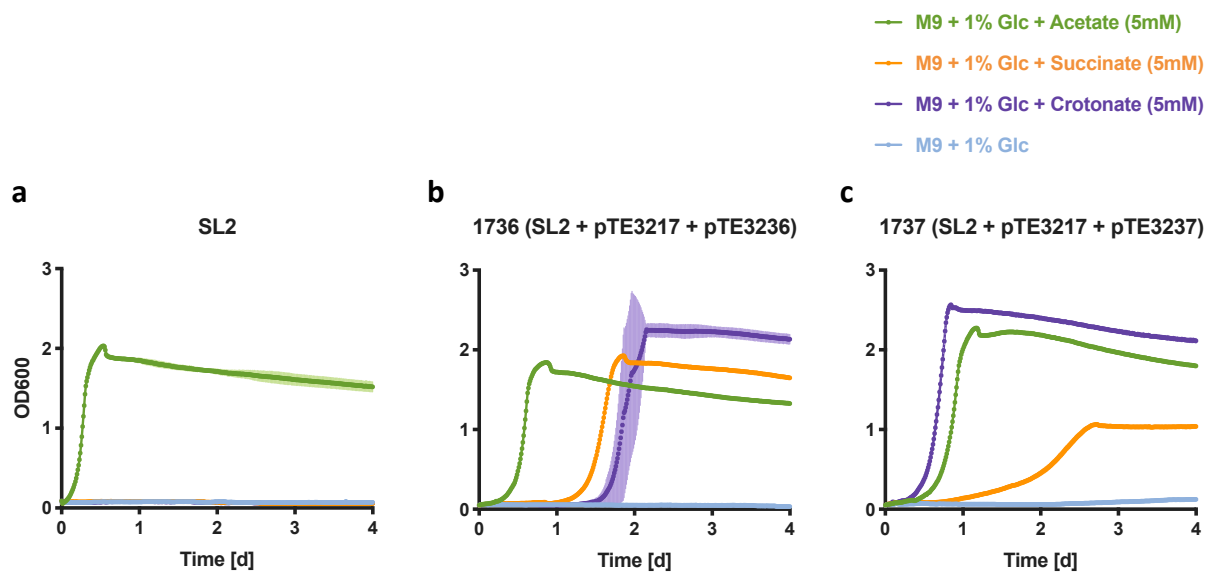
Supplementary Fig. 13. Enzyme activities of overexpressed genes in cell crude extract of JCL301 strain with pTE3225 or with pTE3230. Cell crude extracts of JCL301 and JCL301 with pTE3225 were assayed for activities of SucCD, NADPH-dependent Scr, and NADPH-dependent Ssr. Cell crude extracts of JCL301 and JCL301 with pTE3230 were assayed for activities of SucCD, NADH-dependent Scr, and NADH-dependent Ssr. See the Supplementary Methods section for details of the kinetic assays. *akr7a2* (Hs) and *4hbd* (Pg) encode Ssr; *sucD* (Ck) and *sucD* (Pg) encode Scr. Hs, *Homo sapiens*; Ck, *Clostridium kluyveri*; Pg, *Porphyromonas gingivalis*; Ec, *Escherichia coli*.



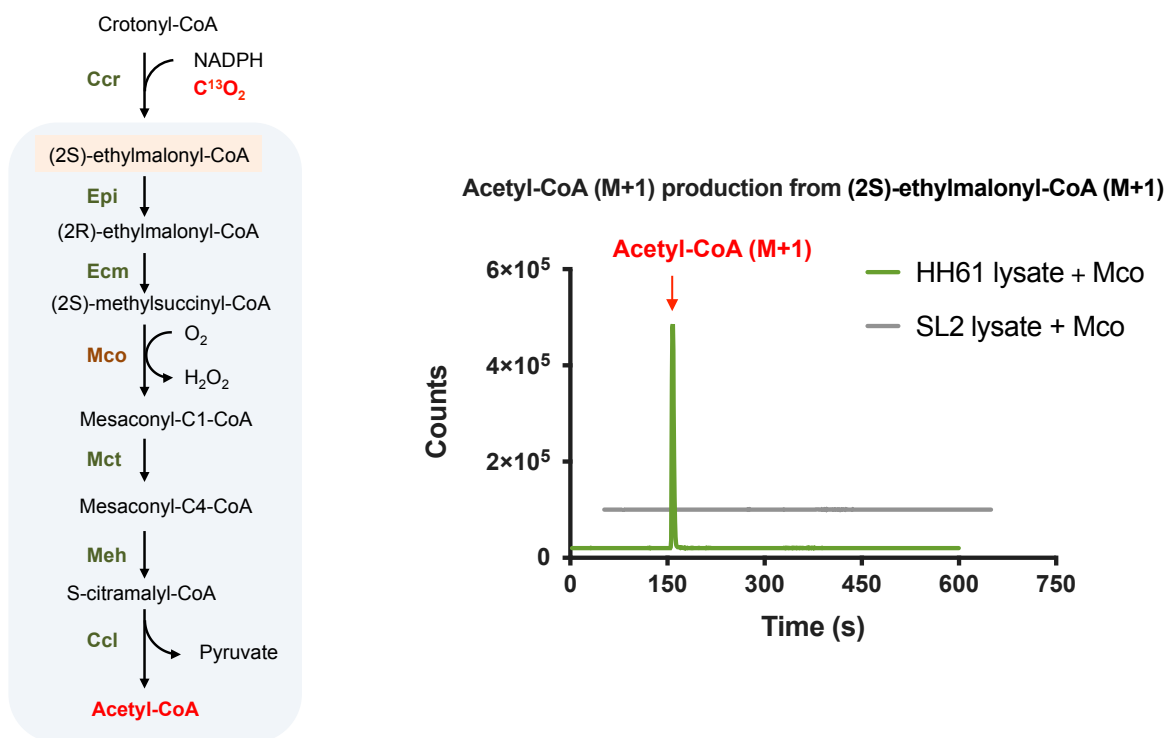
Supplementary Fig. 14. Enzyme activities of overexpressed genes in cell crude extract of JCL301 strain with pTE3226 or with pTE3231. Cell crude extracts of JCL301 and JCL301 with pTE3226 were assayed for activities of Hbd and Hbs. Cell crude extracts of JCL301 and JCL301 with pTE3231 were assayed for activities of Hbd and Cat2. See the Supplementary Methods section for details of the kinetic assays. Nm, *Nitrosopumilus maritimus*; Pg, *Porphyromonas gingivalis*.



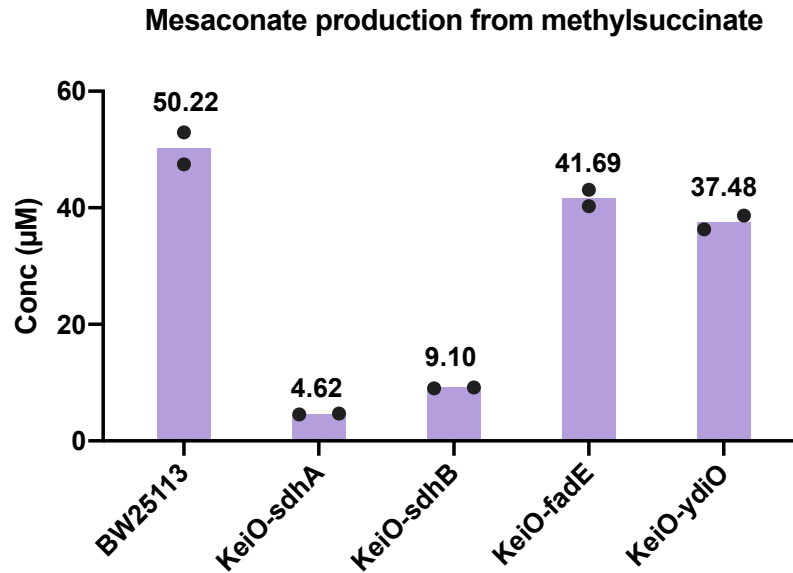
Supplementary Fig. 15. Specific activities of Cat2 and DmdB1 on crotonate and 4-hydroxybutyrate. See the Supplementary Methods section for details of the kinetic assays. The substrate concentration was 0.5 mM in all assays. The data represent the mean $\pm$ s.d. determined from $n = 3$ independent experiments. 4HB, 4-hydroxybutyrate.



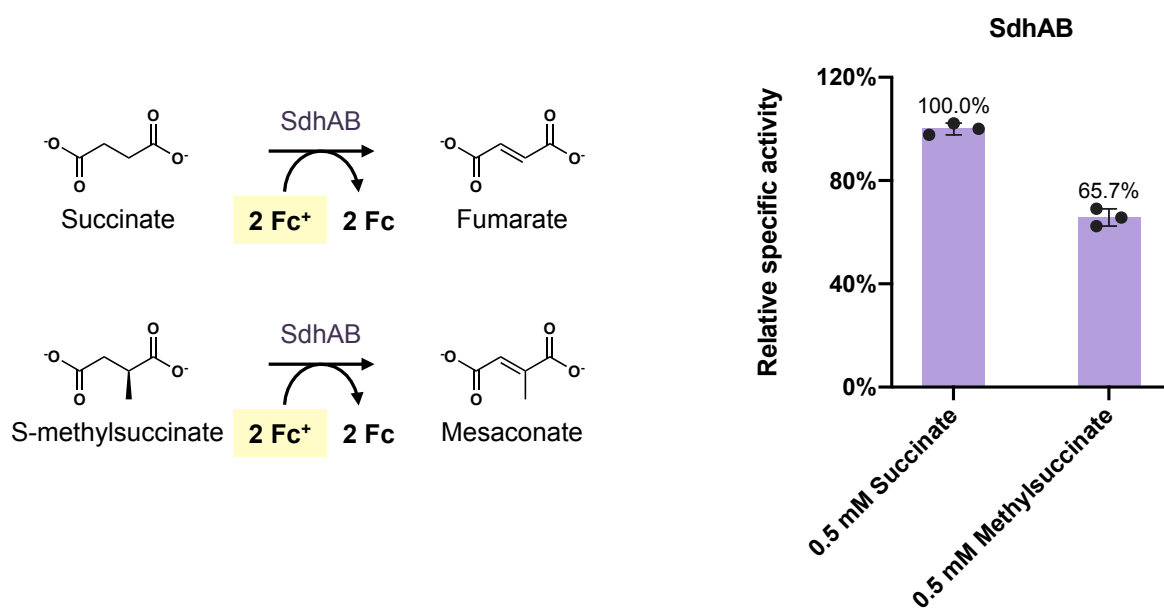
Supplementary Fig. 16. Growth profiles of (a) strain SL2, (b) strain 1736 (SL2 + pTE3217 + pTE3236), and (c) strain 1737 (SL2 + pTE3217 + pTE3237) in four minimal media. Media used in this test: M9 minimal medium with 1% glucose and 5 mM acetate (green line and as the positive control); M9 minimal medium with 1% glucose and 5 mM succinate (orange line); M9 minimal medium with 1% glucose and 5 mM crotonate (purple line); M9 minimal medium with 1% glucose (light blue line and as the negative control). The growth data represent the mean $\pm$ s.d. obtained in n=3 independent experiments.



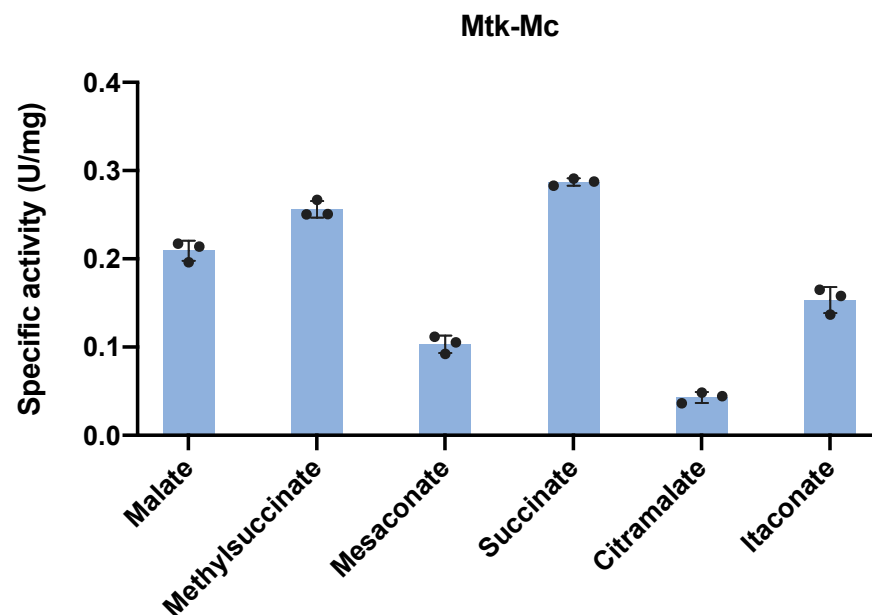
Supplementary Fig. 17. Verification of enzyme activities of integrated genes in HH61 strain with LC-MS. HH61 was created by integrating *epi*, *ecm*, *mct*, *meh*, and *ccl* into the chromosome of SL2 strain. To verify the integrated genes expressed active enzymes in HH61, a LC-MS based assay was performed. The assay mixture (200 μ L) contained 100 mM MOPS pH 7, 5 mM $MgCl_2$, 0.15 mM M+1 (2S)-ethylmalonyl-CoA (synthesized using crotonyl-CoA, $NaH^{13}CO_3$, NADPH, and Ccr), 105 μ g Mco, and 5 μ L cell lysate. The mixture was quenched with 3% formic acid after reacting for 40 min at 30 $^{\circ}C$ and analyzed with LC-MS. The cell lysate of HH61 and Mco together converted M+1 (2S)-ethylmalonyl-CoA to M+1 acetyl-CoA, indicating all enzymes of integrated genes were active in HH61. Before assays, cell lysates were incubated with 0.1 mM Coenzyme B12 in dark on ice for 1 h for Ecm activation.



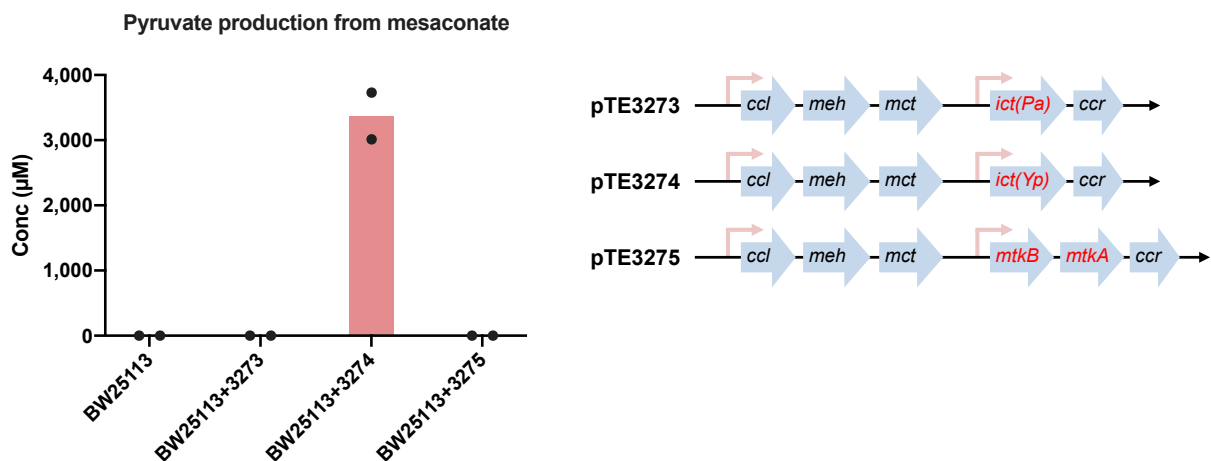
Supplementary Fig. 18. Mesaconate production of BW25113 and four Keio strains in M9 minimal medium with 1% glucose, 10 mM acetate, and 10 mM methylsuccinate. *sdhA* encodes the SdhA subunit of succinate dehydrogenase; *sdhB* encodes the SdhB subunit of succinate dehydrogenase; *fadE* encodes acyl-CoA dehydrogenase; *ydiO* encodes a putative acyl-CoA dehydrogenase. Knocking out *sdhA* or *sdhB* significantly decreased the mesaconate production titer from methylsuccinate (reduced by 80-90%), indicating succinate dehydrogenase is the main enzyme converting methylsuccinate to mesaconate in *E. coli*. Shown are the results of n=2 independent experiments as mean and individual data points.



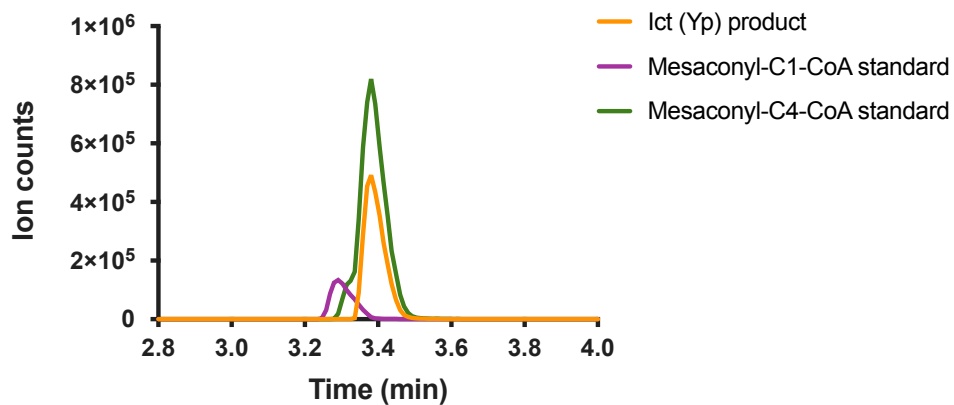
Supplementary Fig. 19. Relative specific activities of SdhAB on succinate and methylsuccinate. See the Supplementary Methods section for details of the kinetic assays. Fc⁺ is used as the artificial electron acceptor of SdhAB. The data represent the mean $\pm$ s.d. determined from n = 3 independent experiments. Fc⁺, ferrocenium hexafluorophosphate; Fc, ferrocene.



Supplementary Fig. 20. Specific activities of Mtk-Mc on malate, methylsuccinate, mesaconate, succinate, citramalate, and itaconate. See the Supplementary Methods section for details of the kinetic assays. The substrate concentration was 0.5 mM in all assays. The data represent the mean $\pm$ s.d. determined from $n=3$ independent experiments. Mtk, malate thiokinase; Mc, *Methylococcus capsulatus*.

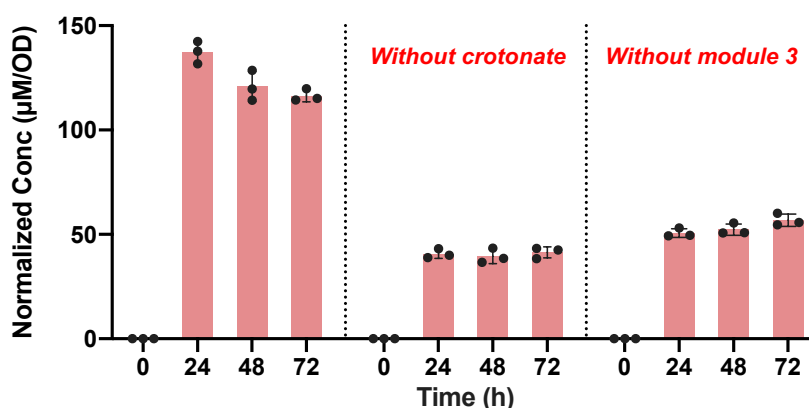


Supplementary Fig. 21. Pyruvate production from mesaconate through Ict(Yp) and the lower part of module 3 in THETA. BW25113 and BW25113 transformed with plasmid pTE3273, pTE3274, or pTE3275 were cultured in M9 minimal medium containing 1% [U-¹³C]glucose and 10 mM mesaconate. Since glucose in the medium was fully labeled, non-labeled pyruvate detected in the medium was produced from non-labeled mesaconate. BW25113 carrying plasmid pTE3274 produced pyruvate from mesaconate, indicating Ict(Yp) and the lower part of module 4 (Meh and Ccl steps) worked *in vivo*. See the right side for details of plasmid pTE3273, pTE3274, and pTE3275. Note that *ccr* and *mct* were included in plasmids for further testing and did not contribute to the pyruvate production. Shown are the results of n=2 independent experiments as mean and individual data points. Yp, *Yersinia pestis*; Pa, *Pseudomonas aeruginosa*.



Supplementary Fig. 22. Verification of the product of Ict (Yp) reaction with LC-MS. The Ict (Yp) reaction mixture (200 μ L) contained 100 mM MOPS pH 7, 5 mM MgCl_2 , 5 mM mesaconate, 0.1 mM acetyl-CoA, and 26 μ g Ict (Yp). The mixture was quenched with 3% formic acid after reacting for 30 min at room temperature and analyzed with LC-MS. As shown, the product of Ict (Yp) is mesaconyl-C4-CoA.

Pyruvate production with module 3 in SL3



Strain: SL3 (Ac-CoA auxotroph with *epi* *ecm* integrated)

pTE4501-ict-ccr-ccl-meh:	+	+	-
pTE4502-sdhCDAB-dmdB1:	+	+	-

Supplementary Fig. 23. Testing of module 3 of THETA in strain SL3. Expression of module 3 (integrated *epi* and *ecm*, pTE4501, and pTE4502) enabled SL3 to produce more non-labeled pyruvate in M9 minimal medium containing 1% [U-¹³C]glucose, 10 mM [U-¹³C]acetate, 2% LB, 0.5 μM coenzyme B12, and 10 mM crotonate, compared to the controls. Two controls were used: SL3 with module 3 in the same medium in the absence of crotonate, and SL3 without module 3 in the same medium containing crotonate. The conversion from pyruvate to acetyl-CoA was blocked in SL3, resulting in pyruvate overproduction in the medium (more than 50 mM pyruvate). Even when using [U-¹³C]glucose (99 atom % ¹³C), SL3 was still able to produce a small amount (30-50 μM) of non-labeled pyruvate in the medium. Hence, SL3 is not suitable for testing module 3 using ¹³C-labeling when the flux of module 3 is low. The data represent the mean ± s.d. determined from n = 3 independent experiments.

Supplementary Table 1. Comparison of THETA with natural and realized synthetic CO₂ fixation pathways.

To produce one molecule of **pyruvate**:

Pathway	Carbon species		CO ₂ -fixing enzyme	ATP	Reducing equivalents			Oxygen sensitivity	Status
	CO ₂	HCO ₃ ⁻			NAD(P)H	FADH ₂	Fd ²⁻ _{red}		
CBB cycle	3	0	RuBisCO	7	5	0	0	No, but side activity	Natural
rTCA cycle	3	0	KOR, Idh, POR	2	2	1	2	Yes	Natural
WL pathway ^[a]	3	0	CODH/ACS, POR	1	2	0	3	Yes	Natural
3HP bicycle	0	3	ACC, PCC	7	6	-1	0	No	Natural
3HP/4HB cycle	0	3	ACC, PCC	9 or 5 ^[b]	6	-1	0	Yes or No ^[c]	Natural
DC/4HB cycle	2	1	Ppc, POR	5	2	1	2	Yes	Natural
rGly pathway ^[d]	3	0	GCS, POR	2	3	0	2 ^[e]	Yes ^[f]	Natural
rAcCoA bicycle	3	0	POR	6	2	0	3	Yes	Synthetic (<i>in vivo</i>) ⁷
GED	3	0	Gnd	6	5	0	0	No	Synthetic (awoken <i>in vivo</i>) ⁸
POAP	1	2	Pyc, POR	10	3	0	2	Yes	Synthetic (<i>in vitro</i>) ⁹
CETCH 5.4	3	0	Ccr	2	9	-4	0	No	Synthetic (<i>in vitro</i>) ⁴
rGPS-MCG	1	2	Ppc, Ccr	5	7	-2	0	No	Synthetic (<i>in vitro</i>) ³
THETA ^[g]	2	1	Ppc, Ccr	4	7	-2	0	No	Synthetic (<i>in vitro</i> & <i>in vivo</i>)

To produce one molecule of **acetyl-CoA**:

Pathway	Carbon species		CO ₂ -fixing enzyme	ATP	Reducing equivalents			Oxygen sensitivity	Status
	CO ₂	HCO ₃ ⁻			NAD(P)H	FADH ₂	Fd ²⁻ _{red}		
CBB cycle	2	0	RuBisCO	7	4	0	0	No, but side activity	Natural
rTCA cycle	2	0	KOR, Idh, POR	2	2	1	1	Yes	Natural
WL pathway ^[a]	2	0	CODH/ACS, POR	1	2	0	2	Yes	Natural
3HP bicycle ^[h]	0	2	ACC, PCC	6	4	0	0	No	Natural
3HP/4HB cycle	0	2	ACC, PCC	6 or 4 ^[i]	4	0	0	Yes or No ^[c]	Natural
DC/4HB cycle	1	1	Ppc, POR	5	2	1	1	Yes	Natural

rGly pathway ^[d]	2	0	GCS, POR	2	3	0	1 ^[e]	Yes ^[f]	Natural
rAcCoA bicycle	2	0	POR	6	2	0	2	Yes	Synthetic (<i>in vivo</i>) ⁷
GED	2	0	Gnd	6	4	0	0	No	Synthetic (awoken <i>in vivo</i>) ⁸
POAP	0	2	Pyc, POR	10	3	0	1	Yes	Synthetic (<i>in vitro</i>) ⁹
CETCH 5.4	2	0	Ccr	2	8	-4	0	No	Synthetic (<i>in vitro</i>) ⁴
rGPS-MCG	0	2	Ppc, Ccr	5	5	-1	0	No	Synthetic (<i>in vitro</i>) ³
THETA	1	1	Ppc, Ccr	4	5	-1	0	No	Synthetic (<i>in vitro</i> & <i>in vivo</i>)

Shown are the numbers of CO₂ and HCO₃⁻ fixed and the numbers of ATP, NAD(P)H, FADH₂ and Fd²⁻_{red} molecules consumed (positive numbers) or generated (negative numbers) during the conversion of CO₂ equivalents into one molecule of pyruvate or acetyl-CoA. Every AMP-forming reaction accounts for two ATP equivalents. The primary product, oxalate, is converted to glyoxylate using oxalate-CoA ligase and glyoxylate dehydrogenase. The primary product, glyoxylate, is converted to pyruvate through the bacterial glycolate catabolic pathway, and pyruvate is further decarboxylated to acetyl-CoA through pyruvate dehydrogenase. ^[a] The variant of WL pathway functioning in acetogens. ^[b] Nine ATP molecules are required in Crenarchaeal HP/HB cycle and five ATP molecules are required in Thaumarchaeal HP/HB cycle. Succinyl-CoA is converted to succinate through succinyl-CoA synthetase. ^[c] The key enzyme, 4-hydroxybutyryl-CoA dehydratase (Hbd), is oxygen sensitive in Clostridia, while Hbd from *Metallosphaera sedula* and *Nitrosopumilus maritimus* are robust in the presence of oxygen. ^[d] The variant of rGly pathway functioning in *Desulfovibrio desulfuricans*. ^[e] Fd²⁻_{red} is assumed to be the electron donor for formate dehydrogenase in this pathway. ^[f] Oxygen tolerant variants of rGly pathway could exist in nature. ^[g] To produce pyruvate, THETA is coupled with 3 steps from the ethylmalonyl-CoA pathway to convert two acetyl-CoA into one crotonyl-CoA. ^[h] To produce acetyl-CoA, 3HP bicycle is coupled with Pps, Ppc, Mdh, and succinyl-CoA:L-malate CoA transferase. ^[i] Six ATP molecules are required in Crenarchaeal HP/HB cycle and four ATP molecules are required in Thaumarchaeal HP/HB cycle. RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; POR, pyruvate:ferredoxin oxidoreductase; KOR, α-Ketoglutarate:ferredoxin oxidoreductase; Idh, Isocitrate dehydrogenase; CODH/ACS, CO dehydrogenase/acetyl-CoA synthase; ACC, acetyl-CoA carboxylase; PCC, propionyl-CoA carboxylase; Ppc, phosphoenolpyruvate carboxylase; Pyc, pyruvate carboxylase; GCS, glycine cleavage system; Gnd, 6-phosphogluconate dehydrogenase; Ccr, crotonyl-CoA carboxylase/reductase; CBB cycle, Calvin-Benson-Bassham cycle; rTCA cycle, reductive citric acid cycle; WL pathway, Wood-Ljungdahl pathway; 3HP bicycle, 3-hydroxypropionate bicycle; 3HP/4HB cycle, 3-hydroxypropionate/4-hydroxybutyrate cycle; DC/4HB cycle, dicarboxylate/4-hydroxybutyrate cycle; rGly pathway, the reductive glycine pathway; rAcCoA bicycle, reductive acetyl-CoA bicycle; Fd²⁻_{red}, reduced ferredoxin.

Supplementary Table 2. Summary of specific activities of enzymes used for building THETA *in vitro*.

Enzyme name	Abbrev.	E.C number	Gene name	Source	Specific Activity (U/mg)	Reference
phosphoenolpyruvate synthetase	Pps	2.7.9.2	<i>pps</i>	<i>Escherichia coli</i>	8.9	10
phosphoenolpyruvate carboxylase	Ppc	4.1.1.31	<i>ppc</i>	Commercial (Sigma C1744)	5.6	
malate dehydrogenase	Mdh	1.1.1.37	<i>mdh</i>	<i>Escherichia coli</i>	838.7	11
malate dehydrogenase	Mdh	1.1.1.37	<i>mdh</i>	Commercial (Sigma M2634)	735	
fumarase	Fum	4.2.1.2	<i>fumC</i>	<i>Escherichia coli</i>	59.4	12
fumarase	Fum	4.2.1.2	<i>fum</i>	Commercial (Sigma F1757)	487	
fumarate reductase (NADH)	Frd	1.3.1.6	<i>frd</i>	<i>Trypanosoma brucei</i>	0.022	13
dihydroorotate dehydrogenase, type 1A	DHOD1a	1.3.98.1	<i>dhod</i>	<i>Trypanosoma cruzi</i>	3.77	This study
dihydroorotate dehydrogenase, type 1B	DHOD1b	1.3.1.14	<i>pyrDK</i>	<i>Bacillus subtilis</i>	2.97	This study
succinyl-CoA ligase	SucCD	6.2.1.5	<i>sucCD</i>	<i>Escherichia coli</i>	12.06	14
succinyl-CoA reductase	Scr	1.2.1.76	<i>sucD</i>	<i>Clostridium kluyveri</i>	29	15
succinic semialdehyde reductase	Ssr	1.1.1.co	<i>akr7a2</i>	<i>Homo sapiens</i>	3.9	4
4-hydroxybutyryl-CoA synthetase	Hbs	6.2.1.56	<i>nmar0206/hbs</i>	<i>Nitrosopumilus maritimus</i>	1.4	16
4-hydroxybutyryl-CoA dehydratase	Hbd	4.2.1.120	<i>nmar0207/hbd</i>	<i>Nitrosopumilus maritimus</i>	25	16
crotonyl-CoA carboxylase/reductase	Ccr	1.3.1.85	<i>ccr</i>	<i>Methylobacterium extorquens</i>	110	4
ethylmalonyl-CoA epimerase	Epi	5.1.99.1	<i>epi</i>	<i>Rhodobacter sphaeroides</i>	440	17
(2R)-ethylmalonyl-CoA mutase	Ecm	5.4.99.63	<i>ecm</i>	<i>Rhodobacter sphaeroides</i>	7	17
methylsuccinyl-CoA oxidase	Mco		<i>mco</i>	<i>Rhodobacter sphaeroides</i>	0.1	4
mesaconyl-C1-CoA-C4-CoA transferase	Mct	5.4.1.3	<i>mct</i>	<i>Chloroflexus aurantiacus</i>	550	18
mesaconyl-C4-CoA hydratase	Meh	4.2.1.153	<i>meh</i>	<i>Chloroflexus aurantiacus</i>	950	18
(S)-citramalyl-CoA lyase	Ccl	4.1.3.25	<i>ccl</i>	<i>Pseudomonas aeruginosa</i>	170	19
malyl-CoA thioesterase	Mcl2	3.1.2.30	<i>mcl2</i>	<i>Rhodobacter sphaeroides</i>	200	20
catalase	Kat	1.11.1.6	<i>katE</i>	<i>Escherichia coli</i>	11740	21
glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49	<i>g6pdh</i>	Commercial (Sigma G5885)	550	
creatine phosphokinase	Cpk	2.7.3.2	<i>cpk</i>	Commercial (Sigma C3755)	338	
adenylate kinase	Adk	2.7.4.3	<i>adk</i>	<i>Escherichia coli</i>	1020	22

Supplementary Table 3. Summary of accession numbers of genes used in building THETA *in vivo*.

	Enzyme	Gene	Source	Accession Number
Module 1	2-oxoglutarate decarboxylase subunit A	<i>sucA</i>	<i>E. coli</i>	AAC73820
	2-oxoglutarate decarboxylase subunit B	<i>sucB</i>	<i>E. coli</i>	AAC73821
	isocitrate lyase	<i>aceA</i>	<i>E. coli</i>	AAC76985
	L-aspartate oxidase (NadB)	<i>nadB</i>	<i>E. coli</i>	QNG33435
	NADH-dependent fumarate reductase (Frd)	<i>frd</i>	<i>T. brucei</i>	AAN40014
Module 2	2-amino-3-ketobutyrate CoA ligase	<i>kbl</i>	<i>E. coli</i>	AAC76641
	low-specificity L-threonine aldolase	<i>ltaE</i>	<i>E. coli</i>	AAC73957
	acetyl-CoA acetyltransferase (AtoB)	<i>atoB</i>	<i>E. coli</i>	AAC75284
	3-hydroxybutyryl-CoA dehydrogenase (Bhbd)	<i>bhbd</i>	<i>C. acetobutylicum</i>	AAA95971
	crotonase (Crt)	<i>crt</i>	<i>C. acetobutylicum</i>	AAA95967
	succinyl-CoA synthetase subunit β (SucC)	<i>sucC</i>	<i>E. coli</i>	AAC73822
	succinyl-CoA synthetase subunit α (SucD)	<i>sucD</i>	<i>E. coli</i>	AAC73823
	succinyl-CoA reductase (Scr)	<i>sucD</i>	<i>C. kluyveri</i>	WP_012103358
	succinyl-CoA reductase (Scr)	<i>sucD</i>	<i>P. gingivalis</i>	WP_005873579
	succinic semialdehyde reductase (Ssr)	<i>akr7a</i>	<i>H. sapiens</i>	AAH04111
	succinic semialdehyde reductase (Ssr)	<i>4hbd</i>	<i>P. gingivalis</i>	WP_004585235
	4-hydroxybutyryl-CoA synthetase (Hbs)	<i>nmar0206/hbs</i>	<i>N. maritimus</i>	ABX12102
	4-hydroxybutyryl-CoA transferase (Cat2)	<i>cat2</i>	<i>P. gingivalis</i>	WP_005874820
	crotonyl-CoA ligase (DmdB1)	<i>dmdB1</i>	<i>P. aeruginosa</i>	AAG07585
	4-hydroxybutyryl-CoA dehydratase (Hbd)	<i>nmar0207/hbd</i>	<i>N. maritimus</i>	ABX12103
Module 3	crotonyl-CoA carboxylase/reductase (Ccr)	<i>ccr</i>	<i>R. sphaeroides</i>	ABA80143
	ethylmalonyl-CoA/methylmalonyl-CoA epimerase (Epi)	<i>epi</i>	<i>R. sphaeroides</i>	ABA79990
	(2R)-ethylmalonyl-CoA mutase (Ecm)	<i>ecm</i>	<i>R. sphaeroides</i>	ABA80144
	acyl-CoA thioesterase	<i>tesB</i>	<i>E. coli</i>	AAC73555
	acyl-CoA thioesterase	<i>yciA</i>	<i>E. coli</i>	AAC74335
	succinate:quinone oxidoreductase subunit A (SdhA)	<i>sdhA</i>	<i>E. coli</i>	AAC73817
	succinate:quinone oxidoreductase subunit B (SdhB)	<i>sdhB</i>	<i>E. coli</i>	AAC73818
	succinate:quinone oxidoreductase subunit C (SdhC)	<i>sdhC</i>	<i>E. coli</i>	AAC73815
	succinate:quinone oxidoreductase subunit D (SdhD)	<i>sdhD</i>	<i>E. coli</i>	AAC73816
	itaconate CoA transferase (Ict)	<i>ict</i>	<i>P. aeruginosa</i>	AAG04271
	itaconate CoA transferase (Ict)	<i>ict</i>	<i>Y. pestis</i>	WP_002212070
	malate thiokinase subunit β (MtkA)	<i>mtkA</i>	<i>M. capsulatus</i>	WP_010960994
	malate thiokinase subunit α (MtkB)	<i>mtkB</i>	<i>M. capsulatus</i>	WP_010960995
	mesaconyl-C1-CoA-C4-CoA transferase (Mct)	<i>mct</i>	<i>C. aurantiacus</i>	ABY33429
	mesaconyl-C4-CoA hydratase (Meh)	<i>meh</i>	<i>C. aurantiacus</i>	ABY33434
	(S)-citramalyl-CoA lyase (Ccl)	<i>ccl</i>	<i>P. aeruginosa</i>	AAG04272

Supplementary Table 4. Summary of mutations and structural variants emerged from evolution.

Mutations identified with Illumina sequencing in the evolved strains (1736 & 1737) compared to their parent strain SL2.

Position (at JCL301)	1736	1737	Annotation	Gene
145,951		C→T	G707S (GGT→AGT)	<i>htrE</i> ←
450,563		IS186 (–) +8 bp	intergenic (+89/-92)	<i>clpX</i> → / → <i>lon</i>
1,758,994	A→G		intergenic (-552/-235)	<i>ydfJ</i> ← / → <i>ydfK</i>
2,449,286	T→G		I129S (ATC→AGC)	<i>atoC</i> →

Structural variations identified with NanoPore sequencing in the evolved strains (1736 & 1737) compared to their parent strain SL2.

Position (at JCL301)	1736	1737	Annotation	Gene
450,574		INS 1326 bp	IS4 transposase	<i>clpX</i>
593,936	DEL 10546 bp	DEL 10547 bp	cryptic prophage DLP12 ^[a]	<i>insC1</i> ← / → <i>repE</i>
1,263,696	INS 1326 bp		IS4 transposase	<i>ndh</i>
4,657,787	INV 296 bp	INV 296 bp	intergenic (+53/-128) ^[b]	<i>fimE</i> → / → <i>fimA</i>
4,696,635	INS 1329 bp		coding (1509/1590 nt)	<i>hsdM</i> ←

Mutations identified with 100% frequency in the evolved strains with constructed JCL301 genome and plasmids sequences as references. No mutation was found in all plasmids. ^[a] This deletion was not detected in Illumina sequencing, indicating prophage DLP12 may still exist in the stains. ^[b] The inversion of *fimA* promoter turned off the production of type 1 fimbriae. DEL, deletion; INS, insertion; INV, inversion.

Supplementary Table 5. Summary of conditions for demonstrating three different modules of THETA *in vivo*.

Module	Testing platform	Plasmids	Testing medium	Result
Module 1	SL1 (Ac-CoA Suc-CoA auxotroph)	pTE3245- <i>frd</i>	M9 + 1% glucose + 10 mM acetate	Growth rescued (verified with ¹³ C-labeling)
		pTE3248- <i>nadB</i>		Growth rescued (verified with ¹³ C-labeling)
Module 2	SL2 (Ac-CoA auxotroph)	pTE3217- <i>atoB-crt-bhbd</i> pTE3236- <i>hbd-cat2-sucD-4hbd-sucCD</i>	M9 + 1% glucose + 5mM succinate	Growth rescued (verified with ¹³ C-labeling)
		pTE3217- <i>atoB-crt-bhbd</i> pTE3237- <i>hbd-dmdB1-sucD-4hbd-sucCD</i>		Growth rescued (verified with ¹³ C-labeling)
Upper part of module 3	HH61 (SL2 with epi ecm mct meh ccl integrated)	pTE3258- <i>ccr</i> pTE3265- <i>dmdB1</i>	M9 + 1% glucose + 10 mM acetate + 10 mM crotonate + 0.5 μM coenzyme B12	Mesaconate and methylsuccinate production from crotonate
Lower part of module 3	HH61 (SL2 with epi ecm mct meh ccl integrated)	pTE3274- <i>ict-ccr-ccl-meh-mct</i>	M9 + 1% [U- ¹³ C]glucose + 10 mM [U- ¹³ C]acetate + 10 mM mesaconate	Non-labeled pyruvate production from mesaconate
Module 3	HH422 (SIJ488 with epi ecm integrated)	pTE4501- <i>ict-ccr-ccl-meh</i> pTE4502- <i>sdhCDAB-dmdB1</i>	M9 + 1% [U- ¹³ C]glucose + 2% LB ^[a] + 10 mM crotonate + 0.5 μM coenzyme B12	Non-labeled pyruvate production from crotonate

All testing media contained 0.5 mM IPTG and appropriate antibiotics. ^[a] To confirm that the non-labeled pyruvate originated from crotonate and not from LB, two negative controls were used: HH422 with module 3 plasmids in the same medium in the absence of crotonate, and HH422 without module 3 plasmids in the same medium containing crotonate. Neither of two negative controls produced non-labeled pyruvate in the medium.

Supplementary Table 6. Summary of production performance of different THETA versions.

Version	Product	Product titer (μM)	Steady-state production rate (μM product/h)	Enzyme conc (mg/ml)	CO ₂ fixation rate (nmol/mg/min)
THETA 1.0	Acetyl-CoA	8.5	8.1	2.3	0.1
THETA 2.0	Acetyl-CoA	37.6	39.1	2.3	0.6
THETA 3.0	Acetyl-CoA	207.3	166.7	2.1	2.7
THETA 3.9.9	Acetyl-CoA	887.0	343.8	7.9	1.4
THETA 3.9.9.mc	Malonyl-CoA	548.1	330.4	8.9	1.9
THETA 3.9.9.gc	Glycolate	760.3	139.1	10.1	0.5

Shown are results from assays started with 200 μM substrate (200 μM pyruvate in THETA 1.0, 2.0, 3.0, 3.9.9, 3.9.9.mc, and 200 μM succinate in THETA 3.9.9.gc).

Supplementary Table 7. List of plasmids used in this study.

Plasmid	Description ^[a]	Reference
pSIJ8	pKD46, rhaRS-prha-FLP, Amp	23
pKD3	R6Ky ori, FRT-Cam-FRT, Cam and Amp	24
pKD4	R6Ky ori, FRT-Kan-FRT, Kan and Amp	24
pCas9cr4	p15A ori, nuclease positive cas9, Cam	25
pKDsg-p15A	pSC101 ori, protospacer targeting p15A ori, Spec	25
pKDsgRNA-sucAB	pSC101 ori, protospacer targeting sucA, Spec	This study
pKDsgRNA-aceA	pSC101 ori, protospacer targeting aceA, Spec	This study
JW1692	pCA24N, pps, Cam, ASKA collection	26
JW3205	pCA24N, mdh, Cam, ASKA collection	26
JW1603	pCA24N, fumC, Cam, ASKA collection	26
JW1721	pCA24N, katE, Cam, ASKA collection	26
JW0463	pCA24N, adk, Cam, ASKA collection	26
JW0718	pCA24N, succ, Cam, ASKA collection	26
JW0717	pCA24N, sucd, Cam, ASKA collection	26
pTE380	pCDFDuet-1, 6xHis-sucD, yihU, Spec	4
p2BP1	pLIC-SGC1, akr7a2, Amp	Addgene # 39130
Nmar0206	pET16b, nmar0206, Amp	16
pTE393	pRSET B, nmar0207 (opt), Amp	4
pTE71	pET16b, ccr, Amp	27
pTE45	pET16b, epi, Amp	17
pTE33A	pET16b, ecm, Amp	17
pTE813	pET16b, mco (mcd [T317G, W315F, E377N]), Amp	4
pMCT_Ca_JZ05	pET16b, mct, Amp	18
pMEH_Ca_JZ_07	pET16b, meh, Amp	18
pBB541	<i>E. coli</i> Chaperones GroEL, GroES, Spec	28
pET16b-Ccl-Pa	pET16b, ccl _{Pa} , Amp	19
pJS12mcl2	pET16b, mcl2, Amp	20
JZ105	pCDFDuet-1, 6xHis-PccB(D407I), PccA, Spec	29
pTE1672	pET51b, gltA, Amp	6
JW1268	pCA24N, acnA, Cam, ASKA collection	26
JW3975	pCA24N, aceA, Cam, ASKA collection	26
pTE1125	pLEGO (A1), gox1801, Amp	30
pET28b-Cat2-Pg	pET28b, cat2 _{Pg} , Kan	This study
pET28a-Mtk-Mc	pET28a, 6xHis-mtkB _{Mc} -rbsA-mtkA _{Mc} , Kan	This study
pET28a-Ict-Yp	pET28a, ict _{Yp} (opt), Kan	This study
pCDF-Duet-SdhAB	pCDFDuet-1, 6xHis-sdhA _{Ec} , sdhB _{Ec} , Spec	This study

pTE3200	pET28a, frd _{Tb} (opt), Kan	This study
pTE3208	pET28a, dhod _{Ef} (opt), Kan	This study
pTE3209	pET28a, dhod _{Ll} (opt), Kan	This study
pTE3210	pET28a, dhod _{Tc} (opt), Kan	This study
pTE3211	pET28a, dhod _{Sc} (opt), Kan	This study
pTE3213	pET28a, pyrD _{Bs} -pyrK _{Bs} (opt), Kan	This study
pTE3214	pET28a, pyrD _{Ll} -pyrK _{Ll} (opt), Kan	This study
pTE3217	p15A ori, P _L lacO ₁ ::atoB _{Ec} -crt _{Ca} -bhbd _{Ca} , Kan	This study
pTE3225	p15A ori, P _L lacO ₁ ::sucD _{Ck} (opt)-akr7a _{Hs} -sucCD _{Ec} , Kan	This study
pTE3226	CloDF13 ori, P _L lacO ₁ ::nmar0206 _{Nm} (opt)-nmar0207 _{Nm} (opt), Spec	This study
pTE3230	p15A ori, P _L lacO ₁ ::sucD _{Pg} -4hbd _{Pg} -sucCD _{Ec} , Kan	This study
pTE3231	CloDF13 ori, P _L lacO ₁ ::nmar0207 _{Nm} (opt)-cat2 _{Pg} , Spec	This study
pTE3236	CloDF13 ori, P _L lacO ₁ ::nmar0207 _{Nm} (opt)-cat2 _{Pg} , P _L lacO ₁ ::sucD _{Pg} -4hbd _{Pg} -sucCD _{Ec} , Spec	This study
pTE3237	CloDF13 ori, P _L lacO ₁ ::nmar0207 _{Nm} (opt)-dmdB1 _{Pa} , P _L lacO ₁ ::sucD _{Pg} -4hbd _{Pg} -sucCD _{Ec} , Spec	This study
pTE3245	p15A ori, P _L lacO ₁ ::frd _{Tb} (opt), Kan	This study
pTE3248	p15A ori, P _L lacO ₁ ::nadB _{Ec} , Kan	This study
pTE3256	CloDF13 ori, SS7::Ps-rbsB-ccl-meh-mct (opt)-FRT-Kan-FRT, Kan, Spec	This study
pTE3257	CloDF13 ori, SS9::Ps-rbsB-epi-ecm-FRT-Kan-FRT, Kan, Spec	This study
pTE3258	p15A ori, P _L lacO ₁ ::ccr _{Rs} , Kan	This study
pTE3260	p15A ori, P _L lacO ₁ ::mco-ccr _{Rs} , Kan	This study
pTE3261	pET28a, dmdB1 _{Pa} , Kan	This study
pTE3265	CloDF13 ori, P _L lacO ₁ ::dmdB1 _{Pa} , Spec	This study
pTE3273	p15A ori, P _L lacO ₁ ::ccl-meh-mct (opt), P _L lacO ₁ ::ict _{Pa} -ccr _{Rs} , Kan	This study
pTE3274	p15A ori, P _L lacO ₁ ::ccl-meh-mct (opt), P _L lacO ₁ ::ict _{Yp} (opt)-ccr _{Rs} , Kan	This study
pTE3275	p15A ori, P _L lacO ₁ ::ccl-meh-mct (opt), P _L lacO ₁ ::mtkB _{Mc} -mtkA _{Mc} -ccr _{Rs} , Kan	This study
pTE3284	CloDF13 ori, P _L lacO ₁ ::dmdB1 _{Pa} , P _L lacO ₁ ::ccl-meh-mct (opt), Spec	This study
pTE4500	CloDF13 ori, P _L lacO ₁ ::sdhC-sdhD-sdhA-sdhB, Spec	This study
pTE4501	p15A ori, P _L lacO ₁ ::ccl-meh, P _L lacO ₁ ::ict _{Yp} (opt)-ccr _{Rs} , Kan	This study
pTE4502	CloDF13 ori, P _L lacO ₁ ::dmdB1 _{Pa} , P _L lacO ₁ ::sdhC-sdhD-sdhA-sdhB, Spec	This study

^[a] Opt, codon optimized for *E. coli* (sequences in Supplementary Table 13). Subscripts indicate the source of the gene as follows: Pa, *Pseudomonas aeruginosa*; Pg, *Porphyromonas gingivalis*; Mc, *Methylococcus capsulatus*; Yp, *Yersinia pestis*; Ec, *Escherichia coli*; Tb, *Trypanosoma brucei*; Ef, *Enterococcus faecalis*; Ll, *Lactococcus lactis*; Tc, *Trypanosoma cruzi*; Sc, *Saccharomyces cerevisiae*; Bs, *Bacillus subtilis*; Ca, *Clostridium acetobutylicum*; Ck, *Clostridium kluyveri*; Hs, *Homo sapiens*; Nm, *Nitrosopumilus maritimus*; Rs, *Rhodobacter sphaeroides*.

Supplementary Table 8. List of primers used for strain construction.

Primer name	Sequence
SS132 sucAB-sgRNA	gccctcaagcaaactactggttttagagctagaaatagcaag
SS135 aceA-sgRNA	gtcgtgccgatcagatccaagtttagagctagaaatagcaag
rec-sucAB	g*a*a*g*gcgataatgccttatccggtctacagtcaggtgaaactaaactacgtgatcccttaagcatcttt ttatgcttacttcgccgtggatactacc
rec-aceA	t*g*c*g*gcgtgaacgccttatccggcctacagtcagcaacgggtgtgtgctgcagatgctccatagttatg tgggtgtttacgctaacaggc
SS143 sucAB_Ver_F	gccatatcaagtcgatgtgttgcaac
SS144 sucAB_Ver_R	ggcataaccacccggtgctg
SS149 aceA_Ver_F	cagctgctggcaccttgatg
SS150 aceA_Ver_R	cagtagagattcccagccgtgatc
SS245 ltaE_KO_F	gctaaggtaaaaagggtggcatttcccgtcataataaggacatgccatgagtgtaggctggagctgcttc
SS246 ltaE_KO_R	gccactggcaccgagaactaaaatgcgttgcggcacgtctctccttaacatatgaatatcctcctta
SS247 kbl_KO_F	agtttgggtaatatgtgctggaatttgcctgtctggagaatcgcaatgcgtgtaggctggagctgcttc
SS248 kbl_KO_R	tgccctctccgcttccagtttggataacgcttcatctcacatcctcagcatatgaatatcctcctta
SS243 ltaE_Ver_F	gaatatacggtgagcagcacaatga
SS251 ltaE_Ver_R	tgcttaactggtacttcacgtagc
SS252 kbl_Ver_F	gcaggcgctgagaattttcc
SS253 kbl_Ver_R	gattaacaccaacgcctatcgtgt
P07_C1_V_R	tgaaagacggtgagctggtg
P08_C2_V_F	gtggtattcactccagagcg
P09_K1_V_R	cagtcatagccgaatagcct
P10_K2_V_F	cggtgccctgaatgaactgc
P13_SS9_F	gcttggtgagaatacgccg
P14_SS9_R	gcctacgattacgcatggctt
P15_SS7_F	tcgtgatgccgaagggcgaattgcg
P16_SS7_R	gcttatatgccaacgcttgg
P19_SS_pro_up	tccaaacctccttaaagttaaacaaaattatttctattaactag
P20_SS_kan_down	gagctgtacaagtaagctagc
Ec031_SS7_Ver_F	ccagttgtgtcaggagatgc
Ec032_SS7_Ver_R	cgctatcacgctgttcatgg
Ec033_SS9_Ver_F	gacaccgagttaaagccacg
Ec034_SS9_Ver_R	agtacgacgatcgctactg
Ec272_yciA_KO_F	tcatagtagcatcgcgctgtgattttccttttaagtcggtttaccatggtgtaggctggagctgcttc
Ec273_yciA_KO_R	aaaagcctccgaccggaggcgtttgactattactcaacaggtaaggcgcggaattagccatggtccatag
Ec274_yciA_V_F	acatcgcatcttgctgc
Ec275_yciA_V_R	cgtaatcagccgcagtatcc
Ec276_tesB_KO_F	tcaactcactttggcttgcgtcgccagcgtttgtactggagagttatatggtgtaggctggagctgcttc
Ec277_tesB_KO_R	ccggacggtttcactccggctatttttaattgtgattacgcacacggaattagccatggtccatag
Ec278_tesB_V_F	caatcgcaaccgctaacc
Ec279_tesB_V_R	ttcgtgaaggtgtgacg

*indicates phosphorothioate bond.

Supplementary Table 9. List of enzyme amounts in THETA assays.

Abbrev.	Source	Concentration (µg/ml) in					
		THETA 1.0	THETA 2.0	THETA 3.0	THETA 3.9.9	THETA 3.9.27	THETA 3.9.16
Pps	<i>Escherichia coli</i>	243.6	121.8	121.8	247.1	82.4	82.4
Ppc	Microbial (Sigma)	357.1	357.1	357.1	1780.0	2225.0	445.0
Mdh	Porcine heart (Sigma)	2.7	2.7	2.7	-	-	-
Mdh	<i>Escherichia coli</i>	-	-	-	42.5	148.8	42.5
Fum	Porcine heart (Sigma)	8.2	8.2	8.2	-	-	-
Fum	<i>Escherichia coli</i>	-	-	-	80.4	40.2	80.4
Frd	<i>Trypanosoma brucei</i>	354.0	354.0	-	-	-	-
DHOD1a	<i>Trypanosoma cruzi</i>	-	-	28.3	302.0	151.0	755.0
DHOD1b	<i>Bacillus subtilis</i>	-	-	10.6	47.0	94.0	47.0
SucCD	<i>Escherichia coli</i>	193.0	193.0	193.0	57.8	115.7	57.8
Scr	<i>Clostridium kluyveri</i>	14.0	28.0	28.0	59.5	59.5	178.5
Ssr	<i>Homo sapiens</i>	42.0	42.0	42.0	103.5	20.7	103.5
Hbs	<i>Nitrosopumilus maritimus</i>	59.4	297.0	297.0	79.5	79.5	159.0
Hbd	<i>Nitrosopumilus maritimus</i>	20.0	30.0	30.0	132.0	330.0	22.0
Ccr	<i>Methylobacterium extorquens</i>	33.6	33.6	33.6	15.1	15.1	45.4
Epi	<i>Rhodobacter sphaeroides</i>	14.8	14.8	14.8	125.0	25.0	25.0
Ecm	<i>Rhodobacter sphaeroides</i>	69.0	115.0	115.0	17.5	140.0	70.0
Mco	<i>Rhodobacter sphaeroides</i>	768.0	512.0	512.0	4155.0	2077.5	6232.5
Mct	<i>Chloroflexus aurantiacus</i>	10.0	15.0	15.0	575.0	230.0	115.0
Meh	<i>Chloroflexus aurantiacus</i>	4.6	4.6	4.6	61.0	15.2	15.2
Ccl	<i>Pseudomonas aeruginosa</i>	10.1	10.1	10.1	25.0	25.0	75.0
Mcl2	<i>Rhodobacter sphaeroides</i>	-	10.3	10.3	220.5	882.0	441.0
Kat	<i>Escherichia coli</i>	115.0	115.0	230.0	69.0	276.0	1380.0
G6PDH	<i>Leuconostoc mesenteroides</i> (Sigma)	-	14.5	14.5	9.0	18.0	36.0
Cpk	Rabbit muscle (Sigma)	-	5.9	5.9	30.0	15.0	75.0
Adk	<i>Escherichia coli</i>	-	-	-	95.3	286.0	95.3
Ca	Bovine erythrocytes (Sigma)	-	-	-	1.0	0.5	2.0

Abbrev.	Source	Concentration (µg/ml) in THETA 3.9.9.mc
Pps	<i>Escherichia coli</i>	247.1
Ppc	Microbial (Sigma)	1780.0
Mdh	<i>Escherichia coli</i>	42.5
Fum	<i>Escherichia coli</i>	80.4
DHOD1a	<i>Trypanosoma cruzi</i>	302.0
DHOD1b	<i>Bacillus subtilis</i>	47.0
SucCD	<i>Escherichia coli</i>	57.8
Scr	<i>Clostridium kluyveri</i>	59.5
Ssr	<i>Homo sapiens</i>	103.5
Hbs	<i>Nitrosopumilus maritimus</i>	79.5
Hbd	<i>Nitrosopumilus maritimus</i>	132.0
Ccr	<i>Methylobacterium extorquens</i>	15.1
Epi	<i>Rhodobacter sphaeroides</i>	125.0
Ecm	<i>Rhodobacter sphaeroides</i>	17.5
Mco	<i>Rhodobacter sphaeroides</i>	4155.0
Mct	<i>Chloroflexus aurantiacus</i>	575.0
Meh	<i>Chloroflexus aurantiacus</i>	61.0
Ccl	<i>Pseudomonas aeruginosa</i>	25.0
Pcc*	<i>Methylobacterium extorquens</i>	1000.5
Mcl2	<i>Rhodobacter sphaeroides</i>	220.5
Kat	<i>Escherichia coli</i>	69.0
G6PDH	<i>Leuconostoc mesenteroides</i> (Sigma)	9.0
Cpk	Rabbit muscle (Sigma)	30.0
Adk	<i>Escherichia coli</i>	95.3
Ca	Bovine erythrocytes (Sigma)	1.0

Abbrev.	Source	Concentration (µg/ml) In THETA 3.9.9.gc
Pps	<i>Escherichia coli</i>	247.1
Ppc	Microbial (Sigma)	1780.0
Cs	<i>Synechocystis sp.</i> PCC 6803	416.7
Acn	<i>Escherichia coli</i>	1587.3
Icl	<i>Escherichia coli</i>	625.0
Gor	<i>Gluconobacter oxydans</i>	19.5
SucCD	<i>Escherichia coli</i>	57.8
Scr	<i>Clostridium kluyveri</i>	59.5
Ssr	<i>Homo sapiens</i>	103.5
Hbs	<i>Nitrosopumilus maritimus</i>	79.5
Hbd	<i>Nitrosopumilus maritimus</i>	132.0
Ccr	<i>Methylobacterium extorquens</i>	15.1
Epi	<i>Rhodobacter sphaeroides</i>	125.0
Ecm	<i>Rhodobacter sphaeroides</i>	17.5
Mco	<i>Rhodobacter sphaeroides</i>	4155.0
Mct	<i>Chloroflexus aurantiacus</i>	575.0
Meh	<i>Chloroflexus aurantiacus</i>	61.0
Ccl	<i>Pseudomonas aeruginosa</i>	25.0
Mcl2	<i>Rhodobacter sphaeroides</i>	220.5
Kat	<i>Escherichia coli</i>	69.0
G6PDH	<i>Leuconostoc mesenteroides</i> (Sigma)	9.0
Cpk	Rabbit muscle (Sigma)	30.0
Adk	<i>Escherichia coli</i>	95.3
Ca	Bovine erythrocytes (Sigma)	1.0

Supplementary Table 10. List of components for optimization and their respective concentrations. After round 3, the concentration grid was adjusted. Changed values are indicated: **Red = removed**; **Green = added**. For AMP regeneration adenylate kinase was added after round 5 (**marked in blue**).

Name	Note	Stock [mM]	Available concentrations [mM]
HEPES		1000	50.0, 75.0, 100.0, 125.0, 150.0, 175.0, 200.0
MgCl2	Hexahydrate	1000	0.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0
CP	Creatine phosphate disodium salt	500	0.0, 2.5, 5.0, 10.0, 20.0, 40.0, 60.0, 80, 100
Bicarbonate	Sodium bicarbonate	1000	2.5, 5.0, 10.0, 20.0, 40.0, 60.0, 80.0
Glc-6-P	Glucose-6-phosphate	500	0.0, 2.5, 5.0, 10.0, 20.0, 40.0, 60, 80
CoA	Coenzyme A trilithium salt	100	1.0, 2.0, 3.0, 8.0, 10.0, 12.0, 14.0
B12	Coenzyme B12	5	0.0, 0.1, 0.2, 0.4
DHO	Dihydro-L-orotic acid	10	0.0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8
ATP	disodium salt hydrate	400	3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0
NADPH	tetrasodium salt	500	0.0, 1.25, 2.5, 3.75, 5.0, 6.25, 7.5, 8.75, 10.0
NADH	disodium salt	500	5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0

Name	Plasmid/Catalog number	Note	Stock [μM]	Available concentrations [μM]
Pps	JW1692	Phosphoenolpyruvate synthase	36.76	0.092, 0.184, 0.459, 0.918, 1.835, 2.753, 4.588
Ppc	Sigma C1744	Phosphoenolpyruvate carboxylase	456.41	1.141, 2.282, 4.564, 5.705, 9.128, 11.410
Mdh	JW3205	Malate dehydrogenase	262.47	0.656, 1.312, 2.625, 4.593, 6.562, 8.530
Fum	JW1603	Fumarate hydratase	319.06	0.798, 1.595, 3.191, 7.977
SucCD	JW0717 / JW0718	Succinyl-CoA ligase	304.47	0.761, 1.522, 3.045, 7.612
Scr	pTE380	Succinyl-CoA reductase	454.46	1.136, 3.408, 6.817, 17.042
Ssr	p2BP1	Succinic semialdehyde reductase	221.33	0.553, 1.660, 2.767, 5.533, 13.833

Hbs	Nmar0206	4-hydroxybutyryl-CoA synthetase	67.86	1.018, 2.036, 4.072, 8.143, 20.359
Hbd	pTE393	4-hydroxybutyryl-CoA dehydratase	145.45	0.364, 1.091, 2.182, 5.455
Ccr	pTE71	Crotonyl-CoA carboxylase/reductase	124.36	0.311, 0.933, 1.865, 4.664
Epi	pTE45	Ethylmalonyl-CoA epimerase	297.8	0.744, 1.489, 2.978, 7.445
Ecm	pTE33A	Ethylmalonyl-CoA mutase	95.98	0.240, 0.960, 1.920, 4.799
Mco	pTE813	Methylsuccinyl-CoA oxidase	1334.08	16.676, 33.352, 66.704, 100.056 , 166.760
Mct	pMCT_Ca_JZ05	Mesaconyl-CoA C1-C4 CoA transferase	243.13	0.608, 2.431, 4.863, 12.156
Meh	pMEH_Ca_JZ_07	Mesaconyl-CoA hydratase	182.1	0.455, 0.910, 1.821, 4.552
Ccl	pET16b-Ccl-Pa	Citramalyl-CoA lyase	317.06	0.793, 2.378, 4.756, 9.512, 15.853 , 23.779
KatE	JW1721	Catalase	328.18	0.820, 3.282, 6.564, 16.409
Cpk	Sigma C3755	Creatine Kinase	35.29	0.088, 0.176, 0.353, 0.618 , 0.882
G6PDH	Sigma G5885	Glucose-6-phosphate dehydrogenase	33.065	0.165, 0.331, 0.661, 0.992, 1.653
Mcl2	pJS12mcl2	Malyl-CoA thioesterase	660.18	3.301, 6.602, 13.204, 33.009
DHOD1a	pTE3210	Dihydroorotate dehydrogenase 1a	834.25	2.086, 4.171, 8.343, 20.856
DHOD1b	pTE3213	Dihydroorotate dehydrogenase 1b	532.58	1.331, 2.663, 5.326, 13.314
Ca	Sigma C3934	Carbonic anhydrase	6.67	0.017, 0.033, 0.067, 0.167
Adk	JW0463	Adenylate kinase	1616.32	0.0 , 4.041 , 8.082 , 12.122 , 16.163 , 20.204

Supplementary Table 11. List of buffer and cofactor conditions in manual assays of optimized THETA versions.

Buffer/Cofactor	Concentration (mM) in				
	THETA 3.9.9	THETA 3.9.27	THETA 3.9.16	THETA 3.9.9.mc	THETA 3.9.9.gc
HEPES Buffer	pH 7.8 125 mM	pH 8 150 mM	pH 7.2 50 mM	pH 7.8 125 mM	pH7.8 125 mM
MgCl ₂	17.50	15.00	15.00	17.50	17.50
Creatine phosphate	40.00	20.00	80.00	40.00	40.00
Bicarbonate	60.00	20.00	10.00	60.00	60.00
Glucose 6-phosphate	5.00	2.50	20.00	5.00	5.00
CoA	7.00	5.00	7.00	7.00	7.00
Coenzyme B12	0.00	0.00	0.00	0.00	0.00
Dihydroorotate	0.20	0.20	0.60	0.20	0.00
ATP	5.00	9.00	5.00	5.00	5.00
NADPH	5.00	8.75	5.00	5.00	5.00
NADH	7.50	5.00	5.00	7.50	7.50

Supplementary Table 12. Strains used in this study.

Strain	Genotype	Reference
MG1655	K-12 F ⁻ λ ⁻ <i>ilvG</i> ⁻ <i>rfb</i> -50 <i>rph</i> -1	Lab collection
SIJ488	MG1655 <i>Tn7::para-exo-beta-gam</i> ; <i>prha</i> -FLP; <i>xylSpm-lscl</i>	23
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r_K^- , m_K^+) <i>phoA supE44</i> λ ⁻ <i>thi</i> -1 <i>gyrA96 relA1</i>	Lab collection
Pir1	F- Δ <i>lac169 rpoS</i> (Am) <i>robA1 creC510 hsdR514 endA recA1 uidA</i> (Δ M1ul):: <i>pir</i> -116	Invitrogen
YYC202	DSM 14335, K-12 Hfr <i>zbi</i> ::Tn10 <i>poxB1</i> Δ (<i>aceEF</i>) <i>rpsL pps-4 pfl-1</i>	31
JCL301	YYC202 Δ <i>pflB</i> Δ <i>aceEF</i> Δ <i>poxB</i>	32
SL1	JCL301 Δ <i>sucAB</i> Δ <i>aceA</i>	This study
SL2	JCL301 Δ <i>kbl</i> Δ <i>ltaE</i>	This study
SL3	SL2 SS9:: <i>epi-ecm</i>	This study
HH61	SL2 SS7:: <i>ccl-meh-mtc</i> SS9:: <i>epi-ecm</i>	This study
HH169	HH61 Δ <i>tesB</i> Δ <i>yciA</i>	This study
HH422	SIJ488 SS9:: <i>epi-ecm</i>	This study
1736	Evolved from SL2 carrying pTE3217 and pTE3236	This study
1737	Evolved from SL2 carrying pTE3217 and pTE3237	This study
BW25113	F ⁻ Δ (<i>araD-araB</i>)567 Δ <i>lacZ</i> 4787(:: <i>rrnB</i> -3) λ ⁻ <i>rph</i> -1 Δ (<i>rhaD-rhaB</i>)568 <i>hsdR514</i>	Lab collection
KeiO-sdhA	BW25113 Δ <i>sdhA</i> :: <i>Kan</i>	33
KeiO-sdhB	BW25113 Δ <i>sdhB</i> :: <i>Kan</i>	33
KeiO-fadE	BW25113 Δ <i>fadE</i> :: <i>Kan</i>	33
KeiO-ydiO	BW25113 Δ <i>ydiO</i> :: <i>Kan</i>	33

Supplementary Table 13. DNA sequences of codon optimized genes.

Gene name	Codon optimized DNA sequence
<i>ict from Y. pestis</i>	ATGGATATTCGCGCGCTGTATGACGAGAACTCACAACGCCAGAAGAGGCCGTATCCTCAATTGCGTCAG GTTACACCTGAGCATGGGTATGTTTGCAGCAGAACCGCCAGCCCTGCTGAAAGCATTAGCGGATCGCG CAACGCGTGGGACATTGGCGACCTTCGCGTTTACTACTTCGAAACGGCGAAGATTGCGGGTGACACTAT TCTGCGGTACGAATTGAACAACCGCATCAAACCCTACTCGATGTTTGTACGGCCGTTGAACGCGCGCTG ATTCTGTCGTGGCATCGAAGATGGAGGTCGGAAGTGGTGAACATATGTGCCCTCTAACTTCCATCAAGCTC CTCGCCTGTTGGCGGAGGAAATTGGCATCGACACCTTCATGCATACCGTATCTCCGATGGATTGCCACGG GTATTTCTCGCTCGGTGTGGTAACGATTACAGCTCACGCATTGCCGTAGTGACGTCGCTTCATTGTG GAAGTGAATCGTTATATGCCACGCGTCCAAGGCCAAGCAGCAGCTATTACATCTCGGAAGTTGATGCGA TTGTCGAAAATCACGTACCCCTTAATCGAGATGCCAGTGCGCTCTGCGATCCCTGAGTACACTTCCATTTC CATATTATCGCAGACCTGGTTCCGGATGGTGCTGTGTGTCAGATGGGCGTGGGAGCCTTGCCGAATCTG GTGTGTGGCGTACTCAAGGATCGTAACGATCTGGGTATCCATACCGAAGTTCTGAATCCGGGCCCTGGTGG ATCTGATCCGTCGTGGTGTGTTACCAATCAGCGGAAAACCTTGGATCGTGGGCGCAGCGTGTTCAGTT TGCTATGGGGCAACAGGAAATGTATGAGTATCTTAACGATCATCCGGCCATTTTCTCGCGTCCGGTAGACT ATGTCAATGATCCGCATATCATTGCGCAGAATGACAACGTCGTGTCCATCAACGCCACTCTTCAGATTGAC TTAACTGGCGCGTGCAACTCTGAACACATGCTTGCCATCAGTATAGCGCAGTGGTGGCCAGCTCGATT TTGTGCGCGGGGCTATGCGAGTAAAGGAGGCCGTAGCATTATTGCGACCCCTAGTACCGCTGCTAAAG GCACAGTTAGCCGCATCATTCCGCGCATTGATGGACCGGTCACCACACCGCGCATCGATACCCACTACAT TGTCACCGAGTTTGGCGCCGTCAATCTGAAAGGTCTGAGCAGCACGGAACGTGCCCTGCGCATCATCGA ACTGGCACATCCGGACTTTCGCGATGAATTAACCCAAGCCGCGAAGAAAATGCATCTGATCTAA
<i>frd from T. brucei</i>	ATGGTTGATGGTCGTAGCAGCGCAAGCATTGTTGCAGTTGATCCGGAACGTGCAGCACGTGAACGTGATG CAGCAGCCCGTGCCTGCTGCAGGATAGTCCGCTGCATACCACCATGCAGTATGCAACCAGCGGTCTGG AACTGACCGTTCGGTATGCACTGAAAGTTGTTGCAAGCGCAGATACCTTTGATCGTGCAAAAAGAAGTTGCA GACGAAGTTCTGCGTTGTGCATGGCAGCTGGCAGATACCGTTCTGAATAGCTTTAATCCGAATAGCGAAG TTAGCCTGGTTGGTCGTCTGCCGTTGGTCAGAAACATCAGATGAGCGCACCGCTGAAACGTGTTATGGC ATGTTGTCAGCGTGTGTATAATAGCAGTGCAGGTTGTTTTGATCCGAGCACCGCACCGGTTGCAAAAGCA CTGCGTGAAATTGCACTGGGTAAAGAACGTAATAATGCATGTCTGGAAGCACTGACCCAGGCATGTACCC TGCCGAATAGTTTTGTTATTGATTTGAAGCAGGCACCATCAGCCGTAAACATGAACATGCAAGCCTTGAT TTAGGTGGTGTTAGCAAAGTTATATCGTGGATTATGTGATCGACAACATTAACTGACGCCGTTTCAGAA CGTGTTTTTTATTGTTGGGTGGTGATTGTCGTGCAAGCGGTATGAATGCACGTAATACCCCGTGGGTGTT GGTATTACCCGTCCGCTAGCCTGGATATGCTGCCGAATCCGCTAAAGAAGCAAGCTATATTAGCGTGGA TTAGCCTGGACAATGAAGCACTGGCGACCGAGCGGTGATTATGAAAATCTGATTTATACCGCAGATGATAAA CCGCTGACCTGTACCTATGATTGGAAGGCAAGAACTGATGAAACCGAGCCAGAGTAATATTGCACAGG TTAGCGTTAAATGTTACAGCGCAATGTATGCAGATGCACTGGCAACCGCATGTTTATCAAACGTGATCCG GCAAAAGTTCGTGAGCTGCTGGATGGTTGGCGTTATGTTCTGTGATACCGTGCGTGATTATCGTGTATGT GCGTGAAAATGAACGTGTGGCCAAAATGTTTGAATGTCACCGAAGATGCAGAAATGCGTAAACGTCGT ATTAGCAATACCTGCCTGCAGTGTTATTGTGGTTGGTGGTCTGGCAGGTCTGAGCGCAGCAATTG AAGCAGCAGGTTGTGGTGCCAGGTTGTGCTGATGGAAGAAAGCAAAATAGGCGGTAATAGCGCAAA AGCAACCACTGGTATTAATGGTTGGGGCACCCGTGCACAGGCAAAAGCATCAATTGTTGATGGTGGCAAA TATTTGAAACGCGATACCTATAAAGCGGCATTGGTGGTAATACCGATCCGGCACTGGTAAAACCTGA GCATGAAATCAGCAGATGCAATTGGTTGGCTGACCAGCCTGGGTGTTCCGCTGACCGTGCTGAGCCAGTT AGGTGGTCATAGTCGTAAACGTACCCATCGTGACCGGATAAAAAAGATGGTACACCGCTGCCGATTGGT TTTACCATCATGAAAACCTGGAAGATCATGTTCTGTTGATCTGAGCGGTCTGATTACCATATTGAAAAA TTGTAGCGTTACCAGCCTGCTGAGCGAAACCAAGAACGTCCGGATGGCACCAACAAATTCGTGTTACC GGTGTGAGTTTACCCAGGCAGGTAGCGGCAAAACCAACATTCTGGCAGATGCAGTTATTCTGGCGACCG GTGGTTTTAGTAATGATAAAACCGCAGATAGCCTGCTGCGTGAACATGCTCCGCATCTGGTTAATTTCCG ACCACAAATGGTCCGTGGGCCACCGGTGATGGTGTTAACTGGCACAGCGTCTGGGTGCACAGCTGGTT GATATGGATAAAGTTGAGCTGCATCCGACCGGTCTGATTAACCCGAAAGATCCTGCAATCCGACCAATT TCTGGGTCCTGAAGCGCTGCGTGGTAGCGGTGGTGTCTGCTGAATAAACAGGGTAAACGTTTTGTGAAT GAACTGGATCTGCGTAGCGTTGTGAGCAAAGCAATTATGGAACAGGGTGCAAGTATCCTGGTAGTGGTG GTAGCATGTTTGCATATTGTGTTCTGAATGCCGCAGCACAGAACTGTTTGGTGTGAGCAGCCATGAGTTC TACTGGAAAAAATGGGTCTGTTTGTAAAGCCGATACCATGCGCGATCTGGCAGCCCTGATTGGTTGTC CGGTTGAAAGCGTTGAGCAGACACTGGAAGAATATGAACGTCTGAGCATTAGCCAGCGTAGCTGTCCGAT

TACACGTAAAAGCGTTTATCCGTGTGTTCTGGGTACAAAAGGTCCGTATTATGTTGCATTTGTTACCCCGA
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CGTGCTCCGCTGAGCCATAGCAATCCGATTCTGGGCCTGTTTGGCGCAGGCGAAGTGACCGGTGGCGTT
CATGGCGGTAATCGCTTAGGTGGTAATCACTGCTGGAATGTGTTGTGTTGGTCGTATTGCCGGTGATC
GTGCCAGTACCATTCTGCAGCGTAAAAGCAGCGCACTGAGCTTTAAAGTTTGGACCACCGTTGTTCTGCG
TGAAGTTCGTGAAGGTGGTGTGTATGGTGCAGGTAGCCGTGTGCTGCGTTTTAATCTGCCTGGTGCAGTG
CAGCGTTCTGGTCTGAGCCTGGGCCAGTTTATTGCAATTCGCGGTGATTGGGATGGTCAGCAGTTAATTG
GTTATTATAGCCCCGATTACGCTGCCGGATGATCTGGGCATGATTGATATTCTGGCACGTAGCGATAAAGG
CACCTGCGTGAATGGATTAGCGCACTGGAACCGGGTGATGCAGTTGAAATGAAAGCATGTGGTGGCCT
GGTTATTGAACGTCGTCTGAGCGATAAACACCTTTGTGTTTATGGGCCACATCATCAACAACTGTGTCTGA
TTGCAGGCGGTACAGGTGTTGCACCGATGCTGCAGATTATCAAAGCAGCATTATGAAACCGTTTCATCGAT
ACCCTGGAAAGCGTGATCTGATCTATGCAGCAGAAGATGTTACCGAACTGACCTATCGTGAAGTGCTGG
AAGAAGCTCGCCGTGAAAGCCGTGGTAAATTCAAAAAACCTTTGTGCTGAATGCTCCGCTCCGCTGTG
GACCGATGGTGTGGTTTTATTGATCGCGGTATTCTGACAAATCATGTTACGCTCCGAGCGATAACCTGC
TGGTTGCCATTTGCGGTCCGCCTGTTATGCAGCGTATTGTGAAAGCAACCCCTGAAAACACTGGGCTATAA
CATGAATCTGGTTCGTACCGTTGATGAAACAGAACCGAGCGGTAGCAGCAAAATTTAA

**dhod from
*E. faecalis***

ATGAGCAAAACCATTTATCGTCGTGTGTTTCTGCTGGATATTAGCGTTGAATTTAGCGAACATAAACTGGC
CAATGTTCTGATGAATGCAAGCGGTATTCATTGCATGACCATCAAAGAAATGGATGAACTGGCAGCAAGCC
AGGCAGGCGCATTTGTTGCAAAAACCGCAACACCGAATCCGCGTCAGGGTAATGAAGAACCGCGTTATTT
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AGATTGCCGTCAGAAAGAATTTCCGGAAGAACTGCGTTTTCTGAGCGTTAGCGGTATGAATTATGAAGAG
AATATCGCCATCCTGAAGAAAGTGCAAGAAAGCGAATATACCGGTGTGACCGAATTTAATCTGAGCTGTCC
GAATCTGCCTGGTAAACCGCAGATTGCCTATGATTTTGAAGTACCGAAAACTGCTGACCGAAGTGTTTC
AGTTTTTTACCAAACCGCTGGGCGTTAACTGCCTCCGTTTTTTGATATTGCACACTTTGATGCAATGGCC
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CCGACCGCACTGGCAATGTGCGTGCAATTTGCACAGCGTCTGAAACCGGAAATCAAAATTATCGGCACCG
GTGGTATTACCTGTGGTAAAGATGTTTTTGAACATCTGCTGTGTGGTGCAACCGTGGTTCAGGTTGGCACC
CAGCTGCATCAAGAAGGTCCGCAGGTATTTGAACGTCTGGCAAAAGAACTGCAAGAAATTATGGCAGCCA
AAGGCTATGAAAGCATGAAGAATTTGCGCGTAAACTGAAAGAAATGTAA

**dhod from
*L. lactis***

ATGCTGAATACCACCTTTGCCAATGCCAAATTTGCCAATCCGTTTATGAATGCAAGCGGTGTTCAATTGCAT
GACCATTGAAGATCTGGAAGAACTGAAAGCAAGCCAGGCAGGCGCATATATTACCAAAAGCAGCACCCCTG
GAAAAACGTGAAGGTAATCCGCTGCCTCGTTATGTTGATCTGGAAGTGGGTAGCATTAAATAGCATGGGTCT
GCCGAATCTGGGCTTTGATTATTATCTGGATTACGTGCTGAAGAACCAGAAAGAAATGCACAAGAAGGTC
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CCTCCATATTTGATCTGGTGCATTTGATATTATGGCCGAAATTCTGAATCAGTTCCTGCTGACCTATGTT
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ATCTGCTGTGTGGTGAACCATGCTGCAGATTGGTACAGCACTGCATAAAGAAGGACCGGCAATTTTTGA
TCGCATCATCAAAGAACTGGAAGAGATCATGAATCAGAAAGGCTATCAGAGCATTGCCGATTTTCATGGTA
AACTGAAAAGCCTGTAA

**dhod from
*T. cruzi***

ATGATGTGCCTGAAACTGAATCTGCTGGATCATGTTTTTGCCAATCCGTTTATGAATGCAGCCGGTGTCT
GTGTAGCACCGAAGAGGATCTGCGTTGTATGACCGCAAGCAGCAGCGGTGCACTGGTTAGCAAAAGCTG
TACCAGCGCACCGCGTGATGGTAATCCGGAACCGCGTTATATGGCATTTCGCTGGGTAGCATTAAATAGC
ATGGGTCTGCCGAATCTGGGCTTTGATTTTTATCTGAAATATGCCAGCGATCTGCACGACTATAGCAAAAA
ACCGCTGTTTCTGAGCATTAGCGGTCTGAGCGTTGAAGAAAATGTTGCAATGGTTCGTCGTCTGGCACCG
GTTGCACAAGGTAAAGGTGTTCTGCTGGAAGTGAACCTGAGCTGTCCGAATGTTCCGGGTAAACCGCAGG
GTGCCTATGATTTTGAAGCAATGCGTACCTATCTGCAGCAGGTAGCCTGGCCTATGGTCTGCCGTTTGG
TGTTAAAATGCCTCCGTATTTTGATATCGCCATTTTGATACCGCAGCAGCCGTTCTGAATGAATTTCTCT
GGTAAAATTTGTGACCTGCGTTAATAGCGTTGGTAATGGTCTGGTTATTGATGCAGAAAGCGAAAGCGTTG
TGATCAAACCGAAACAAGTTTTGGTGGTTTAGGTGGCAAATATATCTGCCGACCGCACTGGCAAATGTT

AATGCATTTTATCGTCGTTGTCCGGATAAACTGGTTTTTGGTTGTGGTGGTGGTTTATAGCGGTGAAGATGC
ATTCTGTCATATTCTGGCAGGCGCAAGCATGGTTCAGGTTGGCACCGCACTGCAAGAAGAAGGTCCGGGT
ATTTTACCCGCTCTGGAAGATGAACTGCTGGAAATTATGGCACGTAAAGGTTATCGTACCCTGGAAGAATT
TCGTGGTCGTGTTAAACCATCGAATAA

***dhod from
S. cerevisiae***

ATGACCGCAAGCCTGACCACCAAAATTTCTGAATAATACCTATGAGAACCCCTTCATGAATGCAAGCGGTGT
TCATTGTATGACCACACAAGAACTGGATGAACTGGCAAATAGCAAAGCCGGTGCATTATTACCAAAAGCG
CAACCACACTGGAACGTGAAGGTAATCCGGAACCGCCTTATATTAGCGTTCCGCTGGGTAGCATTAAATAG
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***pyrD from
B. subtilis***

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***pyrK from
B. subtilis***

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***pyrD from
L. lactis***

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***pyrK* from
*L. lactis***

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***SucD* from
*C. kluyveri***

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***nmar0206/hbs*
from
*N. maritimus***

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from
N. maritimus

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mct from
C. aurantiacus

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