

# Supporting Information

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## SI Text

**Chemicals and Reagents.** All chemicals were purchased from Sigma-Aldrich or Fisher Scientifics unless otherwise specified. iProof high-fidelity DNA polymerase was purchased from Bio-Rad. Restriction enzymes, Phusion DNA polymerase, and ligases were purchased from New England Biolabs (Ipswich, MA). T5-Exonuclease was purchased from Epicentre Biotechnologies. KOD and KOD xtreme DNA polymerases were purchased from EMD biosciences.

**DNA Manipulations.** All chromosomal manipulations were carried out by homologous recombination of plasmid DNA into *S. elongatus* PCC 7942 genome at neutral site I (NSI) (1) and II (NSII) (2). All plasmids were constructed using the isothermal DNA assembly method (3). Plasmids were constructed in *Escherichia coli* XL-1 strain for propagation and storage (Table S1).

**Plasmid Constructions.** The plasmids used and constructed in this work are listed in Table S1 and briefly described below. The sequences of primers used are listed in Table S2. Plasmid pEL29 was synthesized by Genewiz Inc. Plasmid pEL52 was synthesized by DNA 2.0.

Plasmid pEL53 was constructed by assembling a nphT7 fragment and a pEL11 without atoB fragment. nphT7 fragment was amplified by PCR with primers rEL-335 and rEL-336 with pEL52 as template. pEL11 without atoB fragment was amplified by PCR with primers rEL-333 and rEL-334 with pEL11 as template.

Plasmid pEL54 was constructed by assembling a bldh fragment, a yqhD fragment, and a pEL11 without adhE2 fragment. bldh fragment was amplified by PCR with primers rEL-329 and rEL-330 with *Clostridium saccharoperbutylacetonicum* NI-4 genome as template. yqhD fragment was amplified by PCR with primers rEL-331 and rEL-332 with *E. coli* genome as template. pEL11 without adhE2 fragment was amplified by PCR with primers rEL-327 and rEL-328 with pEL11 as template.

Plasmid pEL56 was constructed by assembling a NSII vector fragment and a pEL53 coding sequence fragment. NSII vector fragment was amplified by PCR with primers rEL-217 and rEL-253 with pEL37 as template. pEL53 coding sequence fragment was amplified by PCR with primers rEL-254 and rEL-255 with pEL53 as template.

Plasmid pEL57 was constructed by assembling a NSII vector fragment and a pEL54 coding sequence fragment. NSII vector fragment was amplified by PCR with primers rEL-217 and rEL-253 with pEL37 as template. pEL54 coding sequence fragment was amplified by PCR with primers rEL-254 and rEL-255 with pEL54 as template.

Plasmid pEL59 was constructed by assembling a NSII vector fragment, a pEL54 coding sequence without atoB fragment, and a nphT7 fragment. NSII vector fragment was amplified by PCR with primers rEL-217 and rEL-253 with pEL37 as template. pEL54 coding sequence without atoB fragment was amplified by PCR with primers rEL-352 and rEL-255 with pEL54 as template. nphT7 fragment was amplified by PCR with primers rEL-254 and rEL-351.

Plasmid pEL70 was constructed by assembling a pEL59 without crt.hbd fragment and a phaJ.phaB fragment. pEL59 without crt.hbd fragment was amplified by PCR with primers rEL-390 and rEL-391 with pEL59 as template. phaJ.phaB fragment was amplified by PCR with primers rEL-392 and rEL-393 with pEL29 as template.

Plasmid pEL71 was constructed by assembling a pEL57 without crt.hbd fragment and a phaJ.phaB fragment. pEL57 without crt.hbd fragment was amplified by PCR with primers rEL-390 and rEL-391 with pEL57 as template. phaJ.phaB fragment was amplified by PCR with primers rEL-392 and rEL-393 with pEL29 as template.

Plasmid pEL73 was constructed by assembling a pEL56 without crt.hbd fragment and a phaJ.phaB fragment. pEL56 without crt.hbd fragment was amplified by PCR with primers rEL-390 and rEL-398 with pEL56 as template. phaJ.phaB fragment was amplified by PCR with primers rEL-399 and rEL-393 with pEL70 as template.

Plasmids pEL75, pEL76, pEL77, pEL78, pEL79, and pEL80 were constructed by assembling a pDK26 without *adhE2* fragment and an aldehyde dehydrogenase gene from *Clostridium saccharoperbutylacetonicum* NI-4, *Clostridium Kluyveri*, *Geobacillus thermoglucosidasius*, *Escherichia coli*, *Clostridium beijerinckii NCIMB 8052*, and *Clostridium saccharobutylicum ATCC BAA-117*, respectively. pDK26 without *adhE2* fragment was amplified by PCR using primers rEL-403 and rEL-404 with pDK26 as template. *C. saccharoperbutylacetonicum* NI-4 *bldh* fragment was amplified by primers rEL-332 and rEL-394 with *C. saccharoperbutylacetonicum* NI-4 genome as template. *C. Kluyveri* *bldh* fragment was amplified by primers rEL-405 and rEL-406 with *C. kluyveri* genome as template. *G. thermoglucosidasius* *bldh* fragment was amplified by primers rEL-407 and rEL-408 with *G. thermoglucosidasius* genome as template. *E. coli* *EutE* fragment was amplified by primers rEL-409 and rEL-410 with *E. coli* genome as template. *C. beijerinckii NCIMB 8052* *bldh* fragment was amplified by primers rEL-411 and rEL-412 with *C. beijerinckii NCIMB 8052* genome as template. *C. saccharobutylicum ATCC BAA-117* *bldh* fragment was amplified by primers rEL-413 and rEL-414 with *C. saccharobutylicum ATCC BAA-117* genome as template.

Plasmids pEL90 to pEL96 were constructed by assembling the KASIII-like genes with a vector fragment. Vector fragment was amplified with primers rEL-455 and rEL-456 with pCS27 as the template. *bamb6224* was amplified with primers rEL-457 and rEL-458 with *Burkholderia ambifaria* gDNA as template. *gox0115* was amplified with primers rEL-459 and rEL-460 with *Gluconobacter oxydans* gDNA as template. *hp0202* was amplified with primers rEL-461 and rEL-462 with *Helicobacter pylori* gDNA as template. *lmo2202* was amplified with primers rEL-463 and rEL-464 with *Listeria monocytogenes* gDNA as template. *pae-fabH2* was amplified with primers rEL-467 and rEL-468 with *Pseudomonas aeruginosa* gDNA as template. *sav-fabH4* was amplified with primers rEL-469 and rEL-470 with *Streptomyces avermitilis* gDNA as template. *sco5888* was amplified with primers rEL-471 and rEL-472 with *Streptomyces coelicolor* gDNA as template.

**Strain Construction and Transformation.** The strains used and constructed are listed in Table S1. Briefly, strain EL18 was constructed by recombination of plasmids pEL57 NSII of Strain EL9 (Table S1 for relevant genotypes). Strain EL20 was constructed by recombination of plasmids pEL56 into NSII of strain EL9. Strain EL21 was constructed by recombination of plasmids pEL59 into NSII of strain EL9. Strain EL22 was constructed by recombination of plasmids pEL70 into NSII of strain EL9. Strain EL23 was constructed by recombination of plasmids pEL71 into NSII of strain EL9. Strain EL24 was constructed by recombination of plasmids pEL73 into NSII of strain EL9.

*S. elongatus* PCC 7942 strains were transformed by incubating cells at mid-log phase ( $OD_{730}$  of 0.4 to 0.6) with 2  $\mu\text{g}$  of plasmid DNA overnight in dark. The culture was then spread on BG-11 plates supplemented with appropriate antibiotics for selection of successful recombination. For selection and culture maintenance, 20  $\mu\text{g}/\text{mL}$  spectinomycin and 10  $\mu\text{g}/\text{mL}$  kanamycin were added into BG-11 agar plates and BG-11 medium where appropriate. Colonies grown on BG-11 agar plates were grown in liquid culture. Genomic DNA was then prepared from the liquid culture and analyzed by PCR using gene-specific primers (Table S2) to verify integration of inserted genes into the recombinant strain. In all cases, four individual colonies were analyzed and propagated for downstream tests.

**Protein Purification and SDS/PAGE.** Protein purification was done by using His-Spin Protein miniprep purification kit from Zymo following manufacturer's manual. Overnight culture of XL-1 blue strains harboring individual plasmid of pEL90 to pEL96, each encodes for a particular KAS III-like enzyme, was used to inoculate fresh 20 mL LB. The newly inoculated culture was incubated at 37°C until  $OD_{600\text{nm}}$  reaches 0.6, which was then induced with 1 mM IPTG. The induced culture were then incubated in 30°C shaker for 2 hr to allow protein expression. The culture was then harvested by centrifugation at  $5,250 \times g$  for 20 min. The pellet was then resuspended with 1 mL of 100 mM Tris-HCl pH 7.6 and mixed with 1 mL of 0.1 mm glass beads (Biospec). The sample was then homogenated using mini bead beater (biospec). Total protein was then collected. Soluble protein was collected after centrifugation. Purified protein was collected after His-spin column purification. The protein samples were then ran on SDS/PAGE using commercially available precast gels (Biorad) following standard protocol.

**Enzyme Assays.** Enzyme assays were conducted by using Bio-Tek PowerWave XS microplate spectrophotometer. Thiolase activity was measured via both condensation and thiolysis direction. The enzymatic reaction was monitored by the increase or decrease of absorbance at 303 nm, which corresponded to the result of  $\text{Mg}^{2+}$  coordination with the diketo moiety of acetoacetyl-CoA (4). The enzymatic reaction was initiated by the addition of the enzyme. For purified enzyme reaction, the reaction mixture contained 100 mM Tris-HCl (pH 8.0), 20 mM  $\text{MgCl}_2$ , equimolar acetoacetyl-CoA and CoA. For the crude cyanobacteria extract assay, the same buffer was used with 200  $\mu\text{M}$  acetoacetyl-CoA and 300  $\mu\text{M}$  CoA. Crude extract of strains EL22 (2.7  $\mu\text{g}$ ), EL14 (5.0  $\mu\text{g}$ ), and Wild-type (2.4  $\mu\text{g}$ ) were used for assay. Concentration of acetoacetyl-CoA was calculated based on a constructed standard curve. Cyanobacteria crude extract was obtained using the same bead beater method described above in protein purification. Protein concentration was measured by Coomassie Plus Assay (Thermo Scientific).

Acetoacetyl-CoA synthase activity was measured by monitoring the increase of absorbance at 303 nm, which corresponds to appearance of acetoacetyl-CoA. The reaction buffer is the same as that used for thiolase assay. Equimolar malonyl-CoA and acetyl-CoA were used for purified enzyme assay, while 400  $\mu\text{M}$  of both malonyl-CoA and acetyl-CoA were used for crude extract assay. Crude extract of strains EL22 (27  $\mu\text{g}$ ), EL14 (50  $\mu\text{g}$ ), and wild type (24  $\mu\text{g}$ ) were used for assay.

Enzyme assays for the other enzymes were conducted as previously described (5).

**Alcohol Production by *E. coli* Expressing Butyraldehyde Dehydrogenase.** *E. coli* wild type is based on strain BW25113 (6). Transformed *E. coli* strain JCL299 ( $\Delta adhE$ ,  $\Delta ldhA$ ,  $\Delta frd$ ,  $\Delta pta$ ) was selected on LB plate supplemented with ampicillin (100  $\mu\text{g}/\text{mL}$ ) and kanamycin (50  $\mu\text{g}/\text{mL}$ ). Three colonies were picked from the plate to make overnight preculture. The precultures were then used to inoculate 5 mL of Terrific broth (TB; 12 g tryptone, 24 g yeast extract, 2.31 g  $\text{KH}_2\text{PO}_4$ , 12.54 g  $\text{K}_2\text{HPO}_4$ , 4 mL glycerol per liter of water) supplemented with 20 g/L glucose. Growing culture was then induced with IPTG at  $OD_{600\text{nm}}$  of 0.6 with 0.1 mM final IPTG concentration. After allowing 1 hr of protein expression, the cultures were then switched to anaerobic condition by purging with  $\text{N}_2$  gas using anaerobic chamber. After two days of fermentation, culture sample (2 mL) was centrifuged for 5 min at  $21,130 \times g$ . The supernatant was analyzed by GC following the same method as that described above in 1-butanol quantification.

**1-Butanol Quantification.** Culture samples (5 mL) were centrifuged for 20 min at  $5,250 \times g$ . The supernatant (900  $\mu\text{L}$ ) was then mixed with 0.1% v/v 2-methyl-pentanol (100  $\mu\text{L}$ ) as internal standard. The mixture was then vortexed and directly analyzed on Agilent GC 6850 system with flame ionization detector and DB-FFAP capillary column (30 m, 0.32 mm i.d., 0.25 film thickness) from Agilent Technologies. 1-Butanol in the sample was identified and quantified by comparing to 0.001% v/v 1-butanol standard. 1-Butanol standard of 0.001% v/v was prepared by 100-fold dilution of a 0.1% v/v solution. The GC result was analyzed by Agilent software Chem Station (Rev.B.04.01 SP1). Amount of 1-butanol in the sample was then calculated based on the ratio of its integrated area and that of the 0.001% 1-butanol standard.

Helium gas was used as the carrier gas with 9.52 psi inlet pressure. The injector and detector temperatures were maintained at 225°C. Injection volume was 1  $\mu\text{L}$ . The GC oven temperature was initially held at 85°C for 3 minutes and then raised to 235°C with a temperature ramp of 45°C/min. The GC oven was then maintained at 235°C for 1 min before completion of analysis. Column flow rate was 1.7 ml/min.

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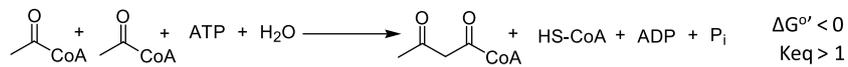
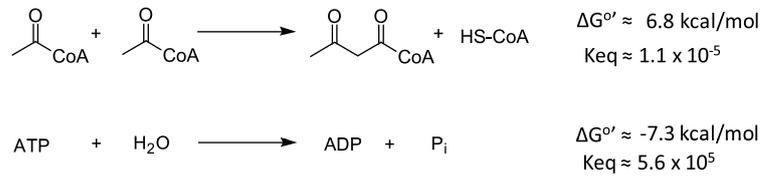


Fig. S1. Combining ATP hydrolysis to thiolase reaction yields a favorable net reaction.

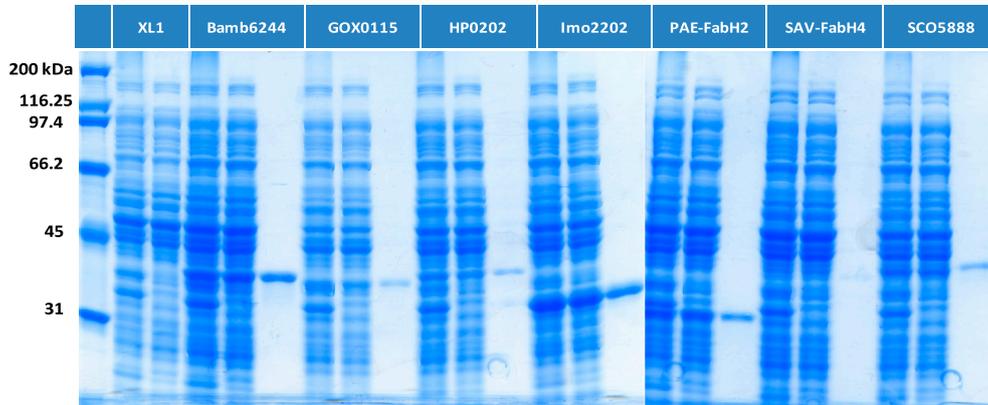


Fig. S2. Protein SDS/PAGE of His-tag purified KASIII-like enzymes.

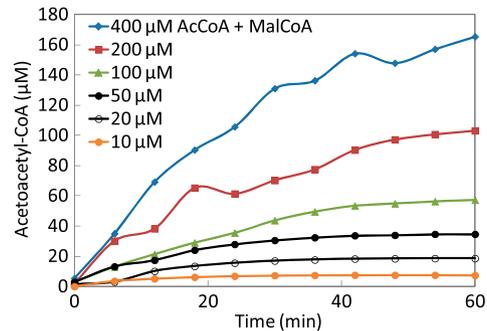


Fig. S3. In vitro assay of purified acetoacetyl-CoA synthase (NphT7). Equimolar acetyl-CoA and 4 malonyl-CoA of different concentration were used with 100 mM Tris-HCl and 20 mM MgCl<sub>2</sub>. Reaction was conducted at room temperature using spectrophotometer.

Table S1 Strain and plasmid list

Strain	Relevant genotypes	Reference
Cyanobacteria strains		
PCC 7942	Wild-type <i>Synechococcus elongatus</i> PCC 7942	S.S.Golden
EL9	P <sub>Trc</sub> ::His-tagged <i>T. denticola ter</i> integrated at NSI in PCC7942 genome	(1)
EL14	P <sub>Trc</sub> ::His-tagged <i>T. denticola ter</i> integrated at NSI and P <sub>LlacO1</sub> :: <i>atoB, adhE2, crt, hbd</i> integrated at NSII in PCC7942 genome	(1)
EL18	P <sub>Trc</sub> ::His-tagged <i>T. denticola ter</i> integrated at NSI and P <sub>LlacO1</sub> :: <i>atoB, bldh, yqhD, crt, hbd</i> integrated at NSII in PCC7942 genome	This work
EL20	P <sub>Trc</sub> ::His-tagged <i>T. denticola ter</i> integrated at NSI and P <sub>LlacO1</sub> :: <i>nphT7, adhE2, crt, hbd</i> integrated at NSII in PCC7942 genome	This work
EL21	P <sub>Trc</sub> ::His-tagged <i>T. denticola ter</i> integrated at NSI and P <sub>LlacO1</sub> :: <i>nphT7, bldh, yqhD, crt, hbd</i> integrated at NSII in PCC7942 genome	This work
EL22	P <sub>Trc</sub> ::His-tagged <i>T. denticola ter</i> integrated at NSI and P <sub>LlacO1</sub> :: <i>nphT7, bldh, yqhD, phaJ, phaB</i> integrated at NSII in PCC7942 genome	This work
EL23	P <sub>Trc</sub> ::His-tagged <i>T. denticola ter</i> integrated at NSI and P <sub>LlacO1</sub> :: <i>atoB, bldh, yqhD, phaJ, phaB</i> integrated at NSII in PCC7942 genome	This work
EL24	P <sub>Trc</sub> ::His-tagged <i>T. denticola ter</i> integrated at NSI and P <sub>LlacO1</sub> :: <i>nphT7, adhE2, phaJ, phaB</i> integrated at NSII in PCC7942 genome	This work
<i>E. coli</i> strains		
BW25113	<i>rrnB</i> <sub>T14</sub> <i>ΔlacZ</i> <sub>WJ16</sub> <i>hsdR514 ΔaraBAD</i> <sub>AH33</sub> <i>ΔrhaBAD</i> <sub>LD78</sub>	(2)
XL-1 blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI</i> <sup>q</sup> <i>ZΔM15 Tn10</i> (Tet <sup>R</sup> )]	Stratagene
JCL299	BW25113 <i>ΔldhA ΔadhE ΔfrdBC Δpta</i> /F' [ <i>traD36, proAB+</i> , <i>lacI</i> <sup>q</sup> <i>ZΔM15</i> (Tet <sup>R</sup> )]	(3)
Plasmid		
	Genotypes	Reference
pCDFDuet	Spec <sup>R</sup> ; CDF ori; P <sub>T7</sub> ::MCS	Novagen
pCDF-nphT7	Spec <sup>R</sup> ; CDF ori; P <sub>T7</sub> :: <i>nphT7</i> (his-tagged)	This work
pCDF-atoB	Spec <sup>R</sup> ; CDF ori; P <sub>T7</sub> :: <i>atoB</i> (his-tagged)	This work
pCS27	Kan <sup>R</sup> ; P15A ori; P <sub>LlacO1</sub> ::MCS	This work
pCS138	Cm <sup>R</sup> ; SC101 ori; P <sub>LlacO1</sub> :: <i>fdh</i>	(3)
pDK26	Amp <sup>R</sup> ; ColE1 ori; P <sub>LlacO1</sub> :: <i>bktB.adhE2.crt.paaH1</i>	This work
pEL11	Amp <sup>R</sup> ; ColE1 ori; P <sub>LlacO1</sub> :: <i>atoB.adhE2.crt.hbd</i>	(3)
pEL29	Kan <sup>R</sup> ; pUC ori; <i>ccr-phaJ-phaB</i>	This work
pEL37	Kan <sup>R</sup> ; NSII targeting; P <sub>LlacO1</sub> :: <i>atoB.adhE2.crt.hbd</i>	(1)
pEL52	Amp <sup>R</sup> ; pUC ori; P <sub>T5</sub> :: <i>nphT7</i>	This work
pEL53	Amp <sup>R</sup> ; ColE1 ori; P <sub>LlacO1</sub> :: <i>nphT7.adhE2.crt.hbd</i>	This work
pEL54	Amp <sup>R</sup> ; ColE1 ori; P <sub>LlacO1</sub> :: <i>atoB.bldh.yqhD.crt.hbd</i>	This work
pEL56	Kan <sup>R</sup> ; NSII targeting; P <sub>LlacO1</sub> :: <i>nphT7.adhE2.crt.hbd</i>	This work
pEL57	Kan <sup>R</sup> ; NSII targeting; P <sub>LlacO1</sub> :: <i>atoB.bldh.yqhD.crt.hbd</i>	This work
pEL59	Kan <sup>R</sup> ; NSII targeting; P <sub>LlacO1</sub> :: <i>nphT7.bldh.yqhD.crt.hbd</i>	This work
pEL70	Kan <sup>R</sup> ; NSII targeting; P <sub>LlacO1</sub> :: <i>nphT7.bldh.yqhD.phaJ.phaB</i>	This work
pEL71	Kan <sup>R</sup> ; NSII targeting; P <sub>LlacO1</sub> :: <i>atoB.bldh.yqhD.phaJ.phaB</i>	This work
pEL73	Kan <sup>R</sup> ; NSII targeting; P <sub>LlacO1</sub> :: <i>nphT7.adhE2.phaJ.phaB</i>	This work
pEL75	Amp <sup>R</sup> ; ColE1 ori; P <sub>LlacO1</sub> :: <i>bktB.bldh.yqhD.crt.paaH1</i>	This work
pEL76	Amp <sup>R</sup> ; ColE1 ori; P <sub>LlacO1</sub> :: <i>bktB.aldh(CK).yqhD.crt.paaH1</i>	This work
pEL77	Amp <sup>R</sup> ; ColE1 ori; P <sub>LlacO1</sub> :: <i>bktB.aldh(GT).yqhD.crt.paaH1</i>	This work
pEL78	Amp <sup>R</sup> ; ColE1 ori; P <sub>LlacO1</sub> :: <i>bktB.eutE.yqhD.crt.paaH1</i>	This work
pEL79	Amp <sup>R</sup> ; ColE1 ori; P <sub>LlacO1</sub> :: <i>bktB.aldh(CB).yqhD.crt.paaH1</i>	This work
pEL80	Amp <sup>R</sup> ; ColE1 ori; P <sub>LlacO1</sub> :: <i>bktB.aldh(BAA117).yqhD.crt.paaH1</i>	This work
pEL90	Kan <sup>R</sup> ; P15A ori; P <sub>LlacO1</sub> :: <i>bamb6224</i> (his-tagged)	This work
pEL91	Kan <sup>R</sup> ; P15A ori; P <sub>LlacO1</sub> :: <i>gox0115</i> (his-tagged)	This work
pEL92	Kan <sup>R</sup> ; P15A ori; P <sub>LlacO1</sub> :: <i>hp0202</i> (his-tagged)	This work
pEL93	Kan <sup>R</sup> ; P15A ori; P <sub>LlacO1</sub> :: <i>lmo2202</i> (his-tagged)	This work
pEL94	Kan <sup>R</sup> ; P15A ori; P <sub>LlacO1</sub> :: <i>pae-fabH2</i> (his-tagged)	This work
pEL95	Kan <sup>R</sup> ; P15A ori; P <sub>LlacO1</sub> :: <i>sav-fabH4</i> (his-tagged)	This work
pEL96	Kan <sup>R</sup> ; P15A ori; P <sub>LlacO1</sub> :: <i>sco5888</i> (his-tagged)	This work

Kan<sup>R</sup>, kanamycin resistance; Amp<sup>R</sup>, ampicillin resistance.

*atoB* (*E. coli*), thiolase; *nphT7* (*Streptomyces* sp. strain CL190), acetoacetyl-CoA synthase; *phaB* (*R. eutropha*), acetoacetyl-CoA reductase; *phaJ* (*A. caviae*), (R)-specific enoyl-CoA hydratase; *hbd* (*C. acetobutylicum*), 3-hydroxybutyryl-CoA dehydrogenase; *crt* (*C. acetobutylicum*), crotonase; *ter* (*T. denticola*), Trans-2-enoyl-CoA reductase; *bldh* (*C. saccharoperbutylacetonicum*), butyraldehyde dehydrogenase; *paaH1* (*R. eutropha*), 3-hydroxybutyryl-CoA dehydrogenase; *yqhD* (*E. coli*), NADP-dependent alcohol dehydrogenase; *adhE2* (*C. acetobutylicum*), bifunctional alcohol/aldehyde dehydrogenase. *bktB* (*R. eutropha*), thiolase; *aldh* (*C. kluyveri*, *C. beijerinckii*, *C. saccharobutylicum*, or *G. thermoglucosidasius*), aldehyde dehydrogenase; *eutE* (*E. coli*), aldehyde dehydrogenase; KASIII-like enzymes: *bamb6224* (*Burkholderia ambifaria*), *gox0115* (*Gluconobacter oxydans*), *hp0202* (*Helicobacter pylori*), *lmo2202* (*Listeria monocytogenes*), *pae-fabH2* (*Pseudomonas aeruginosa*), *sav-fabH4* (*Streptomyces avermitilis*), *sco5888* (*Streptomyces coelicolor*).

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