Additional file 1:

Modification of acetoacetyl-CoA reduction step in *Ralstonia eutropha* for biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from structurally unrelated compounds

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Supporting Information
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Construction of expression plasmids for N-His₆-tagged PhaBs

The coding regions of phaB1, phaB2, and phaB3 were amplified by PCR using primer sets shown in Supplementary Table S4 and R. eutropha gDNA, and the amplified fragments were individually cloned into pUC118. The phaB1 fragment was excised by digestion with NdeI and BamHI, and then inserted into pCold II (Takara Bio) at the corresponding sites to obtain pColdII-phaB1. pET15b-phaB3 was constructed by insertion of an NdeI-BamHI-restricted fragment of phaB3 into pET15b (Novagen), because the gene expression using a pCold II-based vector was not observed by unknown reason. The phaB2 fragment was excised by KpnI and inserted into pCold II at the corresponding site. The extra region between the His₆-tag sequence and phaB2 was removed by inverse PCR and successive self-ligation to obtain pColdII-phaB2.

Preparation of N-His₆-tagged recombinant proteins

E. coli BL21(DE3) transformed with pColdII-phaB1 or pColdII-phaB2 was cultivated in LB medium at 37°C on a reciprocal shaker (115 strokes/min). When the cell growth reached to OD₆₀₀ of 0.4, the culture broth was cooled at 15°C for 30 min and IPTG was added at the final concentration of 0.5 mM for induction of gene expression. The cultivation was continued for further 24 h at 15°C. In the case of expression of phaB3, E. coli BL21(DE3) harboring pET15b-phaB3 was cultivated in LB medium at 37°C on a reciprocal shaker and the gene expression was induced by addition of 0.5 mM IPTG when OD₆₀₀ reached to 0.5, and the cells were cultivated for further 16 h at 37°C.

The cells were harvested, washed and resuspended within 20 mM sodium phosphate buffer (pH7.4) containing 0.5 M NaCl and 30 mM imidazole, and then disrupted by sonication. The soluble fraction prepared by centrifugation and filtration (pore size 0.20 µm) was subjected to Ni-affinity chromatography using HisTrap FF crude 1 mL (GE Healthcare Life Sciences). The N-terminal His₆-tagged recombinant proteins were eluted by linear gradient of imidazole from 30 mM to 500 mM in sodium phosphate buffer (pH7.4) containing 0.5 M NaCl. The protein fraction was desalted using HiTrap Desalting (GE Healthcare Life Sciences) with 50 mM Tris-HCl (pH7.5), and then used for enzyme assay.

Site-directed mutagenesis of PhaB1

NADPH-acetoacetyl-CoA reductase (PhaB) is belonging to a short-chain dehydrogenase/reductase family along with NADPH-3-oxoacyl-ACP reductase (FabG). It is known that the former shows strict specificity to acetoacetyl-CoA, while the latter can accept 3-oxoacyl-ACPs with medium-chain-length as substrates. We had attempted protein engineering of PhaB1 based
on comparison of the crystal structures of PhaB1 and FabG, aiming to obtain the enzyme capable of catalyzing (R)-specific reduction of the C₆ substrate, 3-oxohexanoyl-CoA.

The sequence alignment of three PhaB paralogs from *R. eutropha* (PhaB1, PhaB2, and PhaB3) [1] and FabGs from *E. coli* and *Pseudomonas* sp. 61-3 [2] identified residues that were conserved in the three PhaBs but not in FabGs, and comparison of the three-dimensional structures of FabG from *E. coli* complexed with NADP⁺ (1Q7B) [3] and PhaB1 complexed with NADP⁺ (not containing the substrate, obtained by personal communication from Hokkaido Univ., before deposition of that complexed with NADP⁺ and acetoacetyl-CoA (3VZS) [4]). This suggested that a cavity near from the probable substrate binding pocket was filled by the side chains of asparagine 142 and tyrosine 185 in PhaB1 (Supplementary Fig. S2) possibly forming a hydrogen bond. We therefore replaced these residues by valine and phenylalanine correspondingly conserved in FabGs, respectively.

The site directed mutagenesis was carried out by QuickChange protocol using primer sets N142V_Fw/N142V_Re and Y185F_Fw/Y185F_Re for N142V and Y185F mutations, respectively. The NdeI-BamHI restricted fragments of the mutagenized genes were inserted into pCold II at the corresponding sites. The N-terminal His₆-tagged PhaB1s with N142V, Y185F, or N142V/Y185F mutations (designated as PhaB1NV, PhaB1YF, and PhaB1NVYF, respectively) were produced by recombinant strains of *E. coli* BL21(DE3) harboring each the expression plasmid, and then purified by the same procedure for PhaB1 as described in the main text. NADPH-dependent reduction activities towards acetoacetyl-CoA and 3-oxohexanoyl-CoA were determined as described previously [5].

Unfortunately, expansion of the substrate specificity was not achieved by the site-directed mutagenesis, since the ratios of activity to the C₆ and C₄ substrates of the three mutants were 0.020-0.023 (C₆/C₄) that was only slightly higher than 0.013 of the parent wild-type enzyme. Further characterization of the mutants clarified that catalytic efficiencies for NADPH-dependent reduction of acetoacetyl-CoA were markedly reduced by the mutations (Table 2 in the main text). When compared to wild-type PhaB1, the Km values of the single mutants PhaB1NV and PhaB1YF were remarkably larger (58µM–86 µM), while the Vmax values were similar for PhaB1NV and slightly lower for PhaB1YF. The double mutant PhaB1NVYF exhibited the lowest affinity and reaction rate toward acetoacetyl-CoA among the three mutants.
References

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(A) Glucose transport and phosphorylation

Fig. S1. Modifications of chromosome 1 of *R. eutropha* H16 for assimilation of glucose (A) [Orita I, et al. J Biosci Bioeng. 2011;113:63-9], and enhanced assimilation of glycerol (B) [Fukui T, et al. Appl Microbiol Biotechnol. 2014;98:7559-68].
Fig. S2. Structure of NADP⁺-binding sites in PhaB1 from *R. eutropha* (A) and FabG from *E. coli* (B). Side chains of amino acid residues in interest and NADP⁺ are shown as stick models.
Table S1. P(3HB-co-3HHx) biosynthesis by NSDG-GG-based engineered strains of *R. eutropha* from glucose

| Entry | Strain               | Plasmid               | Cultivation time (h) | Dry cell mass (g/L) | PHA content (wt%) | PHA (g/L) | 3HHx (mol%) | PHA monomer amount in PHA [mmol/l-culture] |
|-------|----------------------|-----------------------|----------------------|--------------------|-------------------|-----------|-------------|------------------------------------------|
|       |                      |                       |                      |                    |                   |           |             | 3HB | 3HHx                 |
| 1     | NSDG-GG             | none                  | 72                   | 4.56 ±0.14         | 83.1 ±2.6         | 3.78 ±0.01| 0           | 44.0 ±0.1 | 0                     |
| 2     | NSDG-GG             |                       |                      | 4.09 ±0.02         | 80.1 ±0.4         | 3.28 ±0.04| 2.3 ±0.2    | 36.9 ±0.5 | 0.9 ±0.1               |
| 3     | NSDG-GG-ΔB1         |                       |                      | 2.74 ±0.15         | 62.5 ±3.3         | 1.71 ±0.16| 22.0 ±0.6   | 14.5 ±1.2 | 4.1 ±0.5               |
| 4     | NSDG-GG-BNV         | pBPP-ccrMeJ4a-emd     | 72                   | 2.66 ±0.10         | 58.7 ±0.7         | 1.56 ±0.07| 15.3 ±0.1   | 14.7 ±0.7 | 2.6 ±0.1               |
| 5     | NSDG-GG-BNVYF       |                       |                      | 2.93 ±0.03         | 63.6 ±0.8         | 1.86 ±0.01| 12.0 ±0.9   | 18.3 ±0.2 | 2.5 ±0.2               |
| 6     | NSDG-GG-B2          |                       |                      | 3.96 ±0.01         | 72.3 ±2.5         | 2.86 ±0.09| 5.9 ±0.1    | 30.7 ±1.0 | 1.9 ±0.1               |
| 7     | NSDG-GG-HC          |                       |                      | 2.99 ±0.24         | 68.7 ±0.2         | 2.05 ±0.16| 26.0 ±0.4   | 16.3 ±1.1 | 5.7 ±0.5               |
| 8     | NSDG-GG             |                       |                      | 3.42 ±0.05         | 76.1 ±0.4         | 2.60 ±0.04| 4.1 ±0.2    | 28.6 ±0.6 | 1.2 ±0.1               |
| 9     | NSDG-GG-ΔB1         |                       |                      | 2.76 ±0.04         | 62.8 ±0.8         | 1.74 ±0.01| 8.3 ±0.2    | 18.0 ±1.6 | 1.6 ±0.2               |
| 10    | NSDG-GG-BNV         | pBPP-ccrMeJAc-emd     | 72                   | 3.06 ±0.02         | 69.9 ±1.1         | 2.14 ±0.03| 8.7 ±0.3    | 22.1 ±0.3 | 2.1 ±0.1               |
| 11    | NSDG-GG-BNVYF       |                       |                      | 3.00 ±0.13         | 66.7 ±2.0         | 2.00 ±0.15| 11.2 ±0.4   | 19.9 ±1.5 | 2.5 ±0.2               |
| 12    | NSDG-GG-B2          |                       |                      | 3.65 ±0.03         | 71.4 ±0.3         | 2.61 ±0.01| 7.0 ±0.2    | 27.6 ±0.2 | 2.1 ±0.0               |
| 13    | NSDG-GG-HC          |                       |                      | 3.71 ±0.13         | 75.3 ±0.4         | 2.79 ±0.08| 12.1 ±0.3   | 27.5 ±0.9 | 3.8 ±0.0               |

The cells were cultivated in a 100 ml MB medium containing 1% (w/v) glucose for 72 h at 30˚C. Standard deviation was shown with each value (*n*=3).
| Entry | Strain               | Plasmid                  | Cultivation time (h) | Dry cell mass (g/L) | PHA content (wt%) | PHA (g/L) | 3HHx (mol%) | Monomer amount in PHA [mmol/l-culture] |
|-------|----------------------|--------------------------|----------------------|--------------------|------------------|-----------|-------------|--------------------------------------|
|       |                      |                          |                      |                    |                  |           |             | 3HB                                  | 3HHx                      |
| 14    | NSDG-GG              | none                     | 72                   | 3.76 ±0.03         | 81.2 ±0.5        | 3.05 ±0.04| 0           | 35.5 ±0.5                            | 0                         |
| 15    | NSDG-GG              |                          |                      | 3.05 ±0.03         | 75.2 ±0.5        | 2.29 ±0.04| 0.9 ±0.1    | 26.4 ±0.4                            | 0.2 ±0.0                  |
| 16    | NSDG-GG-ΔB1          |                          |                      | 2.99 ±0.11         | 63.3 ±1.0        | 1.90 ±0.09| 21.5 ±0.9   | 16.2 ±0.9                            | 4.4 ±0.2                 |
| 17    | NSDG-GG-BNV          | pBPP-ccrMeJ4a-emd         | 72                   | 3.38 ±0.04         | 71.5 ±1.7        | 2.42 ±0.08| 14.8 ±0.4   | 22.8 ±0.8                            | 4.0 ±0.2                 |
| 18    | NSDG-GG-BNVYF        | pBPP-ccrMeJAc-emd         |                      | 3.70 ±0.08         | 73.3 ±1.9        | 2.71 ±0.13| 13.3 ±0.1   | 26.2 ±1.2                            | 4.0 ±0.2                 |
| 19    | NSDG-GG-B2           |                          |                      | 3.90 ±0.02         | 73.0 ±1.8        | 2.85 ±0.06| 10.3 ±0.3   | 28.8 ±0.7                            | 3.3 ±0.1                 |
| 20    | NSDG-GG-HC           |                          |                      | 2.76 ±0.12         | 74.5 ±1.0        | 2.06 ±0.11| 24.2 ±0.8   | 16.8 ±1.1                            | 5.4 ±0.1                 |
| 21    | NSDG-GG              |                          |                      | 2.68 ±0.03         | 69.6 ±2.2        | 1.87 ±0.08| 2.1 ±0.1    | 21.1 ±1.0                            | 0.5 ±0.0                 |
| 22    | NSDG-GG-ΔB1          |                          |                      | 2.93 ±0.03         | 64.1 ±0.3        | 1.87 ±0.01| 14.7 ±0.3   | 17.8 ±0.1                            | 3.1 ±0.1                 |
| 23    | NSDG-GG-BNV          | pBPP-ccrMeJAc-emd         |                      | 3.52 ±0.07         | 75.0 ±0.8        | 2.64 ±0.06| 9.4 ±0.3    | 26.9 ±0.7                            | 2.8 ±0.1                 |
| 24    | NSDG-GG-BNVYF        | pBPP-ccrMeJAc-emd         |                      | 3.67 ±0.10         | 72.5 ±3.9        | 2.66 ±0.11| 10.8 ±0.5   | 26.6 ±1.0                            | 3.2 ±0.3                 |
| 25    | NSDG-GG-B2           |                          |                      | 3.78 ±0.05         | 74.1 ±1.9        | 2.80 ±0.07| 10.0 ±0.3   | 28.4 ±0.6                            | 3.2 ±0.1                 |
| 26    | NSDG-GG-HC           |                          |                      | 3.60 ±0.31         | 78.1 ±0.6        | 2.81 ±0.23| 13.8 ±0.3   | 27.0 ±2.3                            | 4.3 ±0.3                 |

The cells were cultivated in a 100 ml MB medium containing 1% (w/v) fructose for 72 h at 30°C. Standard deviation was shown with each value (n=3).
Table S3. P(3HB-co-3HHx) biosynthesis by NSDG-GG-based engineered strains of *R. eutropha* from glycerol.

| Entry | Strain          | Plasmid             | Cultivation time (h) | Dry cell mass (g/L) | PHA content (wt%) | PHA (g/L) | 3HHx (mol%) | PHA 3HHx [mmol/l-culture] |
|-------|-----------------|---------------------|----------------------|---------------------|-------------------|-----------|-------------|--------------------------|
| 27    | NSDG-GG         | none                | 96                   | 4.53 ± 0.26         | 81.8 ± 1.8        | 3.71 ± 0.29| 0           | 43.1 ± 3.4 0              |
| 28    | NSDG-GG         |                      | 96                   | 3.53 ± 0.04         | 82.0 ± 0.8        | 2.89 ± 0.06| 0.4 ± 0.1   | 33.5 ± 0.7 0.1 ± 0.0      |
| 29    | NSDG-GG-ΔB1     | pBPP-cer<sub>M</sub>J<sub>4a</sub>-emd | 198                 | 1.23 ± 0.02         | 29.4 ± 0.4        | 0.36 ± 0.01| 13.1 ± 0.3 | 3.5 ± 0.1 0.5 ± 0.0       |
| 30    | NSDG-GG-B<sub>NV</sub> | pBPP-cer<sub>M</sub>J<sub>4a</sub>-emd | 198                 | 1.35 ± 0.01         | 30.9 ± 2.1        | 0.42 ± 0.03| 24.0 ± 1.2 | 3.4 ± 0.3 1.1 ± 0.0       |
| 31    | NSDG-GG-B<sub>NVYF</sub> |                       |                      | 1.62 ± 0.15         | 43.9 ± 1.1        | 0.71 ± 0.08| 2.5 ± 0.1   | 8.0 ± 0.9 0.2 ± 0.0       |
| 32    | NSDG-GG-B2      |                     |                      | 1.51 ± 0.02         | 40.2 ± 0.6        | 0.61 ± 0.01| 0.0         | 7.1 ± 0.1 0.0             |
| 33    | NSDG-GG-HC      |                     | 194                  | 1.37 ± 0.03         | 40.2 ± 0.5        | 0.55 ± 0.02| 16.6 ± 0.1 | 5.1 ± 0.2 1.0 ± 0.0       |
| 34    | NSDG-GG         |                     | 96                   | 2.58 ± 0.05         | 66.2 ± 1.6        | 1.71 ± 0.07| 1.7 ± 0.1   | 19.4 ± 0.8 0.3 ± 0.0      |
| 35    | NSDG-GG-ΔB1     |                     |                      | 1.53 ± 0.01         | 40.8 ± 0.6        | 0.62 ± 0.01| 3.8 ± 0.0   | 6.9 ± 0.1 0.3 ± 0.0       |
| 36    | NSDG-GG-B<sub>NV</sub> | pBPP-cer<sub>M</sub>J<sub>4a</sub>-emd | 198                 | 1.57 ± 0.11         | 33.5 ± 1.2        | 0.53 ± 0.06| 22.8 ± 0.4 | 4.4 ± 0.5 1.3 ± 0.1       |
| 37    | NSDG-GG-B<sub>NVYF</sub> |                       |                      | 1.60 ± 0.03         | 45.2 ± 0.2        | 0.72 ± 0.02| 1.8 ± 0.1   | 8.2 ± 0.2 0.2 ± 0.0       |
| 38    | NSDG-GG-B2      |                     |                      | 1.71 ± 0.01         | 44.7 ± 0.9        | 0.76 ± 0.02| 1.5 ± 0.0   | 8.7 ± 0.2 0.1 ± 0.0       |
| 39    | NSDG-GG-HC      |                     | 194                  | 1.79 ± 0.12         | 36.8 ± 0.4        | 0.66 ± 0.05| 3.1 ± 0.1   | 7.4 ± 0.6 0.2 ± 0.0       |

The cells were cultivated in a 100 ml MB medium containing 1% (w/v) glycerol for 96 h or 194-198 h at 30°C. Standard deviation was shown with each value (*n*=3).
Table S4. The sequences of primers used in this study.

| Primer                             | Sequence (5'→3')                      | Note                  |
|------------------------------------|--------------------------------------|-----------------------|
| **Construction of pBPP-ccrMeJAc-emd** |                                       |                       |
| phaJAc-Fw                          | ATGAGCGCAAAATCCTGGAAGTAG             | Amplification of phaJAc |
| phaJAc-Rv                          | TTAAGCGAGCTTGACCACCGGCTTC            |                       |
| phaJ4a-Inv-1                       | GTGAGATGTCTCCTCTTGAGATAAAGC          | Inverse PCR of pBPP-ccrMeJAc-emd |
| phaJ4a-Inv-2                       | GATCCGTTTTTTGTCGCTAGCAGGAGGA         |                       |
| **Construction of phaB-expression plasmids** |                                       |                       |
| PhaB1_ESN-N                        | GGAATTCAGAGATGTAATGACTCAGGCATTGC    | Amplification of phaB1 |
| PhaB1_BE-C                         | GGAATTCGCCCTCTGACCCATGTAGCAGGCCCCGGC |                       |
| PhaB2_KS-N                         | CGGGATACAGGGAGCTGGACATGCCGACAACGC   | Amplification of phaB2 |
| PhaB2_CE-C                         | CGGGATACAGGGATCTCAGCATTGAGGCCCCCGCC |                       |
| PhaB3_ESN-N                        | GGAATTCAGAGATGTAATGACAGGAGCAACGC   | Amplification of phaB3 |
| PhaB3_CE-C                         | GGAATTCGCCCTCTGACGAGTACAGGCCCCCGCC |                       |
| phaB2_inv_his                      | GTGGTGGTGATGATGACAGCAGCCTGTA        | Inverse PCR of phaB2-inserted plasmid |
| phaB2_inv_phha                     | ATGAGCGCAAAATCCTGGAAGTAG            |                       |
| **Site-directed mutagenesis of PhaB1** |                                       |                       |
| N142V_Fw                           | CATCTCTGCTGAGTGgtCGGGCAGAAGGCCC     | N142V mutation         |
| N142V_Re                           | GGCCTTTCTGCCCCGACCAACCGAGGAGATG     |                       |
| Y185F_Fw                           | CGGTCTCTCGGGGCTTGATTCCGCCAGGGC      | Y185F mutation         |
| Y185F_Re                           | GTCGGTGTTAGATGATGACAGCAGCCTGTA      |                       |
| **Construction of plasmids for homologous recombination in R. eutropha** |                                       |                       |
| pk18inv_Fw                         | CCGGCCTGGTTCAACCAGTCGGCA            | Inverse PCR of pk18mobacB-AR |
| del-PhaB1_inv-3                    | GTCGACCTGTCGCCCCGAGCAGGAGATG       |                       |
| phaB1_Fw_ATG                       | ATGAGCTAGGCATTGCGTATG               | Amplification of mutagenized phaB1 |
| phaB1_Rv                           | CAGGTCAGCCTATGCAGGCCCCGCGC         |                       |
| phaB2_Fw                           | ATGAGCGCAAAATCCTGGAAGTAG            | Amplification of phaB2 |
| phaB2_Rv                           | TCACTAGGAGCCTGCCCCCGGCTGTA         |                       |
| A0602-F                            | ATGCAAAATCAGAGGCAAACGTATCA         | Amplification and fusion of had and crt2 |
| A0602-R-Fus                        | ATGATTTTGCCTTACTTGCGCATCGGCCG      |                       |
| A3307-R                            | GCCTAGGCTGCTGAGAATT                 |                       |
| A3307-F-Fus                        | CCAAGTAAAGGCAAATACATAGGAGAGAAGA    |                       |