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Directed evolution and structural analysis of NADPH-dependent acetoacetyl-CoA reductase from *Ralstonia eutropha* reveals two mutations responsible for enhanced kinetics

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\*Running title: *Evolved NADPH-dependent acetoacetyl-CoA reductase*

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1

## 2 **ABSTRACT**

3 Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent acetoacetyl-CoA  
4 reductase (PhaB) is a key enzyme in the synthesis of poly(3-hydroxybutyrate) [P(3HB)],  
5 along with  $\beta$ -ketothiolase (PhaA) and polyhydroxyalkanoate synthase (PhaC). In this study,  
6 PhaB from *Ralstonia eutropha* was engineered by means of directed evolution consisting  
7 of an error-prone PCR-mediated mutagenesis and a P(3HB) accumulation-based *in vivo*  
8 screening system using *Escherichia coli*. Out of approximately twenty thousand mutants,  
9 we obtained two mutant candidates bearing Gln47Leu (Q47L) and Thr173Ser (T173S)  
10 substitutions. The mutants respectively exhibited a 2.4 and 3.5-fold higher  $k_{cat}$  value  
11 compared to that of wild-type enzyme. In fact, the PhaB mutants did exhibit enhanced  
12 activity and P(3HB) accumulation when expressed in recombinant *Corynebacterium*  
13 *glutamicum*. Comparative three-dimensional structural analysis between the wild-type and  
14 highly active PhaB mutants revealed that the beneficial mutations affected the flexibility  
15 around the active site, which in turn play an important role in substrate recognition.  
16 Furthermore, both the kinetic analysis and crystal structure data supported the conclusion  
17 that PhaB forms a ternary complex with NADPH and acetoacetyl-CoA. These results  
18 suggest that the mutations affected the interaction with substrates, resulting in the  
19 acquirement of enhanced activity.

20

## 21 **INTRODUCTION**

22 Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent acetoacetyl-CoA  
23 (AcAcCoA) reductase (PhaB) stereoselectively reduces the 3-ketone group of  
24 acetoacetyl-CoA so as to synthesize (*R*)-3-hydroxybutyryl(3HB)-CoA, which is known to  
25 be a monomer precursor of microbial polyester polyhydroxyalkanoate (PHA) (1-3). The  
26 PhaB-encoding gene is found in many bacteria, including *Ralstonia eutropha* (also known  
27 as *Cupriavidus necator*), and typically located in the *phb* operon together with  
28  $\beta$ -ketothiolase (PhaA) and PHA synthase (PhaC). The three enzymes catalyze the  
29 successive reactions synthesizing P(3HB) from acetyl-CoA (4-5). This pathway has been  
30 extensively utilized for the microbial production of P(3HB) and 3HB-based copolymers (6),  
31 both of which can be used as biobased plastics. For efficient production of these polymers,  
32 the enhancement in the activities of these enzymes is effective (7-8). In several cases, it has  
33 been known that the enhanced expression of either PhaB by itself or both PhaA and PhaB  
34 increased P(3HB) production by as the result of an increase in gene dosage (9) and codon  
35 optimization (10).

36 There are two major strategies to engineer enzymes for acquiring enhanced activity;  
37 structural and non-structural approaches. The three-dimensional structure of PhaB has not  
38 been determined, despite its important role in PHA biosynthesis. PhaB possesses a primary  
39 structure that is similar to NADPH-dependent 3-ketoacyl-acyl-carrier-protein(ACP)  
40 reductase (FabG), which generates the (*R*)-3-hydroxyacyl-ACP involved in fatty acid  
41 biosynthesis. However, although PhaB and FabG presumably have similar protein folding  
42 and reaction mechanisms to some extent, the regions contributing to the activity of PhaB  
43 and FabG have not been identified. Thus, at present we still do not have sufficient

44 information to design the structure-based engineering of PhaB for the activity  
45 enhancement.

46 Therefore, at the initial step in the present study, we attempted to enhance the  
47 enzymatic activity of PhaB using a non-structural strategy of a random mutagenesis of  
48 PhaB and high-throughput screening of the activity-enhanced PhaB mutants. This method  
49 is applicable without structural information, and is a useful means of identifying regions  
50 which exert an effect on the enzymatic activity. In the next step, the wild-type (WT) PhaB  
51 and certain highly active mutants were subjected to crystal structure analysis. The  
52 structural features of the beneficial sites and comparative analysis of the structure of the  
53 WT and activity-enhanced mutants were expected to provide insight into the regions in  
54 PhaB contributing to the reaction rate as well as the reaction mechanism of this enzyme.

55 A technical barrier impeding this goal had been the design of a high-throughput  
56 screening method for selecting highly active PhaB mutants. Our group has performed  
57 extensive *in vitro* evolution of PhaC for the purposes of increasing its activity (11-12) and  
58 altering its substrate specificity (13-14). The *in vivo* screening method used in these studies  
59 is based on polymer accumulation in recombinant *Escherichia coli* expressing the  
60 randomly mutated *phaC* gene together with the *phaAB* genes, and grown on Nile  
61 red-containing agar plates (15-17). The presence of Nile red-stained hydrophobic polymer  
62 granules in the cells allowed visualization of the polymer content in terms of the relative  
63 fluorescent intensity of the colonies. Namely, the colonies with a brighter fluorescence  
64 were suggested to be highly active mutants of the targeted enzyme. However, this method  
65 was not exclusively applicable to the engineering of PhaB, because the activity of the WT

66 PhaB achieved approximately 60 wt% polymer accumulation in *E. coli* (15, 17), which  
67 resulted in the saturated fluorescence and prevented the selection of mutants with a higher  
68 activity. Thus, the optimizing condition of the plate assay should allow for easy  
69 identification of the positive candidates from the other mutants and even the WT enzyme.

70 To meet this challenge, we replaced PhaA with AcAc-CoA synthase (AACS), which  
71 was recently isolated from terpenoid-producing *Streptomyces* sp. strain CL190 (18). AACS  
72 acted as a less efficient AcAc-CoA supplying enzyme than PhaA, and recombinant *E. coli*  
73 harboring the AACS gene, together with the WT *phaB* and *phaC* genes, accumulated a  
74 small amount of P(3HB) (19). The low basal polymer accumulation level, i.e. the weak  
75 fluorescence in the colonies expressing the WT *phaB* gene, would be preferable for  
76 isolating colonies emitting a brighter fluorescence. In fact, this approach successfully  
77 retrieved highly active mutants of PhaB. The kinetics and crystal structure of the obtained  
78 mutants are discussed.

79

## 80 MATERIALS AND METHODS

81 **Plasmid constructions and screening of *phaB* gene.** The *phaB* gene of *R. eutropha*  
82 was amplified under error-prone conditions (20) using pGEM<sup>+</sup>CAB as a template and a  
83 pair of primers, 5'-TTCCGGGGCTCGAGCGGTTG-3' and  
84 5'-CTCAAGCTAGGCATGCAACG-3'. The restriction sites used for the construction are  
85 underlined. The amplified fragment was digested with *XhoI/EcoT22I* and inserted into  
86 *XhoI/EcoT22I* sites of pGEM<sup>+</sup>CAACSB, which harbors AcAc-CoA synthase gene (AACS)  
87 together with the *phaB* and *phaC* genes (19) to replace the *phaB* gene with its mutants. The

88 plasmids were introduced into *E. coli* JM109 and cells were grown on LB agar plates  
89 containing glucose and Nile red (20). Colonies emitting enhanced fluorescence on a  
90 transilluminator were chosen as potential mutants of high activity.

91 To express the selected *phaB* mutant genes in *C. glutamicum*, the plasmids  
92 pPSCAB(Q47L) and pPSCAB(T173S) were constructed as follows. The AACSB gene in the  
93 selected pGEM"CAACSB was replaced with the *phaA* gene from *R. eutropha* using the  
94 *PstI/XhoI* sites. The resultant plasmids pGEM"*phaCAB*(Q47L) and  
95 pGEM"*phaCAB*(T173S) were digested with *Csp45I/BamHI*, and a 4.3-kb fragment was  
96 inserted into the *BstEII/BamHI* site of pPSPTG1 harboring the *cspB* promoter (21) to yield  
97 pPSCAB(Q47L) and pPSCAB(T173S), respectively. pPSCAB (21) bearing the WT *phaB*  
98 gene was used as a control.

99 For enzyme purification, the selected *phaB* gene mutants as well as the wild-type gene  
100 were amplified using a pair of primers; 5'-GTGGGATCCACTCAGCGCATTGCG-3' and  
101 5'-GCCAAGCTTTCAGCCCATATGCAG-3'. The restriction sites used for the construction  
102 are underlined. The amplified fragments were digested with *BamHI/HindIII* and inserted  
103 into pQE30 (Qiagen, USA) to construct the gene encoding the N-terminal His-tag fusion of  
104 PhaB (pQEphaB).

105 **Polymer productions and analysis.** For polymer production in *E. coli*, the  
106 engineered cells harboring pGEM"CAACSB were grown on LB medium containing 2%  
107 glucose and 100 µg/L ampicillin at 37°C for 48 h. Cells were lyophilized and the P(3HB)  
108 content was determined by HPLC, as described previously (22).

109 Transformation of *C. glutamicum* ATCC 13803 was performed by electroporation, as

110 described previously (21). The recombinant cells harboring pPSCAB(WT, Q47L and  
111 T173S) were grown on CM2G medium at 30°C for 12 h, then transferred into MMTG  
112 medium (23) containing 6% glucose and further cultivated at 30°C for 72 h. P(3HB)  
113 content was determined by HPLC, as described (22).

114 **PhaB activity in crude extract.** The PhaB activity in the crude extract of *C.*  
115 *glutamicum* harboring pPSCABs was measured as well. Cells grown on MMTG medium  
116 under the same condition for P(3HB) production were harvested at 24 h. The both cells  
117 were suspended in 125 mM Tris-HCl buffer (pH 8.0) and disrupted by sonication. The  
118 soluble fraction was used for enzymatic assay. The reaction mixture contained 125 mM  
119 Tris-HCl buffer (pH 8.0), 5 mM AcAc-CoA (SIGMA), 20 mM NADPH (Oriental Yeast  
120 Co., LTD, Japan) and 3.125% (vol./vol.) crude extract. The decrease in absorbance at 340  
121 nm was monitored at 30°C to measure the consumption rate of NADPH. The activity was  
122 normalized by the protein concentration determined using Bradford assay (Biorad).

123 **Purification of PhaB and kinetic analysis.** *E. coli* BL21(DE3) harboring pQEphaB  
124 (the WT and mutants) and pREP4 bearing *lacI* repressor gene (Qiagen, USA) was grown  
125 on LB medium containing 100 µg/L ampicillin and 25 µg/L kanamycin at 25°C for 2.5 h.  
126 Then a final 1.0 mM IPTG was added and cells were further cultivated for 10 h.  
127 Purification of the N-terminal His-tag fusion of PhaB was performed using His-Bind Resin  
128 (Novagen), as described previously (24). The eluted solution was replaced with 20 mM  
129 Tris-HCl (pH 8.0) containing 200 mM NaCl using a PD10 column (GE Healthcare) and  
130 stored at -80°C until analysis. The assay was carried out at 30°C using 125 mM Tris-HCl  
131 (pH 8.0) containing 130 - 180 ng enzyme, and varied concentrations of AcAc-CoA (4.2 to

132 12.5  $\mu\text{M}$ ) and NADPH (25 to 125  $\mu\text{M}$ ).

133       **Crystallization and structural analysis.** The His-tag fusion of PhaB was further  
134 purified on a HiLoad 26/60 Superdex 200-pg column (GE Healthcare) pre-equilibrated  
135 with 20 mM Tris-HCl (pH 8.0) and 200 mM sodium chloride. The crystals of PhaB were  
136 obtained from a buffer containing 0.1 M MES (pH 7.1), 1.6 M ammonium sulfate and 10%  
137 1,4-dioxane. Crystals of PhaB complexed with NADP<sup>+</sup> and AcAc-CoA were obtained from  
138 the same buffer containing 0.9 mM NADP<sup>+</sup> and 0.9 mM AcAc-CoA. Crystals of the Q47L  
139 mutant and T137S mutant were grown from a buffer composed of 0.6 – 1.2 M sodium /  
140 potassium tartrate, 0.16 – 0.20 M lithium sulfate and 0.1 M CHES (pH 8.9 – 9.9). X-ray  
141 diffraction experiments were performed at SPring-8 (Harima, Japan) and Photon Factory  
142 (Tsukuba, Japan) under the proposal number of 2010B1460 and 2011G012, respectively.  
143 The X-ray diffraction data set was collected under cryogenic conditions (100 K). Crystals  
144 were soaked in a mother liquor containing 20% glycerol and flash-cooled in a stream of  
145 liquid nitrogen. The diffracted data were indexed, integrated and scaled using the  
146 HKL2000 program package (25) or the XDS (26). The statistical data are shown in Table  
147 S1.

148       The structure of PhaB was determined by the molecular replacement method by  
149 means of the MOLREP program (27) using the structure of FabG from *E. coli* (PDB ID  
150 1I01) as the search probe. To monitor the refinement, a random 5% subset was set aside for  
151 calculation of the R-free factor. After several cycles of manual model fitting and building  
152 with Coot (28) and refinement with REFMAC5 (29), individual atomic coordinate  
153 refinement and individual ADP refinement were performed using phenix.refine (30). The

154 atomic coordinates of WT PhaB, mutant and ternary complex have been deposited in the  
155 Protein Data Bank, [www.pdb.org](http://www.pdb.org) (PDB ID codes 3VZP, 3VZR, and 3VZS). The  
156 refinement statistical data are summarized in Table S1.

157 **Particle size analysis.** The PhaB particle size in solution was analyzed with dynamic  
158 light scattering (DLS) using a Zetasizer Nano-ZS (Malvern). PhaB at 5 mg/ml was filtered  
159 and then analyzed.

160

## 161 **RESULTS**

162 **Selection of beneficial PhaB mutants from the mutant library.** Recombinant *E.*  
163 *coli* harboring the mutagenized *phaB* gene, together with the *AACS* and *phaC* genes, were  
164 grown on Nile red-containing plates for the screening of beneficial PhaB mutants. Out of  
165 the approximately twenty thousand mutant clones, we isolated two colonies emitting a  
166 brighter fluorescence. The selected mutants produced  $7.0 \pm 0.3$  and  $6.8 \pm 0.2$  wt% P(3HB),  
167 respectively, while the WT produced  $6.0 \pm 0.3$  wt% under the same conditions. These  
168 results suggest that the selected mutants were highly active. The gene sequence of the  
169 mutants revealed that the PhaB mutants bore Gln47Leu (Q47L) and Thr173Ser (T173S)  
170 substitutions, respectively.

171 **Enhanced P(3HB) production using highly active PhaB mutants in engineered *C.***  
172 ***glutamicum*.** The effect of the selected PhaB mutants on *in vivo* P(3HB) production was  
173 evaluated using *C. glutamicum*, which is a GRAS (Generally Recognized As Safe)  
174 platform for microbial polyester production (21, 31), because P(3HB) accumulation in  
175 recombinant *E. coli* expressing the wild-type *phaCAB* genes was close to saturation (15,

176 17). Indeed, the recombinant *C. glutamicum* harboring the two mutants exhibited increased  
177 P(3HB) accumulation (Fig. 1A) and the PhaB activities were correlated with the polymer  
178 content (Fig. 1B). This result indicated that the selected PhaB mutants were highly active  
179 and exerted a beneficial effect on P(3HB) production in the *C. glutamicum* strain.

180 **The PhaB mutants exhibited an enhanced turnover rate.** The kinetics of the  
181 selected mutants was analyzed using the N-terminal His-tag fusion forms of PhaB and its  
182 mutants expressed in *E. coli*. The homogeneously purified PhaB and its mutants using one  
183 step Ni-column affinity chromatography were directly used in the analysis. The activity of  
184 the recombinant PhaB and its mutants was measured using the varied concentrations of the  
185 two substrates, AcAc-CoA and NADPH. The kinetic parameters of PhaBs were determined  
186 (Table 1) based on Lineweaver–Burk plots (Fig. S1). The Q47L and T173S mutants had  
187 2.4 and 3.5-fold higher  $k_{cat}$  values than the WT enzyme, respectively. The increase in  $k_{cat}$   
188 was associated with increases in the  $K_{M(NADPH)}$  and  $K_{M(AcAcCoA)}$  values. The  $k_{cat}/K_M$  values  
189 of the Q47L mutant were elevated for  $k_{cat}/K_{M(NADPH)}$ , while  $k_{cat}/K_{M(AcAcCoA)}$  was lower than  
190 that of WT PhaB. In contrast, the  $k_{cat}/K_{M(AcAcCoA)}$  value of T173S was higher than the WT,  
191 although  $k_{cat}/K_{M(NADPH)}$  was decreased by this mutation. These results suggest that the  
192 effects of the two mutations on the interaction between the enzyme and the two kinds of  
193 substrates were different. Namely, the Q47L mutation may improve the recognition of  
194 NADPH rather than AcAc-CoA, while T173S may contribute to the AcAc-CoA recognition.  
195 In addition, kinetic analysis provided insight into the reaction mechanism. The lines  
196 produced from the Lineweaver–Burk plot intersected (Fig. S1), suggesting that PhaB had a  
197 ternary-complex rather than a ping–pong mechanism.

198

199       **Crystal structure analysis of PhaB and certain highly active mutants.** In order to  
200 obtain a better understanding of the beneficial mutation effects, the PhaB crystal structure  
201 was determined at a resolution of 1.8 Å by a molecular replacement method using the  
202 structure of 3-ketoacyl-ACP reductase (FabG) as a search probe. Four molecules of PhaB  
203 were contained in an asymmetric tetramer with the point group being 222, which was  
204 observed also for FabG (Fig. 2A and B). The tetramer of PhaB was superimposed onto the  
205 tetrameric FabG, with an RMSD of 1.85 Å for the 865 C $\alpha$  atoms (Fig. S1). DLS analysis  
206 revealed a radius of approximately 43.6 Å for PhaB in solution, which is in good  
207 agreement with the tetrameric structure in the crystal. These observations indicated that  
208 PhaB exists as a tetramer in solution, as is the case for FabG.

209       The crystal structures of the Q47L and T173S mutants were determined at a resolution  
210 of 2.0 and 2.9 Å, respectively. A single asymmetric unit contained two molecules, and  
211 these formed a crystallographically evident tetramer. The tetrameric structures of these  
212 mutants were superimposable onto that of the WT PhaB (0.62 Å for the 974 Ca atoms of  
213 Q47L and 0.71 Å for the 975 Ca atoms of T173S), although the  $\alpha$ 7 – 8 regions and their  
214 continuous loops exhibited conformational changes (Fig. 2C). The temperature factor of  
215 the  $\alpha$ 7 – 8 regions of the WT structure were significantly high compared with other regions  
216 (Fig. 2D), suggesting that this region has an intrinsically flexible characteristic. DLS  
217 analysis revealed a radius of 44.8 Å and 44.5 Å for the Q47L and T173S mutants,  
218 respectively, clearly indicating that each of these mutants formed a tetramer in solution.  
219 Taken together, it can be concluded that these mutants maintained a tetramer structure that

220 was identical to the WT enzyme, and thus there is a reason other than drastic structural  
221 change for the increase in the enzymatic activity due to these mutations.

222 PhaB formed a ternary complex with AcAc-CoA and NADPH. In order to obtain an  
223 understanding of the contribution of the mutations to the enzymatic activity, the crystal  
224 structure of PhaB in complex with AcAc-CoA and NADP<sup>+</sup> was determined. In the structure  
225 obtained, an obvious electron density pattern corresponding to these substrates was  
226 observed in a large cavity found in all four molecules in a single asymmetric unit, and both  
227 NADP<sup>+</sup> and AcAc-CoA were present (Fig. 3A and B). This structure strongly supports the  
228 finding that the reaction mechanism is effected via a ternary complex as proposed by the  
229 kinetic analysis.

230 The NADP<sup>+</sup> molecule was bound non-covalently in the cavity encircled by loops  
231 between  $\beta 1 - \alpha 1$ ,  $\beta 2 - \alpha 2$ ,  $\beta 3 - \alpha 3$ ,  $\beta 4 - \alpha 4$ ,  $\beta 5 - \alpha 5$  and  $\beta 6 - \alpha 7$ . The corresponding  
232 region of FabG was also used for the NADP(H)-binding pocket (32). NADP<sup>+</sup> was directly  
233 recognized by Arg40, Gly60-Asn61, Gly90-Thr92 and Pro183-Val191 (Fig. 3C). It should  
234 be noted that Pro183-Val191 corresponds to  $\alpha 8$  and its continuous loop, which exhibits  
235 flexibility in the apo form (Fig. 2C and D).

236 An AcAc-CoA molecule was found adjacent to the NADP<sup>+</sup> binding site. The  
237 nicotinamide ring of NADP<sup>+</sup> was in contact with the AcAc moiety, which explains the  
238 catalytic reduction of AcAc-CoA. AcAc-CoA is recognized by Ser140, Thr92, Asp94,  
239 Gln147-Tyr153, Gly184, Tyr185 and Arg195. Gly184, Tyr185 and Arg195 are located in a  
240 flexible portion of  $\alpha 7 - 8$  (Fig. 2C, D, Fig. 3C, and D), suggesting that the flexibility of  
241 this region may have a significant role in both AcAc-CoA and NADP(H) recognition.

242

## 243 Discussion

244 The kinetic analysis indicated that the T173S mutant exhibited a higher  
245  $k_{\text{cat}}/K_{\text{M}(\text{AcAcCoA})}$  than the WT PhaB, whereas  $k_{\text{cat}}/K_{\text{M}(\text{NADPH})}$  was lower than the WT. This  
246 result shows that the reaction with AcAc-CoA was accelerated by the T173S mutation. This  
247 effect was interpreted based on the crystal structure. The residue at the beneficial site 173  
248 of the adjacent subunit (subunit A) was shown to be located close to the adenylyl moiety of  
249 AcAc-CoA, although direct interaction was not observed (Fig. 3D). Moreover, T173 of  
250 another adjacent subunit (subunit B) interacted with the Pro207 residue located at the root  
251 of the  $\alpha 7 - 8$  using the C $\gamma$ 2 atom, which is absent in the T173S mutant. Although the  
252 interaction between Ser173 and Pro207 was maintained in the structure of T173S, the N  
253 atom of Ser173, instead of C $\gamma$ 2, interacted with Pro207. These facts suggest that a change  
254 in the interaction with Pro207, due to a substitution at position 173, may alter the flexibility  
255 of the  $\alpha 7 - 8$  region in the adjacent protomer, and influence the interaction toward  
256 AcAc-CoA as a consequence.

257 On the other hand, kinetic analysis of the Q47L mutant showed that the mutation had  
258 an enhanced  $k_{\text{cat}}/K_{\text{M}(\text{NADPH})}$  value compared with WT PhaB, while  $k_{\text{cat}}/K_{\text{M}(\text{AcAcCoA})}$  was  
259 comparatively lower. This suggests that Q47L influenced the interaction of PhaB with  
260 NADPH. This change in kinetics was also evident by the 3-dimensional structure. Based  
261 on the crystal structure, Q47 was found to be located in the  $\alpha 2$  helix. The loop between  $\beta 2$   
262 and  $\alpha 2$  contributed to NADP<sup>+</sup> recognition through Arg40 (Fig. 3C). Furthermore, the  
263 temperature factor in this region, as well as  $\alpha 7 - \alpha 8$ , was higher than in the others (Fig.

264 2D). These results suggest that the substitution for Q47 may alter the flexibility of this  
265 region so as to be preferable for the interaction with NADP(H). Taking these results  
266 together, it was concluded that the selected mutations influenced the recognition of  
267 NADPH and/or AcAc-CoA indirectly via alterations in the mobility of  $\alpha 2$  and  $\alpha 7 - 8$ . The  
268 crystal structure data did account for the beneficial effects of the mutations. However, it is  
269 worth noting that the mutations were unpredictable from the protein structure, which may  
270 indicate the need for a combined strategy of structural and non-structural based enzyme  
271 engineering.

272 The  $K_{M(\text{AcAcCoA})}$  and  $K_{M(\text{NADPH})}$  values of the native PhaB isolated from *R. eutropha*  
273 were previously reported to be 5 and 19  $\mu\text{M}$ , respectively (33).  $K_{M(\text{AcAcCoA})}$  was consistent  
274 with the result of the present experiment, while the  $K_{M(\text{NADPH})}$  value of native PhaB was  
275 lower than the recombinant protein. This disagreement in the kinetic parameters could be  
276 caused by the difference in the host strain used for PhaB expression and also the presence  
277 of His-tag at the N-terminal of the protein. Moreover, it is reported that *R. eutropha*  
278 possesses multiple PhaB isologs (34). Therefore, the native PhaB isolated from *R. eutropha*  
279 might be composed of multiple proteins having very similar physical properties, which  
280 could be the cause of the differences in the kinetic parameters.

281 The potential variety in the mutated residue was estimated by the database search  
282 (<http://blast.ncbi.nlm.nih.gov/>), which retrieved PhaB homologs assigned as AcAc-CoA  
283 reductase (Fig. 4). Fig. 4 indicates alignment of PhaB homologs, which were chosen based  
284 on the variety of residues 47 and 173, namely many similar sequences are omitted.  
285 According to the alignment of these homologs, residue 173 was found to be located in a

286 highly-conserved region, which suggests that the region around the 173 position plays an  
287 important role in PhaB activity. In contrast, the  $\alpha 2$  region that included residue 47 was not  
288 highly conserved. The position was occupied by a limited variety of amino acid residues,  
289 such as Gln, Glu or Asp, but not Leu. In fact,  $\alpha 2$  was not directly in contact with the  
290 substrate and thus was presumably tolerant to further modification.

291

## 292 **Conclusions**

293 In this study, two novel activity-enhanced mutants of PhaB exhibited enhanced  
294  $k_{\text{cat}}/K_{\text{M}(\text{NADPH})}$  and  $k_{\text{cat}}/K_{\text{M}(\text{AcAcCoA})}$  values, respectively. The design of the plate assay-based  
295 high-throughput screening, namely the effect of AACS on suppressed P(3HB) production,  
296 was a key to obtaining the beneficial mutants. These mutants should be applicable to  
297 obtaining higher P(3HB) production in various platforms, including microbes and plants, if  
298 the monomer-supplying step is rate-limiting, as observed here for recombinant *C.*  
299 *glutamicum*. In addition, the crystal structure analysis of PhaB revealed that the flexible  
300 regions in PhaB contributed to the activity. The structural information reasonably explained  
301 the effect of the mutations on the enzymatic activity, and provides a clue to design the  
302 structure-based engineering of this enzyme, as well as future mutation/selection or other  
303 strategies along similar lines to the one employed in this study.

304

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309

## REFERENCES

1. **Sudesh K, K Bhubalan, JA Chuah, YK Kek, H Kamilah, N Sridewi, YF Lee.** 2011. Synthesis of polyhydroxyalkanoate from palm oil and some new applications. Appl. Microbiol. Biotechnol. **89**:1373-1386.
2. **Keshavarz T, I Roy.** 2010. Polyhydroxyalkanoates: bioplastics with a green agenda. Curr. Opin. Microbiol. **13**:321-326.
3. **Wu Q, Y Wang, GQ Chen.** 2009. Medical Application of Microbial Biopolyesters Polyhydroxyalkanoates. Artif. Cell. Blood Sub. **37**:1-12.
4. **Schubert P, A Steinbüchel, HG Schlegel.** 1988. Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly- $\beta$ -hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. J. Bacteriol. **170**:5837-5847.
5. **Peoples OP, AJ Sinskey.** 1989. Poly- $\alpha$ -hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding  $\alpha$ -ketothiolase and acetoacetyl-CoA reductase. J. Biol. Chem. **264**:15293-15297.
6. **Matsusaki H, H Abe, Y Doi.** 2000. Biosynthesis and properties of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant strains of *Pseudomonas* sp. 61-3. Biomacromolecules **1**:17-22.
7. **Kichise T, T Fukui, Y Yoshida, Y Doi.** 1999. Biosynthesis of polyhydroxyalkanoates (PHA) by recombinant *Ralstonia eutropha* and effects of PHA synthase activity on *in vivo* PHA biosynthesis. Int. J. Biol. Macromol. **25**:69-77.
8. **Jung Y, J Park, Y Lee.** 2000. Metabolic engineering of *Alcaligenes eutrophus* through the transformation of cloned *phbCAB* genes for the investigation of the regulatory mechanism of polyhydroxyalkanoate biosynthesis. Enzyme Microb. Technol. **26**:201-208.
9. **Jo SJ, K Matsumoto, CR Leong, T Ooi, S Taguchi.** 2007. Improvement of poly(3-hydroxybutyrate) [P(3HB)] production in *Corynebacterium glutamicum* by codon optimization, point mutation and gene dosage of P(3HB) biosynthetic genes. J. Biosci. Bioeng. **104**:457-463.
10. **Matsumoto K, K Morimoto, A Gohda, H Shimada, S Taguchi.** 2011. Improved

- polyhydroxybutyrate (PHB) production in transgenic tobacco by enhancing translation efficiency of bacterial PHB biosynthetic genes. *J. Biosci. Bioeng.* **111**:485-488.
11. **Taguchi S, Y Doi.** 2004. Evolution of polyhydroxyalkanoate (PHA) production system by "enzyme evolution": Successful case studies of directed evolution. *Macromol. Biosci.* **4**:145-156.
  12. **Nomura CT, S Taguchi.** 2007. PHA synthase engineering toward superbio-catalysts for custom-made biopolymers. *Appl. Microbiol. Biotechnol.* **73**:969-979.
  13. **Matsumoto K, S Taguchi.** 2010. Enzymatic and whole-cell synthesis of lactate-containing polyesters: toward the complete biological production of polylactate. *Appl. Microbiol. Biotechnol.* **85**:921-932.
  14. **Taguchi S, M Yamada, K Matsumoto, K Tajima, Y Satoh, M Munekata, K Ohno, K Kohda, T Shimamura, H Kambe, S Obata.** 2008. A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme. *Proc. Natl. Acad. Sci. USA* **105**:17323-17327.
  15. **Taguchi S, A Maehara, K Takase, M Nakahara, H Nakamura, Y Doi.** 2001. Analysis of mutational effects of a polyhydroxybutyrate (PHB) polymerase on bacterial PHB accumulation using an *in vivo* assay system. *FEMS Microbiol. Lett.* **198**:65-71.
  16. **Kichise T, S Taguchi, Y Doi.** 2002. Enhanced accumulation and changed monomer composition in polyhydroxyalkanoate (PHA) copolyester by *in vitro* evolution of *Aeromonas caviae* PHA synthase. *Appl. Environ. Microbiol.* **68**:2411-2419.
  17. **Taguchi S, H Nakamura, T Hiraishi, I Yamato, Y Doi.** 2002. *In vitro* evolution of a polyhydroxybutyrate synthase by intragenic suppression-type mutagenesis. *J. Biochem.* **131**:801-806.
  18. **Okamura E, T Tomita, R Sawa, M Nishiyama, T Kuzuyama.** 2010. Unprecedented acetoacetyl-coenzyme A synthesizing enzyme of the thiolase superfamily involved in the mevalonate pathway. *Proc. Natl. Acad. Sci. USA* **107**:11265-11270.
  19. **Matsumoto K, M Yamada, CR Leong, SJ Jo, T Kuzuyama, S Taguchi.** 2011. A new pathway for poly(3-hydroxybutyrate) production in *Escherichia coli* and *Corynebacterium glutamicum* by functional expression of a new acetoacetyl-coenzyme A synthase. *Biosci. Biotechnol. Biochem.* **75**:364-6.
  20. **Takase K, S Taguchi, Y Doi.** 2003. Enhanced synthesis of poly(3-hydroxybutyrate) in recombinant *Escherichia coli* by means of error-prone PCR mutagenesis, saturation mutagenesis, and *in vitro* recombination of the type II polyhydroxyalkanoate synthase

- gene. *J. Biochem.* **133**:139-145.
21. **Jo SJ, M Maeda, T Ooi, S Taguchi.** 2006. Production system for biodegradable polyester polyhydroxybutyrate by *Corynebacterium glutamicum*. *J. Biosci. Bioeng.* **102**:233-236.
  22. **Takase K, K Matsumoto, S Taguchi, Y Doi.** 2004. Alteration of substrate chain-length specificity of type II synthase for polyhydroxyalkanoate biosynthesis by *in vitro* evolution: *in vivo* and *in vitro* enzyme assays. *Biomacromolecules* **5**:480-485.
  23. **Kikuchi Y, M Date, K Yokoyama, Y Umezawa, H Matsui.** 2003. Secretion of active-form *Streptovorticillium mobaraense* transglutaminase by *Corynebacterium glutamicum*: Processing of the pro-transglutaminase by a cosecreted subtilisin-like protease from *Streptomyces albogriseolus*. *Appl. Environ. Microbiol.* **69**:358-366.
  24. **Matsumoto K, F Shozui, Y Satoh, K Tajima, M Munekata, S Taguchi.** 2009. Kinetic analysis of engineered polyhydroxyalkanoate synthases with broad substrate specificity. *Polym. J.* **41**:237-240.
  25. **Otwinowski Z, W Minor.** 1997. Processing of X-ray Diffraction Data Collected in Oscillation Mode. *Methods in Enzymology* **276**:307-326.
  26. **Kabsch W.** 2010. Xds. *Acta Crystallogr D Biol Crystallogr* **66**:125-32.
  27. **Vagin A, A Teplyako.** 1997. MOLREP: an automated program for molecular replacement. *J. Appl. Cryst.* **30**:1022-1025.
  28. **Emsley P, K Cowtan.** 2004. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **60**:2126-32.
  29. **Murshudov GN, P Skubak, AA Lebedev, NS Pannu, RA Steiner, RA Nicholls, MD Winn, F Long, AA Vagin.** 2011. REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr D Biol Crystallogr* **67**:355-67.
  30. **Adams PD, PV Afonine, G Bunkoczi, VB Chen, IW Davis, N Echols, JJ Headd, LW Hung, GJ Kapral, RW Grosse-Kunstleve, AJ McCoy, NW Moriarty, R Oeffner, RJ Read, DC Richardson, JS Richardson, TC Terwilliger, PH Zwart.** 2010. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D Biol Crystallogr* **66**:213-21.
  31. **Song YY, K Matsumoto, M Yamada, A Gohda, CJ Brigham, AJ Sinskey, S Taguchi.** 2012. Engineered *Corynebacterium glutamicum* as an endotoxin-free platform strain for lactate-based polyester production. *Appl. Microbiol. Biotechnol.* **93**:1917-1925.
  32. **Price AC, YM Zhang, CO Rock, SW White.** 2004. Cofactor-induced conformational

- rearrangements establish a catalytically competent active site and a proton relay conduit in FabG. *Structure* **12**:417-428.
33. **Haywood GW, AJ Anderson, L Chu, EA Dawes.** 1988. The role of NADH- and NADPH-linked acetoacetyl-CoA reductases in the poly-3-hydroxybutyrate synthesizing organism *Alcaligenes eutrophus* FEMS Microbiol.Lett. **52**:259-264.
34. **Budde CF, AE Mahan, JN Lu, C Rha, AJ Sinskey.** 2010. Roles of multiple acetoacetyl Coenzyme A reductases in polyhydroxybutyrate biosynthesis in *Ralstonia eutropha* H16. *J. Bacteriol.* **192**:5319-5328.

## FIGURE LEGENDS

**FIG. 1.** P(3HB) production in recombinant *C. glutamicum* harboring the engineered *phaB* genes. (A) Time course of P(3HB) accumulation. Circle: wild-type PhaB, triangle: Q47L, square: T173S. (B) Correlation between the enzymatic activity and P(3HB) content in recombinant *C. glutamicum*. Black bar: enzymatic activity (left axis). Gray bar: P(3HB) content (right axis). The PhaB activity was measured at 24 h. All data is the average of at least three trials.

**FIG. 2.** Crystal structure of the PhaB and mutants. (A) Ribbon diagram of the PhaB monomer. The ribbon model is colored according to the sequence, from blue at the N-terminus to red at the C-terminus. (B) Tetrameric structure of PhaB. The model is colored according to the subunit. Q47 (purple) and T137 (cyan) are also shown as spherical models. (C) Structure superimposition among wild-type (red), Q47L (blue), and T137S (orange). For clarity, only a monomer of the superimposed tetramer is shown. (D) Temperature factor of wild type PhaB. The tube model is colored according to the

temperature factor, from blue at 20 to red at 50. The width of the tube also corresponds to temperature factor. In short, red thick region implies high flexibility and blue thin region low. Q47 is also shown as green sticks. The flexible  $\alpha 7 - 8$  and  $\alpha 2$  are indicated.

**FIG. 3.** Crystal structure of PhaB – AcAc-CoA – NADP<sup>+</sup> ternary complex. (A) The Fo-Fc map (contoured at 1.5 $\sigma$ ) of AcAc-CoA and NADP<sup>+</sup>. AcAc-CoA (yellow) and NADP<sup>+</sup> (green) are also shown as sticks. (B) Ribbon diagram of the tetramer in complex with AcAc-CoA and NADP<sup>+</sup>. The bound AcAc-CoA (yellow) and NADP<sup>+</sup> (green) are shown as spherical models. (C) Close-up view of the substrate-binding site. The bound substrates and their recognition residues are shown in stick models. The flexible  $\alpha 7 - 8$  region and  $\beta 2 - \alpha 2$  are colored purple and brown, respectively. (D) T173 in the adjacent subunits located around the AcAc-CoA binding site. T173 residues in the adjacent subunits are shown as blue spheres. P207 interacting with T173 is shown as a purple sphere. The ribbon diagrams are colored according to the subunits, although the flexible  $\alpha 7 - 8$  regions and their continuing loops are colored purple.

**FIG. 4.** Partial alignment of PhaB from *R. eutropha* and its homologous enzymes. Asterisk indicates the beneficial sites. Secondary structure was shown based on the crystal structure of PhaB from *R. eutropha*. The residue 47 locates in the non-conserved  $\alpha$ -helix, while residue 173 locates in the random coil. The position 47 is often occupied with hydrophilic residues. The position 173 is mostly occupied with Thr, while some PhaB homologs possess Ser residue at this position.

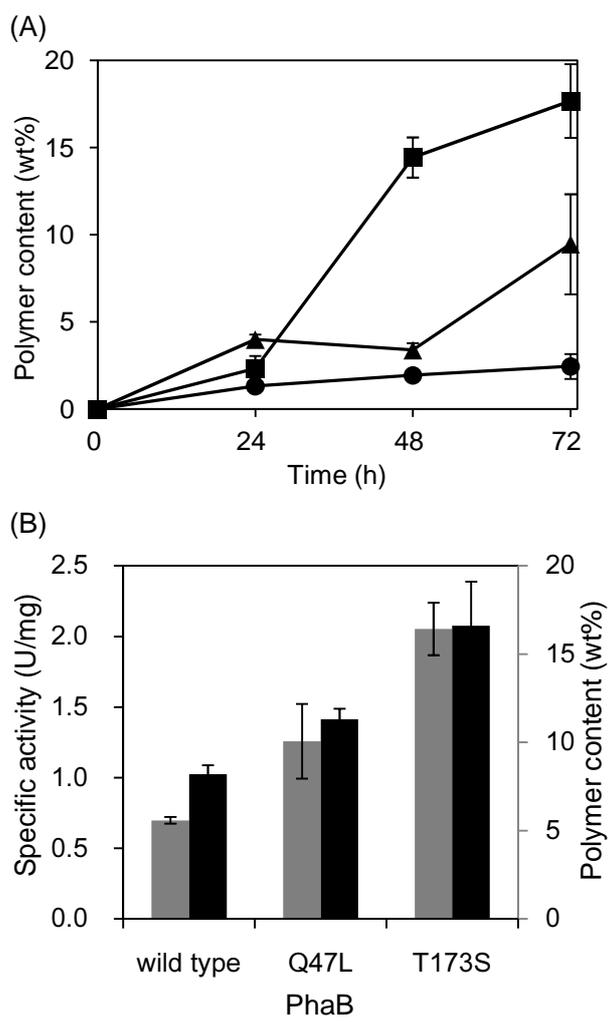


Figure 1

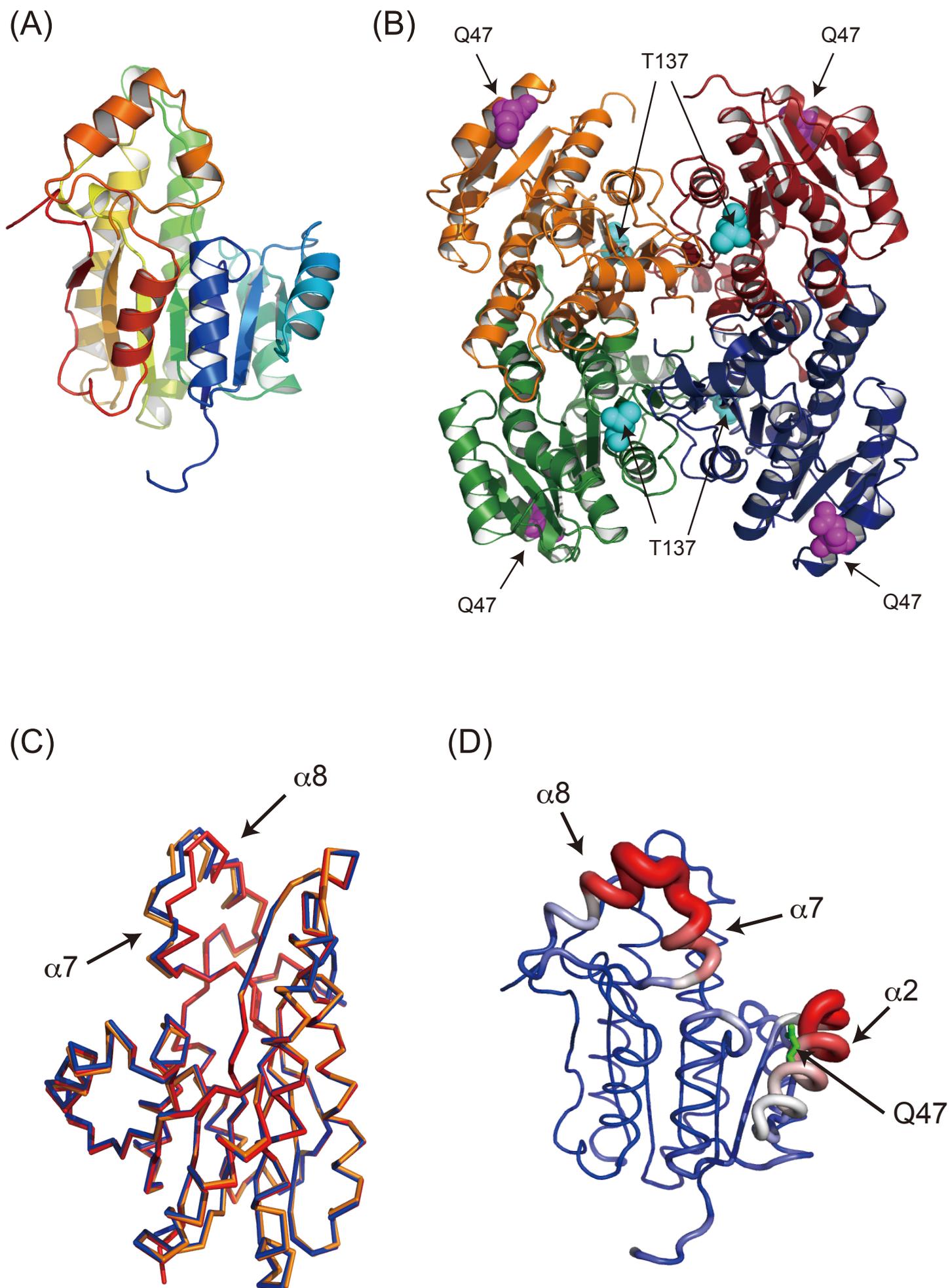


Fig. 2

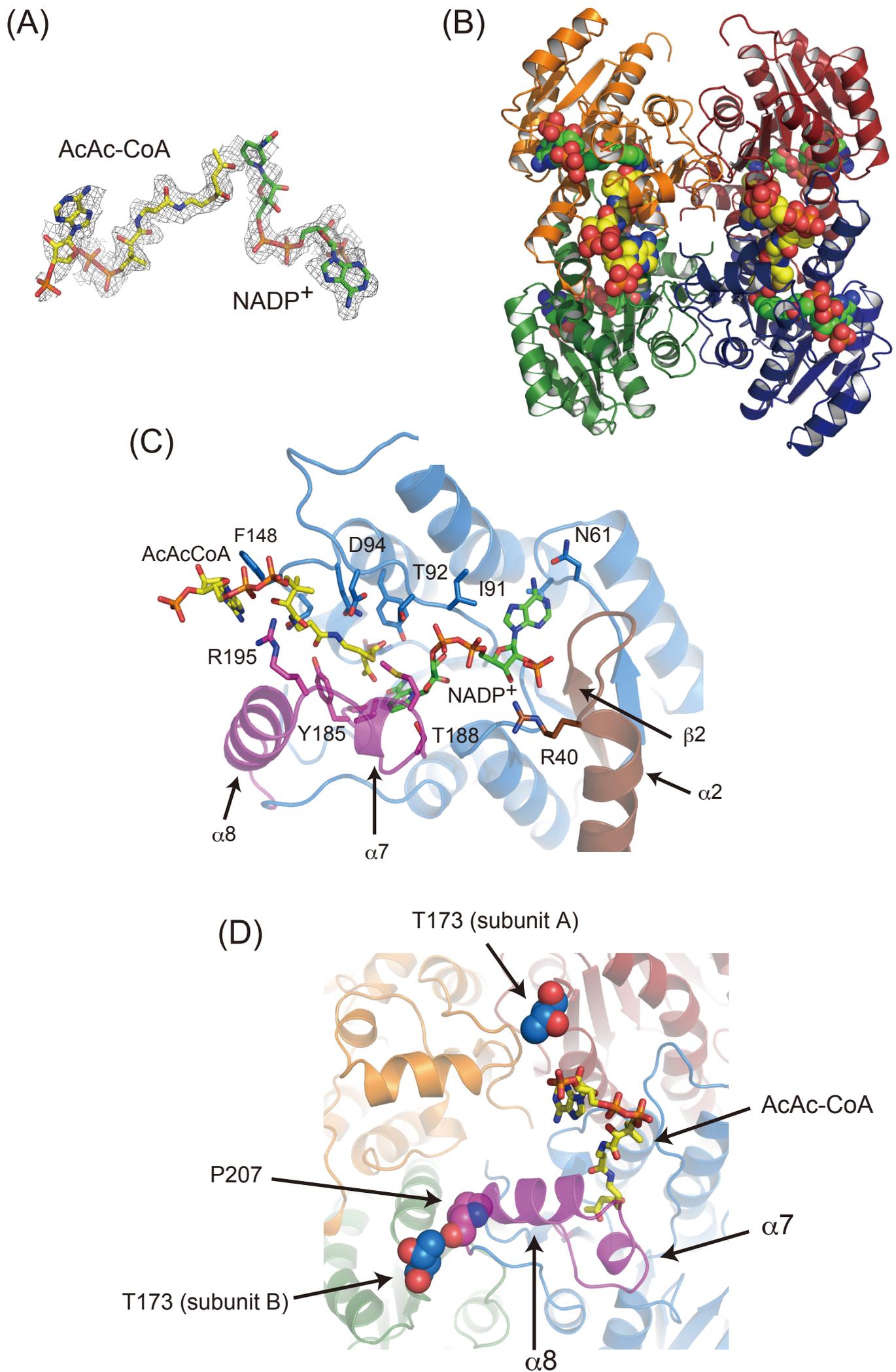


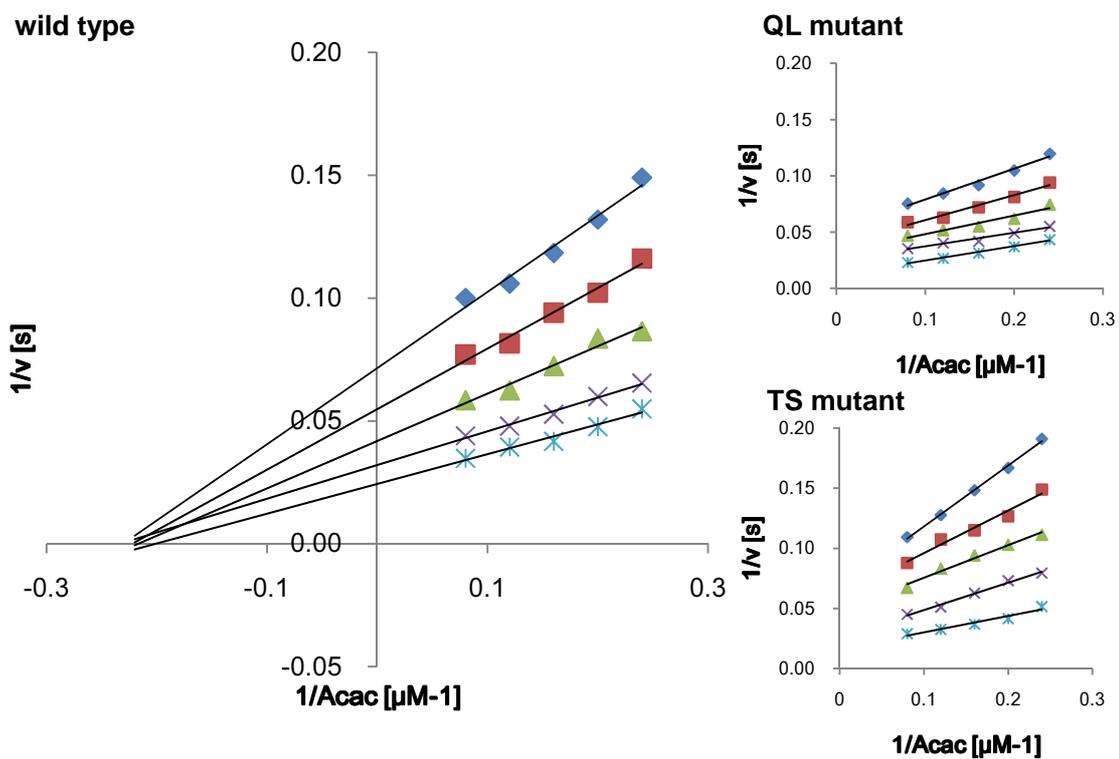
Fig. 3

		<u>β1</u>	<u>α1</u>	<u>β2</u>	<u>α2</u>	<u>β3</u>	
Ralstonia eutropha	1	MTQRIAYVTGGMGGIGTAICQRLAKDGRVAVAGCGPNSPRREKWL				EQKALGFDFIASEG	60
Burkholderia pseudomallei 1710b	1	MSQRIAYVTGGMGGIGTSCQRLHKDGRVAVAGCGPNSPRRVKWL				EDQKALGFDFIASEG	60
Burkholderia multivorans ATCC 17616	1	MSQRIAYVTGGMGGIGTSCQRLSKDGRVAVAGCGPNSPRRVKWL				EDQKALGFDFIASEG	60
Burkholderia thailandensis MSMB43	1	MSQRIAYVTGGMGGIGTSCQRLHKDGRVAVAGCGPNSPRRVKWL				ENQKALGFDFIASEG	60
Burkholderia phytofirmans PsJN	1	MATRIAYVTGGMGGIGTAICQRLHKNFTVVAVAGCGPNSRRARWL				EQKTLGYSFIIASEG	60
Herbaspirillum sp. CF444	1	MVKRIAYVTGGMGGIGTPICARLCKDGYTVVAVAGCGPNSRTRKWL				ASMRQGFDIHASEG	60
Azohydromonas lata	1	MTQKLAYVTGGMGGIGTSMCQRLHKDGRVAVAGCGP-SRDYQKWL				DEQKALGYTFYASVG	60
Limnobacter sp. MED105	2	SEPKVAVVTGGMGGIGTAICKLCEQGYRVIAGCGPNSPRKEKWL				GEMRSAGYEVYASEG	61
Herbaspirillum sp. GW103	1	MSKRIAYVTGGMGGIGTAICTRLCKDGYTVVAVAGCGPNSRTRKWL				ATMRGQGYDIHASEG	60
Bordetella avium 197N	1	MSGKLAYVTGGMGGIGTSCQRLAKDGRVAVAGCGP-SRNYQQWL				DEQAAQGYTFHASEG	60
Herbaspirillum sp. YR522	1	MSKRIAYVTGGMGGIGTSCQRLCKDGYTVVAVAGCGPNSRTRKWL				AMREQGYDIHASEG	60
Achromobacter xylosoxidans	1	MSGKLAYVTGGMGGIGTSCQRLAKEGRVAVAGCGP-SRNYQQWL				DEQAAQGYTFHASEG	60

		<u>α4</u>	<u>β5</u>	<u>α5</u>	<u>α6</u>	<u>β6</u>	
Ralstonia eutropha	121	KQVIDGMADRGWGRIVNISSVNGQKGGQFGQTNYSTAKAGLHGFTMALA				QEVATKGVTVNT	180
Burkholderia pseudomallei 1710b	121	KQVIDGMVERGWGRIINISSVNGQKGGQFGQTNYSTAKAGIHGFTMSLA				QEVATKGVTVNT	180
Burkholderia multivorans ATCC 17616	121	KQVIDGMVERGWGRIINISSVNGQKGGQFGQTNYSTAKAGIHGFTMALA				QEVATKGVTVNT	180
Burkholderia thailandensis MSMB43	121	KQVIDGMVERGWGRIINISSVNGQKGGQFGQTNYSTAKAGIHGFTMSLA				QEVATKGVTVNT	180
Burkholderia phytofirmans PsJN	121	KQVIEGMVQRGWGRIVNISSVNGQKGGQFGQTNYSTAKAGIHGFTMALA				QEVAVKGVTVNT	180
Herbaspirillum sp. CF444	121	KQVIEGMVDRGWGRIINISSVNGQKGGQFGQTNYSTAKAGIHGFTMALA				QEVATKGVTVNT	180
Azohydromonas lata	121	KQVIDGMLDKGWGRIINISSVNGEKGGQFGQTNYSAAKAGMHGFTMALA				QEVAKGVTVNT	180
Limnobacter sp. MED105	122	KQVIGGMVEQGFGRINISSVNGQKGGQFGQTNYSTAKAGLRGFTMALA				QEVASKGVTVNT	181
Herbaspirillum sp. GW103	121	KQVIEGMCERGFGRINISSVNGQKGGQFGQTNYSTAKAGIHGFTMALA				QEVATKGVTVNT	180
Bordetella avium 197N	121	KQVLDSMVDQRGWGRIINISSVNGQKGGQFGQTNYSTAKAGIHGFTMALA				QEVASKGIVTNT	180
Herbaspirillum sp. YR522	121	KQVIEGMIERGFGRINISSVNGQKGGQFGQTNYSTAKAGIHGFTMALA				QEVATKGVTVNT	180
Achromobacter xylosoxidans	121	KQVIDGMVERGWGRIINISSVNGQKGGQFGQTNYSTAKAGIHGFTMALA				QEVASKGVTVNT	180

Figure 4.



**Figure S1.** Lineweaver Burk plots for the wild-type and mutated acetoacetyl-CoA reductase (PhaB). NADPH concentrations are 25 (diamond), 31 (square), 42 (triangle), 62.5 (cross) and 125  $\mu\text{M}$  (asterisk), respectively.

**Table S1.** X-ray data collection and refinement statistics

PDB ID	wild type 3VZP	Q47L 3VZQ	T137S 3VZR	ternary complex 3VZS
Data collection				
Beamline	Photon Factory BL1A	In house (RIGAKU R-AXIS IV)	Spring-8 BL41XU	Photon Factory BL5A
Wavelength	1.0000	1.5418	1.0000	1.0000
Resolution range* (Å)	20.06 - 1.79 (1.90 - 1.79)	45.3 - 2.00 (2.12 - 2.00)	20.00 - 2.90 (3.00 - 2.90)	50.00 - 2.14 (2.18 - 2.14)
Space group	<i>C</i> 222 <sub>1</sub>	<i>C</i> 2	<i>C</i> 222 <sub>1</sub>	<i>C</i> 222 <sub>1</sub>
Unit-cell parameters (Å, °)	a = 67.7, b = 123.7, c = 260.8	a = 93.6, b = 88.6, c = 70.7, β = 104.24	a = 70.7, b = 185.9, c = 75.5	a = 67.4, b = 123.4, c = 260.2
Number of molecule in an asymmetric unit	4	2	2	4
No. of observed reflections	721056	159130	67176	330241
No. of unique reflections	100490	37627	11342	58459
Multiplicity*	7.18 (6.98)	4.23 (4.08)	5.9 (4.8)	5.6 (6.2)
Completeness* (%)	97.7 (89.2)	99.4 (97.8)	99.8 (99.3)	96.9 (98.7)
$\langle I \rangle / \langle \sigma(I) \rangle$ *	17.85 (3.86)	7.07 (3.07)	7.57 (2.15)	25.14 (7.86)
$R_{\text{sym}}$ * (%)	10.1 (61.8)	16.1 (43.4)	17.9 (51.4)	9.1 (30.3)
Refinement				
$R$ -factor / $R$ -free (%)	19.2 / 23.8	23.5 / 26.7	18.9 / 24.0	16.2 / 20.7
No. of atoms	8195	4075	3668	8469
proten	7414	3668	3668	7414
ligands	89	0	0	428
water	692	407	0	627
R.m.s.d bond lengths (Å)	0.007	0.002	0.005	0.003
R.m.s.d bond angles (°)	0.98	0.61	0.84	0.77
Average B-factor	21.3	14.0	24.3	19.6
Ramachandran plot† (%)				
favored	98	98	97	98
ouliers	0	0	0	0

\*Values in parentheses are for outermost shell.

†Ramachandran statistics were calculated with MolProbity.