



Title	Synergy of valine and threonine supplementation on poly(2-hydroxybutyrate-block-3-hydroxybutyrate) synthesis in engineered Escherichia coli expressing chimeric polyhydroxyalkanoate synthase
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19 N-terminal portion of *Aeromonas caviae* PHA synthase and C-terminal portion of  
20 *Ralstonia eutropha* (*Cupriavidus necator*) PHA synthase. PhaCAR has a unique and useful  
21 capacity to synthesize the block PHA copolymer poly(2-hydroxybutyrate-*block*-3-  
22 hydroxybutyrate) [P(2HB-*b*-3HB)] in engineered *Escherichia coli* from exogenous 2HB  
23 and 3HB. In the present study, we initially attempted to incorporate the amino acid-  
24 derived 2-hydroxyalkanoate (2HA) units using PhaCAR and the 2HA-CoA-supplying  
25 enzymes lactate dehydrogenase (LdhA) and CoA transferase (HadA). Cells harboring the  
26 genes for PhaCAR, LdhA, and HadA, as well as for the 3HB-CoA-supplying enzymes  $\beta$ -  
27 ketothiolase and acetoacetyl-CoA reductase, were cultivated with supplementation of four  
28 hydrophobic amino acids—leucine, valine (Val), isoleucine (Ile), and phenylalanine—in  
29 the medium. No hydrophobic amino acid-derived monomers were incorporated into the  
30 polymer, which was most likely because of the strict substrate specificity of PhaCAR;  
31 however, P(2HB-*co*-3HB) was unexpectedly produced with Val supplementation. The  
32 copolymer was likely P(2HB-*b*-3HB) based on proton nuclear magnetic resonance  
33 analysis. Based on the endogenous pathways in *E. coli*, 2HB units are likely derived from  
34 threonine (Thr) through deamination and dihydroxylation. In fact, dual supplementation  
35 with Thr and Val showed synergy on the 2HB fraction of the polymer. Val  
36 supplementation promoted the 2HB synthesis likely by inhibiting the metabolism of 2-

37 ketobutyrate into Ile and/or activating Thr dehydratase. In conclusion, the  
38 LdhA/HadA/PhaCAR pathway served as the system for the synthesis of P(2HB-*b*-3HB)  
39 from biomass-derived carbon sources.

40

#### 41 ■ Introduction

42 Polyhydroxyalkanoates (PHAs) are bacterial storage polyesters and are used as bio-based  
43 and biodegradable plastics (1). Increased interest in these biodegradable plastics in recent  
44 years arises from serious petroleum-based plastic pollution that is typically not degrading  
45 the marine environment and ecosystem (2). Natural PHAs are composed of (*R*)-3-  
46 hydroxyalkanoates (3, 4) and exhibit a variety of properties depending on their monomer  
47 composition. The monomer constituents incorporated into PHAs are limited by the  
48 substrate specificity of PHA synthase.

49 PHAs containing 2-hydroxyalkanoate (2HA) units have never been found in nature;  
50 therefore, they are considered to be artificial. The first 2HA-polymerizing PHA synthase  
51 is an engineered class-II PHA synthase from *Pseudomonas* sp. 61-3 containing two point  
52 mutations [PhaC1<sub>Ps</sub>(STQK)] (5), which incorporates lactate (LA) (6), glycolate (7), and  
53 2-hydroxybutyrate (2HB) units (8) into the polymer chain. In addition, PhaC1<sub>Ps</sub>(STQK)  
54 incorporates hydrophobic amino acid-derived 2HA units hydroxy-4-methylvalerate

55 (2H4MV), 2-hydroxy-3-methylbutyrate (2H3MB), 2-hydroxy-3-methylvalerate  
56 (2H3MV), and 2-hydroxy-3-phenylpropionate (2H3PhP) from leucine (Leu), isoleucine  
57 (Ile), valine (Val), and phenylalanine (Phe), respectively (9). The corresponding monomer  
58 substrates are supplied through lactate dehydrogenase (LdhA) and CoA transferase  
59 (HadA) (Fig. 1) (9). The extremely broad substrate specificity of PhaC<sub>1Ps</sub>(STQK) is  
60 useful for synthesizing a variety of artificial PHAs.

61 PhaC<sub>AR</sub>, which is an engineered chimeric enzyme composed of the N-terminal  
62 portion of *Aeromonas caviae* PHA synthase (PhaC<sub>Ac</sub>) and C-terminal portion of *Ralstonia*  
63 *eutropha* (*Cupriavidus necator*) PHA synthase (PhaC<sub>Re</sub>) (10), is the first class-I enzyme  
64 that efficiently incorporates 2HB units (11). Notably, PhaC<sub>AR</sub> has the unique capacity of  
65 synthesizing block PHAs, while PhaC<sub>1Ps</sub>(STQK) and other PHA synthases, in general,  
66 synthesize random copolymers (11). PhaC<sub>AR</sub> spontaneously synthesizes poly(2HB-*block*-  
67 3-hydroxybutyrate) [P(2HB-*b*-3HB)] from the mixture of 2HB and 3HB supplemented in  
68 the medium. The intact PhaC<sub>Re</sub> and its derivatives carrying A510X mutations also  
69 incorporate 2HB and LA units, respectively, although their capacity for incorporating the  
70 2HB units is lower than that of PhaC<sub>AR</sub> (12, 13).

71 In the present study, we initially attempted to increase the variety of block PHAs  
72 synthesized using PhaC<sub>AR</sub> by introducing other 2HA units into the polymers. We focused

73 on the LdhA/HadA pathway that supplies the amino acid-derived 2HA monomer  
74 substrates (Fig. 1). The 2HA-CoA- and 3HB-CoA-supplying enzymes with Pha<sub>CAR</sub> were  
75 expressed into *Escherichia coli*, and the cells were cultivated together with hydrophobic  
76 amino acids. As a result, under those conditions, P(2HB-*b*-3HB) was unexpectedly  
77 produced. The pathway served as system for the synthesis of P(2HB-*b*-3HB) from  
78 biomass-derived carbon sources.

79

## 80 ■Materials and methods

### 81 **Plasmid construction**

82 pTTQ19ldhAhadA<sub>Cd\_opt</sub> was previously constructed (9). The pBBR1MCS2<sub>CAR</sub>AB that  
83 harbors the genes for PHA synthase (*pha*<sub>CAR</sub>), β-ketothiolase (*pha*<sub>ARe</sub>), and NADPH-  
84 dependent acetoacetyl-CoA reductase (*pha*<sub>BRe</sub>) was constructed by first replacing the  
85 *Eco*NI site in pBSP<sub>Re</sub><sub>CAR</sub>pct<sub>me</sub> (11) with the *Spe*I site using polymerase chain reaction  
86 to yield pBSP<sub>Re</sub><sub>CAR</sub>pct<sub>me</sub>*Spe*I. The *Bam*HI/*Afl*III fragment containing partial *pct* was then  
87 amplified using pBSP<sub>Re</sub><sub>CAR</sub>pct<sub>me</sub> and primers 5'-ATCGGGCCTTTCGAAAGC-3' and  
88 5'-TTTTTCTTAAGTTATTTTTTCAGTC-3', and the *Afl*III/*Spe*I fragment containing  
89 *pha*AB was amplified using pTV118NpctC1(STQK)AB and primers 5'-  
90 TTTTTCTTAAGGAAAGGACTACACAATG-3' and 5'-

91 TTTTTTACTAGTTCAGCCCATATGC-3'. The *Bam*HI/*Afl*III and *Afl*III/*Spe*I fragments  
92 were ligated with the *Bam*HI/*Spe*I-digested pBSP<sub>Re</sub>C<sub>AR</sub>pct<sub>me</sub>*Spe*I to yield  
93 pBSP<sub>Re</sub>C<sub>AR</sub>pct<sub>me</sub>AB and pBSP<sub>Re</sub>C<sub>AR</sub>pct<sub>me</sub>AB was digested with *Eco*RI/*Afl*III, blunted, and  
94 self-ligated to eliminate *pct* and yield pBSP<sub>Re</sub>C<sub>AR</sub>AB. The *Kpn*I/*Xho*I P<sub>Re</sub> fragment from  
95 pBSP<sub>Re</sub>C<sub>AR</sub>AB was inserted into pBBR1MCS2 (14) at the *Kpn*I and *Xho*I sites to yield  
96 pBBR1M2P<sub>Re</sub>. Finally, the *Xho*I/*Spe*I fragments of pBSP<sub>Re</sub>C<sub>AR</sub>AB containing *pha*C<sub>AR</sub>,  
97 *phaA*, and *phaB* were inserted into pBBR1M2P<sub>Re</sub> at the *Xho*I and *Spe*I sites to yield  
98 pBBR1MCS2C<sub>AR</sub>AB.

99

#### 100 **Strain and culture conditions**

101 The engineered *E. coli* DH5 $\alpha$  was grown on 1.5 mL Luria Bertani medium at 30°C for  
102 12 h as the seed culture. One milliliter or 15  $\mu$ L of this culture were used to inoculate 100  
103 mL or 1.5 mL M9 of medium (17.1 g Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 3 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g NaCl, 1 g  
104 NH<sub>4</sub>Cl, 0.02 mol MgSO<sub>4</sub>, and 0.001 mol CaCl<sub>2</sub>/L distilled water), respectively, containing  
105 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside, 20 g/L glucose, and varied concentrations  
106 of amino acids and monomer precursors in a 500-mL shake flask or a 10-mL test tube.  
107 Under all conditions, 100 mg/L ampicillin and 50 mg/L kanamycin were added to the  
108 medium to maintain the plasmids in the cell. The cells were cultivated at 30°C for 72 h,

109 harvested, and rinsed with distilled water to remove any medium components before  
110 lyophilization.

111

## 112 **PHA extraction and analysis**

113 PHA was extracted from the lyophilized cells using chloroform at 60°C for 48 h and  
114 purified by reprecipitation in an excess amount of hexane and subsequently in methanol.

115 The PHA content and monomer composition were determined using gas  
116 chromatography–mass spectrometry (GC–MS, QP2010Plus, Shimadzu) and proton  
117 nuclear magnetic resonance (<sup>1</sup>H NMR) in CDCl<sub>3</sub> (400 MHz, JEOL), as previously  
118 described (6).

119

## 120 **■Results and discussion**

### 121 **Polymer synthesis using PhaC<sub>AR</sub> with supplementing four hydrophobic amino acids**

122 The engineered *E. coli* expressing PhaC<sub>AR</sub> and the monomer-supplying enzymes shown  
123 in Fig. 1 were cultured in a test tube with amino acid supplementation. None of the amino  
124 acid-derived 2HA units (i.e., 2H4MV, 2H3MB, 2H3MV, or 2H3PhP) were incorporated  
125 into the polymer (Fig. S1), which differed from the results of PhaC1<sub>Ps</sub>(STQK) (9).  
126 Unexpectedly, the 2HB units were incorporated into the polymer with Val

127 supplementation (Table 1). The decrease in the dry weight of the cell (0.13-fold) after Val  
128 supplementation was consistent with that in the results of a previous report (15).

129 The monomer sequence of the polymer was estimated using  $^1\text{H}$  NMR (Fig. 2a). The  
130 polymer samples for NMR analysis were obtained using the flask-scale culture (Table S1).  
131 The methyne proton of the 2HB units in P(2HB-*co*-3HB) synthesized with Val  
132 supplementation exhibited a resonance at 5.0 ppm, which was ascribed to the 2HB–  
133 2HB\*–2HB triad; therefore, the polymer contained a P(2HB) segment. Similarly, the  
134 resonance of the methyne proton of the 3HB units at 5.2 ppm corresponded to a P(3HB)  
135 segment. These resonances were identical to those of P(2HB-*b*-3HB) (11) and clearly  
136 distinguishable from those of the P(2HB-*ran*-3HB) random copolymer (Fig. 2b). In  
137 addition, Pha<sub>CAR</sub> is not capable of synthesizing P(2HB) homopolymer (11); therefore, the  
138 obtained polymer was unlikely to be a homopolymer blend of P(2HB) and P(3HB). Based  
139 on these results, the copolymer synthesized with Val supplementation was most likely the  
140 block copolymer P(2HB-*b*-3HB).

141 Generation of the block sequence has been proposed to be the result of changes in  
142 intracellular monomer levels during polymer synthesis (11). The enzymatic properties of  
143 Pha<sub>CAR</sub> and the monomer-supplying enzyme propionyl-CoA transferase (PCT)  
144 potentially contribute to the characteristic kinetics of polymerization in P(2HB-*b*-3HB)

145 synthesis from exogenous 2HB and 3HB. In the present study, 2HB-CoA and 3HB-CoA  
146 were supplied through the LdhA/HadA and PhaA/PhaB pathways, respectively. This  
147 result indicated that these monomer suppliers, as well as PCT, can be used for synthesis  
148 of block PHA.

149

### 150 **Polymer production from threonine**

151 The results prompted identification of the 2HB unit precursor. There is no metabolic  
152 pathway from Val to 2-ketobutyrate in *E. coli* according to the KEGG pathway  
153 (<https://www.genome.jp/kegg/pathway.html>); therefore, Val was unlikely to be a direct  
154 precursor of the 2HB units. To identify the supply route for the 2HB units, the polymer was  
155 synthesized using the empty vector pTTQ19. No 2HB units were incorporated using this  
156 vector with or without Val supplementation (Table 2), which indicated that LdhA and HadA  
157 contributed to the precursor supply route for 2HB. Specifically, 2HB was derived from 2-  
158 ketobutyrate. The result suggests that Thr is a 2HB precursor through deamination and  
159 dihydroxylation (Fig. 3).

160 To verify the precursor role of Thr, the polymer was synthesized using  
161 pTTQ19ldhAhadACd\_opt with Thr supplementation. For this synthesis, *phaA* and *phaB* were  
162 omitted, and 3HB was alternatively supplemented in the medium. The 3HB-CoA supply

163 from exogenous 3HB was less efficient than PhaA and PhaB, and relatively increased the  
164 molar fraction of the 2HB units. These conditions facilitated the detection of the 2HB units  
165 in the polymer. Expectedly, the 2HB fraction increased with Thr supplementation at all  
166 tested concentrations (Table 2), which indicated that they were derived from Thr. The 2HB  
167 fraction was detected without Thr supplementation, which should be due to the endogenous  
168 Thr. Compared to the result in Table 1 obtained from the test-tube culture, the flask scale  
169 culture facilitated the detection of the small amount of 2HB units. The citramalate pathway  
170 has also been previously proposed as a 2HB monomer-supplying route (16), although its  
171 contribution was not fully verified because of the lack of a proper negative control.

172 We then questioned why Val supplementation increased the accumulation of 2HB units.  
173 To verify the role of Val, the dual supplementation of Thr and a small concentration of  
174 Val in the medium was tested. The 2HB content and fraction increased with an increase  
175 in the Val concentration (Table 3). The result indicated that Val enhanced the supply of  
176 2HB derived from Thr. Polymer production decreased with Val supplementation,  
177 especially at levels  $>0.1$  g/L, because of the growth inhibition by Val supplementation.

178 Val reportedly inhibits *E. coli* IlvH involved in the biosynthesis of Ile from 2-  
179 ketobutyrate (17, 18); therefore, Val supplementation potentially reduces the flux from 2-  
180 ketobutyrate to Ile and subsequently increases the flux toward 2HB. In addition, it has

181 been reported that Val allosterically activates Thr deaminase, which catalyzes the  
182 formation of 2-ketobutyrate from Thr (19); therefore, the role of Val should be that of a  
183 regulator of amino acid metabolism and not that of a monomer precursor (Fig. 3).

184

### 185 **Polymer synthesis with 2HHx, 2H4MV, or 2H3PhP supplementation**

186 PhaCAR incorporated no Leu-, Val-, Ile-, and Phe-derived 2HA units into the polymer  
187 (Table 1) most likely because of strict substrate specificity of the enzyme. Substrate  
188 specificity of PhaCAR was investigated using 2-hydroxyhexanote (2HHx), which is a  
189 structural isomer of 2H4MV, with 2H3PhP tested for comparison. As a result, a trace  
190 amount of 2HHx was incorporated into the polymer (Table S2). In contrast, 2H4MV and  
191 2H3PhP units were not detected in the polymer, which indicated that the capacity of  
192 PhaCAR to incorporate medium-chain-length (mcl) 2HA-CoA is considerably lower than  
193 that of PhaC1Ps(STQK).

194

195 In conclusion, P(2HB-co-3HB) was synthesized in *E. coli* using the LdhA/HadA  
196 pathway that supplied 2HB-CoA from Thr (Fig. 1). The copolymer was most likely  
197 P(2HB-b-3HB) based on the <sup>1</sup>H NMR analysis and previous finding that PhaCAR does not  
198 synthesize P(2HB) homopolymer; therefore, this is the first report of P(2HB-b-3HB)

199 synthesis from biomass-derived carbon sources. Dual supplementation with Thr and Val  
200 synergistically increased the 2HB fraction in the polymer. Val likely promoted the  
201 conversion of Thr into 2-ketobutyrate through deamination and dihydroxylation. PhaC<sub>AR</sub>  
202 did not recognize mcl-2HA monomers with the exception of incorporating a trace amount  
203 of 2HHx units. The strict substrate specificity of PhaC<sub>AR</sub> was beneficial for synthesizing  
204 the block PHA with the finely controlled monomer composition compared to synthesis  
205 using PhaC<sub>1Ps</sub>(STQK), which exhibits broad substrate specificity and synthesizes  
206 multicomponent copolymers.

207

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212

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276

277

278 Figure captions

279 FIG. 1. Poly(2-hydroxyalkanoate-*co*-3-hydroxybutyrate) [P(2HA-*co*-3HB)] biosynthesis  
280 pathways in engineered *Escherichia coli* with amino acid supplementation. Amino acids  
281 are also supplied through the endogenous pathways.

282

283 FIG. 2. <sup>1</sup>H NMR analysis of the copolymer poly(8.3 mol% 2-hydroxybutyrate-*co*-3-  
284 hydroxybutyrate) [P(2HB-*co*-3HB)] synthesized with Val supplementation in engineered  
285 *Escherichia coli* harboring pBBR1MCS2C<sub>ARAB</sub> and pTTQldhAhadA<sub>Cd\_opt</sub> (A) and  
286 P(2HB-*random*-3HB) (B). The resonances at 4.8–5.1 ppm are ascribed to the methyne  
287 proton of the 2HB units in the following four triad sequences: 2HB–2HB\*–2HB (i), 2HB–  
288 2HB\*–3HB and/or 3HB–2HB\*–2HB (ii, iii), and 3HB–2HB\*–3HB (iv). The resonance  
289 at 5.2–5.4 ppm is ascribed to the methyne proton of the 3HB units in the dyad sequences  
290 of 3HB–3HB\* and 2HB–3HB\*.

291

292 FIG. 3. Poly(2-hydroxybutyrate-*block*-3-hydroxybutyrate) [P(2HA-*b*-3HB)] biosynthesis  
293 pathways from threonine in engineered *Escherichia coli* and proposed role of valine.  
294 Amino acids are also supplied through the endogenous pathways.

295

Table 1. Polymer production with supplementation of four hydrophobic amino acids<sup>a</sup>

Amino acids	CDW (g/L)	Polymer production (g/L)	Monomer composition(mol%)	
			2HB	3HB
-	4.00 ± 0.68	0.52 ± 0.06	N.D.	100
Leu	5.47 ± 0.09	0.61 ± 0.01	N.D.	100
Val	0.51 ± 0.11	0.38 ± 0.01	3.6	96.4
Ile	3.73 ± 0.52	0.45 ± 0.49	N.D.	100
Phe	5.04 ± 0.31	0.49 ± 0.02	N.D.	100

<sup>a</sup>Polymers were synthesized in recombinant *Escherichia coli* harboring pBBR1MCS2<sub>C<sub>AR</sub>AB</sub> and pTTQ19ldhAhadA<sub>C<sub>d</sub>\_opt</sub> with amino acid supplementation (1 g/L each) in a test tube. CDW, cell dry weight; HB, hydroxybutyrate; N.D., not detected. Data are means ± standard deviations of three independent experiments. Monomer composition was determined by gas chromatography–mass spectrometry (GC–MS).

Table 2. Polymer production in *Escherichia coli* with supplementation of valine (Val) and threonine (Thr)<sup>a</sup>

Plasmids	Val conc. (g/L)	Thr conc. (g/L)	CDW (g/L)	Polymer producti on (g/L)	Monomer composition (mol%) <sup>b</sup>	
					2HB	3HB
pTTQ19 (empty vector),	0	0	3.59	1.51	0	100
pBBR1MCS2 <sub>C<sub>AR</sub>AB</sub>	1	0	0.64	0.37	0	100
	0	0	0.60	0.16	4.6	95.4
pTTQ19ldhAhadA <sub>C<sub>d</sub>_opt</sub> ,	0	1	0.92	0.18	6.7	93.3
pBBR1MCS2 <sub>C<sub>AR</sub></sub> <sup>c</sup>	0	3	0.68	0.16	5.5	94.5
	0	10	0.77	0.23	8.7	91.3
	0	15	0.64	0.20	6.8	93.2

<sup>a</sup>Cells were cultured in a shake flask. <sup>b</sup>Monomer composition was determined by <sup>1</sup>H NMR. HB, hydroxybutyrate. <sup>c</sup>Cells were cultivated with 0.5 g/L (*R,S*)-3HB-Na.

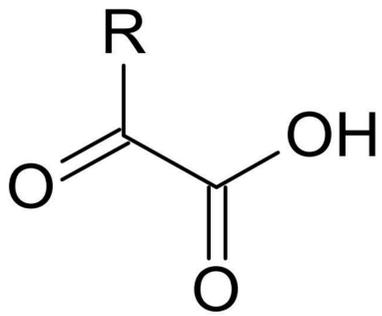
Table 3. Polymer production in *Escherichia coli* with dual supplementation of valine (Val) and threonine (Thr)<sup>a</sup>

Val conc. (g/L)	Thr conc. (g/L)	CDW (g/L)	Polymer production (g/L)	Polymer content (wt%)	2HB content (wt%)	Monomer composition (mol%) <sup>b</sup>	
						2HB	3HB
0	0	2.94 ± 0.28	1.56 ± 0.07	53.7 ± 6.3	0.61	1.0	99.0
0		6.45 ± 1.08	3.57 ± 0.73	55.6 ± 7.4	0.81	1.4	98.6
0.001		7.11 ± 0.44	4.70 ± 0.29	66.7 ± 8.3	0.79	1.4	98.6
0.01	5	4.35 ± 1.82	2.79 ± 1.33	62.2 ± 4.9	1.31	1.9	98.1
0.1		0.56 ± 0.03	0.21 ± 0.01	37.6 ± 1.1	4.20	11.1	88.9
0.5		0.38 ± 0.04	0.13 ± 0.01	35.6 ± 2.4	7.21	18.6	81.4

<sup>a</sup>Polymers were synthesized using *E. coli* harboring pBBR1MCS2C<sub>ARAB</sub> and pTTQ19ldhA<sub>hadA</sub>C<sub>d\_opt</sub> in a shake flask. <sup>b</sup>HB, hydroxybutyrate; 2HB content and monomer composition in the cells were determined by <sup>1</sup>H NMR. Data are the means ± standard deviations of three independent experiments. <sup>1</sup>H NMR was measured using a single sample.

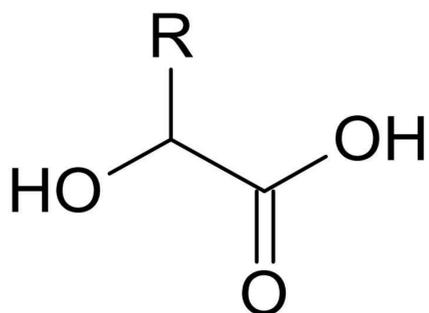
hydrophobic amino acids

glucose



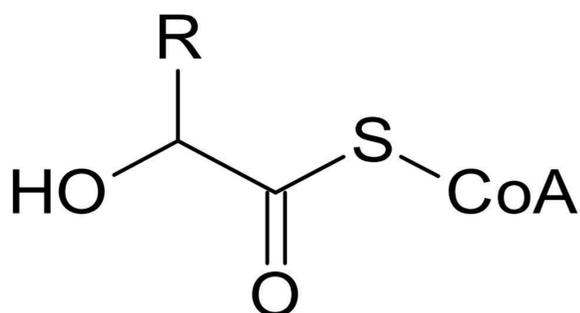
2-ketoalkanoate

LdhA

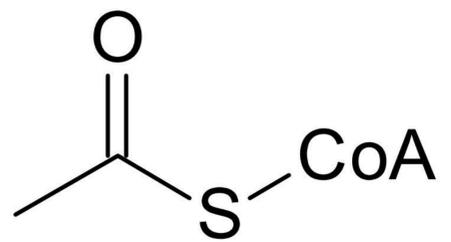


2-hydroxyalkanoate (2HA)

HadA

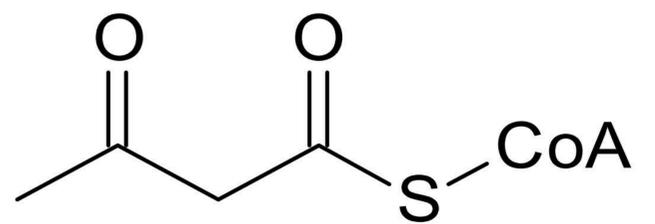


2HA-CoA



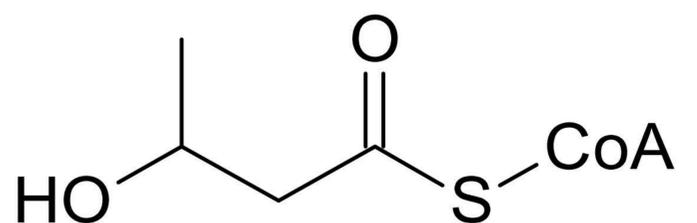
acetyl-CoA

PhaA



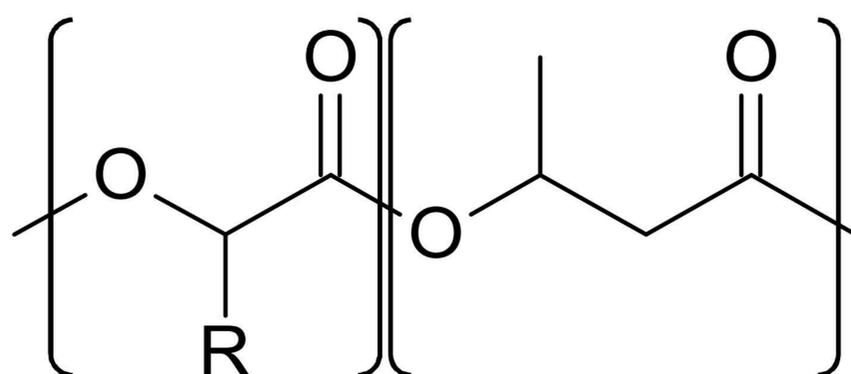
acetoacetyl-CoA

PhaB



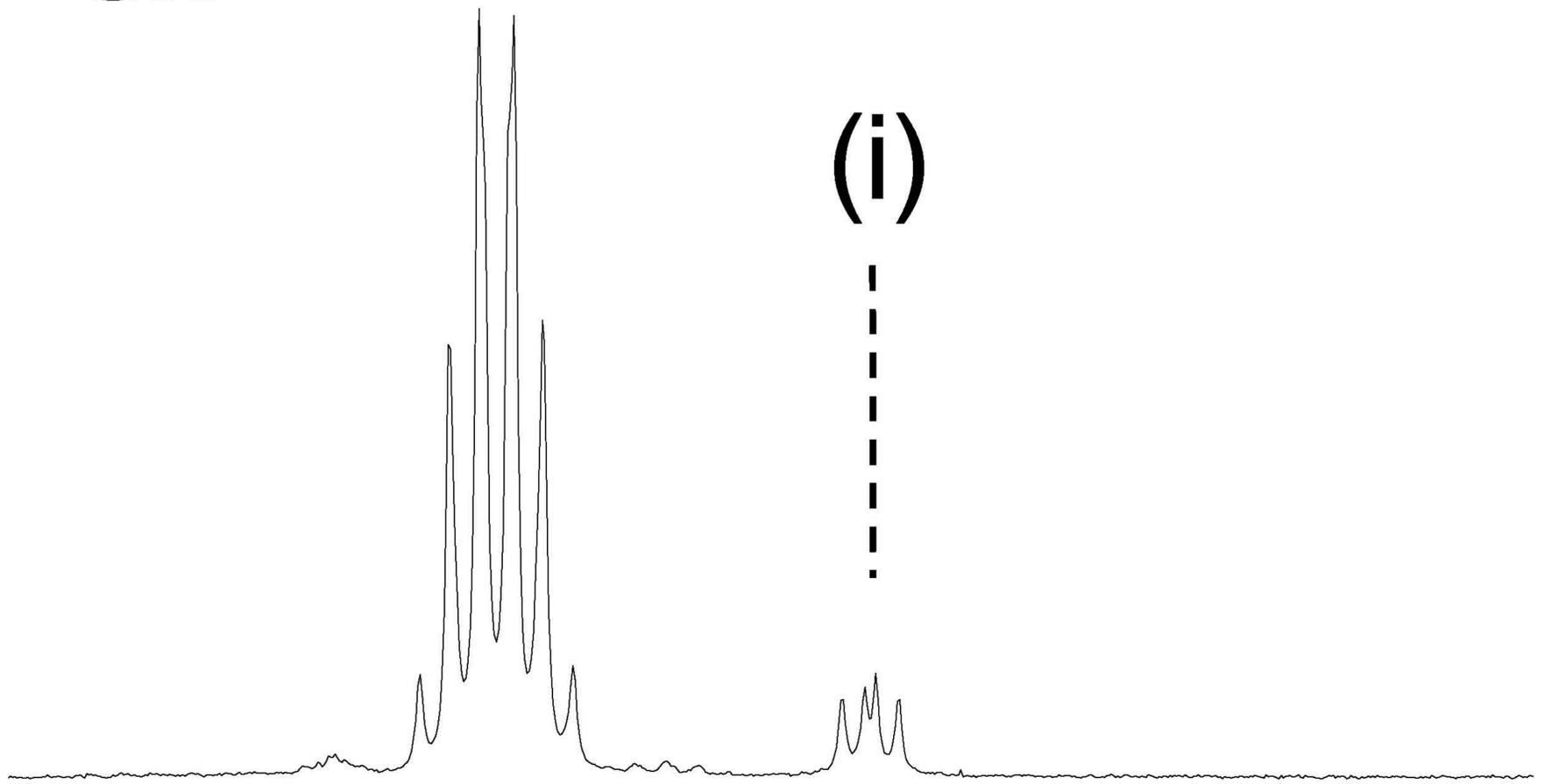
3HB-CoA

PHA synthase

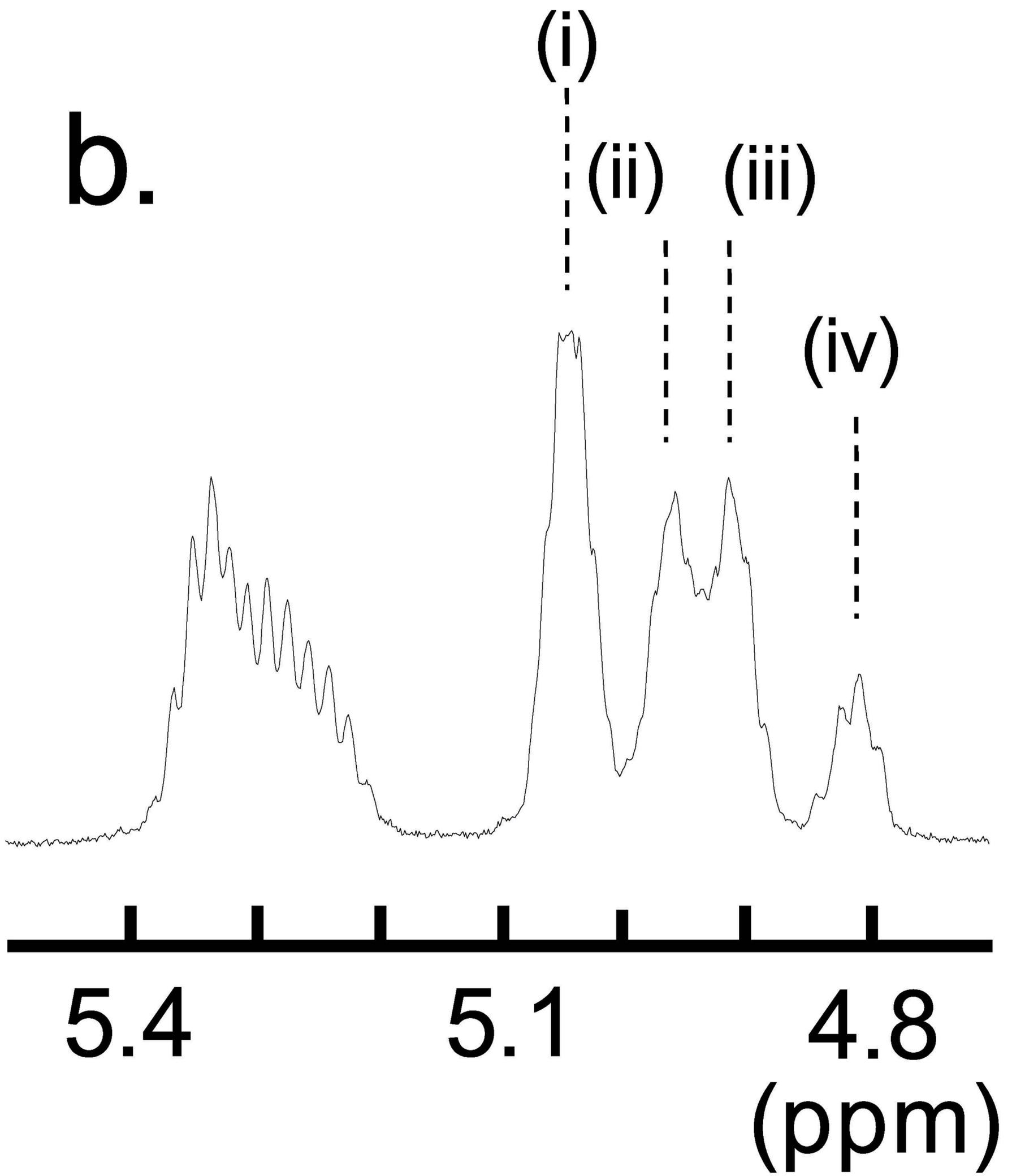


P(2HA-co-3HB)

**a.**



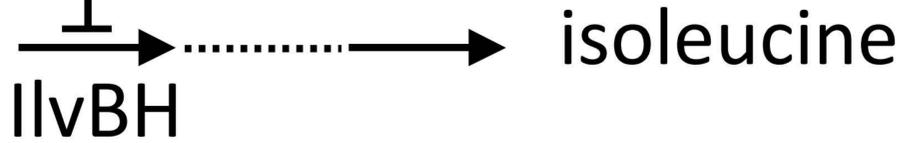
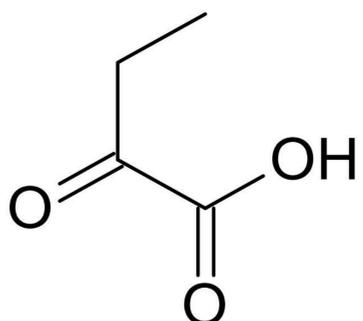
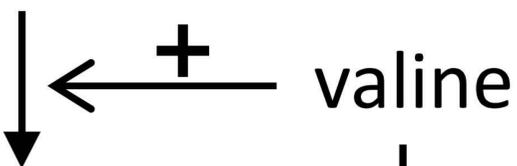
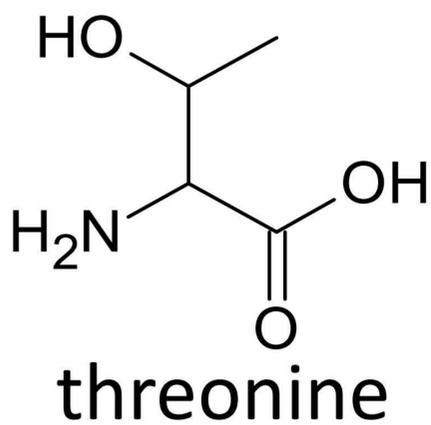
**b.**



threonine

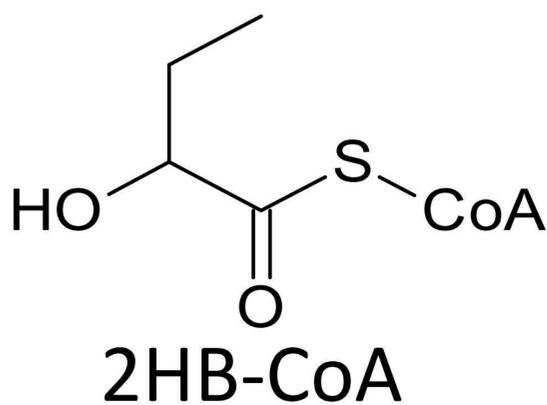
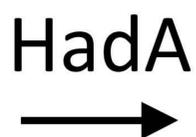
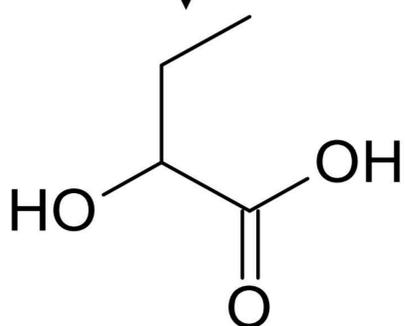
valine

glucose



2-ketobutyrate

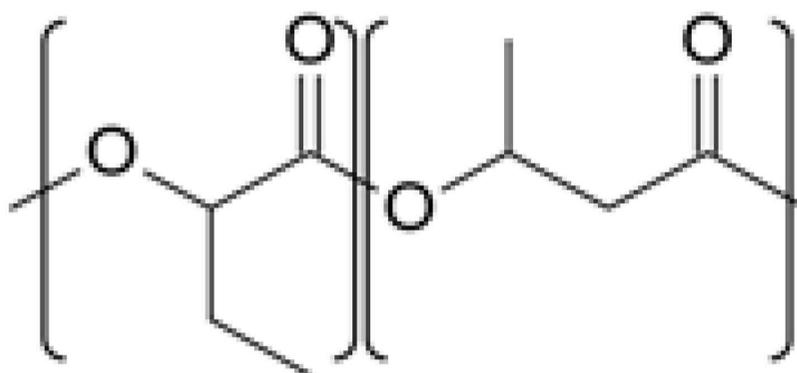
LdhA



3HB-CoA

2-hydroxybutyrate (2HB)

PhaC<sub>AR</sub>



P(2HB-*b*-3HB)