

1 **Manuscript version 2**

2 **Biosynthesis of chiral 3-hydroxyvalerate from single**

3 **propionate-unrelated carbon sources in metabolically**

4 **engineered *E. coli***

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## 23 **Abstract**

### 24 **Background**

25 The ability to synthesize chiral building block molecules with high optical purity is of  
26 considerable importance to the fine chemical and pharmaceutical industries. Production  
27 of one such compound, 3-hydroxyvalerate (3HV), has previously been studied with  
28 respect to the *in vivo* or *in vitro* enzymatic depolymerization of biologically-derived co-  
29 polymers of poly(3-hydroxybutyrate-co-3-hydroxyvalerate). However, production of this  
30 biopolymeric precursor typically necessitates the supplementation of a secondary carbon  
31 source (e.g., propionate) into the culture medium. In addition, previous approaches for  
32 producing 3HV have not focused on its enantiopure synthesis, and thus suffer from  
33 increased costs for product purification.

### 34 **Results**

35 Here, we report the selective biosynthesis of each 3HV stereoisomer from a single,  
36 renewable carbon source using synthetic metabolic pathways in recombinant strains of  
37 *Escherichia coli*. The product chirality was controlled by utilizing two reductases of  
38 opposing stereoselectivity. Improvement of the biosynthetic pathway activity and host  
39 background was carried out to elevate both the 3HV titers and 3HV/3HB ratios. Overall,  
40 shake-flask titers as high as 0.31 g/L and 0.50 g/L of (*S*)-3HV and (*R*)-3HV, respectively,  
41 were achieved in glucose-fed cultures, whereas glycerol-fed cultures yielded up to 0.19  
42 g/L and 0.96 g/L of (*S*)-3HV and (*R*)-3HV, respectively.

### 43 **Conclusions**

44 Our work represents the first report of direct microbial production of enantiomerically  
45 pure 3HV from a single carbon source. Continued engineering of host strains and  
46 pathway enzymes will ultimately lead to more economical production of chiral 3HV.

## 47 **Background**

48           The efficient production of enantiomerically pure chemicals from renewable  
49 resources has gained considerable attention especially in the fine  
50 chemical/pharmaceutical industry. Stereo-selective chemical processes generally employ  
51 expensive chiral catalysts, require harsh physical conditions and solvents, and suffer from  
52 extensive byproduct formation. In contrast, enzyme-catalyzed reactions are highly stereo-  
53 selective and can be performed in aqueous solutions under mild conditions [1]. As a  
54 result, replacing chemical processes by biological ones for the synthesis of chiral  
55 compounds has been extensively investigated not only due to superior stereo-selectivity  
56 of enzymatic reactions but also due to sustainability as an implementation of green  
57 chemistry [2-5]. One example is the production of hydroxyacids, a family of versatile  
58 chiral molecules containing one hydroxyl group and one carboxyl group [6]. These  
59 molecules have the potential to serve as useful chiral building blocks for a diverse range  
60 of products, including polyhydroxyalkanoates (PHAs) (biodegradable polymers) and  
61 optically-active fine chemicals, such as pharmaceuticals, vitamins, antibiotics, and flavor  
62 compounds [7-10]. Naturally, hydroxyacids are primarily found to be polymerized as  
63 PHAs where they serve as intracellular storage materials for numerous microbes. Those  
64 PHAs consist mostly of monomers with 3-hydroxy, 4-hydroxy, and 5-hydroxy groups  
65 with different lengths of main and side chains [11].

66           Among the hydroxyacid monomers, 3-hydroxybutyrate (3HB) is the most prolific,  
67 with several reports on engineering *E. coli* for its production from renewable feedstocks  
68 [5, 12-15]. Biosynthesis of 3HB begins with the condensation of two acetyl-CoA  
69 molecules, a commonly found cellular metabolite regardless of carbon source (Fig. 1).

70 However, economically-feasible production of longer-chain hydroxyacids is complicated  
71 by issues such as low yields and high prices of feedstocks due to the need to supplement  
72 a second carbon source. One example of such hydroxyacids is 3-hydroxyvalerate (3HV).  
73 The production of 3HV has been realized by the hydroxylation of valeric acid through  
74 fermentation of *Candida rugosa* [16]. It has also been reported that 3-  
75 hydroxyvaleronitrile can be converted into 3HV using the nitrilase activity of  
76 *Comamonas testosteroni* [17]. More recently, direct biological production of 3HV was  
77 demonstrated using recombinant *P. putida* KT2440 and levulinic acid as substrate,  
78 although the levulinic acid metabolism pathway in *P. putida* KT2440 has not yet been  
79 fully elucidated [18]. In the aforementioned cases, valeric acid, 3-hydroxyvaleronitrile,  
80 and levulinic acid were supplied as secondary carbon sources (in addition to glucose).  
81 Additionally, the chirality and/or enantiopurity of the 3HV produced in the above-  
82 mentioned studies is unclear as they did not report whether the synthesized 3HV was in  
83 the *R*, *S*, or racemic form. Alternatively, 3HV can be obtained through either the *in vivo*  
84 or *in vitro* enzymatic depolymerization of synthesized poly(3-hydroxybutyrate-co-3-  
85 hydroxyvalerate) (PHBV), a well known biodegradable polymer marketed as Biopol™  
86 which is produced by the natural PHA accumulating bacterium *Ralstonia eutropha* when  
87 grown on glucose and propionate [19]. The production of PHBV has also been reported  
88 in recombinant *E. coli* upon introduction of the PHA biosynthesis genes of *R. eutropha*  
89 and when grown in glucose medium supplemented with valine or threonine [20].  
90 Regardless of whether the end product is 3HV or PHBV, it can be generally concluded  
91 that supplementation of a second carbon source, such as valeric acid, 3-  
92 hydroxyvaleronitrile, levulinic acid, propionate, valine, or threonine in addition to

93 glucose, is necessary to provide the 5-carbon unit precursor of 3HV. Unfortunately, the  
94 high price and/or toxicity of the added second carbon sources could limit industrial  
95 production of 3HV [21]. Therefore, synthesis of 3HV from a single carbon source has  
96 been proposed as an efficient and sustainable avenue in contrast to the above-mentioned  
97 systems.

98 A novel pathway for the production of PHBV solely from glycerol has been  
99 established in recombinant *Salmonella enterica* Serovar Typhimurium, containing a  
100 heterologous pathway that converts succinyl-CoA to propionyl-CoA, the essential  
101 precursor molecule of 3HV-CoA in PHBV synthesis [22]. However, expensive  
102 cyanocobalamin (CN-B<sub>12</sub>) was supplemented to the medium to provide the precursor of  
103 coenzyme B<sub>12</sub> required for the activity of one of the enzymes in the B<sub>12</sub>-dependent  
104 biosynthetic pathway. It should also be noted that the pathway only functioned in *S.*  
105 *enterica*, a pathogen, but not *E. coli*, thus limiting its applicability to other industrially-  
106 relevant host organisms. In this study, we proposed an alternative biosynthetic pathway  
107 that does not require coenzyme B<sub>12</sub> for its functionality to synthesize 3HV from glucose  
108 or glycerol. Specifically, we metabolically engineered *E. coli* to exploit its native  
109 metabolism for endogenous supply of propionyl-CoA via the threonine biosynthesis  
110 pathway, and introduced a heterologous pathway for chiral 3HV biosynthesis using  
111 acetyl-CoA and propionyl-CoA as precursor molecules. As stated above, several previous  
112 methods for producing 3HV did not focus on enantiopure synthesis. Similarly, due to the  
113 stereospecific constraints of PHBV synthesis, in which polymers are composed  
114 exclusively of (*R*)-3HB and (*R*)-3HV monomer units, the synthesis of (*S*)-3HV from

115 PHBV remains effectively impossible. On the contrary, our proposed pathway makes  
116 possible the direct synthesis of both enantiomerically pure (*R*)-3HV and (*S*)-3HV.

117 We have identified a pathway which combines elements of our previously  
118 developed chiral 3HB biosynthesis pathway together with the natural threonine  
119 biosynthesis pathway of *E. coli* for direct biosynthesis of chiral 3HV (Fig. 1). In the  
120 proposed pathway, chiral 3HV is produced from direct hydrolysis of 3HV-CoA catalyzed  
121 by a thioesterase II (encoded by *tesB*) where 3HV-CoA is obtained from condensation of  
122 one acetyl-CoA and one propionyl-CoA to form 3-ketovaleryl-CoA catalyzed by a  
123 thiolase (encoded by *bktB*), followed by a reduction of the 3-ketovaleryl-CoA to 3HV-  
124 CoA catalyzed by a 3-hydroxybutyryl-CoA dehydrogenase. Here, two enantio-selective  
125 3-hydroxybutyryl-CoA dehydrogenases were utilized to control the chirality of 3HV-  
126 CoA produced. The NADPH-dependent dehydrogenase encoded by *phaB* produces (*R*)-  
127 3HV-CoA while the NADH-dependent dehydrogenase encoded by *hbd* produces (*S*)-  
128 3HV-CoA. It should be noted that in order to yield the highest 3HV titers and 3HV/3HB  
129 ratios, BktB was used as the thiolase in this study as opposed to other thiolases such as  
130 PhaA from *R. eutropha* H16 or Thil from *C. acetobutylicum* ATCC 824 because BktB  
131 has been shown to have highest *in vitro* enzyme activity towards the C<sub>5</sub> substrate while  
132 PhaA and Thil were specific towards the C<sub>4</sub> substrate [19]. Next, a pathway allowing for  
133 endogenous propionyl-CoA synthesis from glucose or glycerol, through the threonine  
134 metabolic pathway intermediate 2-ketobutyrate, was introduced to circumvent the need  
135 for feeding propionate. To examine the upstream pathway for endogenous supply of  
136 propionyl-CoA, we used a bottom-up approach where 2-ketobutyrate and threonine were,  
137 at first, fed to provide propionyl-CoA, in addition to glucose, to support 3HV production.

138 In the final stage, a single carbon source of glucose or glycerol was used to provide both  
139 acetyl-CoA and propionyl-CoA to support 3HV biosynthesis in our metabolically  
140 engineered *E. coli*.

141 Overall, in this study we successfully demonstrated the direct biological  
142 production of enantiomerically pure (*R*)-3HV and (*S*)-3HV from a single carbon source.  
143 Improvements of the biosynthetic pathway and *E. coli* host strains have also been carried  
144 out to elevate 3HV titers and 3HV/3HB ratios.

145



## 146 **Results**

### 147 **3HV Synthesis from Glucose and propionate**

148 Acetyl-CoA is an obligate central intermediate occurring in any organism and  
149 under any physiological condition; however, this is not the case for propionyl-CoA,  
150 which is only synthesized under special physiological conditions and from only few  
151 substrates [23]. Therefore, synthesis of 3HV-CoA requires propionyl-CoA biosynthesis.  
152 To validate our 3HV biosynthesis pathway, propionate was initially fed to provide  
153 propionyl-CoA as a precursor molecule to ensure the downstream pathway was capable  
154 of making chiral 3HV. It has been reported that the *R. eutropha* PHA biosynthesis genes  
155 can be functionally expressed in *E. coli*, resulting in homopolymer PHB production from  
156 glucose [24]. However, low levels of 3HV monomer within the synthesized co-polymer  
157 PHBV was observed in recombinant *E. coli* when propionate was co-fed with glucose in  
158 a way analogous to the procedure used for *R. eutropha* [24]. One explanation for the low  
159 content of 3HV monomer is that *E. coli* does not possess an efficient system for  
160 importing and/or converting propionate to propionyl-CoA. Therefore, to address the  
161 propionate utilization problem, a CoA-activation mechanism (encoded by the *ptb-buk*  
162 operon [25]) was incorporated into our previously developed 3HB pathway to investigate  
163 the substrate elasticity of the pathway for 3HV production.

164 Our results show that, in the absence of the CoA-activation mechanism, i.e. Ptb-  
165 Buk, only trace amount of 3HV was produced (Fig. 2). On the contrary, introducing Ptb-  
166 Buk into the pathway yielded up to 2 g/L of both enantiomers of 3HV. It was noted that  
167 for strains expressing Ptb-Buk but leaving out TesB, only (*R*)-hydroxyacids (when PhaB  
168 was employed) were produced, consistent with a previous report that Ptb-Buk forms a

169 reversible, stereo-selective enzyme system [13]. Overall, these results indicate that CoA-  
170 activation was crucial for propionate utilization and, most importantly, all enzymes  
171 originally utilized for 3HB biosynthesis were able to catalyze synthesis of C<sub>5</sub> molecules.

172

### 173 **3HV Synthesis from Glucose and 2-Ketobutyrate**

174 Propionyl-CoA can also be produced from 2-ketobutyrate, a common keto-acid  
175 intermediate for isoleucine biosynthesis, by the action of the endogenous pyruvate  
176 dehydrogenase complex enzyme (encoded by *PDHc*) (Fig. 1) [26]. We first compared  
177 3HV production from glucose and 2-ketobutyrate using pathways with and without over-  
178 expression of the *ptb-buk* operon. The results showed that the presence of Ptb-Buk  
179 reduced production of propionate (only observed in the *R*-isomer construct) and 3HB  
180 while increasing production of acetate and 3HV, yielding (*S*)-3HV and (*R*)-3HV with  
181 titers up to 0.38 g/L and 1.02 g/L, respectively (Fig. 3). The increased production of  
182 acetate and 3HV was presumably due to the promiscuous activity of Ptb-Buk on cleaving  
183 excess acetyl-CoA and activating excess propionate. Given that 3HB production is a  
184 second-order reaction should have a rate proportional to the square of the concentration  
185 of acetyl-CoA, a reduced acetyl-CoA pool resulting from the promiscuous activity of Ptb-  
186 Buk likely caused a significant decrease in 3HB production. In addition, propionyl-CoA  
187 is a competing substrate for BktB, so an increase in propionyl-CoA concentration may  
188 also reduce 3HB production.

189 In an effort to decrease acetate and increase 3HV production, several genes,  
190 including *atoDA* (encoding acetoacetyl-CoA transferase), *poxB* (encoding pyruvate  
191 oxidase), and *ackA-pta* (encoding acetate kinase and phosphate acetyltransferase) were  
192 deleted, and the resulting strain was designated as HCT 20. The production of (*S*)-3HV

193 and (*R*)-3HV was further boosted to titers of 0.60 g/L and 2.04 g/L, respectively, in the  
194 recombinant acetate pathway knockout strains (HCT 20). In general, those strains  
195 produced less acetate and propionate and yielded more 3HB and 3HV compared to  
196 strains without these mutations (based on HCT 10), probably due to preserved acetyl-  
197 CoA and propionyl-CoA pools as a result of the introduced mutations. An empty-plasmid  
198 control experiment has also been conducted in the strain HCT 20 (that was not introduced  
199 with the 3HV pathway), yielding only trace amounts of acetate and propionate when  
200 grown in LB supplemented with glucose and 2-ketobutyrate (data not shown). This  
201 indicates that the production of acetate and propionate in the recombinant HCT 20 was  
202 attributed to the introduced CoA-cleaving activity conferred by *ptb-buk* and *tesB*.

203

### 204 **3HV Synthesis from Glucose and Threonine**

205 The metabolic intermediate 2-ketobutyrate can be produced from threonine by the  
206 action of threonine deaminase. Co-feeding of threonine with glucose, together with over-  
207 expression of *E. coli* threonine deaminase (encoded by *ilvA*), was able to achieve  
208 production of (*S*)-3HV and (*R*)-3HV with titers up to 0.11 g/L and 0.22 g/L, respectively  
209 (Fig. 4). Given that *E. coli* threonine deaminase is subject to feedback inhibition by  
210 isoleucine, a feedback resistant gene from *Corynebacterium glutamicum* [27] was also  
211 used, and the production of (*S*)-3HV and (*R*)-3HV was further boosted to titers of 0.27  
212 g/L and 0.91 g/L, respectively, under the same culture conditions. This experiment has  
213 also been conducted in the recombinant acetate pathway knockout strains (HCT 20);  
214 however, no improvement in production of 3HB and 3HV was observed (data not  
215 shown).

216

### 217 **3HV Synthesis from Glucose**

218 We have demonstrated the production of chiral 3HV from glucose supplemented  
219 with propionate, 2-ketobutyrate, or threonine, in recombinant *E. coli*. The next step is to  
220 construct a threonine over-producing strain in an attempt to achieve 3HV biosynthesis  
221 from a single carbon source. To do so, we up-regulated the threonine biosynthesis  
222 pathway by over-expressing the *thrABC* operon, cloned from the wild type *E. coli* or the  
223 threonine producer *E. coli* ATCC 21277 that has a single amino acid alteration in the  
224 homoserine dehydrogenase (encoded by *thrA*<sup>G1297A</sup>) for relieved feedback-inhibition [28].  
225 Transcriptional attenuation of those genes was removed by replacing the native promoter  
226 with a *T7lac* promoter, allowing for IPTG-inducible expression. In addition, the  
227 pathways that compete with threonine formation as well as degrade threonine were  
228 eliminated by knocking out *metA* (encoding homoserine O-succinyltransferase) and *tdh*  
229 (encoding threonine dehydrogenase) genes, yielding strain HCT 21.

230 Our results showed that there was essentially no difference in 3HV production  
231 between strains expressing the wild type and feedback resistant *thrA* (data not shown)  
232 probably because threonine did not accumulate or its level was not high enough to exert a  
233 feedback inhibition to ThrA. We also compared 3HV production across three different *E.*  
234 *coli* strains, including HCT 10, HCT 20, and HCT 21. The mutants HCT 20 and HCT 21  
235 carrying only empty plasmids significantly reduced acetate production to 0.22 g/L as  
236 opposed to 1.85 g/L by HCT 10 (Fig. 5); however, recombinant mutant HCT 20 or HCT  
237 21 containing the 3HV pathway produced as much acetate as the recombinant HCT 10, a  
238 counterintuitive finding (see Discussion). The deletions of *metA* and *tdh* enhanced (S)-  
239 3HV production by 41% (recombinant HCT 21 relative to recombinant HCT 20), but

240 essentially had no effect on (*R*)-3HV production. Nevertheless, those mutations were able  
241 to boost the ratios of 3HV/3HB by decreasing the 3HB titers and/or increase the 3HV  
242 titers. Overall, titers as high as 0.31 g/L and 0.50 g/L of (*S*)-3HV and (*R*)-3HV were  
243 achieved in the recombinant HCT 21 with 3HV/3HB ratios up to 0.35 and 0.24,  
244 respectively (Fig. 5).

245

### 246 **3HV Synthesis from Glycerol**

247 Glycerol has become a promising and abundant carbon source due to its  
248 generation as an inevitable byproduct of biodiesel production from vegetable oils or  
249 animal fats through a transesterification reaction [29]. There have been several reports on  
250 converting glycerol to more valuable compounds such as thymidine, ethanol, and 1,3-  
251 propanediol [30-32]. Glycerol is also more reduced than glucose, leading to a higher  
252 reduced cofactor pool in the cytoplasm [32]. Therefore, in addition to glucose, we  
253 investigated the ability of our recombinant *E. coli* to convert glycerol to chiral 3HV.  
254 Titers of 0.08 g/L and 0.96 g/L of (*S*)-3HV and (*R*)-3HV, respectively, were achieved in  
255 recombinant HCT 10, while recombinant HCT 21 produced 0.19 g/L and 0.60 g/L of (*S*)-  
256 3HV and (*R*)-3HV, respectively (Fig. 6). As mentioned in the Materials and Methods  
257 section, in this specific experiment, concentration of 3HB was quantified by DAD at 210  
258 nm that had a detection limit at around 0.08 g/L. As a result, the amounts of (*S*)-3HB  
259 produced in both recombinant HCT 10 and HCT 21 strains were too low to be quantified  
260 so that we could not report the 3HV/3HB ratios. Nonetheless, in the case of (*R*)-isomers,  
261 3HV/3HB ratios could be as high as 0.88 and 1.10, respectively, in recombinant HCT 10  
262 and HCT 21 strains. The high 3HV/3HB ratios can be beneficial in terms of product  
263 separation or biosynthesis of PHBV that enables high 3HV content.

264

## 265 **Confirmation of 3HV Stereochemistry**

266           The stereochemistry of the resulting 3HV in the media from these cultures was  
267 determined by methyl esterification of the 3HV present followed by chiral HPLC analysis  
268 using our previously developed method [15]. However, we could not assign an absolute  
269 stereochemistry to each sample due to the unavailability of enantiopure 3HV standards.  
270 However, based on our previous results regarding the product stereochemistry of *phaB*  
271 and *hbd* and the observation that Me-(*R*)-3HB has a faster retention time relative to Me-  
272 (*S*)-3HB, we expect Me-(*R*)-3HV to have a faster retention time than Me-(*S*)-3HV when  
273 analyzed by the same method. Thus, the 6.9 and 9.2 min peaks likely represent Me-(*R*)-  
274 3HV and Me-(*S*)-3HV, respectively (Fig. 7). These results confirm the enantiopurity of  
275 biosynthesized 3HV.

276

## 277 **Discussion**

278           In general, two approaches can be taken to engineer *E. coli* for direct 3HV  
279 production via the threonine biosynthesis pathway. The first is to utilize an existing  
280 threonine producer, such as *E. coli* ATCC 21277 [33], followed by further engineering to  
281 introduce our constructed 3HV pathway. However, this and other available threonine  
282 producing strains have typically been developed through multiple rounds of random  
283 mutagenesis and selection due to the difficulty of engineering this highly regulated and  
284 complex metabolic network. Although there are several successful cases in developing  
285 industrial threonine producers by such approaches, resultant strains usually also suffer  
286 from undesired phenotypes including, for example, growth retardation, low

287 transformation efficiency, and by-product formation as a result of random mutations [34].  
288 In addition, other uncharacterized mutations may hinder further strain development as  
289 often needed. Fortunately, recent advances in computational genomics have allowed for  
290 rational development of production strains [34]. Therefore, as a second approach, a  
291 genetically-defined threonine producing strain was established and introduced with the  
292 3HV pathway to achieve direct microbial production of chiral 3HV from glucose or  
293 glycerol.

294 As seen in Fig 5, acetate is the major byproduct to the production of hydroxyacids  
295 (3HB and 3HV). In an effort to decrease acetate and increase 3HV production, a mutant  
296 strain HCT 20 with deletions on *atoDA*, *poxB*, and *ackA-pta* genes was developed.  
297 Counter-intuitively, the recombinant acetate pathway knockout strains of HCT 20 and  
298 HCT 21 produced slightly more acetate and less 3HB than recombinant HCT 10. We  
299 suspected that the enzymatic activity responsible for acetate production was restored by  
300 Ptb-Buk and TesB in the recombinant HCT 20 and HCT 21. In fact, in a separate  
301 experiment, both enzymes were found to have CoA-removing activities on acetyl-CoA  
302 and propionyl-CoA (data not shown), so an introduction of TesB and/or Ptb-Buk to  
303 strains HCT 20 or HCT 21 would likely restore the ability to produce acetate.

304 Apparently, knocking out enzymes responsible for acetate production failed to  
305 reduce acetate synthesis. Alternatively, to alleviate the substrate promiscuity of TesB and  
306 Ptb-Buk on acetyl-CoA, and thus reduce acetate production, one approach called enzyme  
307 co-localization could be implemented to allow substrate channeling between enzymes  
308 [35]. For example, pathway enzymes of Hbd and TesB, catalyzing successive reactions,  
309 can be co-localized in an attempt to reduce the amount of freely floating TesB that may

310 hydrolyze acetyl-CoA as well as to increase accessibility of 3HV-CoA by TesB. The  
311 spatial organization of the enzymes can be achieved using either the leucine zipper, a  
312 dimer resulting from interaction between leucine residues [36], or the synthetic scaffolds,  
313 constructed from protein–protein interaction domains [37]. Furthermore, expressing  
314 enzymes that would assimilate produced acetate is another way to reduce acetate  
315 accumulation. For example, acetyl-CoA synthetase (encoded by *acs*) from *E. coli* can be  
316 over-expressed to convert acetate to acetyl-CoA with the use of one ATP. While  
317 successfully demonstrated in one work [38], in our case, over-expression of *acs* was  
318 found to have essentially no effect on acetate reduction (data not shown). Additionally, to  
319 overcome the hurdle of acetate reduction, approaches like protein engineering of TesB  
320 and/or Ptb-Buk to alleviate their substrate promiscuity, or utilization of better isozymes  
321 with more stringent substrate specificity could also mitigate the carbon loss in the form of  
322 acetate.

323         Among microbes, NADH and NADPH play a central role in energy metabolism  
324 by providing the cell with the reducing power for a variety of cellular redox reactions.  
325 The availability of such cofactors could impose a huge impact on the functionality of  
326 introduced biosynthetic pathways. In fact, we have previously shown that the  
327 NADPH/NADP<sup>+</sup> ratio was two- to three-fold higher than the NADH/NAD<sup>+</sup> ratio under  
328 the culture conditions examined, presumably affecting *in vivo* activities of PhaB and Hbd  
329 and resulting in greater production of (*R*)-3HB than (*S*)-3HB [15]. Given that our  
330 proposed 3HV pathway was based on the previously established 3HB pathway, it was  
331 also expected to see the same trend of greater production of (*R*)-3HV than (*S*)-3HV, even  
332 though the cofactor dependency of 3HV synthesis may be complicated by the



333 energetically expensive threonine biosynthesis pathway with utilization of both ATP and  
334 NADPH. In an effort to perturb the cofactor balance within the cells, thereby tuning the  
335 production of (*R*)-3HV and (*S*)-3HV, we attempted to use glycerol, a promising,  
336 abundant, and highly-reduced carbon source, to support 3HV production. Based on our  
337 calculation of reducing equivalents ( $e^-$ ) of glucose and glycerol, on the same basis of 2  
338 moles of phosphoenolpyruvate synthesized, glucose and glycerol possess, respectively,  
339 24 and 28 reducing equivalents. Potentially, the additional four reducing equivalents can  
340 be utilized to generate two NADPH or equivalent amount of ATP. In fact, it has been  
341 experimentally confirmed that a higher intracellular NADPH/NADP<sup>+</sup> ratio was observed  
342 when glycerol was used as a carbon source than glucose, and this higher ratio was also  
343 reflected in boosted production of thymidine as its biosynthesis requires NADPH as a  
344 cofactor [32]. Given that both NADPH and ATP play a central role in threonine  
345 biosynthesis, we hypothesized that the use of glycerol, which could generate more  
346 NADPH and ATP (Fig. 1) relative to glucose, may favor threonine biosynthesis by  
347 directing more carbon flux towards production of propionyl-CoA, thus favoring the  
348 formation of 3HV relative to 3HB. In agreement with our hypothesis, a higher 3HV/3HB  
349 ratio was obtained in the (*R*)-3HV production when glycerol was used as the carbon  
350 source (Fig. 6). In addition, much larger ratios of the total (*R*)-hydroxyacids (summation  
351 of (*R*)-3HB and (*R*)-3HV titers) to the total (*S*)-hydroxyacids (summation of (*S*)-3HB and  
352 (*S*)-3HV titers) were observed in glycerol-fed cultures (Fig. 6) compared to glucose-fed  
353 cultures (Fig. 5). We hypothesize that the higher intracellular NADPH/NADP<sup>+</sup> ratio as a  
354 result of the use of glycerol would favor (*R*)-hydroxyacid biosynthesis compared to the

355 use of glucose, thus yielding the larger ratios of total (R)-hydroxyacids to total (S)-  
356 hydroxyacids.

357 As mentioned previously, BktB was chosen as the primary thiolase due to its high  
358 enzymatic specificity towards the C<sub>5</sub> substrate. Given that *E. coli* has an endogenous  
359 thiolase (encoded by *atoB*), a deletion of *atoB* was expected to increase the ratio of  
360 3HV/3HB as AtoB has been shown to prefer to condense two molecules of acetyl-CoA  
361 instead of one propionyl-CoA and one acetyl-CoA [19]. However, our preliminary result  
362 showed that the recombinant HCT 11 with an *atoB* deletion behaved exactly as the  
363 recombinant HCT 10, and the deletion in *atoB* had essentially no effect on 3HV  
364 production (data not shown), implying that *atoB* may not be a constitutively expressed  
365 gene. In addition, it is noteworthy that increased 3HV production in the recombinant  
366 HCT 20 relative to the recombinant HCT 10 was observed only with 2-ketobutyrate  
367 supplementation (Fig. 3) but not with the threonine supplementation, solely glucose, or  
368 solely glycerol experiments (Fig. 4-6); similarly, an accumulation of propionate only  
369 occurred in the 2-ketobutyrate supplementation experiment (Fig. 3), altogether, indicating  
370 that 3HV biosynthesis from glucose or glycerol is most likely limited by the precursor  
371 propionyl-CoA. Therefore, approaches to increase the availability of propionyl-CoA  
372 could enhance the 3HV production.

373

## 374 **Conclusions**

375 Carbon skeletons with even-chain number are naturally found in fatty acid  
376 metabolism, but those with odd-chain number are pretty novel. As a result, there is a  
377 good deal of interest in making odd-carbon chain molecules such as 3HV (C<sub>5</sub>) and

378 propionate (C3) because they are so much harder to get to than even-carbon chain ones  
379 such as acetate (C2) and butyrate/butanol (C4). This paper opens the way for biosynthesis  
380 of the odd-carbon chain molecules from renewable feedstocks. Taking together, our work  
381 represents the first report of direct microbial production of enantiomerically pure 3HV  
382 from a single carbon source. In addition, we have explored the production of each  
383 stereoisomer of 3HV across different genetically altered *E. coli* strains, along with  
384 various enzyme homologs, for enhanced chiral 3HV production. Further engineering of  
385 host strains and pathway enzymes should lead to higher 3HV titers and a more  
386 economical bioprocess for the production of chiral 3HV.

387

## 388 **Methods**

### 389 **Microorganisms**

390 The bacterial strains used are listed in Table 1. *C. acetobutylicum* ATCC 824, *C.*  
391 *glutamicum* ATCC 13032, and a threonine hyper-producer *E. coli* ATCC 21277 were  
392 purchased from the American Type Culture Collection (ATCC, Manassas, VA). *R.*  
393 *eutropha* H16 was provided by Professor Anthony Sinskey of the Department of Biology  
394 at the Massachusetts Institute of Technology (Cambridge, MA, USA). *E. coli* DH10B  
395 (Invitrogen, Carlsbad, CA) and ElectroTen-Blue (Stratagene, La Jolla, CA) were used for  
396 transformation of cloning reactions and propagation of all plasmids. MG1655 (kindly  
397 donated by Professor Gregory Stephanopoulos of the Department of Chemical  
398 Engineering at the Massachusetts Institute of Technology, USA) was used as the parental  
399 strain for genetic modification. Host gene deletions of *endA*, *recA*, *atoDA*, *ackA-pta*,  
400 *poxB*, *tdh*, *metA*, and *atoB* were achieved with P1 transduction using the Keio collection

401 strains as donor cells [39]. The kanamycin cassette was removed using plasmid pCP20 as  
402 described by Datsenko and Wanner [40] and the successfully constructed mutant strains  
403 were verified by colony PCR using appropriate primers. Strains carrying a  $\lambda$ DE3 lysogen  
404 were constructed using a  $\lambda$ DE3 Lysogenization Kit (Novagen, Darmstadt, Germany) for  
405 site-specific integration of  $\lambda$ DE3 prophage into each host.

406

### 407 **Plasmid Construction**

408 Genes derived from *C. acetobutylicum* ATCC 824 (*hbd* and *ptb-buk* operon), *R.*  
409 *eutropha* H16 (*bktB* and *phaB*), *C. glutamicum* ATCC 13032 (*ilvA*), *E. coli* MG1655  
410 (*tesB*, *ilvA*, and *thrABC* opeon), and *E. coli* ATCC 21277 (*thrA*<sup>G1297A</sup>*BC* opeon) were  
411 obtained by polymerase chain reaction (PCR) using genomic DNA (gDNA) templates.  
412 All gDNAs were prepared using the Wizard Genomic DNA Purification Kit (Promega,  
413 Madison, WI). Custom oligonucleotides (primers) were purchased for all PCR  
414 amplifications (Sigma-Genosys, St. Louis, MO) as listed in Table 1. In all cases, Phusion  
415 High Fidelity DNA polymerase (Finnzymes, Espoo, Finland) was used for DNA  
416 amplification. Restriction enzymes and T4 DNA ligase were purchased from New  
417 England Biolabs (Ipswich, MA). Recombinant DNA techniques were performed  
418 according to standard procedures [41]. Three co-replicable vectors, pETDuet-1,  
419 pCDFDuet-1, and pCOLADuet-1 (Novagen, Darmstadt, Germany), were used for  
420 construction of chiral 3HV biosynthetic pathways [42]. All vectors contain two multiple  
421 cloning sites (MCS), each of which is preceded by a *T7lac* promoter and a ribosome  
422 binding site (RBS), affording high-level expression of each individual gene.

423 Plasmids constructed in the present work are listed in Table 1. For cloning genes,  
424 PCR products incorporated with desired restriction sites within the 5' and 3' primers were

425 digested, and the resulting DNA fragments were then cloned into pETDuet-1,  
426 pCDFDuet-1, or pCOLADuet-1. The *bktB* gene was inserted in between the *MfeI* and  
427 *XhoI* sites (MCS II) of pETDuet-1 to create pET-B. The *ptb-buk* gene, digested from  
428 pCDF-PB with *EcoRI* and *NotI* [15], was inserted between the *EcoRI* and *NotI* sites  
429 (MCS I) of pET-B to create pET-PB-B. Plasmid pCDF-H was created by inserting the  
430 *hbd* gene between the *NdeI* and *AvrII* sites (MCS II) of pCDFDuet-1. Cloning the *tesB*  
431 gene between the *NcoI* and *NotI* sites (MCS I) of pCDFDuet-1 resulted in plasmid pCDF-  
432 T. Plasmid pCDF-T-H was then created by inserting the *hbd* gene between the *NdeI* and  
433 *AvrII* sites (MCS II) of pCDF-T. In a similar manner, plasmid pCDF-P was created by  
434 inserting the *phaB* gene between the *MfeI* and *AvrII* sites (MCS II) of pCDFDuet-1.  
435 Plasmid pCDF-T-P was created by inserting the *phaB* gene between the *MfeI* and *AvrII*  
436 sites (MCS II) of pCDF-T. Plasmids of pCOLA-Iec and pCOLA-Icg were constructed by  
437 inserting the *E. coli ilvA* and *C. glutamicum ilvA*, respectively, between the *NdeI* and  
438 *AvrII* sites (MCS II) of pCOLADuet-1. The *thrABC* operon from MG1655 was inserted  
439 in between the *NcoI* and *SalI* sites (MCS I) of pCOLADuet-1 to create pCOLA-Tec.  
440 Plasmid pCOLA-Tec-Icg was then created by inserting the *C. glutamicum ilvA* gene  
441 between the *NdeI* and *AvrII* sites (MCS II) of pCOLA-Tec. To construct plasmid  
442 pCOLA-Tecm-Icg, the *thrA*<sup>G1997A</sup>*BC* operon from *E. coli* ATCC 21277 was inserted in  
443 between the *BamHI* and *SalI* sites (MCS I) of pCOLA-Icg. All constructs were confirmed  
444 to be correct by restriction enzyme digestion and nucleotide sequencing.

445

#### 446 **Culture Conditions**

447 Seed cultures of the recombinant strains were grown in LB medium at 30°C  
448 overnight on a rotary shaker at 250 rpm. For the biosynthesis of chiral 3HV, the seed

449 cultures were used to inoculate 50 mL LB medium supplemented with 20 g/L glucose or  
450 20 g/L glycerol at an inoculation volume of 2% in 250 mL flasks. Cultures were then  
451 incubated at 30°C on a rotary shaker until OD<sub>600</sub> reached 0.8~1.0. At this point, 1 mM  
452 IPTG was added to the cultures to induce recombinant protein expression. Following  
453 induction, cells were cultivated at 30°C and sampled at 24 h intervals for up to 72 h post-  
454 induction for HPLC analysis. We found that both 3HB and 3HV titers did not reach a  
455 plateau until 48 h and that there was essentially no difference in the titers between 48 h  
456 and 72 h. Accordingly, only the peak titers observed at 48 h were reported in this study.  
457 In some experiments as indicated, 20 mM (~1.92 g/L) sodium propionate, 3 g/L sodium  
458 2-ketobutyrate, or 3 g/L threonine was added into the cultures at the same time of  
459 induction. In all cases, LB medium was supplemented with 50 mg/L ampicillin, 50 mg/L  
460 streptomycin, and 30 mg/L kanamycin, as appropriate. In general, experiments were  
461 performed in triplicates, and data are presented as the averages and standard deviations of  
462 the results.

463

#### 464 **Metabolite Analysis**

465 Samples were centrifuged to pellet cells while the aqueous supernatant was  
466 collected for HPLC analysis. Products of interest, including 3HB, 3HV, glucose,  
467 glycerol, 2-ketobutyrate, acetate, and propionate, were analyzed via HPLC using an  
468 Agilent 1100 series instrument equipped with a refractive index detector (RID) and a  
469 diode array detector (DAD). Given that the 3HB peak is overlapped with the glycerol  
470 peak in the RID chromatogram, detection of 3HB in the glycerol-fed cultures was  
471 achieved using the DAD at 210 nm. Analyte separation was achieved using an Aminex®  
472 HPX-87H anion exchange column (Bio-Rad Laboratories, Hercules, CA) with 5 mM

473 H<sub>2</sub>SO<sub>4</sub> as the mobile phase. The mobile phase was pumped at a constant rate of 0.6  
474 mL/min, and the column and detector temperatures were each set at 35°C throughout.  
475 Concentrations were determined by linear extrapolation from calibration of external  
476 standards.

477

### 478 **Chiral Analysis of 3HV**

479 The stereochemistry of 3HV produced was determined by methyl esterification of  
480 the 3HV present in the medium followed by chiral HPLC analysis as described in a  
481 previously reported method [15]. The chiral analysis was performed on an Agilent 1100  
482 Series instrument equipped with a Chiralcel OD-H column (0.46 cm φ x 25 cm)  
483 purchased from Daicel Chemical Industries (West Chester, PA). Methyl-3HV was  
484 detected on a DAD at 210 nm. The mobile phase was 9:1 *n*-hexane:isopropanol and the  
485 flow rate through the column was 0.7 mL/min. Due to unavailability of standards of  
486 Methyl-(*R*)-3HV and Methyl-(*S*)-3HV, these spectra were compared to a racemic 3HV  
487 standard (Epsilon Chimie, Brest, France) derivatized by methyl esterification.

488

### 489 **Competing interests**

490 The authors declare that they have no competing interests.

491

### 492 **Authors' contributions**

493 HCT and KLJP initiated and coordinated the project. HCT performed experiments. CLH  
494 assisted HCT with gene cloning, cell culture, and data analysis. CHM performed the

495 chiral analysis. HCT wrote and KLJP edited the paper. All authors approved the final  
496 version of the manuscript.

497

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502

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- 630  
631  
632

## 633 **Figures**

### 634 **Figure 1 - Schematic representation of chiral 3HV production via the threonine** 635 **biosynthesis pathway in metabolically engineered *E. coli***

636 Genes in bold are overexpressed while disrupted pathway steps are indicated by the “no”  
637 symbols. The carbon sources and main metabolic products in the system are enclosed by  
638 rectangular boxes with thick and thin lines, respectively. For glycerol utilization [43, 44],  
639 a glycerol kinase (GK) phosphorylates glycerol to glycerol-3-phosphate, followed by  
640 oxidation to dihydroxyacetone phosphate that enters glycolysis. The oxidation reaction is  
641 catalyzed by a membrane enzyme called glycerol-3-phosphate dehydrogenase (GlpD)  
642 with concomitant production of ubiquinol (UQH<sub>2</sub>) from ubiquinone (UQ). Electrons  
643 stored in the ubiquinol are then transferred through the aerobic respiratory chain coupled  
644 with proton translocation from cytoplasm to periplasm. Both ATP and NADPH can be  
645 synthesized by an H<sup>+</sup>-driven proton movement from periplasm to cytoplasm, catalyzed by  
646 an ATP synthase and a membrane-bound transhydrogenase (PntAB), respectively.

647

### 648 **Figure 2 - 3HV biosynthesis from glucose and propionate**

649 This figure shows shake-flask production of chiral 3HV by recombinant *E. coli* strain  
650 HCT 10 grown in LB supplemented with 20 g/L glucose and 20 mM sodium propionate.  
651 Over-expressed genes are indicated in the table below the graph.

652

### 653 **Figure 3 - 3HV biosynthesis from glucose and 2-ketobutyrate**

654 This figure shows shake-flask production of chiral 3HV by recombinant *E. coli* grown in  
655 LB supplemented with 20 g/L glucose and 3 g/L sodium 2-ketobutyrate. Effects of  
656 overexpressing *ptb-buk* and using acetate pathway knockout strain HCT 20 (with

657 additional deletions of *ackA-pta*, *poxB*, and *atoDA* genes compared to HCT 10) on 3HV  
658 production are compared. All strains contained the same set of plasmids pET-PB-B, and  
659 pCDF-T-H (for (*S*)-3HV synthesis) or pCDF-T-P (for (*R*)-3HV synthesis).

660

#### 661 **Figure 4 - 3HV biosynthesis from glucose and threonine**

662 This figure shows shake-flask production of chiral 3HV by recombinant *E. coli* strain  
663 HCT 10 grown in LB supplemented with 20 g/L glucose and 3g/L threonine. Chiral 3HV  
664 production using alternative threonine deaminases (encoded by *ilvA*) from *E. coli* and *C.*  
665 *glutamicum* is compared. All strains contained the same set of plasmids pET-PB-B,  
666 pCOLA-Icg or pCOLA-Iec as indicated, and pCDF-T-H (for (*S*)-3HV synthesis) or  
667 pCDF-T-P (for (*R*)-3HV synthesis).

668

#### 669 **Figure 5 - 3HV biosynthesis solely from glucose**

670 This figure shows shake-flask production of chiral 3HV in various knock-out strains as  
671 described in Table 1. Cells were grown in LB supplemented with 20 g/L glucose. The top  
672 and bottom dashed lines represent the acetate titers produced from *E. coli* strain HCT 10  
673 and HCT 20 harboring empty plasmids, respectively. All strains contained the same set of  
674 plasmids pET-PB-B, pCOLA-Tecm-Icg, and pCDF-T-H (for (*S*)-3HV synthesis) or  
675 pCDF-T-P (for (*R*)-3HV synthesis). The recombinant HCT 10 strains carrying an empty  
676 pCOLAduet-1 in place of pCOLA-Tecm-Icg, as control strains, produced essentially no  
677 3HV (data not shown).

678

679 **Figure 6 - 3HV biosynthesis solely from glycerol**

680 This figure shows shake-flask production of chiral 3HV in various knock-out strains as  
681 described in Table 1. Cells were grown in LB supplemented with 20 g/L glycerol. The  
682 amounts of (*S*)-3HB produced in both recombinant HCT 10 and HCT 21 strains were too  
683 low to be quantified due to a low detection limit by DAD at 210 nm; therefore, the  
684 3HV/3HB ratios were not applicable (NA) to the (*S*)-isomer. All strains contained the  
685 same set of plasmids pET-PB-B, pCOLA-Tecm-Icg, and pCDF-T-H (for (*S*)-3HV  
686 synthesis) or pCDF-T-P (for (*R*)-3HV synthesis).

687

688 **Figure 7 - Determination of the stereochemistry of 3HV**

689 HPLC spectra of (A) racemic 3HV standards after boiling in methanol, (B) culture  
690 medium from the recombinant strain HCT 10 expressing *bktB*, *phaB*, *tesB*, and *ptb-buk*  
691 after boiling in methanol, and (C) culture medium from the recombinant strain HCT 10  
692 expressing *bktB*, *hbd*, *tesB*, and *ptb-buk* after boiling in methanol are shown.

693

694



695 **Tables**696 **Table 1 - *E. coli* strains, plasmids and oligonucleotides used**

Name	Relevant Genotype	Reference
<b>Strains</b>		
DH10B	F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> ) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 recA1 endA1 araD139</i> Δ( <i>ara, leu</i> )7697 <i>galU galK λ<sup>-</sup> rpsL nupG</i>	Invitrogen
ElectroTen-Blue	Δ( <i>mcrA</i> )183 Δ( <i>mcrCB-hsdSMR-mrr</i> )173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Kan<sup>r</sup> [F<sup>'</sup> <i>proAB lacI<sup>q</sup>Z</i>ΔM15 Tn10 (Tet<sup>r</sup>)]</i>	Stratagene
MG1655	F <sup>-</sup> λ <sup>-</sup> <i>ilvG- rfb-50 rph-1</i>	ATCC 700926
HCT 10	MG1655 Δ <i>endA</i> Δ <i>recA</i> (DE3)	This study
HCT 11	MG1655 Δ <i>endA</i> Δ <i>recA</i> Δ <i>atoB</i> (DE3)	This study
HCT 20	MG1655 Δ <i>endA</i> Δ <i>ackA-pta</i> Δ <i>atoDA</i> Δ <i>poxB</i> (DE3)	This study
HCT 21	MG1655 Δ <i>endA</i> Δ <i>ackA-pta</i> Δ <i>atoDA</i> Δ <i>poxB</i> Δ <i>meta</i> Δ <i>tdh</i> (DE3)	This study
<b>Plasmids</b>		
pETDuet-1	ColE1(pBR322) <i>ori, lacI, T7lac, Amp<sup>R</sup></i>	Novagen
pCDFDuet-1	CloDF13 <i>ori, lacI, T7lac, Strep<sup>R</sup></i>	Novagen
pCOLADuet-1	COLA <i>ori, lacI, T7lac, Kan<sup>R</sup></i>	Novagen
pET-B	pETDuet-1 harboring <i>bktB</i> from <i>R. eutropha</i> H16	This study
pET-PB-B <sup>a</sup>	pETDuet-1 harboring <i>ptb-buk</i> operon from <i>C. acetobutylicum</i> ATCC 824, and <i>bktB</i> from <i>R. eutropha</i> H16	This study
pCDF-H	pCDFDuet-1 harboring <i>hbd</i> from <i>C. acetobutylicum</i> ATCC 824	This study
pCDF-T-H <sup>a</sup>	pCDFDuet-1 harboring <i>tesB</i> from <i>E. coli</i> MG1655, and <i>hbd</i> from <i>C. acetobutylicum</i> ATCC 824	This study
pCDF-P	pCDFDuet-1 harboring <i>phaB</i> from <i>R. eutropha</i> H16	This study
pCDF-T-P <sup>a</sup>	pCDFDuet-1 harboring <i>tesB</i> from <i>E. coli</i> MG1655, and <i>phaB</i> from <i>R. eutropha</i> H16	This study
pCOLA-Iec	pCOLADuet-1 harboring <i>ilvA</i> from <i>E. coli</i> MG1655	This study
pCOLA-Icg	pCOLADuet-1 harboring <i>ilvA</i> from <i>C. glutamicum</i>	This study
pCOLA-Tec-Icg <sup>a</sup>	pCOLADuet-1 harboring <i>thrABC</i> operon from <i>E. coli</i> MG1655, and <i>ilvA</i> from <i>C. glutamicum</i> ATCC 13032	This study
pCOLA-Tecm-Icg <sup>a</sup>	pCOLADuet-1 harboring <i>thrA</i> <sup>G1297A</sup> <i>BC</i> operon from <i>E. coli</i> ATCC 21277, and <i>ilvA</i> from <i>C. glutamicum</i> ATCC 13032	This study
<b>Primers<sup>b</sup></b>		
	<b>Sequence 5'→3'<sup>c</sup></b>	
bktB_US_EcoRI	<u>GAATTC</u> ATGACGCGTGAAGTGGTAGTG	Sigma-Genosys
bktB_DS_XhoI	<u>CTCGAG</u> CGCAAGGCTAACCTCAGAT	Sigma-Genosys
hbd_US_NdeI	ATT <u>CATATG</u> AAAAAGGTATGTGTTATAGG	Sigma-Genosys
hbd_DS_AvrII	ATT <u>CCTAGG</u> CAGGTCGACTCTAGAACTTA	Sigma-Genosys
phaB_US_MfeI	ATT <u>CAATTG</u> ACGAAGCCAATCAAGGAG	Sigma-Genosys
phaB_DS_AvrII	ATT <u>CCTAGG</u> GGTTCAGCCATATGCAG	Sigma-Genosys
tesB_US_NcoI	ATT <u>CCATGG</u> GCATGAGTCAGGCGCTAA	Sigma-Genosys
tesB_DS_NotI	ATT <u>GCGGCCG</u> CGACTCTAGAGACTTAATTGTG	Sigma-Genosys
ilvAec_US_NdeI	ATT <u>ACATATG</u> GCTGACTCGCAAC	Sigma-Genosys
ilvAec_DS_AvrII	ATT <u>ACCTAGG</u> CATTTTTCCCTAACC	Sigma-Genosys
ilvAcg_US_NdeI	ATT <u>ACATATG</u> AGTGAAACATACGTGTC	Sigma-Genosys
ilvAcg_DS_AvrII	ATT <u>ACCTAGG</u> CCTTCAGCTATGTTTA	Sigma-Genosys
thrABC_US_BamHI	ATT <u>AGGATCC</u> AAGGAGATATATCATGCGAGTGTTGAAG	Sigma-Genosys

thrABC_US_NcoI	ATT <u>ACCATGGGCATGCGAGT</u> GTTGAAG	Sigma-Genosys
thrABC_DS_SalI	ATT <u>AGTCGACGATAATGAATAGATTTT</u> ACTGATG	Sigma-Genosys

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<sup>a</sup> Each gene is under the control of the T7*lac* promoter with a ribosome binding site.

<sup>b</sup> Primers were synthesized at Sigma-Genosys, St. Louis, MO.

<sup>c</sup> Restriction enzyme sites used in the cloning are shown in underlined italics.

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