

1 Running title: Construction of reversed methylotrophy in *E. coli*

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4 **Methanol production by reversed methylotrophy constructed in**
5 ***Escherichia coli***

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17

18 **Abstract**

19 We constructed a reversed methylotrophic pathway that produces methanol, a promising
20 feedstock for production of useful compounds, from fructose 6-phosphate (F6P), which
21 can be supplied by catabolism of biomass-derived sugars including glucose, by a
22 synthetic biology approach. Using *Escherichia coli* as an expression host, we
23 heterologously expressed genes encoding methanol utilization enzymes from
24 methylotrophic bacteria, i.e., the NAD⁺-dependent methanol dehydrogenase (MDH)
25 from *Bacillus methanolicus* S1 and an artificial fusion enzyme of 3-hexulose-6-
26 phosphate synthase and 6-phospho-3-hexuloisomerase from *Mycobacterium gastri*
27 MB19 (HPS-PHI). We confirmed that these enzymes can catalyze reverse reactions of
28 methanol oxidation and formaldehyde fixation. The engineered *E. coli* strain co-
29 expressing MDH and HPS-PHI genes produced methanol in resting cell reactions not
30 only from F6P but also from glucose. We successfully conferred reversed
31 methylotrophy to *E. coli* and our results provide a proof-of-concept for biological
32 methanol production from biomass-derived sugar compounds.

33

34 **Keywords**

35 methanol dehydrogenase, 3-hexulose-6-phosphate synthase, ribulose monophosphate
36 pathway, *Bacillus methanolicus*, *Mycobacterium gastri*

37

38 **Introduction**

39 Much attention has been paid to methanol as an alternative carbon resource to replace
40 fossil fuels, because methanol can be derived from various carbon sources including
41 methane, CO₂, and biomass, and is a key organic chemical used in the production of
42 many kinds of chemicals, plastic materials and other value-added products [1]. In the
43 field of bioindustry, methanol is not only a carbon source for microbial fermentation
44 processes, but also a substrate for biological production of industrial chemicals [2-4]. To
45 date, a variety of methods for methanol production via chemical processes have been
46 developed [1], however, no biological production processes for methanol from biomass
47 constituents such as sugar compounds, that is analogous to bioethanol production, are
48 currently available. Construction of a synthetic biological pathway in a heterologous
49 host using enzymes involved in the metabolism of one-carbon (C1) compounds
50 including methanol can be a possible solution to establish a metabolic pathway that
51 produces methanol from biomass-derived sugar compounds.

52 Methylophilic bacteria, which can use methanol as the sole carbon and energy
53 source, have diverse types of methanol metabolic pathways. Methanol is first oxidized
54 to formaldehyde by methanol dehydrogenases (MDHs). Gram-negative methylophilic
55 bacteria possess MDHs that require pyrroloquinoline quinone (PQQ) as a cofactor [5].
56 In contrast, gram-positive methylophilic bacteria possess NAD(P)⁺-dependent MDHs
57 [6]. For example, the thermophilic methylophilic *Bacillus methanolicus* possesses an
58 NAD⁺-dependent MDH and this type of MDH requires the activator protein Act for
59 efficient methanol oxidation *in vitro* [7].

60 Formaldehyde produced by MDH next undergoes further oxidation to CO₂ or
61 fixation to cell constituents. The two major assimilatory pathways in methylophilic

62 bacteria are the serine pathway and the ribulose monophosphate (RuMP) pathway [8].
63 In the bacteria which use serine pathway for formaldehyde assimilation, the
64 incorporation of a C1 unit into serine involves the tetrahydromethanopterin (H₄MPT)-
65 and glutathione-dependent oxidation of formaldehyde to formate, the conjugation of
66 formate and tetrahydrofolate (H₄F) to produce 5,10-methylene-H₄F, and the transfer of
67 the C1 unit of 5,10-methylene-H₄F to glycine. On the other hand, in the RuMP pathway,
68 formaldehyde is fixed to ribulose 5-phosphate (Ru5P) by 3-hexulose-6-phosphate
69 synthase (HPS), forming *D-arabino*-3-hexulose 6-phosphate (Hu6P), which is then
70 isomerized to fructose 6-phosphate (F6P) by 6-phospho-3-hexuloisomerase (PHI) [9].

71 Recent studies have engineered model bacterium including *Escherichia coli* to
72 incorporate methanol by introducing the enzymes for C1 metabolism [10-15]. These
73 studies have usually employed NAD⁺-dependent MDH, HPS and PHI for their ease of
74 functional production in the host species, because these enzymes do not require any
75 methylotrophy-specific cofactors (PQQ, H₄MPT and H₄F), and the substrate Ru5P and
76 the product F6P exist in almost all organisms, enabling coupling to the endogenous
77 pentose phosphate pathway.

78 Theoretically, the reverse reactions of methanol oxidation and formaldehyde
79 fixation by MDH, HPS and PHI should result in the production of methanol from F6P,
80 which can be derived from sugar compounds (Figure 1). In fact, it has been reported
81 that the NAD⁺-dependent MDH catalyzes the reverse reaction (i.e., reduction of
82 formaldehyde to methanol), which does not require the activator protein Act [16], and
83 the fused form of HPS and PHI (HPS-PHI) found in some hyperthermophilic archaea
84 also catalyzes the reverse reaction (i.e., production of formaldehyde and Ru5P from
85 F6P) [9,17]. Here we describe the construction of a reversed methylotrophic pathway to

86 produce methanol from F6P or glucose in engineered *E. coli* cells that express genes
87 encoding NAD⁺-dependent MDH from *B. methanolicus* S1 (reclassified from *B. brevis*
88 S1) [18,19], and the artificial fusion enzyme HPS-PHI, which was constructed with the
89 *hps* and *phi* genes from *Mycobacterium gastris* MB19 [20]. To our knowledge, this
90 would be the first report of the biotechnological use of the reverse reactions of C1
91 metabolism, and these results provide a starting point towards the industrially relevant
92 biological supply of methanol from biomass sugars.

93

94 **Materials and methods**

95 ***Strains and culture conditions***

96 *E. coli* strains used in this study are listed in Table 1. *E. coli* transformants were grown
97 in Luria-Bertani (LB) medium at 37°C, to which 0.5 mM isopropyl-β-D-
98 thiogalactopyranoside (IPTG) was added at mid-exponential phase (OD₆₁₀ of 0.4-0.6),
99 followed by overnight growth at 16°C to achieve an OD₆₁₀ of 2-3. Ampicillin (50
100 µg/mL) and chloramphenicol (30 µg/mL) were added when applicable.

101

102 ***Plasmid construction***

103 Plasmids used in this study are listed in Table 2. Oligonucleotide primers used in this
104 study are listed in Table 3. The 1.1-kb *mdh* gene from *B. methanolicus* S1 excluding the
105 stop codon was amplified by PCR from the genomic DNA. The 5'-end of each primer
106 contained NheI or HindIII sites. This PCR product and the EcoRV-digested pBluescript
107 II SK(+) were ligated to obtain pBS_{mdh}. pET_{mdh}-His was constructed by ligating the
108 1.1- and 3.6-kb NheI/HindIII fragments of pBS_{mdh} and pET-23a(+). pDmH was
109 constructed by inserting *mdh*-His₆ from pET_{mdh}-His into the NcoI/EcoRI sites of

110 pETDuet-1. pDmHhp was constructed by inserting *hps-phi* from pETHps-phi [20] into
111 the BglIII/KpnI sites of pDmH. The expression vectors were introduced into *E. coli*
112 Rosetta (DE3) by electroporation.

113

114 ***Preparation of cell-free extract***

115 For purification of recombinant *B. methanolicus* S1 MDH tagged with 6xHis, IPTG-
116 induced *E. coli* [pETmdh-His] cells were suspended in buffer A (50 mM potassium
117 phosphate buffer (KPB, pH 7.5), 5 mM MgSO₄ and 1 mM dithiothreitol (DTT)) and
118 then disrupted by French press (Constant cell disruption system one shot model,
119 Constant Systems Ltd., UK). After centrifugation at 5,000 x g for 30 min at 4°C, the
120 resulting supernatant was used as a cell-free extract. For preparation of a cell-free
121 extract of IPTG-induced *E. coli* [pETHps-phi], and *E. coli* [pDmHhp], cells were
122 suspended in buffer B (50 mM KPB (pH 6.5), 1 mM DTT, 0.1 mM
123 phenylmethylsulfonyl fluoride (PMSF) and 5 mM MgCl₂), and disrupted by French
124 press. After centrifugation at 10,000 x g for 10 min at 4°C, resulting supernatant was
125 used as a cell-free extract.

126

127 ***Purification of recombinant B. methanolicus S1 MDH tagged with 6xHis***

128 Cell-free extract (7.5 mL) was loaded onto 2 mL of column-packed Ni-NTA Agarose
129 (QIAGEN, Hilden, Germany) preequilibrated with buffer C (57.5 mM NaH₂PO₄, 300
130 mM NaCl, pH adjusted to 8.0 with NaOH) containing 10 mM imidazole. The column
131 was then washed twice with 2 column volumes of buffer C containing 20 mM
132 imidazole. The column-bound protein was eluted with 2 mL of buffer C containing 250

133 mM imidazole. The eluted fraction was dialyzed against 50 mM KPBS (pH 7.5) and used
134 as purified MDH-His₆.

135

136 *Protein analyses*

137 Protein concentrations were determined using a Bio-Rad protein assay kit (Japan Bio-
138 Rad Laboratories, Tokyo, Japan) with bovine serum albumin as the standard [21]. For
139 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), protein
140 samples (10 µg) were mixed with 3X sample buffer (50 mM Tris-HCl pH 6.8, 30%
141 (v/v) glycerol, 3% (w/v) SDS, 3% (v/v) 2-mercaptoethanol, a dash of bromophenol
142 blue), boiled for 5 min and run on a 12% gel. For total protein staining, GelCode™ Blue
143 Safe Protein Stain (Thermo Scientific, Waltham, MA) was used. For immunoblot
144 analyses, proteins were transferred onto a PVDF membrane by semidry blotting (Bio-
145 Rad, Richmond, CA). 6xHis-tagged proteins were detected using anti-His-tag mAb-
146 HRP-Direct (Medical & Biological Laboratories, Nagoya, Japan) at a 1:5,000 dilution
147 and Western Lightning (Perkin-Elmer Life Science, Waltham, MA). HPS-PHI proteins
148 were detected using rabbit anti-HPS antibody as described previously [20]. The signal
149 was analyzed with LuminoGraph II (ATTO, Tokyo, Japan).

150

151 *Enzyme assay*

152 Formaldehyde reductase (FRD) activity was determined by following the
153 formaldehyde-dependent oxidation of NADH as described previously [16]. The K_m and
154 k_{cat} values of purified MDH-His₆ were determined using a Lineweaver-Burk plot of
155 initial reaction velocity against different concentrations of the substrate formaldehyde.
156 The activity of HPS-PHI to catalyze the forward reaction (F6P synthesis) was

157 determined by following the ribulose-5-phosphate-dependent production of F6P as
158 described previously [20] except that the formaldehyde and ribose 5-phosphate
159 concentrations were 2.5 mM. The activity of HPS-PHI to catalyze the reverse reaction
160 (formaldehyde production) was determined by following the F6P-dependent production
161 of formaldehyde as described previously [9] except that the reaction was performed at
162 30°C. One unit of activity was defined as the amount of enzyme that oxidized 1 μ mol of
163 NADH (FRD reaction), produced 1 μ mol of F6P (HPS-PHI forward reaction), or
164 produced 1 μ mol of formaldehyde (HPS-PHI reverse reaction) per min. Each activity
165 value is presented as mean \pm standard deviation (s.d.) of triplicate measurements.

166

167 ***Production of methanol by whole-cell reaction***

168 For production of methanol from formaldehyde, IPTG-induced *E. coli* [pETmdh-His]
169 and *E. coli* [pET-23a(+)] cells were suspended in 50 mM KPb (pH 6.7), 5 mM MgSO₄,
170 1 mM DTT and 10 mM formaldehyde, to achieve an optical density at 610 nm (OD₆₁₀)
171 of 2.0, and incubated at 37°C. For production of methanol from F6P, IPTG-induced
172 cells of *E. coli* [pDmHhp], *E. coli* [pETDuet], or mixture of *E. coli* [pETmdh-His] and
173 *E. coli* [pETHps-phi] were suspended in 100 mM KPb (pH 6.5), 5 mM MgCl₂ and 100
174 mM F6P, to achieve an OD₆₁₀ of 8.0, and incubated at 37°C. Methanol contained in the
175 purchased F6P (Sigma-Aldrich Japan K.K., Tokyo, Japan) was removed in advance by
176 dissolving F6P in 70% (v/v) acetonitrile and removing the solvent by a centrifugal
177 evaporator. For production of methanol from glucose, IPTG-induced *E. coli* [pDmHhp]
178 and *E. coli* [pETDuet] cells were suspended in 100 mM KPb (pH 6.5), 5 mM MgCl₂
179 and 2% (w/v) glucose, to achieve an OD₆₁₀ of 10, and incubated at 37°C. In these
180 experiments, the reaction volume was 1 mL, and at each time point, 0.1 mL was

181 sampled, centrifuged at 20,000 x g for 2 min at 4°C, and the resulting supernatant was
182 stored at 4°C. The methanol concentration in the supernatant was determined using a
183 GC-2014 (Shimadzu Co, Kyoto, Japan) gas chromatograph equipped with a flame
184 ionization detector and DB-1 column (30 m x 0.25 mm i.d. x 0.25 µm, Agilent
185 Technologies, Santa Clara, CA, USA). Nitrogen gas was used as the carrier. The
186 temperature program of the oven was 40°C for 5 min, then a ramp of 20°C min⁻¹ to
187 200°C (held for 15 min), and the injector and detector were set at 250°C.

188

189 **Results**

190 ***Recombinant B. methanolicus S1 MDH catalyzes NADH-dependent reduction of*** 191 ***formaldehyde to methanol both in vitro and in vivo***

192 To confirm that recombinant MDH from *B. methanolicus* S1 catalyzes NADH-
193 dependent reduction of formaldehyde to methanol as reported for the enzyme from *B.*
194 *methanolicus* C1 [16], we constructed a T7 promoter-based expression vector for the
195 NAD⁺-dependent MDH gene (*mdh*) from *B. methanolicus* S1 tagged with 6xHis and
196 introduced it into *E. coli* Rosetta (DE3). Although efficient methanol oxidation by the
197 NAD⁺-dependent MDH requires the activator protein Act, we did not express it because
198 it is not required for the reverse reaction [16,22]. After production in *E. coli*, we purified
199 MDH-His₆ using the Ni-NTA column (Figure S1). The specific activity of the purified
200 MDH-His₆ to reduce formaldehyde to methanol with NADH was 10.0 units/mg at 37°C.
201 This value was comparable to that of MDH purified from *B. methanolicus* C1 (19.6
202 units/mg at 50°C) [16] or the recombinant *E. coli* strain expressing the MDH gene of
203 strain C1 (3.5 units/mg at 50°C) [23]. K_m and k_{cat} values for our purified MDH-His₆ at
204 37°C were 2.1 mM and 6.8 s⁻¹, respectively. Taken together, recombinant MDH from *B.*

205 *methanolicus* S1 can catalyze NADH-dependent reduction of formaldehyde to methanol
206 *in vitro*.

207 Next, we tested whether *E. coli* cells expressing MDH-His₆ can produce
208 methanol from formaldehyde in whole-cell reactions. After induction of *mdh*-His₆ by
209 IPTG, *E. coli* [pETmdh-His] cells were incubated in buffer containing 10 mM
210 formaldehyde at 37°C. The production of MDH-His₆ protein was confirmed by
211 immunoblot analysis (Figure S2). As a result, we observed an increase in the methanol
212 concentration in the reaction mixture, which was not observed with cells harboring
213 empty vector (*E. coli* [pET-23a(+)]) (Figure 2). Therefore, *E. coli* cells producing
214 recombinant MDH from *B. methanolicus* S1 can produce methanol from formaldehyde.
215 The methanol concentration after 41 h of incubation was 0.82 ± 0.02 mM.

216

217 ***Artificial fusion protein HPS-PHI catalyzes production of formaldehyde from F6P***

218 We next investigated whether the artificial fusion enzyme HPS-PHI derived from *M.*
219 *gastri* MB19 [20] catalyzes formaldehyde production from F6P. We used *E. coli*
220 [pETHps-phi] for production of the enzyme. Cell-free extract of IPTG-induced *E. coli*
221 [pETHps-phi] cells was prepared and subjected to enzyme assays. The specific activities
222 for forward (F6P production) and reverse (formaldehyde production) reactions were 2.0
223 ± 0.6 and $(6.1 \pm 0.1) \times 10^{-1}$ units/mg, respectively. Neither enzyme activity was detected
224 with cells harboring empty vector (*E. coli* [pET-23a(+)]). The production of HPS-PHI
225 protein was confirmed by immunoblot analysis (Figure S3). Therefore, it was confirmed
226 that the artificial HPS-PHI can catalyze both the forward and reverse reactions as
227 reported for HPS-PHI from hyperthermophilic archaea [9,17].

228

229 ***Production of methanol from F6P through sequential reactions catalyzed by HPS-***
230 ***PHI and MDH***

231 To test whether methanol can be produced from F6P through the sequential reactions
232 catalyzed by HPS-PHI and MDH, we constructed a plasmid vector for co-expression of
233 the genes encoding MDH-His₆ and HPS-PHI (pDmHhp), and introduced it into *E. coli*
234 Rosetta (DE3). To confirm the co-expression of these genes, the cell-free extract of
235 IPTG-induced *E. coli* [pDmHhp] cells was subjected to enzyme assays. Specific
236 activities for formaldehyde reduction catalyzed by MDH at 37°C and for formaldehyde
237 fixation catalyzed by HPS-PHI at 30°C were $(3.4 \pm 0.5) \times 10^{-2}$ and $(5.3 \pm 0.3) \times 10^{-1}$
238 units/mg, respectively. Neither enzyme activity was detected with cells harboring empty
239 vector (*E. coli* [pETDuet]). The co-production of MDH-His₆ and HPS-PHI proteins was
240 confirmed by immunoblot analyses (Figure S4). Thus, we succeeded in co-expressing
241 genes encoding these two enzymes.

242 Next, we tested whether cells expressing *mdh*-His₆ and *hps-phi* can produce
243 methanol from F6P in resting cell reactions. IPTG-induced *E. coli* [pDmHhp] cells were
244 incubated in buffer containing 100 mM F6P for 24 h at 37°C. Results showed that
245 methanol accumulated up to 1.5 ± 0.1 mM in the reaction mixture (Figure 3(a)), which
246 was not observed for *E. coli* [pETDuet] cells (Figure 3(a)) or in the absence of F6P
247 (data not shown). Thus, *E. coli* cells co-expressing *mdh*-His₆ and *hps-phi* could produce
248 methanol from F6P. On the other hand, 20 ± 3 μM methanol was detected with *E. coli*
249 [pETmdh-His] cells alone (Figure 3(a), 100% *E. coli* [pETmdh-His]). Nevertheless, the
250 amount of methanol was about 75-fold lower than that by the co-expressing cells. In
251 addition, methanol was not detected with *E. coli* [pETHps-phi] cells (Figure 3(a), 0% *E.*
252 *coli* [pETmdh-His]). Collectively, efficient production of methanol was confirmed to

253 require both MDH-His₆ and HPS-PHI. We also tested the mixture of *E. coli* [pETmdh-
254 His] and *E. coli* [pETHps-phi] cells, expecting that methanol could be produced by *E.*
255 *coli* [pETmdh-His] cells from formaldehyde produced by *E. coli* [pETHps-phi] cells.
256 However, the amount of methanol produced was less than that with the co-expressing
257 cells with all of the mixing ratios tested (Figure 3(a)). This result shows that both
258 enzymes should be produced in the same cell for efficient methanol production. This
259 may be because formaldehyde was converted to methanol before formaldehyde induced
260 the endogenous glutathione-dependent formaldehyde oxidation pathway in the co-
261 expressing cells [24,25]. With *E. coli* [pDmHhp] cells, the methanol concentration
262 reached 2.0 ± 0.01 mM after 72 h of incubation (Figure 3(b)).

263

264 ***Production of methanol from glucose by E. coli expressing mdh and hps-phi***

265 Finally, we tested whether glucose could serve as a substrate for methanol production.
266 Theoretically, glucose incorporated into *E. coli* cells is metabolized in glycolysis,
267 producing as an intermediate F6P, the substrate for methanol production by MDH and
268 HPS-PHI. We suspended *E. coli* [pDmHhp] cells in buffer containing 2% (w/v) glucose,
269 and incubated them at 37°C. We observed the accumulation of methanol that was not
270 observed with the *E. coli* [pETDuet] cells (Figure 4). With the *E. coli* [pDmHhp] cells,
271 methanol accumulated up to 0.25 ± 0.02 mM after 72 h of incubation. As described
272 above, methanol was not produced in the absence of substrate. Taken together, methanol
273 was produced from glucose with the two heterologous enzymes.

274

275 **Discussion**

276 In this study, we established a novel pathway to produce methanol from F6P or glucose
277 in *E. coli* cells via “reversed methylotrophy” by co-expression of genes encoding an
278 NAD⁺-dependent MDH and an artificial fusion protein HPS-PHI. These enzymes have
279 been used to confer synthetic methylotrophy to non-methylotrophic microorganisms
280 [10-15], but we confirmed that these enzymes can catalyze the reverse reactions of C1
281 metabolism both *in vitro* and *in vivo*, and successfully conferred reversed methylotrophy
282 to *E. coli* (Figure 1).

283 The molar yield of methanol from formaldehyde by *E. coli* expressing *mdh* was
284 8.2% (Figure 2). Methanol was likely to have accumulated due to the absence of the
285 MDH activator protein Act, which accelerates the undesired methanol oxidation
286 reaction. Better yield may be achieved by eliminating the endogenous formaldehyde
287 detoxification pathway [24,25]. Next, the molar yield of methanol from F6P by *E. coli*
288 coexpressing *mdh* and *hps-phi* was 2.0% (Figure 3(b)). This low yield can be ascribed
289 to the low expression level of the endogenous sugar phosphate-uptake system [26] or
290 inactivation of MDH-His₆ and HPS-PHI, whose expression was not induced during the
291 incubation with F6P. Finally, the molar yield of methanol from glucose was 0.23%
292 (Figure 4), which was less than that from F6P. The yield may be improved by promoting
293 glucose incorporation or engineering sugar metabolism to increase intracellular
294 concentration of F6P, the substrate for methanol production by MDH and HPS-PHI. In
295 this study, we did not attempt the production of methanol during growth on glucose
296 because we adopted the pET vector system, whose promoter for the gene of interest is
297 repressed in the presence of glucose. The use of a glucose non-repressible promoter will
298 be suitable for methanol production accompanied by the growth on glucose. In spite of

299 the low yield of methanol from glucose, we succeeded in endogenous supply of
300 methanol within the host cells, which has the potential to be utilized for bioproduction
301 of useful compounds that need a methoxy group donor. For example, the supply of
302 methanol is necessary for alcohol acyltransferase-catalyzed production of methyl short-
303 chain esters (methyl acrylate, methyl methacrylate, and other methylester derivatives),
304 which can be used as solvent, plasticizer or lubricant [27]. In such situations, methanol
305 is usually supplied exogenously, however, introducing an endogenous production
306 pathway for methanol and its use as the enzyme substrate within host cells would
307 obviate the need for the exogenous supply and simplify the production process.

308 Because F6P is a ubiquitous metabolic intermediate, the substrate for methanol
309 production can be expanded to other biomass-derived sugars, photosynthetic products,
310 etc. Therefore, this work provides a versatile concept for the biological production and
311 intracellular supply of methanol from various types of substrates that is useful for the
312 production of industrial chemicals including methylesters. Furthermore, this concept is
313 expected to be extended to bioindustrial production of methanol, a promising feedstock,
314 from biomass.

315

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319

320 **Authors' contributions**

321 The experiments were designed by T.T., Y.S., and H.Y. The experiments were performed
322 by T.T., M.Y., D.H., and K.F. The data was analyzed by T.T., M.Y., D.H., K.F., Y.S., and

323 H.Y. The manuscript was written by T.T., Y.S., and H.Y.

324

325 **Disclosure statement**

326 No potential conflict of interest was reported by the authors.

327

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407 **Figure legends**

408 **Figure 1.** Schematic diagram of the reversed methylotrophic pathway to produce
409 methanol from F6P or glucose in recombinant *E. coli* cells. Solid arrows indicate
410 reverse reactions of methanol oxidation and formaldehyde fixation via MDH and the
411 RuMP pathway, respectively. Dashed arrows indicate endogenous metabolic pathways
412 in *E. coli*. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; Ru5P, ribulose 5-
413 phosphate; Hu6P, D-arabino-3-hexulose 6-phosphate; HPS, 3-hexulose-6-phosphate
414 synthase; PHI, 6-phospho-3-hexuloisomerase; MDH, methanol dehydrogenase; PPP,
415 non-oxidative pentose phosphate pathway.

416

417 **Figure 2.** Production of methanol from formaldehyde in a whole-cell reaction of *E. coli*
418 expressing *mdh*-His₆. IPTG-induced *E. coli* [pET-23a(+)] (open circles) and *E. coli*
419 [pETmdh-His] (closed circles) cells were suspended in a reaction mixture containing 10
420 mM formaldehyde and incubated at 37°C for the indicated time. The mean ± s.d. of
421 triplicate incubations are shown. FRD activity of the cell-free extracts of *E. coli* [pET-
422 23a(+)] and *E. coli* [pETmdh-His] cells used in this experiment were not detected and
423 $(1.3 \pm 0.1) \times 10^{-2}$ units/mg at 37°C, respectively. The production of MDH-His₆ protein
424 was confirmed by immunoblot analysis (Figure S2).

425

426 **Figure 3.** Production of methanol from F6P in whole-cell reactions of *E. coli* expressing
427 *mdh*-His₆ and *hps-phi*. (a) Methanol production by *E. coli* [pETDuet] cells (EV), *E. coli*
428 [pDmHhp] cells (co-exp.) or the mixtures of *E. coli* [pETmdh-His] and *E. coli* [pETHps-
429 phi] cells at different ratios. IPTG-induced cells were suspended in a reaction mixture
430 containing 100 mM F6P and incubated at 37°C for 24 h. n.d., not determined. (b) Time-

431 course of methanol production by *E. coli* [pETDuet] cells (open circles) and *E. coli*
432 [pDmHhp] (closed circles). Reaction conditions and the analytical method were the
433 same as in (a), and the cells were incubated for the indicated time. The mean \pm s.d. of
434 triplicate incubations are shown.

435

436 **Figure 4.** Production of methanol from glucose by *E. coli* expressing *mdh*-His₆ and *hps*-
437 *phi*. IPTG-induced *E. coli* [pETDuet] (open circles) and *E. coli* [pDmHhp] (closed
438 circles) cells were suspended in the buffer containing 2% (w/v) glucose to an OD₆₁₀ 10,
439 and incubated at 37°C for the indicated time. The mean \pm s.d. of triplicate incubations
440 are shown. For the samples with the *E. coli* [pETDuet] cells before 96 h, the
441 concentrations of methanol were not determined.

Table 1. *E. coli* strains used in this study.

<i>E. coli</i> strain	Relevant characteristic(s)	Source
Rosetta (DE3)	F ⁻ <i>ompT hsdS_B(rB⁻mB⁻) gal dcm</i> (DE3) pRARE (Cam ^R)	Novagen
Rosetta (DE3) [pET-23a(+)]	Rosetta (DE3) harboring pET-23a(+)	This study
Rosetta (DE3) [pETmdh-His]	Rosetta (DE3) harboring pETmdh-His	This study
Rosetta (DE3) [pEThps-phi]	Rosetta (DE3) harboring pEThps-phi	[20]
Rosetta (DE3) [pETDuet]	Rosetta (DE3) harboring pETDuet-1	This study
Rosetta (DE3) [pDmHhp]	Rosetta (DE3) harboring pDmHhp	This study

Table 2. Plasmids used in this study.

Plasmid	Relevant characteristic(s)	Source
pBluescript II SK(+)	Cloning vector	Stratagene
pBSmdh	pBluescript II SK(+) derivative; <i>mdh</i> from <i>B. methanolicus</i> S1 without the stop codon	This study
pET-23a(+)	T7 promoter-based expression vector	Novagen
pETmdh-His	pET-23a(+) derivative; <i>mdh</i> from <i>B. methanolicus</i> S1 in frame with C-terminal 6xHis-tag	This study
pEThps-phi	pET-23a(+) derivative; <i>hps-phi</i>	[20]
pETDuet-1	T7 promoter-based expression vector for two genes	Novagen
pDmH	pETDuet-1 derivative; <i>mdh</i> -His ₆ from pETmdh-His in one of the two multiple cloning sites	This study
pDmHhp	pDmH derivative; <i>hps-phi</i> in the other multiple cloning site	This study

Table 3. Oligonucleotide primers used in this study.

Primer	Sequence (5'-3')	Purpose
mdh-fw-NheI	CTAGCTAGCATGACAAA CTTTTTCATTCC	Amplification of <i>mdh</i> gene excluding the stop codon
mdh-rv-HindIII(Histag)	CCCAAGCTTCAGAGCG TTTTTGATGATTT	from the genomic DNA of <i>B. methanolicus</i> S1 for construction of pBSmdh
mdh-fw-NcoI	CATGCCATGGGCATGAC AAACTTTTTCATTCC	Amplification of <i>mdh</i> -His ₆ from pETmdh-His for
mdh-His-rv-EcoRI	GGAATTCTCAGTGGTGG TGGTGGTGGTGCT	construction of pDmH
MhMp-BglII	GAAGATCTCATGAAGCT CCAAGTCTCCAT	Amplification of <i>hps-phi</i> from pETHps-phi for
MhMp-KpnI	GGGGTACCTCACTCGA GGTTGGCGTGGCGCG	construction of pDmHhp

Fig. 1

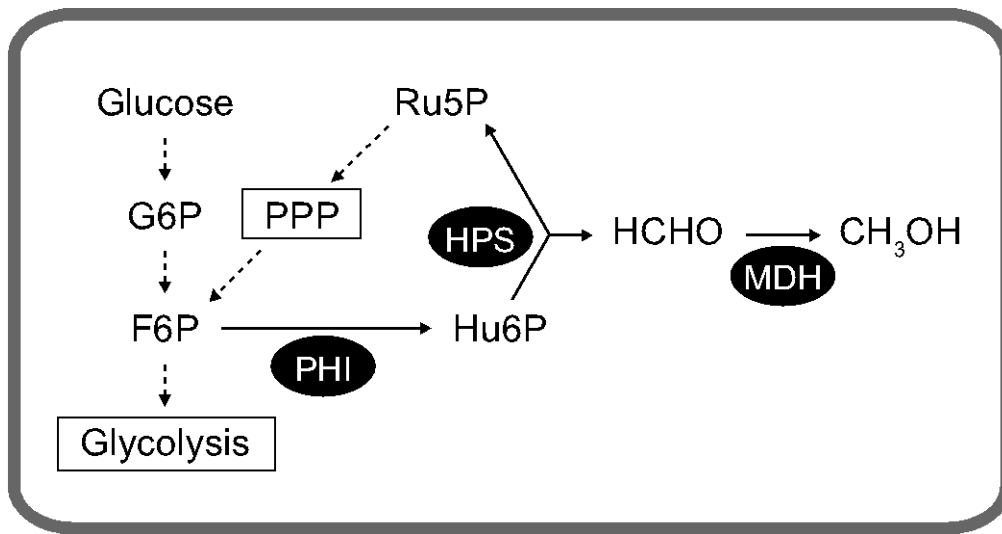


Fig. 2

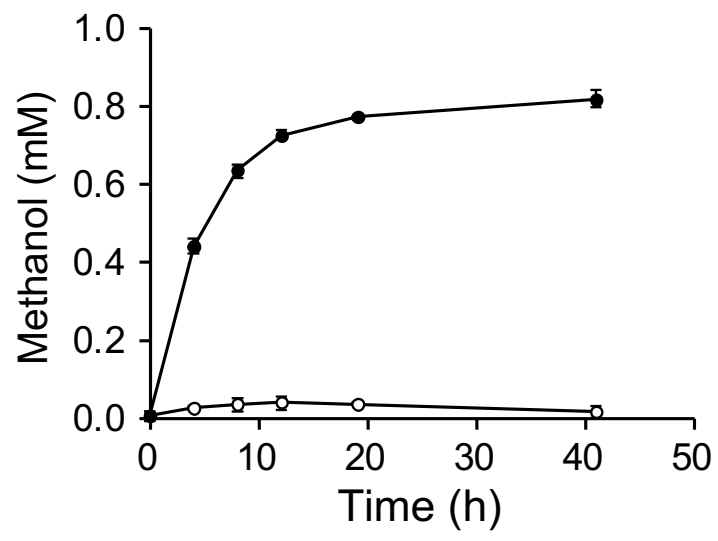


Fig. 3

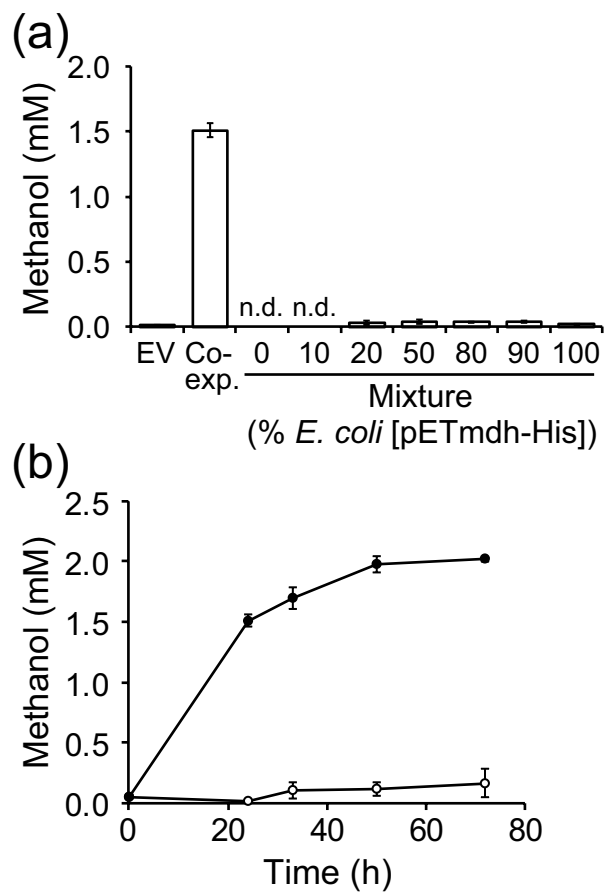
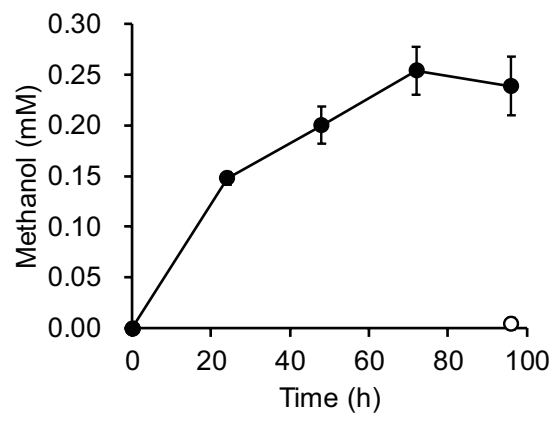


Fig. 4



Supplementary information

Methanol production by reversed methylotrophy constructed in *Escherichia coli*

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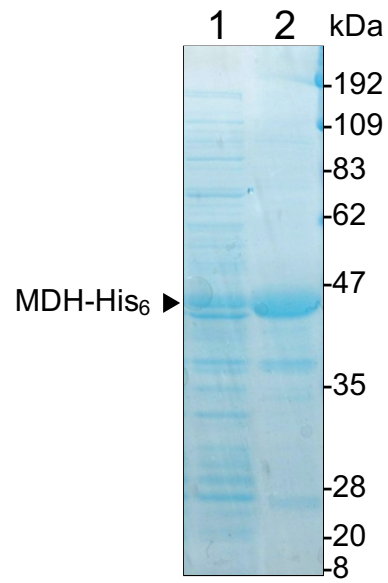


Figure S1. SDS-PAGE analysis of the protein production and purification of recombinant MDH tagged with 6xHis (MDH-His₆) using a Ni-NTA column. Lane 1, Cell-free extract of *E. coli* [pETmdh-His]. Lane 2, Imidazole elution of MDH-His₆ from the Ni-NTA column. The theoretical molecular mass of MDH-His₆ is 42 kDa.

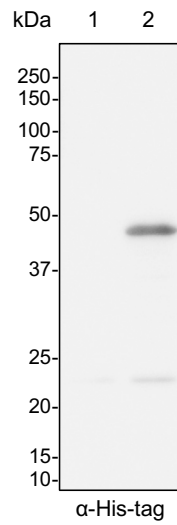


Figure S2. Immunoblot analysis of MDH-His₆ protein in the cell-free extracts of the cells used in Fig. 2. Lane 1, *E. coli* [pET-23a(+)]. Lane 2, *E. coli* [pETmdh-His]. The size of the major band appeared in lane 2 (42 kDa) agreed with that in lane 2 of Fig. S1, confirming the production of MDH-His₆ protein in the *E. coli* [pETmdh-His] cells.

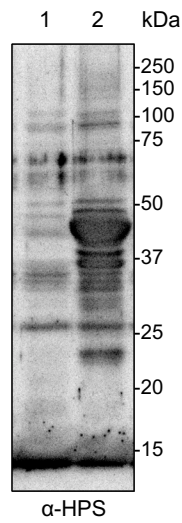


Figure S3. Immunoblot analysis of HPS-PHI protein in the cell-free extracts of *E. coli* [pET-23a(+)] (lane 1) and *E. coli* [pETHps-phi] (lane 2) cells. The size of the major band appeared in lane 2 (42 kDa) agreed with that of purified HPS-PHI observed previously [1], confirming the production of HPS-PHI protein in the *E. coli* [pETHps-phi] cells.

Reference

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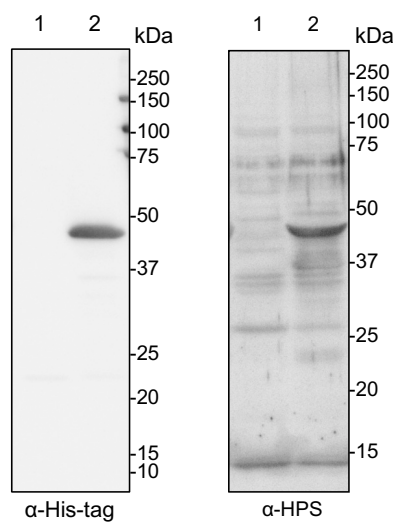


Figure S4. Immunoblot analyses of MDH-His₆ and HPS-PHI proteins in the cell-free extracts of *E. coli* [pETDuet] (lane 1) and *E. coli* [pDmHhp] (lane 2) cells. The sizes of the major bands appeared in lane 2 in α -His-tag and α -HPS-detections agreed with that of Fig. S2 and S3, respectively, confirming the co-production of MDH-His₆ and HPS-PHI proteins.