**Development of Genetic Transformation and Heterologous Expression
System in Carboxydophilic Thermophilic Acetogen *Moorella
thermoacetica***

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30 **Abstract**

31 To develop a microbial production platform based on hydrogen and carbon dioxide,
32 a genetic transformation system for the thermophilic acetogen *Moorella thermoacetica*
33 ATCC39073 was developed. The uracil auxotrophic strain dpyrF was constructed by
34 disrupting *pyrF* for orotate monophosphate decarboxylase. The transformation
35 plasmids were methylated by restriction methylases of *M. thermoacetica* to avoid the
36 decomposition of introduced plasmids by restriction-modification system.
37 Reintroduction of native *pyrF* into the mutant by homologous recombination ensured
38 recovery from uracil auxotrophy. To test heterologous gene expression in dpyrF, the
39 lactate dehydrogenase (LDH) gene (*T-ldh*) from *Thermoanaerobacter pseudethanolicus*
40 ATCC33223 was electroporated into dpyrF with a promoter of the
41 glyceraldehyde-3-phosphate dehydrogenase (G3PD) gene of *M. thermoacetica*
42 ATCC39073. The resulting transformant (C31) successfully transcribed *T-ldh* and
43 exhibited higher LDH activity than ATCC39073 and dpyrF, yielding 6.8 mM of lactate
44 from fructose, whereas ATCC39073 did not produce lactate.

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(Introduction)

Nonrenewable resources such as fossil oil, coal, and gas have been crucial to the development and maintenance of industrialized societies. However, the need for renewable fuels and industrial bulk substrates is escalating because fossil resources are finite and being depleted. Another motivation in the search for renewable energy compounds and technologies is the concern about global warming related to increased CO₂ concentrations in the atmosphere due to the burning of fossil resources. Although the use of biomass has been extensively anticipated as a renewable and sustainable resource, the supply of biomass is not sufficient to satisfy global demand for energy. Moreover, it does not always decrease warming potential to produce biomass because of the extensive use of fertilizers that can increase nitrous oxide emissions (1, 2). Although solar, wind, and water power are also significant renewable energies, they are used mainly for generating electricity, not for producing bulk substrates. New methods, therefore, should be developed for both the production of bulk chemicals and energy from renewable resources that are not restricted to biomass. One approach to solve these problems is the use of H₂ as an energy source and CO₂ as a carbon source. H₂ is a simple and abundant source that will be available even after the complete depletion of fossil resources because it can be generated from various kinds of natural energy via thermal or biological decomposition of biomass (3, 4) [ENREF_4](#) and electrolysis of water (5). If CO₂ can be used as a carbon source, it will contribute to global carbon recycling without emission of CO₂. Thus, we focused on acetogens, a group of anaerobic bacteria that can autotrophically grow on H₂ plus CO₂.

Typically acetogens produce mainly acetate from H₂ plus CO₂ or CO via the

73 acetyl-CoA pathway (6). A large number of mesophilic and thermophilic acetogens
74 have been isolated (6, 7). Among them, mesophilic acetogens such as *Clostridium*
75 *ljungdahlii* (8) and *C. autoethanogenum* (9) can produce ethanol from syngas, mostly a
76 mixture of CO, H₂, and CO₂, derived from biomass. Recently, the whole genome of
77 *Clostridium ljungdahlii* was sequenced, and an electroporation procedure was
78 developed to transform the organism with plasmids bearing heterologous genes to
79 produce butanol (10). This means that mesophilic acetogen can be used as a microbial
80 production platform based on syngas and H₂ plus CO₂.

81 On the other hand, the use of thermophilic acetogens as a microbial
82 production platform besides acetate based on H₂ plus CO₂ (or syngas) has scarcely been
83 considered. However, the genome of *Moorella thermoacetica* has already been
84 sequenced (11), the physiology has been well characterized (12), and we recently
85 reported that *Moorella* sp. HUC-221, a relative of *Moorella thermoacetica*, produced
86 ethanol besides acetate from H₂ plus CO₂ (13, 14)[_ENREF_15](#). The use of
87 thermophilic acetogens for fermentation using H₂ plus CO₂ will facilitate the recovery
88 of alcohols such as ethanol or propanol with a boiling temperature lower than culture
89 broth, thereby enabling the continuous distillation of alcohols. Furthermore,
90 thermophilic bacteria have higher growth and metabolic rates than mesophilic bacteria,
91 and the risk of microbial contamination is lower (15, 16). In this context, thermophilic
92 acetogens are promising candidates for H₂ plus CO₂ (or syngas) fermentation compared
93 to mesophilic bacteria. However, a major limitation for extensive application of
94 thermophilic acetogens has been the lack of genetic transformation systems that would
95 enable the modification of metabolic pathways and microbial physiology for the

96 efficient production of a whole variety of different renewable fuels and chemicals from
97 H₂ plus CO₂. The development of tools for genetic transformation now makes it
98 possible to use *M. thermoacetica* as a novel biotechnological production platform based
99 on H₂ plus CO₂.

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101

102 MATERIALS AND METHODS

103 Bacterial strains and growth conditions

104 *Moorella thermoacetica* ATCC39073 and its derivatives (Table 1) were used in this
105 study. Modified ATCC 1754 PETC medium
106 (<http://www.atcc.org/Attachments/2940.pdf>) was used as the basal medium in 125-ml
107 serum bottles. The basal medium contained 1.0 g of NH₄Cl, 0.1 g of KCl, 0.2 g of
108 MgSO₄·7H₂O, 0.8 g of NaCl, 0.1 g of KH₂PO₄, 0.02 g of CaCl₂·2H₂O, 2.0 g of
109 NaHCO₃, 10 ml of Trace elements (<http://www.atcc.org/Attachments/2940.pdf>), 10 ml
110 of Wolfe's vitamin solution (<http://www.atcc.org/Attachments/2940.pdf>) and 1.0 mg of
111 Resazurin per liter of deionized water. *M. thermoacetica* strains were anaerobically
112 cultured at 55°C with shaking in the medium (initial pH = 6.5). Medium was generally
113 supplemented with H₂ plus CO₂ or 60 mM fructose as the sole carbon source. To
114 culture strains in H₂ plus CO₂, bottles were flushed with a filter-sterilized H₂/CO₂
115 mixture (80:20 v/v) after inoculation to 0.15 MPa final pressure. For lactate
116 production, strains were cultured in medium supplemented with 50 mM phosphate
117 buffer (pH 6.4). *Escherichia coli* HST08 (Takara Bio, Shiga, Japan) was used for
118 plasmid construction and DNA manipulation. *E. coli* TOP10 (Invitrogen, Carlsbad, CA,

119 USA) was used as a host for methyltransferase expression. *E. coli* strains were
120 cultured at 37°C in LB medium supplemented with appropriate antibiotics.

121

122 **Construction of plasmids**

123 To avoid the decomposition of introduced plasmids by the restriction-modification
124 system of *M. thermoacetica* ATCC39073, its putative methyltransferase genes,
125 accession no. CP000232 locus tag Moth_1671–Moth_1672 (Type I) and Moth_2281
126 (Type II) were selected from REBASE (17).

127 PCR primer sets M1672-F/M1672-PstI-R, M1671-PstI-F/M1671-XbaI-R, and
128 M2281-F/M2281-R (Table 2) amplified 1.5, 1.3, and 2.3 kb regions of Moth_1672,
129 Moth_1671, and Moth_2281, respectively, from the genomic DNA of *M. thermoacetica*
130 ATCC39073. Amplified regions of Moth_1672 and Moth_1671 were digested with
131 *PstI* and ligated to the backbone of *SmaI*-digested pBAD33 (18) to obtain pBAD-M12.
132 The amplified region of Moth_2281 was ligated to *SmaI*-digested pBAD33 to obtain
133 pBAD-M81. In plasmids pBAD-M12 and pBAD-M81, a Shine-Dalgarno (SD)
134 sequence region from *M. thermoacetica* ATCC39073 was replaced with that from *E.*
135 *coli*. The 1.3-kb Moth_2281-containing SD sequence region from *E. coli* was
136 amplified by PCR using primer set M2281-XbaI-F/M2281-SphI-R from pBAD-M81,
137 digested with *XbaI/SphI* and ligated to the backbone of *XbaI/SphI*-digested pBAD-M12
138 to obtain pBAD-M1281.

139 To construct a plasmid for the deletion of *pyrF* (accession no. CP000232 locus tag
140 Moth_0883) from the genomic DNA of *M. thermoacetica* ATCC39073, 1.0 kb regions
141 upstream and downstream from *pyrF* from *M. thermoacetica* ATCC39073 were

142 amplified by using primer sets pyrF-up-F/pyrF-up-R and pyrF-dn-F/pyrF-dn-R,
143 respectively (Table 2). Amplified regions were digested with *SpeI* and ligated to the
144 backbone of *SmaI*-digested pK18mob (kindly provided by the National BioResource
145 Project, Japan) (19) to obtain pK18-dpyrF.

146 To construct a plasmid pK18-pyz harboring *pyrF* and a partial fragment of *lacZ* to
147 recover from uracil auxotrophy of dpyrF, the 2.7-kb fragment of *pyrF* was amplified
148 using primer set pyrF-up-F/pyrF-dn-R and ligated to the backbone of the *SmaI*-digested
149 pK18mob (kindly provided by the National BioResource Project, Japan) to obtain
150 pK18-E-pyrF. The 500-bp fragment of *lacZ* (accession no. CP000924, locus tag
151 Teth39_0611) from *T. pseudethanolicus* ATCC33223 was amplified using the primer set
152 of lacZ-500-F/lacZ-500-R. The plasmid pK18-pyz was obtained by cloning the
153 fragment of *lacZ* into the pK18-E-pyrF amplified by inverse PCR using the primer set
154 of pyrF-4-F/pyrF-4-R using an In-Fusion Advantage PCR Cloning Kit (Clontech
155 Laboratories, Mountain View, CA, USA).

156 To construct a plasmid pK18-ldh for heterologous expression of lactate
157 dehydrogenase gene (accession no. CP000924, locus tag Teth39_1997), *T-ldh*, derived *T.*
158 *pseudethanolicus* ATCC33223 in dpyrF, the promoter of glyceraldehyde-3-phosphate
159 dehydrogenase (G3PD) and its SD sequence from *M. thermoacetica* ATCC39073 were
160 amplified using the primer set G3PD-F11/G3PD-SD-R12. The 0.9-kb *T-ldh* was
161 amplified by PCR using the primer set of ldh-F-1/ldh-R-1. PCR fragments of G3PD
162 promoter and *T-ldh* were cloned into pK18-E-pyrF amplified by the primer set of
163 pyrF-in-F1/pyrF-in-R1 using the In-Fusion Advantage PCR Cloning Kit, resulting in
164 plasmid pK18-ldh.

165 For modification of plasmids with putative methylases from *M. thermoacetica*,
166 plasmids pK18-dpyrF, pK18-pyz, and pK18-ldh were introduced into the *E. coli* TOP10
167 harboring plasmid pBAD-M12, pBAD-M18, or pBAD-M1281. The transformants
168 were cultured in LB medium supplemented with 0.005% L-arabinose for
169 methyltransferase expression. Premethylated plasmids were extracted using a NucleSpin
170 Plasmid QuickPure kit (Macherey-Nagel, Düren, Germany).

171

172 **Transformation of *M. thermoacetica* and selection of mutants**

173 For the transformation of *M. thermoacetica* ATCC39073 and the selection of
174 mutants, cells were cultured in the basal medium supplemented with H₂ plus CO₂ and
175 harvested by centrifugation (5,800 × g for 10 min at 4 °C) at OD₆₀₀ = 0.1–0.15. The
176 cells were then washed twice and resuspended in 272 mM sucrose buffer. Plasmids
177 were introduced into *M. thermoacetica* ATCC39073 or dpyrF by electroporation using
178 an Electro Cell Manipulator 620 (BTX, San Diego, CA, USA). Plasmid DNA (1–2
179 µg) was added to 400 µl cell suspensions in an electroporation cuvette with 2 mm gaps.
180 Cells were pulsed at 1.5 kV, 500 Ω, and 50 µF and immediately inoculated into 5-ml
181 basal medium with 2 g/l fructose and 10 µg/ml uracil. The cell suspension was then
182 incubated at 55°C for 48 h; the roll-tube method (20) was used for colony formation.
183 The cell suspensions were inoculated roll-tube vial containing 15ml of basal medium
184 and 2% of agarose. For selecting *pyrF* deletion transformants on roll-tube, 0.2% of
185 5-fluoroorotic acid (5-FOA) and 10 µg/ml of uracil were supplemented into solid
186 medium. Isolated colonies were inoculated into 5-ml of basal medium with 2 g/l
187 fructose and anaerobically cultured at 55°C. Then, cells were screened by PCR using

188 primer sets pyrF-up-F2/pyrF-dn-R2 (Table 2). In all cases, when *pyrF* deletion
189 mutants were cultured, 10 µg/ml of uracil were supplemented into medium.

190

191 **Quantitative reverse transcription PCR (qRT-PCR)**

192 *M. thermoacetica* ATCC39073 and its derivatives were cultured in media
193 supplemented with H₂ plus CO₂ or fructose. Cells were harvested by centrifugation
194 when OD₆₀₀ reached 0.1–0.15 or 0.4–0.8, respectively. Total RNA was extracted from
195 *M. thermoacetica* cells using NucleoSpin RNA II kit (Macherey-Nagel) with
196 RNAProtect bacterial reagent (QIAGEN, Valencia, CA, USA) to stabilize RNA. A
197 One Step SYBR PrimeScript PLUS RT-PCR Kit (Takara Bio) was used for cDNA
198 generation and for qRT-PCR. qRT-PCR was performed using a LightCycler system
199 (Roche Diagnostics, Basel, Switzerland) with reverse transcription at 42°C for 5 min
200 followed by 40 cycles of denaturation (95°C for 5 s), primer annealing (55°C for 30 s),
201 and extension (72°C for 30 s). The primer sets listed in Table 2 were used for the
202 qRT-PCR. DNA gyrase subunit B gene (accession no. CP000232, locus tag
203 Moth_0007), *gyrB*, mRNA was used as an internal standard.

204

205 **Enzyme assays**

206 *M. thermoacetica* ATCC39073 and its derivatives were cultured in the basal
207 medium supplemented with H₂ plus CO₂ or fructose. Cells were harvested by
208 centrifugation (5,800 × g for 10 min at 4 °C) at OD₆₀₀ = 0.1–0.15 or 0.4–0.8,
209 respectively. Cells were then washed, resuspended in 50 mM phosphate buffer (pH
210 6.0), and sonicated. Cell-free extracts were obtained by centrifuging sonicated cells

211 (20,400 × g for 30 min at 4 °C). The protein concentrations in cell-free extracts
212 were determined by the Bradford method. Lactate dehydrogenase activity (LDH) was
213 measured according to the method reported by Contag *et al.* with slight modification
214 (21). LDH activity for pyruvate reduction was spectrophotometrically (A₃₄₀) assayed
215 at 55°C in 50 mM phosphate buffer (1 mM pyruvate, 0.2 mM NADH, and 0.2 mM
216 fructose-1,6-bis phosphate [FBP]; pH 6.0). Enzyme activity (1 U) was defined as the
217 amount of protein that catalyzed the oxidation of 1 μmol NADH per 1 min.

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220

RESULTS

221 Construction of the *M. thermoacetica* ATCC39073 *pyrF* deletion mutant

222 To develop a genetic transformation system for the thermophilic acetogen
223 *Moorella thermoacetica* ATCC39073, a uracil-auxotrophic mutant, dpyrF, was
224 constructed by means of the disruption of *pyrF* for orotate monophosphate
225 decarboxylase (PyrF) (22, 23, 24). PyrF involved in pyrimidine biosynthesis
226 metabolizes 5-FOA to toxic compound, resulting in cell death. Thus, *pyrF* disruption
227 mutant becomes to be resistant to 5-FOA (24). For the disruption of *pyrF*, the
228 wild-type strain was transformed by pK18-dpyrF (Fig. 1A) with upstream and
229 downstream regions of *pyrF* which was methylated by potent restriction methylases of
230 *M. thermoacetica* to avoid the decomposition of introduced plasmids by the
231 restriction-modification system according to the method reported by Yasui et al (25).
232 Among the roughly 100 candidates isolated, only one strain, dpyrF, showed the
233 genotype of *pyrF* deletion; 0.9-kbp and 1.6-kbp DNA fragments were obtained by PCR

234 using total DNA extracted from dpyrF and *M. thermoacetica* ATCC39073, respectively
235 (Fig. 1B). When a uracil auxotrophic test was performed to confirm the phenotype of
236 the mutant dpyrF, it did not grow in a basal medium without uracil supplementation,
237 whereas *M. thermoacetica* ATCC39073 did grow (data not shown). Since these results
238 demonstrated that the dpyrF was a uracil auxotroph and that *pyrF* might be used as a
239 selection marker, dpyrF was used as a transformation host in subsequent experiments.

240

241 **Recovery from uracil auxotrophy of the mutant strain dpyrF by transformation of** 242 **the native *pyrF* gene**

243 To test the genetic transformation system using *pyrF* as the selection marker,
244 plasmid pK18-pyz carrying *pyrF* was introduced into dpyrF by homologous
245 recombination, along with a 500-bp fragment of *lacZ* from *T. pseudethanolicus*
246 ATCC33223 (26) (formerly *T. ethanolicus* 39E) that was constructed as an exogenous
247 DNA tag to distinguish the transformant from the wild-type strain (Fig. 2A). The
248 plasmid was premethylated with a *E. coli* host harboring pBAD-M1281. PCR analysis
249 confirmed the insertion of *pyrF* and the 500-bp DNA tag into the target locus on the
250 genome in all the 30 randomly picked transformants, as shown in Fig. 2B. All of the
251 transformants were recovered from uracil auxotroph. On the other hand, even if
252 pK18mob empty plasmid that is premethylated with pBAD-M1281 as a control was
253 introduced into dpyrF by electroporation, no colonies were observed. These results
254 indicated that it was possible to introduce exogenous DNA into a target locus by using
255 this transformation system.

256

257 **Lactate production by *T-ldh* expression transformants**

258 To demonstrate the modification of the primary metabolic pathway by introducing
259 a heterologous gene into the strain dpyrF, the lactate dehydrogenase (LDH) gene *T-ldh*
260 from *T. pseudethanolicus* ATCC33223 was introduced into dpyrF together with *pyrF* to
261 produce lactate. Although *M. thermoacetica* ATCC39073 possesses the LDH gene
262 *M-ldh* (accession no. CP000232, locus tag Moth_1826) and lactate was produced under
263 nitrate-dissimilating conditions (12), no lactate production by dpyrF was observed under
264 the culture conditions in this study.

265 The plasmid pK18-ldh containing *pyrF* and *T-ldh* constructed to transform dpyrF is
266 illustrated in Fig. 2A. For *T-ldh* expression in dpyrF, the promoter of
267 glyceraldehyde-3-phosphate dehydrogenase (G3PD) from *M. thermoacetica*
268 ATCC39073 was used because the G3PD mRNA transcription level tested by qRT-PCR
269 was relatively high and constitutive in both fructose and H₂/CO₂ (data not shown).
270 After transformation with pK18-ldh premethylated by pBAD-M1281, 30 colonies were
271 randomly picked up, and the insertion of *pyrF* and *T-ldh* into the target locus on the
272 genome was checked by PCR analysis. All transformants possessed *T-ldh*, as shown in
273 Fig. 2C. When lactate production from fructose by the selected transformants was
274 investigated as a preliminary experiment, the transformant strain C31 showed the
275 highest lactate production (data not shown). Thus, C31 was further characterized.

276 To confirm the transcription of exogenous *T-ldh* in C31, qRT-PCR analysis was
277 performed. Simultaneously, the transcription level of *M-ldh*, which *M. thermoacetica*
278 ATCC39073 originally possessed, was also measured. Figures 3A and B show the
279 transcription levels of *M-ldh* and *T-ldh* in strains cultured in the basal medium with

280 fructose or H₂ plus CO₂ as the sole carbon source. *T-ldh* was strongly transcribed only
281 in C31, as expected, while *M-ldh* was equivalently transcribed. The transcription level
282 of *T-ldh* in C31 was 19-fold or 9.5-fold higher than that of *M-ldh* in fructose or H₂ plus
283 CO₂, respectively. These results indicated that the G3PD promoter efficiently operated
284 to translate the *T-ldh* in C31.

285 The specific activity of the LDH in C31 was also assayed (Table 3). When C31
286 was cultured in the basal medium with H₂ plus CO₂ or fructose as the sole carbon
287 source, the specific activity of the NADH-dependent reaction toward pyruvate reduction
288 was 2.68 ± 0.03 U/mg protein and 2.88 ± 0.01 U/mg protein, respectively. These
289 specific activities were 6- to 11-fold higher than those of *M. thermoacetica* ATCC39073
290 and dpyrF. These results demonstrated that heterologous expression was successful in
291 *M. thermoacetica*.

292 When *M. thermoacetica* ATCC39073 and C31 were cultured in the basal medium
293 supplemented with H₂ plus CO₂, neither strain produced L-lactate (data not shown).
294 On the other hand, when *M. thermoacetica* ATCC39073 and C31 were cultured in the
295 basal medium supplemented with 60 mM of fructose, C31 concomitantly produced 6.8
296 mM lactate with acetate production, although profiles of the growth and pH were
297 similar in both strains (Fig. 4A). In contrast, *M. thermoacetica* ATCC39073 did not
298 produce L-lactate (Fig. 4B). This result clearly demonstrates that modification of the
299 metabolic pathway using the tool for molecular biological handling is a powerful
300 technology for the production of useful renewable material in *Moorella* spp.

301

302

Discussion

In this study, we successfully developed a homologous recombination system using *pyrF* as a selection marker for the genetic transformation of *M. thermoacetica* ATCC 39073. The use of *pyrF* for transformation has been reported in yeast, archaea and bacteria, for example, *Saccharomyces cerevisiae* (22), *Thermococcus kodakaraensis* KOD1 (23) and *Clostridium thermocellum* (24), etc. However, transformation system of *M. thermoacetica* ATCC 39073 using similar approach has not been reported. In this context, we constructed the *pyrF* deletion mutant (dpyrF) by means of homologous recombination as the host strain for the gene manipulation. Furthermore, exogenous T-ldh insertion transformant (C31) constructed by the transformation system produced lactate from fructose. This is the first report on production of a non-original metabolite with *M. thermoacetica* by means of genetic engineering.

However, C31 did not produce lactate when the cells were grown with H₂ plus CO₂ unfortunately. Acetogens including *Moorella* strains have a mechanism for CO and CO₂ fixation, known as the reductive acetyl-CoA or the Wood–Ljungdahl pathway (27). *Moorella* strains can grow autotrophically on H₂ plus CO₂ or CO, and usually produce acetate via acetyl-CoA. When C31 was cultured in the basal medium supplemented with fructose as the sole carbon source, a portion of the pyruvate was easily converted to lactate by T-LDH, because fructose is converted to acetyl-CoA via pyruvate by glycolysis. On the other hand, when C31 was grown with H₂ plus CO₂, pyruvate was no longer a major intermediate in the catabolic pathway. It was reported that pyruvate:ferredoxin oxidoreductase (PFOR) of *Clostridium thermoaceticum* (renamed

326 *M. thermoacetica*) catalyzed not only the oxidative decarboxylation of pyruvate to
327 acetyl-CoA and CO₂ but also the reverse reaction, carboxylation of acetyl-CoA and CO₂
328 to pyruvate (28). Pyruvate can be also synthesized from L-malate or oxalate when the
329 cells are cultured in H₂ plus CO₂, because pyruvate is an important building block in
330 biomass synthesis (29). In the case of autotrophic growth with H₂ plus CO₂, however,
331 the intracellular pool of pyruvate might be too small to produce lactate, although
332 detailed information on pyruvate synthesis by *Moorella* strains under the autotrophic
333 condition remains unknown. Further elucidation of the pathways involved in pyruvate
334 synthesis will facilitate the production of not only lactate but also other useful materials
335 via pyruvate from H₂ plus CO₂. The genetic transformation and heterologous
336 expression systems developed in this study are important first steps toward
337 understanding the physiology of *Moorella* strains as a platform for useful material
338 production from H₂ plus CO₂.

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340

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433 *Thermoanaerobium Brockii*, *Clostridium thermosulfurogenes*, and *Clostridium*
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435 *Thermoanaerobacterium thermosulfurigenes* comb. nov., and
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465 **Figure Legends**

466 Fig. 1. Construction of the *M. thermoacetica* ATCC39073 *pyrF* deletion mutant.

467 (A) Premethylated pK18-dpyrF carrying upstream and downstream regions of *pyrF*
468 was introduced into *M. thermoacetica* ATCC39073 by electroporation. *pyrF* in *M.*
469 *thermoacetica* ATCC39073 was deleted by double-crossover homologous
470 recombination with a homologous region of pK18-dpyrF. The *pyrF* deletion mutant
471 dpyrF was selected using 5-FOA. Left and right arrows indicate forward (*pyrF*-up-F2)
472 and reverse (*pyrF*-dn-R2) primers, respectively, for PCR analyses. (B) PCR analysis
473 for checking the deletion of *pyrF* from the microbial genome. Lane 1: DNA ladder
474 marker; Lane 2: 0.9 kb PCR fragment from dpyrF; Lane 3: 1.6 kb PCR fragment from
475 *M. thermoacetica* ATCC39073.

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488 Fig. 2. Complementation of *pyrF* and insertion of exogenous genes into *dpyrF*.

489 (A) Plasmids for transformation were constructed by insertion of the exogenous gene

490 into a downstream region of *pyrF*. The plasmid pK18-pyz was inserted along with a

491 500-bp *lacZ* fragment from *T. pseudethanolicus* ATCC33223 downstream from *pyrF*.

492 The plasmid pK18-ldh was inserted with *T-ldh* from *T. pseudethanolicus* ATCC33223

493 fused with the G3PD promoter and its SD sequence region from *M. thermoacetica*

494 ATCC39073 downstream from *pyrF*. The premethylated plasmids were introduced into

495 *dpyrF* by electroporation and inserted into the target region of *dpyrF* by homologous

496 recombination. Left and right arrows indicate forward (*pyrF*-up-F2) and reverse

497 (*pyrF*-dn-R2) primers, respectively, for PCR analysis. (B) PCR analysis for checking

498 complementation of *pyrF* and insertion of exogenous *lacZ* fragment into *dpyrF*. Lane

499 1: DNA ladder marker; Lane 2: 1.6-kb PCR fragment from *M. thermoacetica*

500 ATCC39073; Lane 3: 0.9-kb PCR fragment from *dpyrF*; Lane 4: 2.3-kb PCR fragment

501 from pK18-pyz transformant. (C) PCR analysis for checking complementation of *pyrF*

502 and insertion of exogenous *T-ldh* into *dpyrF*. Lane 1: DNA ladder marker; Lane 2:

503 1.6-kb PCR fragment from *M. thermoacetica* ATCC39073; Lane 3: 0.9-kb PCR

504 fragment from *dpyrF*; Lane 4: 2.5-kb PCR fragment from C31.

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511 Fig. 3. Transcriptional analysis of the *ldh* genes.

512 The mRNA transcription levels of *M-ldh* and *T-ldh* were normalized to that of the *gyrB*.

513 The mean $\pm$ standard deviations (error bars) for three independent replicates are shown.

514 (A) The *M-ldh* and *T-ldh* transcriptional levels in strains cultured in the basal medium

515 supplemented with H₂ plus CO₂. (B) *M-ldh* and *T-ldh* transcription levels in strains

516 cultured in the basal medium with fructose.

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534 Fig. 4. Growth of *M. thermoacetica* ATCC39073 and dpyrF, and production of lactate
535 and acetate.

536 Each experiment was performed using triplicate samples. The values represent the mean
537 $\pm$ the standard deviation for each point. Basal medium supplemented with 60 mM of
538 fructose as the sole carbon source and 50 mM of phosphate buffer (pH 6.5) was used as
539 the growth medium. Open symbols, *M. thermoacetica* ATCC39073; closed symbols,
540 C31. (A) Time course of growth (circle) and variations in pH (square). (B) Time courses
541 of fructose consumption (circle), acetate production (triangle), and L-lactate production
542 (square).

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Figures

Fig. 1. Kita et al.

Fig. 2. Kita et al.

Fig. 3. Kita et al.

Fig. 4. Kita et al.

24 Table 1. Strains and plasmids used for this study

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Strains or plasmids	Description	Source or reference
Strains		
<i>M. thermoacetica</i> ATCC39073	Type strain	11
<i>M. thermoacetica</i> dpyrF	ATCC39073 Δ <i>pyrF</i>	This study
<i>M. thermoacetica</i> C31	dpyrF derivative, insertion <i>T-ldh</i> from <i>T. pseudethanolicus</i> ATCC33223	This study
<i>E. coli</i> HST08	Cloning host	Takara
<i>E. coli</i> TOP10	Modification host	Invitrogen
Plasmids		
pBAD33	Backbone plasmid for methylation plasmids, Cm ^r	18
pK18mob	Backbone plasmid for transformation plasmids, Km ^r	19
pBAD-M81	pBAD33 carrying the Moth_2281	This study
pBAD-M12	pBAD33 carrying the Moth_1672-Moth_1671	This study
pBAD-M1281	pBAD33 carrying the Moth_1672-Moth_1671-Moth_2281	This study
pK18-dpyrF	pK18mob carrying <i>pyrF</i> 5' and 3' region	This study
pK18-pyz	pK18-dpyrF derivative carrying <i>pyrF</i> and 500bp of <i>lacZ</i> from <i>T. pseudethanolicus</i> ATCC33223	This study
pK18-ldh	pK18-dpyrF derivative carrying <i>pyrF</i> and <i>ldh</i> from <i>T. pseudethanolicus</i> ATCC33223	This study

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33 Table 2. Oligonucleotides used for PCR and qRT-PCR

Primer	Sequence (5' to 3')
Primers for PCR	
M2281-F	TAACAGGAGGAATTAACCATGCGTCGAATATATTCCGGTAATG
M2281-R	ACTGCAGCTCCAGTGAGGCTTCAAAAGG
M1672-F	TAACAGGAGGAATTAACCATGACGGAAAACACCAATATGGAT C
M1672-PstI-R	TGGAACCTGCAGCTTACCCGTTTCAGCCAGCCTCCGAAAC
M1671-PstI-F	GACTGCAGTAACAGGAGGAATTAACCATGGGTAAGGAAGTTA ATG
M1671-XbaI-R	TGGAACCTCTAGAGTTCACTGCCTAAGGGCATC
pyrF-up-F	TGACGTTCTAGACCCTACCTCTCCAAGATTACC
pyrF-up-R	TGACGTACTAGTGGCAAGCAGGCCAGAAG
pyrF-dn-F	TGACGTACTAGTAACTTCGGCCTGCTTTCATGC
pyrF-dn-R	TGACCTGATATCTGTCCAAGCTTATGCACCTTCC
pyrF-up-F2	TGTCCTCAACACCCTCACC
pyrF-dn-R2	TCTTCCCAGGTCCTGTAGG
pyrF-4-F	TCAGTGGTTACTGACTCGGCCTGCTTTCATGCTTG
pyrF-4-R	AGACTAACAACCTTCAAGTTATTATTTACCATCTCTATTTC
lacZ-500-F	TGAAGTTGTTAGTCTTCCTCATGC
lacZ-500-R	GTCAGTAACCACTGATGAACAC
G3PD-F11	GGTGAAATAATAACTGGACGGTTGCCAAGTACC
G3PD-SD-R12	TATGTACTCCTCCTTATATTTATTGTAAACG
pyrF-in-F-1	TCGGCCTGCTTTCATGCTTG
pyrF-in-R1	AGTTATTATTTACCATCTCTATTTC
ldh-F-1	AAGGAGGAGTACATAATGAACAAAATATCTATAATAGGTTTC
ldh-R-1	ATGAAAGCAGGCCGATTATATATCAAGCTCTTGTATTACAC
Primers for qRT-PCR	
(for measurement of <i>gyrB</i> mRNA)	
gyrB-RT-F1	CAGATTCTAGAAGGTCTGGAAGC
gyrB-RT-R1	GTCGATGCTGTTGTCGACC
(for measurement of <i>T-ldh</i> mRNA)	
39E-ldh-RT-F	TCTGGCTTGCCATACGGAAG
39E-ldh-RT-R	AGCTCAGAATCGCCATGCTC
(for measurement of <i>M-ldh</i> mRNA)	
ATCC-ldh-RT-F	ACTCGCCTGGACCTGATC
ATCC-ldh-RT-R	GAGATCTTCCAGGCTACATAGG

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36 Table 3. Enzyme activity of lactate dehydrogenase

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Reaction	Substrate	Strain	Specific activity (U/mg protein) ^a
Pyruvate + NADH → Lactate + NAD ⁺	H ₂ + CO ₂	WT ^b	0.47 ± 0.03
		dpyrF	0.42 ± 0.06
		C31	2.68 ± 0.03
	Fructose	WT	0.25 ± 0.02
		dpyrF	0.28 ± 0.03
		C31	2.88 ± 0.01

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39 ^aEnzyme activity (1 U) was defined as the amount of protein that catalyzed the
 40 oxidation of 1 μmol NADH per 1 min. Values represent the mean and standard deviation
 41 of at least three observations.

42 ^bWT represent *M. thermoacetica* ATCC39073.

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