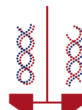

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Development of genetic transformation and heterologous expression system in carboxydotrophic thermophilic acetogen *Moorella thermoacetica*

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

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[**Key words:** Thermophilic; Acetogen; *Moorella*; Transformation; Biomass; Syngas; Fermentation]

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 CO_2 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 H_2 as an energy source and CO_2 as a carbon source. H_2 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) and electrolysis of water (5). If CO_2 can be used as a carbon source, it will contribute to global carbon recycling without emission of CO_2. Thus, we focused on acetogens, a group of anaerobic bacteria that can autotrophically grow on H_2 plus CO_2.

Typically acetogens produce mainly acetate from H_2 plus CO_2 or CO via the acetyl-CoA pathway (6). A large number of mesophilic and thermophilic acetogens have been isolated (6,7). Among them, mesophilic acetogens such as *Clostridium ljungdahlii* (8) and *Clostridium autoethanogenum* (9) can produce ethanol from syngas, mostly a mixture of CO, H_2, and CO_2, derived from biomass. Recently, the whole genome of *C. ljungdahlii* was sequenced, and an electroporation procedure was developed to transform the organism with plasmids bearing heterologous genes to produce butanol (10). This means that mesophilic acetogen can be used as a microbial production platform based on syngas and H_2 plus CO_2.

On the other hand, the use of thermophilic acetogens as a microbial production platform besides acetate based on H_2 plus CO_2 (or syngas) has scarcely been considered. However, the genome of *Moorella thermoacetica* has already been sequenced (11), the physiology has been well characterized (12), and we recently

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reported that *Moorella* sp. HUC-221, a relative of *M. thermoacetica*, produced ethanol besides acetate from H_2 plus CO_2 (13,14). The use of thermophilic acetogens for fermentation using H_2 plus CO_2 will facilitate the recovery of alcohols such as ethanol or propanol with a boiling temperature lower than culture broth, thereby enabling the continuous distillation of alcohols. Furthermore, thermophilic bacteria have higher growth and metabolic rates than mesophilic bacteria, and the risk of microbial contamination is lower (15,16). In this context, thermophilic acetogens are promising candidates for H_2 plus CO_2 (or syngas) fermentation compared to mesophilic bacteria. However, a major limitation for extensive application of thermophilic acetogens has been the lack of genetic transformation systems that would enable the modification of metabolic pathways and microbial physiology for the efficient production of a whole variety of different renewable fuels and chemicals from H_2 plus CO_2. The development of tools for genetic transformation now makes it possible to use *M. thermoacetica* as a novel biotechnological production platform based on H_2 plus CO_2.

MATERIALS AND METHODS

Bacterial strains and growth conditions *M. thermoacetica* ATCC39073 and its derivatives (Table 1) were used in this study. Modified ATCC 1754 PETC medium (http://www.atcc.org/Attachments/2940.pdf) was used as the basal medium in 125-ml serum bottles. The basal medium contained 1.0 g of NH_4Cl, 0.1 g of KCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.8 g of NaCl, 0.1 g of KH_2PO_4, 0.02 g of $CaCl_2 \cdot 2H_2O$, 2.0 g of $NaHCO_3$, 10 ml of Trace elements (http://www.atcc.org/Attachments/2940.pdf), 10 ml of Wolfe's vitamin solution (http://www.atcc.org/Attachments/2940.pdf) and 1.0 mg of Resazurin per liter of deionized water. *M. thermoacetica* strains were anaerobically cultured at 55°C with shaking in the medium (initial pH = 6.5). Medium was generally supplemented with H_2 plus CO_2 or 60 mM fructose as the sole carbon source. To culture strains in H_2 plus CO_2, bottles were flushed with a filter-sterilized H_2/CO_2 mixture (80:20 v/v) after inoculation to 0.15 MPa final pressure. For lactate production, strains were cultured in medium supplemented with 50 mM phosphate buffer (pH 6.4). *Escherichia coli* HST08 (Takara Bio, Shiga, Japan) was used for plasmid construction and DNA manipulation. *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used as a host for methyltransferase expression. *E. coli* strains were cultured at 37°C in LB medium supplemented with appropriate antibiotics.

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

PCR primer sets M1672-F/M1672-PstI-R, M1671-PstI-F/M1671-XbaI-R, and M2281-F/M2281-R (Table 2) amplified 1.5, 1.3, and 2.3 kb regions of Moth_1672, Moth_1671, and Moth_2281, respectively, from the genomic DNA of *M. thermoacetica* ATCC39073. Amplified regions of Moth_1672 and Moth_1671 were digested with *Pst*I and ligated to the backbone of *Sma*I-digested pBAD33 (18) to obtain pBAD-M12. The amplified region of Moth_2281 was ligated to *Sma*I-digested pBAD33 to obtain pBAD-M81. In plasmids pBAD-M12 and pBAD-M81, a Shine-Dalgarno (SD) sequence region from *M. thermoacetica* ATCC39073 was replaced with that from *E. coli*. The 1.3-kb Moth_2281-containing SD sequence region from *E. coli* was amplified by PCR using primer set M2281-XbaI-F/M2281-SphI-R from pBAD-M81, digested with *Xba*I/*Sph*I and ligated to the backbone of *Xba*I/*Sph*I-digested pBAD-M12 to obtain pBAD-M1281.

To construct a plasmid for the deletion of *pyrF* (accession no. CP000232 locus tag Moth_0883) from the genomic DNA of *M. thermoacetica* ATCC39073, 1.0 kb regions upstream and downstream from *pyrF* from *M. thermoacetica* ATCC39073 were amplified by using primer sets pyrF-up-F/pyrF-up-R and pyrF-dn-F/pyrF-dn-R, respectively (Table 2). Amplified regions were digested with *Spe*I and ligated to the backbone of *Sma*I-digested pK18mob (kindly provided by the National BioResource Project, Japan) (19) to obtain pK18-dpyrF.

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

TABLE 2. Oligonucleotides used for PCR and qRT-PCR.

Primer	Sequence (5′–3′)
Primers for PCR	
M2281-F	TAACAGGAGGAATTAACCATGCGTCGAATATATTCCGGTAATG
M2281-R	ACTGCAGCTCCAGTGAGGCTTCAAAAGG
M1672-F	TAACAGGAGGAATTAACCATGACGGAAAACACCAATATGGATC
M1672-PstI-R	TGGAACCTGCAGCTTACCCGTTCAGCCAGCCTCCGAAAC
M1671-PstI-F	GACTGCAGTAACAGGAGGAATTAACCATGGGTAAGGAAGTTAATG
M1671-XbaI-R	TGGAACTCTAGAGTTCACTGCCTAAGGGCATC
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	AGACTAACAACTTCAAGTTATTATTTCACCATCTCTATTTC
lacZ-500-F	TGAAGTTGTTAGTCTTCCTCATGC
lacZ-500-R	GTCAGTAACCACTGATGAACAC
G3PD-F11	GGTGAAATAATAACTGGACGGTTGCCAAGTACC
G3PD-SD-R12	TATGTACTCCTCCTTATATTTATTGTAACG
pyrF-in-F-1	TCGGCCTGCTTTCATGCTTG
pyrF-in-R1	AGTTATTATTTCACCATCTCTATTTC
ldh-F-1	AAGGAGGAGTACATAATGAACAAAATATCTATAATAGGTTC
ldh-R-1	ATGAAAGCAGGCCGATTATATATCAAGCTCTTGTATTACAC
Primers for qRT-PCR	
(For measurement of *gyrB* mRNA)	
gyrB-RT-F1	CAGATTCTAGAAGGTCTGGAAGC
gyrB-RT-R1	GTCGATGCTGTTGTCGACC
(For measurement of *T-ldh* mRNA)	
39E-ldh-RT-F	TCTGGCTTGCCATACGGAAG
39E-ldh-RT-R	AGCTCAGAATCGCCATGCTC
(For measurement of *M-ldh* mRNA)	
ATCC-ldh-RT-F	ACTCGCCTGGACCTGATC
ATCC-ldh-RT-R	GAGATCTTCCAGGCTACATAGG

TABLE 1. Strains and plasmids used for this study.

Strains or plasmids	Description	Source or reference
Strains		
M. thermoacetica ATCC39073	Type strain	11
M. thermoacetica dpyrF	ATCC39073 Δ*pyrF*	This study
M. thermoacetica C31	dpyrF derivative, insertion *T-ldh* from *T. pseudethanolicus* ATCC33223	This study
E. coli HST08	Cloning host	Takara
E. coli 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 *pyrF* 5′ and 3′ region	This study
pK18-pyz	pK18-dpyrF derivative carrying *pyrF* and 500bp of *lacZ* from *T. pseudethanolicus* ATCC33223	This study
pK18-ldh	pK18-dpyrF derivative carrying *pyrF* and *ldh* from *T. pseudethanolicus* ATCC33223	This study

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

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

Transformation of *M. thermoacetica* and selection of mutants For the transformation of *M. thermoacetica* ATCC39073 and the selection of mutants, cells were cultured in the basal medium supplemented with H_2 plus CO_2 and harvested by centrifugation (5800 ×*g* for 10 min at 4°C) at OD_{600} = 0.1–0.15. The cells were then washed twice and resuspended in 272 mM sucrose buffer. Plasmids were introduced into *M. thermoacetica* ATCC39073 or dpyrF by electroporation using an Electro Cell Manipulator 620 (BTX, San Diego, CA, USA). Plasmid DNA (1–2 μg) was added to 400 μl cell suspensions in an electroporation cuvette with 2 mm gaps. Cells were pulsed at 1.5 kV, 500 Ω, and 50 μF and immediately inoculated into 5-ml basal medium with 2 g/l fructose and 10 μg/ml uracil. The cell suspension was then incubated at 55°C for 48 h; the roll-tube method (20) was used for colony formation. The cell suspensions were inoculated roll-tube vial containing 15 ml of basal medium and 2% of agarose. For selecting *pyrF* deletion transformants on roll-tube, 0.2% of 5-fluoroorotic acid (5-FOA) and 10 μg/ml of uracil were supplemented into solid medium. Isolated colonies were inoculated into 5-ml of basal medium with 2 g/l fructose and anaerobically cultured at 55°C. Then, cells were screened by PCR using primer sets pyrF-up-F2/pyrF-dn-R2 (Table 2). In all cases, when *pyrF* deletion mutants were cultured, 10 μg/ml of uracil were supplemented into medium.

Quantitative reverse transcription PCR (qRT-PCR) *M. thermoacetica* ATCC39073 and its derivatives were cultured in media supplemented with H_2 plus CO_2 or fructose. Cells were harvested by centrifugation when OD_{600} reached 0.1–0.15 or 0.4–0.8, respectively. Total RNA was extracted from *M. thermoacetica* cells using NucleoSpin RNA II kit (Macherey–Nagel) with RNAprotect bacterial reagent (Qiagen, Valencia, CA, USA) to stabilize RNA. A One Step SYBR PrimeScript PLUS RT-PCR Kit (Takara Bio) was used for cDNA generation and for qRT-PCR. qRT-PCR was performed using a LightCycler system (Roche Diagnostics, Basel, Switzerland) with reverse transcription at 42°C for 5 min followed by 40 cycles of denaturation (95°C for 5 s), primer annealing (55°C for 30 s), and extension (72°C for 30 s). The primer sets listed in Table 2 were used for the qRT-PCR. DNA gyrase subunit B gene (accession no. CP000232, locus tag Moth_0007), *gyrB*, mRNA was used as an internal standard.

Enzyme assays *M. thermoacetica* ATCC39073 and its derivatives were cultured in the basal medium supplemented with H_2 plus CO_2 or fructose. Cells were harvested by centrifugation (5800 ×*g* for 10 min at 4°C) at OD_{600} = 0.1–0.15 or 0.4–0.8, respectively. Cells were then washed, resuspended in 50 mM phosphate buffer (pH 6.0), and sonicated. Cell-free extracts were obtained by centrifuging sonicated cells (20,400 ×*g* for 30 min at 4°C). The protein concentrations in cell-free extracts were determined by the Bradford method. Lactate dehydrogenase activity (LDH) was measured according to the method reported by Contag et al. with slight modification (21). LDH activity for pyruvate reduction was spectrophotometrically (A_{340}) assayed at 55°C in 50 mM phosphate buffer (1 mM pyruvate, 0.2 mM NADH, and 0.2 mM fructose-1,6-bis phosphate [FBP]; pH 6.0). Enzyme activity (1 U) was defined as the amount of protein that catalyzed the oxidation of 1 μmol NADH per 1 min.

RESULTS

Construction of the *M. thermoacetica* ATCC39073 *pyrF* deletion mutant To develop a genetic transformation system for the thermophilic acetogen *M. thermoacetica* ATCC39073, a uracil-auxotrophic mutant, dpyrF, was constructed by means of the disruption of *pyrF* for orotate monophosphate decarboxylase (PyrF) (22–24). PyrF involved in pyrimidine biosynthesis metabolizes 5-FOA to toxic compound, resulting in cell death. Thus, *pyrF* disruption mutant becomes to be resistant to 5-FOA (24). For the disruption of *pyrF*, the wild-type strain was transformed by pK18-dpyrF (Fig. 1A) with upstream and downstream regions of *pyrF* which was methylated by potent restriction methylases of *M. thermoacetica* to avoid the decomposition of introduced plasmids by the restriction-modification system according to the method reported by Yasui et al. (25). Among the roughly 100 candidates isolated, only one strain, dpyrF, showed the genotype of *pyrF* deletion; 0.9-kbp and 1.6-kbp DNA fragments were obtained by PCR using total DNA extracted from dpyrF and *M. thermoacetica* ATCC39073, respectively (Fig. 1B). When a uracil auxotrophic test was performed to confirm the phenotype of the mutant dpyrF, it did not grow in a basal medium without uracil supplementation, whereas *M. thermoacetica* ATCC39073 did grow (data not shown). Since these results demonstrated that the dpyrF was a uracil auxotroph and that *pyrF* might be used as a selection marker, dpyrF was used as a transformation host in subsequent experiments.

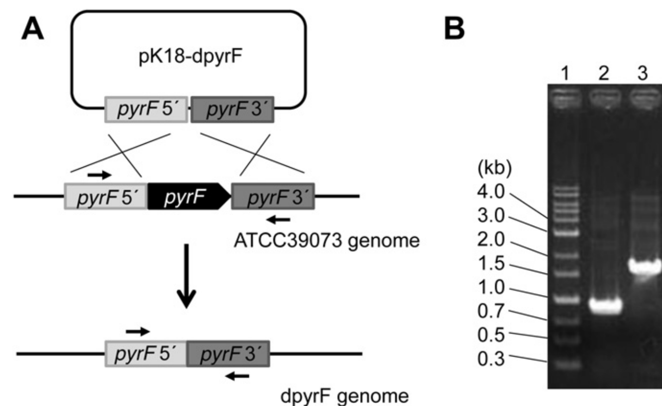

FIG. 1. Construction of the *M. thermoacetica* ATCC39073 *pyrF* deletion mutant. (A) Premethylated pK18-dpyrF carrying upstream and downstream regions of *pyrF* was introduced into *M. thermoacetica* ATCC39073 by electroporation. *pyrF* in *M. thermoacetica* ATCC39073 was deleted by double-crossover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

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

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

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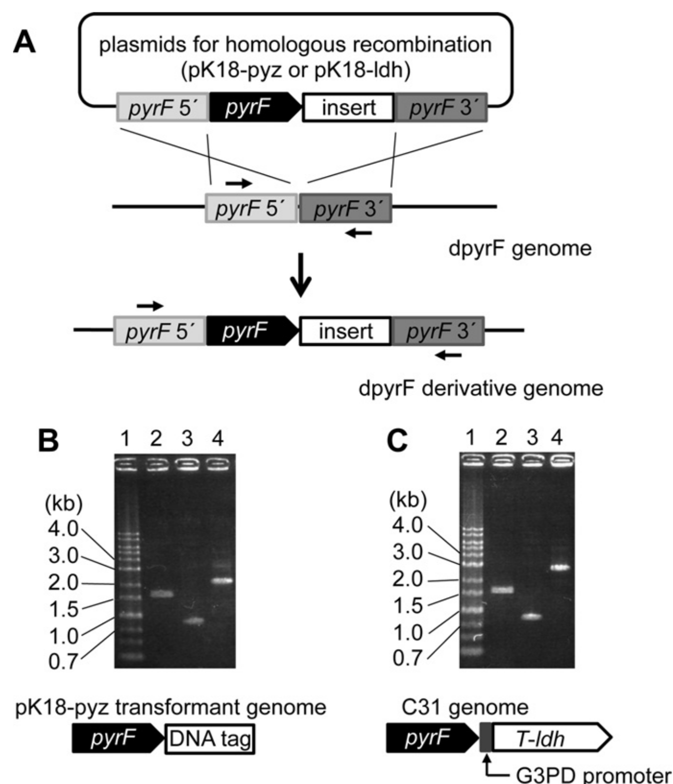

FIG. 2. Complementation of *pyrF* and insertion of exogenous genes into dpyrF. (A) Plasmids for transformation were constructed by insertion of the exogenous gene into a downstream region of *pyrF*. The plasmid pK18-pyz was inserted along with a 500-bp *lacZ* fragment from *T. pseudethanolicus* ATCC33223 downstream from *pyrF*. The plasmid pK18-ldh was inserted with *T-ldh* from *T. pseudethanolicus* ATCC33223 fused with the G3PD promoter and its SD sequence region from *M. thermoacetica* ATCC39073 downstream from *pyrF*. The premethylated plasmids were introduced into dpyrF by electroporation and inserted into the target region of dpyrF by homologous recombination. Left and right arrows indicate forward (pyrF-up-F2) and reverse (pyrF-dn-R2) primers, respectively, for PCR analysis. (B) PCR analysis for checking complementation of *pyrF* and insertion of exogenous *lacZ* fragment into dpyrF. Lane 1: DNA ladder marker; lane 2: 1.6-kb PCR fragment from *M. thermoacetica* ATCC39073; lane 3: 0.9-kb PCR fragment from dpyrF; lane 4: 2.3-kb PCR fragment from pK18-pyz transformant. (C) PCR analysis for checking complementation of *pyrF* and insertion of exogenous *T-ldh* into dpyrF. Lane 1: DNA ladder marker; lane 2: 1.6-kb PCR fragment from *M. thermoacetica* ATCC39073; lane 3: 0.9-kb PCR fragment from dpyrF; lane 4: 2.5-kb PCR fragment from C31.

The plasmid pK18-ldh containing *pyrF* and *T-ldh* constructed to transform dpyrF is illustrated in Fig. 2A. For *T-ldh* expression in dpyrF, the promoter of glyceraldehyde-3-phosphate dehydrogenase (G3PD) from *M. thermoacetica* ATCC39073 was used because the G3PD mRNA transcription level tested by qRT-PCR was relatively high and constitutive in both fructose and H_2/CO_2 (data not shown). After transformation with pK18-ldh premethylated by pBAD-M1281, 30 colonies were randomly picked up, and the insertion of *pyrF* and *T-ldh* into the target locus on the genome was checked by PCR analysis. All transformants possessed *T-ldh*, as shown in Fig. 2C. When lactate production from fructose by the selected transformants was investigated as a preliminary experiment, the transformant strain C31 showed the highest lactate production (data not shown). Thus, C31 was further characterized.

To confirm the transcription of exogenous *T-ldh* in C31, qRT-PCR analysis was performed. Simultaneously, the transcription level of *M-ldh*, which *M. thermoacetica* ATCC39073 originally possessed, was also measured. Fig. 3A and B show the transcription levels of *M-ldh* and *T-ldh* in strains cultured in the basal medium with fructose or H_2 plus CO_2 as the sole carbon source. *T-ldh* was strongly transcribed only in C31, as expected, while *M-ldh* was equivalently transcribed. The transcription level of *T-ldh* in C31 was 19-fold or

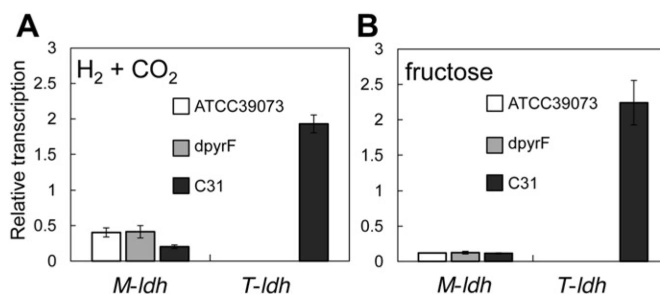

FIG. 3. Transcriptional analysis of the *ldh* genes. The mRNA transcription levels of *M-ldh* and *T-ldh* were normalized to that of the *gyrB*. The mean ± standard deviations (error bars) for three independent replicates are shown. (A) The *M-ldh* and *T-ldh* transcriptional levels in strains cultured in the basal medium supplemented with H_2 plus CO_2. (B) *M-ldh* and *T-ldh* transcription levels in strains cultured in the basal medium with fructose.

9.5-fold higher than that of *M-ldh* in fructose or H_2 plus CO_2, respectively. These results indicated that the G3PD promoter efficiently operated to translate the *T-ldh* in C31.

The specific activity of the LDH in C31 was also assayed (Table 3). When C31 was cultured in the basal medium with H_2 plus CO_2 or fructose as the sole carbon source, the specific activity of the NADH-dependent reaction toward pyruvate reduction was 2.68 ± 0.03 U/mg protein and 2.88 ± 0.01 U/mg protein, respectively. These specific activities were 6- to 11-fold higher than those of *M. thermoacetica* ATCC39073 and dpyrF. These results demonstrated that heterologous expression was successful in *M. thermoacetica*.

When *M. thermoacetica* ATCC39073 and C31 were cultured in the basal medium supplemented with H_2 plus CO_2, neither strain produced L-lactate (data not shown). On the other hand, when *M. thermoacetica* ATCC39073 and C31 were cultured in the basal medium supplemented with 60 mM of fructose, C31 concomitantly produced 6.8 mM lactate with acetate production, although profiles of the growth and pH were similar in both strains (Fig. 4A). In contrast, *M. thermoacetica* ATCC39073 did not produce L-lactate (Fig. 4B). This result clearly demonstrates that modification of the metabolic pathway using the tool for molecular biological handling is a powerful technology for the production of useful renewable material in *Moorella* spp.

DISCUSSION

In this study, we successfully developed a homologous recombination system using *pyrF* as a selection marker for the genetic transformation of *M. thermoacetica* ATCC39073. The use of *pyrF* for transformation has been reported in yeast, archea and bacteria, for example, *Saccharomyces cerevisiae* (22), *Thermococcus kodakaraensis* KOD1 (23) and *Clostridium thermocellum* (24), etc. However, transformation system of *M. thermoacetica* ATCC39073 using similar approach has not been reported. In this context, we constructed the *pyrF* deletion mutant (dpyrF) by means of homologous

TABLE 3. Enzyme activity of lactate dehydrogenase.

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

[a] Enzyme activity (1 U) was defined as the amount of protein that catalyzed the oxidation of 1 μmol NADH per 1 min. Values represent the mean and standard deviation of at least three observations.
[b] WT represent *M. thermoacetica* ATCC39073.

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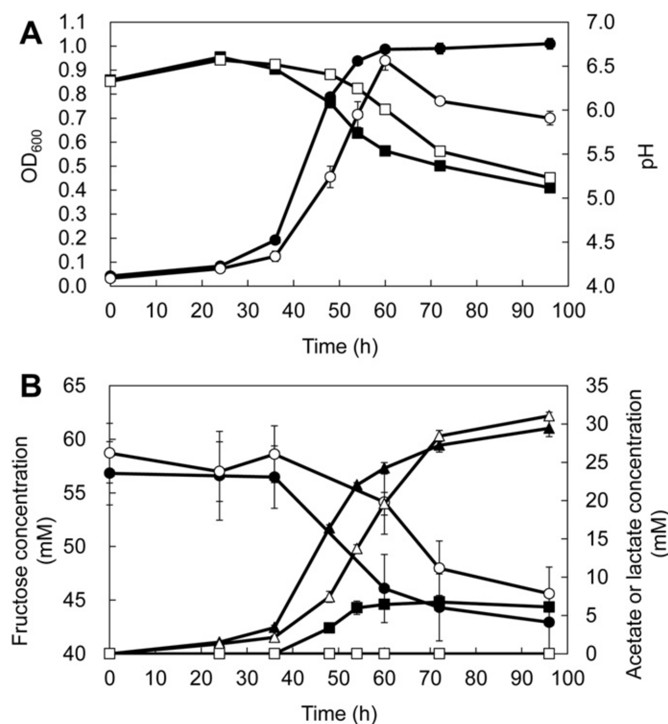

FIG. 4. Growth of *M. thermoacetica* ATCC39073 and dpyrF, and production of lactate and acetate. Each experiment was performed using triplicate samples. The values represent the mean ± the standard deviation for each point. Basal medium supplemented with 60 mM of fructose as the sole carbon source and 50 mM of phosphate buffer (pH 6.5) was used as the growth medium. Open symbols, *M. thermoacetica* ATCC39073; closed symbols, C31. (A) Time course of growth (circles) and variations in pH (squares). (B) Time courses of fructose consumption (circles), acetate production (triangles), and L-lactate production (squares).

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 H_2 plus CO_2 unfortunately. Acetogens including *Moorella* strains have a mechanism for CO and CO_2 fixation, known as the reductive acetyl-CoA or the Wood–Ljungdahl pathway (27). *Moorella* strains can grow autotrophically on H_2 plus CO_2 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 H_2 plus CO_2, pyruvate was no longer a major intermediate in the catabolic pathway. It was reported that pyruvate:ferredoxin oxidoreductase (PFOR) of *Clostridium thermoaceticum* (renamed *M. thermoacetica*) catalyzed not only the oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2 but also the reverse reaction, carboxylation of acetyl-CoA and CO_2 to pyruvate (28). Pyruvate can be also synthesized from L-malate or oxalate when the cells are cultured in H_2 plus CO_2, because pyruvate is an important building block in biomass synthesis (29). In the case of autotrophic growth with H_2 plus CO_2, however, the intracellular pool of pyruvate might be too small to produce lactate, although detailed information on pyruvate synthesis by *Moorella* strains under the autotrophic condition remains unknown. Further elucidation of the pathways involved in pyruvate synthesis will facilitate the production of not only lactate but also other useful materials via pyruvate from H_2 plus CO_2. The genetic transformation and heterologous expression systems developed in this study are important first steps toward understanding the physiology of *Moorella* strains as a platform for useful material production from H_2 plus CO_2.

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