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Construction and Characterization of Fermentative Lactate Dehydrogenase *Escherichia coli* Mutant and Its Potential for Bacterial Hydrogen Production

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Abstract

In *Escherichia coli*, classified as a mixed-acid producer in fermentation, D-lactate is one of the final metabolites from pyruvate. In order to achieve a high efficiency of bacterial hydrogen production from glucose, we have constructed an *E. coli* strain deficient in fermentative lactate dehydrogenase (LDH-A) by P1 transduction. The mutant, designated as MC13-4, entirely lost LDH-A activity while retaining whole formate hydrogenlyase activity. This mutation resulted in an increase in hydrogen production based on glucose consumed. The effect of uptake hydrogenases on the hydrogen production was also discussed.

Index Entries: Metabolic engineering; lactate dehydrogenase; hydrogen production; *Escherichia coli*.

Introduction

The prospect of using hydrogen as an alternative and sustainable energy source has contributed to a surge of interest in hydrogen production by microorganisms. Bacterial hydrogen production based on anaerobic fermentation is an attractive process to reduce organic waste *(1–4)*. A typical and representative research on the production of hydrogen from waste has been carried out using molasses from sugar manufacturing.

Recent progress in molecular biology on the anaerobic metabolism of *Escherichia coli* has elucidated the enzymes and genes responsible for

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fermentative hydrogen production *(5,6)*. Moreover, with the availability of whole genomic information, *E. coli* is an attractive microorganism to form the main framework of an ideal metabolic pathway for bacterial hydrogen production based on metabolic-engineering approaches.

E. coli produces hydrogen by the formate hydrogenlyase (FHL) system. In order to improve the efficiency of bacterial hydrogen production, several metabolic branching points on carbon and/or electron flux from glucose to FHL should be precisely examined. One of such metabolic branches exists in the utilization of pyruvate. In order to utilize pyruvate for the FHL system, it should be consumed by pyruvate formate lyase (PFL). However, pyruvate can also be consumed by the fermentative lactate dehydrogenase (LDH-A). Recently, the structural gene of LDH-A, *ldh*A, was cloned, sequenced, and its biochemical regulation was also elucidated *(7)*. Because the production of lactate by LDH-A is one of the major metabolites during the mixed-acid fermentation of *E. coli*, the elimination of LDH-A activity will make possible the construction of an ideal *E. coli* strain that produces hydrogen with high efficiency.

In this article, we report the construction and characterization of an *E. coli* strain lacking in LDH-A activity, and demonstrate its potential for bacterial hydrogen production.

Materials and Methods

Bacterial Strains and Cultivation

Bacterial strains used in this study are summarized in Table 1. Aerobic cultivation of *E. coli* cells were carried out using Luria broth (LB) at 37°C. Anaerobic cultivation was carried out using a complex medium (1% trypton, 0.5% yeast extract, 0.4% glucose, 100 m*M* potassium phosphate, 15 m*M* sodium formate, 1 µ*M* sodium molybdate, and 5 µ*M* nickel chloride, pH 6.5), in a 30-mL culture flask containing 10 mL medium under Ar atmosphere at 37°C. Chloramphenicol was added to the culture medium at a concentration of 30 mg/mL for the maintenance of the strain harboring chloramphenicol acetyl transferase (CAT) gene.

Construction of an LDH-A Mutant by P1 Transduction

P1 transduction was carried out according to Miller *(9)*. First, an *E. coli* strain KF1344 was used as the donor and FMJ39A as the recipient, and the allele containing the CAT gene of KF1344 was transferred. The resulted transductants were subjected to the LDH-A assay. Among the Cm^R colonies, a clone maintaining LDH-A mutation was selected, and designated as FM#13. Then, using FM#13 as the donor, the second P1 transduction was carried out using MC4100 as the recipient, in order to transfer the allele containing both Cm^R and the LDH-A mutation. Among the colonies showing Cm^R, a clone showing a lack of LDH-A activity was selected and designated as FMJ13-4.

Table 1
Bacterial Strains

Strains	Relevant characteristics	Source or reference
FMJ39	*ldhA* derivatives of LCB898	*(10)*
FMJ39A	*pfl*$^+$ derivative of FMJ39	This study
KF1344	F$^-$ *zda-268*::Is *10*-Cam^r-Is*10 recB21 recC22 sbcA6 lacZ4 gal-44 supE44 endA1 deo-27 [rfa]pX^s-E coliC*	National Institute of Genetics (Japan)
FMJ#13	*zda-268*::Is *10*-Cam^r-Is*10* of FMJ39A	This study
MC4100	F$^-$ *araD* DE(*argF-lac*)*relA rpsL thiA flbB deoC ptsF*	National Institute of Genetics (Japan)
MC13-4	*ldh*A and *zda-268*::Is *10*-Cam^r-Is *10* of MC4100	This study

Hydrogen Production and Metabolite Analyses

E. coli cells were cultivated anaerobically for 5 h, and cells were harvested by centrifugation (3000*g*, 4°C), washed three times in 100 m*M* potassium phosphate buffer, pH 6.5, and resuspended in 41 mL of the same buffer in a 83 mL Schlenk's tube. The cell suspension thus prepared was treated by repeated Ar gas purges and degassing procedures in order to set up Ar atmospheric anaerobic conditions in the reaction tube. The reactions for the hydrogen production were initiated by the addition of 3 m*M* glucose at 37°C. The gas phase of the reaction tube was analyzed every hour using thermal conductivity detector (TCD) type gas chromatography (GC-8AIT, Shimadzu Corp., Kyoto, Japan) equipped with an 1-mL sample loop.

The organic acids in the culture were determined by high-performance liquid chromatography (HPLC; Series 8020, Tosoh, Tokyo, Japan) using a reversed-phase column (Capcell Pak C18 AG120, 4.0 × 250 mm, Shiseido Inc., Yokohama, Japan) and 100 m*M* phosphate as the eluent at 0.5 mL/min. The organic acids were detected by the adsorption at 210 nm. Glucose and ethanol concentrations were determined enzymatically.

Measurement of Enzyme Activities

The measurement of LDH-A was carried out according to Tarmy et al. *(9)*, measuring decrease in absorbance of NADH at 340 nm in the presence of 28 m*M* of pyruvate. Each sample of *E. coli* cells prepared as above was disrupted by an ultrasound disrupter (Bioruptor UCD-200TM, Cosmo-bio Inc., Tokyo, Japan), at 200 W output for about 5 min under ice freezing conditions. The cell lysate was then centrifuged (5000 rpm, 1 h, 4°C) and the nondisrupted cells removed. The supernatant thus obtained was used as the sample for LDH-A assay.

FHL activity was determined by monitoring the rate of hydrogen production from 3 m*M* formate. Cell-sample preparation was carried out in the same way as the preparation of cells for hydrogen production from glucose.

Results and Discussion

Construction and Characterization of an E. coli *Strain Lacking Fermentative Lactate Dehydrogenase*

We first constructed an *E. coli* strain lacking LDH-A. Mat-Jan et al. *(10)* previously reported a mutant strain lacking in both *pfl* and *ldh*A, constructed by chemical mutagenesis, FMJ39. During the successive cultivations of this strain in our laboratory, a spontaneous revertant that lacks only LDH-A was obtained, and designated FMJ39A. Considering that FMJ39A is a chemical mutant strain that might have unknown mutations relating in hydrogenase expression or activity, and was also unstable as it resulted spontaneously from FMJ39, we decided to construct a new LDH-A mutant using *E. coli* MC4100, which we have been using as the parental control strain for hydrogen production studies. Using FMJ39A, we transferred this mutation into MC4100 by P1 transduction, marking the locus (30.3 min) of LDH-A gene. An *E. coli* strain, KF1344, which harbors CAT genes at 31 min, was used as the donor, and FMJ39A was used as the recipient for the P1 transduction. Among the various Cm^R colonies that appeared after P1 transduction, about 13% of the colonies were revealed to be deficient in LDH-A activity. One of these clones, designated FMJ#13, was then used as the donor, and MC4100 was used as the recipient for the second P1 transduction. The resulted strain was named MC13-4.

Table 2 summarizes the LDH-A and FHL activity of various *E. coli* strains. MC13-4 completely lost LDH-A activity. In contrast, MC13-4 showed almost the same FHL activity as its parent strain, MC4100. Therefore, this mutation did not affect the FHL system.

Bacterial Hydrogen Production by an E. coli *LDH-A Mutant*

Figure 1 shows the time courses of hydrogen production from 3 m*M* glucose (123 µmol glucose in a batch reaction), and also the time courses of other metabolites, by the strain MC4100 and its mutant, MC13-4, which lacks LDH-A.

Both strains consumed glucose within 6 h. Because MC13-4 lacks LDH-A, it did not produce lactate, whereas MC4100 accumulated about 40 µmol of lactate. The hydrogen production rate and the amount of hydrogen produced by MC13-4 were at almost the same levels as those achieved by MC4100. After 4 h, the sum of formate and hydrogen reached a maximum, about 120 µmol for MC4100 and about 140 µmol for MC13-4, and maintained their levels for a further 4 h.

Figure 2 summarizes the fluxes of glucose utilized for each metabolite production after 6 h of incubation, when glucose disappeared from the reaction solutions and also the sum of formate and hydrogen reached a plateau.

Glucose is consumed by PFL, subsequently resulting in formate, hydrogen, acetate, and ethanol. Among the glucose consumed by the PFL

Table 2
LDH-A and FHL Activity of Various *E. coli* Strains

Strain	LDH activity (U/mg protein)	FHL activity (μmol/mg protein/h)[a]
FMJ39A	0.00	NT[b]
MC4100	4.47	1.33
MC13-4	0.00	1.11

[a]FHL activity was determined by H_2 production rate (μmol/mg protein/h) of from 3 m*M* formate.
[b]NT, not tested.

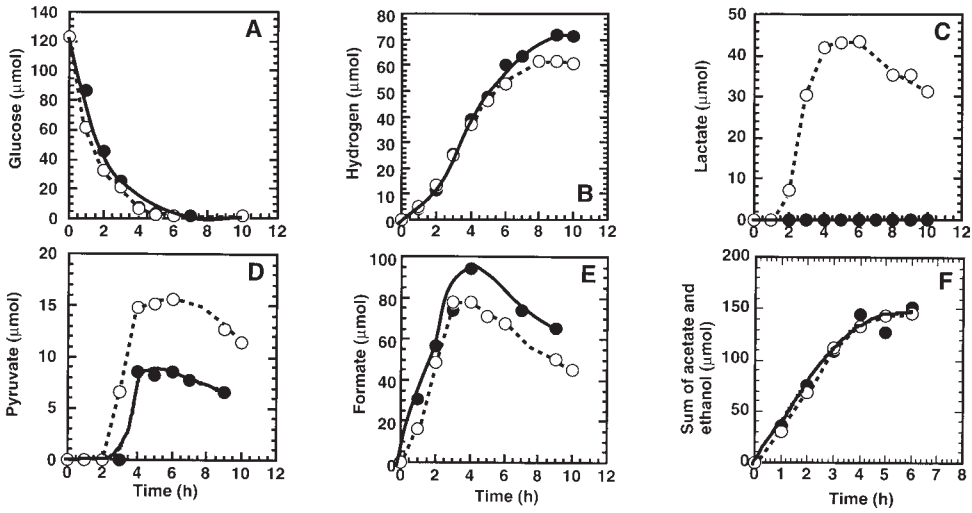

Fig. 1. Time courses of glucose consumption and metabolites production during hydrogen production using the strain MC4100 (open circular) and its LDH-A mutant, MC13-4 (closed circular). **(A)** glucose consumption, **(B)** hydrogen production, **(C)** lactate production, **(D)** pyruvate production, **(E)** formate production, and **(F)** sum of the produced ethanol and acetic acid.

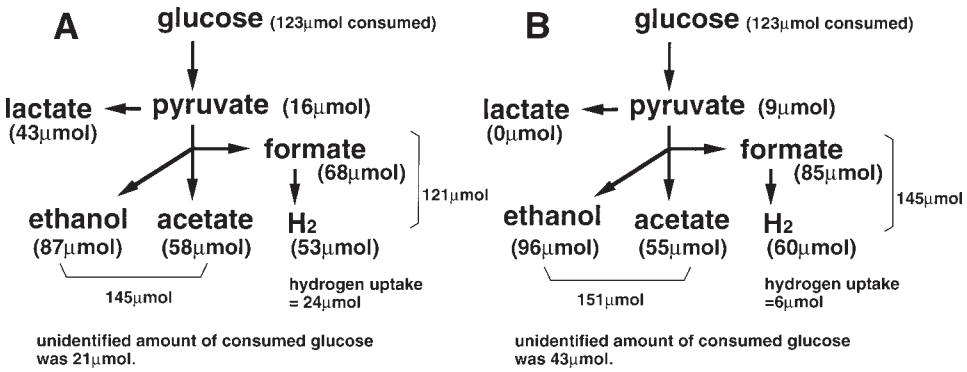

Fig. 2. Metabolic balance for hydrogen production from glucose. **(A)** MC4100, **(B)** MC13-4. These calculations are based on the metabolic fluxes at 6 h of incubation. The amount of formate of MC13-4 is estimated from Fig. 1E.

Table 3
Hydrogen Production Yield and Efficiency[a]

Strains	Theoretical hydrogen yield, %[b]	Hydrogen production efficiency, %[c]	Glucose consumption ratio, %[d]	Hydrogen uptake, %[e]
MC4100	22	26	83	17
MC13-4	24	38	65	3

[a]This calculation is based on that 2 molecules of hydrogen are produced from 1 molecule of glucose. Amount of glucose consumed in PFL-LDH is estimated by the sum of the amount lactate production, pyruvate production, acetate production, and ethanol production; 204 µmol in MC4100 and 160 µmol in MC13-4.

[b]Hydrogen yield = (hydrogen produced/total glucose consumed × 2) × 100.

[c]Hydrogen production efficiency = (hydrogen produced/glucose consumed in PFL-LDH) × 100.

[d]Glucose consumption ratio = (glucose consumed in PFL-LDH/total glucose consumed × 2) × 100.

[e]Hydrogen uptake = [1 – (hydrogen and formate produced/acetate and ethanol produced)] × 100.

system and LDH-A, MC4100 utilized 21% of glucose flux for lactate production and 71% for the FHL system, and 8% for pyruvate. In contrast, MC13-4 utilized 94% of the glucose flux for the FHL system, and the other 6% for pyruvate. Moreover, the sum of formate and hydrogen produced by MC13-4 was higher than that of MC4100. Therefore, the elimination of LDH-A resulted in an increase in the glucose flux that is utilized for formate and hydrogen production. Considering that the amount of hydrogen produced in a batch system was also affected by the equilibrium between formate concentration and hydrogen, MC13-4 may produce a large amount of hydrogen utilizing accumulated formate in an open system compared with MC4100. However, the mutation decreased the flux of glucose utilized in the PFL system. In the strain MC4100, among the total glucose consumed, 83% of the flux was utilized by the PFL system and LDH-A, whereas in the mutant strain MC13-4, only 65% of the total flux was utilized by the PFL system.

On the basis of the accumulated amount of ethanol and acetate, the amount of hydrogen consumed by the uptake hydrogenases, Hyd1 and Hyd2, can be calculated. Assuming that the sum of the accumulated amount of ethanol and acetate should have an equal number of moles to the sum of the hydrogen and formate, MC 4100 should produce 77 µmol of hydrogen and MC13-4 should produce 66 µmol of hydrogen. However, only 53 µmol and 60 µmol of hydrogen were detected by gas chromatography (GC) analyses. Therefore, the rest of the hydrogen was assumed to have been consumed by the uptake hydrogenases, 24 µmol hydrogen for MC4100 and 6 µmol hydrogen for MC13-4. Therefore, LDH-A mutation also repressed the effect of uptake hydrogenase.

Table 3 summarizes the yield and efficiency of hydrogen production by the engineered *E. coli* strain, MC13-4, and its parent strain, MC4100. The

calculation was based on the fact that, ideally, 2 molecules of hydrogen can be produced from one molecule of glucose. The yields of hydrogen based on the total glucose consumed are 22 and 24 % for the parent strain and MC13-4, respectively. On the basis of the calculation of the amount of glucose utilized at the downstream of pyruvate, PFL/LDH/FHL, this mutation yielded a 50% increase, from 26 to 38%. The ratios of the hydrogen consumed by the uptake hydrogenases were 17 and 3% for MC4100 and MC13-4, respectively.

These results suggest that by eliminating LDH-A, hydrogen production efficiency is in principle increased. The physiological roles of uptake hydrogenases are still unknown; however, these enzymes may have an impact on the redox status of anaerobic metabolism and/or formate/hydrogen equilibrium. The LDH-A mutation should result in the accumulation of NADH. Considering that the uptake hydrogenase catalyzes the oxidation of hydrogen, the decrease in uptake hydrogenase activity might complement the LDH-A mutation on the basis of the cellular redox balance of native cells. However, the complementation of LDH-A by the repression of uptake hydrogenase might not be sufficient, and subsequently affect the glucose flux utilized in the PFL system. The further elucidation of the role of uptake hydrogenases is essential in order to advance the improvement of the *E. coli* hydrogen production system.

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