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Expression of Acetaldehyde Dehydrogenase Gene Increases Hydrogen Production and Acetate Consumption by *Rhodobacter sphaeroides*

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Abstract

***Rhodobacter sphaeroides* RV (RV) produces high yields of hydrogen from organic acids in the presence of light. The hydrogen production from acetate is lower than that from lactate, probably because of its low ability to metabolize acetate. In this study, gene of acetaldehyde dehydrogenase (ACDH, EC 1.2.1.10) that catalyzes the reversible conversion of acetaldehyde and CoA to acetyl-CoA with the concurrent reduction of NAD to NADH, is overexpressed in the RV strain. The produced acetyl-CoA can be oxidized to carbon dioxide in the tricarboxylic acid (TCA) cycle, wherein electrons are generated and used for hydrogen production. The byproduct NADH can be used as reducing agent for acetate to produce acetaldehyde by acetate dehydrogenase. The recombinant RV strain (RVAC) expressing the *ACDH* gene showed ACDH activity with a specific activity of 3.2 mU/mg, and the RV and the recombinant RV strain that harbored the intact (empty) plasmid pLP-1.2 (RVI) showed no detectable ACDH activity. The hydrogen yields of the RVAC strain from 21-mM acetate were 1.5-fold higher than that of the wild type RV strain and also that of the RVI strain. In contrast, hydrogen yield from 21-mM lactate was 30% lower than that in the control strains.**

Keywords

Acetate, Lactate, Photosynthetic Bacteria, *Rhodobacter sphaeroides*, Aldehyde Dehydrogenase

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1. Introduction

Hydrogen production by using renewable biomass is an attractive alternative to hydrogen production using fossil feedstocks [1]. Hydrogen can be produced by using two types of fermentation reactions: dark- and photo-fermentation [2]-[5]. In dark fermentation, glucose is used as a substrate and organic acids such as lactate and acetate are produced as byproducts under anaerobic conditions. Photo-fermentation involves the utilization of various organic acids as substrates. Therefore, hydrogen can be efficiently produced by using co-fermentation reactions that involve both dark- and photo-fermentation reactions. *Rhodobacter sphaeroides* RV (RV) is a hydrogen-producing photosynthetic bacterium that is commonly used for co-fermentation [6]-[10]. However, the acetate yield (~21 mM) during dark-fermentation by hydrogen-producing anaerobic bacteria is substantially lower than the acetate concentration (75 mM) required by the RV strain for efficient hydrogen production as previously described [7] [11].

Previously, we have shown that the expression of the gene of aldehyde dehydrogenase (ALDH, EC 1.2.1.3) which catalyzes conversion of acetaldehyde and NAD to acetate and NADH respectively [12], in an RV strain (RVAD) increases hydrogen production by 1.4-fold as compared with the RV strain [7]. The RVAD strain consumes less amount of acetate which is a substrate for hydrogen production as compared with the RV strain during fermentation. These facts suggest that ALDH produces acetate from acetaldehyde and the RV stain include considerable amount of acetaldehyde during fermentation.

Acetaldehyde dehydrogenase (ACDH, EC 1.2.1.10) catalyzes the reversible conversion of acetaldehyde and CoA to acetyl-CoA with the concurrent reduction of NAD to NADH [13] [14]. The produced acetyl-CoA is oxidized to carbon dioxide in the tricarboxylic acid (TCA) cycle, wherein electrons are generated and used for proton reduction to produce hydrogen [15] [16]. Overexpression of ACDH may increase acetyl-CoA and thus increase hydrogen production. However, no *ACDH* gene is found in the four *R. sphaeroides* species (*R. sphaeroides* strains 2.4.1, ATCC17025, ATCC17029, and KD131), whose complete genome sequences are available on the Kyoto encyclopedia of genes and genomes (KEGG) database [17] [18]. Here, the well-studied ACDH (MhpF) from *Escherichia coli* [19] is overproduced in the RV strain. Both the hydrogen production and the retained acetate levels in the ACDH-expressing RV strain (RVAC) were compared with those of RVAD and the wild-type RV strains.

2. Materials and Methods

2.1. Hydrogen Production and Yield

Hydrogen was produced under previously reported culture conditions [7]. The experimental procedure is briefly explained. The hydrogen production medium (pH 7.0) was composed of various concentrations of acetate or lactate. The basal medium contained a 1% (v/v) inorganic solution [1.18 g/L $FeSO_4 \cdot 7H_2O$, 0.28 g/L boric acid, 0.227 g/L $MnSO_4 \cdot 5H_2O$, 75 mg/L $Na_2MoO_4 \cdot 2H_2O$, 24 mg/L $ZnSO_4 \cdot 7H_2O$, 4 mg/L $Cu(NO_3)_2 \cdot 3H_2O$, and 2.0 g/L EDTA(2Na)], a 1% (v/v) vitamin solution (5 mg/L biotin, 5.0 g/L thiamin hydrochloride, 0.15 g/L p-aminobenzoic acid, 5.0 g/L nicotinic acid, and 0.15 g/L nicotinamide), 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 7.33 g/L K_2HPO_4, and 8.66 g/L KH_2PO_4. The bacteria were pre-cultured in a 20-mL screw-cap test tube for 3 d, and then cultured in a 500-mL Roux bottle for another 3 d in the basal medium. The pre-cultured cells were centrifuged at 20,500 × g for 15 min and resuspended in 20 mL of the basal medium at an optical density (OD) of 3 that was measured at 600 nm. The suspension was added to 180 mL of hydrogen production medium (pH 7.0), which consisted of the basal medium, 42 mM phosphate buffer, 75 mM organic acid (lactate or acetate), and 2 mM sodium l-glutamate. Hydrogen was produced at 30˚C under a light intensity of 14.6 W/m^2 that was generated by using halogen lamps (Iwasaki Electric Co., Tokyo, Japan). The produced hydrogen was collected in a measuring cylinder filled with a 1-M sodium hydroxide solution to trap carbon dioxide, which is generated as a by-product. The hydrogen gas in the measuring cylinder was analyzed by using gas chromatography as described previously [7]. Each experiment was repeated more than 3 times.

2.2. Plasmid Construction and ACDH Production

The ORF coding sequence of *Escherichia coli* ACDH (gene name, *mhpF*; NC_007779.1) was obtained from NCBI and was amplified by performing PCR by using chromosomal DNA of *E. coli* BL21 [20]. The primer set, 5'-CGTCTAGACGATGAGTAAGCGTAA-3' (the *Xba*I site is underlined) and 5'-CGGAGCTCTCATGCCG-

CTTCTCCT-3' (the *Sac*I site is underlined) was used for ORF amplification. The PCR products were digested with *Xba*I and *Sac*I, and then ligated into pLP-1.2 [20], which had been digested with the same enzymes. The resultant plasmids (pACDH) expressed the ACDH ORFs under the control of *puf* promoter, which is commonly used for constitutive gene expression. Sequencing studies were performed to identify the plasmids with the ACDH ORF inserts. The conjugal plasmid transfer method was used to perform RV strain transformation. The RV strain and *Escherichia coli* S17-1 harboring the plasmid were mixed on an agar plate with aSy medium consisting of the basal medium, 36 mM sodium succinate, 1 wt% yeast extract, and 9.5 mM ammonium sulfate containing 20 μg/mL of kanamycin and were cultured under a light intensity of 14.6 W/m^2 at 30˚C for 7 d. The recombinant RV strain (RVAC) colonies were then cultured in the presence of 20 μg/mL of kanamycin. The recombinant RV strain harboring the intact (empty) plasmid pLP-1.2 (RVI) was prepared for the control.

2.3. ACDH Assay

For the ACDH assay, the RV cells were cultured under the same conditions as those used for hydrogen production. The cells were cultured in a hydrogen production medium containing 21 mM acetate for 72 h at 30˚C. The cells were suspended in 20 mL culture medium, harvested by centrifugation, and then 1 g (wet weight) cells were suspended in 5 mL of 100 mM potassium phosphate buffer (pH 7.0). The cell suspension was subjected to 20 s sonication cycles of 15 s at 15 W and at 20 kHz frequency, using Smurt NR-50M (Microtech Nichion Co., Funabashi, Japan) fitted with a 3-mm probe. The cells were kept on ice between cycles. Next, the cells were centrifuged at 16,500 × g for 15 min and then the supernatant was used for the enzyme assay. Protein concentrations were estimated by using the Pierce BCA Protein Assay Kit (Thermo-Fisher Scientific, San Jose, CA).

ACDH catalyzes an NAD-dependent conversion of acetaldehyde, CoA, and NAD to produce acetyl-CoA and NADH. The ACDH activity was measured by assessing NADH production, which was monitored by spectrophotometry at a wavelength of 340 nm with an extinction coefficient (ε) of 6220 M^{-1} at 30˚C for 5 min [7]. The standard reaction mixture (0.8 mL) contained a 100-mM potassium phosphate buffer (pH 7.0), 0.15 mM acetaldehyde, 0.15 mM NAD^+, 1 mM CoA, and 20 μL of a crude cell-free extract. One unit of ACDH is defined as the amount of enzyme that is required to catalyze the formation of 1 mol NADH per minute.

2.4. Analytical Methods

The turbidity of the bacterial inoculum that was used for hydrogen production was adjusted to 0.2 at 600 nm wavelength by using a spectrophotometer. The concentrations of lactate and acetate were measured using high-performance liquid chromatography (HPLC) with a TOSOH Agilent 1120 Compact LC instrument and a UV detector that measures readings at 210 nm. The HPLC had a TSKgel SCX column (Tosoh Co., Tokyo, Japan), and water-perchloric acid (1000:1.5, v/v) was used as the eluent at a flow rate of 1.0 mL/min. The produced hydrogen was collected in a measuring cylinder filled with a 1-M sodium hydroxide solution to trap carbon dioxide, which is a reaction byproduct.

3. Results and Discussion

The RV and RVI strains showed no detectable enzyme activity. Together with the fact that the four *R. sphaeroides* species (*R. sphaeroides* strains 2.4.1, ATCC17025, ATCC17029, and KD131) whose genome sequences are available have no *ACDH* gene, the gene may be absent in the RV strain. The crude enzyme from the recombinant RV strain (RVAC) expressing the *ACDH* gene showed a specific activity of 3.2 mU/mg.

The RVAC strain produced 1.5-fold higher amounts of hydrogen than that produced by the wild-type and the RVI strains from 21 mM acetate after 120 h of cultivation (**Figure 1**). The accumulated hydrogen (927 mL/L-broth) was more than that produced by the reported RV strains expressing the ALDH1 (RVAD1; 880 mL/L-broth) and ALDH2 (RVAD2; 910 mL/L-broth) genes [7]. From 43 mM acetate, the RVAC strain produced 1.6-fold higher amounts of hydrogen than the RV strain at 48 h of cultivation, and similar amount of hydrogen at 120 h of cultivation. In contrast, the ALDH1 and ALDH2 produce 0.8-fold amounts of hydrogen as compared to the control strains as described previously [7]. At 64-mM acetate, the RVAC strain produced 0.8-fold amounts of hydrogen, though the ALDH1 and ALDH2 strains produces only 0.3-fold amounts of hydrogen as compared to the control strains. As compared to the RVAD strain which overproduce ALDH, overproduction of ACDH is more efficient in inducing hydrogen production over a wider acetate concentration range. In the RV strain, acetate

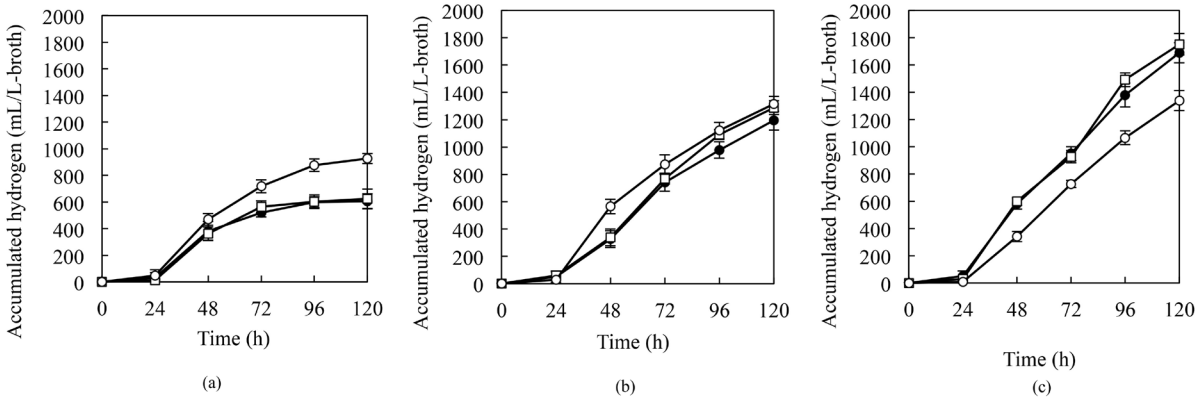

Figure 1. Hydrogen production by the wild-type and recombinant RV strains at various acetate concentrations. RVAC (open circle), RV (open square), and RVI (closed circle) strains were cultured with 21 (a), 43 (b), and 64 (c) mM acetate, at 30˚C under an illumination of 14.6 W/m^2.

may be converted into acetyl-CoA with hydrolysis of ATP by acetate-CoA ligase (EC 6.2.1.1) of which gene is found in four *Rhodobacter sphaeroides* species (*R. sphaeroides* strains 2.4.1, ATCC17025, ATCC17029, and KD131). Production of acetyl-CoA from acetaldehyde without ATP hydrolysis by ACDH can explain the increase in the hydrogen production. At higher concentrations of acetate, advantages for the production of acetyl-CoA from acetaldehyde may be relatively low, and the redundant expression of ACDH could negatively affected the hydrogen production.

The acetate concentrations in the medium were measured (**Figure 2**) to estimate the amount of consumed acetate. After 24 h of cultivation, the acetate level in the RVAC culture was over 10% lower than that in the RV and RVI cultures at 21 and 43 mM acetate. At 64 mM acetate, the acetate level in the RVAC strain was 30% lower than that in the control strains. Thus, our data indicates that the expression of *ACDH* gene increases the acetate consumption, probably because acetaldehyde was converted into acetyl-CoA by ACDH. In contrast, the expression of the ALDH1 and ALDH2 genes increase acetate concentration in the medium, as previously described [7].

Hydrogen production rates (mL/L-broth/h) that were calculated by using the amount of hydrogen produced and the amount of acetate consumed (**Table 1**). The RVAC strain, using 21-mM acetate as the substrate, showed a higher rate of hydrogen production as compared to the control strains. The value of the RVAC strain used 43-mM acetate was slightly higher than that of the control strains.

The hydrogen production from lactate was monitored to elucidate the function of ACDH, which has no activity against lactate (**Figure 3**). Hydrogen production by the RVAC strain was over 20% lower than that by the RV and RVI cultures, at all initial lactate concentrations. These results are consistent with the hypothesis that the redundant expression of ACDH could negatively affect the hydrogen production. In addition, the lactate consumption by the RVAC strain was lower than that of the control strains (**Figure 4**). The RVAC, strain showed a decrease in the average values of hydrogen production rates (**Table 2**).

The hydrogen production efficiency is represented by the ratio of the amount of hydrogen produced to the amount of organic acids consumed (**Table 3**). In the presence of 21-mM acetate, the RVAC strain displayed approximately 1.5 higher yields, compared to the control strain values. The RVAC hydrogen yield was 20% lower than that of the RV and RVI strains for 64-mM acetate, and at all the tested lactate concentrations, 20% - 30% lower than that of the control strains.

4. Conclusion

The RVAC strain produced 1.5-fold higher amount of hydrogen in 21 mM acetate and produced slightly higher hydrogen, even in 43 mM acetate in which the RVAD strains reduce hydrogen production, as compared with the control strains. The expression of *ACDH* gene is better than those of *ALDH* genes for hydrogen production over wider concentration range of acetate. The expression of *ACDH* gene increased acetate consumption, though the expression of *ALDH* gene decreased the consumption. In lactate, the RVAC strain decreased hydrogen production

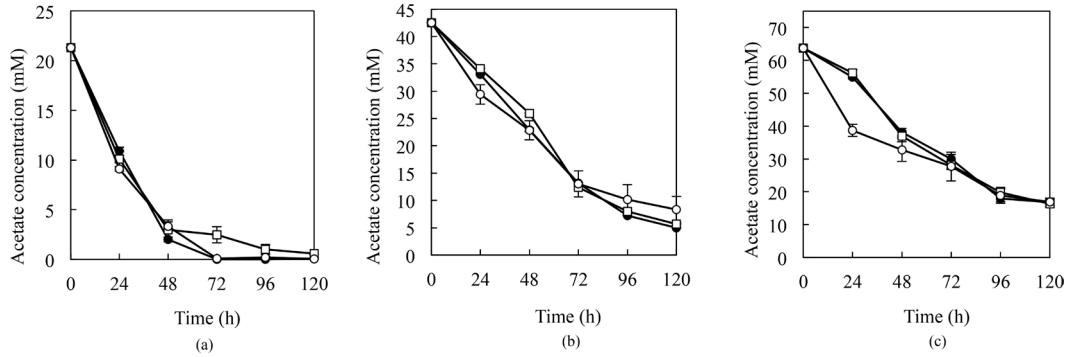

Figure 2. Residual acetate concentrations in the medium for hydrogen production. Acetate concentrations in the medium for hydrogen production by the RVAC (open circle), RV (open square), and RVI (closed circle) strains are shown. 21 mM (a), 43 mM (b), and 64 mM (c).

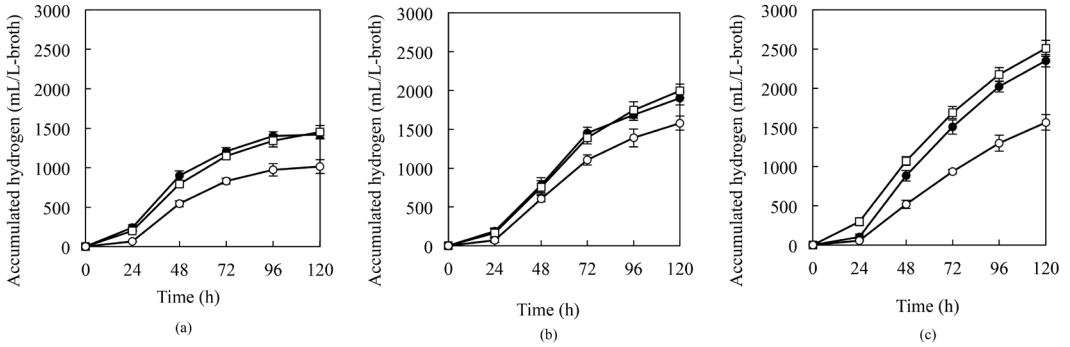

Figure 3. Hydrogen production by the wild-type and recombinant RV strains, at various lactate concentrations. The RVAC (open circle), RV (open square), and RVI (closed circle) strains were cultured under the same conditions as those in **Figure 1**, except that lactate was substituted in place of acetate. 21 mM (a), 43 mM (b), and 64 mM (c).

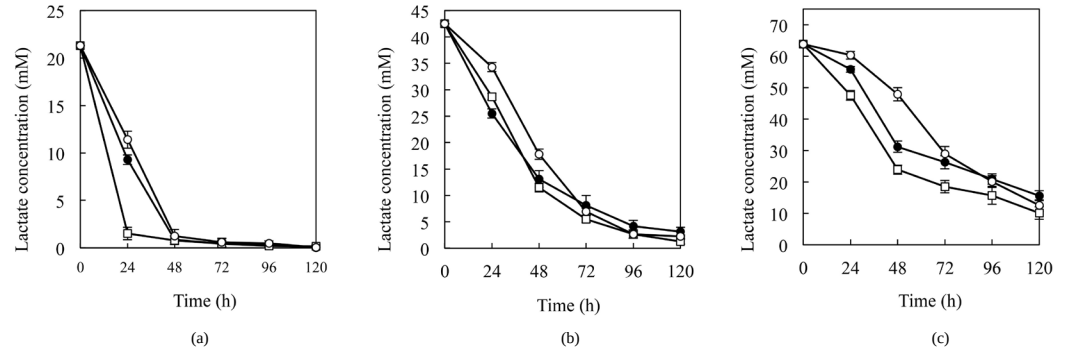

Figure 4. Residual lactate concentrations in the medium for hydrogen production. Lactate concentrations in the medium for hydrogen production by the RVAC (open circle), RV (open square), and RVI (closed circle) strains are shown. 21 mM (a), 43 mM (b), and 64 mM (c).

Table 1. Hydrogen production rates of the RV and recombinant strains at various concentrations of acetate.

Initial acetate concentrations (mM)	21	43	64
RVAC	7.7 ± 0.32	11 ± 0.48	11 ± 0.61
RV	5.2 ± 0.61	11 ± 0.43	15 ± 0.67
RVI	5.1 ± 0.47	10 ± 0.58	14 ± 0.61

The hydrogen production rates (mL/L-broth/h) was calculated from the accumulated hydrogen, assayed using a0.2-L aliquot of the medium grown for 24 h.

Table 2. Hydrogen production rates of the RV and recombinant strains at various concentrations of lactate.

Initial acetate concentrations (mM)	21	43	64
RVAC	8.5 ± 0.72	13 ± 0.76	13 ± 0.83
RV	12 ± 0.17	17 ± 0.74	21 ± 0.85
RVI	12 ± 0.38	16 ± 0.72	20 ± 0.66

The hydrogen production rates (mL/L-broth/h) was calculated from the accumulated hydrogen, assayed using a0.2-L aliquot of the medium grown for 24 h.

Table 3. Hydrogen yields of the RV and recombinant strains at various concentrations of acetate and lactate at 120 h of cultivation.

Strain	Initial substrate concentration (mM)					
	Acetate 21 mM	Acetate 43 mM	Acetate 64 mM	Lactate 21 mM	Lactate 43 mM	Lactate 64 mM
RVAC	1.9 ± 0.18	1.7 ± 0.074	1.3 ± 0.070	2.1 ± 0.18	1.8 ± 0.10	1.4 ± 0.086
RV	1.3 ± 0.15	1.6 ± 0.062	1.6 ± 0.075	3.0 ± 0.04	2.2 ± 0.096	2.1 ± 0.085
RVI	1.3 ± 0.12	1.4 ± 0.083	1.6 ± 0.070	3.0 ± 0.10	2.2 ± 0.098	2.2 ± 0.073

and lactate consumption, as did the RVAD strains.

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