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# A Strategy to Increase Microbial Hydrogen Production, Facilitating Intracellular Energy Reserves

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Overexpression of the genes encoding phosphoeneolpyruvate carboxykinase (*pckA*) and NAD-dependent malic enzyme (*maeA*) facilitates higher intracellular ATP and NAD(P)H concentrations, respectively, under aerobic conditions in *Escherichia coli*. To verify a hypothesis that higher intracellular energy reserves might contribute to $H_2$ fermentation, wild-type *E. coli* strains overexpressing *pckA* and *maeA* were cultured under anaerobic conditions in a glucose minimal medium. Overexpression of *pckA* and *maeA* enabled *E. coli* to produce 3-times and 4-times greater $H_2$ (193 and 284 nmol, respectively) than the wild type (66 nmol $H_2$). The *pckA* and *maeA* genes were further overexpressed in a hydrogenase-3-enhanced *E. coli* strain. The hydrogenase-3-enhanced strain (W3110+*fhlA*) produced 322 nmol $H_2$, whereas the ATP-enhanced strain (W3110+*fhlA*+*pckA*) produced 50% increased $H_2$ (443 nmol). Total $H_2$ in the NAD(P)H-enhanced strain (W3110+*fhlA*+*maeA*) was similar to that in the control strain at 319 nmol $H_2$. Possible explanations for the contribution of the increased cellular energy reserves to the enhanced hydrogen fermentation observed are discussed based on the viewpoint of metabolic engineering strategy.

**Keywords:** Microbial hydrogen production, cellular energy, metabolic engineering, *pckA*, *maeA*, *fhlA*

## Introduction

Hydrogen is a sustainable and clean energy source that is utilized for fuel cells in mobile electronics, power plants, and combustion engines [4]. It is produced from natural gas or the petroleum refining process as a by-product [1]. Biohydrogen has been of interest owing to its sustainable potential and environmentally friendly aspects. It can be produced through either photosynthetic or fermentative processes, depending on whether nitrogenases catalyze the reaction [$8H^+ + 8e^- + 16ATP \rightarrow 4H_2 + 16ADP + 16Pi$] or hydrogenases catalyze the reaction [$2H^+ + 2e^- \rightarrow H_2$ ] [2]. *Escherichia coli*, the best-characterized bacterium that also offers ease of genetic manipulation, produces hydrogen under anaerobic glucose fermentation conditions. Formate, a fermentative product of glycolysis, is metabolized into hydrogen and bicarbonate by the formate hydrogen lyase (FHL) system consisting of hydrogenase 3 (encoded by *hycABCDEFGHI*) and formate dehydrogenase H (encoded by *fdhF*). Many metabolic engineering strategies have been applied to the enhancement of hydrogen production in *E. coli*, such as deleting hydrogenase-3-repressing *hycA* [18], overexpressing hydrogenase-3-activating *fhlA* [16], deleting hydrogen-degrading hydrogenases 1 and 2 [5], and deleting fumarate reductase (*frdC*) and lactate dehydrogenase (*ldhA*) [19]. These studies have focused on increasing the hydrogen-producing enzyme, decreasing hydrogen degradation, and increasing available substrate during fermentation [12].

ATP and NAD(P)H are universal cellular energy reserves in biological processes. We have previously reported that cellular energy reserves can be increased in *E. coli* by overexpressing anaplerotic enzymes under glycolytic conditions. Overexpression of phosphoenolpyruvate carboxykinase (*pckA*), a natural gluconeogenic enzyme, under glycolytic conditions enables *E. coli* to contain twice as much intracellular ATP derived from the reversible reaction [PEP + $CO_2$ + ADP $\rightarrow$ oxaloacetate + ATP] [9], and the

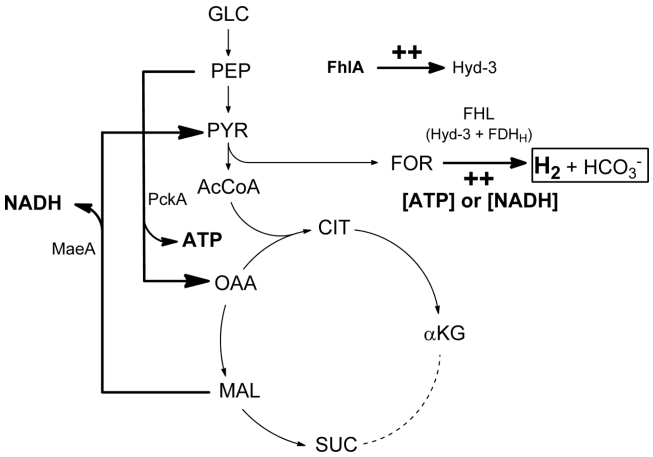


**Fig. 1.** Pathways for facilitating cellular energy reserves and the hydrogen pathway in *E. coli* under anaerobic conditions.
Bold arrows indicate overexpression by induction, and thin arrows indicate pathways. The dashed line indicates the suppressed pathway under anaerobic conditions in *E. coli*. The symbol ++ represents transcriptional activation.

availability of an *E. coli* strain with a higher concentration of ATP is beneficial for recombinant protein synthesis [6, 14]. Overexpression of the NAD-dependent malic enzyme (*maeA*) also contributes to greater intracellular NAD(P)H concentration under glycolytic conditions by the reaction [malate + $NAD(P)^+$ + $H^+$ → pyruvate + $CO_2$ + NAD(P)H] [7]. These results led us to hypothesize that the presence of greater cellular energy reserves in *E. coli* might contribute to the enhancement of fermentative hydrogen production (Fig. 1). To verify this hypothesis, we overexpressed *pckA* and *maeA* to increase the ATP and NAD(P)H concentrations in *E. coli* strains and measured hydrogen production under anaerobic glucose fermentation conditions.

## Materials and Methods

### Strains and Plasmids

All strains, plasmids, and oligonucleotides used are listed in Table 1. Wild-type *E. coli* W3110 (Korean Collection of Type Cultures, 2223) was used for this study. Strain W3110 harboring pUC18Δ*amp*$^R$-*kan*$^R$-*fhlA* was used to express FhlA, a hydrogenase-3 transcriptional activator, leading a hydrogenase-3-enhanced strain. The plasmids pEcPck and pEcMaeA from previous studies were used to enhance the intracellular ATP and NAD(P)H concentrations, respectively [8]. The *flhA* was amplified by the polymerase chain reaction (PCR) using W3110 genomic DNA as the template and the oligonucleotides GGATCCATGTCATAT ACACCGATGAGT (BamHI site underlined) and AAGCTTTTA AATCAATGCCGATTTATCAA (HindIII site underlined) to construct pUC18Δ*amp*$^R$-*kan*$^R$-*fhlA*. After subcloning into the TA vector (TA Cloning Vector; RBC Biosciences Co., Taiwan) and DNA sequencing, the BamHI-HindIII double-digested fragment (2.1 kb) was inserted into the pUC18 cloning vector (Fermentas, Germany) digested with the same enzymes, yielding pUC18-*fhlA* (4.8 kb). This plasmid was digested with AvaII, and the resulting 4.5-kb fragment with a partial *amp*$^R$ deletion was purified and then digested with HindIII (1 + 1.4 kb). The *kan*$^R$ gene was amplified by PCR using pKD13 [3] as a template and the oligonucleotides GGATCCATGATTGAACAAGATGGATTG and

**Table 1.** Strains, plasmids, and oligonucleotides used in this study.

| Strains, plasmids, and oligonucleotides | Relevant characteristics | Reference |
|---|---|---|
| Strains | | |
| W3110 | *E. coli* K-12 wild type | KCTC 2223 |
| Plasmids | | |
| TA vector | TA cloning vector, $Ap^R$ | RBC Co., Taiwan |
| pUC18 | Cloning vector, $Ap^R$ | Fermentas Co. |
| pUC18Δ*amp*$^R$-*kan*$^R$ | Expression vector, $P_{lac}$, $Km^R$ | This study |
| pUC18Δ*amp*$^R$-*kan*$^R$-*fhlA* | pUC18Δ*amp*- *kan*$^R$ with *fhlA* at the BamHI-HindIII sites, $Km^R$ | This study |
| pTrc99A | Expression vector, $p_{trc}$, $Ap^R$ | [15] |
| pEcMaeA | pTrc99A containing *maeA*, $Ap^R$ | [8] |
| pEcPck | pTrc99A containing *pckA*, $Ap^R$ | [9] |
| pKD13 | PCR template for gene knockout, $Ap^R$ | [3] |
| Oligonucleotides | | |
| *fhlA* forward | GGATCCATGTCATATACACCGATGAGT (BamHI site underlined) | This study |
| *fhlA* reverse | AAGCTTTTAAATCAATGCCGATTTATCAA (HindIII site underlined) | This study |
| *kanR* forward | GGATCCATGATTGAACAAGATGGATTG | This study |
| *kanR* reverse | TCTAGATTAGAAGAACTCGTCAAGAAG | This study |

TCTAGATTAGAAGAACTCGTCAAGAAG. The *kan*$^R$ gene fragment (1.3 kb) was subcloned into the TA vector and purified after sequencing and digestion with HindIII. The three HindIII-digested fragments from pUC18-*fhlA* with partially deleted *amp*$^R$ (1 + 1.4 kb) and PCR-amplified *kan*$^R$ were ligated together and transformed. Colonies were positively selected on kanamycin-containing medium and negatively selected on ampicillin-containing medium, and the presence of pUC18Δ*amp*$^R$-*kan*$^R$-*fhlA* was verified by observing the DNA fragmentation pattern after digestion with HindIII.

**Culture Conditions**

Luria–Bertani (LB) medium was used for all DNA manipulations. For hydrogen production, glucose minimal medium was used, consisting of 0.8 g $NH_4Cl$, 0.5 g NaCl, 7.5 g $Na_2HPO_4{\cdot}2H_2O$, 3 g $KH_2PO_4$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2$, 1 mg thiamine, and 9 g glucose per liter. Isopropyl-β-D-thiogalactopyranoside (0.1 mM) was added for induction, and 50 μg/ml antibiotics (ampicillin and kanamycin) were added to maintain the plasmids.

A single colony was inoculated into a 15 ml tube containing 3 ml of LB medium and incubated for 10 h at 37°C and 230 rpm. One milliliter of culture broth was transferred to a 150 ml anaerobic glass serum vial (Bellco Glass, Inc., NJ, USA) containing 100 ml of glucose minimal medium. The vial was tightly sealed with a rubber bung with an aluminum cap, and the head space of the vial was flushed with nitrogen gas repeatedly to remove oxygen prior to inoculation. The inoculated vial was maintained at 37°C and 100 rpm for 72 h. The culture broth and headspace gas samples were taken using a 1 ml syringe to avoid oxygen contamination.

**Analytical Procedure**

Biomass was estimated by measuring the OD at 600 nm, and the result was transformed into dry cell weight using the coefficient $OD_{600nm}$ 1 = 0.31 g/l. Glucose consumption was analyzed using the dinitrosalicylic acid method. A 100 μl aliquot of gas from the head space of the culture vial was injected into a gas chromatography system (Agilent 7890; CA, USA) equipped with a thermal conductivity detector and a packed column (60/80 molecular sieve 5A column; Supelco, PA, USA) to analyze the hydrogen [13]. Argon gas was used as the carrier gas at 35 ml/min. The column, injection, and detector temperatures were 60°C, 150°C, and 200°C, respectively.

The intracellular ATP concentration was immediately determined using an ATP bioluminescent assay kit (FL-AA; Sigma-Aldrich, MO, USA) and a luminometer (Turner BioSystems, CA, USA) after disrupting the cells by sonication (UP200S ultrasonic processor; Hielscher Ultrasonics Co., Germany) at 140 W for 30 sec on ice [9]. Intracellular NADH and NADPH were analyzed using NAD+/NADH and NADP+/NADPH quantification kits (BioVision Research, CA, USA), respectively, and a spectrophotometer (Benchmark Plus; Bio-Rad, CA, USA) at 450 nm [7].

## Results

**Effect of *pckA* and *maeA* Overexpressions on $H_2$ Fermentation in Wild-Type *E. coli***

To verify the assumption that increased intracellular ATP and NADH might contribute to hydrogen production, *E. coli* cells overexpressing *pckA* and *maeA*, respectively, were anaerobically cultured in a glucose minimal medium (Table 2). Wild-type *E. coli* carrying the pTrc99A empty vector (negative control) produced biomass of 12.4 ± 0.2 mg-DCW (dry cell weight) and 66 ± 7 nmol $H_2$ in 72 h. The ATP-enhanced strain (W3110+*pckA*; W3110 carrying pEcPck) and NAD(P)H-enhanced strain (W3110+*maeA*; W3110 carrying pEcMaeA) produced 9.3 ± 1.1 and 11.1 ± 0.3 mg-DCW, respectively, with increased hydrogen fermentations of 193 ± 14.5 and 284 ± 15.5 nmol-$H_2$ under the same conditions. The intracellular energy reserves of the ATP- and NAD(P)H-enhanced strains were greater than those of the wild type in 72 h. The $H_2$ yields per consumed glucose of ATP- and NAD(P)H-enhanced strains were 4-times greater (0.22 ± 0.03 nmol-$H_2$/mol-glucose for the W3110+*pckA* strain and 0.26 ± 0.04 nmol-$H_2$/mol-glucose for the W3110+*maeA* strain) than that of control (0.06 ± 0.01 nmol-$H_2$/mol-glucose). Therefore, increasing the *E. coli* cellular energy reserves by overexpressing either

**Table 2.** Effect of *pckA* and *maeA* overexpression on hydrogen fermentation.

| Description | Strains | [a]DCW (mg-DCW) | [b]$H_2$ (nmol) | Yield (nmol-$H_2$/mol-glucose) | Intracellular energy reserves at the end of fermentation (mmol/ g) | | |
|---|---|---|---|---|---|---|---|
| | | | | | ATP | NADH | NADPH |
| Negative control | W3110/pTrc99A | $12.4_{\pm 0.2}$ | $66_{\pm 7}$ | $0.06_{\pm 0.01}$ | $0.097_{\pm 0.016}$ | $0.072_{\pm 0.006}$ | $0.039_{\pm 0.003}$ |
| ATP-enhanced strain | W3110/pEcPck | $9.3_{\pm 1.1}$ | $193_{\pm 14.5}$ | $0.22_{\pm 0.03}$ | $0.527_{\pm 0.099}$ | $0.041_{\pm 0.014}$ | $0.075_{\pm 0.008}$ |
| NAD(P)H-enhanced strain | W3110/pEcMaeA | $11.1_{\pm 0.3}$ | $284_{\pm 15.5}$ | $0.26_{\pm 0.04}$ | $0.087_{\pm 0.186}$ | $0.033_{\pm 0.011}$ | $0.057_{\pm 0.006}$ |

Data represent the mean$_{\pm SD}$ from at least three independent experiments.

[a]Total dry cell weight at 72 h in 100 ml of culture broth.

[b]Total $H_2$ at 72 h in a 50 ml head space.

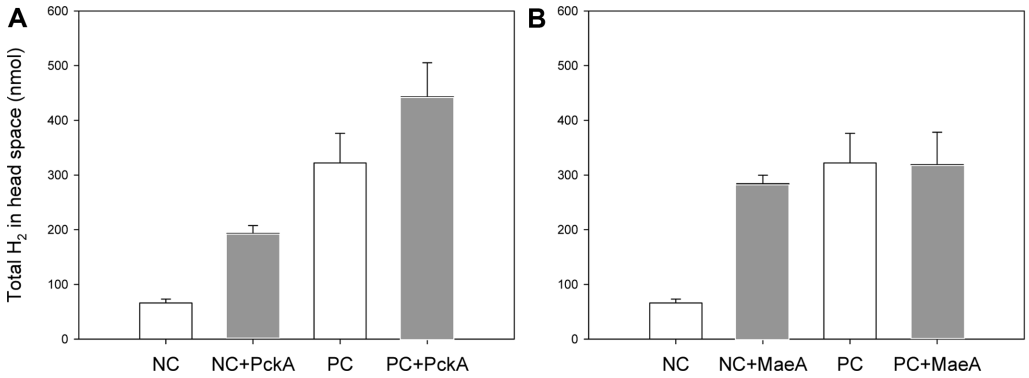


**Fig. 2.** Effect of facilitating cellular energy reserves in *E. coli* strains on hydrogen fermentation.
(**A**) Overexpression of PckA to increase ATP; (**B**) overexpression of MaeA to increase NAD(P)H. NC: negative control, W3110 harboring an empty vector; NC+PckA: W3110 expressing *pckA*; PC: positive control, hydrogenase-3-enhanced W3110 by expressing *fhlA*; PC+PckA, positive control strain simultaneously expressing *pckA*; NC+MaeA: W3110 expressing *maeA*; PC+MaeA: positive control strain simultaneously expressing *maeA*. Bars represent the mean and error bars represent the SD from at least three independent experiments. Negative and positive controls are in open bars; either *pckA* or *maeA* overexpression is in grey bars.

*pckA* or *maeA* resulted in the production of more hydrogen at a higher yield than in the control strain.

### Effect of *pckA* and *maeA* Overexpression on $H_2$ Fermentation in Hydrogenase-3-Enhanced *E. coli*

To confirm the above results, hydrogenase-3 expression was induced in *E. coli* by overexpressing the transcriptional activator gene *flhA*, and increased ATP and NADH production was further facilitated by overexpressing *pckA* and *maeA* (Fig. 2). Overexpression of *fhlA* alone (positive control, W3110+*fhlA*) resulted in a 5-fold greater hydrogen fermentation (322 ± 53 nmol) compared with that of the wild type (negative control) in 72 h. When *pckA* and *flhA* were coexpressed in W3110+*fhlA*+*pckA*, hydrogen production was the highest among the tested (443 ± 62 nmol; a 35% higher hydrogen fermentation than the positive control) (Fig. 2A). W3110+*fhlA*+*maeA* produced a similar amount of hydrogen (319 ± 59 nmol) as W3110+*fhlA*, although the yield (mol-$H_2$/mol-glucose consumption) was 12% greater. W3110+*fhlA*+*pckA* and W3110+*fhlA*+*maeA* contained 68% and 19% more ATP than W3110+*fhlA* (0.824 ± 0.071 μmol-ATP/g-cell) at 72 h, respectively, whereas the concentrations of the reducing equivalents NADH and NADPH were rather similar to the positive control.

## Discussion

Increasing cellular energy reserves by the overexpression of either *pckA* or *maeA* enabled wild-type *E. coli* to enhance fermentative hydrogen production (Table 2). Hydrogen production in hydrogenase-3-enhanced *E. coli* was increased by 38%, due to the overexpression of *pckA* (Fig. 2A), and the yield was improved by 12% via the overexpression of *maeA* (Fig. 2B). Taken together with a recent report showing that the additional cellular energy resources afforded by the overexpression of pyrophosphate and polyphosphate kinase enabled *Enterobacter aerogenes* to enhance hydrogen production [10], these results suggest that increasing cellular energy reserves, either by altering metabolism or by adding an energy resource, could contribute to fermentative hydrogen production. A recent study overexpressing two global regulators, FNR and NarP, in facultative *Enterobacter aerogenes* and *Clostidium paraputrificum* also increased hydrogen the yield by 40%, by causing a more reducing environment inside cells, which also supports the theory of this study [11]. Therefore, development of a strain that contains more ATP and reducing equivalents could be an additional strategy to enhance microbial hydrogen production.

Both intracellular ATP and NAD(P)H increases contributed to the hydrogen production, considering the hydrogen fermentations were 3- and 4-times greater in the strains overexpressing *pckA* and *maeA*, respectively, than that in the negative control strain (Table 2). In the hydrogenase-3-enhanced host background (the positive control in Fig. 2), however, only the ATP increase by *pckA* expression supported the theory (332 nmol-$H_2$ for positive control vs. 443 nmol-$H_2$ for PC+PckA). Unlike the *maeA* expression in the wild-type host, the hydrogen fermentation was not increased in the *maeA* expression in the hydrogenase-3-enhanced strain (332 nmol-$H_2$ for positive control vs. 319 nmol-$H_2$ for PC+PckA). The plausible reason is the

NAD(P)H increase by *maeA* expression might have been limited compared with the ATP increase by *pckA* expression. Extra ATP could have been generated by the *pckA* reaction [PEP + $CO_2$ + ADP → oxaloacetate + ATP] as long as glucose was present. On the contrary, intracellular malate might have been shortened to produce NAD(P)H by the *maeA* reaction [malate + $NAD(P)^+ + H^+$ → pyruvate + $CO_2$ + NAD(P)H ] because of an incomplete TCA cycle under anaerobic conditions in *E. coli*. (Fig. 1). Accumulation of bicarbonate ($HCO_3^-$, co-product of $H_2$) would have further facilitated the *pckA* reaction while inhibiting the *maeA* reaction, and this also explains the limited effect of *maeA* overexpression.

The mechanism by which increased cellular energy results in increased hydrogen production is unclear. Greater amounts of ATP or reducing equivalents may change the intracellular oxidation state to provide more formate (the hydrogen substrate, Fig. 1), resulting in an increase in hydrogen, although the effect must have been marginal considering *pckA* would have reduced the flux to pyruvate whereas *maeA* would have increased the flux to pyruvate [8]. Because the ATP concentration affects the formate dependence of transcriptional activation by FhlA in vitro [17], the formate hydrogen lyase complex may have been more activated in the strains overexpressing *pckA* and *maeA*. Therefore, further studies are needed to elucidate the metabolic flux that occurs when cells produce more hydrogen as a result of an alteration in cellular energy.

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