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

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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 \([8\text{H}^+ + 8\text{e}^- + 16\text{ATP} \rightarrow 4\text{H}_2 + 16\text{ADP} + 16\text{Pi}]\) or hydrogenases catalyze the reaction \([2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2]\) [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 the genes encoding phosphoenolpyruvate 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
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 + CO2 + 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.

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

<table>
<thead>
<tr>
<th>Strains, plasmids, and oligonucleotides</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>E. coli K-12 wild type</td>
<td>KCTC 2223</td>
</tr>
<tr>
<td>TA vector</td>
<td>TA cloning vector, ApR</td>
<td>RBC Co., Taiwan</td>
</tr>
<tr>
<td>pUC18ΔampR-kanR</td>
<td>Expression vector, Pnor, KmR</td>
<td>This study</td>
</tr>
<tr>
<td>pUC18ΔampR-kanR-fhlA</td>
<td>pUC18ΔampR-kanR with fhlA at the BamHI-HindIII sites, KmR</td>
<td>This study</td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td></td>
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</tr>
<tr>
<td>fhlA forward</td>
<td>GGATCCATGTCATATAACCGATGAGT (BamHI site underlined)</td>
<td>This study</td>
</tr>
<tr>
<td>fhlA reverse</td>
<td>AAGCTTTTTAAATCAATACGGGATTTATCAA (HindIII site underlined)</td>
<td>This study</td>
</tr>
<tr>
<td>kanR forward</td>
<td>GGATCCATGATGAAACAGATGGATTG</td>
<td>This study</td>
</tr>
<tr>
<td>kanR reverse</td>
<td>TCTAGATTAGAGAACCTCGTCAAGAG</td>
<td>This study</td>
</tr>
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</table>
TCTAGATTAGAAGAACTCGTCAAGAG. 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 *ampR* (1 + 1.4 kb) and PCR-amplified *kanR* were ligated together and transformed. Colonies were positively selected on kanamycin-containing medium and negatively selected on ampicillin-containing medium, and the presence of pUC18-*ampR-kanR-*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 NH₄Cl, 0.5 g NaCl, 7.5 g Na₂HPO₄·2H₂O, 3 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 0.1 g CaCl₂·1 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 OD₆₀₀nm = 0.31 g/l. Glucose consumption was analyzed using the dinitrosalicylic acid method. A 100 µl aliquot of gas from the headspace 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 5 Å 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 (U200S 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 H₂ 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 H₂ in 72 h. The ATP-enhanced strain (W3110+*pckA*; W3110 carrying pEcPck) and NAD(P)H-enhanced strain (W3110+*maeA*; W3110 carrying pEcMae) 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 H₂ 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 H₂ yields per consumed glucose of ATP- and NAD(P)H-enhanced strains were 4-times greater (0.22 ± 0.03 nmol-H₂/mol-glucose for the W3110+*pckA* strain and 0.26 ± 0.04 nmol-H₂/mol-glucose for the W3110+*maeA* strain) than that of control (0.06 ± 0.01 nmol-H₂/mol-glucose). Therefore, increasing the *E. coli* cellular energy reserves by overexpressing either

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

<table>
<thead>
<tr>
<th>Description</th>
<th>Strains</th>
<th>³²DCW (mg-DCW)</th>
<th>³⁵H₂ (nmol)</th>
<th>Yield (nmol-H₂/mol-glucose)</th>
<th>Intracellular energy reserves at the end of fermentation (nmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ATP</td>
</tr>
<tr>
<td>Negative control</td>
<td>W3110/pTrc99A</td>
<td>12.4±0.2</td>
<td>66.7±0.7</td>
<td>0.06±0.01</td>
<td>0.097±0.016</td>
</tr>
<tr>
<td>ATP-enhanced strain</td>
<td>W3110/pEcPck</td>
<td>9.3±1.1</td>
<td>193±14.5</td>
<td>0.22±0.03</td>
<td>0.527±0.09</td>
</tr>
<tr>
<td>NAD(P)H-enhanced strain</td>
<td>W3110/pEcMaeA</td>
<td>11.1±0.5</td>
<td>284±15.5</td>
<td>0.26±0.04</td>
<td>0.087±0.19</td>
</tr>
</tbody>
</table>

Data represent the mean±SD from at least three independent experiments.

*Total dry cell weight at 72 h in 100 ml of culture broth.

*Total H₂ at 72 h in a 50 ml head space.
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 H₂ 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 fhlA, 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 fhlA 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-H₂/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-H₂ for positive control vs. 443 nmol-H₂ 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-H₂ for positive control vs. 319 nmol-H₂ 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 + CO₂ + ATP → oxaloacetate + ADP] 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 + CO₂ + NAD(P)H] because of an incomplete TCA cycle under anaerobic conditions in *E. coli* (Fig. 1). Accumulation of bicarbonate (HCO₃⁻, co-product of H₂) 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 [17], the formate hydrogen lyase complex may have been more activated in the strains overexpressing maeA overexpression.

Acknowledgments

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