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# Alteration of anaerobic metabolism in *Escherichia coli* for enhanced hydrogen production by heterologous expression of hydrogenase genes originating from *Synechocystis* sp

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ABSTRACT

Metabolic engineering is recognized as one of the most important technologies for improving fermentative hydrogen yield. A vector with hydrogenase genes (*hoxEFUYH*) from *Synechocystis* sp. PCC 6803 under an *alkB* promoter was constructed, and introduced into *Escherichia coli* DH5α to alter the hydrogen metabolism with glucose as the sole carbon source. The recombinant strain reached a highest hydrogen yield of 1.89 mol/mol glucose, which was 95% of the theoretical hydrogen yield of *E. coli*. Hydrogenase activities for hydrogen evolution were increased and formic acid assimilation was accelerated with the expression of *hoxEFUYH*. The expression of *hoxEFUYH* suppressed the transcription of native hydrogenase 1 and hydrogenase 2, which were responsible for hydrogen uptake activity, while it had no influence on the transcription of the hydrogenase 3. Moreover, as the electron donor of HoxEFUYH is NADH, the expressed HoxEFUYH expanded the substrate specificity of the hydrogen-evolving hydrogenase in *E. coli*.



## 1. Introduction

Hydrogen is a promising alternative energy carrier given its advantages as an efficient and clean fuel for powering fuel cells. Converting biomass into hydrogen with biological methods is sustainable, and can be achieved with significantly less energy consumption [1]. Microbial fermentation is amongst the most promising for mass-scale bio-production of hydrogen [2]. But the major factor currently limiting the commercial use of biological hydrogen production is the low yield from biomass [3].

Metabolic engineering has recently been recognized as one of the most important potential technologies to improve fermentative hydrogen yield. Some researches have aimed at improving the yield of originally good hydrogen producers (wild-type *Clostridium* sp., *Enterobacter* sp.), but the genetic manipulation is relatively hard [4–8]. *Escherichia coli*, the best-characterized bacterium, was chosen as the starting model strain to study ways of improving the hydrogen yield by the manipulation of its metabolism [9].

Theoretically, *E. coli* forms 2 mol of hydrogen per mole of glucose, from formate via the formate hydrogenlyase (FHL) complex, which is capable of converting formate to $H^+$ and $CO_2$, and hydrogenase 3 which produces $H_2$ from $2H^+$. Therefore, there are two possible ways to improve the hydrogen yield of *E. coli*. The first is the direct enhancement of the FHL complex. The second involves directing the glucose metabolism toward PFL by disrupting the competing pathways. By combining FHL repressor (HycA) inactivation with FHL activator (FhlA) overexpression, Yoshida and coworkers improved the hydrogen yield from glucose in the *E. coli* SR15 strain from 1.08 to 1.82 mol/mol glucose [10,11]. Wood and coworkers modified the formate pathway of *E. coli* K12 by systematically over-expressing or deleting a series of genes [9,12–14]. More recently, over the engineering of HycE, 9-fold higher yield was achieved [15]. And FhlA was evolved to increase $H_2$ production, a maximum increase in hydrogen production of 9 fold was achieved [16].

However, formic acid is the only electron donor for hydrogen production in *E. coli*, setting an upper absolute limit of $H_2$ yield. To enhance the hydrogen yield above this limit, it is necessary to channel more electrons to the hydrogen pathway. Expressing heterogeneous hydrogenases which have different substrate specificities, such as the FeFe-hydrogenase from *Clostridium* sp. (ferredoxin as electron donor) and the NiFe-hydrogenase from algae (NADH as electron donor) could be promising choices. Recently, *Clostridia* FeFe-hydrogenase was successfully expressed

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**Table 1**
Plasmids used in this study.

| Plasmid | Description | Source | Reference |
|---|---|---|---|
| pBS(Kan)Synhox | 10.7 kb, *lacIq*, $Km^r$ | Kind gift from Professor Wood of Texas A&M University, USA | [22] |
| pCOM10 | 7.6 kb, $Km^r$ | Kind gift from Professor B. Witholt of ETH, Swiss | [29] |
| pCOM10-hox | 14.3 kb, $Km^r$ | This work | – |

**Table 2**
Primers used in this study.

| Primers | Sequence (5′ → 3′) | Target |
|---|---|---|
| hox_F | ATAGGATCCCCATGACCGTTGCCACC | *hoxEFUYH* |
| hox_R | TATAAGCTTATGCCCTCGGTGATGCCG | |
| qhyaA_F | CATCACCGACAAACCCATTA | *hyaA* |
| qhyaA_R | TGGATTCGCTGACCATAGAA | |
| qhyaB_F | TGAATGAACAGGAACGCTACTC | *hyaB* |
| qhyaB_R | CAGCATCGCCTTTGTGAT | |
| qhybB_F | GACCTGGGAAGAACTGTTGAT | *hybB* |
| qhybB_R | ATGCTTTGCTCGCCTCAT | |
| qhybC_F | TACCAGGCGGTTGTTCCAT | *hybC* |
| qhybC_R | TTGCCGTCAGCATCCACTA | |
| qhycE_F | AACGACGAACGCAAACTGA | *hycE* |
| qhycE_R | GCGAACCGTAATCCAACACT | |
| qhycG_F | CAGAACGCTTCGGCATTA | *hycG* |
| qhycG_F | CAGGCACCGTAGGAGATACA | |
| qrsmB_F | GTATGAAGGCGACCGTGAA | *rsmB* |
| qrsmB_R | TATCTGGATGGCGACGAAT | |

The endonuclease sites in the primers were underlined.

in *E. coli* [17,18]. And a synthetic pyruvate:ferredoxin:$H_2$ pathway was constructed [19,20].

To realize the function of FeFe-hydrogenase in *E. coli*, the introduction of ferredoxin is needed, thereby requiring the expression of additional heterogeneous genes [20]. However, the electron donor of *Synechocystis* NiFe-hydrogenase is NADH [21], which is a general cofactor present in *E. coli*. Therefore, only the introduction of *Synechocystis* NiFe-hydrogenase itself is expected to alter the whole metabolism for hydrogen production in *E. coli*. The hydrogenase of *Synechocystis* sp. PCC 6803 was expressed in the strain of *E. coli*, which enhanced hydrogen production presumably by limiting the activity of the native hydrogen uptake hydrogenases in *E. coli*, and the hydrogen productivity was enhanced up to 41-fold [22]. And the expression of HoxEFUYH also showed a distinct $H_2$ production pathway from the native formate dependent one coupling NADH and NADPH pools to $H_2$ [23]. But in these study, as the genes of *hoxEFUYH* were fused to a *lac* promoter, glucose was not contained in the complex medium to avoid the suppression by catabolism repression.

In this study, the hydrogenase genes of *hoxEFUYH* from *Synechocystis* sp. PCC 6803 were first fused to an *alkB* promoter to avoid the influence by carbon catabolite repression [24], and then introduced into *E. coli* DH5α to examine effects of HoxEFUYH on the hydrogen production with glucose as the sole carbon source. The alteration of the metabolism of *E. coli* through the introduction of *Synechocystis* NiFe-hydrogenase and the change of hydrogen yield based on glucose consumption were systematically studied by gene transcription and metabolic flux analysis. From the viewpoint of metabolic network analysis, hydrogen production enhancement was also discussed.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids

*E. coli* DH5α was used as the expression host for hydrogen production in this study.

The plasmids used in the present study are listed in Table 1. The plasmid pCom-hox constructed in this work was used to express HoxEFYUH originated from *Synechocystis* sp. PCC 6803 in *E. coli* DH5α. The primers of hox_F and hox_R (Table 2) for clone *hoxEFYUH* genes from plasmid pBS(Kan) Synhox had the restriction sites of BamHI and HindIII at each end. The cloned *hoxEFYUH* genes were then inserted into pCom10 at the corresponding sites to achieve a new plasmid of pCom-hox.

### 2.2. Cultivation of the recombinant strain for hydrogen production

The recombinant strain of *E. coli* DH5α (pCom-hox) was cultivated for hydrogen production, while the strain of *E. coli* DH5α (pCom10) was cultivated as a control.

The strains were grown in a glucose medium (per liter: 15 g glucose, 5 g tryptone, 14 g $K_2HPO_4$, 6 g $KH_2PO_4$, 2 g $(NH_4)_2SO_4$, and 0.2 g $MgSO_4{\cdot}7H_2O$). A 50 mL bottle containing 20 mL of glucose medium was first degassed to produce anaerobic conditions (purging with nitrogen gas prior to tubing, head-gas replacement, and then autoclaving), and was subsequently inoculated with a 1-d seeding culture at an inoculum size of 2.4% (v/v). Batch cultivation was carried out in a reciprocal shaker, at 150 rpm and 37 °C for 24 h. For the cultivation of the strain of *E. coli* DH5α and *E. coli* DH5α (pCom-hox), *n*-octane with a final concentration of 5‰ was added to induce the expression of HoxEFYUH.

### 2.3. Assay of hydrogenase activities

A 4 ml tailor-made, glass cuvette, which could be tightly closed with a cap and rubber stopper to prevent the ingress of air, was used to detect the hydrogenase activities. The assay was carried out in 2 ml reaction mixture containing 0.4 mM reduced methyl viologen (MV), 0.2–0.8 ml cell suspension and 100 mM HEPES (pH 8), which were all prepared in the anaerobic chamber (Sheldon Manufacturing Inc. USA). The reaction was started by the anaerobic addition of the proper amount of cell suspension. By monitoring the oxidation of reduced MV at 604 nm, the activities of hydrogenase could be assayed. One unit of hydrogenase was defined as the oxidation of 1 mmol reduced MV per min. Note that the hydrogenase enzyme is sensitive to oxygen, so the assays were performed anaerobically.

### 2.4. Analytical methods

The amount of gas produced was measured with a measuring cylinder. The optical density at 600 nm ($OD_{600}$) was measured with a spectrophotometer (Shimadzu UV-1206, Japan) to measure the cell concentration on a dry cell weight basis.

#### 2.4.1. Glucose concentration

1 mL of dinitrosalicylic acid solution was added to 1 mL of sample solution. The mixture was heated in a boiling water bath for 5 min, and then 4 mL of water was added. The absorbance of the sample was measured at 510 nm.

#### 2.4.2. Gas components

The components of the gas evolved were analyzed using a gas chromatography (Shimadzu GC8A, Japan), equipped with a Parapak Q column (80–100 mesh) and a thermal conductivity detector (TCD), with $N_2$ as the carrier gas. The working temperatures of the column and the TCD were 80 °C and 120 °C, respectively.

#### 2.4.3. Metabolites

A 10 mL sample drawn from the cultures was centrifuged (10,200 × *g* for 5 min at 4 °C), and the supernatant was frozen at