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Activation of formate hydrogen-lyase via expression of uptake [NiFe]-hydrogenase in *Escherichia coli* BL21(DE3)

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Abstract

Background: Several recent studies have reported successful hydrogen (H₂) production achieved via recombinant expression of uptake [NiFe]-hydrogenases from *Hydrogenovibrio marinus*, *Rhodobacter sphaeroides*, and *Escherichia coli* (hydrogenase-1) in *E. coli* BL21(DE3), a strain that lacks H₂-evolving activity. However, there are some unclear points that do not support the conclusion that the recombinant hydrogenases are responsible for the in vivo H₂ production.

Results: Unlike wild-type BL21(DE3), the recombinant BL21(DE3) strains possessed formate hydrogen-lyase (FHL) activities. Through experiments using *fdhF* (formate dehydrogenase-H) or *hycE* (hydrogenase-3) mutants, it was shown that H₂ production was almost exclusively dependent on FHL. Upon expression of hydrogenase, extracellular formate concentration was changed even in the mutant strains lacking FHL, indicating that formate metabolism other than FHL was also affected. The two subunits of *H. marinus* uptake [NiFe]-hydrogenase could activate FHL independently of each other, implying the presence of more than two different mechanisms for FHL activation in BL21(DE3). It was also revealed that the signal peptide in the small subunit was essential for activation of FHL via the small subunit.

Conclusions: Herein, we demonstrated that the production of H₂ was indeed induced via native FHL activated by the expression of recombinant hydrogenases. The recombinant strains with [NiFe]-hydrogenase appear to be unsuitable for practical in vivo H₂ production due to their relatively low H₂ yields and productivities. We suggest that an improved H₂-producing cell factory could be designed by constructing a well characterized and overproduced synthetic H₂ pathway and fully activating the native FHL in BL21(DE3).

Keywords: Recombinant hydrogenase, *Escherichia coli* BL21(DE3), Biohydrogen, Formate hydrogen lyase, *Hydrogenovibrio marinus*

Background

Hydrogen (H₂) production via biological means has been considered as a potential source of alternative fuel due to clean and truly renewable processes [1]. Hydrogenases are the key enzymes in microbial H₂ metabolism that catalyze the reversible reduction of protons with electrons [2]. Certain limitations of native hydrogenase systems for H₂ production (i.e., problems related to substrate (electron donor/acceptor) specificity, oxygen (O₂) sensitivity, catalytic bias to H₂ oxidation, electron partitioning, etc.) have been reported in microorganisms [3], and their

properties appear to be unable to meet current needs. Therefore, expression and engineering of hydrogenases in heterologous hosts is generally accepted as the most influential approach to modification of enzyme qualities and H₂ production efficiency for biotechnological applications [3, 4]. Recombinant expression of hydrogenase not only provides the ability to engineer the H₂ metabolism of the host for specific purposes but also could facilitate basic studies on the maturation process of the complex metalloenzyme [4].

Escherichia coli has been widely used as a host microbe for protein expression [5]. This bacterium was also adopted for expression of recombinant hydrogenase in several studies, either for study of hydrogenase

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maturation or for improvement of fermentative H₂ production by coupling to the native electron transfer system of *E. coli* [6–10]. In particular, the strain BL21(DE3) (or BL21), which is an optimized host for protein overexpression, can neither produce nor consume H₂ (no hydrogenase activity) under the general culture conditions where K-12 derivatives do possess the abilities [11–14]. This observation prompted certain researchers to consider this strain as an ideal host for hydrogenase expression and testing for in vivo H₂ production [12–14].

According to the composition of bimetallic active sites, hydrogenases are broadly classified into [FeFe]- and [NiFe]-hydrogenases from the standpoint of biotechnological importance. *E. coli* contains four different [NiFe]-hydrogenases, and among those, hydrogenase-3 is responsible for H₂ production during mixed-acid fermentation [15]. This enzyme forms a formate hydrogen-lyase (FHL) complex together with formate dehydrogenase-H, one of the three formate dehydrogenases of *E. coli* [16].

Recently, certain studies reported that homologous or heterologous expression of the structural (large and small) subunits of uptake [NiFe]-hydrogenases resulted in construction of recombinant BL21(DE3) derivatives that are capable of producing H₂ [17–19]. However, some unclear points arise that do not support the conclusion that the expressed hydrogenases are indeed responsible for the in vivo H₂ production of the recombinant strains. Among these points, the most critical is that all of the engineered hydrogenases engage in H₂ uptake (consumption) and not production in their native hosts [20–22]. In this work, we tackle this problem using simple biochemical and mutant experiments. We suggest that H₂ production in such recombinant systems is almost exclusively dependent on the native FHL of *E. coli*, and thus, careful characterization of the recombinant hydrogenase systems in BL21(DE3) is required, especially for those designed for in vivo H₂ production.

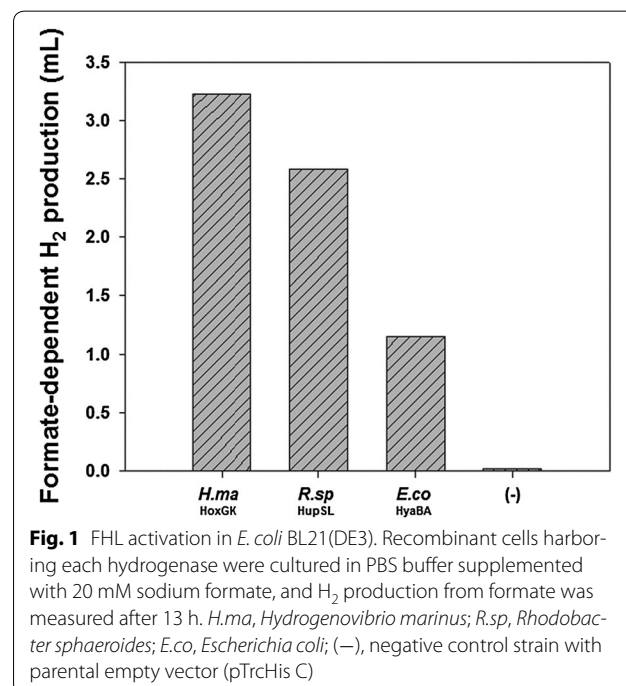
Results and discussion

Activation of FHL activity in recombinant strains

Several efforts have been put forth to engineer uptake [NiFe]-hydrogenases in BL21(DE3) strain [17–19]. In these studies, H₂ production was demonstrated by expressing structural (large and small) subunits of the hydrogenases in the non-H₂ producing *E. coli* strain, and the authors concluded that the engineered, non-native hydrogenases could be used as tools to enhance biohydrogen production in *E. coli*. However, a critical discussion promptly arises related to the fundamental origin of the produced H₂: (1) The engineered hydrogenases are engaged in H₂ uptake and not in H₂ production in their native hosts, which means that standard redox potentials of their respective electron acceptors (e.g.,

cytochrome *b*) are expected to be much higher than that of H₂ (−420 mV) [23]. Additionally, uptake [NiFe]-hydrogenases generally show high catalytic bias to H₂ oxidation [24, 25]. Thus, even if an uptake [NiFe]-hydrogenase is ‘wired’ to an electron transport system in *E. coli*, H₂ produced via the non-native pathway is not expected to highly accumulate in a closed batch culture system [12], which is in contrast to the results of high H₂ accumulation in the previous studies [17–19]. (2) Addition of hypophosphite, an inhibitor of pyruvate formate-lyase, abolished the H₂ production in a recombinant strain expressing *E. coli* HyaBA (hydrogenase-1) [19]. Moreover, addition of formate greatly increased in vivo H₂ production. (3) Full maturation of the expressed hydrogenases is questionable because maturation of [NiFe]-hydrogenase further requires highly specific auxiliary proteins [26].

Putting the theoretical and the experimental clues together, we hypothesized that the BL21(DE3) derivatives produce H₂ via a native FHL pathway that is activated by the expression of the recombinant hydrogenases. A test for H₂ production using formate as a sole electron source showed that the recombinant strains with the heterologous (*H. marinus* HoxGK and *R. sphaeroides* HupSL) or homologous (*E. coli* HyaBA) hydrogenase indeed showed FHL activity, whereas the negative control strain with the parental empty vector exhibited negligible FHL activity as expected (Fig. 1). When we measured formate consumption by the strain with *H. marinus* HoxGK, it was found that the cells consumed 1.6 ± 0.1 mM formate,



whose corresponding calculated H₂ production (3.58 mL) well coincides with the actual amount of H₂ production (3.23 mL). In contrast, the negative control cells showed virtually no consumption of formate (0.0 ± 0.1 mM). These results imply that the FHL pathway was at least partially responsible for the observed in vivo H₂ production in the previously reported recombinant strains.

FHL dependency of H₂ production in the recombinant strains

Measurement of FHL activity was not sufficient to decide whether H₂ production in the recombinant strains originates exclusively from the activated FHL pathway. To examine the FHL-dependency, we constructed two knockout BL21(DE3) strains lacking formate dehydrogenase-H (*fdhF*) and hydrogenase-3 (*hycE*), respectively, both of which constitute essential components of the FHL complex [16] and subsequently tested in vivo H₂ production by expressing the recombinant hydrogenases.

In the case of the *fdhF* mutant, all mutant strains produced small amounts of H₂ that were roughly comparable to that of the negative control (Fig. 2), which clearly demonstrated that H₂ was produced from formate as the only major substrate in the previous reported recombinant strains [17–19]. Similarly, insignificant amounts of H₂ were produced by *hycE* mutants, which indicates that hydrogenase-3 was almost entirely responsible for H₂ production in the reported BL21(DE3) derivatives (Fig. 2). Although H₂ production by both of the mutants with *H. marinus* HoxGK was slightly exceptional (2.1-fold for *fdhF* mutant and 7.5-fold for *hycE* mutant compared with the negative controls), the amounts can still

be considered marginal compared with the positive control. It appears that the expression of HoxGK influenced the other *E. coli* hydrogenase system(s) to evolve H₂ because H₂ was not detected when the *E. coli* MW1001 strain lacking hydrogenase-1, hydrogenase-2, and hydrogenase-3 was transformed with pTrcHoxGK (data not shown). Thus, we concluded that H₂ was produced almost exclusively via the activated FHL pathway in the BL21(DE3) strains with the recombinant hydrogenases. We strongly suspect that the recent report on H₂ production in BL21(DE3) by expression of *Rhodospseudomonas palustris* [NiFe]-hydrogenase [27] falls within this category. It is noteworthy that all recombinant [NiFe]-hydrogenases that activated FHL belong to Group 1 according to the widely used classification of hydrogenases [28].

After in vivo H₂ production in the wild-type and the mutant BL21(DE3) strains with *H. marinus* HoxGK, extracellular formate concentrations were measured and compared with those of negative controls (Table 1). All of the strains with the parent vector showed similar formate level regardless of the FHL mutations. This is not surprising because formate consuming pathways are already impaired in BL21(DE3) [11]. On the other hand, when *H. marinus* HoxGK was expressed, the formate concentration of wild-type BL21(DE3) was lower than those of the mutant strains, indicating that formate was consumed for H₂ production. Notably, the overall formate level was lowered upon the expression of hydrogenase even in the mutants that cannot produce H₂, which implies that formate metabolism (either production or consumption) other than FHL pathway was also affected by the expression of recombinant hydrogenase.

Involvement of each subunit in FHL activation

In an effort to reveal the role of uptake [NiFe]-hydrogenase in FHL activation, we investigated the contribution of each subunit to H₂ production using *H. marinus* hydrogenase as a model enzyme. Expression vectors were constructed for five different combinations of the large subunit (HoxG), small subunit (HoxK), and small subunit without signal peptide (HoxK*) (Fig. 3a), and all of the subunits with His₆-tag were successfully expressed in *E. coli* BL21(DE3) (Fig. 3b). As shown in Fig. 3c, different amounts of H₂ were produced by the different

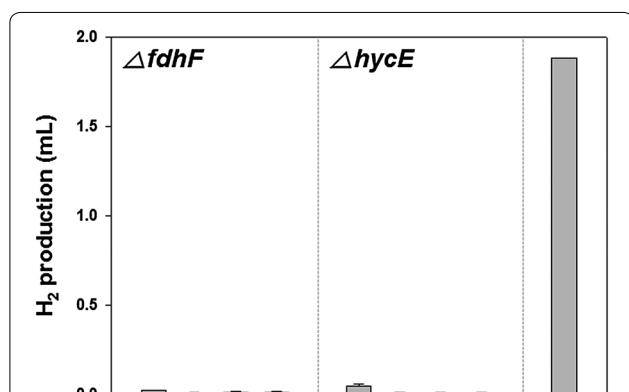
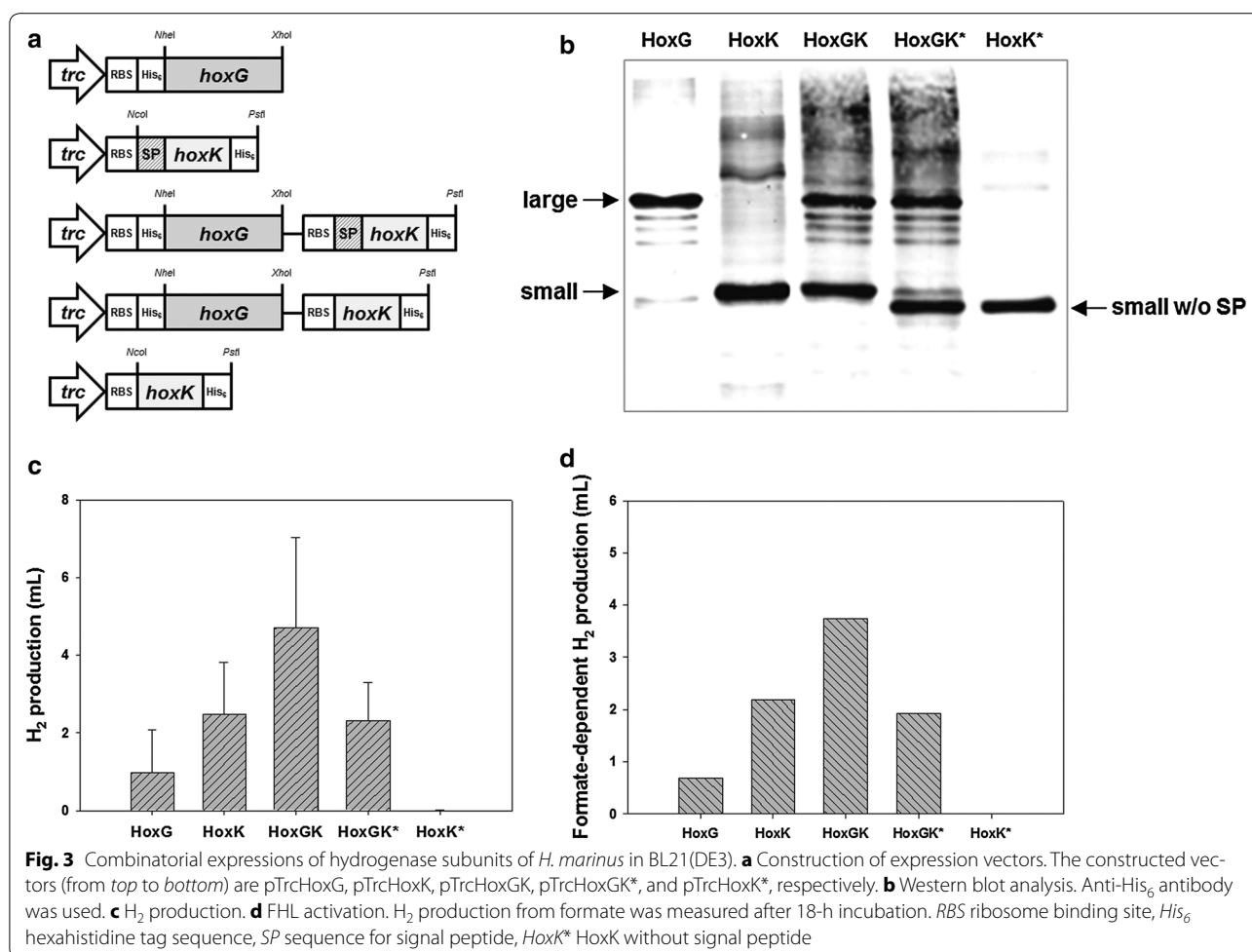


Fig. 2 H₂ production in FHL-deficient mutant BL21(DE3) strains. Strains lacking formate dehydrogenase-H or hydrogenase-3 were used. *H.ma*, *Hydrogenovibrio marinus*; *R.sp*, *Rhodobacter sphaeroides*; *E.co*, *Escherichia coli*; (–), negative control mutant with parental empty vector (pTrcHis C); (+), positive control strain with *R. sphaeroides* HupSL

Table 1 Extracellular concentration of formate (mM) measured after in vivo H₂ production in BL21(DE3) derivatives

Plasmid	Strain		
	Wild-type	Δ <i>fdhF</i>	Δ <i>hycE</i>
pTrcHis C	15.9 ± 0.3	16.5 ± 0.1	16.1 ± 0.4
pTrcHoxGK	10.6 ± 0.2	13.3 ± 1.1	13.1 ± 1.4



combinations. This pattern of H₂ production was well correlated with FHL activity ($R^2 > 0.99$) (Fig. 3d), implying that the different amounts of H₂ production was due to different degrees of FHL activation. Intriguingly, H₂ production was observed in the recombinant strains with HoxG or HoxK alone (Fig. 3c). Because the catalytic active site of [NiFe]-hydrogenase is located in large subunit [28], the result of H₂ production with only the small subunit corroborates the previous conclusion that the recombinant hydrogenase was not the catalyst that produced H₂ in BL21(DE3). Notably, the effects of the two subunits seemed to be additive (Fig. 3c), possibly representing the presence of more than two separate mechanisms for FHL activation. The fact that H₂ was produced with HoxG alone also supports this possibility.

The deletion of signal sequence on HoxK resulted in no H₂ production, indicating that the signal peptide was essential for FHL activation via the small subunit (Fig. 3c). This observation is consistent with the previous report, in which the importance of signal peptide on in vivo H₂ production was shown [17]. Because the signal

peptide is implicated in the interaction with membrane component(s) for protein translocation [29], it is likely that the mechanism by which the small subunit activates FHL involves a membrane component that directly or indirectly affects FHL, which is also a membrane protein complex [16].

A recent study on metabolic deficiencies of BL21(DE3) suggested that the lack of FHL activity in BL21(DE3) can be restored by complementation of a wild type copy of *fnr* gene and a high concentration of metal ions (500 μ M nickel and 1 mM molybdenum) [11]. In our experiments, no additional ions were added except for 30 μ M nickel and iron, and little possibility exists that the expressed subunits can function as FNR. Additionally, the effect of FHL restoration by FNR was only partial when compared with the FHL activity of *E. coli* K-12 strains [11]. Intriguingly, an *fnr* mutant of K-12 strain (PB1000) still possessed 20 % FHL activity of the parent strain [11]. Thus, although we do not offer any clear explanation of how the subunits activate FHL, we suggest the existence of an unknown pathway(s) for FHL activation and regulation

Table 2 Comparison of H₂ production by *E. coli* strains

Host	Genetic modification	H ₂ yield (mol-H ₂ /mol-glucose)	H ₂ productivity (mL-H ₂ /L-culture h)	References
<i>E. coli</i> BL21(DE3)	<i>H. marinus</i> <i>hoxGK</i>	0.65	25.1	[17]
<i>E. coli</i> BL21(DE3)	<i>R. sphaeroides</i> <i>hupSL</i>	0.28	19.7	[18]
<i>E. coli</i> BL21(DE3)	<i>E. coli</i> <i>hyaBA</i>	0.32	12.5	[19]
<i>E. coli</i> BL21(DE3)	<i>R. palustris</i> <i>hupSL</i>	0.32	39.9	[27]
<i>E. coli</i> BW25113	$\Delta hycA \DeltahyaAB \DeltahybBC \DeltaldhA \DeltafrdAB$	1.80	420.7	[30, 31]
<i>E. coli</i> BW25113	$\DeltahyaB \DeltahybC \DeltahycA \DeltafdoG \DeltafrdC \DeltaldhA \DeltaaceE$	1.32	354.8	[32]

of formate metabolism that is distinct from the *fnr*-mediated activation.

Implications for future research

The main purpose of engineering hydrogenase or its relevant pathway is to enhance H₂ yield and/or productivity. Because H₂ production in the recombinant BL21(DE3) strains almost entirely depends on native FHL, in principle, the yield cannot exceed the theoretical maximal H₂ yield from formate (2 mol-H₂/mol-glucose) that has been almost realized with *E. coli* K-12 mutant (Table 2). In terms of productivity, the recombinant strains are also much less effective than previously constructed K-12 derivatives (Table 2). Therefore, in their present form, the reported BL21(DE3) strains with the recombinant uptake [NiFe]-hydrogenases appear to be poorly suited for practical in vivo H₂ production unless non-native FHL-independent H₂ pathways are constructed with the recombinant hydrogenases using synthetic biology and/or metabolic engineering approaches. Thus, we suggest that recombinant hydrogenase systems designed for in vivo H₂ production should be carefully characterized, especially if *E. coli* BL21(DE3) is used as a host; mere observation of in vivo H₂ production doesn't imply successful construction of non-native H₂ pathway.

E. coli BL21(DE3) is an important strain as a general choice for overexpression of recombinant proteins [5] and holds promise for metabolic engineering and biofuel production. Complete elucidation of the mechanisms for FHL activation in BL21(DE3) is important because it could enable the efficient expansion of H₂ yield with high productivity in *E. coli*; H₂ might be produced using more than two substrates simultaneously in BL21(DE3) e.g., via the fully activated FHL pathway and the other FHL-independent H₂ pathway that is robustly constituted by recombinant overexpression of H₂ metabolizing enzymes [8].

Conclusions

In this study, the H₂ production pathway was investigated in recombinant *E. coli* BL21(DE3) strains that express

the structural subunits of uptake [NiFe]-hydrogenase from *H. marinus* (HoxGK), *R. sphaeroides* (HupSL), or *E. coli* (HyaBA). The recombinant strains clearly showed FHL activity, whereas the wild-type strain did not. The H₂ production was not observed in the recombinant strains lacking *fdhF* or *hycE*, thus demonstrating exclusive dependence of the H₂ production on activated native FHL. Formate level was changed upon expression of hydrogenase even in the mutant strains lacking FHL, indicating that formate metabolism other than FHL was also affected. Through combinatorial expression of hydrogenase subunits, it was shown that each subunit could activate FHL independently. In addition, it was revealed that the signal peptide is required for FHL activation by the small subunit. The FHL dependence of the recombinant BL21(DE3) derivatives fundamentally limits the practical use of the strains in applications for biohydrogen production. A more effective system might be constructed by synergetic combination of an overproduced synthetic H₂ pathway with the fully activated FHL pathway in *E. coli* BL21(DE3).

Methods

Strains and plasmid construction

The strains, plasmids, and primers used in this study are listed in Table 3. All of the DNA works were performed using *E. coli* TOP10 (Invitrogen, USA), and *E. coli* BL21(DE3) (Novagen, USA) was used for hydrogenase expression and H₂ production. The plasmid for expression of *Rhodobacter sphaeroides* HupSL (pEMBTL-HJ2) [18] and the *E. coli* mutant strain MW1001 [33] were kindly provided by Dr. Jiho Min (Chonbuk National University, Jeonju, Korea) and Dr. T. K. Wood (Texas A & M University, Texas, USA), respectively. The vectors for expression of the hydrogenase subunits of *Hydrogenovibrio marinus* [34] were constructed by polymerase chain reaction (PCR)-based cloning procedures using genomic DNA of *H. marinus* (DSM 11271) and the listed primers with *NheI*, *NcoI*, *XhoI*, or *PstI* restriction sites. The PCR products were inserted into the pGEM-T Easy vector (Promega, USA) prior to subcloning into pTrcHis C

Table 3 *E. coli* strains, plasmids, and primers used in this study

Strains, plasmids, or primers	Genotypes, relevant characteristics, or sequences	Source or references
Strains		
TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara-leu</i>) 7697 <i>galU</i> <i>galk</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i> , streptomycin-resistant	Invitrogen
BL21(DE3)	F- <i>ompT</i> <i>hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal</i> <i>dcm</i> λ (DE3), carrying the T7 RNA polymerase gene	Novagen
JH0	BL21(DE3) Δ <i>fdhF</i> :: <i>FRT-kan-FRT</i>	This study
JH1	BL21(DE3) Δ <i>hycE</i> :: <i>FRT-kan-FRT</i>	This study
MW1001	<i>lacI^R</i> <i>rrnB</i> _{T14} Δ <i>lacZ</i> _{WJ16} <i>hsdR514</i> Δ <i>araBAD</i> _{AH33} Δ <i>rhaBAD</i> _{LD78} Δ <i>hyaB</i> Δ <i>hycC</i> Δ <i>hycE</i>	[33]
Plasmids		
pGEM-T Easy	<i>bla lacZ</i> , TA cloning vector	Promega
pEMBL-HJ2	Expression vector with T7 promoter carrying <i>R. sphaeroides</i> <i>hupS</i> and <i>hupL</i>	[18]
pTrc-EcH1ABHis	Expression vector with <i>trc</i> promoter carrying <i>E. coli</i> <i>hyaB</i> and <i>hyaA</i>	[19]
pTrcHis C	pBR322 ori <i>bla lacI^R</i> , a parental expression vector with <i>trc</i> promoter	Invitrogen
pTrcHoxGK	pTrcHis C carrying <i>H. marinus</i> <i>hoxG</i> and <i>hoxK</i>	This study
pTrcHoxG	pTrcHis C carrying <i>H. marinus</i> <i>hoxG</i>	This study
pTrcHoxK	pTrcHis C carrying <i>H. marinus</i> <i>hoxK</i>	This study
pTrcHoxGK*	pTrcHis C carrying <i>H. marinus</i> <i>hoxG</i> and <i>hoxK</i> without signal sequence	This study
pTrcHoxK*	pTrcHis C carrying <i>H. marinus</i> <i>hoxK</i> without signal sequence	This study
pKD46	<i>bla</i> γ <i>exo</i> <i>araC</i> , Red recombinase vector containing temperature-sensitive replicon	CGSC
pKD13	<i>bla FRT-kan-FRT</i> , template plasmid used for Red recombination	CGSC
Primers ^a		
hoxG	Forward: <u>GCTAGC</u> ATGAGCGTATTAAACACACC (<i>NheI</i>) Reverse: <u>CTCGAG</u> TTATCGAACCTTGACGGT (<i>XhoI</i>)	This study
hoxK_poly	Forward: <u>CTCGAG</u> TCTGCCCCGATTGCGCGTAAGGAAATCCATTATGTCAT CTCAAGTTGAAAC (<i>XhoI</i>) Reverse: <u>CTGCAG</u> TCAATGGTGATGATGATGATGACCCGCTT TATCTCCTT TCTTTTGAGCC (<i>PstI</i>)	This study
hoxK*_poly	Forward: <u>CTCGAG</u> TCTGCCCCGATTGCGCGTAAGG AAATCCATTATGGCG AACAAAATTGCTCATGC GAT (<i>XhoI</i>) Reverse: <i>ditto</i>	This study
hoxK	Forward: <u>CCATGGG</u> CTCATCTCAAGTTGAAACGTT (<i>NcoI</i>) Reverse: <i>ditto</i>	This study
hoxK*	Forward: <u>CCATGGG</u> CAACAAAATTGCTCATGCGAT (<i>NcoI</i>) Reverse: <i>ditto</i>	This study
fdhF13	Forward: CAATCACGTA CTGCTCGGCGGCGCTGATCGGCGATCGGCTCG ACGCC ATTCCGGGG ATCCGTCGACC Reverse: TCCTGACCCCGCCCTGAAAACCCCATGATCCGTCGCCAGCGT GCGCG TGTAGGCT GGAGCTGCTTCG	This study
hycE13	Forward: TTTTGTGATAAAGTAAACATGGCGATTCTTATTTAGCGGCGA GTTTTT ATTCCGGGG ATCCGTCGACC Reverse: TTAGCGTTCGCTCTCTTGTGCGGCGTGATTAAGAGAGTTTG AGCATG TGTAGGCTGG AGCTGCTTCG	This study
fdhFchk	Forward: GTAGGGAGTAACCAAGTATAA Reverse: AATGACCCACATAAATGT	This study
hycEchk	Forward: CCAGCGGATAAGACGAGGT Reverse: CGTCTTGATATTACTCCGCG	This study

^a Regions that hybridize to the corresponding template sequences are bolded, and restriction sites are underlined

(Invitrogen). For polycistronic expression of both hydrogenase subunits, the primers *hoxK_poly* and *hoxK*_poly* were designed to contain an intergenic sequence with a ribosome binding site (RBS), a slightly modified portion of the intergenic sequence between *lacZ* and *lacY* found in the *E. coli* genome. The plasmid *pTrcHoxGK* was

primarily used throughout the study for expression of *H. marinus* hydrogenase. *E. coli* cells were grown and maintained in Luria–Bertani (LB) medium (Usb Corp., USA) supplemented with the appropriate antibiotics (ampicillin, 50 μ g/mL; streptomycin or kanamycin, 10 μ g/mL) at 37 °C in a shaking incubator at 220 rpm (Jeiotech, Korea).

Construction of mutant strains

The Red recombination system with pKD46 (Coli Genetic Stock Center (CGSC), USA) was adopted for inactivation of chromosomal *fdhF* or *hycE* gene in *E. coli* BL21(DE3). A gene construct composed of kanamycin resistance gene (*kan*) flanked by FLP recognition target (FRT) sites on pKD13 (CGSC) was amplified by PCR using *fdhF*- or *hycE*-specific primers with 50-nt homology extensions. Gene disruption was performed as described in [35] and confirmed by PCR using specific primers that were designed based on the sequences flanking the disrupted region of the genome. The *kan* gene was not cured to avoid contamination in cell culture.

In vivo H₂ production

The recombinant *E. coli* BL21(DE3) derivatives transformed with the expression vectors were cultured in 100 mL of M9 media (6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 1 g/L NH₄Cl, 0.5 g/L NaCl, 2 mM MgSO₄, and 100 μM CaCl₂) supplemented with 5 g/L of casamino acids (BD Bioscience, USA), 5 g/L of glucose, and 50 μg/mL of ampicillin (and 10 μg/mL of kanamycin only for mutant strains) in 165-mL serum bottles (Wheaton, USA) at 37 °C and 220 rpm. When the cell density reached ~0.6 OD at 600 nm, the cultures were induced for hydrogenase expression and H₂ production with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Carbosynth, UK), 30 μM NiSO₄, and 30 μM FeSO₄. The bottles were tightly sealed with rubber stoppers and aluminum caps and cultivated for a further 16 h until H₂ production was measured using gas chromatography (GC; Younglin Instrument, Korea).

FHL activity assay

After in vivo H₂ production, cells were harvested by centrifugation at 4 °C and 4000×g for 10 min and washed with phosphate buffered saline (PBS; 8 g/L NaCl, 1.44 g/L Na₂HPO₄, 0.2 g/L KCl, and 0.24 g/L KH₂PO₄; pH 7.4). They were resuspended in 98 mL of PBS in the serum bottle with addition of 2 mL of 1 M sodium formate. Immediately after brief (~3 min) flushing with N₂ gas, the bottle was sealed with a rubber stopper and an aluminum cap. After incubation at 37 °C and 220 rpm, the production of H₂ from formate was analyzed from the gas phase of the bottle via GC.

H₂ production measurement

The H₂ production was measured as previously described [36]. In brief, a specific volume (usually 100 μL) of gas was sampled from the headspace of culture bottle and analyzed by GC to determine the partial H₂ pressure. The total amount of H₂ was calculated by multiplying the

H₂ concentration by the headspace volume of the bottle (65 mL).

Western blot analysis

Western blot analysis was performed for detection of hexahistidine (His₆)-tagged proteins as previously described [36].

Formate measurement

Formate was measured by enzymatic assay using formate dehydrogenase as previously described [37] with slight modifications. Samples were diluted 1/10 with deionized water. A reaction solution containing 610 μL of 80 mM sodium phosphate buffer (pH 7.0), 300 μL of 10 mM nicotinamide adenine dinucleotide (NAD⁺; Sigma-Aldrich, USA) and 100 μL of formate dehydrogenase (~1 mg/mL; Sigma-Aldrich) was mixed with 25 μL of the diluted sample solution. After 2.5 h reaction at 37 °C, the absorbance change by formate-dependent NAD⁺ reduction was measured at 340 nm. Formate concentration was calculated based on the absorbance change and a standard curve prepared using sodium formate solutions (Sigma-Aldrich) with various concentrations.

Authors' contributions

BHJ and HJC designed the research. BHJ performed the experiments and analyzed the data. BHJ and HJC wrote the paper. Both authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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