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Escherichia coli hydrogenase 3 is a reversible enzyme possessing hydrogen uptake and synthesis activities

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Abstract In the past, it has been difficult to discriminate between hydrogen synthesis and uptake for the three active hydrogenases in *Escherichia coli* (hydrogenase 1, 2, and 3); however, by combining isogenic deletion mutations from the Keio collection, we were able to see the role of hydrogenase 3. In a cell that lacks hydrogen uptake via hydrogenase 1 (*hyaB*) and via hydrogenase 2 (*hybC*), inactivation of hydrogenase 3 (*hycE*) decreased hydrogen uptake. Similarly, inactivation of the formate hydrogen lyase complex, which produces hydrogen from formate (*fhlA*) in the *hyaB hybC* background, also decreased hydrogen uptake; hence, hydrogenase 3 has significant hydrogen uptake activity. Moreover, hydrogen uptake could be restored in the *hyaB hybC hycE* and *hyaB hybC fhlA* mutants by expressing *hycE* and *fhlA*, respectively, from a plasmid. The hydrogen uptake results were corroborated using two independent methods (both filter plate assays and a gas-chromatography-based hydrogen uptake assay). A 30-fold increase in the forward reaction, hydrogen formation by hydrogenase 3, was also detected for the strain containing active hydrogenase 3 activity but no hydrogenase 1 or 2 activity relative to the strain lacking all three hydrogenases. These results indicate clearly that hydrogenase 3 is a reversible hydrogenase.

Keywords *E. coli* hydrogenase 3 · Reversible hydrogenase · Hydrogen production · Hydrogen uptake

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Introduction

Molecular hydrogen is a 100% renewable fuel that burns cleanly and efficiently and that generates no toxic by-products (Hansel and Lindblad 1998). The use of biological methods for hydrogen production promises significant energy reduction costs compared to non-biological methods (Das and Veziroğlu 2001). Biological methods depend on hydrogenases, which catalyze the reaction $2H^+ + 2e^- \leftrightarrow H_2(g)$. Hydrogenases are found in archaea, anaerobic bacteria, and some eukaryotes, such as unicellular green algae, anaerobic ciliates, and anaerobic fungi (Horner et al. 2002). Biohydrogen may be produced through either photosynthetic or fermentative processes by these hydrogenases; in general, fermentative hydrogen production is more efficient than photosynthetic ones (Yoshida et al. 2005).

Hydrogenases are largely classified into three classes based on the metals at their active sites: [FeFe]-hydrogenases from *Clostridium pasteurianum* (Peters et al. 1998) and *Desulfovibrio desufuricans* (Nicolet et al. 1999), [NiFe]-hydrogenases from *Desulfovibrio gigas* (Volbeda et al. 1995), and [Fe]-hydrogenases from *Methanobacterium*

thermoautotrophicum (Zirngibl et al. 1992). [FeFe]- and [NiFe]-hydrogenases have a similar overall structure, as the active site is located within a large subunit and electrons are delivered to this center via iron–sulfur (Fe–S) centers located in the small subunit (Forzi and Sawers 2007). [Fe]-hydrogenases lack Fe–S clusters and are found only in a small group of methanogenic archaea (Zirngibl et al. 1992).

Escherichia coli cells have four hydrogenases (hydrogenases 1, 2, 3, and 4). Hydrogenase 1 and 2 have hydrogen uptake activity only (Ballantine and Boxer 1986; King and Przybyla 1999), hydrogenase 4 appears to be inactive (Self et al. 2004), and hydrogenase 3 is reported to have the only hydrogen production activity (Bagramyan et al. 2002; Mnatsakanyan et al. 2004). To our knowledge, there are no reports indicating *E. coli* hydrogenase 3 is a hydrogen uptake enzyme, but instead it has been reported to be a synthesis enzyme (Böck and Sawers 1996). Hydrogenase 1 (encoded by *hyaABCDEF*; Richard et al. 1999), hydrogenase 2 (encoded by *hybOABCDEFG*; Richard et al. 1999), and hydrogenase 3 (encoded by *hycABCDEFGHI*; Bagramyan and Trchounian 2003) are [NiFe]-hydrogenases that contain two cyanide molecules and a carbon monoxide molecule at the active site (Blokesch et al. 2002); these hydrogenases rely on the auxiliary proteins such as HypABCDEF (metalochaperones for NiFe insertion) and SlyD (nickel insertion) for maturation as well as may possibly rely on the chaperones GroEL/GroES (Zhang et al. 2005). In *E. coli*, hydrogen is produced by the formate hydrogenlyase system (FHL) complex that consists of two separable enzymatic activities: a formate dehydrogenase-H for producing $2H^+$, $2e^-$, and CO_2 from formate and a hydrogenase 3 for synthesizing hydrogen from $2H^+$ and $2e^-$ (Sawers et al. 1985); the source of the two electrons for H_2 is formate (Bagramyan and Trchounian 2003). The FHL system may be used for the regulation of internal pH in cells (Böck and Sawers 1996). *E. coli* hydrogenases are sensitive to oxygen (Glick et al. 1980) like other hydrogenases derived from *D. desufuricans* (Vincent et al. 2005), so their activities are assayed under anaerobic conditions.

It has been reported that the [NiFe]-hydrogenase from *D. gigas* (De Lacey et al. 2000) and *Synechocystis* sp. PCC 6803 (Gutekunst et al. 2005) are enzymes possessing both hydrogen production and uptake activities, i.e., they function physiologically in either direction: hydrogen production from NADH oxidation or NAD^+ reduction with hydrogen as the electron donor. To date, it has been difficult to determine if hydrogenase 3 is a reversible hydrogenase because it was not easy to discriminate between the three different hydrogenase activities. In this paper, we report that hydrogenase 3 is a bidirectional enzyme having significant hydrogen uptake and synthesis activities.

Materials and methods

Bacterial strains, growth, and total protein

Strains are shown in Table 1. *E. coli* cells were initially streaked from −80°C glycerol stocks on Luria–Bertani (LB) agar plates (Sambrook et al. 1989) containing 100 μg/ml kanamycin and 30 μg/ml chloramphenicol where appropriate and incubated at 37°C. After growth on LB agar plates, these strains were cultured at 37°C with shaking at 250 rpm (New Brunswick Scientific, Edison, NJ, USA) from a fresh single colony in complex medium (Rachman et al. 1997) or complex-fructose medium in which fructose (20 g, J.T. Baker Chemical, Phillipsburg, NJ, USA) was substituted for glucose (20 g, Fisher Scientific), and 100 μg/ml kanamycin or 30 μg/ml chloramphenicol was added where appropriate. Plasmids pBS(Kan) (Canada et al. 2002), pBS(Kan)HycE (below), pCA24N (Kitagawa et al. 2005), and pCA24N-FhlA (Kitagawa et al. 2005) were electroporated into the mutants (Table 1). Cell growth was measured using turbidity at 600 nm, and the total protein concentration for *E. coli* was 0.22 mg OD^{-1} ml^{-1} (Protein assay kit, Sigma Diagnostics, St. Louis, MO, USA).

Eliminating kanamycin resistance and P1 transduction

Plasmid pCP20 (Cherepanov and Wackernagel 1995) was used as described previously (Datsenko and Wanner 2000) to eliminate the kanamycin resistance gene (kan^R) from the isogenic BW25113 mutants (Keio strains; Baba et al. 2006). This enabled strains to be generated with multiple mutations via sequential P1 transductions and allowed for the use of the kanamycin-resistant plasmid pBS(Kan)HycE (Table 1). P1 transduction (Silhavy et al. 1984) and pCP20 were used to create *E. coli* MW1000 (*hyaB hybC Δkan*) from BW25113 *hybC Δkan* by transferring *hyaB* kan^R via P1 transduction and using pCP20 to eliminate the kanamycin resistance marker. Similarly, MW1001 (*hyaB hybC hycE Δkan*) and MW1002 (*hyaB hybC fhlA Δkan*) were created from BW25113 *hyaB hybC Δkan* by transferring *hycE* kan^R or *fhlA* kan^R and by eliminating the kanamycin resistance marker.

Construction of pBS(Kan)HycE

E. coli K-12 genomic DNA was obtained as described previously (Zhu et al. 1993). The 1,811-bp chromosomal DNA fragment encoding *hycE* was amplified using *Pfu* polymerase and primers HycEKpnI Front [5′-CTCCTTGCTGGGTACCTGATTAAAGAGAGTTTGAGCATGTC-3′] and HycEEcoRI Rear [5′-GGATAAGACGAATTCGCCGTGCCGGTTTTGATGAC-3′] with 30 cycles and 52°C annealing. The polymerase chain reaction

Table 1 Strains and plasmids used

Strains and plasmids	Genotype	Source
Strains		
E. coli MC4100	F- *araD139 ΔlacU169 rpsL thi fla*	Casadaban 1976
E. coli HD705	MC4100 *ΔhycE*; defective in large subunit of the hydrogenase 3 subunit	Sauter et al. 1992
E. coli BW25113	$lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$	Yale CGSG Stock Center
E. coli BW25113 *ΔfhlA*	*E. coli* JW2701 Km^R; defective in FHL activator	Baba et al. 2006
E. coli BW25113 *ΔhyfG*	*E. coli* JW2472 Km^R; defective in large subunit of hydrogenase 4	Baba et al. 2006
E. coli BW25113 *ΔhyaB Δkan*	*E. coli* JW0955 (Baba et al. 2006) *Δkan*; defective in large subunit of hydrogenase 1	This study
E. coli BW25113 *ΔhybC Δkan*	*E. coli* JW2962 (Baba et al. 2006) *Δkan*; defective in probable large subunit of hydrogenase 2	This study
E. coli BW25113 *ΔhycE Δkan*	*E. coli* JW2691 (Baba et al. 2006) *Δkan*; defective in large subunit of hydrogenase 3	This study
E. coli MW1000	BW25113 *ΔhyaB ΔhybC Δkan*; defective in large subunit of hydrogenase 1 and 2	This study
E. coli MW1001	MW1000 *ΔhycE Δkan*; defective in large subunit of hydrogenases 1, 2, and 3	This study
E. coli MW1002	MW1000 *ΔfhlA* Km^R; defective in large subunit of hydrogenases 1 and 2, and in FHL activator	This study
Plasmids		
pBS(Kan)	Cloning vector; Km^R	Canada et al. 2002
pBS(Kan)HycE	pBS(Kan) *plac*::*hycE*; expresses HycE derived from *E. coli*	This study
pCA24N	Empty vector; Cm^R	Kitagawa et al. 2005
pCA24N-FhlA	pCA24N *pT5-lac*::*fhlA*; expresses FhlA derived from *E. coli*	Kitagawa et al. 2005
pCP20	Ap^R and Cm^R plasmid with temperature-sensitive replication and thermal induction of FLP recombinase	Cherepanov and Wackernagel 1995

Km^R kanamycin resistance, *Cm^R* chloramphenicol resistance, *Ap^R* ampicillin resistance

product was cloned into the multiple cloning site in pBS (Kan) (Canada et al. 2002) after double digestion with *Kpn*I and *Eco*RI to create pBS(Kan)HycE. Plasmid DNA was isolated using a Mini Kit (Qiagen, Chatsworth, CA, USA). The correct plasmid was verified by digesting the plasmid with the restriction enzymes *Eco*RV, *Dra*III, *Nru*I, *Fsp*I, and *Xho*I.

Hydrogen production assay

For all experiments using pBS(Kan)HycE, complex-fructose medium was used to avoid catabolite repression by glucose of the *lac* promoter for expressing *hycE*. Overnight aerobic cultures (25 ml) and fresh media were sparged for 5 min with nitrogen to remove oxygen. Sealed crimp-top vials (27 ml) were sparged for 5 min with nitrogen, and 1 ml of the cell suspension and 9 ml of fresh medium (as needed including 1-mM IPTG) were added to the bottles, which were incubated at 37°C with shaking for 2 to 20 h. The amount of hydrogen generated in the 17-ml head space was measured using a 50-μl aliquot by gas chromatography (GC) as described previously (Maeda et al. 2007).

Hydrogen uptake assay and SDS-PAGE

Three independent hydrogen uptake assays (oxidized methylviologen-based uptake assay, uptake plate assay, and GC-based hydrogen uptake assay) were performed as previously described (Maeda et al. 2007). Expression of complemented proteins from samples was analyzed with standard Laemmli discontinuous sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE; 12%; Sambrook et al. 1989).

Results

Construction of *E. coli* strains defective in hydrogenases 1, 2, and 3 and FHL

The goals of this work were to assay hydrogen uptake and synthesis activities of *E. coli* hydrogenase 3 in a background devoid of competing hydrogenase 1 and 2 activities so that the role of hydrogenase 3 in hydrogen production may be more clearly defined. To achieve this aim, we used the *E.*

coli K-12 BW25113 isogenic single deletion mutants (Keio collection), which were obtained from the Genome Analysis Project in Japan (Baba et al. 2006), as these strains allowed us to introduce multiple mutations into *E. coli* cells through P1 transduction and multiple rounds of selection on kanamycin plates. Each Keio deletion mutant is designed with the ability to eliminate the kanamycin-resistance selection marker by expressing the flippase (FLP) recombinase protein from pCP20 because each kanamycin resistance gene is flanked by an FLP recognition target that is excised by FLP recombinase. Hence, we created a double mutant defective in hydrogenases 1 and 2 (*hyaB hybC*), a triple mutant defective in hydrogenases 1, 2, and 3 (*hyaB hybC hycE*), and a triple mutant defective in hydrogenases 1 and 2 and the FHL system (*hyaB hybC fhlA*; Table 1). For eliminating hydrogenase 1, 2, and 3 activities, the large subunit of each hydrogenase (*hyaB*, *hybC*, and *hycE*) was deleted because the active site of catalysis is located within each large subunit in [NiFe]-hydrogenases. Also, the *fhlA* mutation abolishes hydrogenase 3 activity related to FHL (Sankar et al. 1988).

Hydrogen uptake activity in *E. coli* strains defective in hydrogenases 1, 2, 3, and 4

To assay the hydrogen uptake activity in various *E. coli* cells, the oxidized methylviologen-based hydrogen uptake assay was performed (Table 2). As expected, elimination of hydrogenase 1 and 2, the two known *E. coli* hydrogenases with uptake activity, led to a 3.2±0.4-fold decrease in hydrogen uptake, and the *hyfG* mutation (inactivates vestigial hydrogenase 4) did not decrease hydrogen uptake (Table 2).

Importantly, the hydrogen uptake activity in the hydrogenase 1 and 2 double mutant (*hyaB hybC*) was reduced 2.7±0.4-fold by addition of the *hycE* mutation (*hyaB hybC hycE*) and was reduced 6.0±0.5-fold by addition of the *fhlA* mutation (*hyaB hybC fhlA*); hence, hydrogenase 3 has significant hydrogen uptake activity. Also, corroborating this result, BW25113 *hycE*/pBS(Kan) showed 3.8±0.1-fold lower hydrogen uptake activity than wild-type BW25113/pBS(Kan), and the uptake activity in HD705/pBS(Kan), which is defective in hydrogenase 3, was 4.4±0.4-fold lower than that in wild-type MC4100/pBS(Kan) (Table 2).

Table 2 Hydrogen uptake activity with various *E. coli* BW25113 strains in complex and complex-fructose media as determined by the oxidized methylviologen-based hydrogen uptake assay after 5 min

Strain	Description	Hydrogen uptake	
		nmol min^{-1} mg $protein^{-1}$	Relative
E. coli BW25113	Wild type[a]	76±4	3.2±0.4
E. coli BW25113 *hyfG*	*ΔhyfG* (defective hydrogenase 4)[a]	70±5	2.9±0.4
E. coli BW25113 *hyaB hybC*	*ΔhyaB* and *ΔhybC* (defective hydrogenase 1 and 2)[a]	24±9	1
E. coli BW25113 *hyaB hybC hycE*	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)[a]	9±1	0.4±0.4
E. coli BW25113 *hyaB hybC fhlA*	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defective FHL activator)[a]	4±1	0.2±0.5
E. coli BW25113/pBS(Kan)	Wild type[b]	94±12	4.5±0.1
E. coli BW25113 *hycE*/pBS(Kan)	*ΔhycE* (defective hydrogenase 3)[b]	25±1	1.2±0.1
E. coli BW25113 *hyaB hybC*/pBS(Kan)	*ΔhyaB* and *ΔhybC* (defective hydrogenase 1 and 2)[b]	21±1	1
E. coli BW25113 *hyaB hybC hycE*/pBS(Kan)	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)[c]	1.8±0.4	0.1±0.2
E. coli BW25113 *hyaB hybC hycE*/pBS(Kan)HycE	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)+HycE[c]	11±1	0.5±0.1
E. coli BW25113 *hyaB hybC fhlA*/pCA24N	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defective FHL activator)[c]	3±2	0.1±0.7
E. coli BW25113 *hyaB hybC fhlA*/pCA24N-FhlA	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defective FHL activator)+FhlA[c]	10±3	0.5±0.3
E. coli MC4100/pBS(Kan)	Wild type[b]	35±15	1
E. coli HD705/pBS(Kan)	Defective hydrogenase 3[b]	8±1	0.2±0.4

Standard deviations shown from one representative experiment with two replicates.
[a] Complex medium without IPTG
[b] Complex-fructose medium with 1-mM IPTG
[c] Complex-fructose medium without IPTG

Table 3 Hydrogen production in various *E. coli* BW25113 strains in complex-fructose or complex medium after 2 to 20 h

Strain	Description	Hydrogen production	
		μmol/mg protein	Relative
E. coli BW25113	Wild type[a]	24±1	27.6±0.1
E. coli BW25113 *hycE*	*ΔhycE* (defective hydrogenase 3)[a]	0.87±0.06	1
E. coli BW25113 *fhlA*	*ΔfhlA* (defective FHL activator)[a]	0.99±0.05	1.1±0.1
E. coli BW25113 *hyfG*	*ΔhyfG* (defective hydrogenase 4)[a]	23±1	26.4±0.1
E. coli BW25113 *hyaB hybC*	*ΔhyaB* and *ΔhybC* (defective hydrogenase 1 and 2)[a]	32±3	36.8±0.1
E. coli BW25113 *hyaB hybC hycE*	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)[a]	0.91±0.01	1.0±0.1
E. coli BW25113 *hyaB hybC fhlA*	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defective FHL activator)[a]	0.79±0.00	0.9±0.1
E. coli BW25113/pBS(Kan)	Wild type[b]	45±7	28.1±0.3
E. coli BW25113 *hycE*/pBS(Kan)	*ΔhycE* (defective hydrogenase 3)[b]	1.6±0.4	1
E. coli BW25113 *hycE*/pBS (Kan)HycE	*ΔhycE* (defective hydrogenase 3)+HycE[b]	15±6	9.4±0.5
E. coli BW25113 *hyaB hybC hycE*/pBS(Kan)	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)[b]	1.9±0.1	1.2±0.3
E. coli BW25113 *hyaB hybC hycE*/pBS(Kan)HycE	*ΔhyaB*, *ΔhybC*, and *ΔhycE* (defective hydrogenases 1, 2, and 3)+HycE[b]	23±3	14.4±0.3
E. coli BW25113/pCA24N	Wild type[c]	33±0	36.7±0.1
E. coli BW25113 *fhlA*/pCA24N	*ΔfhlA* (defective FHL activator)[c]	0.9±0.1	1
E. coli BW25113 *fhlA*/pCA24N-FhlA	*ΔfhlA* (defective FHL activator)+FhlA[c]	41±10	45.6±0.3
E. coli BW25113 *hyaB hybC fhlA*/pCA24N	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defective FHL activator)[c]	0.9±0.1	1
E. coli BW25113 *hyaB hybC fhlA*/pCA24N-FhlA	*ΔhyaB*, *ΔhybC*, and *ΔfhlA* (defective hydrogenase 1 and 2, and defective FHL activator)+FhlA[c]	41±1	45.6±0.1

Standard deviations shown from one representative experiment with two replicates.
[a] Complex medium without IPTG after 2 h
[b] Complex-fructose medium without IPTG after 20 h
[c] Complex medium without IPTG after 3 h

Hydrogen synthesis by hydrogenase 3

As shown in Table 3, both mutations for hydrogenase 3 (*hycE* and *fhlA*) nearly abolished hydrogen production activity in the wild-type background in complex medium after 2 h. Furthermore, these two mutations also abolished hydrogen production activity in the *hyaB hybC* background (strain defective in hydrogenase 1 and hydrogenase 2 activities). As expected, inactivating the vestigial hydrogenase 4 via the *hyfG* mutation did not change hydrogen production. These results indicate clearly that the activity of hydrogenase 3 is essential for producing hydrogen in *E. coli*.

Complementation of the *hycE* and *fhlA* mutations for H_2 synthesis

To further demonstrate that *E. coli* hydrogenase 3 has hydrogen uptake activity, pBS(Kan)HycE was constructed to complement the defective *hycE* allele. Also, to complement the defective FHL activator (*fhlA*), pCA24N-FhlA was used. These plasmids were introduced into the single *hycE* or *fhlA* mutant and the triple mutants *hyaB hybC hycE* and *hyaB hybC fhlA*, and hydrogen production in BW25113 *hycE*/pBS(Kan)HycE, *hyaB hybC hycE*/pBS(Kan)HycE, *fhlA*/pCA24N-FhlA, and *hyaB hybC fhlA*/pCA24N-FhlA was assayed. The deficiency in hydrogen production in a *hycE* background was complemented by introducing pBS (Kan)HycE because hydrogen production in both the *hycE* and *hyaB hybC hycE* strains with pBS(Kan)HycE was increased 9.4±0.5- and 14.4±0.3-fold compared to those with the empty vector pBS(Kan) in complex-fructose after 20 h (Table 3). Note that the *plac* promoter is leaky in this vector. Similarly, BW25113 *fhlA* or *hyaB hybC fhlA* with pCA24N-FhlA showed 45.6±0.3- or 45.6±0.1-fold higher hydrogen production activities than those with empty vector pCA24N in complex medium after 3 h (Table 3). These results indicate that these two plasmids are certainly active in *E. coli* and confirm that hydrogenase 3 is responsible for hydrogen synthesis. Also, the expression of both HycE (65.0 kDa) and FhlA (78.5 kDa) from the complementation plasmids was confirmed by SDS-PAGE (data not shown).

Complementation of the *hycE* and *fhlA* mutations for H_2 uptake activity

To ascertain if the two active plasmids can restore the hydrogen uptake activity that was abolished in the two triple mutants *hyaB hybC hycE* or *hyaB hybC fhlA*, hydrogen uptake activity was assayed (Table 2). These results show that BW25113 *hyaB hybC hycE*/pBS(Kan)HycE had 6.1±0.2-fold more hydrogen uptake activity compared to BW25113 *hyaB hybC hycE* with empty vector pBS(Kan) and comparable hydrogen uptake activity relative to BW25113 *hyaB hybC*/pBS(Kan), which has active native hydrogenase 3.

Similarly, hydrogen uptake activity in a *hyaB hybC fhlA* background was restored as indicated by the 3.7±0.9-fold increase in activity by expressing active FhlA (BW25113 *hyaB hybC fhlA*/pCA24N-FhlA vs BW25113 *hyaB hybC fhlA*/pCA24N). These hydrogen uptake results were also corroborated using a plate assay for reversible hydrogenase activity, which showed blue color (showing hydrogen uptake) upon expressing HycE and FhlA in the two triple mutant strains (*hyaB hybC hycE* or *hyaB hybC fhlA*); the mutants without the complementation plasmids remained colorless in this assay. Additionally, a GC-based hydrogen uptake assay indicated that H_2 uptake activity in BW25113 *hyaB hybC fhlA*/pCA24N-FhlA is 10±1-fold more than BW25113 *hyaB hybC fhlA*/pCA24N after. Taken together, these results show that the HycE and FhlA proteins are responsible for hydrogen uptake, i.e., hydrogenase 3 has uptake activity. In addition, these three consistent results clearly indicate that methylviologen can assay hydrogenase activity including hydrogenase 1 and hydrogenase 2 without interference from other non-hydrogenase activities of other present enzymes in *E. coli*.

Discussion

We show here that *E. coli* hydrogenase 3 is a reversible hydrogenase possessing two clear activities: hydrogen production and hydrogen uptake activities. In *E. coli*, there are four [NiFe] hydrogenases (hydrogenase 1, 2, 3, and 4), and hydrogenase 1 and hydrogenase 2 are irreversible hydrogenases equipped with only hydrogen uptake activity because negligible hydrogen production occurred in the *hycE* or *fhlA* background (defective hydrogenase 3), although active hydrogenases 1 and 2 were present (Table 3). These results agree with previous studies (Menon et al. 1994) that found hydrogenase 2 had uptake activity. Complementing *hycE* via pBS(Kan)HycE in BW25113 *hyaB hybC hycE* restored roughly half of the hydrogen uptake activity in BW25113 *hyaB hybC*/pBS(Kan) (Table 2); this agrees with the hydrogen production results in that complementing *hycE* via pBS(Kan)HycE in BW25113 *hycE* restored about one third of hydrogen production of BW25113/pBS(Kan) (Table 3). Hence, these results showed that expression of *hycE* from chromosomal DNA is more effective than that from plasmid pBS(Kan) for both hydrogen production and hydrogen uptake. In contrast, overexpression of *fhlA* via pCA24N-FhlA in BW25113 *fhlA* led to a small (24%) increase in hydrogen production compared to BW25113/pCA24N (Table 3). This indicates that overproducing *fhlA* may trigger increased hydrogen production (note that FhlA is an activator for FHL system; Sankar et al. 1988); however, overexpressing FhlA (BW25113 *hyaB hybC fhlA*/pCA24N-FhlA) only restored one half of hydrogen uptake relative to BW25113 *hyaB hybC*/pBS(Kan) (Table 2). Although the reason is unclear, it may be due to other functions of FhlA because FhlA is a complex regulator protein with at least three functions: controlling the *hyp* operon for protein maturation, controlling the *hyc* operon for hydrogenase 3, and controlling *fdhF* for formate dehydrogenase-H (Schlensog et al. 1994). Note also that the hydrogen uptake and hydrogen production values are roughly comparable; for example, hydrogen production by the wild-type strain (Table 3 with units converted) is 200 nmol min^{-1} mg $protein^{-1}$ in complex medium vs hydrogen uptake by the same strain in the same medium is 76 nmol min^{-1} mg $protein^{-1}$.

Our bioinformatics analysis indicates that there are six conserved metal-binding motifs (L0–L5 motifs; Burgdorf et al. 2002) surrounding the [NiFe] site in the large subunits of *E. coli* hydrogenase 1 and hydrogenase 2, which is similar to the catalytic subunit HoxH in *Ralstonia eutropha*, *D. gigas*, and *Synecocystis* sp. PCC 6803. However, although hydrogenase 3 is a [NiFe] hydrogenase (Drapal and Böck 1998), the large subunit of hydrogenase 3 (and also hydrogenase 4) is different because there is no L0 motif and the order of the remaining five motifs is different than those of hydrogenase 1 and 2. This difference in the [NiFe]-active site in the large subunit of hydrogenase apparently allows hydrogen production for hydrogenase 3.

Previous studies have implied that *E. coli* hydrogenase 3 mainly is responsible for hydrogen formation based on results of low hydrogen productivity in *E. coli* cells with a single mutation for *hycE* (hydrogenase 3 large subunit; Bagramyan et al. 2002; Mnatsakanyan et al. 2004) or *fhlA* (Sankar et al. 1988); however, these results are not completely satisfying in that they were conducted in a hydrogenase 1 and hydrogenase 2 background. In this paper, we provide clear experimental evidence (1) that hydrogenase 1 and hydrogenase 2 have probably no hydrogen synthesis activity (results obtained without hydrogenase 3; Table 3), (2) that hydrogenase 3 is the primary hydrogenase for producing hydrogen in *E. coli* (results obtained without hydrogenase 1 and 2; Table 3), (3) that hydrogenase 3 has significant hydrogen uptake activity (results obtained without hydrogenase 1 and 2 and by using two mutations to eliminate hydrogenase 3; Table 2), and (4)

that hydrogenase 4 is inactive (results obtained without hydrogenase 4; Tables 2 and 3).

Hydrogen in *E. coli* is produced by the collaborative activity between formate dehydrogenase-H, which converts formate into H^+, carbon dioxide, and electrons, and between hydrogenase 3, which converts H^+ and electrons into hydrogen (forward reaction; Bagramyan and Trchounian 2003). Although we show here that hydrogenase 3 has significant hydrogen uptake activity, it is unlikely that formate is synthesized from carbon dioxide and hydrogen by the reverse reaction of hydrogenase 3, but instead it is more likely that the reverse activity of hydrogenase 3 produces H^+ from hydrogen with the concomitant reduction of a species other than carbon dioxide.

Hydrogenase 3 is essential for producing hydrogen in *E. coli* because a single mutation in *hycE* or *fhlA* prevents hydrogen production; these results are consistent with previous studies (Bagramyan et al. 2002). Also, hydrogen production in a double mutant (*hyaB hybC*; defective in hydrogenase 1 and hydrogenase 2) increased up to 32% compared to that in the wild-type strain (Table 3); this result agrees well with our previous study that engineered *E. coli* cells expressing the cyanobacterial hydrogenase HoxEFUYH derived from *Synechocystis* sp. PCC 6803 enhanced hydrogen production by inhibiting the hydrogen uptake activity by hydrogenase 1 and hydrogenase 2 (Maeda et al. 2007). Therefore, if there is no uptake from hydrogenases 1 and 2, more hydrogen may be produced, and this *hyaB hybC* mutant has importance for fermentative hydrogen generation.

It is still not well understood why hydrogenase 4, which is a homolog of reversible hydrogenase 3, does not function in *E. coli*, although our biofilm microarray studies show that transcription of this operon is differentially regulated (Domka et al. 2007). In this paper, we show via an isogenic *hyfG* mutation (defective in hydrogenase 4) that hydrogenase 4 has no detectable hydrogenase activity (both synthesis and uptake) under the experimental conditions used (Tables 2 and 3). In addition, it is not clear why hydrogenases 1 and 2 have hydrogen uptake but not synthesis activity and why there are two of these enzymes. Hence, hydrogen production in *E. coli* is a complex process that is only beginning to be unraveled.

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