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Enhanced hydrogen production from glucose by metabolically engineered *Escherichia coli*

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Abstract To utilize fermentative bacteria for producing the alternative fuel hydrogen, we performed successive rounds of P1 transduction from the Keio *Escherichia coli* K-12 library to introduce multiple, stable mutations into a single bacterium to direct the metabolic flux toward hydrogen production. *E. coli* cells convert glucose to various organic acids (such as succinate, pyruvate, lactate, formate, and acetate) to synthesize energy and hydrogen from formate by the formate hydrogen-lyase (FHL) system that consists of hydrogenase 3 and formate dehydrogenase-H. We altered the regulation of FHL by inactivating the repressor encoded by *hycA* and by overexpressing the activator encoded by *fhlA*, removed hydrogen uptake activity by deleting *hyaB* (hydrogenase 1) and *hybC* (hydrogenase 2), redirected glucose metabolism to formate by using the *fdnG*, *fdoG*, *narG, focA*, *focB*, *poxB*, and *aceE* mutations, and inactivated the succinate and lactate synthesis pathways by deleting *frdC* and *ldhA*, respectively. The best of the metabolically engineered strains, BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*, increased hydrogen production 4.6-fold from glucose and increased the hydrogen yield twofold from 0.65 to 1.3 mol H_2/mol glucose (maximum, 2 mol H_2/mol glucose).

Keywords Enhanced hydrogen production · Metabolic engineering · P1 transduction · Glucose metabolism · Fermentative hydrogen

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Introduction

Hydrogen is the most abundant element in the universe (Dunn 2002), is renewable, efficient, and clean (Hansel and Lindblad 1998), and is utilized for fuel cells in portable electronics, power plants, and the internal combustion engine (Dunn 2002). It is estimated that the global energy system will shift from fossil fuels to hydrogen and methane (Dunn 2002). Most of the hydrogen now produced globally is by the process of steam reforming and the water–gas shift reaction (Yi and Harrison 2005), or as a by-product of petroleum refining and chemical production (Das and Veziroğlu 2001). Use of biological methods of hydrogen production should significantly reduce energy costs, as these processes do not require extensive heating (or extensive electricity as in electrolysis plants; Das and Veziroğlu 2001). Biological methods depend on hydrogenases, which catalyze the reaction $2H^+ + 2e^- \leftrightarrow H_2$ (g) (Evans and Pickett 2003). Hydrogen may be produced through either photosynthetic or fermentative processes, but fermentative hydrogen production is more efficient than photosynthetic production (Yoshida et al. 2005).

Escherichia coli is used in this study for hydrogen production, as it is easy to manipulate genetically, and it is the best-characterized bacterium (Blattner et al. 1997). For example, the glucose glycolytic pathway to phosphoenolpyruvate, pyruvate, acetate, ethanol, and formate via bacterial fermentation is well established (Bagramyan and

Trchounian 2003), and P1 phage transduction allows one to easily introduce mutations into *E. coli* cells. Previously, we (Maeda et al. 2007b) used the isogenic *E. coli* K-12 KEIO collection of the Genome Analysis Project in Japan (Baba et al. 2006), which contains all non-lethal deletion mutations (3985 genes), to introduce as many as six mutations in a single *E. coli* strain for directing cell metabolism from formate to hydrogen without diminishing cell growth. The simple technique consisted of removing the kanamycin antibiotic resistance marker (kan^R) after each round of P1 transduction by using the flanking flippase (FLP) recognition target sequences with FLP recombinase (Datsenko and Wanner 2000).

E. coli produces hydrogen from formate by the formate hydrogen lyase system (FHL) that consists of hydrogenase 3 (encoded by *hycABCDEFGHI*; Bagramyan and Trchounian 2003) and formate dehydrogenase-H (encoded by *fdhF*; Axley et al. 1990); these enzymes catalyze the reaction $HCOO^- + H_2O \leftrightarrow H_2 + HCO_3^-$ (Woods 1936; Fig. 1) and are probably used to help regulate internal pH (Böck and Sawers 1996). FHL activity is repressed by the *hycA* gene product (Bagramyan and Trchounian 2003) and activated by the *fhlA* gene product (Schlensog et al. 1994); hence, the FHL may be manipulated to increase hydrogen by overexpression of *fhlA* (Yoshida et al. 2005) and deletion of *hycA* (Penfold et al. 2003; Yoshida et al. 2005). The evolved hydrogen from the FHL is consumed by *E. coli* hydrogenase 1 (*hyaB* encodes the large subunit; Forzi and Sawers 2007) and hydrogenase 2 (*hybC* encodes the large subunit; Forzi and Sawers 2007; Fig. 1). In *E. coli*, there are also two additional formate dehydrogenases encoded by *fdnG* (α-subunit of formate dehydrogenase-N) and *fdoG* (α-subunit of formate dehydrogenase-O) that serve to consume formate (Rossmann et al. 1991). Also, *focA* (Suppmann and Sawers 1994) and *focB* (Andrews et al. 1997) encode proteins that export formate, and nitrate reductase A (α-subunit encoded

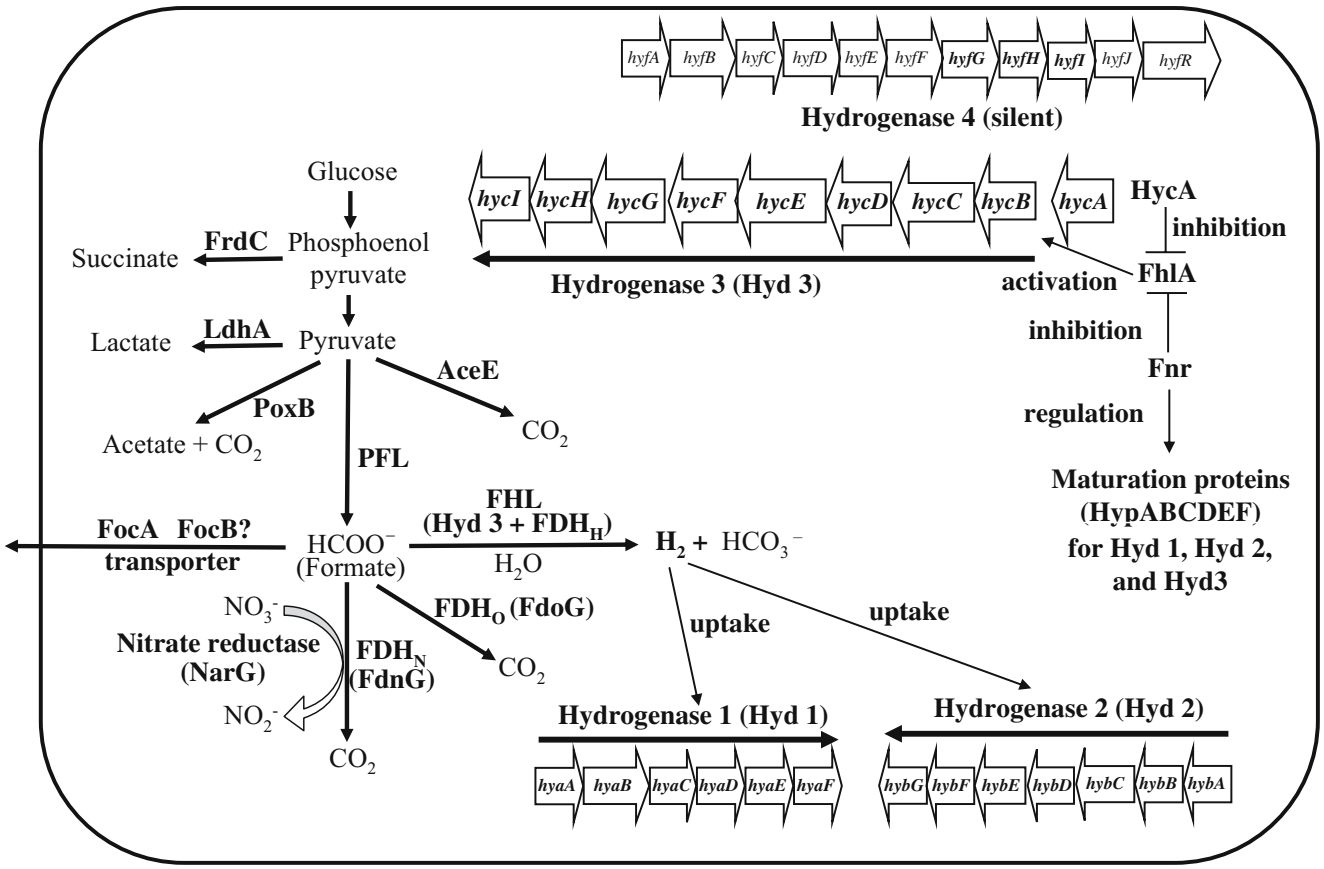

Fig. 1 Schematic of fermentative hydrogen production in *E. coli*. Cells metabolize glucose into phosphoenolpyruvate, pyruvate, and formate. Phosphoenolpyruvate is converted to succinate by fumarate reductase (FrdC), and pyruvate is converted to either lactate by lactate dehydrogenase (LdhA), to carbon dioxide (CO_2) and acetate by pyruvate oxidase (PoxB), to carbon dioxide by pyruvate dehydrogenase (AceE), or to formate by pyruvate formate lyase (PFL). Hydrogen is produced from formate by the formate hydrogen lyase (FHL) system consisting of hydrogenase 3 (Hyd 3) and formate dehydrogenase-H (FDH_H); the FHL is activated by FhlA that is regulated by Fnr and repressed by HycA. Evolved hydrogen is consumed through the hydrogen uptake activity of hydrogenase 1 (Hyd 1) and hydrogenase 2 (Hyd 2). Formate is exported by FocA and/or FocB and is metabolized by formate dehydrogenase-N (FDH_N; FdnG), which is linked with nitrate reductase A (NarG) and formate dehydrogenase-O (FDH_O; FdoG). HypABCDEF are maturation proteins for hydrogenases 1, 2, and 3

by *narG*) consumes formate by converting nitrate into nitrite by using electrons produced from formate by formate dehydrogenase-N (Bertero et al. 2003). Hence, by deleting *hyaB*, *hybC*, *fdnG*, *fdoG*, *focAB*, and *narG*, hydrogen production should be enhanced, and we have found that a quintuple mutant (BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhlA) increases hydrogen production from formate by over two orders of magnitude (Maeda et al. 2007b). In addition, pyruvate dehydrogenase (encoded by *aceE*) and pyruvate oxidase (encoded by *poxB*) consume pyruvate produced from glucose (Abdel-Hamid et al. 2001; Angelides et al. 1979; Fig. 1), so inactivating these genes may be useful for enhancing hydrogen production by preventing pyruvate consumption. Also, the succinate-producing pathway (phosphoenolpyruvate to succinate) and lactate-producing pathway (pyruvate to lactate) may be inactivated to direct glucose metabolism toward hydrogen (Fig. 1); therefore, deletion of fumarate reductase (*frdC*) and lactate dehydrogenase (*ldhA*) increases hydrogen production from glucose (Yoshida et al. 2006).

Because it may be more practical to produce hydrogen from glucose (Kraemer and Bagley 2007) rather than to add or overproduce formate, in this study, we create one septuple mutant (BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*) that produces 4.6-fold more hydrogen than the wild-type strain and that enhances the yield of hydrogen twofold as a result of manipulating the pathway mutations *hyaB*, *hybC*, *hycA*, *fhlA*, *focA*, *focB*, *narG*, *fdoG*, *fdnG*, *frdC*, *ldhA*, *poxB*, and *aceE*. This is the first report of strains harboring these seven mutations for converting glucose to hydrogen (previously, we reported on an *E. coli* strain harboring the *hyaB hybC hycA*, *fdoG* and *fhlA* mutations for converting formate to hydrogen (Maeda et al. 2007b)), and this is the first investigation of the importance of the *poxB* and *aceE* mutations for hydrogen production.

Materials and methods

Bacterial strains, growth rates, 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 (for those with chromosomal kanamycin resistance markers) and 30 μg/ml chloramphenicol (for those containing pCA24N-based plasmids), and incubated at 37°C. After growth on LB agar plates, a fresh single colony was cultured at 37°C with shaking at 250 rpm (New Brunswick Scientific Co., Edison, NJ, USA) in LB medium (Sambrook et al. 1989) or in modified complex glucose medium (Rachman et al. 1997) in which 0.4 mg/l $(NH_4)_6Mo_7O_{24}$ was added; 100 μg/ml kanamycin or 30 μg/ml chloramphenicol were also added where appropriate. Wild-type *E. coli* K-12 BW25113 was obtained from the Yale University CGSC Stock Center, and its isogenic deletion mutants (Keio collection) were obtained from the Genome Analysis Project in Japan (Baba et al. 2006). Plasmids based on pCA24N (Kitagawa et al. 2005) were electroporated into hydrogen-overproducing *E. coli* strains (Table 1). Aerobic cell growth was measured using turbidity at 600 nm from 0.05 to 0.7, and total protein for *E. coli* was 0.22 mg/OD per ml (Protein assay kit, Sigma Diagnostics, St. Louis, MO, USA).

Multiple chromosomal mutations Repeated rounds of P1 transduction (Silhavy et al. 1984) were performed to knockout specific genes by selecting for the kanamycin-resistance gene that is transferred along with each chromosomal deletion that are available from the Keio collection (Baba et al. 2006). Each Keio deletion mutant is designed with the ability to eliminate the kanamycin-resistance selection marker by expressing the FLP recombinase protein from pCP20 (Cherepanov and Wackernagel 1995), as each kanamycin resistance gene is flanked by a FLP recognition target that is excised by FLP recombinase. Hence, plasmid pCP20 (Cherepanov and Wackernagel 1995) was used as described previously (Datsenko and Wanner 2000) to eliminate the kanamycin resistance gene from each isogenic BW25113 mutant allele that was transferred to the chromosome via each P1 transduction so that multiple mutations could be introduced into a single strain.

Hydrogen closed vial assay Overnight, aerobic cultures (25 ml) were used to inoculate 75 ml of the modified complex glucose medium (111 mM glucose) in 250-ml shake flasks, and these cultures were sparged for 5 min with nitrogen, sealed, and incubated anaerobically at 37°C for 6 h. After 6 h, the cultures were poured anaerobically into a 250-mL centrifuge tubes in an anaerobic glove box, and centrifuged (7,350×*g*) for 10 min at 4°C. The supernatant was decanted in the glove box, 20 ml of modified complex medium without glucose was added, and then the cells were suspended to a turbidity of 2.5 at 600 nm. Sealed crimp-top vials (27 ml) were sparged for 5 min with nitrogen, and 9 ml of the cell suspension and 1 ml of 1 M glucose were added to the bottles that were incubated at 37°C with shaking for 30 min to 17 h. The amount of hydrogen generated in the head space of the recombinant system was measured using a 50-μl aliquot by gas chromatography (GC) using a 6890N gas chromatograph as described previously (Maeda et al. 2007c).

Hydrogen-low partial pressure assay Cells (30 ml) were prepared as above for the closed system, sparged, sealed in crimp-top vials (60 ml), 100 mM glucose was added, then the hydrogen gas was allowed to leave the headspace

Table 1 Strains and plasmids used. Km^R, Cm^R and Ap^R are kanamycin, chloramphenicol, and ampicillin resistance, respectively

Strains and plasmids	Genotype	Source
Strains		
E. coli BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Yale CGSG Stock Center
E. coli BW25113 Δ*hyaB*	BW25113 *hyaB* Km^R; defective in large subunit of hydrogenase 1	Baba et al. 2006
E. coli BW25113 Δ*hybC*	BW25113 *hybC* Km^R; defective in large subunit of hydrogenase 2	Baba et al. 2006
E. coli BW25113 Δ*hyaB* Δ*hybC*	BW25113 *hyaB hybC* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA*	BW25113 *hyaB hybC hycA* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, and defective in repressor of FHL	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycE*	BW25113 *hyaB hybC hycE* Δ*kan*; defective in large subunit of hydrogenase 1, hydrogenase 2, and hydrogenase 3	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focA*	BW25113 *hyaB hybC hycA focA* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in formate transporter	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focB*	BW25113 *hyaB hybC hycA focB* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in putative formate transporter	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*narG*	BW25113 *hyaB hybC hycA narG* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in α-subunit of nitrate reductase A	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fnr*	BW25113 *hyaB hybC hycA fnr* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in FNR transcriptional dual regulator	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdnG*	BW25113 *hyaB hybC hycA fdnG* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in α-subunit of formate dehydrogenase-N	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdoG*	BW25113 *hyaB hybC hycA fdoG* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in α-subunit of formate dehydrogenase-O	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*ldhA*	BW25113 *hyaB hybC hycA ldhA* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in lactate dehydrogenase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*frdC*	BW25113 *hyaB hybC hycA frdC* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in fumarate reductase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*frdC* Δ*ldhA*	BW25113 *hyaB hybC hycA frdC ldhA* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in fumarate reductase, and defective in lactate dehydrogenase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdnG* τ*fdoG*	BW25113 *hyaB hybC hycA fdnG fdoG* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in α-subunit of formate dehydrogenase-N and formate dehydrogenase-O	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdnG* Δ*fdoG* Δ*ldhA*	BW25113 *hyaB hybC hycA fdnG fdoG ldhA* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-N and formate dehydrogenase-O, and defective in lactate dehydrogenase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdoG* Δ*focA*	BW25113 *hyaB hybC hycA fdoG focA* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-O, and defective in formate transporter	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdoG* Δ*ldhA*	BW25113 *hyaB hybC hycA fdoG ldhA* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-O, and defective in lactate dehydrogenase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdoG* Δ*frdC*	BW25113 *hyaB hybC hycA fdoG frdC* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-O, and defective in fumarate reductase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdoG* Δ*ldhA* Δ*frdC*	BW25113 *hyaB hybC hycA fdoG ldhA frdC* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-O, defective in lactate dehydrogenase, and defective in fumarate reductase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdoG* Δ*ldhA* Δ*frdC* Δ*aceE*	BW25113 *hyaB hybC hycA fdoG ldhA frdC aceE* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-O, defective in lactate dehydrogenase, defective in fumarate reductase, and defective in pyruvate dehydrogenase	This study

E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdoG* Δ*ldhA* Δ*frdC* Δ*poxB*	BW25113 *hyaB hybC hycA fdoG ldhA frdC poxB* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-O, defective in lactate dehydrogenase, defective in fumarate reductase, and defective in pyruvate oxidase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdnG* Δ*ldhA* Δ*frdC*	BW25113 *hyaB hybC hycA fdnG ldhA frdC* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-N, defective in lactate dehydrogenase, and defective in fumarate reductase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdnG* Δ*ldhA* Δ*frdC* Δ*aceE*	BW25113 *hyaB hybC hycA fdnG ldhA frdC aceE* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-N, defective in lactate dehydrogenase, defective in fumarate reductase, and defective in pyruvate dehydrogenase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdnG* Δ*ldhA* Δ*frdC* Δ*poxB*	BW25113 *hyaB hybC hycA fdnG ldhA frdC poxB* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-N, defective in lactate dehydrogenase, defective in fumarate reductase, and defective in pyruvate oxidase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*fdoG* Δ*fdnG* Δ*ldhA* Δ*frdC*	BW25113 *hyaB hybC hycA fdoG fdnG ldhA frdC* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of formate dehydrogenase-O and formate dehydrogenase-N, defective in lactate dehydrogenase, and defective in fumarate reductase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focA* Δ*focB*	BW25113 *hyaB hybC hycA focA focB* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, and defective in formate transporter and putative formate transporter	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focA* Δ*narG*	BW25113 *hyaB hybC hycA focA narG* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in formate transporter, and defective in α-subunit of nitrate reductase A	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focB* Δ*narG*	BW25113 *hyaB hybC hycA focB narG* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter, and defective in α-subunit of nitrate reductase A	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focA* Δ*focB* Δ*narG*	BW25113 *hyaB hybC hycA focA focB narG* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in formate transporter and putative formate transporter, and defective in α-subunit of nitrate reductase A	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focB* Δ*fdnG*	BW25113 *hyaB hybC hycA focB fdnG* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter, and defective in α-subunit of formate dehydrogenase-N	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focB* Δ*fdoG*	BW25113 *hyaB hybC hycA focB fdoG* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter, and defective in α-subunit of formate dehydrogenase-O	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focB* Δ*fdnG* Δ*fdoG*	BW25113 *hyaB hybC hycA focB fdnG fdoG* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter, and defective in α-subunit of formate dehydrogenase-N and formate dehydrogenase-O	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focB* Δ*ldhA*	BW25113 *hyaB hybC hycA focB ldhA* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter, and defective in lactate dehydrogenase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*focB* Δ*frdC*	BW25113 *hyaB hybC hycA focB frdC* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in putative formate transporter, and defective in fumarate reductase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*narG* Δ*ldhA*	BW25113 *hyaB hybC hycA narG ldhA* Δ*kan*; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of nitrate reductase A, and defective in lactate dehydrogenase	This study
E. coli BW25113 Δ*hyaB* Δ*hybC* Δ*hycA* Δ*narG* Δ*frdC*	BW25113 *hyaB hybC hycA narG frdC* Km^R; defective in large subunit of hydrogenase 1 and hydrogenase 2, defective in repressor of FHL, defective in α-subunit of nitrate reductase A, and defective in fumarate reductase	This study
Plasmids		
pCA24N	Empty vector; Cm^R	Kitagawa et al. 2005
pCA24N-FhlA	pCA24N *pT5-lac*::*fhlA*; expresses FhlA derived from *Escherichia coli*	Kitagawa et al. 2005
pCP20	Ap^R and Cm^R plasmid with temperature-sensitive replication and thermal induction of FLP synthesis	Cherepanov and Wackernagel 1995

through a needle in the septum via tubing that directed the gas through 1 M NaOH (to remove carbon dioxide; Klibanov et al. 1982), and into an inverted graduated cylinder that was used to measure the volume of the gas (Maeda et al. 2007b). The vials were incubated at 37°C with stirring for 15 min, and hydrogen was assayed with a GC. As a negative control, cell suspensions (20 ml) without glucose were also used. Glucose concentrations in complex glucose media were measured using the HK assay (Sigma). For yield calculations, the vials were incubated for 16 h.

Results

Our strategy for metabolic engineering of *E. coli* for enhanced hydrogen production from glucose via formate was sixfold (Fig. 1) and based on our initial success of using some of these mutations for increasing the yield of hydrogen from formate using strain BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhlA (Maeda et al. 2007b); note that all the original mutations had to be reevaluated, as they were originally assayed for their effect on producing hydrogen starting from formate, and two new mutations were evaluated here (*poxB* and *aceE*). We (1) prevented hydrogen consumption by inactivating hydrogenase 1 (HyaB, large subunit) and hydrogenase 2 (HybC, large subunit), (2) inactivated the FHL repressor HycA, (3) overexpressed the FHL activator FhlA (FhlA binds directly to the intergenic region between the *hyc* and *hyp* operons and between the *hycA* and *hycB* genes; Schlensog et al. 1994), (4) eliminated the formate exporters FocA and its homolog FocB (Andrews et al. 1997; Suppmann and Sawers 1994), (5) prevented formate consumption by formate dehydrogenase-N (FdnG, α-subunit) coupled with nitrate reductase A (NarG, α-subunit; Rossmann et al. 1991) and dehydrogenase-O (FdoG, α-subunit; Rossmann et al. 1991), and (6) altered glucose metabolism to efficiently synthesize formate from glucose by preventing lactate and succinate formation, as well as pyruvate consumption. *E. coli* cells metabolize glucose into formate via phosphoenolpyruvate and pyruvate by the glycolytic system (Bagramyan and Trchounian 2003); phosphoenolpyruvate may also be converted into succinate by fumarate reductase (FrdC; Iverson et al. 1999), pyruvate may be converted into lactate by lactate dehydrogenase (LdhA; Sode et al. 1999), and pyruvate may be consumed by pyruvate dehydrogenase (AceE; Angelides et al. 1979) and pyruvate oxidase (PoxB; Abdel-Hamid et al. 2001; Fig. 1). Therefore, deleting *frdC*, *ldhA*, *aceE*, and *poxB* should enhance hydrogen production by increasing formate production.

Another goal was to introduce mutations that did not make the cell less viable so specific growth rates were quantified after each mutation was added. Cell viability was not significantly affected for all strains (46 strains) except the two septuple mutants with the *aceE* mutations (*hyaB hybC hycA fdoG frdC ldhA aceE* and *hyaB hybC hycA fdnG frdC ldhA aceE*) that had a 3.6-fold reduced aerobic specific growth rate compared to the wild type strain in LB medium (Table 2). In addition, the specific growth rate of BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* was 2.7 times lower than that of wild-type cells in complex glucose medium (1.6±0.1 for BW25113 vs 0.59±0.02 1/h for BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*); however, there was no difference in the amount of cellular protein after overnight growth for the low partial pressure/closed hydrogen assay experiments between the wild-type strain and the *hyaB hybC hycA fdoG frdC ldhA aceE* strain (data not shown). These results are primarily in contrast to other approaches in which cell viability has been reduced (Penfold et al. 2006).

To decrease hydrogen uptake activity in *E. coli*, the genes encoding the large subunits of hydrogenase 1 (*hyaB*) and hydrogenase 2 (*hybC*) were deleted because the active site of catalysis is located within each large subunit for these [NiFe]-hydrogenases (Forzi and Sawers 2007). As expected, the double mutant (*hyaB hybC*) showed a significant decrease in hydrogen uptake activity (Maeda et al. 2007a) that led to a 1.4-fold increase in hydrogen production compared to the wild-type strain in complex glucose medium after 30 min (Table 2); however, there was only a slight change in hydrogen production rates for each single mutation (*hyaB* or *hybC*). Also, adding the *hycA* mutation to the *hyaB hybC* double mutant did not show a significant increase in hydrogen production from glucose (Table 2), although BW25113 *hyaB hybC hycA* produced 1.5-fold more hydrogen from formate compared to cells defective in both hydrogenase 1 (*hyaB*) and hydrogenase 2 (*hybC*; Maeda et al. 2007b).

Formate, which is the substrate for producing hydrogen in *E. coli*, is depleted by two non-hydrogen-producing pathways: (1) excretion by the formate transporter FocA (Suppmann and Sawers 1994) and its homolog FocB (Andrews et al. 1997), and (2) degradation by formate dehydrogenase-N (coupled with nitrate reductase A) and degradation by formate dehydrogenase-O, which convert formate to ATP (Wang and Gunsalus 2003). Hence, formate transport, formate dehydrogenase-N/nitrate reductase A activity, and formate dehydrogenase-O activity may be deleted to enhance hydrogen production. Based on this strategy, five quadruple mutants (*hyaB hybC hycA focA*, *hyaB hybC hycA focB*, *hyaB hybC hycA narG*, *hyaB hybC hycA fdnG*, and *hyaB hybC hycA fdoG*) were constructed by introducing a *focA*, *focB*, *narG*, *fdnG*, and *fdoG* mutation to the triple mutant (*hyaB hybC hycA*), and then hydrogen production was assayed. The addition of the *focB* and *narG* mutations to the *hyaB hybC hycA* mutant increased

Table 2 Effect of metabolic mutations on hydrogen production from glucose by *E. coli* BW25113 in the closed system

Strain	Growth rate		H_2 production[a]		H_2 production[b]	
	1/h	Relative	μmol/mg protein	Relative	μmol/mg protein	Relative
BW25113	1.6±0.1	1	15±4	1	35±1	1
BW25113 *hyaB*	1.42±0.01	0.9	15.6±0.5	1.1	35±1	1
BW25113 *hybC*	1.6±0.1	1	17.5±0.04	1.2	37±1	1.1
BW25113 *hyaB hybC*	1.6±0.1	1	21±1	1.4	38.3±0.6	1.1
BW25113 *hyaB hybC hycA*	1.4±0.2	0.9	21±1	1.4	36±2	1
BW25113 *hyaB hybC hycE*	1.5±0.1	0.9	0.57±0.02	0.04	1.74±0.01	0.05
BW25113 *hyaB hybC hycA focA*	1.6±0.1	1	20±2	1.4	35±1	1
BW25113 *hyaB hybC hycA focB*	1.58±0.01	1	24±1	1.6	32±5	0.9
BW25113 *hyaB hybC hycA narG*	1.46±0.00	0.9	24±4	1.6	34±4	1
BW25113 *hyaB hybC hycA fnr*	1.6±0.2	1	0.6±0.2	0.04	1.7±0.1	0.05
BW25113 *hyaB hybC hycA fdnG*	1.6±0.1	1	20.7±0.8	1.4	34.2±0.2	1
BW25113 *hyaB hybC hycA fdoG*	1.44±0.05	0.9	25±3	1.7	35±1	1
BW25113 *hyaB hybC hycA ldhA*	1.58±0.06	1	22±1	1.5	43±1	1.2
BW25113 *hyaB hybC hycA frdC*	1.6±0.1	1	20.5±0.8	1.4	35.4±0.5	1
BW25113 *hyaB hybC hycA frdC ldhA*	1.51± 0.03	0.9	30±1	2.0	43±3	1.2
BW25113 *hyaB hybC hycA fdnG fdoG*	1.46±0.07	0.9	25.3±0.5	1.7	36.3±0.8	1
BW25113 *hyaB hybC hycA fdnG fdoG ldhA*	1.53±0.05	1	27.4±0.7	1.8	45.4±0.9	1.3
BW25113 *hyaB hybC hycA fdoG focA*	1.5±0.2	0.9	23.0±0.2	1.6	34.6±0.2	1
BW25113 *hyaB hybC hycA fdoG ldhA*	1.5±0.1	0.9	26.8±0.7	1.7	52±12	1.5
BW25113 *hyaB hybC hycA fdoG frdC*	1.55±0.07	1	26±1	1.7	41±11	1.2
BW25113 *hyaB hybC hycA fdoG ldhA frdC*	1.5±0.2	0.9	29.1±0.9	1.9	44.64±0.01	1.3
BW25113 *hyaB hybC hycA fdoG ldhA frdC aceE*	0.44±0.03	0.3	32.5±0.8	2.2	45.2±0.3	1.3
BW25113 *hyaB hybC hycA fdoG ldhA frdC poxB*	1.40±0.09	0.9	30±1	2.0	43±1	1.2
BW25113 *hyaB hybC hycA fdnG ldhA frdC*	1.49±0.02	0.9	30±1	2.0	42.9±0.4	1.2
BW25113 *hyaB hybC hycA fdnG ldhA frdC aceE*	0.46±0.01	0.3	32.4±0.2	2.2	44.6±0.7	1.3
BW25113 *hyaB hybC hycA fdnG ldhA frdC poxB*	1.2±0.1	0.8	28.9±0.5	1.9	41± 0.5	1.2
BW25113 *hyaB hybC hycA fdoG fdnG ldhA frdC*	1.48±0.05	0.9	15.7±0.1	1.0	45±2	1.3
BW25113 *hyaB hybC hycA focA focB*	1.3±0.2	0.8	22.3±0.5	1.5	36.0±0.6	1
BW25113 *hyaB hybC hycA focA narG*	1.3±0.1	0.8	20.7±0.2	1.4	33.0±0.1	0.9
BW25113 *hyaB hybC hycA focB narG*	1.4±0.1	0.9	19±2	1.3	34.1±0.1	1
BW25113 *hyaB hybC hycA focA focB narG*	1.4±0.3	0.9	9.7±0.8	0.7	26.3±0.6	0.8
BW25113 *hyaB hybC hycA focB fdnG*	1.6±0.1	1	1.2±0.1	0.08	3.6±0.1	0.1
BW25113 *hyaB hybC hycA focB fdoG*	1.5±0.1	0.9	1.2±0.1	0.08	3.6±0.2	0.1
BW25113 *hyaB hybC hycA focB fdnG fdoG*	1.54±0.09	1	1.2±0.1	0.08	3.7±0.2	0.1
BW25113 *hyaB hybC hycA focB ldhA*	1.44±0.05	0.9	0.86±0.03	0.06	4.0±0.2	0.1
BW25113 *hyaB hybC hycA focB frdC*	1.47±0.04	0.9	0.87±0.00	0.06	3.9±0.4	0.1
BW25113 *hyaB hybC hycA narG ldhA*	1.54±0.01	1	23±2	1.5	47±3	1.3
BW25113 *hyaB hybC hycA narG frdC*	1.5±0.1	0.9	22.0±0.3	1.5	29.7±0.4	0.8
BW25113/pCA24N	1.46±0.03	1	12.4±0.3	1	30±2	1
BW25113/pCA24N-FhlA	1.47±0.03	1	13.2±0.0	1.1	31±1	1
BW25113 *hyaB hybC hycA*/pCA24N	1.39±0.08	1	18±2	1.5	35±4	1.2
BW25113 *hyaB hybC hycA*/pCA24N-FhlA	1.39±0.02	1	18±2	1.5	31±6	1
BW25113 *hyaB hybC hycA focA*/pCA24N-FhlA	1.4±0.1	1	16±1	1.3	33.4±0.2	1.1
BW25113 *hyaB hybC hycA focB*/pCA24N-FhlA	1.4±0.2	1	16.8±0.2	1.4	33.61±0.03	1.1
BW25113 *hyaB hybC hycA narG*/pCA24N-FhlA	1.38±0.06	0.9	16.6±0.3	1.3	33.8±0.4	1.1
BW25113 *hyaB hybC hycA focB narG*/pCA24N-FhlA	ND[c]	-	15±4	1.2	27±7	0.9
BW25113 *hyaB hybC hycA focA focB narG*/pCA24N-FhlA	ND	–	4±3	0.3	8±5	0.3
BW25113 *hyaB hybC hycA fdoG*/pCA24N	1.5±0.2	1	19.9±0.3	1.6	28±3	0.9
BW25113 *hyaB hybC hycA fdoG*/pCA24N-FhlA	1.47±0.03	1	22.0±0.9	1.8	29±1	1
BW25113 *hyaB hybC hycA fdoG ldhA frdC*/pCA24N	1.47±0.09	1	21.6±0.8	1.7	42.7±0.1	1.4
BW25113 *hyaB hybC hycA fdoG ldhA frdC*/pCA24N-FhlA	1.52±0.01	1	20.6±0.5	1.7	44±1	1.5

[a] Thirty minutes in complex glucose medium
[b] Seventeen hours in complex glucose medium
[c] Not determined

hydrogen production 1.6-fold compared to the wild-type strain in complex glucose medium after 30 min (Table 2). Also, hydrogen production in BW25113 *hyaB hybC hycA fdoG* was increased 1.7-fold compared to that in the wild-type cells (Table 2). The *focA* and *fdnG* mutation were not effective for producing more hydrogen in the *hyaB hybC hycA* background.

To further test the combination of the *focA*, *focB*, *narG*, *fdnG*, and *fdoG* mutations, seven quintuple strains (*hyaB hybC hycA focA focB*, *hyaB hybC hycA focA narG*, *hyaB hybC hycA focB narG*, *hyaB hybC hycA focB fdnG*, *hyaB hybC hycA focB fdoG*, *hyaB hybC hycA fdnG fdoG*, and *hyaB hybC hycA fdoG focA*) and two sextuple strains (*hyaB hybC hycA focA focB narG* and *hyaB hybC hycA focB fdnG fdoG*) were constructed, and hydrogen production was assayed. Three quintuple mutants (*hyaB hybC hycA fdnG fdoG*, *hyaB hybC hycA fdoG focA*, and *hyaB hybC hycA focA focB*) produced 1.5–1.7 times more hydrogen than the wild-type strain; hydrogen production in two quintuple mutants BW25113 *hyaB hybC hycA focA narG* and *hyaB hybC hycA focB narG* was the same level with that in BW25113 *hyaB hybC hycA*. On the other hand, two quintuple mutants (*hyaB hybC hycA focB fdnG* and *hyaB hybC hycA focB fdoG*) and two sextuple strains (*hyaB hybC hycA focA focB narG* and *hyaB hybC hycA focB fdnG fdoG*) had lower hydrogen production activity than the wild-type cells.

To test the effect of deleting the succinate-producing pathway (*frdC*) and the lactate-producing pathway (*ldhA*), two quadruple mutants (*hyaB hybC hycA ldhA* and *hyaB hybC hycA frdC*), seven quintuple mutants (*hyaB hybC hycA ldhA frdC*, *hyaB hybC hycA fdoG ldhA*, *hyaB hybC hycA fdoG frdC*, *hyaB hybC hycA focB ldhA*, *hyaB hybC hycA focB frdC*, *hyaB hybC hycA narG ldhA*, and *hyaB hybC hycA narG frdC*), three sextuple mutants (*hyaB hybC hycA fdnG fdoG ldhA*, *hyaB hybC hycA fdoG ldhA frdC*, and *hyaB hybC hycA fdnG ldhA frdC*), and one septuple mutant (*hyaB hybC hycA fdoG fdnG ldhA frdC*) were constructed, and hydrogen production was assayed. One quintuple (BW25113 *hyaB hybC hycA frdC ldhA*) and two sextuple mutants (BW25113 *hyaB hybC hycA fdoG ldhA frdC* and *hyaB hybC hycA fdnG ldhA frdC*) produced twofold more hydrogen than the wild-type strain after 30 min in complex glucose medium (Table 2). Also, hydrogen production in all strains harboring the *ldhA* mutation was increased by 20–50% compared to that in the wild-type cells after 17 h in complex glucose medium (Table 2). One septuple mutant (*hyaB hybC hycA fdoG fdnG ldhA frdC*) showed lower hydrogen production than two sextuple mutants (*hyaB hybC hycA fdoG ldhA frdC* and *hyaB hybC hycA fdnG ldhA frdC*) that have high hydrogen production potential (Table 2).

Previously, we found that expressing the FhlA protein (FHL activator) led to a ninefold increase in hydrogen production in medium containing formate (BW25113/pCA24N-FhlA vs BW25113; Maeda et al. 2007b). Hence, to boost hydrogen productivity further, plasmid pCA24N-FhlA was added to the metabolically engineered strains, and hydrogen production was assayed (Table 2). Unexpectedly, the expression of *fhlA* did not lead to a significant increase of hydrogen production from the modified complex-glucose medium in BW25113, BW25113 *hyaB hybC hycA*, BW25113 *hyaB hybC hycA fdoG*, and BW25113 *hyaB hybC hycA fdoG ldhA frdC* in the closed hydrogen assay. Also, overexpressing FhlA by adding isopropylthiogalactoside (IPTG; 0.01 to 1 mM) led to a significant decrease in hydrogen production; hydrogen production with 1 mM IPTG was threefold less than that without IPTG (data not shown).

To investigate whether pyruvate consumption by the PoxB and AceE pathways (Fig. 1) is significant for hydrogen production, four septuple mutants (BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*, BW25113 *hyaB hybC hycA fdoG frdC ldhA poxB*, BW25113 *hyaB hybC hycA fdnG frdC ldhA aceE*, and BW25113 *hyaB hybC hycA fdnG frdC ldhA poxB*) were constructed, and then hydrogen production was assayed. Two septuple strains with the *aceE* mutation (BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* and BW25113 *hyaB hybC hycA fdnG frdC ldhA aceE*) had a slight increase of hydrogen production (8 to 12%) compared to BW25113 *hyaB hybC hycA fdoG frdC ldhA* or BW25113 *hyaB hybC hycA fdnG frdC ldhA*; hydrogen production in these two strains was 2.2 times higher than that in wild-type strain (Table 2).

With BW25113 *hyaB hybC hycA fdoG frdC aceE*, we also tested whether the enhanced hydrogen production was from the added glucose. As expected, this strain produced hydrogen only slightly from complex medium that lacked glucose (4.4% of that from complex glucose medium). This indicates that hydrogen from complex glucose is derived from glucose.

Because the accumulation of hydrogen in the headspace in the closed system reduces hydrogen production (Kraemer and Bagley 2007), hydrogen production for the nine best strains was measured using an anaerobic system that maintained low hydrogen headspace pressure, and the results are shown in Table 3. BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* produced 4.6-fold more hydrogen than the wild-type strain, whereas BW25113 *hyaB hybC hycA fdnG frdC ldhA*, BW25113 *hyaB hybC hycA fdnG frdC ldhA aceE*, and BW25113 *hyaB hybC hycA fdoG frdC ldhA* had 4.1- to 4.3-fold higher hydrogen production. Similarly, BW25113 *hyaB hybC hycA frdC*, BW25113 *hyaB hybC hycA ldhA*, and BW25113 *hyaB hybC hycA frdC ldhA* synthesized 2.9- to 3.3-fold more hydrogen relative to the wild-type strain (Table 3). As a negative control, BW25113 *hyaB hybC hycE*, which lacks an active hydrogenase 3, showed negligible hydrogen production (8.8-fold less) than that of the

Table 3 Hydrogen production from glucose by metabolically-engineered *E. coli* strains using the low partial pressure assay and hydrogen yields

Strain	Description	Hydrogen production rate[a]		Hydrogen yield	
		μmol/mg protein/h	Relative	mol/mol[b]	Relative
BW25113	wild type	7±1	1	0.65±0.08	1
BW25113 *hyaB hybC hycE*	Δ*hyaB,* Δ*hybC*, and Δ*hycE* (defective hydrogenases 1, 2, and 3)	0.8±0.1	0.1	0.02±0.00	0.03
BW25113 *hyaB hybC hycA*	Δ*hyaB,* Δ*hybC*, and Δ*hycA* (defective hydrogenase 1 and 2, and defective FHL repressor)	16±4	2.3	0.78±0.06	1.2
BW25113 *hyaB hybC hycA frdC*	Δ*hyaB,* Δ*hybC*, Δ*hycA*, and Δ*frdC* (defective hydrogenase 1 and 2, defective FHL repressor, and defective fumarate reductase membrane protein)	23.7±0.8	3.4	0.89±0.01	1.4
BW25113 *hyaB hybC hycA ldhA*	Δ*hyaB,* Δ*hybC*, Δ*hycA*, and Δ*ldhA* (defective hydrogenase 1 and 2, defective FHL repressor, and defective D-lactate dehydrogenase)	22.9±0.7	3.3	1.15±0.03	1.8
BW25113 *hyaB hybC hycA fdoG*	Δ*hyaB,* Δ*hybC*, Δ*hycA*, and Δ*fdoG* (defective hydrogenase 1 and 2, defective FHL repressor, and defective formate dehydrogenase-O)	16±7	2.3	0.81±0.06	1.2
BW25113 *hyaB hybC hycA frdC ldhA*	Δ*hyaB,* Δ*hybC*, Δ*hycA*, Δ*frdC*, and Δ*ldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective fumarate reductase membrane protein, and defective D-lactate dehydrogenase)	20±5	2.9	1.34±0.04	2.1
BW25113 *hyaB hybC hycA fdnG frdC ldhA*	Δ*hyaB,* Δ*hybC*, Δ*hycA*, Δ*fdnG*, Δ*frdC*, and Δ*ldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-N, defective fumarate reductase membrane protein, and defective D-lactate dehydrogenase)	29±3	4.1	1.35±0.06	2.1
BW25113 *hyaB hybC hycA fdnG frdC ldhA aceE*	Δ*hyaB,* Δ*hybC*, Δ*hycA*, Δ*fdnG*, Δ*frdC*, Δ*ldhA,* and Δ*aceE* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-N, defective fumarate reductase membrane protein, defective D-lactate dehydrogenase, and defective pyruvate dehydrogenase)	30±2	4.3	1.28±0.01	2.0
BW25113 *hyaB hybC hycA fdnG frdC ldhA poxB*	r*hyaB,* r*hybC*, r*hycA*, r*fdnG*, r*frdC*, r*ldhA,* and r*poxB* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-N, defective fumarate reductase membrane protein, defective D-lactate dehydrogenase, and defective pyruvate oxidase)	25.2±0.7	3.6	1.33±0.02	2.0
BW25113 *hyaB hybC hycA fdoG frdC ldhA*	Δ*hyaB,* Δ*hybC*, Δ*hycA*, Δ*fdoG*, Δ*frdC*, and Δ*ldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O, defective fumarate reductase membrane protein, and defective D-lactate dehydrogenase)	29±3	4.1	1.23±0.05	1.9
BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*	Δ*hyaB,* Δ*hybC*, Δ*hycA*, Δ*fdoG*, Δ*frdC*, Δ*ldhA,* and Δ*aceE* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O, defective fumarate reductase membrane protein, defective D-lactate dehydrogenase, and defective pyruvate dehydrogenase)	32±6	4.6	1.32±0.04	2.0
BW25113 *hyaB hybC hycA fdoG frdC ldhA poxB*	Δ*hyaB,* Δ*hybC*, Δ*hycA*, Δ*fdoG*, Δ*frdC*, Δ*ldhA,* and Δ*poxB* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O, defective fumarate reductase membrane protein, defective D-lactate dehydrogenase, and defective pyruvate oxidase)	22±4	3.1	1.20±0.04	1.8
BW25113 *hyaB hybC hycA fdoG fdnG frdC ldhA*	Δ*hyaB,* Δ*hybC*, Δ*hycA*, Δ*fdoG*, Δ*fdnG*, Δ*frdC*, and Δ*ldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O and formate dehydrogenase-N, defective fumarate reductase membrane protein, and defective D-lactate dehydrogenase)	8±1	1.1	1.15±0.05	1.8
BW25113/pCA24N	wild type	5±2	1	0.49±0.02	1
BW25113/pCA24N-FhlA	wild type + FhlA	4.1±0.6	0.8	0.57±0.06	1.2
BW25113 *hyaB hybC hycA*/pCA24N	Δ*hyaB,* Δ*hybC*, and Δ*hycA* (defective hydrogenase 1 and 2, and defective FHL repressor)	13±3	2.6	0.74±0.04	1.5
BW25113 *hyaB hybC hycA*/pCA24N-FhlA	Δ*hyaB,* Δ*hybC*, and Δ*hycA* (defective hydrogenase 1 and 2, and defective FHL repressor) + FhlA	13±1	2.6	0.77±0.03	1.6
BW25113 *hyaB hybC hycA fdoG frdC ldhA*/pCA24N	Δ*hyaB,* Δ*hybC*, Δ*hycA*, Δ*fdoG*, Δ*frdC*, and Δ*ldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O, defective fumarate reductase membrane protein, and defective D-lactate dehydrogenase)	24.1±0.4	4.8	1.13±0.04	2.4
BW25113 *hyaB hybC hycA fdoG frdC ldhA*/pCA24N-FhlA	Δ*hyaB,* Δ*hybC*, Δ*hycA*, Δ*fdoG*, Δ*frdC*, and Δ*ldhA* (defective hydrogenase 1 and 2, defective FHL repressor, defective formate dehydrogenase-O, defective fumarate reductase membrane protein, and defective D-lactate dehydrogenase) + FhlA	22±0.7	4.4	1.2±0.1	2.5

[a] An initial hydrogen production rate was calculated from a 15-min incubation in complex glucose medium.
[b] Hydrogen yields were calculated as mol-H_2/mol-glucose from the results after 16 h.

wild-type cells for both the low partial pressure (Table 3), as well as the closed hydrogen assays (Table 2). Also, BW25113 *hyaB hybC hycA fdoG ldhA frdC* with pCA24N or pCA24N-FhlA produced up to 4.8-fold higher hydrogen than BW25113/pCA24N, although the over expression of FhlA protein did not lead to a significant increase of hydrogen production.

Along with hydrogen production, hydrogen yields are important. For BW25113 *hyaB hybC hycA frdC ldhA*, BW25113 *hyaB hybC hycA fdnG frdC ldhA*, BW25113 *hyaB hybC hycA fdnG frdC ldhA aceE*, BW25113 *hyaB hybC hycA fdnG frdC ldhA poxB*, BW25113 *hyaB hybC hycA fdoG frdC ldhA*, and BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*, the hydrogen yield increased by twofold compared to that in BW25113 wild-type cells. In addition, BW25113 *hyaB hybC hycA fdoG frdC ldhA* with pCA24N and pCA24N-FhlA had 2.5-fold higher hydrogen yields than BW25113/pCA24N. Also, the yield of BW25113 *hyaB hybC hycA frdC* and BW25113 *hyaB hybC hycA ldhA* increased 1.4 to 1.8-fold compared to BW25113 *hyaB hybC hycA* (Table 3), indicating that these two mutations (*frdC* and *ldhA*) are effective for enhancing hydrogen yields from glucose. Deleting *aceE* in BW25113 *hyaB hybC hycA fdoG frdC ldhA* (i.e., BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*) had a slight increase (up to 7%) in hydrogen yield compared to that in BW25113 *hyaB hybC hycA fdoG frdC ldhA* (Table 3). Assaying glucose in complex glucose medium demonstrated clearly that the septuple strain consumed over 97% of glucose after 16 h.

Discussion

In this work, we show that a fermentative *E. coli* strain with seven mutations, BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*, produces 4.6-fold more hydrogen than the wild-type strain (~32 μmol/h per mg protein vs 7 μmol/h per mg protein) as a result of inactivating hydrogen consumption by hydrogenase 1 (*hyaB*) and hydrogenase 2 (*hybC*), activation of FHL by deleting the FHL repressor (*hycA*), inactivation of formate dehydrogenase-O (*fdoG*) to prevent formate consumption, inactivation of the succinate synthesis (*frdC*) and lactate synthesis (*ldhA*) pathways, and inactivation of pyruvate dehydrogenase (*aceE*) to prevent pyruvate consumption. Also, the hydrogen yield with BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* by the strain increased twofold (~1.32 vs 0.7 mol H_2/mol glucose).

We used the *E. coli* Keio collection in this study to introduce as many as seven mutations into a single strain. Thus, the use of this library is a breakthrough in that it has been difficult to make strains with multiple mutations using other methods that depend on different selection makers for each gene inactivated (Lee et al. 2005; Yoshida et al. 2006). This method is general and simple (repetition of resistance-gene elimination and P1 transduction) and may be used to engineer *E. coli* for many applications where multiple chromosomal genes must be eliminated.

Previously, three groups have enhanced hydrogen production in *E. coli*. Inactivation of the FHL repressor (HycA) and overexpression of the FHL activator (FhlA) led to a 2.8-fold increase of hydrogen production from formate (Yoshida et al. 2005). Deleting the twin-arginine translocation system for transporting proteins into the periplasm resulted in twofold higher hydrogen production from glucose by indirectly inactivating hydrogenase 1, hydrogenase 2, formate dehydrogenase-N, and formate dehydrogenase-O; however, this mutation led to a significant decrease in cell viability (Penfold et al. 2006). Also, deletions of lactate dehydrogenase (*ldhA*) and fumarate reductase (*frdBC*) resulted in only a 1.4-fold increase in hydrogen production compared to the wild-type strain from glucose (Yoshida et al. 2006). In comparison, our metabolically engineered *E. coli* cells have as much as 4.6-fold greater hydrogen production, and the method remains robust, as it is still possible to introduce further mutations to enhance hydrogen production. Because of the ease of its genetic manipulation, *E. coli* may also be a better model than other hydrogen-producing strains such as *Citrobacter* sp. Y19 (Oh et al. 2003), *Rhodopseudomonas palustris* JA1 (Archana et al. 2003), *Rhodopseudomonas palustris* P4 (Jung et al. 1999), and *Klebsiella oxytoca* HP1 (Minnan et al. 2005) that have high maximum hydrogen activity (up to 65 μmol/mg per h).

The deletion of succinate-producing pathway (*frdC*) and lactate-producing pathway (*ldhA*) in the *hyaB hybC hycA* background led to a threefold higher increase of hydrogen production rate and a twofold higher hydrogen yield compared to the wild-type strain (Table 3); these results are consistent with the results described previously (Sode et al. 1999; Yoshida et al. 2006). Because the two quadruple mutants (BW25113 *hyaB hybC hycA frdC* and BW25113 *hyaB hybC hycA ldhA*) increased hydrogen production 1.5- and 1.4-fold, respectively, vs BW25113 *hyaB hybC hycA* (Table 3) and resulted in a 1.1- or 1.5-fold higher hydrogen yield relative to BW25113 *hyaB hybC hycA*, both the *frdC* and *ldhA* mutations are important for hydrogen production from glucose, but the *ldhA* mutation is more effective than the *frdC* mutation for increasing the hydrogen yield (Table 3).

It has been reported that the deficiency of formate dehydrogenase-N leads to an accumulation of intracellular formate and activation of the FHL pathway (Suppmann and Sawers 1994); hence, mutating *fdnG* should be effective for enhancing hydrogen production. As expected, the deletion of *fdnG* was significant as seen by comparing hydrogen production between BW25113 *hyaB hybC hycA frdC ldhA* vs BW25113 *hyaB hybC hycA fdnG frdC ldhA* (Table 3); the additional *fdnG* deletion led to a 45% increase in the

hydrogen production rate. Similarly, deleting *fdoG* also increased hydrogen production by about 45% (BW25113 *hyaB hybC hycA frdC ldhA* vs BW25113 *hyaB hybC hycA fdoG frdC ldhA*, Table 3); however, the effect was not as large as the effect for growth on formate where there was a 2.2-fold increase in hydrogen production (BW25113 *hyaB hybC hycA* vs BW25113 *hyaB hybC hycA fdoG* in a closed system assay; Maeda et al. 2007b). On the other hand, deleing both formate dehydrogenase-N and formate dehydrogenase-O led to a significant decrease in the hydrogen production rate (Tables 2 and 3), although these mutations did not influence hydrogen yield. These results show that either active formate dehydrogenase-N or formate dehydrogenase-O is essential for producing hydrogen from glucose, whereas increasing hydrogen production from formate requires inactivation of formate dehydrogenase-O (Maeda et al. 2007b).

Because our metabolically engineered *E. coli* strains had a 1.3 mol H_2/mol glucose of hydrogen yield instead of the theoretical hydrogen yield for facultative anaerobes of 2 mol H_2/mol glucose (Yoshida et al. 2006), the *E. coli* cells metabolize glucose by pathways other than those remaining to make formate. For example, *E. coli* cells have three lactate dehydrogenases (*ldhA*, *dld*, and *lldD*) and two of them are membrane-bound flavoproteins linked with the respiratory chain (Mat-Jan et al. 1989); hence, these other two lactate dehyrogenases may prevent the cell from producing even more hydrogen.

The deletion of pyruvate oxidase (*poxB*) in the BW25113 *hyaB hybC hycA fdoG frdC ldhA* and BW25113 *hyaB hybC hycA fdnG frdC ldhA* backgrounds was not effective for enhancing hydrogen production and hydrogen yields (Tables 2 and 3). The reason may be that PoxB is more important under aerobic conditions (Abdel-Hamid et al. 2001). Note that *E. coli* cells require anaerobic conditions to synthesize hydrogen, as *E. coli* hydrogenases are sensitive to oxygen (Glick et al. 1980); therefore, PoxB product may not be important for enhanced hydrogen production. On the other hand, the inactivation of pyruvate dehydrogenase (AceE) was effective for enhancing both hydrogen production and hydrogen yield, although the effect is slight (BW25113 *hyaB hybC hycA fdoG frdC ldhA* vs BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE*, Table 3); this may be caused by the increased metabolic flux to formate during glucose metabolism.

E. coli is robust because many technologies are available for its manipulation; for example, classical chemical mutagenesis followed by genome breeding (Patnaik et al. 2002), which may provide other important genes for enhanced hydrogen production, as there are indubitably unanticipated interactions in the metabolic pathways and their regulators. Microarray analysis (Maeda et al. 2007c) would then enable the molecular basis of the beneficial mutations to be discerned. Such approaches may hold promise for constructing even better strains for enhanced hydrogen production in glucose metabolism.

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