

Metabolic engineering of *Escherichia coli* to enhance hydrogen production from glycerol

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Received: 18 December 2013 / Revised: 5 February 2014 / Accepted: 6 February 2014
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Abstract Glycerol is an attractive carbon source for biofuel production since it is cheap and abundant due to the increasing demand for renewable and clean energy sources, which includes production of biodiesel. This research aims to enhance hydrogen production by *Escherichia coli* from glycerol by manipulating its metabolic pathways via targeted deletions. Since our past strain, which had been engineered for producing hydrogen from glucose, was not suitable for producing hydrogen from glycerol, we rescreened 14 genes related to hydrogen production and glycerol metabolism. We found that 10 single knockouts are beneficial for enhanced hydrogen production from glycerol, namely, *frdC* (encoding for fumarate reductase), *ldhA* (lactate dehydrogenase), *fdnG* (formate dehydrogenase), *ppc* (phosphoenolpyruvate carboxylase), *narG* (nitrate reductase), *focA* (formate transporter), *hyaB* (the large subunit of hydrogenase 1), *aceE* (pyruvate dehydrogenase), *mgsA* (methylglyoxal synthase), and *hycA* (a regulator of the transcriptional regulator FhlA). On that basis, we created multiple knockout strains via successive P1 transductions. Simultaneous knockouts of *frdC*, *ldhA*, *fdnG*, *ppc*, *narG*, *mgsA*, and *hycA* created the best strain that produced 5-fold higher hydrogen and had a 5-fold higher hydrogen yield than the parent strain. The engineered strain also reached the theoretical maximum yield of 1 mol H₂/mol glycerol after 48 h. Under

low partial pressure fermentation, the strain grew over 2-fold faster, indicating faster utilization of glycerol and production of hydrogen. By combining metabolic engineering and low partial pressure fermentation, hydrogen production from glycerol was enhanced significantly.

Keywords *Escherichia coli* · Metabolic engineering · Anaerobic fermentation · Glycerol · Hydrogen

Introduction

Glycerol is the main by-product from biodiesel production, accounting for roughly 10 % of the products (w/w). The production of biodiesel (and therefore glycerol) is increasing, and it is estimated that the world production of biodiesel and crude glycerol will reach 37 and 4 billion gallon by 2016 (Anand and Saxena 2012). As a result, the surplus of crude glycerol has been negatively affecting the refined glycerol market in recent years. To assure the sustainable development and commercial competitiveness with other fuel sources for both the glycerol and biodiesel industries, by-product glycerol should be converted into other value added chemicals and fuels such as ethanol and hydrogen (Deckwer 1995). Given its low carbon structure, glycerol has been used to produce bio-hydrogen and bio-ethanol via the fermentative process with a significant and promising production (Hu and Wood 2010; Murarka et al. 2008).

Hydrogen is a compelling future energy source because it does not produce greenhouse gases or other pollutants since the products of hydrogen oxidation are only water and energy. Furthermore, hydrogen has higher energy content than fossil fuels by 2.75 times. Bio-hydrogen can be produced via four main ways: (a) bio-photolysis of water using algae and cyanobacteria, (b) photodecomposition of organic compounds

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by photosynthetic bacteria, (c) fermentative hydrogen production from organic compounds, and (d) hybrid systems using photosynthetic and fermentative bacteria (Das and Veziroğlu 2001). The first two processes require photosynthesis where hydrogen production is dependent on light while the third is a dark fermentation or anaerobic process, which is strictly light independent. Note that fermentative hydrogen production is more efficient for industrial production compared to the other methods because it can utilize organic pollutants as input sources, requires less energy consumption, and requires simple technology (Das and Veziroğlu 2001; Maeda et al. 2008; Navarro et al. 2009; Ust'ak et al. 2007; Vardar-Schara et al. 2008). Recently, hydrogen production by both dark fermentative and photosynthesis organisms such as *Enterobacter*, *Bacillus*, *Clostridium*, and green alga has been well studied and steadily enhanced (Fabiano and Perego 2002; Hawkes et al. 2002; Kalia et al. 1994; Srirangan et al. 2011; Yokoi et al. 1998, 2002). For example, the highest hydrogen yield of 2.81 mol H₂/mol glucose from *Clostridium beijerinckii* L9 has been reported (Lin et al. 2007). However, it is far lower than a theoretical stoichiometric yield of 4 mol H₂/mol glucose. Among various fermentative hydrogen producing microorganisms, *Escherichia coli* bears a high potential for hydrogen production at a large scale because it is easily manipulated genetically, can achieve a high hydrogen yield, and sustains a rapid growth (Maeda et al. 2008; Vardar-Schara et al. 2008).

Recently, the fermentation flux in *E. coli* has been improved for hydrogen production, typically from glucose metabolism (Bagramyan et al. 2002; Blattner 1997; Maeda et al. 2007a, 2008), with the goal of producing formate, which is converted to CO₂ and H₂. Inactivation of *hycA*, a negative regulator of the FHL define complex, together with the overexpression of the FHL complex (encoded by *fhlA*), results in a significant increase in hydrogen production (Kim et al. 2009; Wang et al. 2012; Yoshida et al. 2005). So far, the role of hydrogenase 3 (Hyd-3) is clear since a defect in *hycE*, which encodes the large subunit of Hyd-3, significantly impairs the hydrogen yield (Maeda et al. 2007b). Furthermore, the FHL complex is essential because its inactivation is detrimental to hydrogen production (Dharmadi et al. 2006). *E. coli* converts pyruvate into CO₂, lactate, and acetate by pyruvate dehydrogenase (encoded by *aceE*), D-lactate dehydrogenase (encoded by *ldhA*), and pyruvate oxidase (encoded by *poxB*), respectively (Bunch et al. 1997; Chang and Cronan 1983; Jiang et al. 2001). Regarding the respiratory pathway, two formate dehydrogenases, formate dehydrogenase N (encoded by *fdnG*) and formate dehydrogenase-O (encoded by *fdoG*), play a pivotal role in formate oxidation to CO₂ (Abaibou et al. 1995; Jormakka et al. 2002; Rossmann et al. 1991; Sawers et al. 1991). The cell exports formate by the activation of the formate transporter (encoded by *focA*) to

prevent acidification when the pH in the cytoplasm drops (Suppmann and Sawers 1994). Consequently, the concentration of formate, which is a precursor of hydrogen production, is reduced. Hence, in addition to the FHL complex, redirection of both metabolic and respiratory pathways should enhance hydrogen production from glycerol.

To date, the production of hydrogen in *E. coli* from glucose has been achieved by applying metabolic engineering (Maeda et al. 2007a, 2008). From glycerol, inactivation of fumarate reductase (*ΔfrdA*) increases the production of hydrogen and ethanol (Shams Yazdani and Gonzalez 2008). Hydrogen production could be also increased by improving the conversion of glycerol. In principle, glycerol is converted to dihydroxyacetone (DHA), which is the first intermediate product in the glycolytic pathway by glycerol dehydrogenase (encoded by *gldA*; Truniger and Boos 1994). Apparently, glycerol dehydrogenase is one of the most important enzymes in the glycerol metabolism since either its overexpression or inactivation highly affects hydrogen production in *E. coli* (Hu and Wood 2010; Sanchez-Torres et al. 2013; Shams Yazdani and Gonzalez 2008). Hence, blocking key pathways and overexpression of the glycerol dehydrogenase should improve the hydrogen yield from glycerol. In addition, CO₂ has a positive role in glycerol conversion, while H₂ partial pressure negatively affects hydrogen production and cell growth (Dharmadi et al. 2006; Gonzalez et al. 2008). Therefore, a low partial pressure fermentation is often applied to enhance hydrogen production (Dharmadi et al. 2006; Kim et al. 2006; Maeda et al. 2008; Mizuno et al. 2000).

Although many of the metabolic pathways in *E. coli* are well studied (Blattner 1997), there remain some gaps in our knowledge base when glycerol is used as a substrate. Recently, some studies have shown that those genes, which are either beneficial or related to hydrogen production from glucose, might not necessarily have a similar effect or even may have the opposite effect when glycerol is used as the substrate. For example, in *E. coli*, Hyd-1 and Hyd-2 are hydrogen uptake enzymes during glucose metabolism (Menon et al. 1991, 1994), whereas they are reversible enzymes in the presence of glycerol (Sanchez-Torres et al. 2013; Trchounian and Trchounian 2009). In addition, anaerobic growth in the presence of glycerol is quite lower than that of glucose (Hu and Wood 2010).

Our previous engineered strain, BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* increased hydrogen production by 4.6-fold from glucose and increased hydrogen yield by 2-fold from 0.65 to 1.3 mol H₂/mol glucose (Maeda et al. 2007a), while the maximum yield in glucose metabolism is 2 mol H₂/mol glucose. However, it could not produce a relatively high amount of hydrogen from glycerol due to the difference in metabolism of glucose and glycerol. Here, we aim to create better metabolic flux for enhancing hydrogen production from glycerol (Fig. 1).

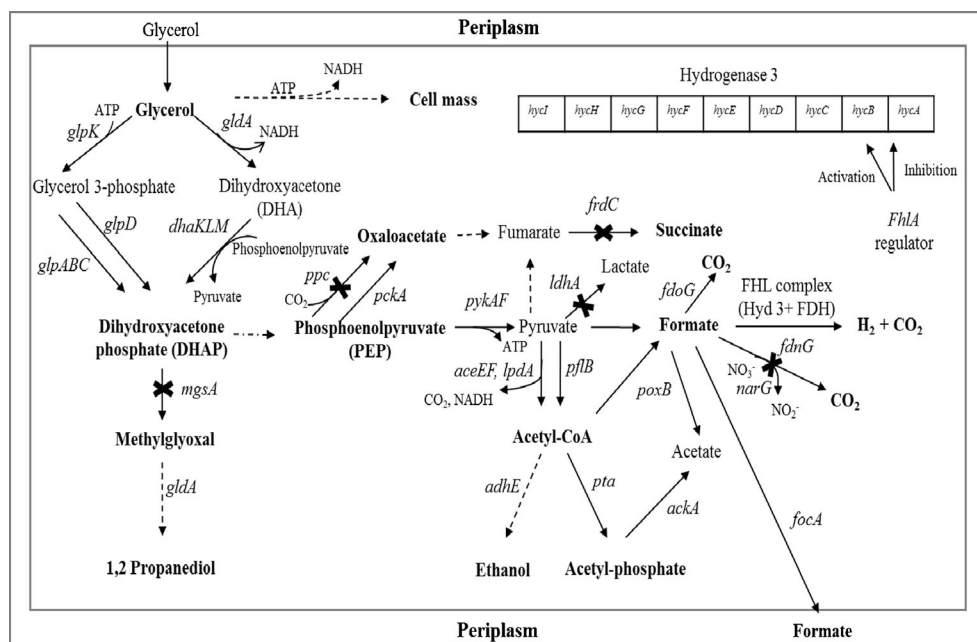


Fig. 1 The metabolic and respiratory pathway in *Escherichia coli* (Altaras and Cameron 1999; Chao et al. 1993; Cooper 1984; Dharmadi et al. 2006; Gonzalez et al. 2008; Murarka et al. 2008; Rossmann et al. 1991; Saikusa et al. 1987; Truniger and Boos 1994). Genetic manipulations are illustrated by cross symbols. The engineered strain is inactivated in fumarate reductase (encoded by *frdC*), D-lactate dehydrogenase (encoded by *ldhA*), formate dehydrogenase N, α subunit (encoded by

fdnG), phosphoenolpyruvate carboxylase (encoded by *ppc*), nitrate reductase A, α subunit (encoded by *narG*), methylglyoxal synthase (encoded by *mgsA*) and regulator of the transcriptional regulator FhlA (encoded by *hycA*). Dashed lines and bold words illustrate multiple metabolic steps and the main products. Abbreviations: DHA (dihydroxyacetone) PEP (phosphoenolpyruvate); FHL (formate hydrogen-lyase); *gldA* (glycerol dehydrogenase)

Materials and methods

Strains, P1 transduction, plasmids, and primers

The parent strain, BW25113, was obtained from the Yale Coli Genetic Stock Center (the USA), while single KEIO and ASKA strains were provided from the KEIO library (National Institute of Genetics, Japan). P1 transduction (Cherepanov and Wackernagel 1995) was used to create the multiple deletion strains (Table 1). Prior to the P1 transduction, the kanamycin resistance gene of the recipient strain was removed by helper plasmid, pCP20, which is able to facilitate homologous DNA recombination with the flanking repeated site (FRT) located at either end of the resistance gene. The helper plasmid containing the chloramphenicol (Cm^R) antibiotic marker was then cured by growing at 43 °C due to its sensitivity to this temperature (Cherepanov and Wackernagel 1995; Datsenko and Wanner 2000). After each round of P1 transduction, the knockout strain with its newly disrupted gene was verified by PCR using a set of specific primers (Table 1).

Four plasmids were used in this study including pCA24N as the empty vector; pCA24N-FdhF, expressing the subunit of formate dehydrogenase; pCA24N-FhlA, expressing the regulator of the FHL complex; and pCA24N-GldA, expressing *E. coli* glycerol dehydrogenase. These plasmids were originated from Kitagawa et al. (2005). A set of primers was designed for the confirmation purpose.

Culture medium, growth condition, and chemicals

Luria-Bertani (LB) medium and LB agar plates containing appropriate antibiotics were used to grow the strains when conducting P1 transduction. Minimal glycerol medium supplemented with 10 g/L of glycerol, 0.02 mg/L of NiCl_2 , and 0.172 mg/L of Na_2SeO_3 (Murarka et al. 2008; Sanchez-Torres et al. 2013) and appropriate antibiotics was used as the medium for preparing the overnight culture and fermentation. Note that selenium is toxic to the cell, however, a small amount of selenium is essential for the synthesis of selenocysteine, which is one of the constituents of the FDH H (Sawers 1991, 1994; Takahata et al. 2008). Furthermore, a low concentration of selenite (below 5 mM) does not affect the growth of *E. coli* (Bebien et al. 2002). The pH of the medium was adjusted by using 5 M NaOH to 7.5. All the experiments were conducted at 37 °C at 120 rpm in a bio-shaker (BR-180LF, Taitec). Chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Dojindo Molecular Technologies, Inc. (Kumamoto, Japan) and Sigma-Aldrich Co. LLC (Tokyo, Japan) and Nacalai Tesque, Inc. (Kyoto, Japan).

Growth rate and hydrogen assay

For the growth rates, the strains were streaked from the -80 °C glycerol stock on LB agar plates, and then incubated overnight

Table 1 Strains, plasmids, and primers used in this work

Strains, plasmids and primers	Genotype/description	Sources
Strains		
BW25113	F ⁻ Δ (<i>araD-araB</i>)567Δ <i>lacZ</i> 4787(:: <i>rrnB-3</i>)λ ^{rph} -1Δ(<i>rhaD-rhaB</i>)568 <i>hsdR</i> 514.	Yale Coli Genetic Stock center
BW25113Δ <i>frdC</i> Ω <i>kan</i> ^R	Defective in fumarate reductase.	(Baba et al. 2006)
BW25113Δ <i>ldhA</i> Ω <i>kan</i> ^R	Defective in D-lactate dehydrogenase.	(Baba et al. 2006)
BW25113Δ <i>fdnG</i> Ω <i>kan</i> ^R	Defect in α-subunit of formate dehydrogenase-N.	(Baba et al. 2006)
BW25113Δ <i>ppc</i> Ω <i>kan</i> ^R	Defective in phosphoenolpyruvate carboxylase.	(Baba et al. 2006)
BW25113Δ <i>focA</i> Ω <i>kan</i> ^R	Defective in formate transporter.	(Baba et al. 2006)
BW25113Δ <i>narG</i> Ω <i>kan</i> ^R	Defective in α-subunit of nitrate reductase A.	(Baba et al. 2006)
BW25113Δ <i>poxB</i> Ω <i>kan</i> ^R	Defective in pyruvate oxidase.	(Baba et al. 2006)
BW25113Δ <i>fdoG</i> Ω <i>kan</i> ^R	Defective in α-subunit of formate dehydrogenase-O.	(Baba et al. 2006)
BW25113Δ <i>hyaB</i> Ω <i>kan</i> ^R	Defective in large subunit of hydrogenase 1.	(Baba et al. 2006)
BW25113 Δ <i>adhE</i> Ω <i>kan</i> ^R	Defective in pyruvate formate-lyase deactivase.	(Baba et al. 2006)
BW25113Δ <i>aceE</i> Ω <i>kan</i> ^R	Defective in subunit of E1p component of pyruvate dehydrogenase complex.	(Baba et al. 2006)
BW25113Δ <i>hycA</i> Ω <i>kan</i> ^R	Defective in a repressor of the FHL complex.	(Baba et al. 2006)
BW25113 Δ <i>hybC</i> Ω <i>kan</i> ^R	Defective in large subunit of hydrogenase 2	(Baba et al. 2006)
BW25113Δ <i>mgsA</i> Ω <i>kan</i> ^R	Defective in methylglyoxal synthase.	(Baba et al. 2006)
BW25113Δ <i>hyaB hybC hycA frdC ldhA fdoG aceE</i> Ω <i>kan</i> ^R	Defective in large subunit of hydrogenase 1, large subunit of hydrogenase 2, a repressor of the FHL complex, fumarate reductase, D-lactate dehydrogenase, α-subunit of formate dehydrogenase-O, subunit of E1p component of pyruvate dehydrogenase complex.	(Maeda et al. 2007a)
BW25113Δ <i>frdC</i> Δ <i>kan</i> ^R	Defective in fumarate reductase.	This study
BW25113Δ <i>ldhA</i> Δ <i>kan</i> ^R	Defective in D-lactate dehydrogenase.	This study
BW25113Δ <i>fdnG</i> Δ <i>kan</i> ^R	Defect in α-subunit of formate dehydrogenase-N.	This study
BW25113Δ <i>ppc</i> Δ <i>kan</i> ^R	Defective in phosphoenolpyruvate carboxylase.	This study
BW25113Δ <i>focA</i> Δ <i>kan</i> ^R	Defective in formate transporter.	This study
BW25113Δ <i>narG</i> Δ <i>kan</i> ^R	Defective in α-subunit of nitrate reductase A.	This study
BW25113Δ <i>hyaB</i> Δ <i>kan</i> ^R	Defective in large subunit of hydrogenase 1.	This study
BW25113Δ <i>hycA</i> Δ <i>kan</i> ^R	Defective in a repressor of the FHL complex.	This study
BW25113Δ <i>mgsA</i> Δ <i>kan</i> ^R	Defective in methylglyoxal synthase.	This study
BW25113Δ <i>frdC ldhA</i> Ω <i>kan</i> ^R and BW25113Δ <i>frdC ldhA</i> Δ <i>kan</i> ^R	Defective in fumarate reductase, D-lactate dehydrogenase.	This study
BW25113Δ <i>frdC ldhA fdoG</i> Ω <i>kan</i> ^R and BW25113Δ <i>frdC ldhA fdoG</i> Δ <i>kan</i> ^R	Defective in fumarate reductase, D-lactate dehydrogenase, α-subunit of formate dehydrogenase-N	This study
BW25113Δ <i>frdC ldhA fdoG ppc</i> Ω <i>kan</i> ^R and BW25113Δ <i>frdC ldhA fdoG ppc</i> Δ <i>kan</i> ^R	Defective in fumarate reductase, D-lactate dehydrogenase, α-subunit of formate dehydrogenase-N, phosphoenolpyruvate carboxylase.	This study
BW25113Δ <i>frdC ldhA fdoG ppc narG</i> Ω <i>kan</i> ^R and BW25113Δ <i>frdC ldhA fdoG ppc narG</i> Δ <i>kan</i> ^R	Defective in fumarate reductase, D-lactate dehydrogenase, α-subunit of formate dehydrogenase-N, phosphoenolpyruvate carboxylase, α-subunit of nitrate reductase A	This study
BW25113Δ <i>frdC ldhA fdoG ppc narG mgsA</i> Ω <i>kan</i> ^R and BW25113Δ <i>frdC ldhA fdoG ppc narG mgsA</i> Δ <i>kan</i> ^R	Defective in fumarate reductase, D-lactate dehydrogenase, α-subunit of formate dehydrogenase-N, phosphoenolpyruvate carboxylase, α-subunit of nitrate reductase A, methylglyoxal synthase.	This study
BW25113Δ <i>frdC ldhA fdoG ppc narG mgsA hycA</i> Ω <i>kan</i> ^R and BW25113Δ <i>frdC ldhA fdoG ppc narG mgsA hycA</i> Δ <i>kan</i> ^R	Defective in fumarate reductase, D-lactate dehydrogenase, α-subunit of formate dehydrogenase-N, phosphoenolpyruvate carboxylase, α-subunit of nitrate reductase A, methylglyoxal synthase, repressor of the FHL complex.	This study
BW25113Δ <i>frdC ldhA fdoG ppc narG mgsA hycA focA</i> Ω <i>kan</i> ^R and BW25113Δ <i>frdC ldhA fdoG ppc narG mgsA hycA focA</i> Δ <i>kan</i> ^R	Defective in fumarate reductase, D-lactate dehydrogenase, α-subunit of formate dehydrogenase-N, phosphoenolpyruvate carboxylase, α-subunit of nitrate reductase A, methylglyoxal synthase, repressor of the FHL complex, formate transporter.	This study
BW25113Δ <i>frdC ldhA fdoG ppc narG mgsA hycA aceE</i> Ω <i>kan</i> ^R	Defective in fumarate reductase, D-lactate dehydrogenase, α-subunit of formate dehydrogenase-N, phosphoenolpyruvate	This study

Table 1 (continued)

Strains, plasmids and primers	Genotype/description	Sources
BW25113Δ <i>frdC lhdA fdnG ppc narG mgsA hycA</i> Ωkan ^R /pCA24N-FdhF	carboxylase, α-subunit of nitrate reductase A, methylglyoxal synthase, repressor of FHL, subunit of E1p component of pyruvate dehydrogenase. Defective in fumarate reductase, D-lactate dehydrogenase, α-subunit of formate dehydrogenase-N, phosphoenolpyruvate carboxylase, α-subunit of nitrate reductase A, methylglyoxal synthase, repressor of FHL, subunit of E1p component of pyruvate dehydrogenase complex; over-expression of formate dehydrogenase.	This study
BW25113Δ <i>frdC lhdA fdnG ppc narG mgsA hycA</i> Ωkan ^R /pCA24N-FhlA	Defective in fumarate reductase, D-lactate dehydrogenase, α-subunit of formate dehydrogenase-N, phosphoenolpyruvate carboxylase, α-subunit of nitrate reductase A, methylglyoxal synthase, repressor of FHL, subunit of E1p component of pyruvate dehydrogenase complex; over-expression of formate hydrogen lyse system activator.	This study
BW25113Δ <i>frdC lhdA fdnG ppc narG mgsA hycA</i> Ωkan ^R /pCA24N-GldA	Defective in fumarate reductase, D-lactate dehydrogenase, α-subunit of formate dehydrogenase-N, phosphoenolpyruvate carboxylase, α-subunit of nitrate reductase A, methylglyoxal synthase, repressor of FHL, subunit of E1p component of pyruvate dehydrogenase complex; over-expression of glycerol dehydrogenase.	This study
Plasmids		
pCP20	Ap ^R and Cm ^R plasmid; thermal induction of FLP synthesis.	(Cherepanov and Wackernagel 1995)
pCA24N	Empty vector; Cm ^R	(Kitagawa et al. 2005)
pCA24N-FdhF	pCA24N pT5- <i>lac::fdhF</i> ; Cm ^R ; expression of FdhF	(Kitagawa et al. 2005)
pCA24N-FhlA	pCA24N pT5- <i>lac::fhlA</i> ; Cm ^R ; expression of FhlA;	(Kitagawa et al. 2005)
pCA24N-GldA	pCA24N pT5- <i>lac::gldA</i> ; Cm ^R ; expression of GldA;	(Kitagawa et al. 2005)
Primers		
K1	5'-CAGTCATAGCCGAATAGCCT-3'	(Datsenko and Wanner 2000)
K2	5'-CGGTGCCCTGAATGAACTGC-3'	(Datsenko and Wanner 2000)
<i>frdC</i> confirm-f	5'-GAAACCACGCTAAGGAGTGC-3'	This study
<i>frdC</i> confirm-r	5'-ATGATGGCGCTCCACATACC-3'	This study
<i>ldhA</i> confirm-f	5'-GGTTGCGCCTACACTAAG-3'	This study
<i>ldhA</i> confirm-r	5'-GAGGATGAAAGGTCATTGGG-3'	This study
<i>fdnG</i> confirm-f	5'-TTCCTCGCGCAGTAATACCC-3'	This study
<i>fdnG</i> confirm-r	5'-AGCCGATACAGGTGGAAACG-3'	This study
<i>focA</i> confirm-f	5'-CTGTTTTAGCGGAGGATGCG-3'	This study
<i>focA</i> confirm-r	5'-GCGGTGTCAAAGTCAACTGG-3'	This study
<i>ppc</i> confirm-f	5'-GCCGCAATAATGTCGGATGC-3'	This study
<i>ppc</i> confirm-r	5'-GAAAACCCTCGCGCAAAAGC-3'	This study
<i>narG</i> confirm-f	5'-ACGCTGTTTCAGAGCGTTAC-3'	This study
<i>narG</i> confirm-r	5'-ATTTTACCCAGCAGCATGGC-3'	This study
<i>poxB</i> confirm-f	5'-TCCGGTGAATATACGGTGAGC-3'	This study
<i>poxB</i> confirm-r	5'-AAATGGCGGCTTCTTTACCG-3'	This study
<i>fdoG</i> confirm-f	5'-GAGCCAATTCTGGACCTTTGC-3'	This study
<i>fdoG</i> confirm-r	5'-GTTATTGCCGACGGTATCGC-3'	This study
<i>mgsA</i> confirm-f	5'-CAGTGGGCGGAAAAACAACC-3'	This study
<i>mgsA</i> confirm-r	5'-CGTCATCATCGTTGGCTTGC-3'	This study
<i>hycA</i> confirm-f	5'-AGCTGGCATCTCTGTAAACG-3'	This study
<i>hycA</i> confirm-r	5'-GTCATTTTCGACACTCATCGAC-3'	This study
<i>aceE</i> confirm-f	5'-ATCGCCATCTGGCCTTTATCG-3'	This study
<i>aceE</i> confirm-r	5'-AGGCTTTGTGCGCTTCTACG-3'	This study

Kan^R, Cm^R, and Ap^R indicate kanamycin, chloramphenicol, and ampicillin resistance, respectively

at 37 °C. After 12–18 h, a single colony was inoculated into a 250-mL flask (Iwaki, Japan) containing 25 mL of minimal glycerol medium, and the strains were grown aerobically. Cell turbidity was measured at 600 nm wavelength by using a UV/VIS spectrophotometer (JASCO V-530, Tokyo, Japan).

For the hydrogen assay, sealed crimp top vials (34 or 68 mL) were used for screening hydrogen production of the single mutant strains and for examining the kinetic growth, glycerol utilization, and hydrogen production of the highest hydrogen producing strains and the wild type strains. The pre-culture, closed vials, and fresh minimal glycerol medium were sparged with nitrogen gas for 5 and 10 min to remove oxygen. Inside the anaerobic chamber, an appropriate volume of overnight culture was added to vials containing sparged minimal glycerol medium with 0.1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG). The initial optical density at 600 nm (OD₆₀₀) of the fermentative culture was adjusted to 0.05–0.06. Samples were then grown anaerobically at 37 °C, 120 rpm. After each 24 h, 50 μ L of gas from the headspace of the fermentation vial was consecutively measured by a gas chromatograph (Agilent 6890 Series GC System) equipped with a thermal conductive detector (Maeda et al. 2007c). The cell mass was calculated based on the value of OD₆₀₀ with the amount of 0.22 mg protein/OD/mL (Fishman et al. 2005). Hydrogen production and productivity was calculated based on the method reported elsewhere (Maeda et al. 2007c).

Hydrogen low partial pressure assay

The pre-culture and closed vial assays were performed as above using a 68-mL-sealed crimp top vial. To reach a low partial pressure condition, the fermentative vials were connected to an inverted cylinder and the generated gases such as H₂ and CO₂ were released into the headspace of the vial prior to the inverted cylinder (Maeda et al. 2008). The fermentation vials were slightly stirred and kept at 37 °C. Hydrogen was measured after 24 h when the fermentation likely reached the log phase. The generated hydrogen volume was calculated as the sum of headspace volume of the fermentative vial and the inverted cylinder, and the volume of the connecting tube.

Quantification of organic acids, cell, and glycerol consumed

The fermentation culture was filtered by a 0.2- μ m membrane (Sartorius, Germany) for organic acids and glycerol quantification. Organic acids were measured after 24, 48, 72, 96, and 120 h of fermentation by high performance liquid chromatography (8 mm $\times$ 300 mm, Shimadzu Co., Tokyo, Japan) using a Shim-Pack SCR-102H column and a CDD-6A electric conductivity detector (Maeda et al. 2009). The mobile phase was set to be 5 mM p-toluenesulfonic acid monohydrate with a flow rate of 0.8 mL/min, and the column was set at 40 °C. The

organic acids were quantified after 24 h of fermentation when the hydrogen low partial pressure was applied. The Free glycerol determination Kit (Sigma, San Louis, MO) was used to determine glycerol concentration. Ethanol and 1, 2 propanediol (1, 2-PDO) were quantified by a GC 2025 gas chromatograph equipped with a CBP20-M25-025 capillary column and a flame ionization detector (Shimadzu, Japan). The temperature of the column, oven, and detector were set at 50 °C, 250 °C, and 250 °C, respectively (Sanchez-Torres et al. 2013). Product yields (hydrogen, cell, organic acids, 1, 2-PDO, and ethanol) were calculated in mmole of product per mmole glycerol consumed. Cell yield was calculated based on the estimated molecular weight of 26.535 g provided by the formula of CH_{1.94}O_{0.52}N_{0.25}P_{0.025} of *E. coli* dry mass (Villadsen et al. 2011).

Results

Screening of hydrogen production of the single mutant strain

In our previous study (Maeda et al. 2007a), we created the metabolically engineered strain, BW25113 *hyaB hybC hycA frcD ldhA fdxG aceE*, which produces 4.6-fold more hydrogen than wild-type BW25113 during glucose metabolism. However, its hydrogen productivity increased only 1.8-fold over the parent strain during glycerol metabolism (Table 2). Furthermore, the growth rate of this strain decreased by 2.7-fold with glycerol. Therefore, we hypothesized that the enzymes of the related pathways may play different roles in glycerol metabolism. Hence, we investigated the pathways most suitable to delete to increase hydrogen production under glycerol metabolism. Next, beneficial genes were introduced to enhance hydrogen production. Based on the knowledge on the metabolic pathway of *E. coli* under both glucose and glycerol metabolism (Fig. 1), we investigated 14 single KEIO mutant strains individually (Table 2).

Table 2 shows that except for BW25113 *hybC*, the growth rate of the other 13 single knockout strains decreased from 1.1 to 3.3-fold. Noticeably, introduction of multiple gene disruptions does not necessarily lower the growth rate of the mutant strains. In fact, the highest hydrogen producing mutant-septuple strain grew negligibly lower than strain BW25113 with the growth rate of 0.31/h with glycerol.

Similar to previous work, inactivation of fumarate reductase (Δ *frcD*) led to a 1.6-fold increase in hydrogen production with glycerol (Shams Yazdani and Gonzalez 2008). Disruption of *ldhA*, *fdxG*, *ppc*, *narG*, *focA*, *hyaB*, and *hycA* have been reported to have a positive effect on the production of either intermediates or end products such as succinate or hydrogen in glucose metabolism (Kabir et al. 2005; Maeda et al. 2007a; Redwood et al. 2008; Wang and Gunsalus 2003; Yoshida et al. 2005). Single disruption of these genes boosted

Table 2 Growth rate and hydrogen production of *E. coli* from minimal glycerol

Strain	Growth rate ^a		Hydrogen productivity ^b	
	1/h	Relative	μmol/mg protein	Relative
BW25113	0.35±0.02	1	28.8±0.3	1
BW25113Δ <i>hyaB</i> <i>hybC</i> <i>hycA</i> <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>aceE</i> Ω kan^R	0.13±0.01	0.38	53±2	1.8
BW25113Δ <i>frdC</i> Ω kan^R	0.22±0.03	0.6	45±5	1.6
BW25113Δ <i>ldhA</i> Ω kan^R	0.23±0.01	0.7	31±4	1.1
BW25113Δ <i>fdoG</i> Ω kan^R	0.28±0.01	0.8	37.7±0.5	1.3
BW25113Δ <i>focA</i> Ω kan^R	0.31±0.01	0.9	31±4	1.1
BW25113Δ <i>ppc</i> Ω kan^R	0.26±0.01	0.7	41±5	1.4
BW25113Δ <i>narG</i> Ω kan^R	0.27±0.01	0.8	33±4	1.1
BW25113Δ <i>poxB</i> Ω kan^R	0.3	0.8	16±1	0.6
BW25113Δ <i>fdoG</i> Ω kan^R	0.27±0.01	0.8	28±3	0.96
BW25113Δ <i>aceE</i> Ω kan^R	0.21	0.6	62±3	2.1
BW25113Δ <i>hyaB</i> Ω kan^R	0.32±0.01	0.9	32.3±0.2	1.1
BW25113Δ <i>adhE</i> Ω kan^R	0.27±0.01	0.8	0	0
BW25113Δ <i>mgsA</i> Ω kan^R	0.31	0.9	36±1	1.2
BW25113 <i>hycA</i> Ω kan^R	0.31±0.01	0.9	35±13	1.2
BW25113Δ <i>hybC</i> Ω kan^R	0.42	1.2	0	0
BW25113Δ <i>frdC</i> <i>ldhA</i> Ω kan^R	0.27	0.8	31±4	1.1
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> Ω kan^R	0.29±0.01	0.8	34±2	1.2
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> Ω kan^R	0.31±0.01	0.9	91±4	3.2
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> <i>focA</i> Ω kan^R	0.29±0.01	0.8	88±6	3.1
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> <i>hyaB</i> Ω kan^R	0.29±0.01	0.8	39.5±0.7	1.4
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> <i>narG</i> Ω kan^R	0.29±0.01	0.8	109±11	3.8
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> <i>narG</i> <i>mgsA</i> Ω kan^R	0.28±0.01	0.8	124±5	4.3
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> <i>narG</i> <i>mgsA</i> <i>hycA</i> Ω kan^R	0.31±0.01	0.9	158±2	5.5
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> <i>narG</i> <i>mgsA</i> <i>hycA</i> <i>focA</i> Ω kan^R	0.23±0.01	0.66	83±7	2.9
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> <i>narG</i> <i>mgsA</i> <i>hycA</i> <i>aceE</i> Ω kan^R	0.12±0.01	0.3	33±1	1.1
BW25113/pCA24N	0.37±0.02	1	25±1	0.9
BW25113/pCA24N-FdhF	0.2	0.6	0	0
BW25113/pCA24N-FhlA	0.28	0.6	13±2	0.4
BW25113/pCA24N-GldA	0.2	0.6	7±2	0.3
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> <i>narG</i> <i>mgsA</i> <i>hycA</i> Ω kan^R /pCA24N	0.33±0.01	0.9	90±4	3.1
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> <i>narG</i> <i>mgsA</i> <i>hycA</i> Ω kan^R /pCA24N-FdhF	0.2	0.6	99±7	3.4
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> <i>narG</i> <i>mgsA</i> <i>hycA</i> Ω kan^R /pCA24N-FhlA	0.33±0.01	1	89±4	3.1
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdoG</i> <i>ppc</i> <i>narG</i> <i>mgsA</i> <i>hycA</i> Ω kan^R /pCA24N-GldA	0.25±0.02	0.7	90±3	3.1

Values represent the mean and standard deviation of at least three independent samples

^a Growth rate of all strains were measured aerobically in minimal glycerol pH 7.5

^b Hydrogen fermentation was conducted anaerobically in minimal glycerol pH 7.5. Calculation of hydrogen productivity indicated in the “Materials and methods” section

hydrogen production from 1.1 to 1.4-fold during glycerol metabolism. Consistent with the results that have been previously reported in glucose metabolism (Maeda et al. 2007a), single inactivation of pyruvate oxidase (encoded by *poxB*) was detrimental to the hydrogen production and reduced viability with glycerol. In contrast to that with glucose (Maeda et al. 2007a), inactivation of formate dehydrogenase O (encoded by *fdoG*) did not improve hydrogen production

with glycerol. Additionally, blocking the synthesis pathway to alcohol by inactivating dehydrogenase (encoded by *adhE*) severely impaired hydrogen production and cell growth and this result is consistent with those reported previously with glycerol (Blankschien et al. 2010; Murarka et al. 2008). In agreement with previous studies with glycerol, inactivation of the large subunit of Hyd-2 (encoded by *hybC*) resulted in almost no hydrogen produced indicating that Hyd-2 plays

the main role in hydrogen production from glycerol under alkaline pH (Sanchez-Torres et al. 2013; Trchounian et al. 2011; Trchounian and Trchounian 2009). In *E. coli*, 1, 2-PDO is the end product of glycerol metabolism, and it is synthesized by the conversion of dihydroxyacetone phosphate into methylglyoxal prior to other intermediates (Fig. 1). Therefore, in order to direct the metabolic flux toward hydrogen production, we blocked this sub-pathway by inactivating methylglyoxal synthase (encoded by *mgsA*). This disruption resulted in 1.2-fold increase of hydrogen productivity.

From these screening results with single mutants, we conducted successive P1 transductions by introducing multiple disruptions into the host strain. Surprisingly, there was little increase in hydrogen production for the double and the triple knockout strains namely, BW25113 *frdC ldhA* and BW25113 *frdC ldhA fdnG*. However, hydrogen productivity remarkably increased by 3.2-fold in the quadruple knockout strain BW25113 *frdC ldhA fdnG ppc*. Single inactivation of formate transporter (Δ *focA*) and the large subunit of Hyd-1 (Δ *hyaB*) somewhat increased hydrogen production, though, when disrupted in the quadruple mutant strain, they did not contribute to any improvement of hydrogen production (Table 2). Rather, disruption of *focA* in the septuple mutant strain also decreased by 1.9-fold of hydrogen productivity, indicating that the formate transporter is necessary for increasing hydrogen production in the multiple mutant strain, and this finding agrees with those reported previously for the conversion of glucose into hydrogen (Maeda et al. 2007a). Successive transductions of *narG* and *mgsA* increased hydrogen production with glycerol by 3.8 and 4.3-fold, respectively. Since we were not able to remove the kanamycin maker gene for BW25113 *aceE*, disruption of *aceE* was made the final step. Hydrogen productivity significantly increased by 5.5-fold when the negative regulator of the *hyc* operon-*hycA* was inactivated. As mentioned above, hydrogen productivity of the strain BW25113 *aceE* increased by 2.1-fold. However, when this gene was disrupted in the septuple mutant strain, hydrogen productivity dramatically dropped due to its extremely slow growth rate. The possible reason for this decrease of both hydrogen production and cell growth is a deficiency in electron acceptors when the metabolic pathway of *E. coli* was highly engineered. The best strain was the septuple mutant, BW25113 *frdC ldhA fdnG ppc narG mgsA hycA*, which had a reasonable growth rate and 5.5-fold higher hydrogen productivity than that of its parent strain.

Since the conversion of glycerol is highly mediated by glycerol dehydrogenase (encoded by *gldA*) and the conversion of formate into H₂ and CO₂ by the FHL complex (Fig. 1), there is a high possibility that overexpression of these enzymes would enhance hydrogen production. Nevertheless, like under glucose metabolism (Maeda et al. 2007a), hydrogen productivity for both the parent and the septuple mutant strain

significantly decreased when *fhlA* was overexpressed (Table 1). Likewise, overexpression of the subunit of formate dehydrogenase (pCA24N-FhdF) also decreased hydrogen productivity by 1.6-fold compared to the septuple mutant strain. This decrease in hydrogen production might result from the selective pressure of the antibiotic added (30 μ M chloramphenicol). As previously reported, overexpressing *gldA*, which converts glycerol into DHA, did not increase pyruvate production, and did not enhance formate and hydrogen production (Blankschien et al. 2010; Gonzalez et al. 2008; Hu and Wood 2010; Truniger and Boos 1994). In fact, overexpression of *gldA* in the parent strain and in the septuple mutant strain decreased by 3.9 and 1.8-fold of hydrogen productivity, respectively.

Glycerol consumption, cell growth, product yield, and productivity

We also examined the kinetic cell growth, glycerol utilization as well as product yields of the septuple mutant strain, BW25113 *frdC ldhA fdnG ppc narG mgsA hycA* after 120 h. As reported previously, *E. coli* consumes glycerol at a very slow rate (Dharmadi et al. 2006; Shams Yazdani and Gonzalez 2008). Starting from a glycerol concentration of 10 g/L, the parent strain and the septuple mutant strain consumed glycerol in a similar rate after the first 24 h of fermentation (Fig. 2). However, strain BW25113 utilized glycerol in a faster manner compared to the engineered strain after 48 h and later due to its faster cell growth. It steadily reached the late stage of the log phase after 96 h, while the septuple mutant strain slowly grew and reached the highest cell growth after 96 h before declining (Fig. 2).

As expected, by enhancing the production of formate by blocking the metabolic pathways for the synthesis of succinate (Δ *frdC*) and oxaloacetate (Δ *ppc*; Fig. 1), production of these

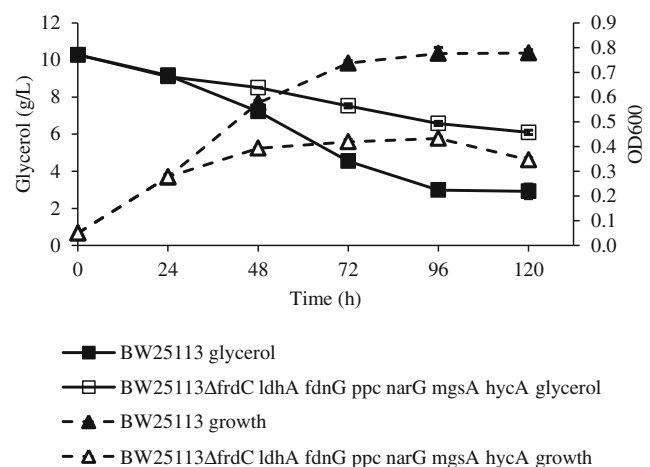


Fig. 2 Glycerol utilization and kinetic growth of strain BW25113 and strain BW25113 Δ *frdC ldhA fdnG ppc narG mgsA hycA* under anaerobic glycerol metabolism at pH 7.5

organic acids, especially succinate of the septuple mutant strain, significantly decreased by 7.2-fold compared to the wild-type strain, BW25113, after 120 h of fermentation (Fig. 3a). A slight decrease in acetate production of the mutant strain also indicated that inactivation of phosphoenolpyruvate carboxylase via disrupting *ppc* gene negligibly affected the synthesis of acetate and oxalate (data not shown). This was no surprise since acetate may be synthesized from other precursors such as formate and acetyl phosphate. Lactate was not detected in the fermentation broths of both BW25113 and the septuple mutant strain, indicating that D-lactate dehydrogenase (encoded by *ldhA*), played a minor role in the production of lactate from glycerol at an alkaline pH condition. This finding agrees with those previously reported (Sanchez-Torres et al. 2013).

Our metabolic engineering should lead to a high level of accumulated formate. However, Fig. 3b shows that formate production in the septuple mutant strain was much lower than that of strain BW25113. We speculate that accumulated formate in the septuple mutant was converted into CO₂ and H₂ at a higher rate compared to strain BW25113. Thus, there were lower concentrations of formate in the fermentation culture for the septuple strain.

As indicated in Fig. 1, ethanol is one of the main products from glycerol metabolism in *E. coli*. In agreement with a previous study (Shams Yazdani and Gonzalez 2008), the ethanol yield of the wild-type strain was close to the theoretical maximum after 48 h (0.92 mol ethanol/mol glycerol). Meanwhile, the septuple mutant strain achieved 0.88 mol ethanol/mol glycerol after 120 h of fermentation (Table 3). This was no surprise since the wild-type strain anaerobically grew faster than the mutant strain did (Fig. 2), resulting a higher rate of glycerol conversion into other products. In other aspects, a disruption in methylglyoxal synthase (encoded by *mgx*A), which is required for the synthesis of methylglyoxal, significantly lowered 1, 2-PDO production in the septuple mutant strain (Table 3). The existence of 1, 2-PDO in the septuple mutant is possibly derived from the conversion of dihydroxyacetone into methylglyoxal (Gonzalez et al. 2008).

Critically, after 24 h, the hydrogen yield of the septuple reached 0.67 mol/mol glycerol utilized which is 4.5-fold higher than that of the wild-type BW25113 (Table 3). The mutant strain then reached the maximum theoretical yield of 1 mol H₂ formed per 1 mol glycerol consumed after 48 h. To date, this is the highest H₂ yield in *E. coli* from glycerol at pH 7.5 and equal to those conducted at a more favorable acidic condition to co-produce H₂ and ethanol (Shams Yazdani and Gonzalez 2008). In principle, the FHL complex is highly active at the acidic condition under both glucose and glycerol metabolism (Bagramyan et al. 2002; Dharmadi et al. 2006; Gonzalez et al. 2008). Meanwhile the conversion efficiency of glycerol depends on glycerol dehydrogenase and dihydroxyacetone kinase, which are most active at neutral to slightly alkaline pH (Dharmadi et al. 2006; Gonzalez et al. 2008; Truniger and Boos 1994). In addition, the intermediate products prior to hydrogen are mainly organic acids that lower intracellular pH during the fermentation. Consequently, acidic pH conditions in the later step during the fermentation would help increase hydrogen production. Therefore, we used glycerol medium starting at pH 7.5 to achieve both objectives: improvement of glycerol conversion in an early period of fermentation and increase in hydrogen production in the later stage under non-pH-controlled fermentation.

Due to the inactivation of those genes required for the synthesis of acetate (encoded by *ppc*) and succinate (encoded by *frdC*), disruption of these genes resulted in a lower product yield of acetate and succinate in the mutant strain. Nevertheless, these negligible reductions suggest that the synthesis of acetate and succinate in *E. coli* under glycerol metabolism did not heavily rely on the activation of phosphoenol pyruvate and fumarate reductase. The productivity rate of either product formed or glycerol utilized of both parent and mutant strain achieved maximum level after 24 h.

Hydrogen low partial pressure

To further improve, hydrogen production, we conducted a low partial pressure fermentation. Unlike under the normal condi-

Fig. 3 Production of succinate (a) and formate (b) for the BW25113 strain and the septuple mutant strain BW25113Δ*frdC* *ldhA* *fdnG* *ppc* *narG* *mgx*A *hyc*A after 120 h of anaerobic glycerol metabolism. Data represent the mean and standard deviation of three independent samples

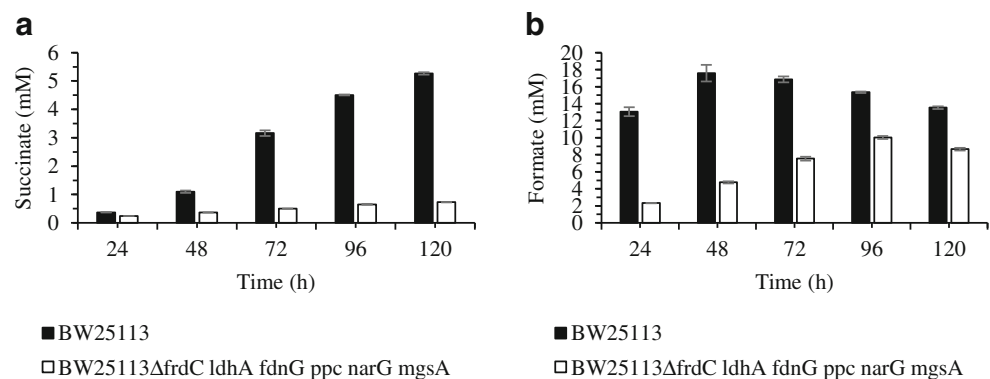


Table 3 Anaerobic fermentation parameters under normal condition

Strain	Time ^a (h)	Yield (mmol product/mmol glycerol) ^b					Productivity rate (mmol product/g cell/h) ^c								
		H ₂	Cell	Succinate	Formate	Acetate	1,2 Propanediol	Ethanol	H ₂	Glycerol	Succinate	Formate	Acetate	1,2 Propanediol	Ethanol
BW25113	24	0.15	0.19	0.03	1.09±0.04	0.12	0.032±0.007	0.67±0.05	1.2	8.2±0.1	0.25±0.01	9.0±0.2	0.99±0.04	0.26±0.06	5.5±0.5
	48	0.72±0.04	0.14±0.01	0.03	0.53±0.05	0.05	0.013±0.002	0.92±0.05	3.9±0.1	5.5±0.3	0.18±0.01	2.9±0.1	0.26	0.067±0.015	4.6±0.7
	72	0.74±0.03	0.1	0.05	0.27±0.01	0.02	0.007±0.002	0.48±0.02	3.9±0.1	5.3±0.2	0.27	1.44±0.02	0.13	0.038±0.008	2.62±0.04
	96	0.76±0.02	0.08	0.06	0.19	0.02	0.005	0.46±0.02	3.7±0.2	4.8±0.2	0.28±0.01	0.94±0.02	0.10	0.026±0.001	2.21±0.02
	120	0.88±0.04	0.08	0.07	0.17	0.02	0.008±0.001	0.61±0.04	3.4	3.9±0.2	0.26	0.66±0.01	0.08	0.03±0.002	2.64±0.29
BW25113Δ <i>frdC</i>	24	0.67±0.05	0.16	0.02	0.16	0.1	0.028±0.01	0.45±0.03	6.6±0.5	9.91±0.04	0.16	1.6	0.95±0.03	0.22±0.03	4.6±0.3
	48	1.05±0.02	0.17	0.02	0.25±0.01	0.07	0.013±0.001	0.66±0.08	4.8±0.1	4.61±0.01	0.09	1.15±0.04	0.32	0.06±0.01	3.3±0.5
	72	1.11±0.03	0.12	0.02	0.25±0.01	0.05	0.01±0.001	0.53±0.02	5	4.5±0.1	0.08	1.14±0.02	0.21	0.045±0.004	2.37±0.07
	96	1.10±0.03	0.09	0.02	0.25±0.01	0.04	0.009±0.001	0.65±0.07	4.85±0.03	4.4±0.1	0.07	1.1	0.16±0.01	0.04	2.85±0.32
	120	1.15±0.05	0.06	0.02	0.19	0.03	0.008	0.88±0.07	5.7±0.2	5.0±0.3	0.08	0.95±0.04	0.16	0.04	4.4±0.2

Data represent the mean and standard deviation of three independent samples in each time course interval

^a Anaerobic fermentation was conducted in 120 h; And fermentation parameters were measured after each 24 h

^b Product yields were calculated in mmol product per mmol glycerol consumed. Calculation of hydrogen and cell yield were mentioned in the "Materials and methods" section

^c Productivity rate indicates either mmol product was formed or mmol of glycerol was consumed per gram of cell per hour during a given period of time

tion, *E. coli* grew and utilized glycerol in a remarkably faster manner. After 24 h, the BW25113 and the septuple mutant strain reached the middle log phase (OD₆₀₀~0.6), which is about over 2-fold faster than that compared to those under the normal condition.

Under a low partial pressure condition, hydrogen yield and productivity of the mutant strain slightly increased from 0.67 to 0.7 mol H₂/mol glycerol and from 6.6 to 6.9 mmol H₂/g cell/h, respectively (Table 4). Meanwhile, the formate yield and productivity rate significantly increased over 2-fold. On the contrary, the formate yield and productivity of strain BW25113 tended to decrease which is understandable due to a higher growth rate achieved under low partial pressure conditions.

Results from organic acids quantification show that production of all main organic acids significantly improved under low partial pressure conditions (Fig. 4). Of these, formate production of strain BW25113 and the septuple mutant strain increased by 1.9 and 5.4-fold. A small amount of lactate was detected in the fermentation culture of strain BW25113 (0.03 mmol lactate/mmol glycerol), while it was not detected in those of the septuple mutant strain. These results agree with that under anaerobic and at low pH condition, *E. coli* converts pyruvate into D-lactate by D-lactate dehydrogenase (encoded by *ldhA*) (Bunch et al. 1997; Jiang et al. 2001). At the end of the fermentation, the pH became acidic (data not shown) that is a favorite environment for the expression of D-lactate dehydrogenase. Consequently, lactate was formed in the fermentation culture of the parent strain. This result indicates that disruption of *ldhA* would be more effectively for the hydrogen production at a low pH condition.

Discussion

We previously created a novel strain (BW24113 *hyaB hybC hycA frdC ldhA fdoG aceE*), which so far produced highest hydrogen production and yield from glucose (Maeda et al. 2007a). Nevertheless, this engineered strain is not suitable for producing a relatively high amount of hydrogen with fast growth rate from glycerol (Table 2). Hence, different pathways (compared to glucose) must be optimized to produce hydrogen from glycerol. Therefore, based on current knowledge on the metabolic and respiratory pathway of *E. coli*, we investigated various genes related to hydrogen production from glycerol to figure out what genes are beneficial.

In total, we screened the hydrogen producing capacity of 14 single mutant strains under glycerol metabolism (Fig. 1; Tables 1 and 2). We found that 10 genes, namely *frdC*, *ldhA*, *fdoG*, *ppc*, *narG*, *focA*, *hyaB*, *mgsA*, *aceE*, and *hycA* were critical for hydrogen production in *E. coli* (Table 2). In principle, the anaerobic respiratory pathways of formate oxidation

Table 4 Anaerobic fermentation parameters under low partial pressure condition

Strain	Yield (mmol product/mmol glycerol) ^a					Productivity rate (mmol product/g cell/h) ^b				
	H ₂	Cell	Succinate	Formate	Acetate	H ₂	Glycerol	Succinate	Formate	Acetate
BW25113	0.19	0.13±0.01	0.02	0.66±0.03	0.11	2.2±0.2	12±1	0.24	7.8±0.4	1.3±0.1
BW25113Δ <i>frdC</i> <i>ldhA</i> <i>fdnG</i> <i>ppc</i> <i>narG</i> <i>mgsA</i> <i>hycA</i>	0.7±0.1	0.16±0.02	0.01	0.37±0.02	0.10	6.9±0.3	10±1	0.08	3.8±0.5	1.0±0.1

Fermentation parameters were measured after 24 h of fermentation. Data represent the mean and standard deviation of three independent samples. Product yield and productivity rate were calculated as mentioned above

and nitrate reduction are regulated by two enzymes formate dehydrogenase (FDH) and terminal nitrate reductase (NAR-A; Abaibou et al. 1995; Sawers et al. 1991; Sawers 2005). Inactivation of formate dehydrogenase N (encoded by *fdnG*) and terminal nitrate reductase (encoded by *narG*), consistent with those under glucose metabolism, hydrogen production of multiple mutant strains also increased (Maeda et al. 2008). It is thought that formate dehydrogenase O (encoded by *fdoG*) shares a similar subunit structure and amino acid sequence to formate dehydrogenase N (FDH-N). Thus, it would have a similar role in converting formate into CO₂. However, inactivation of this enzyme did not improve hydrogen production when using glycerol as a substrate. Since the biochemical characterization of this enzyme is still limited, the possible reason for this may come from its location on the periplasmic side of the cytoplasmic membrane and its activation favors aerobic conditions (Abaibou et al. 1995; Jormakka et al. 2002). In fact, microbial production is inhibited by the accumulation of methylglyoxal—a toxic element synthesized by methylglyoxal synthase under anaerobic fermentation (Saikusa et al. 1987; Subedi et al. 2008; Zhu et al. 2001). Hence, we hypothesized that blocking this metabolic route might help increasing cell growth and producing more precursors for hydrogen production. Interestingly, the result showed that inactivation of methylglyoxal synthase (encoded

by *mgsA*) enhanced about 1.2-fold hydrogen productivity. To date, this is the first report on this matter where blocking of the metabolic pathway to the synthesis of methylglyoxal could enhance hydrogen production in *E. coli*.

From this foundation, we conducted successive transductions aiming to create a novel strain, which is able to produce high hydrogen production as well as to sustain the cell growth. In the final stage, except for *focA*, *hyaB*, and *aceE*, which encode for a formate transporter, the large subunit of hydrogenase 1 and pyruvate dehydrogenase, respectively, multiple disruptions of the other genes contributed to a significant improvement in hydrogen productivity. The novel strain with defects in *frdC*, *ldhA*, *fdnG*, *ppc*, *narG*, *mgsA*, and *hycA* had a 5.5-fold higher hydrogen productivity rate and 4.5-fold higher hydrogen yield than that of strain BW25113 (Table 2). Although disruption of *focA*, *hyaB*, and *aceE* alone could enhance hydrogen production, disruption of these genes together with the other beneficial genes in the multiple mutant strains detracted from hydrogen production and cell growth. Hence, blocking some pathways may positively affect hydrogen production as single mutations, but not necessarily have the same effect in the multiple mutant strains. We speculate that in the presence of a high level of accumulated formate resulting from the disruption of the formate transporter in the septuple mutant strain led to uncoupling of the membrane potential, and thus, it was toxic to the cell growth (Beyer et al. 2013; Kirkpatrick et al. 2001; Russell and Diez-Gonzalez 1997). Meanwhile, inactivation of *aceE* caused less cell viability due to insufficient electron acceptors resulted from redox imbalance. In turn, both of these disruptions lowered hydrogen production in the septuple mutant strain. Under normal pressure conditions, the strain BW25113 *frdC* *ldhA* *fdnG* *ppc* *narG* *mgsA* *hycA* could achieve the theoretical maximum yield after 48 h (1 mol hydrogen/1 mol glycerol), while the specific maximum yield of the parent strain was just 0.88 mol hydrogen/mol glycerol after 120 h (Table 3). To our knowledge, the hydrogen yield of our engineered strain is superior to those reported previously utilizing the co-overexpression of glycerol dehydrogenase and dihydroxyacetone kinase together with the disruption of fumarate reductase at alkaline pH and is equivalent to those achieved under acidic conditions (Shams Yazdani and Gonzalez 2008).

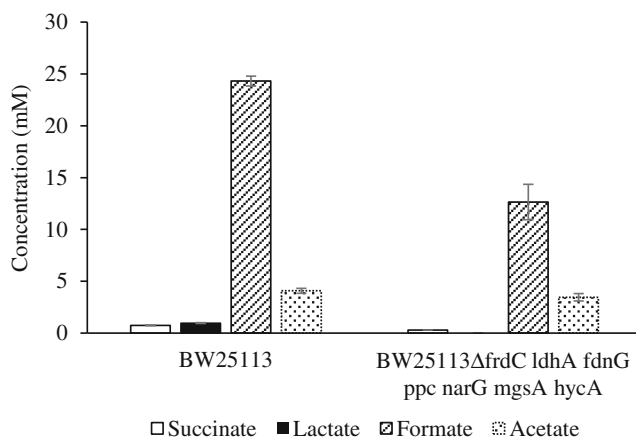


Fig. 4 Organic acids production by BW25113 and BW25113Δ*frdC* *ldhA* *fdnG* *ppc* *narG* *mgsA* *hycA* strains after 24 h of growth with glycerol under low partial pressure fermentation

Given that glycerol dehydrogenase (encoded by *gldA*) is responsible for the conversion of glycerol into glycolytic intermediates and is activated highly at slightly alkaline pH, in agreement with previous studies, our results show that individual overexpression of this enzyme does not improve hydrogen production (Fig. 1; Table 2) (Gonzalez et al. 2008; Truniger and Boos 1994). However, by co-overexpressing glycerol dehydrogenase and dihydroxyacetone kinase (encoded by *dhaKLM*), Shams Yazdani and Gonzalez (2008) have created a strain that has remarkably improved glycerol utilization, thus enabling it to produce higher hydrogen production. Therefore, together with such genetic disruptions, hydrogen yield and productivity of *E. coli* can be enhanced via co-overexpression of these two primary enzymes. We also overexpressed the FHL complex to enhance the conversion efficiency of formate into H₂ and CO₂ by transforming two different plasmids that constitute the FHL complex: pCA24N-FhlA and pCA24N-FdhF. Nevertheless, overexpression of these genes in both the wild type and the septupe strain lowered hydrogen production and this result is consistent with the previous report on glucose metabolism (Table 2; Maeda et al. 2007a). Possibly, IPTG, an inducer of the FHL complex, and the antibiotic (chloramphenicol) inhibited the cell growth because of their toxicity and selective pressure, respectively.

Aiming to enhance hydrogen production and yield in a shorter time course, we applied a low partial pressure assay for the glycerol metabolism. Despite the minor improvements in hydrogen yield and productivity, it is a step forward since both strains grew much faster than under the closed hydrogen assay (Table 4). Therefore, faster growth of the strain has a potential application for production at a large scale. Additionally, production of formate, a precursor of hydrogen, sharply increased up to 5.4-fold (Table 4). To some extent, hydrogen productivity and yield were not totally formate production dependent. They also depend on various factors such as intracellular pH, extracellular pH, and the diffusion of CO₂ into and out of the cell (Kozliak et al. 1995; Merlin et al. 2003; Murarka et al. 2008).

In addition to the metabolic engineering approach, pH is also a critical factor for improving hydrogen production in *E. coli*. In general, the activation of four hydrogenases is complicated and depends on external pH, F₀F₁-ATPase and carbon source (Bagramyan et al. 2002; Dharmadi et al. 2006). Hyd-3 is highly active under acidic conditions, enabling it to convert more efficiently H⁺ into H₂ (Bagramyan et al. 2002; Dharmadi et al. 2006; Murarka et al. 2008; Trchounian et al. 2012). In contrast, Hyd-2 is most responsible for hydrogen production at neutral and slight alkaline pH (Trchounian and Trchounian 2009). Besides, glycerol dehydrogenase and dihydroxyacetone kinase that determine the efficiency of glycerol conversion are highly active at alkaline pH (Gonzalez

et al. 2008). Therefore, to improve hydrogen yield and productivity in *E. coli* from glycerol, together with metabolic engineering and pressure control, the external pH should be considered. Clearly, metabolic engineering in *E. coli* has advantages over other hydrogen-producing microorganisms because it can reach the theoretical maximum yield, while sustaining reasonable growth. Hopefully, further improvements in terms of glycerol utilization will lead to large scale application for the production of hydrogen.

Acknowledgements Special acknowledgment goes to the NBRP-*E. coli* at the National Institute of Genetics (Japan) for providing KEIO mutants and ASKA clones. The authors would like to thank the Japan Student Services Organization for the scholarship of K. T. Tran during this study. This research was supported by the JGC-S scholarship foundation.

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