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An evolved *Escherichia coli* strain for producing hydrogen and ethanol from glycerol

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ABSTRACT

Glycerol is an attractive feedstock for biofuels since it accumulates as a byproduct during biodiesel operations; hence, here we consider converting glycerol to hydrogen using the formate hydrogen lyase system of *Escherichia coli* which converts pyruvate to hydrogen. Starting with *E. coli* BW25113 *frdC* that lacks fumarate reductase (this mutation reduces repression of hydrogen synthesis during glycerol fermentation) and by using both adaptive evolution and chemical mutagenesis combined with a selection method based on increased growth on glycerol, we obtained an improved strain, HW2, that produces 20-fold more hydrogen in glycerol medium (0.68 ± 0.16 mmol/L/h). HW2 also grows 5-fold faster (0.25 ± 0.01/h) than BW25113 *frdC* on glycerol, so it achieves a reasonable anaerobic growth rate. Corroborating the increase in hydrogen production, glycerol dehydrogenase activity in HW2 increased 4-fold compared to BW25113 *frdC*. In addition, a whole-transcriptome study revealed that several pathways that would decrease hydrogen yields were repressed in HW2 (*fbp*, *focA*, and *gatYZ*) while a beneficial pathway which encodes enolase was induced. Ethanol production was also increased 5-fold in the evolved HW2 strain.

Introduction

Diminishing supplies of fossil fuels along with environmental concerns for global warming and pollution are exerting pressure to search for new renewable sources [1]. Biodiesel, methyl or ethyl esters of fatty acids made from renewable biological resources, has attracted much attention [1] because it generates less CO_2 compared to fossil fuels [2]. As a result, biodiesel production is increasing; for example, the annual production capacity in the US and the EU were 1.2 and 5.7 million metric tons in 2007 and will double by 2012 [3].

Biodiesel is most often produced by reacting a fat or oil (triglycerides) with methanol or ethanol in the presence of an alkali catalyst [2]. The production of 10 kg of biodiesel yields about 1 kg of glycerol [1]. The rapid growth of the biodiesel industry has created excess glycerol, which has resulted in a significant decrease (about 10-fold) in crude glycerol prices and has become a burden to the biodiesel industry [4].

Although crude glycerol can be used as boiler fuel and as a supplement for animal feed, the market value of crude glycerol is still very low [4]. Thus, there is an urgent need to develop a practical process for converting glycerol to a useful product. Glycerol can be used as a carbon source by a number of microorganisms and can be converted into various interesting products such as 1,3-propanediol, succinic acid, dihydroxyacetone, and ethanol [5].

Another possible route is conversion of glycerol to hydrogen. Hydrogen is efficient, clean, and utilized for fuel cells as an energy source in portable electronics, power plants, and for the internal combustion engine [6]. It has been reported that hydrogen can be produced by the pyrolysis of glycerol; however, this process requires high temperatures, and the product gas has a complex composition [7]. Production of hydrogen by microorganisms may be more desirable than the current conventional thermal and electrolytic processes for hydrogen production since biological production requires less energy [8].

Conversion of glycerol to hydrogen by photo-fermentation with *Rhodopseudomonas palustris* has been investigated [1]; however, the long fermentation process (200–300 h) [1], the difficulty in light penetration [9], and the difficulty in uniform light distribution [9] hinder its practical use. Compared to photosynthetic processes, fermentative hydrogen production generally gives two orders of magnitude higher rates of hydrogen production [8]. Moreover, anaerobic fermentation of glycerol to produce hydrogen is attractive because it produces not only hydrogen but also other biofuels such as ethanol [10]. Hydrogen production from glycerol by fermentation has been studied with *Enterobacter aerogenes* [10,11], *Escherichia coli* [12,13], *Klebsiella pneumoniae* [14], and mixed cultures [15]. However, hydrogen production is repressed at high glycerol concentrations and little ethanol is produced using

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E. aerogenes [10]. Although hydrogen production is improved by using an electrode system with thionine as an electron transfer mediator [11], the complex system may be impractical [1]. *K. pneumoniae* has been investigated to produce 1,3-propanediol from glycerol [4,5]; however, metabolic flux analysis shows that it may not be suitable for hydrogen production because the production of hydrogen and ethanol are dramatically decreased at higher concentrations of glycerol [16]. In addition, industrial use of *K. pneumonia* would be limited because of its classification as an opportunistic pathogen which can cause human infections [4]. Furthermore, mixed culture fermentations are very complex and are difficult to optimize due to the variable compositions of the microbial communities [17].

Escherichia coli ferments glycerol anaerobically [12,13]; however, it has a very low specific growth rate in glycerol [12], which is about 40-fold lower than aerobic growth in LB medium. Nonetheless, *E. coli* is very promising for glycerol utilization because it is one of the most commonly used host organisms for metabolic engineering and industrial applications. It is easy to manipulate genetically, can produce a wide variety of anaerobic fermentation products, and it is the best-characterized bacterium [6]. Furthermore, we have created *E. coli* bacteria capable of converting glucose [6] and formate [18] into hydrogen using metabolic engineering as well as have used protein engineering for hydrogenase 3 [19] and the hydrogen transcriptional activator FhlA [20] to improve hydrogen yields.

Adaptive evolution is a set of environmentally-induced mutations that confer growth advantages to the cell [21]. An organism is subjected to serial or continuous cultivation for many generations to which it is not optimally adapted to select more fit genetic variants [22]. Hence, the adaptive evolution method has been used to select improved strains from *E. coli* [23].

The aims of this study were to obtain a mutant that grows both faster on glycerol and which has higher hydrogen productivity as well as to investigate the mechanism by which these two traits are improved. By combining adaptive evolution with chemical mutagenesis, we obtained an evolved strain which has 5-fold faster growth, 20-fold greater hydrogen productivity, and 5-fold greater ethanol production. We also determined key enzymes that were affected by the mutagenesis.

Materials and methods

Bacterial strains, media, and growth conditions. The isogenic *E. coli* BW25113 strains and plasmids used in this study are listed in Table 1. For deleting and overexpressing genes, we used the Keio collection [24] and the ASKA library [25], respectively. Kanamycin (50 μg/mL) was used for pre-culturing the isogenic knock-outs. Chloramphenicol (30 μg/mL) was used for the strains harboring pCA24N and its derivatives. All experiments were conducted at 37 °C.

The glycerol medium used for hydrogen production was based on minimal medium that was supplemented with 10 g/L glycerol, 2 g/L tryptone, 5 mM sodium selenite, and 1.32 mM Na_2HPO_4 in place of K_2HPO_4 [12]. The final concentration of MOPS (morpholinopropanesulfonic acid) was 50 mM, and the pH was adjusted to 6.3 by concentrated NaOH. All chemicals were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma–Aldrich Co. (St. Louis, MO).

Growth rates and the glycerol dehydrogenase assay. The specific growth rates of the *E. coli* wild-type strain and the evolved mutants were determined in Luria–Bertani (LB) medium (aerobically) and glycerol medium (anaerobically) by measuring the turbidity at 600 nm for two independent cultures of each strain as a function of time with values less than 0.7. The activity of glycerol dehydrogenase for glycerol was measured as described previously [13] using 100 mM potassium carbonate buffer (pH 9.4); this assay is based on the change of absorbance at 340 nm due to the conversion of NAD^+ into NADH by glycerol dehydrogenase.

Adaptive evolution and chemical mutagenesis. Adaptive evolution of BW25113 *frdC* was conducted anaerobically by successive batch fermentations in glycerol medium supplemented with 50 μg/mL of kanamycin using sealed crimp-top vials (60 mL) with shaking at 250 rpm for 78 passages. For each batch culture, the turbidity was measured at 600 nm, cells were inoculated into vials with fresh glycerol medium (20 mL), the vials were sealed, and the headspace was purged with nitrogen for 5 min. The volume of the inoculum for each passage (5–50 μL) was adjusted due to changes in the growth rate of the evolved strain to ensure that cultures would be in the exponential growth phase during the complete fermentation. Cultures were frozen and stored at regular intervals throughout adaptive evolution.

Table 1
Escherichia coli bacterial strains, plasmids, and primers used in this study.

Strains and plasmids	Genotype/relevant characteristics	Source
Strains		
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR*514 Δ*varaBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	[24]
BW25113 *frdC*	BW25113 Δ*frdC* Ω Km^R	[24]
BW25113 *gldA*	BW25113 Δ*gldA* Ω Km^R	[24]
HW1	BW25113 *frdC* with adaptive evolution	This study
HW2	HW1 with chemical mutagenesis	This study
Plasmids		
pCA24 N	Cm^R; *lacI*^{q}, pCA24 N	[25]
pCA24 N-*gldA*	Cm^R; *lacI*^{q}, pCA24 N P$_{T5\text{-}lac}$::*gldA*$^+$	[25]
Primers for qRT-PCR		
glpT_F	5′-GATGGCCTGGTTCAATGACT-3′	This study
glpT_R	5′-CAGCAGTTTGTTCGGCAGTA-3′	This study
focA_F	5′-TGTTGTTGCTAAGGCGAGTG-3′	This study
focA_R	5′-GGCGGTTTGTAGGACGTTTA-3′	This study
gatZ_F	5′-AATGACACCGGCAGACTTTC-3′	This study
gatZ_R	5′-ATTCCTTTACCAGCTCGACG-3′	This study
fbp_F	5′-TGTCGTCTTTGAAGGCTGTG-3′	This study
fbp_R	5′-ACCAGGCTGGAGGAAATCTT-3′	This study
eno_F	5′-GCTCCGTCAGGTGCTTCTAC-3′	This study
eno_R	5′-AGCCAGAGATACAGCCAGGA-3′	This study
rpoA_F	5′-CGCGGTCGTGGTTATGTG-3′	This study
rpoA_R	5′-GCGCTCATCTTCTTCCGAAT-3′	This study

Km^R and Cm^R are kanamycin and chloramphenicol resistance, respectively. F indicates forward primer and R indicates reverse primer.

Chemical mutagenesis of HW1 with nitrosoguanidine was carried out as described previously [26]. After chemical mutagenesis, all cells were added to a sealed vial (60 mL) with 20 mL of glycerol medium with 50 μg/mL of kanamycin to start the second adaptive evolution process that was used for 15 passages.

Hydrogen and ethanol assay. Hydrogen and ethanol yields were quantified using anaerobic fermentations. Overnight aerobic cultures in LB (25 mL), inoculated from single colonies, were sparged for 5 min with nitrogen. Sealed crimp-top vials (27 mL) were also purged for 2 min with nitrogen. Inside an anaerobic glove-box, 10 mL of sparged, glycerol medium and 1 mL of sparged overnight culture were added to each vial. The amount of hydrogen generated in the head space was measured after anaerobic incubation for 5 and 24 h by gas chromatography using a 6890N gas chromatograph (Agilent Technologies Inc., Santa Clara, CA) as described previously [27].

For the ethanol assay, after 5 and 24 h of anaerobic fermentation, one milliliter of each culture was centrifuged at 13,000 rpm for 1 min, the supernatants were filtered (0.22 μm), and the samples were stored at −20 °C for the ethanol assay. Ethanol was measured using the gas chromatograph, a packed metal column (0.10% AT-1000, 1.83 m × 3.175 mm, 2.159 mm thickness, Alltech Associates Inc., USA), and a flame ionization detector. The inlet, detector, and oven temperature were controlled at 180 °C, 210 °C, and 90 °C, respectively.

Whole-transcriptome analysis. The *E. coli* GeneChip Genome 2.0 array (Affymetrix, P/N 900551) was used to analyze differential gene expression for the HW2 evolved mutant vs. the BW25113 *frdC* mutant. RNA was isolated from cells grown anaerobically for 5 h as for the hydrogen experiments except 60 mL sealed crimp-top vials were used with 33 mL cultures. The initial/final turbidity of the *frd*C mutant and HW2 were 0.55/0.58 and 0.52/0.64, respectively; hence, there was little growth for the two stains. The cDNA synthesis, fragmentation, and hybridizations were performed as described previously [28]. The probe array images were inspected for any image artifacts. Background values, noise values, and scaling factors of both arrays were examined and were comparable. The intensities of polyadenosine RNA controls were used to monitor the labeling process, and signals of the *araA* and *rhaA* deleted genes of BW25113 (Table 1) were low. If the gene with the larger transcription rate did not have a consistent transcription rate based on the 11–15 probe pairs (*P*-value less than 0.05), these genes were discarded. A gene was considered differentially expressed when the *P*-value for comparing two chips was lower than 0.05 (to assure that the change in gene expression was statistically significant and that false positives arise less than 5%) and if the fold change was higher than the standard deviation for the whole genome [29]. The expression data were deposited in the NCBI Gene Expression Omnibus and are accessible as GSE18972.

Quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR was performed using the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Total RNA (100 ng) was used for the qRT-PCR using the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Applied Biosystems, Foster City, CA). The primers that were used are listed in Table 1 (the annealing temperature was 60 °C), and the housekeeping gene *rpoA* was used to normalize the gene expression data.

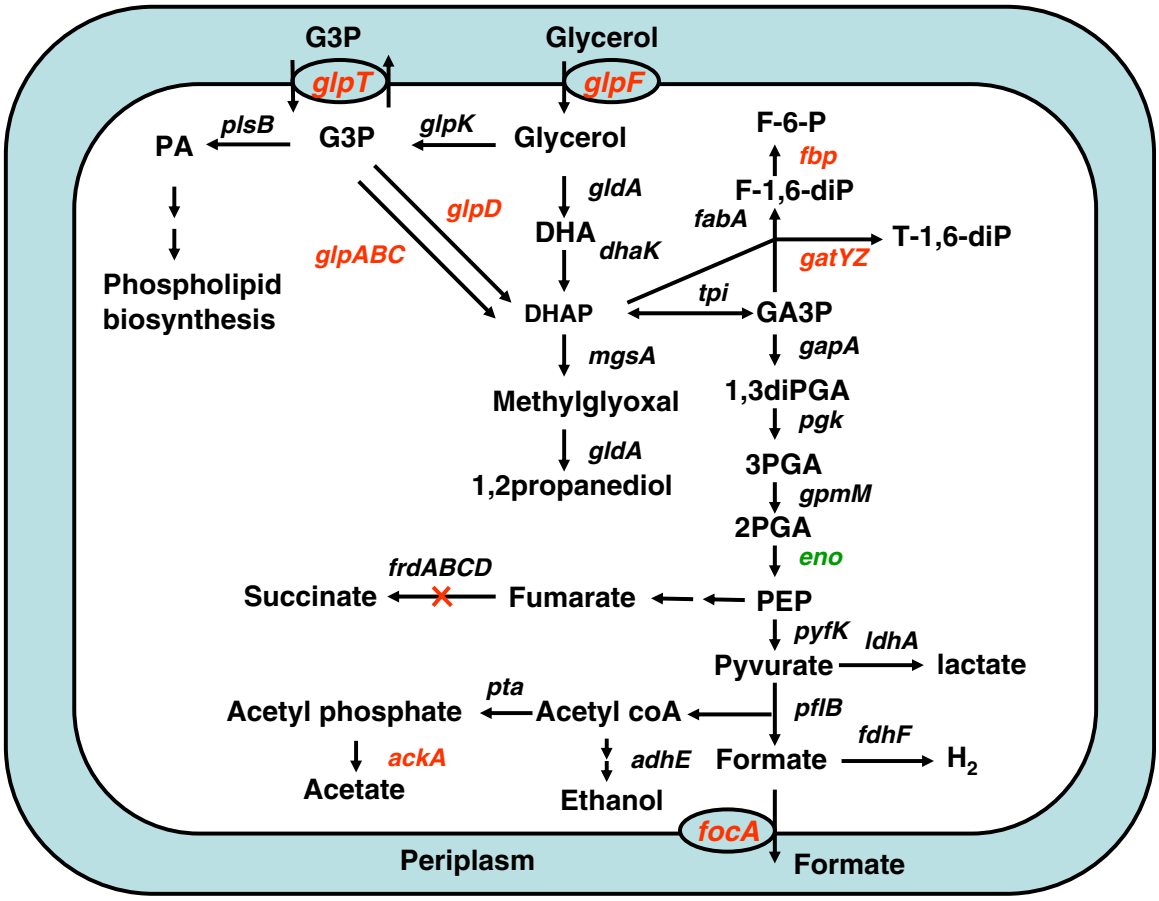

Fig. 1. Major differential gene expression patterns for hydrogen-related pathways for HW2 vs. the wild-type strain. Red indicates repressed genes and green indicates induced genes. G3P: glycerol-3-phosphate, DHA: dihydroxyacetone, DHAP: dihydroxyacetone phosphate, F-1,6-dip: fructose-1,6-diphosphate, F-6-p: fructose-6-phosphate, GlpF: glycerol facilitator, GlpT: sn-glycerol-3-phosphate transporter, GA3P: glyceraldehyde-3-phosphate, 1,3-diPGA: glycerate 1,3-bisphosphate, 3PGA: glycerate 3-phosphate, 2PGA: glycerate 2-phosphate, PA, phosphatydic acid, PEP: phosphoenolpyruvate, and T-1,6-dip: tagatose-1,6-diphosphate.

Results

Adaptive evolution and chemical mutagenesis of BW25113 frdC

frdC encodes a subunit of fumarate reductase, and its inactivation eliminates the negative effect that hydrogen has on glycerol fermentation in closed vessels [12]. In addition, the *frdC* mutation minimizes the synthesis of succinate, a competing byproduct for hydrogen production [30] (Fig. 1), and the kanamycin resistance of this mutant may be used to prevent contamination during the long evolution period. Furthermore, the *frdC* mutant has similar values to the wild-type strain for hydrogen yield and volumetric productivity (data not shown). Therefore, the BW25113 *frdC* mutant was used to initiate the adaptive evolution process.

Before creating an improved *E. coli* strain for glycerol fermentation, we first investigated growth and hydrogen production by the BW25113 wild-type strain and its isogenic *frdC* mutant. Both the wild-type strain and the *frdC* mutant grew very slowly (0.05 ± 0.01/h) on glycerol medium with anaerobic conditions (Table 2) which agrees with that of MG1655, which is 0.040/h [12].

The rationale for the adaptive evolution was that mutations that give rise to better growth on glycerol, a poor energy source, would be selected since cells would outcompete those that lacked the beneficial changes. After three months of adaptive evolution (78 passages), four colonies were isolated and each was found to have similar hydrogen production in glycerol medium; one was designated as HW1. The specific growth rate of HW1 on glycerol reached 0.09 ± 0.01/h which was 1.8-fold higher than that of the *frdC* mutant (Table 2). However, HW1 had a hydrogen yield of 0.87 ± 0.03 μmol/mg protein and a volumetric productivity of 0.024 ± 0.001 mmol/L/h after 5 h, which were less than that of the *frdC* mutant (1.6 ± 0.3 μmol/mg protein and 0.035 ± 0.006 mmol/L/h). After 24 h, the hydrogen yield and volumetric productivity of HW1 were 36.8 ± 0.6 μmol/mg protein and 0.28 ± 0.01 mmol/L/h which were 1.5- and 2.2-fold higher than those of the *frdC* mutant, respectively. These results indicate that HW1 produces more hydrogen only after long incubation times.

To improve HW1 further, chemical mutagenesis with nitrosoguanidine and a second round of adaptive evolution were conducted. After mutagenesis and 15 successive fermentations, four colonies were isolated, and all four strains had similar hydrogen productivity; one was designated as HW2. The specific growth rate of HW2 reached 0.25 ± 0.01/h and was thereby increased 2.8- and 5.0-fold compared to HW1 and the *frdC* mutant, respectively (Table 2). Note there was no significant difference among the three strains during aerobic growth rate in LB medium.

Critically, the hydrogen yield of HW2 after 5 h was 21 ± 3 μmol/mg protein, which was 25- and 14-fold higher than those of HW1 and the *frdC* mutant, respectively. In addition, the hydrogen volumetric productivity of HW2 at 5 h was 0.68 ± 0.16 mmol/L/h, which was 28- and 20-fold higher than those of HW1 and the *frdC* mutant, respectively. A similar trend in hydrogen yield and volumetric productivity occurred at 24 h. These results indicate that combining chemical mutagenesis with adaptive evolution may be used to create a strain, HW2, which can grow faster and have a higher hydrogen production rates.

Table 2
Specific growth rates in LB medium (aerobic) and glycerol medium (anaerobic).

Strain	Specific growth rate, 1/h	
	LB, aerobic	Glycerol, anaerobic
BW25113 wild-type	1.55 ± 0.01	0.05 ± 0.01
BW25113 *frdc*	1.61 ± 0.05	0.05 ± 0.01
HW1	1.49 ± 0.01	0.09 ± 0.01
HW2	1.42 ± 0.04	0.25 ± 0.01

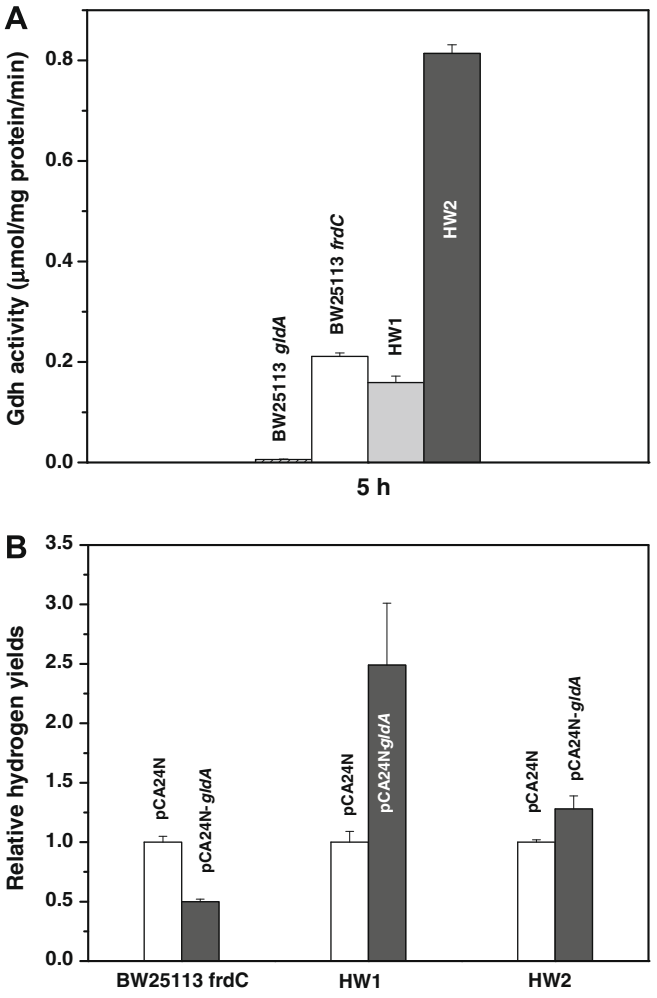

Fig. 2. Glycerol dehydrogenase (Gdh) activity and effect of overexpression of *gldA* on hydrogen yields. Cells were cultured anaerobically in glycerol medium for 5 h (A). Cells were cultured anaerobically in glycerol medium for 22 h with *gldA* induced with 0.1 mM IPTG (B). Results are the average of two independent cultures, and one standard deviation is shown.

Ethanol production

Since glycerol fermentation by *E. coli* produces not only hydrogen but also ethanol (Fig. 1) [10], the ethanol production of the three strains were also measured. The ethanol yield of HW2 at 5 h was 2.1 ± 0.3 mg/mg protein, which was 5- and 6-fold more than those of the *frdC* mutant (0.41 ± 0.05 mg/mg protein) and HW1 (0.36 ± 0.04 mg/mg protein), respectively. A similar trend in ethanol occurred at 24 h. These results indicate clearly that chemical mutagenesis with adaptive evolution may be used to engineer a strain that can produce more ethanol.

Effect of gldA on glycerol fermentation in HW2

To investigate the mechanism by which hydrogen production is increased with HW2, we checked the activity of the first enzyme in the glycerol to hydrogen pathway (Fig. 1); glycerol is oxidized to dihydroxyacetone by glycerol dehydrogenase (Gdh) encoded by *gldA* [31]. BW25113 *gldA* was used as the negative control. The activity of HW2 compared to the *frdC* mutant was 4-fold at 5 h (Fig. 2A) and 5-fold at 24 h. Therefore, HW2 metabolizes glycerol better due to enhanced Gdh activity.

To provide corroborating evidence of the importance of the evolved mutants, we overexpressed *gldA* in the *frdC* mutant,

HW1, and HW2 using plasmid pCA24N. The relative hydrogen productivity of HW1/pCA24N-*gldA* at 22 h increased by 2.5-fold compared to HW1/pCA24N, while HW2/pCA24N-*gldA* increased by 1.3-fold (Fig. 2B). However, in agreement with prior results [32], overexpression of *gldA* in the *frdC* mutant did not result in increased hydrogen production; here, hydrogen productivity was reduced by 50%. Therefore, the increase in hydrogen production for HW1 and HW2 may due to higher Gdh activity in HW1 and HW2.

Whole-transcriptome analysis

To investigate further why HW2 produced more hydrogen than the *frdC* mutant, a whole-transcriptome analysis was used to determine differential gene expression for the two strains in glycerol medium after 5 h; the primary results are summarized in Fig. 1. It was found that the evolved strain repressed the transcription of *fnr* by 3.8-fold (Supplementary Table S1); Fnr is a global transcription factor which is essential for the regulation of many genes involved in anaerobic respiration [30]. Also, genes were repressed related to glycerol uptake (*glpF*), glycerol-3-phosphate transport (*glpT*), anaerobic glycerol-3-phosphate dehydrogenase activity (*glpA*, *glpB*), and aerobic glycerol-3-phosphate dehydrogenase activity (*glpD*). These results confirm that glycerol dissimilation under anaerobic condition relies on the glycerol dehydrogenase and dihydroxyacetone pathway [13] rather than the glycerol kinase and anaerobic glycerol-3-phosphate dehydrogenase pathway encoded by *glpK-glpABC*. In addition, genes involved in gluconeogenesis (*fbp*), galactitol catabolism (*gatABCDRYZ*), formate transportation (*focA*), and acetate synthesis (*ackA*) were repressed, while the genes involved in lipopolysaccharide biosynthesis (*rfaBIJKPSYZ*) and enolase synthesis (*eno*) were induced in HW2. These results indicate that the regulation of many genes related to glycerol metabolism is changed after adaptive evolution and chemical mutagenesis. However, the transcriptional level of the *gldA* and *dhaMKL* were not changed in HW2.

qRT-PCR

qRT-PCR was used to corroborate the whole-transcriptome results for the HW2 relative to the *frdC* mutant. Transcription of *gatZ*, *glpT*, and *focA* in HW2 was repressed by 6.4-, 1.4-, and 1.3-fold compared to the *frdC* mutant using qRT-PCR vs. 6.5-, 5.7-, and 4.0-fold with the whole-transcriptome study. Similarly, the transcription of *eno* was induced by 1.4-fold using qRT-PCR vs. 2.6-fold with the whole-transcriptome study.

Discussion

Although glycerol may be converted into many products by microorganisms, fuels, such as hydrogen and ethanol may be the best choices since they have a sufficiently large market; i.e., the demand for fuels is nearly inexhaustible [1]. Microaerobic metabolism of glycerol in *E. coli* to produce ethanol has been studied recently [23,33] to overcome the drawback of low growth rates under anaerobic conditions. However, the oxygen levels must be controlled in a complex manner to alter the competition between ethanol production and biomass synthesis [23]. In addition, the need for aeration typically increases the operational expenses by 40% compared with anaerobic processes [4].

By using adaptive evolution and chemical mutagenesis, here we obtained a mutant that both grew faster on glycerol and produces both more hydrogen (20-fold) and ethanol (5-fold) under anaerobic conditions. Chemical mutagenesis is a powerful tool for the development of strains with improved traits. However, the screening process for desirable mutants is usually labor-intensive and time-consuming. With glycerol fermentation, hydrogen production is related to cell growth via increasing ATP production [32]; therefore, we used the method of adaptive evolution to select the mutant which had high growth and found subsequently that the hydrogen productivity was increased. Compared to the conventional screening methods, this was relatively facile. The specific growth rate of HW2 strain was 5-fold higher than those of strains studied previously [12]. To our knowledge, it is the fastest growing *E. coli* strain on glycerol.

As shown in Fig. 1, *eno* was induced in HW2 while *ackA*, *fbp*, *focA*, and *gatZ* were repressed. Enolase (encoded by *eno*) catalyzes the dehydration of 2-phosphoglycerate to phosphoenolpyruvate (PEP), which is essential for dihydroxyacetone phosphorylation to dihydroxyacetone phosphate (DHAP) in glycerol metabolism [34]. In addition, FocA is a putative formate exporter and may be responsible for regulating the intracellular formate pool [35]; *focA* mutants have increased intracellular levels of formate and decreased secretion of formate [35]. Based on these results, we hypothesize that HW2 grows faster and produces more hydrogen than the *frdC* mutant since increased expression of *eno* may provide more PEP for both DHA phosphorylation and for formate; therefore, glycerol may be more effectively converted to DHAP by the high activity of GldA and sufficient PEP. Moreover, repression of *gatYZ* and *fbp* may reduce the consumption of glyceraldehyde-3-phosphate by the gluconeogenesis pathway; therefore, there may be increased metabolic flux to pyruvate and hydrogen. Furthermore, repression of *focA* may increase the intracellular level of formate, which then can be converted to hydrogen by the formate hydrogen lyase system [35]. Also, the repression of *ackA* in HW2 may decrease the production of acetate to relieve its inhibition on cell growth [30]. Beyond the specific changes that lead to the 20-fold increase in hydrogen production, our approach shows that random changes based on a strong selection method may be used to enhance strains for engineering applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.12.013.

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