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Beneficial knockouts in *Escherichia coli* for producing hydrogen from glycerol

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Abstract Glycerol is an inexpensive and abundant source for biofuel production on a large scale. *Escherichia coli* is a robust bacterium for producing hydrogen; however, its hydrogen productivity from glycerol is low. In this study, we conducted random transposon mutagenesis to identify uncharacterized genes whose inactivation is beneficial for hydrogen production from glycerol. Through screening, four mutant strains were found that are able to have from 1.3- to 1.6-fold higher hydrogen productivity (μmol H_2/mg protein) than that of their parent strain ($p<0.05$). These mutations were identified as *aroM*, *gatZ*, *ycgR*, and *yfgI*. The hydrogen yield (mol H_2/mol glycerol consumed) of the *aroM*, *gatZ*, *ycgR*, and *yfgI* strains was 1.7-, 1.4-, 2.4-, and 2.1-fold higher than that of their parent strain, respectively. Moreover, a single disruption in these genes resulted in a faster cell growth and glycerol consumption under anaerobic conditions. In *E. coli*, AroM is predicted to be involved in the shikimate pathway, GatZ is tagatose-1,6-bisphosphate aldolase 2 which converts dihydroxyacetone phosphate to 1,6-biphosphate, and YcgR acts as a molecular brake limiting the swimming speed and ATP consumption. So far, the function of YfgI in general and in hydrogen production in particular remains unknown.

Keywords Glycerol · Hydrogen production · *Escherichia coli* · Transposon mutagenesis

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Introduction

Fossil fuels such as oil and coal are forecasted to be depleted in the next 50–100 years, while the global thirst for energy will only increase. Biofuels are considered an ideal alternative because they can be easily and renewably produced from available and inexpensive biomass sources such as byproducts or wastes released from agricultural and industrial practices. Among biofuels, hydrogen is attractive because it does not evolve into greenhouse gases or other pollutants after combustion. Furthermore, hydrogen has 2.75-fold higher energy content compared to fossil fuel (Das and Veziroğlu 2001; Kim and Kim 2011).

As a result of an increasing demand for biodiesel production, the global production of crude glycerol is expected to continue to increase (Anand and Saxena 2012). Meanwhile, the industrial purification of glycerol is likely not economically viable, since the surplus of crude glycerol has negatively impacted the glycerol market in recent years (Dobson et al. 2012; Yang et al. 2012). Moreover, crude glycerol from biodiesel production contains high amounts of salt, methanol, and soap that would be a burden to the environment (Hansen et al. 2009). Therefore, to assure the sustainable development of the biodiesel industry, crude glycerol as well as other byproducts from the biodiesel production should be utilized as a feedstock

for other chemicals. In fact, dozens of studies have been recently conducted with the goal of converting crude glycerol into other value-added chemicals and biofuels. Of these, glycerol has been intensively used for hydrogen production via anaerobic fermentation (Blankschien et al. 2010; Clomburg and Gonzalez 2011, 2013; Mazumdar et al. 2013; Murarka et al. 2008; Tokumoto and Tanaka 2012; Tran et al. 2014; Trinh and Srienc 2009).

Escherichia coli is a potential microorganism for hydrogen production at the industrial scale because it can sustain a high growth rate, requires simple technology, and could achieve a high hydrogen yield and production (Maeda et al. 2007, 2008a; Shams Yazdani and Gonzalez 2008; Tran et al. 2014). Although the genome of *E. coli* is well characterized, only 54 % is experimentally determined, while the rest is either uncharacterized or computationally predicted (Riley et al. 2006). The metabolic flux of *E. coli* has been gradually elucidated and more new genes have been characterized (Blattner 1997; Gonzalez et al. 2008; Maeda et al. 2007; Sanchez-Torres et al. 2013). More recently, some pseudogenes which are regarded as nonfunctional DNA sequences and uncharacterized genes were determined to play a role in hydrogen production in *E. coli* (Mohd Yusoff et al. 2013). Hence, there is a gap in the current knowledge of the unknown metabolic pathways or genes related to hydrogen production of *E. coli* under glycerol metabolism. In addition, anaerobic growth of *E. coli* in the presence of glycerol is quite low (Hu and Wood 2010; Tran et al. 2014) unlike that of glucose (Maeda et al. 2007, 2008a).

Transposon mutagenesis is considered a powerful genetic tool to create random mutant strains, and this method has been intensively applied to characterize the function of unknown genes in various microorganisms (Ahmed 1985; Hamer et al. 2001; Liu et al. 2012; Nakata 2002; Pannekoek et al. 1980). In fact, random transposon mutagenesis has been used in various hydrogen-producing microorganisms such as *Pantoea agglomerans*, *Rhodobacter*, or *Rhodovulum sulfidophilum* to identify beneficial knockouts for hydrogen production, and this approach has gained momentum (Cai and Wang 2014; Liu et al. 2012; Ma et al. 2012a). Based on the aforementioned issues, this study aims to identify uncharacterized genes that are beneficial for hydrogen production in *E. coli* under glycerol metabolism by applying random transposon mutagenesis and screening.

Materials and methods

Random transposon mutagenesis

A single colony of *E. coli* BW25113, which is obtained from Yale Coli Genetic Stock Center (New Heaven, USA) (Table 1), was inoculated in Luria-Bertani (LB) medium without sodium chloride at 37 °C, 120 rpm overnight. The cell pellets were harvested by centrifugation at 7000 rpm for 10 min. Transposon mutagenesis was performed as described previously with some slight modifications (Liu et al. 2012; Nakata 2002). A Tnp Transposome™ Kit EZ-TN*5* <KAN-2> Tnp Transposome™ Kit (Epicentre, Madison, WI, USA) was used to introduce random insertions into the host cell. The transformation was performed by electroporation at the parameters 1.2 kV and 200Ω with a 0.1-cm electroporation gap cuvette. The electroporated cells were immediately transferred into 1 mL of SOC medium (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) and incubated at 37 °C for 1 h to facilitate cell outgrowth. The number of transformants was counted by spreading 100 μL of electroporated cells on a LB plate with kanamycin (final conc 50 μg/mL). The transformation efficiency was calculated as the number of colony forming unit (cfu) over the amount of transposon used (Nakata 2002).

Preliminary screening of hydrogen production

The hydrogen assay was conducted anaerobically. A sealed crimp top vial (68 mL) which was sparged with nitrogen gas for 5 min was used for the fermentation with the rationale being that the fastest growing cells on glycerol would be selected. About 100 μL of electroporated cells were added into five separated sealed crimp top vials containing 20 mL of glycerol minimal medium (Murarka et al. 2008) supplemented with 50 μg/mL of kanamycin and incubated at 37 °C, 120 rpm for 2 days. The fermentation batches were successively cultured for five passages for mutant enrichment. In the final step, the fermentation broths were stocked and kept at −80 °C for screening purposes.

Single colonies were obtained by streaking the adapted culture stock onto LB plates containing kanamycin and were incubated overnight at 37 °C. To prevent missing any beneficial mutants for hydrogen production, the Taro Yamane formula was applied to calculate the population sample or the numbers of single mutant strain needed for investigation with the level of significance of 0.1 (Yamane 1967). A single *E. coli* strain was randomly selected for screening hydrogen production (below).

Identification of insertion site

The candidate mutant strains were cultivated overnight in LB medium containing kanamycin (50 μg/mL). Cell pellets were harvested and then the chromosomal DNA was extracted by UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). A two-round arbitrary PCR with some modifications was consecutively conducted to amplify the transposon flanking site (Das et al. 2005; Knobloch et al. 2003; Ma et al. 2012b). The arbitrary, internal

and external primers used are listed in Table 1. First round arbitrary PCR (PCR1) was conducted using the arbitrary primer 1 together with the internal primer under the following conditions: 94 °C for 5 min; 6 cycles of 94 °C for 30 s, 30 °C for 30 s, for 72 °C 1.5 min; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1.5 min; and 72 °C for 5 min. Samples were held at 4 °C. The PCR1 product was purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). About 100 ng of purified PCR1 was used as the DNA template for the second round PCR (PCR2), where the arbitrary primer 2 and the external primer were used with the following conditions: 95 °C for 1 min; 30 cycles of 95 °C for 30 s, 65 °C for 30 s, and 72 °C for 1.5 min; and 72 °C for 4 min.

The PCR2 products were run on 0.8 % agarose gel at 100 V for 25 min. A single band with a target size from 700 to 1200 base pairs was subjected for gel cutting. QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was used for gel extraction. The extracted DNA fragment was then run on 0.8 % agarose gel to assure that only a single band was attained prior to the DNA sequencing. A basic local alignment search tool (BLAST) from NCBI (http://www.ncbi.nlm.nih.gov/) (Wheeler and Bhagwat 2007) and Ecogene (http://www.ecogene.org) (Zhou and Rudd 2013) were mutually used for the alignment between the chromosomal DNA sequence of the amplified fragment and the genome database of *E. coli* strain, respectively. Only alignments with a high score value (above 98 % of similarity) were considered for the hydrogen confirmation and other analysis.

Hydrogen assay, growth condition, and confirmation

Based on the nucleotide BLAST (blastn) results, the candidate strains were obtained from the Keio library (National Institute of Genetics, Japan) as listed in the Table 1. These Keio strains were used to investigate their hydrogen production from glycerol. Minimal glycerol medium at pH 7.5 was used for both screening and confirmation of hydrogen production. Details of the hydrogen assay, growth condition, and medium were previously reported (Tran et al. 2014). Due to the requirement of a large sample population for parametric data analysis, hydrogen production of each strain was conducted for 10 independent batches. A test of analysis of variance (ANOVA) and Tukey post hoc with the significance level of 0.05 were conducted using a Statistical Package for the Social

Table 1 Strains and primers used in this study

Strains and primers	Genotype/description	Sources
Strains		
BW25113	F⁻Δ (*araD-araB*)567Δ*lacZ4787(::rrnB-3)*λ⁻ rph-1Δ*(rhaD-rhaB)568hsdR514*	Yale Coli Genetic Stock Center
BW25113Δ*aroMΩkan*^{R}	Defective in shikimate kinase II	Baba et al. (2006)
BW25113Δ*garRΩkan*^{R}	Defective in tartronate semialdehyde reductase	Baba et al. (2006)
BW25113Δ*gatYΩkan*^{R}	Defective in tagatose-1,6-bisphosphate aldolase 2	Baba et al. (2006)
BW25113Δ*gatZΩkan*^{R}	Defective in tagatose-1,6-bisphosphate aldolase 2	Baba et al. (2006)
BW25113Δ*pitAΩkan*^{R}	Defective in tellurite importer; phosphate/arsenate + symporter	Baba et al. (2006)
BW25113Δ*ycgRΩkan*^{R}	Defective in flagellar velocity braking protein, c-di-GMP-regulated	Baba et al. (2006)
BW25113Δ*yfgIΩkan*^{R}	Defective in nalidixic acid resistance protein	Baba et al. (2006)
BW25113Δ*yfhKΩkan*^{R}	Defective in sensor protein kinase regulating glmY sRNA in a two-component system with response regulator GlrR	Baba et al. (2006)
BW25113Δ*yjhUΩkan*^{R}	Defective in putative DNA-binding transcriptional regulator	Baba et al. (2006)
Primers		
Arbitrary primer 1	5′-GGCCAGGCCTGCAGATGATGNNNNNNNN NNGTAT-3′	Ma et al. (2012b)
Arbitrary primer 2	5′-GGCCAGGCCTGCAGATGATG-3′	Ma et al. (2012b)
Internal primer	5′-CTGGCAGAGCATTACGCTGACTTGAC-3′	This study
External primer	5′-ACCTACAACAAAGCTCTCATCAACC-3′	EZ-Tn*5*™ <KAN-2> Tnp Transposome™ Kit, Epicenter

Kan^R kanamycin resistance

Sciences (SPSS) software (IBM, USA) to determine whether there is any difference among the strains regarding hydrogen production.

Quantification of organic acids, glycerol, and ethanol

Prior to the quantifications, the fermentation broth was filtered by a 0.2-μm membrane (Sartorious, Göttingen, Germany) to remove cells. A high-performance liquid chromatograph (8 mm × 300 mm, Shimadzu Co., Tokyo, Japan) using a Shim-Pack SCR-102H column and a CDD-6A electric conductivity detector was used to quantify organic acids; 5 mM *p*-toluenesulfonic acid monohydrate with a flow rate of 0.8 mL/min was used as the mobile phase (Maeda et al. 2009; Tran et al. 2014). To determine the glycerol consumption and yield, glycerol was quantified after 24, 48, 72, and 96 h of fermentation by the Free Glycerol Reagent Kit (Sigma, St. Louis, MO, USA). Ethanol was measured by GC 2025 gas chromatograph using CHP20-M25-025 capillary column (Shimadzu, Tokyo, Japan) (Sanchez-Torres et al. 2013; Tran et al. 2014). Data were collected from and represented as the mean of at least three biological replicate samples in each experiment.

Results

Random mutagenesis, preliminary screening, DNA sequencing, and BLAST analysis

The transposon Tn*5* was successfully introduced into the genome of the strain *E. coli* BW25113. After 16 h of incubation at 37 °C, the numbers of cfu were roughly 3200 cfu/mL. The transformation efficiency was 1.6×10^5 cfu/μg. By applying the Taro Yamane formula with the significance level of 0.1, the population sample needed for the screening was 97. In other words, 97 individual colonies were required for the screening of hydrogen production. In fact, over 100 different colonies were randomly selected for screening of hydrogen production from glycerol. In the preliminary screening of hydrogen production, this study found over 30 single colonies that likely produced higher hydrogen than their parent strain. Besides, we also found some mutant strains whose hydrogen productions were significantly lower than that of strain BW25113 (data not published). Such strains were subjected for identification of the insertion site via arbitrary PCR and DNA sequencing. After two rounds of arbitrary PCR and gel extraction, single amplified DNA fragments containing kanamycin flanking sequence were obtained and ready for the DNA sequencing. Empirically, DNA template samples were successfully sequenced when the size ranges from 700 to 1200 base pairs.

Finally, excluding failed samples of DNA sequencing, low alignment score, and similar insertion sites, this study found nine different insertions by which each of the nine mutant strains produced 1.1–1.6-fold higher hydrogen production than the wild-type strain. These insertion sites were individually determined as *aroM*, *garR*, *gatY*, *gatZ*, *pitA*, *ycgR*, *yfgI*, *yfhK*, and *yjhU* (Table 2).

Confirmation of hydrogen production, glycerol consumption, and cell growth

The nine candidate strains selected from the preliminary screening were attained from the Keio library (Japan) and their hydrogen production ability was confirmed. Due to a small difference between the mutant strains and the wild-type strain in terms of hydrogen production, about 10 independent fermentation batches were conducted for each strain. Prior to the data analysis, to assure the consistency of the data, the outliers were removed. Additionally, the Kolmogorov-Smirnov test was conducted which indicated that the data were normally distributed and ready for parametric data analyses ($p > 0.05$).

A test of ANOVA and Tukey post hoc analysis were conducted to judge whether these differences were statistically significant or not. The statistical analysis showed that the hydrogen productivity of four out of the nine candidate strains, namely *aroM*, *gatZ*, *ycgR*, and *yfgI*, were statistically significantly different from that of the wild-type strain ($p < 0.05$) (Table 2). Although they had higher hydrogen production than the wild-type strain, strains *garR*, *gatY*, *pitA*, *yfhK*, and *yjhU* were not statistically different from BW25113 ($p > 0.05$). In other words, a single disruption in *garR*, *gatY*, *pitA*, *yfhK*, and *yjhU* was not beneficial for hydrogen production in *E. coli* from glycerol.

Among the mutant strains whose hydrogen productivity was significantly different from their parent strain, *aroM* has the highest hydrogen productivity—1.6-fold higher than that of the wild-type strain. Interestingly, all mutant strains grew anaerobically from 1.2- to 1.3-fold faster than their parent strain (Table 2). Hence, the respective disruptions were not only beneficial for hydrogen production, but also for biomass synthesis, which is an advantage for overcoming low cell growth during glycerol metabolism (Gonzalez et al. 2008; Murarka et al. 2008; Poladyan et al. 2013).

The kinetics of hydrogen production shows that strains *aroM*, *gatZ*, *ycgR*, and *yfgI* gradually increased their production over time and almost reached the maximum level by 96 h of fermentation. While strain *aroM* has the highest hydrogen production during the early log phase (after 48 h), strain *gatZ* was likely most active during the middle log phage or after 72 h (Fig. 1).

Under anaerobic conditions, strains *aroM*, *gatZ*, *ycgR*, and *yfgI* grew steadily and likely reached the log phase after 72 h.

Table 2 Hydrogen production of *E. coli* strains under glycerol metabolism after 24 h of anaerobic fermentation

Strains	H_2 productivity[a]		Growth rate (1/h)[b]			
	μmol H_2/mg protein	Relative	Aerobic	Relative	Anaerobic	Relative
BW25113	31±6	1	0.32±0.01	1	0.018	1
aroM	49±8*	1.6	0.24	0.8	0.023±0.001	1.3
garR	38±7	1.2	0.25	0.8	0.014±0.001	1
gatY	35±6	1.1	0.28	0.9	0.015±0.001	1
gatZ	46±7*	1.5	0.41±0.01	1.3	0.024±0.001	1.3
pitA	37±2	1.2	0.23	0.7	0.017	1.1
ycgR	41±8*	1.3	0.28	0.9	0.022	1.2
yfgI	44±9*	1.4	0.22	0.7	0.022±0.001	1.2
yfhK	40±12	1.3	0.37	1.1	0.016±0.001	1.1
yjhU	36±6	1.1	0.2	0.6	0.017±0.001	1.1

[a] Hydrogen fermentation was conducted anaerobically in minimal glycerol pH 7.5 at 37 °C. Calculation of hydrogen productivity and statistical analysis were mentioned in the "Materials and methods" section. The asterisk symbol indicates the statistical significance at the 0.05 level

[b] Data represent the mean and standard deviation of three independent cultures. Growth rate of all strains was measured aerobically and anaerobically under minimal glycerol medium pH 7.5 at 37 °C

Unlike the parent strain, all the mutant strains reached the stationary phase after 72 h of fermentation. Additionally, four mutant strains with higher hydrogen productivity also consumed glycerol in a faster manner compared to the strain BW25113. After 96 h of fermentation, about 60 % of glycerol was consumed (Fig. 2).

Hydrogen yield

All strains whose hydrogen productivities were statistically significantly higher than those of the wild-type strain were analyzed for glycerol consumption. Hence, glycerol quantification was conducted for the four mutant strains: *aroM*, *gatZ*, *ycgR*, and *yfgI* (Table 3). Consistent with previous studies, the hydrogen yield of the wild-type strain was about 0.2 mol H_2 produced per mol of glycerol consumed (Tran et al. 2014). Among the four strains, the *ycgR* mutant had the highest hydrogen yield—0.49 mol H_2/mol glycerol consumed (Table 3). Even though the hydrogen yield of the four mutant strains were far lower than the theoretical maximum of 1 mol H_2 formed per 1 mol glycerol consumed, these results are a considerable improvement in *E. coli* under glycerol fermentation; however, this yields less than for the best engineered strain, BW25113 Δ*frdC ldhA fdnG ppc narG mgsA hycA* which produces 0.67 mol H_2/mol glycerol after 24 h under the same condition (Tran et al. 2014).

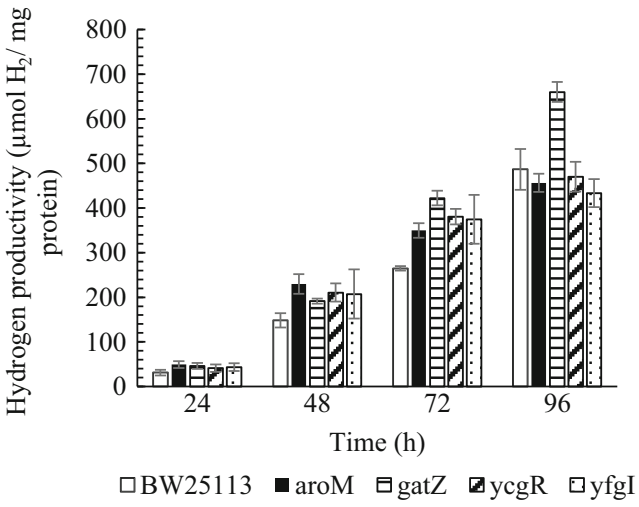

Fig. 1 Kinetics of hydrogen production of strains BW25113, aroM, gatZ, ycgR, and yfgI during 96 h of fermentation. Data represent the mean of three independent cultures; *error bars* represent standard deviation of the mean

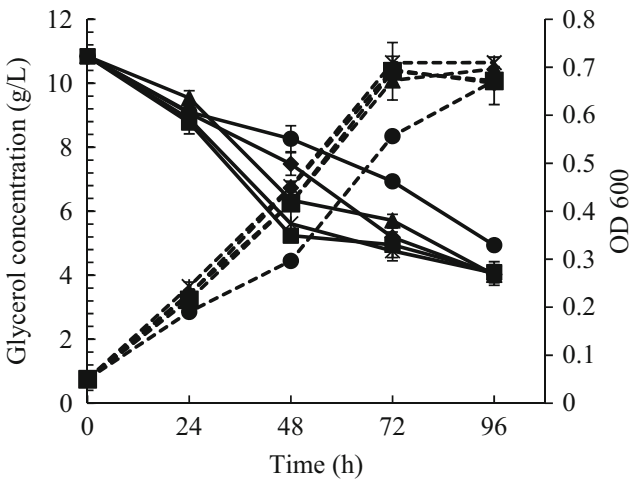

Fig. 2 The kinetics of glycerol consumption (*straight line*) and cell growth (*dashed line*). Strain BW25113 (*round*), aroM (*rectangular*), gatZ (*diamond*), ycgR (*triangle*), and yfgI (*asterisk*). The data present the mean and standard deviation of at least three independent cultures

Organic acids and ethanol production

Together with hydrogen, ethanol, formate, and acetate were the main products during the glycerol metabolism. In fact, formate is the precursor of hydrogen production in that it is converted into hydrogen and carbon dioxide by the formate hydrogen lyase complex system (FHL) and the formate dehydrogenase (encoded by *fdhF*) (Axley et al. 1990; Bagramyan et al. 2002; Enoch and Lester 1975). Results from the organic acid measurements showed that a higher level of formate was detected in the fermentation broth of the mutant strains compared with that of the parent strain (Table 4, Fig. 3a). The results suggest that all four mutant strains, *aroM*, *gatZ*, *ycgR*, and *yfgI*, produced more formate than the wild-type strain during the fermentation.

Similar to previous studies, the wild-type strain produced more ethanol and somewhat slightly higher acetate than the mutant strains (Table 4, Fig. 3b) (Tran et al. 2014). This result is understandable since the intermediate products in the mutant strains were directed toward hydrogen production; thus, a reduced amount was converted into other end products such as ethanol and acetate.

Discussion

Generally, the metabolism of *E. coli* is rather complicated and the metabolites vary with each carbon source. Regarding hydrogen production, some knockouts are beneficial under glucose metabolism but they are not necessarily beneficial for glycerol or other carbon sources (Maeda et al. 2008a; Tran et al. 2014). With persistent effort, the metabolic pathway of *E. coli* for producing hydrogen has been gradually elucidated. Recently, hydrogen production in *E. coli* has been improved by applying metabolic engineering and by optimizing the fermentation conditions (Kim et al. 2006, 2009; Kim and Kim 2011; Maeda et al. 2007; Shams Yazdani and Gonzalez 2008; Tran et al. 2014). For example, in our previous study, we were able to create an engineered *E. coli* strain, BW25113 Δ*frdC ldhA fdnG ppc narG mgsA hycA*, which produced 5-fold higher hydrogen yield than the parent strain, and this strain could reach the theoretical maximum of 1 mol H_2 formed per 1 mol of glycerol consumed after 48 h of fermentation (Tran et al. 2014). However, the hydrogen production of *E. coli* from glycerol is not yet industrially viable due to a slow cell growth and glycerol consumption. Therefore, additional effort to enhance hydrogen production in *E. coli* is necessary to overcome the emerging issues of overproduced glycerol and an increasing demand for renewable and clean energy.

In other aspects, controlling the metabolism of *E. coli* remains a challenge since only half of the genome of this species has been empirically characterized (Riley et al. 2006). Thus, there is potential to find out unknown genes or metabolic routes that are beneficial for hydrogen production. In addition to the well-characterized genes that play a critical role in hydrogen production, the current research aimed to find uncharacterized genes via random transposon mutagenesis.

A higher accumulation of formate together with a higher hydrogen production of the four mutant strains suggests that the deletions in *aroM*, *gatZ*, *ycgR*, and *yfgI* lead to an increase in the conversion of glycerol to formate. Therefore, the mutant strains were able to produce more hydrogen than the wild type. Consistent with previous studies, formate accounted for the highest amount of detected organic acids. It is unknown why formate accumulation occurs in glycerol metabolism but not in glucose metabolism (Sanchez-Torres et al. 2013; Tran et al. 2014; Zhang et al. 2010). Perhaps in glycerol metabolism, formate is highly excreted from the intracellular to the extracellular by the formate transporter (encoded by *focA*). In turn, a high level of formate was detected.

To our knowledge, *aroM* is cotranscribed with *aroL* which is required for the synthesis of shikimate kinase II. In general, the phosphoenol pyruvate is converted into shikimate that is a precursor of aromatic compounds (Ely and Pittard 1979; Herrmann 1995) (Fig. 4). Thus, blocking the shikimate pathway would result in an increase of phosphoenol pyruvate, and logically, more intermediates are available for the synthesis of formate. A higher level of accumulated formate detected in the

Table 3 Hydrogen yields after 24 h of anaerobic fermentation

Strain	Hydrogen yield (mmol H_2/mmol glycerol)	Relative
BW25113	0.20±0.03	1
aroM	0.34±0.09	1.7
gatZ	0.29±0.06	1.4
ycgR	0.5±0.2	2.4
yfgI	0.41	2.1

Data present the mean and the standard deviation of at least three independent cultures

Table 4 Productivity of some end products after 24 h of anaerobic fermentation[a]

Strain	Productivity (μmol product/mg protein)		
	Ethanol	Formate	Acetate
BW25113	443±26	165±2	31.5±0.5
aroM	260±67	195±4	30.5±0.7
gatZ	252±67	194±6	31.9±0.7
ycgR	296±62	201±6	29.6±0.5
yfgI	168±55	185±5	25±2

Data represent the mean and standard deviation of at least three independent cultures

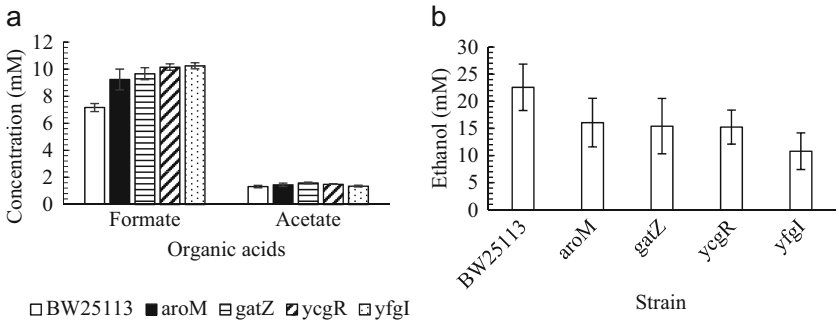

Fig. 3 Production of organic acids (**a**) and ethanol (**b**) after 24 h of fermentation. Data represent the mean of three independent cultures; *error bars* represent 1 standard deviation of the mean

fermentation broth of *aroM* mutant strain is evidence for this argument (Table 4, Fig. 3a). However, so far, the function of *aroM* in the metabolic pathway is not experimentally characterized (DeFeyter and Pittard 1986).

gatYZ encodes for the tagatose-1,6-bisphosphate aldolase, which catalyzes D-tagatofuranose 1,6-bisphosphate into dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate (Brinkkötter et al. 2002; Nobelmann and Lengeler 1995, 1996; Reizer et al. 1996). Blocking the pathway to the synthesis of tagatose-1,6-bisphosphate aldolase 2 (encoded by *gatYZ*) was shown to enhance hydrogen in *E. coli* (Hu and Wood 2010). However, in this study, a disruption in *gatY* did not make any significant difference in hydrogen production. In general, *gatY* requires *gatZ* for full activity and stability of adolases (Brinkkötter et al. 2002). Moreover, due to a considerable higher hydrogen production and growth rate, *gatZ* likely plays a bigger role than *gatY* does in the glycerol metabolism.

YcgR controls flagella motility in enterobacteria (Ko and Park 2000). It is predicted that the interaction between the YcgR-c-di-GMP complex and the flagella motor results in an impairment to the motility. Therefore, inactivation of *ycgR* enhances motility in the *hns*-deficient cell (Girgis et al. 2007; Ko and Park 2000). In other words, the c-di-GMP-binding protein YcgR acts as a backstop brake in enterobacter (Armitage and Berry 2010; Boehm et al. 2010; Paul et al. 2010). In this study, it is not clear why a disruption in *ycgR* led to an increase in hydrogen production. YfgI is an uncharacterized protein and the *yfgI* mutant is sensitive to the nalidixic acid, which is a DNA-damaging agent (Škunca et al. 2013). Hence, our knowledge about the *yfgI*-encoding protein and its role remains insufficient and it should be determined in future studies.

Hydrogen production of *E. coli* can be further improved by various methods such as controlling the pH and atmospheric condition and metabolic and protein engineering (Durnin et al. 2009; Kim et al. 2006; Maeda et al. 2008a, b; Sanchez-Torres et al. 2009; Tran et al. 2014; Trchounian et al. 2011). Undoubtedly, the findings of four new genes in this study could significantly contribute to the enhancement of hydrogen production in *E. coli.*

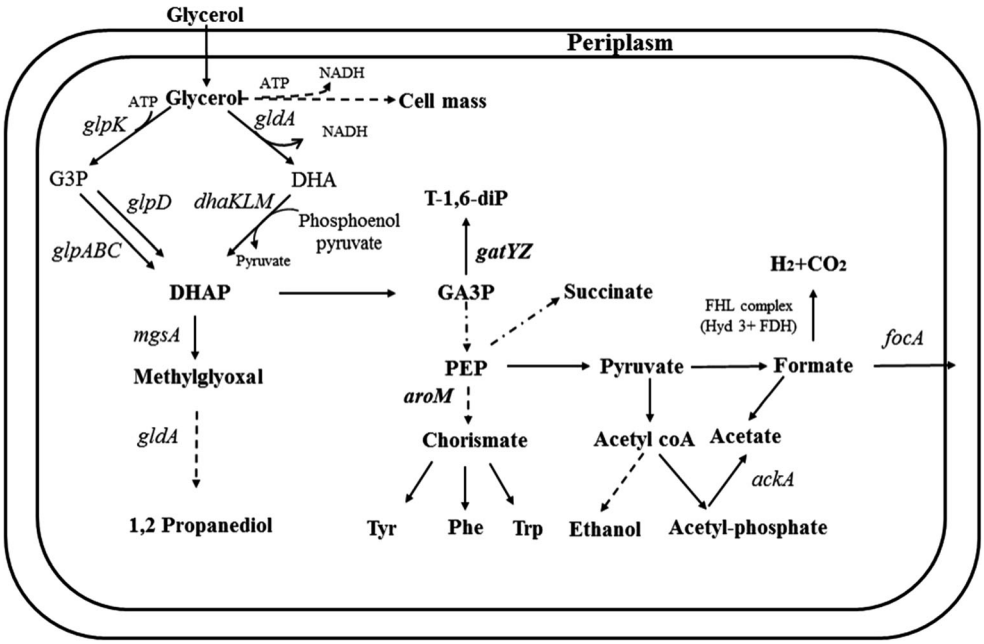

Fig. 4 The metabolic pathway of *E. coli* under glycerol metabolism (Altaras and Cameron 1999; Bongaerts et al. 2001; Chao et al. 1993; Cooper 1984; Gonzalez et al. 2008; Herrmann 1995; Hu and Wood 2010; Saikusa et al. 1987; Tran et al. 2014; Truniger and Boos 1994). *DHA* dihydroxylacetone, *DHAP* dihydroxylacetone phosphate, *G3P* glycerol-3-phosphate, *G3AP* glyceraldehyde-3-phosphate, *PEP* phosphoenol pyruvate, *T-1,6-diP* tagarose-1,6-diphosphate

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Conflict of interest We declare that we do not have any conflict of interest.

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