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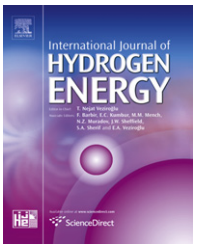

Influence of *Escherichia coli* hydrogenases on hydrogen fermentation from glycerol

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ABSTRACT

Since the actual role of *Escherichia coli* hydrogenases on fermentation from glycerol has not been clear, we evaluated the effect of inactivation of each *E. coli* hydrogenase on cell growth, hydrogen production, organic acids production, and ethanol production. Inactivation of hydrogenase 2 and hydrogenase 3 reduced cell growth, hydrogen and succinate production as well as glycerol utilization while acetate increased. Inactivation of hydrogenase 2 in minimal medium at pH 7.5 impaired hydrogen production, but no significant effect occurred at pH 6.5 or in complex medium. Inactivation of hydrogenase 3 impaired hydrogen production in minimal and rich medium, pH 6.5 and pH 7.5 accumulating formate in all conditions. Therefore during fermentation from glycerol, hydrogenase 3 is the main hydrogenase with hydrogen synthesis activity through the formate hydrogen lyase complex. Hydrogenase 2 seems mainly required for optimum glycerol metabolism rather than hydrogen synthesis. There were no significant impacts by inactivating hydrogenase 1 and hydrogenase 4.

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1. Introduction

Hydrogen is a promising energy carrier, suitable for portable applications where fuel cells can be used to generate electricity [1]. Biological hydrogen production using low price renewable carbon sources, such as glycerol, may be a convenient alternative for large scale hydrogen production as 60% of overall costs for hydrogen production corresponds to feedstock cost [2]. Glycerol prices have being decreasing in recent years due to the increased glycerol production as a byproduct of biodiesel production [3].

Improvements in hydrogen production by engineered *Escherichia coli* strains have been obtained before using several renewable feed stocks including glycerol [4,5]. However, *E. coli* cell growth and hydrogen production rates using glycerol are still low under anaerobic conditions [5]. In order to continue engineering *E. coli* for enhanced hydrogen production from glycerol it would be beneficial to have a comprehensive understanding of the genes required for glycerol utilization and hydrogen production. For instance, we recently reported that two previously uncharacterized proteins are essential for hydrogen production from glucose [6].

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Under anaerobic conditions, *E. coli* converts glycerol to dihydroxyacetone phosphate (DHAP) via the GldA–DhaKLM route, or the GlpK–GlpABC anaerobic respiratory route [7]. DHAP is converted to pyruvate, which is further converted into formate and acetyl-coenzyme-A by pyruvate formate lyase (encoded by *pflB*) [8]. Finally, the formate hydrogen lyase (FHL) complex converts formate into hydrogen and carbon dioxide [9].

E. coli possesses four [Ni–Fe] hydrogenases; which have a small subunit containing iron-sulfur clusters, involved in electron transfer, and a large subunit containing the nickel-iron active site [10]. Generally, hydrogenase 1 (Hyd-1) and hydrogenase 2 (Hyd-2) oxidize H_2 [11]. Hydrogenase 3 (Hyd-3) is a part of the FHL complex, which produces H_2 from formate [9]. Hydrogenase 4 (Hyd-4) seems to be inactive since the genes encoding Hyd-4 are not expressed at significant levels [12].

The role of *E. coli* hydrogenases on anaerobic fermentation from glycerol is not completely understood. At pH 7.5, Hyd-2 and to a lesser extent Hyd-1, were reported as having hydrogen production activity, since single deletion mutants of the genes encoding the corresponding large subunit (*hybC* and *hyaB*) have lower hydrogen production [13]. It is not clear if this reduction in hydrogen production is due to deficient formate production by *pflB*, reduced FHL activity, or inactivation of a new hydrogen-producing pathway that depended on the function of Hyd-1 and Hyd-2 in the presence of glycerol. Even though Hyd-1 is an uptake hydrogenase on fermentation from glucose, hydrogen synthesis activity was detected *in vitro* and *in vivo* by a recombinant *E. coli* BL21 expressing Hyd-1 genes [14]. Moreover, hydrogen synthesis by Hyd-1 and Hyd-2 is opposite to the hydrogen uptake activity [11] observed by these hydrogenases during fermentation from glucose. On the contrary, for fermentation from glycerol at pH 6.5, the *hybC* and *hyaB* mutants have slightly higher hydrogen production [15] indicating the usual hydrogen uptake activity. Also, the impact of deleting the large subunit of Hyd-3 (*hycE*) and the large subunit of Hyd-4 (*hyfG*) for hydrogen production from glycerol at pH 6.5 and pH 7.5 has not been evaluated. Therefore, the purpose of this study was to determine the role of each *E. coli* hydrogenase on fermentative hydrogen production, cell growth, organic acids production, and ethanol production using glycerol as a main carbon source at pH 6.5 and 7.5.

2. Materials and methods

2.1. Bacterial strains and cell growth

The *E. coli* strains used in this study are listed in Table 1. Single deletion mutants of the parental strain *E. coli* K-12 BW25113 were obtained from the Keio collection [16]. The double deletion strain BW25113 *hybC hycE* was constructed via P1 transduction [17] and verified via polymerase chain reaction (PCR) using primers listed in Table 1.

Experiments were conducted at 37 °C in minimal medium [18] supplemented with 10 g L^{-1} glycerol, 0.172 mg L^{-1} $NiCl_2$,

Table 1 – *E. coli* strains and primers used in this study.

Strains and plasmids	Genotype/relevant characteristics[a]	Source
Strains		
BW25113	F⁻ Δ(*araD-araB*)*567* Δ*lacZ4787*(::rrnB-3) λ⁻ *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*; parental strain for the Keio collection.	Yale Coli Genetic Stock Center
BW25113 *hyaB*	BW25113 Δ*hyaB787*::*kan* Km^R	[16]
BW25113 *hybC*	BW25113 Δ*hybC767*::*kan* Km^R	[16]
BW25113 *hycE*	BW25113 Δ*hycE725*::*kan* Km^R	[16]
BW25113 *hyfG*	BW25113 Δ*hyfG735*::*kan* Km^R	[16]
BW25113 *pflB*	BW25113 Δ*pflB727*::*kan* Km^R	[16]
BW25113 *fhlA*	BW25113 Δ*fhlA735*::*kan* Km^R	[16]
BW25113 *fdhF*	BW25113 Δ*fdhF774*::*kan* Km^R	[16]
BW25113 *gldA*	BW25113 Δ*gldA732*::*kan* Km^R	[16]
BW25113 *glpA*	BW25113 Δ*glpA721*::*kan* Km^R	[16]
BW25113 *hybC hycE*	BW25113 Δ*hybC867hycE725*::*kan* Km^R	This study
Primers for verification of strains		
hybC-confirm-f	5′-TCCCGACCTGGGAAGAACTG-3′	This study
hybC-confirm-r	5′-GTGCCGCCATCGAGGATCTC-3′	This study
hycE-confirm-f	5′-GTGGTCGGCGTCCTGGTTATCG-3′	This study
hycE-confirm-r	5′-CTGCTCTGGCTTACCACGGAAG-3′	This study
k1	5′-CAGTCATAGCCGAATAGCCT-3′	[28]
k2	5′-CGGTGCCCTGAATGAACTGC-3′	[28]
kanrev	5′-ATCACGGGTAGCCAACGCTATGTC-3′	[29]
Primers for qRT-PCR		
rrsG-f	5′-TATTGCACAATGGGCGCAAG-3′	[30]
rrsG-r	5′-ACTTAACAAACCGCCTGCGT-3′	[30]
RT-*gldA*-for	5′-AAATGAGATCGACCGTCTGC-3′	This study
RT-*gldA*-rev	5′-CTCACCCTCATCGGTGTAGATAAC-3′	This study
RT-*dhaK*-for	5′-GCCACTCAATAGGTATCGCTCT-3′	This study
RT-*dhaK*-rev	5′-GGTATGAGCCATTTACCAGCAG-3′	This study

a Km^R is kanamycin resistance.

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and 0.02 mg L^{-1} Na_2SeO_3; or in rich glycerol medium, which has the same composition as minimal glycerol medium, but supplemented with 5 g L^{-1} of tryptone and 5 g L^{-1}yeast extract. Kanamycin (final concentration 50 μg mL^{-1}) was used for streaking the deletion mutants on agar plates. Cell growth was monitored by spectrophotometric measurement of the optical density at 600 nm (OD_{600}) using at least two independent cultures for each strain. Aerobic growth was measured in crimp-top vials with a loosened cap and anaerobic growth was measured in sealed crimp-top vials sparged with nitrogen.

2.2. Hydrogen assay

Overnight aerobic cultures (50 mL), and fresh medium (pH 6.5 or pH 7.5) were sparged for 5 min with nitrogen to remove oxygen. Inside an anaerobic glove box, 9 mL of sparged fresh medium and 1 mL of overnight culture were added to sealed crimp-top vials sparged with nitrogen gas (volumetric capacity 34 mL). The vials were incubated at 37 °C, 120 rpm, for 48 h. At least two independent cultures of each strain were assayed. The amount of hydrogen generated in the headspace was measured by gas chromatography using a 6890 gas chromatograph equipped with an AT 19095P-NS5 column (Agilent Technologies Inc., Santa Clara, CA) as described previously [19]. Total protein was calculated as 0.22 mg mL^{-1} OD_{600}^{-1} [20].

2.3. Quantification of organic acid, ethanol, and glycerol

Anaerobic cultures of BW25113 and the hydrogenase mutants were prepared as the above hydrogen assay, but inoculating 100 μL of overnight cultures into 20 mL of medium in 64 mL crimp-top vials. Organic acids (acetate, lactate, succinate, and formate) were quantified by high performance liquid chromatography using a Shim-Pack SCR-102H column (Shimadzu Corp., Kyoto, Japan) and a CDD-6A detector (Shimadzu Co., Tokyo, Japan). The mobile phase was 5 mM p-toluenesulfonic acid monohydrate at 0.8 mL min^{-1} and the column temperature was 40 °C [21]. Ethanol was measured by gas chromatography using a GC-2025 gas chromatograph equipped with a CBP20-M25-025 capillary column and a flame ionization detector (Shimadzu Corp., Kyoto, Japan). The oven and detector were maintained at 250 °C and the column at 50 °C. N_2 was used as carrier gas at 1.94 mL min^{-1}. Glycerol concentrations were quantified using the Free Glycerol Determination Kit (Sigma, San Louis, MO).

2.4. Quantitative real-time reverse transcription PCR (qRT-PCR)

Anaerobic cultures of BW25113, and the *hybC* and *hycE* mutants were prepared in minimal glycerol medium at pH 7.5 under the same conditions used in the above hydrogen assay. Cell pellets for RNA extraction were obtained after 48 h of incubation, using RNALater (Applied Biosystems Foster City, CA). Total RNA was extracted using a bead beater model 301 1b (Wakenyaku Co. Ltd., Japan) and the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA). For qRT-PCR, the housekeeping gene *rrsG* (16S rRNA) was used to normalize the expression data. The expression of *gldA* and *dhaK* was analyzed using 100 ng of total RNA, the primers listed in Table 1, the Power SYBR green RNA-to-CT 1-Step Kit (Applied Biosystems, Foster City, CA), and the StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA). At least two biological replicates with three technical replicates were evaluated.

3. Results and discussion

3.1. Effect of inactivation of hydrogenases on cell growth

Each of the four *E. coli* hydrogenases was inactivated by deleting the gene encoding the corresponding large subunit: *hyaB* for Hyd-1, *hybC* for Hyd-2, *hycE* for Hyd-3, and *hyfG* for Hyd-4 [10]. To evaluate the importance of each hydrogenase for cell fitness on glycerol as a substrate, cell growth was monitored in minimal glycerol medium at pH 6.5 and pH 7.5 under aerobic and anaerobic conditions. Under aerobic conditions, the specific growth rate was similar for the BW25113 parental strain and the single hydrogenase mutants. At pH 6.5, the average aerobic specific growth rate for all the strains tested was 0.51 ± 0.03 h^{-1} and at pH 7.5 the average specific growth rate was 0.70 ± 0.04 h^{-1}, respectively. Under anaerobic conditions, the specific growth rates were at least 10-fold lower. For BW25113, the anaerobic specific growth rate was 0.05 ± 0.01 h^{-1} at both pH 6.5 and pH 7.5; this result agrees with the specific growth rate value reported previously [18]. Note that only slight cell growth was observed in minimal medium without glycerol; hence, the growth of strains tested relies on glycerol present in the medium.

Deletion of *hyaB* or *hyfG* did not affect cell growth under anaerobic conditions in minimal glycerol medium. In contrast, the OD_{600} after 120 h of the *hybC* and *hycE* cultures at pH 6.5 was roughly 2-fold lower than BW25113 (Fig. 1a). At pH 7.5, the *hycE* culture had 50% higher OD_{600} than at pH 6.5, while the *hybC* culture had similar growth to that at pH 6.5 (Fig. 1a and b). The *hybC hycE* double mutant had lower growth than each *hybC* and *hycE* culture, and 3-fold lower growth than BW25113 after 120 h (Fig. 1a and b).

In rich glycerol medium at pH 7.5 (Fig. 1c), BW25113 and the hydrogenase mutants had exponential growth during the first 6 h, with an average growth rate of 0.26 ± 0.04 h^{-1}. After this period, the growth curves followed a similar pattern to minimal glycerol medium (Fig. 1b) with the *hybC*, *hycE*, and *hybC hycE* mutants reaching stationary phase at a lower OD_{600} than BW25113 and the *hyaB* and *hyfG* mutants. Therefore, in order to have optimum growth using glycerol as a sole carbon source, Hyd-2 and Hyd-3 activity are required. Hyd-3 activity via FHL may be required to supply the CO_2 necessary for cell growth [22].

3.2. Effect of inactivation of hydrogenases on hydrogen production

Hydrogen production for BW25113 and the hydrogenase mutants was evaluated to reveal the impact of each hydrogenase on hydrogen production from glycerol in minimal and rich media (Fig. 2a). In general, hydrogen productivity was lower in rich glycerol medium than in minimal glycerol medium probably because cell growth was about 4-fold higher in rich glycerol medium than in minimal glycerol

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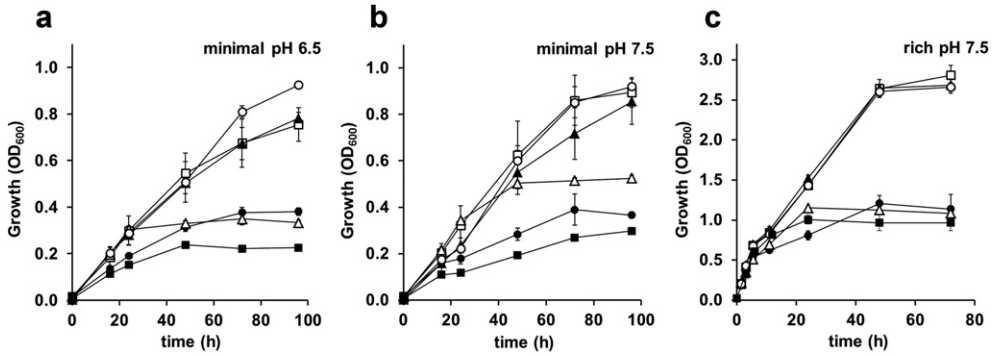

Fig. 1 – Anaerobic growth curves. (a) Minimal glycerol medium at pH 6.5. (b) Minimal glycerol medium at pH 7.5. (c) Rich glycerol medium at pH 7.5. BW25113 (open square), *hyaB* (full triangle), *hybC* (full circle), *hycE* (open triangle), *hyfG* (open circle), *hybC hycE* (full square).

medium (Fig. 1). Hyd-1 and Hyd-4 do not seem to participate in hydrogen production from glycerol since the deletion of *hyaB* or *hyfG* did not significantly alter hydrogen production. In minimal glycerol medium, hydrogen production by the *hybC* mutant decreased 1.4-fold at pH 6.5 and 16-fold at pH 7.5 after 48 h relative to BW25113. However, there was no significant difference in rich glycerol medium (Fig. 2a). Since hydrogen production at pH 7.5 by the *hybC* mutant was recovered in rich medium, Hyd-2 may be important for glycerol metabolism in minimal glycerol medium at slight basic conditions by contributing to glycerol consumption pathways and cell fitness.

The *hycE* mutant had reduced hydrogen production for all conditions tested, minimal or rich glycerol medium, and pH 6.5 or pH 7.5 (Fig. 2a). These results indicate that Hyd-3 contributes to the synthesis of hydrogen under anaerobic fermentation from glycerol. Hydrogen was not detected in minimal glycerol medium (pH 6.5 or pH 7.5) for the double mutant *hybC hycE* (Fig. 2a) suggesting that Hyd-2 and Hyd-3 may work independently. It is not clear if in minimal medium at pH 7.5 Hyd-2 has hydrogen synthesis activity, or indirectly contributes to hydrogen production.

To verify that hydrogen was produced from the consumption of glycerol, hydrogen gas was measured for BW25113 and the single hydrogenase mutants in minimal medium without glycerol at pH 6.5 and pH 7.5. At pH 6.5, the hydrogen production after 48 h for BW25113 and the *hyaB*, *hybC*, and *hyfG* mutants had an average value of 3.2 ± 0.2 μmol mg protein^{-1}, about 27-fold lower than that with glycerol. For the *hycE* mutant, hydrogen production was negligible in minimal medium without glycerol at pH 6.5. Furthermore, none of the strains tested produced hydrogen from minimal medium without glycerol at pH 7.5. Hydrogen produced in minimal medium without glycerol at pH 6.5 should proceed from fermentation of tryptone (2 g L^{-1}). Similarly, hydrogen production was evaluated for BW25113 and the single hydrogenase mutants in rich medium without glycerol at pH 6.5. BW25113 and the *hyaB*, *hybC*, and *hyfG* mutants had similar hydrogen production (15 ± 2 μmol mg protein^{-1}) in rich medium without glycerol; this is about 3-fold lower than the hydrogen production in rich medium with glycerol. For the *hycE* mutant, hydrogen production was not detected in rich medium without glycerol at pH 6.5. Hence, these results indicated that most of the hydrogen produced in minimal and rich glycerol media comes from the consumption of glycerol.

3.3. *Effect of inactivation of FHL, GldA, and GlpA on hydrogen production*

Under fermentative conditions, pyruvate is converted into formate by pyruvate formate lyase (PFL) [23]; subsequently, formate is converted to hydrogen and carbon dioxide by FHL [9]. Hydrogen production in minimal glycerol medium was evaluated for single deletion mutants defective in *pflB* (encoding PFL) [23], *fhlA* (encoding the transcriptional activator of genes encoding FHL and hydrogenase maturation proteins) [24], and *fdhF* (encoding formate dehydrogenase H which is part of FHL [25]); in order to evaluate the importance of formate availability and FHL activity for hydrogen production from glycerol at pH 6.5 and pH 7.5. Fig. 2b indicates that at pH 6.5, deletions of *pflB*, *fhlA*, and *fdhF* reduced hydrogen production similarly to the *hycE* mutant (Fig. 2a), suggesting that hydrogen gas generated from glycerol is produced mainly through the PFL pathway and the FHL pathway as well as that from glucose. However, the metabolic route for the small amount of hydrogen produced by the *pflB*, *fhlA*, *fdhF*, and *hycE* mutants still remains unknown.

Hydrogen production was also evaluated for mutations perturbing the anaerobic glycerol dissimilation pathways: deletion of *gldA* affect the fermentative route, and deletion of *glpA* affects the anaerobic respiratory route [7]. Deletion of both *gldA* and *glpA* impaired hydrogen production at both pH 6.5 and pH 7.5 indicating that both metabolic pathways are required for hydrogen production from glycerol under the conditions tested.

3.4. *Effect of inactivation of hydrogenases on glycerol consumption*

The glycerol concentration for anaerobic cultures of BW25113 and the hydrogenase mutants was monitored during 72 h to determine if each hydrogenase activity of *E. coli* is related to glycerol metabolism. In minimal glycerol medium at pH 6.5

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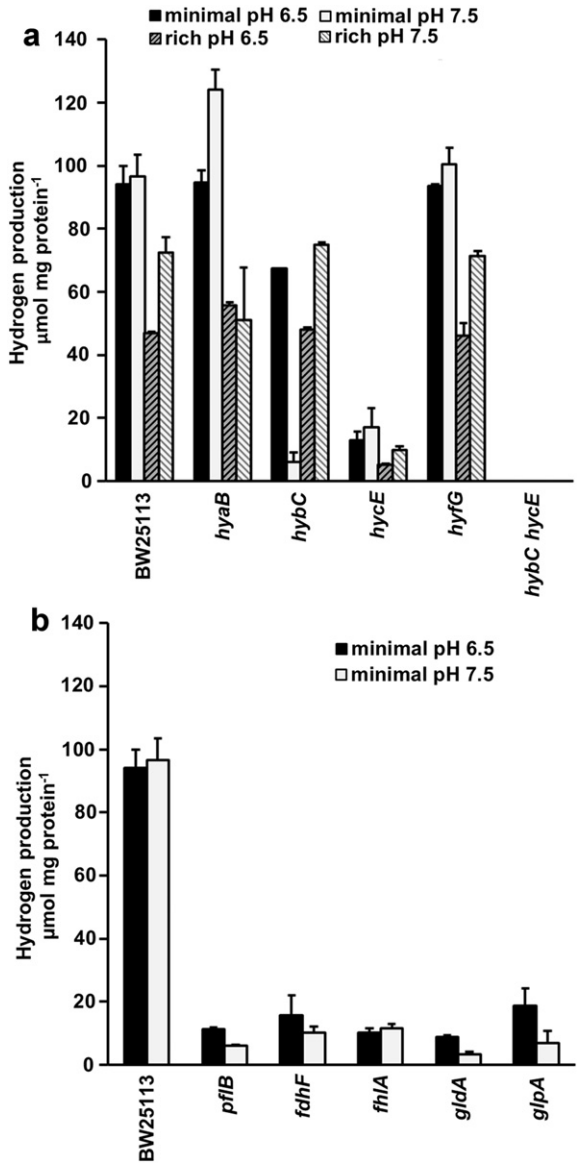

Fig. 2 – Hydrogen production from glycerol after 48 h. (a) BW25113 and hydrogenase mutants in minimal and rich glycerol medium. (b) BW25113, *pflB*, *fdhF*, *fhlA*, *gldA*, and *glpA* in minimal glycerol medium.

(Fig. 3a) and pH 7.5 (Fig. 3b), glycerol consumption curves followed a similar trend for BW25113, and the *hyaB* and *hyfG* mutants. After 72 h, BW25113 had the highest glycerol consumption (60%). In rich medium at pH 7.5, BW25113, and the *hyaB* and *hyfG* mutants had lower glycerol consumption than in minimal medium probably due to the preferential utilization of another carbon sources such as tryptone and yeast in the rich medium (Fig. 3c). However, after 48 h glycerol consumption was higher in rich medium than minimal medium reaching to 80% consumption after 72 h (Fig. 3c).

The *hybC* mutant had 2-fold lower glycerol consumption than BW25113 in minimal medium at pH 6.5 (Fig. 3a) and in rich medium at pH 7.5 (Fig. 3c), while *hybC* glycerol consumption was 4-fold lower than BW25113 in minimal medium at pH 7.5 (Fig. 3b). These results suggest that glycerol consumption and hydrogen production in minimal glycerol medium are directly correlated with the data of *hybC* mutant, which had 2-fold lower hydrogen production than BW25113 at pH 6.5 and almost negligible at pH 7.5 (Fig. 2a). On the other hand, the *hycE* mutant had roughly 3-fold lower and the *hybC hycE* mutant 5-fold lower glycerol consumption than BW25113 in minimal and rich medium (Fig. 3). The *hybC*, *hycE*, and the *hybC hycE* mutants had reduced glycerol consumption and also reduced cell growth (Fig. 1), thus suggesting that glycerol utilization is directly related to biomass production. Previously, it was proved that during glycerol fermentation about 20% of the carbon incorporated into proteins comes from glycerol [18].

3.5. *Effect of inactivation of Hyd-2 and Hyd-3 on* gldA *and* dhaK *expression*

A dynamic model of the kinetics of fermentation from glycerol, predicted that the main steps controlling the glycolytic flux are the reactions catalyzed by the glycerol dehydrogenase (encoded by *gldA*) and the dihydroxyacetone kinase (encoded by *dhaKLM*) [26]. Therefore, we performed qRT-PCR to determine whether the reduced glycerol consumption by the *hybC* and the *hycE* mutants (Fig. 3) is due to a lower expression of *gldA* and *dhaK*. For 48 h anaerobic cultures in minimal glycerol pH 7.5, the *hybC* mutant had 1.8-fold lower expression of *gldA* and 2.7-fold lower expression of *dhaK* compared to BW25113. However, for the *hycE* mutant the expression of *gldA* and *dhaK* was similar to BW25113. These results support the hypothesis that Hyd-2 is mainly required for optimum glycerol metabolism, while the role of Hyd-3 on anaerobic fermentation from glycerol is mainly due to the role as FHL function.

3.6. *Organic acids and ethanol produced during fermentation from glycerol*

Organic acids (acetate, lactate, succinate, and formate) and ethanol were quantified after 48 h for BW25113 and the hydrogenase mutants cultured in minimal glycerol medium pH 6.5 and pH 7.5. We sought to determine how the carbon flux was affected by the deleted genes. All the strains had similar production of acetate, with an average of 1.2 ± 0.2 mM at pH 6.5 and 1.8 ± 0.5 mM at pH 7.5. However, if we consider the productivity of organic acids based on the total amount of protein (Table 2), acetate production increased 1.5-fold for the *hybC* mutant, and about 2-fold for the *hycE* and *hybC hycE* mutants.

Lactate was not detected for any of the strains as a product of anaerobic fermentation from glycerol. Succinate production decreased 4-fold at pH 6.5 and 5-fold at pH 7.5 for the *hybC* mutant, 3-fold at pH 6.5 and 2-fold at pH 7.5 for the *hycE* mutant, and 4-fold at pH 6.5 and 5-fold at pH 7.5 for the *hybC hycE* mutant (Fig. 4a).

Formate production was higher at pH 7.5 than at pH 6.5 for all the strains except the *hybC hycE* mutant, in the case of BW25113 formate production at pH 7.5 was 10-fold higher than at pH 6.5 (Fig. 4b). As expected, inactivation of FHL via *hycE* deletion increased formate concentration 10-fold at pH 6.5 and 3-fold at pH 7.5 relative to BW25113 (Fig. 4b, Table 2) due to the inability to convert formate into hydrogen and carbon dioxide. Deletion of *hybC* did not affect formate accumulation at pH 6.5

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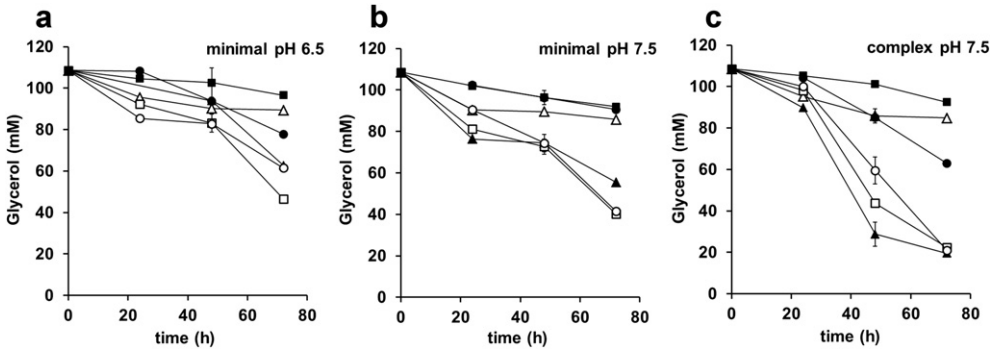

Fig. 3 – Glycerol consumption curves. (a) Minimal glycerol medium at pH 6.5. (b) Minimal glycerol medium at pH 7.5. (c) Rich glycerol medium at pH 7.5. BW25113 (open square), *hyaB* (full triangle), *hybC* (full circle), *hycE* (open triangle), *hyfG* (open circle), *hybC hycE* (full square).

and at pH 7.5 the normalized formate production increased 1.7-fold (Fig. 4b, Table 2). Moreover, formate accumulation for the *hybC hycE* mutant was lower than the *hycE* mutant (Fig. 4b, Table 2). These results indicate that although inactivation of Hyd-2 decreases hydrogen production (Fig. 2a) from glycerol, especially at pH 7.5, Hyd-2 does not possess a major hydrogen-producing activity from formate.

Ethanol was the primary product of fermentation from glycerol which agrees with a previous study [22]. At pH 6.5, ethanol production decreased 2-fold for the *hybC* and *hycE* mutants, and 3-fold for the *hybC hycE* mutant. At pH 7.5, the *hybC* and *hybC hycE* mutants decreased ethanol production about 4-fold; however, deletion of *hycE* did not affect ethanol production (Fig. 4c). However, considering the ethanol production normalized by the total protein (Table 2), there were not significant differences between the hydrogenase mutants and BW25113. Similarly to cell growth, hydrogen production, and glycerol consumption, the organic acids and ethanol concentrations for the *hyaB* and *hyfG* mutants were close to BW25113 (Fig. 4); thus, further demonstrating that Hyd-1 and Hyd-4 are not key hydrogenases for fermentation from glycerol.

3.7. *Products of fermentation from glycerol and redox balance*

Glycerol utilization in the absence of external electron acceptors is favored by the production of metabolites synthesized through a redox balanced pathway [7]. Conversion of glycerol to succinate [27], glycerol to ethanol and formate, and glycerol to ethanol, H_2, and CO_2 occurs through redox balanced pathways, where NADH is reduced back to NAD^+ [5]. When 1 mol of glycerol is converted to pyruvate 1 mol ATP is produced, but 2 mol of reducing equivalents in the form of NADH are also produced. If pyruvate is converted to ethanol the 2 mol of NADH are regenerated to NAD^+. If pyruvate is converted to acetate 1 additional mol of ATP is produced, but NADH is not regenerated. If pyruvate is converted to lactate only 1 mol of NADH is regenerated [18]. Thus, the conversion of glycerol to ethanol has the advantage that in a redox balanced pathway 1 net ATP is produced [18]. Since the *hybC*, *hycE*, and *hybC hycE* mutants had increased acetate production (Table 2), and reduced succinate (Table 2) and hydrogen production (Fig. 2a); these strains may have a $NADH/NAD^+$ ratio different than BW25113 stalling anaerobic growth and glycerol consumption.

The degree of reduction, k, is a measure of the number of available electrons per carbon. Glycerol has $k = 4.67$ and biomass has an average $k = 4.3$; therefore, the synthesis of cell mass from glycerol generates reducing equivalents [1]. In order to maintain redox balance, a fermentation product more reduced than glycerol must be produced [7]. Even though ethanol has a degree of reduction higher than glycerol ($k = 6$), its production occurs through a redox balanced pathway where formate, or H_2 and CO_2 are coproduced. Previously, it was reported that *E. coli* is able to produce 1,2-propanediol ($k = 5.33$) from fermentation from glycerol, this pathway allows the consumption of the excess reducing equivalents [18].

Table 2 – Productivity of organic acids and ethanol normalized by total protein.

Strain	Acetate[a]		Succinate[a]		Formate[a]		Ethanol[a]	
	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5	pH 6.5	pH 7.5
BW25113	10 ± 1	15 ± 1	10.8 ± 0.3	11 ± 2	21 ± 1	135.2 ± 0.4	257 ± 35	306 ± 26
hyaB	11.0 ± 0.4	13.8 ± 0.2	10.7 ± 0.3	8.0 ± 0.9	7 ± 1	150 ± 5	185 ± 17	238 ± 67
hybC	16 ± 2	22.6 ± 0.3	5.2 ± 0.4	4.6 ± 0.2	20 ± 2	236 ± 21	208 ± 56	190 ± 8
hycE	22 ± 2	24 ± 0	6 ± 1	5.8 ± 0.1	241 ± 1	422 ± 5	189 ± 39	331 ± 42
hyfG	11 ± 1	13.78 ± 0.03	12.7 ± 0.9	7.7 ± 0.1	25 ± 5	185 ± 9	285 ± 4	288 ± 7
hybC hycE	18.2 ± 0.2	31.79 ± 0.04	5.0 ± 0.2	7.5 ± 0.1	207 ± 3	286 ± 34	198 ± 3	235 ± 35

a Fermentation products were measured as described in the Materials and methods section, values (μmol mg cell protein^{-1}) based on 20 mL fermentation volume are reported as the average ± standard deviation.

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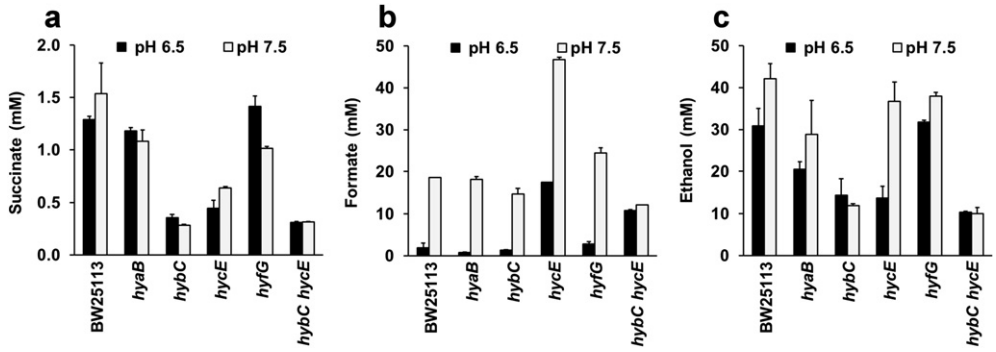

Fig. 4 – Fermentation products after 48 h in minimal glycerol medium. (a) Succinate. (b) Formate. (c) Ethanol.

4. Conclusions

Hyd-1 and Hyd-4 are not the main hydrogenase enzymes for hydrogen production in minimal glycerol medium since deletion of the corresponding large subunits did not affect cell growth, hydrogen production, glycerol consumption, organic acid production, or ethanol production. In contrast, Hyd-2 and Hyd-3 activity are required for optimum glycerol metabolism. Deletions of *hybC* and *hycE* reduced cell fitness, glycerol consumption, and succinate production; while it increased acetate production at pH 6.5 and pH 7.5. Inactivation of Hyd-3 impaired hydrogen production and increased formate production at both pH 6.5 and pH 7.5. Inactivation of Hyd-2 impaired hydrogen production at pH 7.5, but it has no effect on hydrogen at pH 6.5. Therefore, during anaerobic fermentation from glycerol, Hyd-3 is the main hydrogenase responsible for hydrogen production via FHL. Hyd-2 had a major influence on anaerobic fermentation from glycerol in minimal glycerol medium at pH 7.5, and it affects mainly glycerol utilization rather that hydrogen synthesis activity. Since the *hybC* and *hycE* mutants had some hydrogen synthesis activity, but hydrogen production was not detected for the *hybC hycE* mutant, Hyd-2 may have some hydrogen synthesis activity, or influence hydrogen production in an indirect way. The distribution of the fermentation products, the glycerol consumption, and the evaluation of cell growth, suggest that Hyd-2 and Hyd-3 activity seems to be required for optimal utilization of glycerol and the maintenance of redox balance.

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