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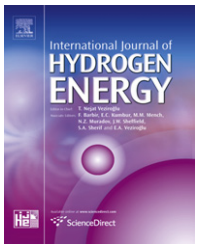

Escherichia coli hydrogenase activity and H_2 production under glycerol fermentation at a low pH

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ABSTRACT

Hydrogenase (Hyd) activity and H_2 production by *Escherichia coli* were studied at a low pH. H_2 production at pH 5.5 under glycerol fermentation was shown to be ~1.5-fold higher than that at pH 6.5 or above but less than that under glucose fermentation. It was inhibited by *N,N′*-dicyclohexylcarbodiimide: H_2 production inhibition was increased with decreasing pH and almost maximal inhibition was observed at pH 5.5. The data on H_2 production by single and double mutants with defects in different Hyd-enzymes and in *fhlA* gene suggest that under glycerol fermentation at a low pH, Hyd-1, Hyd-2 and Hyd-4 were operating in a reversed, non-H_2 producing mode. Moreover, a role of *fhlA* gene in Hyd-3 and Hyd-4 activity in H_2 production is proposed under glucose fermentation at a low pH.

1. Introduction

Molecular hydrogen (H_2) is one of the fermentation end products of *Escherichia coli* and other bacteria that can be widely used in rather different branches of industry and human life [1].

Dharmadi et al. [2] have an accelerated publication that glycerol like sugars (glucose) can be fermented by *E. coli* to produce H_2 at acidic pH. This might be novel way for a cheap source for H_2 production. Moreover, glycerol metabolism pathways leading to H_2 formation by bacteria are further suggested [3,4] but have not been established clearly.

E. coli possesses four membrane-bound hydrogenases which are catalyzing reversible oxidation of H_2 to $2H^+$: $H_2 \leftrightarrow 2H^+ + 2e^-$ [5,6]. Hydrogenase 1 (Hyd-1) and 2 (Hyd-2) are H_2 uptake enzymes during glucose fermentation [7,8] and operate in a reverse mode during glycerol fermentation [9]. Hydrogenase 3 (Hyd-3) and 4 (Hyd-4) are H_2 producing enzymes during glucose fermentation [10–12] and function in a reverse mode during glycerol fermentation [9]. Each hydrogenase is likely to function primarily in one direction depending on fermentation substrate, pH and other conditions.

Hyd-1 is encoded by the *hya* operon, expression of which is induced under anaerobic conditions at acidic pH [13] and by the presence of formate but not nitrate [14]. However, *E. coli* does not require Hyd-1 for anaerobic growth [13]. Hyd-2 is encoded by the *hyb* operon [14,15] and its maximal expression is attained in alkaline medium [13]. The *hybA*-encoded protein might be involved in the periplasmic electron-transferring activity of Hyd-2 during catalytic turnover. Moreover, Hyd-2 activity was observed in more reducing environment [16] and absent under aerobic conditions [17]. This has direct relevance for Hyd-1 and Hyd-2 role in bacterial physiology.

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Hyd-3 and Hyd-4 encoded by the *hyc* and *hyf* operons, respectively [10–12], have similarities with each other. Hyd-3 with formate dehydrogenase H (Fdh-H), the component of the formate hydrogen lyase (FHL-1) complex, produces H_2 mostly at acidic pH [10,18]. For pH ~7.5, Hyd-4 with Fdh-H forming the FHL-2 pathway becomes responsible for H_2 production; expression of the *hyf* operon has been shown [18]. Hyd-4 having several subunits more than Hyd-3 might also include proton-translocating pathway [12], but nothing is known about such activity. The latter is also suggested with Hyd-3 [19]. Furthermore, the FHL pathways both are determined by the *hycB* gene, coding the small subunit of Hyd-3 [18], and by the *fhlA* gene, coding transcriptional activator for *hyc* [20,21] and *hyf* [22] operons. Although different ways and factors are studied, FHL activity and especially its regulation are not clear.

Interestingly, the activity of both Hyd-3 and Hyd-4 is related with the F_0F_1-ATPase [18,19,23]. This relationship at pH ~7.5 might result from Hyd-4 interaction with F_0F_1 to supply reducing equivalents ($H^+ + e^-$) for energy transfer to the secondary transport system [18,24,25]. However, the detailed mechanisms have not been determined yet; a role of H^+ gradient generated by F_0F_1 is not ruled out.

As mentioned, activity of different Hyd-enzymes is dependent on pH [2,7,18,26]. Hyd-1 activity is less than that of Hyd-2; pH optimum for Hyd-1 ranges from 6 to 8 whereas maximal activity of Hyd-2 is observed at pH 8 [7]. Gonzalez et al. [4] have shown that FHL plays a role in H_2 production under glycerol fermentation at a low pH. However, hydrogenase responsible for H_2 production as well as interaction between different Hyd-enzymes and various factors under these conditions are not established yet.

In this manuscript, *E. coli* hydrogenase activity and H_2 production during glycerol fermentation at low pH ($pH = 6.5$; $pH < 6.5$) has been studied. To establish which hydrogenase is responsible for H_2 production under the latest conditions, different mutants were constructed. In addition, to understand mechanisms of enzyme activity and regulation as well as relationship with H^+ transport, the inhibitor's effects were determined.

2. Materials and methods

2.1. Bacterial strains and their construction

The *E. coli* wild type and mutant strains used in this study are listed in Table 1.

To construct the *E. coli* BW25113 *fhlA hyfG* and *E. coli* BW25113 *fhlA hycG* double mutants, the kanamycin (Kan) resistance gene was first removed from JW2472 (*hyfG*) and JW2689 (*hycG*) by expressing the FLP recombinase from plasmid pCP20 [29] as described previously [30].

The deletion of the Kan marker from JW2472 and JW2689 was verified via polymerase chain reaction (PCR) using different primers (*hyfG* front: 5′-GCATATTCCACAACCTGTCATCAGG-3′, *hyfG* rev: 5′- CAGGCAATACATTGGCTGGGCATC-3′, *hycG* front: 5′-CCAGAAAGAGATCGACTACGCCATTG-3′, and *hycG* rev: 5′-AGGCTGTAATAGACCACCTGCTGC -3′) flanking the correspondent target locus. The *fhlA* locus was then inactivated in both strains by inserting the Kan resistance marker to the Kan-sensitive strains JWD2472 and JWD2689 via P1 transduction [31]. The constructed strains were verified via PCR using the upstream primer *fhlA* up: 5′- GCTGATTGGTGAAGTGGTGGAACG -3′ and Kan rev: 5′- ATCACGGGTAGCCAACGCTATGTC -3′ which is inside the coding region of the Kan resistance marker.

2.2. Bacterial growth and preparation of bacteria

Bacteria were grown under anaerobic conditions in peptone medium (20 g/l peptone, 15 g/l K_2HPO_4, 1.08 g/l KH_2PO_4, 10 g/l NaCl) with glycerol (10 ml/l) or glucose (2 g/l) at different pH mentioned in the text at 37 °C for 20–22 h [9,18]. Overnight growth medium was supplemented with kanamycin (25 μl/ml) for some mutants where appropriate (see Table 1). The medium pH was measured by a pH-meter with selective pH-electrode (HJ1131B, Hanna Instruments, Portugal) and adjusted as necessary by means of 0.1 M NaOH or HCl. Bacterial growth was monitored by absorbance increase at 600 nm measuring with a Spectro UV–vis Auto spectrophotometer (Labomed, USA).

Table 1 – Characteristics of *E. coli* strains used.

Strains	Genotype	Absent or defective hydrogenase or related protein	Source and/or reference
BW25113	*lacI*^{q} *rrnB*$_{T14}$*ΔlacZ*$_{W116}$ *hsdR514 ΔaraBAD*$_{AH33}$ *Δrha BAD*$_{LD78}$	Wild type	Yale University CGSG Stock Center
JW2701 Km^{Ra}	BW25113 *ΔfhlA*	FHL activator	[26]
JW2472 Km^{Ra}	BW25113 *ΔhyfG*	Large subunit of Hyd-4	[26]
JW2689 Km^{Ra}	BW25113 *ΔhycG*	Small subunit of Hyd-3	[26]
JWD2472	BW25113 *ΔhyfG*	Large subunit of Hyd-4	This work
JWD2689	BW25113 *ΔhycG*	Small subunit of Hyd-3	This work
JW0955 Km^{Ra}	BW25113 *ΔhyaB*	Large subunit of Hyd-1	[26]
JW2962 Km^{Ra}	BW25113 *ΔhybC*	Large subunit of Hyd-2	[26]
MW1000	BW25113 *ΔhyaB ΔhybC*;	Large subunit of Hyd-1 and Hyd-2	[27]
SW1001 Km^{Ra}	BW25113 *ΔfhlA ΔhyfG*	FHL activator and large subunit of Hyd-4	This work
SW1002 Km^{Ra}	BW25113 *ΔfhlA ΔhycG*	FHL activator and subunit of Hyd-3	This work

a Resistant to Kan.

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Whole cells for assay were prepared as described [9,18]. For *N,N′*-dicyclohexylcarbodiimide (DCCD) (Sigma, USA) inhibition studies, cells were incubated with DCCD at 0.5 mM for 10 min at 37 °C. Dry weight of bacteria was determined as previously [9].

2.3. Redox potential determination and hydrogen production assays

Redox potential (E_h) in bacterial suspension was measured using the oxidation-reduction, a titanium–silicate (Ti–Si) (EO-02, Gomel State Enterprise of Electrometric Equipment (GSEEE), Gomel, Belarus) and platinum (Pt) (EPB-1, GSEEE, or PT42BNC, Hanna Instruments, Portugal) electrodes [9,18,32]. In contrast to Ti–Si-electrode measuring the overall E_h, a Pt-electrode is sensitive to H_2 under anaerobic conditions (in the absence of O_2) [33] allowing detection of H_2 production. Therefore, H_2 production rate (V_{H_2}) was calculated through the difference between the initial rates of decrease in Pt- and Ti–Si-electrode readings per time and expressed as mV of E_h per min per mg dry weight of bacteria as described before [9,18,23,34,35]. E_h measurement by means of Pt- and Ti–Si-electrodes was done in the assay buffer where bacterial suspension was transferred and upon glycerol or glucose addition mentioned. No significant differences between Pt- and Ti–Si-electrodes readings were detected in bacterial suspension without glycerol and glucose added; bacterial count alteration in the suspension by ~8–10-fold had no marked effect on E_h value. This determination is closed to the method with Clark-type electrode employed by Noguchi et al. [26]: a correlation between E_h and H_2 production was shown. Note, the electrochemical measurement was reviewed to be indirect for H_2 production determination but can give accurate and reproducible data [5,6].

H_2 production was verified by the chemical assay based on the bleaching of $KMnO_4$ solution in H_2SO_4 with H_2 [34]. This method was suggested for detecting enhanced H_2 production [36]. Using the Durham tube method [18], H_2 production during *E. coli* growth was also estimated by the appearance of gas bubbles in the test tubes over the bacterial suspension.

2.4. Others and data processing

Assays were done in a closed chamber. All chemicals used were of analytical grade. Each data point represented is averaged from independent triplicate cultures, the standard deviations are not more than 5% if not represented.

3. Results and discussion

3.1. Change in E_h and H_2 production by *E. coli* during glycerol fermentation at low pH

E. coli is known can grow well and produce H_2 during glucose fermentation [1,5,6]. This bacterium could also grow slowly by fermenting peptone and although unlike growth with glucose it could not grow by fermentation with glycerol as sole carbon source but it could utilize glycerol in the presence of peptone under anaerobic conditions and grow fast at different pH (not shown). This seems to be in good conformity with data about requirement of rich nutrients in the medium for glycerol fermentation by *E. coli* at acidic pH, whereas low supplementation (as peptone) allowed glycerol fermentation at alkaline pH [2,4]. However, mechanisms of this phenomenon have been not clear yet [4].

E_h, measured by Pt-electrode in the suspension of *E. coli* BW25113 wild type grown in peptone medium on glycerol added at a low pH, washed and transferred into the assay medium, was decreased in the absence of glycerol, but this change was negligible (Fig. 1, dotted curves). However upon addition of glycerol (10 ml/l), E_h dropped markedly from positive values (+146 ± 8 mV at pH 6.5 or +326 ± 18 mV at pH

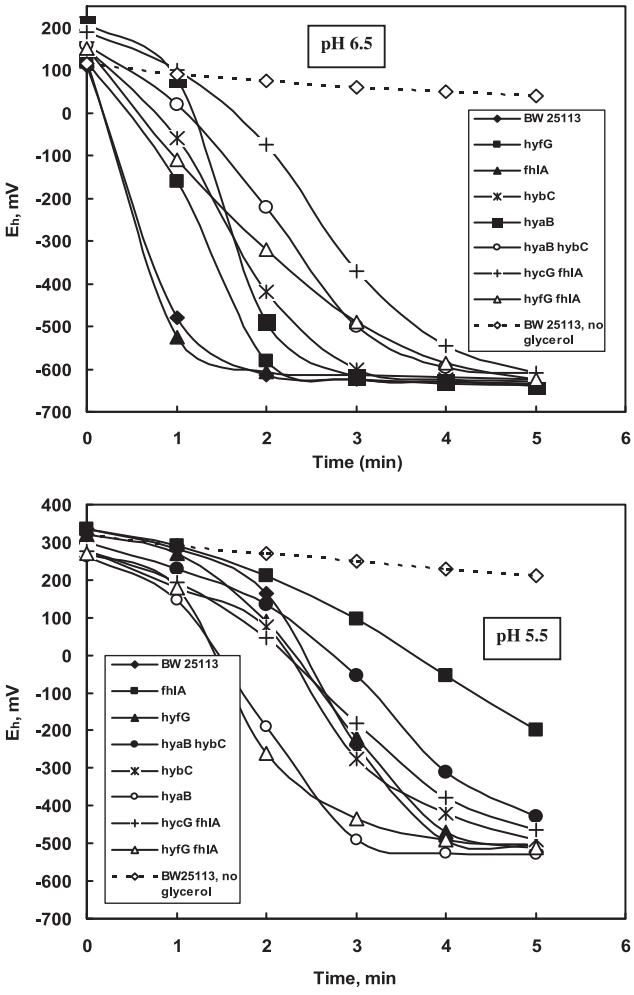

Fig. 1 – Simultaneous changes in E_h for *E. coli* BW25113 wild type and different mutants with defects in hydrogenases. Bacteria grown under glycerol fermentation at a low pH (the initial pH 6.5 or pH 5.5) were transferred into a 200 mM Tris-phosphate (appropriate pH) buffer containing 0.4 mM $MgSO_4$, 1 mM NaCl, 1 mM KCl, and 10 ml/l glycerol was subsequently added at time zero. E_h changes by wild type bacterial suspension in the absence of glycerol added was as dotted curves. Bacterial culture absorbance (after 10-time dilution) measured by a spectrophotometer was ~0.70. E_h was measured with Pt-electrode as in Materials and methods. For strains, see Table 1.

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5.5) down to negative ones (up to −637 ± 24 mV at pH 6.5 or −510 ± 20 mV at pH 5.5) (Fig. 1). Such a drop was not observed with E_h, measured with Ti–Si-electrode: E_h was decreased to −100 ± 10 mV. These findings indicated the strengthening of reduction processes upon glycerol fermentation. They are correlated with E_h decrease during bacterial growth under fermentative conditions [37,38] that has different explanations [39]. Actually, during fermentation of glycerol at a low pH, E_h decreased with lowering pH although different pH values (not shown).

Note, E_h depends on pH by the equation: $E_h = E_0 + (RT/nF) \ln([ox]/[red]) + (RT/nF) \ln[H^+]$ (E_0 is a standard redox potential, R,T and F are known parameters, [ox] and [red] are activities of oxidized and reduced compounds), following E_h decrease with increasing pH [39]. This was considered for two-basic ionizing system and, actually, was observed for the assay mixture without bacteria (see Fig. 1). However, for bacterial suspension under glycerol fermentation, E_h decrease could not be described by the equation above specifying on set and complexity of processes.

Besides, the acidification of medium at different pH was low (~0.2–0.3 pH unit) due to less formation of fermentation end products (organic acids) or their changed proportion as suggested [3,4]. Therefore, input of pH change in E_h drop (see Fig. 1) might not be taken into account.

The marked difference in readings between two redox electrodes indicated H_2 production by *E. coli*: V_{H_2} at pH 5.5 was ~3.36 mV E_h/min/mg dry weight (Fig. 2); it is ~1.5-fold higher than those at pH 6.5 and pH 7.5. However, this value was lower than those during glucose fermentation at appropriate pH (Fig. 2) as shown before [18]. This was in conformity with H_2 production data under external formate [23]. The difference between E_h minimal values measured by Pt- and Ti–Si-electrodes reached during glycerol fermentation for 4–5 min was similar to that at glucose fermentation (not shown) so H_2 yield might be close to each other in spite of different V_{H_2}.

In addition, H_2 production by *E. coli* under glycerol fermentation at different pH was tested and verified using different methods (see Materials and methods).

Besides, the findings above might be interesting for hydrogen metabolism by *E. coli* during glycerol and glucose fermentation when metabolic pathways are still not clear but production of various end products is suggested may have different coupling with H_2 production [3,4,40].

3.2. *H_2 production inhibition by DCCD*

To reveal a relationship of H_2 production with H^+ transport and F_0F_1, effect of DCCD was determined. Note, DCCD, lipid-soluble carbodiimide, is a non-specific inhibitor of F_0F_1 but this reagent becomes specific one for *E. coli* under fermentative conditions [25,32,41].

Indeed, H_2 production by *E. coli* BW25113 during glycerol fermentation was inhibited by DCCD in a concentration dependent manner; clear effects with 0.5 mM concentration were observed (Fig. 2). This fact was in accordance with the DCCD effect observed at pH 7.5 [9]. However, DCCD inhibition of V_{H_2} was increased with decreasing pH and almost maximal inhibition was observed at pH 5.5: V_{H_2} in the presence of DCCD was ~30 fold lower than that without DCCD at pH 5.5 and

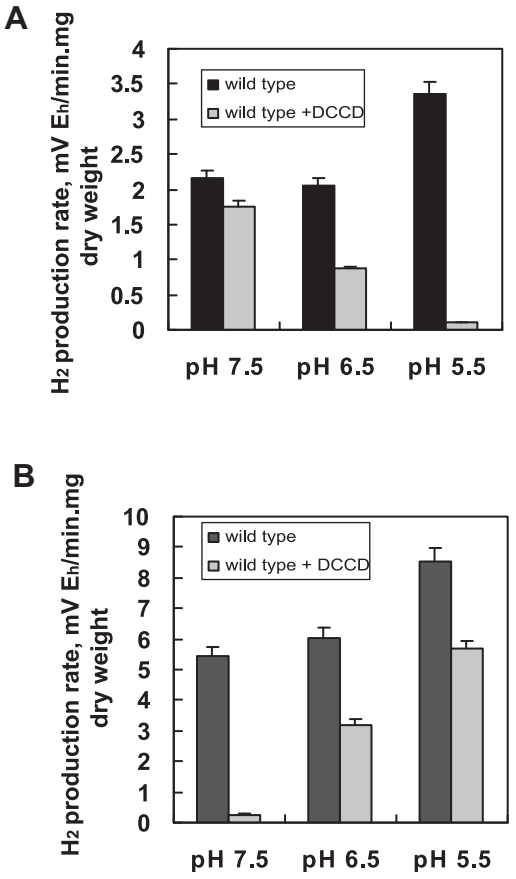

Fig. 2 – H_2 production rates (V_{H_2}) and effect of DCCD for *E. coli* BW25113 wild type and *fhlA* mutants under glycerol (A) and glucose (B) fermentation at different pH. 0.4% glucose was added into the assay buffer. 0.5 mM and 0.2 mM DCCD was used for glycerol and glucose fermentation conditions, respectively. For the others, see Materials and methods and legends to Fig. 1.

~2.5 and ~1.2 fold lower than those at pH 7.5 and 6.5, respectively (see Fig. 2). The results might indicate that H_2 production at a low pH was related with H^+ transport for which F_0F_1 was one of the main pathways [9,18] and was transporting up to 40% H^+ from cytoplasm into the external medium [42]. This seems to be in conformity with that the F_0F_1 activity facilitates the fermentative metabolism of glycerol by *E. coli* at acidic pH enhancing H_2 production [3,4]. It is not ruled out that DCCD might interact with Hyd-enzymes responsible for H_2 production under glycerol fermentation at a low pH; no DCCD-binding and inhibition of *E. coli* Hyd-enzymes have been shown yet.

During glucose fermentation DCCD inhibited H_2 production at different pH (Fig. 2); in contrary to glycerol fermentation this inhibition was more effective at pH 7.5 (comp. Fig. 3A and B). Bagramyan et al. [18,34] have shown that H_2 production by *E. coli* under glucose fermentation was accompanied by H^+ efflux. Again, the results obtained indicate that H_2 production was related with H^+ transport. These findings are in favor with the idea about relationship of Hyd-3 and Hyd-4

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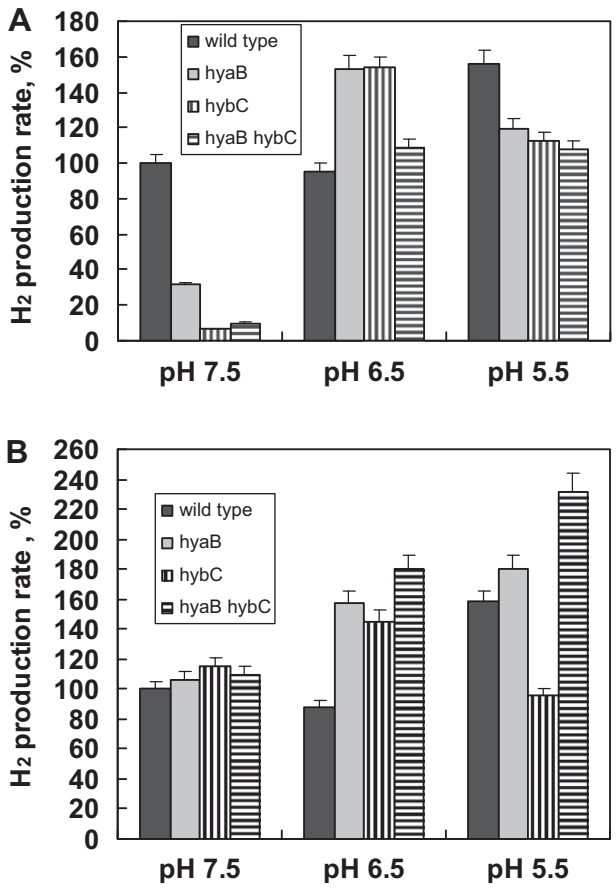

Fig. 3 – V_{H_2} for *E. coli* mutant strains with defective Hyd-1 and Hyd-2 under glycerol (A) and glucose (B) fermentation at different pH. V_{H_2} for wild type under mentioned conditions is 100%. For the others, see legends to Fig. 1.

with F_0F_1 [24,25]. Alternatively, Hyd-3 [19] and Hyd-4 [11,18] might have H^+-translocating function so DCCD inhibition is likely. Moreover, the different DCCD inhibition under glycerol and glucose fermentation could be due to different Hyd-enzymes responsible for H_2 production.

Besides, DCCD inhibition has been shown for H_2 production by *Rhodobacter sphaeroides* [43]. Kars et al. [44] have reported that *Rh. sphaeroides* Hyd-enzyme could be an ATP-dependent one. However, the mechanisms of this inhibition in bacteria are not clear yet.

3.3. H_2 production by E. coli mutants with defects in Hyd-1 and Hyd-2

To establish a role of Hyd-enzymes required for H_2 production during glycerol fermentation at a low pH, mutants with defective Hyd-1 and Hyd-2 (see Table 1) were studied.

A decrease in E_h down to strong negative values was observed with JW0955, JW2962 and MW1000 mutants (see Table 1) at pH 6.5 and 5.5 both (see Fig. 1). This indicates a high total Hyd-activity and H_2 production under the conditions mentioned above. In contrast to pH 7.5 when V_{H_2} by single *hyaB* and *hybC* mutants was less than that with wild type, at pH 6.5 V_{H_2} by these mutants was ~1.6 higher but V_{H_2} by double *hyaB hybC* mutant was lower than that by single *hyaB* and *hybC* mutants and similar to that of the wild type: the latter was unexpected (Fig. 3). At pH 5.5 V_{H_2} by wild type was higher (see Figs. 3 and 1), however it was lowered ~1.3 and ~1.4-fold with the *hyaB* and *hybC* mutants, respectively; the low rate was defined with the double *hyaB hybC* mutant (Fig. 3). The results obtained suggest that, during glycerol fermentation at a low pH, increased H_2 production was probable in the absence of H_2 uptake activity so as Hyd-1 and Hyd-2 were working in a reversed H_2 oxidizing mode and the other Hyd-enzymes were responsible for H_2 production (Fig. 4). Thus, recycling of produced H_2 is required for hydrogen metabolism during glycerol fermentation by *E. coli*. The latter and changed V_{H_2} by double *hyaB hybC* mutant are likely to the results obtained by Redwood et al. [16] about compensatory uptake function during H_2 production under different conditions. Increased H_2 production in a double *hyaB hybC* mutant (compared to the wild type) was associated with the loss of *hyb* and not *hya* genes [16]. Therefore, during glycerol fermentation at a low pH, a role of Hyd-1 and Hyd-2 in recycling of

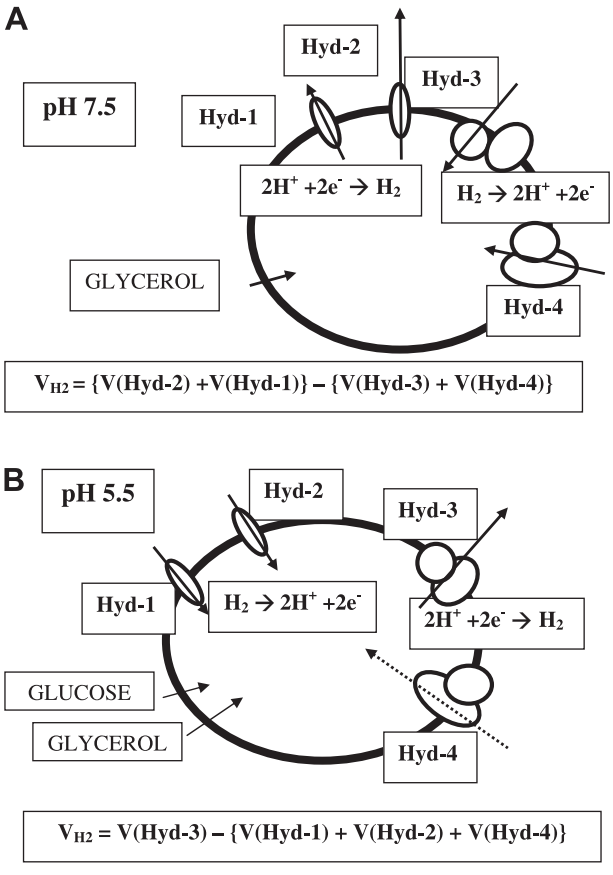

Fig. 4 – Different H_2 producing and H_2 uptaking Hyd-enzymes expressed by *E. coli* under glycerol fermentation at pH 7.5 (A) or pH 5.5 (B). V_{H_2} is H_2 producing rate by whole cells; V(Hyd) is H_2 producing or H_2 oxidizing rate by appropriate Hyd-enzyme. Arrows are for direction of enzyme operation to produce and/or to oxidize H_2. The mode for Hyd-enzymes functioning at pH 5.5 during glucose fermentation is similar with that under glycerol fermentation (B). For the others, see the text.

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produced H_2 could be proposed. This seems to be likely to the conclusion of Murarka et al. [4] about recycling of H_2 evolved by the FHL complex during glycerol fermentation and, in addition, to the suggestion by Zbell and Maier [45] about a role of different levels of Hyd-1 to recycle all H_2 production in *Salmonella enterica* under fermentative conditions. The findings above are also in accordance with the idea of Lukey et al. [17] about two-directional activity of Hyd-2 but under different conditions.

Moreover, at pH 6.5 double *hyaB hybC* deletions in *E. coli* may affect the other Hyd-enzymes expression and activity since a pleiotropic effect of *hyb* genes deletions is suggested at a low pH [46]. Alternatively but with less chance, double *hyaB hybC* deletions resulting in the lack of large subunits of Hyd-1 and Hyd-2 both in the appropriate Hyd complexes in the *E. coli* membrane (see Table 1) could affect protein organization within the membrane and induce conformational changes in appropriate Hyd-enzymes changing the total H_2 producing activity. Interestingly, this is in favor with the suggestion that low level or not proper localization of Hyd-2 or other Hyd-enzyme can result in different coupling of H_2 oxidation to FHL too [45]. In all cases, it is difficult to give a clear explanation for the finding on lower V_{H_2} in double *hyaB hybC* mutant since expression and regulation of Hyd-enzymes are different and very complex so as their role is not well characterized [13,16,17] in spite of long-term study [47,48] and, in addition, contradictory reports with cultures grown under anaerobic and aerobic conditions on different carbon sources have been published [48,49]; further study is required.

Note that the data obtained at pH 7.5 confirm the results reported [9].

To understand the mechanisms of *E. coli* Hyd-enzymes activity and regulation, H_2 production was investigated during glucose fermentation at a low pH. In contrast to pH 7.5 when V_{H_2} by the *hyaB* and *hybC* mutants as well as by the double *hyaB hybC* mutant was similar to the wild type, at pH 6.5 and 5.5 V_{H_2} by these mutants (but not by *hybC* at pH 5.5) was increased significantly ($p < 0.001$, Fig. 3). The increase might occur if Hyd-1 and Hyd-2 are operating as H_2 uptaking and oxidizing Hyd-enzymes. The last statement is in favor with view point about Hyd-1 and Hyd-2 activity mode under fermentation [8,13]. Moreover, the data of V_{H_2} by *hybC* mutant at pH 5.5 could point out a different role of Hyd-2 according to pH when Hyd-1 was major in H_2 uptake. Again, several explanations about different role of *hyaB* and *hybC* mutants similar to those for glycerol fermentation conditions (see above) might be given for these findings. The results are in conformity with the results of a similar pattern of increasing H_2 production with decreasing pH reported [26]. Hyd-3 more than Hyd-4 becomes responsible for H_2 production at a low pH; further study is required.

Importantly, Hyd-enzymes activity and expression at different pH might depend on fermentation substrate (glycerol, glucose) and other factors. A different level of H_2 production and a distinguishing expression and activity of Hyd-enzymes have been reported in our lab [18,23] and by Rossman et al. [50] and confirmed in Slonczewski lab [26]. Glucose (0.2%) or formate (100 mM) used in the growth media [16,18,23,41] and their absence [26] can attribute to this discrepancy; interaction between appropriate genes or between Hyd-enzymes may contribute too.

3.4. *Construction of E. coli strains deleted for fhlA gene and defective in Hyd-3 and Hyd-4*

To understand the mechanism of H_2 production from glycerol, we constructed the double mutant *E. coli* SW1002 (*fhlA hycG*) defective in FhlA and in Hyd-3 large subunit and SW1001 (*fhlA hyfG*) defective in FhlA and Hyd-4 large subunit (see Table 1). The strains were verified via PCR; the PCR products using primers flanking *hycG* have the expected size for BW25113 (Fig. 5, A, lane 1), for the *hycG* mutant JWD2689 after deletion of the Kan resistance marker from JW2689 (Fig. 5, A, lane 2), and SW1002 (Fig. 5, A, lane 3). As expected, no PCR product was obtained for BW25113 (Fig. 5, A, lane 5) using a forward primer upstream of *fhlA* and the backward primer inside the Kan resistance marker, but the expected product size was obtained for SW1002 (Fig. 5A, lane 6).

PCR products of the expected size were also obtained using primers flanking *hyfG* for BW25113 (Fig. 5, B, lane 1), the *hyfG* mutant including the Kan resistance marker JW2472 (Fig. 5B, lane 2), the *hyfG* mutant after deletion of the Kan resistance marker JWD2472 (Fig. 5, B, lane 3) and four independent colonies of SW1001 (Fig. 5, B, lanes 4–7). The insertion of the Kan resistance marker at the *fhlA* locus of SW1001 was also verified for 4 independent colonies (Fig. 5, B, lanes 8–11).

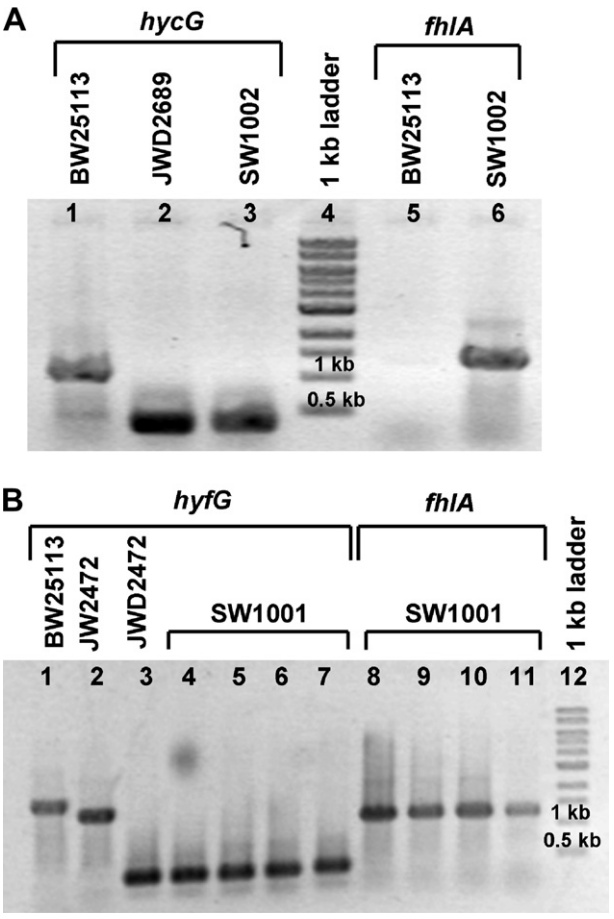

Fig. 5 – Verification of *E. coli* JWD2689 (*hycG*) and SW1002 (*fhlA hycG*) (A) and of JWD2472 (*hyfG*) and SW1001 (*fhlA hyfG*) (B) strains. For strains, see Table 1; for the others, see text. Four independent colonies of SW1001 were tested.

3.5. *Role of fhlA mutation and Hyd-3 and Hyd-4 in E_h change and H_2 production*

A decrease in E_h down to strong negative values was observed with *E. coli* JW2701 as well as *fhlA hycG* and *fhlA hyfG* double mutants at pH 6.5 and 5.5 (see Fig. 1). This indicates a high total Hyd-activity and H_2 production under the conditions above. However, the kinetics of E_h decrease was different: it was less with *fhlA* strain at pH 5.5 (see Fig. 1).

V_{H_2} was mentioned above to depend on pH. This was recently determined for fermentation conditions by Noguchi et al. [26]: unfortunately, fermentation substrate and other conditions were not clarified but they may affect the processes. Upon glycerol fermentation at pH 6.5, V_{H_2} by *fhlA* strain as well as double *fhlA hycG* mutants did not demonstrate large changes (Fig. 6), but at pH 5.5 it was lowered ~2.3-fold with the *fhlA* mutant and increased ~2-fold with the double *fhlA hyfG* mutant (comp. with wild type, see Fig. 2). The results point out that the appropriate gene products, FhlA protein and Hyd-4 both, might affect H_2 production and Hyd-4 can work in a reverse mode probably as H_2 uptaking and oxidizing Hyd-enzyme. Maeda et al. [28] have shown that *E. coli* Hyd-3 operates as a reversible enzyme during glucose fermentation at pH 6.8. Moreover, Trchounian and Trchounian [9] have established reversible mode with Hyd-2 under glycerol fermentation but at pH 7.5 (see Fig. 4). As noted, two-directional Hyd-2 has been also suggested for different conditions by Lukey et al. [17]. In addition, these results suggest that each Hyd-enzyme operating in one direction might change its direction depending on pH and carbon source. Hyd-4 functioning upon glycerol fermentation at a low pH could be unexpected novel finding in addition to that with the operation of this enzyme upon glucose fermentation at pH 7.5 [18,23] (see Fig. 4).

Interestingly, DCCD effectively inhibited H_2 production by *fhlA* strain as well as double *fhlA hycG* and *fhlA hyfG* mutants in spite of different values for V_{H_2} (Fig. 6). This did not add anything novel to the DCCD inhibition findings discussed above for wild type.

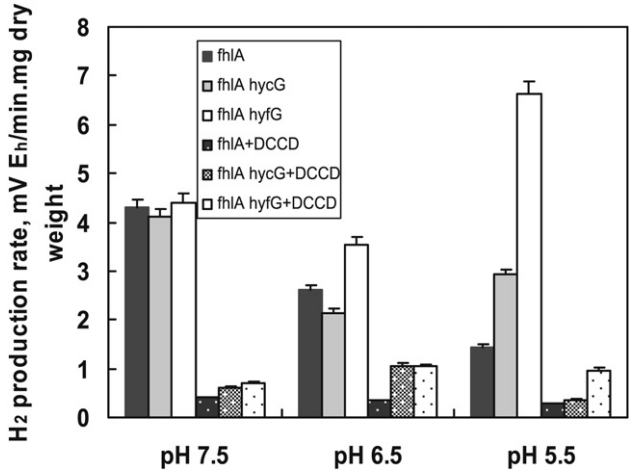

Fig. 6 – V_{H_2} and effect of DCCD for *E. coli* mutants with absent FhlA, defective Hyd-3 and Hyd-4 during glycerol fermentation at different pH. For the others, see legends to Figs. 1 and 2.

Besides, in contrast to glycerol fermentation, during glucose fermentation by *fhlA* strain and by *fhlA hycG* and *fhlA hyfG* double mutants, H_2 production was lowered at pH 6.5 and it was less at pH 5.5 (not shown). At pH 6.5, V_{H_2} by the double *fhlA hycG* and *fhlA hyfG* mutants was ~1.5-fold lower than that by the *fhlA* strain (not shown). The results show a responsibility of Hyd-3 and Hyd-4 and suggest a role of the *fhlA* gene or appropriate gene product, FhlA, in Hyd-3 and Hyd-4 expression and activity in H_2 production under glucose fermentation at a low pH. This is in conformity with data about Hyd-3 responsibility in H_2 production during glucose fermentation at different pH; the *hycE* gene (coding a large subunit of Hyd-3 [10]) expression was demonstrated to be increased when pH decreased, whereas the activity of Hyd-3 was unchanged with a shift in pH [26].

4. Concluding remarks

In this study, *E. coli* hydrogenase activity and H_2 production were studied at a low pH. V_{H_2} during glycerol fermentation at pH 5.5 was shown to be ~1.5-fold higher than that at pH 6.5 or above but less than that under glucose fermentation at appropriate pH. It was inhibited by DCCD; however DCCD inhibition of H_2 production was increased with pH decreasing. DCCD inhibition of Hyd-enzymes is interesting to understand a role of H^+ transport through the bacterial membrane including that via F_0F_1, since defects in F_0F_1 might stimulate H_2 production at pH 7.5 [9].

H_2 production study with *hyaB* and *hybC* mutants with defective Hyd-1 and Hyd-2, correspondingly (see Fig. 3), suggests that during glycerol fermentation at pH 6.5, Hyd-1 and Hyd-2 were working in a reversed, non-H_2 producing mode. Under glycerol fermentation at pH 6.5, H_2 production by *fhlA* strain as well as double *fhlA hycG* and *fhlA hyfG* mutants at a low pH (see Fig. 6) indicate that the appropriate gene products FhlA and Hyd-4 both affect H_2 production and Hyd-4 can operate in a reverse mode.

In contrast to glycerol fermentation, upon glucose fermentation H_2 production by *fhlA* strain as well as double *fhlA hycG* and *fhlA hyfG* mutants was lowered at pH 6.5; it was less at pH 5.5 (see Fig. 6). The results show a responsibility of Hyd-3 and Hyd-4 and suggest a role of *fhlA* gene or FhlA in regulation of Hyd-3 and Hyd-4 activity for H_2 production under glucose fermentation at a low pH; this might be due to appropriate transcription activation.

Followed from this and different groups [9,17,28] study it could be concluded that reversibility is likely to be a property of Hyd-enzymes having a key role in regulation of hydrogen metabolism under different environment. This would be important for construction of new mutants, stimulating H_2 production and biotechnological application of glycerol.

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