ՀՀ ԿՐԹՈՒԹՅԱՆ ԵՎ ԳԻՏՈՒԹՅԱՆ ՆԱԽԱՐԱՐՈՒԹՅՈՒՆ
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ԹՌՉՈՒՆՅԱՆ ԿԱՐԵՆ ԱՐՄԵՆԻ

ՀԻԴՐՈԳԵՆԱԶԱՅԻՆ ԱԿՏԻՎՈՒԹՅՈՒՆԸ ԵՎ H_2-Ի ԱՐՏԱԴՐՈՒԹՅԱՆ ԱՌԱՆՁՆԱՀԱՏԿՈՒԹՅՈՒՆՆԵՐԸ *ESCHERICHIA COLI*-ՈՒՄ ԳԼԻՑԵՐՈԼԻ ԽՄՈՐՄԱՆ ԸՆԹԱՑՔՈՒՄ

Գ.00.02- կենսաֆիզիկա և Գ.00.14- կենսատեխնոլոգիա մասնագիտություններով
կենսաբանական գիտությունների թեկնածուի
գիտական աստիճանի հայցման ատենախոսության

ՍԵՂՄԱԳԻՐ

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MINISTRY OF EDUCATION AND SCIENCE OF THE REPUBLIC OF ARMENIA
YEREVAN STATE UNIVERSITY

TRCHOUNIAN KAREN ARMEN

HYDROGENASE ACTIVITY AND PECULIARITIES OF H_2 PRODUCTION BY *ESCHERICHIA COLI* DURING GLYCEROL FERMENTATION

Dissertation for the scientific degree of
Candidate of Biological Sciences
specialties 03.00.02 – Biophysics and 03.00.14 - Biotechnology

S Y N O P S I S

Yerevan 2013

Ատենախոսության թեման հաստատվել է Երևանի պետական համալսարանում

Գիտական ղեկավար՝	ՀՀ ԳԱԱ թղթակից անդամ, կենսաբանական գիտությունների դոկտոր, պրոֆեսոր Ա. Հ. Թրչունյան
Պաշտոնական ընդդիմախոսներ՝	ՀՀ ԳԱԱ ակադեմիկոս, կենսաբանական գիտությունների դոկտոր, պրոֆեսոր Է. Գ. Աֆրիկյան ֆիզիկամաթեմատիկական գիտությունների դոկտոր, պրոֆեսոր Վ. Բ. Առաքելյան
Առաջատար կազմակերպություն՝	Հայ-Ռուսական (Սլավոնական) համալսարան

Ատենախոսության պաշտպանությունը տեղի կունենա 2013թ. հոկտեմբերի 22 -ին, ժամը 14^{00}-ին, Երևանի պետական համալսարանում գործող ՀՀ ԲՈՀ-ի Կենսաֆիզիկայի 051 Մասնագիտական Խորհրդի նիստում (0025, Երևան, Ալեք Մանուկյան 1, ԵՊՀ, կենսաբանության ֆակուլտետ):

Ատենախոսությանը կարելի է ծանոթանալ Երևանի պետական համալսարանի գրադարանում:

Ատենախոսության սեղմագիրն առաքված է 2013թ. սեպտեմբերի 20-ին:

051 Մասնագիտական Խորհրդի գիտական քարտուղար,
կենսաբանական գիտությունների դոկտոր, պրոֆեսոր՝ Լ. Հ. Նավասարդյան

Dissertation title is approved at Yerevan State University

Academic advisor:	Corresponding Member of NAS RA, Doctor of Biological Sciences, Professor A. H. Trchounian
Official opponents:	Academician of NAS RA, Doctor of Biological Sciences, Professor E.G. Afrikyan Doctor of Physico-Mathematical Sciences, Professor V. B. Arakelyan
Leading organization:	Russian-Armenian (Slavonic) State University

The dissertation defense will take place on 22nd of October 2013 at 14^{00} during 051 Biophysics Specialized Board meeting of SSC RA at Yerevan State University (0025 Yerevan, Alex Manoogian 1, YSU, Faculty of Biology).

The dissertation can be found at library of Yerevan State University.

The synopsis of dissertation has been sent on September 20h 2013.

Scientific Secretary of the 051 Specialized Board,
Doctor of Biological Sciences, Professor L. H. Navasardyan

INTRODUCTION

Topic relevance. Molecular hydrogen (H_2) as a renewable and alternative energy source has high impact of interest because from its burning ~142 kJ/g energy is released. Moreover, H_2 is 100% ecologically clean renewable energy source. Application of H_2 in everyday human life and industry is getting more and more. Meanwhile, research and industry are trying to find cheap sources for producing H_2 to apply it in different industrial technologies.

H_2 can be produced by different ways, one of which is chemical – from water or carbohydrates electrolysis or by heating. But the chemical way of producing H_2 is economically inefficient. Therefore, H_2 production from bacteria fermenting certain (glucose, glycerol, formate) substrates is in demand. H_2 is produced by bacteria during mixed-acid fermentation or during photo-fermentation. This process is catalyzed by special membrane-associated enzymes named hydrogenases (Hyd) catalyzing reversible oxidation of H_2: $H_2 \rightarrow 2H^+ + 2e^-$. Hyd have been determined in many prokaryotes and some eukaryotes and play important role in anaerobic metabolism (Adams and Stiefel, 1998; Frey, 2002). *Escherichia coli* has four Hyd enzymes (Sawers, 1994; Bagramyan and Trchounian, 2003; Poladyan and Trchounian, 2009; Kim and Kim, 2011; Trchounian et al., 2012b). It is suggested that dependent on environmental conditions two of them operate in producing- and the other two- in oxidizing- mode. Such working mode for Hyd is indicative to form new **H_2 cycling**. But the biosynthesis, genetic regulation, activity and working mechanisms of these enzymes are not clear yet. Revealing the regulation of enzyme activity and biosynthesis, it might be possible to apply Hyd in H_2 production biotechnology. Certain bacteria ferment sugars and produce lactate, acetate succinate, other organic acids, ethanol, CO_2 and H_2. This process is well known and in different environmental conditions it has been shown which Hyd is responsible for H_2 production and these have stimulated for constructing via metabolic and genetic engineering special *E. coli* strain with high H_2 production yield (Maeda et al., 2008; Hu and Wood, 2010).

Recently, it has been discovered that glycerol can be fermented by *E. coli* (Dharmadi et al., 2006; Gonzalez et al., 2008). This is interesting phenomenon not only for fundamental studies but also for industry because glycerol is very cheap source for producing H_2 compared to sugars. Gonzalez's group (Dharmadi et al., 2006; Gonzalez et al., 2008) has shown that during glycerol fermentation at pH 6.3 H_2 gas is also detected. But it has negative impact on the cell growth and glycerol fermentation.

The application of glycerol in industry is suggested to have wide spectrum for producing different compounds (Khanna et al., 2012; Clomburg and Gonzalez, 2013). But this process must be investigated. The mechanisms of Hyd biosynthesis, determination of their specific activity and working mechanisms during glycerol fermentation at different conditions would be of great importance.

Research goals and tasks. The main goal of the work was dedicated to the investigation of H_2 production by *E. coli* at different pH during glycerol fermentation.

Constituted tasks of the research were to:

- reveal Hyd responsible for H_2 production in whole cells using different Hyd defective mutants;

- determine hydrogenase specific activity in the cell extracts at different pH;
- investigate effect of different factors such as inhibtors, osmotic stress on H_2 production;
- determine the proton motive force and understand the link between H_2 production and F_0F_1-ATPase;
- do comparative analysis of H_2 production during glucose and glycerol fermentation;
- study H_2 production during mixed carbon (glucose and glycerol) fermentation.

Topic scientific novelty and applied value. During the conducted research it has been revealed which Hyd is (are) responsible for H_2 production during glycerol fermentation at slightly alkaline pH. It was determined that mainly Hyd-2 and to some extent Hyd-1 are responsible for H_2 production. The other two Hyd which were responsible for H_2 production during glucose fermentation work, in the above mentioned conditions, in reverse H_2 uptake mode. It was shown that H_2 production is slightly inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD) but the link between Hyd-1 and the F_0F_1-ATPase was suggested. For slightly acidic and acidic pHs FHL-1 complex is responsible for H_2 production. Moreover, H_2 production was inhibited more at acidic pH and thus the important role of F_0F_1 was proposed. pH dependent differences of Hyd specific activities were shown during glucose or glycerol as well as mixed carbon fermentation.

The results obtained has unique significance in H_2 production biotechnology and the application of glycerol as a cheap carbon source is of importance.

Main points to present at defense.

1. At pH 7.5 glycerol is fermented and H_2 gas is produced by *E. coli*: Hyd enzymes are reversible and Hyd-2 mostly is responsible for H_2 production.
2. The F_0F_1-ATPase is required for Hyd activity during glycerol or glucose fermentation at different pHs.
3. Hyd are involved in proton motive force generation during glycerol fermentation at different pHs.

Work approbation. Main results of the dissertation were discussed at seminars in Department of Biophysics and Department of Microbiology, Plants and Microbes Biotechnology, Biology Faculty of Yerevan State University (Armenia) and at different scientific congresses and meetings, namely 15th EBEC Conference in Dublin, Ireland (2008), 53rd Annual Meeting of the US Biophysical Society in Boston, USA (2009), 110th General Meeting of American Society for Microbiology in San Diego, USA (2010), 35th FEBS Congress in Gothenburg, Sweden (2010), 4th FEMS Congress Geneva, Switzerland (2011), 8th EBSA Congress in Budapest, Hungary (2011), 112th General Meeting of American Society for Microbiology in San Francisco, USA (2012), 37th FEBS & 22nd IUBMB Congress in Sevilla, Spain (2012), 17th EBEC Congress in Freiburg, Germany (2012), 38th FEBS Congress in St. Petersburg, Russia (2013), 5th FEMS Congress in Leipzig, Germany (2013).

Publications. Based on experimentally obtained data 27 works, including 12 full papers in international peer reviewed journals and book were published.

Volume and structure of dissertation. Dissertation contains of introduction, literature review (Chapter 1), experimental part (Chapter 2), results and discussion (Chapter 3),

conclusions and cited references (total 146 papers and books). The document consists of 128 pages, contains 10 tables and 48 graphs and figures.

MATERIALS AND METHODS

Bacteria: All experiments were conducted on *E. coli* BW25113 or MC4100 wild type (wt) strains and appropriate mutants (Table 1). Strains with multiple deletions were generally constructed by introduction of mutations from *E. coli* donor strains into recipient strains of BW25113 by P1*kc* phage transduction according to Miller (1972).

Table 1. Characteristics of the *E. coli* strains used in this study

Strain	Genotype	Absent or defective gene product	Reference
BW 25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{W116}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rha BAD*$_{LD78}$	Wild type	Maeda et al. (2007)
JW 0955*	BW 25113 Δ*hyaB*	Large subunit of Hyd-1	Maeda et al. (2007)
JW 2472*	BW 25113 Δ*hyfG*	Large subunit of Hyd-4	Andrews et al. (1997)
JW 2701*	BW 25113 Δ*fhlA*	FHL activator	Baba et al. (2006)
JW 2962*	BW 25113 Δ*hybC*	Large subunit of Hyd-2	Maeda et al. (2007)
MW 1000	BW 25113 Δ*hyaB* Δ*hybC*	Large subunits of Hyd-1 and Hyd-2	Maeda et al. (2007)
SW1001*	BW 25113 Δ*hyfG* Δ*fhlA*	FHL activator and large subunit of Hyd-4	Trchounian et al. (2011b)
SW1002*	BW 25113 Δ*hycG* Δ*fhlA*	FHL activator and large subunit of Hyd-3	Trchounian et al. (2011b)
KT 2110	BW 25113 Δ*hyaB* Δ*hybC* Δ*selC*	Large subunits of Hyd-1, Hyd-2 and tRNA^{sec}	Trchounian et al. (2012a)
MC 4100	*araD139 ΔlacU169 rpsL thi fla*	Wild type	Redwood et al. (2008)
HDK 103	MC 4100 Δ*hya* Δ*hyc*	Hyd-1 and Hyd-3	Laurinavichene et al. (2002)
HDK 203	MC 4100 Δ*hyb* Δ*hyc*	Hyd-2 and Hyd-3	Laurinavichene et al. (2002)

FM 460*	MC 4100 Δ*selC*	$tRNA^{sec}$	Trchounian et al. (2012a)
FTD 147	MC 4100 Δ*hyaB* Δ*hybC* Δ*hycE*	Large subunits of Hyd-1, Hyd-2 and Hyd-3	Redwood et al. (2008)
FTD 150	MC 4100 Δ*hyaB* Δ*hybC* Δ*hycE* Δ*hyfG*	Large subunits of Hyd-1, Hyd-2, Hyd-3 and Hyd-4	Redwood et al. (2008)
DHP-F2	MC4100 Δ*hypF*	Maturation of all hydrogenases	Paschos et al. (2002)
DK8	*bglR thill rell* Δ*(uncB-uncC) ilv::Tn 10*	F_0F_1-ATPase	Trchounian et al. (2011a)

[a] Resistant to Kan

Bacterial cultivation, preparation for assays. *E. coli* were cultivated at 37^{o}C for 18-22 h in anaerobic conditions by direct transfer from nutrient agar surface in Petri dish into high buffered liquid peptone growth medium containing peptone 2%, K_2HPO_4 1.5%, KH_2PO_4 0.1%, NaCl 0.5%, pH 7.5; peptone 2%, K_2HPO_4 0.75%, KH_2PO_4 0.86%, NaCl 0.5%, pH 6.5; peptone 2%, K_2HPO_4 0.1%, KH_2PO_4 1.5%, NaCl 0.5%, pH 5.5. 5 ml/l 40% glucose and/or 10 ml/l glycerol was added. Kanamycin (25 μg/ml final concentration) was added where appropriate (see Table 1). In some experiments bacteria were incubated for 5-7 minutes with DCCD or washed with 0,8 M sucrose for the following hypo-osmotic stress.

Preparation of cell extracts. Harvested cells were washed in MOPS-buffer (50 mM MOPS at either, pH 5.5, 6.5 or 7.5) by centrifugation and the cell pellet was re-suspended typically in three volumes of 50 mM MOPS buffer, pH 7.5, including 5 μg DNase/ml and 0.2 mM phenylmethylsulfonyl fluoride. 1-2 g wet weight of cells were disrupted by sonication (30 W power for 5 min with 0.5 s pulses). Unbroken cells and cell debris were removed by centrifugation for 30 min at 50,000Xg and at 4^{0}C. Protein concentration was determined (Lowry et al., 1951) with bovine serum albumin as standard.

Determination of total hydrogenase enzyme activity. Hyd enzyme activity (H_2-dependent reduction of benzyl viologen (BV)) was measured according to (Ballantine and Boxer, 1985) except that the buffer used was 50 mM MOPS, pH 7.0. The wavelength used was 578 nm and an E_M value of 8,600 M^{-1} cm^{-1} was assumed for reduced BV. One unit of activity corresponded to the reduction of 1 μmol of hydrogen per min.

Non-denaturing polyacrylamide gel electrophoresis and in-gel hydrogenase activity staining. Non-denaturing PAGE was performed using 7.5% (w/v) polyacrylamide gels, pH 8.5 and included 0.1% (v/v) Triton X-100 in the gels and Hyd activity-staining was done as described (Ballantine and Boxer, 1985) except the buffer used was 50 mM MOPS pH 7.0.

Determination of redox potential and H_2 production assays. Redox potential (E_h) in bacterial suspension was measured using the redox, a titanium-silicate (Ti-Si) and platinum (Pt)

electrodes (Trchounian et al., 2011b). 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) allowing detection of H_2. H_2 production rate (V_{H2}) 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 (Trchounian et al., 2009; 2011b; Trchounian, 2012). H_2 production was verified by the chemical assay (Maeda and Wood, 2008) and Durham tube method (Bagramyan et al., 2002). Dry weight of bacteria was determined as described (Trchounian and Vassilian, 1994).

Determination of proton motive force and intracellular pH. Δp was calculated as a sum of Δφ and ΔpH according to $\Delta\mu_{H+}/F=\Delta\varphi-Z\Delta pH$ (negative value in mV) (Skulachev et al., 2010), where Z is RT/F equal 61.1 mV at 37^0C. Δφ was measured determining tetraphenylphosphonium cation (TPP^+) distribution between the bacterial cytoplasm and the external medium at a steady-state level as described (Zakharyan and Trchounian, 2001). The assay was done in a thermo-stated vessel of 2 ml with 150 mM Tris-HCl buffer containing 1 μM TPP^+. The changes in the TPP^+ concentration were determined by using a TPP^+-selective electrode. The pH_{in} was determined by the distribution of weak base - 9-aminoacridine (9-AA) across the membrane according to ΔpH (Puchkov et al. 1983). The assay was done in the buffer as above but containing 10 μM 9-AA. The 9-AA fluorescence was measured with a spectrofluorimeter (SPEX Fluoro Max, France) with excitation at 324 nm and emission at 451 nm.

RESULTS AND DISCUSSION

H_2 production by *E. coli* during glycerol fermentation at different pH and in the presence of inhibitors or during osmotic stress.

H_2 is one of the fermentation end products of *E. coli* and other bacteria that can be widely used in rather different branches of industry and human life (Momirlan and Veziroglu, 2005; Trchounian, 2013). Dharmadi et al. (2006) have discovered 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 but have not been established clearly.

E. coli possesses 4 membrane-bound hydrogenases (Hyd) catalyzing reversible oxidation of H_2 to $2H^+$. Hyd-1 and Hyd-2 are H_2 uptake enzymes during glucose fermentation and operate in a reverse mode during glycerol fermentation (Trchounian and Trchounian, 2009). Hyd-3 and Hyd-4 are H_2 producing enzymes during glucose fermentation and function in a reverse mode during glycerol fermentation (Trchounian and Trchounian, 2009). Each Hyd is likely to function primarily in one direction depending on fermentation substrate, pH and other conditions (Trchounian et al., 2012b).

Hyd-1 is encoded by the *hya* operon, expression of which is induced under anaerobic conditions at acidic pH (King and Przybyla, 1999). Hyd-2 is encoded by the *hyb* operon (Laurinavichene et al., 2002) and its maximal expression is attained in alkaline medium (King and Przybyla, 1999). Hyd-3 and Hyd-4 encoded by the *hyc* and *hyf* operons, respectively

(Trchounian et al., 2012b), 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. For neutral and alkaline pH, Hyd-4 with Fdh-H forming the FHL-2 pathway becomes responsible for H_2 production (Trchounian et al., 2012b).

Interestingly, the activity of Hyd-3 and Hyd-4 both is related with the F_0F_1-ATPase (Trchounian et al., 2012b). 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 (Trchounian, 2004). To establish which Hyd is responsible for H_2 production under the latest conditions, different mutants were constructed. In addition, to understand mechanisms of Hyd activity and regulation as well as relationship with H^+ transport, the inhibitor's effects were determined.

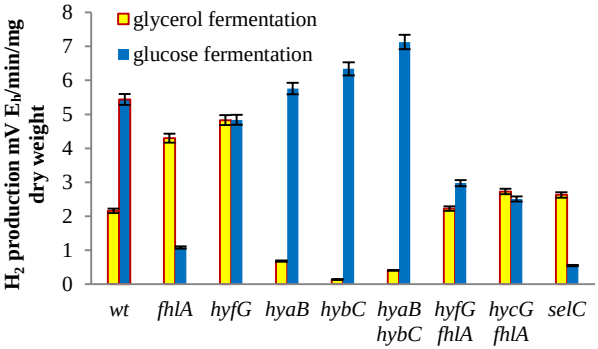

Fig. 1. H_2 production by *E. coli* wt and different Hyd defective mutants during glucose or glycerol fermentation at pH 7.5. For others see Materials and Methods. Here and in other figures the experiments were carried out 3-5 times and standart deviations are not more than 3%.

V_{H2} at pH 7.5 was ~2.2 mV E_h/min/mg dry weight (see Fig. 1). This value was ~2.5-fold lower than that with bacteria grown on glucose. These data are consistent with previous work with either whole cells or protoplasts (Bagramyan et al., 2002; Trchounian et al., 2012b). It is interesting that during *E. coli* growth on glycerol for 18–22 h medium pH decreased from 7.5 to 7.25 although upon glucose fermentation pH decreased from 7.5 to 6.8. This difference may indicate lower acidification of the medium due to ethanol extrusion and less formation of different acids or their changed proportion by glycerol fermentation as suggested (Murarka et al., 2008). Furthermore, V_{H2} was markedly (~2-fold) increased (see Fig. 1) for the *fhlA* mutant. The result obtained might point out that Hyd-3 at least is operating in a reverse mode under glycerol fermentation at pH 7.5. If H_2 production results from activity of different Hyd, and its rate is increased under insignificant Hyd-3 and Hyd-4 activity due to *fhlA* deletion, it might be suggested that Hyd-3 and Hyd-4 operate in H_2 uptaking but not in H_2 producing mode. V_{H2} was shown to be lowered (~3-fold) for the *hyaB* mutant and to be less or only residual in the *hybC* mutant (see Fig. 1). In contrast, during glucose fermentation V_{H2} was almost the same for wt and mutants studied. These data point out clearly that under glycerol fermentation Hyd-2 is mostly responsible for H_2 production; a less participation of Hyd-1 is probable. Moreover, the results suggest that Hyd-2 can be a reversible enzyme to operate in H_2 producing mode under glycerol fermentation whereas this enzyme has been shown (Trchounian et al., 2012b) to be

functioning as re-oxidizing H_2 under sugar fermentation. H_2 production by *E. coli* upon glycerol fermentation was inhibited by DCCD (Fig. 2).

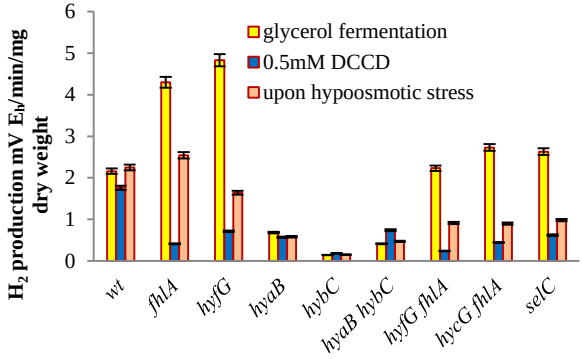

Fig. 2. H_2 production inhibition by DCCD or with hypo-osmotic stress in *E. coli* wild type and different Hyd mutants. The DCCD was in 0.5mM concentration and osmotics stress was performed with 0.8M sucrose. For others see legends to Fig. 1.

However, in contrast with DCCD effect on H_2 production under glucose fermentation, higher concentration of DCCD (0.5 mM) was effective. Thus, DCCD inhibition of V_{H2} might indicate an involvement of F_0F_1 in H_2 production or otherwise that might occur due to inhibition of Hyd. Under hyper- and hypo-osmotic stress H_2 production was the same for the *E. coli* wt. These data may put forward a new property of Hyd activity by *E. coli* regarding osmotic stress sensitivity of these enzymes under glycerol fermentation.

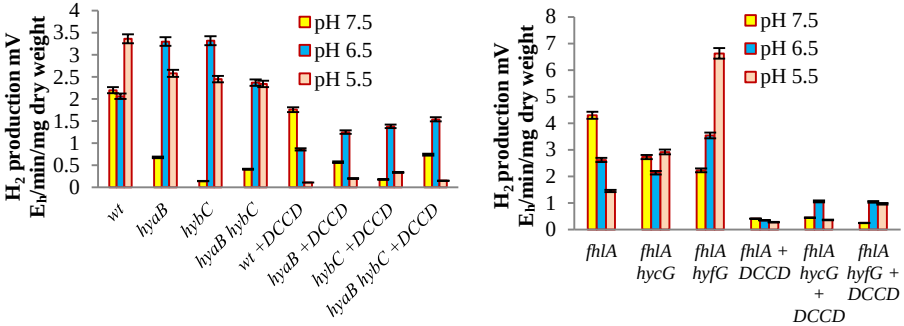

Fig. 3. H_2 production by *E. coli* wt and Hyd-1 and Hyd-2 or Hyd-3 and Hyd-4 mutants during glycerol fermentation at pH 7.5, 6.5 and 5.5. For others see legend to Fig. 1.

V_{H2} 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 compared to glucose fermentation at appropriate pH (see Fig. 3). It was inhibited by DCCD; however, DCCD inhibition of H_2 production was increased with pH decreasing. DCCD inhibition of Hyd is interesting to understand a role of H^+ transport through the membrane including that via F_0F_1, since defects in F_0F_1 might stimulate H_2 production at pH 7.5. H_2 production study with *hyaB* and *hybC* mutants 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. 3) 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 and double *fhlA hycG* and *fhlA hyfG* mutants was lowered at pH 6.5; it was less at pH 5.5. These results show a responsibility of Hyd-3 and Hyd-4 and suggest a role of *fhlA* gene in regulation of Hyd-3 and Hyd-4 activity for H_2 production under glucose fermentation at a low pH. It could be concluded that reversibility is likely to be a property of Hyd having a key role in regulation of H_2 metabolism under different environment.

Hydrogenase specific activity during glucose or glycerol fermentation at different pHs. The activity of Hyd enzymes can be assessed by measuring the H_2-dependent reduction of the electron acceptor BV (Ballantine and Boxer, 1985). During growth on glucose wt at pH 7.5 yielded a total Hyd specific activity of 3.34 U (mg of protein)$^{-1}$ (Fig. 4), while the activities after growth at pH 6.5 and 5.5 were 2.05 U and 0.82 U (mg of protein)$^{-1}$, respectively (Fig. 5). Thus, a direct correlation between medium pH and total Hyd activity was observed, with the lowest activity being measured after growth at low pH. Analysis of an extract derived from *hyaB* mutant, clearly showed that Hyd-1 contributed little to the Hyd specific activity during growth at pH 7.5. On the other hand in *fhlA* mutant or in *fhlA hyfG* double mutant an approximate 60% reduction in total Hyd activity (1.38 U (mg of protein)$^{-1}$; Fig. 4) was observed, indicating that Hyd-3 of the FHL complex made a significant contribution to total Hyd activity under these conditions. Hyd-2 was responsible for the rest of the activity because a mutant (*hyaB hybC selC*) phenotypically devoid of Hyd-1, Hyd-2 and the FDH-H component of the FHL complex essentially lacked Hyd activity at pH 7.5 (Fig. 4).

After growth at pH 7.5 on glucose the total Hyd enzyme activity in a mutant lacking Hyd-2 was only approximately 30% of the wt, yet a mutant lacking Hyd-1 and Hyd-2 both retained 60% of the wt activity (see Fig. 4).

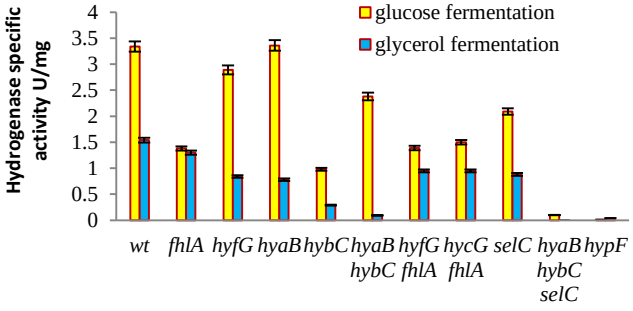

Fig. 4. Hydrogenase specific activity of *E.coli* wt and different Hyd mutants grown during glucose or glycerol fermentation at pH 7.5. For others see Materials and Methods.

This finding suggests that the enzymes necessary for [NiFe] cofactor biosynthesis common to all three Hyd enzymes limited the synthesis of active Hyd-3 and this limitation was removed by preventing Hyd-1and Hyd-2 synthesis. Growth at pH 6.5 revealed the contribution of Hyd-1 to total Hyd activity at acidic pH, where deletion of the *hyaB* gene reduced activity to 65% (1.36 U (mg of protein)$^{-1}$) of wt (Fig. 5,A). Deletion of the *hybC* gene reduced activity to 25%

of wt type but combining the *hyaB* and *hybC* mutations did not reduce Hyd activity. A further important observation from the growth studies at pH 6.5 was the finding that in *hyaB hybC selC* triple mutant did not abolish Hyd activity completely, 15% of wt was retained (Fig.5,A). This finding suggests that at slightly acidic pH Hyd-3 was stabilized and retained activity in the absence of the FDH-H component. A mutant unable to synthesize the FHL complex retained between approximately 45–60% Hyd activity (Fig. 5,A). This level of Hyd was due to the combined activities of Hyd-1 and Hyd-2 (Fig. 5,A). At pH 6.5 and above, the *selC* mutation caused reduction of the total Hyd activity by 20–30%, presumably due to partial destabilization of the FHL complex.

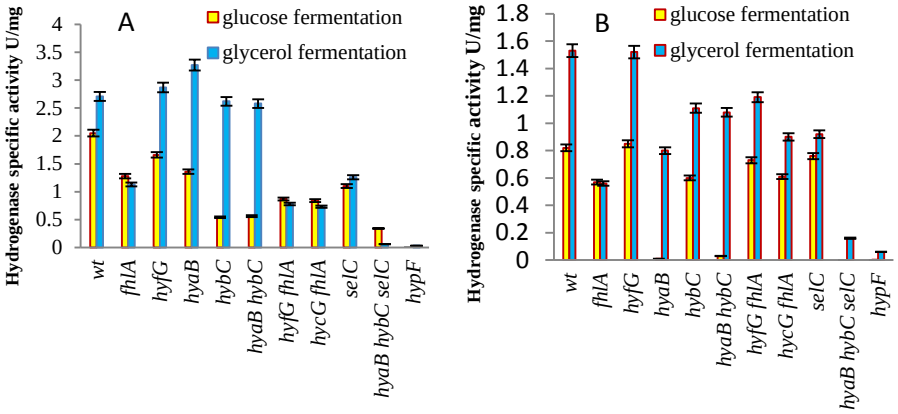

Fig. 5. Hydrogenase specific activity of *E.coli* wt and different Hyd mutants grown during glucose or glycerol fermentation at pH 6.5 (A) and 5.5 (B). For others see Materials and Methods.

At pH 5.5 the *selC* mutation had no effect on Hyd activity, suggesting that Hyd-3 contributed little to the overall Hyd activity at this low pH (see Fig. 5,B). This assumption was supported by the limited reduction in total Hyd activity in a mutant unable to synthesize Hyd-3 (see Fig. 5,B). A mutant unable to synthesize active Hyd-2 also retained 75% of the total Hyd activity compared to wt grown at pH 5.5 (see Fig. 5,B). An unexpected finding was the complete dependence on active Hyd-1 for H_2-oxidizing activity; a mutation in the *hyaB* gene or all combinations of multiple mutations in which *hyaB* was also deleted resulted in a strain without measurable Hyd activity (see Fig. 5,B). These findings indicate that during growth at low pH Hyd-1 has an essential role in H_2 metabolism. The nature of this pH dependence on Hyd-1 at low pH is unclear but might be related to a role of the enzyme in $\Delta\mu_H^+$ maintenance (King and Przybyla, 1999).

During growth on glycerol the highest Hyd specific activity for wt (2.7 U (mg of protein)$^{-1}$) was observed after growth at pH 6.5 (see Fig. 5,A), which contrasts with what was observed for glucose fermentation. Growth of wt at pH 7.5 or pH 5.5 resulted in a similar total Hyd specific

activity. It should be noted that the total Hyd activity of wt grown in peptone medium without addition of glycerol or glucose was 0.2 U (mg of protein)$^{-1}$, 0.3 U (mg of protein)$^{-1}$ and 0.44 U (mg of protein)$^{-1}$ after growth at pH 7.5, 6.5 and 5.5, respectively. This indicates that supplementation of peptone-based growth medium with glycerol resulted in a 6- to 10-fold increase in total Hyd enzyme activity, depending on pH. In contrast to what was observed for glucose-grown cells, in cells grown in the presence of glycerol at pH 7.5 Hyd-1 and Hyd-2 were the main contributors to total Hyd enzyme activity (see Fig. 4) because the specific activity measured after growth of a *hyaB hybC* double mutant was approximately 5% of the activity observed in wt. Deletion of the *hybC* gene resulted in an 80% decrease in enzyme activity (see Fig. 4), while in a single *hyaB* mutant the Hyd specific activity decreased by 50%. Analysis of Hyd-1 and Hyd-2 activities using the in-gel assay revealed that the activity band associated with Hyd-1 in wt was rather weak in intensity (compare lanes D1 and wt in Fig. 6,F), despite the fact that the *hyaB* mutation caused a significant decrease in total Hyd activity (see Fig. 4). Hyd-2 revealed an additional, rapidly migrating activity band in cells grown in glycerol-peptone medium at pH 7.5 (see band labeled 20 in Fig. 6,F), which was still observed in a *hyaB* background but was absent in a *hyaB hybC* double mutant.

At pH 6.5 wt had the highest Hyd specific activity (2.7 U (mg of protein)$^{-1}$) and when the genes encoding the large subunits of Hyd-1 and Hyd-2 were deleted either individually or in combination, little effect was observed on total Hyd activity (see Fig. 5,A). The residual activity appeared to be Hyd-3-dependent because a mutant with combined deletions of *hyaB, hybC* and *selC* essentially abolished Hyd activity (see Fig. 5, A). The activity bands of Hyd-2 were also observed to be weak after growth in glycerol-peptone medium at pH 6.5 (Fig. 6, E), consistent with the bulk of the activity measured in the liquid assay resulting from the activity of Hyd-3. Furthermore, growth at pH 5.5 revealed that Hyd-1 and Hyd-2 made similar overall contributions to total Hyd activity (Figs. 5,B, 6,D,) and deletion of the *hyaB* and *hybC* genes reduced the total activity by approximately 30%, which indicates that Hyd-1 in particular had a much less significant impact on total Hyd activity at this pH compared with growth on glucose at pH 5.5 (see Fig. 5,B). It is conceivable that in the absence of either Hyd-1 or Hyd-2, more Hyd-3 can be matured by the Hyp proteins (Bock et al., 2006), resulting in an apparently less significant impact of the double *hyaB hybC* null mutations (see Fig. 5,B). Total Hyd activity was essentially abolished in the *hyaB hybC selC* triple mutant (Fig. 5,B).

pH-dependent activity of Hyd-1 and Hyd-2 revealed by activity-staining after native-PAGE. The activity of Hyd-1 and Hyd-2 can be readily visualized by in-gel staining for Hyd activity after native-PAGE; the labile nature of Hyd-3 means that the activity of this enzyme cannot be detected after electrophoresis. Thus, extracts derived from wt and mutants lacking Hyd-3 or Hyd-4 but retaining Hyd-1 and Hyd-2, show the same pattern of activity bands as observed for the lane labeled Δ3 (Fig. 6,A). Hyd-1 can be identified as a single activity band, which is absent in a *hyaB* mutant. Hyd-2, in contrast, migrates more slowly than Hyd-1 and exhibits two active species; in a mutant deleted for the *hybC* gene, the two active Hyd-2 bands cannot be visualized but the activity band due to Hyd-1 was retained (Fig. 6). Extracts of a

mutant deleted for *hyaB* and *hybC* both showed neither Hyd-1 nor Hyd-2 activity bands (lane Δ (1+2) in Fig. 6,A). A similar phenotype was observed in a strain carrying a deletion in the *hypF* gene, which consequently was unable to synthesize any active Hyd (see lanes labelled ΔF, Fig. 6).

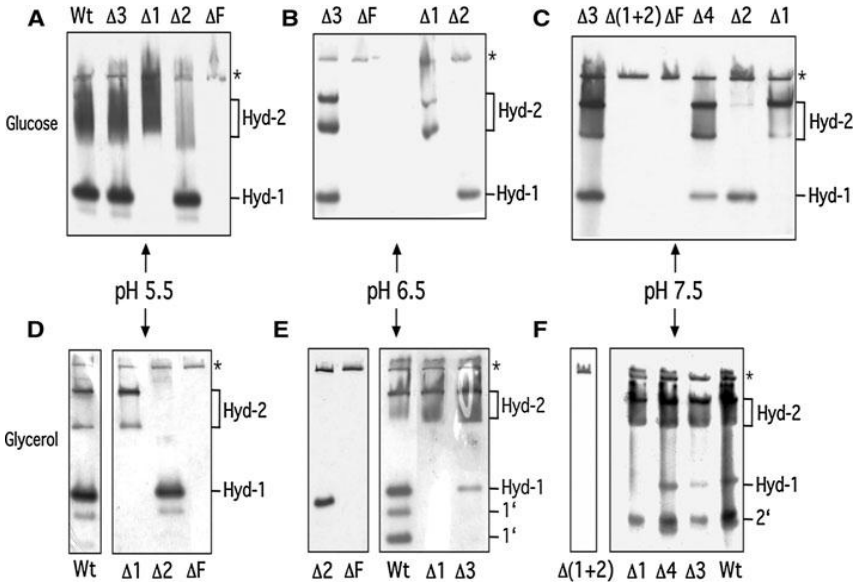

Fig. 6. Identification of active Hyd-1 and Hyd-2 by activity staining after native-PAGE. Crude extracts derived from *E. coli* wt and different mutants grown on glucose (A-C) or glycerol (D–F) at different pH were analyzed. The locations of Hyd-1 and Hyd-2 in the gels are shown on the right of each panel. Where $1^{/}$ is signified this indicates a rapidly migrating form of Hyd-1 and where $2^{/}$ is shown, this signifies a more rapidly migrating form of Hyd-2. The asterisk near the top of each gel designates a hydrogenase-independent activity band. To simplify the nomenclature of the strains wt and mutants were given the following phenotypic designations: Δ1 (*hyaB*); Δ2 (*hybC*); Δ3 (*fhlA*); Δ4 (*hyfG*); Δ(1+2) (*hyaB hybC*); ΔF (*hypF*).

The weak, slowly migrating activity band observed in all extracts, including that of the *hypF* mutant, is independent of the [NiFe]-Hyd but is caused by a side-activity of FDH-N and -O (Soboh et al., 2011).

The increase in activity of Hyd-1 after growth at low pH compared to growth at high pH can be readily observed for glucose-containing peptone medium (see Fig. 6,A-C). Although after growth at pH 5.5 total Hyd activity was dependent on Hyd-1, an extract derived from a *hyaB* mutant retained a weak activity of Hyd-2 (see Fig. 6,C) that appeared as a smear on the gel rather than as the clearly defined activity bands observed at higher pH.

Dependence on the F_0F_1-ATP synthase for the activities of the H_2-oxidizing hydrogenases 1 and 2 during glucose and glycerol fermentation at high and low pHs. Initially, the Hyd activity in the mutant DK8 (see Table 1), was determined during glucose fermentation and in response to the pH of the medium. Previous studies have shown that the *E. coli* F_0F_1-ATPase has the highest activity at alkaline pH (pH>7.0) (Bagramyan et al. 2002). Upon glucose fermentation at pH 7.5 no Hyd activity in DK8 could be measured while a high Hyd activity was measured in wt (Fig. 7,A). Analysis of Hyd-1 and Hyd-2 enzyme activity by in-gel staining revealed that there were no activity bands corresponding to Hyd-1 or Hyd-2 in DK8 (Fig. 8,A), which corroborated the findings of the enzyme assays. A Hyd-independent activity migrating near the top of the gel (marked by an asterisk in Fig. 8) was unaffected by the *atp* mutation, and this activity acted as a loading control in further experiments. Growth of DK8 at pH 6.5 resulted in a Hyd activity of 0.15 U (mg protein)$^{-1}$ while after growth with glucose at pH 5.5, a Hyd activity of 0.19 U (mg protein)$^{-1}$ was determined. Wt grown under the same conditions had activities of 2.05 and 0.82 U (mg protein)$^{-1}$, respectively (Fig. 7,A). These data indicate an inverse correlation between medium pH and Hyd activity in DK8 (Fig. 7,A).

In the in-gel Hyd activity assay, very weak activity bands corresponding to Hyd-1 and Hyd-2 were observed only after growth at pH 6.5 in DK8, while wt showed strong activity bands, particularly for Hyd-1 (Fig. 8,B). After growth of DK8 at pH 5.5 activity of Hyd-2 could not be observed and that of Hyd-1 was barely detectable, indicating that the activity of both H_2-oxidizing enzymes was severely affected in *atp* mutant, particularly at extreme pHs (Fig. 8,C).

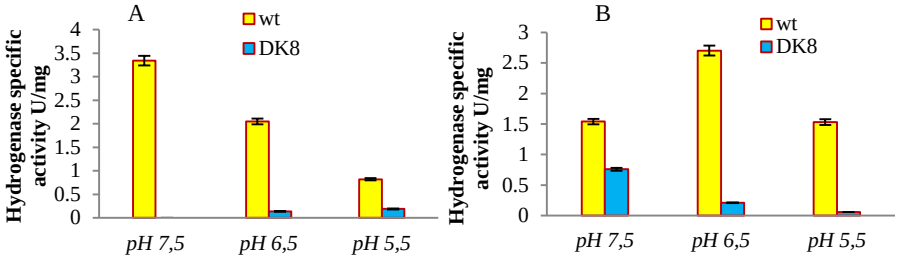

Fig. 7. Hydrogenase activity of *E. coli* wt and DK8 mutant strains grown and assayed at different pH on peptone medium supplemented with glucose (A) or glycerol (B). For others see Materials and Methods.

As a control, *hyaB hybC* double mutant failed to reveal any activity band (Fig. 8,C). Notably, the in-gel staining data indicated that the weak Hyd-independent enzyme activity due to FDH, and designated by an asterisk, was observed under all growth conditions with equal intensity in wt and *atp* mutant. This indicates that the effects observed in DK8 grown on glucose at pH 5.5 did not affect all oxidoreductases and that the residual Hyd activity in the mutant was contributed by Hyd-3. These findings are also in accordance with the strong inhibitory effect of DCCD on the activity of FDH-H and H_2 production at pH 7.5 (Trchounian et al., 2011b). This effect could be due either to the lack of active F_0F_1 directly or may be mediated by a deficient $\Delta\mu_{H^+}$ (Trchounian, 2004). During growth on glycerol, a double mutant

lacking both Hyd-1 and Hyd-2 had elevated F_0F_1-ATPase activity at pH 7.5 but not at pH 5.5 (Blbulyan et al., 2011). These results have been interpreted to indicate that the activity of Hyd-1 and Hyd-2 has a strong influence on the activity of F_0F_1. At pH 7.5, DK8 had a lower Hyd-activity (by 50%) than wt (see Fig. 7,B).

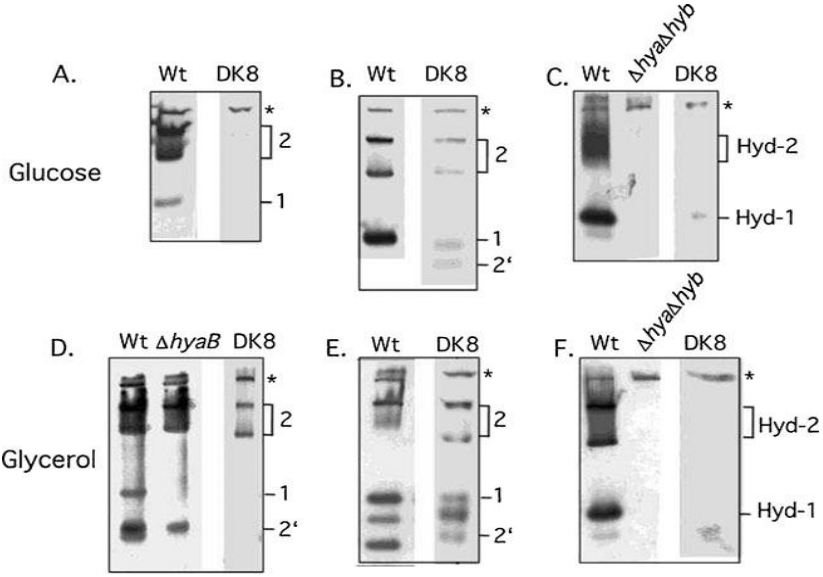

Fig. 8. Analysis of active Hyd-1 and Hyd-2 in *E. coli* by activity staining after non-denaturing-PAGE. Strains were grown either with glucose (A, B, C) or glycerol (D, E, F) as indicated and at pH 7.5 (A, D), pH 6.5 (B, E) or pH 5.5 (C, F). For others see legends to Fig. 6.

In-gel activity staining revealed that this activity was mainly due to Hyd-2 but not Hyd-1 (see Fig. 8,D). Under these conditions the activity of Hyd-3 is usually absent. After growth of wt at pH 6.5 the total Hyd activity was similar to that during glucose fermentation and the mutant had a value of approximately 10% of the activity in wt (see Fig.7,B). The in-gel assay demonstrated that Hyd-2 and Hyd-1 both were active under these conditions (see Fig. 8,E). Growth of wt at pH 5.5 with glycerol resulted in a Hyd specific activity of 1.5 U (mg protein)$^{-1}$ (see Fig. 7,B). In contrast, the Hyd activity of DK8 was barely detectable (see Fig. 7,B). In-gel activity-staining revealed that neither Hyd-1 nor Hyd-2 activity could be detected (see Fig. 8,F). The results with F_0F_1-negative mutant point to a requirement for active F_0F_1 for the activity of Hyd-1 and Hyd-2 during glucose or glycerol fermentation. Moreover, the data obtained indicate that there is an inverse correlation between Hyd activity and pH during fermentative growth on glucose and a direct correlation between Hyd activity and pH during glycerol fermentation. While both enzymes retain low activity during growth at pH 6.5 in the *atp* mutant, both are inactive at more extreme pHs. These data demonstrate a metabolic link between F_0F_1 activity and H_2-oxidizing activity and underscore the key role of Hyd-1 and Hyd-2 in energy conservation.

Anaerobic growth of *E. coli* on mixed carbon (glucose+glycerol) sources fermentation at pH 6.5, E_h drop and H_2 production in the assays supplemented with glucose or glycerol. *E. coli* wt and mutant cells grown during mixed carbon (glucose and glycerol) fermentation at pH 6.5 yielded an OD of ~1.0 at 20–22 h. V_{H2} in the assays supplemented with glucose was ~2.35 mV E_h/min/mg dry weight for wt grown during mixed carbon fermentation (Fig. 9) (Trchounian, 2012). This was ~2 fold lower than that in wt grown on glucose, and it was almost same for the cells grown on glycerol. In the assays with glycerol, V_{H2} was only ~0.43 mV E_h/min/mg dry weight (Fig. 9).

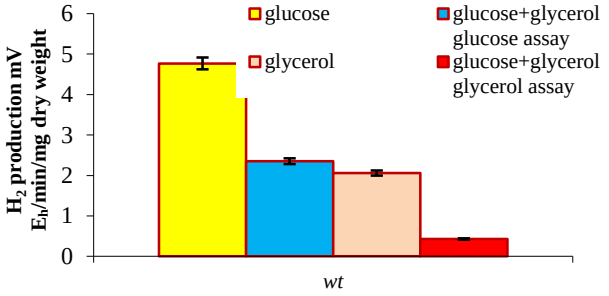

Fig. 9. H_2 production by *E. coli* wt during mixed carbon (glucose and glcyerol) fermentation at pH 6.5. Bacteria were grown in glucose, glycerol or mixed carbon (glucose and glycerol). For others see Materials and Methods.

This was ~4.5 fold lower than that for the cells grown on glycerol. Thus, *E. coli* produced H_2 during mixed carbon fermentation at pH 6.5, its production rate depended on the supplemented carbon source during the assays. These findings may have different explanations based on glucose and glycerol transport and biochemical pathways of their oxidation; however, a further study is required.

V_{H2} by *hyaB*, *hybC* single and *hyaB hybC* double mutants was lowered ~1.5 fold, compared to wt when glucose was added whereas it was increased in the cells grown on glucose only (Fig. 10,A). H_2 production by these mutants was similar or less than the cells grown on glycerol or mixed carbon, respectively, when glycerol was supplemented (Fig. 10,B). These data showed that Hyd-1 and Hyd-2, might operate in reverse, H_2 producing mode, when cells were grown on mixed carbon. Alternatively, they might be involved in H_2 recycling and supply H^+ to Hyd-3 and Hyd-4 for H_2 production. This suggestion agrees with the idea proposed by Lukey et al. (2010) concerning the compensatory uptake functions of Hyd-1 and Hyd-2. In *fhlA*, *hyfG* single and *fhlA hyfG* double mutants grown on mixed carbon, V_{H2} was decreased ~2 fold compared to wt when glucose was supplemented (Fig. 10,A). These data point out that Hyd-3 and Hyd-4 play key role in H_2 production. In addition, Hyd-4 could be proposed to be a compensatory or reserve Hyd enzyme under mixed carbon fermentation that is becoming active at different conditions (Trchounian et al., 2012b). The results are significant and suggest a new role of Hyd-4 in H_2 production during mixed carbon fermentation. In this respect, it is interesting that H^+ transport becomes sensitive to DCCD in *hyf* but not *hyc* mutants and DCCD-sensitive H^+ release into the medium of the *hyf* mutant is ~50% (Trchounian et al., 2009). This could be considered as another finding that Hyd-4 plays some role at pH 6.5. Different data for *fhlA* and

fhlA hyfG mutants grown during glycerol fermentation were obtained with H_2 production when glycerol was supplemented.

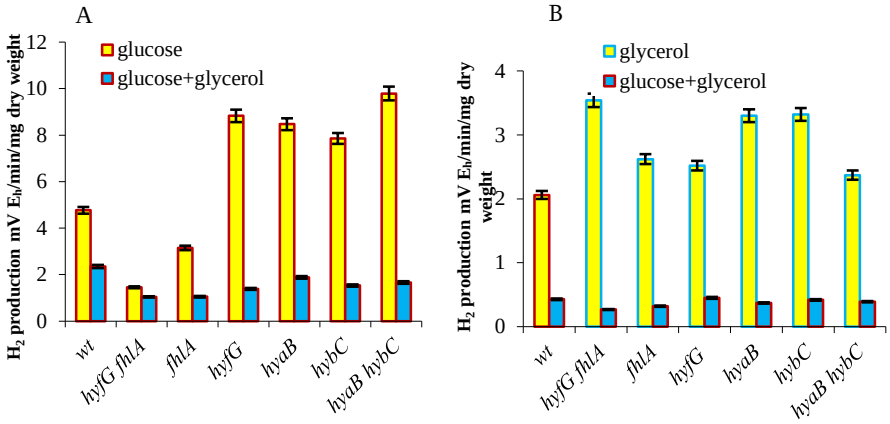

Fig. 10. H_2 production by *E. coli* wt and Hyd-1 and Hyd-2 or Hyd-3 and Hyd-4 mutants during mixed carbon fermentation at pH 6.5. Bacteria were grown in glucose, mixed carbon (glucose and glycerol) and assayed with adding glucose (A) or grown on glycerol, mixed carbon (glucose and glycerol) and assayed with adding glycerol (B). For others, see legends to Fig.9.

V_{H2} was increased by ~1.3 and ~1.7 fold, respectively (see Fig. 10,B). An explanation for these results might be followed if Hyd-3 and Hyd-4 serve as H_2 oxidizing enzymes instead of Hyd-1 and Hyd-2 under those mutations: this could be regulated by the FhlA protein. The interactions between Hyd enzymes forming H_2 cycling and other proteins seems to be probable (Redwood et al., 2008; Trchounian and Trchounian, 2009; Zbell and Maier, 2009; Trchounian et al., 2011ab; 2012ab).

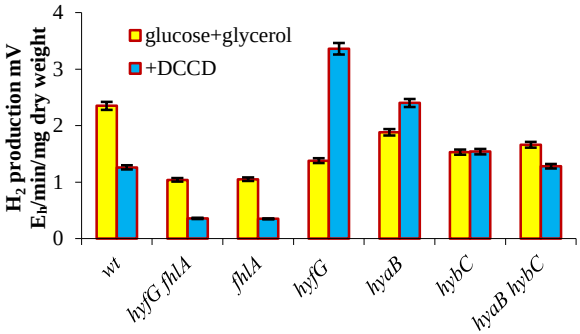

Fig .11. H_2 production inhibition by DCCD in *E. coli* wild type and different Hyd mutants grown during mixed carbon fermentation at pH 6.5. 0.3 mM DCCD was used. For others, see legends to Fig. 9.

In wt V_{H2} was inhibited ~2 fold when glucose was supplemented (see Fig. 11). This might indicate a role of F_0F_1 in H_2 production. DCCD inhibition of H_2 production was observed only

in *fhlA* and *fhlA hyfG* mutants; this reagent had no inhibitory effect on H_2 production for the other mutants used (see Fig. 11). These results indicate that F_0F_1 is not playing major role in H_2 production during mixed carbon fermentation at pH 6.5. Alternatively, the *fhlA* gene deletion can break the possible interaction of FhlA with F_0F_1. In *fhlA*, more than in the other mutants, F_0F_1 becomes involved in H_2 production since the *fhlA* mutation was shown to significantly increase ATPase activity in the absence of formate; this mutation can affect the conformational interaction between formate binding and ATP hydrolysis (Korsa and Bock, 1997). Thus, all these data suggest that FhlA could be associated with F_0F_1 for supplying H^+ to different Hyd for producing H_2.

Role of Hyd enzymes in Δp generation. Δp generation by *E. coli* during glycerol fermentation was first studied. At pH 7.5, the increase of Δφ was established to be only partially compensated by a reversed ΔpH, resulting in a low Δp (Fig. 12). It should be noted that pH_{in} and Δφ were lower and consequently Δp was also not higher when compared with glucose fermentation. This difference between glycerol and glucose fermentation might be due to distinguished mechanisms for H^+ transport, especially Hyd, besides of F_0F_1. Therefore, a role of Hyd in Δp generation during glycerol fermentation can be suggested. That could be different depending on pH.

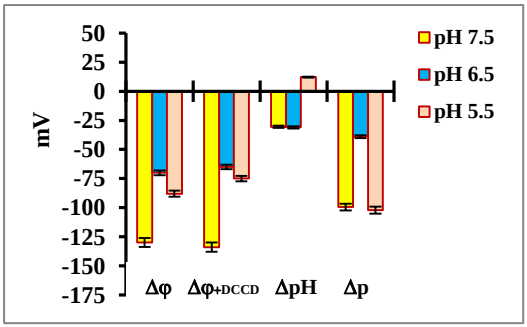

Fig. 12. The values of Δφ, ΔpH and Δp by *E. coli* wt whole cells grown during glycerol fermentation at different pHs. The DCCD (0.3 mM) was added into the assays mixture when indicated. ΔpH was calculated in mV (ZΔpH). For the others, see Materials and methods.

CONCLUSIONS

1. Different Hyd enzymatic activity and H_2 production rate were shown for *E. coli* wild type and mutants with defects in genes coding Hyd enzymes and its maturation as well as F_0F_1 during glycerol fermentation depending on pH.
2. During glycerol fermentation DCCD was demonstrated to inhibit H_2 production at acidic (pH 5.5) but not slightly alkaline (pH 7.5) medium and DCCD inhibition was reversed during glucose fermentation. Moreover Hyd-3 and Hyd-4 were shown to be sensitive to osmotic stress during glycerol fermentation at different pHs.
3. Hyd activity was inferred by native page electrophoresis to be dependent on the active F_0F_1 during glycerol or glucose fermentation, especially at extreme pHs.
4. Proton motive force generation with its chemical (ΔpH) and electrical components (Δφ) was detected during glycerol fermentation at different pHs. The pH_{in} and Δp was lower at pH 7.5

compared during glucose fermentation. Hyd impact on overall proton motive force generation was disclosed.

5. Hyd-2 mostly and Hyd-1 partially were shown to be responsible for H_2 production during glycerol fermentation at pH 7.5; Hyd-3 and Hyd-4 can work in H_2 oxidizing mode.
6. Hyd activity was established to be only Hyd-1 dependent during glucose fermentation at pH 5.5.
7. Hyd-4 activity was revealed during mixed carbon (glycerol and glucose) sources fermentation at pH 5.5 or pH 6.5.

LIST OF PUBLICATIONS AS A PART OF DISSRETATION TOPIC

1. ***Trchounian K.**, Soboh B., C., Sawers G., Trchounian A.* (2013) Contribution of hydrogenase 2 to stationary phase H_2 production by *Escherichia coli* during fermentation of glycerol. *Cell Biochem. Biophys*. **66**, 103-108.
2. ***Trchounian K.**, Blbulyan S., Trchounian A.* (2013) Hydrogenase activity and proton-motive force generation by *Escherichia coli* during glycerol fermentation. *J. Bioenerg. Biomembr*. **45**, 253-260.
3. ***Trchounian K.,** Trchounian A.* (2013) *Escherichia coli* hydrogenase 4 (*hyf*) and hydrogenase 2 (*hyb*) contribution in H_2 production during mixed carbon (glucose and glycerol) fermentation at pH 7.5 and pH 5.5. *Int. J. Hydrogen Energy* **38**, 3919-3927.
4. ***Trchounian K.*** (2012) Transcriptional control of hydrogen production during mixed carbon fermentation by hydrogenases 4 (*hyf*) and 3 (*hyc*) in *Escherichia coli*. *Gene* **506**, 156-160.
5. ***Trchounian K**., Poladyan A., Vassilian A., Trchounian A*. (2012) Multiple and reversible hydrogenases for hydrogen production by *Escherichia coli*: Dependence on fermentation substrate, pH and the F_0F_1-ATPase. *Crit. Rev. Biochem. Mol. Biol.* **47**, 236-249.
6. ***Trchounian K**., Pinske C., Sawers G., Trchounian A.* (2012) Characterization of *Escherichia coli* [NiFe]-hydrogenase distribution during fermentative growth at different pHs. *Cell Biochem. Biophys*. **62**, 433-440.
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ԹՌՉՈՒՆՅԱՆ ԿԱՐԵՆ ԱՐՄԵՆԻ

Ամփոփագիր

Հանգուցային բառեր՝ *Escherichia coli*, հիդրոգենազներ (Հիդ), հիդրոգենազային ակտիվություն, պրոտոնային F_0F_1-ԱԵՖազ, մոլեկուլային ջրածնի (H_2) արտադրություն, գլիցերոլի խմորում, ածխածնի աղբյուրների խառը խմորում, pH

Այս աշխատանքը նվիրված է *E. coli*-ում միջավայրի pH-ի տարբեր արժեքներում գլիցերոլի խմորման ընթացքում Հիդ-ային ակտիվության և H_2-ի արտադրության ուսումնասիրմանը: Նշված հիմնախնդիրների լուծման համար ստացվել և օգտագործվել են Հիդ-ները, ինչպես նաև F_0F_1-ԱԵՖազը որոշող գեներում խանգարումներով տարբեր մուտանտներ: Ուսումնասիրվել է նաև, թե ինչպես են ազդում տարբեր արգելակիչներն ու օսմոսային սթրեսը H_2-ի արտադրության գործընթացի վրա, որոշվել է պրոտոնաշարժ ուժը և ուսումնասիրվել H_2-ի արտադրության կապը F_0F_1-ԱԵՖազի հետ: Կատարվել է, գլիցերոլի խմորման ընթացքում ստացված արդյունքների, համեմատական վերլուծություն գլյուկոզի, ինչպես նաև ածխածնի խառը աղբյուրների (գլիցերոլի և գլուկոզի) խմորման հետ: Այս խնդիրների լուծման նպատակով օգտագործվել են Հիդ-ային ակտիվության որոշման կենսաքիմիական, H_2-ի արտադրության և բջջի թաղանթում պրոտոնաշարժ ուժի որոշման մանրէաբանական և կենսաէլեկտրաքիմիական մեթոդներ:

Կատարված հետազոտությունների արդյունքում գլիցերոլի (10 մլ/լ) խմորման պայմաններում թույլ հիմնային միջավայրում (pH 7,5) բացահայտվել են այն Հիդ-ները, որոնք պատասխանատու են H_2-ի արտադրության համար: Պարզվել է, որ H_2-ի արտադրության համար պատասխանատու են Հիդ-2-ը և մասամբ Հիդ-1-ը: Այն Հիդ-ները, որոնք պատասխանատու են H_2-ի արտադրության համար գլյուկոզի խմորման ընթացքում՝ այսինքն Հիդ-3-ը և Հիդ-4-ը, գլիցերոլի խմորման դեպքում գործում են հակադարձ՝ H_2-ի օքսիդացման ուղղությամբ:

Ցույց է տրվել, որ թույլ հիմնային pH-ում H_2-ի արտադրությունը թույլ է ճնշվում է *N,N'*-դիցիկլոհեքսիլկարբոդիիմիդի (ԴՑԿԴ) ազդեցությամբ. ենթադրվում է, որ Հիդ-1-ը կապված է F_0F_1-ԱԵՖազի հետ: Թույլ թթվային (pH 6,5) և թթվային pH-ներում (pH 5,5) H_2-ի արտադրության համար պատասխանատու է ՄՋԼ-1 համակարգը, որի բաղադրիչ է Հիդ-3-ը: Ավելին, H_2-ի արտադրությունն առավել ճնշվում է ԴՑԿԴ-ի ազդեցությամբ թթվային pH-ում և այստեղ կարևորվում է F_0F_1-ԱԵՖազի որոշիչ դերը:

Բացահայտվել է Հիդ-ների զգայունությունը բացասական օսմոսային սթրեսի նկատմամբ գլիցերոլի խմորման ընթացքում: Առանձհատուկ է գլիցերոլի խմորման պայմաններում բջջի թաղանթով պրոտոնաշարժ ուժի ստեղծումը և ներբջջային pH-ի ցածր արժեքը, որը կարող է ներգործել Հիդ-ների աշխատանքի վրա:

Ցույց են տրվել Հիդ-ային ակտիվության էական տարբերությունները գլիցերոլի և գլյուկոզի խմորման ընթացքում՝ կախված միջավայրի pH-ից, ինչպես ժելում, այնպես էլ քանակական արժեքներով: Բացահայտվել են տարբեր Հիդ-ների ակտիվությունը և H_2-ի արտադրության առանձհատկությունները ածխածնի աղբյուրների խառնուրդի (գլիցերոլ և գլյուկոզ) խմորման ընթացքում:

Ստացված արդյունքներն էական նշանակություն ունեն Հիդ-ների ակտիվության կարգավորման և H_2-ի արտադրության կենսատեխնոլոգիայի հետագա մշակման, ինչպես նաև դրանում գլիցերոլի որպես ածխածնի էժան աղբյուրի օգտագործման ընդլայնման համար:

ТРЧУНЯН КАРЕН АРМЕНОВИЧ

РЕЗЮМЕ

Ключевые слова: *Escherichia coli,* гидрогеназы (Гид), гидрогензная активность, протонная F_0F_1-АТФаза, производство молекулярного водорода (H_2), брожение глицерина, смешанное брожение источников углерода, pH

Эта работа посвящена исследованию Гид-ной активности и производства H_2 у *E. coli* при сбраживании глицерина при различных pH. Для решения этих основных задач получены и использованы различные мутанты с дефектами в генах, кодирующих Гид-ы, а также протонную F_0F_1-АТФазу. Также изучены эффекты разных ингибиторов и осмотического стресса на производство H_2, определена протонодвижущая сила и связь производства H_2 с F_0F_1-АТФазой. Был проведен сравнительный анализ с брожением глюкозы, а также со сбраживанием смешанных источников углерола (глицерина и глюкозы). Для решения этих задач использовали биохимические методы

определения Гид-ной активности, микробиолгические и биоэлектрохимические методы определения производства H_2.

В результате исследований было показано, какие Гид-ы ответственны за производство H_2 при брожении глицерина (10 мл/л) в слабощелочной среде (pH 7,5). Выяснено, что за производство H_2 ответственны Гид-2 и частично Гид-1. Те Гид-ы, которые были ответственны за производство H_2 при брожении глюкозы, в условиях сбраживания глицерина действуют как окислители H_2, то есть работают в обратном направлении.

Было показано, что производство H_2 при слабощелочном pH практически не ингибируется с помощью N,N'-дициклогексилкарбодиимида (ДЦКД), однако это не исключает связи Гид-1 с F_0F_1-АТФазой. В слабокислой (pH 6,5) и кислой (pH 5,5) среде за производство H_2 ответственен комплекс формиат водород лиазы (ФГЛ-1), компонентом которого является Гид-3. Кроме того, производство H_2 ингибируется с помощью ДЦКД при кислом pH, что предполагает ключевую роль F_0F_1-АТФазы.

Обнаружена чувствительность Гид к отрицательному осмотическому стрессу при брожении глицерина. Определены особенности генерирования протон-движушей силы на мембране клеток и низкого значения внутриклеточного pH в условиях сбраживания глицерина, что может влиять на работу Гид.

Более того, были выявлены существенные различия Гид-ой активности при брожении глицерина или глюкозы при разных pH как в количественном виде, так и в полиакриламидных гелях. Выявлена активность Гид и показаны особенности производства H_2 при сбраживании смешанных источников углерода (глицерина и глюкозы).

Полученные результаты имеют важное значение, как в регуляции активности Гид и совершенствования производства H_2 в биотехнологии, так и для дальнейшего расширения использования глицерина как дешевого источника углерода.