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REVIEW ARTICLE

Multiple and reversible hydrogenases for hydrogen production by *Escherichia coli*: dependence on fermentation substrate, pH and the F_0F_1-ATPase

Karen Trchounian[1], Anna Poladyan[1], Anait Vassilian[2], and Armen Trchounian[1,3]

[1]*Department of Biophysics,* [2]*Department of Ecology and Nature Protection, and* [3]*Department of Microbiology and Biotechnology of the Biology Faculty, Yerevan State University, Yerevan, Armenia*

Abstract

Molecular hydrogen (H_2) can be produced via hydrogenases during mixed-acid fermentation by bacteria. *Escherichia coli* possesses multiple (four) hydrogenases. Hydrogenase 3 (Hyd-3) and probably 4 (Hyd-4) with formate dehydrogenase H (Fdh-H) form two different H_2-evolving formate hydrogen lyase (FHL) pathways during glucose fermentation. For both FHL forms, the *hycB* gene coding small subunit of Hyd-3 is required. Formation and activity of FHL also depends on the external pH ($[pH]_{out}$) and the presence of formate. FHL is related with the F_0F_1-ATPase by supplying reducing equivalents and depending on proton-motive force. Two other hydrogenases, 1 (Hyd-1) and 2 (Hyd-2), are H_2-oxidizing enzymes during glucose fermentation at neutral and low $[pH]_{out}$. They operate in a reverse, H_2-producing mode during glycerol fermentation at neutral $[pH]_{out}$. Hyd-1 and Hyd-2 activity depends on F_0F_1. Moreover, Hyd-3 can also work in a reverse mode. Therefore, the operation direction and activity of all Hyd enzymes might determine H_2 production; some metabolic cross-talk between Hyd enzymes is proposed. Manipulating of different Hyd enzymes activity is an effective way to enhance H_2 production by bacteria in biotechnology. Moreover, a novel approach would be the use of glycerol as feedstock in fermentation processes leading to H_2 production, reduced fuels and other chemicals with higher yields than those obtained by common sugars.

Keywords: Bacteria, glucose and glycerol fermentation, formate, hydrogenases, gene expression and regulation, pH, F_0F_1-ATPase, bioenergy

Introduction

Molecular hydrogen as potential fuel and its production by bacteria during mixed-acid fermentation

H_2 has a great potential as an ecologically-clean, renewable and capable fuel. It is generating no toxic by-products producing only water. H_2 has higher (~3-fold) energy content (~140 MJ/kg) than hydrocarbon fuels as oil (Momirlan & Veziroglu, 2005; Maeda *et al.*, 2007c). The most of H_2 is now produced from the water by the process of steam reforming or as a by-product from petroleum refining or chemicals production (Das & Veziroglu, 2001). However, H_2 may be produced from agricultural products and organic wastes by bacteria during either fermentative or photosynthetic processes. But fermentative H_2 production is more efficient than photosynthetic one. Significant reduction of energy costs is also important as these processes do not require heating or extensive electricity.

Among H_2-producing bacteria, *Escherichia coli* is the best-characterized bacterium, having established metabolic pathways and, importantly, there are many strains to manipulate genetically (Maeda *et al.*, 2007a,b,c; 2008a,b; 2011; Sanchez-Torres *et al.*, 2009; Hu & Wood, 2010).

During fermentation, the oxidation of common sugar (glucose) proceeds via consequent biochemical pathways (Figure 1); at the stage of phosphoenolpyruvate, some intermediates may be used for succinic acid formation, whereas all other end products are formed from pyruvate (Clark, 1989; Bock & Sawers, 2006; Poladyan & Trchounian,

Address for Correspondence: Prof. A. Trchounian, Department of Microbiology and Biotechnology of the Biology Faculty, Yerevan State University, 1 A. Manoukian Str., 0025, Yerevan, Armenia. Tel: 37410–570591. E-mail: Trchounian@ysu.am

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2009). The ratio of the forming products is variable, and it depends on the concentration of glucose, external pH ($[pH]_{out}$), oxidation–reduction potential (E_h) and other factors (Clark, 1989; Riondet *et al.*, 2000). It should be noted that the production of H_2 reduces organic acid formation and represents, therefore, an important factor in regulation of cytoplasmic pH ($[pH]_{in}$) in bacteria too.

Further oxidation of formic acid (HCOOH) to H_2 and carbon dioxide (CO_2) (Figure 1) is catalyzed by formate hydrogen lyase (FHL) pathway consisting of formate dehydrogenase H (Fdh-H) and hydrogenase (Hyd) (Peck & Gest, 1957). H_2 production and FHL pathway have intrigued microbial biochemists and biotechnologists for many decades: the study has led to the discovery of multiple and reversible [Ni-Fe] Hyd enzymes in *E. coli* (Bock & Sawers, 2006; Poladyan & Trchounian, 2009), of which Hyd-3 is a H_2-producing one (Sauter *et al.*, 1992). This has provided new insights into our understanding of the FHL, especially Hyd, formation and activity, its cofactors and other regulatory components.

Besides, formate is a highly reducing (E_h for formate:H_2 couple is –420 mV) and potentially high energy compound. Moreover, the change of standard free energy in this reaction is ~3.5 kJ/mol but in *vivo* conditions, it has the value of ~22 kJ/mol (Andrews *et al.*, 1997), so FHL reaction can be coupled to energy conservation. However, energetics of this reaction has not studied well.

Recently, it has been shown that *E. coli* can also utilize glycerol during anaerobic fermentative conditions in the absence of external electron acceptor with production of H_2; the latter is observed at acidic (Dharmadi *et al.*, 2006; Murarka *et al.*, 2008) and alkaline $[pH]_{out}$ (Trchounian & Trchounian, 2009; Trchounian *et al.*, 2011c). Gonzalez's group (Murarka *et al.*, 2008) has confirmed that the utilization of glycerol occurred in a fermentative manner, even when a low supplement (tryptone or mixture of amino acids) medium was used. Relevantly, no *E. coli* cell growth was observed when glycerol was omitted, and tryptone was shown not to serve as a source of electron acceptors (Murarka *et al.*, 2008). Glycerol fermentation is the novel intriguing finding which contradicts to an idea about inability of these bacteria to grow on glycerol under anaerobic conditions in the absence of fumarate as an electron acceptor (Kistler & Lin, 1971; Varga & Weiner, 1995; Booth, 2006). Glycerol metabolism represents a relatively simple cluster of biochemical reactions leading to glyceraldehyde-3-phosphate or to pyruvate, the entry points to the lower section of glycolysis (Figure 1). Succinic, acetic and formic acids and ethanol are shown to be produced under acidic conditions; no lactic acid was detected (Murarka *et al.*, 2008). This is linked to the availability of CO_2, which is produced by the formate oxidation through FHL and required for glycerol fermentation to proceed; H_2 has negative impact on glycerol metabolism (Dharmadi *et al.*, 2006; Gonzalez *et al.*, 2008; Murarka *et al.*, 2008). But glycerol metabolism during anaerobic fermentative conditions requires further physiological and biochemical study. It should be noted that glycerol is a cheap, abundant and highly reduced carbon compared to sugars and offers the opportunity to obtain H_2 and other reduced products by bacteria.

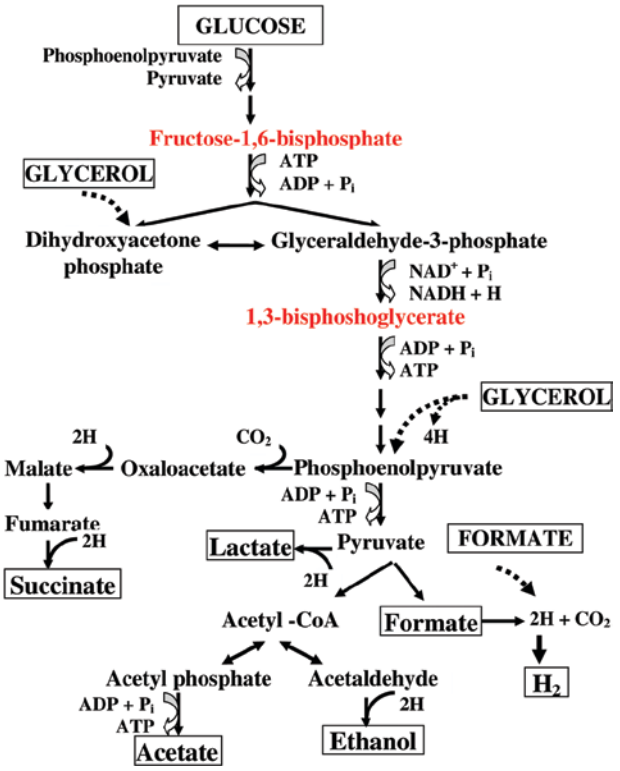

Figure 1. Mixed-acid fermentation pathways scheme in *E. coli*. Glucose oxidation follows via the Embden-Meyerhoff-Parnas pathway. Formation of lactic, formic, acetic, succinic acids and the other end products as well as further oxidation of formate resulting in formation of CO_2 and H_2 are shown. On the ways from phosphoenolpyruvate to pyruvate or from acetyl phosphate to acetate, ATP is synthesized on the level of substrate phosphorylation. Substrates involved in ATP formation are shown. Glycerol can be entered into the pathways on different steps shown; exogenic formate can be also oxidized (broken arrows).

H_2 production by *E. coli* grown at slightly alkaline $[pH]_{out}$ has been recently determined during glycerol fermentation: a different hydrogenase (Hyd-2 but not Hyd-3) is suggested to be mainly involved in such process (Trchounian & Trchounian, 2009). But Hyd function seems to be similar during glycerol and glucose fermentation at low $[pH]_{out}$ (Trchounian *et al.*, 2011c). However, it is necessary to note that mechanisms for H_2 formation during glycerol fermentation are not clear yet.

Hydrogenases and pathways for H_2 production by *E. coli* during glucose fermentation

Multiple and reversible hydrogenases

E. coli possesses four Hyd enzymes, which are catalyzing the simple oxidation–reduction reaction of $2H^+ + 2e^- \rightarrow H_2$; three of these (Hyd-1, Hyd-2 and Hyd-3) are characterized well.

Hyd-3 consists of several large and small subunits, denominated as Hyc, which are involved in intra-molecular electron transfer between oxidation–reduction centers. One of the large subunits is a Ni-containing [Fe-S]-protein, HycE, with molecular mass of 65 kDa; some of the small subunits contain $[4Fe\text{-}4S]^{2+}$ clusters (Bohm *et al.*, 1990; Sauter *et al.*, 1992; Rossmann *et al.*, 1994). The other Hyd enzyme, Hyd-4 denominated as Hyf, was postulated by Andrews's group (Andrews *et al.*, 1997). It is possible that Hyd-4 is also a Ni-containing protein that includes [Fe-S] clusters. Some Hyd-4 subunits are homologous to Hyd-3 subunits; they have not been isolated and studied yet. Interestingly, amino acid sequences of some small subunits of Hyd-3 and Hyd-4 are homologous to components of the electron transport chains (ETC) of bacteria, mitochondria, and plastids (e.g. NADH-ubiquinone-oxidoreductase, complex I) (Bohm *et al.*, 1990; Andrews *et al.*, 1997). Thus, Hyd-3 and Hyd-4 both are [Ni-Fe]-containing enzymes forming one group of Hyd enzymes; this group includes Hyd from archaea (Kunkel *et al.*, 1998) and many other bacteria (Vignais & Colbeau, 2004; Forzi & Sawers, 2007; Poladyan & Trchounian, 2009).

Two other Hyd enzymes, Hyd-1 and Hyd-2, oxidize H_2 and contribute electrons to the quinone pool (Ballantine & Boxer, 1985; 1986; Sawers & Boxer, 1986; Sargent *et al.*, 1998; Richard *et al.*, 1999). Hyd-1 is a Ni-containing protein, consisted of 3 different subunits (Sawers *et al.*, 1986). This enzyme affects H^+ translocation across the membrane and could be inhibited, for instance, by azide (Sawers *et al.*, 1986).

Hyd-1 is encoded by the *hya* operon; *hyaA, hyaB* and *hyaC* genes encode large and small subunits (Menon *et al.*, 1991). Four open reading frames *hyaD* through *hyaF* could encode different polypeptides (Menon *et al.*, 1990); however, their function is unknown: they probably have a role in modification of structural subunits and activity level of Hyd-1. The expression of *hya* is induced during fermentation under anaerobic conditions in acidic medium (King & Przybyla, 1999) and in the presence of formate but not nitrate (NO_3^-) during growth on glucose (Brondsted & Atlung, 1994; Richard *et al.*, 1999). However, Hyd-1 is not required for anaerobic growth; but this Hyd enzyme is required for the response to $[pH]_{out}$ shift from alkaline to acidic conditions (King & Przybyla, 1999; Trchounian *et al.*, 2011b). Since the proton-motive force (Δp) is changed upon $[pH]_{out}$ shift (Trchounian, 1997), Hyd-1 is suggested to function to maintain Δp in an energy-conserving manner.

Hyd-2 contains four different large and small subunits (Dubini *et al.*, 2002). The large subunit, HybC, has similarity with a large subunit of Hyd-1 (Ballantine & Boxer, 1986). It is oxidizing H_2 to H^+ and probably involved in their translocation by the other subunit, being an integral protein (Laurinavichene & Tsygankov, 2001). HybO small subunit with HybC forming the core enzyme is associated with two other Hyb proteins to complete the Hyd-2 complex (Dubini *et al.*, 2002).

Hyd-2 is encoded by the *hyb* operon (Menon *et al.*, 1994; Richard *et al.*, 1999). Its maximal expression is attained in alkaline medium (King & Przybyla, 1999) which is in agreement with the reported pH optimum of the purified enzyme (Ballantine & Boxer, 1986). Some *hyb* gene products are suggested to be involved in the maturation of Hyd-2 and Hyd-1 large subunits (Menon *et al.*, 1994; Blokesch *et al.*, 2001; Hube *et al.*, 2002). Therefore, metabolic cross-talk between Hyd-1 and Hyd-2 on the level of gene expression and enzyme activity is proposed. This cross-talk is also suggested when Hyd-2, but not Hyd-1, activity is enhanced upon the loss of Hyd-3 activity or when Hyd-1, but not Hyd-2, activity is reduced upon abolishing Hyd-3 activity (Menon *et al.*, 1994). Moreover, Hyd-3 activity is increased when *hyb* but not *hya* is deleted (Redwood *et al.*, 2008). Altogether is understood when interaction between Hyd enzymes is towards to recycle H_2; however, further detailed study is required.

The Hyd-1 and Hyd-2 activities exhibit a reciprocal dependence on the pH of the medium, when cells are grown on glucose (Trchounian *et al.*, 2011b). Indeed, Hyd-1 activity has an essential role in H_2 metabolism at low $[pH]_{out}$. The nature of this dependence on Hyd-1 is unclear.

Certain evidence has been obtained that these Hyd-1 and Hyd-2 both operate preferentially under different conditions (Laurinavichene et al., 2001). Unlike Hyd-1, Hyd-2 content is reduced by formate (Sawers *et al.*, 1985; Ballantine & Boxer, 1986). Hyd-2 is also sensitive to oxygen (Laurinavichene & Tsygankov, 2001; Lukey *et al.*, 2010) and much content of this protein is observed under anaerobic conditions (Richard *et al.*, 1999; Laurinavichene & Tsygankov, 2001). The study of oxidation–reduction properties of these Hyd-1 and Hyd-2 enzymes under glucose fermentation has revealed maximal Hyd-1 activity at oxidizing environment (E_h of +30 mV to +100 mV) whereas, Hyd-2 maximal activity appears in more reducing conditions (E_h below –80 mV) (Laurinavichene & Tsygankov, 2001; Laurinavichene *et al.*, 2001; 2002; Redwood *et al.*, 2008). The latter is confirmed by the absence of Hyd-2 activity under aerobic conditions (Lukey *et al.*, 2010).

Interestingly, Wood's group (Maeda *et al.*, 2007a) has recently obtained that Hyd-3 in *E. coli* might operate in a reverse direction having significant H_2 uptake activity like Hyd-1 and Hyd-2 during sugar fermentation. Moreover, in their turns, Hyd-1 and Hyd-2 can also function in reversible mode upon glycerol fermentation (Trchounian & Trchounian, 2009). Each Hyd enzyme is, therefore, likely to function in one direction, depending on fermentation substrate and other conditions; this is toward to recycle H_2.

Thus, Hyd-1 has an energy-conserving role, possibly acting to scavenge H_2 at low $[pH]_{out}$ (Sawers & Boxer, 1986; King & Przybyla, 1999). But Hyd-2 might reversibly oxidize H_2 (Lukey *et al.*, 2010), and functioning principally in H_2 oxidation, it has the potential to function as a "valve" to release excess reducing equivalents in the form of H_2.

Formate hydrogen lyases: expression of genes encoding subunits, FHL structure, different forms and functioning mechanisms

Genes encoding FHL subunits and their expression; FHL enzyme complex organization

The *E. coli* genome includes different genes organized in *fdh*, *hyc, hyf, hya* or *hyb* and other operons, products of which are involved in H_2 metabolism. First of all, the *fdh* genes encode Fdh-H and *fdhF* encodes the large subunit of Fdh-H (Wu & Mandrand-Berthelot, 1987). Protein products of *fdhD* and *fdhE* are required for catalytic activity of Fdh-H; the first is suggested to be a protease involved in formation or maturation of large subunit of Fdh-H, whereas the second one is essential for membrane binding.

Hyd-3 subunits are encoded by the *hyc* operon, which according to the Bock's group (Rossmann *et al.*, 1991; 1994) consists of 9 genes. The *hycA* gene is a transcriptional regulator for the whole operon and its protein product, HycA, antagonizes expression of other genes, particularly *fdhF* (Sauter *et al.*, 1992). The effect of HycA is possibly realized via binding to a formate-sensitive protein, FhlA (Maupin & Shanmugam, 1990; Schlensog & Bock, 1990; Schlensog *et al.*, 1994). The large subunit of Hyd-3 is encoded by the *hycE* gene (Bohm *et al.*, 1990), whereas the *hycB, hycF,* and *hycG* genes encode the small subunits of Hyd-3 (Sauter *et al.*, 1992). It is possible that *hycB* causes pleiotropic effects. Its protein product, HycB, is suggested by Sawers (1994) to be the small subunit of Fdh-H. This interesting idea was supported by the results that the protein product of this gene is required for the activity of Hyd-4 (Bagramyan *et al.*, 2002). Furthermore, the other protein product of this operon, HycH, is not a constituent part of Hyd-3, but is required for FHL formation (Sauter *et al.*, 1992). HycI forms a protease required for the Hyd-3 large subunit maturation (Rossmann *et al.*, 1994). Furthermore, the *hyc* operon expression depends on formate and $[pH]_{out}$ (Rossmann *et al.*, 1991) and, in addition, on molybdenum (Mo) (Self *et al.*, 1999); such regulation has been studied for the last years as well. Moreover, oxygen and NO_3^- suppress the expression of both the *hycB* and *fdhF* genes (Wu & Mandrand-Berthelot, 1987; Sauter *et al.*, 1992).

The *hyf* operon formed by 12 genes is predicted by Andrews *et al.* (1997) to encode Hyd-4. Nine of these genes encode subunits that are homologous to seven Hyd-3 subunits. The *hyfG* and *hyfI* genes encode large and small subunits, respectively; the *hyfR* gene encodes a formate-sensitive regulatory protein, and the terminal gene *focB* encodes formate permease. Protein products of the *hyfD, hyfE,* and *hyfF* genes probably represent integral membrane proteins that lack analogs in Hyd-3. These three proteins are assumed to give H^+-translocating activity to Hyd-4 (Andrews *et al.*, 1997), however such activity has not been shown yet although it is determined with Hyd-3 (Bagramyan *et al.*, 2002; Hakobyan *et al.*, 2005). It is possible, that these proteins might underline characteristic functions of Hyd-4 if they actually exist; this would be important for FHL energetics.

Thus, the *hyc* and *hyf* operons both include structural genes encoding small and large subunits of Hyd-3 and Hyd-4, respectively. There is also a whole set of genes encoding regulatory proteins definitely required for FHL maturation.

Moreover, different gene products in *E. coli* are required for appropriate gene expression, synthesis and maturation of FHL subunits, and formation of FHL complex (Bock *et al.*, 2006; Forzi & Sawers, 2007). They may be important for synthesis of ligands, coordination of Fe in active site, incorporation of Ni and Mo into corresponding subunits, the proteolytic maturation of large subunits and others (Hasona *et al.*, 1998; Magaon & Bock, 2000; Self *et al.*, 2001; Skibinski *et al.*, 2002; Rangarajan *et al.*, 2008). This is likely to be a remarkable complex pattern of most novel pathways. It is not ruled out that they might be involved in repression of other genes encoding components of ETC under aerobic or anaerobic conditions in the presence of oxygen or NO_3^-. In fact, *E. coli* cells contain various systems sensing aerobic, anaerobic or fermentative conditions such as the Arc or Fnr systems and others (Lin & Iuchi, 1991; Unden *et al.*, 2002; Kovacs *et al.*, 2005). However, their relationship to FHL remains unclear.

Based on structure of proteins, membrane localization of enzyme and expression of genes encoding its subunits, different models of FHL enzyme complex organization in *E. coli* have been proposed (Sauter *et al.*, 1992; Andrews *et al.*, 1997; Bagramyan & Trchounian, 2003; Poladyan & Trchounian, 2009). One model of FHL complex has been proposed by Sauter *et al.* (1992). According to this model, two catalytic components, Fdh-H and large subunit of Hyd-3, associated with the cytoplasmic side of the membrane, interact with other Hyd-3 subunits. The model requires experimental validation because of the little information about small subunits and precise pathways of reducing equivalents transfer within FHL. It is possible that functionally competent FHL complex includes other proteins too.

In spite of limited information about Hyd-4 subunits, the other model also suggests location of Fdh-H and large Hyd-4 subunit in cytoplasmic side, which bind to membrane via other subunits of Hyd-4 (Andrews *et al.*, 1997). Moreover, the *hycB* gene product has been shown to be required for the activity of Hyd-4 at neutral and slightly alkaline pH upon glucose fermentation (Bagramyan *et al.*, 2002) and would be, therefore, considered as a part of FHL functional complex. This could explain why *E. coli* mutants lacking Hyd-1, Hyd-2 and Hyd-3 do not produce H_2 (Sauter *et al.*, 1992; Self *et al.*, 2004; Hakobyan *et al.*, 2005).

Both these models are still rather speculative because of limited experimental data but they stimulate further study.

FHL enzyme complex structure, different forms and functioning mechanisms

In *E. coli*, FHL is a complex that consists of two enzymes, Fdh-H and Hyd; the latter is in two forms, Hyd-3 and

Hyd-4. FHL containing Hyd-3 is considered as FHL-1 whereas, the enzyme containing Hyd-4 is considered as FHL-2 (Andrews *et al.*, 1997; Bagramyan *et al.*, 2002; Mnatsakanyan *et al.*, 2004). This is likely during glucose fermentation or upon external formate added, but it might be different under other conditions.

Fdh-H is established to be a Se-cysteine (Stadtman *et al.*, 1991) and Mo-containing protein located at the cytoplasmic side of the membrane. It may have several subunits denominated as Fdh; the large subunit, FdhF, has a molecular mass of ~80 kDa (Pecher *et al.*, 1985; Gladyshev *et al.*, 1996) and exhibits catalytic properties (Cox *et al.*, 1981). FdhF contains one $[4Fe\text{-}4S]^{2+}$ cluster (Axley *et al.*, 1990; Axley & Grahame, 1991), which involves NAD^+ in the oxidation of formic acid to H_2 and CO_2. The protein has been already crystallized (Gladyshev *et al.*, 1996; Boyington *et al.*, 1997), the crystal structure is reinterpreted (Raaijmakers & Romao, 2006): Se-cysteine-140, a ligand of Mo in the original work (Boyington *et al.*, 1997), and essential for catalysis, is no longer bound to the metal after reduction of the enzyme with formate.

Fdh-H is stable; pH optimum for Fdh-H activity is 8.0 (Gladyshev *et al.*, 1996). Decrease in pH from 7.5 to 6.0 has been shown to be accompanied by the reduction of Fdh-H activity on 60%, which is also decreased in the presence of NO_3^- or azide (Axley *et al.*, 1990; Axley & Grahame, 1991). However, such dependence on pH could not be determined when Fdh-H operates in FHL (Bagramyan *et al.*, 2002; Trchounian *et al.*, 2011c).

There is a little information about active centers, dynamics and mechanisms of electron transfer in Fdh-H, Hyd-3 and Hyd-4, but principles revealed with different bacteria (Vignais & Colbeau, 2004; Poladyan & Trchounian, 2009) may be employed to image functioning mechanisms in *E. coli* (Figure 2). So, Ni-Fe active site in Hyd-3, for example, may be bridging to [4Fe-4S] clusters; cysteine residues are probably responsible for such bridges. The $[4Fe\text{-}4S]^{4+}$ clusters are able to transfer electrons to Ni^{2+} or Fe^{2+}; $[4Fe\text{-}4S]^{2+}$ state could be possible. These electrons interacting with H^+ can form bonds between Ni and Fe through H, and this may allow further transformation of $2H^+ \rightarrow H_2$ (Figure 2). Interestingly, the interaction of electrons with active center could be considered as an autocatalytic step in the reaction cycle and, moreover, two possible autocatalytic schemes as prion- and product-activation type are suggested (Osz & Bagynka, 2005).

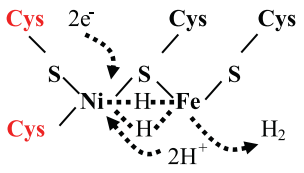

Figure 2. Electrons and H^+ transfers in [Ni-Fe]-containing active site of Hyd-3 or other proteins to form H_2 (Poladyan & Trchounian, 2009).

Since Hyd-3 and Hyd-4 are encoded by distinguished operons and characterized by different subunit composition and organization in the membrane, it is reasonable to assume that different FHL forms are functionally active under different conditions and therefore they play distinct roles in bacteria.

Factors determining functional activity of multiple membrane systems in bacteria include $[pH]_{out}$, E_h, the concentration of substrates and/or products of fermentation, the presence of some exogenous electron acceptors and the ratios of end products (Trchounian, 1997; 2004; Poladyan & Trchounian, 2009). These conditions probably determine FHL different forms (Bagramyan *et al.*, 2002; 2003; Mnatsakanyan *et al.*, 2002b; 2004).

Actually, our study with *E. coli* grown during glucose fermentation at neutral and slightly alkaline $[pH]_{out}$ has shown that H_2 production is not observed in *fdhF* and *hyf* mutants (Bagramyan *et al.*, 2002). Interestingly, H_2 production is detected in various *hyc* mutants lacking large and small subunits of Hyd-3, but it is not formed in the *hycB* mutant (Bagramyan *et al.*, 2002). These results suggest that the production of H_2 by *E. coli* at neutral and slightly alkaline medium involves FHL-2. The physiological role of FHL-2 is uncertain, but it may be required for generation of CO_2 for use in the formation of oxaloacetate from phopshoenolpyruvate during fermentation (Figure 1).

Formate concentration may also act as a regulatory factor (Rossmann *et al.*, 1991; Mnatsakanyan *et al.*, 2002b; 2004; Bagramyan *et al.*, 2003); various forms of FHL are active at formate micromolar and relatively high millimolar (≥30 mM) concentrations. Moreover, succinate (Figure 1) may also regulate FHL activity; addition of succinate together with glucose causes 2-fold increase in H_2 production (Nandi *et al.*, 2001).

Formic acid as a substrate for formation of H_2; generation and transportation of formate by FocA protein

Formic acid is derived primarily from pyruvate in a reaction (Figure 1) catalyzed by pyruvate formate lyase (PFL) (Sawers, 2005). The latter in *E. coli* is activated only under microaerobic or anaerobic conditions (Alexeeva *et al.*, 2000).

If formate is left to accumulate in the cytoplasm, this would result of acidification in a cell and uncouple Δp. Controling formate metabolism therefore is crucial checkpoint where the potentially deleterious effects of formate excess must be balanced against the loss of an important source of reducing power and optimization of energy generation (Leonhartsberger *et al.*, 2002). Analysis of formate levels in the fermenting *E. coli* culture reveals that formate is initially exported out of the cells to prevent acidification of the cytoplasm and accumulates to levels as high as 10 mM. Once $[pH]_{out}$ drops below 6.8, formate is rapidly and completely imported back into the cells where it is metabolized to produce H_2 by FHL.

So far, one protein, FocA (formate channel) has been identified in *E. coli* to have a role in formate transport across the membrane (Rossmann *et al.*, 1991; Suppmann & Sawers, 1994). This protein is encoded by the *focA* gene, which is regulated with the *pfl* gene in a way that both gene products are present in the cell at the same time (Sawers, 2005). FocA represents a new class of organic acid transport protein. Although nothing is known about energetics of formate transport, it is clear that during fermentation, the cells are energy limited but, anyway, a symport of formate with H^+ is a distinct possibility.

Hydrogenases and H_2 production by *E. coli* during glycerol fermentation

E. coli is suggested to process different Hyd enzymes having H_2 production activity during fermentation conditions with glycerol as with glucose (Figure 1). Two novel principal findings have been already reported for glycerol fermentation: (1) H_2 production is pH dependent (Murarka *et al.*, 2008; Trchounian *et al.*, 2011c), it is significant at different pH with the highest rate at $[pH]_{out}$ 5.5 (Trchounian *et al.*, 2011c). (2) At neutral and slightly alkaline $[pH]_{out}$, Hyd-2 mostly and Hyd-1 partially are involved in H_2 production, no relation with FHL activity is observed (Trchounian & Trchounian, 2009). Whereas at acidic $[pH]_{out}$, FHL complex consisting of rather Hyd-3 than Hyd-4 is required for H_2 production (Gonzalez *et al.*, 2008; Trchounian *et al.*, 2011c). This has been confirmed recently by results obtained from Hyd enzyme activity studies. For instance, in cells grown in the presence of glycerol at $[pH]_{out}$ 7.5, the specific Hyd enzyme activity measured after growth of a *hyaB hybC* double mutant was approximately 5% of the activity observed in the wild type strain (Trchounian *et al.*, 2011b). Deletion of the *hybC* gene encoding the large subunit of Hyd-2 (Menon *et al.*, 1994) resulted in an 80% decrease in enzyme activity, while in a single *hyaB* mutant the Hyd specific activity decreased by 50% (Trchounian *et al.*, 2011b). Hyd-1 and Hyd-2 are, therefore, shown to be the main contributors to total Hyd enzyme activity. All these are in favor with Hyd-2 induction to a higher level upon growth in the presence of glycerol during anaerobic respiration shown many years ago (Sawers *et al.*, 1985). Importantly, the finding of Hyd-2 functioning in H_2-producing mode at neutral and slightly alkaline $[pH]_{out}$ contradicts to an idea that Hyd-2 is irreversible enzyme equipped with only H_2 uptake activity (Maeda *et al.*, 2007a). This contradiction might be due to highly reduced state of glycerol, which provided twice the reducing equivalents produced during glucose fermentation (Dharmadi *et al.*, 2006) and probably changed redox regulation of enzymes.

However, there remains a considerable amount that we do not understand concerning the physiological benefit of the reversibility of Hyd enzymes and H_2 recycling as well. Possibly, the reversible activity of different Hyd enzymes is likely of importance in maintaining *E. coli* survival in the intestinal tract and it has a role in establishment of the pathogenic state, as has been demonstrated for other bacteria (Maier, 2005).

Moreover, at acidic $[pH]_{out}$, FhlA and Hyd-4 both affect H_2 production and the requirement of Hyd-3 and Hyd-4 is different: Hyd-3 evolves H_2 although Hyd-4 might

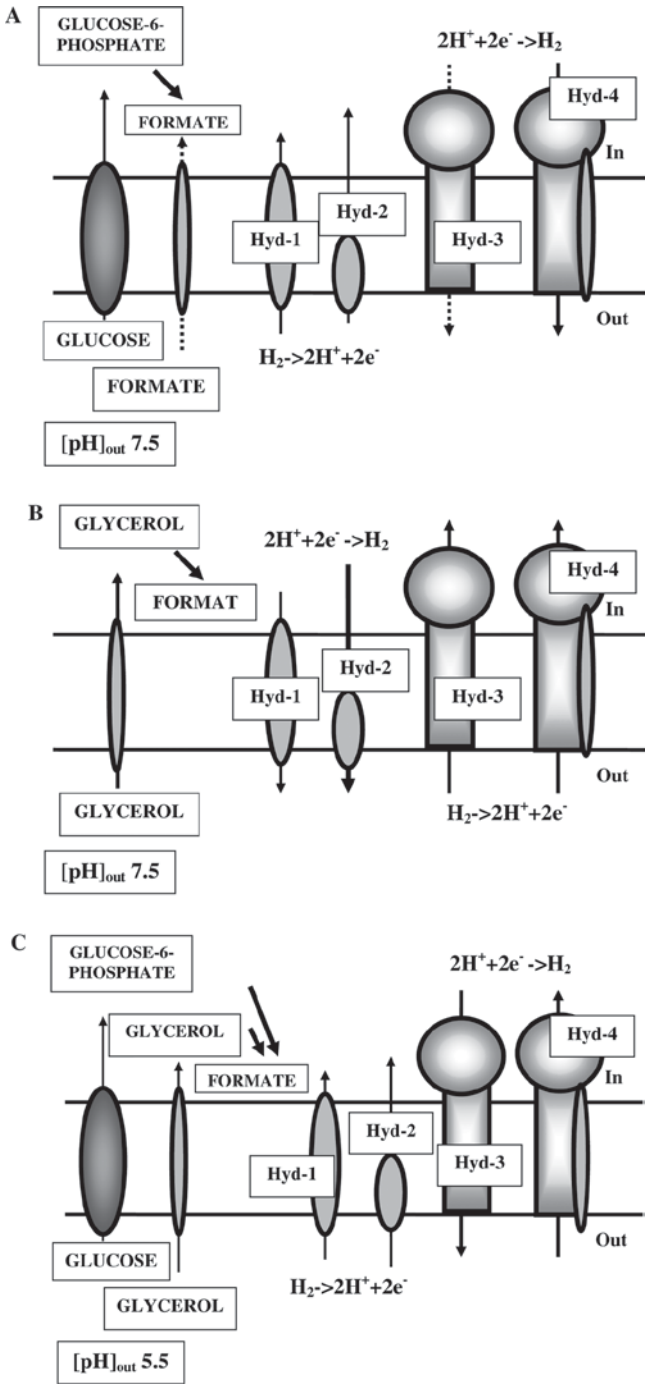

Figure 3. Reversibility of Hyd enzymes in *E. coli* depending on fermentation substrate and $[pH]_{out}$. Glucose fermentation at $[pH]_{out}$ 7.5 (A), glycerol fermentation at $[pH]_{out}$ 7.5 (B), glucose and glycerol fermentation at $[pH]_{out}$ 5.5 (C). The direction of enzyme operation to produce and/or to oxidize H_2 is shown (arrows) (Trchounian *et al.*, 2011c). Exogenous formate translocated through FocA or FocB increases Hyd-3 activity (A, dotted arrows).

operate in a reverse mode (Trchounian *et al.*, 2011c). Interestingly, increased H_2 production at a low $[pH]_{out}$ is probable when H_2 uptake activity is absent, so as Hyd-1 and Hyd-2 worked in a reversed H_2-oxidizing mode have low activity (Trchounian *et al.*, 2011c). By these results and other data obtained with different mutants during glycerol fermentation (compared to glucose), the reversibility with different Hyd-enzymes operation could be presented (Figure 3).

The recycling of produced H_2 is, therefore, required for hydrogen metabolism during glycerol fermentation. The H_2 recycling suggested and, in addition, changed H_2 production rate by double *hyaB hybC* mutant (compared to the wild type) shown (Trchounian *et al.*, 2011c) are in favor with the results obtained by Redwood *et al.* (2008) about compensatory uptake function during H_2 production under different conditions. Moreover, increased H_2 production in a double *hyaB hybC* mutant might be associated with the loss of *hyb* but not *hya* genes (Redwood *et al.*, 2008). This seems to be also likely to the conclusion of Murarka *et al.* (2008) about recycling of H_2 evolved by the FHL complex during glycerol fermentation and, in addition, to the suggestion by Zbell & Maier (2009) about a role of different levels of Hyd-1 to recycle all H_2 produced in *Salmonella enterica* under fermentative conditions.

However responsibility of different Hyd enzymes for H_2 production and regulation of their activity require further comparative study with new mutants deleted for large subunits of Hyd-1 and Hyd-2 and for FHL transcriptional activators as well.

Regulation of hydrogenases and formate hydrogen lyases activity, its coupling with H^+ transport and dependence on the F_0F_1-ATPase

Role of formate and pH in induction of the enzymes

Formate has a dual role in *E. coli* FHL regulation as a substrate also determining pH of the medium (together with one of the products of FHL reaction). Increasing formate concentration derived in fermentation or presence of exogenous formate induces FHL-1 (Rossmann *et al.*, 1991; Mnatsakanyan *et al.*, 2002b; 2004) (Figure 3A), although acidification of the medium also promotes induction of this enzyme (Rossmann *et al.*, 1991). Such induction was observed at slightly alkaline medium too (Mnatsakanyan *et al.*, 2002b; 2004). It should be noted that some relation between concentration of formate added and change in $[pH]_{out}$ is established (Bagramyan *et al.*, 2002; Hakobyan *et al.*, 2005), however it seems to be not simple to calculate such change in $[pH]_{out}$ because of complex situation.

The mechanism of such induction might be a result of increased concentration of fermentation products, organic acids, causing a decrease in pH. This also may be attributed to formate transport from an external medium into the cell via formate permease like FocA (Rossmann *et al.*, 1991). Formate is susceptible to activation (Figure 4) by formate-sensitive protein, FhlA (Self & Shanmugam, 2000). Formate may bind to FhlA, which interacts with FocA (Self *et al.*, 2001). Interestingly, FhlA exhibits ATPase activity that might be stimulated by formate (Figure 4); direct binding of formate is probable (Hopper & Bock, 1995; Self *et al.*, 2001; Mnatsakanyan *et al.*, 2002b). Then these proteins act as a cascade in expression of corresponding genes encoding components of FHL; they belong to formate regulon (Figure 4). Furthermore, expression induction of the *fdhF* and *hycB* genes by formate (Wu & Mandrand-Berthelot, 1987; Hopper *et al.*, 1994) involves FhlA (Schlensog & Bock, 1990; Rossmann *et al.*, 1991; Schlensog *et al.*, 1994; Hopper *et al.*, 1996) (Figure 4) and Mo (Rosentel *et al.*, 1995; Self & Shanmugam, 2000; Self *et al.*, 2001). Regulation by FhlA takes place at different $[pH]_{out}$; it has been recently shown that H_2 production by *fhlA* strain as well as double *fhlA hycG* mutant is lowered at $[pH]_{out}$ 6.5 and less at $[pH]_{out}$ 5.5 (Trchounian *et al.*, 2011c). In all cases, the presence of formate inside the cells is ultimately required for expression of corresponding genes and synthesis of subunits for FHL-1. Moreover, formate can also induce the *hyf* operon expression by binding of HyfR, the homologue of FhlA (Skibinski *et al.*, 2002). However, Self *et al.* (2004) has confirmed that FhlA and HyfR are able to activate *hyf* transcription but they have further reported that formate is not needed for expression of this operon. Thus, a comparatively simple model for the control of *fdhF, hycB* and the other gene expression by formate (Figure 4) can be proposed. This is based on controlled synthesis of the formate anion, multiple routes of formate metabolism and pH-dependent control of transport processes (Sawers, 2005).

Anyway, although information on regulation of FHL components synthesis by formate and pH becomes increasingly available, a general mechanism for this regulation still has not been proposed. However, recently it has been demonstrated that high *fdh* expression might

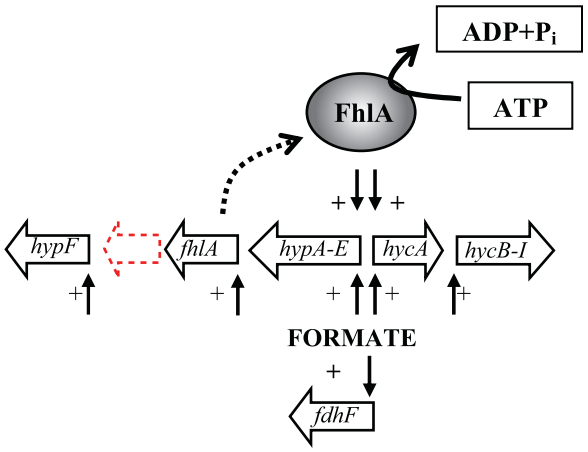

Figure 4. The gene expression induced by formate and regulated by the FhlA protein in *E. coli*. Positive control in gene expression and ATP required for FhlA activity are shown (arrows). FhlA has ATPase activity.

occur even without formate (Takahata *et al.*, 2008). Moreover, Hayes *et al.* (2006) have reported that *hycB* expression under aerobic conditions is greater (~6-fold) at pH 8.7 than pH 5.0. But these authors do not give any explanation to the finding. They seem to indicate that *hycB* has a role in strengthening bacterial adaptation to alkaline medium. On the other hand, this may specify again an important and unusual role of *hycB* in FHL-2 under anaerobic conditions at neutral and high $[pH]_{out}$ (Bagramyan *et al.*, 2002) and in $[pH]_{in}$ regulation as well.

The F_0F_1-ATPase requirement for enzymes functioning and H^+ transport

The most important aspect in regulation of Hyd enzymes, FHL and its energetics is the requirement of F_0F_1, which is the main mechanism in H^+ transport generating Δp. H_2 production by *E. coli* during glucose (Bagramyan & Martirosov, 1989; Bagramyan *et al.*, 2002) or glycerol (Trchounian & Trchounian, 2009; Trchounian *et al.*, 2011c) fermentation at neutral and slightly alkaline $[pH]_{out}$ has been shown to be sensitive to *N,N′*-dicyclohexylcarbodiimide (DCCD). H_2 production inhibition by DCCD is revealed during glycerol fermentation at low $[pH]_{out}$ too (Trchounian *et al.*, 2011c). It should be noted that DCCD is a nonspecific inhibitor of F_0F_1; it can also inhibit other systems involved in H^+ translocation. However, a point mutation in the *atpB* gene leading to nonfunctional F_0F_1 results in DCCD-resistant H^+ efflux (Martirosov & Trchounian, 1983). The latter is shown with *E. coli* whole cells grown under aerobic conditions (Martirosov & Trchounian, 1986) or under anaerobic conditions but in the presence of NO_3^- (Trchounian *et al.*, 1998). These data point out selective inhibition of F_0F_1 by DCCD. In addition, H_2 production upon fermentation of glucose is not detected in some *atp* mutants lacking functional F_0F_1 whereas, the other *atp* mutants having functional F_0F_1 are able to produce H_2, but with different characteristics (Bagramyan *et al.*, 2002; Mnatsakanyan *et al.*, 2002a). The production of H_2 inhibited by DCCD is also found in *E. coli* protoplasts (with increased membrane permeability) in the presence of ATP and formate (Trchounian *et al.*, 1997). Moreover, in the presence of arsenate and protonophores, which are decreasing Δp, H_2 is not produced (Bagramyan & Martirosov, 1989). On the other side, the F_0F_1-ATPase activity of membrane vesicles is markedly increased by formate when bacteria were grown on glucose (Bagramyan *et al.*, 2003). However, that activity disappears in *atp*-mutants as well as in *hyf* but not *hyc* mutants grown in the absence of formate and in *hyc* but not in *hyf* mutants in the presence of formate (Bagramyan *et al.*, 2003; Mnatsakanyan *et al.*, 2004). Thus, the results obtained and summarized (Table 1) indicate the requirement of F_0F_1 for H_2 production by FHL and by Hyd-1 and Hyd-2 in *E. coli* depending on fermentation substrate, respectively. This is supported by the results of independent studies by Barrett's group (Sasahara *et al.*, 1997) who has demonstrated that the DCCD-inhibited production of H_2 by *S. typhimurium* is not observed in *atp*-mutants lacking functional F_0F_1.

How can such requirement for F_0F_1 be explained? It is possible that during fermentation F_0F_1 couples ATP hydrolysis with H^+ translocation through the membrane and, therefore, it provides transformation of energy accumulated in ATP into Δp possibly required for FHL and Hyd enzymes functioning. Such possibility is quite reasonable due to probability of functionally active enzyme complex formation at certain Δp or at H^+ transport. But the results obtained using *atp*-mutants (Trchounian *et al.*, 1997; Bagramyan *et al.*, 2002) and also the other

Table 1. The effects of different factors on H_2 production rate by *E. coli* during glucose and glycerol fermentation at neutral or slightly alkaline $[pH]_{out}$.

Strains	Factors, conditions	H_2 production rate, %	
		Glucose fermentation*	Glycerol fermentation**
Wild type	Certain conditions	100	100
Wild type	K^+ depletion	10	no effect
Wild type	Osmotic shock (800 mosM)	18.5	100
Wild type	DCCD	7.6 (0.1 mM)***	81 (0.5 mM)***
Wild type	Protonophore	3	ND****
Wild type	Reducer	118	150*****
Wild type	Formate (30 mM)	105	ND
fdhF mutant	Comp. with wild type under certain conditions	34*****	45*****
hycB mutant	Comp. with wild type under certain conditions	21.2	ND
hyaB hybC mutant	Comp. with wild type under certain conditions	115	12
hyfB-R	Comp. with wild type under certain conditions	11.5	ND
fhlA mutant	Comp. with wild type under certain conditions	20.4	206
atpB-C mutant	Comp. with wild type under certain conditions	11.1	76.5*****

*Data are from Bagramyan *et al.* (2002); Mnatsakanyan *et al.* (2004); Trchounian & Trchounian (2009).
**Data are from Trchounian & Trchounian (2009); Trchounian *et al.* (2011c).
***DCCD concentration used.
****Not determined.
*****Not published yet.

data (Trchounian, 2004; Kirakosyan *et al.*, 2008) suggest direct involvement of F_0F_1 by association with FHL-2 to form some multi-enzyme *supercomplex* within the membrane (Figure 5). The requirement of Fdh-H, HycB and Hyd-4 proteins for F_0F_1 in supercomplex results from data that the activities of these proteins were dependent on each other; moreover, formate, ATP and reducing equivalents (NAD^+ + NADH) were required (Trchounian *et al.*, 1997; Bagramyan *et al.*, 2002; Mnatsakanyan *et al.*, 2002a; Bagramyan *et al.*, 2003; Mnatsakanyan *et al.*, 2004). There are no evidences about direct interactions between the proteins in supercomplex yet but the results above made arguments about structural interactions (Martirosov *et al.*, 1988; Trchounian, 1997; 2004) stronger. These arguments include the fixed stoichiometry for H^+-K^+-fluxes during F_0F_1 and TrkA system operation under different assays conditions (pH, K^+ activity, temperature, osmotic stress, etc) (Martirosov & Trchounian, 1983; 1986; Trchounian *et al.*, 1998) and K^+-dependent F_0F_1 activity (Martirosov *et al.*, 1988; Trchounian *et al.*, 1992) altered by defects in TrkA (Trchounian & Vassilian, 1994). It is important that this fixed stoichiometry has no other physical interpretation. Moreover, in such supercomplex, F_0F_1 also interacts with TrkA to supply the energy for a large K^+ uptake. F_0F_1 is, therefore, considered as part of the sophisticated metabolic network and energy conversion during fermentation. It is suggested that in this supercomplex, the energy could be transferred from F_0F_1 to TrkA by reducing equivalents (Trchounian, 2004). They can be donated from formate through FdhF and via HycB. The subsequent transfer of H^+ and electrons through F_0F_1 to TrkA may lead to energy release, used for the work of counter-gradient K^+ uptake. 2H can then be employed for H_2 evolution by Hyd-4. This model (Figure 5) seems to be well-grounded and already employed in a different approach (Verma *et al.*, 2007). Furthermore, it is in favor to an idea about *interactomes* with ATPase superassemblies in animals and plants that two or more proteins are better (Seelert & Dencher, 2011). However, protein–protein supercomplexes organization and its interplay with metabolism should be further studied to understand molecular functional details.

Besides, in acidic medium when H_2 is formed due to activity of FHL-1, F_0F_1 has been shown to be also necessary (Bagramyan *et al.*, 2002; 2003). For this case, especially with formate oxidation under anaerobic conditions, the other possibility with F_0F_1 operating without multi-enzyme complex to synthesize ATP seems to be probable (Figure 6). FHL-1 translocating H^+ as suggested (Hakobyan *et al.*, 2005) might generate Δp which could drive F_0F_1 to provide additional ATP for cell. This ATP might be also used by K^+ uptake TrkA system which is shown to have lower activity in acidic medium (Trchounian & Kobayashi, 1999) and to function, therefore, in a separate manner without complex with F_0F_1. It should be noted that changed activity of K^+ uptake TrkA system at different $[pH]_{out}$ (Trchounian & Kobayashi, 1999; 2000; Trchounian *et al.*, 2009) might result from its different relationship with FHL and, therefore, from a different mode of function. The idea (Figure 6) is likely to the proposal for the archaeon *Thermococcus onnurineus*, which generates a $\Delta\mu_{H^+}$ driven by formate oxidation via a FHL complex (Kim *et al.*, 2010).

The requirement for F_0F_1 on the activities for the H_2-oxidizing Hyd-1 and Hyd-2 has been recently examined with *E. coli* (Trchounian *et al.*, 2011a). During fermentative growth on glucose at $[pH]_{out}$ 7.5, H_2-oxidizing Hyd activity is lacked in *atp* mutant whereas, at $[pH]_{out}$ 5.5, it is only 20% that of the wild type. Using in-gel activity

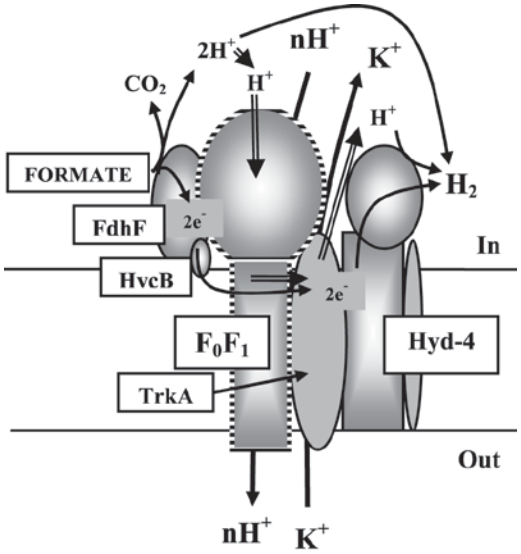

Figure 5. Proposed H_2-producing protein–protein supercomplex in *E. coli*, formed by association of F_0F_1, FHL-2 consisted of Fdh-H, HycB and Hyd-4, and TrkA. This mode is suggested for cells during mixed-acid fermentation of sugars at neutral and slightly alkaline $[pH]_{out}$ (Bagramyan & Trchounian, 2003; Trchounian, 2004). As reducing equivalents, electrons and H^+ transfers through the complex leading to H_2 formation, H^+ and K^+ translocation through F_0F_1 and via TrkA but not ATPase activity are shown (arrows).

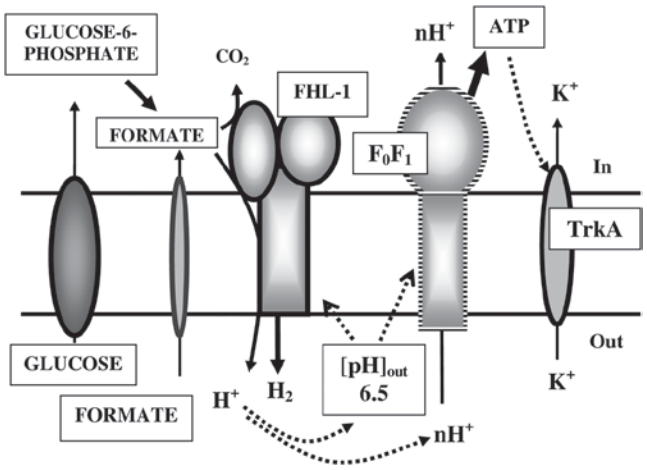

Figure 6. Interaction between F_0F_1, FHL-1 and TrkA in *E. coli* during mixed-acid fermentation of sugars upon formate supplied at slightly acidic $[pH]_{out}$. FHL-1 can translocate H^+ (Hakobyan *et al.*, 2005) and F_0F_1 operates to synthesize ATP (arrows). TrkA is dependent on ATP (dotted arrow). See the text.

staining, it could be demonstrated that in the mutant, both Hyd-1 and Hyd-2 are essentially inactive at these pHs (Trchounian *et al.*, 2011a), indicating that the residual activity at $[pH]_{out}$ 5.5 could be due to the H_2-evolving Hyd-3 (Trchounian *et al.*, 2011c). During fermentative growth in the presence of glycerol, H_2-oxidizing Hyd activity in *atp* mutant is highest at $[pH]_{out}$ 7.5 attaining a value of ~50% of wild type activity, and Hyd-2 is only partially active at this $[pH]_{out}$, while Hyd-1 is inactive (Trchounian *et al.*, 2011a). Essentially no H_2-oxidizing Hyd activity is measured at $[pH]_{out}$ 5.5 during growth with glycerol. Taken together, these results demonstrate for the first time that the activity of the H_2-oxidizing Hyd enzymes depends on an active F_0F_1.

Interestingly, the requirement of F_0F_1 or Δp generated by this ATPase for Hyd activity and H_2 production by *E. coli* during glycerol fermentation has been followed the DCCD-inhibited H^+ flux study. At $[pH]_{out}$ 7.5, H^+ efflux has been determined to be stimulated in *fhlA* and lowered in *hyaB* or *hybC* mutants and *hyaB hybC* double mutant: DCCD-sensitive H^+ efflux is observed (Blbulyan *et al.*, 2011). At $[pH]_{out}$ 5.5, H^+ efflux in wild type is lowered compared with that at $[pH]_{out}$ 7.5; it is increased in *fhlA* and absent in *hyaB hybC* mutants. Furthermore, ATPase activity of *E. coli* membrane vesicles is lowered in wild type glycerol fermented cells at $[pH]_{out}$ 7.5 compared with the glucose fermented cells. The ATPase activity is decreased in *hyaB* and less in *hybC*, stimulated in *hyaB hybC* and suppressed in *fhlA* mutants; DCCD inhibits ATPase activity. At $[pH]_{out}$ 5.5, ATPase activity of *hyaB*, *hybC* mutants membrane vesicles is with similar rates and higher compared with that in wild type; it is suppressed in *hyaB hybC* and *fhlA* mutants. Therefore, as per these results obtained recently (Blbulyan *et al.*, 2011), in addition to the requirement of F_0F_1 (Trchounian *et al.*, 2011a), it could be probable to suggest that Hyd-1 and Hyd-2 might be involved in H^+ transport across the membrane.

Regulation of cytoplasmic pH by hydrogenases

Bacteria maintain $[pH]_{in}$ in narrow range and for this they employ different mechanisms. Bock & Sawers (2006) have proposed that FHL in *E. coli* is involved into regulation of $[pH]_{in}$. Such hypothesis is based on the following observations. First of all, FHL catalyzes decomposition of formic acid to H_2 and CO_2 (Peck & Guest, 1957) so, the enzyme neutralizes acidic products of fermentation. This process depends on formate concentration within the cell and $[pH]_{out}$ (Rossmann *et al.*, 1991). Then, formic acid is a weak acid that may act as an uncoupling factor dissipating Δp. Decrease of $[pH]_{out}$ potentiates such effect of acids formed during fermentation.

It becomes clear why *E. coli* possesses two forms of FHL (Andrews *et al.*, 1997; Bagramyan *et al.*, 2002; Mnatsakanyan *et al.*, 2004) and why decrease of $[pH]_{out}$ increases expression of genes encoding components of FHL-1 (Rossmann *et al.*, 1991). However, even in this case FHL is also involved in regulation of $[pH]_{in}$. It could be suggested that a decrease of $[pH]_{out}$ also increases expression of genes encoding subunits of Hyd-4 (Andrews *et al.*, 1997). Although such expression has not been demonstrated yet, it is hard to assume that both forms of FHL are simultaneously activated in response to $[pH]_{out}$.

It seems unlikely that FHL catalyzing terminal reaction of mixed-acid fermentation in *E. coil* (Figure 1) operates independently from other fermentation enzymes. This viewpoint can be supported by the following arguments. First, FHL functioning requires catalytically competent F_0F_1, however FHL may interact with the K^+ uptake system TrkA in *E. coli* (Trchounian, 1997; 2004). Lack of H_2 production during K^+ depletion was demonstrated long time ago (Bagramyan & Martirosov, 1989), and this phenomenon could be explained in various ways. Mutations in TrkA are accompanied by changes in H_2 production at slightly alkaline $[pH]_{out}$ (Trchounian *et al.* 1998). Under these conditions, TrkA forms H^+-K^+-exchange pump in complex with F_0F_1 (Trchounian, 1997; 2004); energy transfer from this ATPase to TrkA requires reducing equivalents (Trchounian, 1997; 2004; Bagramyan *et al.*, 2002; Mnatsakanyan *et al.*, 2002a; Kirakosyan *et al.*, 2008). As already mentioned, FdhF may provide these equivalents that are further utilized by Hyd-4 for H_2 formation (Figure 5). Thus, FHL activity and H_2 production depend on K^+ concentration and therefore in K^+-depleted cells, H_2 production is blocked. Another scenario of fermentation impairment in response to decreased intracellular K^+ includes reduction of phosphofructokinase and pyruvate kinase activity (Puchkov *et al.*, 1982), and this also blocks H_2 production.

Thus, interaction of FHL with other enzymes involved into fermentation may be well explained within the concept on multi-enzyme complexes controlling metabolic fluxes (*metabolon*) in different cells (Lyubarev & Kurganov, 1989; Kholodenko *et al.*, 1992; Seelert & Dencher, 2011). However, arrangement of sugar and glycerol metabolism pathways, especially terminal fermentation reactions (Figure 1), includes formation of functional membrane-bound complexes including FHL (Figure 5); this is very important from structural and energetic viewpoints for regulation under anaerobic conditions.

Concluding remarks: responsible hydrogenases and further study to enhance H_2 production

H_2 is stated to be produced by *E. coli* and other bacteria during mixed-acid fermentation due to Hyd enzymes and FHL. *E. coli* possesses four Hyd enzymes encoded by different operons and two FHL pathways composed of different Hyd enzymes. The effects of different mutations in these operons on H_2 production are important being dependent upon environmental conditions. It is clear that expression of multiple Hyd enzymes and their reversible function depend on fermentation substrate and $[pH]_{out}$. These properties of Hyd enzymes appear to

play important role in increasing the fitness of bacterium in order to survive a variety of growth environments.

Optimal conditions for H_2 production by bacteria are under study now, however it is interesting that production could be more if glucose is replaced with the other sugars and glycerol in complex medium (Maeda *et al.*, 2007c). The other important study would relate to interplay between biofilm formation and H_2 production by bacteria (Domka *et al.*, 2007; Maeda *et al.*, 2007c). Different gene expressions of four hydrogenases in *E. coli* has been found during biofilm formation and that may change H_2 production activity.

It can be concluded that our knowledge on Hyd enzymes and FHL functioning in H_2 production by bacteria is not exhaustive although mechanisms of terminal stages of fermentation are rather clear. Further study would clarify the factors regulating H_2 production and role of Hyd enzymes and FHL pathways in energy transformation in the membrane, regulation of $[pH]_{in}$ and adaptation of bacteria to different environment, as well as FHL complex formation and its binding to other transport systems and enzymes, formation of functional assemblies and their effectiveness in catalysis of fermentation reactions. Moreover, the requirement of the F_0F_1-ATPase for Hyd enzymes and FHL activity is established, but mechanisms of this requirement should be studied.

Besides, recently Wood' group (Maeda *et al.*, 2007b,c; 2008a,b; 2011; Sanchez-Torres *et al.*, 2009; Hu & Wood, 2010) have used *E. coli* K-12 library containing all non-lethal deletion mutations to rapidly construct multiple, precise deletions in the *E. coli* genome to re-direct the metabolic flux toward H_2 production (Figure 1). Removing H_2 uptake by inactivating Hyd-1 and Hyd-2 by deleting *hyaB* and *hybC,* respectively, is among simple approaches for metabolically engineering *E. coli* to enhanced H_2 production during glucose fermentation (Maeda *et al.*, 2008a,b). Manipulating with *hyaB, hybC, hycA, fdoG* and other genes and overexpressing of *fhlA* to re-direct formate metabolism, a single fermentative *E. coli* strain has been engineered that produces ~141-fold more H_2 from formate and ~3-fold more H_2 from glucose than the wild type strain (Maeda *et al.*, 2008b). Different *E. coli* strains were also developed to enhance H_2 production by the other group (Kim *et al.*, 2009). Using of recombinant DNA technology in strain construction and other new and more effective approaches are recently reviewed for potential strategies with whole-cell and cell-free systems compared (Maeda *et al.*, 2011).

However, glycerol fermentation might be a useful pathway to produce H_2 by bacteria. In this case, the effects of cultivation conditions and $[pH]_{out}$ on fermentation of glycerol and end products by *E. coli* (Murarka *et al.*, 2008), the establishing of Hyd enzymes responsible for H_2 production (Trchounian & Trchounian, 2009; Trchounian *et al.*, 2011c) may be also usefully implicated for enhanced production of fuels and reduced chemicals.

All of the above become significant for H_2 production technology from organic wastes: absolutely new strategy to regulate Hyd enzymes and FHL activity should be developed when mixed carbon (glucose, formate and glycerol at least) is present.

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Declaration of interest

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