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Escherichia coli hydrogen gas production from glycerol: Effects of external formate

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ABSTRACT

Hydrogen gas (H_2) production was studied in bacteria cultured at different pH conditions, and on different carbon substrates. H_2 production by *Escherichia coli* was first studied during glycerol fermentation when external formate (10 mM) was supplemented. Changes in H_2 production rate (V_{H2}) were determined when in the assays glycerol and/or formate were supplemented at pH 7.5 and pH 6.5, using single and double mutants coding large subunits of different hydrogenases (Hyd-1, Hyd-2 and Hyd-3) as well as *selC* (coding for formate dehydrogenases), *hyaB hybC* (coding for large subunits of Hyd-1 and Hyd-2, respectively) *selC* or *hyaB hybC hycE* (coding for large subunit of Hyd-3) triple mutants at both pHs. The results point out that Hyd-3 becomes mainly responsible for H_2 production by *E. coli* during glycerol fermentation when external formate is added; Hyd-4 can also contribute to H_2 production. Besides, in the glycerol supplemented assays, three hydrogenases can work in H_2 producing mode and only deletion of three of them decreases the production of H_2 which might be due to disturbance of H_2 cycling. This is of significance in application of different carbon sources in renewable energy production technology using bacteria.

1. Introduction

The current state with reducing fossil fuels has led to find out alternative and renewable energy sources. One of these energy sources is biodiesel which is produced mostly by algae without a big impact on carbon dioxide emission [1,2]. The other energy source can be hydrogen gas (H_2) which is ecologically clean, effective and renewable one; H_2 can be produced from glycerol during microbe-mediated biological conversion [3]. Glycerol is a main waste (about 10% w/w) of biodiesel production; it has increased amount during the last years [4]. Moreover, glycerol becomes a cheap substrate, since this carbon source gradually changes from a chemical commodity to a chemical waste. Therefore, H_2 production from agricultural, industrial, kitchen, water and other kinds of wastes is of great interest for finding out other substrates or cheap sources for energy production which are available in nature and environment [5].

Gonzalez et al. [6] have established absolutely novel phenomenon that, in addition to different sugars, glycerol can be fermented by bacteria, especially *Escherichia coli*, at slightly acidic pH. Interestingly, among the end products of mixed-acid fermentation of glycerol H_2 is detected not only at acidic but also at slightly alkaline pH [7,8]. However, no clear data are available about glycerol metabolic pathways and fermentation end products by *E. coli*, their dependence on external pH and different chemicals during co-fermentation of glycerol with other carbon substrates and on other external factors. Nowadays already several studies are ongoing using different mixtures of carbon sources like sugars (glucose, xylose, mannitol etc.) and glycerol to enhance H_2 production by increased bacterial biomass and utilization of cheap carbon sources which are present in the mixtures [9–12]. Interestingly, formate is one of the end products of glycerol mixed-acid fermentation in *E. coli* and can be oxidized to H_2 and CO_2 or exported into external medium. Recently, it has been shown that external formate can pass into the cell and affect H_2 production [13]. This depends on pH and other factors and might be important for regulation of H_2 production.

It is well-known that H_2 is evolved by *E. coli* via special enzymes named hydrogenases (Hyd), which catalyze the reaction of

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$H_2 \leftrightarrow 2H^+ + 2e^-$ [14–16]. The latter is simple redox reaction but responsible enzymes as well as mechanisms of activity and regulation are complex. The distinguished properties of Hyd enzymes are their multiplicity and reversibility [17]. *E. coli* has the capacity to encode four membrane-associated [Ni–Fe]-hydrogenases [17]. Hyd-1 and Hyd-2 are reversible Hyd enzymes: during glycerol or glucose fermentation they operate in H_2 evolving or uptake mode, respectively [17–19]. Hyd-3 and Hyd-4 are H_2 producing Hyd enzymes under glucose fermentation but these enzymes both are able to work in reverse mode during glycerol fermentation [17–21]. The mode or direction of Hyd enzymes operation depends not only on fermentation substrate but also on external pH and other factors [17–21].

In addition, Hyd enzymes of *E. coli* have complex mechanisms for genetics, maturation and regulation. Hyd-1 is encoded by the *hya* operon; it consists of three different subunits [22]. The *hya* gene is expressed under anaerobic conditions at acidic pH [22] and by formate [24] though *E. coli* hasn't any requirement of Hyd-1 for growth under anaerobic conditions [23]. Hyd-2 is encoded by the *hyb* operon; it consists of four different subunits three of which are similar to Hyd-1 subunits [25]. Unlike *hya*, the *hyb* operon is maximally expressed at alkaline pH [23] in the cultures grown on H_2 and fumarate. This is in accordance with pH optimum of this enzyme [26]. Moreover, Hyd-2 has activity in more reducing environment [27]; however it's activity is absent under aerobic conditions [28]. The role of Hyd-1 in bacterial physiology is yet unclear, but it has been propounded to shuttle electrons from formate to fumarate during reduction [29] or to contribute electrons to the quinones pool when oxidizing of H_2 [30]. On the other hand, Hyd-2 may reversibly oxidize H_2 *in vitro* [28]. But this Hyd enzyme has potential to function as a "valve" to release excess reducing equivalents ($H^+ + e^-$) in the form of H_2. Therefore, Hyd-2 might have a role in physiology under anaerobic conditions at alkaline pH. These findings on Hyd-1 and Hyd-2 might have some input in investigating the exact functions of these enzymes which can be applied in H_2 production biotechnology. However, their functions and role are still not defined clearly.

Two other Hyd enzymes - Hyd-3 and Hyd-4 are encoded by the *hyc* and *hyf* operons, respectively [31–33]. Hyd-3 consists of different large and small subunits and with formate dehydrogenase (FDH-H) forms the formate hydrogen lyase (FHL-1) complex, producing H_2 preferably at acidic pH [20]. On the other hand, Hyd-3 has been shown to work in reverse, H_2 oxidizing mode under certain conditions [34]. Hyd-4 also consists of different large and small subunits and together with FDH-H is suggested to form the second FHL-2 complex which must have functional HycB protein, a small subunit of Hyd-3, to evolve H_2 at slightly alkaline pH [20,32]. Interestingly, the subunit compositions of these FHL complexes are not confirmed yet and their mechanisms and regulation pathways are under the on-going study. For both FHL-1 and FHL-2 complexes the *fhlA* gene is required: it is coding transcriptional activator for the *hyc* [35,36] and *hyf* [37] operons – FhlA and its expression might be dependent on formate. In addition, Hyp proteins and other factors are required for the maturation and assembly of Hyd enzymes [38]. Some interaction with each other or metabolic cross-talk is also proposed for these enzymes [17,39], however the nature of the link between different Hyd enzymes is not known at all.

Beforehand, it has been established that H_2 production by *E. coli* during glucose or glycerol fermentation was inhibited by *N,N′*-dicyclohexylcarbodiimide (DCCD), inhibitor of the proton F_0F_1-ATPase [20]. This finding suggests some link of Hyd enzymes or FHL complexes with F_0F_1. Especially, under glucose fermentation at pH 7.5 a link between Hyd and F_0F_1 could result from Hyd-4 interaction with F_0F_1 to supply reducing equivalents ($H^+ + e^-$) for energy transfer to the secondary transport system, especially to potassium uptake TrkA system [40]. The metabolic cross-talk between H_2 producing Hyd-1 and Hyd-2 and F_0F_1 is also suggested during glycerol fermentation at high and low pHs [41]. Importantly, the relationship between different Hyd enzymes and F_0F_1 or proton gradient ($\Delta\mu_H^+$) generated by F_0F_1 has been also demonstrated [18,20,42]. Such relationship has been also shown by Kim et al. [43] for the archaeon *Thermococcus onnurineus*, generating $\Delta\mu_H^+$, which is driven by formate disproportionation via FHL complex. Moreover, Sasahara et al. [44] have independently revealed a relationship between FHL complex and F_0F_1 during thiosulfate reduction by *Salmonella typhimurium*.

Furthermore, it has been shown in different studies [6,13,17–20,39,45] that *E. coli* Hyd activity is pH dependent. The latter seems to be very important property in regulating Hyd enzymes activity to enhance H_2 production efficiency. Currently, growing interest of H_2 production by bacteria in different environment and of developing H_2 bio-production technology is with mixed carbon sources fermentation or co-fermentation since different carbon substrates, including glycerol and different organic acids (namely formate), could be found in agricultural, industrial, kitchen, water and other wastes but a little is available on the fermentation of mixed carbon sources, metabolic pathways, responsible Hyd enzymes and H_2 production as well.

In the present paper *E. coli* H_2-producing activity by Hyd enzymes during glycerol fermentation upon formate supplemented (mixed carbon sources fermentation) has been studied at both slightly alkaline and acidic pHs. The possibilities of modifying metabolic pathways, coordinating appropriate genes and regulatory factors to perform some biochemical functions related to the production of H_2 were chosen during mixed carbon substrates fermentation. Moreover, novel functions of different Hyd enzymes especially Hyd-3 and Hyd-4 were revealed.

2. Materials and methods

2.1. Bacterial strains, growth and preparation for assays

E. coli BW25113 or MC4100 wild type and mutant strains with deletions in the genes coding subunits for different Hyd enzymes were used in the study. The strains used are listed in Table 1.

Bacteria from an overnight (O/N) growth culture were transferred into the fresh buffered liquid peptone medium containing 20 g/l peptone, 15 g/1 K_2HPO_4, 1.08 g/l KH_2PO_4, 5 g/l NaCl (pH 7.5) or 20 g/l peptone, 7.4 g/1 K_2HPO_4, 8.6 g/l KH_2PO_4, 5 g/l NaCl (pH 6.5) and supplemented with glycerol (10 g/l) and/or sodium formate (0.68 g/l). O/N medium was supplemented with kanamycin (25 μl/ml) for some kanamycin-resistant mutants when appropriate (see Table 1). Bacteria were grown in batch culture for 18–22 h at 37 °C; anaerobic conditions were described previously [13,18–20]. The medium pH was measured by a pH-meter using a selective pH-electrode (ESL-63-07, Gomel State Enterprise of Electrometric Equipment (GSEEE), Gomel, Belarus; or HJ1131B, Hanna Instruments, Portugal) and adjusted by 0.1 M NaOH or 0.1 N HCl. Bacterial growth was monitored by measuring bacterial culture absorbance at 600 nm with a spectrophotometer (Spectro UV–Vis Auto, Labomed, USA). The growth specific rate was determined as before [13,18–20].

2.2. Redox potential determination and hydrogen production assays

Redox potential (E_h) in bacterial suspension was assayed using the oxidation-reduction, titanium-silicate (Ti–Si) (EO-02, Gomel State Enterprise of Electrometric Equipment (GSEEE), Gomel, Belarus) and platinum (Pt) (EPB-1, GSEEE, or PT42BNC, Hanna