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Short Communication

Hydrogen producing activity by *Escherichia coli* hydrogenase 4 (*hyf*) depends on glucose concentration

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ABSTRACT

Escherichia coli produces molecular hydrogen (H_2) during glucose fermentation. This production of H_2 occurs via multiple and reversible membrane-associated hydrogenases (Hyd). Dependence of H_2 producing rate (V_{H_2}) by Hyd-4 (*hyf*) on glucose concentration was studied at different pHs. During growth on 0.2% glucose at pH 7.5 in JRG3615 (*hyfA-B*) and JRG3621 (*hyfB-R*) mutants (V_{H_2}) was decreased ~6.7 and ~5 fold, respectively, compared to wild type. Only in JRG3621 mutant at pH 6.5 and 5.5 (V_{H_2}) was severely decreased ~7.8 and ~3.8 fold, respectively. But when cells were grown on 0.8% glucose no difference between wild type and mutants was detected at any of the tested pHs. The results indicate Hyd-4 H_2 producing activity inhibition by high concentration of glucose mainly at pH 7.5. This is of significance to regulate Hyd activity and H_2 production by *E. coli* during fermentation.

Introduction

Currently substantial mitigation of fossil fuels needs to find cheap, ecologically clean alternative energy sources. One of these sources is molecular hydrogen (H_2) which can be produced from microbial and algal biomass [1]. H_2 production from different sugars or organic carbon-containing industrial, agricultural, water and other kind wastes has been set on well and the biotechnology has been already elaborated. Moreover, co-fermentation of different carbon sources by different bacteria resulting H_2 production has been shown [2–5].

It is well known that H_2 is produced via special membrane-associated enzymes named hydrogenases (Hyd) which reversibly oxidize H_2 to $2H^+$. *Escherichia coli* has the capacity to encode four [Ni–Fe]-hydrogenases, three of which are biochemically and genetically characterized well [6] but the activity of Hyd-4 (*hyf*) is described vague [7]. Hyd-3 (*hyc*) and Hyd-4 with formate dehydrogenase H (FDH-H) are suggested to form formate hydrogen lyase (FHL)-1 and FHL-2 pathways,

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respectively [6]. Hyd-1 (*hya*) and Hyd-2 (*hyb*) are reversible Hyd enzymes which can operate in different mode depending on carbon source: during glucose or glycerol fermentation they operate in H_2 uptake or producing mode, respectively [6,8]. Thus, the important features of Hyd enzymes are their multiplicity and reversibility.

It was demonstrated that Hyd-4 is primarily active at slightly alkaline pH and this enzyme is mainly responsible for H_2 production by *E. coli* [9]. However, the conditions when Hyd-4 activity is observed should be studied. Moreover, it was detected that glucose has inhibitory effect on *hyf* genes expression [10]. In addition, the stimulatory role of glucose on phosphotransferase transport system in *E. coli* has been established [11]. But the mechanism of glucose inhibitory effects on Hyd-4 is not clear.

Nowadays the advanced interest of H_2 production by *E. coli* for developing H_2 bio-production technology is to detect and to control the conditions of different enzymes activities. The main goal is to demonstrate the effect of glucose concentration on H_2 producing activity of Hyd-4.

Materials and methods

Bacterial strains and growth

The *E. coli* MC4100 wild type and different *hyf* mutant strains were used in this study (Table 1). Bacteria from an overnight growth culture were transferred into the fresh liquid medium (20 g L^{-1} peptone, 15 g L^{-1} K_2HPO_4, 1.08 g L^{-1} KH_2PO_4, 5 g L^{-1} NaCl) with different concentrations of glucose (0.2% or 0.8%) at pH 5.5, 6.5 or 7.5. Overnight growth culture was the same as fresh liquid medium at appropriate pH mentioned and at 37 °C; 1 ml of overnight culture per 100 ml of fresh medium was transferred. Bacteria were grown under anaerobic conditions at 37 °C for 18–22 h as described [4–6]. For anaerobic conditions glass vessels with plastic press caps were used; O_2 and N_2 dissolved in liquid medium were bubbled out of the media by autoclaving, after which the vessels were closed by press caps. pH was determined by a pH-meter with a selective pH-electrode (ESL-63-07, Gomel State Enterprise of Electrometric Equipment (GSEEE), Gomel, Belarus; or HJ1131B, Hanna Instruments, Portugal) and adjusted using 0.1 M NaOH or HCl.

Analytical methods

H_2 production assays were done by redox potential (E_h) determination. The latter was done using a pair of redox, titanium-silicate (Ti–Si) (EO-02, GSEEE) and platinum (Pt) (EPB-1, GSEEE; or PT42BNC, Hanna Instruments, Portugal) electrodes as described in details previously [4–6,9]. H_2 production rate (V_{H_2}) was calculated as the difference between the initial decreases in Pt- and Ti–Si-electrodes readings per time. It was expressed as mV of E_h per min per mg dry weight of bacteria. This approach is close to the method employed by Fernandez [12] and different groups [13–15] with a Clark-type electrode: a correlation between E_h and H_2 production was shown. Using the Durham tube method [9], H_2 production during the growth of *E. coli* was also estimated by the appearance of gas bubbles in the test tubes over the bacterial suspension.

H_2 production by the cells grown on various concentrations of glucose was assayed with either 0.2% or 0.8% glucose.

Preparation of whole cells for H_2 production assays was described before [4–6,9]. The cells were washed with distilled water and then transferred into the assays mixture (100 mM Tris-phosphate buffer (appropriate pH) containing 0.4 mM $MgSO_4$, 1 mM NaCl and 1 mM KCl); glucose was supplemented. The assays were performed in a thermo-stated chamber at 37 °C; bacterial suspension in the closed vessel was mixed with a magnetic stirrer bar. Dry weight of bacteria was determined as described previously [4,9].

Agar, glucose, peptone, Tris (Carl Roths GmbH, Germany), and the other reagents of analytical grade were used for bacterial growth and hydrogen production assays.

Each data point represented is averaged from independent triplicate cultures; the standard deviations calculated as described [4,9] are not more than 3% if they are not represented. The validity of differences between experimental and control data is evaluated by Student's criteria (p) [16]; $p < 0.01$ or less if this is not represented, otherwise $p > 0.5$ if the difference is not valid.

Results

H_2 production during 0.2% glucose fermentation by E. coli *wild type and Hyd-4 mutants at different pHs*

During fermentative growth of *E. coli* in the presence of 0.2% glucose and in the assays supplemented with 0.2% glucose at pH 7.5 JRG3615 (*hyfA-B*) and JRG3621 (*hyfB-R*) mutant strains (see Table 1) had H_2 production rate (V_{H_2}) ~6.7 fold and ~5 fold less, respectively, than wild type cells (Fig. 1). These data are in good conformity with previously obtained results [9]. In the same conditions addition of 0.8% glucose in the assays had the same effect on H_2 generation. At pH 6.5 in JRG3615 and JRG3621 strains (V_{H_2}) was decreased ~2.2 fold and ~7.8 fold, respectively, compared to wild type (see Fig. 1). But at pH 5.5 in JRG3621 strain it was decreased ~3.8 fold, compared to wild type (see Fig. 1). These findings point out that at pH < 7.0 only the deletion of the most of Hyd-4 operon genes disturbs H_2 production, unless(V_{H_2}) of JRG3615 is ~3.5 fold higher than

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

Strains	Genotype	Source and reference
MC4100	araD139 Δ(argF-lac)U169 ptsF relA1 fib5301 rpsL150	S.C. Andrews (The University of Reading, Reading, UK) [9]
JRG3615[a]	MC4100 Δ*(hyfA-B)::spc*	S.C. Andrews [9]
JRG3621[a]	MC4100 Δ*(hyfB-R)::spc*	S.C. Andrews [9]

[a] Resistant to spectinomycin.