Biology

THE ROLE OF PROTON ATPASE SPECIFIC INHIBITOR *N,N'*-DICYCLOHEXYLCARBODIIMIDE AND EXTERNAL FORMATE CONCENTRATION ON *E. COLI* GROWTH DURING MIXED CARBON SOURCES FERMENTATION AT DIFFERENT PHs

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This research is focused on the investigation of specific growth rate changes of *E. coli* wild type and mutant strains with defect of Hyd, FDH enzymes and FhlA regulatory protein in the presence of *N,N'*-dicyclohexylcarbodiimide (DCCD) and external formate various concentration during co-fermentation of glucose, glycerol and formate at pHs 5.5–7.5. The highest value of SGR was observed at pH 7.5. It was revealed that SGR depends on external formate concentration at all pHs. DCCD inhibitory effect was shown mainly at pH 7.5 and partially at pH 6.5 and 5.5. In the case of the F_0F_1-ATPase inhibition, FhlA compensatory effect on SGR was revealed.

Introduction. Bacteria are used for many purposes, such as production of food, drinks, chemicals, biofuels, etc. [1]. From this perspective the study of bacterial growth is significant to regulate various industrial processes. Bacterial growth depends on various environmental factors: composition of growth medium, pH, temperature, oxygen level, toxins, etc. [2]. In nature, bacteria do not live in an environment with ideal conditions for the growth. However, in laboratory conditions, it is possible to create favorable conditions for the growth of bacteria and characterize growth properties. The results of bacterial growth studies can be expressed by the specific growth rate (SGR, μ). *Escherichia coli* is a biotechnologically applicable bacteria [3, 4]. The ability to grow on different carbon sources increases the importance of these bacteria. Furthermore, *E. coli* produces compounds with great economic value, such as molecular hydrogen (H_2), ethanol, lactate and other chemicals [5]. It is important to study the role of enzymes involved in H_2 metabolism during growth for further improving hydrogen production technology.

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E. coli has four reversible membrane-associated [Ni-Fe]-hydrogenases (Hyd), which reversibly oxidize H_2 to $H^+ + 2e^-$. Hyd-3 and Hyd-4 with formate dehydrogenase H (Fdh-H) form formate hydrogen lyase-1 and -2 complexes (FHL), respectively [6]. FhlA is transcriptional activator of FHL complexes. This protein is required for induction of expression of the Fdh-H and Hyd-3 structural genes. FhlA activates expression of *hyf* operon (encodes Hyd-4) [7]. The central domain of FhlA regulatory protein is responsible for ATP hydrolysis once formate is bound [8]. For synthesis of the FHL component Fdh-H *selC* gene is required, which gene-product selenocysteinyl-tRNA inserts selenocysteine in the mRNA of FDHs genes [9]. Expression of *fdhF* is induced by formate and the absence of external electron acceptors [10]. HypF is one of a number of proteins required for maturation of Hyd enzymes [9, 11].

Numerous studies have shown that Hyd can have metabolic cross-talk with proton-translocating F_0F_1-ATPase depending on fermentation substrate and extracellular pH [9, 12, 13]. F_0F_1-ATPase is the main enzyme during fermentation in bioenergetics terms. Secondary transporters might function together with F_0F_1 to maintain Δp [6]. *N,N'*-dicyclohexylcarbodiimide (DCCD) is specific inhibitor of the F_0F_1-ATPase [9]. It was demonstrated, that in *E. coli* F_0F_1-ATPase has major input in overall ATPase activity during glycerol fermentation [13]. F_0F_1-ATPase had a relationship with FDHs and Hyd enzymes during utilization of glucose, glycerol and formate at pH 5.5 and pH 7.5 [9]. It was shown that proton ATPase activity depends on the presence of formate during mixed carbon fermentation at low pH [14].

Many studies have shown that Hyds can have a function in the growth of bacteria. It was suggested that *E. coli* H_2 producing Hyd-3 and Hyd-4 have a roll in cell growth during anaerobic utilization of lactose [15]. During glycerol fermentation all Hyd enzymes have a significant role for bacterial growth and can be regulated by external formate in *E. coli*. Disturbance of the cycle due to lack of one Hyd enzyme might have an effect on bacterial growth [16].

It is important to investigate the role of the interaction between H_2 metabolism involving enzymes and F_0F_1-ATPase on bacteria growth. In this study it was investigated SGR of *E. coli* wild type and mutant strains with defect of all Hyds, FDHs and FHL complexes and role of the F_0F_1-ATPase in SGR during mixed carbon sources (glucose, glycerol, formate) fermentation at different pHs (7.5, 6.5, 5.5) and various external formate concentrations (1 *mM*, 5 *mM*, 10 *mM*).

Materials and Methods.

Bacterial Strain and Growth Medium. The *E. coli* wild type and mutant strains used in this study are listed in Tab. 1.

Overnight anaerobically grown bacterial culture was added into the high buffered peptone growth medium. *E. coli* bacteria were grown under anaerobic fermentative conditions at 37°C. Growth medium had the following composition: 20 *g/L* peptone, 15 *g/L* 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/L* K_2HPO_4, 8.6 *g/L* KH_2PO_4, 5 *g/L* NaCl (pH 6.5) or 20 *g/L* peptone, 15 *g/L* KH_2PO_4, 1.08 *g/L* K_2HPO_4, 5 *g/L* NaCl (pH 5.5) with addition of glucose (11.1 *mM*), glycerol (137 *mM*) and sodium formate (1, 5, 10 *mM*). Medium pH was determined by pH-meter via selective pH-electrode (HI1131, Hanna Instruments, Portugal) and adjusted by 0.1 *M* NaOH or HCl.

Table 1

Characteristics of E. coli wild type and mutant strains used

Strain	Genotype	Absent related protein	Ref.
BW 25113 (wild type)	*rrnB ΔlacZ4787 HsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1* (old genotype: *lacI^q rrnB_{T14}ΔlacZ_{WJ16} hsdR514 ΔaraBAD_{AH33} Δrha BAD_{LD78}*)	Wild type	[9]
MC4100	*araD139 ΔlacU169 rpsL thi fla*	Wild type	[9]
JW2701[a]	BW25113 *ΔfhlA*	FHL activator	[17]
DHP-F2	MC4100 *ΔhypF*	Maturation of all hydrogenases	[9]
FM460[a]	MC 4100 *ΔselC*	tRNA^{sec}	[9]

[a] resistant to kanamycin.

Bacterial growth was monitored by measuring bacterial culture absorbance by spectrophotometric method (600 nm, Spectro UV-Vis Auto (Labomed, US). SGR was determined by regularly measurement of optical density (OD) until the stationary phase by the following formula [16]:

$$\mu = \frac{\ln (OD_1) - \ln (OD_2)}{t_2 - t_1},$$

where the values of $\ln (OD_1)$ and $\ln (OD_2)$ were recorded at t_1 and t_2 hours, respectively.

0.2 *mM N,N′*-dicyclohexylcarbodiimide was added to the growth medium as F_0F_1-ATPase specific inhibitor to show the role of proton ATPase or the interplay between proton ATPase and H_2 metabolism enzymes in SGR of *E. coli* bacteria. To determine the impact of external formate concentration on SGR, 1 *mM*, 5 *mM* and 10 *mM* respective amounts of sodium formate were used.

Results and Discussion.

Effect of DCCD and Different Concentrations of Formate on Growth of E. coli Wild Type and Mutant Strains During Mixed Carbon Sources Fermentation at Slight Alkaline pH. *E. coli* is able to utilize the mixture of glucose, glycerol and formate anaerobically, as shown before [9, 18]. The SGR of *E. coli* bacteria was higher at pH 7.5, than at pH 6.5 and 5.5, which was shown during fermentation of other carbon sources, previously [13].

To understand the role of proton ATPase in SGR, the effect of DCCD was studied during glucose, glycerol and formate co-fermentation at pH 7.5 (Fig. 1). It was shown, that the SGR in the presence of DCCD was decreased in all strains. DCCD suppressed the SGR of wild type by ~35%, ~36% and ~47% with addition of 1 *mM*, 5 *mM* and 10 *mM* formate, respectively. Whereas, overwhelming effect of DCCD was increased in *hypF* and *selC* mutant strains and was ~ 44%, ~ 53%, ~ 56% and ~43%, ~54%, ~60%, accordingly, in the presence of respective amounts of formate (1 *mM*, 5 *mM*, 10 *mM*). Moreover, effect of DCCD was severe in *fhlA* mutant strain as SGR was decreased by ~89%, ~89% and 86% when 1 *mM*, 5 *mM* and 10 *mM* formate was added in the growth medium.

Overwhelming effect of DCCD on SGR highlights the essential role of F_0F_1-ATPase, particularly in the lack of Hyd and FDH enzymes, when H_2 generation was absent and H^+ outflow and intracellular pH regulation occurred via F_0F_1 pump were

not active. In *fhlA* mutant strain, when both Hyds and FDHs are absent, the growth was almost completely suppressed. Hence, the separate interaction between Hyds or FDHs and proton ATPase is not considerable. Mentioned membrane-bound enzymes are in interaction, which was shown earlier [9], and this interplay has a function in bacteria growth and is not dependent on external formate concentration. On the other hand, FhlA protein has its own ATP binding and ATPase activity [8], and when the *fhlA* mutant lacks the FhlA protein and the proton ATPase is suppressed, it is suggested that the energy required for bacterial growth is provided less.
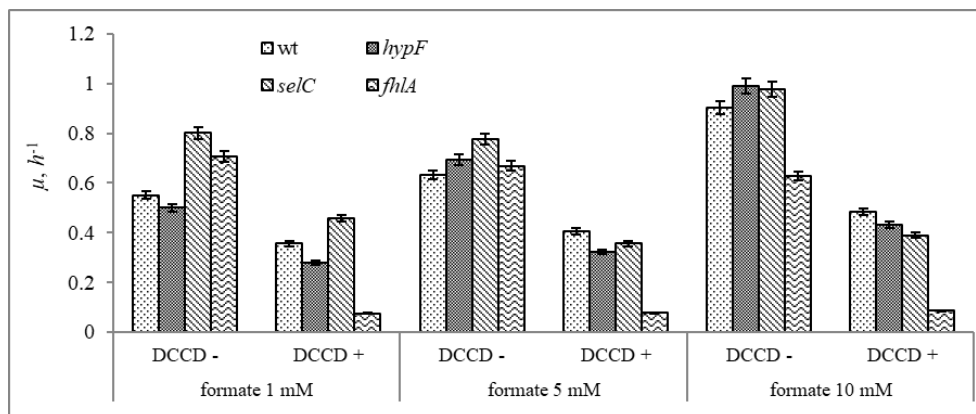

Fig. 1. SGR of *E. coli* wild type and mutant strains during fermentation of mixed carbon sources (glucose (11.1 *mM*), glycerol (137 *mM*) and formate (1 *mM*, 5 *mM*, 10 *mM*)) at pH 7.5. DCCD was added to the growth medium with 0.2 *mM* final concentration. For mutant strains see Tab. 1. For growth condition and other details see Methods.

The SGR of wild type is less by ~39% and ~30% in the presence of 1 *mM* and 5 *mM* external formate, respectively, compared to 10 *mM* formate concentration (see Fig. 1). Therefore, formate has a positive effect on bacteria growth during utilization with glucose and glycerol at pH 7.5. The promoting effect of external formate concentration was mentioned in *hypF* and *selC*, but not in *fhlA* mutant strain. It is suggested that the effect of external formate on the growth of bacteria is mediated by FhlA regulatory protein at pH 7.5.

It was shown that the SGR of *selC* and *fhlA* mutant strains (see Tab. 1) was ~31% and ~22% higher compared with wild type, accordingly, when 1 *mM* formate concentration was added in the batch culture. However, there was no difference between SGR of *hypF* and wild type. Consequently, in the presence of less amount of formate FDHs, but not Hyd enzymes, have a negative role in bacterial growth at pH 7.5. *fhlA* mutant strain showed reduced SGR by ~30% in the presence of 10 *mM* formate, in comparison with wild type (Fig. 1). Unlike, similar data were not detected in the case of *hypF* and *selC* mutant strains. Thus, Hyds and FDHs perhaps have a positive influence on *E. coli* growth but only in the composition of FHL complexes.

Effect of DCCD and Different Concentrations of Formate on Growth of E. coli Wild Type and Mutant Strains During Mixed Carbon Sources Fermentation at Slight Acidic and Acidic pHs. The effect of DCCD was less at pH 6.5, than it was at pH 7.5, and was expressed only in addition of 10 *mM* formate (Fig. 2). Nevertheless, in *fhlA* mutant strain DCCD reduced the SGR value by ~19%,

~21%, ~38% correspondingly in the presence 1 *mM*, 5 *mM* and 10 *mM* of external formate. Here, as at pH 7.5, the compensatory role of FhlA protein in proton ATPase inhibition is also evident. This phenomenon is dependent on formate concentration at pH 6.5, which is probably due to the fact that ATP hydrolysis occurs when formate is bound to FhlA protein [8].

The positive effect of formate was observed at pH 6.5 as the SGR of *E. coli* wild type was higher by ~30% and ~36% in the presence of 5 *mM* and 10 *mM* formate, accordingly, in comparison to 1 *mM* (Fig. 2). The lack of FDHs caused the SGR increase by ~22% during the availability of 1 *mM* external formate. So, these enzymes separately have repressive effect on bacterial growth at the mentioned conditions.
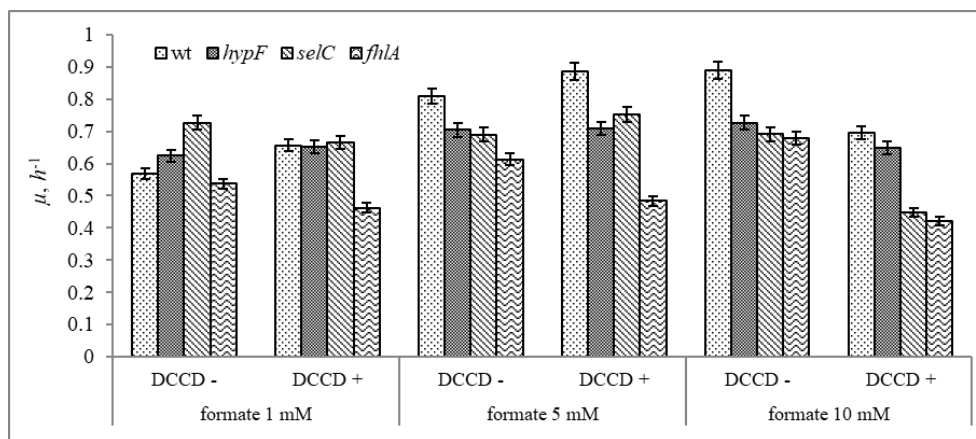

Fig. 2. SGR of *E. coli* wild type and mutant strains during fermentation of mixed carbon sources (glucose (11.1 *mM*), glycerol (137 *mM*) and formate (1 *mM*, 5 *mM*, 10 *mM*)) at pH 6.5. For other see legends to Fig. 1.

The SGR was decreased in *fhlA*, *hypF* and *selC* mutant strains by ~24%, ~13%, ~15% and ~24%, ~18%, ~22% respectively in the presence of 5 *mM* and 10 *mM* formate. Consequently, Hyds and FDHs interact in the bacterial cell membrane in the case of relatively high formate amount (5 *mM* and 10 *mM*) and have influence on growth processes either separately or in the complex.

The inhibitory influence of DCCD on bacterial growth was shown in the mutant strain absent of FDH enzymes and this effect is dependent on formate concentration at pH 5.5. DCCD weak inhibitory effect might be explained by that F_OF_1-ATPase possibly has such a conformation and position in the membrane that inhibitors, such as DCCD are not able to bond. Another point of view is availability of mechanisms to regulate $\Delta\mu_{H+}$ which are targeted to resist at acidic pH.

In the contrary at pH 7.5 and 6.5, external formate high concentration had an overwhelming effect on bacterial growth at pH 5.5, as SGR reduced value by ~20% and ~37% was determined in the presence of 5 *mM* and 10 *mM* formate, in comparison to 1 *mM* (Fig. 3).

There was no difference between the SGR of wild type and *selC* mutant strain, whereas in *hypF* and *fhlA* mutant strains it was decreased by ~13% and ~19% in the case of 5 *mM* formate suggesting that FDHs only in FHL complexes with Hyds can

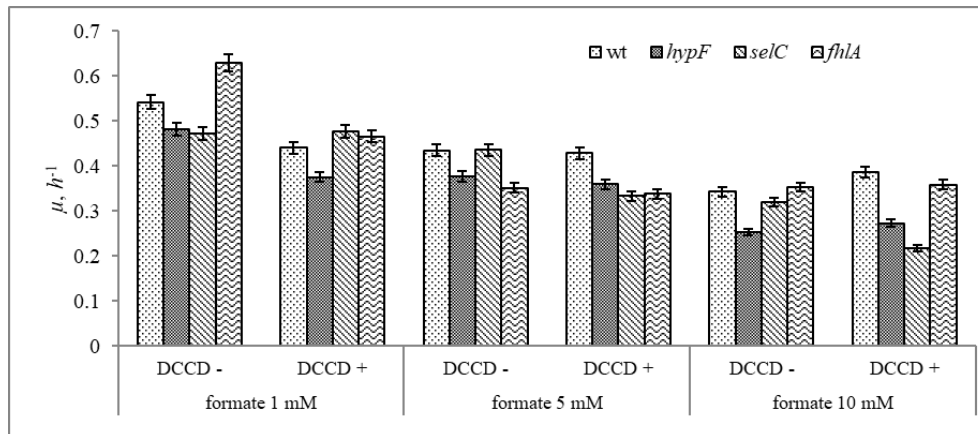have influence on bacteria growth. Hyds had a significant role ~26% in SGR when 10 *mM* formate was added.

Fig. 3. SGR of *E. coli* wild type and mutant strains during fermentation of mixed carbon sources (glucose (11.1 *mM*), glycerol (137 *mM*) and formate (1 *mM*, 5 *mM*, 10 *mM*)) at pH 5.5. For other see legends to Fig. 1.

Conclusion. Summarizing the data received it is significant to emphasize, that pH 7.5 is the optimum condition for *E. coli* bacterial growth during the co-fermentation of glucose and glycerol in the presence of formate. Furthermore, the influence of DCCD is considerable at pH 7.5. It is weaker at pH 6.5 and observed only in the case of 10 *mM* formate. Hyds do not have a role on bacterial growth in the presence of 1 and 5 *mM* external formate at pH 7.5. The mediated role of FhlA in the promotion of bacterial growth in the presence of external formate is suggested. It is demonstrated FhlA regulatory protein compensatory effect on SGR in the terms of proton ATPase inhibition by DCCD at pH 7.5 and 6.5 with addition of 10 *mM* formate. Hyds and FDHs have shown an important role on bacteria growth at pH 6.5 separately or in the FHL complex. Moreover, the SGR is formate concentration dependent at all pHs as the amount of external formate increase leads to SGR increase simultaneously at pH 7.5 and 6.5. The opposite effect is observed at pH 5.5. Thereby, the study, conducted in the condition of utilization of a mixture of carbon sources, provide important information for the improvement of bacterial biomass production. The data found in this research will be applicable to the development of bio-hydrogen technology.

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ՊՐՈՏՈՆԱՅԻՆ ԱԵՖազի ՄԵՆԱՀԱՏՈՒԿ ԱՐԳԵԼԱԿԻՉ
N,N'-ԴԻՑԻԿԼՈՀԵՔՍԻԼԿԱՐԲՈԴԻԻՄԻԴԻ ԵՎ ԱՐՏԱՔԻՆ
ՄՐՋՆԱԹԹՎԻ ՏԱՐԲԵՐ ԿՈՆՑԵՆՏՐԱՑԻԱՆԵՐԻ ԴԵՐԸ *E.COLI*-ի
ԱՃՄԱՆ ԳՈՐԾԸՆԹԱՑՈՒՄ ԱԾԽԱԾՆԻ ԽԱՌՆ ԱՂԲՅՈՒՐՆԵՐԻ
ԽՄՈՐՄԱՆ ԵՎ ՏԱՐԲԵՐ pH-ի ՊԱՅՄԱՆՆԵՐՈՒՄ

Տվյալ հետազոտությունն ուղղված է *E. coli* վայրի տիպի, Հիդ ու Մդի ֆերմենտների և FhlA կարգավորիչ սպիտակուցի խախտումներով մուտանտների աճման տեսակարար արագության փոփոխության ուսումնասիրությանը *N,N'*-դիցիկլոհեքսիլկարբոդիիմիդի (ԴՑԿԴ) և արտաքին մրջնաթթվի տարբեր խտությունների առկայության պայմաններում գլյուկոզի, գլիցերոլի և մրջնաթթվի համախմորման ընթացքում pH 5.5–7.5 արժեքներում: *E. coli-ի* աճման տեսակարար արագության ամենաբարձր արժեքը դիտվել է pH-7.5-ում: Բացահայտվել է, որ աճման տեսակարար արագությունը կախված է արտաքին մրջնաթթվի խտությունից բոլոր pH-ներում: ԴՑԿԴ-ի ճնշող ազդեցությունը նկատվել է առավելապես pH 7.5-ում և մասնակիորեն pH 6.5 և 5.5 արժեքներում: F_0F_1-ԱԵՖազի արգելակման պայմաններում բացահայտվել է FhlA-ի փոխհատուցողական դերը բակտերիաների աճման տեսակարար արագության մեջ:

Г. Х. ГЕВОРГЯН, А. В. ВАСИЛЯН, К. А. ТРЧУНЯН

РОЛЬ СПЕЦИФИЧЕСКОГО ИНГИБИТОРА ПРОТОН–АТФазы
N,N'-ДИЦИКЛОГЕКСИЛКАРБОДИИМИДА И КОНЦЕНТРАЦИИ
ВНЕШНЕГО ФОРМИАТА В РОСТЕ *E.COLI* ВО ВРЕМЯ БРОЖЕНИЯ
СМЕШАННЫХ ИСТОЧНИКОВ УГЛЕРОДА ПРИ РАЗЛИЧНЫХ pH

Данное исследование направлено на изучение изменений удельной скорости роста *E. coli* дикого типа и мутантов с дефектом ферментов гидрогеназ, формиат дегидрогеназы и регуляторного белка FhlA в присутствии *N,N'*-дициклогексилкарбодиимида (ДЦКД) и различных концентраций внешнего формиата при ферментации глюкозы, глицерина и формиата при pH 5.5–7.5. Самое высокое значение роста *E. coli* наблюдалось при pH 7.5. Выявлено, что рост зависит от концентрации внешнего формиата при всех значениях pH. Эффект ингибирования ДЦКД проявлялся в основном при pH 7.5 и частично – при pH 6.5 и 5.5. В условиях ингибирования F_0F_1-АТФазы выявлен компенсаторный эффект FhlA на удельную скорость роста бактерий.