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Impact of membrane-associated hydrogenases on the F_OF_1-ATPase in *Escherichia coli* during glycerol and mixed carbon fermentation: ATPase activity and its inhibition by *N,N′*-dicyclohexylcarbodiimide in the mutants lacking hydrogenases

Syuzanna Blbulyan, Armen Trchounian *

Department of Microbiology & Plants and Microbes Biotechnology, Faculty of Biology, Yerevan State University, 1 A. Manoukian Str., Yerevan 0025, Armenia

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ABSTRACT

Escherichia coli is able to ferment glycerol and to produce molecular hydrogen (H_2) by four membrane-associated hydrogenases (Hyd) changing activity in response to different conditions. In this study, overall ATPase activity of glycerol alone and mixed carbon sources (glucose and glycerol) fermented *E. coli* wild type and different Hyd mutants and its inhibition by *N,N′*-dicyclohexylcarbodiimide (DCCD) were first investigated. ATPase activity was higher in glycerol fermented wild type cells at pH 7.5 compared to pH 6.5 and pH 5.5; DCCD inhibited markedly ATPase activity at pH 7.5. The ATPase activity at pH 7.5, compared with wild type, was lower in *selC* and less in *hypF* single mutants, suppressed in *hyaB hybC selC* triple mutant. Moreover, total ATPase activity of mixed carbon fermented wild type cells was maximal at pH 7.5 and lowered at pH 5.5. The ATPase activities of *hypF* and *hyaB hybC selC* mutants were higher at pH 5.5, compared with wild type; DCCD inhibited markedly ATPase activity of *hypF* mutant. These results demonstrate that in *E. coli* during glycerol fermentation the membrane proton-translocating F_OF_1-ATPase has major input in overall ATPase activity and alkaline pH is more optimal for the F_OF_1-ATPase operation. Hyd-1 and Hyd-2 are required for the F_OF_1-ATPase activity upon anaerobic fermentation of glycerol. The impact of Hyd-1 and Hyd-2 on the F_OF_1-ATPase is more obvious during mixed carbon fermentation at slightly acidic pH.

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Introduction

It is well known that many bacteria can ferment different carbon sources like sugars (glucose) and glycerol, the mixtures of which are available in many industrial, agricultural and other organic substrates containing wastes as well as in human gut [1–3]. Nowadays different projects are focused on understanding the role of sole and mixed carbons in bacterial cell physiology during fermentation; particularly, it is of interest to investigate metabolic pathways of molecular hydrogen (H_2) production by bacteria upon utilization of mixed carbon sources (glucose and glycerol) [2,3]. Recently, it has been found that *Escherichia coli* has ability to anaerobically ferment glycerol at a pH-depended manner [4–8] leading to H_2 evolution. The glucose and glycerol mixed fermentation by *E. coli* is investigated: the results already obtained indicate that H_2 production is inhibited by glucose in a concentration-dependent manner during glucose fermentation [9] but glucose in combination with glycerol might enhance H_2 production during mixed carbon fermentation [3,10].

Under anaerobic conditions, *E. coli* converts glycerol to dihydroxyacetone phosphate (DHAP) which is converted to pyruvate [11]. The latter is further converted to formate and acetyl coenzyme-A. Finally, formate is oxidized to H_2 and carbon dioxide by formate hydrogen lyase (FHL) [12].

In *E. coli* FHL is membrane-associated protein complex consisted of selenocysteine- and Mo-cofactor-containing formate dehydrogenase-H (Fdh-H), [Ni–Fe] hydrogenase (Hyd) and other electron-transferring components [13]. The complex containing Hyd-3 (*hyc*) is considered as FHL-1, whereas Hyd-4 (*hyf*) containing complex is considered as FHL-2 [13,14]. These two FHL complexes are active upon glucose fermentation, but they might be different under glycerol fermentative conditions [13]. Besides, *E. coli* synthesizes the other Hyd enzymes (Hyd-1, Hyd-2), which are also functional in anaerobic fermentative conditions [13,15]. Hyd-1 (*hya*) and Hyd-2 (*hyb*) oxidize H_2 during glucose fermentation and work

* Corresponding author.
E-mail address: Trchounian@ysu.am (A. Trchounian).

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in a reverse mode upon glycerol fermentation [6]. For biosynthesis of Fdh-H and Hyd enzymes two factors are important. The mRNA for Fdh-H contains termination codon which is decoded by the product of *selC* gene [16]. The latter is necessary for incorporation of selenocysteine in Fdh-H [17]. Moreover, Hyd synthesis and maturation require different proteins, especially HypF, which is encoded by *hypF* gene [18]. HypF is essential accessory protein which is involved in the assembly of ligands on the large subunits of Hyd enzymes; this protein is coupled with ATP hydrolysis [19].

The activities of FHL and, in addition, proton-translocating F_OF_1-ATPase, key membrane enzyme of bioenergetic relevance, have been also found out to affect the fermentative metabolism of glycerol in *E. coli* [13,20]. Some requirement of F_OF_1 and its relationship with Hyd enzymes in *E. coli* were suggested upon both glucose and glycerol fermentations [7,13]. It was shown that Hyd-3 and Hyd-4 activities are related with the F_OF_1-ATPase at pH 7.5 and this relationship may be due to Hyd-4 interaction with the F_OF_1-ATPase to provide reducing equivalents ($H^+ + e^-$) for energy transfer to the secondary transport system [21,22]. Indeed, it has been also established that H_2 production by *E. coli* during glucose and glycerol fermentation at slightly alkaline and acidic pHs, respectively, is inhibited by *N*,*N*′-dicyclohexylcarbodiimide (DCCD), an inhibitor of the F_OF_1-ATPase [7]. The results, obtained by using *E. coli atp* mutant lacking functional F_OF_1, indicate a requirement of active F_OF_1 for the activity of Hyd-1 and Hyd-2 during glucose or glycerol fermentation [7,23]. Moreover, double mutant lacking Hyd-1 and Hyd-2 had the increased F_OF_1-ATPase activity at pH 7.5, but not at pH 5.5 [24]. It was suggested a metabolic link between F_OF_1 activity and Hyd operation, and especially the key role of Hyd-1 and Hyd-2 in energy conservation [7]. However, impact of Hyd enzymes on F_OF_1 during glycerol and especially mixed carbon sources fermentation and appropriate mechanisms for this functional link are not clear and require further study using different mutants lacking all four Hyd enzymes.

In the present paper, the impact of different Hyd enzymes with F_OF_1 in *E. coli* was investigated during glycerol and mixed carbon (glucose and glycerol) fermentation. The overall ATPase activity of membrane vesicles, isolated from glycerol alone and in combination with glucose fermented *E. coli* wild-type and different Hyd mutant strains, and its inhibition by DCCD were determined at different pHs.

Materials and methods

Bacteria, bacterial growth, membrane vesicles

The *E. coli* strains used in this study are listed in Table 1.

Bacteria were grown under anaerobic conditions at 37 °C for 18–20 h in highly buffered peptone medium (20 g L^{-1} peptone, 15 g L^{-1} K_2HPO_4, 1.08 g L^{-1} KH_2PO_4, 10 g L^{-1} NaCl) with glycerol (10 g L^{-1}) and glucose and glycerol together (2 g L^{-1} and 10 g L^{-1}) at different pHs during 20–24 h. The medium was filled in glass vessels; oxygen was bubbled out from medium during autoclaving, and then vessels were closed with plastic press caps, as described elsewhere [23–26]. To check anaerobic conditions, H_2 production was tested, as before [25–27]. The growth medium pH was measured by a pH-meter with a selective pH-electrode (HJ1131B, Hanna Instruments, Portugal) and adjusted using of 0.1 M KCl or 0.1 N NaOH. The specific growth rate was determined measuring the change of culture absorbance, as detailed previously [23–26].

Membrane vesicles were isolated from bacteria treated with l ysozyme–ethylenediaminetetraacetic acid by osmotic lysis of spheroplasts [28] or by disrupting the cells with a French press [29,30], as described previously [24,31–33].

Membrane vesicles ATPase assay

ATPase activity of membrane vesicles was determined by the amount of inorganic phosphate (P_i) produced in the reaction of membrane vesicles with 5 mM ATP (pH 7.5, 6.5 and 5.5) [24–26,31–33] in the assay mixture (50 mM Tris–HCl buffer (pH 7.5, 6.5 and 5.5) containing 1 mM $MgSO_4$). ATP can reach ATPase in right-side-out vesicles due to membrane peculiarities of cells grown under the mentioned conditions [24,32] or the presence of in-side-out vesicles in the preparations, as suggested [29,34]. The ATPase activity was expressed in nMol P_i (min μg protein)$^{-1}$. P_i was determined spectrophotometrically (with UV–VIS Auto PS scanning spectrophotometer, LaboMed, USA) by the method, as described [31–33]. The DCCD-inhibited ATPase activity was calculated as difference between activities in the absence and in the presence of the inhibitor.

Others, reagents and data processing

Protein levels were measured by the method of Lowry [24] using bovine serum albumin (BSA) as a standard. Membrane vesicles were incubated with 0.5 mM DCCD (ethanol solution) for 10 min prior assays; ethanol in the final concentration of 0.5% was used as a blank; no effect on growth and ATPase activity was observed. All assays were done at 37 °C.

Agar, peptone, glycerol, Tris (Carl Roth GmbH, Germany), ATP (Tris salt), BSA, DCCD, lysozyme (Sigma, USA) and other reagents of analytical grade were used.

Average data obtained from 3 independent assays are represented, and standard deviations of values do not exceed 3% if not given. Student criteria (p) is employed to validate the difference in average data between various series of experiments, as described previously [3,24]; the difference is valid when $p < 0.5$ or less if not otherwise shown.

Results and discussion

Growth of E. coli mutants lacking Hyd enzymes at different pHs

E. coli wild type and different Hyd mutant strains (see Table 1) can grow well in peptone medium fermenting glycerol only or

Table 1
Characteristics of the *E. coli* wild type and mutant strains used.

Strain	Genotype	Appropriate absent or defective proteins	References
BW 25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{W116}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rha* *BAD*$_{LD78}$	Wild tye	[6,12]
KT 2110	BW 25113 Δ*hyaB* Δ*hybC* Δ*selC*	Large subunits of Hyd-1 and Hyd-2, tRNA^{sec}	[23]
FM 460	MC 4100 Δ*selC*	tRNA^{sec}	[23]
DHP-F2	MC 4100 Δ*hypF*	All four Hyd enzymes	[18]

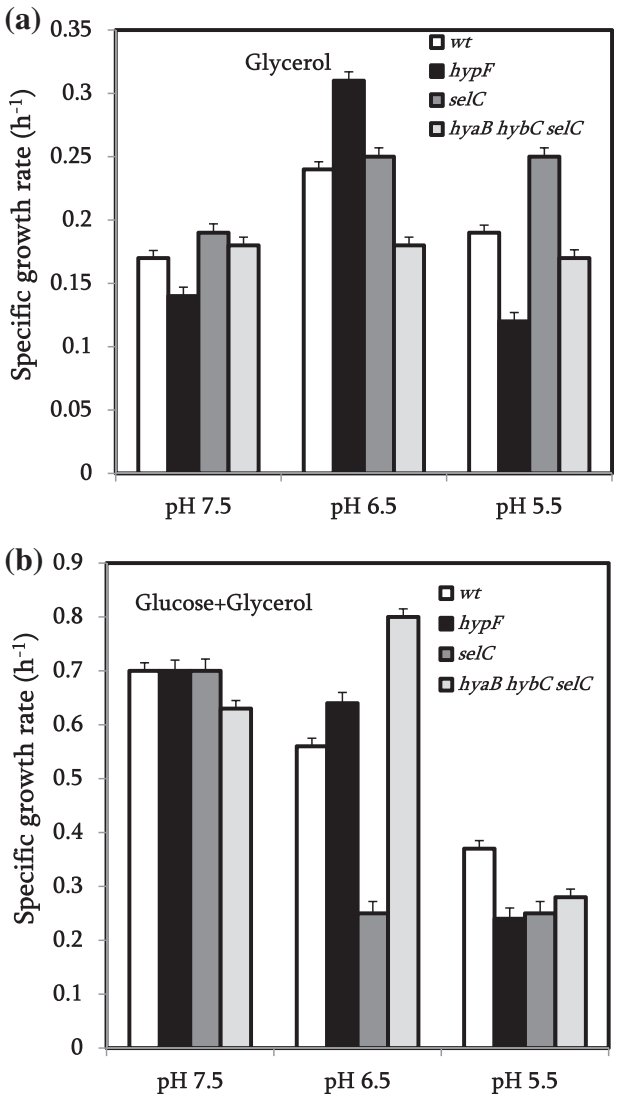

Fig. 1. Growth of *E. coli* wild type and *selC*, *hyaB hybC selC* and *hypF* mutant strains at different pHs. Bacteria were grown during glycerol (a) and glycerol with glucose (b) fermentation. For the strains see Table 1; for others, see Materials and methods.

glycerol added to glucose at pH 7.5, 6.5 and 5.5 (Fig. 1). The cells growth was more during mixed carbon fermentation of glycerol added to glucose. This might be explained by different membrane mechanisms for glycerol and glucose transfer into the cells and some differences in their metabolic pathways [9,25]. pH 6.5 was optimal for growth of *E. coli* on glycerol when specific growth rate was ~1.4-fold higher (see Fig. 1a). Interestingly, specific growth rate was lowered in *E. coli* DHP-F2 *hypF* mutant strain lacking all Hyd enzymes [18] at pH 7.5 and pH 5.5 but not pH 6.5 (see Fig. 1a).

Moreover, specific growth rate during mixed carbon fermentation on glycerol added to glucose was markedly low at pH 5.5 ($p < 0.01$) (see Fig. 1b). In the case with *selC* mutant, the specific growth rate was low at pH 6.5 too ($p < 0.01$) (see Fig. 1b).

These data suggest the role of Hyd enzymes in bacterial growth on glycerol only or glycerol and glucose mixture depending on pH. This interesting idea seems to be not simple and might have different explanations. Hyd enzymes can affect the production of fermentation end products and they might impact on the F_OF_1-ATPase, maintaining a proton-motive force [13,22]. Therefore, the relationship of Hyd enzymes with the F_OF_1-ATPase was studied during the glycerol and mixed carbon sources fermentation.

ATPase activity of glycerol fermented E. coli membrane vesicles at different pHs

The overall ATPase activity of membrane vesicles from glycerol-fermented *E. coli* BW25113 wild type and different Hyd mutants (see Table 1) and its inhibition by DCCD were investigated with membrane vesicles of different types at different pHs. ATPase activity of right-side-out vesicles was 223 ± 8 nMol P_i (min μg protein)$^{-1}$ (Table 2); this value was closed to data on ATPase activity reported before [24]. Interestingly, this ATPase activity was ~74% of that in in-side-out membrane vesicles (see Table 2). Moreover, ATPase activity of both types of membrane vesicles was markedly inhibited by DCCD (see Table 2). Note, ATPase activity of right-side-out vesicles from *E. coli* grown during glucose fermentation was determined previously [24–26,31–33]: it was ~70% of that in in-side-out ones, as shown by Adler and Rosen [34] and confirmed by Bagramyan et al. [32]. Moreover, DCCD has been shown to inhibit the F_OF_1-ATPase in *E. coli* since effect of DCCD is absent in the *atp* mutant lacked this ATPase [23] and, in addition, *E. coli* has no bc_1 complex which could be inhibited by DCCD [21,34]. Therefore, right-side-out vesicles were valuable to study ATPase activity in the mutants lacking Hyd enzymes and to compare results with those obtained during glucose fermentation.

Indeed, ATPase activity of membrane vesicles from glycerol-fermented *E. coli* was ~3-fold higher at pH 7.5 in wild type compared with that at pH 6.5 (see Fig. 2a and b). 0.5 mM DCCD inhibited markedly (10-fold, $p < 0.001$) ATPase activity of wild type at pH 7.5; but the inhibition was less (1.3-fold) at pH 6.5 (see Fig. 2a and b). It should be mentioning that no ATPase activity was detected in supernatant upon preparation of membrane vesicles (data not shown). These data confirm that alkaline pH is more optimal for F_OF_1 operation in *E. coli* during glycerol fermentation, as shown before [35].

Then, compared with the wild type cells, ATPase activity of *hypF* mutant was ~2.2-fold decreased at pH 7.5. These findings might be explained by direct relationship of F_OF_1 and Hyd enzymes during glycerol fermentation at slightly alkaline pH. Both, *hypF* mutant and wild type demonstrated the similar ATPase activity at pH 6.5 (see Fig. 2b). DCCD inhibited ATPase activity by ~1.4-fold in *hypF* mutant at pH 7.5 but this reagent unaffected ATPase activity at pH 6.5 (see Figs. 2a and b, Table 3). However, at pH 5.5 total ATPase activity of wild type and *hypF* mutant strains was lower compared with those at pH 7.5 and again had similar values ($p > 0.5$) (see Fig. 2c). Thus, a correlation between overall ATPase activity and medium pH value was observed in wild type: the lowest ATPase activity was determined during growth on glycerol at pH 5.5.

During growth on glycerol at pH 7.5, the total ATPase activity of *E. coli* FM460 *selC* mutant, which was unable to synthesize seleno-proteins of FHL (with defective Fdh-H; see Table 1), was approximately 60–65% of the wild type (see Fig. 2a). This effect of *selC* mutation on overall ATPase activity suggests that at pH 7.5 Fdh-H or FHL might affect the F_OF_1-ATPase directly or probably

Table 2
ATPase activity in right-side-out and in-side-out membrane vesicles *E. coli* BW25113 grown during glycerol fermentation at pH 7.5 and its inhibition by DCCD.

Membrane vesicles[a]	ATPase activity, nMol P_i/min/μg protein	
	Overall	DCCD-inhibited[b]
Right-side-out	223 ± 8	203 ± 7
In-side-out	300 ± 12	256 ± 10

[a] For preparation of membrane vesicles, see Materials and methods.
[b] DCCD-inhibited ATPase activity was calculated as the difference between the overall activity in the absence of DCCD and the activity in the presence of DCCD (0.5 mM).

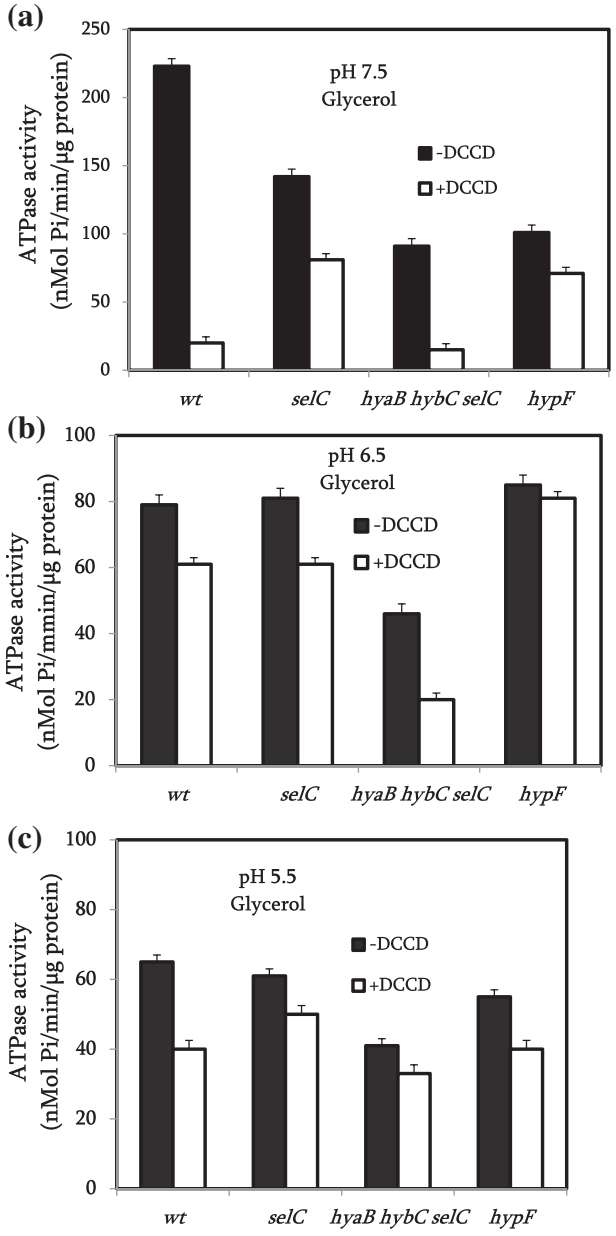

Fig. 2. ATPase activity of membrane vesicles of *E. coli* wild type and *selC*, *hyaB hybC selC* and *hypF* mutant strains at pH 7.5 (a), pH 6.5 (b) and pH 5.5 (c). The DCCD (0.5 mM) was added into the assay medium when indicated. The assays pH was the same as growth pH. Right-side-out membrane vesicles were used. For the strains see Table 1; for others, see Materials and methods and legends to Fig. 1.

indirectly due to some relation of Hyd-3 to Hyd-1, as suggested [36]. This seems to be likely to that Fdh-H is required to stabilize Hyd-3 but it is not essential for activity [38].

Then, the overall and DCCD-inhibited ATPase activity of membrane vesicles of *E. coli* KT2110 *hyaB hybC selC* triple mutant grown under glycerol fermentation at pH 7.5 was markedly decreased compared with the wild type (see Fig. 2a, Table 3). In comparison with only *selC* mutation, the triple deletions of *hyaB*, *hybC selC* significantly decreased (1.56-fold) and increased (1.24-fold) the overall and DCCD-inhibited ATPase activity, respectively. These findings indicate that Hyd-1 and Hyd-2 are more than Hyd-3 required for the F_OF_1-ATPase activity. This is in good conformity with that no Hyd-1 or Hyd-2 activities were determined in *E. coli atp* mutant lacking the F_OF_1-ATPase [7].

At pH 6.5 wild type, *selC* and *hypF* mutants had similar ATPase activity but *hyaB hybC selC* mutant had ~1.7-fold lower ATPase activity than the wild type; the ATPase activity was suppressed ~2.3-fold in this mutant by DCCD ($p < 0.01$) (see Fig. 2b, Table 3). Note, DCCD had the same effect on wild type and *selC* ATPase activities (see Fig. 2b, Table 3). These results suggest that there is no direct relationship between Hyd-3 and the F_OF_1-ATPase upon the anaerobic fermentation of glycerol at slightly acidic pH.

The ATPase activities were lower at pH 5.5 (see Fig. 2c) than at pH 7.5 and pH 6.5 for the mutants used; in the case of *hyaB hybC selC* mutant ATPase activity at pH 5.5 was similar with that at pH 6.5 (see Fig. 2c). At acidic pH DCCD lowered ~1.3-fold ATPase activity; a stronger effect was observed in *hyaB hybC selC* mutant (see Fig. 2c, Table 3). These results indicated that acidic pH is not optimal for ATPase activity upon glycerol fermentation. Low overall and DCCD-inhibited ATPase activity of *hyaB hybC selC* mutant again suggests the requirement of Hyd-1 and Hyd-2 for the F_OF_1-ATPase activity.

The data obtained demonstrate that there is a correlation between the F_OF_1-ATPase activity and pH value during glycerol fermentation: the highest activity was observed at pH 7.5. Moreover, Hyd-1 and Hyd-2 are required for the F_OF_1-ATPase activity under glycerol fermentation.

ATPase activity of E. coli membrane vesicles under mixed carbon fermentation at different pHs

The ATPase activity of mixed carbon fermented *E. coli* wild type and Hyd mutants (see Table 1) was determined at different pHs. It is worth mentioning that the all strains during mixed carbon sources (glucose and glycerol) fermentation showed higher ATPase activity at pH 7.5, 6.5 and 5.5 compared with ATPase activity of cells grown under glycerol only fermentation at appropriate pHs. Membrane vesicles of mixed carbon fermented *E. coli* wild type cells demonstrated significant overall ATPase activity of 255 ± 2 nMol P_i (min μg protein)$^{-1}$: DCCD at the same

Table 3
DCCD-inhibited ATPase activities of right-side-out membrane vesicles of *E. coli* wild type and mutants grown under glycerol or mixed carbon sources (glucose and glycerol) fermentation.

Strain	DCCD-inhibited ATPase activity, %[a]					
	pH 7.5		pH 6.5		pH 5.5	
	Glycerol	Glucose + Glycerol	Glycerol	Glucose + Glycerol	Glycerol	Glucose + Glycerol
BW 25113	90 ± 3	60 ± 1	25 ± 2	65 ± 1	33 ± 1	77 ± 1
DHP-F2	30 ± 2	87 ± 2	5 ± 2	50 ± 1	16 ± 2	86 ± 1
KT2110	83 ± 2	14 ± 2	56 ± 1	60 ± 1	19 ± 2	28 ± 2
FM 460	43 ± 2	67 ± 1	25 ± 1	66 ± 1	21 ± 2	63 ± 1

[a] For strains see Table 1; for the others see Materials and methods; DCCD-inhibited ATPase activity was calculated as percentage of overall ATPase activity which was 100% for each strain at appropriate pH during either glycerol or mixed carbon fermentation.

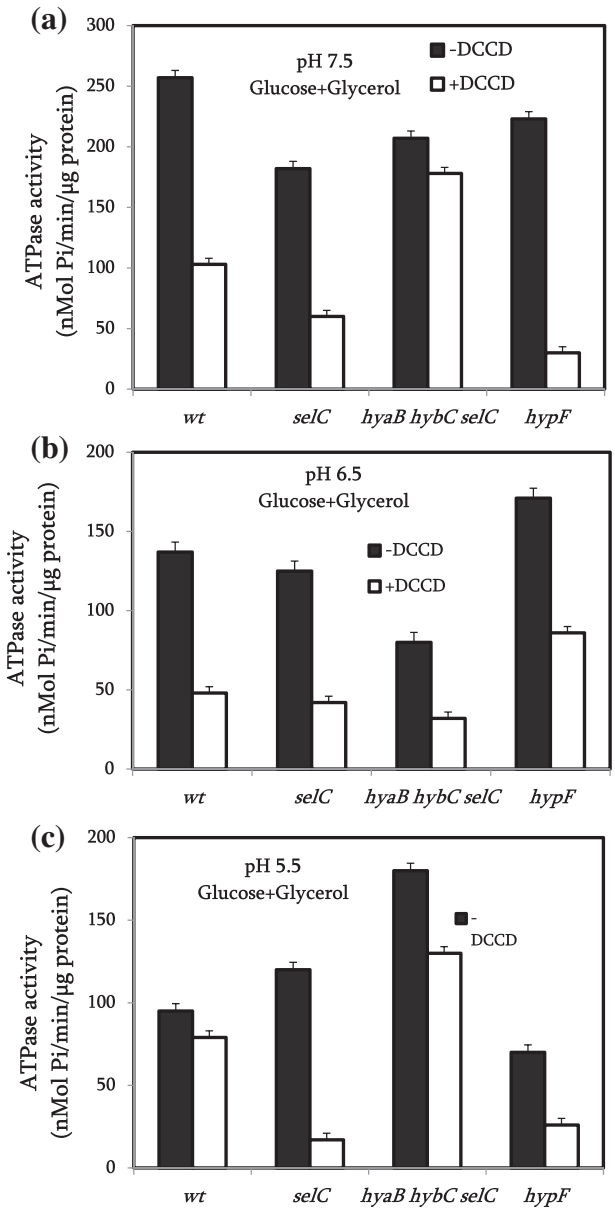

Fig. 3. ATPase activity of membrane vesicles of *E. coli* wild type and mutant strains at pH 7.5 (a), pH 6.5 (b) and pH 5.5 (c). Bacteria were grown during mixed carbon sources (glucose and glycerol) fermentation at different pHs. For the others, see Materials and methods and legends to Fig. 2.

concentration as that used with the glycerol fermented cells, inhibited ATPase activity of wild type by 2-fold ($p < 0.01$) (see Fig. 3a, Table 3). Interestingly, ATPase activity upon mixed carbon fermentation compared with glycerol fermentation was stimulated (~2.2-fold, $p < 0.01$) in *hypF* mutant at pH 7.5, this activity was closed to the wild type (see Fig. 3a). The membrane vesicles demonstrated the marked inhibition (~7-fold, $p < 0.001$) of ATPase activity in the presence of DCCD so the F_OF_1-ATPase activity was increased (see Fig. 3a, Table 2). These results indicate the major input of (see Fig. 3a, Table 2) in overall ATPase activity upon mixed carbon fermentation at pH 7.5. Deletion of *hypF* did not affect significantly overall ATPase activity but increased the F_OF_1-ATPase activity; thus suggesting that Hyd enzymes have some impact on the F_OF_1-ATPase at slightly alkaline pH during mixed carbon fermentation in contrast to glycerol only fermentation.

Besides, in the mixed carbon fermented cells, compared with glycerol only fermented ones, ATPase activity at pH 7.5 was higher ~1.3-fold in *selC* and ~2.3-fold ($p < 0.01$) in *hyaB hybC selC* mutants. In spite of a higher ATPase activity in *hyaB hybC selC* mutant, DCCD inhibition was less (see Fig. 3a, Table 3) whereas DCCD inhibited ATPase activity ~3-fold in *selC* mutant (see Fig. 3a, Table 3). These results suggest that during mixed carbon fermentation at slightly alkaline pH FHL and probably Hyd-3 are more necessary for F_OF_1 activity than Hyd-1 and Hyd-2.

The absence of all four Hyd enzymes had no effect on ATPase activity at pH 6.5 during the fermentation of glucose and glycerol: *hypF* mutant had the highest total ATPase activity (see Fig. 3b). The wild type had ~2-fold ($p < 0.01$) lower ATPase activity at pH 6.5 than at pH 7.5. Thus, correlation between total ATPase activity and growth medium pH was also observed upon mixed carbon fermentation as for glycerol only fermentation.

It should be noted that in the mixed carbon fermented *hyaB hybC selC* membrane vesicles overall and DCCD-inhibited ATPase activity was lower (2.5-fold, $p < 0.01$) at pH 6.5, compared with that at pH 7.5 (see Fig. 3b). The total ATPase activity of triple mutant was ~55% of wild type activity, while *selC* mutant retained ~90% of the wild type activity. These data allow suggesting that during mixed carbon fermentation at slightly acidic pH Hyd-1 and Hyd-2 but not Hyd-3 are required for the F_OF_1-ATPase activity. DCCD inhibition of ATPase activity was the same (~3-fold) in *selC* mutant at pH 6.5 and pH 7.5 (see Fig. 3a and b, Table 3).

The situation upon mixed carbon fermentation was different at pH 5.5. The wild type had ~3-fold lower ($p < 0.01$) overall ATPase activity than at pH 7.5, while ATPase activities of *hypF* and *hyaB hybC selC* mutants were higher compared with the wild type (see Fig. 3c). Note, DCCD inhibited significantly (7-fold) and less (1.4-fold) ATPase activity of *hypF* and *hyaB hybC selC* mutants at acidic pH, respectively (see Fig. 3c, Table 3). Probably, in these conditions (glucose and glycerol fermentation) when end products of fermentation, such as formate and other acids, might be accumulated within the cell and, therefore, decrease intracellular pH, the F_OF_1-ATPase is playing an important role in regulation of intracellular pH by detoxification of different acids formed during fermentation, specifically of formic acid.

Conclusions and remarks

The data obtained indicate a correlation between membrane ATPase activity in *E. coli* and pH; alkaline pH is more optimal for the DCCD-inhibited F_OF_1-ATPase activity during glycerol fermentation. This is probably related with bacterial optimal growth at pH 7.5. Moreover, the F_OF_1-ATPase has a different role in maintaining proton motive force depending on Hyd enzymes as suggested [13,22]. The results with the *hyaB hybC selC* triple mutant suggest the requirement of Hyd-1 and Hyd-2 for the activity of the F_OF_1-ATPase during glycerol fermentation, and cooperation or interaction between these enzymes is more obvious at slightly alkaline pH (see Fig. 2a). The higher ATPase activity of *hyaB hybC selC* and *selC* mutants upon the mixed carbon (glucose and glycerol) fermentation at pH 7.5 (see Fig. 3a) presumably can be explained by that, during mixed carbon fermentation, the F_OF_1-ATPase is involved in the formation of H_2 cycling through the membrane as suggested [22]. It is possible that, when Hyd-3 is not active as a part of FHL, the F_OF_1-ATPase operates for regulation of intracellular pH.

Moreover, the higher ATPase activity of *hypF* mutant during the mixed carbon fermentation probably is a response of cell to the absence of all Hyd enzymes. F_OF_1 is functioning to balance proton cycle and regulate cell's energetic requirements. Interestingly, conservation of energy by *E. coli* during glycerol fermentation might be different from that at glucose [37], although stoichiometry of ATP/glucose was constructed to depend on the concentration of glucose over a wide range of H_2 production [39].

Then, Hyd-1 and Hyd-2 impact on the F_OF_1-ATPase is more essential at slightly acidic pH (pH 6.5) upon mixed carbon fermentation by *E. coli*. The higher ATPase activity of *hyaB hybC selC* mutant during mixed carbon fermentation at acidic pH might be explained by the F_OF_1-ATPase role in neutralization of fermentation end products. Probably, the F_OF_1-ATPase is functioning as additional mechanism for detoxification of organic acids, specifically formic acid formed during fermentation. Interestingly, *E. coli* is suggested to have a mechanism to respond to different carbon sources and aerobic or anaerobic conditions by regulating the composition, the activity and the functions of the F_OF_1-ATPase [33,40], so a further special study is required to clarify different functions of the F_OF_1-ATPase depending on glycerol and glucose. Differential expression of genes coding the F_OF_1-ATPase should be not ruled out. But this is a very complex problem.

Thus, the present results are important in understanding the role of the F_OF_1-ATPase and Hyd enzymes in cells and especially the functional link between Hyd-1, Hyd-2 and the F_OF_1-ATPase during anaerobic fermentation of glycerol and mixed carbon sources. It is also worth mentioning that, depending on fermentation substrate and pH, the form of relationship between these enzymes could be different.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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