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The roles of hydrogenases 3 and 4, and the F_0F_1-ATPase, in H_2 production by *Escherichia coli* at alkaline and acidic pH

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Abstract **The *hyc* operon of *Escherichia coli* encodes the H_2-evolving hydrogenase 3 (Hyd-3) complex that, in conjunction with formate dehydrogenase H (Fdh-H), constitutes a membrane-associated formate hydrogenlyase (FHL) catalyzing the disproportionation of formate to CO_2 and H_2 during fermentative growth at low pH. Recently, an operon (*hyf*) encoding a potential second H_2-evolving hydrogenase (Hyd-4) was identified in *E. coli*. In this study the roles of the *hyc*- and *hyf*-encoded systems in formate-dependent H_2 production and Fdh-H activity have been investigated. In cells grown on glucose under fermentative conditions at slightly acidic pH the production of H_2 was mostly Hyd-3- and Fdh-H-dependent, and Fdh-H activity was also mainly Hyd-3-dependent. However, at slightly alkaline pH, H_2 production was found to be largely Hyd-4, Fdh-H and F_0F_1-ATPase-dependent, and Fdh-H activity was partially dependent on Hyd-4 and F_0F_1-ATPase. These results suggest that, at slightly alkaline pH, H_2 production and Fdh-H activity are dependent on both the F_0F_1-ATPase and a novel FHL, designated FHL-2, which is composed of Hyd-4 and Fdh-H, and is driven by a proton gradient established by the F_0F_1-ATPase.** **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Hydrogenase; F_0F_1-ATPase; H_2 production; Fermentation; *Escherichia coli*

1. Introduction

Escherichia coli growing on glycolytic carbon sources under anaerobic conditions in the absence of electron acceptors carries out a mixed-acid fermentation resulting in the excretion of formate (via the formate channel, FocA), acetate, succinate, lactate and ethanol. The formate produced can be further metabolized to H_2 and CO_2 by a membrane-associated formate hydrogenlyase (FHL-1) system consisting of formate dehydrogenase H (Fdh-H) and the hydrogenase 3 complex (Hyd-3) [1]. This pathway is only active at low pH and high formate concentrations, and is thought to provide a detoxification/de-acidification system countering the buildup of formate during fermentation. The recent discovery of a new, potential H_2-evolving hydrogenase (Hyd-4) in *E. coli* has led to the suggestion that *E. coli* may possess a second formate hydrogenlyase (FHL-2) composed of Fdh-H and Hyd-4 [2]. It was proposed that, unlike FHL-1, FHL-2 may be energy transducing since, like the proton-translocating NADH:ubiquinone oxidoreductase complex and unlike Hyd-3, the Hyd-4 complex possesses three ND2/ND4/ND5-like subunits [2]. However, until now no activity has been ascribed to Hyd-4 and so its function remains uncertain.

The seven subunits of Hyd-3 are encoded by the *hycABCDEFGHI* operon [3]. The *hycB* and *hycF* genes encode small subunits thought to function in electron transfer within the FHL complex, the *hycE* and *hycG* genes encode the hydrogenase large subunit (containing the Ni–Fe center) and the hydrogenase small subunit, respectively, and the *hycC* and *hycD* genes encode polytopic membrane proteins. The *hycH* gene encodes a hydrophilic protein likely to comprise part of the Hyd-3 complex. *hycA* encodes a repressor (anti-activator) of the *hyc* operon, and *hycI* encodes a protease required for maturation of the Hyd-3 large subunit (and, possibly, the Hyd-4 large subunit) [4,5]. The *hyc* operon appears to be regulated solely in response to formate concentration at low pH. This regulation is mediated by the FhlA protein which is a σ^{54}-dependent activator of the formate regulon [1,6,7].

The 10 putative subunits of Hyd-4 are encoded by the *hyfABCDEFGHIJR-focB* operon [2]. The *hyf* operon encodes homologues of all seven Hyd-3 subunits, and in addition contains genes (*hyfDEF*) apparently specifying three integral-membrane subunits with no direct counterpart in Hyd-3 [2]. The *hyfR* gene encodes an FhlA homologue, and *focB* encodes a putative formate transporter homologous with FocA [2].

In *Salmonella typhimurium*, fermentative gas (H_2 and H_2S) production is absent in *atp* mutants possessing defective F_0F_1-ATPase, and Fdh-H activity is greatly reduced by both *atp* mutations and the F_0F_1-ATPase inhibitor, *N*,*N′*-dicyclohexylcarbodiimide (DCCD) [8]. Fermentative gas (H_2) production in *E. coli* is also DCCD-inhibited [9] and absent in *atp* mutants [10]. Furthermore, FHL activity in *E. coli* is not observed when arsenate or protonophores are used to lower the transmembrane proton electrochemical gradient ($\Delta\mu_{H^+}$) [2]. These observations strongly suggest that FHL activity is

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Abbreviations: F_0F_1, the H^+-translocating ATP synthase; Fdh-H, formate dehydrogenase H; FHL, formate hydrogenlyase; Hyd-1, Hyd-2, Hyd-3 and Hyd-4, hydrogenases 1, 2, 3 and 4, respectively; ND2, ND4, ND5, denoted subunits of NADH dehydrogenase of NADH:ubiquinone oxidoreductase complex; DCCD, *N*,*N′*-dicyclohexylcarbodiimide; $\Delta\mu^+_H$, the transmembrane proton electrochemical gradient

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both F_0F_1-ATPase-dependent and sensitive to perturbations in $\Delta\mu_{H^+}$.

The results reported here show that H_2 production and Fdh-H activity are largely F_0F_1-ATPase- and Hyd-4-dependent at alkaline pH, but are independent of these factors at low pH.

2. Materials and methods

2.1. Bacterial strains, genetic methods

The *E. coli* strains used in this study are listed in Table 1. To investigate the effects of *atp* deletion on the expression of the *hyc* and *hyf* operons and the *fdhF* gene, the *atpB-D* deletion of CAG18431 was transferred to the corresponding *lacZ* fusion strains in a two-step process: first, the *ilv500*::Tn*10* mutation of CAG18431 [11] was transferred via P1-mediated transduction [12] to strain TK2538 to generate the tetracycline-resistant strain, AT1 (*ilv500*::Tn*10* Δ*atpB-D*), which was identified by its inability to grow on minimal medium containing 40 mM succinate plus 100 mM K^+; and second, the *atpB-D* deletion and *ilv500*::Tn*10* mutation of AT1 were co-transduced into the corresponding *lacZ* fusion strains, MC10613, DS5 and M9s (Table 1), to generate the Lac^+ strains AT2, AT3 and AT4, which were identified as described for AT1.

2.2. Growth and preparation of bacteria, protoplasts

Bacteria were grown under anaerobic conditions at 37°C in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or peptone (2% peptone, 0.5% NaCl) growth medium with 0.2% glucose and 0.1 M potassium phosphate (pH 7.5) (unless otherwise stated). Note that the pH of the medium decreased from 7.5 to 6.9 during the course of fermentative growth. The *atp* strains were able to grow well under fermenting conditions, as noted previously [17–19]. Cells were harvested at pH ~7.0 (unless otherwise noted). Preparation of whole cells and protoplasts for the H_2 production and Fdh-H assays was as described previously [20]. To increase membrane permeability for ATP, whole cells and protoplasts were treated with a small amount of toluene (15–20 µl/mg protein and 2 µl/ml, respectively) for 10 min and then used immediately for assays. By the treatment ATP could cross well the membrane to drive ATP-dependent transport and enzymatic activity [10]. For DCCD inhibition studies, cells and protoplasts were incubated with DCCD at 0.5 µmol/mg protein and 0.02 mM (unless otherwise stated), respectively, for 10 min at 37°C.

2.3. H_2 production assay

H_2 production by *E. coli* whole cells and protoplasts was assayed using a pair of the oxidation–reduction, platinum and titanium–silicate electrodes (Gomel State Enterprise of Electrometric Equipment, Gomel, Belarus) as previously described [9,10,21]. H_2 production rates were determined as the difference between the rates of decrease in oxidation–reduction potentials for the platinum and titanium–silicate electrodes (mV per min per mg dry weight). Dry weights were determined as previously described [18].

Molecular hydrogen gas production was also tested using the chemical assay described by Bagramyan and Martirosov [9] and the Durham tube method [22] for bacterial cultures grown for 8–10 h. No H_2 production was detected during anaerobic growth on glucose in the presence of 50 mM nitrate.

2.4. Determination of H^+ secretion

H^+ efflux through the bacterial membrane was measured using a pH selective electrode as described elsewhere [9,17–19]. Small changes in external H^+ activity were recorded using a potentiometer and calibrated by titration with 0.05 mM HCl.

2.5. Fdh-H assay

Measurement of Fdh-H activity was performed with whole cells by monitoring formate-dependent benzyl viologen reduction at 600 nm as described by Sawers et al. [23], except for the following modifications: in some measurements, 100 mM Tris-phosphate pH 7.5 was used instead of 100 mM potassium phosphate, 5 mM $MgSO_4$ was included in the buffer, and oxidized benzyl viologen was added to give a final concentration of 2 mM. Rates of reduction of benzyl viologen were measured in a Helios-α spectrometer (UNICAM). Fdh-H activity was found to be directly proportional to the amount of cells added. Protein content of whole cells treated with 5% sodium dodecyl sulfate was determined by Bio-Rad *DC* protein assay with a SPECTRAmax 340pc microplate reader (Molecular Devices) using bovine γ globulin as a standard. Specific Fdh-H activities (µmol of benzyl viologen reduced per min per mg of protein) are represented.

Table 1
E. coli strains

Strain	Genotype	Source and/or reference
AT1	TK2538 *ilvD500*::Tn*10*	This work
AT2	M9s Δ*atpB-D trkD1* Δ*trkA ilvD500*::Tn*10*	This work
AT3	MC10613 Δ(*atpB-D*) *trkD1* Δ*trkA trkD1 ilvD500*::Tn*10*	This work
AT4	DS5 Δ(*atpB-D*) *ilvD500*::Tn*10*	This work
CAG18431	*rph-1 ilvD500*::Tn*10*	C.A. Cross via M. Berlyn, *E. coli* Genetic Stock Center (Yale University, New Haven, CT, USA) [11]
DS5	MC4100 [λRS45::*hyfA′-lacZ*]	W. Skibinski, P. Golby, S.C. Andrews (The University of Reading, Reading, UK)
FM911	MC4100 Δ*fdhF recA56*	A. Bock (Munich University, Munich, Germany) [13]
FRAG90	*lacZ gal kdpABC5 trkD1*	W. Epstein (The University of Chicago, Chicago, IL, USA) [14]
FRAG115	FRAG90 Δ(*atpB-D*)	W. Epstein
HD702	MC4100 Δ*hycB*	A. Bock [3]
HD705	MC4100 Δ*hycE*	A. Bock [3]
HD706	MC4100 Δ*hycF*	A. Bock [3]
HD707	MC4100 Δ*hycG*	A. Bock [3]
JRG3615	MC4100 Δ(*hyfA-B*)::*spc*	Y.S. Chang, P. Golby, S.C. Andrews
JRG3618	MC4100 Δ*hyfR*::*spc*	Y.S. Chang, P. Golby, S.C. Andrews
JRG3621	MC4100 Δ(*hyfB-R*)::*spc*	Y.S. Chang, P. Golby, S.C. Andrews
M9s	MC4100 *fdhF*::Mu*d1*(*lac* Ap^r)	A. Bock [15]
MC4100	*araD139* Δ(*argF-lac*)*U169 ptsF relA1 fibB5301 rpsL150*	M. Casadaban via A. Bock [16]
MC10613	F^- Δ(*ara-leu*)*7697 araD139 hsdR* Δ(*lacIPOZY*)*X74 galK15 galE16 rpsL* [λTS102::*hycB′-lacZ*]	A. Bock
TK2420	*thi rha nagA lacZ* Δ(*kdpABC*)*5 trkD1* Δ(*trkA*)	W. Epstein
TK2538	TK2420 Δ(*atpB-D*)	W. Epstein

2.6. β-Galactosidase assay

Preparation of cell extracts and measurement of β-galactosidase activity and protein content were performed on samples taken during the mid-logarithmic to stationary growth phase as described by Golby et al. [24]. β-Galactosidase specific activities (μmol of *o*-nitrophenyl-β-D-galactopyranoside per minute per mg of protein) were determined for samples taken from two independent cultures. Each of the samples was assayed in duplicate.

3. Results and discussion

3.1. H_2 production in hyc and hyf mutants at alkaline and acidic pH

H_2 production under fermentative conditions at acidic pH (pH 6.5) was detected, by oxidation–reduction potential measurements, in the wild-type (MC4100) and the *hyf* mutants (JRG3615, JRG3618 and JRG3621), but very little H_2 production was observed in the *hyc* mutants (HD705, HD706 and HD707) (Fig. 1 and Table 2). These findings are consistent with previous work of Sauter et al. [3] showing that Hyd-3 is the major benzyl viologen-reducing hydrogenase at pH ∼6.5. Surprisingly, inclusion of 30 mM formate in the growth medium did not subsequently increase hydrogen production rates, at either pH 6.5 or 7.5. In addition, hydrogen production in the wild-type was only slightly affected by pH (production was ∼1.2-fold higher at pH 6.5 than at pH 7.5). These findings are unexpected since formate and low pH are known to induce expression of the FHL-1 encoding genes (*hyc* and *fdhF*) [1,6,7]. This lack of induction of hydrogen production by these factors may be related to incomplete incorporation of nickel into Hyd-3 [25]. However, nickel (10 μM) added had no effect on hydrogen production (data not shown).

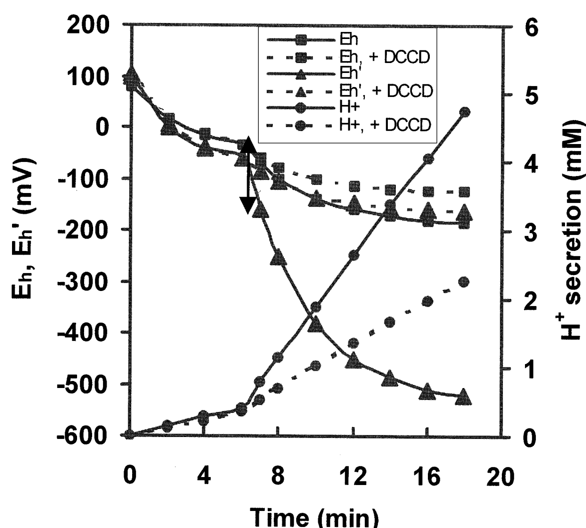

Fig. 1. Simultaneous changes in oxidation–reduction potentials and external H^+ activities for *E. coli* MC4100 grown fermentatively. MC4100 cultures grown fermentatively at pH 7.5 were transferred into a 200 mM Tris-phosphate (pH 7.5) buffer containing 0.4 mM $MgSO_4$, 1 mM NaCl and 1 mM KCl (giving 10^{10} cells/ml) at time zero, and glucose (20 mM) was subsequently added (arrow) at 6 min. Oxidation–reduction potentials (E_h and E_h') and H^+ secretion were measured as in Section 2. The difference between stationary values of E_h' and E_h was used to determine H_2 production [9,10,21]. Dotted curves represent results obtained in the presence of 0.2 mM DCCD. Each data point is averaged from two or three assays, the standard deviations are not more than 5% (not represented).

Interestingly, H_2 production was detected at pH 7.5 and this was not affected by *hycE*, *hycF* or *hycG* mutations indicating that Hyd-3 is not involved in H_2 production at high pH, although this conclusion is somewhat contradicted by the marked reduction (five-fold) in H_2 production in the *hycB* mutant at pH 7.5 (Table 2). Furthermore, H_2 production at pH 7.5 was virtually abolished (up to 17-fold reduced) in all three *hyf* mutants, suggesting that Hyd-4 is required for H_2 production at alkaline pH. The H_2 production levels were verified by chemical assay and by the Durham tube method (data not shown). As expected, no significant H_2 production was detected in the *fdhF* mutant (FM911) at either pH 6.5 or 7.5 (Table 2).

The above data indicate that H_2 production by *E. coli* at pH 7.5 is mediated by Hyd-4 and Fdh-H, whereas H_2 production at pH 6.5 is mostly Hyd-3- and Fdh-H-dependent (as previously reported [3]). The requirement for both Fdh-H and Hyd-4 for H_2 production at pH 7.5 suggests that these enzymes combine to form a second FHL complex (FHL-2) which functions mainly at alkaline pH. It should be noted that the *hyf* mutations had no major effect on growth under the fermentative growth conditions employed here.

Although H_2 production by *E. coli* at pH 7.5 was found to be independent of at least three of the Hyd-3 subunits, the HycB subunit was still required for this activity. It is not obvious why this should be so. HycB is thought to serve in the transfer of electrons from Fdh-H to Hyd-3, and would thus couple formate dehydrogenation with H_2 production. It is possible that HycB fulfills a similar function in the proposed FHL-2 complex. Alternatively, *hycB* gene might have a pleiotropic effect on *hyf* genes expression.

Sauter et al. [3] previously reported that no hydrogenase activity is detectable in an *E. coli* triple mutant containing defects in the Hyd-1, -2 and -3 systems, suggesting that there are only three hydrogenases in *E. coli*. However, Sauter et al. [3] performed measurements on *E. coli* grown at pH ∼6.5 and did not consider FHL activity following growth at higher pH. Furthermore, the hydrogenase assay employed involved the use of the unnatural electron acceptor, benzyl viologen. The method used here measures the fermentative production of H_2 directly and therefore is likely to be more reliable. In summary, the experiments described above clearly indicate the presence of a fourth hydrogenase (Hyd-4), active at pH ∼7.5 and encoded by the *hyf* operon.

H_2 production at pH 7.5 in the wild-type was accompanied by H^+ efflux (Fig. 1) and was almost completely inhibited by the F_0F_1-ATPase inhibitor, DCCD (Fig. 1; Table 2), as previously observed [9,10,21]. H^+ efflux was also inhibited (∼50%) by DCCD (Fig. 1), probably as a result of inhibition of F_0F_1-ATPase activity [10,17]. Previous work has shown that DCCD does not affect H^+ efflux under conditions of nitrate or aerobic respiration where F_0F_1-ATPase functions as an ATP synthase rather than an ATP-driven H^+ pump [9,10,17,19]. H_2 production at pH 6.5 was not inhibited by DCCD (Table 2). However, H_2 production at pH 7.5 was greatly reduced (nine-fold) in the *atpB-D* (F_0F_1-ATPase) mutant, but was only slightly reduced (< two-fold) at pH 6.5 (Table 2). The inhibition of H_2 production by DCCD, together with the very low hydrogen production in the *atpB-D* mutant, suggest that Hyd-4 activity at pH 7.5 is dependent on

Table 2
Rates of H_2 production by fermenting wild-type, *hyc* and *hyf* strains of *E. coli* grown at pH 6.5 and 7.5

Strain	Genotype	H_2 production rate (mV/min/mg dry weight)			
		pH 6.5[a]		pH 7.5[a]	
		−DCCD[b]	+DCCD	−DCCD	+DCCD
MC4100	Wild-type	6.0 ± 0.2	5.8 ± 0.4	5.2 ± 0.2	0.4 ± 0.1
		5.7 ± 0.3[c]	2.2 ± 0.2[c]	5.5 ± 0.3[d]	2.8 ± 0.2[d]
		6.0 ± 0.2[e]		5.2 ± 0.3[e]	
FM911	*fdhF*	0.3 ± 0.1		0.2 ± 0.0	
HD702	*hycB*	0.2 ± 0.0		1.1 ± 0.1	
HD705	*hycE*	0.1 ± 0.0		3.0 ± 0.2	0.1 ± 0.0
HD706	*hycF*	0.5 ± 0.1		5.9 ± 0.4	0.3 ± 0.1
HD707	*hycG*	0.3 ± 0.1		5.1 ± 0.3	0.3 ± 0.1
JRG3615	*hyfA-B*	4.8 ± 0.3	5.0 ± 0.4	0.3 ± 0.1	
JRG3618	*hyfR*	5.3 ± 0.4	4.7 ± 0.4	0.3 ± 0.1	
JRG3621	*hyfB-R*	4.6 ± 0.2	4.5 ± 0.3	0.6 ± 0.1	
FRAG90	Wild-type	5.4 ± 0.1	5.2 ± 0.4	5.2 ± 0.3	0.3 ± 0.1
FRAG115	*atpB-D*	2.8 ± 0.2	2.7 ± 0.2	0.6 ± 0.1	

Standard deviation values and averages are shown for duplicate/triplicate measurements (in all tables).
[a]Both growth and H_2 production measurements were performed at the same initial pH (either 6.5 or 7.5), as indicated.
[b]DCCD was at 0.2 mM.
[c]H_2 production was assayed at pH 7.5.
[d]H_2 production was assayed at pH 6.5.
[e]30 mM formate was added to the growth medium.

F_0F_1-ATPase activity. As for the wild-type, H^+ efflux in the *hyf* mutants was partially (∼50%) inhibited by DCCD (data not shown), which is indicative of F_0F_1-ATPase activity. However, DCCD-resistant H_2 production (the reagent was effective to inhibit H^+ efflux, data not shown) at pH 6.5 might point out that Hyd-3 activity is not F_0F_1-ATPase-dependent. Alternatively the effect of DCCD on H_2 production may be compensated by a low pH.

3.2. H_2 production by protoplasts

In order to more closely examine the relationship between H_2 production and F_0F_1-ATPase activity, H_2 production was measured in protoplasts. The wild-type grown at pH 7.5 produced H_2 upon addition of ATP in the presence of formate but this production was inhibited by DCCD (Table 3). Good H_2 production and DCCD inhibition were also exhibited by the *hycE* mutant (Table 3), and the *hycF* and *hycG* mutants (data not shown). However, the *hyfA-B* mutant (Table 3) and the *hyfB-R* and *hyfR* mutants (data not shown) produced ∼five-fold less H_2 than the wild-type and the *hyc* strains, and furthermore their residual H_2 production was not affected by DCCD. The *atpB-D* mutant also produced very little H_2 (Table 3). In contrast, although the wild-type grown and assayed at pH 6.5 produced H_2 upon addition of formate, its H_2 production was not affected by ATP or DCCD (Table 3).

The above findings provide further support for the requirement of Hyd-4 in H_2 production at pH 7.5, and the lack of involvement of the Hyd-3 system in this process. They also support the notion that the F_0F_1-ATPase is required for H_2 production at alkaline pH by Hyd-4, and suggest that the observed ATP stimulation of H_2 production by Hyd-4 is dependent on the F_0F_1-ATPase.

3.3. Fdh-H activity is partly F_0F_1-ATPase- and Hyd-4-dependent at alkaline pH

Previous work showed that the formate-dependent reduction of benzyl viologen is mediated jointly by Fdh-H and Hyd-3 (following fermentative growth at pH 6.5) [3]. Therefore, we investigated the possibility that Fdh-H may be active following growth at pH 7.5, and that any such activity may be Hyd-3-, Hyd-4- and/or F_0F_1-ATPase-dependent. Weak but significant Fdh-H activity was indeed detected in the wild-type grown on glucose fermentatively at pH 7.5 (Table 4). This weak activity was ∼four-fold increased by ATP when assayed at pH 7.5 (Table 4). When the assay pH was lowered to 6.5, Fdh-H activity was four-fold higher but was no longer affected by the presence of ATP (Table 4). When the wild-type was grown at pH 6.5 (rather than pH 7.5) on glucose under fermentative conditions, Fdh-H activity was up to seven-fold higher and was not significantly stimulated by ATP when

Table 3
Rates of H_2 production by protoplasts of *E. coli* wild-type, *hyc*, *hyf* and *atp* strains grown at pH 7.5

Assay conditions[a]	H_2 production (mV/min/mg dry weight)				
	MC4100 (wild-type)		HD705 (*hycE*)	JRG3615 (*hyfA-B*)	FRAG115[b] (*atpB-D*)
	pH 6.5	pH 7.5			
	0.8 ± 0.1	0.8 ± 0.0	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
+formate	6.1 ± 0.3	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	0.8 ± 0.0
+formate+ATP	6.2 ± 0.2	5.6 ± 0.3	4.9 ± 0.2	1.1 ± 0.1	0.8 ± 0.0
+formate+ATP+DCCD	5.9 ± 0.4	0.3 ± 0.0	0.3 ± 0.1	0.9 ± 0.1	0.8 ± 0.1

[a]Concentrations used were: 3 mM ATP, 5 mM formate, and 0.1 mM DCCD, where present, and 3 mM DL-dithiothreitol was present in all assays.
[b]H_2 production by FRAG90 (FRAG115 *atpB-D*$^+$) was similar to that of MC4100.

Table 4
Effects of pH on Fdh-H activity in *E. coli* MC4100

Growth pH[a]	Assay pH[b]	Fdh-H activity (μmol benzyl viologen/min/mg protein)	
		−ATP	+ATP[c]
7.5	7.5	0.036 ± 0	0.162 ± 0.009
	6.5	0.156 ± 0.027	0.172 ± 0.005
6.5	7.5	0.265 ± 0.038	0.323 ± 0.044
	6.5	1.058 ± 0.062	0.640 ± 0.096

Bacteria were grown on glucose under anaerobic conditions.
[a]Initial value of the pH of the growth medium.
[b]0.1 M potassium phosphate buffer.
[c]ATP was added to the assay mixture to give a final concentration of 10 mM.

assayed at either pH 7.5 or 6.5 (Table 4). Addition of DCCD inhibited the ATP stimulation of Fdh-H activity for cells grown at pH 7.5, but had no effect on Fdh-H activity in the absence of ATP or on cells grown at pH 6.5 (Table 5). The levels of Fdh-H activity (~1.05 μmol benzyl viologen/min/mg protein for MC4100) obtained at pH 6.5 were ~three-fold lower than those (2.83 μmol/min/mg protein for MC4100) previously reported by Sauter et al. [3], probably because of differences in growth conditions employed such as the absence or presence of formate (30 mM).

Fdh-H activity at pH 7.5 was just ~0.05 μmol/min/mg protein for the wild-type (MC4100) in the absence of ATP. Similar levels (0.03–0.05 μmol/min/mg protein) were detected for the *hycE*, *hyfA-B*, *hyfR*, *hyfB-R*, and *hycE hyfB-R* mutants, but no activity was detected in the *fdhF* mutant (data not shown). Measurement of the Fdh-H activity of the *atpB-D* mutant (TK2538) and its isogenic parent (TK2420) at pH 7.5 in the absence of ATP revealed only a slight decrease in activity as a result of the absence of functional F_0F_1-ATPase (0.07 and 0.10 μmol/min/mg protein, respectively). However, when ATP was included in the assay mixture, Fdh-H activity increased by 0.10–0.14 μmol/min/mg protein in the wild-types (MC4100 and TK2420) and the *hycE* mutant, but was only slightly increased (0.005–0.02 μmol/min/mg protein) in the *hyf* mutants and the *hyc hyf* double mutant (Fig. 2). No ATP-stimulated increase in Fdh-H activity was observed for the

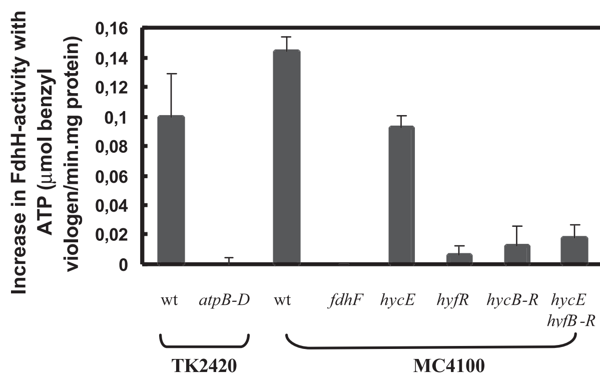

Fig. 2. Increase in Fdh-H activity with ATP in *E. coli* wild-type, *atp*, *hyc* and *hyf* mutants. The cells were grown and assayed at pH 7.5. The assay mixture contained 0.1 M potassium phosphate pH 7.5. Increase in enzyme activity was the difference between activities in the presence and absence of ATP, 10 mM. Error bars represent the standard deviations from duplicate or triplicate cultures. For wild-type and mutant strains, see Table 1.

atpB-D and *fdhF* mutants (Fig. 2). These data therefore indicate that the formate-dependent benzyl viologen reduction at pH 7.5 is fully Fdh-H-dependent, and the ATP-mediated stimulation of Fdh-H activity is wholly dependent on the F_0F_1-ATPase and largely dependent on Hyd-4. Therefore, both Fdh-H activity and H_2 production at pH 7.5 are at least partly dependent on Fdh-H, Hyd-4 and F_0F_1-ATPase. In addition, Fdh-H activity was affected in the *hycB* mutant (the level of this activity in the absence of ATP was 2.2-fold less than that for the wild-type, and ATP-dependent increase in the activity was not obtained, data not shown). This is in correlation with the notion that the gene is required for H_2 production (see Section 3.1).

Fdh-H activity at pH 6.5 was 2.5-fold reduced in the *hycE* mutant (data not shown), which supports the studies of Sauter et al. [3] showing that Fdh-H activity following growth at pH 6.5 is Hyc-dependent. However, activity at pH 6.5 was not affected by the *hyf* mutations (data not shown) indicating that Fdh-H activity is Hyf-independent at this pH.

It has previously been shown that the formate-dependent reduction of benzyl viologen is only ~85% dependent on Hyd-3 (at pH 6.5) and the reaction can be directly catalyzed by the Fdh-H protein alone [1,3]. It has been suggested that interaction of Fdh-H and Hyd-3 increases formate oxidation and/or benzyl viologen reduction [3]. It is likely that Hyd-4 acts similarly to increase Fdh-H activity (at pH 7.5) in the presence of both ATP and active F_0F_1-ATPase, but that Fdh-H possessed residual activity at pH 7.5 in the absence of these factors. The physiological relevance of the residual activity of Fdh-H is uncertain [1–3].

3.4. Expression of hyc, hyf and fdhF in F_0F_1-ATPase deficient strains

The above results and previous studies in *S. typhimurium* and *E. coli* show that fermentative gas production (and Fdh-H activity) is impaired in strains lacking a functional F_0F_1-ATPase [8–10]. To determine whether this effect is due to a reduction in the expression of the genes encoding Hyd-3, Hyd-4 or Fdh-H, the expression levels of *fdhF-lacZ*, *hycB-lacZ* and *hyfA-lacZ* fusions were measured in *atp* mutants and in the corresponding atp^+ parental strains (Fig. 3). The strains were grown under fermentative conditions with 0.2% glucose at an initial pH of 7.5, conditions that result in F_0F_1-ATPase-dependent gas production for the wild-type (Table 2). No significant differences in expression were observed between the

Table 5
Effects of DCCD on Fdh-H activity in *E. coli* MC4100 grown fermentatively on glucose at pH 7.5 or 6.5

Conditions	Fdh-H activity (μmol benzyl viologen/min/mg protein)		
	pH 7.5		pH 6.5
	−ATP	+ATP[a]	−ATP
No additions	0.051 ± 0.005	0.193 ± 0.010	0.927 ± 0.115
DCCD (μmol/mg protein)			
0.5	0.058 ± 0.007	0.110 ± 0.009	ND[b]
1.25	0.057 ± 0.005	0.070 ± 0.008	ND
2.5	ND	ND	0.937 ± 0.103

The assay pH was the same as the initial value of the pH of the growth medium.
[a]ATP was added to the assay mixture at a concentration of 10 mM.
[b]ND, not determined.

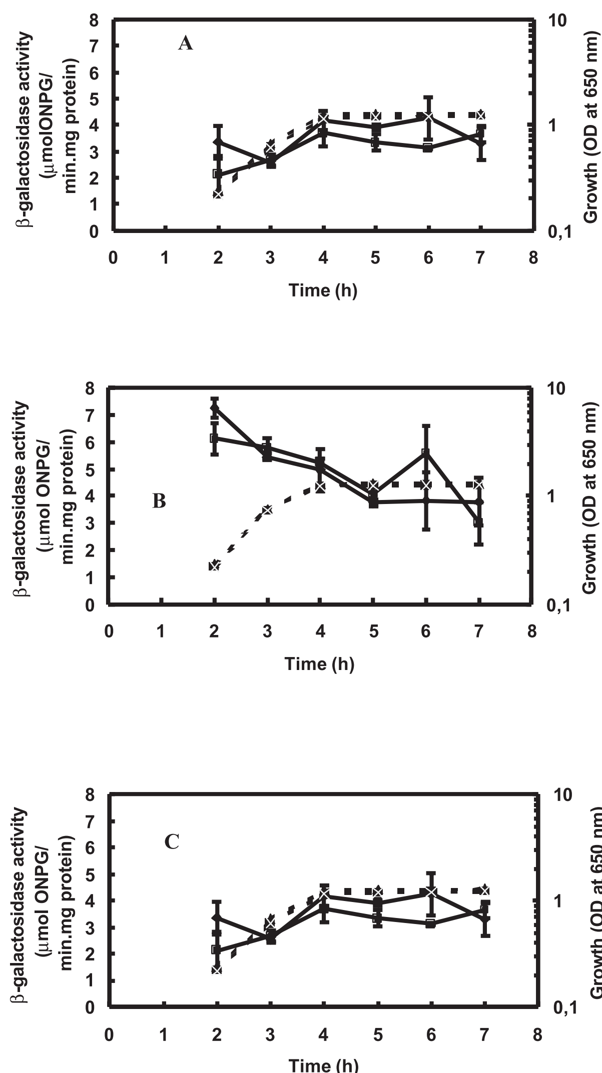

Fig. 3. Effect of *atp* mutation on the expression of the *hyc* and *hyf* operons, and the *fdhF* gene. The β-galactosidase activities (solid lines) and culture densities (broken lines) are shown for corresponding atp^+ (diamond symbols) and atp^- (square symbols) strains: A: M9s (*fdhF-lacZ*) and AT2 (*atp fdhF-lacZ*); B: MC10613 (*hycB-lacZ*) and AT3 (*atp hycB-lacZ*); and C: DS5 (*hyfA-lacZ*) and AT4 (*atp hyfA-lacZ*). Growth was in LB containing 0.2% glucose and 0.1 M potassium phosphate (pH 7.5) under fermentative conditions. Error bars represent the standard deviations of duplicate cultures.

atp^+ and atp^- strains, indicating that the *atp* mutation does not affect expression of the genes encoding FHL components and that the lack of H_2 production in F_0F_1-ATPase deficient strains is not due to weak *hyc*, *hyf* or *fdhF* expression. The expression levels of *fdhF-lacZ*, *hycB-lacZ* and *hyfA-lacZ* fusions were enhanced at a low pH (S. Andrews, personal communication) but it is irrelevant to the results described.

4. Conclusions

The results reported here clearly indicate that the Hyd-4 system, along with F_0F_1-ATPase and Fdh-H, is required for most of the H_2 production observed at pH ∼7.5. Fdh-H activity at pH ∼7.5 is also dependent, in part, on Hyd-4 and F_0F_1-ATPase. It is likely that Fdh-H and Hyd-4 combine to form a second FHL system (FHL-2) in *E. coli* that is driven by the proton gradient established by F_0F_1-ATPase. The physiological purpose of the FHL-2 pathway is uncertain, but it may be required for the generation of CO_2 during fermentation at high pH (above pH ∼7) [26] for use in the generation of oxalate by phosphoenolpyruvate carboxylase that in turn could be used for biosynthesis or for the consumption of reducing equivalents and for H_2-dependent fumarate respiration. FHL-1 and FHL-2 would appear to have distinct functions during fermentation. FHL-1 acts in formate detoxification at acidic pH whereas FHL-2 acts at alkali pH possibly in supplying CO_2 for oxalate formation. It is unclear why the FHL-2 reaction should be energy-dependent. This may relate to the need to generate CO_2 under fermentative conditions when formate concentrations are relatively low.

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