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Genetic Regulation of Formate Hydrogenlyase of *Escherichia coli*: Role of the *fhlA* Gene Product as a Transcriptional Activator for a New Regulatory Gene, *fhlB*†

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A new gene whose product is required for the production of formate hydrogenlyase (FHL) has been identified in *Escherichia coli*. This gene, termed *fhlB*, maps between the *frdA* (94.4 min) and *argI* (96.6 min) genes on the *E. coli* chromosome and is transcribed in a clockwise direction toward *argI*. Biochemical analysis of an FhlB$^-$ mutant, strain SE-2011 [Φ(*fhlB-lacZ*$^+$)], revealed that the mutant lacks formate dehydrogenase activity associated with FHL (FDH-H) and hydrogenase activity. As a result of these defects, fermentative hydrogen production and hydrogen uptake reactions were undetectable in strain SE-2011. Fumarate reductase activity of this mutant was also reduced to about 15% of the levels of the parent (strain MC4100), and strain SE-2011 did not produce succinate as a fermentation end product. Regulation of expression of the *fhlB* gene, studied as production of β-galactosidase activity by strain SE-2011, revealed that the operon is expressed at low levels under aerobic conditions. Under anaerobic growth conditions, this activity increased by two- to threefold. Addition of formate enhanced the differential rate of synthesis of the *fhlB* gene product to as high as 130 U of β-galactosidase specific activity per μg of cell protein, but only under anaerobic conditions. Formate-dependent expression of Φ(*fhlB-lacZ*$^+$) required the σ^{54} subunit of RNA polymerase and the *fhlA* gene product. The concentration of formate required for maximum expression of the *fhlB* gene was about 15 mM; this value decreased to about 3 mM in the presence of plasmid pSE-133, which carries the *fhlA* gene in a multicopy plasmid. DNA sequence analysis of the *fhlA* gene showed that the FhlA protein is 686 amino acids long and has an anhydrous molecular weight of 78,086. On the basis of sequence homology with other transcriptional activators such as NtrC, HydG, and *Klebsiella pneumoniae* NifA proteins, the FhlA protein was deduced to be a transcriptional activator controlling the production of FHL. It is proposed that formate interacts with the FhlA protein and that this active complex initiates transcription of the *fhlB* gene. The FhlA and FhlB proteins act as a cascade in regulating the production of FDH-H and the FHL-linked hydrogenase and ultimately the production of FHL and fermentative hydrogen.

Under anaerobic growth conditions, *Escherichia coli* ferments glucose to yield acetate, ethanol, succinate, lactate, and formate (9). Formate is further metabolized by formate hydrogenlyase (FHL) to dihydrogen and carbon dioxide (13). Various components of the FHL complex, a unique formate dehydrogenase isoenzyme (FDH-H) and a specific hydrogenase isoenzyme (HYD3), have been identified, and the genes coding for these proteins (*fdhF* and *hyd-17*, respectively) have been described (10, 24, 32, 41, 45). These genes are transcribed only under anaerobic conditions and require formate as an inducer (4, 25, 26, 42, 44). Several other genes whose products are essential for production of one or both of the enzyme activities have also been described. These include the *hyd*, *fhlA*, *chl*, *mol*, and *rpoN* genes (3, 6, 18, 23, 25, 28–31, 33, 37, 38, 40, 43). Among these genes, mutations in the *fhlA* and *rpoN* genes affect only the production of FHL activity, and these gene products are needed for transcription of the *fdhF* and *hyd-17* genes (3, 28, 29, 33).

This report presents evidence of an additional gene, *fhlB*, which is also needed for production of FHL activity of *E. coli*. Transcription of this gene requires the FhlA protein. The role of the FhlA protein as a putative transcriptional activator in regulation of the *fhlB* gene was deduced from the DNA sequence and homology to other transcriptional activators of two-component regulatory systems (35).

MATERIALS AND METHODS

Enzyme activities. The following enzyme activities were determined by using whole cells as described previously (17): Hydrogenase (HYD) activity, measured as tritium exchange; hydrogen uptake (ability to reduce benzyl viologen or fumarate with dihydrogen as electron donor); FDH-H, formate-dependent reduction of benzyl viologen (FDH activity associated with FHL); FDH-N, formate-dependent reduction of phenazine methosulfate (FDH activity associated with nitrate respiration).

Bacterial strains, plasmids, and culture conditions. The bacterial strains used are derivatives of *E. coli* K-12 (Table 1). Plasmid pSE-133, which carries the *hydB*$^+$ and *fhlA*$^+$ genes, was described previously (29). The two transposon Tn*5* derivatives of plasmid pSE-133 were constructed as described before (30). The relevant genotype of plasmid pSE-133-1 is *hydB*::Tn*5* *fhlA*$^+$; pSE-133-2 carries the transposon in the *fhlA* gene (*hydB*$^+$ *fhlA*::Tn*5*).

Cultures were grown at 37°C in LB medium supplemented as needed with glucose (0.3%) or sodium formate (0.5%). For anaerobic induction of β-galactosidase activity, 120 ml of medium in a 160-ml Wheaton bottle was inoculated (1%, vol/vol) with a 1.5-h-old aerobic culture grown at 37°C in a shaker at 250 rpm. The bottles were closed with rubber stoppers and sealed with aluminum seals. The gas phase was

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TABLE 1. Bacterial strains

Strain	Relevant genotype	Source or reference
E. coli		
MC4100	*araD139* Δ(*argF-lacU*)*205 ptsF25 relA1 rpsL150 deoC1 flb-5301*	CGSC 6152
BW545	Δ(*lacU*)*169 rpsl*	G. Walker (43)
CSH26	*ara* Δ(*lac-pro*) *thi*	Laboratory collection
M2508	Hfr *relA1 spoT1 metB1 melA7*	CGSC 4926
SE-1000	*cysC43 srl-3000*::Tn*10 thr-1 leu-6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1, proA2 his-4 argE3 rpsL31 tsx-33 supE44*	Laboratory collection
M9s	MC4100, Φ(*fdhF-lacZ*$^+$)	A. Böck (25)
PC0287	*thr-20 leu-32 proA35 argF58 argI60 lacY1 gal-6 rpsL125 tonA48 tsx-70 supE44*	CGSC 5404
LCB898	*thr-1 leuB6 pfl-1 thi-1 lacY1 rpsL175 tonA21*	CGSC 6161
JRG780	*trpA9761 frdA11 trpR72 gal-25 rpsL195*	CGSC 5916
RK5278	*narL215*::Tn*10*	V. Stewart
SE-1162	LS853, *zif-4*::Tn*10*	Laboratory collection
SE-1174	*fhlA*::Tn*10*	Laboratory collection (29)
SE-1188	JRG861a, *zcj-5*::Tn*10*	Laboartory collection
SE-1265	LCB898, *zba-6*::Tn*10*	Laboratory collection
SE-2011	MC4100, Φ(*fhlB-lacZ*$^+$)	This study
JRG861a	*gal trpA9761 iclR trpR rpsL fnr*	J. Guest
LS853	*trpA9605 his-85 cya-2 trpR55*	CGSC 5381
YMC18	*endA thi hsdR* Δ(*lacU*)*169 rpoN*:Tn*10*	B. Magasanik
SE-67-1	*hydF102 cys*::Tn*10*	Laboratory collection
MJ-2	Φ(*fhlB-lacZ*$^+$) *hydF102 cys*::Tn*10*	P1 transduction (SE-2011 × SE-67-1)
MJ-3	MC4100, Φ(*fhlB-lacZ*$^+$)	P1 transduction (MC4100 × SE-2011)
MJ-4	BW545, Φ(*fhlB-lacZ*$^+$)	P1 transduction (BW545 × SE-2011)
MJ-5	Φ(*fhlB-lacZ*$^+$) *rpoN*::Tn*10*	P1 transduction (SE-2011 × YMC18)
MJ-6	Φ(*fhlB-lacZ*$^+$) *fnr zcj-5*::Tn*10*	P1 transduction (SE-2011 × SE-1188)
MJ-7	Φ(*fhlB-lacZ*$^+$) *narL215*::Tn*10*	P1 transduction (MJ-4 × RK5278)
MJ-8	Φ(*fhlB-lacZ*$^+$) *cya-2 zif-4*::Tn*10*	P1 transduction (SE-2011 × SE-1162)
MJ-9	Φ(*fhlB-lacZ*$^+$) *pfl-1 zba-6*::Tn*10*	P1 transduction (SE-2011 × SE-1265)
MJ-18	MJ-19, Hfr PO(*fhlB*)	Conjugation (MJ-19 × TT627)
MJ-19	CSH26, Φ(*fhlB-lacZ*$^+$)	P1 transduction (CSH26 × SE-2011)
MJ-20	Φ(*fhlB-lacZ*$^+$) *fhlA*::Tn*10*	P1 transduction (SE-2011 × SE-1174)
MJ-21	SE-1000, *metB1 melA7 thr*$^+$ *arg*$^+$ *leu*$^+$ F$^-$	Conjugation (SE-1000 × M2508)
Salmonella typhimurium		
TT627	*strA1 pyrC7* (F'ts114 *zzf*::Tn*10*)	J. Roth (8)

replaced with argon. Samples were removed at different time periods with a syringe and needle, and growth of the culture and β-galactosidase activity of the cells were determined. The amount of β-galactosidase activity present was determined as described by Miller (19) after permeabilization with sodium dodecyl sulfate and chloroform. The specific activity of the enzyme is expressed as nanomoles of *o*-nitrophenol produced per minute per milligram of cell protein. The differential rate of synthesis of β-galactosidase activity was calculated as units of activity per microgram of cell protein and represents the increase in the amount of β-galactosidase activity produced by the culture in relation to the increase in total cell protein.

In another set of experiments, the aerobic cultures were used to inoculate (1%, vol/vol) the appropriate medium in screw cap tubes (13 by 100 mm) filled to the top. Cells from these cultures were harvested after 4 h of incubation at 37°C (standing) and used for β-galactosidase assays. All other methods, including enzyme assays, were as described previously (17, 30). Genetic experiments were performed according to Miller (19) or by using procedures previously described (17, 18).

Isolation of mutants. Strain MC4100, grown in LB-0.3% maltose medium, was mutagenized with λ *plac*Mu53 and λ pMu507 as described by Bremer et al. (5). Kanamycin-resistant mutants were replicated to LB medium supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (19) and incubated under aerobic or anaerobic conditions (GasPak anaerobic system). Mutants that were Lac$^+$ only under anaerobic growth conditions were identified and inoculated into 1 ml of LB-0.3% glucose medium in tubes (12 by 75 mm). The tubes were sealed with serum stoppers, and the gas phase was replaced with dinitrogen. After 16 h of incubation at 37°C, dihydrogen in the gas phase of culture tubes was determined by using Varian gas chromatograph (model 920) fitted with a 0.5-nm molecular sieve column. From a total of 68 potential mutants, 13 dihydrogen production-defective mutants (Fhl$^-$) were identified. One such mutant, strain SE-2011, which lacked both FDH-H and HYD activities, was used in these studies.

DNA sequence determination. DNA sequence was determined by the dideoxy method with double-stranded plasmid DNA (14, 27). The plasmids used in the sequencing experiments, pSE-130, pSE-132, and pSE-133, were described previously (29, 30). Plasmid pSE-190 carries a 1.0 kilobase *Sal*I-*Pst*I internal fragment of the *fhlA* gene from plasmid pSE-128 (29) in vector plasmid pUC19. Both strands of chromosomal DNA present in these plasmids were sequenced by using appropriate primers. New oligonucleotide primers were synthesized as needed, on the basis of the partial DNA sequence of the *fhlA* gene, by the DNA synthesis core laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida. Commercially available sequencing primers were obtained from U.S. Biochemical Corp., Pharmacia-LKB, or New England BioLabs, Inc. DNA sequence was determined by using T7 DNA

TABLE 2. Biochemical characteristics of strain MC4100 and an *fhlB* mutant, strain SE-2011

Strain	HYD ($^3H_2$ exchange)[a]	Sp act					
		Hydrogen uptake		FHL[b]	FDH-H[c]	FDH-N[d]	Fumarate reductase[e]
		H_2 to BV[c]	H_2 to fumarate[e]				
MC4100 (parent)	1,200	700	290	170	490	47	600
SE-2011 Φ(*fhlB-lacZ*$^+$)	44	29	UD[f]	UD	38	100	100

[a] Expressed as nanomoles of ^3H_2O produced per minute per milligram of cell protein.
[b] Expressed as nanomoles of H_2 produced per minute per milligram of cell protein.
[c] Expressed as nanomoles of benzyl viologen reduced per minute per milligram of cell protein.
[d] Expressed as nanomoles of phenazine methosulfate per minute per milligram of cell protein.
[e] Expressed as nanomoles of H_2 consumed per minute per milligram of cell protein.
[f] UD, Undetectable.

polymerase (Sequenase) obtained from either U.S. Biochemical Corp. or Pharmacia-LKB, using the protocols supplied by the manufacturer. The DNA sequence was manipulated and homology with other known sequences in the GenBank and EMBL data bases was determined by using the computer programs provided by Genetics Computer Group, University of Wisconsin (11, 39), and Genepro (Hoeffer Scientific).

Materials. Biochemicals were purchased from Sigma Chemical Co. Analytical-grade inorganic and organic chemicals were from Fisher Scientific Co.

RESULTS

Strain SE-2011, lacking fermentative dihydrogen production, was isolated as a *lac* operon fusion derivative of strain MC4100, using λp*lac*Mu53 (5). Upon detailed analysis, this strain was found to be defective in the production of both HYD and FDH-H activities (Table 2). The lack of tritium exchange activity in strain SE-2011 shows that all three HYD isoenzymes are absent in this strain. As a consequence of this defect, both FHL and hydrogen uptake activities were not detectable in this strain. The FDH-H activity in strain SE-2011 was less than 10% of the levels observed in the parent strain MC4100; on the basis of this property, strain SE-2011 can be distinguished from all known *hyd* mutants, which produce FDH-H activity. This defect is unique for FDH-H, since this strain produces FDH-N and is normal for nitrate respiration. The level of fumarate reductase activity in strain SE-2011 was also lower (less than 20% of the parent value). The same phenotype was observed when this mutation was transduced into strain MC4100 or other *lac* deletion mutants of *E. coli* (strains CSH26 and BW545), indicating that the mutational effect is due to a single gene defect in these genetic backgrounds. The altered gene is termed *fhlB* since the gene is formate inducible (see below) and thus probably plays a major role in the production of FHL activity, although a defect in the production of all three HYD isoenzymes can be readily detected in strain SE-2011.

The aerobic and anaerobic growth of strain SE-2011 was comparable to that of the parent, strain MC4100, in LB medium supplemented with different sugars. However, this strain failed to grow in glucose-minimal medium, probably as a consequence of the low fumarate reductase activity. Strain SE-2011 also failed to produce succinate as a fermentation product.

Genetic map location of the *fhlB* gene. The approximate map location of the altered gene in the *E. coli* chromosome was determined by Hfr-mediated conjugation analysis. For these experiments, an Hfr derivative of strain SE-2011 in which the origin of DNA transfer is the *fhlB* gene was constructed, using *lac* homology, as described before (8, 18). In a 30-min conjugation period, strain MJ-18 transferred the *argI* gene (96.6 min; 1) and not the *frdA* gene (94.4 min; 1), indicating that the *fhlB* gene is located between these two genes (Fig. 1). Both *thr* and *leu* were also transferred at high frequency during this 30-min duration. Since the orientation of the *lac* operon with respect to the origin of transfer is known (8), results of these experiments were also used to determine the direction of transcription of the *fhlB* gene. The *fhlB* gene is transcribed in a clockwise direction, toward *argI*, *thr*, and *leu* (Fig. 1).

The *fhlB* gene is located near the *fdhF* gene, which maps at 92.4 min (24, 42), and the two mutant phenotypes can be readily distinguished by the hydrogen uptake characteristics of the strains. In experiments similar to those presented in Table 2, strain M9s, a known *fdhF* mutant (24), produced 75 to 85% of the hydrogen uptake activity of parent strain MC4100, measured either as benzyl viologen or fumarate reduction. The HYD activity of this strain, measured as tritium exchange, was close to 100% of the parent level. Strain M9s produced elevated levels of FDH-N (6.6-fold) and lower levels of fumarate reductase (28%) than did the parent, strain MC4100. These values, which are consistent with the phenotype described by Pecher et al. (25), are quite distinct from the properties of strain SE-2011 (Table 2).

Requirements for formate for expression of the *fhlB* gene. Regulation of expression of the *fhlB* gene was monitored by measuring the levels β-galactosidase activity produced by strain SE-2011 from the *fhlB* promoter. When cultured under strict aerobic conditions, strain SE-2011 produced about 50 U of β-galactosidase activity (nanomoles per minute per milligram of cell protein) irrespective of medium composition. Upon transfer to anaerobic conditions, the β-galactosidase activity of the culture increased exponentially with time, an increase which paralleled the growth of the culture. The specific activity of the enzyme reached the maximum value during the early stationary phase of growth and remained constant over an additional 8 h of incubation. In

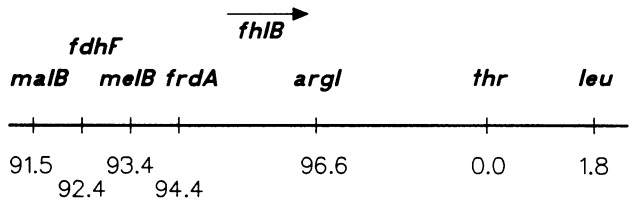

FIG. 1. Genetic map location of the *fhlB* gene on the *E. coli* chromosome. Numbers represent map distance in minutes. The arrow indicates the direction of transcription of the *fhlB* gene.

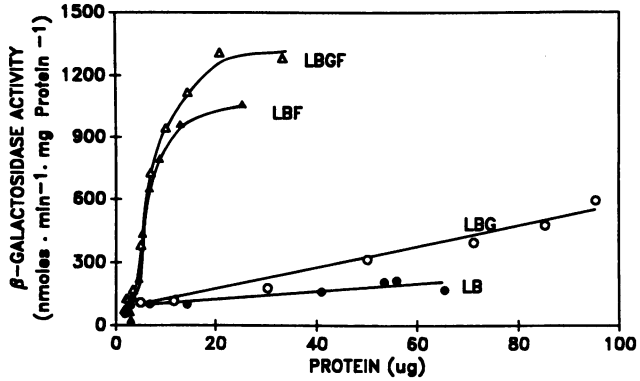

FIG. 2. Differential rate of synthesis of β-galactosidase activity by Φ(*fhlB-lacZ*$^+$) strain SE-2011 grown in LB medium with different supplements. LBG, LBF, and LBGF represent LB-glucose, LB-formate, and LB-glucose-formate media, respectively. β-Galactosidase and protein activities are expressed as units per milliliter and micrograms per milliliter, respectively.

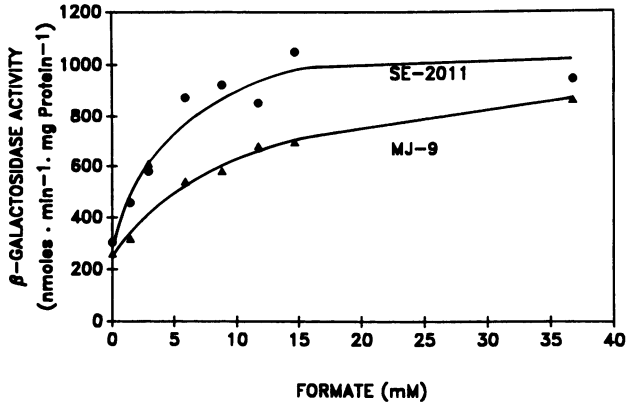

FIG. 3. Effect of formate on the induction of β-galactosidase activity by Φ(*fhlB-lacZ*$^+$) strain SE-2011 and a *pfl* derivative, strain MJ-9. Specific activity represents the maximum value observed at each formate concentration.

LB medium, the maximum activity observed was about 200 U (Fig. 2). In this medium, the increase in specific activity of the enzyme was coupled to growth, and the differential rate of induction was about 1.0. In LB medium supplemented with glucose, the differential rate of β-galactosidase production increased exponentially during growth, probably because of continued production of formate by the growing culture, since the amount of formate produced by the culture was proportional to the cell density. The maximum activity reached was about 600 U during the early stationary phase of growth, when the cell density was about 100 μg of protein per ml. With the addition of formate to the medium, the growth rate and final cell yield decreased, although the differential rate of synthesis of β-galactosidase was increased to as high as 130 U/μg of cell protein. In this medium, the maximum activity produced by the culture increased to about 1,000 U. In LB medium supplemented with both glucose and formate, the differential rate of induction of β-galactosidase activity was similar to the values obtained with LB-formate cultures, but the total amount of the enzyme produced by strain SE-2011 was higher (about 1,300 U). The final cell yields of the culture in the latter two media were comparable. These experiments clearly show that transcription of the *fhlB* operon is dependent on formate, either produced internally or added externally to the medium.

The amount of β-galactosidase activity produced by the Φ(*fhlB-lacZ*$^+$) strain increased linearly with increasing formate concentration up to about 5 mM (Fig. 3). The activity continued to increase at a lower rate until the maximum was reached at about 15 mM. For these experiments, strain SE-2011 was grown under anaerobic conditions in LB-formate medium, and the cells were harvested after 4 h for enzyme assays. Similar results were obtained with strain MJ-9 [Φ(*fhlB-lacZ*$^+$) *pfl*], which lacks the ability to produce formate internally as a result of a defect in pyruvate formate-lyase. At 15 mM formate, strain MJ-9 produced only about 60% of the β-galactosidase activity observed in the *pfl*$^+$ parent strain. At higher formate concentrations (about 30 mM), the specific activity of β-galactosidase activity produced by strain MJ-9 was comparable to that of the *pfl*$^+$ strains. These results suggest that the internally produced formate plays a significant role in transcription of the *fhlB* operon.

Genetic regulation of the *fhlB* operon. Expression of the enzymes in dihydrogen metabolism requires the products of several genes: *hyd*, *fnr*, *fhlA*, and *rpoN* (3, 6, 17, 18, 25, 28–31, 33, 36–38, 40, 43). To study the role of these gene products in the expression of Φ(*fhlB-lacZ*$^+$), appropriate double-mutant strains were constructed in which one of the putative regulatory genes is defective. Analysis of these double-mutant strains revealed that the *fhlA* and *rpoN* gene products are needed for anaerobic induction of Φ(*fhlB-lacZ*$^+$) (Table 3). The differential rate of expression of β-galactosidase activity in the double mutants, strains MJ-5 and MJ-20 (*rpoN* and *fhlA*, respectively), was unity, indicating that induction and cell growth are coupled and that the enhancing effect of formate was absent. In an *fnr* mutant (strain MJ-6), although the differential rate of expression was comparable to that of strain SE-2011, the maximum activity was reduced by about 35%. The reduction in the amount of β-galactosidase activity produced by the *fhlB fnr* double mutant could be a consequence of lower cell yield of the culture, since the production of β-galactosidase activity by Φ(*fhlB-lacZ*$^+$) required growth of the organism. The *hydF* and *cya* genes had no apparent effect on expression of the Φ(*fhlB-lacZ*$^+$) operon. Addition of nitrate to the growth medium had no effect on the formate-dependent production

TABLE 3. Expression of Φ(*fhlB-lacZ*$^+$) in different genetic backgrounds[a]

Strain	Relevant genotype	β-Galactosidase activity	
		Differential rate of induction (U/μg of protein)[b]	Maximum activity[c]
SE-2011	Φ(*fhlB-lacZ*$^+$)	130	1,300
MJ-5	Φ(*fhlB-lacZ*$^+$) *rpoN*	1	110
MJ-20	Φ(*fhlB-lacZ*$^+$) *fhlA*	1	150
MJ-2	Φ(*fhlB-lacZ*$^+$) *hydF*	ND[d]	1,200
MJ-6	Φ(*fhlB-lacZ*$^+$) *fnr*	110	840
MJ-8	Φ(*fhlB-lacZ*$^+$) *cya*	160[e]	1,300

[a] All cultures were grown anaerobically at 37°C in LB-formate medium except strain MJ-6, which was grown in LB medium with formate and glucose to enhance cell yield.

[b] Calculated as the amount of enzyme activity produced by the culture in relation to the increase in total cell protein.

[c] Expressed as nanomoles of *o*-nitrophenol produced per minute per milligram of protein.

[d] ND, Not determined.

[e] Poor cell growth.

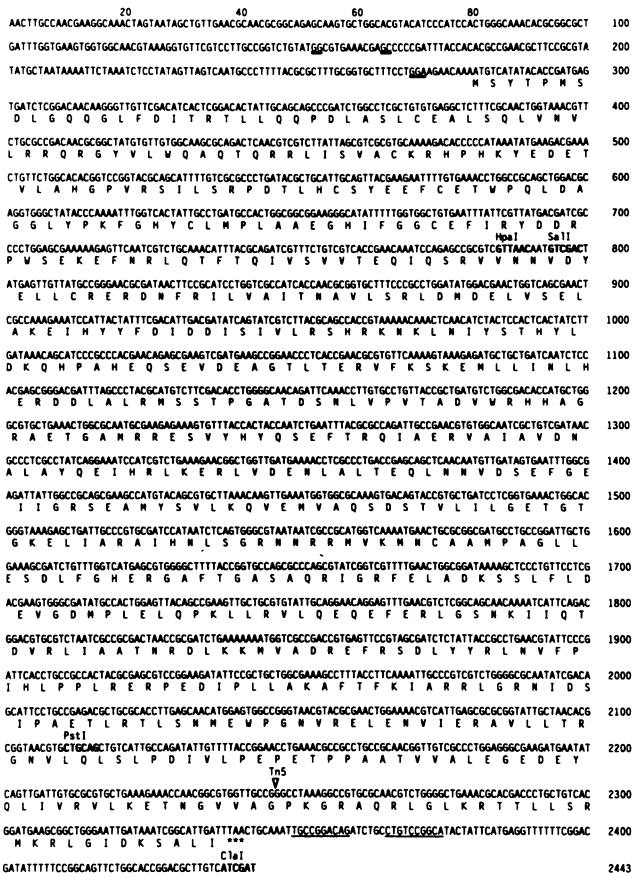

FIG. 4. Nucleic acid and predicted protein sequences of the *fhlA* gene. The termination codon is indicated by three asterisks. The Shine-Dalgarno sequence (GGA) and −12 and −24 region of the gene are double underlined. The inverted repeat in the 3′ end of the gene is underlined. Restriction sites for some of the enzymes are highlighted. The inverted triangle between positions 2,246 and 2,247 represents the position of transposon Tn*5* in plasmid pSE-133-2, as determined by DNA sequence analysis.

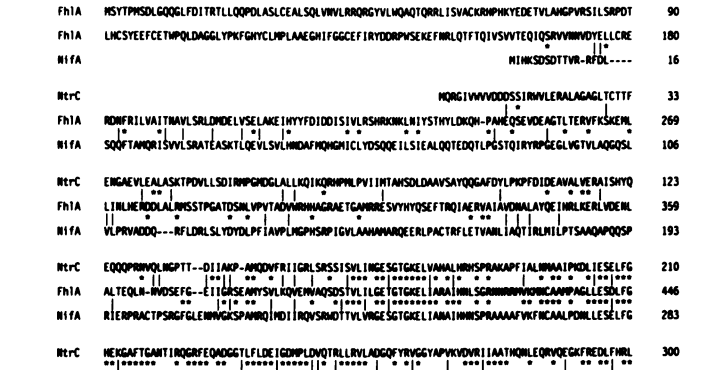

FIG. 5. Alignment of the predicted sequences of FhlA, NtrC, and *K. pneumoniae* NifA proteins. Symbols: *, identical amino acid; |, functionally similar amino acid between FhlA and NtrC or NifA. Gaps were introduced in the sequences to maximize the alignment.

of β-galactosidase activity by Φ(*fhlB-lacZ*$^+$) (740 and 720 U in the absence and presence of 15 mM nitrate, respectively). Introduction of *narL*::Tn*10* (34) into strain SE-2011 did not alter the amount of β-galactosidase activity produced by the culture, either in the presence (710 U) or in the absence (770 U) of nitrate in the medium.

Primary structure of the *fhlA* gene. Previous experiments identified the *fhlA* gene product as a putative regulatory element of both the *fdhF* and *hyd-17* genes (28, 29, 33). The results presented above (Table 3) also show that the FhlA protein is a needed regulatory element for the *fhlB* operon. Because of these observations, the DNA sequence of the *fhlA* gene was determined to identify the characteristics of the gene and its product. The *fhlA* gene codes for a protein of 686 amino acids with an anhydrous molecular weight of 78,086 (Fig. 4), which is comparable to the apparent molecular weight of 78,000 obtained by maxicell experiments (29). This protein contained no significant hydrophobic regions, indicating that the primary location of the protein is the cytoplasm. Eight base pairs from the end of translational stop codon (position 2341), the coding region is followed by an inverted repeat (underlined in Fig. 4; positions 2350 to 2359 and 2366 to 2375) that can produce a 10-base-pair stem and a 6-base (positions 2360 to 2365) loop structure. This region is followed by a stretch of six thymine residues at a distance of 14 bases (positions 2390 to 2395). Five more thymine residues can be found nine bases from the first set of thymines (positions 2405 to 2409). This segment of DNA, in appropriate configuration, can function as a Rho-independent transcription termination site. The 5′ end of the putative coding region is preceded by a typical ribosome-binding site (GGA, starting at position 270; 16). A −12 and −24 region that is unique for σ^{54} promoters is also indicated in Fig. 4. According to the DNA sequence, the *fhlA* gene resides between 2,867 and 2,870 kilobases of *E. coli* chromosomal DNA as described by Kohara et al. (15), and the direction of transcription is clockwise toward the *cys* operon at 59 min (1).

FhlA protein is a transcriptional activator. The FhlA protein has sequence homology with known transcriptional activators such as the NtrC protein of *E. coli* and the NifA protein of *Klebsiella pneumoniae* (Fig. 5; 12, 20, 35). The overall homology is about 30% between the three proteins, although the FhlA protein is considerably larger than the other two. Significant regions of the FhlA protein were also found to be homologous with other transcriptional activators of two-component regulatory systems, including the recently described HydG protein (35, 37). Homology with this class of proteins suggests that the FhlA protein functions as a transcriptional activator of the *fhlB* operon. Among this class of proteins, the FhlA protein is the largest (686 amino acids) and is 162 amino acids larger than the *K. pneumoniae* NifA protein (524 amino acids) (12, 35). On the basis of the sequence homology with these proteins, the FhlA protein can be divided into five regions. Region I is unique to FhlA and includes the first 370 amino acids. The next region of about 24 amino acids (region II; amino acids 370 to 393) is about 60% homologous with the HydG protein and is less homologous (46%) with the NtrC and NifA proteins. Region III contains about 211 amino acids (positions 395 to 605) and is highly conserved (between 60 and 65% homology) with all

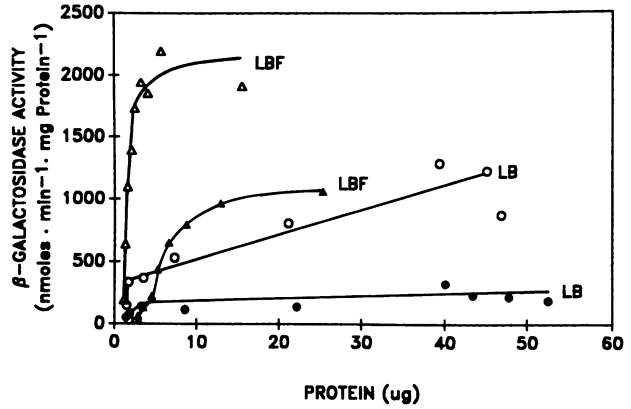

FIG. 6. Differential rate of induction of the Φ(*fhlB-lacZ*$^+$) fusion to strain SE-2011 in the presence and absence of plasmid pSE-133. LB, LB medium; LBF, LB medium supplemented with 30 mM formate. Closed symbols, strain SE-2011; open symbols, strain SE-2011(pSE-133). β-Galactosidase and protein activities are expressed as units per milliliter and micrograms per milliliter, respectively.

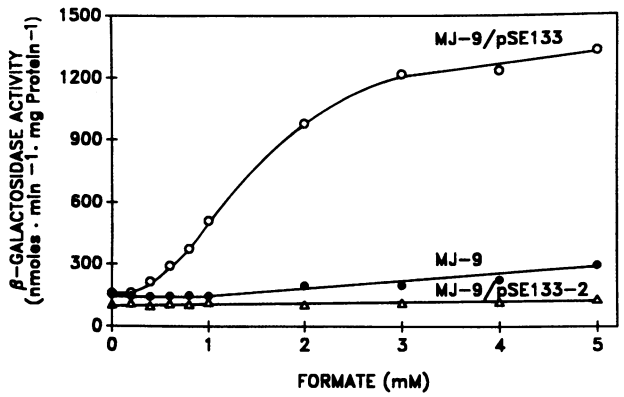

FIG. 7. Effect of formate concentration on the levels of β-galactosidase activity produced by a Φ(*fhlB-lacZ*$^+$) *pfl* double mutant, strain MJ-9, in the presence of plasmids pSE-133 and pSE-133-2 (*fhlA*::Tn*5*). Cultures were grown for 4 h in LB medium with appropriate concentrations of formate for 4 h under anaerobic conditions before the assay.

transcriptional activators of the two-component regulatory systems. This region is analogous to domain D of the NifA protein (12). The next region (IV) of about 55 amino acids (positions 606 to 660), with homology of about 22 to 27% with the HydG, NtrC, and NifA proteins, is unique to the FhlA protein, although the four proteins probably diverged from the same or a similar ancestral protein. Region V, consisting of 17 amino acids, (amino acids 661 to 677), includes the suggested DNA-binding domain (helix-turn-helix motif; 7, 22) of the HydG and NifA proteins (12, 36). This segment of the FhlA protein is 59% homologous with the HydG protein; 8 of the 17 amino acids are the same. The extent of homology of this segment of the FhlA protein with the NtrC and NifA proteins is considerably lower (about 30%). A typical helix-turn-helix motif is not predictable from the amino acid sequence of the FhlA protein, especially in the C-terminal end of the protein. However, a possible DNA-binding motif with the crucial amino acids, Ala-5, Gly-9, Leu-15, and hydrophobic amino acids at positions 4, 8, and 10 (22), can be detected starting at position 657 (PKGRA QRLGL KRTTL LSRMK) with one exception. At position 4, instead of a hydrophobic amino acid, arginine is present. This segment of the protein is crucial for the biological activity of the FhlA protein. Insertion of transposon Tn*5* between amino acids 656 and 657 (between the DNA bases G and G at positions 2246 and 2247 in plasmid pSE-133-2; Fig. 4) completely abolished the formate-dependent expression of Φ(*fhlB-lacZ*$^+$).

Effect of multiple copies of the *fhlA* gene on *fhlB* transcription. The effect of increasing the copy number of the *fhlA* gene and thus the putative transcriptional activator on the expression of Φ(*fhlB-lacZ*$^+$) was investigated by using plasmid pSE-133, which carries the complete *fhlA*$^+$ and *hydB*$^+$ genes (29, 30). In the presence of plasmid pSE-133, the differential rate of production of Φ(*fhlB-lacZ*$^+$) was about 700 U of β-galactosidase activity per μg of cell protein in LB-formate medium (Fig. 6). This value is more than five times the rate of about 130 U/μg of cell protein for strain SE-2011 cultured in the same medium. The maximum activity produced by strain SE-2011(pSE133) was also more than two times the values obtained with strain SE-2011; this increase was detected immediately after establishing anaerobic conditions. The maximum activity observed in strain SE-2011(pSE-133), grown in LB medium without formate supplementation, was also increased to about 1,100 U of β-galactosidase activity. This level of activity is comparable to the values obtained with strain SE-2011 grown in LB medium with 30 mM formate (about 1,000 U), although the differential rate of induction observed with strain SE-2011(pSE-133) in LB medium was lower. Insertion of transposon Tn*5* into the *fhlA* gene in plasmid pSE-133-2 (Fig. 4) abolished this enhancing effect of plasmid pSE-133, whereas a *hydB*::Tn*5* mutation in plasmid pSE-133-1 had no effect, indicating that the plasmid-mediated increase was due to the *fhlA* gene. Transfer of an F′ element carrying the *fhlA*$^+$ gene (F143-1) into strain SE-2011 did not significantly alter the rate or the level of β-galactosidase activity. These results suggest that increasing the copy number of the *fhlA*$^+$ gene either decreased the concentration of formate required for transcription of Φ(*fhlB-lacZ*$^+$) or eliminated the need for formate.

To distinguish between the two possibilities, plasmid pSE-133 was transferred to strain MJ-9 [Φ(*fhlB-lacZ*$^+$) *pfl*], and the amount of β-galactosidase activity produced was determined after culturing the cells in either LB or LB-formate medium. Strain MJ-9(pSE-133) produced about 100 U of β-galactosidase activity when grown in LB medium and about 1,300 U when grown in LB-formate medium (Fig. 7). The optimum amount of formate needed for this transcription was about 3 mM. At formate concentrations higher than 3 mM, the amount of β-galactosidase activity produced by strain MJ-9(pSE-133) increased slowly, reaching a maximum value of about 1,500 U at about 30 mM. The amount of β-galactosidase activity produced by strain MJ-9(pSE-133-2) (*fhlA*::Tn*5*) was actually lower than that of strain MJ-9 itself; under the conditions used in these experiments, this activity never exceeded 300 U. These results show that in the presence of multiple copies of the *fhlA* gene, the concentration of formate required for optimum expression of the *fhlB* gene was considerably reduced but was still a needed inducer.

At this lower formate concentration (3 mM), the differential rate of synthesis of β-galactosidase activity by strain SE-2011 was increased from about 20 to about 330 U/μg of protein if the *fhlA* gene was also present in a multicopy plasmid (pSE133-1) (Fig. 8). Immediately after anaerobic

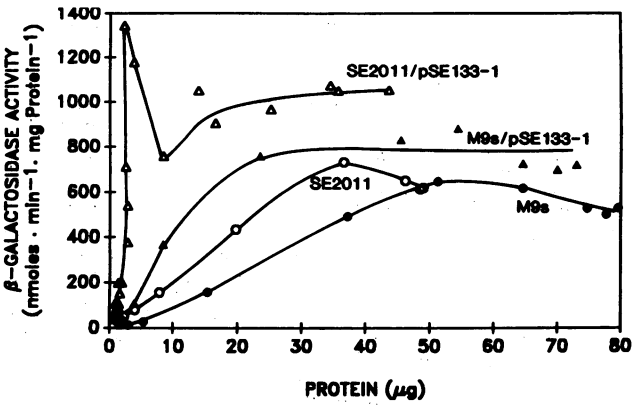

FIG. 8. Differential rate of synthesis of Φ(*fdhF-lacZ*$^+$) (strain M9s) and Φ(*fhlB-lacZ*$^+$) (strain SE-2011) in LB medium supplemented with 3 mM formate in the presence and absence of the *fhlA*$^+$ gene in a multicopy plasmid (pSE-133-1). β-Galactosidase and protein activities are expressed as units per milliliter and micrograms per milliliter, respectively.

conditions were established, the *fhlB*-mediated β-galactosidase activity increased to about 1,400 U within a generation time. During the second generation, this activity decreased to about 50% of the observed peak value. The β-galactosidase activity reached about 1,000 U and was maintained at that level. On the other hand, strain M9s with the same plasmid [M9s(pSE133-1)] produced β-galactosidase activity after an initial lag. The differential rate of synthesis was about 10% of the value of strain SE-2011(pSE133-1) and was only about threefold higher than that of strain M9s without the plasmid. The levels of β-galactosidase activity produced by strains SE-2011 and M9s, both in the presence and in the absence of plasmid pSE-133-1, were not altered by including Mo or Se in the LB medium, although FDH-H is a Mo- and Se-containing protein. The amount of formate needed for optimum expression of Φ(*fdhF-lacZ*$^+$)(pSE-133-1) was about 3 mM, similar to that required for strain MJ-9(pSE-133).

DISCUSSION

Production of the FHL complex by *E. coli* requires anaerobiosis, formate, and presumably a lower pH value (4, 25, 26, 42–45). At least two genes coding for the components of the complex have been identified (24, 25). The *fhlA* gene has been proposed as a regulatory gene controlling expression of both the *fdhF* and *hyd-17* genes (29, 33). The DNA sequence clearly shows that the *fhlA* gene is a transcriptional activator in the category of two-component regulatory systems (Fig. 5).

Besides the FhlA protein, the FhlB protein is also needed for production of active FHL complex by *E. coli* (Table 2). Transcription of the *fhlB* gene requires the *fhlA* gene product, formate, and the σ^{54} subunit of RNA polymerase (Fig. 2; Table 3), and in this regard *fhlB* is similar to the *fdhF* and *hyd-17* genes (3, 29, 33). Although strain SE-2011 is also Hup$^-$, production of hydrogen uptake activity is not known to require formate (32). Transcription of *hup* genes from Φ(*hup-lacZ*$^+$) is also independent of formate (J. W. Wendt and K. T. Shanmugam, unpublished observations). So far, only the genes involved in dihydrogen evolution have been found to require formate for transcription (2, 44). The possibility that the FhlB protein is involved in cofactor (Ni) biosynthesis and processing could be discounted because of the need for formate for transcription. The *hydC* and *hydE* mutants whose products are needed for Ni transport and processing also produced FDH-H activity. A. Böck and co-workers (personal communication) have identified a complex operon (*hyd*-17) which, on the basis of DNA sequence, probably codes for all of the needed electron transport proteins, ruling out the possibility that the FhlB protein is involved in electron transport.

If the Hyd$^-$ phenotype of strain SE-2011 is due to a second mutation, this altered *hyd* gene should be very close to the *fhlB* gene, since upon transduction into strain MC4100, the *fhlB* transductants were found to be Fhl$^-$ and Hyd$^-$. A second alternative, that strain MC4100 carries a silent mutation whose Hyd$^-$ phenotype is expressed only in the presence of the *fhlB* mutation, cannot be ruled out at this time. A third alternative is that the lack of hydrogen uptake and fumarate reductase activities could be a direct effect of the mutation or a physiological effect induced by the absence of the FHL pathway in this unique mutant strain. Additional experiments are needed to distinguish between these alternatives. However, it is highly likely that the FhlB protein plays a role, yet unknown, in expression of the genes in dihydrogen metabolism.

The optimum concentration of formate needed for transcription of Φ(*fhlB-lacZ*$^+$) depends on the FhlA protein concentration. When the *fhlA* gene is present only in the chromosome, the differential rate of *fhlB* gene transcription is considerably lower than when the *fhlA* gene is also expressed from a multicopy plasmid. Obviously, the FhlA protein is rate limiting for expression of the *fhlB* operon. Although the rates of transcription of both the *fhlB* and *fdhF* genes are enhanced by multiple copies of the *fhlA* gene, the rate of *fdhF* gene expression is not as high as the rate of *fhlB* gene expression (Fig. 8). It is also known that the FHL activity of wild-type cells is not increased by plasmids carrying the *fhlA* gene (28), probably because of the cellular location of the FHL complex in the membrane. The finite space in the membrane would preclude overproduction of the proteins in the FHL complex (FDH-H and FHL-HYD) if the transcription and translation of FHL are coupled to membrane synthesis. On the other hand, the rate of expression of cytoplasmic proteins can be enhanced without detrimental effect on cellular physiology. According to this analogy, the high rate of transcription of the *fhlB* gene (Fig. 6 and 8) would suggest that the FhlB protein is cytoplasmic and functions in the regulation of the enzymes in the dihydrogen metabolism.

In analogy with other regulatory components of two-component regulatory systems, the FhlA protein, a putative transcriptional activator for the *fhlB* operon, would be expected to undergo phosphorylation or dephosphorylation, depending on the presence or absence of the environmental stimulus, formate. However, analysis of the protein sequence failed to identify any significant homology with the possible phosphorylation sites found in the N-terminal region of the other proteins (21, 35). In this regard, the FhlA protein is similar to the NifA protein of *K. pneumoniae* and is distinct from the NtrC protein. The availability of additional mutants with phenotypes similar to that of *fhlA* (unpublished data) raises the possibility that a formate sensor-regulator pair similar to the known two-component regulatory systems may also exist in the cell. The *fhlC* gene located near the *hydA* gene (unpublished data) or the *fdv* gene (29) could fulfill this role.

Two lines of evidence suggest that formate could interact with the FhlA protein and that formate-FhlA protein is the

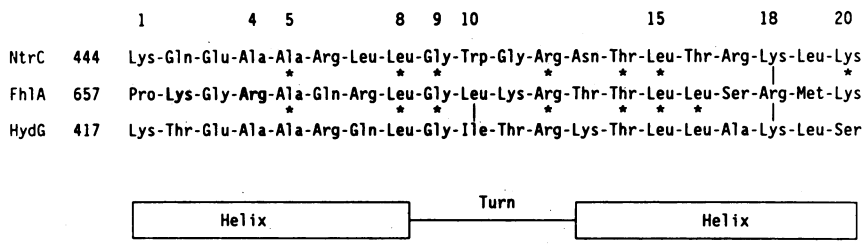

FIG. 9. Alignment of possible DNA-binding motifs in the NtrC, FhlA, and HydG proteins. Symbols: *, identical amino acid; |, functionally similar amino acid. The NtrC and HydG sequences are from Miranda-Rios et al. (20) and Stoker et al. (37), respectively. See text for details.

active complex. Equilibrium kinetics would predict that the amount of formate needed to produce the amount of stable complex necessary to maximally express the *fhlB* gene can be reduced by increasing the concentration of the FhlA protein, which is in agreement with the observed results. Increasing the copy number of the *fhlA* gene by introducing plasmid pSE133, which raised the level of the FhlA protein in the cell, decreased the needed formate concentration from 15 to 3 mM. If formate is a biochemical effector for modulation of the FhlA protein, transcription of the *fhlA* gene would be independent of formate. In agreement with this possibility, we have isolated a Φ(*fhlA-lacZ*$^+$) mutant in which production of β-galactosidase activity is formate independent. If formate indeed binds to the FhlA protein, this domain may lie within the unique N-terminal 370 amino acids, in the C-terminal end of the FhlA protein, or in a structure combining the two regions of the protein.

In this connection, it is interesting to note that between positions 657 and 676, lysine and arginine account for 35% of the total amino acids (Fig. 9). This segment of the protein is 45% homologous to the putative DNA-binding region (helix-turn-helix motif; 22) of the HydG and NtrC proteins (Fig. 9). In this stretch of 20 amino acids, arginine is present at the crucial position 4, in which a hydrophobic amino acid is generally found. This arginine is preceded by glycine and lysine. Formate could interact at this location by forming hydrogen bonds with the $-NH_3^+$ side chains of arginine and lysine and thus modify the basic characteristics of these amino acids. Such a combination could lead to a secondary structure of the protein which promotes the interaction with the *fhlB* promoter. Mutational alteration of the critical amino acids in this region is needed to evaluate this possibility.

As a working model, we would like to propose that the *fhlA* gene product is a transcriptional activator for the *fhlB* gene and that the *fhlB* gene product in turn controls expression of the *fdhF* and FHL-HYD genes, coding for the structural components of the FHL complex. This regulation is controlled by the level of formate and the concentration of the FhlA protein. The FhlA protein binds formate, and this complex is probably the actual control element for *fhlB* gene transcription, which in turn interacts with the upstream regulatory sequence of the *fdhF* gene (2). The FhlA and FhlB proteins act as a cascade, probably in association with other, as yet unidentified proteins (e.g., the FhlC protein) in controlling the production of FHL by the cell. Formate plays a critical role in modulation of this cascade.

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