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Expression and Regulation of a Silent Operon, *hyf*, Coding for Hydrogenase 4 Isoenzyme in *Escherichia coli*†

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On the basis of *hyf-lacZ* fusion studies, the *hyf* operon of *Escherichia coli*, noted for encoding the fourth hydrogenase isoenzyme (HYD4), is not expressed at a significant level in a wild-type strain. However, mutant FhlA proteins (constitutive activators of the *hyc*-encoded hydrogenase 3 isoenzyme) activated *hyf-lacZ*. HyfR, an FhlA homolog encoded by the *hyfR* gene present at the end of the *hyf* operon, also activated transcription of *hyf-lacZ* but did so only when *hyfR* was expressed from a heterologous promoter. The HYD4 isoenzyme did not substitute for HYD3 in H_2 production. Optimum expression of *hyf-lacZ* required the presence of cyclic AMP receptor protein-cyclic AMP complex and anaerobic conditions when HyfR was the activator.

Three hydrogenase isoenzymes have been identified, purified, and characterized from *Escherichia coli* (6, 10, 16, 27, 37). The structural subunits and accessory proteins needed for these three isoenzymes are encoded by the *hya, hyb*, *hyc*, and *hyp* operons (9–11, 26, 29, 30, 34). The *hya* operon, *hyaABC DEF* (30), encodes the hydrogenase 1 (HYD1) isoenzyme and other accessory proteins required for processing of these subunits into the active form. This operon is induced under anaerobic conditions in the presence of formate or fumarate, repressed in the presence of nitrate, and requires acidic pH, ArcA, and AppY for optimal expression (12, 21, 32). However, *hya* mutants have no detectable phenotype (31). The *hyb* operon, *hybABCDEFG* (29), encodes the structural subunits of HYD2 as well as the needed accessory proteins (9). Based on genetic and physiological studies, HYD2 is responsible for uptake of hydrogen as an electron donor during anaerobic respiration, with fumarate serving as an electron acceptor (24, 29, 45).

The *hyc* operon encodes the structural subunits and necessary enzyme components to link HYD3 (36) to a unique formate dehydrogenase isoenzyme (FDH-H, encoded by *fdhF*) (46) to produce active formate hydrogenlyase complex (FHL) (10). This protein complex catalyzes the cleavage of formate to dihydrogen and carbon dioxide. Transcription of the *hyc* operon and *fdhF* requires the FhlA protein, a formate-dependent transcriptional activator (28, 38). In addition to FhlA-formate, molybdate is also required for transcription of the *hyc* operon, and this requirement is in part due to the need for the ModE-molybdate complex as a secondary activator (40). ModE, initially characterized as a molybdate-dependent repressor of the *modABC* operon carrying high-affinity molybdate transport genes (18), has subsequently been shown to act as a positive transcriptional regulator of the *hyc* operon (HYD3) as well as of the *narXL* operon (40), encoding a nitrate-responsive two-component regulatory system which activates transcription of *narGHJI* (respiratory nitrate reductase) (17). Additionally, optimal expression of *hyc* also requires the catalytic product of MoeA, a protein implicated in the activation of Mo during Mo-cofactor biosynthesis (19, 20). Mutated forms of FhlA that are independent of formate and/or molybdate have been described previously (42). These mutations are localized in the unique N-terminal region of the FhlA protein (23, 42). Deletion of the N-terminal 350 amino acids also produced an effector-independent transcriptional activator (FhlA165) (25, 41).

The *E. coli* genome sequence (8) revealed a 10-gene cluster (*hyfABCDEFGHIJ*), which is recognized as being the *hyf* operon (the fourth hydrogenase) based on similarity to corresponding Hyc proteins (2, 3). The proteins encoded by the *hyf* operon are proposed to constitute a proton-translocating formate hydrogenlyase (2). In support of this proposal, Bagramyan et al. (4, 5) reported an H^+-K^+ exchange reaction in osmotically stressed *E. coli* cells which was absent in a *hyf* mutant. On the basis of these and other studies, these investigators proposed that Hyf catalyzes dihydrogen production and ion transport when the cells are grown at a starting pH of 7.5. Skibinski et al. (43) reported that *hyf-lac* was expressed in wild-type *E. coli* in a formate-dependent manner, with FhlA serving as the activator. However, the maximum level of β-galactosidase activity produced by *hyfA-lac* was less than 100 U. Only when HyfR was produced from a multicopy plasmid was *hyfA-lac* expressed at a high level. It has been observed that mutant strains lacking all three known hydrogenases failed to produce hydrogenase activity assayed either by viologen reduction or by a more sensitive tritium exchange assay (J. C. Wendt and K. T. Shanmugam, unpublished data). These results suggest that the fourth hydrogenase encoded by the *hyf* operon is not produced in *E. coli* and that the *hyf* operon is silent in this organism. In this communication, we report that *hyf-lacZ* is not expressed to significant levels in wild-type *E. coli*, and this fact is independent of medium and growth conditions. We further report that *hyfA-lacZ* can be activated in the presence of effector-independent mutated forms of FhlA (FhlA132 and

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TABLE 1. *E. coli* strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Relevant genotype	Source or reference
Bacterial strains		
BN4020	*fur-1*::Tn*5*	CGSC no. 7540
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{W116}$ *hsdR514 araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	B. Wanner
RK4353	Δ(*argF-lac*) *U169 rpsL150*	Laboratory collection
YMC18	*endA thi hsdR* Δ*lac rpoN*::Tn*10*	B. Magasanik
JW138	Δ*hya* Δ*hyb* Φ(*hycB′-lacZ*)	Laboratory collection
SE1174	*fhlA102*::Tn*10*	Laboratory collection
SE1931	*fnr zcj*::Tn*10*	Laboratory collection
SE1265	*pfl-1 zba*::Tn*10*	Laboratory collection
SE1910	Δ(*modE*-Km)*2*	18
SE1989	Δ*cya*-Km CRP*	Laboratory collection
SE2147	*moeA113 zbi*::Tn*10*	19
VJS720	*modB247*::Tn*10*	V. Stewart
WS219	RK4353 Δ(*hyfB* to *hyf*G)-Cm	This study
WS127	Δ(*srl-fhlA*) Δ*lac* λWS1-(*hycA-lac*)	41
Φ(*hyfA′-′lacZ*) derivatives		
WS222	RK4353 λWS4	This study
WS228	WS222 *fhlA*::Tn*10*	WS222 × (P1)SE1174
WS229	WS222 *rpoN*::Tn*10*	WS222 × (P1)YMC18
WS230	WS222 *fnr zcj*::Tn*10*	WS222 × (P1)SE1931
WS231	WS222 *moeA113*	WS222 × (P1)SE2147
WS232	WS222 Δ(*hyfB* to *hyfG*)-Cm	WS222 × (P1)WS219
WS233	WS222 *pfl-1 zba*::Tn*10*	WS222 × (P1)SE1265
WS235	WS222 *modB247*::Tn*10*	WS222 × (P1)VJS720
WS236	WS222 Δ(*modE*-Km)*2*	WS222 × (P1)SE1910
WS266	RK4353 λWS10	This study
WS267	WS266 *modB247*::Tn*10*	WS222 × (P1)VJS720
WS268	WS266 *pfl-1 zba*::Tn*10*	WS266 × (P1)SE1265
WS269	WS266 *rpoN*::Tn*10*	WS266 × (P1)YMC18
WS270	WS266 *fhlA*::Tn*10*	WS266 × (P1)SE1174
WS271	WS266 Δ(*modE*-Km)*2*	WS266 × (P1)SE1910
WS272	WS266 Δ*cya*-Km	WS266 × (P1)SE1989
WS273	WS266 *fnr zcj*::Tn*10*	WS266 × (P1)SE1931
WS274	WS266 *moeA113*	WS266 × (P1)SE2147
WS275	WS266 Δ(*hyfB* to *hyfG*)-Cm	WS266 × (P1)WS219
WS280	WS266 *fur*::Tn*5*	WS266 × (P1)BN4020
AH266	WS222 Δ(*hyfA* to *hyfJ*), Km	This study
AH267	WS222 Δ(*hyfR*), Km	This study
Phages		
P1	Tn*9* Cm^r *clr*-100	Laboratory collection
λRZ5	λ*′bla ′lacZ lacY*$^+$	Laboratory collection
λWS4	λ*bla*$^+$ Φ(*hyfA′-lacZ*) *lacY*$^+$	This study
λWS10	λ*bla*$^+$ *lacI*^{q} *hyfR*$^+$ Φ(*hyfA′-lacZ*) *lacY*$^+$	This study
Plasmids		
PKD4	FRT-*kan*$^+$-FRT *bla*$^+$	B. Wanner
pWS2	pACYC184-*fhlA*$^+$	This study
pWS132	pACYC184-*fhlA132*	This study
pWS165	pACYC184-*fhlA165*	This study
pWS42	pBR322 *bcp hyfABCDEFGHIR′*	This study
pWS43	pBR322 *nlpB dapA gcvR bcp hyfA′*	This study
pWS44	pBR322 *nlpB dapA gcvR bcp hyfA′-lacZ*	This study
pWTS3	pWS42 Δ*hyf*-Cm	This study
pWTS36	pBR322-*lacI*^{q} *hyfR*$^+$ *bcp hyfA′-lacZ*	This study
pWTS37	pUC19-*hyf* promoter region	This study
pZCam	pZ1918-Cm^r	This study

FhlA165) or native HyfR produced from a heterologous promoter, even when the gene is at single-copy level. In the presence of these activators, *hyf* expression is dioxygen sensitive and subject to catabolite repression.

Bacterial strains. The bacterial strains, phages, and plasmids used in this study are listed in Table 1. All strains are derivatives of *E. coli* K-12.

Media, growth conditions, and materials. Media used for bacterial growth were previously described (33). Luria broth (LB) (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl), which served as rich medium, was supplemented with glucose (0.3%), sodium formate (15 mM), or sodium molybdate (1 mM) as needed. Glucose-minimal medium included 44 mM Na_2HPO_4, 5.5 mM KH_2PO_4, 34 mM NaCl, 41 μM Na_2MoO_4, 36 μM

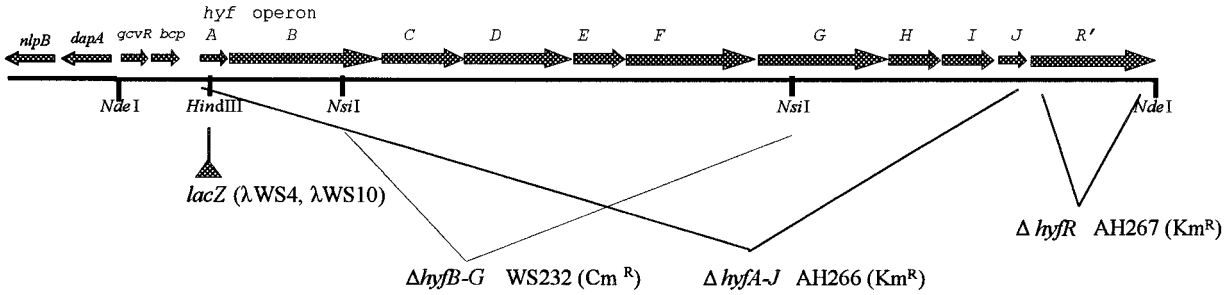

FIG. 1. The *hyf* DNA from *E. coli*. Individual genes and direction of transcription are indicated above the line. Promoterless *lacZ* was inserted into the *Hin*dIII site in the *hyfA* gene in the construction of λWS4 and λWS10. The extent of deletion in each of the deletions and the corresponding strain are indicated.

$FeSO_4$, 7.5 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, and 83 mM glucose. Antibiotics, when included, were used at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 30 μg/ml; chloramphenicol, 50 μg/ml (plates) and 10 μg/ml (liquid); and kanamycin, 50 μg/ml.

Transduction with phages P1 and λ was performed as previously described (33). Genetic and molecular biological experiments were carried out essentially as previously described (40). Biochemicals were purchased from Sigma Chemical Co. Other organic and inorganic chemicals came from Fisher Scientific and were of analytical grade. Restriction endonucleases and DNA-modifying enzymes were purchased from New England BioLabs and Promega.

Enzyme assays. β-Galactosidase activity assays were carried out using cells in late exponential phase with cells that were permeabilized with sodium dodecyl sulfate and chloroform as previously described (33, 40). Units are expressed as nanomoles · $minute^{-1}$ · (milligram of cell protein)$^{-1}$. Under our experimental conditions, a Δ*lac* mutant of *E. coli* was assayed at high cell density and produced enough *o*-nitrophenol to account for about 20 to 50 U of β-galactosidase activity. Due to this extremely low level of *o*-nitrophenyl-β-D-galactopyranoside hydrolysis, we used a value of 50 U of β-galactosidase activity as the basal level. Specific activity values represent the average of at least three independent experiments and varied by less than 15%. FHL activity of the cultures was determined by using whole cells to minimize dioxygen inactivation of FHL, with formate used as the electron donor (24). The amount of formate-dependent dihydrogen produced was determined by gas chromatographic methods (Varian gas chromatography with thermal conductivity detector and a 5-Å molecular sieve column).

Construction of Φ(*hyfA-lacZ*). In order to construct a *lacZ* operon fusion for transcriptional analysis of the *hyf* operon, a 4.3-kb *Eco*RI-*Hin*dIII fragment from Kohara λ clone no. 424 (22), which carries the *hyfA′* gene and 3.7 kb of upstream DNA, was cloned into plasmid pBR322 within the unique *Eco*RI and *Hin*dIII sites. The resulting plasmid, pWS43, was modified by inserting a 3.2-kb *Hin*dIII fragment from plasmid pZ1918 (39), which carries a promoterless *lacZ* gene, into the *Hin*dIII site. The resulting plasmid, pWS44, carries a *hyfA′-lacZ* fusion which is adjacent and opposite in orientation to the *bla* gene. In this plasmid, the *lac* fusion is located 296 bp downstream of the *hyfA* translation start site. This *hyfA′-lacZ* fusion was recombined in vivo with λRZ5 as previously described (33) in order to yield λWS4 (Fig. 1).

Construction of Φ(*hyfA-lacZ*) *hyfR*$^+$. For the construction of λWS10, which carries the *hyfA′-lacZ* operon as well as the *hyfR*$^+$ gene, the *hyfR* gene was amplified from plasmid pLC32-45 (14) by using two primers, 5′-ACTGTCCATGGCT ATGTCAGACGAG-3′ and 5′-AAAAGAAGCTTACAACA CCTCGCGA-3′. This PCR product was engineered to incorporate an *Nco*I site into the start codon (ATG) of the *hyfR* gene and a *Hin*dIII site past the translation stop codon. After amplification by Vent polymerase (New England Biolabs) and hydrolysis by *Nco*I and *Hin*dIII, the PCR product was ligated into the *Nco*I-*Hin*dIII sites of vector pTrc99A (1). The resulting plasmid, pWTS35, which also carries *lacI*^{q}, expressed the *hyfR* gene from the *trc* promoter at low levels even in the absence of inducers of the *lac* operon (13). The *lacI*^{q} and *hyfR* genes were removed from plasmid pWTS35 as an *Nsi*I-*Sca*I fragment (3.5 kb) and ligated to an *Nsi*I-*Sca*I fragment from plasmid pWS44, which carries the *hyfA′-lacZ* DNA. This construct, plasmid pWTS36, contains the *hyfR*$^+$ gene and *hyfA′-lacZ* as well as 3.7 kb of *hyf* upstream DNA. In this construct, *hyfR* is still expressed from the *trc* promoter in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG). The *E. coli* DNA in plasmid pWTS36 was recombined in vivo with λRZ5 as described previously (33) in order to produce λWS10 (Fig. 1).

For the construction of a plasmid which expresses *hyfR* and is also chloramphenicol resistant (pWTS34), a Cm^r cartridge from plasmid pZCam was removed as a 988-bp *Hin*cII fragment and cloned into the *Fsp*I site of pTrc99A, thus creating pTrc99A-Cm. As per the construction of plasmid pWTS35, the PCR-amplified *hyfR* gene was cloned into the *Nco*I-*Hin*dIII sites of plasmid pTrc99A-Cm, resulting in plasmid pWTS34.

Construction of Δ*hyf* and Δ*hyfR*. Two different deletions of the *hyf* operon were constructed. The first, with an internal deletion between *hyfB* and *hyfG*, was constructed by starting with a 12-kb *Nde*I fragment from Kohara λ clone no. 424 (22), which was cloned into the *Nde*I site in plasmid vector pBR322. This plasmid, pWS42, which carries *hyfABCDEFGHIJR′*, was hydrolyzed with restriction enzyme *Nsi*I so as to release a 5.6-kb internal fragment between the *hyfB* and *hyfG* genes (Fig. 1). This fragment was replaced with a 1.0-kb *Pst*I fragment from pZCam carrying a Cm^r gene cartridge. The resultant plasmid, pWTS3, carries the gene for chloramphenicol resistance in an orientation opposite to that of the *hyf* operon

transcription between the *hyfB* and *hyfG* genes. The Cm^r gene cartridge was expected to have a polar effect on the expression of downstream *hyf* (*hyfHIJ* and possibly *hyfR*) genes. In order to replace the wild-type *hyf* DNA in the chromosome with Δ(*hyfB* to *hyfG*)-Cm DNA, an 8.0-kb *Nde*I fragment from pWTS3 containing the Cm^r gene and the neighboring *hyf* genes was removed and self-ligated by using T_4 DNA ligase. This circular DNA lacks the *bla* gene and the origin of replication. Approximately 1 μg of the self-ligated 8.0-kb *Nde*I fragment was transformed into strain RK4353, and Cm^r transformants were selected. One stable Cm^r clone, strain WS219, was used in further studies. Cotransduction of the Cm^r gene with a *narQ*::Tn*10* mutation by P1 phage transduction confirmed that the gene for Cm^r had recombined into the *hyf* operon.

The second deletion, which removed the entire *hyf* operon, was constructed as described previously (15). Hybrid primers that are complementary to *E. coli hyfA* and *hyfJ* and to the kanamycin gene in plasmid pKD4 (Hyf1, 5′-CGCTTTGTGG TGGCCGAACCACTGTGGTGTACAGGATGTAATACG TGTAGGCTGGAGCTGCTTC-3′, and Hyf2, 5-GGTCAAC AGGGCGGTGTGGCTGGCGTCAATAACAATCTCACC AACATATGAATATCCTCCTTAG-3′) were obtained from Sigma-Genosys. Plasmid pKD4 was used as the template for PCR amplifications. About 1 μg of PCR product was electroporated into *E. coli* strain BW25113 with plasmid pKD46 pregrown in arabinose in order to induce the red recombinase. The resulting deletion (of *hyfA* to *hyfJ*) was confirmed by PCR. This mutation was transduced into strain WS222 for further studies (AH266).

A deletion which removed the entire *hyfR* gene was constructed by using the same method described above based on the procedures described by Datsenko and Wanner (15). The two primers used for PCR amplification of DNA and deletion of the *hyfR* gene were HyfR1 (5′-AAAAATTGCGTGAGAA GGATTTCTCATTAATAAGGACTGTTGATGGTGTAGG CTGGAGCTGCTTC-3′) and HyfR2 (5′-CCATTGGTTTCT CGCAATACCTGAACAATGCGCTGACGTTCTTCCATA TGAATATCCTCCTTAG-3′). Upon construction, the Δ*hyfR* was transduced into strain WS222 (strain AH267).

The *hyf* operon is not expressed to significant levels in wild-type *E. coli*. Based on genomic analysis, Andrews et al. (2) proposed that the Hyf hydrogenase, together with the FDH-H, couples formate oxidation to proton translocation. Recently, Bagramyan et al. (4, 5) reported that *E. coli* produced dihydrogen from formate which was Hyf dependent and inhibited by *N*,*N*′-dicyclohexylcarbodiimide. Production of dihydrogen, catalyzed by the fourth hydrogenase, required both growth of the culture at a starting pH of 7.5 and exposure to hyperosmotic stress before the assay. This hydrogenase activity was also proposed to be responsible for H^+-K^+ exchange. These results suggest that the *hyf* operon is expressed and that HYD4 isoenzyme is produced by *E. coli* during anaerobic growth at an alkaline starting pH.

Although the Hyf proteins are similar to the Hyc proteins, *hyc* and *fhlA* mutants are defective in dihydrogen production (28, 35, 38). *E. coli* mutants lacking all three known hydrogenase isoenzymes did not produce hydrogenase activity as determined by either dihydrogen-dependent dye reduction or by a more sensitive tritium exchange assay (Wendt and Shanmugam, unpublished). In the present study, *E. coli* mutants carrying a deletion within the *hyf* operon (AH266 and WS232) were cultured at a constant pH of 7.5 or 6.5, and the level of FHL activity in the cells was determined (Table 2). The parent and the deletion strains produced comparable levels of FHL activity when grown at pH 7.5. Although the FHL activity of cultures grown at a constant pH of 6.5 was higher, again, no significant difference in the levels of FHL activity between the parent and deletion strains could be observed. These results clearly show that the FHL activity observed in the pH 7.5 culture (constant pH) was derived from the HYD3 isoenzyme. In this regard, the *hyf* mutant is similar to the *hya* mutant, which also lacks a detectable phenotype (31). However, the HYD1 produced by the *hya* operon has been purified and characterized (16, 37), while a hydrogenase corresponding to Hyf was not detected in *E. coli* cells or extracts.

TABLE 2. Formate hydrogenlyase activities of *E. coli* cultures grown in a pH-stat[a]

Strain	Relevant genotype	Growth medium	FHL activity (nmol · min^{-1} · mg of cell protein^{-1})	
			pH 7.5	pH 6.5
WS222[b]	Wild type	LB + Glu (0.3%)	15	48
AH266	Δ(*hyfA* to *hyfJ*)		23	56
AH267	Δ(*hyfR*)		15	24
WS222	Wild type	LB + Glu (0.1%) + formate (15 mM)	40	148
AH266	Δ(*hyfA* to *hyfJ*)		50	129
WS232	Δ(*hyfB* to *hyfG*)		66	136
AH267	Δ(*hyfR*)		83	103

[a] Cultures, in the indicated media, were grown anaerobically under argon gas phase at the indicated pH in a pH-stat. pH was maintained by the addition of 2 N KOH. FHL activity was determined as formate-dependent dihydrogen production by using a gas chromatograph. β-Galactosidase activities of all these cultures were below the detection limit of 50 nmol · min^{-1} · (mg of cell protein)$^{-1}$.

[b] Strain WS222 carries Φ(*hyfA-lacZ*) via λWS4.

In order to evaluate the possibility that the fourth hydrogenase is produced in *E. coli* when cultured under specific medium composition (5), a *hyc* mutant (strain WS127) was grown in rich or minimal medium with or without glucose at a starting pH value of 6.5, 7.0, 7.5, or 8.0 (±0.1 M phosphate buffer) without pH control under a gas phase N_2 atmosphere. These and other cultures grown with NaCl (0.2 or 0.3 M) at pH 7.5 or 8.0 did not produce any detectable dihydrogen measured as H_2 by gas chromatography (data not presented). Strain JW138, lacking the three known hydrogenase isoenzyme genes (*hya, hyb*, and *hyc*), grown under similar conditions (initial medium pH value of 6.5, 7.0, 7.5, or 8.0 [±0.1 M phosphate buffer and ±0.3 M NaCl]) also did not produce detectable H_2.

Maturation of the three known hydrogenases requires chaperone-like proteins, and the three proteins are interconnected at this level (9). It is possible that the inability to detect the fourth hydrogenase activity in a mutant lacking the other three hydrogenases is related to a need for such a chaperone-like protein or a specific protease produced by either the *hya*, *hyb*, or *hyc* operon for processing the fourth hydrogenase precursor protein to become the active enzyme. In previous studies, we

TABLE 3. Regulation of expression of Φ(*hyfA-lacZ*) in the presence of various *fhlA* alleles and HyfR

Mutated gene[a]	β-Galactosidase activity[b]		
	FhlA$^+$	FhlA165	HyfR
None (wild type)	<50	2,500	8,200
fhlA	<50	2,500	9,900
rpoN	<50	<50	<50
hyf	ND[c]	ND	7,200
pfl	<50 (<50)[d]	2,700 (2,700)[d]	7,000 (7,500)[d]
modB	<50 (<50)[e]	3,400 (3,400)[e]	9,800 (11,000)[e]
modE	<50	3,300	12,500
moeA	<50	1,800	9,000
fur	<50	ND	11,000
fnr	<50	2,600	22,000

[a] All strains with FhlA plasmids are derivatives of *E. coli* strain WS222, which carries Φ(*hyfA-lacZ*) via λWS4, and all are listed in Table 1. For experiments with HyfR, mutant derivatives of strain WS266, which carries Φ(*hyfA-lacZ*) *hyfR*$^+$ via λWS10, were used (Table 1). Cultures were grown in LBG (LB with glucose) medium under anaerobic conditions.

[b] β-Galactosidase activity is expressed as nanomoles · minute^{-1} · (milligram of cell protein)$^{-1}$.

[c] ND, not determined.

[d] Formate was added to the growth medium at an initial concentration of 15 mM (values in parentheses).

[e] Molybdate was present in the medium at an initial concentration of 1 mM (values in parentheses).

have observed that the transcription of *hycA-lac* is unaffected by deleting the entire *srl-fhlA* region of the chromosome, which includes the *hyp* and *hyc* operons (41). By analogy, transcription of the *hyf* operon is expected to be independent of the ability of the coded proteins to function in the cell and thus should permit analysis of *hyf* expression as β-galactosidase activity by using a *hyf-lacZ* derivative.

In order to evaluate the level of transcription of the *hyf* operon, a λ phage carrying the *hyfA′-lac* fusion was constructed and inserted into the *E. coli* chromosome (strain WS222). Strain WS222 was cultured in a pH-stat at either pH 7.5 or 6.5, and irrespective of culture pH, β-galactosidase activity was not detected in these cells (data not presented). StrainWS222 did not produce detectable levels of β-galactosidase activity when cultured in either rich medium or minimal medium under a variety of anaerobic and aerobic growth conditions, including growth at pH 8.0 and in the presence of 0.3 M NaCl—conditions that, according to Bagramyan et al. (5), support Hyf-dependent activity (data not shown). Likewise, plasmid pWS44 (*hyfA′-lacZ* construct) used in the construction of this λ phage also did not support production of β-galactosidase activity either in the wild type or in various *E. coli* mutant strains tested (*modE*, *moeA*, *fur*, *fnr*, and *crp* mutants; data not shown). Attempts to isolate point mutations within the putative promoter region of *hyf*, which allowed expression of *hyf-lacZ* in a wild-type background, were also unsuccessful. The lack of expression of *hyfA-lacZ* suggests that this operon is not expressed under the physiological conditions tested and is apparently a silent operon. Skibinski et al. (43) reported that *E. coli* with a *hyf-lacZ* fusion produced about 15 U [nanomoles · minute^{-1} · (mg of protein)$^{-1}$] of β-galactosidase activity, which was increased to about 50 U in the presence of formate. Under our experimental conditions, strain WS222 produced less β-galactosidase activity (Table 3) than did a *lac* deletion mutant without the λ phage carrying the fusion. These results show that the *hyf* operon is not expressed to significant levels in wild-type *E. coli*, and thus this operon should be considered a silent operon. However, the possibility that the *hyf* operon is expressed in the presence of an effector(s) which is not present in the cytoplasm when *E. coli* is cultured in the laboratory cannot be ruled out.

FhlA132 and FhlA165 proteins activate expression of the *hyf* operon. The lack of expression of the *hyf* operon in *E. coli* could be due to the absence of an appropriate activator protein. A gene coding for a putative transcriptional activator, HyfR, is located at the end of the *hyf* operon (2). HyfR is similar to the FhlA protein (44% identical and 54% similar), which is the formate- and molybdenum-dependent activator of the *hyc* operon. HyfR, a protein with 663 amino acids, is missing the amino acids corresponding to the first 43 amino acids of the FhlA protein, which contains the region similar to the ABC-ATPases (41). Except for a stretch of about 60 amino acids (139 to 195 in HyfR and 179 to 234 in FhlA) in which the two proteins are 56% identical, HyfR and FhlA are dissimilar in their unique N-terminal regions. This N-terminal segment of the FhlA protein was proposed to be essential for formate binding in vivo (23, 41, 42). These differences in the N-terminal domain of the two proteins may be responsible for the inability of FhlA to activate *hyfA-lacZ* since the *fhlA* gene is constitutively expressed in anaerobic *E. coli.* Even when the copy number of *fhlA*$^+$ was increased by introducing a plasmid carrying the *fhlA*$^+$ gene (plasmid pWS2), the *hyfA-lacZ* expression was below the detection limit. It is apparent that the FhlA protein, either with or without formate and molybdate, is not an activator for the *hyf* operon. Although Skibinski et al. (43) reported that the FhlA activated *hyfA-lac*, the level of β-galactosidase activity produced by these cultures was only about 20 nmol · min^{-1} · (mg of protein)$^{-1}$, and this was increased to about 50 U of activity in the presence of formate in the growth medium.

Both point mutations and deletions in the N-terminal domain of the FhlA protein were found to be effector independent, and some of the deletion derivatives activated *hyc* to a higher level than did the native protein (23, 25, 41, 42). Furthermore, the N-terminal domain of FhlA has also been reported to inhibit *hyc* activation by the deletion derivatives of FhlA (25). Since the central and C-terminal domains of FhlA and HyfR are more than 60% identical (70% similar), it is possible that the effector-independent forms of the FhlA protein would activate *hyfA-lacZ*. In the presence of FhlA132, which carries two point mutations (42), *hyf-lac* was expressed, and the level of β-galactosidase activity produced by the strain WS222(pWS132) was 330 U. The FhlA165 protein, which lacks the unique N-terminal region (amino acids 5 to 374) (41), increased activation of *hyf-lac* by about eight times, to about 2,500 U of β-galactosidase activity (Table 3). Although FhlA132 and FhlA165 activated *hyc-lac* expression at comparable levels (2,900 and 3,500 U of β-galactosidase activity, respectively) (41, 42), FhlA132 is only minimally effective with the *hyf* operon. This may be a consequence of the N-terminal domain (although carrying point mutations) significantly affecting the activation of transcription of *hyf* by the C-terminal domain of FhlA.

Activation of *hyf* by HyfR. In a separate experiment, the *hyfR* gene was cloned and expressed from a heterologous promoter

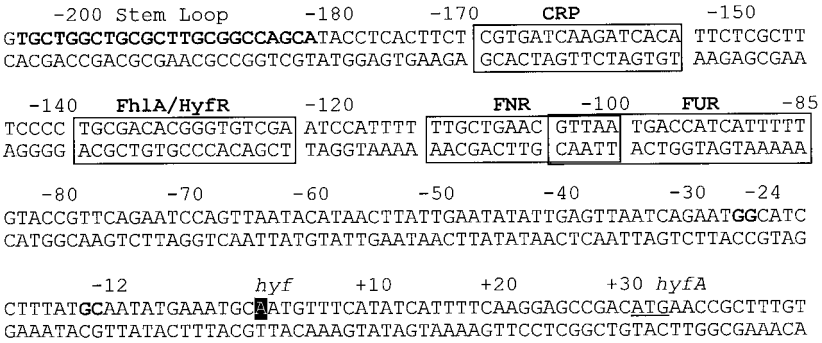

FIG. 2. The *hyf* operon upstream DNA. The putative consensus sequences for the CRP, FNR, Fur, and FhlA/HyfR proteins are enclosed by rectangles. A possible stem-loop between the stop codon of the upstream *bcp* gene and *hyf* promoter is shown in boldface type. The transcription start site is in reverse type. The −12 and −24 positions are also in shown in boldface type. A putative start codon for *hyfA* is both underlined and enclosed within a rectangle.

to determine whether HyfR, once produced within the cell, would activate the expression of *hyfA-lacZ*. In this experiment, the *hyfR* gene was cloned into phage λ, which also carries the *hyfA-lacZ* fusion (λWS10), in order to minimize the copy number effect. The HyfR protein, produced independent of its native control system, activated the *hyf* operon, and the level of β-galactosidase activity produced by strain WS266 (with λWS10) was about 8,000 U (Table 3). This level of expression is more than threefold higher than the value obtained with a strain carrying multiple copies of plasmid pWS165 coding for FhlA165. These results also confirm that the lack of transcription of *hyf* in wild-type *E. coli* is due to the absence of HyfR, the activator protein. Once produced in the cell, HyfR is an effective activator of the *hyf* operon (Table 3). However, HyfR failed to activate the *hyc* operon coding for the HYD3 isoenzyme, as evidenced by the lack of β-galactosidase activity from *hyc-lacZ* (strain WS127) or by dihydrogen production by a *fhlA* mutant (strain SE1174) carrying a plasmid expressing the *hyfR*$^+$ gene (plasmid pWTS35) (data not presented). This difference is apparently due to the differences in the unique N-terminal domains of the two proteins. These results are in agreement with those of Skibinski et al. (43).

Even upon activation by either the FhlA165 or the HyfR protein, a *hyc* mutant that is *hyf*$^+$ failed to produce detectable dihydrogen under any of the growth conditions tested. These results show that the Hyf proteins, although similar to the Hyc proteins, could not substitute for the HYD3 isoenzyme and other proteins of the FHL complex.

Formate and molybdate are not needed for *hyf* expression. As expected, in the presence of FhlA165 as the activator, *hyf-lac* expression was not significantly affected by the presence or absence of either formate or molybdate (Table 3). Similar results were also obtained with HyfR, indicating that the *hyf* operon expression was not affected in strains carrying mutations in the production of formate (*pfl*) or molybdate transport (*modB*). A slight increase in *hyf-lac* expression in a *modB* mutant (8,200 versus 9,800 U of β-galactosidase activity) and a further increase when molybdate was added to the medium (9,800 to 11,000 U) both suggest that the observed effect is physiological. If molybdate is required for *hyf-lac* expression, the level is expected to be lower in a *modB* mutant and restored by molybdate addition, as was seen with other operons such as *hyc-lac* (40–42). A modest increase in the level of expression of *hyf-lac* occurred in a *modE* mutant compared to that of the wild-type parent, suggesting a potential repression by ModE. However, the upstream region of the *hyf* operon lacks a ModE consensus sequence (Fig. 2), and the observed effect is apparently physiological. A mutation in *moeA* had a minimal effect on *hyf-lac* expression with FhlA165 and no effect with HyfR as the activator. These results suggest that molybdenum apparently had a minimal indirect effect on *hyf* expression (Table 3). Although a consensus sequence for the iron-dependent control protein Fur can be found upstream of the transcription start site, a *fur* mutation had only a minimal effect on the level of expression of *hyf-lac* (Table 3). However, a mutation in *fnr* increased the level of β-galactosidase activity produced by the culture about threefold with HyfR as the activator (Table 3) but had no significant effect on expression activated by the mutant FhlA protein. A putative FNR consensus sequence (44) in the *hyf* upstream DNA can be detected between positions −111 and −98 (Fig. 2). It is possible that binding of FNR at this site may not have an impact on binding of the smaller constitutive activator FhlA165 but may minimize the ability of the larger HyfR protein to bind at the target sequence, which is only about 25 bases upstream of the predicted FNR site. This possibility will be tested in future experiments by using purified HyfR, FhlA, and FhlA165 proteins.

CRP-cAMP is required for activation of *hyf* operon. A cyclic AMP receptor protein-cyclic AMP complex (CRP-cAMP) consensus sequence is also present in the *hyf* upstream sequence centered at position −160.5 (Fig. 2), which is 30 bp upstream of the HyfR/FhlA consensus. In order to evaluate the significance of this sequence, the level of expression of *hyf* was determined in wild-type and *cya* mutant strains in the absence of added glucose to evaluate the role of CRP-cAMP with HyfR as the activator (Table 4). When the culture was grown in LB without glucose, the level of β-galactosidase activity increased 3.5-fold to about 28,000 U from a value of 8,200 U of activity in the presence of glucose (Table 4). In the presence of the *cya* mutation, the level of β-galactosidase activity produced by the LB culture of strain WS272 decreased ninefold to about 3,000 U, and the addition of 3 mM cAMP restored *hyf-lac* expression to a level higher than that observed with the wild type grown in the same medium. These results show that *hyf* expression is

TABLE 4. Regulation of HyfR-dependent expression of Φ(*hyfA-lacZ*) requires cAMP for maximal activation and is repressed in aerobically grown cells

Strain	Relevant genotype[a]	β-Galactosidase activity[b]		
		LBG	LB	LB + cAMP
WS266	Wild type	8,200	27,800	26,200
WS272	*cya*	5,100	3,260	39,800
WS266 (aerobic)	Wild type	ND	180	ND

[a] All strains are derivatives of WS266, which carries Φ(*hyfA-lacZ*) *hyfR*$^+$ via λWS10. Cultures were grown under anaerobic conditions, except where indicated. cAMP, when present, was added at a final concentration of 3 mM.

[b] β-Galactosidase activity is expressed as nanomoles · minute^{-1} · (milligram of cell protein)$^{-1}$. ND, not determined.

subject to catabolite repression. It should be noted that although FhlA132 and FhlA165 activated *hyf-lac* expression and apparently bind to the *hyf* upstream DNA at the same location as does HyfR, the *cya* mutation had a slightly positive effect on *hyf-lacZ*-dependent β-galactosidase activity produced in the presence of FhlA165 (data not presented). This difference in the responses between FhlA165 and HyfR to CRP-cAMP is probably related to the absence of N-terminal domain in the smaller protein, FhlA165.

HyfR does not activate transcription of *hyf-lacZ* in aerobically grown cultures. Although strain WS266, with HyfR as the activator, produced more than 25,000 U of β-galactosidase activity when grown anaerobically in LB medium, aerobic cultures produced less than 200 U of β-galactosidase activity (Table 4). However, FhlA132 and FhlA165 did activate transcription of *hyf-lacZ* under aerobic conditions to levels comparable to those of the anaerobically grown cultures (data not presented). These mutant proteins have previously been shown to activate *hyc* transcription aerobically, so their activation of *hyf* is not unexpected (41). The lack of expression of *hyf-lac* by HyfR when the cells were grown aerobically demonstrated that when activated by HyfR, expression of *hyf* is oxygen sensitive. In the unique N-terminal domain of HyfR, a cysteine-rich amino acid sequence can be detected (200-**C**SDLSASH**C**A**C** LPR**C**-214). This segment of the protein may potentially play a role in redox-dependent regulation of the *hyf* operon, as has been shown previously for the well-studied FNR protein (7). Although FhlA165 successfully activated transcription of *hyf-lacZ* in an in vitro transcription-translation experiment, aerobically purified HyfR protein was unable to activate transcription in vitro (data not shown), a finding that was in agreement with the putative oxygen sensitivity of the protein. Biochemical experiments with HyfR protein purified under aerobic and anaerobic conditions will help identify the oxygen-sensitive nature of *hyf* expression.

Although the *hyf* operon is apparently silent in wild-type *E. coli*, two mutant FhlA proteins (FhlA132 and FhlA165) and constitutively expressed HyfR protein were able to activate transcription of *hyf*. The ability of mutated forms of FhlA proteins to activate this operon represents a unique way to activate transcription of what seems to be a vestigial, unexpressed operon. Appropriate altered forms of known regulatory proteins may help activate corresponding silent genes or operons in *E. coli* or other organisms in order to elucidate the potential physiological role(s) of these proteins in the cell.

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