N-terminal truncations in the FhlA protein result in formate- and MoeA-independent expression of the hyc (formate hydrogenlyase) operon of Escherichia coli

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The formate hydrogenlyase complex of Escherichia coli catalyses the cleavage of formate to CO₂ and H₂ and consists of a molybdoenzyme formate dehydrogenase-H, hydrogenase 3 and intermediate electron carriers. The structural genes of this enzyme complex are activated by the FhlA protein in the presence of both formate and molybdate; ModE-Mo serves as a secondary activator. Mutational analysis of the FhlA protein established that the unique N-terminal region of this protein was responsible for formate- and molybdenum-dependent transcriptional control of the hyc operon. Analysis of the N-terminal sequence of the FhlA protein revealed a unique motif (amino acids 7–37), which is also found in ATPases associated with several members of the ABC-type transporter family. A deletion derivative of FhlA lacking these amino acids (FhlA9-2) failed to activate the hyc operon in vivo, although the FhlA9-2 did bind to hyc promoter DNA in vitro. The ATPase activity of the FhlA9–2–DNA–formate complex was at least three times higher than that of the native protein–DNA–formate complex, and this degree of activity was achieved at a lower formate level. Extending the deletion to amino acid 117 (FhlA167) not only reversed the FhlA− phenotype of FhlA9-2, but also led to both molybdenum- and formate-independence. Deleting the entire N-terminal domain (between amino acids 5 and 374 of the 692 amino acid protein) also led to an effector-independent transcriptional activator (FhlA165), which had a twofold higher level of hyc operon expression than the native protein. Both FhlA165 and FhlA167 still required ModE-Mo as a secondary activator for an optimal level of hyc–lac expression. The FhlA165 protein also had a twofold higher affinity to hyc promoter DNA than the native FhlA protein, while the FhlA167 protein had a significantly lower affinity for hyc promoter DNA in vitro. Although the ATPase activity of the native protein was increased by formate, the ATPase activity of neither FhlA165 or FhlA167 responded to formate. Removal of the first 117 amino acids of the FhlA protein appears to result in a constitutive, effector-independent activation of transcription of the genes encoding the components of the formate hydrogenlyase complex. The sequence similarity to ABC-ATPases, combined with the properties of the FhlA deletion proteins, led to the proposal that the N-terminal region of the native FhlA protein interacts with formate transport proteins, both as a formate transport facilitator and as a cytoplasmic acceptor.

Keywords: FhlA mutations, molybdenum, operon regulation

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Abbreviations: FDH-H, formate dehydrogenase isoenzyme; FHL, formate hydrogenlyase enzyme complex; Pₕyc, hyc operon promoter.
INTRODUCTION

*Escherichia coli* catalyses the cleavage of intracellular formate to H₂ and CO₂ via the formate hydrogenlyase enzyme complex (FHL) (reviewed by Böck & Sawers, 1996). This complex includes a formate dehydrogenase isoenzyme (FDH-H; encoded by the *fdhF* operon), a hydrogenase isoenzyme (HYD3; encoded by the *byc* operon), and intermediate electron carriers (also encoded by the *byc* operon). FHL is only produced under anaerobic conditions and in the absence of alternative terminal electron acceptors, such as nitrate. Reduced levels of expression of the *byc* operon under these conditions have been attributed to a reduced pool of intracellular formate (Rössmann et al., 1991). This lack of available formate affects the transcriptional activator FhlA, as it requires formate as an effector molecule (Maupin & Shanmugam, 1990; Schlensog et al., 1994).

Molybdenum is required for FHL activity and is present in the FDH-H protein as molybdopterin guanine dinucleotide (Boyington et al., 1997). Molybdenum is transported in the form of molybdate by a high-affinity transporter encoded by the *modABC* operon (reviewed by Grunden & Shanmugam, 1997). Previous studies have shown that the expression of both the *byc* and *fdhF* operons also requires molybdenum for optimal transcription (Rosentel et al., 1995; Hasona et al., 1998b). ModE protein, originally characterized as a molybdate-dependent repressor of the *modABC* operon (Grunden et al., 1996), was also identified as a secondary transcriptional activator of the *byc* operon (Self et al., 1999). The FhlA protein has been proposed to serve as a molybdenum sensor (Hasona et al., 1998b), possibly interacting with the product of molybdate activation by the MoeA protein (Hasona et al., 1998a).

The FhlA protein has three distinguishable domains based, in part, on sequence similarity with other transcriptional activators, such as NtrC from *E. coli* (Nixon et al., 1986) and NifA from *Klebsiella pneumoniae* (Drummond et al., 1986). The C-terminal region contains a helix–turn–helix motif which constitutes a DNA-binding domain; this was confirmed by the DNA-binding activity of a C-terminal fragment of FhlA containing the amino acids 379–692 (Self, 1998; Leonhartsberger et al., 2000). The central region of FhlA is conserved among all members of the NtrC/NifA family of activators and contains a nucleotide-binding motif (Weiss et al., 1991): this region is probably responsible for formate- and DNA-dependent ATP hydrolysis and interaction with the e³¹ form of RNA polymerase in the initiation of transcription (Hopper & Böck, 1995; Korsa & Böck, 1997). The N-terminal segment of the FhlA protein appears to contain a region which modulates the transcriptional activity of the C-terminal segment. In many proteins of the NtrC family, the unique N-terminal region (of varying length) contains a receiver domain which must be phosphorylated by a cognate sensor-protein for activity (Keener & Kustu, 1988). Some members of this family of activators are not known to be phosphorylated, and are presumed to interact with appropriate effector molecules directly.

The N-terminal region of FhlA is one of the largest (380 aa), when compared to other response regulators of the NtrC family. The unique N-terminal region of the FhlA protein displays no significant sequence similarity to any known protein, and is not believed to be phosphorylated (Böck & Sawers, 1996). Point mutations in the N-terminal region of FhlA lead to partial independence from formate (Korsa & Böck, 1997) and molybdenum (Self & Shanmugam, 2000) in the activation of the *byc* operon. Based on these studies, it has been proposed that the N-terminal region of FhlA binds the effector molecule formate and may also interact with the product of the MoeA protein (activated molybdenum).

Removal of the first 378 amino acids of the 692 aa FhlA protein led to a formate-independent transcriptional activator (Self, 1998; Leonhartsberger et al., 2000). The N-terminal half of the protein was also shown to inhibit the ATPase activity of the C-terminal half of the protein and to inhibit the formate-dependent ATPase activity of the native protein. Since the FhlA protein has been proposed to interact with the MoeA product for optimum transcription of the *byc* operon, and mutations in the unique N-terminal domain of FhlA lead to molybdenum-independence, it is possible that removing the N-terminal domain would also impart molybdenum-independence to the truncated FhlA protein. It is also not known which part of the nearly 400 aa N-terminal segment of the protein is essential for formate- and molybdenum-dependence for *byc* activation. Therefore, several deletion derivatives extending to varying lengths of the N-terminal domain of FhlA were constructed to identify the critical region(s) responsible for effector-independence, and the results of this study are reported here.

METHODS

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

**Media and growth conditions.** Media used for bacterial growth were as described by Rosentel et al. (1995). L-broth (LB; Miller, 1972) was supplemented with glucose (0.3%), sodium formate (30 mM), sodium molybdate (1 mM) or sodium nitrate (30 mM). Antibiotics were added to the media, as required, at the following concentrations: 100 µg ampicillin ml⁻¹; 30 µg tetracycline ml⁻¹; 50 µg chloramphenicol ml⁻¹ (plates), 10 µg chloramphenicol ml⁻¹ (broth); 50 µg kanamycin ml⁻¹.

Transduction with phages P1 and λ was performed as described by Grunden et al. (1996). Genetic and molecular experiments were performed essentially as previously described (Self et al., 1999). Plasmids pWS2, pWS164 and pWS165 are derivatives of the low-copy vector pACYC184. Plasmid pWS167 is a derivative of pT7-7. For experiments involving *pbnB* mutant (Lopilato et al., 1986), the appropriate *fhlA* and *fhlA165* DNAs were transferred to plasmid vector pBR322 in the construction of pWS3 and pWS165-2, respectively. Plasmid pWS165-2 also carries the gene for spectinomycin resistance from pPS1240 (Kim et al., 1998).
Table 1. Bacterial strains, phages and plasmids used in this study

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* Cm, chloramphenicol; Km, kanamycin; Ap, ampicillin; Sp, spectinomycin.
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Isolation of N-terminal deletion fhlA alleles. Internal deletion mutations within the fhlA gene were isolated by Bal31 nuclease treatment of pWS2 (Self & Shanmugam, 2000) after HpaI restriction endonuclease hydrolysis. HpaI hydrolyses pWS2 approximately in the centre of the DNA encoding the unique N-terminal region of the FhlA protein. The reactions were terminated by the addition of ethanol (70% final concn) after 5, 10 and 15 min of nuclease treatment. The DNA was precipitated and subsequently ligated with T4 DNA ligase. The ligation mixture was transformed into strain SE1174 (fhlA : Tn10), and colonies expressing FDH-H activity were selected by the dye-overlap procedure as described by Lee et al. (1985). Plasmids from selected clones were isolated and the size of the deletion was determined using restriction endonucleases. Three plasmids with a deletion of ≥1 kb were selected for further analysis. The extent of the deletion in each mutant fhlA allele was determined by sequencing the DNA using the Sanger dyeoxy sequencing method (Sanger et al., 1977).
Purification of the FhlA165 protein.

pWS9-2 did not carry any additional mutations, besides the fhlA167 allele. The region was sequenced to confirm that the coding DNA as a Pfml–Smal fragment and cloning it into a Smal-digested vector, pT7-7. This construct, pWS41, carries a deletion of the first 117 aa of the N-terminus, and an extra 7 aa in the N-terminus derived from the vector DNA sequence (MARIRAL) which also provided the translation start site. Plasmid pWS16 was constructed by inserting a chloramphenicol-resistance gene cartridge into the Scal site of pWS41. The plasmid DNA was sequenced to confirm the deletion. DNA sequences were analysed using genePRO software (Riverside Scientific).

For the construction of pWS9-2, plasmid pWS9 was modified by introducing a chloramphenicol-resistance gene cartridge from plasmid pBR325 into the Sspl–DraI sites, to generate pWS9-1, followed by PCR-mediated deletion of the fhlA DNA corresponding to amino acids 7–37 of the FhlA protein. The fhlA gene on both sides of the deletion was amplified using two primer pairs (pair one, 5′-AATGTCATATACCCGGATTCTCTCCTGCGGCATTGCTTGTCGTTGCTTTCTGTCGCCGAG-3′ and 5′-GGACTTCGCGTACCACTCGGCTGTTGGTAAACCGTATTACCGCCT-3′; pair two, 5′-CGAGCGCGAGAACCGTATACCCATATGACATTGTCGTCACCGCCT-3′ and 5′-TGTGATAACCGATTCGTTGCA-3′). The PCR products were ligated together to create the deletion derivative (plasmid pWS9-2), and the PCR-amplified region was sequenced to confirm that the fhlA gene in plasmid pWS9-2 did not carry any additional mutations, besides the intended deletion.

**Purification of the FhlA165 protein.** Purification of the FhlA165 protein was essentially as described for the native FhlA protein by Schlensof et al. (1994). Briefly, pWS16, which carries the fhlA165 allele under the control of phage T7 promoter, was constructed by ligating a 1.4 kb BstEl–ClaI fragment from pWS16 into the ClaI site in the expression vector pT7-7 (Tabor & Richardson, 1985). Fresh transformants of BL21(DE3) with pWS16 were grown with shaking (225 r.p.m.) at 37 °C. When the culture reached OD600 = 2, IPTG was added (0.1 mM final concn) to the culture, and incubation was continued at 23 °C with shaking (225 r.p.m.) for an additional 6 h. Incubating the culture at this temperature allowed a larger fraction of the newly synthesized FhlA to stay in solution. Cells were harvested at 4 °C and washed once with Tris buffer (50 mM Tris, pH 8.0; 0.5 mM EDTA; 1 mM benzamidine; 1 mM DTT). The cells were then resuspended in 12 ml Tris buffer and broken by passage through a French pressure cell [20000 p.s.i. (138 MPa)]. The crude extract was centrifuged at 30000 g for 2 h and the supernatant fraction was loaded onto Q-Sepharose column (50 cm x 2.5 cm; Pharmacia). The column was then washed with Tris buffer and the proteins were eluted with a linear gradient of NaCl (0–0.4 M). The FhlA protein eluted at approximately 0.37 M NaCl. FhlA-containing fractions, as determined by SDS-PAGE analysis, were pooled and dialysed overnight in Tris buffer. This protein mixture was loaded onto a Hi-trap heparin column (Pharmacia) and eluted using a linear NaCl gradient (0–0.4 M); the FhlA protein eluted at approximately 0.3 M NaCl. FhlA-containing fractions were pooled and dialysed overnight in HEPES buffer (30 mM HEPES, pH 7.0; 0.5 mM EDTA; 1 mM benzamidine and 1 mM DTT). The FhlA protein was further purified using a 1 ml High S column (cation exchange, Bio-Rad) equilibrated with HEPES buffer and eluted with a linear gradient of 0–0.5 M NaCl. The FhlA protein eluted at approximately 0.25 M NaCl. Fractions containing FhlA protein were pooled and dialysed overnight in HEPES buffer. The final purification step required chromatography on a 1 ml High Q column (anion exchange, Bio-Rad) equilibrated with HEPES buffer and elution with a linear gradient of sodium citrate (0–0.3 M). The pure FhlA protein, as judged by SDS-PAGE analysis, was dialysed in storage buffer (25 mM HEPES, pH 7.0; 0.8 mM based on SDS-PAGE analysis. Fractions containing the FhlA165 protein were pooled and dialysed overnight in Tris buffer (25 mM, pH 7.5) with 1 mM EDTA and 50% glycerol (v/v). The FhlA165 protein was stored at −20 °C.

FhlA167 and FhlA9-2 proteins were purified essentially as described for FhlA165. However, the two proteins obtained after the Q-Sepharose step were subjected to further chromatography on a Q-Sepharose column with sodium citrate as the eluent. Both proteins eluted at a citrate concentration of 0.15 M. The pure proteins were dialysed against Tris buffer (50 mM, pH 7.5) and stored on ice. Freshly prepared proteins were used immediately.

**Purification of native FhlA protein.** The purification procedure similar to the one used for FhlA165 protein led to the characteristic precipitation of the native protein during dialysis after the ammonium sulfate precipitation step (Schlensof et al., 1994). The FhlA protein required high salt (0.2 M KCl) to remain in solution; thus, it may not have been present in the native form. In addition, most of the FhlA protein produced upon expression from a T7 promoter was present in inclusion bodies. Therefore, the conditions used for expression of FhlA and purification of the native FhlA were modified, and the method is described below.

The fhlA* gene was cloned into the expression vector pT7-7, by removing a 2.4 kb SpeI–ClaI fragment from plasmid pSE133 (Sankar et al., 1988) and ligating this DNA to a 2.4 kb XbaI–ClaI fragment from pT7-7 (Tabor & Richardson, 1985). The resultant plasmid, pWS9, carried the fhlA gene under the control of phase T7 gene 10 promoter. Fresh BL21(DE3) transformants of pWS9 were inoculated into 1 litre LB containing ampicillin, in a 2.8 litre Fernbach flask. The culture was grown with shaking (225 r.p.m.) at 37 °C until an OD600 of 0.7 was reached. IPTG was added (0.5 mM final concn) to the culture, and incubation was continued at 23 °C with shaking (225 r.p.m.) for an additional 6 h. Incubating the culture at this temperature allowed a larger fraction of the newly synthesized FhlA to stay in solution. Cells were harvested at 4 °C and washed once with Tris buffer (50 mM Tris, pH 8.0; 0.5 mM EDTA; 1 mM benzamidine; 1 mM DTT). All subsequent operations were carried out at 4 °C. Cells were resuspended in 25 ml Tris buffer and broken by passage through a French pressure cell at 20000 p.s.i. (138 MPa). The crude extract was centrifuged at 30000 g for 2 h and the supernatant fraction was loaded onto Q-Sepharose column (50 cm x 2.5 cm; Pharmacia). The column was then washed with Tris buffer and the proteins were eluted with a linear gradient of NaCl (0–0.4 M): the FhlA protein eluted at approximately 0.37 M NaCl. FhlA-containing fractions, as determined by SDS-PAGE analysis, were pooled and dialysed overnight in Tris buffer. This protein mixture was loaded onto a Hi-trap heparin column (Pharmacia) and eluted using a linear NaCl gradient (0–0.4 M); the FhlA protein eluted at approximately 0.3 M NaCl. FhlA-containing fractions were pooled and dialysed overnight in HEPES buffer (30 mM HEPES, pH 7.0 with 0.5 mM EDTA; 1 mM benzamidine and 1 mM DTT). The FhlA protein was further purified using a 1 ml High S column (cation exchange, Bio-Rad) equilibrated with HEPES buffer and eluted with a linear gradient of 0–0.5 M NaCl. The FhlA protein eluted at approximately 0.25 M NaCl. Fractions containing FhlA protein were pooled and dialysed overnight in HEPES buffer. The final purification step required chromatography on a 1 ml High Q column (anion exchange, Bio-Rad) equilibrated with HEPES buffer and elution with a linear gradient of sodium citrate (0–0.3 M). The pure FhlA protein, as judged by SDS-PAGE analysis, was dialysed in storage buffer (25 mM HEPES, pH 7.0; 0.8 mM
DTT; 10% glycerol). FhIA protein was quickly frozen in liquid nitrogen and stored at −70 °C, in aliquots.

**Enzyme assay.** FHL activities [expressed as nmol (mg cell protein)−1 min−1] of late-exponential phase cultures were determined as described by Lee et al. (1985). All experiments were carried out with whole cells to minimize QO inactivation of the enzyme. β-Galactosidase activities [expressed as nmol (mg cell protein)−1 min−1] were determined in late-exponential- to early-stationary-phase cultures as described by Rosenthal et al. (1995).

**ATPase activity.** ATPase activity associated with the FhIA protein was determined as described by Korsa & Böck (1997), and expressed as pmol ATP hydrolysed (pmol FhIA)−1.

**DNA electrophoretic mobility-shift experiments.** DNA mobility-shift experiments were performed essentially as described by Grunden et al. (1996). Briefly, DNA containing the byc−hyc intergenic region was PCR-amplified from plasmid pGHH100, using two primers: 5′-CCGGATCCGTTATTTCCGAGCATATC-3′ and 5′-CCCTGCGATTTAAGCTAAAGATGAA-3′. This amplified DNA fragment, after digestion with PsI and BamHI, was cloned into plasmid pUC19, also digested with PsI and BamHI. The resulting plasmid, pWS40, was the source of a 154 bp PsI–BamHI fragment used in the electrophoretic mobility-shift experiments. The 154 bp DNA fragment, after isolation from a 10–30% sucrose gradient, was labelled with [32P]dNTPs by filling in the recessed 3′ end of the BamHI site with the DNA polymerase Klenow fragment. FhIA or FhIA165 protein was pre-incubated in binding buffer (10 mM Tris, pH 7.5; 1 mM EDTA; 1 mM DTT; 1 mM ATP) for 5 min at 4 °C before the labelled DNA was added. Binding reaction mixtures (total volume 20 µl) were incubated for 20 min at 37 °C. Polyacrylamide gels (5%) were pre-run (100 V, 60 min, room temperature), with circulation of TBE buffer (Tris, 89 mM, pH 8.3; borate, 89 mM; EDTA, 2.5 mM). Samples were loaded and the gels were run for a further 60 min (100 V), in TBE buffer. Buffer circulation re-started after the samples had been loaded. The gels were then dried and exposed to pre-flashed X-ray film. The band intensity was determined using a phosphorimager (Molecular Dynamics).

**Materials.** Biochemicals were purchased from Sigma. Other organic and inorganic chemicals were from Fisher Scientific and were of analytical grade. Restriction endonucleases and DNA-modifying enzymes were purchased from Promega and New England Biolabs.

**RESULTS AND DISCUSSION**

**Isolation of deletions within the N-terminal domain of FhIA**

A number of deletion derivatives of fhlA which allowed the production of dihydroxybenzaldehyde by an fhlA mutant strain, *E. coli* SE1174 (fhlA : Tn10) were isolated. After analysis of the size of the fhlA gene in these plasmids and screening for the ability to support FHL production, four fhlA mutations with large deletions within the unique N-terminal domain of FhIA were selected for further analysis. Based on DNA sequence data, all four alleles (fhlA164, ∆26–370, L371V; fhlA165, ∆5–374; fhlA166, ∆36–314; fhlA167, Δ1–117) carried large deletions in the unique N-terminal domain of FhIA. Although fhlA164 and fhlA166 supported production of FHL activity in a fhlA mutant, the level of byc–lac expression in the presence of these two alleles was only about 25% of the values obtained with native fhlA [approximately 350 units β-galactosidase activity, compared to approximately 1800 units activity for the strain with native FhIA]. The alleles fhlA165 and fhlA167, which supported the highest level of β-galactosidase production (Fig. 1), were studied in detail.

**FhIA165 and FhIA167 are formate- and molybdate-independent**

In the presence of native FhIA protein (pWS2), the optimum level of byc–lac expression required both
molybdate and formate (Table 2). Most notably, in the mod pfl double mutant, strain WS118(pWS2), hyc–lac expression was very low (140 units β-galactosidase activity) in the absence of molybdate and formate. The addition of formate to the growth medium increased the level of hyc–lac expression to approximately 50% of the maximal level (840 units); supplementing the medium with molybdate alone increased the level of expression by approximately threefold (470 units). A level of hyc–lac expression equivalent to that of wild-type E. coli was attained only when both formate and molybdate were present in the growth medium of the mod pfl double mutant.

In contrast, activation of hyc–lac expression by FhlA165 and FhlA167 was no longer dependent on formate and molybdate: this was demonstrated by the high level of β-galactosidase activity present in all three strains carrying pWS165 or pWS167 (Table 2). Although both deletion derivatives led to molybdenum–formate-independence, only FhlA165 supported a higher level of hyc–lac expression than the wild-type (4000 and 2000 units β-galactosidase activity, respectively; Table 2). The higher than expected level of expression in the presence of FhlA165 suggests that the N-terminal half of the FhlA protein negatively regulates the activation of the hyc operon by the native FhlA protein, even in the presence of the effectors formate and molybdate. Removal of the N-terminal half of FhlA165 allowed the C-terminal DNA-binding segment to activate the hyc operon maximally; this was in agreement with a previous report by Leonhartsberger et al. (2000).

The FhlA167 mutant carried a deletion of amino acids 1–117, while the deletion in FhlA165 was of amino acids 5–374. These results show that removal of the first 117 aa is sufficient to produce an effector-independent transcriptional activator, but that it is not sufficient to overcome the inhibitory effect of the N-terminal domain of FhlA on the transcription of the hyc operon by the C-terminal domain. The critical structure responsible for modulating the activity of the C-terminal DNA-binding domain appears to reside between amino acids 118 and 374.

The product of the first gene of the hyc operon, hycA, appears to act by negatively modulating the level of hyc operon expression (Sauter et al., 1992). The nature of this apparent repression is yet to be determined. Even in the presence of FhlA165 protein as activator, hyc–lac expression was reduced by HycA by the same level as molybdate.

**FhlA165 and FhlA167 activate transcription of P_{hyc} lacZ in a modE moeA double mutant**

Both ModE and MoeA proteins are required for the molybdate-dependent transcription of the hyc operon (Hasona et al., 1998b). ModE was shown to be a secondary transcriptional activator and directly interacts with hyc promoter (P_{hyc}) DNA (Self et al., 1999). The role of MoeA in this regulation is not known but it has been proposed that the FhlA protein binds the catalytic product of MoeA (activated molybdenum; Hasona et al., 1998a) and modulates its activity (Self & Shanmugam, 2000). Since FhlA165 and FhlA167 activated transcription of hyc–lacZ even in the absence of molybdate, the need for ModE and MoeA proteins for the expression of hyc–lac with these deletion derivatives was determined.

**A modE moeA double mutant containing the native FhlA protein, strain WS198(pWS2), produced a measurable, but low, level of β-galactosidase activity (approximately 180 units) when cultured in L-broth containing glucose (Table 3); the addition of molybdate to the medium had no effect on hyc–lac expression. In contrast, the same double mutant containing the FhlA165 protein, E. coli strain WS198(pWS165), produced an approximately 15-fold more β-galactosidase activity (2600 units): this level of activity is also approximately 2.2-fold higher than the activity of a modE strain containing the native FhlA, strain WS127(pWS2). The FhlA167 protein also overcame the requirement of ModE and MoeA proteins for hyc operon expression, and the level of expression of hyc–lac was similar to that of the modE strain with native FhlA (approximately 1500 units). As expected, addition of molybdate had no significant effect on the expression of the hyc operon when FhlA165 or FhlA167 was used as the activator. Although hyc–lac expression in the modE moeA mutant was higher in the presence of FhlA165 or FhlA167, the level of β-galactosidase activity produced was only approximately**

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**Table 3. Expression of P_{hyc} lacZ by FhlA165 and FhlA167 requires ModE-Mo**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>WS127(pWS2)</td>
<td>mod*</td>
<td>1200</td>
</tr>
<tr>
<td>WS182(pWS2)</td>
<td>ΔmodE (fhlA*)</td>
<td>800</td>
</tr>
<tr>
<td>WS179(pWS2)</td>
<td>moeA113(fhlA*)</td>
<td>1400</td>
</tr>
<tr>
<td>WS198(pWS2)</td>
<td>ΔmodE moeA113(fhlA*)</td>
<td>180</td>
</tr>
<tr>
<td>WS127(pWS165)</td>
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<td>3500</td>
</tr>
<tr>
<td>WS182(pWS165)</td>
<td>ΔmodE (fhlA165)</td>
<td>2000</td>
</tr>
<tr>
<td>WS179(pWS165)</td>
<td>moeA113(fhlA165)</td>
<td>3500</td>
</tr>
<tr>
<td>WS198(pWS165)</td>
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<td>2600</td>
</tr>
<tr>
<td>WS127(pWS167)</td>
<td>mod*</td>
<td>2000</td>
</tr>
<tr>
<td>WS182(pWS167)</td>
<td>ΔmodE (fhlA167)</td>
<td>1250</td>
</tr>
<tr>
<td>WS179(pWS167)</td>
<td>moeA113(fhlA167)</td>
<td>2500</td>
</tr>
<tr>
<td>WS198(pWS167)</td>
<td>ΔmodE moeA113(fhlA167)</td>
<td>1600</td>
</tr>
</tbody>
</table>

* All strains carry P_{hyc} lacZ via λWS1 and a deletion of the srl–fhlA region.
65–75% of the value obtained with the mod+ strain with these mutated forms of the FhlA proteins [WS127(pWS165); WS127(pWS167)]. This lower than expected value was apparently a consequence of the absence of ModE protein, a required secondary activator, since a mutation in modE alone accounted for this difference. In the presence of either FhlA165 or FhlA167 the moeA mutation had no effect on byc–lac expression. These results show that the deletions in FhlA165 and FhlA167 overcame the need for the MoeA-catalysed product for byc operon expression, but not that for ModE-Mo, a required secondary activator of the byc operon.

In a separate study, expression of byc–lac was found to be limited by the concentration of FhlA in the cell when the protein was produced from a chromosomal copy of fhlA (J. A. Maupin & K. T. Shanmugam, unpublished data). Under the present experimental conditions, the amount of β-galactosidase activity produced by a culture carrying hyclacZ (byc–lac) and a chromosomal copy of fhlA+ was approximately 600 units. This value did not change significantly when the fhlA+ gene was introduced from a plasmid (pWS3; vector pBR322) in a pcnB mutant background which limits the copy-number of the plasmid (Lopilato et al., 1986). In a pcnB+ strain, the level of β-galactosidase activity produced by a culture carrying plasmid pWS2 (vector pACYC184), which contains fhlA+, was approximately 1300 units, and in the presence of plasmid pWS3 (vector pBR322) the level of β-galactosidase activity produced by byc–lac increased further to approximately 3000 units. This observed copy-number effect is not related to the proposed antagonistic effect of HycA on byc–lac expression (Sauter et al., 1992), as the strain used in these experiments carries a deletion of the entire srl–fhlA region. In the experiments described above (Tables 2 and 3), the fhlA gene was present in a pBR322-based plasmid and it is possible that the observed independence from MoeA could be a result of overexpression of the fhlA mutant allele. However, it should be noted that in a modE background, the moeA mutation severely limited byc–lac expression even in the presence of multiple copies of the fhlA gene. To rule out the effect of multiple copies of fhlA, byc–lac expression was investigated in a pcnB mutant background with the fhlA165 allele.

When the overall level of byc–lac expression was reduced to approximately 600–700 units β-galactosidase activity (in a pcnB mutant, strain WS244) in the presence of native FhlA, the requirement for ModE-Mo and MoeA for byc–lac expression could be readily detected. In the presence of FhlA165, the modE mutation reduced the level of byc–lac expression by about 33%. Even in a modE moeA double mutant, the level of byc–lac expression catalysed by FhlA165 was only reduced by about 30% compared to the corresponding isogenic moeA mutant, strain WS246. However, with FhlA165, a moeA mutation alone had a positive effect on byc–lac expression (1.4-fold higher than with the native FhlA protein) in this pcnB mutant, in contrast to a negative effect of the moeA mutation (27% lower) in the presence of FhlA+. This positive effect of MoeA in the presence of the FhlA165 protein could be a result of a large pool of ModE-Mo in the cell, since molybdate is not processed in the moeA mutant combined with the MoeA-independence of the FhlA165 protein. The large pool of Mod-E-Mo could increase the level of expression of byc–lac in the presence of FhlA165, but could not overcome the MoeA requirement for optimal activation by native FhlA. These results further demonstrate that transcription of the byc operon by the N-terminal deletion derivative of FhlA is independent of the MoeA protein.

**Activation of Pbyc-lacZ by FhlA165 and FhlA167 is O2- and nitrate-independent**

Böck et al. previously proposed that the lack of byc operon expression during conditions of aerobic (O2) or anaerobic respiration (nitrate) was due to a lack of sufficient levels of formate to interact with FhlA (Rossmann et al., 1991). The formate-independence of byc–lacZ expression in the presence of FhlA165 and FhlA167 should lead to the production of significant β-galactosidase activity even under aerobic growth conditions.

Strain WS127(pWS2) with wild-type fhlA+ did not produce β-galactosidase activity when grown under aerobic growth conditions (Table 4). Addition of formate to the medium increased the level of β-galactosidase activity only to approximately 200 units and this represents 21% of the anaerobic level (Table 4). Exogenously added formate appears to be unable to support byc expression to an optimum level when the cells are cultured under aerobic conditions. Even in the presence of O2, Pbyc-lacZ was activated by FhlA165 protein to a level higher than that of the wild-type strain grown anaerobically in L-broth containing glucose (1600 and 1200 units β-galactosidase activity, respectively). However, the level of β-galactosidase activity produced by WS127(pWS165) was still only about 50% of the same strain grown under anaerobic conditions. Including formate in the aerobic growth medium did not increase the level of Pbyc-lacZ expression in the presence of FhlA165. Since FhlA165 is formate-independent for the transcription of the byc operon, this O2 effect could be due to a reduction in the expression of the fhlA gene. Previous work from our laboratory showed that fhlA–lacZ expression under aerobic growth conditions is only approximately 15% of the values obtained with anaerobic cultures (J. A. Maupin & K. T. Shanmugam, unpublished data). Recently, the oxyS small RNA species was shown to play a role in the regulation of fhlA, and this could account for O2-dependent control of the fhlA and fhlA165 genes (Altuvia et al., 1998) which indirectly controls the level of FHL in the cell. However, transducing an oxyS deletion mutation into strain WS127 did not affect the level of expression of byc–lac or fhlA–lac in the presence of O2 (data not shown). There appears to be an as yet unidentified O2-
Table 4. Effect of O2 and nitrate on the FhlA165- and FhlA167-mediated expression of P_{hyc}lacZ

Cultures were grown in the following media: LB, L-broth; LBF, L-broth with sodium formate (30 mM); LBG, L-broth with glucose (0.3%); LBGN, L-broth with glucose (0.3%) and nitrate (30 mM); LBGFN, L-broth with glucose (0.3%), sodium formate (30 mM) and nitrate (30 mM). Unless indicated (+O2), cultures were grown under anaerobic conditions. β-Galactosidase activity is expressed as nmol (mg cell protein)^{-1} min^{-1}.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype*</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td>LB</td>
</tr>
<tr>
<td>WS127(pWS2)</td>
<td>p/fhlA+</td>
<td>1100</td>
</tr>
<tr>
<td>WS127(pWS165)</td>
<td>p/fhlA165</td>
<td>3000</td>
</tr>
<tr>
<td>WS127(pWS167)</td>
<td>p/fhlA167</td>
<td>1400</td>
</tr>
</tbody>
</table>

ND, not determined.
*All strains carry P_{hyc}lacZ and a deletion of the srl–fhlA region.

Fig. 2. Alignment of the N-terminal region of the FhlA protein with ATPases from various E. coli ABC transport systems. Amino acids 7–37 of the FhlA N-terminal region are shown on the top line. Below are proteins which all act as energizer proteins (cytoplasmatic ATPases) in ABC-type transport systems within E. coli. The bottom line represents a consensus sequence derived from analysis of all nine proteins. References for each of the proteins are as follows: FhlA (Maupin & Shanmugam, 1990; Schlenseg & Böck, 1990); PotA (Kashiwagi et al., 1993); ModF (Grunden et al., 1996); MalK (Shuman & Silhavy, 1981); XylG (Rosenfeld et al., 1984); LviG (Adams et al., 1990); HisP (Ames & Lever, 1970); ModC (Maupin-Furlow et al., 1995); PotG (Pistocchi et al., 1993).

A sequence in the N-terminal region of FhlA is similar to a conserved region in ATPases of ABC transporters

As the first 117 amino acids of FhlA had been shown to be responsible for effector-mediated control of the hyc operon, this region of the FhlA sequence was compared with the sequences of other known E. coli proteins. This comparison revealed significant similarity between the N-terminal amino acid sequence of FhlA and sequences found in the energizer proteins of ABC transporters (Fig. 2). The C-terminal end of the presented ABC protein sequences corresponds to the ‘Walker’ B site, and the N-terminal end represents the unique ABC signature sequence (Linton & Higgins, 1998). Upstream of this

dependent control of the hyc operon in E. coli. Although both the native FhlA and FhlA165 are produced to the same level in the cell, it is possible that there is a difference in the affinity of the two proteins for the P_{hyc} which may account for the difference in the level of expression of hyc–lac in the aerobic cell.

As formate is also an electron donor for nitrate reduction, the presence of nitrate in vivo is expected to decrease the formate pool and consequently reduce the expression of the hyc operon. As expected, hyc–lacZ was expressed at a very low level in the presence of nitrate when native FhlA served as the activator (Table 4). Supplementing the medium with formate restored hyc–lacZ expression. However, with FhlA165 as the activator, nitrate had no detectable effect on the expression of the hyc operon, supporting the model that formate is not a required effector for FhlA165.

It is interesting to note that although FhlA167 supported effector-independent expression of hyc–lac when the cells were grown anaerobically, in an aerobic cell this allele supported the production of only approximately 200 units β-galactosidase activity (approximately 15% of the anaerobic level). The addition of formate to the medium had a minimal effect on FhlA167-mediated hyc expression in the aerobic cell: this is in agreement with its formate independence. Addition of nitrate to the anaerobic culture also reduced the hyc–lac expression in the presence of FhlA167, but the nitrate effect was minimal compared to that of O2. This difference in the response of FhlA165 and FhlA167 to terminal electron acceptors suggests that the amino acids between 117 and 374 play a significant role in sensing the redox properties of the environment. Alternatively, the affinity of the two proteins for the P_{hyc} may be different.
ATPase-like sequence resides a helical domain which interacts with the membrane component of the ABC transport system (Hunke et al., 2000). Although the critical aspartate and proline amino acids, present in all “Walker” B sites of the ABC proteins and the unique ABC signature sequence, were not present in the FlhA protein 18 of the 31 amino acids within this sequence (between amino acids 7 and 37) were similar or identical to a derived consensus sequence of the ABC proteins (Fig. 2). The observed sequence similarity between the FlhA and ABC-ATPases raised interesting questions about the role of this N-terminal region of the FlhA protein. Does this region of FlhA along with the central domain of the protein contribute to formate-dependent ATP hydrolysis? However, it should be noted that the N-terminal domain of FlhA by itself has no detectable ATPase activity (Leonhartsberger et al., 2000).

To evaluate the significance of the N-terminal region of the FlhA protein, the DNA sequence corresponding to the amino acids 7–37 was deleted from the 

flA9 gene, and the resulting 

flA9-2 allele was used in 

hyc expression studies (Fig. 1). The 

flA9-2 allele failed to support 

hyc expression or production of FHL activity, even when the medium was supplemented with formate at levels as high as 30 mM and/or 1 mM molybdate. These results show that this segment of the FlhA protein is critical for 

in vivo activity of the FlhA protein. It is possible that the FlhA9-2 protein is still formate-dependent for its activity and the inability to produce a FlhA9-2–formate complex 

in vivo apparently negated its activity. Removal of an additional 80 amino acids (to amino acid 117) completely reversed this defect, as FlhA167 is formate-independent (Fig. 1, Table 2).

Production of active FHL is independent of formate in the presence of FlhA165

Since transcription of the 

hyc operon was at its highest in the presence of FlhA165, the need for formate in the conversion of apo-FDH-H to active FDH-H was determined. Strain SE2007, which carries a mutation in the chromosomal 

flA gene, produced approximately 125 units FHL activity with plasmid pWS165. This value was slightly higher than the 110 units FHL activity produced by strain SE2007(pWS2) with wild-type 

flA. However in a 

flA/pfl double mutant, only the strain carrying 

flA65 produced FHL activity (approximately 90 units), again confirming the formate-independence for 

hyc and 

fdhF expression in the presence of FlhA165. These results further demonstrate that formate is only required for transcriptional activation of 

fdhF and 

hyc, and not for maturation and activation of the apoproteins to activate the FHL.

FlhA165 has a higher affinity for hyc DNA

The higher level of 

hyc–lac expression when activated by FlhA165 (Table 2) prompted investigation into the affinity of FlhA165 protein for the target DNA. Wild-type FlhA (250 nM) shifted the electrophoretic mobility of the P

hyc DNA and completely shifted the DNA at a concentration of 1 µM FlhA (Fig. 3). In contrast, the FlhA165 protein shifted the mobility of the DNA at a concentration of 100 nM and completely shifted the DNA at 500 nM FlhA165, suggesting that FlhA165 had a higher affinity for the target DNA sequence. Based on phosphorimager analysis of the DNA electrophoretic mobility shift presented in Fig. 3, the apparent Kd for the binding of FlhA165 to DNA was calculated to be 125 nM, while the value for the native FlhA protein was 270 nM. In similar mobility shift experiments, including formate in the binding reaction had no effect on the apparent Kd of the interaction between DNA and protein (data not shown). The higher affinity of FlhA165 for the P

hyc DNA could explain the higher level of 

hyc expression seen in the presence of the FlhA165 protein (Tables 2 and 4), compared to the level of expression when activated by native FlhA. However, it could not be ruled out that FlhA165 had an increased ability to activate transcription.

FlhA167 had a very low affinity for P

hyc DNA (Fig. 3D), although this allele activated 

hyc–lac to the same level as the native protein, 

in vivo. The addition of formate to the binding reaction or to the electrophoresis conditions had no effect on the FlhA167–P

hyc DNA interaction. In contrast, the FlhA9-2 allele, which did not support 

hyc operon expression, did bind to the P

hyc DNA 

in vitro and shifted the electrophoretic mobility of the P

hyc DNA (Fig. 3C). This change in the electrophoretic mobility of the P

hyc DNA saturated at a protein concentration of approximately 100 nM FlhA9-2, and at this concentration only a small fraction of the total
DNA was protein-bound. At a protein concentration of 150 nM, most of the DNA was found in larger DNA–protein complexes.

**ATPase activity of FhlA mutant derivatives**

Members of the NtrC/NifA family of proteins including FhlA have a promoter DNA-dependent ATPase activity (Weiss et al., 1991; Hopper & Böck, 1995). This activity is associated with the formation of the DNA-open complex, a prelude to the initiation of transcription (Wedel & Kustu, 1995). Since the FhlA protein alleles had differing binding characteristics, the \( P_{hyc} \)-dependent ATPase activity of the various FhlA alleles was determined.

Purified native FhlA protein had a basal ATPase activity of 250 units (Fig. 4). This activity increased to 320 units in the presence of formate, and the optimum concentration of formate was between 10 and 25 mM. At 50 mM formate, the ATPase activity of FhlA protein decreased. In the presence of \( P_{hyc} \) DNA the ATPase activity of the FhlA protein increased by over 200 pmol ATP hydrolysed (pmol FhlA)\(^{-1}\) (approximately 25 mM formate present) (Fig. 4a). This characteristic formate- and DNA-dependent increase in ATPase activity of the FhlA protein is similar to that reported by Hopper & Böck (1995).

The basal ATPase activity of the FhlA165 protein was only 60 pmol ATP hydrolysed (pmol FhlA)\(^{-1}\) (Fig. 4), and as expected this activity was not increased by formate. In the presence of \( P_{hyc} \) DNA, the level of ATPase activity also increased by approximately 200 pmol ATP hydrolysed (pmol FhlA)\(^{-1}\). In contrast to the native protein, which responded positively to lower concentrations of formate, the FhlA165 reacted negatively to formate in the ATPase assay. The negative response to formate seen *in vitro* could also account for the formate-dependent decrease in \( hyc-lacZ \) activation in the *mod* mutant, strain WS113(pWS165) (Table 2). The similar response of ATPase activity of the native FhlA and FhlA165 proteins to DNA suggested that these proteins, once bound to the \( hyc \) upstream DNA, activated transcription at the same level, and the observed difference in the \( hyc \) operon expression could be attributed to the differences in the affinity of the proteins to the \( P_{hyc} \) DNA.

In these experiments the native FhlA protein had a higher than expected basal ATPase activity. This is in contrast to the ATPase activity of FhlA protein reported by Hopper & Böck (1995). This difference could be due to variations in the protein purification methods employed in these two studies. The absence of formate-dependent ATPase activity in FhlA165 suggested that the deletion of the unique N-terminal domain of FhlA removed a previously unknown ATPase activity, which may be specific for formate binding.

The FhlA167 protein had an ATPase activity of approximately 300 units which was not altered either by formate or by \( P_{hyc} \) DNA. This is in agreement with its lower affinity to \( P_{hyc} \) DNA (Fig. 4b). The FhlA9-2 protein also produced approximately 300 pmol ATP hydrolysed (pmol FhlA)\(^{-1}\) ATPase activity which was not influenced by the presence of formate. However, the ATPase activity of this protein increased by more than threefold in the presence of \( P_{hyc} \) DNA. Addition of both \( P_{hyc} \) DNA and formate to the reaction further increased the ATPase activity of the FhlA9-2 protein by twofold. In the presence of 10 mM formate and \( P_{hyc} \) DNA the ATPase activity was over 1500 pmol ATP hydrolysed (pmol FhlA)\(^{-1}\), the highest among the four FhlA alleles tested.

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**Fig. 4.** ATPase activity of native FhlA protein and mutant forms of the FhlA protein in the presence of formate and \( P_{hyc} \) DNA. ATPase activities are given in relative units [pmol ATP hydrolysed (pmol FhlA)\(^{-1}\)]. DNA (plasmid pFGH100) was added to give a final concentration of 0.1 μM. (a) Native FhlA protein and FhlA165. ○, FhlA (final concn 1 μM) and DNA; □, FhlA165 (final concn 2 μM) and DNA; ▲, FhlA165 only (final concn 2 μM). (b) FhlA9-2 and FhlA167. ●, FhlA9-2 (final concn 0.13 μM) and DNA; ○, FhlA9-2 only (final concn 0.13 μM); ■, FhlA167 (final concn 0.13 μM) and DNA; □, FhlA167 only (final concn 0.13 μM).
Increasing the formate concentration beyond 10 mM inhibited the DNA-dependent ATPase activity. The ability of FhlA9-2 to bind to \( P_{hyc} \) DNA in vitro and to hydrolyse ATP in a DNA-dependent manner shows that the FhlA9-2 protein is active in vitro. The formate-dependent increase in ATPase activity of this allele is similar to that of the native FhlA protein and is in contrast to that of FhlA165 and FhlA167. These results suggest that the FhlA9-2 protein requires formate for activity and the FhlA9-2-formate complex is not formed in vivo because of the deletion of the amino acids 7–37. The inability of the FhlA9-2 protein to activate the expression of the \( hyc \) operon in vivo combined with the higher than expected activity in vitro raises the possibility that the \( fhlA9-2 \) is not transcribed in vivo. To test this, the upstream region of the \( fhlA \) gene in both pWS9 and pWS9-2 was sequenced. The two sequences were identical for approximately 500 bases upstream of the translation start site. In a separate experiment, total RNA was isolated from strains carrying either pWS9-2 or its parent pWS9 grown under anaerobic conditions in L-broth containing glucose (0.3%). A cDNA corresponding to the \( fhlA \) gene was synthesized from both samples using Moloney Murine Leukaemia virus reverse transcriptase (Superscript II; Life Technologies). The cDNA was quantitated by real-time PCR using SYBR green (iCycler; Bio-Rad). The results of these experiments showed that the level of cDNA produced from the total RNA from strains with plasmid pWS9 or pWS9-2 was comparable, indicating that the \( fhlA9-2 \) allele was also transcribed in \( E. coli \). The inability to activate the \( hyc \) operon is apparently due to the deletion of amino acids in the protein.

**CONCLUSION**

Results from this study show that removal of the first 117 amino acids from the FhlA protein (FhlA167) leads to effector-independence for expression of the \( hyc \) operon. Extending the deletion to amino acid 374 (FhlA165) increases the ability of the protein to bind \( P_{hyc} \) DNA in vitro, enhances the level of \( hyc \) operon expression in vitro, and also overcomes the redox-dependent control of \( hyc \) operon expression. The amino acids between 117 and 374 could play a significant role in modulating the level of \( hyc \) operon transcription via the central ATP-binding domain and the C-terminal domain (amino acids 375–692 of the FhlA protein). Although the need for MoeA for expression of the \( hyc \) operon is overcome by either of the two deletion derivatives FhlA165 and FhlA167, ModE-Mo is still required as a secondary activator for the optimum expression of the \( hyc \) operon. These results further support the proposal that the MoeA-catalytic product interacts with the native FhlA protein for optimum expression of the \( hyc \) operon. Removing the sequence of amino acids with similarity to the sequences found in the ATPase components of ABC-type transport systems eliminates the in vivo activity, but not the formate and \( P_{hyc} \) DNA dependent in vitro ATPase activity. This suggests that in vivo the FhlA9-2 protein is unable to produce a formate-bound complex. The FhlA protein could accept formate directly from the transporter, and the formate-dependent ATPase activity may signify this interaction. Direct transfer of formate to the FhlA protein would also minimize formate accumulation in the cytoplasm. The amino acids 7–37 may play a role in transferring formate from the transporter to the appropriate location in the FhlA protein. The sequence similarity with the ABC-ATPases, combined with the absence of formate-dependent ATPase activity in FhlA165 and FhlA167, suggests that the native FhlA protein contains two ATPase activities: one associated with DNA-binding and transcription activation and the other related to the interaction with formate.

**ACKNOWLEDGEMENTS**

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