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# H-NS-Dependent Regulation of Flagellar Synthesis Is Mediated by a LysR Family Protein

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**H-NS regulates the flagellar master operon (*flhDC*) and thus is necessary for flagellation of *Escherichia coli*. However, the molecular mechanism of its regulation has remained unknown. Genetic screening of a transposon insertion abolishing the H-NS effect revealed a previously unidentified gene, named *hdfR*, encoding a LysR family protein. Binding of purified HdfR to the *flhDC* promoter was demonstrated by a DNA mobility shift assay, indicating that HdfR is a transcriptional regulator for the flagellar master operon. Furthermore, the expression of the *hdfR* gene was shown to be negatively regulated by H-NS.**

The *Escherichia coli* flagellar system consists of more than 40 genes whose products are required for flagellar assembly, function, and sensory signaling (12). Expressions of the genes are regulated in a cascade mode. At the top of the hierarchy is the *flhDC* operon, encoding the FlhD and FlhC proteins, which are essential for expression of downstream flagellar genes (11). Flagellar expression is affected by various environmental conditions (20), perhaps involving at least some transcriptional regulators. In most cases, flagellar expression is modulated at the transcriptional level of the *flhDC* operon. The cyclic AMP (cAMP) receptor protein-cAMP complex and the OmpR protein are known to affect the expression of the *flhDC* operon by binding to its promoter region (21, 23).

H-NS, a nucleoid protein (7, 25), affects the expression of many unrelated genes, including *proVWX*, *bgl* (9), *appY* (1), and *fimB* (6) of *E. coli* or *Salmonella enterica* serovar Typhimurium, and also affects expression of some virulence genes of *Salmonella* serovar Typhimurium and *Shigella* spp. (8, 16, 13). The majority of affected genes are negatively regulated by H-NS, although some, including the flagellar regulon, are positively regulated. It has been reported that H-NS-deficient cells are nonflagellated because of reduced transcription of *flhDC* (4). Although it was assumed that H-NS positively affects *flhDC* transcription, its regulation has not been clearly demonstrated. In an assay of in vitro transcription of the *flhDC* operon (23), purified H-NS did not enhance the transcription. Thus, it was suspected that the regulation of *flhDC* by H-NS might be indirect.

In this study, we isolated an insertion enabling cells to enhance *flhDC* expression even in the absence of H-NS. The insertion was found in a gene, named *hdfR*, encoding a LysR family protein, which has a helix-turn-helix DNA-binding motif. HdfR binds to the promoter region of the *flhDC* operon, and its expression was negatively regulated by H-NS, suggesting that the apparent activation of *flhDC* transcription by H-NS is mediated through the negative regulator HdfR.

**A transposon insertion abolishing the repression of *flhDC-lacZ* due to an H-NS defect.** Genetic screening employing random transposon insertion was performed to search for a putative mediator involved in the H-NS-dependent regulation of *flhDC*. As a tool to monitor *flhDC* expression, the *flhDC-lacZ* protein fusion contained in bacteriophage λSS10 (21) was used. The lower level of LacZ activity of the protein fusion made it possible to screen for a clone with a distinguishable phenotype on an indicator plate, which was impractical with the transcriptional fusion used later. A pool of random transposon insertions obtained from CP807 (*E. coli* K-12 Δ*lacZ thr leu his met*) (21) infected with phage λ::Tn*phoA132* (*tet*) (26) was transferred to MS368 (MC4100 *flhD*$^+$ λSS10 *hns*::*neo*) (27) by P1. Among the derivatives of MS368 containing random insertions, a clone showing derepression of *flhDC-lacZ* even in the absence of H-NS was selected on indicator agar plates (Luria-Bertani agar containing 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal]/ml) and exhibited a consistent increase in LacZ activity (14) when transferred back to MS368.

To monitor the transcriptional activities of the *flhDC* operon, the insertion was transferred to strains MS358 (MC4100 *flhD*$^+$ λMS205) and MS359 (MS358 *hns*::*neo*) lysogenized with λMS205, containing the *flhDC-lacZ* transcriptional fusion. The λMS205 phage was constructed by subcloning the *flhDC* region including −544 to +593 from the transcriptional start (23) into pRS415 (promoterless *lacZYA*; *bla*), which was double recombined into λRS45 (*'lacZAY bla'*) using *lacZAY* and *bla* homologies (22). A strain with a single prophage, confirmed by PCR (17), was used to monitor the transcriptional activities of the *flhDC-lacZ* fusion. As shown in Fig. 1, the LacZ activity of MS372 containing the transposon insertion, later designated *hdfR*::Tn*phoA132*, was higher than that of the parent strain and was not affected by an *hns*::*neo* mutation. This indicates that the transposon insertion abolished the *hns* effect on *flhDC* transcription.

**The insertion was found in a novel gene named *hdfR*.** The site of the transposon insertion was identified by an inverse PCR. After digestion of chromosomal DNA with *Sau*3A1, PCR amplification was performed for the ligated DNA with a pair of outwardly directed primers complementary to the regions in the transposon. The DNA amplified was sequenced to search for homology in the *E. coli* genome database. The result revealed an insertion site in the putative gene *yifA* at 84 min (Fig. 2). In the *E. coli* genome database for strain MG1655, *yifA* is located next to another gene, *pssR*, named under the presumption that it locates in the same region where *pssR1* was mapped (24). The *pssR1* strain was isolated as a mutant exhibiting an elevated expression of *pssA*, encoding the phosphatidylserine synthase.

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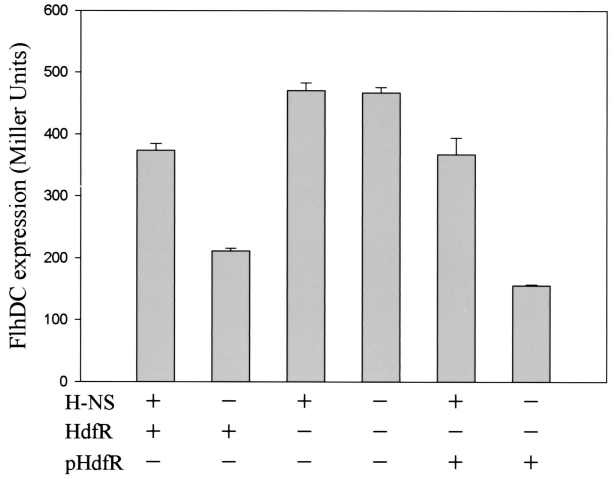


FIG. 1. Effects of *hns* and *hdfR* mutations on the transcription of the *flhDC* operon. β-Galactosidase activities expressed from the strains containing the *flhDC-lacZ* transcriptional fusion in λMS205 were measured in cells grown in TB medium (1% tryptone, 0.25% NaCl) at 35°C to an optical density at 600 nm of 0.4 to 0.5. Strains used were MS358 (λMS205 *hns*$^+$ *hdfR*$^+$), MS359 (MS358 *hns*), MS372 (MS358 *hdfR*), MS373 (MS358 *hns hdfR*), MS372/pMS272ΔH (*hdfR*/pHdfR), and MS373/pMS272ΔH (*hns hdfR*/pHdfR). The activities of β-galactosidase are presented in Miller units (14), with standard deviations (error bars) estimated from three independent samples.

The GenBank report for *pssR* (accession no. AAC77484) predicts an open reading frame (ORF) encoding a protein of 133 amino acids which contains a helix-turn-helix DNA-binding motif found in the LysR family proteins. However, the PssR protein was unusually small compared to other LysR family proteins of about 300 amino acids (18). Moreover, *pssR* is overlapped (157 bp) with the neighboring *yifA* gene. Thus, we suspected that there might be a sequencing error around that region. When we sequenced the junction between *pssR* and *yifA*, we found an additional G just after the 80th codon of *pssR* in our strains derived from W3110 or MC4100. Accordingly, the *pssR* and *yifA* genes merged into a single ORF of 279 amino acids, which was named *hdfR*, for *hns*-dependent *flhDC* regulator. The predicted size of the protein matches well with the band on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (data not shown). In this protein, the helix-turn-helix motif is found in the region between residues 17 and 47. The site of transposon insertion was found immediately after amino acid residue 168 (Fig. 2). Recently, GenBank filed an *hdfR* homologue (STMD1.99 [accession no. AF233324]) in *Salmonella* serovar Typhimurium LT2, encoding a 282-amino-acid protein (82% identical to the *E. coli* HdfR).

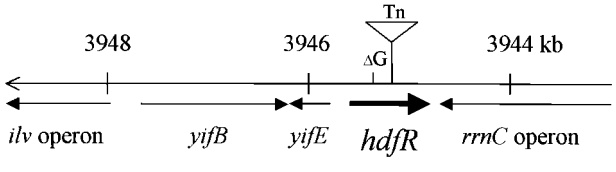


FIG. 2. Chromosomal location of the *hdfR* gene. The *hdfR* gene was previously divided into two ORFs, *pssR* and *yifA*, by *E. coli* genome sequencing for strain MG1655. Numbers indicate nucleotide positions, in kilobases. Arrows indicate directions of the ORFs. The nucleotide and deduced amino acid sequences of the *hdfR* gene have been submitted to GenBank (accession no. AF25103). ΔG indicates the location of the missing guanine nucleotide in the reported sequence (accession no. AE000453). The insertion site of Tn*phoA132* (Tn) is also indicated.

In order to confirm that the insertion effect is solely due to the disruption of the *hdfR* gene, a complementation experiment was conducted with the *hdfR*-containing plasmid. The DNA fragment containing *hdfR* originated from the Kohara library (10), in which the gene is expressed by its own promoter. Introduction of the plasmid into strains containing *hdfR*::Tn*phoA* restored the normal level of *flhDC-lacZ* transcription (Fig. 1, pHdfR), although the plasmid expression of HdfR exhibits a copy effect, as seen in the rightmost bar of Fig. 1.

**Flagellar regulation by *hdfR* seems independent of membrane phospholipid.** As described previously, *hdfR* was found in a region where *pssR*, which is involved in the expression of *pssA*, was mapped. On the other hand, it was reported that the *pssA* and *psd* genes, encoding enzymes for the synthesis of phosphatidylethanolamine (PE), are required for the expression of the *flhDC* operon (19). Thus, one might suspect that flagellar regulation and phospholipid synthesis might be associated at either the genetic or the physiological level.

In order to test whether *hdfR* is allelic to *pssR*, whose mutation increases the expression of *pssA*, we examined the effect of *hdfR* insertion on the expression of the *pssA* gene using a P*pssA-lacZ* fusion on pMS330. The plasmid contained the promoter fragment, including 440 bp upstream to 174 bp downstream from the translation initiation site of *pssA*. The β-galactosidase activities of pMS330 in MS296 (MC4100 *flhD*$^+$), MS299 (MS296 *hns*::*neo*), MS377 (MS296 *hdfR*::Tn*phoA132*), and MS380 (MS296 *hns*::*neo hdfR*::Tn*phoA132*) were similar within the ranges between 11,300 and 14,300 Miller units (14). This result suggests that the *hdfR* gene may not regulate *pssA* and thus differs from *pssR*. We also measured the proportions of PE among the total cellular phospholipids from the wild type (MS296) and *hns* mutant (MS299) strains using thin-layer chromatography (15). The two strains contained similar ratios of PE: 71.2% for MS296 and 72.4% for MS299. This implies that the *hns* effect on the transcription of *flhDC* is not due to a PE depletion.

**Purified HdfR binds to the promoter region of *flhDC*.** In order to test the possibility of HdfR serving as a transcriptional regulator, a gel shift assay was performed with purified HdfR for the promoter region of the *flhDC* operon, including −626 to +185 from the transcription start. HdfR with a C-terminal His tag (HdfR-$His_6$) was expressed under the control of the T7 promoter from the pET-HdfR plasmid derived from pET-21b (Novagen). After induction with isopropyl-β-D-thiogalactopyranoside (IPTG) in the BL21(DE3) strain, the amount of the HdfR-$His_6$ protein was estimated to be about 10% of the total soluble fraction that appeared on a Coomassie-stained SDS-polyacrylamide gel. For purification of HdfR with an additional 22 amino acids including the His tag at the C terminus, a His-bind resin (Novagen) was loaded with an addition of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (Sigma) to the binding buffer at a concentration of 0.25% because HdfR-$His_6$ bound much better under mildly denaturing conditions than under native conditions. The purified protein exhibits a size of about 35 kDa with more than 98% purity on an SDS-polyacrylamide gel. The binding of purified HdfR-$His_6$ on the DNA fragment shifted mobility on gel electrophoresis (Fig. 3), suggesting that the protein functions as a transcriptional regulator for the *flhDC* operon.

**Expression of the *hdfR* gene is negatively regulated by H-NS.** The results so far suggest a possibility that HdfR is a mediator for H-NS-dependent regulation of the *flhDC* operon. The next question would be how *hdfR* is regulated by H-NS. We directly examined the expression of *hdfR* by H-NS by subcloning the fragment of *hdfR* containing its promoter region (flanked by

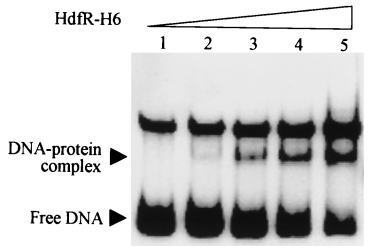


FIG. 3. Gel mobility shift of the DNA fragment containing the *flhDC* promoter by purified HdfR-$His_6$. The probe DNA corresponding to the region from −626 to +185 bases from the transcription start was obtained by PCR and labeled with [$\gamma$-$^{32}$P]ATP using polynucleotide kinase (Boehringer Mannheim). For a 20-μl reaction volume, 1 ng of DNA probe (about 3,000 cpm), 2 μg of sheared salmon sperm DNA, and 0 to 100 ng of purified proteins were used. The buffer contained 50 mM KCl, 20 mM Tris-Cl (pH 7.9), 1 mM dithiothreitol, 10% glycerol, and 125 μg of bovine serum albumin/ml. The whole mixture was stored on ice for 30 min and loaded onto a 5% low-ionic-strength polyacrylamide gel (2). The DNA species are appropriately marked. The unlabeled upper band appears to be a denatured form of the probe generated during the purification step. Amounts of purified HdfR-$His_6$ proteins used are none (lane 1), 10 ng (lane 2), 20 ng (lane 3), 50 ng (lane 4), and 100 ng (lane 5).

*Sau3*AI) to *lacZ* (preceded by a *Bam*HI site) in pMC1396 (5). The resulting plasmid (pMS274) carries a *lacZ* translational fusion, whose β-galactosidase activity was increased about twofold by a deletion of *hns* (*hns*::*neo*), from 1,022.3 ± 5.6 Miller units for the wild type (MS296/pMS274) to 2,181.1 ± 20.1 for its *hns*::*neo* drivative (MS299/pMS274). This result indicates that H-NS negatively modulates the expression of *hdfR*, although we still cannot exclude the possibility of indirect interaction between H-NS and the promoter of *hdfR*.

We describe here a new LysR family protein, HdfR, which is involved in the H-NS-dependent regulation of the flagellar master operon by binding to the upstream region of the operon. The expression of *hdfR* was negatively controlled by H-NS, which explains the negative effect on flagellation of an H-NS mutation. When H-NS is inactive, the expression of *hdfR* gene is increased, resulting in an overproduction of HdfR, which will reduce the transcription of *flhDC* by binding to its upstream region. In a previous report (23), the observation of *flhDC* activation in vivo by H-NS was not correlated with an in vitro transcription assay. Our model involving HdfR provides an alternative to the previous conjecture that H-NS serves as a direct activator for *flhDC*. Our model is more consistent with the fact that the modes of H-NS regulation in most cases were characterized as transcriptional silencers. It would be interesting in the near future to reveal the physiological signal to which HdfR is responding. The global nature of transcriptional regulation by the LysR family protein may also apply to the regulation involving HdfR. Therefore, a future study of HdfR is likely to uncover a novel global network comprising the flagellar regulon.

**Nucleotide sequence accession number.** The nucleotide and deduced amino acid sequences of the *hdfR* gene have been submitted to GenBank under accession no. AF25103.

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