Regulation of ndh expression in Escherichia coli by Fis

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The Escherichia coli ndh gene encodes NADH dehydrogenase II, a primary dehydrogenase used during aerobic and nitrate respiration. The anaerobic transcription factor FNR represses ndh expression by binding at two sites centred at −94.5 and −50.5. In vivo transcription studies using promoter fusions with 5′ deletions confirmed that both FNR sites are required for maximum repression under anaerobic conditions. The histone-like protein Fis binds to three sites [centred at −123 (Fis I), −72, (Fis II) and +51 (Fis III)] in the ndh promoter. Using ndh::lacZ promoter fusions carrying 5′ deletions, or replacement mutations it is shown that Fis III is a repressing site and that Fis I and II are activating sites, with the greatest contribution from Fis II. Deletion of the C-terminal domain of the RNA polymerase α-subunit abolished Fis-mediated activation of ndh expression, suggesting that ndh has a Class I Fis-activated promoter. In accordance with the established pattern of Fis synthesis, ndh transcription was greatest during exponential growth. Thus, it is suggested that Fis enhances ndh expression during periods of rapid growth, by acting as a Class I activator, and that the binding of tandem FNR dimers represses ndh expression by preventing interaction of the RNA polymerase α-subunit with DNA and Fis.

INTRODUCTION

Escherichia coli is a facultative anaerobe with two genetically distinct NADH dehydrogenases that serve as primary dehydrogenases in the aerobic respiratory chain (Calhoun & Gennis, 1993). The nuoA-N operon encodes NADH dehydrogenase I (NdhI), a membrane-associated, multi-subunit, proton-translocating enzyme, similar to Complex I of eukaryotic mitochondria (Weidner et al., 1992). The ndh gene encodes NADH dehydrogenase II (NdhII) which is a membrane-bound but non-proton-translocating monomeric flavoprotein (Young et al., 1981; Matsushita et al., 1987). During glucose-limited aerobic growth, significant electron flow is directed through both enzymes (Calhoun et al., 1993). During aerobic and nitrate respiration, NdhII is the preferred NADH dehydrogenase, whereas NdhI is used during fumarate respiration (Tran et al., 1997). Because the affinity of NdhII for NADH is relatively low (Hayashi et al., 1989) it has been suggested that NdhII may operate to regulate the NADH pool independently of energy generation and is thus likely to be important when the capacity of the bacteria to generate energy exceeds demand (Calhoun et al., 1993; Green & Guest, 1994; Neijssel & Teixeira de Mattos, 1994).

Both NADH dehydrogenases are controlled at the transcriptional level by complex regulatory networks. Expression of the nuoA-N operon responds to oxygen and nitrate availability via the two-component sensor–regulators ArcB-A (anaerobic repression) and NarX-L (nitrate activation) and to C4-dicarboxylates via an uncharacterized regulator acting at a far upstream site between −277 and −899 (Bongaerts et al., 1995). The ndh gene is subject to anaerobic repression (Spiro et al., 1989) by the direct interaction of the oxygen-responsive transcription factor FNR with two sites in the ndh promoter, FNR I and FNR II (Fig. 1; Green & Guest, 1994; Meng et al., 1997). Both FNR sites are required for full repression of ndh expression (Meng et al., 1997). As well as being subject to FNR-mediated repression, the ndh promoter responds to growth phase and it has been suggested that this response is regulated by the growth-phase-responsive transcription factor Fis (Green et al., 1996). The ndh promoter region has three Fis-binding sites (Fig. 1; Green et al., 1996) and because of the relative affinities of Fis for each site it was suggested that binding to the high-affinity Fis I site might mediate activation, whereas progressive occupation of the lower affinity sites might lead to repression (Fig. 1; Green et al., 1996). Here we show that Fis bound at the high-affinity Fis I site has a small positive effect on ndh expression, but that a greater contribution is made by Fis occupying the lower affinity Fis II site. The location of the Fis II site (centred 72 bp upstream of the transcript start) and the observation that Fis-mediated activation of ndh expression is dependent on the C-terminal domain of the RNA polymerase α-subunit, indicates that the ndh promoter is a Class I Fis-activated promoter. Furthermore, it is confirmed that both FNR sites contribute to FNR-mediated repression under anaerobic conditions.
et al (Lodge using standard techniques. The low-copy-number vector pRW50 the RK5279 were used as the recipients for P1-mediated transduction of uncertain, as indicated by the question marks.

Guest, 1994; Meng et al FNR II) in the promoter region (Sharrocks et al under anaerobic conditions by binding at two sites (FNR I and

The transcription factor FNR is known to repress

Table 1. Bacterial strains and plasmids. The bacterial strains and plasmids used along with their relevant characteristics are listed in Table 1. Escherichia coli RK4353 (lac) and the isogenic fnr derivative RK5279 were used as the recipients for P1-mediated transduction of the fis::kan mutation to create JRG4656 and JRG4203, respectively, using standard techniques. The low-copy-number vector pRW50 (Lodge et al, 1992), which contains a promoterless lacZ gene, was used to create a series of ndh::lacZ fusions with specific deletions (pGS1756–pGS1762) and replacements (pGS1529, pGS1530, pGS1541) in the ndh promoter region. The rpoA expression plasmid, pGS1209, and the corresponding plasmid containing a subgene encoding RpoA lacking the C-terminal domain were derivatives of ptac85 (Marshall et al, 2001).

Growth conditions and assay of β-galactosidase. The rich growth medium was tryptone (10 g l⁻¹), yeast extract (5 g l⁻¹), NaCl (5 g l⁻¹) containing 0·2 % (w/v) glucose and antibiotics when required: tetracycline (35 mg l⁻¹); kanamycin (25 mg l⁻¹); ampicillin (200 mg l⁻¹). The minimal glucose medium was M9 (Sambrook & Russell, 2001) supplemented with arginine (30 mg l⁻¹). Cultures were grown from colonies on agar plates at 37 °C, either aerobically in conical flasks (5 ml in a 250 ml flask, shaking at 250 r.p.m.) or anaerobically in sealed bottles (7 ml without shaking). Except where indicated, cultures were grown for 16 h before measuring β-galactosidase activities according to Miller (1972) using at least three independent cultures. To determine whether the different genetic backgrounds altered the copy number of the lacZ reporter plasmids the method of Taylor & Brose (1988) was used. It was shown that plasmid copy number was not significantly affected.

Plasmid construction and mutagenesis. The ndh promoter fragments used were amplified by PCR from pGS41B (Sharrocks et al, 1991) using the EXPAND Hi-fidelity system (Roche). After restriction digestion at unique EcoRI and BamHI sites introduced into the PCR primers, the products were ligated into EcoRI/BamHI-digested pRW50 (Lodge et al, 1992). Overlap PCR using specific mutagenic primers was used to replace the Fis sites with unrelated DNA sequences (see below). The authenticity of the constructs was confirmed by DNA sequencing.

RESULTS

Deletion analysis of the ndh promoter

The starting point for this work was the observation that Fis specifically interacts with three regions of the ndh promoter (Fig. 1; Green et al, 1996). Moreover, from the patterns of

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>JRG4203</td>
<td>RK5279 fis; KanR</td>
<td>This work</td>
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<tr>
<td>JRG4656</td>
<td>RK4353 fis; KanR</td>
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<td>RJ1802</td>
<td>fis767; KanR</td>
<td>Johnson et al. (1988)</td>
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<td>RK4353</td>
<td>Δ(argF-lac)U69</td>
<td>V. Stewart†</td>
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<tr>
<td>RK5279</td>
<td>Δ(argF-lac)U69 furl-250</td>
<td>V. Stewart†</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td>pRW50</td>
<td>A low-copy-number vector for lac-based promoter fusions; TetR</td>
<td>Lodge et al. (1992)</td>
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<tr>
<td>pGS1209</td>
<td>A ptac85-based rpoA expression plasmid; AmpR</td>
<td>Marshall et al. (2001)</td>
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<tr>
<td>pGS1374</td>
<td>A ptac85-based rpoA256 expression plasmid: encodes an RNA polymerase α subunit lacking the C-terminal domain; AmpR</td>
<td>Marshall et al. (2001)</td>
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<tr>
<td>pGS1529</td>
<td>As pGS1533, but the Fis I site is replaced by an unrelated sequence (see text)</td>
<td>This work</td>
</tr>
<tr>
<td>pGS1530</td>
<td>As pGS1533, but the Fis II site is replaced by an unrelated sequence (see text)</td>
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<tr>
<td>pGS1533</td>
<td>A pRW50 derivative containing a 422 bp EcoRI–BamHI ndh promoter fragment</td>
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<tr>
<td>pGS1541</td>
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<tr>
<td>pGS1756-1762</td>
<td>A series of ndh::lacZ promoter fusions in pRW50 in which upstream DNA is progressively deleted as shown in Fig. 2</td>
<td>This work</td>
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*AmpR, Ampicillin resistance; KanR, kanamycin resistance; TetR, tetracycline resistance.
†University of California, Davis, USA.
Fis and ndh expression during the E. coli growth cycle it was suggested that ndh expression is activated when the intracellular concentration of Fis is low (late exponential/stationary phase) and repressed when the intracellular concentration of Fis is high (early exponential phase) (Green et al., 1996). Based on relative binding affinities, it was suggested that occupation of the high-affinity Fis site centred at −123 (Fis I; Fig. 1) might enhance ndh expression, whereas occupation of the lower affinity sites (Fis II and III; Fig. 1) might reduce ndh expression (Green et al., 1996). To test this hypothesis a series of ndh::lacZ gene fusions (N1–N7) were created in which ever larger sections of the ndh promoter were deleted such that the upstream Fis I, FNR I, Fis II and FNR II sites were sequentially removed (Fig. 2a). Cultures of E. coli RK4353 (lac) carrying the indicated ndh::lacZ fusions on the low-copy-number plasmid pRW50 (Lodge et al., 1992) were grown in L-broth under anaerobic conditions and promoter activity was estimated by measuring β-galactosidase activities.

Previous site-directed mutagenesis of the FNR sites within the ndh promoter indicated that both were required for maximal repression (Meng et al., 1997). Accordingly, removal of the section of the ndh promoter containing the upstream FNR I site (fusion N5) partially relieved anaerobic repression in RK4353 and repression was further relieved by the removal of the FNR II site (fusion N7; Fig. 2b). Thus, the promoter deletion analysis supports the conclusion that both FNR sites contribute to the anaerobic repression of ndh expression.

To investigate the role of Fis in the absence of FNR-mediated repression, β-galactosidase activities from the ndh::lacZ fusions in anaerobic cultures of RK5279 (fnr lac) were measured. The data revealed that deletion of the far upstream Fis I site (fusion N4) reduced ndh expression, suggesting that Fis bound at this site enhances ndh expression (Fig. 2c). Deletion of the Fis II site (fusion N6) reduced ndh transcription still further, suggesting that Fis also acts positively at this site (Fig. 2c).

To show that Fis was responsible for the effects observed, β-galactosidase activities were measured in a fis lac mutant strain (JRG4656) and in an fnr fis lac mutant strain (JRG4203). These investigations confirmed that under anaerobic conditions both FNR sites contribute to FNR-mediated repression (Fig. 2d). Moreover, in the absence of Fis and FNR, deletion of the Fis and FNR sites did not significantly alter ndh expression, showing that, as expected, deletion of the upstream Fis-binding sites has no effect on ndh expression in a fis background (Fig. 2e).

The experiments described above suggested that Fis bound at two upstream sites within the ndh promoter activates ndh expression under anaerobic conditions. The ndh gene is normally expressed under aerobic conditions when FNR is inactive. Therefore, the effects of the ndh promoter deletions were tested under aerobic conditions. To ensure that the bacteria contained no residual active FNR protein, aerobic cultures of RK5279 (fnr lac) and JRG4203 (fnr fis lac) were used. For RK5279 the data show that deletion of the far upstream Fis I site (fusion N4) reduced ndh expression, which was further reduced by deletion of the Fis II site (Fig. 3a). In the absence of Fis, deletion of the Fis sites did not affect ndh expression, which was ~5-fold lower than the levels observed in the presence of Fis (Fig. 3b). Comparison of expression in the fis fnr double mutant in the absence (Fig. 2e) and presence (Fig. 3b) of oxygen suggests that at least one other regulatory protein modulates ndh expression. Previous work indicated that IHF, HU and an uncharacterized protein, Arr, interact with ndh promoter DNA (Green et al., 1997). Thus, one or more of these transcription factors could be responsible for the threefold-lower levels of ndh expression observed under aerobic conditions in the absence of Fis. In conclusion, the experiments

![Image of diagram](http://mic.sgmjournals.org)
under aerobic conditions confirmed that Fis acts as a positive regulator of \textit{ndh} expression by interaction with two sites located upstream of the basic promoter elements.

**Site-directed mutagenesis of Fis sites in the \textit{ndh} promoter**

The experiments using stationary-phase cultures described above established that Fis affects \textit{ndh} expression and that these effects correlate with the presence of the previously identified upstream Fis sites. Because Fis is more abundant in growing bacteria (Ishihama, 1999), any effect of Fis is likely to be more apparent in exponential-phase cultures. Therefore, exponential-phase cultures were used to assign specific roles to the Fis sites in the \textit{ndh} promoter (Fig. 1). Site-directed mutagenesis was used to generate three \textit{ndh} promoter variants: \textit{P}_{\text{ndh}}^{\text{retained Fis II and Fis III sites}}, but the Fis I site was altered by replacing 26 bp (−137 to −112, AATTGCTCAATAATAACCAATAA) with an unrelated sequence (GTCGGCGGGTGCTGTGGGC-TGGA); \textit{P}_{\text{ndh}}^{\text{Fis I and Fis III sites, but the Fis II site was altered by replacing 16 bp (−76 to −62, TATCTTTTGCAACA}) by an unrelated sequence (GCGGGTGCTGTGGGC); \textit{P}_{\text{ndh}}^{\text{Fis I and Fis II sites but the Fis III site was altered by replacing 25 bp (+39 to +63, AGCTATTGTAATAACCATTAAT- TAA) by an unrelated sequence (TGCAGCGGCTGCTGTGGGC-GTGGGGCTGGC). Initial experiments indicated that mutation of the low-affinity Fis III site (Fig. 1) appeared to slightly enhance \textit{ndh} expression in aerobic cultures of RK4353 (2138±183 Miller units) compared to the unaltered \textit{ndh}::\textit{lacZ} fusion (1790±115 Miller units). Thus, as expected from the low affinity of this site for Fis and its location relative to the transcript start, the Fis III site had only a small negative effect on \textit{ndh} transcription in vivo and attention was therefore focused on the two upstream Fis sites. Exponential-phase cultures of strains carrying the unaltered \textit{ndh} promoter (Fig. 4) exhibited higher levels of expression than the equivalent stationary-phase cultures (Figs 2 and 3), consistent with the known enhanced levels of Fis in growing cultures. The altered \textit{ndh}::\textit{lacZ} fusions in the parental strain grown under anaerobic conditions in rich medium showed that impairment of the Fis I site caused a small reduction in expression, whereas impairment of the Fis II site caused a greater reduction (Fig. 4). As expected, in the absence of FNR, expression from all the promoters was derepressed, but the pattern of relative expression was similar to that of the parental strain, with impairment of Fis II yielding the greatest reduction in expression (Fig. 4). In the absence of Fis, the transcriptional activities for all the promoter fusions tested were similar, indicating that the mutagenesis of the Fis sites had not altered the basal activity of the promoter. Moreover, the activities of \textit{P}_{\text{ndh}}^{++} in both \textit{fis} and \textit{fnr fis} strains were similar to the corresponding parental strains, indicating that impairment of Fis II affected \textit{ndh} expression to the same extent as a \textit{fis} lesion. Similar patterns of expression were observed for equivalent cultures grown in minimal medium (not shown). Thus, it was concluded that Fis acts as a positive regulator of \textit{ndh} expression mainly by interacting with the Fis II site. It is unlikely that Fis acts as an antirepressor by preventing FNR from fulfilling its role as a repressor of \textit{ndh} expression, because altering the Fis II site lowers \textit{ndh} expression even in a \textit{fnr} mutant. In accord with the observations made under anaerobic conditions, replacement of the Fis I site in aerobic cultures had only a small effect on \textit{ndh} expression, but replacement of Fis II had a marked effect on transcription of \textit{ndh} (Fig. 4). In the absence of Fis, expression of the unaltered \textit{ndh}
Regulation of *ndh* expression by Fis

Fis is a Class I activator of *ndh* expression

Many transcription factors act by forming direct contacts with RNA polymerase to assist in transcription initiation. Regulated promoters have been classified according to the location of their activator-binding sites (Busby & Ebright, 1994, 1999). At Class II promoters the activator-binding site is located further upstream. The Class I promoter architecture allows RNA polymerase to establish only one protein:protein contact, and this is between the activator and the C-terminal domain of the α-subunit of RNA polymerase. Therefore, expression of a gene encoding an α-subunit lacking the C terminus should diminish *ndh* expression to the levels observed in a fis mutant because the activating contact cannot be made. To maintain bacterial viability, unaltered RNA polymerase has to be available in the cell. Thus, a chromosomal copy of wild-type *rpoA* is retained in the strains used in these experiments and the altered *rpoA* gene is introduced on a multicopy plasmid. This causes a proportion of the RNA polymerase to be assembled with the altered α-subunit in place of the wild-type. Therefore, *fur* and *fis* mutants carrying *ndh*:*lacZ* fusion N1 (pGS1756) were transformed with either pGS1209 (carrying a wild-type *rpoA* gene) or pGS1374 (carrying an *rpoA* gene lacking the coding region for the C-terminal domain). Expression of *ndh*:*lacZ* was then measured in three independent anaerobic cultures grown in rich medium plus glucose. Expression of *ndh*:*lacZ* expression in the *fur* mutant transformed with pGS1374 was ~2-fold lower (318 ± 17 Miller units) than that observed with the same strain containing pGS1209 (601 ± 24 Miller units). Expression of *ndh*:*lacZ* expression in the *fis* double mutant transformed with pGS1374 was similar (265 ± 27 Miller units) to that observed with the same strain containing pGS1209 (269 ± 9 Miller units). Thus, in the presence of the mutant *rpoA* gene, or in the absence of *fis*, *ndh* expression was lowered by a similar amount, supporting the conclusion that Fis acts as a Class I activator of *ndh* expression.

DISCUSSION

The experiments described here lead to three conclusions. First, Fis stimulates *ndh* expression under both aerobic and anaerobic growth conditions. Second, this activation is dependent upon Fis acting as a Class I activator by binding at a site (Fis II) located between the two FNR sites. Third, under anaerobic conditions both FNR sites within the *ndh* promoter contribute to FNR-mediated repression.

Fis is a multifunctional nucleoid-associated protein and is the most abundant transcriptional activator in exponential-phase cells (Ishihama, 1999). Fis is a homodimeric protein that binds to DNA through a C-terminal helix–turn–helix motif (Osuna et al., 1991). Because the DNA recognition helices of the Fis dimer are unusually close, a severe bend is induced in the DNA upon Fis binding (Pan et al., 1996). The bending of target DNA is thought to be important for the function of Fis. To our knowledge this is only the second report showing that Fis can act as a Class I transcription activator, the other example being at *rrn* P1 promoters (Aiyar et al., 2002). Class I activators bind to promoter DNA upstream of the −10 and −35 elements. The binding sites are located on the same face of the helix as RNA polymerase. Thus, Class I regulators may be located at, or close to −61,
−71, −82 bp, etc., relative to the transcript start (Busby & Ebright, 1994). At the ndh promoter the major Fis effect is mediated from a site 72 bp upstream of the transcript start. At the rrnB promoter Fis stimulates transcription from a site centred at −71.5 (Aiyar et al., 2002). Specific protein : protein contacts are established between the C-terminal domain of the α-subunit of RNA polymerase and a small surface-exposed patch of Fis consisting of residues Q68, R71, G72 and Q74 (Aiyar et al., 2002). These interactions involve only one of the α-C-terminal domains and the downstream subunit of the Fis dimer (Aiyar et al., 2002). The location of the Fis II-binding site, and the observation that introduction of an RNA polymerase α-subunit lacking the C-terminal domain abolishes Fis-mediated activation, suggests that stimulation of ndh expression by Fis is mediated by identical protein : protein contacts to those established at the rrn promoters.

Investigation of the roles of the Fis I and Fis III sites revealed that the Fis I site has a small positive effect on ndh expression, whereas Fis III site had a small negative effect. Therefore, we are now able to suggest the effects of Fis acting at each of the three binding sites within the ndh promoter. Thus, the question marks in Fig. 1 can be replaced to indicate positive regulation from Fis sites I and II and negative regulation from site III.

Expression of nuoA-N, encoding the proton-translocating NADH dehydrogenase, is also positively regulated by Fis (Wackwitz et al., 1999). It has been suggested that this ensures high ATP yields at the outset of growth (Wackwitz et al., 1999). This suggestion is consistent with the presence of a low-affinity repressing Fis site (Fis III) at the ndh promoter, which should ensure that Ndh I is used in preference to Ndh II when intracellular Fis levels are highest. As growth proceeds into exponential phase, Fis levels begin to fall. This will release the brake applied to ndh expression by Fis bound at Fis III, allowing Fis-mediated activation of ndh, and also lower Fis-mediated activation of nuoA-N expression. Such a scheme would be consistent with the relative affinities of Fis (estimated from DNase I footprinting reactions) for the nuo promoter (Fis 1, ~20 nM; Fis 2, ~40 nM; Fis 3, ~100 nM; Wackwitz et al., 1999), which are lower than those for the ndh promoter (Fis I, ~0.1 nM; Fis II, ~1 nM; Fis III, ~10 nM; Green et al., 1996), and provides a possible explanation for the presence of Fis III in the ndh promoter.

Previous work used site-directed mutagenesis to show that both FNR sites within the ndh promoter are necessary for full anaerobic repression of ndh expression (Meng et al., 1997). The data obtained here from deletion analysis confirms that this is the case. Recent work to investigate regulation of yfdD gene expression, and studies with a series of model promoters with tandem FNR sites, suggested that appropriately spaced tandem FNR dimers act together to repress transcription (Marshall et al., 2001; Barnard et al., 2003). Thus, although an FNR dimer located at −50 : 5 would be expected to interfere with the docking of the z-subunit of RNA polymerase with DNA and thus partially occlude the ndh promoter, the binding of a second upstream FNR dimer would appear to enhance repression, perhaps by stabilizing the FNR : DNA complex, allowing it to effectively compete with the RNA polymerase α-C-terminal domains for this region of the promoter. An α-C-terminal domain that is not bound to either DNA or to a transcriptional activator is thought to inhibit transcription. Moreover, it has been shown here that Fis bound at the Fis II site is a positive regulator of ndh expression. Therefore, by occupying sites located immediately upstream and downstream of the Fis II site, FNR acts as a physical barrier, preventing the formation of the Fis : RNA polymerase contacts necessary for Fis-mediated transcription activation. Thus, it is likely that interactions between the tandem FNR dimers conspire to inhibit ndh transcription at several levels, i.e. by preventing interaction between the α-C-terminal domains of polymerase with DNA and with Fis. This promoter architecture must also prevent the formation of contacts between FNR and RNA polymerase that result in activation of transcription.

In conclusion, it is shown that ndh expression is driven from a Class I Fis-activated promoter and that Fis-mediated activation is inhibited under anaerobic conditions by FNR binding to two sites immediately flanking the region occupied by Fis. Activation of ndh transcription by Fis could account for the enhanced level of ndh expression during periods of rapid growth. This could be necessary to recycle NADH and achieve redox balance independently of energy generation during periods of rapid growth.

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REFERENCES


