A Promoter Region Binding Protein and DNA Gyrase Regulate Anaerobic Transcription of nifLA in Enterobacter cloacae

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Our work provides evidence that a sequence characteristic of FNR binding sites, when interacted with by a trans-acting factor, activates anaerobic transcription of the nifLA operon in Enterobacter cloacae. DNA gyrase activity has been found to be important for the anaerobic transcription of the nifLA promoter. Our results suggest that anaerobic regulation of the nifLA operon is mediated through the control of the promoter region-binding trans-acting factor at the transcriptional level, while DNA supercoiling functions in providing a topological requirement for the activation of transcription.

In the nitrogen-fixing (nif) enteric bacteria Klebsiella pneumoniae and Enterobacter cloacae, the nif genes are regulated by the activity of the ntrC, ntrA, nifA, and nifL genes at the transcriptional level (2, 3, 4, 24). nifL and nifA constitute an operon which is regulated by the product of ntrC or autoregulated by the product of nifA, NifA (5, 9). NifA acts as a positive regulator in conjunction with NtrA to activate nif genes (19), while the product of nifL, NifL, acts as a repressor of nif genes under oxygen or in an excess of fixed nitrogen (13).

Our previous investigations showed that the nifLA promoter is highly sensitive to oxygen and that NifA is inactivated by NifL under oxygen (5, 15, 23). Accordingly, we hypothesized that nif regulation by oxygen is mediated at two different levels. First, at the transcriptional level, the oxygen sensitivity of the nifLA promoter ensures that the nifLA promoter and, hence, all other nif promoters are repressed by oxygen; second, at the posttranslational level, NifL interacts with NifA in the presence of oxygen. As the result of a shortage of active NifA, the expression of other nif genes is blocked.

Direct interaction of NifL and NifA has been demonstrated by the two-hybrid system test (16, 23). As to the question of aerial regulation of the nifLA promoter, most investigators have claimed that neither FNR nor the oxrC gene product is involved in the expression of nifLA in response to oxygen (14).

Since the activity of the nifLA promoter is proposed as a mechanism for aerobic regulation of the nifLA promoter, we also proved that DNA gyrase is truly important for anaerobic transcription of the nifLA promoter. A mechanism for aerobic regulation of the nifLA promoter is proposed.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this work are listed in Table 1.

DNA manipulations. Preparation of plasmids DNA, endonuclease digestion, ligation, and transformation were carried out essentially as described by Sambrook et al. (21).

Construction of nifLA promoter mutants. Initially, the E. cloacae nifLA promoter (Fig. 1) was cloned in vector M13mp19. The resultant clone is referred to as M13mp19-nifLAp. The M13mp19-nifLAp DNA was digested with BglI and HpaI, filled in with Klengow polymerase, and ligated to yield M13mp19-nifLAp-D, where the FNR site consensus sequence upstream of the nifLA promoter was deleted. Promoter mutant clones M13mp19-nifLAp-m1 and M13mp19-nifLAp-m2, with a mutation in the FNR site consensus sequence, were constructed by oligonucleotide-mediated mutagenesis as described by Sambrook et al. using a 0.5-kb BamHI-To-HindIII fragment of the nifLA promoter region carried in M13mp19-nifLAp-D as a template. Two 26-mer mutant primers (5'-ACAGGGCTTAAATCGATCCAGAT-3') complementary to bases 63 to 38 with the four most conserved bases mismatched with respect to the FNR consensus was synthesized and used to construct M13mp19-nifLAp-m1. Another 24-mer mutant primer (5'-ACAGGGCTTAAATCGATCCAGAT-3') complementary to bases 62 to 39 with one most conserved base mismatched was synthesized and used to construct M13mp19-nifLAp-m2. The 26-mer primer and the 24-mer primer create a unique restriction ApaI or ClaI endonucleases site sequence in each case. It can be used to screen the desired mutants.

Promoter mutant clone pUC18-nifLAp-mc, with alteration of base pairs outside the FNR site consensus, was constructed by PCR (1). The first-round PCR product was generated with mutant primer 5'-GGGGGGCGCGCGTGACGAAACTCTGTGGCCG3', which complements bases 99 to 128 upstream of the nifLA transcription start site, and primer 5'-AGCGGTAACAATTTCACAGGA3', which complements the other side of M13. M13mp19-nifLAp double-stranded DNA was used as the template in both PCR rounds. The final PCR product containing the mutations was then cloned in vector pUC18 to give rise to pUC18-nifLAp-mc.

All of the mutants obtained as described above were rescreened by DNA sequencing, nifLAp-lacZ translational fusions pWP296, pWP-D926, pAP296, pCL926, and pST926, respectively, were constructed by digesting M13mp19-nifLAp, M13mp19-nifLAp-D, and M13mp19-nifLAp-m2 with BamHI and HindIII. The resultant 0.5-kb fragments, each of which contains the nifLA promoter region, were then cloned into the same restricted plasmid, pG926.

Assay of β-galactosidase. Bacteria were grown aerobically for 24 h at 28°C in nitrogen-free minimal medium supplemented with 0.01% casein hydrolysate, 1 mg of vitamin B12 per liter, 20 mg of glutamine per liter, and appropriate antibiotics (5). Cells were pelleted, washed, and resuspended in the same medium and then grown under aerobic or anaerobic (flushing with nitrogen) conditions for 8 h. β-Galactosidase activity was assayed as described by Miller (17).

DNA binding assay. Binding of protein to the FNR site consensus sequence was monitored by measuring the reduction in the electrophoretic mobility of the labeled probe DNA fragments as described by Fried and Crothers (11). In a 20-μl total sample volume, 3.5 pmol of an [α-32P]dATP-labeled double-stranded probe was incubated with 14 μg of protein extract containing 3 μg of calf DNA for 25 min at 25°C. A DNA fragment with the sequence 5'-TATATGGCCTCG AAATACCGATCGATCAGTTAAGCGGCTGGT3', followed by the nifLA promoter containing the FNR binding site consensus sequence, and a DNA fragment with the sequence 5'-TATATGGCCTCG AAATACCGATCGATCAGTTAAGCGGCTGGT3', followed by the nifLA promoter with the FNR binding site consensus sequence mutated, were synthesized as probes. Protein-DNA binding was performed in 30 mM sodium phosphate buffer pH 7.5 (250 mM NaCl, 2 mM DTT, 0.02% NaN3).
complexes were separated by 12% polyacrylamide gel electrophoresis and then autoradiographed.

RESULTS AND DISCUSSION

A sequence upstream of the nifLA promoter and its role in the activity of the nifLA operon. As reported previously for E. cloacae E26, there are three cis-acting elements residing in the region upstream of the nifLA operon: the $\sigma^{54}$-RNA polymerase recognition site at -24 to -12, the NtrC binding site at -171 to -135, and the NifA binding site at positions +44 to +59 from the transcription start site (TSS) (5). Through analysis of the DNA sequence of the nifLA promoter, we found a sequence, CCGAT-ATCAA, at positions -69 to -48 from the TSS (Fig. 1) which is characteristic of the sequence of an FNR binding site (10, 22). In order to know the role of this defined sequence in the activity of the nifLA operon under anaerobic conditions, we constructed a promoter mutant with a 48-bp BglII-to-HpaI fragment including the consensus FNR binding site upstream of the TSS deleted and cloned it into plasmid pGD926 to form a nifLAp-lacZ translational fusion. After it was introduced into E. cloacae E26, or Escherichia coli YMC9, the $\beta$-galactosidase activity of the fusion was measured. As shown in Table 2, deletion of the consensus FNR site produced low activity under anaerobic conditions, whereas mutation at sites outside of this sequence did not affect the anaerobic expression of the fusion (Table 2). These results substantiate the evidence that the consensus FNR site upstream of the nifLA promoter is important for regulation of the nifLA operon.

A trans-acting factor binds to the defined sequence upstream of the nifLA promoter. To test if there is a trans-acting factor bound to the defined sequence upstream of the nifLA promoter which enhances the anaerobic expression of the nifLA operon, a gel mobility shift assay was conducted. The Table 2 show that either deletion or alteration of the sequence of the consensus FNR site produced low activity under anaerobic conditions, whereas mutation at sites outside of this sequence did not affect the anaerobic expression of the fusion (Table 2). These results substantiate the evidence that the consensus FNR site upstream of the nifLA promoter is important for regulation of the nifLA operon.
TABLE 2. Expression of E. cloacae nifLA::p-lacZ fusions in E. cloacae and E. coli strains

<table>
<thead>
<tr>
<th>Strain (plasmid)</th>
<th>Genotype of relevant host (plasmid)</th>
<th>β-Galactosidase activity</th>
</tr>
</thead>
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<tr>
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<td>Air</td>
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<td>E26(pGD926)</td>
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<tr>
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</table>

a β-Galactosidase assays were performed as described in Materials and Methods, and values are means of results from at least three independent measurements.

b Constitutively expressed.

fragments encompassing the consensus FNR site or its variants were incubated with the soluble protein extracts from E. cloacae or E. coli and assayed for DNA-protein complex formation. As shown in Fig. 2, a slower-migrating complex was detected following incubation of the probe containing the FNR site consensus sequence with the cell extracts. In contrast, no complex was detected following incubation of the probe containing the mutated consensus sequence of the FNR site with the cell extracts. We thus concluded that a trans-acting factor binding to the FNR site consensus sequence is present in E. cloacae and also in E. coli.

When plasmid pPW926 carrying the E. cloacae nifLA::p-lacZ fusion was transferred to E. coli fnr mutant strain JRG2865, which fails to produce FNR, the fusion was just as active as it was in the wild-type E. coli strain (Table 2). As assessed by a gel mobility shift assay using the DNA fragment encompassing the FNR site consensus sequence, followed by incubation with protein extract of E. coli fnr mutant strain JRG2865, a DNA-protein complex was also consistently formed, as it was when the DNA fragment was incubated with the protein extract of the wild-type strain of E. coli (Fig. 2). From these results, we concluded that a trans-acting factor other than FNR binds to the putative FNR site and operates in the expression of the nifLA promoter in E. coli. However, we cannot exclude the possibility that FNR is capable of binding to the consensus sequence of the FNR site to activate the nifLA promoter in E. cloacae.

Superhelical status of DNA and activity of the nifLA promoter. Before the identification of a regulatory factor responding to oxygen status, it has been reported that the K. pneumoniae nifLA promoter requires a specific degree of negative supercoiling for expression, which is only possible under anaerobic conditions (6, 8). To examine this possibility, we tested the influence of gyrase activity on the transcription of the E. cloacae nifLA promoter by introducing the nifLA::p-lacZ fusion into an E. coli DH5α gyrA mutant or into gyrB⁺ strains of E. coli and E. cloacae in the presence of a gyrase inhibitor. The known gyrase-dependent K. pneumoniae nifLA::p-lacZ fusion (6, 8) was also run as a control. The results showed that expression of the nifLA::p-lacZ fusion has been markedly halted both in the gyrA mutant and in the gyrB⁺ strains with the presence of the gyrase inhibitors under aerobic or anaerobic conditions (Tables 2 and 3). When a plasmid carrying constitutively expressed gyrA was introduced into the gyrA DH5α mutant harboring the nifLA::p-lacZ fusion, the activity of the fusion was restored. However, a gyrB clone did not have the same effect (Table 2). Curiously, the nifH operon of Rhizobium melliloti, which is known to be insensitive to oxygen (unpublished data), appears to be DNA gyrase dependent too. These results confirm the earlier inference that DNA gyrase activity is crucial for transcription of the nifLA operon and possibly other nif genes.

These findings have significantly advanced our understanding of the mechanism of oxygen regulation of nifLA transcription. The trans-acting factor, which responds to the redox status, activates the nifLA promoter when bound to the defined sequence upstream of the nifLA promoter, while DNA supercoiling produced by the activity of gyrase functions in providing a topological requirement for the bound trans-acting factors.

FIG. 2. Gel mobility shift assay of a DNA fragment containing the FNR site consensus sequence incubated with protein extracts from E. cloacae E26 (lanes 2 to 5), E. coli YMC9 (lanes 7 and 9), and E. coli JRG2865 (lane 6). Lanes: 1 and 8, labeled DNA fragment; 2, 3, 6, and 7, labeled DNA fragment incubated with protein extract from anaerobic culture; 4, labeled DNA fragment incubated with protein extract from aerobic culture; 5 and 9, labeled DNA fragment containing mutated FNR site consensus sequence incubated with protein extract from an anaerobic culture. The reaction mixture in lane 2 also contained 300 pmol of unlabeled probe DNA as a specific competitor.
presumably through the process of looping of DNA between the sites of the NtrC and the trans-acting factors, thus enhancing the cooperative interaction between those bound trans-acting factors for activation of transcription.

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REFERENCES


