Repression of the Inner Membrane Lipoprotein NlpA by Rns in Enterotoxigenic Escherichia coli

Maria D. Bodero, M. Carolina Pilonieta, and George P. Munson*

Department of Microbiology and Immunology, University of Miami Miller School of Medicine, Florida

Received 6 November 2006/Accepted 14 December 2006

The expression of the inner membrane protein NlpA is repressed by the enterotoxigenic Escherichia coli (ETEC) virulence regulator Rns, a member of the AraC/YviS family. The Rns homologs CfaD from ETEC and AggR from enteropathogenic E. coli also repress expression of nlpA. In vitro DNase I and potassium permanganate footprinting revealed that Rns binds to a site overlapping the start codon of nlpA, preventing RNA polymerase from forming an open complex at nlpA-4p. A second Rns binding site between positions −152 and −195 relative to the nlpA transcription start site is not required for repression. NlpA is not essential for growth of E. coli under laboratory conditions, but it does contribute to the biogenesis of outer membrane vesicles. As outer membrane vesicles have been shown to contain ETEC heat-labile toxin, the repression of nlpA may be an indirect mechanism through which the virulence regulators Rns and CfaD limit the release of toxin.

Enterotoxigenic E. coli (ETEC) is a major cause of profusewatery diarrhea and in underdeveloped nations is a significantcause of infant and childhood mortality (23). There are twotypes of ETEC exotoxins that produce diarrheal disease, a heat-stable toxin (ST) and a heat-labile toxin (LT). Whereassome ETEC strains express both toxins, other strains produceonly one toxin, as either toxin is sufficient to cause diarrhea.The STs are small polypeptides consisting of 18 amino acids(STIa), 19 amino acids (STIb), or 48 amino acids (STII). Inthe host cell, STI activates guanylate cyclase, which leads to a dramatic increase in cyclic GMP levels in the cytoplasm. Themechanism of action of STII has not been determined yet.Internalization of LT ultimately leads to activation of adenylatecyclase and overproduction of cyclic AMP. LT is a largeperiplasmic protein that is attached to the inner membraneand streptomycin.

The ST and LT preproteins are transported across the bacterialinner membrane by the signal peptide-dependent general exportpathway. In the periplasm, the preproteins are processed and theheteromeric LT is assembled. Both STI and STII cross the outermembrane, it does not transport LT (10).

Early studies of LT secretion seemed to produce contradictoryfindings. Some studies showed that as much as 55% of a cell’s total LT was bound to the outer membrane, presumablyafter transport (29). Other studies showed that nearly all ofthe LT produced by a bacterium was contained in its periplasmand that the LT was not membrane bound (10). It has sincebeen shown that a type II secretion system is required for thetransport of LT across the outer membrane (28). The lack ofthis system in E. coli K-12 explains why studies using K-12strains showed that LT accumulates in the periplasm (10),while studies using ETEC strains showed that LT is transportedand bound to the outer membrane (29). An interactionbetween the B subunit of LT and lipopolysaccharide anchors thetoxin to the outer membrane (11).

Eventually, membrane-bound LT becomes a surface componentof outer membrane vesicles (OMVs) as they are shed from thebacterium (12). The biogenesis of OMVs is not fullyunderstood, but a recent study has shown that disruption ofnlpA decreases the production of OMVs (16). NlpA is a periplasmic protein that is attached to the inner membrane(34). In this study we found that transcription of nlpA is repressed(34). In this study we found that transcription of nlpA is repressedby the ETEC virulence regulators Rns and CfaD.

MATERIALS AND METHODS

Strains and plasmids. Plasmid pHKLac1 is a promoterless lacZ reporter and integration plasmid with a pir-dependent origin of replication. It was constructed by cloning a 5.5-kb BamHI-MfeI fragment from pRS550 carrying lacZYA (27) into pH144 (accession no. AY048731) (8). Transcriptional terminators flank lacZYA in the resulting plasmid. It also carries attP39053 for Int39053-mediated integration at attB39053 in pir hosts and adaA for selection with spectinomycin and streptomycin.

The nlpA-yicS intergenic region was amplified from ETEC strain HK10407 (CfaD+ CFA/I+) (4) with primers nlpA-F1 (GCGGATCCATGCTTCATCGCAGATGAGG) and nlpA-r1 (GCGGGAATTCCTGACCGTGAGCTTTGCGTG) and nlpA-r2 (GTCGCAACCTGCGCTGCCTG). The PCR products were digested with BamHI and EcoRI and then ligated into the same sites of pHKlac1 to construct pHKalac1 [nlpA::pHKLac1] (numbering is relative to the start codon of nlpA). Each reporter plasmid was integrated into the chromosome of MC4100 (F– arudH39 ΔargF–lacI) by triparental mating with pHKLac1 as the donor andHK10407 as the recipient. The PCR products were digested with BamHI and EcoRI and then ligated into the same sites of pHKlac1 to construct pHKalac1 [nlpA::pHKLac1] (numbering is relative to the start codon of nlpA).

Received 6 November 2006/Accepted 14 December 2006

Published ahead of print on 22 December 2006

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Miami Miller School of Medicine, P.O. Box 016960 (R-138), Miami, FL 33101. Phone: (305) 243-5317. Fax: (305) 243-4623. E-mail: gmunson@miami.edu.

† Published ahead of print on 22 December 2006.
Plasmid pGPMRs expresses mc from lacp. It was constructed by ligating a 0.9-kb RsaI fragment from pEU2030 (6) into the Smal site of pNEB193 (New England Biolabs). To construct pMBPRns1, which expresses an isoaryl-β-D-thiogalactopyranoside (IPTG)-inducible maltose binding protein (MBP)-Rns fusion protein from lacp, mc was amplified from pEU2005 (1) with primers RnsNcoI (AGGTATAACC ATGGACTTAAATACATGCA) and Rsam-Bam (GCGGATCCTGTTTTTAT CCACCTTAA). The 0.9-kb PCR product was then digested with BamHI and ligated into pMalC2 (New England Biolabs) previously digested with BamHI and XmnI. Other plasmids used in this study include pNTP503, which expresses CfaD (26, 31), and pGMPN52, which expresses AggR (22).

**Purification of RNA.** Strain GPM1080 was cultured aerobically in 10 ml of Luria-Bertani (LB) medium at 37°C. After the absorbance at 550 nm reached 1.0, 2 ml of PE buffer (5% (vol/vol) phenol, 95% (vol/vol) ethanol) was added to the culture, and the cells were pelleted. The cell pellet was suspended in 10 ml of RNA wash buffer (0.75% (vol/vol) NaCl, 0.8% (vol/vol) phenol, 15.8% (vol/vol) ethanol) and then centrifuged. The resulting pellet was resuspended in 500 μl of 0.9% (vol/vol) NaCl and shaken at room temperature for 30 min after addition of 500 μl water-saturated, nonbuffered phenol. Subsequently, 50 μl of a chloroform-isooamyl alcohol (24:1) solution was added, and the preparation was incubated for an additional 15 min. The solution was then chilled on ice for 5 min and centrifuged in a microcentrifuge at the maximum setting for 5 min. The supernatant was recovered, and the RNA was ethanol precipitated and then resuspended in 22 μl of RNAse-free water.

**Primer extension.** Two PCR products of Hsp-ended labeled oligonucleotide pnpA-1 (GCAGATTCGGCCCGACAATATG) and pnpA-2 (GCGGATCCTGTTTTTATCCACCTTAA) were generated by the Maxam-Gilbert method (15). The PCR products were then heat denatured and separated on sequencing gels. GA sequencing ladders (24) were used according to the supplier's protocol (Invitrogen). Heat-denatured aliquots of each sample were then heat denatured and separated on sequencing gels. GA sequencing ladders (24) were used according to the supplier's protocol (Invitrogen). Heat-denatured aliquots of each sample were then combined with 1.0, 2 ml of PE buffer (5% (vol/vol) phenol, 95% (vol/vol) ethanol) and 100 μg/ml ampicillin. After cells reached the mid-log phase, the culture was transferred to a 30°C shaking water bath, and expression of MBP-Rns was induced by addition of IPTG to a final concentration of 300 μM. After several hours of induction, cells were harvested at 4°C and centrifuged in a microcentrifuge at the maximum setting for 5 min. The supernatant was recovered, and the RNA was ethanol precipitated and then resuspended in 22 μl of RNAse-free water.

**Purification of MBP-Rns.** Strain K1001/pRare2/pMBPRns1 was used for expression of MBP-Rns. The gene encoding Prc (Tsp) protease is disrupted in strain K1001 [F lacP lacO pro insu(lac-pro) &com:kan eda51::Tn10 gateway psp thi-1 arg4]. Plasmid pRARE2 (Novagen) provides several rare tRNAs to supplement the rare codon usage of argI in M. After several hours of induction, cells were harvested at 4°C and centrifuged in a microcentrifuge at the maximum setting for 5 min. The supernatant was recovered, and the RNA was ethanol precipitated and then resuspended in 22 μl of RNAse-free water.

**Enzymatic assays.** Reporter strains GPM1080, GPM1092, and GPM1095 transformed with pGPMRs (Rns+ bla) were used in enzymatic assays. Enzymatic assays were performed as previously described (20).

**RESULTS**

**Characterization of nlpAp.** The virulence of some ETEC strains is dependent upon Rns, which positively regulates its own expression and the expression of CS1, CS2, and CS3 pili. Rns recognizes the same binding sites as CfaD (18), a regulator typically found in strains of ETEC that express CFA/I pili (2). We determined the nucleotide sequence of CS1-CFA/I regulatory region predicted to be overlapping the start codon of nlpA by sequencing the genomes of E24377A (Rns+ CS1+ CS3− ST+ LTr; accession no. AAIZ00000000), H10407 (CfaD+ Cfa/I+ ST− LT+), and MG1655 (K-12; accession no. U00096) for sites similar to known Rns binding sites (19, 20). In silico sequence analysis predicted a Rns binding site (GATAAAAAATG) partially conserved in the 221-bp intergenic region between the divergently encoded yicS and nlpA genes. Potentially, yicS encodes a 11-kDa secreted protein of unknown function. A 28-kDa inner membrane lipoprotein is encoded by nlpA (32). The last two nucleotides of the putative Rns binding site are the first two nucleotides of nlpA’s ATG start codon.

**Potassium permanganate footprinting.** MBP-Rns and 32P-end-labeled nlpA promoter DNA was equilibrated for 20 min at 37°C in potassium permanganate buffer [10 mM Tris-Cl (pH 7.6 at room temperature), 50 μM KCl, 1.2 ng/μl poly(dI-dC), 0.2 μM MgCl2, 10 μg/ml bovine serum albumin]. The reaction was quenched by addition of 0.5 volume of potassium permanganate stop buffer (750 mM sodium acetate, 500 mM β-mercaptoethanol, 50 μg/ml RNAse). The DNA was precipitated by addition of 5 volumes of 95% (vol/vol) ethanol, pelleted, washed with 70% (vol/vol) ethanol, and dried. Pipetidine cleavage and subsequent steps were performed as previously described (20).

**Enzymatic assays.** Reporter strains GPM1080, GPM1092, and GPM1095 transformed with pGPMRs (Rns+ bla) were used in enzymatic assays. Enzymatic assays were performed as previously described (20).

**RESULTS**

**Characterization of nlpAp.** The virulence of some ETEC strains is dependent upon Rns, which positively regulates its own expression and the expression of CS1, CS2, and CS3 pili. Rns recognizes the same binding sites as CfaD (18), a regulator typically found in strains of ETEC that express CFA/I pili (2). We determined the nucleotide sequence of CS1-CFA/I regulatory region predicted to be overlapping the start codon of nlpA by sequencing the genomes of E24377A (Rns+ CS1+ CS3− ST+ LTr; accession no. AAIZ00000000), H10407 (CfaD+ Cfa/I+ ST− LT+), and MG1655 (K-12; accession no. U00096) for sites similar to known Rns binding sites (19, 20). In silico sequence analysis predicted a Rns binding site (GATAAAAAAATG) partially conserved in the 221-bp intergenic region between the divergently encoded yicS and nlpA genes. Potentially, yicS encodes a 11-kDa secreted protein of unknown function. A 28-kDa inner membrane lipoprotein is encoded by nlpA (32). The last two nucleotides of the putative Rns binding site are the first two nucleotides of nlpA’s ATG start codon.

**Potassium permanganate footprinting.** MBP-Rns and 32P-end-labeled nlpA promoter DNA was equilibrated for 20 min at 37°C in potassium permanganate buffer [10 mM Tris-Cl (pH 7.6 at room temperature), 50 μM KCl, 2.0 ng/μl poly(dI-dC), 0.2 μM MgCl2, 10 μg/ml bovine serum albumin]. RNA polymerase (RNAP), preequilibrated at 37°C, was then added, and the solutions were incubated for an additional 20 min. KNO3 was then added to a final concentration of 2 mM for 2 min at 37°C. The reaction was quenched by addition of 0.5 volume of potassium permanganate stop buffer.
attempt to map the TSS of \( nlpA \) in the presence of Rns because, as shown below, Rns represses \( nlpAp \) at the transcription initiation step.

We next confirmed that the predicted Rns binding site overlapping the start codon of \( nlpA \) is an actual binding site by in vitro DNase I footprinting with an MBP-Rns fusion protein (Fig. 2). For in vitro work it is necessary to use MBP-Rns instead of Rns because it is severalfold more soluble than Rns. Fortunately, MBP does not interfere with the activity of Rns in vivo (19). We found that MBP-Rns protects a region from \(+5\) to \(+40\) (numbering relative to the TSS of \( nlpAp \)), thus encompassing the predicted Rns binding site from \(+16\) to \(+26\). The DNase I footprint of MBP-Rns is larger than the core sequence of highly conserved nucleotides comprising the predicted binding site because steric occlusion prevents DNase I cleavage within several nucleotides of bound MBP-Rns.

Although sequence analysis predicted only one Rns binding site in the \( nlpA-yicS \) intergenic region, an additional MBP-Rns binding site was observed from \(-152\) to \(-195\) relative to the TSS of \( nlpAp \) (Fig. 2). This second site was not identified by our in silico analysis because it is the most divergent of all known Rns binding sites. For simplicity, the two Rns binding sites are referred to below as \( nlpAo \) and \( yicSo \) based on their proximity to \( nlpA \) and \( yicS \). Qualitatively, it appears that the affinity of Rns for \( nlpAo \) is higher than the affinity of Rns for \( yicSo \) because \( nlpAo \) is saturated by lower concentrations of MBP-Rns than \( yicSo \). As shown below, only \( nlpAo \) is required for the repression of \( nlpAp \).

FIG. 2. Identification of two Rns binding sites in the \( nlpA-yicS \) intergenic region. DNase I footprinting of MBP-Rns bound to the coding and noncoding strands of \( nlpA \) revealed two Rns binding sites in the \( nlpA-yicS \) intergenic region. The Rns binding sites are designated \( nlpAo \) and \( yicSo \) based on their proximity to \( nlpA \) and \( yicS \). Straight arrows indicate the relative positions of \( nlpA \) and \( yicS \). The numbering is relative to the transcription start site of \( nlpA \), which is indicated by a wavy arrow. Lanes GA and TC contained Maxam-Gilbert sequence ladders.
Repression of nlpA by Rns, CfaD, and AggR. To assess whether Rns regulates the expression of nlpA, we constructed nlpA-lacZ fusions that were integrated into the chromosome of K-12 strain MC4100. In the absence of Rns, CfaD, and AggR, β-galactosidase was expressed at moderate levels from nlpA::lacZ (numbering relative to the TSS) (Table 1). In contrast, we observed nearly complete repression of nlpA in the presence of Rns (Table 1). Although a slight decrease in Rns-independent expression from nlpAPETEC::lacZ was observed compared to the expression from nlpAPETEC::lacZ, repression of nlpA was not dependent upon yicS since yicS was deleted from nlpAPETEC::lacZ (Table 1). These results demonstrate that Rns functions as a repressor of nlpA transcription and that the Rns binding site nlpA is sufficient for repression.

In transcriptional activation assays, Rns was shown to be functionally interchangeable with CfaD, which activates the expression of CFA/I pili in some strains of ETEC, and the enteroaggregative virulence regulator AggR (18). As shown in Table 1, both CfaD and AggR also repressed transcription from nlpAPETEC::lacZ. Although the nlpA-yicS loci are identical in K-12 and ETEC strains, this locus is not as well conserved in the prototypical EAEC strain 042. To determine if nlpA is regulated in EAEC, an additional reporter strain was constructed using the nlpA promoter cloned from EAEC strain 17-2 (22). Strain 17-2 was repressed. Thus, nlpA is repressed by Rns or CfaD in ETEC and by AggR in EAEC.

Mechanism of repression. Potassium permanganate, a reagent that is reactive with unpaired thymine nucleotides in transcription bubbles (9, 25), was used to determine if Rns interferes with the formation of RNAP-open (RPo) complexes or a later step of transcription, such as transcript elongation. In the absence of Rns, potassium permanganate reactivity was detected from −8 to +17 (numbering relative to the TSS of nlpA) (Fig. 3). This region of reactivity corresponds to single-stranded DNA within an RPo complex at nlpA as it is dependent upon RNAP. Although the region of reactivity is consistent with the transcription start site of nlpA (Fig. 1), we noted that it extends further downstream than the usual 4 nucleotides reported for other promoters (25). One possible explanation for the extended permanganate footprint is the AT richness (85%) from position 5 to position 17, which might contribute to DNA melting beyond the expected region. Regardless, potassium permanganate reactivity at nlpA was not observed in the presence of MBP-Rns (Fig. 3). These results demonstrate that Rns represses nlpA at the initiation step of transcription.

**TABLE 1. Repression of nlpA by Rns, CfaD, and AggR**

<table>
<thead>
<tr>
<th>nlpA</th>
<th>Position relative to: TSS</th>
<th>Open reading frame</th>
<th>β-Galactosidase activity (Miller units) in the presence of*:</th>
</tr>
</thead>
<tbody>
<tr>
<td>nlpAPETEC::lacZ</td>
<td>−367 to +82</td>
<td>−391 to +58</td>
<td>479 ± 10</td>
</tr>
<tr>
<td>nlpAPETEC::lacZ</td>
<td>−162 to +82</td>
<td>−186 to +58</td>
<td>348 ± 15</td>
</tr>
<tr>
<td>nlpAPAEAC::lacZ</td>
<td>ND</td>
<td>−366 to +58</td>
<td>223 ± 6</td>
</tr>
</tbody>
</table>

*a* The values are means and standard deviations (n = 3).

*b* ND, not determined.

FIG. 3. Rns represses nlpA by preventing the formation of an open complex at nlpA. Potassium permanganate footprinting of RNAP bound to the noncoding strand of nlpA in the presence and absence of MBP-Rns is shown. A straight arrow indicates the position of the transcription start site of nlpA. The numbering is relative to the transcription start site of nlpA, which is indicated by a wavy arrow. Lanes GA and TC contained Maxam-Gilbert sequence ladders. Lane q.c. was not treated with potassium permanganate and was a control for the quality of the DNA template.
because it prevents the formation of an RPo complex. Given the proximity of \( nlpAo \) to \( nlpAp \), it seems likely that Rns bound at \( nlpAo \) sterically occludes RNAP from \( nlpAp \). These results also demonstrate that Rns is sufficient for repression of \( nlpAp \) and that other factors are not required.

In addition to the RPo complex observed at \( nlpAp \), other RNAP-dependent regions of potassium permanganate reactivity were observed in the \( nlpA-yicS \) intergenic region (Fig. 3). One or more of the RPo complexes may be at the promoter or promoters for \( yicS \). Alternatively, they may be the result of RNAP binding to pseudopromoter sequences. In either case, the additional RPo complexes were not investigated further since they were not affected by MBP-Rns and do not correspond to the known TSS of \( nlpA \) (Fig. 1).

**DISCUSSION**

Our results demonstrate that the virulence regulators Rns and CfaD of ETEC and AggR of EAEC repress the transcription of \( nlpA \). Two Rns binding sites were identified by DNase I footprinting; these sites were \( nlpAo \) from +5 to +40 and \( yicSo \) from −152 to −195 (numbering relative to the TSS of \( nlpA \)). The \( nlpAo \) binding site was sufficient for repression of \( nlpA \) in vivo, and Rns binding to this site prevented RNAP from forming an open complex at \( nlpAp \). We have not yet determined the function, if any, of \( yicSo \), but additional studies are planned. It is possible that Rns binding to \( yicSo \) affects the expression of \( yicS \), a gene upstream of and divergently encoded from \( nlpA \). Rns is undoubtedly repressed in all strains of ETEC that carry Rns or CfaD or one of the functional homologs (CsvR, CsfR, or CswR) with which they are interchangeable (18, 30). Although Rns is also interchangeable with VirF from *Shigella flexneri* (18), we did not investigate the regulation of \( nlpA \) by VirF because \( nlpA \) has been disrupted in each of the three *S. flexneri* strains for which a genomic sequence is available (strain 2457T [accession no. AE014073], strain 301 [accession no. AE005674], and strain 8401 [accession no. CP000266]).

Until now, Rns, CfaD, and AggR were only known to function as activators, but our results demonstrate that these regulators are bifunctional. However Rns, CfaD, and AggR do not switch functions in response to effector ligands since they repress \( nlpA \) under the same conditions that they activate expression of pilin genes. This differentiates them from other AraC/XylS family members that have different activities in response to effector molecules (7). The factor that is most likely to determine the effect of Rns, CfaD, and AggR at a given promoter is the precise position of the regulator’s binding site relative to RNAP. Binding sites that occlude RNAP binding to promoter elements function as repressor sites, while other binding sites may have a stimulatory effect. The location of a Rns binding site downstream of the TSS is not sufficient to predict whether it functions as a repressor or activator because Rns has been shown to bind downstream of its own promoter, where it is an activator (20). However, Rns binds further downstream from its own promoter than from \( nlpAp \).

In *E. coli*, NlpA is a periplasmic protein bound to the inner membrane. Original investigations of a \( nlpA::kan \) mutant revealed no significant differences in growth, cell morphology, or chemical sensitivity compared to a wild-type strain (32). However, in a recent screen for transposon insertions that affect the production of OMVs, it was observed that a \( nlpA::Tn5(\text{kan}) \) mutant produces less OMVs than an isogenic wild-type strain of *E. coli* (16). Although the mechanism by which NlpA contributes to the formation of OMVs is not known, it has been suggested that NlpA may have a direct role in OMV biogenesis (16).

The OMVs produced by ETEC contain nearly all of the pathogen’s secreted LT, and OMVs deliver the toxin into eukaryotic cells (11–13). Our studies raise the possibility that the ETEC virulence regulators Rns and CfaD indirectly control the release of LT by repressing the transcription of \( nlpA \), thus limiting the production of OMVs. Since these regulators are not found in all ETEC strains, this may also account, at least in part, for the reported variability of OMV production between strains (14).

**ACKNOWLEDGMENTS**

We thank J. R. Scott, J. P. Nataro, and B. L. Wanner for kindly providing strains and plasmids. Preliminary sequence data for EAEC strain 042 was produced by the Pathogen Sequencing Unit at the Sanger Institute and was obtained from the website http://www.sanger.ac.uk/Projects/Escherichia_Shigella/ Preliminary sequence data for ETEC strain H11007 was produced by the Sanger Institute in collaboration with I. Henderson and M. Pullen and was obtained from http://www.sanger.ac.uk/Projects/E_coli_H11007/.

This work was supported by Public Health Service award AI 057648 from the National Institutes of Health and by the University of Miami Miller School of Medicine.

**REFERENCES**


