

A Positive Regulatory Loop Controls Expression of the Locus of Enterocyte Effacement-Encoded Regulators Ler and GrlA

Jeannette Barba,¹ Víctor H. Bustamante,¹ Mario A. Flores-Valdez,¹ Wanyin Deng,²
B. Brett Finlay,² and José L. Puente^{1*}

Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México,¹ and Michael Smith Laboratories, University of British Columbia, Vancouver, British Columbia, Canada²

Received 8 June 2005/Accepted 31 August 2005

The formation of attaching and effacing (A/E) lesions on intestinal epithelial cells is an essential step in the pathogenesis of human enteropathogenic and enterohemorrhagic *Escherichia coli* and of the mouse pathogen *Citrobacter rodentium*. The genes required for the development of the A/E phenotype are located within a pathogenicity island known as the locus of enterocyte effacement (LEE). The LEE-encoded transcriptional regulators Ler, an H-NS-like protein, and GrlA, a member of a novel family of transcriptional activators, positively control the expression of the genes located in the LEE and their corresponding virulence. In this study, we used *C. rodentium* as a model to study the mechanisms controlling the expression of Ler and GrlA. By deletion analysis of the *ler* and *grlA* regulatory regions and complementation experiments, negative and positive *cis*-acting regulatory motifs were identified that are essential for the regulation of both genes. This analysis confirmed that GrlA is required for the activation of *ler*, but it also showed that Ler is required for the expression of *grlA*, revealing a novel regulatory loop controlling the optimal expression of virulence genes in A/E pathogens. Furthermore, our results indicate that Ler and GrlA induce the expression of each other by, at least in part, counteracting the repression mediated by H-NS. However, whereas GrlA is still required for the optimal expression of *ler* even in the absence of H-NS, Ler is not needed for the expression of *grlA* in the absence of H-NS. This type of transcriptional positive regulatory loop represents a novel mechanism in pathogenic bacteria that is likely required to maintain an appropriate spatiotemporal transcriptional response during infection.

Enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and *Citrobacter rodentium* belong to a family of bacterial pathogens causing a destructive lesion of the intestinal enterocyte, called the attaching and effacing (A/E) lesion, as well as gastrointestinal disorders in infected hosts (reviewed in references 28 and 33). EPEC is an important etiological agent of childhood diarrhea in developing countries, whereas EHEC is the cause of frequent outbreaks of food and water poisoning in the developed world. In addition to causing diarrhea, an EHEC infection can result in severe complications, such as hemorrhagic colitis and hemolytic-uremic syndrome (reviewed in reference 33). Due to the specificity of EPEC and EHEC for human hosts, a corresponding small-animal infection model does not exist. Thus, most of the current models to explain EHEC and EPEC pathogen-host interactions, such as those for A/E lesion formation, have been developed based on *in vitro* studies performed with infected cultured epithelial cells. In recent years, *C. rodentium* has become accepted as a representative infection system to study the mechanisms leading to the production of the A/E lesion and A/E-associated pathogenesis (12, 13, 47).

The A/E lesion is characterized by a localized loss of microvilli from the surfaces of epithelial cells and important cytoskeleton rearrangements beneath the adherent bacteria, leading to the formation of actin-rich cup-like structures and intimate bacterium-host cell interactions. Intimate adherence is mediated by the interaction between Tir (translocated intimin receptor), a bacterial protein that is translocated and inserted into the host cell membrane, and intimin, a bacterial outer membrane adhesin (reviewed in reference 7). The genes required for the formation of the A/E lesion in EPEC, EHEC, and *C. rodentium* are located within a pathogenicity island known as the locus of enterocyte effacement (LEE), where they are organized in five polycistronic operons (*LEE1-LEE5*), two putative bicistronic operons, and four monocistronic units (8). The *LEE1* to *LEE3* operons encode mostly structural components of a type III secretion system (Esc and Sep), the *LEE4* operon encodes proteins involved in protein translocation (EspA, B, and D and SepL), and the *LEE5* operon encodes the proteins required for intimate attachment (intimin and Tir). The genes encoding effector proteins, chaperones, and transcriptional regulators are scattered along the LEE (reviewed in references 7 and 8). During A/E lesion formation, several LEE-encoded proteins (Tir, Map, EspF, EspG, EspH, and EspZ), as well as non-LEE-encoded proteins (NleA/EspI, EspFu/TccP, EspJ, and Cif), are translocated by the type III secretion apparatus into the host epithelial cells, where they affect different signaling processes (reviewed in references 10 and 20).

* Corresponding author. Mailing address: Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad 2001, Cuernavaca, Morelos 62210, México. Phone: (52) (777) 329-1621. Fax: (52) (777) 313-8673. E-mail: puente@ibt.unam.mx.

Several studies have shown that Ler (*LEE*-encoded regulator), a 15-kDa protein encoded by the first gene of the *LEE1* operon, is a central positive regulator needed for the expression of the *LEE* genes (5, 16, 19, 31) as well as the non-*LEE*-carried gene *espC* (32). Ler belongs to the H-NS family of nucleoid-associated proteins, exhibiting high amino acid identity with the carboxy termini of these proteins, which contain the DNA binding domain (16). The global regulator H-NS (14) represses the expression of several *LEE* genes, and Ler induces the expression of these genes by counteracting the H-NS-mediated repression (5, 24, 46). Thus, Ler is primarily an antirepressor needed to conduct gene expression (5, 24, 46).

Different studies of EPEC and EHEC have shown that *ler* expression is regulated by a complex assortment of global and A/E-specific regulators. The global regulator integration host factor (IHF), which directly binds to a DNA region upstream of the *ler* promoter, is essential for *ler* activation (19). *ler* is also positively regulated by other global regulators, such as BipA, a member of the ribosome-binding GTPase superfamily (23); Fis (factor for inversion stimulation), a bacterial nucleoid-associated protein (21); and QseA (quorum-sensing *E. coli* regulator A), a factor involved in regulation via quorum sensing (42). H-NS and Hha play a negative role in *ler* expression, with both binding directly to its regulatory region (40, 46). In addition, specific regulators such as PerC, the product of the third gene of the *per* locus located in the EPEC adherence factor plasmid, can directly activate the expression of *ler* (5, 31, 35, 36). PerC-like proteins have also been identified in EHEC and are involved in *ler* expression (25). GadX regulates the expression of the *perABC* operon and thus indirectly regulates the expression of *ler* (41). It has been reported that Ler binds to its own regulatory region and autorepresses its transcription in a concentration-dependent manner (2). The negative regulation of *LEE* gene expression is also mediated by YhiE and YhiF (44) as well as by EtrA (*E. coli* type III secretion system 2 regulator A) and EivF (49) by mechanisms that remain to be defined. We have recently identified two novel *LEE*-encoded regulators, GrIA (global regulator of *LEE* activator; formerly called Orf11) and GrIR (Grl repressor; formerly called Orf10), which are highly conserved in all A/E pathogens (12). These proteins are encoded by the putative *grlRA* operon located between the *rorf3* gene and the *LEE2* operon in the *LEE*. GrIA is a positive regulator of *ler* expression (12). The closest GrIA homologue is the putative product of an uncharacterized gene found in different *Salmonella enterica* serotypes. In addition, GrIA is 23% identical to CaiF, a regulatory protein responsible for the carnitine-dependent induction of the *cai* and *fix* *E. coli* operons under anaerobic conditions and the best-characterized member of this novel family of transcriptional regulators (15). A motif search of GrIA has also revealed the presence of a putative helix-turn-helix DNA binding motif at its N-terminal domain, where most of the similarity with CaiF and the *Salmonella* GrIA homologue (Sgh) is found (12). GrIR has a significant negative effect on *LEE* gene expression, probably acting as a negative regulator of *ler* (12, 26, 27), although its mechanism of action remains to be defined. PSI-BLAST searches have identified only one other GrIR homologue, located next to a GrIA homologue in *Salmonella bongori* (34). For the present study, we used *C. rodentium* as a model to study the mechanisms controlling the expression of the genes

encoding the positive regulators Ler and GrIA. Although *C. rodentium* has been used as a model organism to study EPEC and EHEC, there is little known about the regulation of its *LEE* gene expression. Here we characterize the regulatory regions of the *C. rodentium ler* and *grlRA* genes in detail. Furthermore, we demonstrate that Ler and GrIA regulate each other, forming a transcriptional positive regulatory loop that, to our knowledge, represents a novel mechanism controlling gene expression in bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are described in Table 1. Luria-Bertani (LB) broth (37) or Dulbecco's modified Eagle's medium (DMEM) containing glucose (0.45% [wt/vol]) and L-glutamine (584 mg liter⁻¹), but not sodium pyruvate (Gibco BRL Life Technologies), was used for static cultures at 37°C in a 5% CO₂ atmosphere. When required, antibiotics were added at the following concentrations for LB cultures: ampicillin (Amp), 100 µg ml⁻¹; carbenicillin (Cb), 100 µg ml⁻¹; kanamycin (Km), 25 µg ml⁻¹; tetracycline (Tc), 12 µg ml⁻¹; and streptomycin (Stp), 100 µg ml⁻¹. The following antibiotic concentrations were used for DMEM cultures, when required: Amp, 50 µg ml⁻¹; Cb, 50 µg ml⁻¹; and Tc, 5 µg ml⁻¹. Test cultures were inoculated as described before (13). Culture samples to determine chloramphenicol acetyltransferase (CAT) activity were collected at 6 h. At this time point, all strains reached similar optical densities. Each experiment was done independently in duplicate at least three times.

DNA manipulations. Recombinant DNA techniques were performed according to standard protocols (1, 37). Restriction enzymes were obtained from Invitrogen or New England Biolabs and used according to the manufacturer's instructions. The oligonucleotides used for amplification by PCR and for primer extension experiments (Table 2) were synthesized by the oligonucleotide synthesis facility at our institute. PCRs were performed in 100-µl reaction mixtures containing a 1.5:1 mixture of AmpliTaq and Pfu DNA polymerases, using an Eppendorf mastercycler gradient thermocycler.

Construction of *ler-cat* and *grlRA-cat* transcriptional fusions. Oligonucleotides were designed for PCR amplification of different fragments spanning the *ler* regulatory region and the *rorf3-grlRA* region (Table 2). PCRs were performed using these oligonucleotides, with *C. rodentium* DBS100 chromosomal DNA as the template. The PCR fragments were double digested with BamHI and HindIII and ligated into pKK232-8 (Pharmacia LKB Biotechnology), which contains a promoterless *cat* gene, digested with the same enzymes. Combination of the forward primers CRler-260, CRler-200, CRler-160, CRler-120, CRler-80, and CRler-40 with the reverse primer Orf1-H3-R was used for the construction of the fusions pCRler-260, -200, -160, -120, -80, and -40, respectively. Fusions pCRgrlRA-1, -2, and -3 were constructed using the forward primer CR-ORF10-BHI in combination with the reverse primers CR-ORF10-HIII-A, CR-ORF10-HIII-B, and CR-ORF11-H3, respectively. pCRgrlRA-4 was constructed using primers CR-ORF11-BHI and CR-ORF11-H3. The forward primer CR-RORF3-BH and the reverse primers CR-ORF10-HIII-A and CR-ORF11-H3 were used to construct pCRgrlRA-5 and -6, respectively. The nucleotide sequences of the *ler-cat* and *grlRA-cat* fusions were determined in the sequencing facility at our institute.

Construction of *E. coli* MC4100 Δ*hns*::Km mutant. Deletion of the *hns* gene from *E. coli* MC4100 was performed by the one-step mutagenesis procedure for bacterial genes described by Datsenko and Wanner (9). The deletion eliminated 131 codons out of the 137 codons of the *hns* gene, which were replaced with a Km resistance marker. Primers *hns*H1P1 and *hns*H2P2 and DNA of plasmid pKD4 were used to generate the deletion cassette. The replacement of *hns* by the Km resistance marker was confirmed by PCR using primers *hns*M and *hns*G. The resulting strain was designated JPMC1 (Table 1).

PCR cloning of *ler* and *grlA*. The primer pairs Cler-RBS-F (BamHI)/ClerOrf1-R (HindIII) and CROrf11Xho/EpCiorf11R-H3 were used to amplify the *C. rodentium ler* and *grlA* genes, respectively. The resulting PCR products were digested with the BamHI-HindIII and XhoI-HindIII restriction enzymes, respectively, and ligated into pMPM-T3 (30) digested with the same enzyme combinations, generating plasmids pTCRLer4 and pTCRGrIA1 (Table 1). The identity of the inserts was confirmed by DNA sequencing. The plasmids contain the promoterless *ler* or *grlA* gene plus the putative ribosome-binding sites and are expressed from the vector *lac* promoter.

CAT assay. CAT assays and protein quantification to calculate CAT specific activities were performed as described previously (29).

TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Description ^a	Reference or source
<i>C. rodentium</i> strains		
DBS100	Wild type (ATCC 51459)	39
Δ ler	DBS100 carrying an in-frame deletion of <i>ler</i>	12
Δ orf11/ <i>grlA</i>	DBS100 carrying an in-frame deletion of <i>grlA</i>	12
<i>E. coli</i> strains		
MC4100	F' <i>araD139</i> Δ (<i>argF-lac</i>)U169 <i>rpsL150 relA1 flb5301 deoC1 ptsF25 rbsR</i>	6
JPMC1	MC4100 Δ <i>hns</i> ::Km	This study
BL21/pLys21	F ⁻ <i>ompT</i> (<i>lon</i>) <i>hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (λ DE3)	Invitrogen
N99	<i>E. coli</i> K12 F ⁻ <i>galK2 rpsLl</i>	22
K5185	N99 Δ <i>himA82</i>	18
Plasmids		
pKD46	Red recombinase system under <i>araB</i> promoter; Ap ^r	9
pKD4	Template plasmid containing the Km cassette for lambda Red recombination	9
pMPM-T3	Low-copy-number cloning vector; p15A derivative; Tc ^r	30
pTCRLer4	pMPM-T3 derivative carrying the <i>ler</i> structural gene and ribosome binding site under the control of the <i>lac</i> promoter	This study
pTCRGr1A1	pMPM-T3 derivative carrying the <i>grlA</i> structural gene and ribosome binding site under the control of the <i>lac</i> promoter	This study
pMPM-T6	Cloning vector containing an arabinose-inducible promoter; p15A derivative; Tc ^r	30
pT6HNS	pMPM-T6 derivative expressing H-NS-His ₆ under the control of the arabinose-inducible promoter	Unpublished
pT6Ler	pMPM-T6 derivative expressing Ler-His ₆ under the control of the arabinose-inducible promoter	Unpublished
pKK232-8	pBR322 derivative containing a promoterless chloramphenicol acetyltransferase (<i>cat</i>) gene	Pharmacia LKB Biotechnology
pLEE2-CAT	pKK232-8 derivative carrying <i>C. rodentium</i> <i>LEE2-cat</i> transcriptional fusion from nucleotides -375 to +121	12
PCRler-260	pKK232-8 derivative carrying <i>C. rodentium</i> <i>ler-cat</i> transcriptional fusion from nucleotides -265 to +216	This study
pCRler-200	<i>CRler-cat</i> transcriptional fusion from nucleotides -197 to +216	This study
pCRler-160	<i>CRler-cat</i> transcriptional fusion from nucleotides -163 to +216 (pLEE1-CAT)	12
pCRler-120	<i>CRler-cat</i> transcriptional fusion from nucleotides -123 to +216	This study
pCRler-80	<i>CRler-cat</i> transcriptional fusion from nucleotides -86 to +216	This study
pCRler-40	<i>CRler-cat</i> transcriptional fusion from nucleotides -44 to +216	This study
pCRgrlRA-1	pKK232-8 derivative carrying <i>C. rodentium</i> <i>grlRA-cat</i> transcriptional fusion from nucleotides -420 to +152	This study
pCRgrlRA-2	<i>CRgrlRA-cat</i> transcriptional fusion from nucleotides -420 to +397	This study
pCRgrlRA-3	<i>CRgrlRA-cat</i> transcriptional fusion from nucleotides -420 to +565	This study
pCRgrlRA-4	<i>CRgrlRA-cat</i> transcriptional fusion from nucleotides +212 to +565	This study
pCRgrlRA-5	<i>CRgrlRA-cat</i> transcriptional fusion from nucleotides -135 to +152	This study
pCRgrlRA-6	<i>CRgrlRA-cat</i> transcriptional fusion from nucleotides -135 to +565	This study

^a The coordinates for *cat* transcriptional fusions are indicated with respect to the *ler* or *grlR* transcriptional start site.

RNA isolation and primer extension analysis. Total RNAs were isolated from samples of cultures grown for 6 h in DMEM at 37°C in a 5% CO₂ atmosphere without agitation, using an RNeasy kit (QIAGEN) according to the manufacturer's instructions. The RNA concentration and quality were determined by measuring the *A*₂₆₀-to-*A*₂₈₀ ratio and by gel electrophoresis. Primer extension reactions were performed as described previously (29). Briefly, oligonucleotides complementary to the *grlR* (CR-ORF10-HIII-A) or *ompA* (*ompA*PE) (Table 2) coding region were end labeled with [γ -³²P]dATP, using T4 polynucleotide kinase, and annealed with 8 μ g (for *grlR*) or 0.8 μ g (for *ompA*) of total RNA in 0.37 M NaCl-0.035 M Tris-HCl (pH 7.5) by heating for 3 min at 90°C and then cooling slowly to 50°C. Reverse transcription reactions were performed at 42°C for 2 h with 10 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) in avian myeloblastosis virus buffer containing 1 mM dithiothreitol, a 0.3 mM concentration of each deoxynucleoside triphosphate, and 50 U of RNase inhibitor (Invitrogen). The reverse transcription products were cleaned and concentrated using a Microcon YM-30 microconcentrator (Amicon) according to the specifications of the manufacturer, denatured by heating to 95°C for 5 min in loading buffer, and resolved by electrophoresis through an 8% polyacrylamide-7 M urea-Tris-borate-EDTA sequencing gel. The gel was analyzed using

a PhosphorImager scanner (Molecular Dynamics). The transcriptional start site was determined by comparison with a DNA ladder obtained by sequencing plasmid pCRgrlRA-3 (Table 1), using primer CR-ORF10-HIII-A (Table 2).

Expression and purification of His-tagged H-NS and Ler proteins. *E. coli* BL21/pLys21 harboring the pT6HNS or pT6Ler plasmid (Table 1), expressing H-NS-His₆ or Ler-His₆, respectively, was grown to mid-logarithmic phase at 37°C. L-(+)-Arabinose (Sigma-Aldrich) was added to a final concentration of 0.1%, and the bacteria were further incubated for 4 h at 30°C and 250 rpm. Cells were then pelleted by centrifugation at 4°C, resuspended in urea buffer (pH 8.0) (8 M urea, 20 mM NaH₂PO₄, and 2 M Tris-HCl), and disrupted by sonication. The suspension was centrifuged at 4°C, and the supernatant was filtered through a 0.22- μ m membrane (Millipore) and applied to a HiTrap Ni²⁺-chelating column, which was loaded with 100 mM NiSO₄ and connected to a minichromatographer ÄKTA prime system (Amersham Pharmacia Biotech). Proteins were eluted with a pH gradient (pH 8.0 to 4.5) of urea buffer (8 M urea, 20 mM NaH₂PO₄, and 2 M Tris-HCl). Fractions containing purified H-NS-His₆ or Ler-His₆ were selected based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The selected fractions were loaded into a Slide-A-Lyzer 10K cassette (Pierce) and gradually dialyzed at 4°C in a buffer containing 50 mM

TABLE 2. Primers used for this study

Primer	Sequence ^a (5'-3')
Orf1-H3-R	gctctatAagctTaagtatg
CRler-260	gaaaaatggAtCggttactg
CRler-200	cctggaTCCttgatctga
CRler-160	caatacggAtcCggcgagccg
CRler-120	attaatggaTCCacaata
CRler-80	actagctGGatcCttataat
CRler-40	tttttaattggGatCCtttt
CR-ORF10-HIII-A	cccacaggaGcttcattac
CR-ORF10-HIII-B	ctgacataaGcTtcaacaataac
CR-ORF11-H3	tatacagaAgctTaccattgtaa
CR-ORF10-BHI	tgcaccacaggGatccacg
CR-ORF11-BHI	atttcctctgtGatcCggggg
CR-RORF3-BH	aaacaatcagaagGatCCcaaaagttagt
Cler-RBS-F	catgtaaggatCCgcttggtaa
ClerOrf1-R	gttcagttaaGCTtattcattta
CRorf11Xho	cagatttCtgaGccgttaattat
EpCiorf11R-H3	tactaagaAagcttcgtctaactctc
ompAPE	tttgcgctcttatcatcaa
hnsH1P1	caccaataataagtttgagattactacaatgag cgaagctgtaggctggagctgcttcg
hnsH2P2	gattttaagcaagtgcaatctacaagaattat tgcttcatatgaatatcctcctt
hnsM	tgcgagctcatcggtgtaa
hnsG	ttgctggcaaaaacctccg

^a Capital letters indicate changes in the oligonucleotide sequence with respect to the wild-type sequence, designed to introduce restriction enzyme sites.

Tris-HCl (pH 7.5), 10 mM MgCl₂, 20% glycerol, 0.5 M NaCl, 0.1% Triton X-100, and various amounts of urea (4, 1, and 0.2 M), which was changed every hour. The final dialysis was done in storage buffer containing 30 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 20% glycerol, 240 mM NaCl, 0.1% Triton X-100, and 3 mM EDTA, and aliquots of the purified proteins were stored at -70°C. Protein concentrations were determined by the Bradford procedure.

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed as follows. Approximately 100-ng samples of PCR-generated DNA fragments corresponding to the inserts carried by the *grlA-cat* fusions were mixed with increasing concentrations of purified Ler-His₆ or H-NS-His₆ protein in a buffer containing 11.7 mM Tris-HCl, pH 7.5, 0.975 mM EDTA, 78 mM NaCl, 9.75 mM 2-mercaptoethanol, 0.975 mM dithiothreitol, and 6.5% glycerol. The reactions were incubated for 30 min at room temperature and then separated by electrophoresis in 4% polyacrylamide gels in 0.45× Tris-borate-EDTA buffer at room temperature. The DNA bands were stained with ethidium bromide and visualized with an Alpha-Imager UV transilluminator (Alpha Innotech Corp.). A fragment containing the *ler* structural gene of EPEC was used as a negative control when evaluating H-NS-DNA interactions, as previously described (17).

RESULTS

cis-acting elements involved in transcriptional regulation

of *C. rodentium* *ler*. We constructed a series of transcriptional fusions to the *cat* reporter gene in plasmid pKK232-8, encompassing different lengths of the *ler* 5' upstream regulatory region, to determine the *cis*-acting elements controlling its expression (Fig. 1A). The promoterless *cat* reporter gene has proven to be a reliable system for analyzing gene expression in A/E pathogens (5, 29, 38). The *ler-cat* fusions were called pCRler-260, -200, -160, -120, -80, and -40 according to the positions of their 5' ends with respect to the transcriptional start site (12). All of the *ler-cat* fusions contained a common 3' end at position +216 with respect to the transcriptional start site (Fig. 1A). The plasmids containing the fusions were transformed into *C. rodentium* DBS100, the prototype wild-type strain (Table 1), and the CAT specific activity was determined from bacterial cultures grown under inducing conditions for

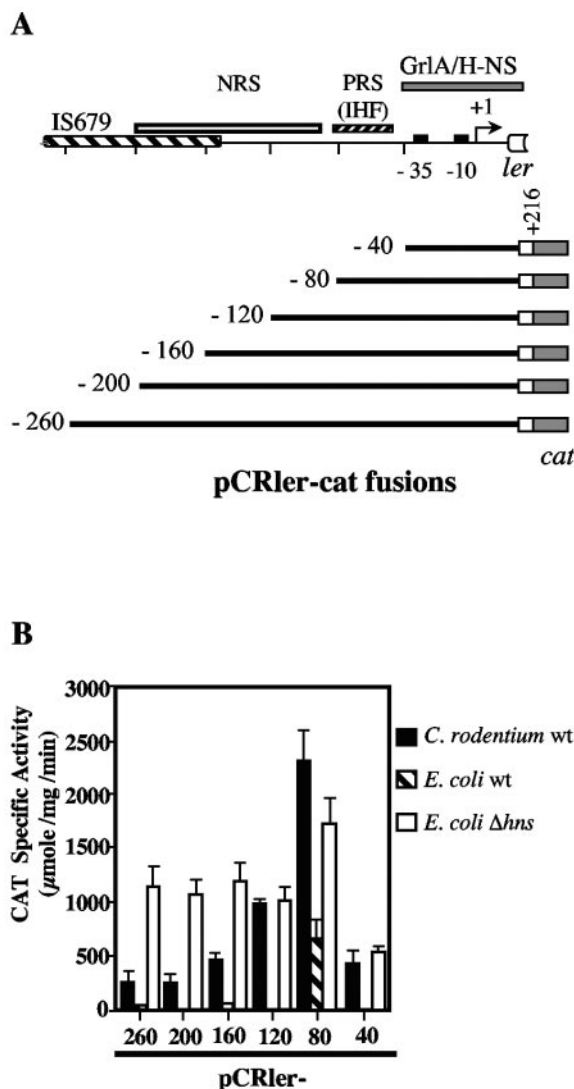


FIG. 1. Expression of *C. rodentium* *ler* is regulated by global and specific regulators. (A) Schematic representation of the *ler* regulatory region. The bent arrow indicates the previously reported transcriptional start site (+1) (12). -35 and -10 consensus sequences are shown as black boxes. A large hatched box represents the insertion sequence element (IS679) localized at the 5' end of the *C. rodentium* LEE (11). Open and hatched boxes indicate the approximate positions of negative and positive regulatory sequences (NRS and PRS), respectively, revealed by expression analysis of *ler-cat* transcriptional fusions. The PRS contains the putative IHF binding site. A gray box indicates a region required for GrlA and H-NS-mediated regulation of *ler*. Schematic representations of the *ler-cat* transcriptional fusions are shown below the diagram of the *ler* regulatory region. The *ler-cat* fusions were named pCRler and numbered according to the position of the 5' end of the *ler* region contained in each fusion with respect to the transcriptional start site. (B) Expression of the *ler-cat* fusions was monitored in *C. rodentium* DBS100, *E. coli* MC4100, and *E. coli* MC4100 Δhns. The CAT specific activity was determined from samples collected from bacterial cultures grown for 6 h in DMEM at 37°C without agitation in a 5% CO₂ atmosphere. The values are the means of at least three independent experiments performed in duplicate. Standard errors are shown with error bars.

the expression of *C. rodentium* LEE genes, as described in Materials and Methods. Fusions pCRler-260 and pCRler-200 expressed similar levels of CAT (Fig. 1B), whereas pCRler-160, -120, and -80 showed a gradual increase in CAT activity with respect to the expression shown by pCRler-200 (Fig. 1B). These results indicate that the region between positions -200 and -80 contains *cis*-acting elements that negatively control *ler* expression. The fusion pCRler-40, which still contains the *ler* promoter, showed an approximately sixfold reduced activity with respect to the expression shown by pCRler-80 (Fig. 1B), indicating the presence of positive regulatory *cis*-acting elements between positions -80 and -40. This region is equivalent to the one containing the IHF binding site previously found to be essential for *ler* expression in EPEC (19), suggesting that IHF plays a similar role in the expression of *C. rodentium* *ler*. In agreement with this hypothesis, the pCRler-80 fusion, which renders significant levels of expression in an *E. coli* K-12 strain (Fig. 1B), was no longer active in an isogenic *E. coli* *ihf* mutant (data not shown).

H-NS negatively regulates the expression of *C. rodentium* *ler*.

It has previously been reported that H-NS represses the expression of *ler* in EPEC (46). To further characterize the role of H-NS and the elements controlling the expression of *C. rodentium* *ler*, the plasmids containing the *ler*-*cat* fusions were transformed into *E. coli* K-12 and its isogenic *hns* mutant, and the CAT activity was measured after the strains were grown under inducing conditions. Expression in *E. coli* K-12 was close to the background level for all fusions except for pCRler-80 (Fig. 1B), further supporting the notion that *C. rodentium* contains specific positive regulatory factors for *ler* expression that are not present in *E. coli* K-12. In contrast, all fusions were expressed in the *E. coli* *hns* mutant at a similar or even higher level than that in *C. rodentium* (Fig. 1B), confirming the role of H-NS as a repressor of *ler* expression. Interestingly, the fusion pCRler-40, containing only the promoter, was still partially expressed in *C. rodentium* and in the *E. coli* *hns* mutant, but not in wild-type *E. coli* K-12 (Fig. 1B). This indicates that *cis*-acting elements required for positive regulation by a *C. rodentium* factor and for H-NS-mediated repression are present in the region between positions -40 and +216 of *ler*.

GrlA positively regulates the expression of *C. rodentium* *ler*.

In addition to the set of global regulators currently known to regulate *ler* expression in A/E pathogens, we have recently reported that the expression of *ler*, and thus of the LEE genes involved in the development of the A/E lesion, requires a second LEE-encoded regulator called GrlA (12). To define the regulatory region required for the GrlA-mediated activation of *ler*, we analyzed the CAT activity driven from three representative *ler* fusions (pCRler-200, -80, and -40) in wild-type *C. rodentium* and its Δ *grlA* derivative. According to the results shown in Fig. 1, pCRler-200 contains all of the regulatory elements involved in *ler* regulation, pCRler-80 lacks putative negative regulatory elements located upstream of the putative IHF binding site and showed a 10-fold increase in activity with respect to the longest fusions in the wild-type strain, and pCRler-40 contains the promoter and downstream elements involved in positive and negative regulation. In the *grlA* mutant, the transcriptional activity of pCRler-200 was reduced to background levels, confirming the requirement of GrlA for *ler* expression (Fig. 2A). The activity of pCRler-80 showed a

threefold decrease in the *grlA* mutant compared to that in the wild-type strain, indicating that even in the absence of negative *cis*-acting regulatory elements, GrlA was still needed for full *ler* promoter activation. Interestingly, the expression of pCRler-40 was also abolished in the absence of GrlA (Fig. 2A). To further confirm the direct positive role of GrlA on *ler* expression, the CAT activities of these three fusions were determined in the nonpermissive *E. coli* K-12 strain in the presence of a plasmid carrying *grlA* (pTCRgrlA1) expressed from the *lac* promoter on the vector. As shown in Fig. 2B, GrlA activated high levels of expression of fusions pCRler-200 and pCRler-40 and further increased (approximately fivefold) the activity of pCRler-80, while no changes were observed with the vector alone. Together, these results strongly suggest that GrlA is directly involved in *ler* activation, probably interacting with *cis*-acting elements located between positions -40 and +216 (Fig. 1A). In addition, these results indicated that sequences located upstream of position -40, including the putative IHF binding site, are not required for the GrlA-mediated activation of the *ler* promoter. Nonetheless, the presence of the sequence up to position -80 enhances the GrlA-dependent expression of the *ler* promoter as well as the level of GrlA-independent *ler* expression.

Autoregulation of *C. rodentium* *ler*. The autoregulation of *ler* expression was examined by performing a similar analysis of the pCRler-*cat* fusions in the *C. rodentium* *ler* mutant strain as well as in *E. coli* K-12 carrying a plasmid expressing Ler. The expression of pCRler-200, pCRler-80, and pCRler-40 showed a 4-, 1.4-, and 10-fold reduction, respectively, in the *ler* mutant compared with the expression in the wild-type strain (Fig. 2A). The high levels of expression of pCRler-80 in the Δ *ler* strain were roughly the same in the wild-type strain, supporting the proposal that this fusion lacks a negative regulatory motif that is required for repression of the *ler* promoter (see above). The results obtained with pCRler-200 and pCRler-40 suggested that *ler* expression could be directly autoregulated by its own product or indirectly regulated through an additional regulator encoded by a Ler-regulated gene. To discriminate between these two possibilities, we measured the expression of fusions pCRler-200, -80, and -40 in *E. coli* K-12 containing a plasmid carrying the *ler* gene (pTCRLer4). In contrast to the strong GrlA-mediated activation of *ler* expression in the nonpermissive *E. coli* background, the presence of Ler did not increase *ler*-*cat* expression (Fig. 2B). Conversely, the GrlA-independent expression of the *ler* promoter in pCRler-80 was reduced sevenfold in the presence of a plasmid expressing Ler (Fig. 2B), supporting the notion that Ler may negatively autoregulate its own expression to optimize its cellular levels, preventing the uncontrolled expression of LEE genes, as recently proposed (2). As a control, the expression of a transcriptional fusion to the *LEE2* promoter (pLEE2-*cat*), whose expression is Ler dependent, was measured. As expected, this fusion was not active in the presence of plasmid-encoded GrlA, while as previously shown (5), its expression was increased significantly in the presence of plasmid-encoded Ler (Fig. 2B).

Taken together, these results rule out a direct positive autoregulation of *ler* expression by Ler itself, at least in the absence of other A/E-specific factors, and suggest that Ler could be involved in regulating a positive regulatory loop by reciprocally controlling GrlA expression (see below).

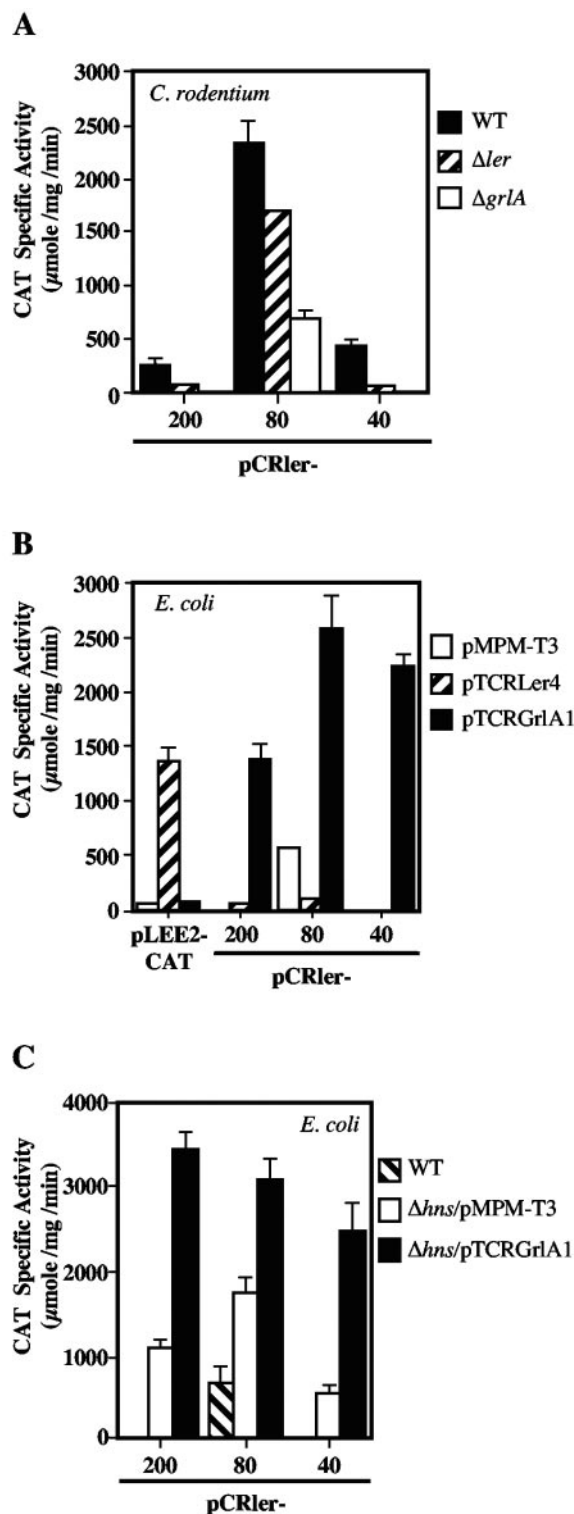


FIG. 2. GrlA is required for expression of *C. rodentium* *ler*. The expression of representative *ler-cat* fusions was monitored in *C. rodentium* DBS100, *C. rodentium* Δ *ler*, and *C. rodentium* Δ *grlA* (A) or in *E. coli* MC4100 containing the pMPPM-T3 vector or its derivative pTCRLer4 or pTCRGrIA1, expressing Ler or GrlA, respectively. As a control, the expression of a *LEE2-cat* fusion (pLEE2-CAT) was analyzed in the same strains (B). The expression of representative *ler-cat* fusions was monitored in *E. coli* MC4100 and its isogenic *hns* mutant containing plasmid pMPPM-T3 or pTCRGrIA1 (C). The CAT

Effect of GrlA on *ler* expression in the absence of H-NS. The expression of pCRler-40, which lacks the sequences upstream of the *ler* promoter, was abolished in a *C. rodentium* *grlA* mutant and restored in *E. coli* K-12 by a plasmid expressing GrlA (Fig. 2A and B). In addition, this fusion was active in the absence of H-NS (Fig. 1B) but did not reach the levels seen in wild-type *E. coli* K-12 carrying the plasmid expressing GrlA (Fig. 2B). These results led us to believe that both regulators (GrlA and H-NS) perform their function by interacting with elements located downstream of position -40 and that GrlA, although it can in part counteract H-NS-mediated repression, is essential for the efficient activation of the *ler* promoter, even in the absence of H-NS. In order to investigate this hypothesis, the expression of fusions pCRler-200, -80, and -40 in *E. coli* K-12 Δ *hns* containing plasmid pTCRGrIA1 was determined. As shown in Fig. 2C, the presence of GrlA further increased the expression of pCRler-200, -80, and -40 approximately three-, two-, and fivefold, respectively, compared to the activity observed in the *E. coli* K-12 *hns* mutant strain carrying the vector. Although other scenarios cannot be excluded at this point, two possibilities may explain this result. In addition to H-NS, another factor could also partially repress *ler* expression, and thus GrlA could counteract the total repression exerted by more than one negative regulator. Alternatively, GrlA may counteract the H-NS-mediated repression but also promote the interaction of the RNA polymerase with the *ler* promoter.

To further define the mechanism by which GrlA induces the expression of *ler*, GrlA fused to a six-His or maltose binding protein (MBP) tag was purified. Both fusion proteins restored protein secretion in the *C. rodentium* *grlA* mutant when expressed *trans* (data not shown). However, when using the purified proteins, we were unable to detect GrlA binding to DNA fragments containing the regulatory region of *ler* by EMSA, even with protein concentrations as high as 25 μ M (data not shown).

Identification of *cis*-acting elements involved in the regulation of *grlA* expression. As described above, Ler does not directly regulate its own expression, but could indirectly autoregulate it in a positive manner by reciprocally regulating GrlA expression. In order to test this hypothesis, the regulation of the *grlR* and *grlA* genes was studied using a series of transcriptional fusions containing different segments of the 5' upstream region of *grlR* and *grlA* fused to the *cat* reporter gene (Fig. 3A). Expression was measured in wild-type *C. rodentium* and its isogenic *ler* and *grlA* mutants. The tandem organization of the *grlR* and *grlA* genes suggested that they were transcribed as an operon from a promoter located upstream of *grlR*. In support of this notion, a transcriptional fusion between the *grlR-grlA* intergenic region and the *cat* reporter gene (pCRgrlIRA-4) was inactive in all three strains tested, while a fusion carrying the intergenic region between *grlR* and the divergently transcribed *rorf3* gene (pCRgrlIRA-5) was highly active in the wild-type strain (Fig. 3B). In addition, the

specific activity was determined as described for Fig. 1. The values are the means of at least three independent experiments performed in duplicate. Standard errors are shown with error bars.

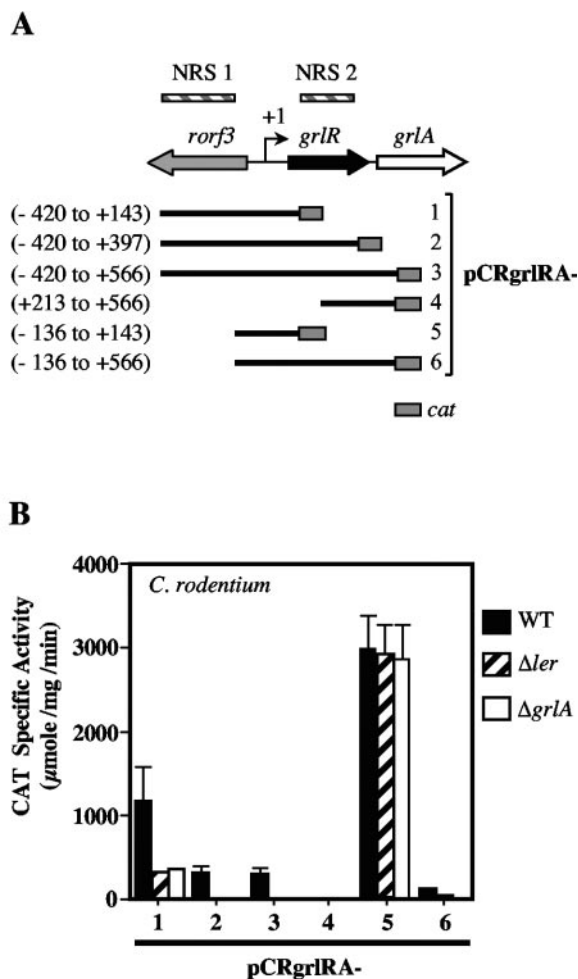


FIG. 3. Ler is required for *C. rodentium* *grlRA* expression. (A) Schematic representation of the *rorf3-grlRA* region. Hatched boxes indicate negative regulatory sequences (NRS) revealed by expression analysis of the *grlRA-cat* transcriptional fusions. The bent arrow indicates the transcriptional start site (+1) for *grlRA* determined in this study. Schematic representations of the *grlRA-cat* transcriptional fusions are shown below the diagram of the *rorf3-grlRA* region. The positions for the 5' and 3' ends of the *rorf3-grlRA* region contained in each fusion, with respect to the transcriptional start site of *grlRA*, are shown to the left of the fusions. The *grlRA-cat* fusions were named pCRgrlRA and numbered consecutively as shown at the right of the diagram. (B) Expression of the *grlRA-cat* fusions was monitored in *C. rodentium* DBS100 and its isogenic *ler* and *grlA* mutants. The CAT specific activity was determined as described for Fig. 1. The values are the means of at least three independent experiments performed in duplicate. Standard errors are shown with error bars.

expression of pCRgrlRA-5 was Ler and GrlA independent, as it was equally active in the wild-type and mutant strains (Fig. 3B). The presence of further upstream sequences in fusion pCRgrlRA-1 with respect to pCRgrlRA-5 decreased the expression of the *grlRA* promoter about 2.5-fold in the wild-type strain. In addition, the activity of this fusion was further decreased in the *ler* and *grlA* mutants, suggesting that the region from -420 to -136 with respect to the transcriptional start site (see below) contains a negative *cis*-acting element, which we named NRS1 (negative regulatory sequence 1), and a putative Ler binding region. The presence of further downstream elements in fusions pCRgrlRA-2 (down to the end of *grlR*) and

pCRgrlRA-3 (down to the 5' end of *grlA*) with respect to pCRgrlRA-1 (Fig. 3A) reduced their transcriptional activity about fourfold in the wild-type strain, but they were still Ler and GrlA dependent, as their expression was abolished in the mutant strains (Fig. 3B). Since the activities of pCRgrlRA-2 and pCRgrlRA-3 were very similar, these results suggested the presence of a second negative regulatory element (NRS2) between positions +143 and +397 with respect to the *grlR* transcriptional start site. In agreement with these observations, fusion pCRgrlRA-6, which contains the *rorf3-grlRA* intergenic region carried by pCRgrlRA-5 plus the NRS2 motif, was 36-fold less active in the wild-type strain than was pCRgrlRA-5 (Fig. 3B).

Taken together, this analysis demonstrated that *grlR* and *grlA* form an operon under the control of a promoter located upstream of *grlR*. In addition, it revealed that sequences flanking the *grlRA* operon promoter, named NRS1 and NRS2 in this study, are involved in its negative regulation as well as its Ler- and GrlA-dependent activation. In the absence of these elements, *grlRA* expression becomes constitutive, resembling the regulation of other Ler-dependent promoters (5, 24, 38).

To further support the role of Ler and GrlA in the regulation of the *grlRA* promoter in *C. rodentium* and to map the promoter, primer extension analysis was performed using total RNAs purified from the wild-type strain and the *ler* and *grlA* mutants. A predominant primer extension product was detected for the wild-type strain (Fig. 4A), revealing that the transcriptional start site of the *grlRA* promoter corresponds to the T residue located 102 bp upstream of the *grlR* start codon (Fig. 4B). Putative promoter sequences which show identity to the consensus -10 (five of six [TATAT]) and -35 (four of six [TTGAA]) sequences of sigma 70-dependent promoters were found upstream of the *grlRA* transcriptional start site (Fig. 4B). This promoter closely matches the promoter previously reported for EPEC orf10/*grlR* (31).

In contrast, a primer extension product was not detected in the *ler* mutant, in agreement with a previous report showing that the expression of the orf10 (*grlR*) transcript in EPEC was reduced in the absence of Ler (16). Similarly, *grlRA* transcription was reduced in the *grlA* mutant (Fig. 4A). To control the RNA load size and integrity, primer extension was performed in parallel to detect the expression of *ompA*, a constitutively expressed gene coding for an outer membrane protein (17). As shown in Fig. 4A, the *ompA* transcript was detected at similar levels in the wild-type strain and the *ler* and *grlA* mutants.

Ler directly regulates the expression of the *grlRA* operon. To further confirm the role of Ler on *grlRA* regulation, the expression of fusions pCRgrlRA-1, -3, and -6 was analyzed in the nonpermissive *E. coli* K-12 strain in the presence of a plasmid expressing Ler (pTCRLer4) or GrlA (pTCRGrIA1). The expression levels of these fusions were slightly above the background in the presence of only the vector or the plasmid expressing GrlA (Fig. 5A). In contrast, significant levels of expression were obtained in the presence of Ler (Fig. 5A). This pattern of expression resembles the regulation of the *LEE2-cat* control fusion (Fig. 5A), which is directly regulated by Ler (5).

Taken together, these results highlight the existence of a novel positive regulatory loop where GrlA and Ler reciprocally regulate each other to modulate the expression of LEE genes.

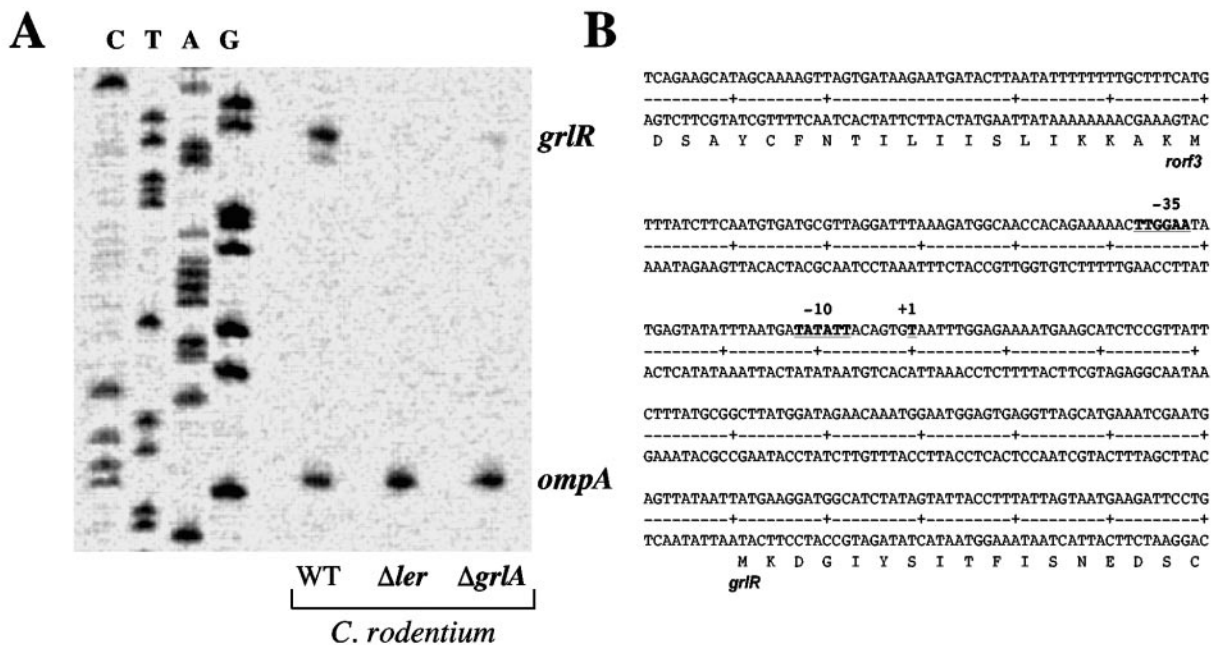


FIG. 4. Primer extension analysis of the *C. rodentium* *grlRA* promoter region. (A) Total RNAs were obtained from culture samples of *C. rodentium* wild-type (WT), Δ ler, and Δ grlA strains grown for 6 h in DMEM at 37°C without agitation in a 5% CO₂ atmosphere. Primer extension assays were performed with purified total RNA and a primer specific for the *grlR* structural gene or a primer specific for *ompA*, which was used as a control. (B) Sequence of the intergenic region between *rorf3* and *grlRA*. The transcriptional start site (+1) and the -10 and -35 promoter sequences for *grlRA* are shown with bold underlined letters.

H-NS is a negative regulator of *grlRA* expression. Previous reports indicated that Ler induces LEE gene expression by counteracting the repression exerted by H-NS on their promoters (5, 24, 46). The results described above indicated that *grlRA* is positively regulated by Ler and subjected to negative regulation resembling that of other Ler-regulated genes. In order to evaluate whether H-NS is involved in the negative regulation of *grlRA*, we measured the expression of the *grlRA-cat* fusions (Fig. 3A) in the *E. coli* K-12 strain and its isogenic *hns* mutant. Increased CAT activity was observed for all fusions in the *hns* mutant, except for pCRgrlRA-4 (which lacks the *grlRA* promoter), indicating that H-NS negatively regulates *grlRA* expression (Fig. 5B). However, the fact that the *grlRA-cat* fusions were expressed at different levels in the *hns* mutant suggested that, in addition to H-NS, other regulators could be involved in repressing *grlRA* expression. In this regard, compared to pCRgrlRA-1, the pCRgrlRA-2 and pCRgrlRA-3 fusions were between three- and sixfold less active in the *hns* mutant. This difference could be due to the presence of the *grlR* gene in these fusions, either because the structural sequence contains *cis*-acting negative regulatory motifs or because the expression of GrlR, which has been shown to act as a repressor of LEE gene expression (12, 26, 27), has a negative effect on the expression of its own promoter. However, further studies are needed to distinguish between these possibilities.

Fusion pCRgrlRA-5 was also expressed in *E. coli* K-12, further supporting the notion that it lacks negative *cis*-acting regulatory elements; however, its expression was further increased (approximately fivefold) in the *hns* mutant (Fig. 5B). This observation suggests that H-NS negatively controls *grlRA* expression by interacting with the *rorf3-grlRA* intergenic region

in the vicinity of the promoter between positions -136 and +143. The presence of Ler did not further increase the expression of the *grlRA-cat* fusions in the *E. coli hns* mutant (Fig. 5B), strongly suggesting that Ler induces *grlRA* expression by mainly counteracting the H-NS-mediated repression of this promoter.

Since different attempts to delete or interrupt the *C. rodentium hns* gene have so far been unsuccessful (despite our success in the generation of deletion mutants in *C. rodentium* [12]), the experiments described above were performed with *E. coli* strains. The *C. rodentium hns* gene, as provided by the Wellcome Trust Sanger Institute, codes for a protein sharing 96% identity with *E. coli* H-NS, with six amino acid changes located outside functional domains (data not shown). This high degree of conservation suggests that the two proteins are functionally equivalent. In order to confirm the role of H-NS in the transcriptional repression of the *grlRA* promoter in *C. rodentium*, we took advantage of the dominant-negative effect shown by *E. coli* H-NS mutants that are defective in the ability to repress transcription but not in the ability to interact with other H-NS monomers (45). Plasmids expressing *E. coli* H-NS and the H-NS R12C and G113D mutants under the control of an arabinose-inducible promoter (4, 5) were introduced into *C. rodentium* Δ ler carrying the fusion plasmid pCRgrlRA-1 to determine the CAT activity in the presence or absence of arabinose. The expression of the *grlRA* promoter in the Δ ler strain was further repressed when wild-type H-NS was induced in *C. rodentium* Δ ler. In contrast, when the R12C or G113D H-NS mutant was induced, a dominant-negative effect that allowed the expression of the *grlRA* promoter was observed

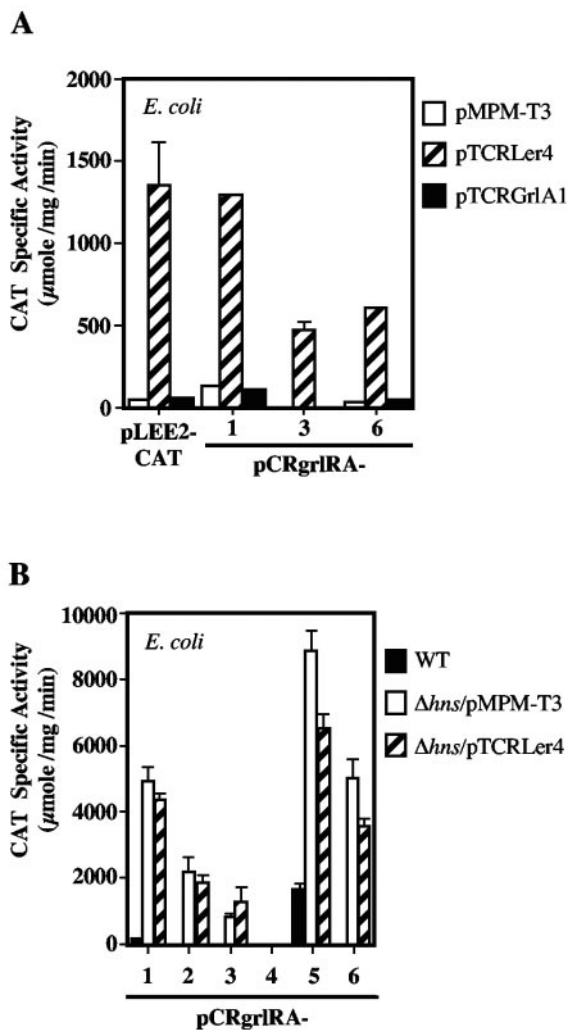


FIG. 5. Ler activates and H-NS represses the expression of *grlRA-cat* fusions in an *E. coli* K-12 strain. (A) The expression of representative *grlRA-cat* fusions was monitored in *E. coli* MC4100 containing plasmid pMPM-T3 (vector), pTCRLer4, or pTCRGrIA1. As a control, the expression of pLEE2-CAT was analyzed in the same strains. (B) H-NS mediates repression of *grlRA* expression. The expression of *grlRA-cat* fusions was monitored in *E. coli* MC4100 and its isogenic *hns* mutant containing plasmid pMPM-T3 (vector) or pTCRLer4 (*ler*). The CAT specific activity was determined as described for Fig. 1. The values are the means of at least three independent experiments performed in duplicate. Standard errors are shown with error bars.

(data not shown). These results are in agreement with those obtained using *E. coli* strains (Fig. 5B).

Ler and H-NS bind to different motifs in the *rorf3-grlRA* region. In order to identify the DNA binding sites of Ler and H-NS in the *grlRA* region, EMSAs with purified Ler-His₆ and H-NS-His₆ proteins and PCR products corresponding to the fragments contained in the *grlRA-cat* fusions were performed (Fig. 6A). These experiments demonstrated that Ler binds to DNA fragments corresponding to those present in pCRgrlRA-2, pCRgrlRA-3, and pCRgrlRA-6, starting at a concentration of 480 nM, whereas no binding was detected to pCRgrlRA-4 or -5 fragments, even at a concentration of 1.4 µM (Fig. 6B). The common region between pCRgrlRA-2, -3, and -6 which

is not present in pCRgrlRA-4 and -5 is located within the *grlR* structural sequence between positions +143 and +213 (Fig. 6A), indicating that this region contains sequences recognized by Ler.

Binding of Ler to the fragment contained in pCRgrlRA-1 at higher concentrations (Fig. 6B, bottom panel) revealed the presence of an additional lower-affinity binding site. In agreement with this observation, this fusion was still regulated by Ler (Fig. 3B and 5A). The lack of binding to the fragment contained in pCRgrlRA-5 at the same protein concentrations (Fig. 6B, bottom panel) suggested that this putative Ler binding site is located between positions -420 and -136, within the structural sequence of *rorf3*. Ler binding to fragment 3 generates at least two distinctive complexes (Fig. 6B), suggesting that the binding of Ler to the higher-affinity binding site precedes subsequent binding to the lower-affinity binding site. The expression analysis of *grlRA-cat* fusions described above suggested that both Ler binding sites could independently mediate *grlRA* induction, since the expression of *grlRA-cat* fusions containing only one of these binding sites (pCRgrlRA-1 or pCRgrlRA-6) was still Ler dependent (Fig. 3B and 5A). More defined deletions and site-directed mutagenesis will be required to further map the Ler binding sites involved in *grlRA* expression, since footprinting analysis has shown that Ler binds to extended regions, complicating the definition of primary binding sites (2, 24, 43; our unpublished results).

Using the same approach, we showed that H-NS binds to the fragments carried by fusions pCRgrlRA-3, -4, and -6, at concentrations ranging from 430 to 750 nM, but not to fragments contained in fusions pCRgrlRA-1, -2, and -5 or to a DNA fragment corresponding to the *ler* structural gene, which was used as a negative control for Ler and H-NS binding (Fig. 6C and data not shown). Fragments pCRgrlRA-3, -4, and -6 share a common region that is absent in pCRgrlRA-1, -2, and -5, localized between positions +397 and +566 spanning the last codons of *grlR* and the first codons of *grlA*, indicating that this region contains sequences recognized by H-NS. However, considering that fusions pCRgrlRA-2 and pCRgrlRA-3 have very similar regulatory patterns (Fig. 3B and 5B), it is likely that this binding site does not play a major role in the negative regulation of *grlRA* expression.

Since fusions pCRgrlRA-1 and pCRgrlRA-5 are still strongly regulated by H-NS (Fig. 5B), another EMSA was performed using higher concentrations of H-NS to explore the existence of lower-affinity binding sites in the vicinity of the *grlRA* promoter region. At concentrations between 1.6 and 2.3 µM, H-NS bound to the DNA fragments corresponding to pCRgrlRA-1 and -5, but not to the negative control (Fig. 6C, bottom panel), indicating that the sequence contained in pCRgrlRA-5 spanning positions -136 to +143 is bound by H-NS to repress *grlRA* expression.

DISCUSSION

Different studies have demonstrated that Ler is the primary positive regulator of virulence gene expression in A/E bacterial pathogens (12, 16, 31). Ler expression is finely regulated by a myriad of regulatory factors, as described in the introduction. In addition to all of the regulatory proteins shown thus far to be involved in *ler* regulation, it was recently shown that *ler* expression, and thus the expression of

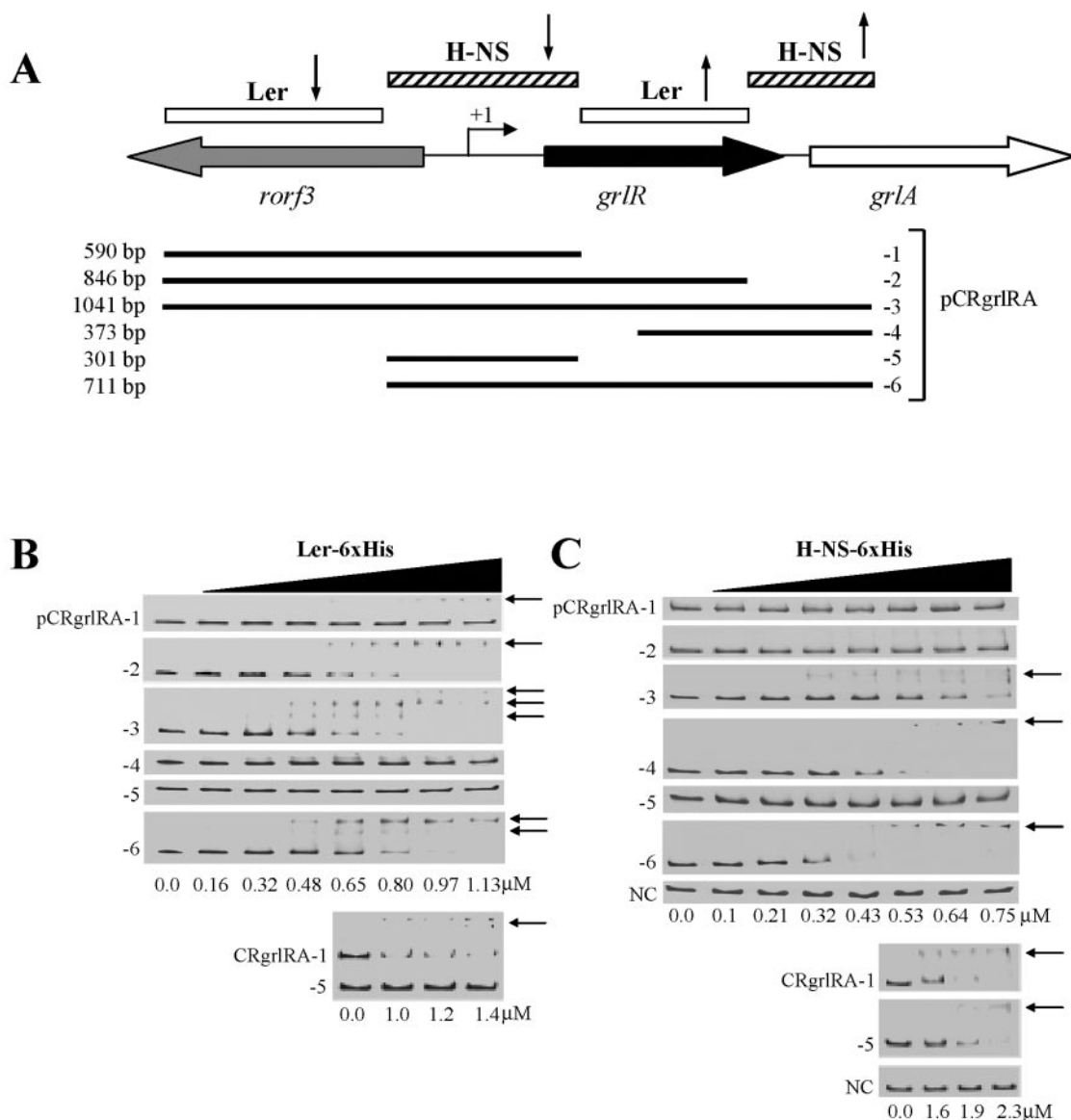


FIG. 6. Binding of Ler and H-NS to the *rorf3-grlRA* region. (A) Schematic representation of the *rorf3-grlRA* region. The bent arrow indicates the *grlRA* transcriptional start site determined in this study. The binding regions for H-NS and Ler revealed by EMSAs are represented by hatched and open boxes, respectively. The DNA fragments used in EMSAs are represented below the diagram of the *rorf3-grlRA* region. The sizes of the fragments are indicated to the left, and the corresponding fusion numbers are indicated to the right. Increasing concentrations of purified Ler-His₆ (B) or H-NS-His₆ (C) protein were incubated with the PCR-generated DNA fragments represented in panel A, resolved in 4% polyacrylamide gels, and stained with ethidium bromide. The fragments correspond to the pCRgrlRA transcriptional fusions, as indicated to the left of the gels. The EPEC *ler* structural gene was used as a negative control (NC) for the EMSAs. Arrows indicate DNA-protein complexes.

other LEE genes, requires the additional LEE-encoded regulator GrIA (12).

In the present study, we demonstrate that GrIA and Ler positively regulate each other's expression, forming a novel transcriptional positive regulatory loop. This notion is supported by the results showing that *ler* expression was severely reduced in a *C. rodentium* *grlA* mutant and restored by GrIA in the nonpermissive *E. coli* K-12 background (Fig. 2), while the expression of the *grlRA* operon was impaired in a *C. rodentium* *ler* mutant (Fig. 3 and 4) and restored in *E. coli* K-12 by Ler (Fig. 5A). The complementation experiments with *E. coli* K-12 clearly reproduced the reciprocal regulation between GrIA and

Ler observed in the experiments performed with *C. rodentium* mutants.

Our results also indicate that Ler positively regulates the expression of *grlRA* by counteracting, at least in part, the H-NS-mediated repression of its promoter (Fig. 5B). In this regard, we and other groups have shown that H-NS exerts a global repressing effect on EPEC LEE promoters and that Ler acts as an antirepressor counteracting this negative effect (5, 24, 46). For example, H-NS-mediated repression of the divergently transcribed *LEE2* and *LEE3* operons involves the binding of H-NS to silencer regulatory sequences 1 and 2 (SRS1 and -2) flanking the *LEE2* and *LEE3* promoters, which favors

the formation of a repressor nucleoprotein complex that is probably stabilized by H-NS–H-NS bridging interactions (4, 5). Specific binding of Ler to SRS1 destabilizes the repressor nucleoprotein complex and releases the expression of the *LEE2* and *LEE3* operons. The expression of both operons is constitutive and is no longer affected by Ler in the absence of any of the SRSs or of H-NS (4, 5). A similar model has been proposed for the regulation of the *LEE5* operon (24). However, overcoming transcriptional repression by H-NS is a common mechanism for inducing virulence gene expression in pathogenic bacteria and involves different families of transcriptional activators (reviewed in reference 14).

In agreement with their role in *grlRA* regulation, H-NS and Ler bind to nonoverlapping sites in the *rorf3-grlRA* region. DNA binding assays showed that a higher-affinity H-NS-binding site is located between the 3' end of *grlR* and the 5' end of *grlA* and a lower-affinity H-NS-binding site is located in the intergenic region between *rorf3* and *grlRA*. In contrast, for Ler a lower-affinity binding site is contained within the *rorf3* structural gene and a higher-affinity Ler binding site is located at the beginning of the *grlR* structural gene flanking the *grlRA* promoter (Fig. 6). The lower-affinity H-NS-binding site, but not the higher-affinity H-NS-binding site, seems to be the one involved in the repression of the *grlRA* promoter, as all the *grlRA-cat* fusions containing the *rorf3-grlRA* intergenic region were derepressed in the Δhns background (Fig. 5B). In contrast, both Ler binding sites could independently mediate *grlRA* induction by Ler, as fusions carrying one or the other were still regulated in a Ler-dependent manner (Fig. 3B and 5A). It is likely that the binding of Ler to sequences flanking the *grlRA* promoter region, where the H-NS-binding site resides, induces structural changes that may destabilize H-NS binding, thus releasing promoter expression. However, H-NS is not fully responsible for the negative regulation, since activation of the different pCR*grlRA* fusions showed different degrees of derepression in its absence (Fig. 5B). The fact that derepression was only partial in the presence of one or both NRS elements in the Δhns background suggests that an additional factor or mechanism which is not yet defined is required for a second level of repression. Thus, in contrast to the case for the *LEE2* and *LEE3* promoters, full strength Ler-independent expression of the *grlRA* promoter is only achieved in the absence of H-NS and both NRSs. Considering the putative role of GrlR as a repressor of LEE gene expression (12, 27), we cannot rule out the possibility that the presence of the *grlR* gene in some of the pCR*grlRA* fusions has a negative influence on its own expression. H-NS also represses the expression of *ler* in *E. coli* K-12, but in contrast to the *grlRA* and *LEE2-LEE3* promoters, the *ler* promoter does not become fully constitutive (e.g., GrlA independent) in the absence of H-NS or negative *cis*-acting regulatory elements.

It has been previously reported that H-NS represses *ler* expression at 27°C, but not at 37°C, as a mechanism controlling thermoregulation (46). Our observations confirm the role of H-NS in *ler* regulation, but they also show that H-NS can exert its negative effect even at 37°C in the absence of *ler*-specific activators. They also indicate that both H-NS and GrlA require sequences located in close proximity to the *ler* promoter to exert their functions.

The results reported here indicate that GrlA is required for

promoter activation, probably favoring productive interactions of the RNA polymerase with the *ler* promoter, as well as for counteracting H-NS repression. Similar double functions have been observed, for example, for the regulator ToxT in the expression of *ctx* and *tcp* (48). GrlA contains a putative helix-turn-helix motif potentially involved in DNA binding (12). Mutations of this domain at residues that are conserved in CaiF and the *Salmonella* GrlA homologue abolish GrlA's ability to activate *ler* expression (unpublished observations). However, despite all the evidence implicating GrlA in binding to DNA, we have not yet been able to detect GrlA binding to the *ler* promoter region by EMSAs using purified MBP-GrlA and GrlA-His₆ fusion proteins, which fully complement the *C. rodentium* *grlA* mutant strain (data not shown). The lack of binding in vitro may be the result of different situations, including the possibility that GrlA may become inactive upon purification or that it requires another factor for DNA binding. Correlating with the second possibility, it has been shown that CaiF, the only characterized homologue of GrlA, binds more efficiently to the intergenic *cai-fx* regulatory region when CRP is present (3) and also counteracts H-NS repression (15).

Furthermore, IHF has been shown to be essential for *ler* expression in EPEC (19) and for pCR*ler-cat* fusion expression in *E. coli* K-12 (unpublished results), making it a candidate for acting synergistically with GrlA to activate *ler* expression. However, our results suggest that IHF is not necessary for the GrlA-mediated activation of *ler*, since in the absence of the putative IHF binding site, as for pCR*ler-40*, GrlA was still able to activate *ler* expression (Fig. 2). Similarly, a transcriptional fusion of the EPEC *ler* regulatory region lacking the IHF binding sequence was still activated in a GrlA-dependent manner (unpublished results). It is worth noting that pCR*ler-80* rendered significant levels of GrlA-independent expression of the *ler* promoter in *C. rodentium* $\Delta grlA$ and *E. coli* K-12 (Fig. 1B and 2B). These observations suggest that upstream of position –80, there is a putative negative regulatory motif that negatively modulates *ler* repression. In support of this notion, it has been shown that Hha negatively regulates *ler* expression in EHEC and interacts with its regulatory region (40). These results also suggest that binding of IHF to its putative binding site, located between position –80 and the *ler* promoter, may generate architectural changes that partially counteract the negative regulation mediated by, for example, H-NS and/or facilitate RNA polymerase productive interactions with the *ler* promoter in the absence of GrlA.

It is not yet possible to determine whether Ler or GrlA is responsible for initiating the feedback regulatory loop. However, it is tempting to suggest that under inducing conditions, preexisting basal levels of Ler and/or GrlA adopt a transcriptionally proficient conformation that allows the reciprocal activation of the *grlRA* or *ler* promoter, respectively. Alternatively, or in parallel, the initial increase in *ler* or *grlRA* expression could be mediated by DNA structural changes that set the promoters to a more competent transcriptional state or by additional regulatory proteins in response to specific environmental cues. In this way, the active feedback loop will increase the cellular concentration of Ler, which then specifically counteracts the H-NS-mediated repression of several LEE and non-LEE promoters. To prevent the detrimental accumulation of Ler or of the proteins encoded by Ler-regu-

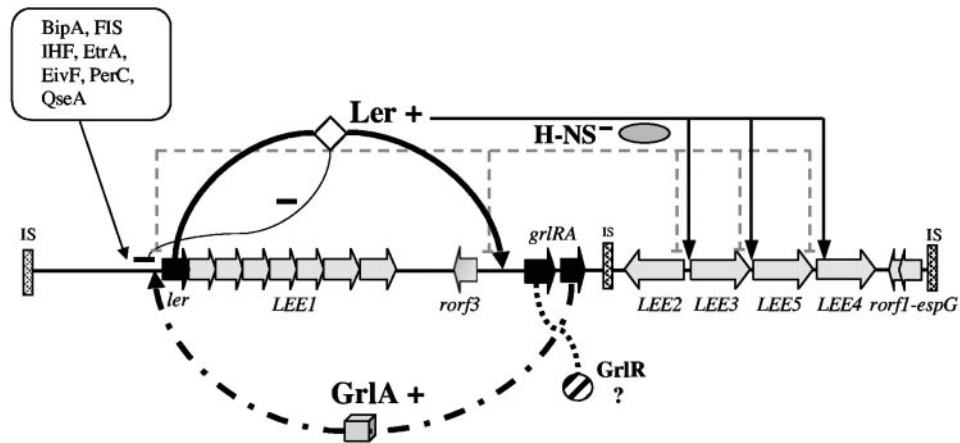


FIG. 7. Model for the regulation of LEE genes in A/E pathogens. Ler positively regulates LEE gene expression by counteracting the H-NS-mediated repression of LEE gene promoters. The expression of *ler* is tightly regulated by specific regulators, such as GrlA, GrlR, and PerC, as well as by global regulators, such as IHF, Fis, BipA, EtrA, EivF, and QseA. Appropriate levels of *ler* expression are maintained by a positive regulatory loop formed by Ler and GrlA, which could be negatively modulated by GrlR through a mechanism that is still not well understood or by the ability of Ler to negatively autoregulate its own expression (see the text).

lated genes in the cell, the Ler-GrlA feedback loop could be negatively modulated when Ler reaches the threshold concentration that represses *ler* transcription, as recently proposed (2). Alternatively, other elements could establish a checkpoint to prevent Ler overexpression. One candidate is GrlR, a protein encoded by the first gene of the *grlRA* operon that has shown to be involved in the negative regulation of *ler* expression and thus of Ler-regulated genes (12, 26, 27). Intriguingly, as shown here, *grlR* is cotranscribed with *grlA* in a Ler-dependent manner, suggesting that, while the feedback loop is active, GrlR may reach a concentration that down regulates the feedback loop to set it back to the steady-state level. We propose that the Ler-GrlA positive regulatory loop is functionally similar in all A/E pathogens, since the expression of LEE-encoded proteins is also abolished in EPEC and EHEC *grlA* mutants (unpublished results) and since *grlRA* (*orf10-11*) expression is abolished in *ler* mutants (16; unpublished results). In this way, the concentration of Ler required for the appropriate induction of the LEE genes in A/E pathogens would be maintained by the combined action of positive and negative regulatory loops. A model for the regulation of LEE genes, with emphasis on the positive and negative regulatory loops controlling the expression of Ler, is depicted in Fig. 7.

In summary, we identified a novel regulatory mechanism involving a reciprocal positive regulatory circuit integrated by the LEE-encoded positive regulatory proteins Ler and GrlA. Although the role of this regulatory loop during infection remains to be elucidated, it is probably required to maintain appropriate levels of different regulatory proteins to achieve a precise and optimal spatiotemporal response to the host environment. This would allow the successful colonization of the preferred niche and prevent the disproportionate production of virulence factors that could potentially jeopardize subsequent stages of the infectious process.

ACKNOWLEDGMENTS

We are particularly thankful to R. Oropeza and K. Carrillo for their support with H-NS and Ler purification, to J. A. Ibarra for help with early

primer extension experiments, to C. Lara and M. I. Villalba for their help with GrlA purification and binding assays, to J. M. Tellez for his advice, and to A. Vázquez and F. J. Santana for their excellent technical assistance. We also thank E. Calva for helpful discussions and continuous support.

J.B. is supported by a Ph.D. fellowship from CUNACyT. J.L.P. is funded by Dirección General de Asuntos del Personal Académico (DGAPA), Consejo Nacional de Ciencia y Tecnología (CONACyT), and the Howard Hughes Medical Institute (HHMI). B.B.F. is supported by grants from Canadian Institutes of Health Research (CIHR) and the HHMI. J.L.P. and B.B.F. are HHMI International Research Scholars, and B.B.F. is a CIHR Distinguished Scientist and the Peter Wall Distinguished Professor.

REFERENCES

1. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1989. Current protocols in molecular biology. John Wiley and Sons, New York, N.Y.
2. Berdichevsky, T., D. Friedberg, C. Nadler, A. Rokney, A. Oppenheim, and I. Rosenshine. 2005. Ler is a negative autoregulator of the *LEE1* operon in enteropathogenic *Escherichia coli*. *J. Bacteriol.* **187**:349–357.
3. Buchet, A., W. Nasser, K. Eichler, and M. A. Mandrand-Berthelot. 1999. Positive co-regulation of the *Escherichia coli* carnitine pathway *cai* and *fix* operons by CRP and the CafF activator. *Mol. Microbiol.* **34**:562–575.
4. Bustamante, V. H., J. A. Ibarra, K. Carrillo, A. Vazquez, and J. L. Puente. 2004. The locus of enterocyte effacement-encoded regulator (Ler) overcomes repression of type III secretion operons by modifying a nucleoprotein complex formed by H-NS. Presented at the 104th General Meeting of the American Society for Microbiology, New Orleans, La., 23 to 27 May 2004.
5. Bustamante, V. H., F. J. Santana, E. Calva, and J. L. Puente. 2001. Transcriptional regulation of type III secretion genes in enteropathogenic *Escherichia coli*: Ler antagonizes H-NS-dependent repression. *Mol. Microbiol.* **39**:664–678.
6. Casadaban, M. J. 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J. Mol. Biol.* **104**:541–555.
7. Chen, H. D., and G. Frankel. 2005. Enteropathogenic *Escherichia coli*: unravelling pathogenesis. *FEMS Microbiol. Rev.* **29**:83–98.
8. Clarke, S. C., R. D. Haigh, P. P. Freestone, and P. H. Williams. 2003. Virulence of enteropathogenic *Escherichia coli*, a global pathogen. *Clin. Microbiol. Rev.* **16**:365–378.
9. Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640–6645.
10. Dean, P., M. Maresca, and B. Kenny. 2005. EPEC's weapons of mass subversion. *Curr. Opin. Microbiol.* **8**:28–34.
11. Deng, W., Y. Li, B. A. Vallance, and B. B. Finlay. 2001. Locus of enterocyte effacement from *Citrobacter rodentium*: sequence analysis and evidence for

- horizontal transfer among attaching and effacing pathogens. *Infect. Immun.* **69**:6323–6335.
12. Deng, W., J. L. Puente, S. Gruenheid, Y. Li, B. A. Vallance, A. Vazquez, J. Barba, J. A. Ibarra, P. O'Donnell, P. Metanikov, K. Ashman, S. Lee, D. Goode, T. Pawson, and B. B. Finlay. 2004. Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proc. Natl. Acad. Sci. USA* **101**:3597–3602.
 13. Deng, W., B. A. Vallance, Y. Li, J. L. Puente, and B. B. Finlay. 2003. *Citrobacter rodentium* translocated intimin receptor (Tir) is an essential virulence factor needed for actin condensation, intestinal colonization and colonic hyperplasia in mice. *Mol. Microbiol.* **48**:95–115.
 14. Dorman, C. J. 2004. H-NS: a universal regulator for a dynamic genome. *Nat. Rev. Microbiol.* **2**:391–400.
 15. Eichler, K., A. Buchet, R. Lemke, H. P. Kleber, and M. A. Mandrand-Berthelot. 1996. Identification and characterization of the *cafF* gene encoding a potential transcriptional activator of carnitine metabolism in *Escherichia coli*. *J. Bacteriol.* **178**:1248–1257.
 16. Elliott, S. J., V. Sperandio, J. A. Giron, S. Shin, J. L. Mellies, L. Wainwright, S. W. Hutcheson, T. K. McDaniel, and J. B. Kaper. 2000. The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect. Immun.* **68**:6115–6126.
 17. Flores-Valdez, M. A., J. L. Puente, and E. Calva. 2003. Negative osmoregulation of the *Salmonella ompS1* porin gene independently of OmpR in an *hns* background. *J. Bacteriol.* **185**:6497–6506.
 18. Friden, P., K. Voelkel, R. Sternglanz, and M. Freundlich. 1984. Reduced expression of the isoleucine and valine enzymes in integration host factor mutants of *Escherichia coli*. *J. Mol. Biol.* **172**:573–579.
 19. Friedberg, D., T. Umanski, Y. Fang, and I. Rosenshine. 1999. Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic *Escherichia coli*. *Mol. Microbiol.* **34**:941–952.
 20. Garmendia, J., G. Frankel, and V. F. Crepin. 2005. Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: translocation, translocation, translocation. *Infect. Immun.* **73**:2573–2585.
 21. Goldberg, M. D., M. Johnson, J. C. Hinton, and P. H. Williams. 2001. Role of the nucleoid-associated protein Fis in the regulation of virulence properties of enteropathogenic *Escherichia coli*. *Mol. Microbiol.* **41**:549–559.
 22. Gottesman, M. E., and M. B. Yarmolinsky. 1968. Integration-negative mutants of bacteriophage lambda. *J. Mol. Biol.* **31**:487–505.
 23. Grant, A. J., M. Farris, P. Alefounder, P. H. Williams, M. J. Woodward, and C. D. O'Connor. 2003. Co-ordination of pathogenicity island expression by the BipA GTPase in enteropathogenic *Escherichia coli* (EPEC). *Mol. Microbiol.* **48**:507–521.
 24. Haack, K. R., C. L. Robinson, K. J. Miller, J. W. Fowlkes, and J. L. Mellies. 2003. Interaction of Ler at the *LEE5* (*tir*) operon of enteropathogenic *Escherichia coli*. *Infect. Immun.* **71**:384–392.
 25. Iyoda, S., and H. Watanabe. 2004. Positive effects of multiple *pch* genes on expression of the locus of enterocyte effacement genes and adherence of enterohaemorrhagic *Escherichia coli* O157:H7 to HEp-2 cells. *Microbiology* **150**:2357–2371.
 26. Iyoda, S., and H. Watanabe. 2005. ClpXP protease controls expression of the type III protein secretion system through regulation of RpoS and GrlR levels in enterohemorrhagic *Escherichia coli*. *J. Bacteriol.* **187**:4086–4094.
 27. Lio, J. C., and W. J. Syu. 2004. Identification of a negative regulator for the pathogenicity island of enterohemorrhagic *Escherichia coli* O157:H7. *J. Biomed. Sci.* **11**:855–863.
 28. Luperchio, S. A., J. V. Newman, C. A. Dangler, M. D. Schrenzel, D. J. Brenner, A. G. Steigerwalt, and D. B. Schauer. 2000. *Citrobacter rodentium*, the causative agent of transmissible murine colonic hyperplasia, exhibits clonality: synonymy of *C. rodentium* and mouse-pathogenic *Escherichia coli*. *J. Clin. Microbiol.* **38**:4343–4350.
 29. Martinez-Laguna, Y., E. Calva, and J. L. Puente. 1999. Autoactivation and environmental regulation of *bfpT* expression, the gene coding for the transcriptional activator of *bfpA* in enteropathogenic *Escherichia coli*. *Mol. Microbiol.* **33**:153–166.
 30. Mayer, M. P. 1995. A new set of useful cloning and expression vectors derived from pBlueScript. *Gene* **163**:41–46.
 31. Mellies, J. L., S. J. Elliott, V. Sperandio, M. S. Donnenberg, and J. B. Kaper. 1999. The Per regulon of enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). *Mol. Microbiol.* **33**:296–306.
 32. Mellies, J. L., F. Navarro-Garcia, I. Okeke, J. Frederickson, J. P. Nataro, and J. B. Kaper. 2001. *espC* pathogenicity island of enteropathogenic *Escherichia coli* encodes an enterotoxin. *Infect. Immun.* **69**:315–324.
 33. Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* **11**:142–201.
 34. Pallen, M. J., S. A. Beatson, and C. M. Bailey. 2005. Bioinformatics analysis of the locus for enterocyte effacement provides novel insights into type-III secretion. *BMC Microbiol.* **5**:9.
 35. Porter, M. E., P. Mitchell, A. Free, D. G. Smith, and D. L. Gally. 2005. The *LEE1* promoters from both enteropathogenic and enterohemorrhagic *Escherichia coli* can be activated by PerC-like proteins from either organism. *J. Bacteriol.* **187**:458–472.
 36. Porter, M. E., P. Mitchell, A. J. Roe, A. Free, D. G. Smith, and D. L. Gally. 2004. Direct and indirect transcriptional activation of virulence genes by an AraC-like protein, PerA from enteropathogenic *Escherichia coli*. *Mol. Microbiol.* **54**:1117–1133.
 37. Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 38. Sanchez-SanMartin, C., V. H. Bustamante, E. Calva, and J. L. Puente. 2001. Transcriptional regulation of the *orf19* gene and the *tir-cesT-ee* operon of enteropathogenic *Escherichia coli*. *J. Bacteriol.* **183**:2823–2833.
 39. Schauer, D. B., and S. Falkow. 1993. Attaching and effacing locus of a *Citrobacter freundii* biotype that causes transmissible murine colonic hyperplasia. *Infect. Immun.* **61**:2486–2492.
 40. Sharma, V. K., and R. L. Zuerner. 2004. Role of *hha* and *ler* in transcriptional regulation of the *esp* operon of enterohemorrhagic *Escherichia coli* O157:H7. *J. Bacteriol.* **186**:7290–7301.
 41. Shin, S., M. P. Castanie-Cornet, J. W. Foster, J. A. Crawford, C. Brinkley, and J. B. Kaper. 2001. An activator of glutamate decarboxylase genes regulates the expression of enteropathogenic *Escherichia coli* virulence genes through control of the plasmid-encoded regulator, Per. *Mol. Microbiol.* **41**:1133–1150.
 42. Sircii, M. P., M. Walters, L. R. Trabulsi, and V. Sperandio. 2004. Modulation of enteropathogenic *Escherichia coli* virulence by quorum sensing. *Infect. Immun.* **72**:2329–2337.
 43. Sperandio, V., J. L. Mellies, R. M. Delahay, G. Frankel, J. A. Crawford, W. Nguyen, and J. B. Kaper. 2000. Activation of enteropathogenic *Escherichia coli* (EPEC) *LEE2* and *LEE3* operons by Ler. *Mol. Microbiol.* **38**:781–793.
 44. Tatsuno, I., K. Nagano, K. Taguchi, L. Rong, H. Mori, and C. Sasakawa. 2003. Increased adherence to Caco-2 cells caused by disruption of the *yhiE* and *yhiF* genes in enterohemorrhagic *Escherichia coli* O157:H7. *Infect. Immun.* **71**:2598–2606.
 45. Ueguchi, C., T. Suzuki, T. Yoshida, K. Tanaka, and T. Mizuno. 1996. Systematic mutational analysis revealing the functional domain organization of *Escherichia coli* nucleoid protein H-NS. *J. Mol. Biol.* **263**:149–162.
 46. Umanski, T., I. Rosenshine, and D. Friedberg. 2002. Thermoregulated expression of virulence genes in enteropathogenic *Escherichia coli*. *Microbiology* **148**:2735–2744.
 47. Wiles, S., S. Clare, J. Harker, A. Huett, D. Young, G. Dougan, and G. Frankel. 2004. Organ specificity, colonization and clearance dynamics *in vivo* following oral challenges with the murine pathogen *Citrobacter rodentium*. *Cell. Microbiol.* **6**:963–972.
 48. Yu, R. R., and V. J. DiRita. 2002. Regulation of gene expression in *Vibrio cholerae* by ToxT involves both antirepression and RNA polymerase stimulation. *Mol. Microbiol.* **43**:119–134.
 49. Zhang, L., R. R. Chaudhuri, C. Constantinidou, J. L. Hobman, M. D. Patel, A. C. Jones, D. Sarti, A. J. Roe, I. Vlisidou, R. K. Shaw, F. Falciani, M. P. Stevens, D. L. Gally, S. Knutton, G. Frankel, C. W. Penn, and M. J. Pallen. 2004. Regulators encoded in the *Escherichia coli* type III secretion system 2 gene cluster influence expression of genes within the locus for enterocyte effacement in enterohemorrhagic *E. coli* O157:H7. *Infect. Immun.* **72**:7282–7293.