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*

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Regulation of virulence gene expression in enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) is incompletely understood. In EPEC, the plasmid-encoded regulator Per is required for maximal expression of proteins encoded on the locus of enterocyte effacement (LEE), and a LEE-encoded regulator (Ler) is part of the Per-mediated regulatory cascade upregulating the *LEE2*, *LEE3*, and *LEE4* promoters. We now report that Ler is essential for the expression of multiple LEE-located genes in both EPEC and EHEC, including those encoding the type III secretion pathway, the secreted Esp proteins, Tir, and intimin. Ler is therefore central to the process of attaching and effacing (AE) lesion formation. Ler also regulates the expression of LEE-located genes not required for AE lesion formation, including *rorf2*, *orf10*, *rorf10*, *orf19*, and *espF*, indicating that Ler regulates additional virulence properties. In addition, Ler regulates the expression of proteins encoded outside the LEE that are not essential for AE lesion formation, including TagA in EHEC and EspC in EPEC. Δ ler mutants of both EPEC and EHEC show altered adherence to epithelial cells and express novel fimbriae. Ler is therefore a global regulator of virulence gene expression in EPEC and EHEC.

Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are important enteric pathogens for humans. EPEC is the most common bacterial cause of diarrhea in infants (35), while EHEC, especially those of serotype O157:H7, are important emerging pathogens causing diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (35). Central to the pathogenesis of both EPEC and EHEC infections is the formation of attaching and effacing (AE) lesions on infected host intestinal epithelial cells. The AE lesion is characterized by the loss of microvilli (effacement) and the induction of a pedestal of polymerized actin and other cytoskeletal elements that forms underneath and around the infecting bacterium (13, 24, 35). In EPEC strain E2348/69, the AE phenotype is encoded by a 35.6-kb pathogenicity island, the locus of enterocyte effacement (LEE) (31, 32). The LEE contains genes encoding an outer membrane protein (intimin), a type III secretion system (Esc, Sep, and Ces proteins), secreted proteins (Esp), and the translocated intimin receptor (Tir), as well as a number of open reading frames of undetermined function (8). These genes are also found in the same organization on the LEE of EHEC (36) and are necessary but not sufficient for AE lesion formation by EHEC in vitro (11).

In addition to the LEE pathogenicity island and the AE

phenotype, other parts of the genome in both EPEC and EHEC encode additional virulence factors and pathogenic mechanisms. The EPEC virulence plasmid encodes the regulator Per (18) and the type IV bundle-forming pili (BFP) (16), which are necessary both for in vitro EPEC adherence to Hep-2 cells in the characteristic localized-adherence pattern and for full virulence in humans (2). The EHEC virulence plasmid encodes a large number of known or potential virulence factors (4) including an RTX cytotoxin-hemolysin, Hly, and the autotransporter toxin, EspP (3), and contains *tagA*, which encodes a lipoprotein homologous to the cryptic ToxR-activated TagA of *Vibrio cholerae*. The EHEC, but not EPEC, chromosome contains phages encoding Shiga toxins 1 and/or 2, which are central to the pathogenesis of both hemorrhagic colitis and hemolytic-uremic syndrome (35). The EPEC chromosome contains an additional small pathogenicity island encoding the autotransporter toxin EspC (43; J. L. Mellies, F. Navarro-Garcia, J. P. Nataro, and J. B. Kaper, submitted for publication).

The way in which EPEC and EHEC regulate the expression of these multiple virulence genes is not well understood, and regulation studies have been largely confined to BFP and the LEE-encoded genes. It has been shown that BFP (16) and EspC (26) (in EPEC) and the LEE-encoded Esps (26) are maximally secreted when bacteria are grown in tissue culture media. The genetic basis for regulation has focused, in EPEC, on the role of the plasmid-encoded regulator, Per (18), which upregulates the expression of BFP and the LEE-encoded

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Details	Reference or source ^a
Strains		
E2348/69	Wild-type EPEC O127:H6	29
EDL933	Wild-type EHEC O157:H7	37
85-170	EHEC O157:H7 Δ stx	45
86-24	Wild-type EHEC O157:H7	20
SE941	O55:H7 strain	CVD diarrheal isolate
SE796	EPEC E2348/69 Δ ler:kan	This study
SE1099	EHEC 85-170 Δ ler:kan	This study
SE1101	EHEC 86-24 Δ ler:kan	This study
SE860	SE796(pCVD456)	This study
SE1104	SE796(pSE1100)	This study
SE1110	SE796(pSE1092)	This study
SE1140	SE1099(pSE1092)	This study
TE2680	MC4100 <i>recD::Tn10</i>	12
Plasmids		
pCVD456	pMOB::ler <i>orf2345</i> _{EPEC}	31
pSE1092	pACYC184::ler _{EHEC}	This study
pSE1093	pBR322::ler _{EHEC}	This study
pSE1100	pBR322::ler _{EPEC}	This study
pTHK113	<i>hns</i>	25
pJG9	<i>ori_{is} cat sacB</i>	J. Galen, unpublished data
pRS551	<i>lacZ</i> reporter gene fusion vector	41

^a CVD, Center for Vaccine Development.

genes in EPEC (18, 33, 44). An analogous specific regulator has not been described for EHEC. It has recently been demonstrated that quorum sensing is also involved in the regulation of EHEC and EPEC LEE genes (42).

In contrast to EPEC and EHEC, much more is known about regulation in related pathogens and other type III secretory systems (reviewed in references 7 and 21). A common theme is gene activation by an AraC-like protein and repression with a second DNA binding protein such as YmoA (in *Yersinia*) or H-NS. H-NS, the "histone-like nonstructural protein," binds strongly to curved (i.e., usually AT-rich) DNA, causing changes in supercoiling and packing and influencing gene expression (7, 21). In *Shigella*, H-NS counters the upregulatory effect of VirF and represses transcription of *virB*, while in enterotoxigenic *E. coli*, H-NS represses CfaD-mediated activation of *cfa* (7). H-NS is important for flagellar synthesis, F1-fimbrial phase switching, and regulation of *proU* and *csgA* among other genes (1, 5, 7, 21). H-NS can also negatively regulate its own expression (6).

A large family of H-NS-like proteins has been described which includes orthologs such as BpH3 in *Bordetella* (19) and the paralogous *E. coli* protein StpA (40). These proteins diverge significantly from H-NS and may have a different spectrum of activity but can functionally substitute for H-NS in several assays (1).

A gene whose predicted protein product has similarity to the H-NS family of DNA binding proteins was recently found in the LEE of EPEC (8). Originally termed *orf1*, this open reading frame is shown here to encode a protein able to regulate virulence gene expression but to be functionally distinct from H-NS. We recently reported that *orf1* in EPEC is part of a regulatory cascade involving the AraC homolog Per and renamed this gene *ler* (for "LEE-encoded regulator") (33). *ler* was found to activate the transcription of several LEE operons.

Another group has also recently found that *ler* can activate transcription from LEE operons and is required for expression of LEE-encoded proteins (14). They also demonstrated that integration host factor binds upstream of *ler* and is required for *Ler* expression.

We report here that *ler* also regulates LEE genes in EHEC O157:H7 and, more surprisingly, affects the expression of phenotypes encoded elsewhere in the genome of both EPEC and EHEC O157:H7. These results expand the role of *Ler* as a global regulator of virulence gene expression in both EPEC and EHEC.

MATERIALS AND METHODS

Bacterial strains, plasmids, and PCR primers. Bacterial strains and plasmids used in this study are listed in Table 1, and PCR primers are listed in Table 2. Unless otherwise stated, bacteria were grown at 37°C in Luria broth. In experiments where minimal essential medium (MEM; Life Technologies, Bethesda, Md.) was used, bacteria were grown overnight at 37°C in MEM prior to inoculation into fresh MEM and grown until an optical density at 600 nm of 1.0 (late log phase) was reached. The growth medium was supplemented with ampicillin (200 µg/ml), chloramphenicol (25 µg/ml), kanamycin (25 µg/ml), or nalidixic acid (100 µg/ml) as needed.

Molecular techniques. Where cloning required PCR amplification, the proof-reading polymerase *Pwo* (Boehringer-Mannheim) was used and the resultant clones were examined for fidelity by sequencing. All other PCR amplifications were performed using *Taq* polymerase (Life Technologies). Automated sequencing was performed at the University of Maryland Biopolymer Core Facility. All other molecular techniques were performed by standard methods. DNA analysis was performed with DNAsis v5 (Hitachi) and with the suite of programs provided by the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Homology searches were performed using PSI-BLAST (<http://www.ncbi.nlm.gov/blast/psiblast.cgi>) with the filter off and gap function activated. Protein localization was predicted using the PSORT algorithm (<http://www.psort.nibb.ac.jp>).

Cloning and mutagenesis. The *ler* regions from EPEC and EHEC were cloned into a variety of vectors. pCVD456 (31) is a 3.1-kb *EcoRI* fragment from the

TABLE 2. Primers used in this study

Name	Sequence (5' to 3')
K590	AAG ACA TTC TAC CCC GGG AAA ATA TTT AAC
K591	CTG GCT TTC AGG ATC CTT ATT TTG GC
K592	GTG AAT TAG TTT CCC GGG TCA TAA TAA ATA
K593	CTT CAC ATT TTG GGA TCC TAT CTC TC
K803	GAC ATA TCA TCA TGG ATC CTG AAT AAT GC
K848	GCG TTA ATT GCT GAG ATT C
K874	TGC GAT CCT TCA TAA TCA T
K893	CGT ACC TAG CGT AGG TT
K1226	CGG GAT CCG CGG TTA CTT GTT CAG CTA
K1229	CGG GAT CCT TAT CCT CTG GTA TGA TAT C
K1230	CGG GAT CCA AAG CGA GCT CGA CAG CAG GA
K1370	AGA GGA TTC CTC TTC ACC ATA TGT GTA CCC CTC AA
K1371	TAT TTA TTA CCC GGG CCG CTG AAA AAT ATT TAA CAT GAA AT
K1372	CCG GAA TTC CTG TAA CTC GAA TTA AGT AGA GT
K1373	TTT CAG CGG CCC GGG TAA TAA ATA ATC TCC GCA TGC TTT
K1420	CGC GGA TCC AGC TCA CGT TAT CGT TAT CAT T
K1502	CGC GGA TCC AGC TAC AGG AAG CTC ATC CTT
K1503	ACA TGA ATT CAG CGA TGC TGC CCA TGA A
K1547	ACT TTC TCC GAC AGC ACC
K1548	TCA GAC GCA GAC TGG TAG
K1938	GAG GGA TCC AGT TCG GAT ACG CAA TCA
K1939	GCA GAA TTC ATC ATG GCT CCG GGA GAG AGA
K1942	GCAGAATTCTGACTCGTATGACAACGCGA
K1943	AGA GGA TCC GGT AGT TTT ACG GTA GGA GCC A
K1944	AGC GGA TCC AAC GAA CCC TGC AGA TCA T
K1945	AGA GGA ATT CAG GAT AAT GAG CTT ACC CAG CA
K1964	AGC GAT TAA CCC TCC TGT A
K1965	AAC ACA TTG GCG GAC TCG AT
K1966	TCC AAT GTT ATC CCA AAC GTA
K1967	GAT TCT CCT ATC TGG TTT GTA

LEE of EPEC E2348/69 cloned into pMOB and containing *ler* *orf2345*. Primers K1372 and K1420 were used to amplify *ler* from the EPEC chromosome, which was cloned into pBR322 digested with *EcoRI* and *BamHI*, creating pSE1100. pSE1092 was constructed by amplification of the *ler* region from EHEC 85-170 using primers K1372 and K1370 and cloning as an *EcoRI*-blunt fragment into an *EcoRI*-*PvuII* fragment of pACYC184. In all cases the *ler* region was transcriptionally isolated from plasmid promoters, since we observed that clones containing *ler* under the control of a strong plasmid promoter grew poorly and the plasmid was unstable.

A nonpolar in-frame deletion mutation of *ler* was constructed in both EPEC E2348/69 and EHEC O157:H7 strains by allelic exchange. For EPEC, primers K590 through K593 were used to amplify ca. 500-bp regions flanking *ler* which contained a *SmaI* site at the deletion junctions into which was cloned the promoterless kanamycin resistance cassette, *aphA3* (hereafter referred to as *kan*). This fragment, containing the EPEC *ler* promoter upstream and the resistance cassette and *orf2* downstream, was initially cloned as an *EcoRI*-*BamHI* fragment in pBluescript and was then moved as a *PvuII* fragment into the suicide vector pJG9. pJG9 contains a temperature-sensitive replicon and *sacB* gene for counter selection. The resultant plasmid, pSE774, was electroporated into E2348/69, and, via allelic exchange, the wild-type chromosomal *ler* gene was replaced with an in-frame, nonpolar *kan* cassette, generating strain SE796. The site of insertion was confirmed by PCR and Southern hybridization.

To construct a *ler* mutation in the LEE of EHEC, primers K1370 through K1373 were used to PCR amplify and ligate regions flanking *ler* from the chromosome of EHEC strain 85-170, using a strategy similar to that employed for pSE774. The ultimate construct of pSE1096 was used to mutate the *ler* gene on the EHEC chromosome, as was previously done with EPEC. The mutation was confirmed with PCR and by sequencing the flanking regions. pSE1096 was used to mutate *ler* in EHEC O157:H7 strain 85-170, generating SE1099, and strain 86-24, generating SE1101. The *ler* regions in both these strains are identical to that of EDL933. The mutant phenotypes exhibited by SE1099 and SE1105 were complemented by cloned *ler*.

Primer extension. Primer extension was performed as described previously (33). Briefly, primers hybridizing to the sense strand between 20 and 50 nucleotides downstream of the ATG start codon were end labeled using T4 DNA kinase and [γ -³²P]ATP. Labeled primers were hybridized with 35 μ g of DNA-free total bacterial RNA and reverse transcribed for 1 h at 42°C using Life Technologies Superscript II reverse transcriptase and the recommended buffers, reagents, and methods. The resultant cDNA mix was treated with RNase H, precipitated, and resolved through a 6% acrylamide-urea sequencing gel, and the bands were visualized by autoradiography.

Assays for virulence-associated phenotypes. The fluorescent actin stain (FAS) test (27) utilizes fluorescein isothiocyanate-phalloidin to visualize the accumulation of actin beneath and around bacteria attached to Hep-2 cells. A weak or unfocused accumulation of actin underneath bacteria appears as a faint halo of fluorescence known as the shadow phenotype (27). The assay for Tir translocation has been previously described (38). EspA filaments were visualized using anti-EspA antibodies and immunofluorescence microscopy, as described previously (28). Expression of bacterial proteins was examined in supernatants and purified membrane and cytoplasmic fractions prepared as outlined previously (9, 23). Following separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were either stained with Coomassie blue or blotted to polyvinylidene difluoride and Western blotted with monospecific polyclonal rabbit antibodies against Tir, intimin, and all EPEC secreted proteins as previously described (9, 23).

Adherence was examined by the modified method of Scaletsky et al. (40) as previously described (10). Shiga toxin production was assessed by quantitative killing of Vero cells as previously described (15). Bacterial motility was examined in Craigie tubes containing 0.25% agar in Luria broth. Fimbriae were examined by electron microscopy on negatively stained bacteria as previously described (17).

Assessing promoter activity with *lacZ* fusions. To assay the effect of *ler* on gene expression, regions containing the promoter and at least 200 bp of flanking DNA were amplified with *Pwo* polymerase and cloned into plasmid pRS551, which contains a promoterless *lac* operon (41). To generate single-copy *lacZ* fusions, these plasmids were linearized with *XhoI* and the linear DNA was transformed into *E. coli* K-12 strain TE2680 and integrated into the chromosome as previously described (12). Promoter activity (in Miller units) was assayed by quantification of β -galactosidase activity in bacterial cultures as previously described (34). For promoters from the EHEC LEE, *bla* and *stx*, we used previously constructed fusions (33, 42). New promoter fusions were constructed with the following primers: *bfp* (K1546 and K1548), *espC* (K1948 and K1939), *tagA* (K1944 and K1945), *espP* (K1942 and K1943), and *hly* (K1502 and K1503).

Examination of Ler for properties similar to those of H-NS. To examine if *ler* is functionally related to *hns*, we examined the ability of *ler* to rescue and/or interfere with *hns* function by using *lacZ* reporter strains as described by Donato et al. (5). pCVD456, containing *ler orf234*, or pTHK113, containing *hns*, was transformed into THK60 (*hns*⁺ *proU::lacZYA*), THK62 (Δ *hns::tet proU::lacZYA*), THK88 (*hns*⁺ *fimB::lacZYA*), and THK90 (Δ *hns::tet fimB::lacZYA*) and assayed for β -galactosidase activity (in Miller units) as described above.

RESULTS

Characterization of *ler* and the *ler* gene product. *ler*, previously known as *orf1*, is the first in a series of codirectional genes in the LEE (Fig. 1). It has been recently demonstrated by reverse transcription-PCR that these genes form a polycistronic operon denoted *LEE1* in EPEC O127:H6 strain E2348/69 (33). The *LEE1* operon contains nine genes, *ler orf2345 escRSTU*, and is highly conserved with respect to EHEC O157:H7 (36). Primer extension has demonstrated that the promoter for *LEE1* in EPEC is different from that in EHEC O157:H7 and is 169 nucleotides upstream (33, 36), although the region upstream of *ler* is conserved. Alignment of these two regions demonstrated the duplication of a 6-nucleotide (ATAAGG) sequence in EPEC O127:H7 compared to the same region in O157:H7 strain EDL933 (Fig. 1). This duplication in EPEC falls in the region predicted as the -10 region for the *LEE1* promoter in O157:H7, disrupting this corresponding area in EPEC. This duplication may be responsible for the inactivity of the downstream promoter in EPEC.

Sequencing of the *ler* region from two other EHEC O157:H7 strains and an EPEC O55:H7 isolate (Table 1) demonstrated that the *ler* region in these strains was 100% identical to that of EHEC O157:H7 strain EDL933 and presumably contained identical promoters. Unlike EPEC O127:H6, which belongs to the EPEC1 evolutionary group, these four strains are all members of the EHEC1 evolutionary group, with the O55:H7 serotype believed to be a progenitor of the O157:H7 lineage (48).

To demonstrate the ability of *orf1/ler* to encode a protein product, *ler* was cloned into and expressed from the T7 expression vector pET21. A 14-kDa protein was detected, which is consistent with the 15.1-kDa mass predicted from the amino acid sequence (results not shown).

Comparison of the predicted Ler sequence with previously described proteins by using PSI-BLAST demonstrated that Ler is distantly related to proteins that are members of the H-NS family of DNA binding proteins, including BpH3 from *Bordetella* (29% identical, 47% similar over the homologous region, and 41% similar over the entire length), StpA from *E. coli* (23% identical, 48% similar over the homologous region, and 40% similar over the entire length) and, more distantly, H-NS (36% identical, 55% similar over the homologous region, but only 20% similar over the entire length) (Fig. 2). The highest similarity between Ler and its homologs is found in the DNA binding C-terminal domain, and Ler possessed the conserved DNA binding motif of H-NS family proteins (TWTGXGRXP) (5, 46). No homology was seen within the N-terminal oligomerization domain (6). Both Ler and H-NS have similar predicted pI values (5.86 and 5.29, respectively), and both were predicted by the PSORT algorithm to be localized in the cytoplasm. Together, these predictions are consistent with the classification of Ler as a member of the H-NS-family of DNA binding proteins, although somewhat distant from *E. coli* H-NS itself.

Mutation of *ler* disrupts functions associated with the LEE. An in-frame nonpolar deletion mutation of *ler* was constructed in EPEC O127:H6 and both *stx*⁺ and *stx* strains of EHEC O157:H7, as described in Materials and Methods. For biosafety reasons, the *stx* EHEC strain was used for all experiments unless otherwise stated.

EPEC and EHEC Δ *ler* mutants were defective in the formation of AE lesions on Hep-2 cells as determined by the FAS test (Table 3). EHEC Δ *ler* mutants were negative in the FAS assay, and a shadow FAS phenotype was observed after 6-h incubations of EPEC Δ *ler* on Hep-2 cells (data not shown) which suggests that the ability to form AE lesions was not completely abolished in this strain but was, rather, strongly

FIG. 1. The LEE, showing the structures of the *LEE1* through *LEE4* and *tir* operons and the mapped promoters. The promoter driving *ler* expression (P_{LEE1}) has been expanded to show the differences between the promoter of EPEC O127:H6 and those of EHEC O157:H7 and EPEC O55:H7. EPEC contains an ATAAGG duplication that disrupts the region corresponding to the -10 sequence found in EHEC (33, 42). Open reading frames have been shaded to distinguish those encoding secreted proteins from those involved in type III secretion or other functions. In the alignment of the sequences, /.../ represents an area deleted from the figure for presentation and - represents a nucleotide missing in that sequence when compared with the other sequence.

diminished. The FAS phenotype in both EPEC and EHEC mutants was restored by complementation with *ler* from their respective parent strains cloned on a multicopy plasmid vector (Table 3), and complemented strains exhibited a FAS reaction that was visibly enhanced over that of the wild type. Interestingly, the cloned EHEC *ler* was also able to restore FAS in the EPEC Δler mutant (Table 3), indicating that EHEC *ler* is able to functionally substitute for EPEC *ler*.

The coordinated expression of multiple virulence factors, including the Esp proteins, Tir, and intimin, is necessary for AE lesion formation. We investigated which of these factors is regulated by Ler. A comparison of the wild-type, Δler mutant, and complemented strains revealed that *ler* is necessary for EPEC and EHEC to secrete Esp proteins into the supernatant (Fig. 3A) and for production of the EspA filament by EPEC, since the filament was not observed in mutant strains (Stuart Knutton, personal communication). Similarly, Tir was not secreted by the *ler* mutants (Fig. 3B) and was not translocated by EPEC Δler into HEp-2 cells as determined by staining HEp-2 cell lysates with antiphosphotyrosine antibodies (data not shown). Immunoblotting of membrane preparations demonstrated that intimin levels were markedly reduced in the absence of *ler* (Fig. 3C). In addition to the lack of Esp secretion observed in the Δler mutants, Esp proteins were not found at detectable levels in the whole cell as determined by Western blot analyses performed on lysates, probed with antibodies against secreted Esp proteins (data not shown).

The LEE also encodes a number of proteins unnecessary for AE lesion formation, including EspF and rOrf2. *espF* is co-transcribed with *espADB* on *LEE4*, while *rorf2* is transcribed on an operon divergent from *LEE1* and separate from the main *LEE* operons (8, 33, 36). Production of these proteins was demonstrated to be dependent on Ler since Δler mutants did

not produce them, as determined by Western blot analyses using antisera specific for EspF (Fig. 3D) (a gift of M. Donnenberg) or rOrf2 (Fig. 3E). The antiserum raised against EPEC EspF did not react with EHEC EspF, presumably reflecting the high sequence divergence in EspF between EPEC and EHEC (36).

Ler affects the level of proteins encoded outside the LEE. The *ler* gene product was found to control the expression of genes encoded outside the LEE in both EPEC and EHEC. We observed high-molecular-mass (~ 110 kDa) proteins in the supernatants of wild-type and complemented strains grown in MEM but not in supernatants from Δler mutants (Fig. 4A). In EPEC, this band has been identified as EspC, an autotransported toxin encoded by a separate chromosomal pathogenicity island (43; Mellies et al., submitted). Western blotting with an antiserum that recognizes EspC (22) supported this identification (Fig. 4B).

Similarly, Ler regulated a high-molecular-mass protein(s) in EHEC, observed as a doublet in supernatants (Fig. 4A). These bands may correspond either to one full-length protein and a breakdown product of the same protein or two distinct secreted proteins coregulated by Ler. An EspC-analogous high-molecular-mass autotransporter, EspP, has been identified in EHEC supernatants (3), but we lack a sufficiently specific antiserum to confirm this identity.

The major virulence factor produced by EHEC but not EPEC is Shiga toxin (Stx). Ler does not affect the production of Stx since the wild-type EHEC 86-24 ($Stx1^+ Stx2^+$) produced toxin in amounts equivalent to those produced by its Δler derivative, SE1101, when assayed on Vero cells (A. O'Brien, personal communication). Similarly, Ler did not appear to affect the production of enterohemolysin from EHEC, as assayed on 5% washed sheep erythrocyte agar (data not shown).

FIG. 3. Mutation of *ler* affects the production of LEE-encoded proteins. Western blots of secreted proteins probed with antibodies against all-EPEC secreted proteins (A) or Tir (B), membrane preparations stained with antibodies against the outer membrane protein intimin (C), and whole-cell lysates stained with antisera against EspF (D) or rOrf2 (E) indicate that multiple LEE-encoded virulence factors are regulated by Ler. wt, wild type.

6A and B). The complemented mutant, E2348/69 Δ ler(pLER), did not express these novel fimbriae (data not shown) and displayed the wild-type phenotype.

Immunoelectron microscopy using antibodies conjugated to gold particles was used to confirm that the novel fimbriae were not derived from BFP. Antibodies directed against BfpA reacted to BFP but not to fimbriae with novel morphologies (data not shown). Further, no differences were found in the levels of BfpA between wild-type and Δ ler strains in Western blot analyses using anti-BfpA antiserum against whole-cell lysates of bacteria grown both in MEM and on agar (data not shown).

When we examined EHEC, we also observed that mutation of *ler* was associated with enhanced fimbrial expression. Wild-type 85-170 typically expressed few fimbriae (data not shown). The Δ ler mutant SE1099, however, produced long fine fimbriae (Fig. 6C) that were not observed in the wild type or the complemented strain (data not shown). It is not yet known if these fimbriae are identical to the morphologically similar long fine fimbriae observed in the EPEC Δ ler mutant. We are currently characterizing these novel Ler-regulated fimbriae from EPEC and EHEC.

Ler activates promoters in the absence of other EPEC- or EHEC-specific genes. Ler is able to activate promoters from the LEE and elsewhere in the genome in the absence of other EPEC- or EHEC-specific genes. This was demonstrated by fusing promoters to a *lacZ* reporter gene and introducing them as single copies into the chromosome of *E. coli* K-12. These strains were then transformed with pSE1093 (pBR322 containing *ler* from EHEC) when the promoter was derived from EHEC or pSE1100 (pBR322 containing *ler* from EPEC) when the promoter was EPEC derived. Control strains containing only the pBR322 vector were also constructed and tested.

The genes within the LEE are arranged in at least five large

polycistronic operons designated *LEE1* through *LEE4* and *tir* (Fig. 1), and the transcription start sites have been determined by primer extension (9, 33, 42). Using previously constructed reporter fusions (9, 42), we demonstrated that Ler upregulated transcription from EHEC LEE promoters for the *tir*, *LEE2*, and *LEE3* operons in the range of about eightfold (Table 4). These data demonstrate that Ler upregulates the expression of several virulence-associated proteins and demonstrate that Ler acts directly as an activator of transcription of LEE operons from EHEC and EPEC, including those containing *esc/sep* and *tir*. Ler did not regulate *P_{LEE1}* (i.e., the *ler* promoter) or *eae*, indicating that intimin expression is controlled via the *tir* promoter, which regulates the *tir cesT eae* polycistronic operon. Similar results have been previously reported by us for EPEC LEE operons *LEE1*, *LEE2*, and *LEE3* (33). Interestingly, Ler did not regulate the *LEE4* operon in EHEC and caused only a twofold upregulation of the *LEE4* operon in EPEC (33). The observation that Ler is, at best, a weak activator of the *LEE4* promoter contrasts with the dramatic increase in EspABD protein levels in the presence of Ler (Fig. 3).

A number of genes found outside the LEE also were regulated by Ler in a K-12 background (Table 4). Ler strongly activated transcription from promoters for the EHEC plasmid-located *tagA* gene (20-fold) and the EPEC chromosomally located *espC* gene (30-fold). In contrast, promoters for EHEC genes *stx* (on a chromosomally integrated phage) and plasmid gene *hly* were not activated by Ler in a K-12 host background. These results agree with phenotypic observations, Western blotting results, or preliminary data from a (not shown) DNA array. Interestingly, the *espP* promoter was not regulated by Ler. This suggests that the high-molecular-mass Ler-regulated secreted protein in EHEC is not EspP or that *espP* is regulated in EHEC via a second regulator.

Finally, the promoter for the EPEC plasmid operon *bfp* was not regulated by Ler in either K-12 or EPEC backgrounds (Table 4). This result confirms Western blot data and supports the identity of novel, Ler-regulated EPEC fimbriae as distinct from BFP.

Primer extension identifies additional genes regulated by Ler in wild type EPEC and EHEC. Primer extension was used to find whether particular mRNA transcripts were synthesized in EPEC and EHEC in either the presence or absence of Ler. We have previously (33) used primer extension to demonstrate that Per upregulates the *LEE1* and (via Ler) *LEE2* transcripts. We now report that Ler is absolutely necessary for full transcription of the *LEE4* operon in both EPEC and EHEC (Fig. 7), supporting our observations that levels of EspADB and EspF are markedly reduced in Δ ler mutants. This finding is in contrast with data from *LEE4::lacZ* reporters in an *E. coli* K-12 background and strongly implies the presence of a specific

FIG. 4. Regulation of high-molecular-mass secreted proteins by Ler. (A) Coomassie blue-stained gels of secreted protein preparations from EPEC and EHEC demonstrated that ~110-kDa proteins were not secreted from Δ ler mutants. (B) Antibodies identify this protein in EPEC supernatants as EspC. wt, wild type.

FIG. 5. Altered adherence phenotypes of Δler mutants. (A to C) EPEC (A) and the complemented Δler mutant (C) exhibited LA and formed large microcolonies on the surface of the HEp-2 cells; the EPEC Δler mutant (B) displayed a complex mixture of DA and AA patterns with some LA microcolony formation. (D to F) With EHEC 85-170, the wild type adhered to HEp-2 cells in a DA-LA pattern (D) while the Δler mutant displayed increased adherence, especially to glass, and an AA pattern (E); complementation of the Δler mutation with plasmid-encoded *ler* restored the wild-type adherence phenotype (F). Magnification, $\times 2,500$.

LEE4 regulator that is present in EPEC and EHEC but not K-12.

We also found that mRNA transcripts for *rorf2*, *orf10*, *rorf10*, *orf19*, and *escD* (*rorf11*) were absent or strongly reduced in the absence of Ler. Therefore, Ler regulates all major operons within the LEE. Finally, we examined *espP* transcription and could not find evidence of Ler regulation. This agrees with our *espP::lacZ* fusion data and suggests that the high-molecular-mass Ler-regulated protein from EHEC is not EspP but another, as yet unidentified protein.

Ler is distinct from H-NS. Alleles of H-NS may have divergent amino acid sequences but nonetheless be able to functionally substitute for H-NS in *hns* mutant strains, as has been observed with BpH3 (19), StpA (40), and others (1). At the same time, some H-NS homologs, such as StpA, form het-

erodimers with H-NS that may affect the normal DNA binding properties of H-NS, and so StpA action is more fully understood in an *hns*⁺ background (6). We examined to what extent Ler might be functionally analogous to H-NS or whether Ler could alter H-NS activity by using reporter fusions of *lacZ* to either *proU* or *fim* (5). *hns*⁺ and *hns* fusion strains were transformed with pCVD456 (*ler*) or pTHK113 (*hns*). We found that while cloned H-NS affected the expression of *proU::lacZ* or *fim::lacZ* up to 18-fold (Table 5), Ler had less than a 2-fold effect, which we do not consider significant. Therefore, Ler is neither functionally equivalent to H-NS nor able to have dominant negative effects on H-NS function.

Mutation in *hns* has been reported to repress flagellar synthesis and therefore motility (1, 7, 19). In contrast, Ler did not affect motility, since the motility of EPEC Δler mutants was not

FIG. 6. Novel fimbriae expressed by Δler mutants. (A and B) The EPEC Δler mutant expressed a number of novel fimbriae (A, a and c) that are clearly distinguishable from BFP (b) and include numerous LFF (c) as well as "bent" fimbriae (b) and shorter fine fimbriae (B, arrow). (C) EHEC 85-170 does not express fimbriae (not shown), but the Δler mutant exhibited long fine fimbriae (arrow). Bar = 200 μm .

TABLE 4. Activation of gene expression by Ler

Operon	Source of promoter	β-Galactosidase activity ^a		Fold activation ^b
		pBR322	pLER	
<i>LEE1</i>	EHEC LEE	111 ± 6	100 ± 6	0.9
<i>LEE2</i>	EHEC LEE	27 ± 4	207 ± 14	7.6 ^c
<i>LEE3</i>	EHEC LEE	22 ± 5	175 ± 22	8.1 ^c
<i>tir</i>	EHEC LEE	10 ± 1	80 ± 16	7.6 ^c
<i>eae</i>	EHEC LEE	34 ± 9	33 ± 2	0.9
<i>LEE4</i>	EHEC LEE	861 ± 51	942 ± 38	1.1
<i>stx</i>	EHEC phage	15 ± 4	20 ± 3	1.3
<i>espP</i>	EHEC plasmid	230 ± 11	218 ± 8	0.9
<i>hly</i>	EHEC plasmid	1,169 ± 54	951 ± 62	0.8
<i>tagA</i>	EHEC plasmid	12 ± 1	245.3 ± 20	19.5 ^c
<i>espC</i>	EPEC chromosome	33 ± 3	1,027 ± 70	31.0 ^c
<i>bfp</i>	EPEC plasmid	85 ± 1	61 ± 0.2	0.7
<i>bfp</i> ^d	EPEC plasmid	2,836 ± 81	2,476 ± 170	0.9
<i>bla</i>	control	160	170	1.1

^a Mean β-galactosidase activity (in Miller units) present in bacteria containing indicated promoter-reporter fusion ± standard error. EHEC promoters were fused with a promoterless *lacZ* reporter and introduced as single-copy fusions into the chromosome of *E. coli* K-12 strain TE2680. These reporter strains were transformed with pBR322 or pSE1093, except for *espC* and *bfp* fusions, which were transformed with pSE1100 containing EPEC *ler*.

^b β-Galactosidase activity of pLER divided by activity of pBR322.

^c Significant at *P* = 0.05 compared to vector-only control.

^d Plasmid pRS551 containing *bfp::lacZ* fusion in EPEC *Δler* or EPEC wild-type backgrounds, respectively.

diminished from that of the wild type as assessed in the Craigie tube, semisolid agar, and hanging-drop methods (data not shown). The results of all of these experiments indicate that Ler is functionally distinct from H-NS.

DISCUSSION

The *ler* locus encodes a regulator of virulence gene expression in both EPEC and EHEC O157:H7, directly regulating genes within the LEE elements and elsewhere in the genome. *ler* was originally described as *orf1* and was proposed as a DNA binding protein on the basis of homology to the H-NS family of regulators (8, 30). Once it was demonstrated that the locus fulfills the functions of a regulator by activating transcription of LEE operons, it was renamed *ler*, the LEE-encoded regulator (33).

Ler is essential for the formation of AE lesions, since all the genes known to be important for AE lesion formation are regulated by *ler* or, in the case of the *LEE1* operon, coregulated with *ler*. Both EPEC O127:H6 and EHEC O157:H7, with nonpolar deletions of *ler*, were unable to form AE lesions on HEp-2 cells, form the EspA filament, express intimin, translocate Tir, or secrete into the supernatant the type III secreted proteins EspA, EspB, EspD, or Tir. In addition, *Δler* mutants failed to express the type III secreted proteins, in contrast to mutants with mutations in *escN* or some other genes involved in type III secretion, which produce Esp and Tir proteins normally but are unable to secrete or translocate these proteins (9, 22, 23, 47). Therefore, the *Δler* phenotype is consistent with Ler as a regulator of LEE-located genes. Ler was then directly demonstrated to act as a regulator able to activate the transcription of LEE promoters in the absence of other EPEC- or EHEC-specific genomic elements with the use of *lacZ* reporter fusions in an *E. coli* K-12 background. This has been demonstrated for several EPEC promoters (33), and we have now demonstrated it for EHEC LEE promoters, including that of *tir*.

FIG. 7. Regulation of multiple EPEC and EHEC genes by Ler. Primer extension was performed on mRNA extracted from *Δler* mutants (–) and Ler⁺ complements (+) of EHEC (*) or EPEC (no *) by using primers K848 through K1967, which are directed against transcripts from genes *ror2* through *espP*, as shown. The resultant cDNA transcripts were visualized by autoradiography.

The level of induction observed in a K-12 background for *LEE2,3* and *tir* promoters, from both EPEC and EHEC, was ca. eightfold. It is possible that induction of these operons is more dramatic in the wild-type strain due to contributions of accessory factors, modifications of Ler, or loss of topological features in the single-copy fusions. This is clearly the case with the promoter for *LEE4*. In a K-12 background, *LEE4* is strongly expressed in the absence of Ler and is induced at most twofold by Ler. In a wild-type background, by contrast, *LEE4* is not expressed in *Δler* mutants and is strongly induced by Ler, as assessed from the levels of EspADBF proteins and from primer extension experiments. This implies both the presence of EPEC- and EHEC-specific accessory factors and the normal repression of *LEE4* in the wild type. Further, it suggests that an accessory factor normally represses *LEE4* and that Ler may function as a derepressor of expression from this operon. For example, lack of type III secretion may feed back to inhibit transcription.

While Ler is clearly responsible for regulating the expression of the elements involved in the AE phenotype, it also regulates other LEE-encoded factors not involved in AE lesion formation. Based on primer extension, Ler increased transcription from *ror2*, *orf10*, *ror10*, *orf19*, *escD*, and *LEE4* operons. From the operon structure of the LEE (8, 33), this also predicts increased transcription of *rorf1*, *orf11*, and *orf27* through *espF*. We observed increased levels of rOrf2 and EspF proteins in strains containing Ler, consistent with the results predicted from primer extension. Ler therefore is potentially able to activate the entire LEE.

Our findings compare with those recently published by Friedberg et al. (14) who screened, in an EPEC background, a multicopy plasmid library containing random fragments of the EPEC LEE fused to a *gfp* reporter. They demonstrated that Ler was necessary for production of EspADB, Tir, intimin, and EspF and could activate promoters for *LEE2*, *LEE3*, and *eae* in the range of 5- to 44-fold. They found that Ler did not activate *rorf2*, nor did they find evidence that *LEE4* or other LEE genes were activated by Ler. While the use of reporter fusions in a wild-type background has certain advantages, our laboratory has found that the use of multicopy plasmids con-

TABLE 5. Inability of Ler to complement *hns* mutation

Strain	Genotype	β-Galactosidase activity ^a		
		No plasmid	pHNS ^b	pLER ^c
THK60	<i>hns</i> ⁺ <i>proU::lacZ</i>	200 ± 16	27 ± 2	137 ± 9
THK62	<i>hns</i> <i>proU::lacZ</i>	553 ± 16	30 ± 6	932 ± 17
THK88	<i>hns</i> ⁺ <i>fimB::lacZ</i>	74 ± 2	26 ± 2	54 ± 1
THK90	<i>hns</i> <i>fimB::lacZ</i>	417 ± 8	30 ± 3	475 ± 7

^a β-Galactosidase activity (in Miller units) present in bacteria containing indicated promoter-reporter fusion ± standard error.

^b pTHK113 encoding H-NS.

^c pCVD456 encoding Ler.

FIG. 8. The *ler* regulon. *Ler* regulates the expression of many genes both within the LEE and elsewhere on the genome. Some genes are directly regulated by *Ler*, as shown in gene fusion studies in *E. coli* K-12 (indicated by solid lines), while other genes are indirectly regulated or direct regulation has not been demonstrated (dashed lines). Expression of *Ler* is activated by quorum sensing in both EPEC and EHEC and additionally by *Per* in EPEC. Genes in the top half of the figure apply to EPEC O127:H6, and genes in the bottom half apply to EHEC O157:H7.

taining strong promoters fused to a toxic protein such as green fluorescent protein in a *RecA*⁺ background may mislead. For example, highly expressed genes may be toxic in this system and cannot be cloned, and so they are missed in a genetic screen. Furthermore, DNA topology is an important factor in the function of H-NS-like proteins, and studying regulation of a chromosomal gene cloned into a multicopy plasmid may result in misleading conclusions (such as the level of activation) due to differences in DNA topology between supercoiled plasmids and chromosomal genes. Consequently, we continue to use stable, chromosomally integrated fusions to the nontoxic *lacZ* reporter and support or extend our findings with Western blots, primer extensions, and other assays of activity in the wild-type host. We believe that the differences observed in the levels of activation between the two studies and the failure of Friedberg et al. (14) to observe *Ler* activation of *rorf2*, *orf10*, *rorf10*, *orf19*, *escD*, and *LEE4* reflect differences in methodology.

In addition to the effects of *Ler* on LEE-located genes, we found that *Ler* regulates the expression of phenotypes and proteins encoded outside the LEE. We observed that *Ler* strongly activates (31-fold) the *espC* promoter and increases the levels of *EspC* secreted from EPEC. The 110-kDa secreted protein *EspC* is encoded on a second chromosomal pathoge-

nicity island in EPEC and has recently been demonstrated to be an enterotoxin in vitro (Mellies et al., submitted). The homologous EHEC O157:H7 protein *EspP* has been identified in EHEC culture supernatants and is encoded on the EHEC virulence plasmid. We observed that *Ler* regulated the levels of a large secreted protein(s) from EHEC but could not demonstrate *Ler* activation of an *espP*::*lacZ* fusion in K-12 or activation of *espP* transcription in the wild type. This suggests that *Ler* regulates another, as yet unidentified autotransporter and that the protein observed in EHEC 85-170 supernatants is not *EspP*. Another gene carried on the EHEC plasmid, *tagA* (4), was, however, regulated by *ler*, as judged by *tagA*::*lacZ* fusions in K-12. *tagA* has no known function in EHEC or in *V. cholerae*, where it was first described as a ToxR-activated protein. It is interesting that the function of *TagA* remains cryptic yet it is activated by major virulence regulons in two unrelated enteric pathogens.

Ler also regulates fimbrial expression and adherence phenotypes. In EHEC, mutation of *ler* was associated with enhanced adherence to tissue culture monolayers, altered adherence patterns, and expression of long fine fimbriae (LFF). These parallels suggest that *Ler* is a repressor of LFF (or perhaps an activator of another repressor) and that these fimbriae mediate the DA-AA pattern of adherence observed in

vitro. No fimbrial adhesin has yet been clearly defined for EHEC O157:H7, and we are conducting further investigation into the identity and properties of these fimbriae. It should be noted that it is difficult to determine the roles of particular Ler-regulated fimbriae in altered adherence phenotypes since other factors involved in adherence, such as intimin and the type III secretion system, are not expressed in Δler strains.

The EPEC Δler mutant exhibited a mixture of both DA-AA and LA to HEP-2 cells, while the wild type and the complemented strain exhibited LA. Electron microscopy demonstrated that the EPEC Δler mutant expressed a number of fimbriae of morphologic types not observed in the wild type and not previously described in EPEC or EHEC. These fimbriae in EPEC were unrelated to BFP since they were expressed under conditions normally nonpermissive for BFP production, they were demonstrated by immunoelectron microscopy to be distinct from BFP, and both Western blots and *bfp::lacZ* fusions demonstrated that Ler does not regulate BFP production. We are currently characterizing these novel fimbriae, and it is possible that the LFF observed in the EPEC Δler are identical to those observed in EHEC Δler and may represent a common EPEC and EHEC adhesin mediating DA-AA adherence to HEP-2 cells.

Ler therefore can have both negative and positive effects and thereby can play a central role in regulating many virulence and virulence-related phenotypes including AE lesion formation, adherence, and toxin production in both EPEC and EHEC. The fact that Ler coregulates so many genes suggests that a number of cryptic genes may play important roles in pathogenesis. We propose an entire *ler* regulon (Fig. 8), which suggests a pattern of gene expression in pathogenesis. Low levels of Ler expression are associated with enhanced production of fimbriae and/or adhesins which could be involved in initial colonization. High levels of Ler expression are associated with upregulation of genes involved in AE lesion formation, intimate adherence, and toxin production. This would imply at least two distinct phases in the regulation of virulence genes involved in pathogenesis.

Since Ler expression is central to regulation of virulence genes, it follows that the regulation of Ler expression is important to pathogenesis. Ler expression in EPEC is activated by Per (33) and IHF (14). Per is not present in EHEC, but there is at least one shared regulatory pathway, since we have recently shown that quorum sensing activates the *LEE1* promoter in both EPEC and EHEC (42). Differences in the pathways of Ler activation reflect differences in EPEC and EHEC pathogenesis. EHEC infects the large intestine and so could potentially use autoinducer secreted by the large number of resident bacteria to signal activation of virulence gene expression. EPEC normally infects the small intestine, where the concentration of bacteria is low, and may overcome this deficit by the presence of Per, which may respond to other environmental signals.

The structure of the Ler regulon also reflects the evolutionary history of EPEC and EHEC as proposed by Whittam and McGraw (48), in which the LEE elements were inherited first and other virulence factors added to the genome in later evolution. It would appear that many of the later elements have come under the control of Ler as they have been acquired, including other virulence loci and the large virulence plasmid (in EHEC). In contrast, it would appear that in EPEC the Per regulator may have evolved on the BFP plasmid first to regulate BFP and subsequently to regulate Ler.

The mechanism by which Ler regulates the expression of these genes remains to be determined. Ler is distantly related to the H-NS family of proteins but is functionally distinct from

H-NS, as demonstrated by several assays (Table 5), and neither protein can functionally substitute for the other. Therefore, while H-NS is an important global regulator of housekeeping genes, Ler appears to be specific for virulence-associated genes and is encoded in a pathogenicity island. Furthermore, Ler also appears to be different from other H-NS homologs that appear to act as antagonists or modifiers of H-NS action, since Ler could not suppress H-NS-mediated phenotypes. This suggests that Ler may represent a new member of the H-NS family of DNA binding proteins but one that is neither analogous nor antagonistic to H-NS.

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