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# Role of *hha* and *ler* in Transcriptional Regulation of the *esp* Operon of Enterohemorrhagic *Escherichia coli* O157:H7

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The locus of enterocyte effacement (LEE), which includes five major operons (*LEE1* through *LEE4* and *tir*), enables enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 to produce attaching and effacing lesions on host cells. Expression of *LEE2*, *LEE3*, and *tir* is positively regulated by *ler*, a gene located in *LEE1*. Transcriptional regulation of the *esp* operon (*LEE4*), however, is not well defined. Transposon mutagenesis was used to identify transcriptional regulators of the *esp* operon by screening for mutants with increased  $\beta$ -galactosidase activity in an EHEC O157:H7 strain harboring an *esp::lac* transcriptional fusion. All mutants with significant increases in  $\beta$ -galactosidase activity had transposon insertions in *hha* (*hha::Tn*). Specific complementation of the *hha::Tn* mutation with a plasmid-encoded copy of *hha* reduced  $\beta$ -galactosidase activity to the level expressed in the parental *esp::lac* strain. Purified Hha, however, bound poorly to the *esp* promoter, suggesting that Hha might repress the transcription of a positive regulator of *esp*. Transposon mutagenesis of a  $\Delta$ *hha* *esp::lac* strain expressing elevated levels of  $\beta$ -galactosidase resulted in *ler* mutants with reduced  $\beta$ -galactosidase activity. Purified Hha bound to the *ler* promoter with a higher affinity, and complementation of a  $\Delta$ *hha* mutation in a  $\Delta$ *hha* *ler::lac* strain repressed  $\beta$ -galactosidase activity to the level expressed in a *ler::lac* strain. A positive regulatory role of *ler* in *esp* expression was demonstrated by specific binding of Ler to the *esp* promoter, reduced expression of  $\beta$ -galactosidase in  $\Delta$ *ler* *esp::lac* strains with and without *hha*, and severalfold-increased transcription of *ler* and *espA* in strains lacking *hha*. These results indicate that *hha*-mediated repression of *ler* causes reduced expression of the *esp* operon.

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7, a Shiga toxin-producing *E. coli*, causes a broad spectrum of diseases, including uncomplicated diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (21). In addition to Shiga toxins, which act on vascular endothelial cells to produce hemolytic uremic syndrome, EHEC O157:H7 harbors genes mediating its adherence to intestinal epithelial cells by a characteristic attaching-and-effacing mechanism (31). The attaching and effacing lesions are characterized by the loss of microvilli and the formation of pedestals of polymerized actin and other cytoskeletal elements underneath and around the infecting bacterial cells (10). The attaching and effacing phenotype requires concerted action of several genes contained within a pathogenicity island, called the locus of enterocyte effacement (LEE) (32). LEE encodes a type III secretion system, an outer membrane protein called intimin, a translocated intimin receptor called Tir, and secreted proteins EspA, EspD, and EspB. The genes *espA*, *espD*, and *espB*, which encode Esp proteins, are organized into a single operon (2) and are secreted by the type III secretion apparatus. EspA forms a novel filament-like structure which is essential for early bacterial attachment to epithelial cells and translocation of EspB and Tir into host cells (11, 22).

Nucleotide sequence analysis of the LEE region of EHEC O157:H7 strain EDL 933 (37) revealed the presence of 41 open reading frames (ORFs), most of which are organized into five polycistronic operons named *LEE1* through *LEE4* and *tir*.

The genetic organization of the LEE-encoded ORFs of EHEC O157:H7 is similar to that reported for the LEE of enteropathogenic *E. coli* (EPEC) O127:H6 (13). The Ler protein, encoded by the gene *ler* of the *LEE1* operon, is essential for transcriptional activation of the *LEE2*, *LEE3*, and *tir* operons, which encode many of the protein factors required for the production of the attaching and effacing phenotype by EHEC and EPEC strains on host cells (12). Ler has also been shown to be a weak regulator of *esp* operon (*LEE4*) expression in EPEC (28).

Ler shows some similarities with H-NS (histone-like nucleoid-structuring protein) and StpA (suppressor of *td*<sup>-</sup> phenotype A) of *Salmonella enterica* and *E. coli*, predominantly with the DNA-binding domain near the carboxy terminus (3, 13). Binding of Ler to the *LEE2* regulatory region is required for activation of both the *LEE2* and *LEE3* operons (43), and Ler counteracts the negative regulation exerted by some H-NS proteins on the expression of *LEE2* and *LEE3* in EPEC (6). However, the genetic factors required for expression of the *esp* operon in EHEC O157:H7 are not fully understood.

The objective of this study was to identify transcriptional regulators of *esp* (*LEE4*). Our approach was to use transposon mutagenesis of EHEC O157:H7 strain 86-24  $\Delta$ *stx2*  $\Delta$ *lac* carrying an *esp::lac* transcriptional fusion to identify mutants with altered  $\beta$ -galactosidase activity compared to the parent strain and then characterize the genes altered by the transposon insertions. With this strategy, *hha* was identified as an indirect negative regulator of *esp*. The gene *hha* is implicated in the negative regulation of  $\alpha$ -hemolysin expression in pathogenic *E. coli* (33). We provide several lines of data that show that Hha

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TABLE 1. Bacterial strains and plasmids<sup>a</sup>

Strain or plasmid	Relevant genotype and description	Source or reference
<i>E. coli</i>		
EHEC 86-24	<i>stx2</i> <sup>+</sup> EHEC strain (serotype O157:H7)	18
EHEC $\Delta$ <i>stx2</i>	86-24 deleted of <i>stx2</i>	This study
EHEC $\Delta$ <i>stx2</i> $\Delta$ <i>lac</i>	86-24 deleted of <i>stx2</i> and <i>lac</i> operon	This study
EHEC <i>esp::lac</i>	86-24 $\Delta$ <i>stx2</i> $\Delta$ <i>lac</i> containing chromosomal <i>esp::lac</i> transcriptional fusion	This study
EHEC <i>esp::lac/TnKn</i>	Transposon mutant of EHEC <i>esp::lac</i>	This study
EHEC <i>esp::lac hha::TnKn</i>	86-24 <i>esp::lac</i> containing transposon TnKn in <i>hha</i>	This study
EHEC <i>esp::lac</i> $\Delta$ <i>hha</i>	86-24 <i>esp::lac</i> deleted of <i>hha</i>	This study
EHEC $\Delta$ <i>hha</i>	86-24 deleted of <i>hha</i>	This study
TOP 10	<i>endA1 recA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> ) <i>supE44</i> $\phi$ 80 <i>dlacZ</i> $\Delta$ <i>M15</i> $\Delta$ ( <i>lacZYA-argF</i> )	Invitrogen
DH10B	<i>endA1 recA1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> ) <i>supE44</i> $\phi$ 80 <i>dlacZ</i> $\Delta$ <i>M15</i> $\Delta$ ( <i>lacZYA-argF</i> )	Gibco-BRL
BL21	F <sup>-</sup> <i>ompT hsdS</i> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) <i>gal dcm</i>	Amersham Biosciences
Plasmids		
pCR2.1	Cloning vector	Invitrogen
pBR322	Cloning vector	4
pAM450	Suicide vector	27
pUC18	Cloning vector	45
pGEX-4T-3	Protein expression vector	Amersham Biosciences
pSM71	pAM450 derivative used for deleting <i>stx2</i> in EHEC 86-24	This study
pSM80	pAM450 derivative used for deleting the <i>lac</i> operon in 86-24 $\Delta$ <i>stx2</i>	This study
pSM102	pAM450 derivative used for constructing an <i>esp::lac</i> transcriptional fusion	This study
pSM116	pBR322 containing a cloned copy of <i>hha</i>	This study
pSM122	pAM450 derivative used for deleting <i>hha</i>	This study
pSM138	pGEX-4T-3 carrying an <i>hha</i> ORF for producing GST-Hha fusion protein	This study
pSM142	pCR2.1 containing <i>esp</i> promoter	This study
pSM143	pCR2.1 containing <i>hilA</i> promoter	This study
pSM146	pCR2.1 containing <i>ler</i> promoter	This study
pSM163	pGEX-4T-3 carrying <i>ler</i> ORF for producing GST-Ler fusion protein	This study
pSM188	pAM450 derivative used for deleting <i>ler</i>	This study
pSM197R	pCR2.1 containing <i>hha</i>	This study
pSM212	pAM450 derivative used for constructing an <i>esp::lac</i> transcriptional fusion	This study

<sup>a</sup> Detailed descriptions of the bacterial strains and plasmids listed in this table are provided in Materials and Methods.

represses *ler* transcription and Ler is a positive regulator of *esp* expression.

(A preliminary account of this work was presented at the 102nd Annual Meeting of the American Society for Microbiology, Salt Lake City, Utah, 19 to 23 May 2002 [V. K. Sharma and R. W. Morgan, Abstr. 102nd Annu. Meet. Am. Soc. Microbiol., abstr. B269, p.78, 2002].)

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The strains and plasmids used in this study are listed in Table 1. All *E. coli* strains were propagated on Luria-Bertani (LB) agar at 37°C. For liquid cultures, well-isolated colonies from LB agar plates were inoculated into LB broth and incubated at 37°C unless stated otherwise in an orbital shaker at 200 rpm. Dulbecco's modified Eagle's medium (DMEM) containing 0.45% glucose was purchased from Invitrogen, Carlsbad, Calif.. Medium were supplemented, when required, with ampicillin at 50  $\mu$ g/ml or kanamycin at 50  $\mu$ g/ml.

**Primer design, PCR amplification, and DNA sequencing.** The primers for PCR amplification of the nucleotide sequences of EHEC O157:H7 strain 86-24 are listed in Table 2. These primer sequences were selected from the published sequence of EHEC O157:H7 EDL 933 (38). Primers were synthesized by Integrated DNA Technologies (Coralville, Iowa). PCR amplifications were per-

formed in 50  $\mu$ l containing 5  $\mu$ l of DNA (0.2  $\mu$ g) and 0.3  $\mu$ M each of the forward and reverse primers. The AmpliTaq Gold (PE Biosystems, Foster City, Calif.) or Failsafe PCR kit (Epicenter Technologies, Madison, Wis.) was used to amplify DNA fragments of <2.0 and >2.0 kb, respectively, according to the instructions provided by the manufacturers of these kits. PCR-amplified products were purified either with the Qiagen PCR purification kit or by first resolving PCR samples on agarose gels followed by extraction of DNA from agarose slices with a QIAquick gel extraction kit (Qiagen, Valencia, Calif.).

DNA sequencing was performed with a Thermo Sequenase dye terminator cycle sequencing kit (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) and an ABI 377 DNA sequencer (PE Applied Biosystems, Foster City, Calif.).

**Deletion of *stx2* and *lac* operons.** All DNA manipulations were performed by standard methods (40). To create deletions of target genes in the chromosome of EHEC O157:H7 strain 86-24 *hha*<sup>+</sup> *lac*<sup>+</sup>, a 1.5-kb sequence located upstream (termed US for upstream sequence) and a 1.5-kb sequence located downstream (termed DS for downstream sequence) of the genes to be deleted were isolated by PCR. The primer sets (VS174-XbaI and VS178-SmaI and VS175-XbaI and VS177-SmaI to amplify US and DS fragments, respectively, for the *stx2* deletion; VS244-XbaI and VS245-SalI and VS246-SalI and VS247-XbaI to amplify US and DS fragments, respectively, for the *lac* deletion) used in PCR amplification incorporated restriction sites for XbaI at the 5' and SmaI (for *stx2* deletion) or SalI (for *lac* deletion) at the 3' end of fragments containing the US, and SmaI (for *stx2* deletion) or SalI (for *lac* deletion) at the 3' and XbaI at the 5' end of fragments carrying the DS. The 3' end of the US fragment was joined to the 5'

TABLE 2. Primers used for PCR and sequencing<sup>a</sup>

Primer	Nucleotide sequence (5'-3')	Position (bp)/gene/accession no./reference
VS174	CATCTAGAGGAAGCAGTAGACGAAATCG	19931-19950/L0097/AF125520
VS175	CATCTAGAGGTCCTCTCCGGTTTGTTTC	24224-24205/L0105/AF125520
VS177	ATCCCGGGTGAGGCATAACCTGATTCGTG	22700-22720/region between <i>stx2</i> and L0105/AE125520
VS178	ATCCCGGGTTCATATACAGGTGTTCTTTTGG	21466-21443/region between <i>argO</i> and <i>stx2</i> /AE125520
VS244	ACGTCTAGATGAGCAAGATGCTGAATCACTG	2688-2667/Z0443/AE005214
VS245	CGAGTCGACCATTACCACCCTGAATTGAC	1174-1194/ <i>lacI</i> -Z0442 intergenic region AE005214
VS246	CGAGTCGACAGAGATTACAAAGTTGAATCGTCAG	6257-6231/ <i>lacA</i> /AE005213
VS247	ACGTCTAGAACTGTTACAGATGATCCCACTGC	4685-4706/ <i>cynS</i> /AE005213
VS256	CAGTCTAGACAGGTAAGGTTTATCTG	15241-15221/ <i>escD</i> /AE071034
VS257	CAGGTCGACTATATACCTTTGATAATTTTC	13742-13765/ <i>sepL</i> - <i>espA</i> intergenic/region AE071034
VS264	CAGGTCGACGCAACATCCGTTGTTAATGTTGAG	13726-13704/ <i>espA</i> /AE071034
VS265	CAGTCTAGATCCCGGCAGAACGAATACCGTTC	12256-12278/ <i>espD</i> /AE071034
VS266	CAGGTCGACAATTTACACAGGATACAGCTATG	11304-11287/ <i>lacZ</i> /AE005213
VS268	CAAATTGCCACTCACCATTGAC	11100-11121/ <i>lacZ</i> /AE005213
VS269	TGGAATTATGGCCACACCAG	8451-8431/ <i>lacZ</i> /AE005213
VS270	TGTATTGTAACAGTGGCCGGAAG	7854-7876/ <i>lacY</i> /AE005213
VS275	CCGGTTATTTACCAAGGGCATATTTG	13159-13183/ <i>espA</i> /AE071034
VS276	AAATGGCGCCTCTTTACTTGACTG	14266-14242/ <i>sepL</i> /AE071034
VS280	CAGGTCGACCCTGATAAGCGAAGCGTATCAGGC	6191-6214/ <i>cynX</i> - <i>lacA</i> intergenic region/AE005213
VS303	CATCTAGAGATTCCGTTCTCCGTTATGC	3045-3026/ <i>acrB</i> /AE005213/AE005225
VS304	CAGTTCGACTCGGACATACTTCTACCCATG	1630-1650/ <i>ybaJ</i> - <i>hha</i> intergenic region/AE005225
VS305	CAGTTCGACACTACTGAACAACATAAAGGTG	1771-1792/ <i>ybaJ</i> /AE005225
VS306	CAGTTCGACTCGCTTTCCGAGCTATAACCG	1409-1388/ <i>ylaD</i> - <i>hha</i> intergenic region/AE005225
VS307	CATCTAGAGCTGGCAGGAGATAAGGAGGT	1-20/downstream of <i>ylaC</i> /AE005225
VS308	ACGGATCCATGGTATTAATATGCAGGATC	1769-1746/ <i>hha</i> /AE005225
VS309	CAGGATCCCGGTTATAGCTCCGAAAG	1389-1409/ <i>hha</i> /AE005225
VS317	ACGTTCGACCCGGTTATAGCTCCGAAAG	1389-1409/ <i>hha</i> /AE005225
VS318	ACGAATTCATGTCGAAAAACCTTTAACG	1637-1619/ <i>hha</i> /AE005225
VS319	CCGTTGAAGTGAAGACGGTTC	1453-1474/ <i>gapA</i> /AE005401
VS320	AACCACTTTCTTCGCACCAGC	1638-1618/ <i>gapA</i> /AE005401
VS323	TGTGGATGCCAAAATTGCTG	13493-13474/ <i>espA</i> /AE071034
VS324	CGCCTTCACTGTTTGCAGATC	13322-13342/ <i>espA</i> /AE071034
VS327	CATCTAGAACAGGCTCTAAAACGGTTAAC	41697-41677/ <i>LEE</i> /AE071034
VS328	CAGTTCGACGCTTTAATATTTAAGCTATTAGCG	40200-40222/ <i>LEE</i> /AE071034
VS330	CATCTAGACTTATCCTCTGGTATGATATCTTC	38596-38619/ <i>LEE</i> /AE071034
VS331	CAGGATCCATGCGGAGATTATTTATTATG	40199-40173/ <i>ler</i> ORF/AE071034
VS332	CAGTTCGACATGTTAAATATTTTTAGCGG	39806-39827/ <i>ler</i> ORF/AE071034
VS339	AAAGACATGATATCGGCACAGC	2018-1918/ <i>ybaJ</i> /AE005225
VS340	TGAGCATATTGCGACCTTCG	1882-1864/ <i>ybaJ</i> /AE005225
VS341	CGGAATTCGTCCAGATGACA	8153-8168/ <i>hilA</i> <sub>promoter</sub> /AE008831.1/14
VS342	TGGGGTGTAATGCTGCTT	8627-8609/ <i>hilA</i> <sub>promoter</sub> /AE008831.1/14
VS353	GCTTAACCACCTCATCCTTCG	13639-13659/ <i>espA</i> <sub>promoter</sub> /AE071034
VS354	GACTGGTTGCTGGAGAGTCC	13982-13963/ <i>espA</i> <sub>promoter</sub> /AE071034
VS355	GAATGTATGGACTTGTGTATGTG	40139-40162/ <i>ler</i> <sub>promoter</sub> /AE071034
VS356	TTGTTTATGCAATGAGATCTATC	40526-40505/ <i>ler</i> <sub>promoter</sub> /AE071034
VS376	AGTCCATCATCAGGCACATTAG	39985-40006/ <i>ler</i> ORF/AE071034
VS371	CAGGATCCATGAAGAAGATGTTTATGGCGG	22433-22454/ <i>stx2B</i> /AF125520
VS372	CAGTTCGACTCAGTCATTATTAACCTGCACCTCAG	22702-22676/ <i>stx2B</i> /AF125520
VS377	CATACAACAAGTCCATACATTC	40160-41139/ <i>ler</i> ORF/AE071034
VS398	GAGTTCGACCATGAAATAATTAATGATAACG	30809-39787/ <i>LEE</i> /AE071034
VS415	GGAGTTCGACGAATAAAAACCAAGCTGAACC	40534-40554/ <i>LEE</i> /AE071034
VS426	GCAGTTCCTTTGCTTCATG	39642-39662/ <i>LEE</i> /AE071034
VS427	GCCTTCCTGTAACCTCGAATTAAG	40593-40571/ <i>LEE</i> /AE071034

<sup>a</sup> The position of the primer sequence represents the location in the published sequence deposited under the indicated accession number at NCBI. Underlined sequences GGATCC, GAATTC, GTCGAC, CCCGGG, and TCTAGA represent restriction sites for BamHI, EcoRI, SalI, SmaI, and XbaI, respectively.

end of the DS fragment through SmaI (for *stx2* fragments) or SalI (for *lac* fragments) to generate a 3-kb fragment containing the US and DS fragments (US-DS).

The 3-kb US-DS fragment, containing restriction sites for XbaI at its 5' and 3' ends, was cloned at the XbaI site of temperature-sensitive, ampicillin-resistance-encoding plasmid pAM450 (27) to generate plasmids pSM71 (for *stx2* deletion)

and pSM80 (for *lac* deletion). Plasmid pSM71 was electroporated into EHEC O157:H7 strain 86-24 with a Gene Pulser (Bio-Rad, Richmond, Calif.) at settings of 200 Ω, 25 μF, and 2.5 kV, and an isolate containing pSM71 was cultured under conditions (27) that facilitated exchange of the cloned 3-kb fragment of pSM71 with sequences flanking the *stx2* operon to create a deletion of *stx2*. The chromosomal deletion for *stx2* was confirmed by PCR with the primer set VS174 and

VS175. Plasmid pSM80 was electroporated into EHEC O157:H7 strain 86-24  $\Delta$ stx2 to delete the *lac* operon by the procedure described above. The *lac* operon deletion in the chromosome was confirmed by PCR with primers VS244 and VS247. In addition, isolates confirmed to have lost the *lac* operon were tested for  $\beta$ -galactosidase activity.

**Determination of  $\beta$ -galactosidase activity.** An overnight culture was diluted 1:100 in LB broth or DMEM and incubated at 37°C. Samples were taken at different time intervals to measure the optical density at 600 nm (OD<sub>600</sub>) and  $\beta$ -galactosidase activity (30).

**Construction of *esp::lac* and *ler::lac* transcriptional fusions.** To introduce *esp::lac* and *ler::lac* transcriptional fusions into the chromosome of strain 86-24  $\Delta$ stx2  $\Delta$ lac, we used the same procedure that we employed for deleting the *stx2* and *lac* operons from the chromosome. Briefly, a 1.5-kb sequence located upstream (US) and a 1.5-kb sequence located downstream (DS) of the start codons for the *espA* and *ler* ORFs were isolated by PCR. The primer sets (VS256-XbaI and VS257-SalI and VS264-SalI and VS265-XbaI, for amplifying the US and DS fragments, respectively, for constructing the *espA::lac* fusion and VS327-XbaI and VS328-SalI and VS330-XbaI and VS398-SalI to amplify the US and DS fragments, respectively, for constructing the *ler::lac* fusion) used in PCR amplification incorporated restriction sites for XbaI at the 5' and SalI at the 3' end of fragments containing the US and SalI at the 3' and XbaI at the 5' end of fragments carrying the DS.

The 3' end of the US fragment was joined to the 5' end of the DS fragment through SalI to generate a 3-kb US-DS fragment. A 5.1-kb SalI fragment containing the *lacZ*, *lacY*, and *lacA* ORFs (*lac* cassette) was isolated from strain 86-24  $\Delta$ stx2 *lac*<sup>+</sup> by PCR with primers VS266-SalI and VS280-SalI and cloned at the SalI site (present at the junction of the US and DS) of the 3-kb US-DS fragment to generate an 8.1-kb US-*lac*-DS fragment, flanked at its 5' and 3' ends by XbaI and containing the *lacZ* ORF immediately downstream of the *espA* or *ler* promoter. The 8.1-kb fragment containing an *esp::lac* or *ler::lac* transcriptional fusion was cloned at the XbaI site of pAM450 to produce plasmids pSM102 and pSM212, respectively. Plasmids pSM102 and pSM212 were introduced into strain 86-24  $\Delta$ stx2  $\Delta$ lac by electroporation, and isolates containing pSM102 or pSM212 were cultured under conditions (described above) to facilitate integration and excision events for generating *espA::lac* and *ler::lac* transcriptional fusions by replacement of chromosomal sequences in the *esp* and *ler* regions, respectively, by the sequence present on the 8.1-kb fragment of pSM102 and pSM212, respectively. The presence of a chromosomal *esp::lac* or *ler::lac* transcriptional fusion was confirmed by PCR with primers VS276 and VS270 and VS269 and VS275 to amplify the 3.6-kb (*escD-lacZ lacY* region) and 2.6-kb (*lacY lacA-esp* region) fragments from the chromosomally generated *esp::lac* fusion or with primers VS270 and VS427 and VS269 and VS426 to amplify the 3.8-kb (5' of the *ler* ORF-*lacZ lacY* region) and 2.3-kb (*lacY lacA-3'* of the *ler* ORF region) fragments from the chromosomally generated *ler::lac* fusion, respectively. Isolates carrying the *esp::lac* or *ler::lac* fusion were tested for their  $\beta$ -galactosidase activities.

**Transposon mutagenesis.** Strain 86-24  $\Delta$ stx2  $\Delta$ lac containing a chromosomal *esp::lac* or *ler::lac* fusion was transformed with a transposon encoding kanamycin resistance (TnKn; EZ::Tn<KAN-2>Tnp; Epicenter Technologies, Madison, Wis.). Transformed cells were plated on LB agar containing kanamycin and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal). After overnight incubation at 37°C, plates were visually screened for colonies appearing either lighter or darker blue than the majority of isolates on the plate. Differently colored colonies were selected for quantitative measurement of  $\beta$ -galactosidase activity.

**Determination of transposon insertion sites.** Genomic DNAs of transposon mutants were digested with EcoRI (the transposon lacks EcoRI sites) and cloned at the EcoRI site of pUC18 (45). The recombinant plasmids were subjected to DNA sequencing with primers complementary to the 5' (KAN-2 FP-1) and 3' (KAN-2 RP-1) ends of the transposon (EZ::Tn<KAN-2>Tnp). The deduced DNA sequences were compared to nucleotide sequences in the database at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

**Construction of in-frame *hha* and *ler* deletions.** A 1.3-kb sequence located upstream (US) and a 1.5-kb sequence located downstream (DS) of *hha* and a 1.17-kb sequence located upstream (US) and a 1.2-kb sequence located downstream (DS) of *ler* were isolated by PCR. The primer sets (VS303-XbaI and VS305-SalI and VS306-SalI and VS307-XbaI for amplifying the US and DS fragments, respectively, of *hha*, and VS327-XbaI and VS415-SalI and VS398-SalI and VS330-XbaI, for amplifying the US and DS fragments, respectively, of *ler*) used in PCR amplification incorporated restriction sites for XbaI at the 5' and SalI at the 3' end of fragments containing the US and SalI at the 3' and XbaI at the 5' end of fragments carrying the DS. The 3' end of fragment US was joined to the 5' end of fragment DS through SalI to generate 2.8-kb (for the *hha*

deletion) and 2.37-kb (for the *ler* deletion) US-DS fragments, which were cloned at the XbaI site of pAM450 to generate pSM122 and pSM188, respectively. Plasmids pSM122 and pSM188 were introduced into strain 86-24  $\Delta$ stx2  $\Delta$ lac or 86-24 carrying the *esp::lac* or *ler::lac* fusion, and an isolate containing pSM122 or pSM188 was cultured under the conditions described above to generate a *hha* or *ler* deletion. The presence of the *hha* or *ler* deletion was confirmed by PCR with primers VS309 and VS340 or VS426 and VS427, respectively.

**Cloning of *hha*.** The *hha* gene (380 bp) was isolated by PCR from strain 86-24 with primers VS308 and VS309, and the amplified product was digested with BamHI and cloned at the BamHI site of pBR322 to generate pSM116. A 630-bp fragment containing *hha* was also isolated from 86-24 with primers VS309 and VS339 and cloned in pCR2.1-TOPO to construct pSM197R. Plasmids pSM116 and pSM197R were used in complementation of *hha::TnKn* and  $\Delta$ *hha* mutants of 86-24.

**Purification of Hha and Ler.** A 263-bp Hha-encoding fragment was PCR amplified from strain 86-24 with primers VS317 and VS318, and the amplified fragment was digested with SalI and EcoRI and cloned in expression vector pGEX-4T-3 (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) to generate an in-frame translational fusion of *hha* with the 3' end of the sequence encoding glutathione S-transferase (GST). The plasmid containing the GST-Hha fusion (pSM138) was introduced into *E. coli* BL21 (Amersham Pharmacia Biotech Inc., Piscataway, N.J.). The GST-Hha fusion protein from *E. coli* BL21 containing pSM138 was purified according to the instructions of the supplier of the glutathione-Sepharose matrix used for purifying GST-target protein fusions (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) and by the procedure described previously (41). The Hha moiety was cleaved from GST-Hha fusion protein by thrombin treatment. Hha was stored at -20°C in phosphate-buffered saline containing 50% glycerol.

For Ler purification, a 380-bp fragment was PCR amplified from the strain 86-24 with primers VS331 and VS332, digested with BamHI and SalI, and cloned in pGEX-4T-3 to generate a translational fusion (pSM163) between GST and Ler. The GST-Ler fusion was purified from *E. coli* BL21 containing pSM163 by the procedure described above. The Hha and GST-Ler protein preparations were analyzed on a sodium dodecyl sulfate-12% polyacrylamide gel to determine their molecular weights and purity. The conditions for polyacrylamide gel electrophoresis, staining, and destaining of gels were as described in the manual for the Mini-Protein II electrophoresis cell (Bio-Rad, Hercules, Calif.). Hha and GST-Ler protein concentrations were estimated with the Bio-Rad protein assay kit (Bio-Rad, Hercules, Calif.).

**Electrophoretic mobility shift assays.** The 350-bp *esp* promoter fragment was isolated with primers VS353 and VS354. Because Hha proteins from *E. coli* and *S. enterica* serovar Typhimurium are almost identical in their amino acid sequences, it has been speculated that Hha produced by *E. coli* should bind to the 450-bp *hilA* promoter of *S. enterica* serovar Typhimurium (14). A 450-bp sequence upstream of *hilA* was also isolated by PCR with primers VS341 and VS342 (14) and used as a positive control in an electrophoretic mobility shift assay. The 388-bp fragment containing the *ler* promoter was isolated by PCR with primers VS355 and VS356. The fragments containing the *espA*, *hilA*, and *ler* promoters were cloned in the pCR2.1 Topo TA cloning vector to generate plasmids pSM142, pSM143, and pSM146, respectively. The cloned fragments were isolated from recombinant plasmids with EcoRI, resolved on a 4% agarose gel (BMA, Rockland, Maine), and recovered from gel slices. End labeling of the recessed 3' termini of promoter fragments with [ $\alpha$ -<sup>32</sup>P]dATP, purification of labeled fragments from unincorporated deoxynucleotides, and determination of the binding of labeled fragments to Hha and GST-Ler in mobility shift assays were performed according to previously described procedures (8, 41). A 280-bp *stx2B* ORF-encoding sequence was also isolated from 86-24 by PCR with primers VS371 and VS372 for cloning in pCR-XL-Topo TA cloning vector to generate pSM156. The 280-bp fragment recovered from pSM156 after EcoRI treatment was used as a nonspecific competitor DNA in the electrophoretic mobility shift assay.

**RT-PCR analysis.** EHEC O157:H7 strains 86-24  $\Delta$ stx2  $\Delta$ lac and 86-24  $\Delta$ stx2  $\Delta$ lac  $\Delta$ *hha* were cultured in LB broth at 37°C with shaking to an OD<sub>600</sub> of 0.8. Bacterial cells were collected by centrifugation at 6,000  $\times$  g and processed for RNA isolation with the RNeasy kit (Qiagen, Valencia, Calif.) according to the directions of the manufacturer. The isolated RNA was treated with 40 U of RNase-free DNase (Stratagene, La Jolla, Calif.) at 37°C, followed by heating of the sample to 95°C for 15 min to inactivate the DNase. The RNA was analyzed on an agarose gel to verify its integrity by observing the presence of distinct 23S and 16S rRNA bands, and the concentration of RNA was determined spectrophotometrically. A single-step reverse transcription (RT)-PCR kit (PE Biosystems, Foster City, Calif.) was used according to the directions of the manufacturer for detecting gene-specific transcripts. Primer sets VS319 and VS320,

VS323 and VS324, and VS376 and VS377 facilitated detection of transcripts specific to *gapA*, *espA*, and *ler*, respectively. The samples were analyzed on a 4% Nusieve agarose gel containing ethidium bromide (Cambrex Corporation, East Rutherford, N.J.), and the gel was visualized with the Alpha Innotech Image documentation system (Alpha Innotech Corporation, San Leandro, Calif.).

## RESULTS

**Construction of an EHEC O157:H7  $\Delta$ *stx2*  $\Delta$ *lac* mutant carrying an *esp::lac* fusion.** To identify transcriptional regulators of the *esp* operon by transposon mutagenesis, EHEC O157:H7 strain 86-24 was deleted of *stx2* (to reduce accidental exposure to Stx2) and *lac* operons (to remove endogenous  $\beta$ -galactosidase activity). The isolates deleted of *stx2* produced a 3-kb fragment, and the parent 86-24 *stx2*<sup>+</sup> produced a 4.2-kb fragment (data not shown). Deletion of the *lac* operon was confirmed by the lack of amplification of *lacZ lacY* and *lacY lacA* fragments by PCR with primers VS244 and VS268 and VS269 and VS280, respectively (data not shown). Strain 86-24  $\Delta$ *stx2* deleted of the *lac* operon produced white colonies on LB agar containing X-Gal and no detectable  $\beta$ -galactosidase activity in an *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG)-based assay. Strain 86-24 containing the *lac* operon, on the other hand, produced blue colonies on X-Gal agar and 327 U of  $\beta$ -galactosidase activity in an ONPG-based assay.

Strain 86-24  $\Delta$ *stx2*  $\Delta$ *lac* was then used for constructing an *esp::lac* transcriptional fusion by allelic replacement of the *esp* gene on the chromosome with the *esp::lac* fusion present on pSM102. The generation of this fusion in the chromosome was confirmed by PCR with primers VS276 and VS270 and VS269 and VS275. These primers produced amplicons of 3.6 kb (*escD-lacZ lacY* region) and 2.6 kb (*lacY lacA-esp* region) from the chromosomally generated *esp::lac* fusion (data not shown). A graphic representation of the *esp::lac* fusion and its location relative to some of the other LEE-encoded genes are shown in Fig. 1A.

**Transposon mutagenesis to identify transcriptional regulators of *esp*.** Strain 86-24  $\Delta$ *stx2*  $\Delta$ *lac* carrying an *esp::lac* fusion was subjected to transposon mutagenesis to identify the gene(s) affecting transcription of the *esp* operon. A total of five kanamycin-resistant colonies that appeared darker blue on LB agar containing kanamycin and X-Gal from a total of more than 3,000 colonies also produced elevated levels of  $\beta$ -galactosidase activity in ONPG-based assays. The  $\beta$ -galactosidase activity of one representative TnKn mutant selected for further analysis is shown in Fig. 1B.  $\beta$ -Galactosidase production was elevated in the mutant strain by 17-fold (655 U h<sup>-1</sup>) compared to the parent 86-24 *hha*<sup>+</sup> *esp::lac* strain (39 U h<sup>-1</sup>) after 4.5 h of growth. These results indicated that the transposon insertion abolished the function of a gene product that was involved in negative regulation of the *esp::lac* fusion.

Nucleotide sequence analysis of the DNA at the transposon insertion sites in TnKn mutants of 86-24  $\Delta$ *stx2*  $\Delta$ *lac* *esp::lac*, expressing  $\beta$ -galactosidase at elevated levels, resulted in identification of an ORF with 100% homology to *hha* in four and *yaaJ* in one of the five independent mutant isolates. The gene *hha*, implicated in the negative regulation of  $\alpha$ -hemolysin in pathogenic *E. coli* (33), encodes a 72-amino-acid (8.6-kDa) protein and is a member of the Hha/YmoA/RmoA family of proteins, which modulate virulence gene expression (Hha and

YmoA) (9, 33) and conjugative transfer of broad-host-range plasmid R100 (RmoA) (35).

To confirm the effect of *hha* on *esp* expression, the isolate 86-24  $\Delta$ *stx2*  $\Delta$ *lac* *esp::lac* *hha::TnKn* was transformed with pSM116 (pBR322 containing a cloned copy of *hha*). Transformants had reduced  $\beta$ -galactosidase activity, similar to that produced by the parent 86-24 *hha*<sup>+</sup> *esp::lac*, confirming that Hha acted as a negative regulator of *esp* expression (Fig. 1B).

**Effect of growth medium and osmolarity on *esp* transcription.** Strain 86-24  $\Delta$ *stx2*  $\Delta$ *lac* *esp::lac* was deleted of *hha* by the allelic replacement method (described above) with pSM122. The *hha* deletion was confirmed by the lack of *hha* amplification in PCR with primers VS309 and VS340 (data not shown). Since expression of LEE-encoded genes has been shown to be enhanced by growth of EHEC O157:H7 in minimal medium of high osmolarity (2), we cultured 86-24  $\Delta$ *stx2*  $\Delta$ *lac* *esp::lac* with and without *hha* in LB broth and minimal medium (DMEM) of low or high osmolarity to determine if *hha*-mediated repression of the *esp* operon is more pronounced in LB broth than in DMEM.

Samples from these cultures were evaluated for the production of  $\beta$ -galactosidase at various time intervals. After 5 h of growth, strain 86-24  $\Delta$ *hha* *esp::lac* produced severalfold-higher  $\beta$ -galactosidase activity in LB (74-fold higher; Fig. 2A) or DMEM (281-fold higher; Fig. 2B) containing 100 mM NaCl compared to the parent 86-24 *esp::lac* carrying a functional copy of *hha*. Although the  $\beta$ -galactosidase activity of 86-24  $\Delta$ *hha* *esp::lac* was higher in LB (13-fold higher) and DMEM (38-fold higher) containing 400 mM NaCl than the parent 86-24 *esp::lac*, the higher salt concentration appeared to have a negative effect on growth and the expression of  $\beta$ -galactosidase activity. It is apparent from these results that growth of the 86-24 *esp::lac* strain lacking *hha* in DMEM of low or high osmolarity resulted in higher levels of  $\beta$ -galactosidase than were produced in LB broth of low or high osmolarity. DMEM containing a high salt concentration also resulted in 3.6-fold-higher  $\beta$ -galactosidase activity of the parent 86-24 *esp::lac* than DMEM containing low salt concentrations.

**Binding of *hha* to the *esp* promoter.** Amino acid sequence analysis of Hha from 86-24 revealed no classical helix-turn-helix motif (5), although a 10-amino-acid domain (SAADHRLAEL) with no known function is present at the C terminus (14). Hha of 86-24 exhibited 100, 99, 82, and 50% homology to Hha of *E. coli*, *S. enterica* serovar Typhimurium (14), YmoA of *Yersinia enterocolitica* (9), and RmoA of *E. coli* (35), respectively. Purified Hha was incubated with a 350-bp end-labeled fragment containing the previously mapped *esp* promoter region (2), and the reaction mixture was analyzed on a non-denaturing polyacrylamide gel. As shown in Fig. 3, Hha bound poorly to the *esp* promoter fragment, as only a small fraction of the promoter was bound at the highest concentration of Hha ( $3.5 \times 10^{-10}$  mol). On the other hand, as a control, Hha bound to the *hilA* promoter fragment with a higher affinity, and more than half of the promoter fragment was bound to Hha at the highest concentration of the protein tested ( $3.5 \times 10^{-10}$  mol).

**Identification of the gene directly affected by *hha*.** The results described above suggested that Hha represses *esp* transcription indirectly, that is, that Hha may repress a transcriptional activator of *esp*. To identify genes having a positive effect on *esp* transcription in the absence of Hha, a *hha* deletion was

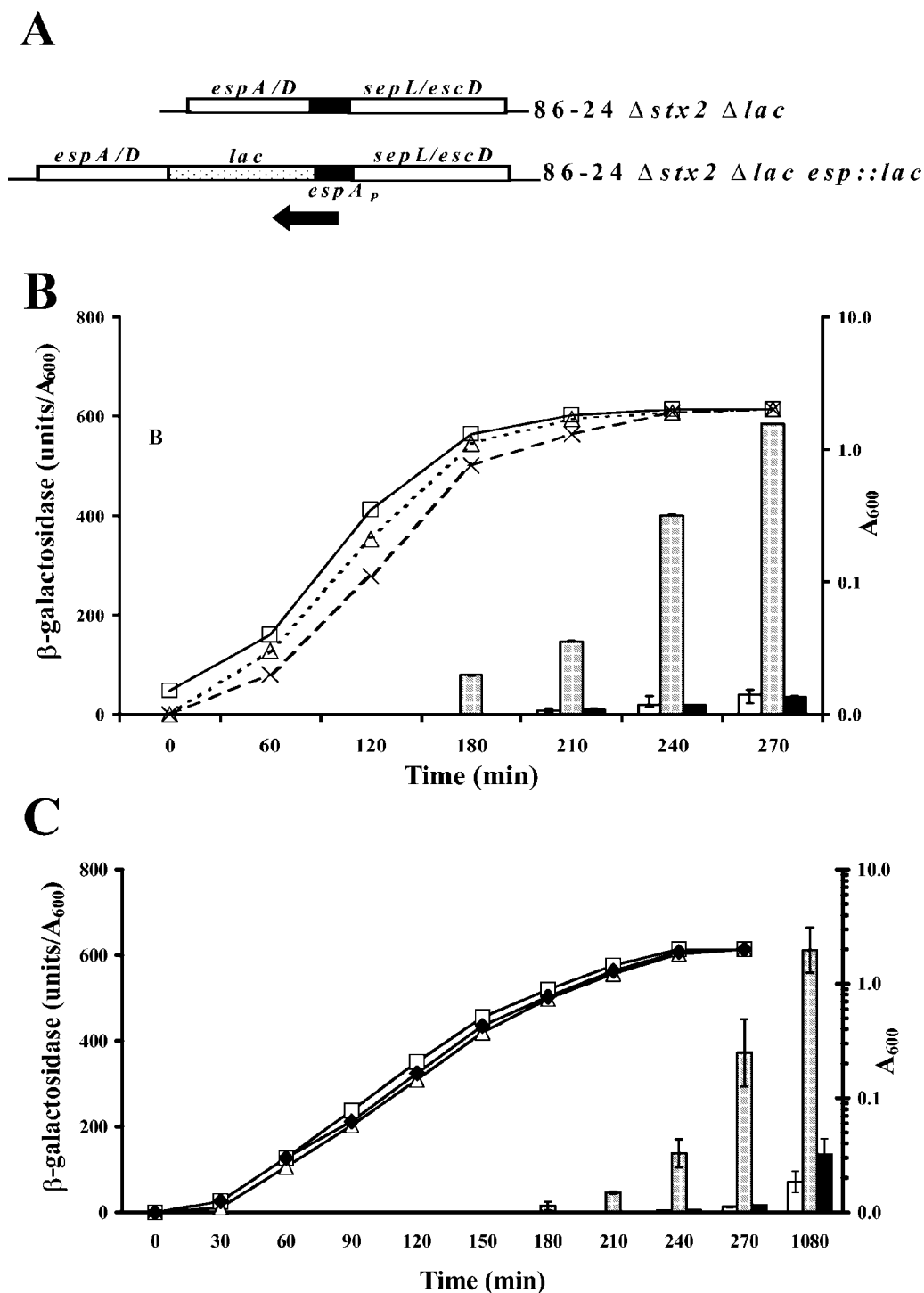


FIG. 1. (A) Map of the chromosomal *esp::lac* transcriptional fusion. The arrow indicates the direction of transcription from the *esp* promoter (solid rectangle) into the *lacZ* ORF of the *lac* cassette. (B)  $\beta$ -Galactosidase activity of EHEC O157:H7 strain 86-24 *esp::lac* and a transposon (TnKn) mutant of 86-24 *esp::lac* with and without complementation with plasmid pSM116. Line graphs represent the OD<sub>600</sub> values of *esp::lac* ( $\square$ ), *esp::lac*/TnKn ( $\Delta$ ), and *esp::lac*/pSM116 ( $\times$ ).  $\beta$ -Galactosidase activities are shown for the *esp::lac* (open bars), *esp::lac*/TnKn (hatched bars), and *esp::lac*/pSM116 (solid bars) strains. (C) Determination of  $\beta$ -galactosidase activity of the 86-24  $\Delta$ *hha* *esp::lac* (carrying a TnKn insertion in an unknown gene), 86-24  $\Delta$ *hha* *esp::lac*, and 86-24 *esp::lac* strains. Line graphs represent the OD<sub>600</sub> for the *esp::lac* ( $\square$ ), *esp::lac*  $\Delta$ *hha* ( $\Delta$ ), and *esp::lac*  $\Delta$ *hha* carrying TnKn in an unknown gene ( $\blacklozenge$ ) strains.  $\beta$ -Galactosidase activities are shown for the *esp::lac* (open bars),  $\Delta$ *hha* *esp::lac* (hatched bars), and  $\Delta$ *hha* *esp::lac* TnKn:unknown gene (solid bars) strains. Bacterial strains were grown in LB broth at 37°C with shaking, and samples were taken at different times to measure the OD<sub>600</sub> and  $\beta$ -galactosidase activity with an ONPG-based assay. Error bars indicate standard deviations based on three measurements.

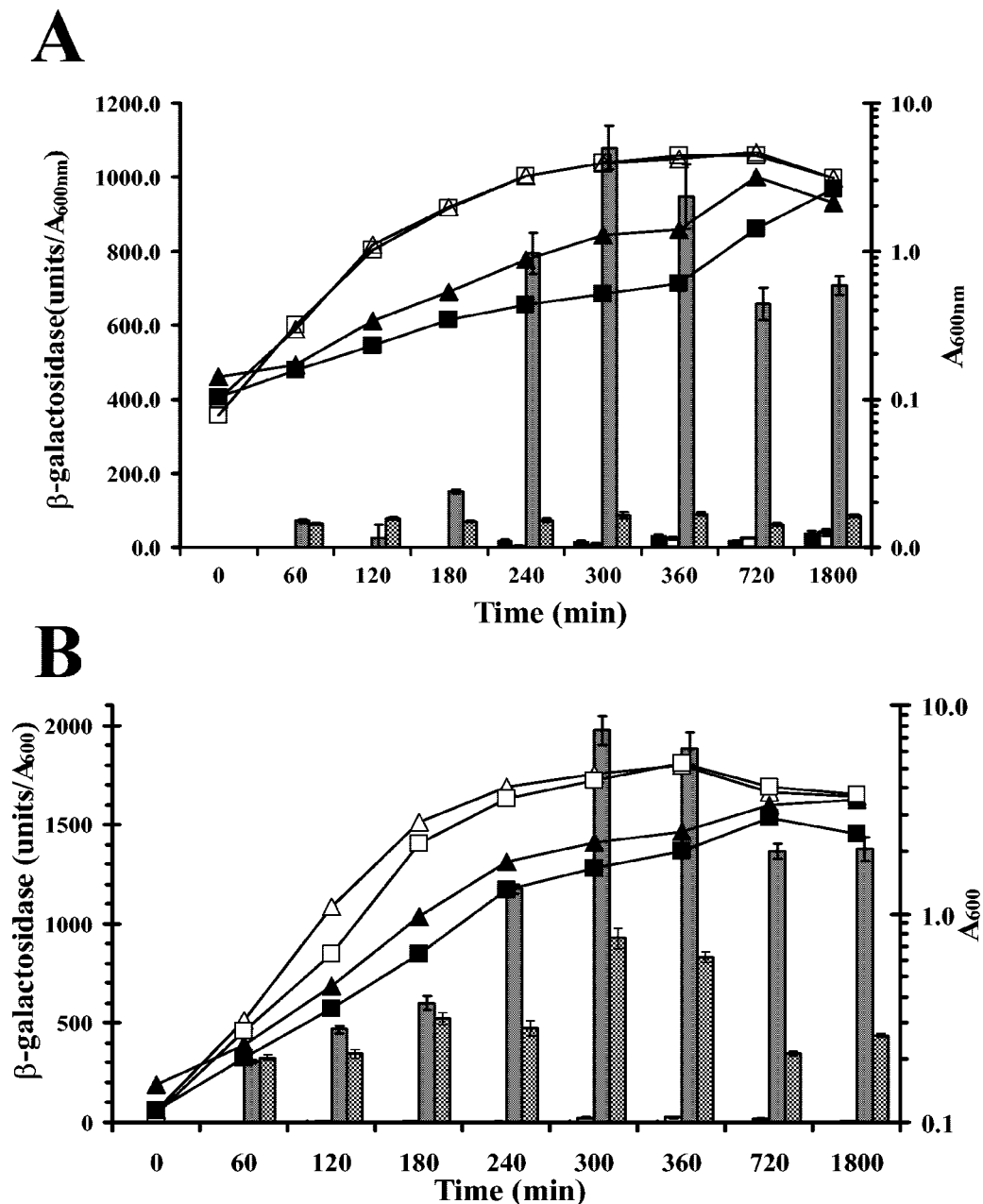


FIG. 2. Effect of growth medium and NaCl on the expression of  $\beta$ -galactosidase activity. EHEC O157:H7 strains 86-24 *esp::lac* and 86-24  $\Delta hha$  *esp::lac* were cultured in LB broth (A) or DMEM (B) in the presence of 100 or 400 mM NaCl. Samples from cultures grown at 37°C (with shaking at 200 rpm) were taken at the indicated times for measuring the  $OD_{600}$  and  $\beta$ -galactosidase activity with an ONPG-based assay. The  $OD_{600}$  is represented as line graphs for the *esp::lac* fusion in 100 mM ( $\Delta$ ) or 400 mM ( $\blacktriangle$ ) NaCl and the  $\Delta hha$  *esp::lac* strain in 100 mM ( $\square$ ) or 400 mM ( $\blacksquare$ ) NaCl.  $\beta$ -Galactosidase activities are shown as solid (100 mM NaCl) or open (400 mM NaCl) bars for *esp::lac* and small checkerboard bars (100 mM NaCl) or large checkerboard bars (400 mM NaCl) for the  $\Delta hha$  *esp::lac* fusion strain. Error bars indicate standard deviations based on three measurements.

constructed in strain 86-24  $\Delta stx2$   $\Delta lac$  *esp::lac*. The resulting strain, 86-24  $\Delta hha$  *esp::lac*, had elevated  $\beta$ -galactosidase activity (Fig. 1C), similar to 86-24 *esp::lac* *hha::TnKn*. Strain 86-24  $\Delta stx2$   $\Delta lac$   $\Delta hha$  *esp::lac* was subjected to transposon mutagenesis, which resulted in the identification of seven kanamycin-resistant colonies that were light blue in color and one kanamycin-resistant colony appearing white out of about 4,000

colonies that were dark blue on kanamycin-X-Gal-LB agar plates.

Analysis of the DNA at the transposon insertion sites in three of seven light blue TnKn isolates resulted in identification of an ORF with 100% homology to the *ler* gene of EHEC O157:H7 (13). The white colony isolate had the transposon inserted in *lacY*, and of the remaining four light blue isolates,



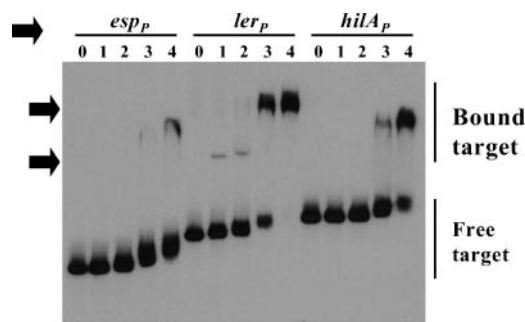


FIG. 3. (A) Detection of Hha binding to DNA fragments with gel shift assays.  $^{32}\text{P}$ -labeled DNA fragments (7,500 cpm) containing the *espA*, *ler*, and *hilA* promoter regions were incubated with 0 mol (lane 0),  $5.8 \times 10^{-11}$  mol (lane 1),  $1.16 \times 10^{-10}$  mol (lane 2),  $1.74 \times 10^{-10}$  mol (lane 3), and  $3.5 \times 10^{-10}$  mol (lane 4) of Hha. Samples containing promoter DNA and purified protein were incubated at  $30^\circ\text{C}$  for 30 min and resolved on a nondenaturing polyacrylamide gel, and DNA bands were visualized by autoradiography. Vertical lines on the right indicate the positions of free and bound DNA, and arrows on the left indicate the positions of two complexes formed by the binding of Hha to the *ler* promoter.

two isolates had transposon insertions in *truA*, one in *tyrU*, and one in *fruR*. Since *ler* is known to be a positive regulator of several LEE- and non-LEE-encoded genes, we selected one of the three  $\Delta hha$  *esp::lac* *ler::TnKn* isolates to determine if *hha* directly affects *ler* transcription. The expression of  $\beta$ -galactosidase activity in the  $\Delta hha$  *esp::lac* *ler::TnKn* isolate was similar to that in the 86-24  $\Delta stx2$   $\Delta lac$  *esp::lac* strain containing a wild-type copy of *hha* (Fig. 1C).

**Downregulation of *ler* expression by *hha*.** An isolate of strain 86-24  $\Delta stx2$   $\Delta lac$  containing a *ler::lac* transcriptional fusion was constructed with the allelic replacement plasmid pSM212. The presence of a chromosomal *ler::lac* fusion was confirmed by PCR with primers VS270 and VS427 and VS269 and VS426, which resulted in the amplification of a 3.8-kb (5' *ler* ORF-*lacZ* *lacY* region) and a 2.3-kb (*lacY* *lacA*-3' *ler* ORF region) fragment, respectively, from the chromosomally generated *ler::lac* fusion (data not shown). Strain 86-24  $\Delta stx2$   $\Delta lac$  *ler::lac* was then deleted of *hha* by the allelic replacement method (described above) with plasmid pSM122. The *hha* deletion was confirmed by the lack of *hha* amplification by PCR with primers VS309 and VS340 (data not shown). As shown in Table 3, 86-24  $\Delta hha$  *ler::lac* produced 22% more  $\beta$ -galactosidase than the parent 86-24 *ler::lac*. The introduction of pSM197R, a pCR2.1 derivative carrying a cloned copy of *hha*, into 86-24  $\Delta hha$  *ler::lac* reduced its  $\beta$ -galactosidase activity to a level 23% lower than that of the parent 86-24 *hha*<sup>+</sup> *ler::lac* strain.

**Interaction of Hha with the *ler* promoter region.** A 388-bp fragment containing the *ler* promoter region was incubated with various amounts of purified Hha, and the reaction mixtures were analyzed by nondenaturing polyacrylamide gel electrophoresis. Hha bound to the *ler* promoter fragment with a higher affinity than to the *hilA* promoter fragment at equivalent concentrations of Hha (Fig. 3). Moreover, binding of Hha to the *ler* promoter fragment produced two types of complexes (indicated by the arrows); a fast-migrating complex appeared at lower Hha concentrations ( $5.8 \times 10^{-11}$  to  $3.5 \times 10^{-10}$  mol

TABLE 3. Downregulation of *ler* expression by *hha*<sup>a</sup>

Strain 86-24 genotype	Plasmid <sup>b</sup>	$\beta$ -Galactosidase activity/ $A_{600}$	% change <sup>c</sup>
<i>ler::lac</i>		$1,389.6 \pm 36.3$	0
$\Delta hha$ <i>ler::lac</i>		$1,700 \pm 46.0$	+22
$\Delta hha$ <i>ler::lac</i>	pSM197R	$1,080 \pm 39.1$	-23
$\Delta hha$ <i>ler::lac</i>	pCR2.1	$1,646 \pm 89.1$	+18

<sup>a</sup> The effect of *hha* on the expression of *ler* was monitored by determination of the  $\beta$ -galactosidase activity of a chromosomal *ler::lac* fusion in strain 86-24 and its derivatives containing or deleted of *hha*. Strains were cultured in LB broth, and samples for monitoring  $\beta$ -galactosidase activity and  $A_{600}$  were taken after 270 min of growth.

<sup>b</sup> pSM197R; harboring a cloned copy of *hha* in pCR2.1, was used for complementing the lack of *hha* function in  $\Delta hha$  *ler::lac* strains.

<sup>c</sup> Increases (+) and decreases (-) in the level of  $\beta$ -galactosidase activity compared to that produced by the parent *ler::lac* fusion strain are shown. The assay variability was within 5% of the mean of three independent measurements.

of Hha), and a slow-migrating complex at higher Hha concentrations ( $1.74 \times 10^{-10}$  to  $3.5 \times 10^{-10}$  mol of Hha).

**Effect of *hha* on transcription of the *esp* and *ler* genes.** Purified RNAs from strains 86-24 *hha*<sup>+</sup> and 86-24  $\Delta hha$  were analyzed by RT-PCR to determine the relative amounts of *espA*- and *ler*-specific transcripts. A housekeeping gene, *gapA*, served as a positive control, as its expression was not affected by the presence or absence of *hha*. As shown in Fig. 4, *ler*- and *esp*-specific amplified products were detected at  $\geq 10$ -fold and  $\geq 100$ -fold-higher levels, respectively, in a  $\Delta hha$  background than in the *hha*<sup>+</sup> strain. The amount of *gapA*-specific amplification products was almost identical in both strains.

**Positive regulatory role of *ler* in *esp* gene expression.** The preceding results suggested that *hha* is a negative regulator of *ler* and that removal of *hha* led to elevated *esp* transcription, presumably by the positive action of Ler. To test this hypothesis, the expression of  $\beta$ -galactosidase from an *esp::lac* strain was tested in the presence and absence of *hha* and the presence and absence of *ler*. The *ler* deletion was introduced into strains 86-24  $\Delta stx2$   $\Delta lac$  *esp::lac* and 86-24  $\Delta stx2$   $\Delta lac$   $\Delta hha$  *esp::lac* with pSM188 and the allelic replacement method described above. The loss of *ler* was confirmed by PCR with the primer set VS426 and VS427. The strain deleted of *ler* produced a 230-bp amplicon, and the strain harboring *ler* produced a 940-bp amplicon (data not shown). As shown in Table 4, 86-24  $\Delta hha$  *ler*<sup>+</sup> *esp::lac* produced 3818% higher  $\beta$ -galactosidase activity than the parent 86-24 *hha*<sup>+</sup> *ler*<sup>+</sup> *esp::lac*. In contrast, 86-24  $\Delta hha$   $\Delta ler$  *esp::lac* showed only a 200% increase in  $\beta$ -galactosidase activity compared to the 86-24 *hha*<sup>+</sup> *ler*<sup>+</sup> *esp::lac* control strain. These results suggested a positive requirement for *ler* in the expression of *esp* genes. The 86-24  $\Delta ler$   $\Delta hha$  *esp::lac* strain produced a 200% higher level of  $\beta$ -galactosidase activity than 86-24 *hha*<sup>+</sup>  $\Delta ler$ <sup>+</sup> *esp::lac*, suggesting that *hha* may also have a negative effect, albeit a milder one, on expression of the *esp* operon.

**Interaction of Ler with the *esp* promoter region.** A 350-bp end-labeled fragment (5 ng) containing the *esp* promoter was incubated with  $1.5 \times 10^{-11}$  mol of GST-Ler fusion protein. At this protein concentration (Fig. 5), more than 50% of the labeled fragments were bound (bound target) with the GST-Ler fusion protein, resulting in a single slow-migrating complex (lane 3) compared to the band (free target) that resulted when the labeled fragment was incubated without the Ler protein

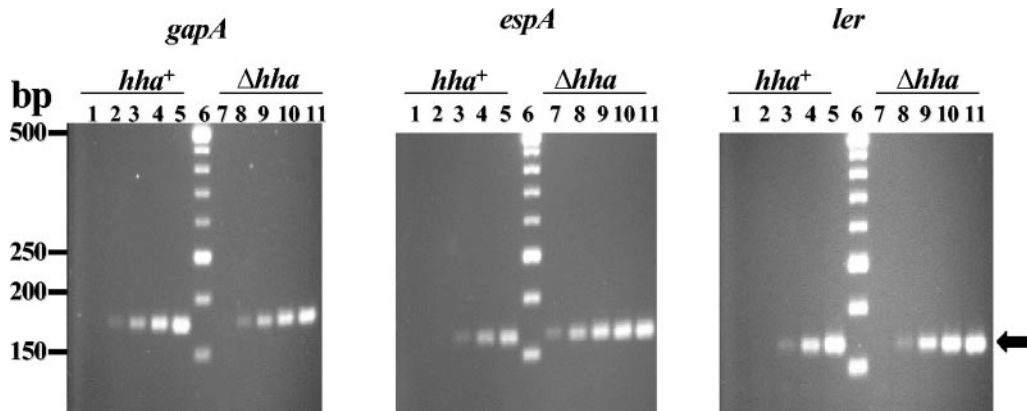


FIG. 4. Determination of transcriptional levels of *esp* and *ler* with RT-PCR. Total RNA purified from 86-24  $\Delta stx2 \Delta lac$  and 86-24  $\Delta stx2 \Delta lac \Delta hha$ , which were grown in LB broth at 37°C (with shaking at 200 rpm), was used in RT-PCR assays containing primer sets for specific amplification and detection of *gapA*-, *esp*-, and *ler*-specific transcripts. *gapA*, a housekeeping gene, was used as a control. Amplified DNA was resolved on 4% agarose gels containing ethidium bromide, and DNA bands were visualized with the Alpha Innotech Image documentation system. Lanes 1 to 5, RT-PCR conducted in the presence of  $7.5 \times 10^{-4}$ ,  $7.5 \times 10^{-3}$ ,  $7.5 \times 10^{-2}$ ,  $7.5 \times 10^{-1}$ , and  $7.5 \mu\text{g}$ , respectively, of total RNA from 86-24  $\Delta stx2 \Delta lac$ ; lane 6, DNA size markers (sizes listed on the left); lanes 7 to 11, RT-PCR conducted in the presence of  $7.5 \times 10^{-4}$ ,  $7.5 \times 10^{-3}$ ,  $7.5 \times 10^{-2}$ ,  $7.5 \times 10^{-1}$ , and  $7.5 \mu\text{g}$ , respectively, of total RNA from 86-24  $\Delta stx2 \Delta lac \Delta hha$ . The arrow on the right points to the position of amplified products specific for *gapA*, *espA*, and *ler*.

(lane 1) or with the GST protein only (lane 2). When the labeled promoter fragment (5 ng) was mixed with increasing amounts (10 to 500 ng) of the unlabeled *esp* promoter fragment (specific competitor) prior to addition of the GST-Ler protein, an increasing amount of labeled DNA migrated as free DNA (lanes 4 to 8), indicating that the GST-Ler protein-*esp* promoter interactions were specific. On the other hand, addition of 100 to 500 ng of an unlabeled nonspecific DNA (lanes 9 and 10) to the reactions containing the labeled *esp* promoter fragment and the GST-Ler fusion did not alter the specific interactions between the *esp* promoter and Ler.

## DISCUSSION

The *esp* operon of EHEC O157:H7 encodes secreted proteins EspA, EspD, and EspB, and it is analogous to *LEE4* of EPEC O126:H6 (26). The Esp proteins are essential for the formation of attaching and effacing lesions by EHEC and EPEC on intestinal epithelial cells (20). The promoter element driving the expression of the *esp* operon in EHEC is virtually identical to the promoter governing the expression of *LEE4* in EPEC, and transcription activities at these promoter regions

are dependent on environmental stimuli (2). The transcriptional regulation of several LEE- and non-LEE-encoded operons in EPEC and EHEC is under the positive regulation of Ler, a protein with similarity to the H-NS family of DNA binding proteins (12). Moreover, recent studies have also shown that Ler is absolutely necessary for full transcription of the *esp* (*LEE4*) operon in both EPEC and EHEC (12), but the role of *ler* in transcriptional regulation of the *esp* operon is not

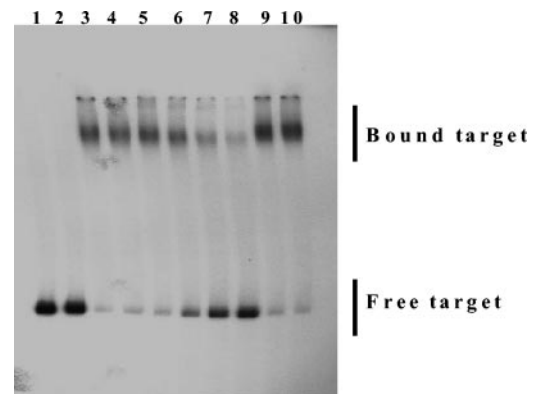


FIG. 5. Gel shift assay demonstrating specificity of binding of purified Ler to the *esp* promoter.  $^{32}\text{P}$ -labeled *esp* promoter DNA (5 ng containing 7,500 cpm) was mixed either with unlabeled *esp* promoter DNA (specific competitor) or an unrelated DNA fragment (nonspecific competitor) and  $1.5 \times 10^{-11}$  mol of purified GST-Ler fusion protein. Lane 1, labeled *esp* promoter incubated with  $2.25 \times 10^{-10}$  mol of GST protein; lane 2, labeled *esp* promoter incubated with GST-Ler fusion protein; lanes 3 to 8, labeled *esp* promoter incubated with GST-Ler and 0, 5, 25, 50, 100, and 500 ng, respectively, of unlabeled *esp* promoter DNA; lanes 9 and 10, labeled *esp* promoter incubated with GST-Ler and 100 or 500 ng, respectively, of unlabeled nonspecific competitor DNA. Samples containing promoter DNA and purified protein were incubated at 30°C for 30 min and resolved on a non-denaturing polyacrylamide gel, and DNA bands were visualized by autoradiography. Vertical lines on the right indicate the positions of free and bound DNA.

TABLE 4. Dependence of *esp* expression on *ler*<sup>a</sup>

Strain 86-24 genotype	$\beta$ -Galactosidase activity/ $A_{600}$	% change <sup>b</sup>
<i>hha</i> <sup>+</sup> <i>ler</i> <sup>+</sup> <i>esp::lac</i>	29.7 $\pm$ 3.6	0
$\Delta hha$ <i>ler</i> <sup>+</sup> <i>esp::lac</i>	1,134 $\pm$ 48.4	+3,818
$\Delta hha \Delta ler$ <i>esp::lac</i>	59.4 $\pm$ 6.3	+200
$\Delta ler$ <i>hha</i> <sup>+</sup> <i>esp::lac</i>	29.2 $\pm$ 4.2	-1.69

<sup>a</sup> Effect of *ler* on the expression of *esp* operon was monitored by the determination of  $\beta$ -galactosidase activity of a chromosomal *esp::lac* fusion in 86-24 and its derivatives deleted either of *ler* or *hha* or both *ler* and *hha*. Strains were cultured in LB broth and samples for monitoring  $\beta$ -galactosidase activity and  $A_{600}$  were taken after 270 min of growth.

<sup>b</sup> See Table 3, footnote c. The assay variability was within 4 to 15% of the mean of three independent measurements. High variability was normally seen in samples expressing very low  $\beta$ -galactosidase activity.

completely understood. Similarly, the requirement of additional, albeit unknown, factors in transcriptional regulation of the *esp* operon has not been completely ruled out.

In order to identify transcriptional factors affecting transcription of the *esp* operon, strain 86-24  $\Delta$ *stx2*  $\Delta$ *lac* carrying an *esp::lac* transcriptional fusion was subjected to transposon mutagenesis. A transposon mutant that produced 17-fold more  $\beta$ -galactosidase than the parent *esp::lac* fusion strain was identified. The maximum expression of  $\beta$ -galactosidase was observed as bacterial cells entered the stationary phase. Nucleotide sequence analysis of DNA flanking the transposon insertion site resulted in the identification of *hha*. The *hha* gene encodes an 8.5-kDa protein (Hha) that is implicated in the regulation of  $\alpha$ -hemolysin in pathogenic *E. coli* (33) and *S. enterica* serovar Typhimurium (14). A plasmid-cloned copy of *hha* reduced the highly increased expression of the *esp::lac* fusion in the *hha::TnKn* mutant to the level expressed in the parent 86-24 *esp::lac* strain, confirming that *hha* acted as a negative regulator of *esp* gene expression.

We also observed that 86-24 *esp::lac*  $\Delta$ *hha* produced severalfold-higher  $\beta$ -galactosidase activity than the parent *esp::lac* strain in LB broth or DMEM-HEPES of low or high osmolarity. With a highly sensitive assay for measuring  $\beta$ -galactosidase activity, Beltrametti et al. (2) observed only a fourfold increase in the level of  $\beta$ -galactosidase activity of an *esp::lac* fusion strain of EHEC O157:H7 in DMEM-HEPES or in high-osmolarity M9 minimal medium. In our studies, we noticed that 86-24 *esp::lac*  $\Delta$ *hha* produced 294-fold more  $\beta$ -galactosidase activity than the parent *esp::lac* strain after 5 h of growth in DMEM-HEPES (100 mM NaCl), indicating that the presence of *hha* downregulated the expression of the *esp* genes in the *esp::lac* strain. Although the growth rates of the 86-24 *esp::lac* and *esp::lac*  $\Delta$ *hha* strains were reduced in DMEM-HEPES of high osmolarity (400 mM NaCl), the merely fourfold increase in the  $\beta$ -galactosidase activity of the parent strain compared to the 138-fold increase in the *hha* mutant strain after 5 h of growth in high-osmolarity DMEM-HEPES reiterates the negative role of *hha* in *esp* gene expression.

Hha homologs are involved in the regulation of virulence genes in *Y. enterocolitica* (YmoA) (9) and conjugative plasmid transfer in *E. coli* (RmoA) (35) and constitute an Hha-YmoA-RmoA family of proteins. These proteins are environment-dependent modulators of gene expression, and mutations in the genes encoding these proteins produce pleiotropic effects. Since environment dependency and pleiotropism are also characteristics of H-NS, a well-characterized nucleoid-associated protein (1), Hha and related proteins are also classified as nucleoid-associated proteins. Experimental evidence supporting the nucleoid association of Hha is implicit in findings that *hha* mutants display alterations in reporter plasmid topology (7) and overexpression of Hha increases the frequency of transposition of insertion elements in *E. coli* (7, 29).

Recent studies have demonstrated that Hha acts as a negative modulator of gene expression either by binding directly to a specific sequence located in the promoter region of a target gene (*hilA* regulation in *S. enterica* serovar Typhimurium) (14) or by oligomerizing with H-NS protein before binding to a specific regulatory sequence in the target gene (hemolysin expression in *E. coli*) (25, 34). However, the Hha protein purified from strain 86-24 bound poorly to the fragment containing the

*esp* promoter in a gel shift assay but showed greater affinity for the fragment containing the *hilA* promoter region of *S. enterica* serovar Typhimurium. The poor binding of Hha to the *esp* promoter observed in gel shift assays and only a 200% increase in the expression of  $\beta$ -galactosidase of 86-24  $\Delta$ *hha*  $\Delta$ *ler* *esp::lac* compared to 86-24  $\Delta$ *ler* *hha*<sup>+</sup> *esp::lac* might be indicative of poor binding of Hha to the *esp* promoter under in vivo conditions. However, the lack of efficient binding of Hha to the *esp* promoter could not be attributed to the promoter sequence that we selected for use in the electrophoretic mobility shift assay because this promoter region was identified based on primer extension data that clearly defined the transcriptional start site immediately upstream of *espA* and on the ability of this promoter to direct the expression of *lacZ* fusions, providing strong evidence that this promoter directed transcription of the polycistronic *espADB* mRNA (2).

A second promoter, located upstream of *sepL*, has also been demonstrated to direct the synthesis of a polycistronic transcript extending from *sepL* through *espD* that may be processed into a *sepL* monocistronic mRNA and a larger *espADB* transcript (23, 28, 36). However, the relative contribution of transcripts from the *sepL* promoter versus the *espA* promoter in the synthesis of EspA, EspD, and EspB is unclear. Roe et al. (39) also reported that expression of the *esp::lac* translation fusion was detected only when this fusion was transcribed from the *sepL* promoter, but that study involved partial comparison of nonisogenic isolates of *E. coli* O157:H7, and it is unclear if the promoter regions of these isolates are identical to those studied here or as reported by Beltrametti et al. and Kresse et al. (2, 23). It is reasonable to assume that both promoters can direct transcription of *espADB* but that these promoters may have different strengths and may be under different forms of regulation. The apparent direct interaction between Ler and the *espA* promoter that we have demonstrated in our studies provides additional evidence that this promoter likely has an important role in *esp* expression.

One possible explanation for the lack of Hha binding to the *esp* promoter with a higher affinity could be an absolute need by Hha to form nucleoprotein complexes with H-NS or H-NS-like proteins in order for it to bind and completely repress transcription from the *esp* promoter. Data from transcriptional studies involving *esp::lac* transcriptional fusions and identification of putative H-NS-binding sites in the *esp* promoter region of EHEC O157:H7 EDL933 have implicated H-NS in the negative regulation of *esp* operon (2).

An alternative explanation is that Hha represses the expression of another gene that serves as a positive regulator of *esp* transcription. We identified transposon mutants of 86-24  $\Delta$ *hha* *esp::lac* that no longer expressed high levels of  $\beta$ -galactosidase activity. In fact, the  $\beta$ -galactosidase activity of these mutants was similar to the level expressed by 86-24 *hha*<sup>+</sup> *ler*<sup>+</sup> *esp::lac*. These mutants carried transposon insertions in *ler*, suggesting that Hha might repress *ler*, and repression of *ler* by Hha results in reduced expression of *esp*. A negative effect of Hha on the transcription of *ler* was demonstrated by the following observations. (i) Increased production of  $\beta$ -galactosidase activity in 86-24  $\Delta$ *hha* *ler::lac*. This increase in the  $\beta$ -galactosidase activity of  $\Delta$ *hha* *ler::lac* (1.2-fold higher than that of the *ler::lac* strain), however, was much lower than that observed in the  $\Delta$ *hha* *esp::lac* strain (17-fold higher than the *esp::lac* strain). A small

increase in  $\beta$ -galactosidase activity of the  $\Delta hha\ ler::lac$  strain could be attributed to the fact that the parent  $ler::lac$  fusion strain produced a very high basal level of  $\beta$ -galactosidase activity. A higher basal-level expression of  $\beta$ -galactosidase activity from the  $ler::lac$  fusion strain has also been observed in other studies (44).

(ii) The presence of pSM197R (pCR2.1 carrying a cloned copy of *hha*) in 86-24  $\Delta hha\ ler::lac$  reduced  $\beta$ -galactosidase activity to a level lower (presumably due to the presence of multiple copies of the plasmid-cloned *hha*) than that expressed by the parent 86-24  $ler::lac$  strain. (iii) RT-PCR assays showed a  $\geq 10$ -fold increase in the level of *ler*-specific transcripts in 86-24 carrying an in-frame *hha* deletion. (iv) Purified Hha bound to the *ler* promoter with the highest affinity. Hha not only bound to the *ler* promoter but also generated a binding pattern that was strikingly different from the one observed with the *hilA* promoter of *S. enterica* serovar Typhimurium (14). Hha formed a single complex with the *hilA* promoter, while two discrete complexes of different sizes were observed for the *ler* promoter at different concentrations of Hha.

Although we have not presented direct evidence, the pattern of Hha binding to the *ler* promoter suggests a possible cooperative mode of binding, which presumably requires multimerization of Hha. The cooperative mode of DNA binding is exhibited by regulatory proteins that contain an N-terminal oligomerization domain to recognize and bind to specific regulatory sequences for modulating the transcriptional activity of downstream genes (19, 24). N-terminal domains possessing structural features facilitating adoption of coiled-coil formation for protein oligomerization have been identified in H-NS and H-NS-like proteins (3) and Hha (23, 32).

A positive regulatory role of *ler* in expression of the *esp* genes was corroborated by the findings that strain 86-24  $\Delta hha\ esp::lac$  deleted of *ler* could no longer produce higher levels of  $\beta$ -galactosidase activity and purified Ler exhibited specific binding to the *esp* promoter. Previous reports have shown that H-NS plays a negative role in the transcription of *esp* genes and that putative H-NS binding sites exist in the promoter region of the *esp* operon (2). Bustamante et al. (6) demonstrated that Ler acts as an antirepressor of H-NS and presumably activates the expression of the divergently transcribed *LEE2* and *LEE3* operons by competing with H-NS for binding to the promoter element located upstream of *LEE2*. The activation of *esp* expression observed in EHEC 86-24  $\Delta hha$ , which also showed increased expression of Ler, could also result from the antirepressor properties of Ler that facilitate preferential binding of Ler over H-NS to the *esp* promoter region.

In summary, the results presented in this study clearly demonstrate that Ler acts as a positive regulator of *esp* expression and the level of *esp* expression is regulated by the degree of repression exerted by Hha on *ler* transcription. Several regulatory factors that affect the expression of *ler* in both EPEC and EHEC have been identified. In EPEC, expression of *ler* is under the positive regulation of the Per regulon (28), and the quorum-sensing *E. coli* regulator A (QseA) activates the transcription of *ler* in both EPEC and EHEC (42). Transcription of *ler* in EPEC is also dependent on integration host factor (15), Fis (16), and BipA (17). Since quorum sensing appears to be the major pathway that activates the expression of *ler* in EHEC O157:H7, a better understanding of the factors that modulate

the expression of *hha* and facilitate enhanced expression of *ler* would increase our understanding of virulence gene expression in EHEC O157:H7.

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