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Transcriptional Regulation of the *orf19* Gene and the *tir-cesT-eae* Operon of Enteropathogenic *Escherichia coli*

CLAUDIA SÁNCHEZ-SANMARTÍN, VÍCTOR H. BUSTAMANTE, EDMUNDO CALVA, AND JOSÉ LUIS PUENTE*

Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos 62251, México

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To establish an intimate interaction with the host epithelial cell surface, enteropathogenic *Escherichia coli* (EPEC) produces Tir, a bacterial protein that upon translocation and insertion into the epithelial cell membrane constitutes the receptor for intimin. The *tir* gene is encoded by the locus for enterocyte effacement (LEE), where it is flanked upstream by *orf19* and downstream by the *cesT* and *eae* genes. With the use of a series of *cat* transcriptional fusions and primer extension analysis, we confirmed that *tir, cesT*, and *eae* form the *LEE5* operon, which is under the control of a promoter located upstream from *tir*, and found that the *orf19* gene is transcribed as a monocistronic unit. We also demonstrated that the LEE-encoded regulator Ler was required for efficient activation of both the *tir* and the *orf19* promoters and that a sequence motif located between positions −204 and −157 was needed for the Ler-dependent activation of the *tir* operon. Sequence elements located between positions −204 and −97 were determined to be required for the differential negative modulatory effects exerted by unknown regulatory factors under specific growth conditions. Upon deletion of the upstream sequences, the *tir* promoter was fully active even in the absence of Ler, indicating that *tir* expression is subject to a repression mechanism that is counteracted by this regulatory protein. However, its full activation was still repressed by growth in rich medium or at 25°C, suggesting that negative regulation also occurs at or downstream of the promoter. Expression of *orf19*, but not of the *tir* operon, became Ler independent in an *hns* mutant strain, suggesting that Ler overcomes the repression exerted by H-NS (histone-like nucleoid structuring protein) on this gene.

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of acute and persistent infantile diarrhea and a leading cause of infant death in developing countries (35, 43, 45). The interaction of EPEC with the host cell, which has been the subject of several recent reports, has been divided into three different stages characterized by two distinctive phenotypes, localized adherence and the attaching-and-effacing (A/E) lesion (reviewed in references 9, 11, 18, and 43). The virulence determinants required for the induction of the A/E lesion in EPEC are encoded in a 35.6-kb pathogenicity island, denoted LEE (for locus of enterocyte effacement), which contains 41 predicted open reading frames (16, 38, 39). Based on recent studies and sequence analyses, most of the LEE-encoded genes have been divided into three functional regions: the *esc* and *sep* genes, which code for a type III secretion-translocation apparatus (25); the *tir, cesT*, and *eae* genes, coding for the proteins involved in intimate attachment (1, 14, 26, 28); and the *esp* genes, which encode effector proteins that are involved in the formation of a translocon for delivering effector molecules to the host cell (17, 29, 30, 32, 33, 51).

Recent studies have indicated that the LEE-encoded genes are organized into five major operons (14, 41): the *LEE1, LEE2*, and *LEE3* operons, which contain the *esc* and *sep* genes; the *LEE4* operon, which encodes secreted Esp proteins; and the *tir, cesT*, and *eae* cluster, herein denoted *LEE5*. Ler acts as a positive transcriptional regulator of the *LEE2, LEE3, LEE4*, and *LEE5* operons (5, 19, 41). Furthermore, integration host factor (IHF) (19) and a quorum-sensing autoinducer (LuxS) (47) are also required for efficient activation of the LEE-encoded genes. In addition, it has been proposed that Ler overcomes the negative regulation exerted by H-NS (histone-like nucleoid structuring protein) on the expression of at least the *LEE2, LEE3*, and *LEE4* operons (5).

We studied the transcriptional organization and regulation of the *orf19, tir, cesT*, and *eae* genes of EPEC. The *tir* gene codes for Tir (translocated intimin receptor), which is transferred by the type III secretion system into host cells, where it is phosphorylated and inserted into the host cell membrane (28). Tir is the receptor for intimin, an *eae*-encoded outer membrane protein necessary for intimate attachment to epithelial cells (26). *cesT*, previously known as *orfU*, codes for a chaperone that is required for the stable secretion of Tir (1, 14). The *orf19* gene encodes a protein that exhibits similarity to IpgB of *Shigella flexneri* (16) and to TrcA and TrcP of EPEC (48, 50). In the present study we have demonstrated that *tir, cesT*, and *eae* constitute an operon and that *orf19* is a monocistronic unit. We show that transcription of the *tir* operon and the *orf19* gene requires Ler, which seems to overcome the negative regulation exerted by a repressor protein that is also present in *E. coli* K-12. In addition, we show that distinct *cis*-acting elements are involved in negative and positive regulation of *tir* expression by different regulatory elements and environmental cues.

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* Corresponding author. Mailing address: Departamento de Microbiología Molecular, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca, Morelos 62251, México. Phone: (52) 73 291 621. Fax: (52) 73 138 673. E-mail: puente@ibt.unam.mx.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
E2348/69	Wild-type EPEC O127:H6	36
JPN15	E2348/69 lacking the EAF plasmid	26
E2348/69Δler	E2348/69; Δ*ler* mutant	5
B171-8	Wild-type EPEC O111:NM	44
B171-10	B171-8 lacking the EAF plasmid	4
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 prsL150 relA1 deoC1 rbsR fthD5301 fruA25*λ$^-$	7
RO64	MC4100 *lrp-201*::Tn*10*	34
MCG007	MC4100 *rpoS*::Tn*10*	C. Gómez
MCG009	MC4100 *fis*::*kan*	C. Gómez
GS480Δ900	MC4100 *malP*::*neo* Δ(*envZ-malP*)*900*	20
CSH56	Δ*lac-pro supD nalA thi*	52
CSH56 Δ*hns*	CSH56 carrying a deletion of *hns*	52
N99	F$^-$ *galK2 rpsL* λ$^-$	22
K5185	N99 Δ*himA82*	42
ET8000	*rbs gyrA hutC*$_K$ *lacZ*::IS*1* Mu *cts62*	23
ET8045	ET8000 *rpoN208*::Tn*10*	8
SM796	F$^-$ *araD139* Δ(*araABC-leu*)*7697 galE galK* Δ(*lac*) *X74 rpsL thi phoA* ΔPvull *phpR*	6
SBC796	SM796 *fur*::Tn*5*	6
Plasmids		
pKK232-8	pBR322 derivative containing a promoterless *cat* gene	3
pCAT232	pKK232-8 derivative containing a *bfpA-cat* transcriptional fusion from nt −232 to +36	44
pKORF1	pMPM-K3 derivative carrying the *ler* gene expressed under the control of the *tac* promoter	5
pCS-TVW	pMPM-K3 derivative carrying the *per* (*bfpTVW*) locus expressed under the control of the *tac* promoter	Sánchez-SanMartín et al. (unpublished)
pORF19	pKK232-8 derivative containing the *orf19-cat* transcriptional fusion from nt −175 to +166	This study
pTIR394	*tir-cat* transcriptional fusion from nt −394 to +138	This study
pTIR243	*tir-cat* transcriptional fusion from nt −243 to +138	This study
pTIR204	*tir-cat* transcriptional fusion from nt −204 to +138	This study
pTIR157	*tir-cat* transcriptional fusion from nt −157 to +138	This study
pTIR122	*tir-cat* transcriptional fusion from nt −122 to +138	This study
pTIR97	*tir-cat* transcriptional fusion from nt −97 to +138	This study
pTIR80	*tir-cat* transcriptional fusion from nt −80 to +138	This study
pTIR45	*tir-cat* transcriptional fusion from nt −45 to +138	This study
pTIR22	*tir-cat* transcriptional fusion from nt −22 to +138	This study
pTIREAE	*tir-cat* transcriptional fusion from nt −294 of *tir* to the fourth codon of *eae*	This study
pTIR-EAE-DEL1	*tir-cat* transcriptional fusion from nt −294 of *tir* to the 15th codon of *cesT*	This study
pTIRCAM-10	pTIR-EAE derivative in which the −10 promoter hexamer sequence of *tir* was replaced by a *Sac*I recognition sequence	This study
pTIRCAM-10-DEL1	pTIRCAM-10 derivative from which the *cesT-eae* intergenic region was deleted	This study
pCEST	*cesT-cat* transcriptional fusion containing the intergenic region between *tir* and *cesT*	This study
pEAE1800	*eae-cat* transcriptional fusion containing 1,800 bp upstream from the *eae* start codon	This study
pEAE1629	*eae-cat* transcriptional fusion containing 1,629 bp upstream from the *eae* start codon	This study
pCEST-EXT-EAE	pCEST derivative, equivalent to pEAE1629	This study
pEAE1422	*eae-cat* transcriptional fusion containing 1,422 bp upstream from the *eae* start codon	This study
pEAE532	*eae-cat* transcriptional fusion containing 532 bp upstream from the *eae* start codon	This study

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used in this study are listed in Table 1. Overnight cultures were grown at 37°C in Luria-Bertani (LB) broth medium (46). Dulbecco's modified Eagle's medium (DMEM) containing 0.45% (wt/vol) glucose and L-glutamine (584 mg/l) without sodium pyruvate (Gibco Life Technologies) and supplemented with pyridoxal (4 μg/ml) was used for growth at 37°C. Where indicated, 20 mM ammonium sulfate was added. An overnight LB culture was pelleted, and the bacteria were resuspended in phosphate-buffered saline, pH 7.4, to an optical density at 600 nm (OD_{600}) of 1. Fifty milliliters of DMEM or LB was inoculated with 1 ml of the bacterial phosphate-buffered saline suspension and incubated in an orbital shaker water bath (Amerex Instruments) at 200 rpm and various temperatures. When necessary, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; nalidixic acid, 15 μg/ml, kanamycin, 40 μg/ml, chloramphenicol, 50 μg/ml, gentamicin, 15 μg/ml; and tetracycline, 25 μg/ml. Samples were collected every hour, or when the cultures reached OD_{600}s of 0.8, 1.0, 1.2, and 1.4, to determine chloramphenicol acetyltransferase (CAT) activity or for RNA extraction.

Molecular biology techniques. DNA manipulations were performed according to standard protocols (46). Restriction and DNA-modifying enzymes were obtained from Boehringer Mannheim, New England Biolabs, or Gibco BRL and used according to the manufacturer's instructions. [α-^{32}P]dCTP (3,000 Ci mmol^{-1}) was purchased from Amersham Corp. Oligonucleotides were purchased from BioSynthesis or provided by the Oligonucleotide Synthesis Facility at our institute. PCRs were performed in 100- or 50-μl volumes, with AmpliTaq (Perkin-Elmer) being used according to the manufacturer's instructions. Double-stranded DNA sequencing of the plasmids generated in this work was carried out by the dideoxy-chain termination procedure with a Thermo Sequenase cycle sequencing kit according to the manufacturer's (Amersham) instructions.

Construction of *cat* transcriptional fusions. PCR fragments of different lengths that spanned the region between *orf19* and *eae* were amplified using as a

TABLE 2. Primers used in this study

Primer	Sequence (5′-3′)
CAM10TIR2R	ACCAGAGCTCTCACAGTATAATTTTTG
CAM10TIR1F	GTGAGAGCTCTGGTTTATGCTCAGTTTG
EAE1669REG	GGGATAAAAAGCTTGAGTA
EAEREG06	GTTCGCTCAGATCCTAAATTCTG
EAEREG1052L	CAACGTTGCAGCATGGGTAAC
ORFU-H3R	CCAACACCAATTTTTTCCGC
ORF19-H3R	CCGCCTAAGCTTACGCTCTAC
RORF10-H3R	TGTTATCCCAAGCTTATGT
TIR-H3R	CTGTTTGTGAAGCTTGTGGC
TIR22F-E23	CATCAAAAAGGATCCTGTGATTTATTTG
TIR45F-E23	CATTTCTGGATCCTTATTTTGC
TIR80F-E23	GATTTTGATTATGGGATCCTTTA
TIR97F-E23	GATAATTATAGGATCCTTTTGATTATG
TIR122F-E23	CATATAAAAATTAGGATCCTTTTTTTTC
TIR157F-E23	ATATTATTTTTGGATCCACAATTAAATTTC
TIR204F-E23	TTTTTTCTGGATCCAAAAAGGTCTC
TIR243F-E23	GATACTCGGATCCAGGGGGAAAC
16S	CACAGATTGTCTGATAAATTG

template chromosomal DNA from wild-type EPEC strain E2348/69. The forward and reverse oligonucleotides were designed to introduce *Bam*HI or *Hin*dIII restriction sites, respectively (Table 2). The PCR-amplified fragments were digested with *Bam*HI and *Hin*dIII and cloned into pKK232-8, a vector, digested with the same enzymes, containing a promoterless CAT gene (*cat*) (Pharmacia LKB Biotechnology). Each ligation reaction product was electroporated into *E. coli* MC4100, after which ampicillin-resistant colonies were selected. The resulting plasmids (Table 1) were sequenced to confirm the fidelity of the PCR amplification and introduced into different strains by electroporation, using a Gene Pulser apparatus (Bio-Rad) at settings of 2.5 kV, 25 μF, and 200 Ω, or by $CaCl_2$ transformation according to standard protocols (46).

Mutagenesis of the −10 promoter sequence. Oligonucleotide CAM10tirR plus pKKampi and oligonucleotide CAM10tirF plus pKKcat (Table 2) were used to amplify two fragments encompassing the *tir* regulatory region contained in pTIREAE. Oligonucleotides CAM10tirR and -F introduce a *Sac*I restriction site that replaces the −10 promoter hexamer. Both fragments were digested with *Sac*I and with either *Bam*HI (left fragment) or *Hin*dIII (right fragment) and cloned into pKK232-8 digested with *Bam*HI and *Hin*dIII, creating pTIRCAM-10. The insert in this plasmid was sequenced to verify that only the −10 hexamer sequence was modified.

RNA isolation and primer extension analysis. Total bacterial RNA from samples obtained from DMEM or LB cultures was isolated using a commercial kit (RNeasy [Qiagen] or Boehringer Mannheim High Pure RNA isolation kit). End-labeled oligonucleotides complementary to the 5′ end of the *cat* structural gene or the *tir, cesT, eae*, or *orf19* structural gene were used for primer extension reactions as previously described (5, 37). The extended products were purified with a Microcon-10 microconcentrator (Amicon) and analyzed by electrophoresis in 8% polyacrylamide–urea gels. Primer extension using a 16S rRNA-specific oligonucleotide was included as a control to monitor RNA quality and loading concentration. Sequence ladders were generated with the same primers and DNA of the different pKK232-8 derivatives or plasmids carrying the genes being studied in this work.

CAT assay. The CAT assay was performed as described previously (5, 37, 44).

RESULTS

Transcriptional organization of the *orf19, tir, cesT*, and *eae* genes. The start codon of *tir* is located 326 bp downstream from the stop codon of *orf19*, while the *tir-cesT* and *cesT-eae* intergenic regions consist of 137 and 63 bp, respectively (Fig. 1). To confirm and further analyze the transcriptional organization of the *orf19, tir, cesT*, and *eae* genes and to identify intergenic regions with promoter activity, a series of transcriptional fusions to the *cat* reporter gene was constructed (Fig. 1). These plasmids were transformed into EPEC wild-type strains E2348/69 and B171-8, and CAT activity directed by each fusion was determined from samples collected from cultures grown in DMEM at 37°C, conditions that induce expression of different virulence factors in EPEC (5, 27, 37, 44). The fusions carried by plasmids pTIR394, pTIREAE, and pTIREAE-DEL1, which all contain the 5′ upstream region of *tir*, expressed significant levels of CAT (Fig. 1). In contrast, fusions pEAE1800, pEAE1629, pEAE1422, and pEAE520, which contain different lengths of the region upstream from the *eae* start codon but lack the *tir* promoter region, expressed only background levels of CAT activity (Fig. 1 and data not shown). These results confirmed that *tir, cesT*, and *eae* constitute an operon that is expressed under the control of a promoter located upstream of *tir* and indicated that *eae* does not have an independent promoter.

To confirm that the *eae* gene is transcribed from the *tir* promoter, the putative −10 hexamer (see below) was replaced by the CTCGAG sequence in pTIREAE, as described in Materials and Methods, generating pTIRCAM-10. As expected, this fusion did not express CAT (Fig. 1). Fusion plasmid pORF19, which contains 308 bp upstream from the translational start codon of *orf19*, was active (Fig. 1), indicating that this gene is transcribed from its own promoter.

A fusion containing just the *tir-cesT* intergenic region (pCEST) rendered activity levels that suggested the existence of an additional active promoter for the *cesT* gene (Fig. 1). However, fusions pEAE1800, pEAE1629, and pEAE1422, containing the *tir-cesT* and *cesT-eae* intergenic regions, were inactive (Fig. 1), suggesting a role for the *cesT-eae* intergenic region in terminating transcription originating at the *cesT* putative promoter. To further analyze this possibility, the *cesT-eae* intergenic region was cloned into pCEST to generate plasmid pCEST-EXT-EAE (see Materials and Methods). In this case, no activity was detected (data not shown). When this region was deleted from the inactive fusions carried by plasmids pEAE1629 and pTIRCAM-10, thus recreating the pCEST fusion and generating pTIRCAM-10-DEL1 (which lacks a functional *tir* promoter), respectively, the *cesT* transcriptional activity was recovered (Fig. 1).

Two transcriptional start sites were identified for the putative *cesT* promoter when primer extension experiments were performed with total RNA of EPEC E2348/69 carrying either pCEST, pEAE1629, or pTIRCAM-10 fusions (Fig. 2C). These results confirmed the existence of active *cesT* promoters in these fusions and suggested that the *eae* upstream region contains elements involved in terminating transcription originating from the *cesT* promoter.

Determination of the transcriptional start sites of the *orf19* and *tir* genes. The potential transcriptional activity from the 5′ upstream regions of the four genes considered for this study was tested by primer extension analysis (Fig. 2 and data not shown). A transcriptional start site corresponding to a T residue was located 85 bp upstream from the translational start codon of *tir* (Fig. 2A), one base further downstream from where it was previously mapped (14).

Examination of the 5′ upstream region revealed the presence of putative −35 (TTGCAT) and −10 (TTTATT) promoter sequences (Fig. 3A). When the putative −10 promoter sequence was replaced by the CTCGAG sequence, fusions carrying this mutation became inactive (Fig. 1), indicating that this sequence motif was essential for transcriptional activation of the *tir* operon. An additional transcriptional start site for *tir*,

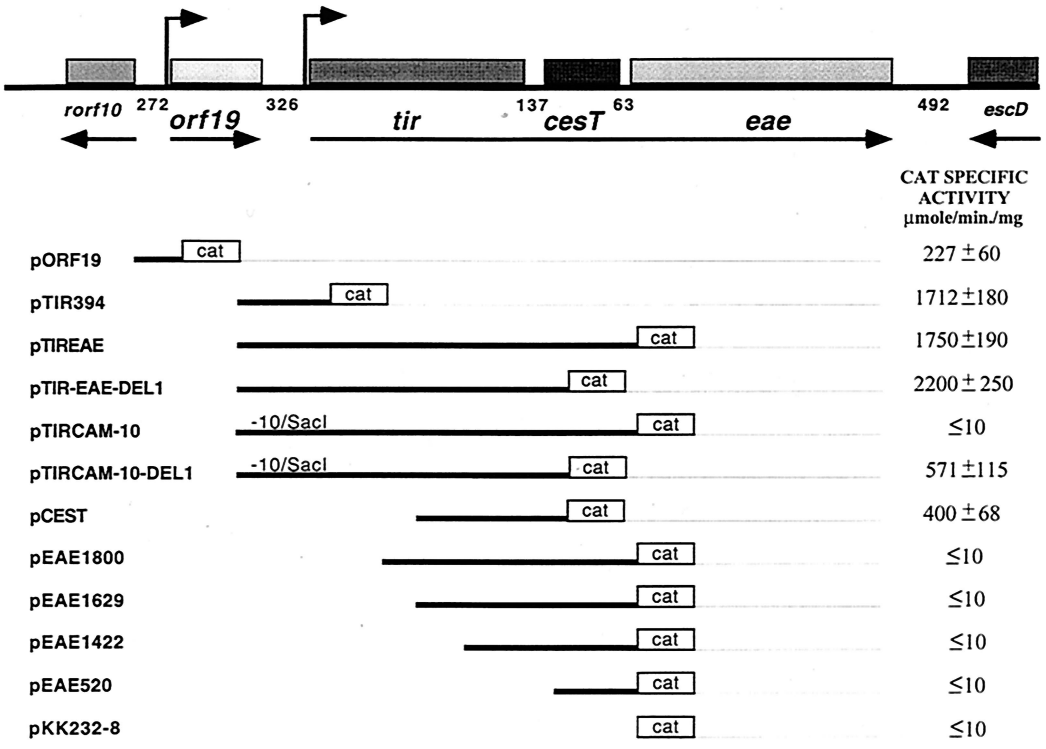


FIG. 1. *tir, cesT*, and *eae* constitute an operon. (A) Schematic representation of the organization of the *orf19, tir, cesT*, and *eae* genes and of the transcriptional fusions constructed to study their regulation. The horizontal arrows indicate the direction of transcription. Bent-tailed arrows denote the transcriptional start sites identified in this work (see the text). The sizes (in base pairs) of the intergenic regions are shown below the thick horizontal line. Plasmid denominations are indicated in the left-hand column below. The fragments cloned into the promoterless *cat* gene vector pKK232-8 are denoted by solid lines, and the *cat* gene is indicated by an open box at the end of each fragment. The −10/SacI label indicates the presence of a mutation that replaced the putative *tir* −10 promoter hexamer by a *Sac*I restriction site. The right-hand column shows the CAT activity expressed by each fusion in EPEC E2348/69 grown in DMEM at 37°C. The data obtained for EPEC B171-8 are not shown, for simplicity, but rendered the same conclusions. The CAT specific activity was determined from cells harvested at an OD_{600} of 1.4. The results reported are the averages ± standard deviations of data from at least four different experiments.

corresponding to a T residue, was identified 24 bp upstream from the first reported site (Fig. 2A); however, the relevance of this weaker potential promoter is unknown. A transcriptional start site was also identified for *orf19*, on an A residue located 133 bp upstream from its translational start codon (Fig. 2B). Analysis of its 5′ upstream region revealed the presence of putative −35 (TTGCAT) and −10 (ATAAAT) promoter sequences (Fig. 3B). In contrast, no transcriptional start site was observed for the chromosomal *cesT* and *eae* genes under the conditions tested.

Ler is required for transcriptional activation of *tir* and *orf19*. As described above, *tir-cat* fusions were active in two different EPEC wild-type strains (Fig. 1). However, when plasmid pTIR394, carrying the *tir-cat* fusion, was transformed into *E. coli* MC4100 (a K-12 derivative laboratory strain), its transcriptional activity was reduced (Fig. 4A), indicating that full expression of the *tir* operon required a regulatory factor that was present only in wild-type EPEC. To identify the activator(s) involved in *tir* expression, we first examined the potential role of the EAF plasmid by transforming pTIR394 into EPEC strains JPN15 and B171-10 (pEAF-minus derivatives of EPEC wild-type strains E2348/69 and B171-8, respectively) (Table 1). Expression of the *tir-cat* fusion in the plasmid-cured strains rendered levels of CAT activity similar to those obtained in the wild-type strains (Fig. 4A and data not shown), indicating that the transcriptional activation of *tir* was independent of the EAF plasmid. This observation was confirmed by determining that the transcriptional activities of the chromosomal *tir* promoters of wild-type EPEC and its pEAF-minus derivative were similar when compared by primer extension (Fig. 2A, lanes 1 and 2).

It has been shown that the product of the *ler* gene (previously known as *orf1*) is a positive regulator of the expression of *sepZ* and *orf12*, two genes also located in the LEE (5, 41). This observation prompted us to investigate the role of Ler in *tir* expression by performing primer extension experiments with total RNA obtained from DMEM cultures of an EPEC Δ*ler* strain which carries an in-frame deletion of *ler* (5). Expression of *tir* was considerably reduced in this mutant, while complementation with *ler* on a plasmid restored its expression (Fig. 2A, lanes 3 and 4). In agreement with these results, the activity directed by the *tir-cat* fusion (pTIR394) was reduced about 10-fold in the Δ*ler* mutant strain, while its activation was restored and enhanced by supplementing *ler* in *trans* with plasmid pKORF1 (Fig. 4A). Furthermore, activation was restored in *E. coli* MC4100 when supplemented with pKORF1, but not with a plasmid carrying the entire *per* locus (pCS-TVW), which, in

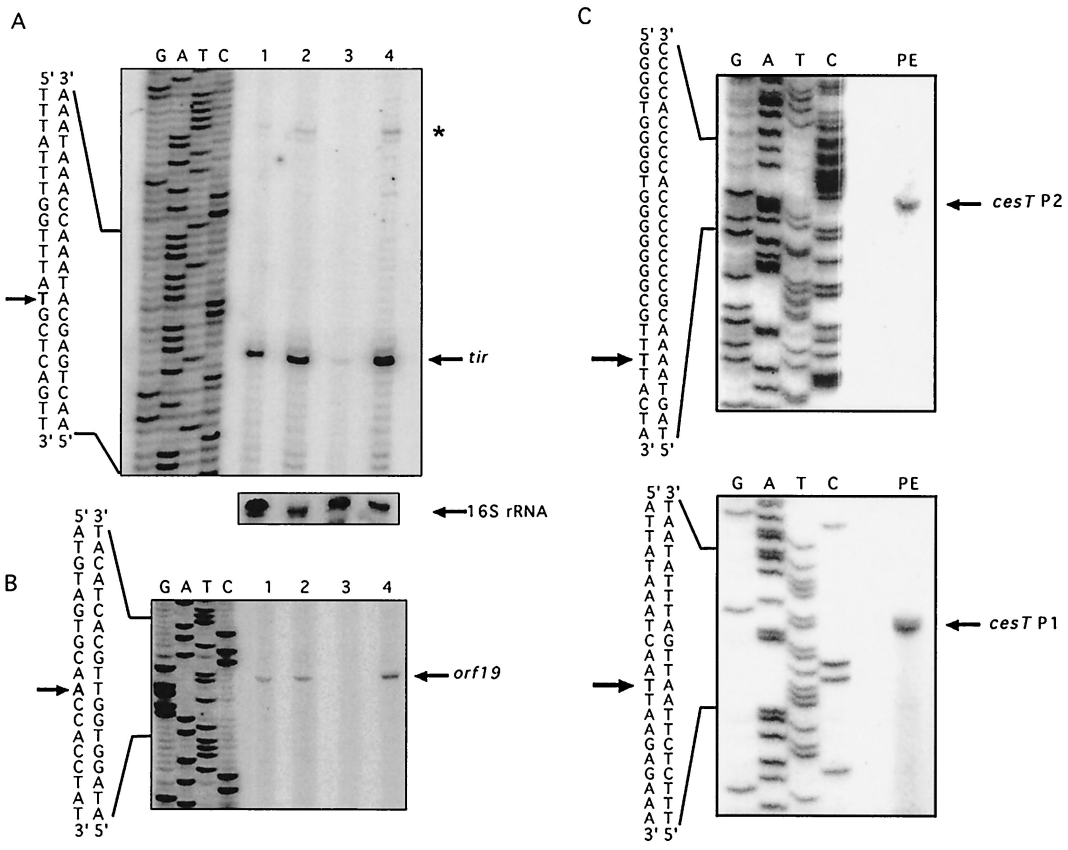


FIG. 2. Primer extension analysis of the *tir, orf19*, and *cesT* promoter regions. (A) Total RNA was obtained from culture samples of strains EPEC E2348/69 wild type (lane 1), JPN15 (pEAF cured) (lane 2), EPEC E2348/69Δ*ler* (lane 3), and EPEC E2348/69Δ*ler* carrying pKORF1 (lane 4) growing in DMEM at 37°C (OD_{600} = 0.8). A primer specific for the *tir* structural gene was used, and primer extension was performed as described in Materials and Methods. A primer extension assay using a primer specific for the 16S rRNA gene was performed as a control. (B) Primer extension analysis was performed as described for panel A but with a primer specific for the *orf19* structural gene. (C) Total RNA from EPEC E2348/69 carrying pCEST (*cesT-cat* fusion) and a *cat*-specific primer were used for primer extension reactions. The upper and bottom panels show the two extended products. Lanes G, A, T, and C correspond to the DNA sequence ladder obtained with the corresponding primer. The sequences spanning the transcription start site are shown, and the transcription start sites (in bold) are marked with arrows.

contrast, complemented the expression of the *bfpA-cat* fusion that was used as a control (Fig. 4A).

Consistent with these results, primer extension analysis revealed that Ler-dependent activation of the *tir-cat* fusion in pTIR394 was directed by the same putative promoter predicted for the wild-type gene (Fig. 4B). Similar experiments, using the same set of strains carrying the pORF19 fusion, revealed that *orf19* expression also required a functional *ler* gene (Fig. 2B; see also Fig. 7A). Taken together, these results indicated that Ler was required for the positive regulation of *tir* and *orf19* expression.

Analysis of the *tir* promoter region. To define the minimal regulatory sequence required for *tir* expression, a series of fusions containing segments of the *tir* 5′ promoter region ranging from 394 to 22 bp in length, with respect to the transcriptional start site (Fig. 3 and 5), was constructed. Fusions containing sequences up to position −204 (pTIR204) or longer were found to express similar amounts of CAT in EPEC E2348/69 (Fig. 5A). However, fusions to position −157 (pTIR157) or −122 (pTIR122) showed a significant reduction of CAT activity compared with that obtained with pTIR394 (Fig. 5A). Fusions containing up to position −97, −80, or −45 (pTIR97, pTIR80, and pTIR45, respectively) expressed a twofold increase in CAT activity in comparison to that obtained with pTIR394 (Fig. 5A). As expected, a fusion containing up to position −22 which lacks the putative −35 promoter hexamer did not express CAT.

In the absence of Ler (in EPEC Δ*ler* or *E. coli* MC4100), *tir-cat* fusions in pTIR394, pTIR243, and pTIR204 showed only background levels of *tir* promoter activity (Fig. 5A). In the absence of sequence elements located between positions −157 and −97, activation of the *tir* promoter became Ler independent (Fig. 5A). These results suggested that Ler could interact with a DNA sequence motif located between positions −204 and −157 and that this interaction overcame the repressing effect exerted by a negative regulatory factor, also present in *E. coli* K-12, that interacts with sequence elements located between positions −157 and −97. To rule out the possibility that an additional promoter was responsible for the Ler-independent activity shown by the shorter fusions, primer extension experiments were performed with total RNA obtained from culture samples of strains EPEC E2348/69, EPEC Δ*ler*, and *E. coli* MC4100 carrying fusions pTIR204, pTIR157, pTIR97, or pTIR45. The resulting primer extension products demon-

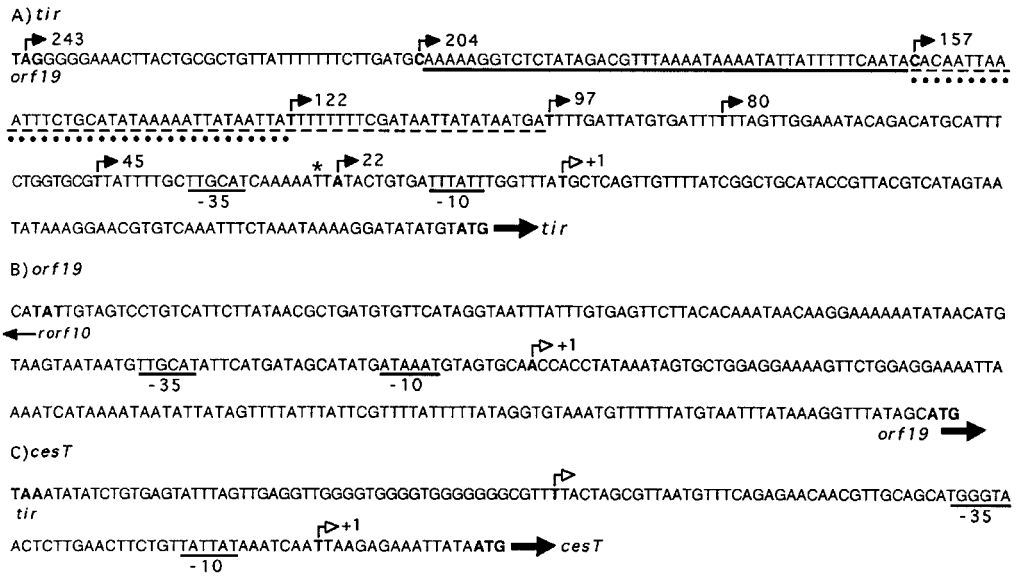


FIG. 3. Nucleotide sequences of the *orf19-tir* (A), *rorf10-orf19* (*r*, reverse) (B), and *tir-cesT* (C) intergenic regions. The transcriptional start sites (+1) are indicated by the bent-tailed open-headed arrows. The bent-tailed filled-head arrows indicate the 5′ ends of the different *tir-cat* fusions. The predicted −10 and −35 promoter sequences are underlined. The sequences containing motifs potentially involved in Ler activation and ammonium-dependent repression, in negative regulation by an as-yet-unidentified repressor, and in repression at high temperature are indicated by solid, broken, and dotted underlines, respectively.

strated that the transcriptional activity observed for these fusions was directed by the same promoter predicted for the wild-type gene (Fig. 5B and data not shown).

H-NS represses the expression of *orf19* but not *tir*. The experiments described above suggested that the putative repressor for *tir* and *orf19* was conserved between EPEC and *E. coli* K-12. To identify putative repressors, different *E. coli* K-12-derived strains carrying mutations in well-characterized genes coding for global regulators were transformed with fusion plasmids pORF19 and pTIR394. Only background levels of CAT activity were obtained for both fusions in strains carrying mutations in the genes coding for LRP (leucine-responsive regulatory protein), IHF (integration host factor), Fis (factor for inversion stimulation), StpA (suppressor of td^- phenotype A), factor, FIS, StpA, RpoN, RpoS, and OmpR as well as in their corresponding parental strains (Table 1 and data not shown). In contrast, expression of the *orf19-cat* fusion resulted in a 25-fold increase in CAT activity in *E. coli* CSH56 Δ*hns* (470 ± 36 CAT units [mean ± standard deviation]) in comparison with its parental strain, CSH56 (18 ± 5 CAT units). Repression was restored when a plasmid carrying the *hns* gene was introduced into CSH56 Δ*hns*/pORF19 (11 ± 7 CAT units). The absence of H-NS did not have any effect on expression of the *tir-cat* fusion.

Regulation of *tir* and *orf19* expression in response to environmental conditions. Expression and secretion of different virulence factors in EPEC are optimal in tissue culture medium (DMEM) at 37°C and are negatively regulated in response to growth in a rich medium such as LB at temperatures above or below 37°C or in the presence of ammonium salts (4, 5, 27, 37, 44). To determine whether *tir* expression was modulated by changing the growth conditions, EPEC E2348/69 carrying plasmid pTIR394 was grown in DMEM at 37°C, in LB at 37°C, in DMEM containing 20 mM ammonium sulfate at 37°C, and in DMEM at 25°C and 40°C, as described previously (44). *tir* expression was significantly reduced by growth in LB or in DMEM containing ammonium, as well as by growth at temperatures above or below 37°C (Fig. 6).

When the same experiment was carried out with EPEC E2348/69 carrying the shorter *tir-cat* transcriptional fusions, it was observed that growth in LB (Fig. 6A) and in DMEM at 25°C (Fig. 6B) resulted in negative modulation of the expression of all the active fusions (pTIR243 to pTIR45). This indicated that repression under these conditions acts on or downstream of the promoter. In contrast, the repression mediated by the presence of ammonium or by growth at 40°C was observed only for fusions containing the sequence between positions −204 and −157 (Fig. 6A) or between positions −157 and −122 (Fig. 6B), respectively. These observations suggested that *tir* expression is subjected to different levels of negative regulation, probably involving one or more factors that act on different segments of the *tir* regulatory region. In addition, CAT activity assays of samples obtained from cultures of EPEC E2348/69/pORF19 grown under the conditions described above revealed that expression of *orf19* is regulated coordinately with the expression of the *tir* operon (Fig. 7B) and suggested that Orf19 plays an important role in EPEC pathogenesis.

DISCUSSION

In this work, we studied the regulation of the *tir-cesT-eae* operon (henceforth referred as the *LEE5* operon), whose expression is directed by a promoter located upstream of the *tir* gene (Fig. 1 to 3). Recently, it was reported that these genes are expressed in the same transcript, which initiates 86 nucleotides (nt) upstream of the *tir* start codon (14), one base upstream from where the transcriptional start site was determined to be located (Fig. 3A). The existence of an *eae* tran-

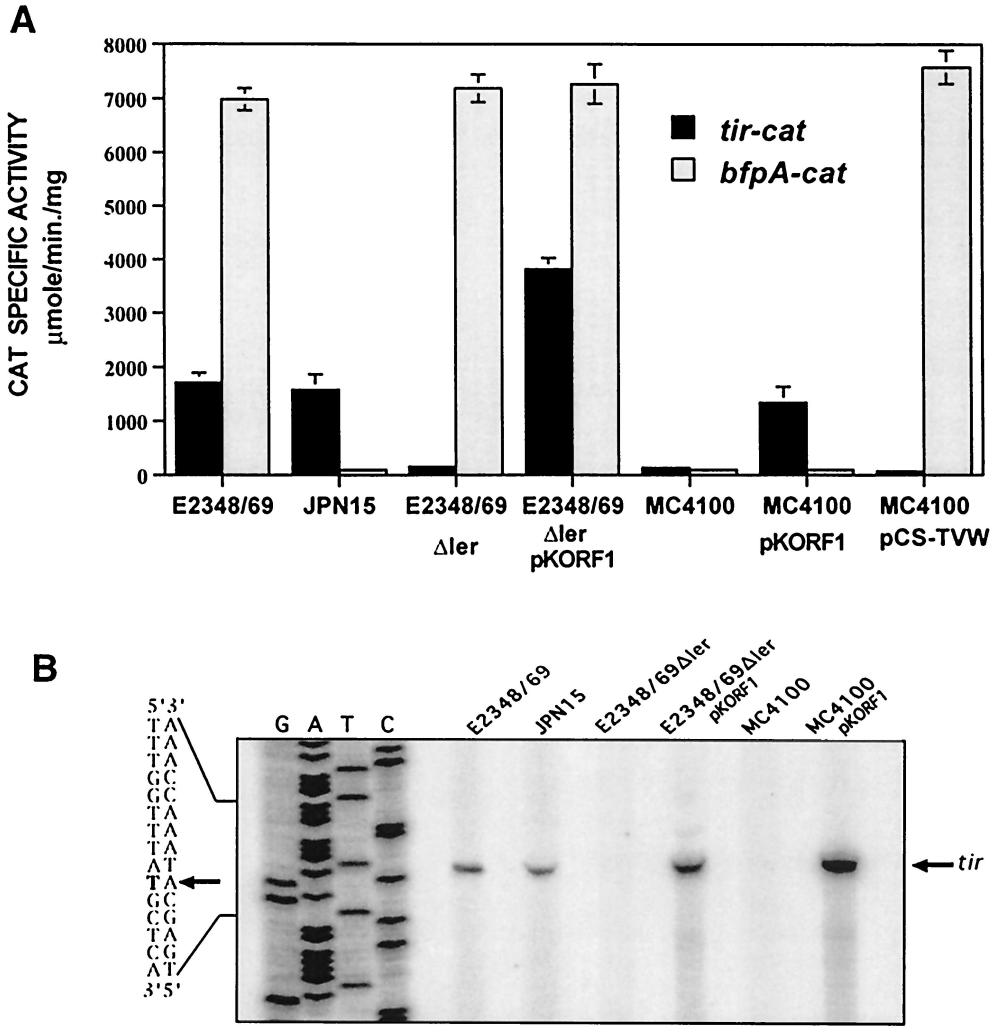
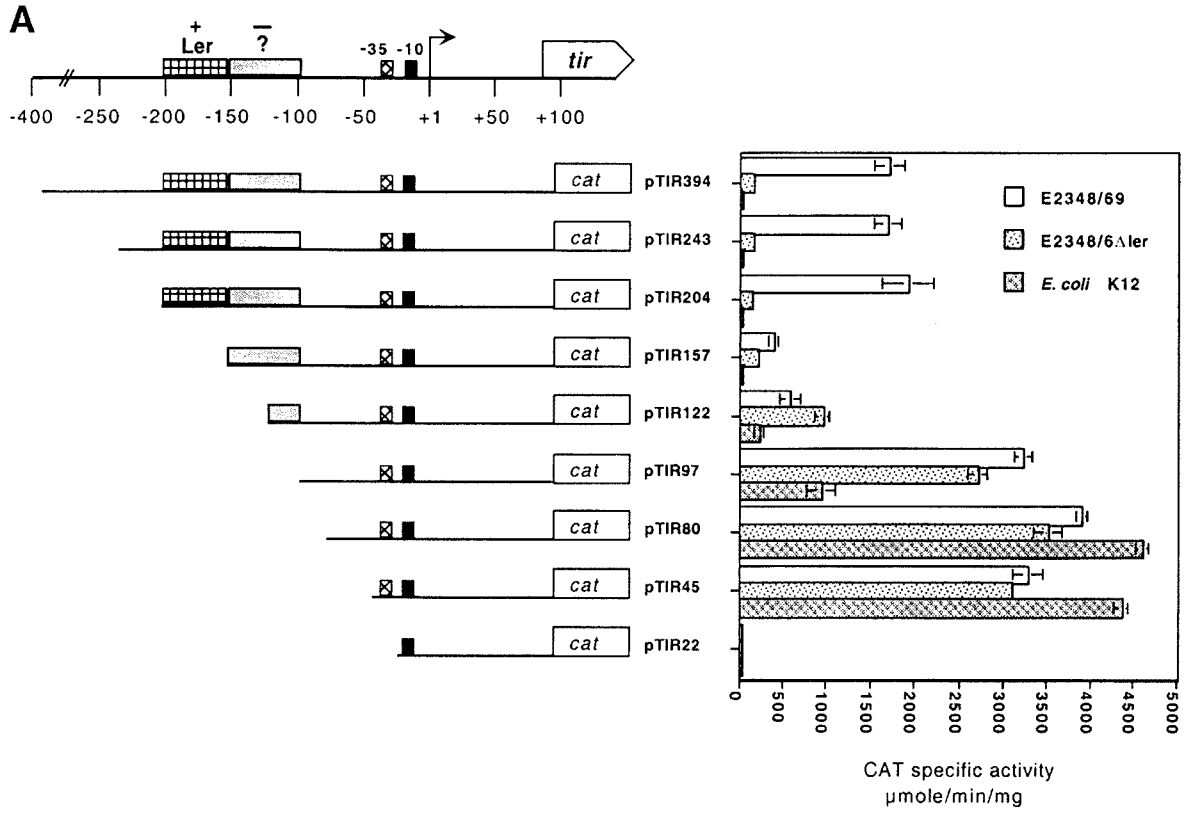


FIG. 4. Expression of *tir* requires the Ler protein. (A) The transcriptional activity directed by the *tir-cat* fusion in pTIR394 and the *bfpA-cat* fusion contained in pCAT232 was tested in EPEC strains E2348/69 (wild type), JPN15 (an EAF-minus derivative), and Δ*ler* (a *ler* in-frame deletion mutant of E2348/69) carrying or not carrying pKORF1 (*ler*$^+$), as well as in *E. coli* K-12 strain MC4100 (wild type) carrying or not carrying either pKORF1 (*ler*$^+$) or pCS-TVW (*per*/*bfpTVW*$^+$). The CAT specific activity was determined from cells grown in DMEM at 37°C and harvested at an OD_{600} of 1.4. The data are the averages of results from at least three different experiments. Error bars indicate standard deviations. (B) Primer extension analysis of the *tir-cat* fusion in pTIR394 was carried out with total RNA extracted from samples obtained at an OD_{600} of 0.8 from the cultures described above. The primer extension reactions were performed as described in the legend to Fig. 2, using a primer specific for *cat*. The arrow on the left indicates the transcriptional start site of *tir-cat*.

scriptional start site located inside the structural *cesT* gene was previously proposed (21). However, neither fusions carrying different fragments of the *eae* upstream region (pEAE1800, pEAE1629, pEAE1422, and pEAE520) nor a *tir-eae-cat* fusion (pTIRCAM-10) carrying a mutation in the −10 *tir* promoter sequence demonstrated promoter activity (Fig. 1). Different attempts to define a transcriptional start site for *eae* by primer extension rendered only a variable ladder of undefined reverse transcription products (data not shown). We cannot rule out the possibility that additional promoters allow the differential expression of the components of the *LEE5* operon under different conditions. However, our initial data suggest that the observed *eae* primer extension products could be the result of posttranscriptional mRNA processing events.

A transcriptional start site for *cesT* has been reported (14). Consistent with this observation, *cesT-cat* fusions carrying sequences of the upstream region of *cesT* without the *tir* promoter (pCEST and pTIRCAM-10-DEL1) directed the expression of significant levels of CAT activity (Fig. 1). Primer extension experiments with the *cat*-specific primer revealed two different transcriptional start sites (Fig. 2C). The first transcriptional start site was located 15 nt upstream of the *cesT* start codon and allowed the prediction of a good putative −10 (TATTAT) promoter sequence and a poor −35 (TGGGTA) hexamer. The sequence preceding the second start site did not show any homology with known promoter sequences (Fig. 3C). Because of its weak activity, we were unable to detect primer extension products derived from the transcript of the chromosomal *cesT* gene. This Ler-independent promoter activity does not seem to read through the *eae* gene, since the presence of



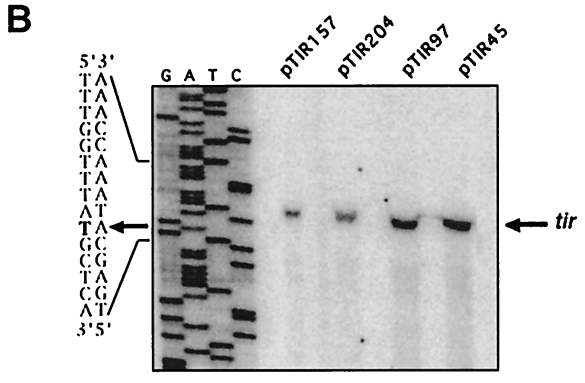


FIG. 5. *cis*-acting elements involved in *tir* expression. (A) Schematic representation of the *tir* regulatory region and *tir-cat* fusions. Numbering is relative to the transcription start site, which is indicated by a bent-tailed arrow. Regions containing sequences potentially involved in Ler-dependent activation (subdivided boxes) or in negative regulation (shaded boxes) are indicated. EPEC strains E2348/69 and E2348/69Δ*ler*, as well as *E. coli* MC4100, were transformed with different *tir-cat* fusions contained in plasmids pTIR394, pTIR243, pTIR204, pTIR157, pTIR122, pTIR97, pTIR80, pTIR45, and pTIR22. The resulting strains were grown in DMEM at 37°C, and the CAT specific activity was determined from samples obtained at an OD_{600} of 1.4. The plots of the activities determined from samples obtained along the growth curve at other OD_{600} values showed the same pattern. Values are the averages of data from at least three different experiments; error bars indicate standard deviations. (B) Total RNA was obtained from EPEC E2348/69 carrying the *tir-cat* fusions in pTIR204, pTIR157, pTIR80, and pTIR45, and primer extension assays were performed as described in the legend to Fig. 2. The arrow on the left indicates the transcriptional start site of the *tir-cat* fusions, which corresponds to that identified for the wild-type gene.

the *cesT-eae* intergenic region rendered fusions inactive (compare pTIRCAM-10 with pTIRCAM-10-DEL1 and pEAE1422 with pCEST [Fig. 1]). These results suggest that P_{tir}-initiated transcription may be antiterminated while P_{cesT}-initiated transcription is not. Further detailed analysis is required to establish the significance of these observations; however, it is tempting to speculate that the independent expression of *cesT* may ensure the presence of the chaperone when Tir is translated or that CesT has additional, as-yet-undefined functions.

Two major mechanisms regulate virulence gene expression in EPEC (5, 15, 19, 21, 41, 49). One involves a classical activator (PerA/BfpT) that is fully required for the activation of the *bfpA* and *perA* (*bfpT*) promoters (37, 49). *per* is also proposed to be involved in the modulation of *eae* expression (21), protein secretion (27), the down-regulation of intimin during A/E adhesion (31), and the direct activation of the *LEE1* operon, which encodes the *ler* gene (41). The other involves an antagonist protein (Ler) (19, 41, 47) that is required to overcome the repression exerted by negative regulators on the expression of several LEE-encoded genes, which is directed by promoters that in the absence of upstream regulatory elements are constitutively expressed (5). Ler seems to act as a master key which modulates the expression of different virulence factors that allow the intimate colonization of the proximal small intestine and the generation of A/E lesions by EPEC. Based on these observations, we analyzed the role of the *ler* and *per* (*bfpTVW*)-encoded products in *LEE5* and *orf19* expression.

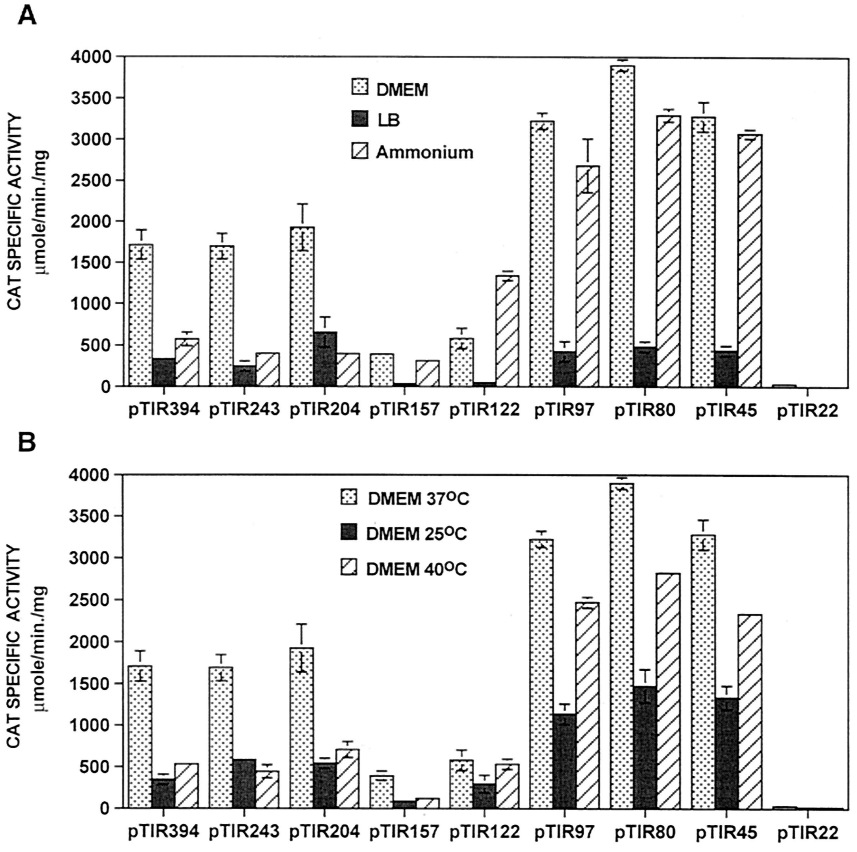


FIG. 6. Effect of the growth medium, the presence of ammonium, and temperature on *tir* expression. EPEC E2348/69 derivatives carrying the *tir-cat* fusions described in the legend to Fig. 5 were grown at 37°C in DMEM, DMEM plus 20 mM ammonium sulfate, or LB (A) or in DMEM at 37, 25, or 40°C (B). CAT specific activities from samples obtained at an OD_{600} of 1.4 were determined and plotted. The plots of the activities determined from samples obtained along the growth curve at other OD_{600} values showed the same pattern. Values are the averages of data from at least three different experiments; error bars indicate standard deviations.

Ler was needed for the efficient expression of both the *LEE5* operon and the *orf19* gene (Fig. 2 and 4 and data not shown). In contrast, wild-type EPEC, its EAF-cured derivative, and a *per* mutant strain did not show significant differences in their expression (Fig. 2 and 4 and data not shown). Hence, the *LEE5* operon and the *orf19* gene are part of the Ler regulon.

Deletion analysis of the *LEE5* upstream regulatory region indicated that nucleotides up to position −45, which include only the *tir* promoter, were sufficient for maximal activation even in the absence of Ler (Fig. 5). Full activation was maintained with sequences up to position −97, but not with sequences up to position −122 or −157, which sustained low levels of activation. This suggested that a sequence motif involved in negative regulation is located upstream of position −97 and that the negative regulator was conserved in nonpathogenic *E. coli* strains. Fusions up to position −204 or longer were activated in a Ler-dependent manner but only reached intermediate levels of activity with respect to those directed by the shorter fusions (Fig. 5). This established that sequences between positions −204 and −157 were necessary for Ler-dependent activation of the *LEE5* promoter, which was still modulated by a putative negative regulator that probably interacts with the region between positions −157 and −97.

Ler shows amino acid similarity to the DNA-binding global transcriptional regulator H-NS and its paralogue, StpA (12, 16, 41). The H-NS protein has been implicated in the negative regulation of several virulence factors and housekeeping genes (2). Considering its similarity to H-NS, it is likely that Ler binds DNA sequences between positions −204 and −157. The H-NS protein is involved in the negative regulation of the *LEE2* and *LEE3* operons (5) and the *orf19* gene but not *LEE5* expression (see Results). Ler might overcome the repression exerted by H-NS or an unknown factor by directly or indirectly (e.g., changing the DNA topology) interfering with its binding. However, further work is required to distinguish between these and other possibilities.

Environmental conditions regulate the expression of several virulence factors in different bacteria, such as *Vibrio cholerae, Shigella* spp., *Yersinia* spp., and pathogenic *E. coli* strains (10, 24, 40). In EPEC, the expression of virulence factors is also regulated in response to culture conditions that mimic in vivo regulatory signals (5, 27, 37, 44). We found that distinct regulatory sequences in the *LEE5* promoter region are involved in the regulatory response to these conditions (Fig. 6). Negative regulation by growth in rich medium or at temperatures below 37°C involves factors that act directly on the promoter (Fig. 6). The repression mechanism mediated by temperatures above 37°C and by the presence of ammonium in the culture medium

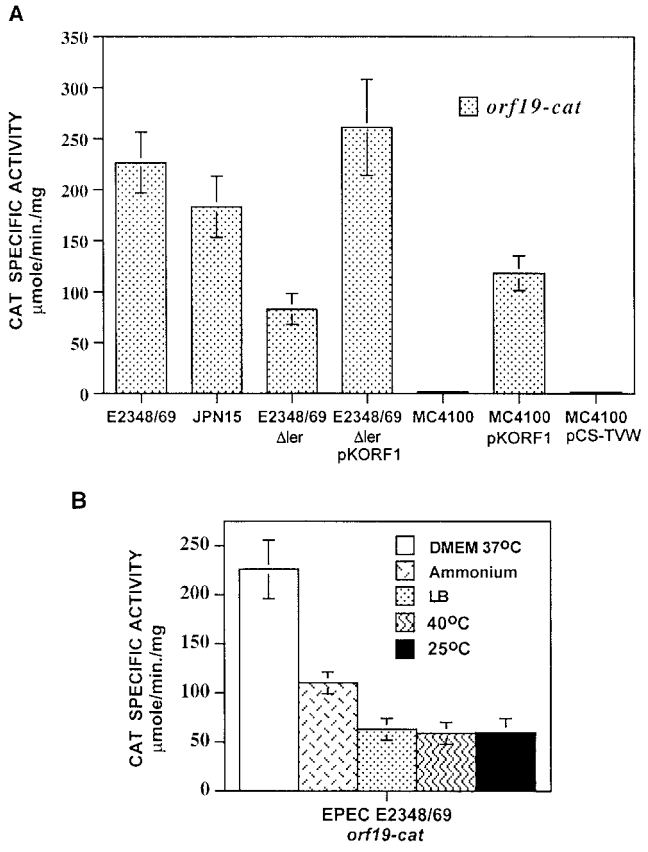


FIG. 7. Regulation of *orf19*. (A) Expression of *orf19* requires the Ler protein. The transcriptional activity directed by the *orf19-cat* fusion in pORF19 was tested in different strains as described in the legend to Fig. 4A. (B) Effect of the growth medium, the presence of ammonium, and temperature on *orf19* expression. EPEC E2348/69 carrying the *orf19-cat* fusion (pORF19) was grown under different conditions to determine CAT activity, as described in the legend to Fig. 6.

is dependent on sequences located upstream from the putative promoter, between positions −157 and −122 and positions −204 and −157, respectively (Fig. 6). These motifs overlap with the sequence presumably required for negative regulation by an unknown factor and the putative Ler-binding region, respectively (Fig. 3A), suggesting the existence of different regulatory mechanisms that probably involve additional *trans*-acting factors. Neither ammonium nor high temperature is a common regulatory signal for the regulation of virulence factors. However, both can be found during transit along the intestinal lumen, and they may represent signals indicating harmful or inappropriate niches for colonization (13, 44).

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