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Identification of a Negative Regulator for the Pathogenicity Island of Enterohemorrhagic *Escherichia coli* O157:H7

Joaquim Chan-Wang Lio Wan-Jr Syu

Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan

Key Words
EHEC · EPEC · LEE · Ler · Regulation

Abstract
Enterohemorrhagic *Escherichia coli* (EHEC) forms histological lesions termed attaching and effacing lesions (A/E lesions) on infected large intestine tissue. The major virulence factors involved in A/E lesions reside on a locus of enterocyte effacement (LEE), a pathogenicity island. The LEE comprises 41 specific open reading frames, of which most are organized in 5 major operons, *LEE1*, *LEE2*, *LEE3*, *LEE4*, and *tir (LEE5)*. The expression of LEE genes is regulated in a complicated manner by environmental factors such as temperature, osmolarity, and quorum sensing. Current knowledge is that regulation is hierarchical: a pivotal positive regulator, *ler*, is first stimulated, which in turn activates the expression of other operons. Herein, we report on the presence of a negative regulation protein located within the LEE. *L0044* is 372 bp in length and is located outside of the 5 major operons. An isogenic *L0044* deletion mutant displayed loss of the repression phenotype and increased synthesis of several LEE proteins when bacteria were cultured under repressive conditions that disfavor expression of LEE proteins. Reciprocally, *trans* expression of *L0044* suppressed the expression of the LEE. Furthermore, mRNA of *ler* increased as a result of deleting *L0044*, and disrupting *ler* in a *L0044*-deleted background reversed the loss of the repression phenotype. Thus, *L0044* plays a role in regulating the expression of virulence genes in EHEC by modulating the activation of *ler*.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is the major etiological agent of hemorrhagic diarrhea, which can proceed to a severe illness called hemolytic-uremic syndrome and which occasionally results in patient death [31]. To infect a host, this bacterium firmly attaches to an epithelial cell of the large intestine and causes a histological change termed an attaching and effacing lesion (A/E lesion) [6]. The pathogenesis of formation of A/E lesions by this organism depends on the bacterial type III secretion system (TTSS) [6]. With this system, many effector molecules are translocated from the bacterium directly into host cells. Thus, the cellular signals are disturbed, and the normal processes of the host cells are subverted. In EHEC, and also in enteropathogenic *E. coli* (EPEC), the TTSS, translocators, and effector molecules are encoded in a pathogenicity island known as the locus of enterocyte effacement, or LEE [15, 33].

The EHEC LEE is 43,359 bp in length and annotated into 56 open reading frames (ORFs) [33]. The first 15 ORFs are from the sequences of a putative prophage. The other ORFs encode the components of the TTSS *(esc,*

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Wan-Jr Syu, PhD
Institute of Microbiology and Immunology, National Yang-Ming University
155 Sec. 2, Li-Nong Street, Beitou
Taipei 112 (Taiwan)
Tel. +886 2 28267003, Fax +886 2 28212880, E-Mail wjsyu@ym.edu.tw

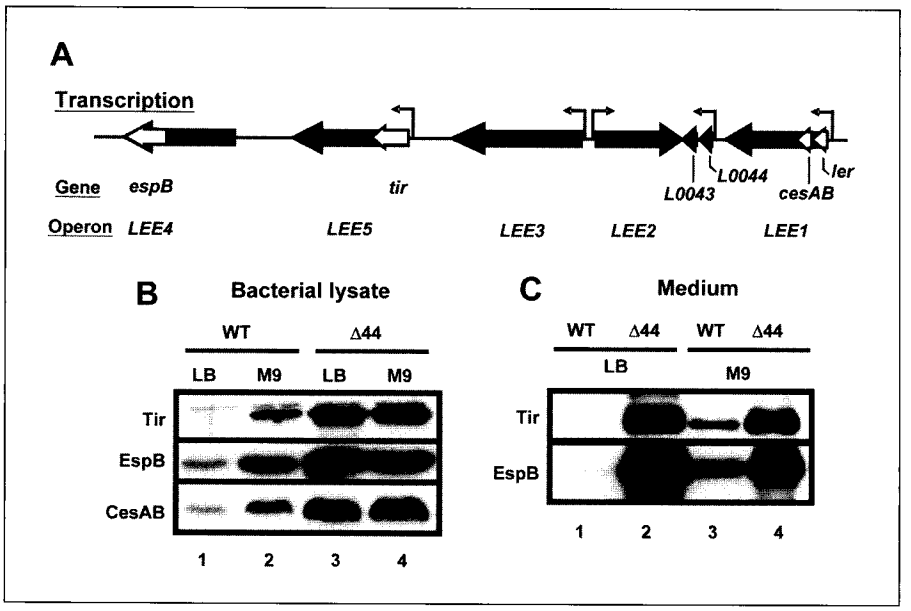

Fig. 1. Expression and secretion of EHEC LEE proteins affected by deletion of *L0044*. **A** Schematic illustration of the EHEC LEE. Operons have the transcription direction labeled, and among the 41 genes encoded, only those discussed in the text are shown. The wild-type parental EHEC (WT) and the *L0044-deletion* mutant (Δ44) were grown for 3 h in LB or M9 medium to an OD_{600} of 0.8. Proteins from bacterial lysates (**B**) and spent media (**C**) were analyzed for the representative proteins by Western blotting with the specific antisera indicated. These included Tir, an effector protein encoded by the *LEE5* operon, EspB, a translocator encoded by *LEE4*, and CesAB, a non-secreted chaperone encoded by *LEE1*.

sep); secreted proteins such as EspA, EspB, EspD, EspF, EspG, EspH, and Map [11, 12, 25–27, 29, 38]; chaperones (*ces*), such as CesT for Tir and Map [1, 7], CesD for EspB and EspD, CesD2 for EspD, CesF for EspF, and CesAB for EspA and EspB [7, 9, 13, 32, 40]; a transcriptional regulator, Ler (LEE-encoded regulator) [30]; an adhesin molecule, intimin *(eae)* [22, 41], and a translocated receptor, Tir, for intimin [24]. Apparently, most of these ORFs fall into the category of TTSS, and such components have been defined by correlation with other TTSS, such as that of *Yersinia* [21]. However, to the present, the exact functions of several ORFs remain to be characterized.

Although TTSS components are highly conserved among many gram-negative bacteria, several ORFs in the LEE have no homologue except in other A/E pathogens. *L0044* is one of the ORFs with an unknown function, which has a counterpart named *orf10* in EPEC. *L0044* is 372 bp in length and encodes a putative 13.9-kDa protein with a pI of about 5.04. Furthermore the known regulations of the LEE island occur mainly at the 5 major operons [30]. ORF *L0044* lies between *LEE1* and *LEE2* and is located outside the major operons (fig. 1A). These reasons prompted us to characterize *L0044* and define its role in the pathogenesis of EHEC. By using a homologous recombination, we generated an *L0044*-deleted mutant. We demonstrated that *L0044* encodes a negative regulator of LEE protein expression in EHEC, and disruption of *L0044* resulted in a loss-of-repression phenotype when the bacterium was cultured under repressive conditions. Overexpression of *L0044* enhanced the repression of LEE protein expression. Finally, repression through the effect of the gene product on Ler downregulation was deduced.

Materials and Methods

Bacterial Strains and Growth Conditions

EHEC (ATCC 43888) was obtained from the American Type Culture Collection. Bacteria were regularly grown in LB broth. M9 medium containing 0.4% glucose and 0.2% casamino acid was prepared as described by Sambrook and Russell [35]. When necessary, the medium was supplemented with appropriate antibiotics.

General Recombinant DNA Techniques

Unless otherwise mentioned, restriction endonucleases, DNA-modifying enzymes, and polymerase were purchased from New England Biolabs (Beverly, Mass., USA). Standard DNA manipulation procedures were followed as described [35], or as recommend by the manufacturers. Primers were commercially ordered from MD Bio (Taipei, Taiwan, ROC), and their sequences are listed in table 1. DNA sequencing was carried out by a contract service (Mission Biotech, Taipei, Taiwan, ROC).

Plasmid Construction

Gene *L0044* was amplified from the EHEC genome by PCR with the primers L44F and L44R, and the fragment was cloned into pUC-T (MD Bio) to generate pUC-L0044. *L0044* was re-amplified from pUC-L0044 using the primers PUCT-FB and PUCT-RK. Since PUCT-FB and PUCT-RK are primers annealed to the multiple cloning sites of the cloning vector pUC-T, *Bam*HI and *Kpn*I sites were introduced to the 5′ and 3′ ends of the amplified fragment, respectively. The fragment was then cloned into *Bam*HI/*Kpn*I-digested pRESET-A (Invitrogen, Carlsbad, Calif., USA) to generate the plas-

Table 1. Oligonucleotides used in this study (sequences recognized by restriction enzymes are underlined)

Primer	Sequence (5′ to 3′)
L44F	ATGATTATGAAGGATGGCATC
L44R	TTATTTTAAATAAACTTGTGGCATT
PUCT-FB	ACTAGTGGATCCAGAATT
PUCT-RK	ATGGTACCGCCGCCACTCAT
T5P	CCCGAAAAGTGCCACCTG
PQER	CATTACTGGATCTATCAACAGG
LER-F	GCATGCGGAGATTATTTATTATG
LER-R	CATGTTAAATATTTTTCAGCGGTAT
ESPB-F	TAACTGCAGATGAATACTATTGATAAT
ESPB-R	TGGTACCTTACCCAGCTAAGCGACCCG

mid, pRA-44. From this plasmid, L0044 which was linked to a 6x His tag was expressed. As an alternative, *L0044* was subcloned into *Bam*HI/*Hind*III-digested pQE60, generating pQ-44, in which the recombinant protein carried no additional tag.

To generate pAT44 in which *L0044* was driven by a T5 promoter, PCR was carried out on pQ-44 using the primers T5P and PQER. The resultant PCR product was then phosphorylated using T4 polynucleotide kinase and ligated to *Eco*RV-digested and calf intestinal phosphatase-treated pACYC184.

The gene *ler* was similarly amplified except the primers used were LER-F and LER-R, and the PCR product was cloned into pGEM-T-easy (Promega, Madison, Wisc., USA). In the resulting plasmid, pLac-Ler, *ler* is under the control of the *lac* promoter.

Generation of EHEC Deletion Mutants

To prepare plasmids for homologous recombination [28], *L0044*-flanking DNA was cloned into pKO3, to give pKO3-d44. The pKO3-d44 plasmid was transformed into wild-type EHEC, from which bacteria were selected for deletion of *L0044* through recombination as previously described [5]. The resulting strain, designated Δ44, was verified by PCR and by sequencing the amplified DNA fragment. The results showed a 306-bp in-frame deletion of *L0044* and the introduction of an *Xba*I site at the deletion junction.

The plasmid, pKO3-dLer, was constructed using the same strategy as for pKO3-d44 and was used to transform Δ44 described above to delete *ler* under the *L0044* deletion background. The resulting *ler*/*L0044* double mutant, named Δ44/L, was examined by PCR amplification of the target site and confirmed by sequencing of the amplified product.

Protein Sample Preparations

The bacterial proteins secreted into the media were prepared as described [23] except for a slight modification. Briefly, bacteria were cultured overnight in LB broth supplemented with appropriate antibiotics and were diluted 1:50 in 30 ml of M9. After a 3-hour incubation at 37°C in the presence of 5% CO_2, the bacteria were centrifuged at 12,000 *g* for 15 min at 4°C. Proteins in the bacteria were directly prepared by lysis of the pellets in SDS sample buffer. To prepare proteins from the media, trichloroacetic acid (TCA) was added to a final concentration of 10% (v/v), and the mixtures were incubated on ice for 1 h. The insoluble proteins were collected by centrifugation (20,000 *g*, 1 h), and the pellets were washed with acetone and then resuspended in 50 µl 500 m*M* Tris-base containing 2 *M* urea. To normalize the precipitation efficiency, 20 µg of bovine serum albumin was added to the supernatant before TCA treatment.

Immunoblotting

Immunoblotting analysis was performed as previously described [20]. Samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred to a nitrocellulose membrane. Membranes were then treated with mouse anti-Tir, anti-CesAB, and rabbit anti-EspB [5] as the primary antibodies to detect the corresponding antigens. The membrane-bound antibodies were then detected by species-specific goat secondary antibodies conjugated with horseradish peroxidase (Sigma, St. Louis, Mo., USA). Finally, the membranes were developed using Renaissance Western Blot Chemiluminescence Reagent Plus (NEN Life Science, Boston, Mass., USA), and the signals were detected by X-ray film exposure.

Northern Blotting

Bacteria were cultured at 37°C in 5% CO_2 until an OD_{600} of between 0.7 and 0.8 was reached. Cells (30 ml) were harvested by centrifugation (6,000 *g*), washed once with PBS, and lysed in 1 ml of Trizol reagent (Invitrogen). Thereafter, total RNA was purified by following the instructions of the manufacturer except that the RNA was further extracted twice using 1:1 acidic phenol and chloroform. RNA (10 µg) was then separated using formaldehyde-agarose gel electrophoresis. A comparable loading of RNA on the gel was further ensured by equal intensities of ethidium-bromide-stained rRNA bands. After electrophoresis, the RNA was then transferred to a nylon membrane overnight and cross-linked. The membrane was next pre-hybridized with ExpressHyb hybridization solution (Clontech, Palo Alto, Calif., USA) and hybridized with probes in solution overnight (Clontech). After hybridization, the membrane was washed 4 times in the washing buffer (0.5 × SSC and 0.1% SDS). The signal was then detected using a PhosphoImager (Amersham Biosciences, Piscataway, N.J., USA) and quantified with Personal Densitometer SI (Molecular Dynamics, Sunnyvale, Calif., USA). DNA fragments used for the preparation of the probe were obtained by amplification from *espB* and *ler* by PCR synthesis using the primer pairs ESPB-F/ESPB-R and LER-F/LER-R, respectively. Probes were then labeled with [α-^{32}P]dCTP using the Rediprime II random prime labeling system (Amersham Biosciences).

Results

Deletion of L0044 Displayed a Loss-of-Repression Phenotype

To study the possible function of *L0044* in EHEC, we generated a mutant of EHEC with *L0044* deleted by homologous recombination [28]. Bacteria were then cultured in medium such as LB that disfavors the expression of the LEE [2] (fig. 1B, lane 1) or in M9 (fig. 1B, lane 2) that favors its expression. When bacteria were equally inoculated in the media in the presence of 5% CO_2 and

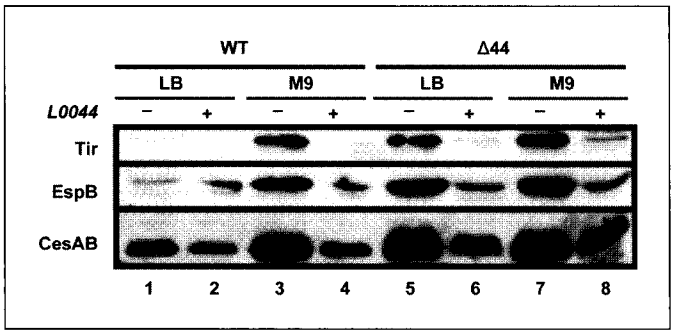

Fig. 2. Recovery of the repression phenotype after Δ44 was complemented with a plasmid expressing *L0044*. Bacteria carrying either the vector control (–) or pAT44 (+) were grown in LB or M9 medium. Bacterial lysates were prepared and analyzed by Western blotting using the specific antisera indicated.

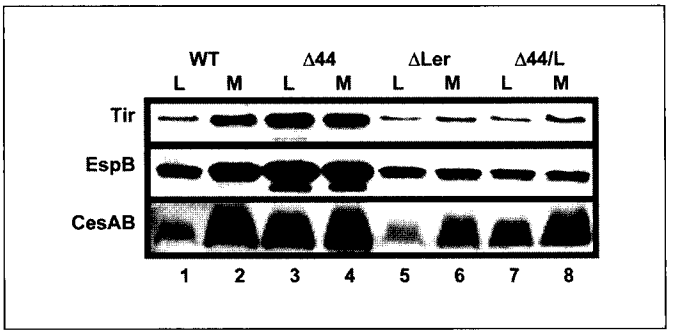

Fig. 4. De-repression of LEE genes in Δ44 dependent on *ler*. Wild-type, Δ44, and Δ44/L, in which both *L0044* and *ler* were deleted, were grown in LB (L) and M9 (M) media. Bacterial lysates were harvested and analyzed for protein expression by Western blotting using specific antisera.

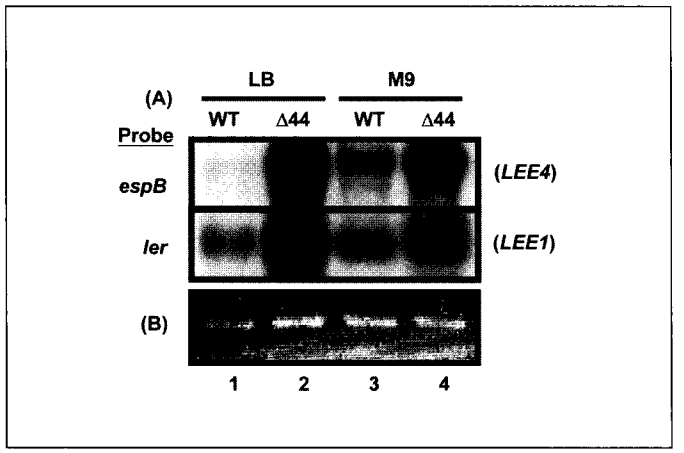

Fig. 3. Upregulation of LEE mRNAs by cultivation in M9 and by deletion of *L0044*. Bacteria were cultured in LB or M9 medium to an OD_{600} of about 0.8, and total RNA was extracted. **A** Total RNA (10 μg) was run on a formaldehyde-containing agarose gel and analyzed by Northern blotting using the probes indicated. **B** Before being transferred to a nylon membrane for reaction with the probes, the RNA was stained with ethidium bromide for 23S rRNA to show comparable sample loading in each lane. The band detected by the *espB* probe was about 3 kb in length, whereas that by the *ler* probe was about 0.5 kb.

cultured for 6 h at 37°C, the final cell densities differed. The bacteria grew to a higher density in M9 than in LB (OD_{600} at 1.8 vs. 1.0). A careful examination of the growth curves revealed that the growth rate was almost identical in the two media during the first 3 h and then it significantly increased in M9 compared to LB (data not shown). For this reason, we used 3-hour cultures in this study in which both bacteria have reached the same density of about OD_{600} 0.8. The bacterial lysate and proteins secreted were then prepared and analyzed for the expression of representative proteins by immunoblotting [5]. Three representative LEE proteins (fig. 1A) were chosen for the analysis. Tir is a protein encoded by *LEE5*. EspB together with EspA and EspD are encoded by *LEE4*. EspB was chosen as the representative protein because it was more consistently detected from batch to batch than either EspA or EspD ([5] and data not shown). CesAB is encoded by *LEE1* and differs from the secreted Tir and EspB by being present only in the bacterial lysate.

As shown in figure 1B, the parental wild-type strain gave lower levels of Tir, EspB, and CesAB in the bacterial lysate when in LB (lane 1) compared to M9 (lane 2). The same effect was also observed in media for the secretion of Tir and EspB (fig. 1C, lanes 1 and 3). However, unlike the parental EHEC, Δ44 produced a similar amount of intracellular proteins at high levels in both LB and M9 (fig. 1B, lanes 3 and 4) and secreted more Tir and EspB into the media than the wild-type strain in either medium (fig. 1C, compare lanes 1 and 2, also lanes 3 and 4). These results suggest that *L0044* is associated with a repression phenotype of the expression and secretion of LEE proteins. In LB, the expression of these LEE proteins was repressed, whereas in M9, this repression seemed to be relieved. Deleting *L0044* greatly promoted the synthesis of these proteins and also the subsequent secretion of these proteins into the media. Since the expression of proteins in bacteria occurs before the secretion event or the two events are coupled together, we thereafter focused only on the analysis of proteins expressed in bacterial lysates.

Complementation with L0044 Restored Suppression of LEE Expression in Δ44

As shown above, disruption of *L0044* resulted in a loss-of-repression phenotype. It is thus possible that *L0044* may encode a protein with the function of repressing the expression of LEE. To examine this possibility, we transformed an expression plasmid pAT44 into the parental strain and Δ44, and the transformants were then cultured and analyzed similarly to that described above. Results in figure 2 show that the additional expression of *L0044* in the wild-type strain resulted in severe suppression of the expression of the representative LEE proteins when bacteria were cultured in M9 (compare lane 3 vs. lane 4). When the bacteria were cultured in LB, there was no difference in LEE protein expressions, and this could simply be explained by the fact that these bacteria were in a repressed state (fig. 2, lanes 1 and 2).

Mutant Δ44, however, repeatedly showed that these LEE proteins were expressed at high levels when cultured in LB (fig. 2, lane 5) as observed in figure 1B. Complementation to express *L0044* in *trans* resulted in restoration of the repression phenotype in LB (fig. 2, lane 6). In M9, a similar suppressive effect could be seen with Δ44 complemented by pAT44 (compare lanes 7 and 8). These results strengthened the hypothesis that deletion of *L0044* results in loss of the suppression phenotype.

Increasing Expression of LEE Gene mRNAs in Δ44

Deletion of *L0044* in EHEC increased LEE protein expression in the repressive medium. Since the proteins are translated from mRNAs, the cognate mRNAs must be transcribed in increasing amounts prior to expression of the proteins. To test this notion, bacteria were cultured in the conditions described above, and then the total RNA was isolated from harvested bacteria. The RNA was next analyzed for the presence of representative mRNAs for *LEE4* and *LEE1* by Northern blotting using probes derived from *espB* and *ler* (fig. 3A), respectively. Consistent with our expectations, *LEE4* mRNA in the wild-type bacteria was upregulated in M9 (lane 3) as compared to LB (lane 1). When *L0044* was deleted (in Δ44), the level of *LEE4* mRNA substantially increased even in LB (compare lanes 1 and 2). The degree of increase was greater than the effect of culturing the wild-type strain in M9 (compare lanes 2 and 3). When the effect of M9 alone (lane 3) was compared with that of the double effect of M9 and the *L0044* deletion (lane 4), the latter was relatively profound.

In EHEC, the central positive regulator, Ler, is required for the upregulation of LEE gene expression [14, 19, 37]. As shown above by immunoblotting, deletion of *L0044* led to increased expression of CesAB (fig. 2), a protein that together with Ler and seven other proteins are encoded by *LEE1* [32]. Since *ler* is the first gene in the *LEE1* operon and it activates the expression of the other operons in LEE, increased expression of CesAB implies a simultaneous increase in Ler and its related mRNA. For this reason, we used a probe for *ler* to detect the corresponding *LEE1* mRNA. The results shown in figure 3A indicate that M9 culture did augment expression of *ler* mRNA (lane 3), and a quantitative estimation indicated there was a 2-fold increase as compared to that in LB (lane 1). Therefore, Ler must accordingly increase and subsequently results in upregulation of the LEE. This fact explains why M9 stimulated the LEE protein expressions seen above. Mutant Δ44 cultured in both LB and M9 further increased the level of *LEE1* mRNA as revealed by *ler* (fig. 3A, lanes 2 and 4) or *cesAB* (data not shown), a trend similar to that observed with the upregulation of *LEE4* mRNA seen above. Furthermore, the increase in *LEE1* mRNA, and thus Ler, suggests that the repression of the promoters of LEE operons by H-NS (histone-like nucleoid structuring protein) [3, 4, 39] might have been relieved.

It is worth noting that Δ44 seemed to have higher levels of both *LEE4* and *LEE1* mRNAs when in LB than in M9 (fig. 3A, lanes 2 and 4). However, this phenomenon was not seen with proteins detected in the bacteria (fig. 1, lanes 3 and 4). The reason for this observation remains to be explored.

Disruption of ler *in Δ44 Suppressed the Loss-of-Repression Phenotype*

Removing *L0044* increased *ler* mRNA expression in Δ44 (fig. 3). Thus, L0044 may function as a repressor by lowering Ler. This prompted us to speculate that L0044 may act upstream of Ler, and that disruption of *ler* in Δ44 should abolish the effect of deleting *L0044*. To test this possibility, *ler* in Δ44 was further deleted by homologous recombination which resulted in a double-mutant strain, namely Δ44/L. This mutant along with Δ44 and the wild-type strains were cultured and analyzed for LEE protein expressions (fig. 4). Deletion of *L0044* alone again produced increased expression of LEE protein in LB (lane 3). Deletion of *ler* alone (in the ΔLer strain) produced a basal level of expression (lane 5). Disruption of *ler* in addition to *L0044* (in the Δ44/L strain) resulted in a phenotype similar to that of ΔLer and that of the wild-type bacteria when the bacteria were cultured in LB (compare lanes 1, 5, and 7). Thus, the lost suppression in M9 due to the deletion of *L0044* (lane 4) reappeared by the additional

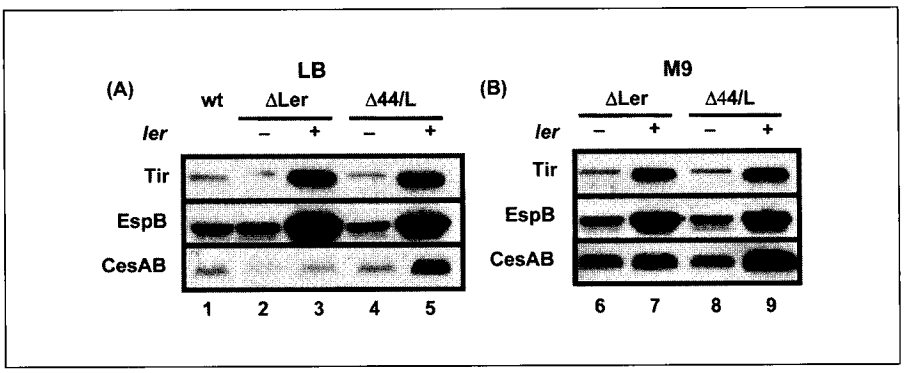

Fig. 5. Effect of the *L0044* gene revealed by complementation of ΔLer and Δ44/L with Ler. The plasmid, pAT-Ler, was transformed into the ΔLer and Δ44/L strains, and the bacteria were grown in different media. Bacterial lysates were prepared and analyzed by Western blotting using specific antisera.

deletion at *ler* (lane 8). Therefore, *ler* is apparently involved in the deletion effect of *L0044* on LEE protein expression.

It is worth noting that CesAB seemed to be under a control mechanism different from that for Tir and EspB. In the wild-type strain, they all appeared in a similar upregulated tendency when induced with M9 (fig. 4, lanes 1 and 2). However, in ΔLer (lanes 5 and 6), M9 induction of the expressions of Tir and EspB was abolished while that of CesAB remained detectable. Simultaneous deletion of *L0044* and *ler* further augmented the differences in CesAB levels (compare lanes 5–8). One possible explanation for these observations is that L0044 normally suppresses the expression of *LEE1* down to a basal level such as in LB. Once there is a signal such as M9 induction to break the suppression, *LEE1* is activated and thus the levels of Ler and CesAB increase. No Ler in ΔLer results in unchanged *LEE4* and *LEE5* expressions when induced with M9, as observed from the levels of EspB and Tir (lanes 5 and 6). However, mutant ΔLer still contains L0044 that remains functionally repressive to *LEE1* and can respond to M9 induction, as seen from the increased level of CesAB (lane 6). No *L0044* in Δ44/L would completely free the repression on *LEE1*, and, thus, the CesAB level further increased (as observed in lanes 7 and 8).

Expression of ler *in the* L0044/ler *Double Mutant Activated the LEE Operons*

To confirm the relationship between *L0044* and *ler*, we examined the expression patterns of the single mutation at *ler* (i.e. ΔLer) and the double mutations at both *L0044* and *ler* (i.e., Δ44/L) in a different way. In theory, both of these mutants have *ler* deleted and differ only by the presence or absence of *L0044*. When cultured in LB, ΔLer and Δ44/L expressed LEE proteins at a basal level (fig. 5A, lanes 2 and 4). When transformed with pAT-Ler, ΔLer and Δ44/L regained Ler, and both strains increasingly expressed Tir and EspB, which are representative proteins of the *LEE5* and *LEE4* operons, respectively (lanes 3 and 5). However, the two transformants differed in their expressions of CesAB encoded by the *LEE1* operon (compare lanes 3 and 5). Enhancement of the expression of CesAB by complementation with Ler was more prominent in Δ44/L (lane 5) than in ΔLer (lane 3). In M9, increased expressions of Tir and EspB were also seen in ΔLer and Δ44/L when the bacteria were complemented with Ler (fig. 5B). However, CesAB expression was greater in Δ44/L (lane 9) than in ΔLer (lane 7). Thus, these results suggested that the presence of *L0044* may have a unique suppressive effect on the *LEE1* operon. This effect subtly differs from those on *LEE4* and *LEE5*.

Coexpression of L0044 *and* ler *Suppressed the Expression of* LEE1

To consolidate the notion above that L0044 has a peculiar suppression effect on the expression of the *LEE1* operon, and thus CesAB, two compatible plasmids, pLac-Ler and pAT44, were used to transform the wild-type EHEC. When Ler was additionally expressed from the plasmid, the three representative LEE proteins were increasingly expressed in LB to a level near that in M9, and the suppressive effect of LB was obviously relieved (fig. 6, compare lanes 1 and 3). Furthermore, additional expression of L0044 in the presence of extra Ler did suppress the expression of CesAB but not those of Tir and EspB (compare lanes 1 and 2, also lanes 3 and 4). Thus, these results strongly support the concept that L0044 overrules the positive effect of Ler and suppresses the expression of CesAB, and thus *LEE1*.

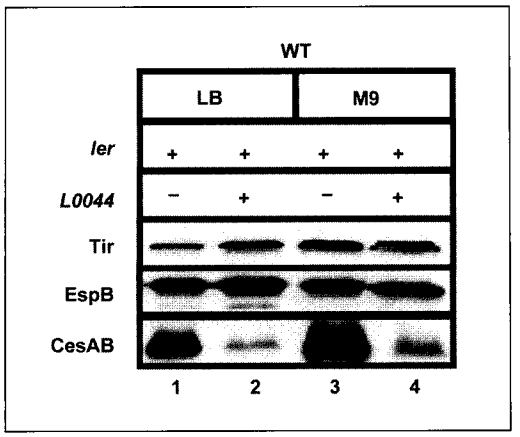

Fig. 6. Repression of the synthesis of CesAB, but not Tir or EspB, by the overexpression of *L0044* and *ler* in EHEC. The wild-type EHEC was transformed with a vector control (–) or with pLac-Ler and pAT44 for expression of Ler and L0044, respectively. Bacteria were cultured in LB or M9, and bacterial lysates were analyzed by immunoblotting.

Discussion

Virulence factors residing on the LEE islands of EHEC and EPEC are regulated by environmental factors including temperature, osmolarity, and quorum sensing [6]. Herein we used a difference in culture conditions between LB and M9 to demonstrate regulation of the EHEC LEE at the level of mRNA and proteins. The data obtained all support the hypothesis that *L0044* on the LEE plays a critical role in this regulation.

When EHEC was cultured in M9, or DMEM, it is known that secretion of the effectors and translocators derived from LEE significantly increased as compared to that in LB. Therefore, in LB, the expression of these LEE products is maintained at basal levels in a repression state [2], while in M9 and without the *L0044* gene product, the repression state of LEE was completely relieved, and synthesis of LEE products probably reached a maximum (fig. 1B). Therefore, the gene product of *L0044* obviously functions as a negative regulator for the transcription of the LEE operons, at least *LEE1* and *LEE4*, as shown by Northern blots (fig. 3). It also represses the expression of *LEE5*, as reflected by the fact that deletion of *L0044* increased the level of Tir (fig. 1B). It is likely that other LEE operons such as *LEE2* and *LEE3* are also indirectly repressed by L0044 since the presence of *L0044* suppressed the expression of *LEE1*, as evidenced by a drastic decrease in CesAB (fig. 6).

Several regulators have been implicated in regulation of the LEE, such as IHF (integration host factor) [16], H-NS [3, 4, 39], FIS (factor for invasion stimulation) [17], QseA (quorum sensing *E. coli* regulator A) [36], and BipA [18]. Of these, only IHF and H-NS have been shown to directly bind to the *LEE1* promoter and result in operon activation and repression, respectively [16, 39]. At low temperatures, H-NS represses the expression of the *LEE1* promoter. When the temperature is raised to body temperature, repression of the *LEE1* promoter is alleviated [39]. Ler was the first gene product translated from the polycistronic mRNA of *LEE1* [30]. Its production then activates other LEE promoters, including those of *LEE2, LEE3, LEE4, LEE5*, and *L0044-L0043*, by relieving the repression of H-NS by an as yet unknown mechanism [4, 14, 19]. To the present, no information has been provided about whether Ler can regulate its own promoter. Our data provide the first evidence that Ler has a function in activation of *LEE1* expression (fig. 5A, lanes 4 and 5); expressing Ler in *trans* not only raised the protein levels of Tir and EspB but also that of CesAB, a product from *LEE1*. However, it is not known whether Ler regulation of *LEE1* is a direct effect or an indirect one through other factors.

In *Yersinia*, the negative regulator, LcrQ/YscM, suppresses the synthesis of Yops to reduce expression of virulence genes. When the environment is appropriate, such as with a low level of calcium ions, the negative regulator is then secreted, and repression is relieved. *Yersinia* then actively expresses the virulence factors [34]. In the case of EHEC *L0044*, the putative protein was not detected in the concentrated spent media of either LB or M9, nor was the product detected in the media by overexpressing L0044 in the wild-type EHEC (data not shown). These observations suggest that the negative regulation mechanism of EHEC L0044 may differ from that of *Yersinia's* LcrQ/YscM. While determining how L0044 confers its repression remains to be explored, one possibility is that the activity of L0044 might be antagonized by a product from a second ORF in *L0043* (fig. 1A). Previously, the EPEC homologue of *L0044*, *orf10*, was reported to possibly give rise to a product with self-to-self interaction or interaction with the product from *orf11*, a *L0043* homologue in EPEC [8]. Assuming that L0044 interacts with the product of *L0043*, then the repression effect of L0044 must be diverted. If so, *L0043* may regulate the expression of the LEE in a way opposite to that of *L0044*. Our preliminary data on overexpression of the product of *L0043* in the wild-type EHEC indicated relief of the suppression effect observed in LB, as evidenced by increased expressions of

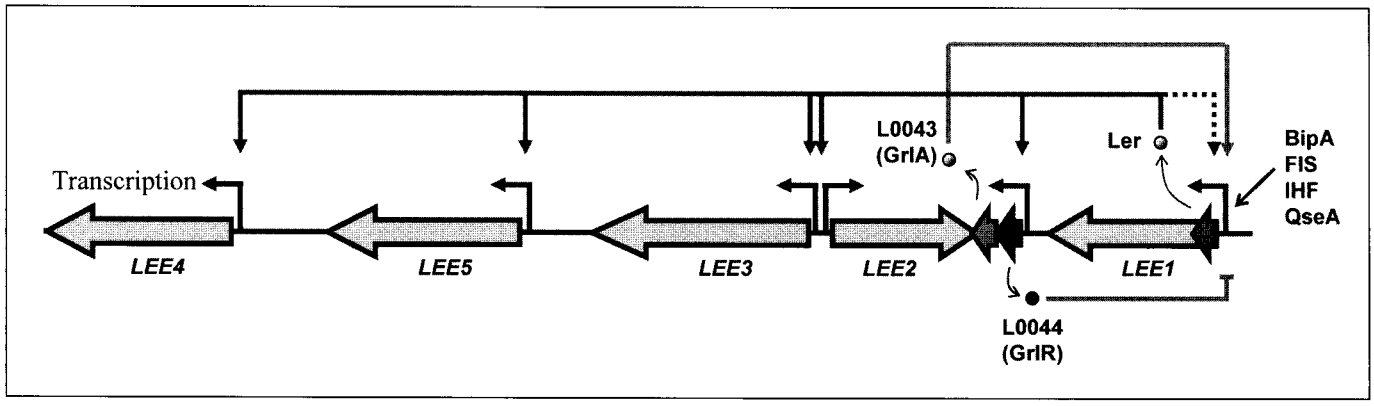

Fig. 7. A proposed model for LEE regulation. In repressive conditions, such as at low temperatures or in M9 medium, all the major LEE operons are repressed by H-NS, and L0044, described in the text or generally named GrlR [10], specifically downregulates the expression of *LEE1*. When the environment is appropriate, the expression of *LEE1* is activated. The expressed Ler, in turn, activates expression of the major LEE promoters (*LEE1* to *LEE5*), possibly by antagonizing H-NS. Activation of *LEE1* by *ler* may be indirect, as shown in the broken arrow line. BipA, FIS, IHF, and QseA may activate the expression of *LEE1*; IHF and QseA bind directly to the *LEE1* promoter, whereas the mechanism of BipA and FIS remains unclear. L0044 may also be activated by Ler and may downregulate the expression of *LEE1*, perhaps by acting on the *LEE1* promoter or by counteracting with another activator of *LEE1*, i.e. ORF L0043 or previously named GrlA [10].

Tir, EspB, and CesAB (data not shown). Recently, Deng et al. [10] demonstrated that *orf10* and *orf11* of *Citrobacter rodentium* are negative and positive regulators of the LEE, and the products are named GrlR (global regulator of the LEE, repressor) and GrlA (global regulator of the LEE, activator), respectively. Although our studies were based on EHEC rather than C. *rodentium*, we reached the same conclusions for *L0044* as they reported for *orf10*. For this reason, L0044 should be named after GrlR. Furthermore our measurements monitored the effects on expression with *LEE1*, *LEE4*, and *LEE5*, whereas they determined those with *LEE2* and *LEE5*. These partially complementary data increasingly provide evidence that the suppression effect of GrlR may occur globally across the island. More importantly, our finding that GrlR suppresses the expression of CesAB in a unique way strongly suggests that *LEE1* is regulated differently from the other LEE operons.

Apparently, regulation of the LEE is far more complicated than has previously been thought. A model based on previous research and our current findings is as follows (fig. 7). A feedback loop may exist to coordinate expression of the LEE island. In the repressive condition, L0044 produced at a basal level represses the expression of *LEE1*, and thus Ler. In inductive conditions, L0044 is inactivated, perhaps by interaction with other factors such as L0043, and, as a result, *LEE1* expression is induced. The increase in Ler then activates expression of the LEE operons including *LEE1*, although the activation of the latter might be indirect [14]. Activation of *LEE1* then results in more-active synthesis of Ler, which in turn enhances LEE protein expression. One step within this loop that remains to be defined is whether the promoter of *L0044* is also activated by Ler. If so, the actively expressed L0044 would feed back and repress the production of Ler. Although the product encoded by *L0043* needs to be better characterized and more regulatory factors have to be explored and considered, our current model should provide a framework for understanding how multiple factors regulate the LEE. In conclusion, it is clear that pathogens need to orchestrate the synthesis and delivery of their virulence factors in a very precise but complicated manner as seen here.

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