DNA looping-dependent autorepression of *LEE1* P1 promoters by Ler in enteropathogenic *Escherichia coli* (EPEC)

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Ler, a homolog of H-NS in enteropathogenic *Escherichia coli* (EPEC), plays a critical role in the expression of virulence genes encoded by the pathogenic island, locus of enterocyte effacement (LEE). Although Ler acts as an antisilencer of multiple *LEE* operons by alleviating H-NS–mediated silencing, it represses its own expression from two *LEE1* P1 promoters, P1A and P1B, that are separated by 10 bp. Various in vitro biochemical methods were used in this study to elucidate the mechanism underlying transcription repression by Ler. Ler acts through two AATT motifs, centered at position −111.5 on the coding strand and at +65.5 on the noncoding strand, by simultaneously repressing P1A and P1B through DNA-looping. DNA-looping was visualized using atomic force microscopy. It is intriguing that an antisilencing protein represses transcription, not by steric exclusion of RNA polymerase, but by DNA-looping. We propose that the DNA-looping prevents further processing of open promoter complex (RP_O) at these promoters during transcription initiation.

DNA-bending | first phosphodiester bond formation | road block transcript

Bacterial transcription initiation is a multistep process, during which the RNA polymerase (RNAP)-promoter complex undergoes a series of conformational changes, from an initial closed (RP_C) to an open (RP_O) complex, all stages of which are potentially subjected to regulation. It is generally perceived that the formation of open complex is likely to be rate-determining in the initiation of transcription for many promoters because triphophate binding and subsequent initiation reaction is rapid (1–4). The traditional model of transcriptional repression involves competition between a repressor and RNAP for binding to DNA at overlapping target sites (5, 6). In this model, steric exclusion because of repressor binding at or near the promoter prevents RNAP access to the promoter. An alternative model proposes that binding of a repressor to bipartite operators flanking a promoter creates DNA looping, thereby resulting in conformational changes in the promoter DNA and transcription repression (7, 8). Repression requiring DNA looping has been experimentally shown in several operons in *Escherichia coli*, including *ara*, *lac*, *gal*, *deo*, and *nag*, which are reviewed in ref. 8. The best-studied example, however, would be that of *gal* promoters in *E. coli*. The dimeric GalR represses two *gal* promoters separated by 5 bp, P1 and P2, through simultaneous binding to upstream (O_E) and downstream (O_I) operators, centered at −60.5 and at +53.5, respectively (7). In this case, a bacterial histone-like protein, HU, is required to stabilize the looped complex (9, 10). Furthermore, in a limited number of cases it has been proposed that a direct interaction between repressor- and promoter-bound RNAP results in transcription repression (11, 12).

Enteropathogenic *E. coli* (EPEC) is a pathogenic Gram-negative bacterium. EPEC causes attaching and effacing intestinal lesions. The genes involved in the formation of attaching and effacing lesions are encoded within a chromosomal pathogenicity island, locus of enterocyte effacement (LEE). The LEE region carries five major operons, *LEE1*, *LEE2*, *LEE3*, *LEE4*, and *LEE5*, which are repressed by the global regulator H-NS (13–15). Ler (LEE-encoded regulator) is a member of the H-NS protein family encoded by the first gene of the *LEE1* operon. Although Ler induces the expression of *LEE2–5* by counteracting H-NS–mediated silencing, it acts as a specific autorepressor of *LEE1* transcription. The autoregulation limits the steady-state level of Ler to concentrations that are just sufficient to counteract H-NS–mediated silencing of the LEE promoters (16). It has been reported that the lack of specific autorepression domain, but having autocompensatory modulations for mutations, allows Ler to restore its biological functions by alternative mechanisms (17).

The mechanism of Ler-mediated antisilencing has been extensively studied (14, 18–27). It was suggested that Ler counteracts H-NS by displacing H-NS from specific promoter regions; however, the mechanism of repression by Ler has not been elucidated. In this study, the mechanism underlying Ler-mediated repression of *LEE1* P1 was investigated by using various in vitro methods, including high-resolution atomic force microscopy (AFM). We report that the H-NS homolog Ler represses transcription from *LEE1* P1A and P1B (28) simultaneously through DNA looping. The data suggest that oligomeric Ler (29) acts on the two operator sites flanking *LEE1* P1 promoters and loops out the intervening DNA, in which RNAPs are trapped as

Significance

Ler [locus of enterocyte effacement (LEE)-encoded regulator], encoded by the first gene of the LEE1 operon in enteropathogenic *Escherichia coli* (EPEC), represses its own transcription driven by two promoters separated by 10 bp. We found that Ler does this repression through a DNA loop of 16 helical turns, in which RNA polymerase is trapped as open promoter complex, although this complex should be most readily transformed into productive initiation complex.

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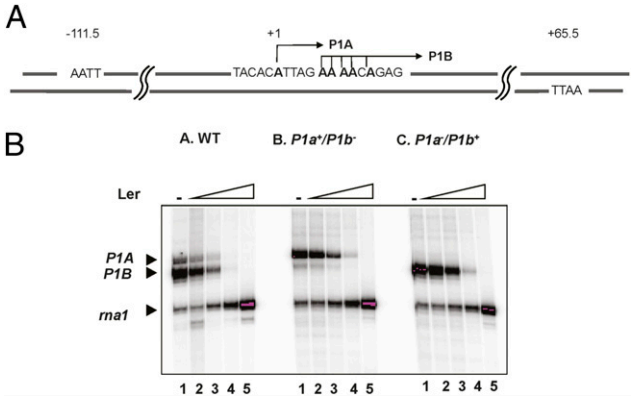

Fig. 1. Regulatory effect of Ler on *LEE1* P1. (*A*) Line scheme showing the transcription start sites of P1A and P1B. P1A is assigned as +1. The positions of two AATT Ler recognition sequences are shown (see below). (*B*) In vitro transcription assays using *LEE1* P1 DNA templates carrying wild-type promoter or $P1A^{-}/P1B^{+}$ (A^{-12} to G) or $P1A^{+}/P1B^{-}$ (A^{-2} to G) mutant promoter DNA, were carried out in the absence or presence of 7,15, 30, and 60 nM Ler (lanes 1–5 in each panel). Transcripts were separated on an 8% (wt/vol) denaturing gel.

an open promoter complex (RP_O), as demonstrated by AFM and other methods that dissect protein–DNA interactions. Interestingly, we observed in vitro that in the absence of upstream operator, downstream operator-bound Ler restores autorepression likely by road-blocking the transcription elongation.

Results

Repression of *LEE1* P1 by Ler. Expression of *LEE1* in EPEC is driven by two promoters separated by 10 bp, P1A and P1B (Fig. 1*A*) (28). To elucidate the mechanism underlying *LEE1* P1 regulation by Ler, multiple-round in vitro transcription assays using purified components, followed by analysis on 8% (wt/vol) polyacrylamide DNA sequencing gels were used. *LEE1* P1 DNA from EPEC strain E2348/69 (−193 to +87) (Fig. 1*A*) was PCR-amplified and cloned into pSA508 between the EcoR1 and Pst1 sites, located immediately upstream of the 54-bp Rho-independent transcription terminator of the *rpoC* gene of *E. coli* (30, 31). [α-^{32}P] UTP was included in the reaction to detect nascent RNA. Multiple transcripts were generated, with sizes ranging from ∼125–135 nt, in addition to the 105-nt *rna1* transcript from the origin of plasmid replication (Fig. 1*B*) (31). It has been shown previously that the transcripts are products of two P1 promoters, A^{+1} from P1A, and $A^{+8}A^{+9}A^{+10}A^{+11}$ and A^{+13} from P1B (28). In the presence of increasing concentrations of purified Ler, transcripts originating from both P1A and P1B disappeared simultaneously. Mutant *LEE1* P1, lacking either one of the two promoters, was produced by replacing critical A residues with G residues in the −10 hexamer of each promoter, generating $P1A^{+}/P1B^{-}$ (A^{-2} to G) and $P1A^{-}/P1B^{+}$ (A^{-12} to G) (28). Each mutant promoter generated transcripts from the respective functional promoter (first lanes in each group of Fig. 1*B*). Addition of Ler resulted in a reduction of the transcripts from the respective promoters, as with intact wild-type *LEE1* P1. Thus, Ler acts on the two promoters simultaneously, which is unlikely to be the result of simple steric exclusion (7).

Identification of Ler Action Sites. ***Promoter deletion analysis in vitro.*** To identify the Ler action sites, *LEE1* P1 DNA was serially deleted from the 5′ end and assayed for Ler-mediated repression in vitro (Fig. 2 *A* and *B*). Repression of P1A and P1B by Ler was nearly the same with the constructs truncated at −193, −143, and −102: over 90% repression was observed with 15 nM Ler, and at 30 nM Ler full repression was seen (Fig. 2*C*). However, loss of repression was observed at 15 and 30 nM of Ler in the construct deleted to −82, which required an excessive of 60 nM Ler to attain full repression. *LEE1* P1 was then truncated from the 3′ end to +67 and +47, with the 5′ end remaining fixed at −193, and assayed for Ler-mediated repression. Although transcripts from P1A with the construct truncated at +67 partially overlapped *rna1*, careful examination revealed that the repression was normal. No repression was observed for the construct truncated at +47. We concluded that Ler acted at both upstream (−143 and −82) and downstream (+47 and +67) sites in *LEE1* P1 and that repression observed for the construct −82 to +87, with the highest concentration of Ler, was mediated by the Ler bound to downstream action site (32).

Identification of operator sites in vivo. A recent NMR study suggested that Ler recognizes a certain structural pattern in the DNA minor groove associated with an AATT motif (33). Introduction of TpA steps within the AATT motif has been shown to disrupt

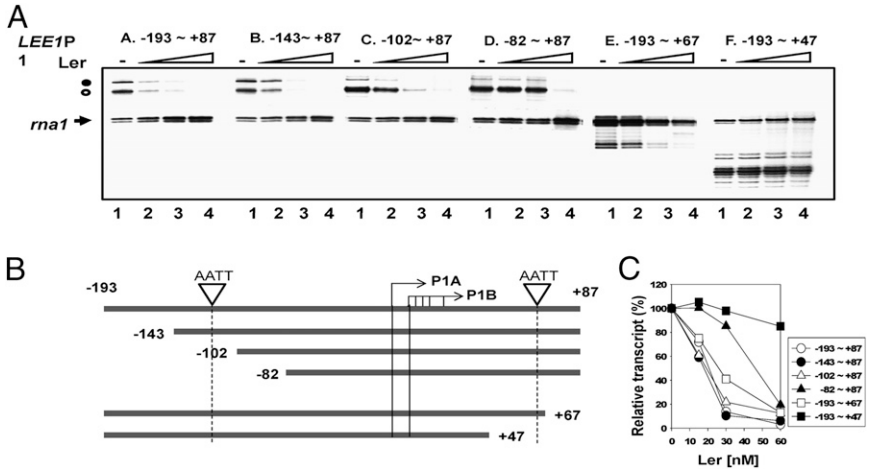

Fig. 2. Promoter deletion analysis. (*A*) *LEE1* P1 was truncated at the indicated upstream and downstream sites and cloned into pSA508. In vitro transcription assays were carried out in the presence of 0, 15, 30, and 60 nM Ler (lanes 1–4 in each panel) and separated on an 8 M urea/8% (wt/vol) polyacrylamide gel. (*B*) Schematic representation of LEE1 promoter deletion analysis shown in *A*. (*C*) The graph shows the amounts of P1A and P1B RNA in lanes 1–4 in *A* relative to RNA generated in the absence of Ler. The RNA transcripts quantified by determining counts per minute (cpm) with a scanner were averaged and plotted as a function of Ler concentration.

Ler binding to their target sequences. Nucleotide sequence analysis of *LEE1* P1 DNA revealed a presence of AATT motifs, centered at −111.5 on the coding strand (O_R1) and at +65.5 on the noncoding strand (O_R2) of *LEE1* P1 DNA. To determine Ler action through O_R1 and O_R2, the *LEE1* P1-carrying mutations destroying AATT motifs at the operator sites (AATT→TTAA substitution) were constructed and tested in vivo using a laboratory strain of *E. coli* K-12 (MG1655). *LEE1* P1 carrying AATT→TTAA substitutions were cloned in pRS415 carrying promoterless *lacZ* (34) and subsequently moved to construct respective λ lysogens in the MG1655 test tube strain background (35). The *ler* cloned under the arabinose-inducible *araBAD* promoter in pBAD33 (36) were transformed into the above lysogenic strains and β-Galactosidase activities were assessed during exponential growth of bacteria in Luria-Bertani (LB) media in the presence or absence of 0.2% L-arabinose (Table 1). The wild-type *LEE1* P1 were repressed about eightfold when promoter activities were compared in the presence or absence of L-arabinose. The activities of the *LEE1* P1 promoter carrying TTAA substitutions were about the same as the wild-type promoter in the absence of L-arabinose. *LEE1* P1 carrying the mutations at O_R1 or O_R2 were repressed 0.96-fold and 1.19-fold, respectively. Thus, the losses of repression in the absence of O_R1 or O_R2 compared with that of the wild-type construct suggested interdependency or cooperativity between the operators (8). The repression in the absence of upstream operator observed in vitro (Fig. 2) suggested that a different mechanism predominates in vitro with purified components.

Independent binding of Ler to either of the operators. A gel mobility-shift analysis was carried out to determine independent binding of Ler to two operator sites in *LEE1* P1. PCR amplified DNA fragments (−193 to +1 and +27 to +87), each carrying either of the operators (Fig. 3, panels A and B, respectively) and purified Ler was used for the experiment. Migration of protein-bound DNA to shifted positions on a 5% gel in the presence of increasing amounts of Ler suggested that Ler binds to the two operator sites independently of each other to repress the two promoters simultaneously.

Ler-Binding Induced Changes in *LEE1* Promoter DNA. DNase I protection was carried out to investigate changes in *LEE1* promoter DNA induced by Ler binding to two operator sites as well as to verify the operator sites. *LEE1* P1 DNA fragments were incubated with increasing concentrations of Ler, followed by DNase I treatment. A segment of ∼11 bp, from position −112 to −102, was protected by Ler in the coding strand (Fig. 4 *A* and *F*), whereas the region from +61 to +68 was protected in the noncoding strand (Fig. 4 *D* and *I*); these sites containing the AATT motif corresponded to the two operator sites, O_R1 and O_R2. In addition to protection at the operator site, the coding strand displayed a pattern of alternating enhanced and diminished DNaseI-sensitive bands in the presence of Ler (Fig. 4*A*). One example is a hypersensitive band at position −101 followed by less-sensitive bands at positions −97 and −94. Relatively extensive enhanced cleavage was observed from the band at −86 to that at −69, separated by diminished cleavage at −77. Changes in the DNase I sensitivity were more pronounced in the noncoding strand. Upon Ler binding, simultaneous but periodic protected regions followed by short regions of hypersensitivity were seen starting from nucleotide position +29, which are situated 31 bp away from the primary Ler binding site, O_R2. Although Ler occupied O_R1 weakly in the coding strand at a concentration of 60 nM, strong protection was seen in O_R2 at 30 nM Ler. Further increases in Ler concentration resulted in enhanced cleavage by DNase I. A pattern of alternating enhanced and diminished DNase I-sensitive bands between two primary binding sites suggests DNA looping that are a typical to the DNA loops with 100–200 bp (7, 37–42). In a DNA loop, the enhanced cleavages outside of the loop and diminished cleavages inside the loop can be attributed to curvature of the DNA backbone. This pattern on *LEE1* P1 DNA could therefore display cooperative binding of Ler to O_R1 and O_R2. However, the possibility that the multiple periodic footprints were a result of extended Ler binding to DNA at the primary binding sites could not be ruled out. Thus, we examined the DNase I sensitivity pattern of *LEE1* P1 DNA lacking either of the two operators. The O_R1-deleted coding strand (−93 to +87) (Fig. 4 *B* and *G*) lacked the dark and light bands that were present in Fig. 4*A*, whereas its noncoding counterpart showed very weak binding of Ler to the O_R2 and lack of multiple protection and hypersensitive band pattern reasserting the loss of cooperativity (Fig. 4 *C* and *H*). The abolition of cooperativity and loss of consecutive enhanced and diminished band patterns in the mutant DNA further suggested the occurrence of DNA looping (38). Interestingly, the O_R2-deleted DNA (−134 to +47 on the noncoding strand) (Fig. 4 *E* and *I*) showed an altered band patterns compared with the wild-type noncoding strand (Fig. 4*D*).

For example, a loss of protection at the first three bases (+29 to +26) compared with Fig. 4*D*, wherein a complete protection was seen from position +29 to +18. The protection pattern was gradual and weak from positions +26 to +18, +12 to +2, and −3 to −18, with increasing concentrations of Ler. The hypersensitive bands at positions +31 and +32 disappeared but those at positions +14, +15, +16, and +17 were reduced in intensity even at the highest concentration of Ler. Furthermore, new hypersensitive bands appeared at positions −36, −45, and −46. Thus, the change in the binding pattern and the degree of occupancy at the above mentioned positions suggested an alternative mode of Ler binding in the absence of O_R2.

Table 1. Effect of operator mutation on *LEE1* P1 expression in MG1655

	Miller Unit		
Strain	Ler$^-$	Ler$^+$	Fold repression
AB5024 (wild-type)	327.2	40.7	8.0
AB5033 (O_R1^-)	230.6	239.2	0.96
AB5034 (O_R2^-)	250.3	209.4	1.19

Ler was supplied from pBAD33::*ler*; Miller unit = A_{420}·min·mL/A_{600} × 1,000.

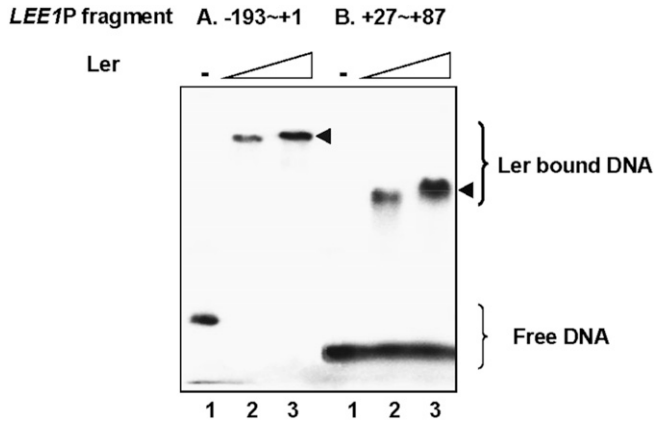

Fig. 3. Gel mobility-shift assay. Ler binding to *LEE1* P1 DNA sequences −193 to +1 (A) and +27 to +87 (B). Ler was included in the preincubation mix at a concentration of 0, 30, and 60 nM (lanes 1–3). Samples were analyzed by electrophoresis in a 5% (vol/vol) native polyacrylamide gel.

Effect of Phase Change on Ler–Mediated Repression. It is known that short stretches of DNA resist torsional changes (43–45) and that the proper angular orientation of two binding sites is necessary

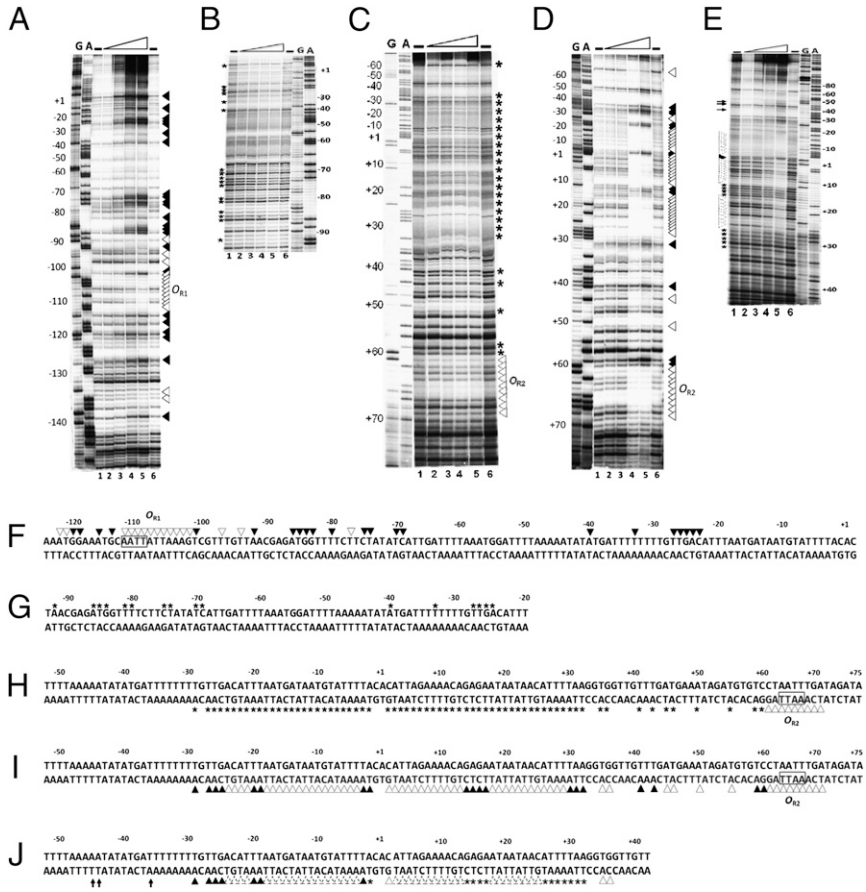

Fig. 4. DNase I footprint analysis. The results of coding strands from −193 to +87 (*A*), −93 to +87 (*B*) and noncoding strands from −93 to +87 (*C*), −134 to +87 (*D*), and −134 to +47 (*E*) are presented. [γ-32P] ATP labeled PCR products were incubated with increasing concentrations of Ler followed by DNase I treatment. The concentrations of Ler were 0 (lanes 1 and 6) and 7, 15, 30, and 60 nM in lanes 1–5. G/A sequencing markers are shown. O_R1 and O_R2 are the two primary operator sites occupied by Ler. The data presented in *A–E* are aligned with the *LEE1* P1 sequence in *F–J*, respectively. The AATT motifs in O_R1 and O_R2 are boxed. Closed and open triangles represent enhanced and diminished bands, respectively. Asterisks represent disappeared band patterns that were present in *A* or *D* and dashed open triangles represent loss of protections that were present in *D*. Arrows represent appearance of new bands.

for the looping of a short DNA fragment by bound protein (7). To explore the possibility that Ler proteins bound to the sites centered at −111.5 and +65.5 associate to induce DNA looping, mutant *LEE1* P1 sequences, carrying a 5-bp insertion (a half helical turn) or a 10-bp insertion (a full helical turn) at position −63, were created and assayed in vitro (Fig. 5). With the 5-bp insertion, the extent of repression of P1A and P1B transcripts by Ler was significantly reduced. Partial repression could be attributed to Ler bound to the downstream site, as observed with the −82 to +87 construct in Fig. 2 (32). With the 10-bp insertion, Ler-mediated repression of P1A and P1B transcripts was as efficient as that of wild-type *LEE1* P1 DNA. These results are consistent with the DNA looping model, which predicts that no repression should occur when the angular orientation of the two sites is shifted by the addition of a one-half DNA helical turn, and full repression should occur when the orientation of two sites is restored by the addition of a full helical turn. This mechanism closely resembles that underlying the repression of *gal* promoters in *E. coli*, where GalR bound to two operators flanking two promoters induces DNA looping (7).

Visualization of DNA Looping. High-resolution AFM was used to visualize Ler-bound *LEE1* P1 DNA (−193 to +107). DNA was prepared by PCR amplification using primers bound at −255 and +561 relative to the +1 position of *LEE1* P1, thereby adding additional sequences to the upstream and downstream ends (Fig. S1). Closed circular DNA was used to avoid nonspecific binding of Ler to DNA fragment ends. Closed circular DNA was prepared by PCR amplification using 5′-biotin–derivatized primers, which resulted in DNA biotinylation at both ends. Added streptavidin bound to the biotin residues, circularized the DNA strands, and provided a visual reference (Fig. 6*A*). *LEE1* P1-Ler complexes were formed under in vitro reaction conditions, deposited on mica and Ler-induced changes in DNA conformation were observed by AFM (*Materials and Methods*). Fig. 6*B* shows a representative picture on the mica: 14 molecules with DNA loop and 14 molecules without DNA loop. Small loops (Fig. 6*B*, green arrowheads) were observed approximately at a third position from the streptavidin molecules (Fig. 6*B*, yellow arrowheads) on the DNA circles, where *LEE1* P1 should be located. Looped DNA molecules were collected and the distance of DNA arms from the knots at the entry of loops to the streptavidin was determined (Fig. 6*C*). The average length of the short and long arms was 45 nm and 140 nm, respectively. The average length of each base pair, estimated from the length (259 nm) of the 816-bp strand of closed circular DNA, was 0.317 nm. Thus, the short and long arms would consist of 141 and 441 bp, respectively. The positions located at −265 and +551 relative to the +1 position of *LEE1* P1 would correspond to −117 and +107. This estimate is a close approximation of what is derived from the studies presented in Figs. 2 and 3 (i.e., −115.5 and +65.5). Taken together, these data suggest that the multimeric Ler interacts with operator

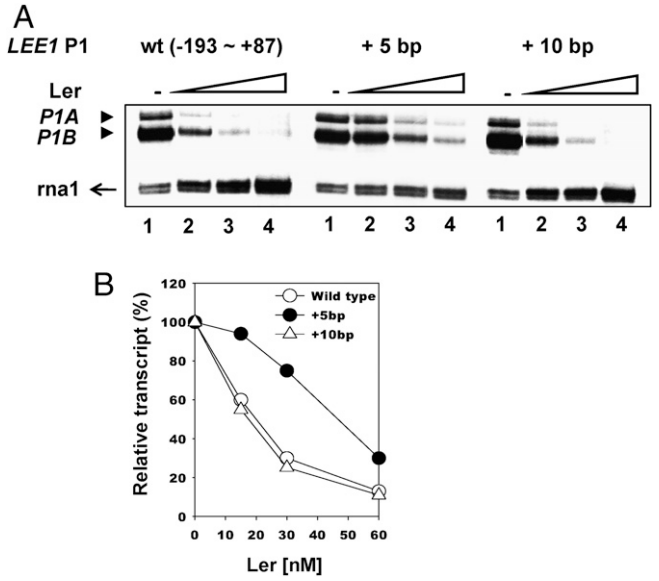

Fig. 5. Effect of operator site spatial registration on Ler-mediated repression of *LEE1* P1. Ler was titrated in vitro using wild-type DNA and mutant DNA carrying either a 5-bp (ACTAA) or a 10-bp (ACTAATATGT) insertion at −63. The results of in vitro transcription in the presence of 0, 15, 30, and 60 nM Ler (lanes 1–4), followed by electrophoresis in an 8% (wt/vol) denaturing gel, are shown in *A*. (*B*) The amounts of P1A and P1B RNA in lanes 1–4 relative to RNA generated in the absence of Ler.

sites by recognizing AATT motifs on two opposite strands of DNA simultaneously to induce DNA looping.

Repression of the Transcription Initiation Stage by Ler. ***A gel-shift analysis of initiation complex.*** The conventional steric exclusion model of transcription repression assumes that repressor binding prevents occupation of the promoter by RNAP (6, 31). However, the results described so far in this report have implicated DNA looping as a primary mechanism underlying repression of *LEE1* P1 by Ler. To determine whether RNAP was present on the Ler-induced DNA loop in *LEE1* P1, a gel-retardation assay was carried out. Ler or RNAP were incubated with *LEE1* P1 DNA (−193 to +87) for 10 min before loading onto a 5% (vol/vol) native gel (Fig. 7). To distinguish between RNAPs binding to P1A and P1B, mutants devoid of one of the two promoters were used; these were $P1A^{+}/P1B^{-}$ (A^{-2} to G in Fig. 7*A*) and $P1A^{-}/P1B^{+}$ (A^{-12} to G in Fig. 7*B*). Ler (15 kDa) and RNAP (500 kDa) formed complexes with *LEE1* P1 DNA that migrated only slightly to the position on the gel near the top (Fig. 7, lanes 2–4). The complexes formed with *LEE1* P1 DNA in the presence of both Ler and RNAP produced a gel-migration pattern (Fig. 7, lanes 5 and 6) that was indistinguishable from those of RNAP-DNA or Ler-DNA binary complexes. In the gel-retardation experiments, looped DNA–protein complexes exhibit high retardation (42, 46, 47). High retardation of this Ler–DNA complex contrasted clearly with the mobility of Ler bound to the DNA containing either one of the operators (Fig. 3). Furthermore, sharply shifted bands with no smearing also indicated that the Ler–DNA complexes were highly stable. When the Western blot analysis was carried out using antibodies specific for Ler or the RNAP α-subunit, both antibodies recognized the same band (black arrowheads with dashed lines). There was no change in the intensity of the anti-RNAP antibody-positive band with increasing Ler concentration, suggesting that Ler did not destabilize the RNAP–*LEE1* P1 complex at both promoters. Bands (gray arrowheads in Fig. 7) below the major bands were taken to be unbound RNAP. These data suggest that Ler does not interfere with RNAP binding to either P1A or P1B, but forms a ternary complex with RNAP.

Analysis of* LEE1 *P1 DNA-Ler-RNAP complex by DNase I footprinting. DNase I protection assays were carried out with *LEE1* P1 DNA in the presence or absence of Ler and RNAP (Fig. 8). Distinct patterns were observed on the noncoding strand in the presence of RNAP or Ler or both. The DNA backbone adjacent to nucleotides at positions +15, +11, +10, +2, +1, −13, −15, −16, −24, −25, and −26 was much more sensitive to DNase I in the presence of RNAP and Ler together, compared with either protein alone. In contrast, the DNA backbone near nucleotides at +24, +18, +17, +13, +3, −6, −8, −21, −50, and −51 was less sensitive to DNase I in the presence of RNAP and Ler together than in the presence of either protein alone. The pattern obtained with RNAP and Ler was clearly different from that obtained with Ler alone, which is not what would be expected if Ler binding prevented RNAP binding. The experiment was repeated to visualize downstream of +20 and the density plot was generated accordingly (Fig. S2). The results indicate that Ler and RNAP bind simultaneously to *LEE1* P1 DNA, suggesting that transcription repression occurs at a step subsequent to RP_C formation, such as RP_O formation or subsequent step in the initiation process (11).

Analysis of* LEE1 *P1 DNA-Ler-RNAP complex by KMnO4 footprinting. The $KMnO_4$ assay detects unpaired bases at the −10 region in RP_O (48–50), and was used to identify reactive bases generated by RNAP in the absence and presence of increasing concentrations of Ler (Fig. 9). We previously reported KMnO4-reactive bands for P1A and P1B using mutants defective in either one of the two promoters (Fig. 4) (28). Binding of RNAP induced the opening of several base pairs in and around the promoter core: T^{-19}, T^{-20}, and T^{-21} for P1A; T^{+4} and T^{+5} for P1B (Fig. 9, lane 2) compared with the control containing no protein (Fig. 9, lane 1). These sites are unique features of the RP_O on each promoter; other sites, including T^{-4}, T^{-5}, and T^{-6}, are reactive in both promoters. The intensities of the $KMnO_4$-hypersensitive bands created by RNAP were not decreased in the presence of increasing concentrations of Ler (Fig. 9, lanes 3 and 4). These results indicate that Ler does not prevent RNAP from forming an RP_O at both *LEE1* P1 promoters. Thus, DNA looping induced by Ler appears to block a step subsequent to RP_O formation at these promoters, but presumably before the first phosphodiester bond formation.

Discussion

We have shown that Ler represses simultaneously both P1A and P1B promoters, which are separated by one helical turn (10 bp) (28), by binding to two primary operator sites positioned at −111.5 and +65.5. Mutation of either of the operator sites led to complete loss of Ler-mediated repression in vivo, suggesting cooperativity between the two operator sites (Table 1). The observed cooperativity was tested in vitro and verified by AFM analysis, which encouraged us to propose DNA looping as the notable cause of repression. We have seen that Ler represses completely at a concentration of 30 nM in the constructs containing intact upstream and downstream operators, whereas the construct deleted of O_R1 demanded up to 60 nM Ler to achieve full repression (Fig. 2). Moreover the DNase I footprinting using DNA fragments deleted of O_R1 showed weak binding of Ler to O_R2 and lack of periodic hypersensitive/diminished band pattern (Fig. 4 *B* and *C*), suggesting loss of cooperativity (8). However, near full-length repression was observed in vitro under O_R1-lacking condition, as exemplified by the construct deleted to −102 (Fig. 2). We speculate that this could be a result of the "road-block" effect of Ler-bound downstream operator (O_R2), which may be predominant in vitro (11, 32, 51, 52). The construct deleted of O_R2 (−193 ~ +47) showed complete loss of repression in the Fig. 2 even at highest Ler concentration, yet a weak and diminished band pattern was seen in the Fig. 4*E*. The altered band pattern observed only at highest concentration of Ler

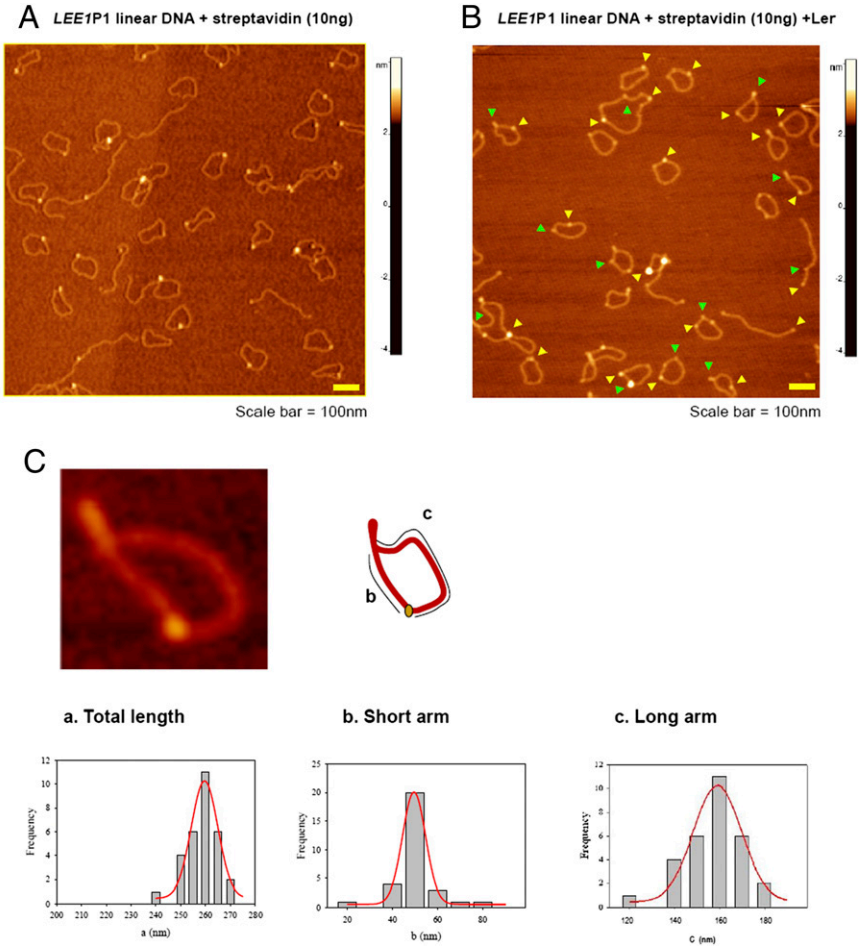

Fig. 6. Analysis of DNA looping by AFM. (*A*) An AFM image of closed circular DNA created upon addition of streptavidin to biotinylated DNA. (*B*) DNA loop (green arrowheads) formed upon addition of Ler to closed circular DNA carrying lerP. Yellow arrowheads indicate streptavidin molecules. (*C*) Representative image and a drawing showing short (*b*) and long (*c*) arms relative to the reference strepatividin molecule (oval). Histograms show total length (*a*) and the lengths of the short (*b*) and long (*c*) arms estimated from 30 molecules with the DNA loop.

(Fig. 4*E* and *Results*) could be because of the binding of multiple Ler molecules that stretches toward the core promoter region. If repression involved a protein–protein interaction and steric exclusion, it is unlikely that the deletions would have affected the two promoters in the same way. Further support for the involvement of DNA looping in the repression of the two promoters by Ler is provided by the observation that Ler action sites at approximate positions −111.5 and +65.5 are located at too great a distance to exert a direct effect on RNAP at the two promoters.

We propose herein that the C-terminal domain of Ler interacts with AATT motifs on two opposite strands of DNA (33) and that the N-terminal domains of two Ler molecules associate to form a dimer (17, 18). A recent NMR study of the DNA-binding domain of Ler (residues 70–116) suggests that Ler recognizes a preexisting structural pattern in the DNA minor groove formed by two consecutive regions that are narrower and wider, respectively, than standard B-DNA (33). The compressed region associated with an AATT motif is sensed by the side chain of Arg90 of Ler and the expanded groove allows the approach of the loop in which Arg90 is located. Arg90 is present in the DNA-binding motif sequence TWSGVGRQP. Consistently, we observed AATT motifs centered at position −111.5 in the coding strand and +65.5 in the noncoding strand of *LEE1* P1, suggesting that dimeric Ler binds to these sites in a parallel orientation. Interruption of the AATT motif by introducing TpA steps has been shown to affect the stability of CT-Ler–DNA complex, and TpG step is preferred over TpA for the stability of the complex (33). By mutating AATT→TTAA, we have introduced $T^{-111}pA^{-110}$ in O_R1 and $T^{+64}pA^{+65}$ in O_R2 and observed a complete loss of repression by Ler (Table 1). Taken together, these data suggest that dimeric Ler binds to AATT on opposite strands, with rotation of intervening DNA bringing the two binding sites facing each other in space to interact. Looping of short (177 bp) segments of DNA should require significant curvature of the sugar-phosphate backbone in the intervening DNA; this was shown by the results of the DNase I protection assay (Fig. 4), where alternating DNase I-sensitive sites occurred at periodical intervals (38–40). Taking the results of this study into account, we interpret the periodic DNase I protection pattern as evidence of DNA looping rather than binding of multiple Ler molecules.

Repression of *LEE1* P1 by a mechanism involving DNA looping is both similar to and distinct from the mechanism underlying repression of *hdeAB*p by H-NS in *E. coli* (53). It has been suggested that the DNA looping created by association of H-NS bound to upstream and downstream sites of *hdeAB*p DNA requires the presence of RNAP at the promoter. In this context, RNAP binding results in crossing over of the DNA arms that are sealed off by H-NS molecules. In contrast, in the case of *LEE1* P1, DNA-looping by Ler takes place in the absence of other factors, such as RNAP (Fig. 6). It has been reported that circularization of DNA segments of less than 500 bp with cohesive ends by T4 DNA ligase requires proper helical orientation of the

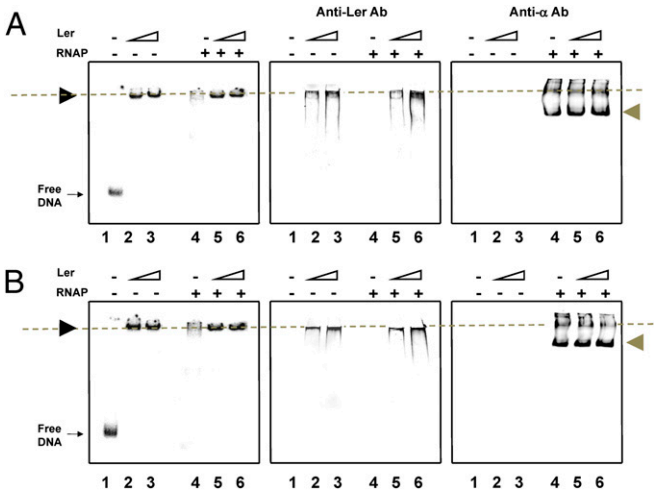

Fig. 7. Gel mobility-shift and Western analysis of simultaneous binding of Ler and RNAP to *LEE1* P1 DNA (−193 to +87). Mobility shift assays were carried out with the 2-nM mutant promoter P1A^{+}/P1B^{-} (*A*) or P1A^{-}/P1B^{+} (*B*) in the presence of 0 (lanes 1 and 4), 30 (lanes 2 and 5), and 60 nM (lanes 3 and 6) Ler. *LEE1* P1 DNA was incubated with Ler and 20 nM RNAP for 10 min. Panels show an image of a 5% (vol/vol) native PAGE gel (*Left*) and the corresponding PVDF membranes probed with antibody against Ler (*Center*) or RNAP α-subunit (*Right*). Bound antibodies were detected by enhanced chemiluminescence. The dark arrowheads with dashed lines indicate ternary complexes of DNA, RNAP, and Ler. The gray arrowheads indicate unbound RNAP.

two ends because of torsional inflexibility of the DNA (7, 54). If the number of base pairs in the DNA fragment is not an integral multiple of the helix repeat, the need to twist the DNA helix to make the ends meet reduces the probability of circularization. This finding is consistent with our observation that addition of one half helical turn between the two binding sites of Ler reduces its repressive effect on transcription (Fig. 5).

In general, a DNA segment smaller than 150 bp is virtually impossible to circularize (55) in the absence of another factor, such as negative supercoiling or a protein with DNA-bending capability. DNA looping by GalR requires HU (7, 9) and the looping of *hdeAB*p DNA by H-NS requires RNAP (Esigma^{70}) (53). Despite the high cost in free energy, however, Ler circularizes LEE P1 DNA in the absence of other factors, as observed with LacI (7). In the case of LacI, the tetrameric protein binds, with apparent K_D of 10^{-13} M, to a bipartite operator, O_R1 and O_R2, on *lac*P separated by nine helical turns on supercoiled DNA (46, 56). It therefore is intriguing to observe DNA looping by Ler, a homolog of H-NS that binds to DNA rather weakly, apparent K_D ranging from 10^{-8} to 10^{-7}M depending on the binding sequences (57, 58), even though distance between the bipartite operators is 177 bp, slightly longer than the persistence length of DNA, 150 bp (55). It is conjectured that the intervening sequence contains highly bendable DNA motifs (59). Circularization of DNA results in a perturbation of the DNA helix (60). Perturbation of DNA structure within the promoter region would create a kinetic or energetic barrier for the conformation change that accompanies RNAP–promoter complex formation during transcription initiation. Apparently, the modes of DNA-looping of *LEE1* P1 by Ler and of *hdeAB*p by H-NS are distinct. However, in both cases, RNAP remains bound to the promoters as an open promoter complex (RP_O) (Figs. 7–9), which should most readily transform into productive initiation complex (1). It is possible that the DNA loop commonly forms an energetic barrier to the RNAP–promoter complex, resulting in failure to form the first phosphodiester bond. Alternatively, the open complexes, formed in the presence of Ler, may be too stable to process into the elongation mode (61).

Autoregulation of *LEE1* P1 has been proposed as a critical factor for maintaining a balance between two apparently opposed processes (i.e., maximizing bacterial colonization via increases in the expression of virulence genes and minimizing the immune responses of the host by limiting the expression of LEE-encoded genes) (16). In this study, we demonstrated that Ler regulates its own expression through DNA looping. This function of Ler must be separate from its function as a transcription antisilencer, achieved by displacing H-NS. Autorepression is frequently observed with transcription activators belonging to the AraC-XylS family, which often bind to multiple DNA sites (62). It is generally presumed that the autorepression occurs by a steric-exclusion mechanism, whereby the activator binds to a site near or overlapping its own promoter, resulting in transcription repression (5). In the case of *crp* autoregulation, the cAMP–CRP complex stimulates binding of a RNAP to a partially overlapping divergent promoter and this binding of RNAP interferes the occupation of RNAP at the *crp* promoter (63). The *fnr* is autoregulated by FNR binding to −7 to +7, which blocks RNAP from binding to its own promoter (64). Autorepression by DNA-looping has been reported with $araP_C$ by AraC, which regulates the expression of the nine genes arranged in five operons required for the uptake and catabolism of the sugar L-arabinose (65). Although

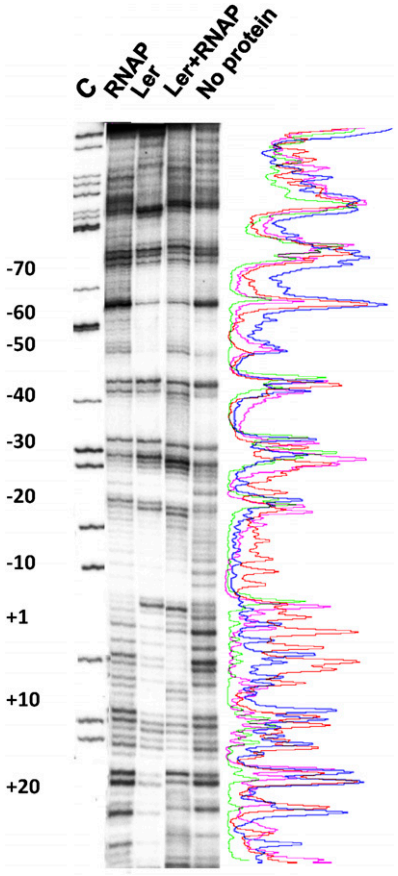

Fig. 8. DNase I protection assay with RNAP in the presence or absence of Ler on the *LEE1* P1 noncoding strand as determined by primer extension. [γ-32P] ATP-labeled PCR products (−134 to +87) were treated with DNase I in the presence of 20 nM RNAP or 60 nM Ler or Ler plus RNAP, or no protein as indicated above each lane. Sequencing marker lane is indicated. The density plot on the right shows the pattern in the presence of no protein (red), Ler (green), RNAP (blue), and Ler plus RNAP (magenta).

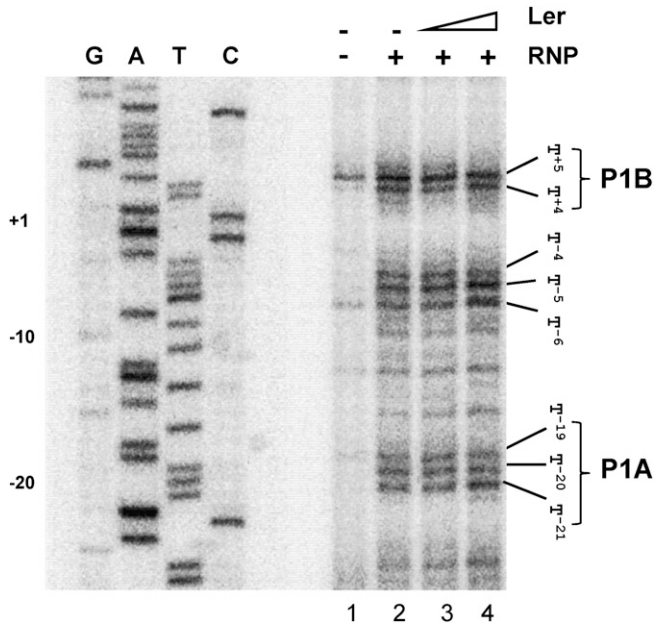

Fig. 9. Ler blocks the step subsequent to RP_O formation on *LEE1* P1. *LEE1* P1 DNA (−193 to +87) was end-labeled with [γ-32P] ATP and reacted with 20 nM RNAP in the absence (lane 2) or presence of 30 nM (lane 3) or 60 nM (lane 4) Ler. The DNA-protein complex was probed with $KMnO_4$. Lane 1 is a protein-free control. The DNA sequencing ladder is shown on the left. $KMnO_4$-hyperreactive bases of P1A and P1B in RP_O are indicated.

AraC binds to multiple sites flanking *araPc*, the promoter-downstream site (O_2) is essential for *araC* autoregulation (66). It has been suggested that AraC binds to different promoter-upstream high-affinity sites depending on the presence or absence of arabinose, which allows cooperative binding of AraC to the low-affinity $araO_2$, resulting in a DNA looping. The autorepression could be from the AraC or the DNA loop interfering with RNAP binding or AraC bound to $araO_2$ blocking the RNAP elongation (67, 68). In this regard, Ler is unique in that it regulates its own expression via DNA looping, in which the RNAP trapped in the DNA loop would act as a repressor.

Materials and Methods

Strains and Plasmids. The primers and plasmids used in this study are listed in Table S1 and Table 2, respectively. Cultures of *E. coli* or EPEC 2348/69 were grown in LB medium or DMEM, respectively, with appropriate antibiotics (ampicillin 100 μg/mL^{-1}, chloramphenicol 35 μg/mL^{-1}). *LEE1* P1 DNA was obtained by PCR amplification of EPEC strain E2348/69 chromosomal DNA (69). Single-copy *lac*Z fusion promoter constructs under the MG1655 strain background were obtained by cloning *LEE1* P1 fragments into pRS415 and subsequent homologous recombination with bacteriophage λRS45 (34). The lysogen constructs were transformed with pBAD33::*ler*.

Promoter Constructions. The promoter constructs used for in vitro assays were obtained by cloning *LEE1* P1 DNA fragments between the EcoR1 and Pst1 sites of the transcriptional vector pSA508 (31). *LEE1* P1 fragment from −193 to +152 was PCR amplified and inserted between EcoR1 and BamH1 sites of pRS415. Mutant promoters P1A$^+$/P1B$^-$ (A^{-2} to G) and P1A$^-$/P1B$^+$ (A^{-12} to G) were generated by cloning the synthetic DNA oligomers as in ref. 28. Promoters carrying 5-bp or 10-bp insertions or AATT to TTAA substitutions were generated by oligo-directed mutagenesis followed by overlapping PCR (Table S2). pBAD33::*ler* and *10lys-ler-6his* were constructed by inserting PCR amplified *ler* gene into the EcoR1 and Kpn1 sites of pBAD33 and pBAD24, respectively.

β-Galactosidase Assay. Briefly, λ lysogens under the MG1655 strain background were transformed with pBAD33::*ler* and the cultures were grown in LB medium supplemented with appropriate antibiotics in aeration condition and in presence or absence of 0.2% L-arabinose at 37 °C. Cultures were sampled at OD_{600} and β-Galactosidase assay was performed as described in ref. 69. Assays were performed in triplicates and the values (in Miller units) were expressed in mean ± SD.

In Vitro Transcription Assay. Transcription reactions were carried out as described in ref. 31. Briefly, 2 nM DNA template, 1 mM ATP, 0.1 mM GTP, 0.1 mM

Table 2. Plasmids and strains used in this study

Plasmids or strains	Description	Source
Plasmids		
pSA508*	Transcriptional vector, Amp ^{r}	(27)
pAB528	*LEE1* P1 (−193 to +87) in pSA508	Present work
pAB517	*LEE1* P1 (−193 to +67) in pSA508	Present work
pAB518	*LEE1* P1 (−193 to +47) in pSA508	Present work
pAB524	*LEE1* P1 (−82 to +87) in pSA508	Present work
pHJ75	*LEE1* P1 (−143 to +87) in pSA508	Present work
pAB529	*LEE1* P1 (−102 to +87) in pSA508	Present work
pHJ76	*LEE1* P1 (−93 to +87) in pSA508	Present work
pAB530	∇5bp at −63 in pAB528	Present work
pAB531	∇10bp at −63 in pAB528	Present work
pHJ77	*LEE1* P1 (-193 to +87) A^{-2} substituted by G in pSA508	(24)
pHJ78	*LEE1* P1 (-193 to +87) A^{-12} substituted by G in pSA508	(24)
pAB543	*LEE1* P1 (−193 to +152) in pRS415	Present work
pAB547	AATT substituted by TTAA at −112 in pAB543	Present work
pAB548	AATT substituted by TTAA at +62 in pAB543	Present work
pBAD33	*araC*-P_{BAD} promoter low copy vector system, Cm ^{r}	(32)
pBAD33::*ler*	*Ler* ORF in pBAD33	Present work
Strains		
MG1655	*E. coli* wild-type	Waterborne Virus Bank (Seoul, Korea)
2348/69	Wild-type EPEC O127:H6	(63)
CH1018	MG1655, [*argF-lac*]$_\Delta$	(73)
AB5027	CH1018, Φ*Lee1* P1 (-193 ~ +152)::*lacZYA*	Present work
AB5033	CH1018, Φ*Lee1* P1 (−193 ~ +152, AATT substituted by TTAA at -112)::*lacZYA*	Present work
AB5034	CH1018, Φ*Lee1* P1 (−193 ~ +152, AATT substituted by TTAA at +62)::*lacZYA*	Present work

CTP, 0.01 mM UTP, and 10–20 μCi of [α-^{32}P] UTP were preincubated in 20 mM Tris·acetate, pH 7.8, containing 10 mM magnesium acetate, 200 mM potassium glutamate at 37 °C for 10 min. Different concentrations of Ler were added and incubated further for 5 min. Transcription was initiated by the addition of 20 nM RNA polymerase in a total volume of 10 μL and terminated after 10 min at 37 °C by the addition of an equal volume of RNA loading buffer consisting of 80% (vol/vol) deionized formamide, 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA), 0.025% bromophenol blue, and 0.025% xylene cyanole. The mixture was separated by electrophoresis in an 8 M urea/8% (wt/vol) polyacrylamide sequencing gel (40 cm × 0.4 mm). RNA polymerase holoenzyme from strain BL21 was purchased from Epicentre. The RNA transcripts were quantified by determining cpm with a β-scanner (FLA3000, Fuji Instrument).

DNase I Footprint Analysis. The experiments were performed as described in ref. 70. The reaction conditions (10 μL total volume) were same as in in vitro transcription assay except that nucleotides were omitted. Next, 2–3 nM [γ-^{32}P]ATP labeled PCR DNA was preincubated along with buffer containing 20 mM Tris·acetate, pH 7.8, containing 10 mM magnesium acetate, 200 mM potassium glutamate, and BSA (200 μg/μL) at 37 °C for 10 min. DNase I (1 ng/μL) was then added and incubated for 20 s. The reaction was stopped by adding stop buffer containing 0.5 M sodium acetate, pH 5.2, 50 mM EDTA and tRNA (100 μg/μL). Modified bases were analyzed by primer extension analysis (see main text). The primers were annealed to upstream (−193-CGGAATTCCAGCTTGGTTTTTATTCTG; −93-CGGAATTCTAACGAGATGGTTTTCTTCT; −134-CGCTTAACTAAATGGAAATGC) and downstream (+87-CGGGATCCGAGATAACGTTTAT CTATC; +47-GGTT CTGCAGCATCAAACAACCACCTTA). The band intensities were quantified by determining cpm with a β-scanner (FLA3000).

Primer Extension Analysis. Primer extension analysis used the alkaline denaturation procedure described by ref. 50. Sequencing analysis was done using AccuPower and Top DNA Sequencing Kit, Bioneer.

$KMnO_4$ Assay. *LEE1* P1 DNA (−193 to +87) was amplified by PCR with the 27-mer primers 5′-CGGAATTCCAGCTTGGTTTTTATTCTG-3′, 5′-CGGGATCCGAGATAACGTTTATCTATC-3′ and labeled using [γ-^{32}P]ATP. $KMnO_4$ reactions were carried out as described in ref. 50. The reaction conditions were the same as for the in vitro transcription reactions except that nucleotides were omitted. Bases modified by $KMnO_4$ were analyzed by sequencing.

Gel Mobility-Shift Assay. Gel mobility-shift assays were carried out as described in ref. 71. Assays were performed using various PCR fragments of the *Ler* promoter that were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega). Reaction mixtures contained 2 nM end-labeled *LEE1* P1 DNA fragments in transcription buffer and different concentrations of Ler or RNAP. The mixture was incubated for 10 min at 37 °C and then separated in a 5% (vol/vol) native polyacrylamide gel (50:1) at 100 V for 1.5 h.

Western Analysis. DNA–protein complexes were transferred from the native polyacrylamide gel to a nitrocellulose membrane and probed with monoclonal antibody against the α-subunit of RNAP (Neoclone) and anti-Ler antibody prepared from mouse serum. Secondary antibody conjugated to alkaline phosphatase was used for visualization and was detected using BCIP/NBT (Sigma-Aldrich).

Expression and Purification of Ler. Wild-type *E. coli* MG1655 cells expressing the plasmid were grown in LB medium in the presence of 0.2% arabinose to induce *ler*. The bacterial cultures were centrifuged at 26,817 × *g* for 30 min and the pellet was resuspended in sample buffer containing 20 mM pH 7.5 Tris and 500 mM NaCl. Cells were lysed using a FRENCH pressure cell press (Thermo Electron) and the lysate was passed through His Akta Prime Plus (Amersham Biosciences). Proteins were eluted with 20 mM Tris, pH 7.5, containing 500 mM NaCl and 500 mM imidazole. Fractions containing Ler were identified by electrophoresis of each fraction followed by Western analysis with anti-Ler antibody. Ler-containing fractions were concentrated using a 10,000 molecular weight cut-off CENTRICON filter (Millipore). Protein concentration was estimated by the Bradford assay (Sigma-Aldrich).

AFM Analysis. The biotinylated DNA fragment containing *LEE1* P1 was prepared by PCR amplification using 5′-biotin-derivatized primers of vector sequence as described in ref. 72. The DNA product was mixed with 10 ng streptavidin and incubated in transcription buffer at 37 °C for 30 min. The DNA–protein complex was prepared by incubating 3 ng Ler and 2 nM biotinylated DNA fragment coupled to streptavidin in buffer containing 20 mM Tris·acetate, pH 7.8, 10 mM magnesium acetate, and 200 mM potassium glutamate at 37 °C for 10 min in a total volume of 10 μL. The whole reaction mixtures were deposited onto freshly cleaved mica and incubated at room temperature for 15 min. The mica disk was rinsed with distilled water and dried under nitrogen gas. AFM images of DNA-protein complexes were obtained under air with a NanoscopeIIIa or IV (Digital Instruments) in tapping mode with the cantilever (OMCL-AC160TS-W2, Olympus). The cantilever used was 129 μm in length with spring constant of 33–62 N/m. The microscope was equipped with a type E scanner. Images (512 × 512 pixels) were collected with a scan size of 2 mm at a scan rate of one scan line per second. Images were processed for analysis with Femtoscan software (Advanced Technologies Center).

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