Mutational Analysis of the Locus of Enterocyte Effacement-Encoded Regulator (Ler) of Enteropathogenic *Escherichia coli*[▽][†]

Gal Yerushalmi, Chen Nadler, Tatiana Berdichevski, and Ilan Rosenshine*

*Department of Molecular Genetics and Biotechnology, The Hebrew University, Faculty of Medicine, POB 12272,
Jerusalem 91120, Israel*

The locus of enterocyte effacement (LEE) pathogenicity island of enterohemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC, respectively) comprises a cluster of operons encoding a type III secretion system and related proteins, all of which are essential for bacterial colonization of the host intestines. The *LEE1* operon encodes Ler, which positively regulates many EPEC and EHEC virulence genes located in the LEE region and elsewhere in the chromosome. In addition, Ler is a specific autorepressor of *LEE1* transcription. To better understand the function of Ler, we screened for Ler mutants defective in autorepression. We isolated 18 different point mutations in Ler, rendering it defective in autorepression and in DNA binding. Among these mutants were those defective in positive regulation as well as in autorepression, dominant-negative mutants, and a mutant deficient in oligomerization. Importantly, a group of Ler autorepression mutants complemented an EPEC *ler* deletion mutant for transcription activation in a dosage-dependent manner, suggesting that Ler and possibly other autorepressors have an intrinsic compensatory mechanism that enables them to sustain mutations. In addition, the phenotypes of the different mutants identified by the screen define a novel domain in Ler that is required for oligomerization.

Enterohemorrhagic *Escherichia coli* and enteropathogenic *E. coli* (EPEC) represent a major global health problem, causing hemorrhagic colitis and hemolytic-uremic syndrome (enterohemorrhagic *E. coli*) and watery diarrhea of infants (EPEC). Their virulence is mediated by the concerted activity of an array of virulence factors. A major player among these factors is a type III protein secretion system (TTSS), through which these pathogens inject (translocate) toxic proteins (effectors) into host cells, thereby causing "attaching and effacing" intestinal lesions (22). The attaching-and-effacing histopathology is characterized by a localized destruction of the brush border microvilli and an assembly of highly organized pedestal-like actin structures in the epithelial cells beneath the intimately attached bacteria (11). The genes coding for the TTSS components, as well as for several of the effectors translocated by the TTSS, are clustered in a 35-kb chromosomal region, termed the locus of enterocyte effacement (LEE) (18, 19). In the LEE, genes are organized into five major operons (designated *LEE1* to *LEE5*) and several smaller transcriptional units (reviewed in reference 16). Ler, encoded by the first gene in the *LEE1* operon, positively regulates the expression of most of the *LEE* genes. Transcription activation by Ler is achieved by alleviating abundant DNA binding protein H-NS-mediated repression of the *LEE* promoters (6, 31). Ler activates and represses additional virulence genes located outside the LEE (1, 9, 17).

In addition to its role in activating the transcription of the *LEE* genes, Ler also functions as an autorepressor, by repressing the transcription of the *LEE1* promoter (P_{LEE1}) (3). Autorepression is a common motif in bacterial regulatory networks, shared by over 40% of the transcription factors in *E. coli* K12 (29). In addition to autorepression, Ler expression (P_{LEE1} activity) is tightly controlled by a plethora of regulators, including IHF, Fis, PerC, BipA, GrlA, GrlR, GadX and quorum sensing (7, 12–15, 21, 24, 25, 27), attesting to its central role in virulence gene regulation.

In this work, we subjected *ler* to random mutagenesis and screened for mutants defective in autorepression. We isolated and characterized 18 different point mutations that interfere with autorepression. All of the mutations caused a significant decrease in DNA binding. Most of the mutants were defective in transcription activation of the *LEE* genes, and some exhibited a dominant-negative effect over wild-type Ler. One of the mutants was defective in oligomerization, harboring a mutation in a region never predicted to be involved in oligomerization. Four mutants were able to successfully complement a *ler*-deleted strain, activating *LEE* transcription in a dosage-dependent manner. The latter results suggest that Ler harbors an intrinsic compensatory mechanism that enables it to sustain mutations, and this mechanism may be general to autorepressed genetic systems.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria-Bertani (LB) broth at 30°C and diluted 1:50 into Dulbecco's modified Eagle's medium (DMEM) or modified Casamino-DMEM (3). When needed, LB was supplemented with ampicillin (Amp) at 100 μg/ml, kanamycin (Kan) at 40 μg/ml, or 20 mM $(NH_4)_2SO_4$ (to repress *LEE1* expression).

Plasmid construction. The primers used for plasmid construction are listed in Table S1 in the supplemental material. To construct pGY1, a DNA fragment containing P_{LEE1} and *ler* (starting from position −159 compared to the transcriptional start site determined in reference 21) was amplified using primers

TABLE 1. List of strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
E. coli strains		
E2348/69	EPEC wild type	J. Kaper
TU1403	E2348/69 carrying a chromosomal *ler-His₆*	3
DF2	E2348/69 *ler::kan*	12
A70	CC102 (*ara* Δ(*lac-proB*)$_{XIII}$ *thi* F′-*lacI378 lacZ461 proA⁺B⁺*) *mutS215*::Tn*10*	S. Altuvia
W3110	F⁻ λ⁻ *thyA36 deoC2* IN(*rrnD-rrnE*)*1*	A. Oppenheim
Plasmids		
pIR1	pKK177-3 derivative containing *gfp*mut3	12
pGY1	pIR1 containing a transcriptional fusion of P$_{LEE1}$, *ler*, and *gfp*mut3 (P$_{LEE1}$-*ler-gfp*mut3)	This study
pGY2180	pGY1 carrying P$_{LEE1}$-*ler(L16P)*-*gfp*mut3	This study
pGY2206	pGY1 carrying P$_{LEE1}$-*ler(L23R)*-*gfp*mut3	This study
pGY2189	pGY1 carrying P$_{LEE1}$-*ler(L56P)*-*gfp*mut3	This study
pGY2190	pGY1 carrying P$_{LEE1}$-*ler(Y77H)*-*gfp*mut3	This study
pGY2191	pGY1 carrying P$_{LEE1}$-*ler(Y77C)*-*gfp*mut3	This study
pGY2192	pGY1 carrying P$_{LEE1}$-*ler(G82V)*-*gfp*mut3	This study
pGY2207	pGY1 carrying P$_{LEE1}$-*ler(G82E)*-*gfp*mut3	This study
pGY2208	pGY1 carrying P$_{LEE1}$-*ler(W85R)*-*gfp*mut3	This study
pGY2209	pGY1 carrying P$_{LEE1}$-*ler(G87S)*-*gfp*mut3	This study
pGY2210	pGY1 carrying P$_{LEE1}$-*lerG87D)*-*gfp*mut3	This study
pGY2211	pGY1 carrying P$_{LEE1}$-*ler(G89D)*-*gfp*mut3	This study
pGY2193	pGY1 carrying P$_{LEE1}$-*ler(P92S)*-*gfp*mut3	This study
pGY2194	pGY1 carrying P$_{LEE1}$-*ler(W94R)*-*gfp*mut3	This study
pGY2195	pGY1 carrying P$_{LEE1}$-*ler(L95P)*-*gfp*mut3	This study
pGY2212	pGY1 carrying P$_{LEE1}$-*ler(A98V)*-*gfp*mut3	This study
pGY2196	pGY1 carrying P$_{LEE1}$-*ler(G102R)*-*gfp*mut3	This study
pGY2213	pGY1 carrying P$_{LEE1}$-*ler(G102E)*-*gfp*mut3	This study
pGY2214	pGY1 carrying P$_{LEE1}$-*ler(L109P)*-*gfp*mut3	This study
pGY2501	pGY1 carrying P$_{LEE1}$-*ler(E4Stop)*-*gfp*mut3	This study
pGY3682	pGY1 carrying P$_{LEE1}$-*ler(N50Stop)*-*gfp*mut3	This study
pGY3683	pGY1 carrying P$_{LEE1}$-*ler(G65Stop)*-*gfp*mut3	This study
pSA10	pKK177-3 derivative containing *lacI*^{q}	23
pGY2746	pSA10 carrying *ler*	This study
pGY2742	pSA10 carrying *ler(L23R)*	This study
pGY2743	pSA10 carrying *ler(G82E)*	This study
pGY2744	pSA10 carrying *ler(A98V)*	This study
pGY2745	pSA10 carrying *ler(G102R)*	This study
pGY3576	pSA10 carrying *ler(G89D)*	This study
pQE-70	Expression of C-terminally His₆-tagged proteins	Qiagen
pDF5	pACYC184 containing *lacI*^{q} and *ler* expressed from the *tac* promoter	12
pDF11	pQE-70-based, expressing Ler-His₆ from the P$_{LEE1}$promoter	This study

specified in Table S1 in the supplemental material; this amplified fragment was digested by XbaI and BamHI and cloned into pIR1 digested by the same enzymes. pGY1 derivatives carrying *ler* mutants and the plasmids carrying the truncated *ler* variants N50Stop and G65Stop (pGY3682 and pGY3683, respectively) were generated using the QuikChange site-directed mutagenesis kit (Stratagene) with the primers specified in Table S1 in the supplemental material. For pGY3682 and pGY3683, an additional step of PCR and self-ligation to delete the *ler* C-terminal coding region after the newly inserted stop codon followed. To construct a plasmid expressing Ler under the transcriptional regulation of P*tac* (pGY2746), a DNA fragment containing *ler* was amplified from pGY1, digested by EcoRI and SalI, and cloned into pSA10, which was digested by the same enzymes. In order to generate plasmids expressing LerL23R, LerG82E, LerA98V, LerG102R, and LerG89D under the transcriptional regulation of P*tac* (pGY2742, pGY2743, pGY2744, pGY2745, and pGY3576, respectively), similar fragments were amplified from the relevant pGY1 derivatives (pGY2206, pGY2207, pGY2212, pGY2196, and pGY2211, respectively) and cloned in a similar manner. To construct pDF11, which contains *ler* fused in-frame to a His₆ tag at the C terminus (expressed from its native promoter and ribosome binding site), a DNA fragment containing P$_{LEE1}$ and *ler* was amplified using the primers specified in Table S1 in the supplemental material; this amplified fragment was digested by XhoI and BamHI and cloned into pQE-70, which was digested by the same enzymes.

Fluorescence microscopy. To test for the formation of actin pedestals and *gfp* expression from P$_{LEE1}$, HeLa cells were seeded and grown overnight on glass coverslips in 24-well plates. The cells, in 1 ml of DMEM, were infected with 10 μl of overnight LB standing bacterial cultures. Infection was stopped after 3.5 h by fixing the infected cells in phosphate-buffered saline (PBS) containing 2% paraformaldehyde for 30 min. The fixed cells were washed with PBS, permeabilized for 5 min with 0.1% Triton X-100 in PBS, and washed as before. The actin filaments were stained by overlaying the coverslips with 20 μl (0.1 μg/ml in Tris-buffered saline) of phalloidin-rhodamine (Sigma). After 1 h of incubation, the samples were washed, mounted on glass slides, and analyzed.

Measurement of gene expression. Strains containing the *gfp*-expressing plasmids pIR1 and pGY1 and the pGY1 derivatives carrying *ler* mutants were grown overnight at 30°C in LB supplemented with 20 mM (NH₄)₂SO₄. The cultures were washed and diluted 1:50 in Casamino-DMEM and then grown in 96-well plates in a microplate reader at 37°C (SPECTRAFluor Plus; Tecan). The fluorescence intensity (filter set at a 485-nm excitation wavelength and a 535-nm emission wavelength) and optical density at 600 nm (OD$_{600}$) were read and collected using Magellan version 5.0 software (Tecan). To determine the protein levels, strains were grown in DMEM at 37°C up to an OD$_{600}$ of 0.3 to 0.4. When indicated, different concentrations of IPTG (isopropyl-β-D-thiogalactopyranoside) were added. The cultures were centrifuged and the bacteria lysed by boiling in sodium dodecyl sulfate (SDS) loading buffer. Protein concentrations in the samples were adjusted, and then the samples were subjected to immunoblot analysis. Blots were developed with polyclonal anti-Tir, anti-EspB, or anti-Ler antibodies.

Genetic screen to identify mutants in autorepression. The pGY1 plasmid was introduced into an *E. coli mutS* strain (A70). Plasmids were prepared from an overnight culture of the *mutS* strain, introduced into EPEC *ler::kan*, and plated

to give ~500 individual colonies per plate, and colonies that exhibited high green fluorescence were picked (108 colonies). Those that did not express Ler or expressed truncated Ler proteins were identified by immunoblot analysis with an anti-Ler antibody and omitted, leaving 45 mutant strains. The plasmids from these strains were prepared, and the *LEE1* regulatory region and the *ler* coding region were sequenced. Each of these plasmids was found to contain a single mutation, and several of them carried the same mutation. The screen rendered 17 distinct mutations; each was regenerated on a plasmid that had not been previously introduced into the *mutS* strain. For this, a QuikChange site-directed mutagenesis kit (Stratagene) was used with the primers specified in Table S1 in the supplemental material. The L23R mutation added to this group was previously termed LerL29R (3, 27), and we renamed it LerL23R, given the identification of the second methionine of Ler as its translation start site.

Analysis of the Ler translation start site. The pDF11 plasmid containing the *ler* native promoter and regulatory region and *ler* fused to a His$_6$ tag at the region encoding the C terminus was introduced into wild-type EPEC. Bacteria were grown in DMEM at 37°C to an OD$_{600}$ of 0.4, and the Ler-His$_6$ protein was purified by metal affinity chromatography using Talon beads (Clontech), followed by SDS-polyacrylamide gel electrophoresis (PAGE) gel and blotting onto a polyvinylidene difluoride membrane, and then subjected to N-terminal amino acid sequencing.

Protein-DNA binding assay. A modified enzyme-linked DNA/protein interaction assay (ELDIA) (2) was employed. Briefly, three types of 5′-biotinylated PCR fragments were used: the *LEE1* regulatory region (prepared using the primers BioLEE1reg2-F and 5R), the *LEE2* regulatory region (prepared using the primers BioLEE2reg2-F and 31R; see Table S1 in the supplemental material) and the *etk* (*yccC*) coding region (a negative control; prepared using the primers BioYccC-F and YccC-R [see Table S1 in the supplemental material]). A total of 100 μl of 500 pmol/ml of each of the fragments in PT buffer (PBS supplemented with 0.05% Tween 20 and 1 mM EDTA) were bound separately to streptavidin-coated 96-well plates (Sigma) by incubating for 1 h. Unbound DNA was removed by washing with PT. Whole-cell extracts were prepared by sonicating (in 50 mM Tris-HCl [pH 7.4], 70 mM KCl, 5 mM EDTA, 1 mM dithiothreitol) EPEC *ler*::*kan* bacteria harboring the plasmids pIR1 and pGY1 or pGY1 derivatives, and glycerol and Tween 20 were added to final concentrations of 6% and 0.05%, respectively. The extracts were cleared by centrifugation. A total of 100 μl of the cell extracts were added to the fragment-bound wells for 1 h and then unbound proteins were removed by washes with PT buffer. Polyclonal anti-Ler antibodies (100 μl) were added for 1 h, the wells were washed with PT, and then 100 μl of alkaline phosphatase-conjugated secondary anti-rabbit immunoglobulin G antibodies (Sigma) were added for 1 h. The wells were washed with PT and later with a 1 M Tris (pH 9.5) and 0.5 mM MgCl$_2$ buffer. Finally, 100 μl of this buffer containing 10 mg/ml *p*-nitrophenyl phosphate were added to each well, and the plate was immediately inserted into a microplate reader (SPECTRAFluor Plus; Tecan). The OD$_{405}$ was read every minute, and data were collected by Magellan version 5.0 software (Tecan). The slopes of the OD$_{405}$ values at the first 25 min (before the reaction was saturated) were chosen to represent the level of binding.

Ler oligomerization assay. The oligomerization of wild-type Ler was tested using the pDF5 plasmid, from which two forms of Ler are expressed: the native Ler and Ler fused at its N terminus to a 16-amino-acid peptide containing His$_6$ (6His-10AA-Ler; the exact sequence of the tag is MRGSHHHHHHPRRLFI-Ler). *E. coli* W3110 containing pDF5 was grown under expression conditions, proteins were extracted, and 6His-10AA-Ler was purified by metal affinity chromatography. The purified protein was analyzed by SDS-PAGE and Coomassie staining or by transfer into nitrocellulose, which was stained with Coomassie or used for immunoblot analysis using anti-His antibodies.

The oligomerization of the Ler mutants was assessed by introducing the pGY1-derived plasmids into TU1403, a strain containing a chromosomal fusion of *ler* to His$_6$ at the region encoding the C terminus. Bacteria were grown in DMEM at 37°C to an OD$_{600}$ of 0.4, sonicated, and subjected to metal affinity chromatography using Talon beads (Clontech). Ler-His$_6$ expressed from the chromosome was expected to bind the beads, along with the Ler mutants that were capable of oligomerization. The beads were treated with 8 M urea solution. Under these conditions Ler-His$_6$ remained bound to the beads, but protein-protein interactions were disrupted, eluting the proteins bound to Ler-His$_6$. The residual Ler-His$_6$ in the eluted samples were reabsorbed and completely removed from the preparations with fresh Talon beads in 8 M urea solution. The eluted material was subjected to immunoblot analysis with anti-Ler antibodies.

RESULTS

Determination of the Ler N terminus. Before utilizing the mutagenesis approach for analyzing Ler activity, we first wished to determine its native N terminus, as it was ambiguous as documented in the databases and never determined experimentally. The putative N-terminal sequence of EPEC Ler according to GenBank (AF022236) is **M**RRLFI**M**N**M**ETNSH TTSPYIQ, containing methionine residues at positions 1, 7, and 9 (which are in boldface type). To experimentally determine the native N terminus, we constructed a plasmid (pDF11) expressing *ler* fused to a His$_6$ tag at its C terminus, under the regulation of its native *LEE1* promoter (P$_{LEE1}$-*ler*-His$_6$). The Ler-His$_6$ protein was purified by metal affinity chromatography. N-terminal amino acid sequencing analysis of the purified tagged Ler revealed only one protein, whose N-terminal sequence was MNMETNSHTTSPYIQ. This redefined the second methionine (M7) as M1, the translation start site of Ler (see Fig. S1 in the supplemental material). The designations of all the mutations in this paper are according to this newly defined translation start site.

Isolation of Ler mutants defective in autorepression. To establish a method that will allow rapid screening of a large number of Ler mutants defective in transcription regulation, we constructed the medium-copy-number plasmid pGY1. This plasmid contains a bicistronic operon of *ler* and *gfp* expressed from the native *LEE1* promoter (P$_{LEE1}$-*ler*-*gfp*) (Fig. 1A). Mutagenesis was accomplished by introducing pGY1 into a mismatch-repair-deficient *E. coli* mutant (*mutS*, A70) and growing the bacteria overnight to allow the accumulation of plasmids with random spontaneous point mutations. The plasmids were then recovered from the *mutS* strain and introduced into EPEC *ler*::*kan*, and the transformants were plated to give ~500 individual colonies per plate. Colonies containing plasmids deficient in autorepression were expected to overexpress *gfp*, exhibiting increased fluorescence, as P$_{LEE1}$ was no longer subjected to Ler autorepression (Fig. 1A). About 100 colonies exhibiting strong fluorescence were isolated, representing 0.1 to ~1.0% of the population. The corresponding cultures were analyzed for Ler production by immunoblot analysis with an anti-Ler antibody, and their corresponding plasmids were recovered and sequenced. In all the selected colonies, mutations were mapped to the *ler* coding region and none were found in the *LEE1* regulatory region. Plasmids expressing a truncated Ler or Ler with more than one mutation and redundant mutations were eliminated from further analysis. The remaining 17 mutations were located throughout the *ler* coding region (Fig. 1B). The 17 *ler* mutants, each containing a single mutation, were further analyzed and were shown to be strongly defective in P$_{LEE1}$ autorepression by immunoblot analysis of their Ler production (data not shown). To reconfirm these results, and to make sure that the effect was not due to other mutations in regions that were not sequenced, we regenerated each of the 17 mutations in vitro by site-directed mutagenesis on the pGY1 plasmid. We also added to this collection the previously described LerL23R mutant (3). These plasmids, designated the pGY1 derivatives (Table 1), were introduced into EPEC *ler*::*kan* and showed the derepression of P$_{LEE1}$ activity similarly to that seen in the original isolates (Fig. 1C and D and data not shown; see also Fig. S2 in the supplemental
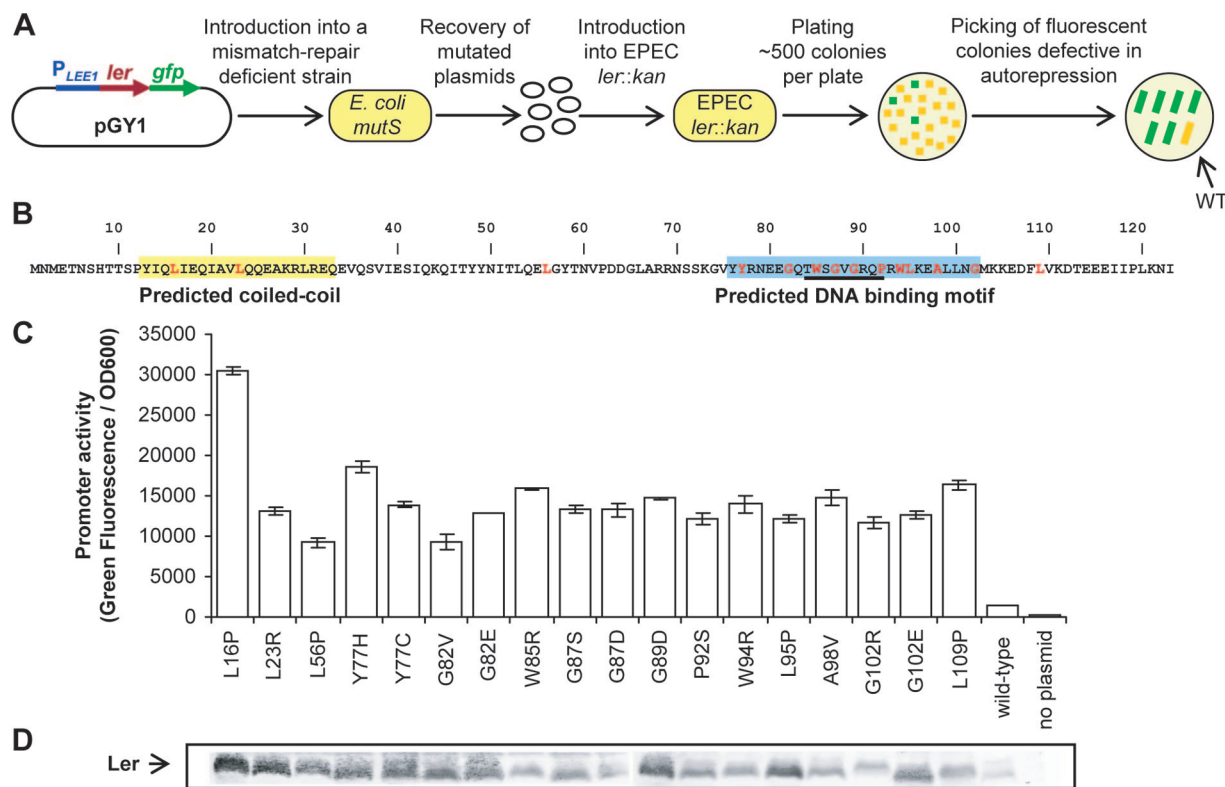
FIG. 1. Identification and initial characterization of 18 *ler* mutants in autorepression. (A) The pGY1 plasmid, harboring a *ler* transcriptional fusion to *gfp* under the control of the native *ler* promoter (P*LEE1*), was introduced into a *mutS* mismatch-repair-deficient strain. The mutated plasmids were recovered and introduced into an EPEC *ler::kan* strain, and mutants in autorepression were identified as fluorescent colonies. Eighteen mutants were further analyzed. These mutations (B, marked in red) are scattered throughout the Ler amino acid sequence. The predicted coiled-coil and DNA binding motifs are in yellow and light blue, respectively. The core DNA binding domain is underlined. (C) The 18 mutants were reconstructed in pGY1 and the expression of green fluorescent protein, representing the transcription from P*LEE1*, was measured at late exponential-growth phase. (D) Protein levels of the Ler mutants were analyzed using immunoblot analysis with an anti-Ler antibody.

material). In some cases, the derepression of *gfp* expression appears stronger than that of *ler* (Fig. 1C and D), which might reflect reduced reactivity of the mutated Ler with the anti-Ler antibody or reduced stability of the mutated Ler. To conclude, we obtained 18 distinct point mutations in *ler*, each causing a reduction in autorepression.

All the Ler autorepression-deficient mutants show low affinity to the *LEE1* and *LEE2* regulatory regions. We next tested the capacity of the different Ler mutants to bind DNA, using a modified ELDIA (2). Whole bacterial extracts, which were generated from EPEC *ler::kan* harboring pGY1-derived plasmids expressing the different Ler mutants, were added to wells previously coated with different DNA sequences (the *LEE1* and *LEE2* regulatory regions and the *etk* coding region, which served as a negative control). The unbound proteins were washed, and the amount of bound Ler was determined using anti-Ler antibodies and secondary alkaline-phosphatase-conjugated antibodies and normalized according to the expression level from P*LEE1* by the different mutants (Table 2). Whole bacterial extracts are used in this assay for better reflection of the Ler binding capacity in vivo. All the Ler mutants exhibited a reduced affinity to the DNA binding sites in the *LEE1* and the *LEE2* regulatory regions (Fig. 2). Since several of the mutations were located outside the predicted DNA binding domain (Fig. 1B), these results suggest that in addition

to this domain, other Ler domains are also required for DNA binding, possibly by promoting Ler oligomerization or by affecting the stability of the protein.

Ler forms oligomers. Ler oligomerization was predicted to be involved in DNA binding (27), but this prediction was never tested. To examine this assumption, we tested the binding of native Ler to His₆-Ler, both expressed from the pDF5 plasmid. Proteins were purified from *E. coli* W3110 containing pDF5 by metal affinity chromatography. The purified proteins were analyzed by SDS-PAGE and Coomassie staining to give two highly purified bands (Fig. 3A; see also Fig. S3 in the supplemental material). A comparison of the immunoblot analyses, using anti-His antibodies with Coomassie-stained nitrocellulose membrane, revealed that the larger protein was His₆-Ler (Fig. 3B). The smaller copurified protein was subjected to N-terminal amino acid sequencing, and the obtained sequence was MNMETNSHTT, identifying it as native Ler (see Fig. S1 in the supplemental material). These results suggest that Ler forms homooligomers. The Ler oligomers contained only His₆-Ler and Ler, as no other proteins were copurified with the complex (Fig. 3).

Identification of a mutant deficient in oligomerization. We next subjected all the Ler mutants to an oligomerization assay, in which we introduced the different pGY1-derived plasmids into an EPEC strain containing the *ler* gene fused to His₆ at the

TABLE 2. Summary of the characterization of Ler mutants deficient in autorepression

Mutant	No. of occurrences[a]	Transcription level[b]	Secretion of EspB	Pedestal formation	Dominant negative[c]	Group[d]
LerL16P	4	11	−	−	?	?
LerL23R		9	+	+	−	CID
LerL56P	2	8	−	−	−	N
LerY77H	3	9	−	−	−	N
LerY77C	2	10	−	−	−	N
LerG82V	1	10	−	−	+	DN
LerG82E	2	12	+	+	−	CID
LerW85R	1	6	−	−	+	DN
LerG87S	1	9	−	−	+	DN
LerG87D	1	7	−	−	+	DN
LerG89D	2	8	−	−	+	DN
LerP92S	1	8	−	−	+	DN
LerW94R	2	9	−	−	+	DN
LerL95P	1	9	−	−	+	DN
LerA98V	5	11	+	+	−	CID
LerG102R	1	7	+	+	−	CID
LerG102E	1	10	−	−	−	N
LerL109P	1	11	−	−	+	DN
Wild type		1	+	+	−	

[a] The number of identical mutants picked in the screen. The LerL23R mutant was not identified by the screen; it was described in reference 3.
[b] Fluorescence/OD$_{600}$ compared to that of the wild type at exponential phase.
[c] Expression of EspB and Tir (from the *LEE4* and *LEE5* promoters, respectively) when the plasmid harboring the Ler mutant was introduced into a wild-type EPEC strain.
[d] N, null; DN, dominant negative; CID, compensation by increased dosage (see Discussion).

region encoding the C terminus (Ler-His$_6$). The coprecipitation of the different Ler mutants with Ler-His$_6$ was tested by metal affinity chromatography and immunoblot analysis with anti-Ler antibodies as described in Materials and Methods. Interestingly, the mutants in the Ler coiled-coil domain (L16P and L23R) still bound to Ler-His$_6$ (Fig. 4A), implying that this domain does not function as the sole oligomerization domain

of Ler. A possible explanation for that can be that the N-terminal coiled-coil domain might be involved in other protein-protein interactions, possibly in higher-order Ler oligomerization or interactions of Ler with other proteins. An alternative possibility is that the coiled-coil domain retained some oligomerization capacity despite these mutations. Only one mutant (L56P) did not coprecipitate with Ler-His$_6$, identifying a do-
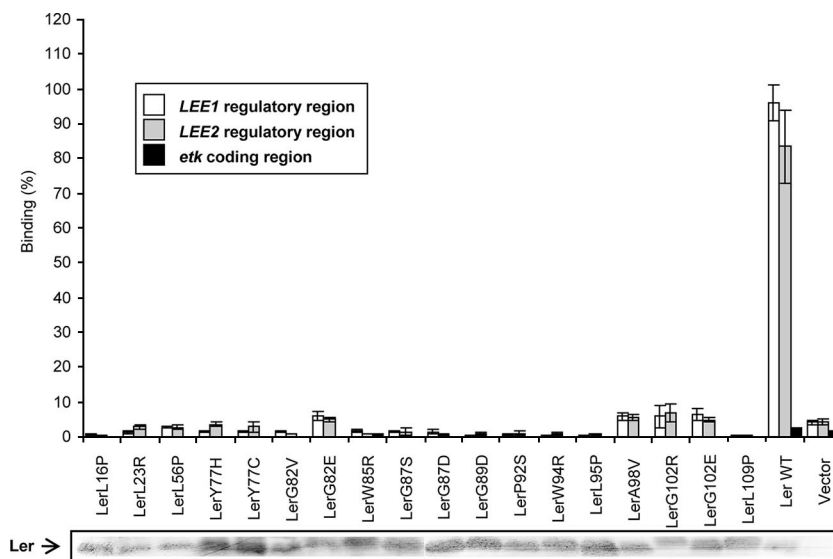

FIG. 2. DNA binding by the Ler autorepression-defective mutants. The binding of Ler mutants to the *LEE1* and *LEE2* regulatory regions and to the *yccC* (*etk*) coding region (used as a negative control) was tested using an ELDIA. Whole bacterial extracts, generated from EPEC *ler::kan* containing plasmids encoding the different Ler mutations (pGY1 and pGY1-derived plasmids) were used. As a positive control, we used pGY1 expressing native Ler (Ler WT), and as a negative control, we used a strain containing the vector, which is not expressing Ler (Vector). The mutations of the different Ler proteins are indicated below the columns. DNA binding is expressed as a percentage of the maximum binding. Protein levels in the extracts used for the experiments were analyzed by immunoblot analysis using the anti-Ler antibody, shown below the graph. Similar results were obtained in three independent experiments.
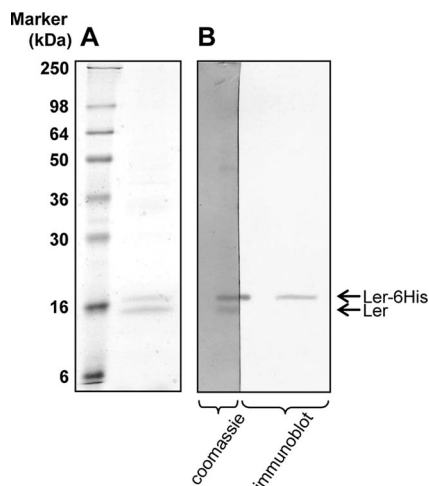
FIG. 3. Ler forms homooligomers. (A) The pDF5 plasmid, from which two forms of Ler are expressed (the native Ler and Ler fused at its N terminus to a 16-amino-acid peptide containing His$_6$ [MRGSH HHHHHPRRLFI]-Ler) was introduced into *E. coli* W3110. The expressed His$_6$-Ler was purified by metal affinity chromatography, and proteins retained on the column were eluted and analyzed by SDS-PAGE and Coomassie staining (right lane). The left lane contains size markers, and the different sizes are indicated. Two highly purified protein bands are located in the right lane near the 16-kDa marker. (B) Proteins from two lanes of a similar gel were transferred onto a nitrocellulose membrane, and the membrane was cut along one of the lanes. The left half of the membrane was developed by Coomassie staining and thus appears darker. The right half of the membrane was used for immunoblot analysis and developed with anti-His$_6$ antibody. Only the upper band reacted with the anti-His$_6$ antibody.
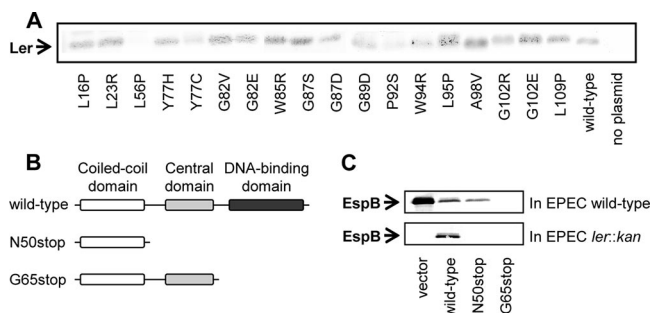

FIG. 4. Oligomerization of the Ler autorepression-defective mutants and truncation analysis. (A) Oligomerization of the Ler autorepression-defective mutants was assessed by introducing the plasmids encoding them into an EPEC strain chromosomally encoding Ler-His$_6$. Ler-His$_6$ and associated proteins were pulled down from cleared extracts with Talon beads, and proteins associated with Ler-His$_6$ were eluted with 8 M urea. Under these conditions, Ler-His$_6$ remained attached to the beads. The eluted samples were further treated with fresh Talon beads to remove any residual Ler-His$_6$ and were then analyzed by SDS-PAGE, followed by an immunoblot analysis with anti-Ler antibodies. pGY1 expressing native Ler (wild-type) was used as a positive control, and the strain expressing Ler-His$_6$ was used as a negative control. The different mutations are indicated below the corresponding lanes. (B) Schematic representation of the N50Stop and G65Stop truncated Ler variants. (C) EPEC wild-type (top panel) or EPEC *ler::kan* (bottom panel) containing the plasmids pIR1 (vector), pGY1 expressing wild-type *ler* (wild-type), and pGY1-derived plasmids expressing a truncated Ler containing only the first 50 amino acids [*ler(N50stop)*] or only the first 65 amino acids [*ler(G65stop)*]. To test the ability of the different plasmids to restore or block EspB secretion (the latter is via dominant negativity), the supernatant of bacterial cultures of these strains grown in LEE-inducing conditions was subjected to immunoblot analysis with anti-EspB antibody.

main around residue L56 as essential for oligomerization or as a residue essential for N-terminal oligomerization.

A domain around L56 mediates a dominant-negative effect on wild-type Ler. To further examine the role of the domain around L56 in oligomerization, we generated two truncated variants of Ler, in which N50 or G65 was changed into a stop codon (LerN50Stop and LerG65Stop, respectively). The LerN50Stop variant contained only the coiled-coil domain, and the LerG65Stop variant contained the coiled-coil domain and the central domain, including L56. Both LerN50Stop and LerG65Stop lack the C-terminal DNA binding domain (Fig. 4B), and in the case of the latter, we also experimentally confirmed its DNA binding deficiency (see Fig. S4 in the supplemental material). We hypothesized that if these truncated variants were able to oligomerize, they would have exerted a dominant-negative effect on wild-type Ler. To test this prediction, plasmids harboring each of the two truncated variants were introduced into wild-type EPEC and EPEC *ler::kan*, and the expression of the LEE-encoded genes (data not shown) and the secretion of EspB (Fig. 4C) were then examined. The latter served as a readout for TTSS gene expression and the assembly of a functional TTSS. As expected, neither of the truncated variants complemented the EPEC *ler::kan* strain to restore the expression of the TTSS genes and EspB secretion (Fig. 4C and data not shown). However, the LerG65Stop variant, but not the LerN50Stop variant, showed a dominant-negative effect on wild-type Ler (Fig. 4C). These results show that amino acids 50 to 65 have a role in the ability of LerG65Stop

to interfere with wild-type Ler function, probably through the formation of oligomers deficient in DNA binding. These results further support the notion that the central domain of Ler is required for oligomerization in vivo.

Complementation of EPEC *ler::kan* by four of the autorepression-deficient Ler mutants. The EPEC *ler::kan* mutant strain is defective in producing most of the *LEE*-encoded proteins and consequently cannot assemble a functional TTSS. Complementation of this mutant with a plasmid expressing wild-type *ler* restores *LEE* gene expression and the assembly of a functional TTSS (12, 21). We therefore tested the capacity of the different *ler* mutants (expressed from the pGY1-derived plasmids) to complement EPEC *ler::kan*. To assess the complementation and the assembly of a functional TTSS, we applied three assays: (i) we examined the production of the LEE-encoded proteins EspB and Tir (encoded by the *LEE4* and *LEE5* operons, respectively), (ii) we tested for TTSS-dependent EspB secretion to the growth medium, and (iii) we examined the bacterial capacity to induce the formation of actin pedestals in infected HeLa cells. Two categories of mutants were found in these experiments. The first included 14 mutants (the L16P, L54P, Y77H, Y77C, G82V, W85R, G87S, G87D, G89D, P92S, W94R, L95P, G102E, and L109P mutants), which were unable to complement EPEC *ler::kan* in the secretion of EspB and the induction of actin pedestal formation (Fig. 5A to C). Some of these mutants showed a residual ability to activate transcription, but this was not sufficient to restore EspB secretion and the induction of pedestal formation, either
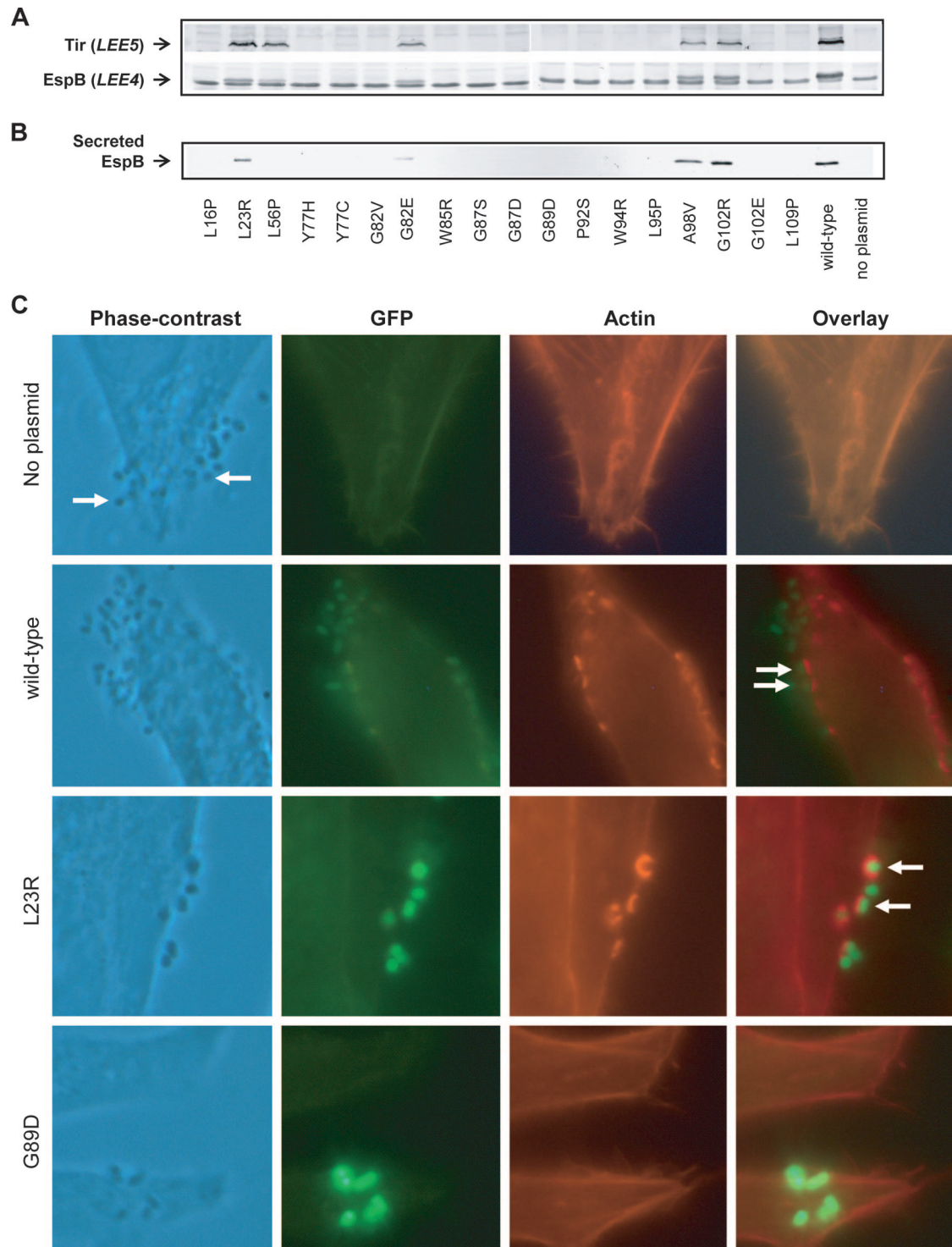
FIG. 5. Complementation experiments with the Ler autorepression defective mutants. Plasmids expressing each of the Ler mutants (pGY1 or a pGY1 derivative) were introduced into EPEC *ler::kan*. The capacity of the mutated Ler to restore the expression of Tir and EspB (expressed from the promoters P*LEE5* and P*LEE4*, respectively) (A), EspB secretion (B), and the formation of actin pedestals (C) was determined. (A) Expression of Tir and EspB was tested using immunoblot analysis with antibodies raised against these proteins. (B) To test for EspB secretion, the different strains were used to infect HeLa cells for 3 h. The growth medium was recovered, cleared, and analyzed by immunoblot analysis using anti-EspB antibodies. The *ler* mutations in the complementing plasmids are indicated below the lanes. The names of the LEE-encoded proteins are indicated on the left-hand side of the blots, and the specific operon carrying each gene is in parentheses. The EspB protein in the bacterial lysate (in panel A) is the upper band out of the seen doublet. The origin of the lower band that cross-reacted with the anti-EspB antibody is not known, and it was seen also in strains deleted of the *espB* gene (data not shown). (C) To test for the formation of actin pedestals, EPEC *ler::kan* containing different complementing plasmids was used to infect HeLa cells for 3 h. The infected cells were fixed and actin filaments were stained with rhodamine-phalloidin. A few representative examples are shown, including an uncomplemented *ler::kan* mutant (no plasmid), a *ler::kan* mutant complemented with pGY1 (wild-type), and mutants complemented with pGY1-*ler*(*L23R*) (L23R) or with pGY1-*ler*(*G89D*) (G89D). Phase-contrast images of the infected cells are shown in the first panel, GFP produced by the bacteria in the second panel (green), and actin in the third panel (red). The fourth panel shows overlays of the red and green images. Arrows indicate some actin pedestals.
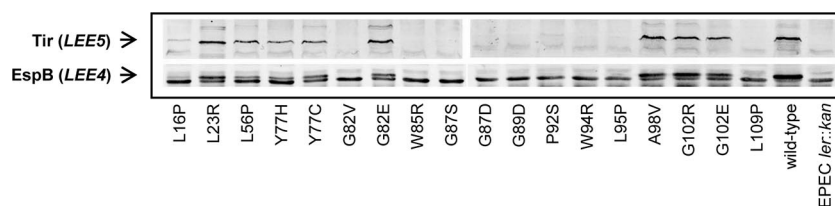
FIG. 6. Identification of dominant-negative Ler autorepression-defective mutants. pGY1 or a pGY1-derivative plasmid expressing each of the Ler mutants was introduced into wild-type EPEC. The expression of Tir and EspB (natively expressed from the Ler-activated promoters P$_{LEE5}$ and P$_{LEE4}$, respectively) was determined using immunoblot analysis with antibodies raised against these proteins. The *ler* mutations are indicated below the lanes. The names of the LEE-encoded proteins are indicated on the left-hand side of the blots and the specific operon carrying each gene is written in parentheses.

because the expression level of the TTSS proteins was not sufficient or because not all of the TTSS proteins were produced (e.g., Y77C and G102E; Fig. 5A). The second category included four mutants, LerL23R, -G82E, -A98V, and -G102R, which successfully complemented the EPEC *ler*::*kan* strain according to all of the aforementioned assays (Fig. 5A to C). These four mutations are located in different regions of the Ler protein (Fig. 1B).

Identification of dominant-negative Ler mutants. To further characterize the *ler* mutants, we tested the mutant alleles for a dominant-negative effect on wild-type Ler. The pGY1-derived plasmids were introduced into wild-type EPEC, and the production of the LEE proteins was tested as an indication for wild-type Ler activity (Fig. 6). The results show that some *ler* mutants had no effect on wild-type Ler, while others were clearly dominant negative. These include the G82V, W85R, G87S, G87D, G89D, P92S, W94R, L95P, and L109P mutants, all residing within the putative Ler DNA binding region. Since all of the dominant-negative mutants were able to form oligomers with wild-type Ler (Fig. 4A) and did not bind DNA (Fig. 2), we suggest that they cause the dominant-negative effect by forming heterooligomers with wild-type Ler that cannot bind DNA. Interestingly, some of the mutants (LerL16P, -Y77H, -Y77C, and -G102E) were able to oligomerize and did not bind DNA but were not dominant negative. It is possible that in these cases, heterooligomers, but not homooligomers, retained the DNA binding capacity.

The increased dosages of the L23R, G82E, A98V, and G102R Ler mutants are responsible for the recovery of their biological activity. As was mentioned earlier, four mutants, LerL23R, -G82E, -A98V, and -G102R, were found to be defective in autorepression but otherwise restored the wild-type phenotype upon complementation of the EPEC *ler*::*kan* strain (Fig. 5). This intriguing phenotype led us to further investigation of these four mutants. A possible explanation for this phenotype is that these Ler mutants possess residual DNA binding activity and that upon their overexpression (due to reduced autorepression), they reach a concentration that compensates for their reduced affinity to DNA (Fig. 7A). This compensatory mechanism hypothesis predicts that the ability of these four mutants to complement EPEC *ler*::*kan* is dosage dependent. To test this prediction, the four mutations (L23R, G82E, A98V, and G102R) were cloned into the pSA10 expression vector, placing them under a tightly regulated *tac* promoter (P*tac*) that can be induced for expression by IPTG. These pSA10-derived plasmids were then introduced into the EPEC

ler::*kan* strain, and the amount of expressed Ler was adjusted by using different IPTG concentrations. The capacity of the expressed Ler to restore the expression of the *LEE* genes and to enable the assembly of a functional TTSS was tested under the different expression levels. The TTSS-dependent EspB secretion to the growth medium was used as an indicator of TTSS activity. Wild-type Ler was used as a positive control and LerG89D, a mutant that did not complement EPEC *ler*::*kan* (Fig. 5 and Table 2), was used as a negative control. We found that a very low concentration of wild-type Ler (its basal level in the absence of IPTG) was sufficient to restore EspB secretion, and maximal secretion was evident upon induction with 5 μM IPTG (Fig. 7B). In the case of the L23R, G82E, A98V, and G102R Ler mutants, an increased amount of IPTG (20 to 50 μM), resulting in an ∼5- to >10-fold increase in the Ler concentration, was required to restore EspB secretion. The increase in the Ler concentration varied between the mutants, indicating that some mutations also affected Ler translation or its stability (Fig. 7C). Our model predicts that at high concentrations, these Ler mutants restore some of their DNA binding functionality, enabling them to activate transcription of the *LEE* promoters. To test this prediction, we determined the DNA binding affinity of wild-type Ler and LerL23R, revealing that LerL23R still specifically binds to the *LEE2* regulatory region but at a lower affinity and thus that higher concentrations of the mutant were needed in order to bind the regulatory *LEE2* DNA (see Fig. S5 in the supplemental material). In the case of the G89D mutant, which served as a negative control, even strong overexpression could not restore EspB secretion (Fig. 7B). These results support the hypothesis that in the case of the L23R, G82E, A98V, and G102R Ler mutants, a compensatory mechanism involving an increased Ler dosage is operating. These mutants will therefore be referred to as the CID mutants, for *c*ompensation by *i*ncreased *d*osage.

DISCUSSION

A simple and rapid method for identifying important residues in regulators. We have described here a simple method that allows rapid, high-throughput screens for regulators defective in autorepression. Since autorepression is a very common network motif in bacteria, this approach can be easily adopted for the functional analysis of many other regulators. Eight of the mutations picked up by our screen occurred more than once (and up to five times for A98V), indicating that our screen was close to saturation. The 18 Ler mutations causing
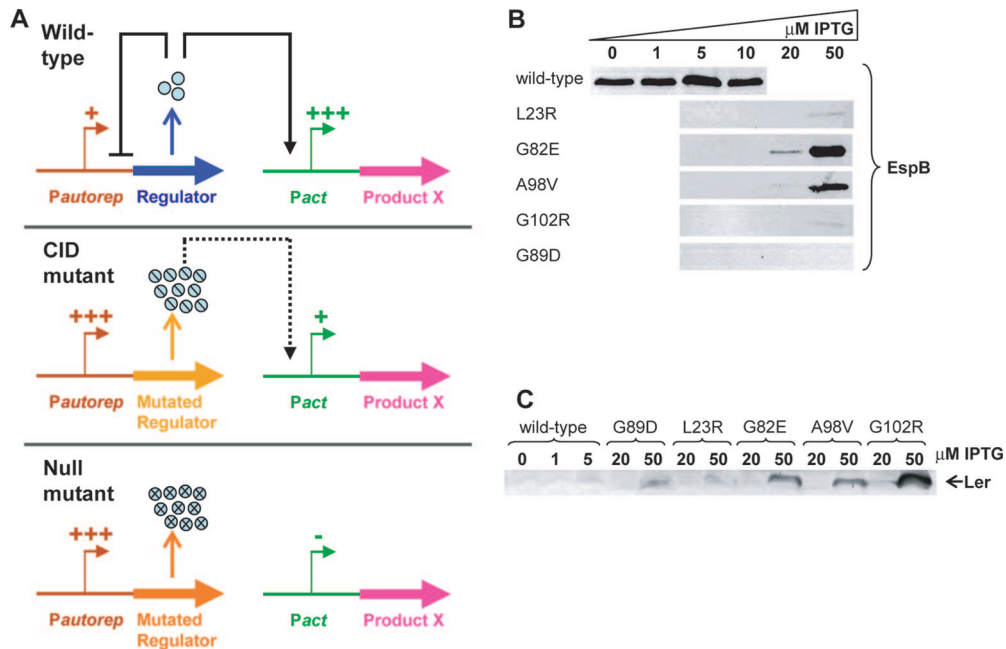
FIG. 7. Increased dosage of the CID mutants is required for their function. (A) A schematic representation of the compensatory mechanism: an autorepressor represses its own transcription (from the P*autorep* promoter) and activates transcription from other promoters (P*act*). A mutant autorepressor can either retain a partial function like the CID mutants or no function. A CID mutation (middle panel) alleviates autorepression, causing an increase in the concentration of the mutated regulator, which in turn compensates for its reduced DNA binding affinity, enabling it to activate the transcription from P*act*. This situation demonstrates the compensatory mechanism embedded in autorepressed regulatory systems. When a mutation completely abolishes the regulator's function (see the "Null mutant" bottom panel), even a high concentration cannot compensate for the mutation. (B) The L23R, G82E, A98V, and G102R CID mutants were cloned under the regulation of the P*tac* promoter, from which their expression level could be controlled (plasmids pGY2742, pGY2743, pGY2744, and pGY2745, respectively). Wild-type Ler and LerG89D (a DN mutant) were also subjected to the same analysis (plasmids pGY2746 and pGY3576, respectively). The plasmids were electroporated into EPEC *ler::kan*, and the strains were grown with increasing concentrations of IPTG (0, 1, 5, 10, 20, and 50 µM). The secretion of EspB, representing the expression and assembly of a functional TTSS, was tested by subjecting the supernatant of the cultures to an immunoblot analysis using anti-EspB antibody. (C) The expression level of Ler was determined by subjecting the pellet of the cultures to an immunoblot analysis using anti-Ler antibody (only two or three IPTG concentrations for each Ler mutant are shown).

the deficiency in autorepression described in this paper (Table 2) were not located in a distinct domain in Ler, revealing that there is no specific Ler domain that functions as an "autorepression domain" (Fig. 8). This implies that Ler binds to different DNA regions by the same mechanism, and it is the binding context that defines the outcome: gene induction or repression. This is probably the case with most other regulators which are also autorepressors. Therefore, the described screening

approach should reveal residues important to the fundamental aspects of most regulators: oligomerization and DNA binding.

Mutants' classification. We found that all the isolated mutants are deficient in autorepression and in binding DNA of the regulatory regions of both repressed and activated promoters (P$_{LEE1}$ and P$_{LEE2}$, respectively). However, these mutants differ in other aspects, and these differences were used for their classification (Table 2). Most of the mutations in the Ler pu-
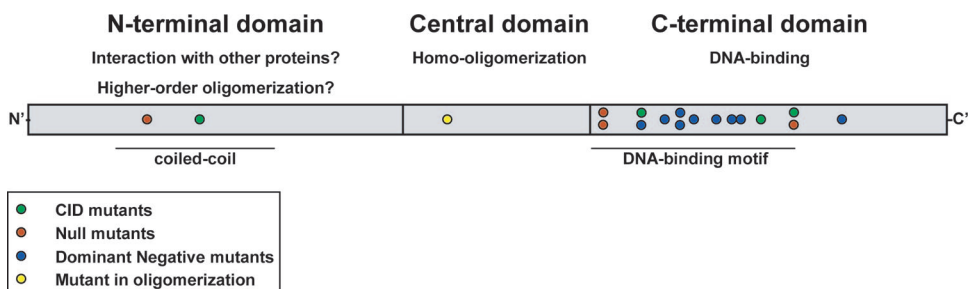

FIG. 8. A schematic of Ler domains. Based on the comparison to H-NS together with our results, we suggest the following model for Ler domains: an N terminus that functions either as a high-order oligomerization domain or as a domain that interacts with other proteins, a central domain that functions as a homooligomerization domain, and a C-terminal DNA binding domain. The three domains are essential for DNA binding and transcription regulation.

tative DNA binding domain resulted in a dominant-negative form of Ler. Other mutants could not support the activation of the *LEE* promoters, but they were not dominant negative. Interestingly, the third class (the CID mutants) restored a wild-type phenotype upon complementation of the EPEC *ler::kan* mutant with a plasmid encoding these alleles under their native promoter.

The intrinsic compensatory mechanism associated with Ler CID mutants. The analysis of the CID mutants elucidated an interesting auto-compensatory mechanism, which possibly functions in the case of other autorepressors. The binding of Ler, as well as other DNA binding proteins, is affected by two factors: K_d (dissociation constant) and concentration. Importantly, autoregulation is expected to be the link between the two: strong DNA binding (low K_d) should result in strong autorepression and a low concentration of the regulator. In contrast, weaker DNA binding (increased K_d) due to a point mutation should result in decreased autorepression and an increase in the regulator concentration. The latter increase in concentration might compensate for the reduced binding affinity to allow efficient binding of the regulator to other promoters (Fig. 7A). In the case of Ler, the latter promoters include those of operons *LEE2* and *LEE5*. Consequently, this compensatory mechanism might rescue the biological function of the mutated regulator. Our data support the notion that CID mutants restored the wild-type phenotype (Fig. 5) due to an increase in the steady-state level of the mutated Ler. This compensatory mechanism possibly protects regulators such as Ler from deleterious mutations. This mechanism is predicted to be particularly efficient in cases of cooperativity in DNA binding, as shown for Ler (our unpublished data), where a small increase in the regulator concentration may result in strong DNA binding. This proposed mechanism is not expected to function in cases of a severe reduction in DNA binding as seen in our results (Fig. 7A, bottom panel).

Different putative Ler domains. Ler is a paralog of H-NS, which induces DNA condensation (26). H-NS and related proteins consist of an N-terminal oligomerization domain and a C-terminal DNA binding domain separated by a flexible linker (28). The N-terminal oligomerization domain of H-NS, which has low sequence similarity with Ler, contains three α-helical segments: the first two stabilize the structure, and the third and longest α-helix forms the core of a coiled-coil configuration (5, 10, 28). This coiled-coil region is essential for H-NS oligomerization (30, 32). Using PSIPRED (20), we found that the first two helices of H-NS have no counterparts in Ler, whereas a counterpart to the coiled-coil region was found in the Ler N terminus, as previously predicted (27). Two of the isolated Ler mutations (L16P and L23R) were located within this N-terminal coiled-coil domain. Unexpectedly, neither of these mutations prevented oligomerization, suggesting that this motif is not the sole determinant of Ler oligomerization. It is possible that in Ler, this domain is required for higher-order oligomerization or for the interaction of Ler with other proteins, possibly regulators. Importantly, these mutations caused a strong deficiency of DNA binding of the homooligomers, but they did not impose a dominant-negative effect upon wild-type Ler. How this domain contributes to DNA binding is not yet clear.

The H-NS C-terminal DNA binding domain is conserved and highly similar to that of Ler (27), containing the consensus

DNA binding motif: Y-X(6)-[GS]-[ED]-X(0,2)-T-W-[TS]-G-[QR]-G-[RK]-X-P-X(4,5)-A-X(3,4)-G (4, 8). It was consequently suggested that this domain might be the Ler DNA binding domain (27). In agreement with this prediction, most of the mutants isolated in our screen were clustered in this region. These mutations were either dominant-negative mutations, null mutations, or CID mutations (Fig. 8). Interestingly, in two cases, the same residue could be mutated to give different phenotypes. The G82V mutation caused a dominant-negative effect, whereas the G82E mutation was a CID mutation. Another case is G102R, which was a CID mutation, while G102E had a null phenotype.

The central flexible linker of H-NS is not essential for oligomerization but has been suggested to be involved in higher-order oligomerization (5). The central regions of H-NS and Ler are very different; still, we identified that the central region of Ler, around L56, is essential for oligomerization. L56P is the only mutation that completely prevented oligomerization. Moreover, truncation analysis suggests that this region is essential for the dominant-negative effect, suggesting that it is involved in oligomerization in vivo. These differences in the oligomerization mechanism and the dissimilarity between the N-terminal and central regions of H-NS and Ler may reflect some functional differences. Ler alleviates the H-NS silencing of only a few specific promoters that are flanked by one or two Ler DNA binding regions. Therefore, Ler probably does not function by forming Ler–H-NS heterodimers. In contrast, other proteins, closely related to H-NS, like StpA, can function by forming StpA–H-NS heterooligomers (32).

In conclusion, the results described in this report, together with the comparison of Ler to H-NS, suggest that Ler has three domains (Fig. 8): a C-terminal domain that functions as a DNA binding domain, a central domain that functions as a homo-oligomerization domain, and an N-terminal domain that may have a role in interacting with other proteins or in higher-order oligomerization. All three domains are essential for the DNA binding activity of Ler and for its role as a regulator.

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