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Original Article

GrlA of enterohemorrhagic *Escherichia coli* O157:H7 activates *LEE1* by binding to the promoter region

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Background and Purpose: The locus of enterocyte effacement (LEE) of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 encodes virulence factors that lead cooperatively to an attaching and effacing lesion on host large intestine cells. Global regulator of LEE activator (GrlA), encoded by the open reading frame 3 in the EHEC LEE, is known to serve as a positive regulator of LEE expression. However, how it functions to orchestrate gene expression remains unclear.

Methods: A *grlA*-deleted mutant strain was created, and the determinants needed for the LEE activation were addressed by complementation experiments. A DNA electrophoresis mobility-shifting assay was used to test a hypothesis that the activation occurs via a direct binding on the putative promoter region.

Results: Activation of the major LEE operons could be rescued by an over-expression of LEE-encoded regulator (Ler), except for the *LEE1* operon, expression of which absolutely required GrlA. Consistent with the latter observation, GrlA bound specifically to the putative *LEE1* promoter region. Furthermore, determinants critical for this activity have been mapped to the N-terminal region of GrlA.

Conclusion: GrlA upregulates the expression of LEE through binding of the *LEE1* promoter, which in turn increases the level of Ler allowing it to function as a downstream activator. The opposing effect of global regulator of LEE repressor (GrlR) is explainable by in vitro findings that GrlR interacts with GrlA, suppressing the specific binding of GrlA on the *LEE1* promoter.

Key words: Enterohemorrhagic *Escherichia coli*; Enteropathogenic *Escherichia coli*; EspD protein, *Escherichia coli*; *Escherichia coli* proteins; Etiology; Gene expression regulation

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) causes a pathogenic presentation called the attaching and effacing lesion [1], a property shared in infections of enteropathogenic *E. coli* and *Citrobacter rodentium*. Genes involved in this lesion are clustered in the bacterial chromosome and organized at the locus of enterocyte effacement (LEE) [1-3]. Within the LEE island, there are 41 open reading frames, and most of them are clustered into five major operons: *LEE1*, *LEE2*, *LEE3*, *LEE4* and *LEE5* [4]. Between *LEE1* and *LEE2*, there are two small genes named *grlR* (global regulator of LEE repressor [GrlR]) and *grlA* (global regulator of LEE activator [GrlA]), that encode negative and positive factors, respectively, and are critical to the global regulation of the LEE; it is presumed that they share the same transcript and a common promoter. On the other hand, *LEE1* comprises *ler* (LEE-encoded regulator [Ler]), *cesAB* (open reading frame 3 of the LEE) and six other genes [5] driven by the same promoter.

Ler, GrlR, GrlA, and Mpc (multiple point controller; a product encoded by the first gene on *LEE3*) are currently known to regulate LEE expression, but only Ler has been demonstrated to autoregulate *LEE1* [6-9]. Recently, GrlA has been demonstrated to activate *LEE1* by a reporter assay in *C. rodentium*, but no direct binding has been seen [10]. Here, we provide evidence that GrlA does bind to the promoter region of *LEE1*. Furthermore, we mapped the region of GrlA that is required for this binding activity.

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Methods

Plasmid construction

The oligonucleotides used in this study are listed in Table 1. Polymerase chain reaction (PCR) amplification, restriction enzyme digestion, DNA ligation, and plasmid transformation were carried out by standard procedures. DNA sequencing was performed by a contract service with Mission Biotech (Taipei, Taiwan). The gene *grlA* was amplified from chromosomal DNA of the strain 43888 [11] by PCR using the primers GRLA-F and GRLA-R and cloned into pUCmT (MD Bio., Taipei, Taiwan) to generate pUC-GrlA. To generate pGrlA and pGexA, *grlA* was re-amplified from pUC-GrlA using the primers PUCT-FB and PUCT-RK, and the PCR product was digested with *Bam*HI and *Kpn*I. This *Bam*HI/*Kpn*I-digested product was cloned into pQE80 (Qiagen, Hilden, Germany) or pGex-2KS [12] that had been pretreated with the same enzymes.

Two primer pairs, GRLA-F-BamHI/GRLA-R-XhoI and GRLR-F-BamHI/GRLR-R-XhoI, were used to amplify *grlR* fragments from the strain 43888 chromosomal DNA, and the PCR products were treated with *Bam*HI/*Xho*I and separately ligated with pBT and pTRG (Stratagene, La Jolla, CA, USA) digested with the same enzymes. The resulting plasmids were named pBA and pTR, respectively. A similar strategy was used to create pBR and pTA, except that the inserts and vectors were switched during the ligation.

To construct plasmids expressing variously truncated *grlA* fragments, pGrlA and pGexA were inversely amplified using primer pairs GRLA39-60-F/GRLA39-60-R and GRLA80-108-F/GRLA80-108-R. The amplified fragments were then self-ligated, producing $pGrlA_{delta(\Delta)39\text{-}60}$, $pGrlA_{\Delta 80\text{-}108}$, $pGexA_{\Delta 39\text{-}60}$, and $pGexA_{\Delta 80\text{-}108}$, respectively. The *grlA* sequence in pBA and pTA was similarly shortened to give $pBA_{\Delta 39\text{-}60}$, $pBA_{\Delta 80\text{-}108}$, $pTA_{\Delta 39\text{-}60}$, and $pTA_{\Delta 80\text{-}108}$. To create $pGrlA_{\Delta 107\text{-}138}$, the corresponding *grlA* fragment was amplified directly from the strain 43888 chromosomal DNA, using primers GRLA-F-BamHI and GRLA107-138-R. This PCR product was treated with *Bam*HI and *Xho*I and cloned into *Bam*HI/*Xho*I-digested pQE80 to generate $pGrlA_{\Delta 107\text{-}138}$. The same product was similarly cloned into pGex-2KS, pBT, and pTRG to give $pGexA_{\Delta 107\text{-}138}$, $pBA_{\Delta 107\text{-}138}$, and $pTA_{\Delta 107\text{-}138}$, respectively. To create pGexR, the entire *grlR* fragment was obtained by digesting pQ-44 [8] with *Bam*HI/*Kpn*I and ligated into pGex-2KS that was treated with the same enzymes.

Plasmid pACgrlA was created to differ from pGrlA by taking advantage of the distinct and compatible *ori* in pAC184. To construct this plasmid, PCR was used to amplify the *grlA* fragment from pGrlA using the primers T5P and PQER. The PCR product was first phosphorylated by T4 polynulceotide kinase and then ligated with *Eco*RV-digested, calf intestinal phosphatase-treated pACYC184. To assay for promoter activation activity, we used a previously used system [9], in which *lacZ* is driven by the inserted sequence to be addressed. The *LEE1* promoter region was amplified with primers KM1-F and KM1-R using strain 43888 chromosomal DNA as the template. After digestion

Table 1. Oligonucleotides used in this study

Oligonucleotides	Sequence (5' to 3'; restriction enzyme site underlined)
PUCT-FB	ACTAGTGGATCCAGAATT
PUCT-RK	ATGGTACCGCCGCCACTCAT
T5P	CCCGAAAAGTGCCACCTG
PQER	CATTACTGGATCTATCAACAGG
LEE1-F	GCTCTAGAGAGAAACGCTTAACTAAATGG
LEE1-R	GCTCTAGATAATCTCCGCATGCTTTA
GRLA-F	ATGGAATCTAAAAATAAAAATGGCG
GRLA-R	CTAACTCTCCTTTTTCCGCC
GRLA-F-BamHI	CGGGATCCATGGAATCTAAAAATAAAAATGGC
GRLA-R-XhoI	CCGCTCGAGACTCTCCTTTTTCCGCCTCAT
GRLR-F-BamHI	CGGGATCCATGATTATGAAGGATGGCATC
GRLR-R-XhoI	CCGCTCGAGTTTTAAATAAACTTGTGGCAT
GRLA39-60-F	TATATATCGAGAAGAAAAGAAAAAATTTC
GRLA39-60-R	CCATTTCTCCTGCAATTTAC
GRLA80-108-F	GGACCAAAAAGAAAAACCTACCG
GRLA80-108-R	ACCATAACTAACATATCTGACACG
GRLA107-138-R	CCGCTCGAGGGTTGTTCCAGGACTTTC

with *Xba*I, the PCR product was cloned into pKM that had been pre-cleaved with the same enzymes to give pLEE1.

Western blotting

Proteins from bacterial lysates or spent media were prepared as previously described [11]. Proteins in the samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane, and then analyzed with specific antibodies. Anti-Tir, anti-EspD, anti-CesAB, anti-His_{x6} tag, and anti-glutathione S-transferase (GST) were of mouse origin, whereas anti-EspB and anti-EspA were derived from rabbit sera [8,13]. Species-specific antibodies conjugated with horseradish peroxidase (Sigma, St. Louis, MO, USA) were used as the secondary antibodies. After binding and washing, the membranes were finally developed with Renaissance Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA, USA), and the signals were captured by exposure on X-ray films.

Promoter activity assay

Overnight cultured transformants were diluted 1:50 into fresh media and grown until an absorbance of 0.5 to 0.6 was obtained at 600 nm. After reading the absorbance, beta (β)-galactosidase activity of the bacteria was measured and calculated as described [9,14].

Electrophoresis mobility-shifting assay

GST-fusion proteins expressed in *E. coli* JM109 carrying various plasmids were purified by affinity binding towards glutathione-conjugated agarose [13]. To prepare the probe, a control promoter fragment from pBtuB containing the 5' promoter region of *btuB* (a gift from ST Hu, National Yang-Ming University), was isotope-labeled with [gamma-32P]-adenosine triphosphate using T4 polynucleotide kinase. A similar method was used to prepare the probe for the *LEE1*-promoter region, except that the DNA fragment was obtained by PCR amplification of the bacterial chromosomal DNA using primers LEE1-F and LEE1-R. After removing the free nucleotides, the probes were incubated with the test proteins dissolved in the binding buffer (100 mM sodium chloride, 20 mM Tris-hydrochloride, pH 7.5 and 100 μg/mL herring sperm DNA) for 30 min at room temperature. After the incubation, the mixtures were electrophoretically separated on 5% polyacrylamide gel, and radiographic images were taken by exposing the dried gels to X-ray films.

GST pull-down assay

E. coli JM109 strain transformed with either the control vector or pGrlA was cultured in Luria-Bertani broth overnight and then diluted 1:50 to 200 mL. The bacteria were harvested and re-suspended in phosphate-buffered saline (PBS). Cells were broken, and the soluble fraction was collected after centrifugation. Equal volumes of the soluble fractions containing comparable amounts of His_{x6}-tagged GrlA and GST-GrlR, respectively, were mixed and incubated for 3 h at 4°C. Glutathione agarose beads were then added, and the mixtures were rotated on a rolling drum for 1 h at 4°C. After washing the agarose beads thoroughly with cold PBS, the proteins retained on the beads were eluted with 10 mM glutathione in PBS.

Bacterial two-hybrid assay

The bacterial two-hybrid assay has been described previously [9]. In brief, pBT that encodes lambda cI protein was used to produce a bait protein fused with lambda cI, and pTRG was used to generate a target protein fused to the C-terminus of an N-terminal domain of RNA polymerase alpha subunit. A strong interaction of the proteins in the system would then result in a bacterial phenotype displaying carbenicillin resistance and a high level of β-galactosidase expression, the activity of which was measured as described above.

Results

GrlA activates the *LEE1* operon

The activation effect of GrlA [7] in EHEC was first established by Western blotting (Fig. 1A). EHEC strain 43888, referred to as the wild-type parental strain, and a strain with *ler* deleted (Δler) have been described previously [8]. A strain with *grlA* deleted (ΔGrlA) was created similarly by homologous recombination [15]. From Δler, *grlA* was further deleted to result in a *ler* and *grlA* doubly deleted (ΔA/L) mutant. The deletion mutants grew with no apparent difference from the parental strain in either Luria-Bertani broth or M9 medium in the presence of 5% carbon dioxide [8].

When *grlA* was deleted from the LEE island (i.e., in strain ΔGrlA), four representative products from the LEE were poorly detected in the bacterial lysate (Fig. 1A, lane 2), a result reflecting the minimum expression of the operons. When the mutant was complemented with GrlA expressed from pGrlA, the synthesis of the LEE proteins was restored to a level similar to that seen with the parental strain (compare lanes 1 and 3).

CesAB is encoded by *LEE1* and was used to reflect the regulation exerted on *LEE1*. To avoid unwanted complexity with regard to Ler, the effect of GrlA on *LEE1* was examined on the *ler* and *grlA* doubly deleted mutant (ΔA/L) [Fig. 1B]. Fig. 1B (lanes 1 and 2) shows that an extra supply of plasmid-derived GrlA to strain 43888 did not have much effect on the expression of Tir and EspB but caused a slight increase in the levels of both CesAB and EspA. On the other hand, a complete lack of both GrlA and Ler set a baseline level for the expressed LEE proteins. Using this as background, complementation with GrlA recovered the high-level expression of CesAB, but not of the other proteins (Fig. 1B, lanes 3 and 4). Therefore, CesAB expression was definitely stimulated by GrlA, a fact suggesting that *LEE1* transcription is upregulated by GrlA. To substantiate this notion, pACgrlA expressing GrlA was co-transformed with pLEE1 carrying the putative promoter in front of *lacZ*. From the levels of β-galactosidase activities measured in the transformed bacteria, the degree of promoter activation by GrlA was then monitored. The *E. coli* 43888 ΔGrlA mutant strain was used as the host for this promoter activation assay. GrlA expressed together with the putative promoter of *LEE1* increased the β-galactosidase activity about 4-fold compared to the vector (pAC184) control (data not shown), a result consistent with the conclusion derived from the Western blotting analysis.

Binding of GrlA to the *LEE1* promoter

To determine whether GrlA activates the promoter regions through direct binding, an in vitro DNA binding using electrophoresis mobility-shifting assay (EMSA) was performed. PCR fragments containing the putative promoters were end-labeled with [gamma-^{32}P]-adenosine triphosphate and incubated with affinity-purified GST-GrlA. The radiographic results after separating the mixtures on gels followed by X-ray film exposure are shown in Fig. 1C. In the presence of GST, the probes migrated in a fashion similar to that with the control protein (compare lanes 1 and 2). When the probe containing the putative *LEE1* promoter was reacted with GST-GrlA (Fig. 1C, upper panel), gel retardation of the bands occurred. With increasing concentration of GST-GrlA, the slow-migrating complexes progressively increased in intensity and band numbers (Fig. 1C, lanes 3-6). Although some signals were found, clearly defined binding pattern was not seen with the probe derived from the putative promoter of *btuB* (Fig. 1C, lower panel). These results indicated

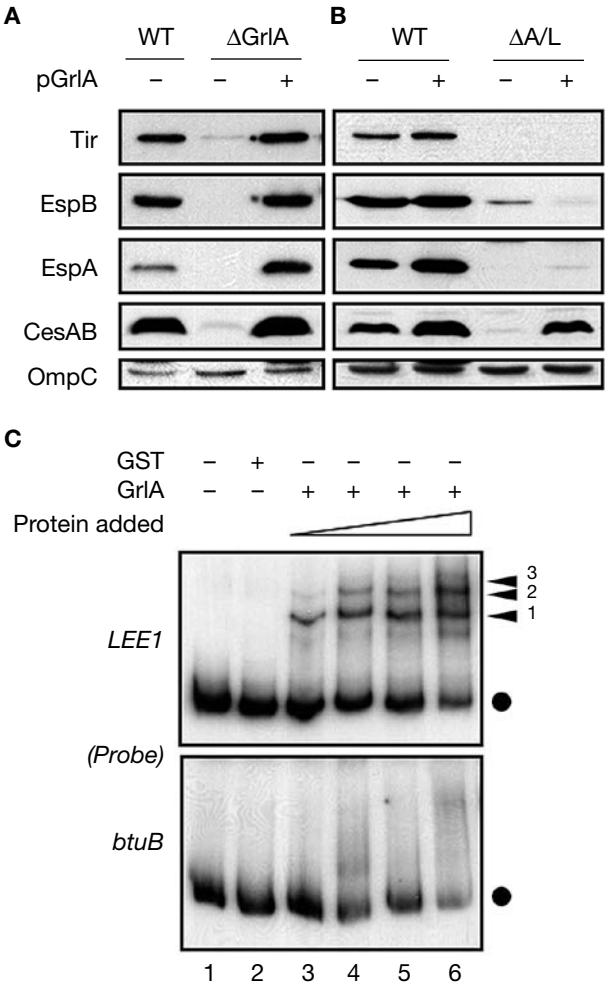

Fig. 1. Functional activities of global regulator of LEE (locus of enterocyte effacement) activator (GrlA). (A) Expression differences of LEE among enterohemorrhagic *Escherichia coli* strain 43888 (WT), the *grlA*-deleted 43888 mutant (delta[Δ] GrlA), and the plasmid-complemented mutant. (B) Effect of plasmid-expressed GrlA on LEE protein expression when *ler* (LEE-encoded regulator [Ler]) and *grlA* were doubly deleted in strain ΔA/L. Proteins from the bacterial lysates were analyzed by Western blotting using specific antibodies: Tir, an effector from *LEE5*; EspB and EspA, two translocators from *LEE4*; CesAB, an *LEE1* protein. Outer membrane protein C (OmpC) serves as a protein-loading control. Note: in ΔA/L, pGrlA transformation rescued the expression of CesAB (open reading frame 3 of the LEE). (C) Binding of GrlA to specific DNA as evidenced by electrophoresis mobility-shifting assay. DNA fragments were end-labeled with [gamma-^{32}P]-adenosine triphosphate and incubated with various amounts of the affinity-purified glutathione S-transferase (GST)-GrlA fusion protein. The mixtures were resolved in 5% polyacrylamide gel. The bands of the original probe are indicated by dots, whereas bands shifted by increasing concentrations of recombinant GrlA are labeled with arrowheads. Proteins added: GST, 2.2 μg; recombinant GrlA, from lanes 3 to 6 with an increment of 0.55 μg between each.

that a specific binding of GrlA to the *LEE1* promoter region generated complexes. The intensities of bands 2 and 3 increased along with the GrlA concentration gradient, while that of band 1 seemed to stay constant. The band 1 complex might represent an initial complex formed by contact of GrlA with DNA, whereas the complexes seen as bands 2 and 3 might reflect the formation of advanced structures when sufficient amounts of GrlA are present.

Mode of interaction of GrlR with GrlA

GrlR of EPEC has been demonstrated to interact with GrlA in a yeast two-hybrid system [16]. To validate the interaction between GrlA and GrlR in EHEC, a GST pull-down assay followed by Western blotting was carried out, as previously described [9]. As shown in Fig. 2A, His-tagged GrlA was retained by glutathione-agarose beads, to which GST-GrlR fusion protein was first bound, and detected as one of the eluted products after adding glutathione. However, this retention did not occur in the control, in which the fusion partner GST was used (compare lanes 2 and 4). This definite interaction between GrlR and GrlA raised the question as to whether GrlA remains active in binding to the target promoter after complex formation with GrlR. To address this question, an EMSA experiment similar to that for Fig. 1C was performed. Fig. 2B shows that neither GST nor GST-GrlR bound to the probe of the *LEE1* promoter region as GST-GrlA did (compare lanes 1-5). After increasing the amount of GrlR relative to GrlA, which had been kept at a constant amount sufficient to form advanced DNA-protein complexes, the band patterns were analyzed by gel electrophoresis and shown to change progressively. The intensity of band 3 gradually decreased, followed by band 2, whereas band 1 remained unchanged or increased in intensity (Fig. 2B, lanes 5-9). These facts together suggest that GrlR is not a direct binding competitor for the *LEE1* promoter, but rather that it may interact with GrlA and prevent GrlA from forming advanced complexes with the target DNA, detected as bands 2 and 3.

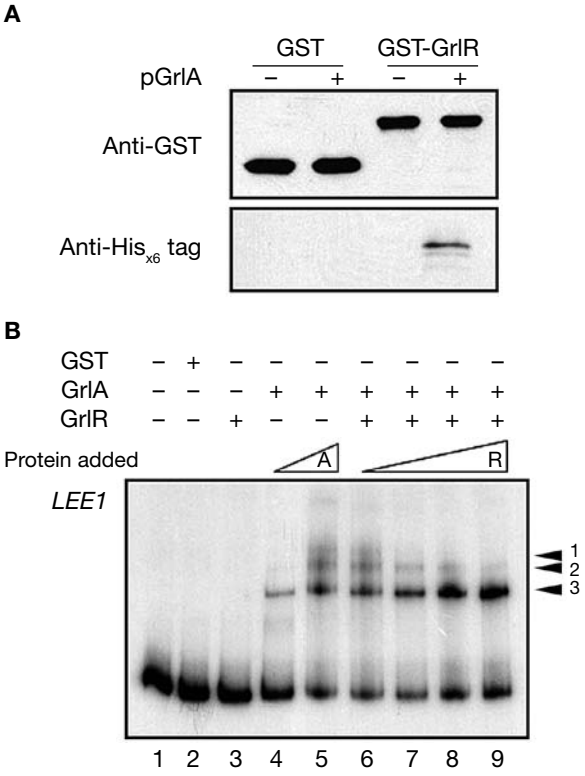

Fig. 2. Interaction between global regulator of LEE (locus of enterocyte effacement) activator (GrlA) and global regulator of LEE repressor (GrlR) and the resulting effect on DNA binding ability. (A) Glutathione S-transferase (GST) pull-down assay to follow the interaction between GrlA and GrlR. Strain 43888 delta(Δ)GrlA was transformed with pGrlA, and the soluble cytosolic proteins of the bacteria were prepared. The proteins were mixed with GST-GrlA (or control GST) that was then bound on to glutathione agarose. After washing, the associated proteins were eluted and analyzed for the presence of GST fusion proteins and His-tagged GrlA by Western blotting. (B) Electrophoresis mobility-shifting assay analysis to detect GrlA-bound ^{32}P-DNA complexes derived from the *LEE1* promotor. Experiments were carried out as in Fig. 1C, except that before incubating with ^{32}P-labeled DNA, increasing amounts of the following proteins were added: A, GrlA; R, GrlR. In lane 4, 1.1 μg GrlA was added, and in lanes 5 to 9, GrlA was held constant at 2.2 μg per reaction while an increment of 0.7 μg GrlR was increasingly added. Note: arrowheads indicate the bands resulting from the interactions of GrlA and DNA fragment; bands 2 and 3 decreased in intensity in the presence of increasing amounts of GrlR.

Mapping the interaction domains of GrlA

In addition to the function of global LEE activation, GrlA showed binding activity with the *LEE1* promoter and it interacted with GrlR. To examine these activities at a molecular level, three truncated GrlA constructs were generated (Fig. 3A). Plasmids expressing these molecules were first transformed into *E. coli* 43888 ΔGrlA (similar to Fig. 1A), and the abilities of the individual constructs to complement the activity of deleted GrlA were examined. It was found that $GrlA_{\Delta 107\text{-}318}$ nearly restored LEE expression as if it were a full-length GrlA (Fig. 3B, lanes 4 and 5) whereas the other two deletions were totally incapable of doing so and gave undetectable activation (Fig. 3B,

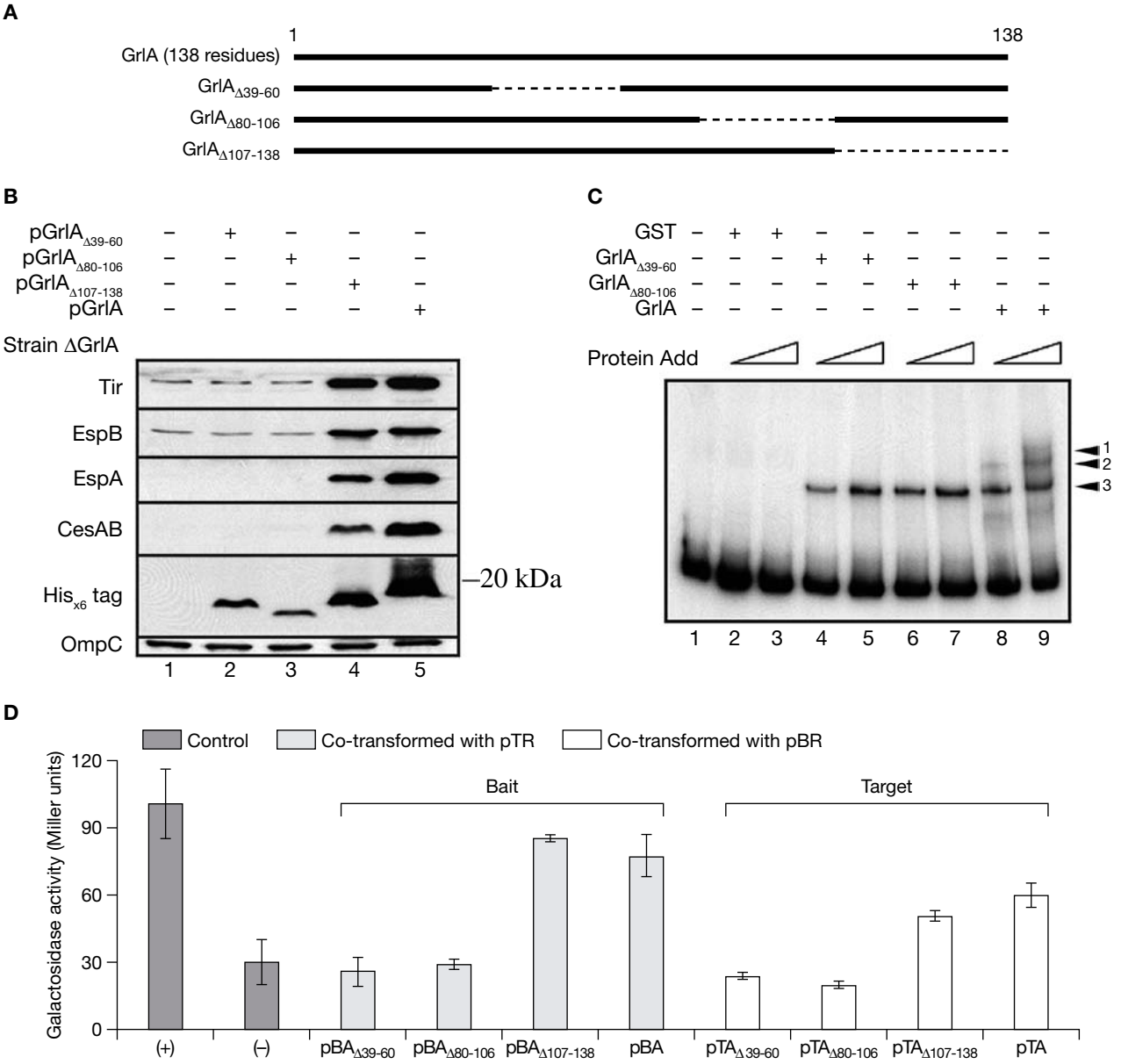

Fig. 3. Mapping the functional determinants of global regulator of LEE (locus of enterocyte effacement) activator (GrlA). (A) Schematic illustration of various GrlA constructs. (B) Region of GrlA necessary for LEE activation. Truncated GrlA was expressed in strain 43888 delta(Δ)GrlA, and the resulting expression of LEE proteins was monitored from the bacterial lysates by Western blotting analysis, as described in Fig. 1. Expression of the His$_{x6}$-tagged GrlA variant from a plasmid is driven by T5 promoter, induced with isopropylthiogalactoside, and traced by a tag-specific antibody. (C) Protein-DNA complexes analyzed by electrophoresis mobility-shifting assay. The two forms of truncated GrlA that failed to activate LEE in (B) were tested for binding to ^{32}P-labeled *LEE1* DNA fragment as that in Fig. 1C. (D) Interaction between truncated GrlA and a full-length GrlR as determined by a bacterial two-hybrid system. GrlR was expressed as a bait or target protein from plasmids pBR and pTR, respectively. The plasmid was then co-transformed into *Escherichia coli* XL1 Blue MRF' Kan with a second plasmid expressing GrlA in various truncated forms. The degree of association of the co-expressed proteins was revealed by measuring beta-galactosidase activities of the bacteria.

lanes 1 to 3). Therefore, a deletion C-terminal to residue 107 had no effect on GrlA activation activity, whereas a deletion at residues 80-106 or at residues 39-60 resulted in an activation-incompetent GrlA.

Activation of GrlA on the LEE was deduced in the above experiments via the direct binding to the putative *LEE1* promoter. It is thus expected that those truncated GrlA constructs that failed to activate the

LEE should also fail to form advanced complexes with the target DNA. Therefore, EMSA (Fig. 3C) was performed in which GST (lanes 2 and 3) and GrlA (lanes 8 and 9) were used as negative and positive controls, respectively. Fig. 3C shows that the two constructs ($GrlA_{\Delta 39\text{-}60}$ and $GrlA_{\Delta 80\text{-}106}$) which failed to activate the LEE in Fig. 3B also failed to form advanced complexes (lanes 4-7), as seen with bands 2 and 3 in lanes 8 and 9, but the primary DNA-protein complex (band 1) was formed.

Interaction of GrlR with the truncated GrlA was examined by the bacterial two-hybrid system (Stratagene) [9], in which a positive contact is reflected by an increase in the bacterial β-galactosidase activities measured (Fig. 3D). The truncated GrlA was first expressed as a bait protein to test for the interaction with a targeted GrlR. When $GrlA_{\Delta 107\text{-}138}$ (in $pBA_{\Delta 107\text{-}138}$) was used as a bait protein, the β-galactosidase activity was increased to about 3-fold that of the negative control and was comparable to that of a full-length GrlA (in pBA).

On the other hand, both $GrlA_{\Delta 39\text{-}60}$ and $GrlA_{\Delta 80\text{-}106}$ gave low levels of β-galactosidase, similar to that of the negative control. Thus, the regions including residues 39-60 and 80-106 are likely to be involved in interaction with GrlR. In reciprocal experiments using truncated GrlA as the target and GrlR as the bait protein, the same conclusion was consistently observed (Fig. 3D).

Discussion

The control of GrlA over the expression of *LEE1*, and thus the level of Ler, is evidentially supported from the above data. This result from EHEC is consistent with the recent report from *C. rodentium* [10], which found that GrlA not only counteracts the repression of H-NS on *LEE1* but also plays a vital role for optimal expression of Ler. The results of this study further develop knowledge of the relationship between the protein function of GrlA and the gene activation of *LEE1* in terms of a direct binding of GrlA to the promoter region of *LEE1*. The critical region of GrlA that confers the ability of forming advanced complexes with the *LEE1* promoter (Fig. 3C) appears to overlap with the region that interacts with GrlR (Fig. 3D). Thus, a simple explanation to account for the inhibitory role of GrlR on LEE expression [8,10] is a blocking effect of GrlR that keeps GrlA from binding to the *LEE1* promoter.

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