

The GrlR-GrlA Regulatory System Coordinately Controls the Expression of Flagellar and LEE-Encoded Type III Protein Secretion Systems in Enterohemorrhagic *Escherichia coli*

Sunao Iyoda,^{1*} Nobuo Koizumi,¹ Hitomi Satou,¹ Yan Lu,¹ Takehito Saitoh,²
Makoto Ohnishi,¹ and Haruo Watanabe¹

Department of Bacteriology¹ and Infectious Diseases Surveillance Center,² National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan

Received 12 March 2006/Accepted 30 May 2006

The gene function of the locus of enterocyte effacement (LEE) is essential for full virulence of enterohemorrhagic *Escherichia coli* (EHEC). Strict control of LEE gene expression is mediated by the coordinated activities of several regulatory elements. We previously reported that the ClpX/ClpP protease positively controls LEE expression by down-regulating intracellular levels of GrlR, a negative regulator of LEE gene expression. We further revealed that the negative effect of GrlR on LEE expression was mediated through GrlA, a positive regulator of LEE expression. In this study, we found that the FliC protein, a major component of flagellar filament, was overproduced in *clpXP* mutant EHEC, as previously reported for *Salmonella*. We further found that FliC expression was reduced in a *clpXP grlR* double mutant. To determine the mediators of this phenotype, FliC protein levels in wild-type, *grlR*, *grlA*, and *grlR grlA* strains were compared. Steady-state levels of FliC protein were reduced only in the *grlR* mutant, suggesting that positive regulation of FliC expression by GrlR is mediated by GrlA. Correspondingly, cell motility was also reduced in the *grlR* mutant, but not in the *grlA* or *grlR grlA* mutant. Because overexpression of *grlA* from a multicopy plasmid strongly represses the FliC level, as well as cell motility, we conclude that GrlA acts as a negative regulator of flagellar-gene expression. The fact that an EHEC strain constitutively expressing FlhD/FlhC cannot adhere to HeLa cells leads us to hypothesize that GrlA-dependent repression of the flagellar regulon is important for efficient cell adhesion of EHEC to host cells.

Enterohemorrhagic *Escherichia coli* (EHEC) is an important class of diarrheagenic *E. coli* associated with severe diseases, such as hemorrhagic colitis and hemolytic uremic syndrome (reviewed in references 20 and 55). The majority of EHEC strains share with enteropathogenic *E. coli* (EPEC) and *Citrobacter rodentium* an island of pathogenicity determinants within their genomes termed the locus of enterocyte effacement (LEE) (52). The LEE is responsible for the production of attaching and effacing lesions, which are characterized by disruption of microvilli and formation of actin-accumulated pedestal-like structures beneath bacteria attached to intestinal epithelial cells (53).

The LEE encompasses 41 genes, which encode structural components of the specialized type III protein secretion system (T3SS), translocator (EspA, -D, and -B) and effector proteins secreted through the LEE-encoded T3SS, chaperones, adhesin (intimin), and the intimin receptor, Tir (16, 34–37). The LEE genes are organized into at least five operons and are under strict regulatory control (54). Three regulators, Ler, GrlR, and GrlA, also are encoded within the LEE: Ler is a central activator necessary for the transcription of most LEE genes (17, 54); GrlA and GrlR are positive and negative regulators, respectively, of *ler* transcription (3, 13, 47). The negative effect of GrlR on LEE expression depends on the GrlA function (33).

Consistent with this evidence, GrlR has been considered to interact with GrlA (11). Therefore, in the absence of GrlR, free GrlA up-regulates LEE gene expression through Ler (3, 13, 33, 47). Furthermore, intracellular protein levels of GrlR are regulated in a growth phase-dependent manner; GrlR levels are minimal as the EHEC cells enter the stationary phase (33), when LEE gene expression is induced (60).

Since LEE expression has been shown to be under the control of various regulatory elements encoded outside the locus (8, 21, 23, 24, 60, 62, 65, 66, 68–70, 74, 78), its regulatory mechanism seems to be complicated and awaits clarification. However, it has been demonstrated that multiple *pch* genes (*pchABC*) encode positive regulators important for LEE expression and the adhesion phenotype of EHEC (32, 58). PchABC positively regulate *ler* transcription and act as key regulators in response to various environmental stimuli (33). For example, the RcsCDB phosphorelay system has been shown to control the expression of LEE both positively and negatively in EHEC (70); a multicopy plasmid carrying *rscB* or *rscD* activated transcription of LEE through a newly identified positive regulator, GrvA, while deletion of *rscB* resulted in increased LEE expression via activation of *pchA* and *pchC* transcription (70). In addition, the ATP-dependent protease complex ClpX/ClpP positively regulates LEE expression in EHEC by regulating intracellular protein levels of GrlR and RpoS (33, 72); the latter negatively affects LEE expression by reducing the transcription of *pchA* by a currently unknown mechanism (33).

ClpXP similarly regulates the stability of many cellular pro-

* Corresponding author. Mailing address: Department of Bacteriology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1111. Fax: 81-3-5285-1163. E-mail: siyoda@nih.go.jp.

teins, including several transcriptional regulators (18). In *Salmonella*, ClpXP negatively controls flagellar synthesis by altering the stability of FlhD/FlhC master regulators (71, 73), which are essential for the expression of all other flagellar genes (28, 29, 42, 48).

The supramolecular structure of the flagellar-hook-basal-body complex resembles the needle structure of T3SS, and they are evolutionarily related (40). Several lines of evidence suggest that regulatory genes for the flagellar regulon also control the expression of T3SS and subsequent virulence phenotypes in some bacteria (7, 14, 26, 31, 49, 57). However, the biological significance and molecular mechanism of this cross-regulation remain unclear. Here, we report that a LEE-encoded positive regulator, GrlA, negatively regulates flagellar-gene expression in EHEC by reducing transcription of the *flhD* operon. Our results indicate that GrlA is a key regulator of inversely regulated flagellar and LEE-encoded T3SS expression in EHEC, and this regulation may be important for efficient cell adhesion of EHEC.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are summarized in Table 1. SKI-5142 is a Lac-negative derivative of EHEC O157:H7 strain Sakai, described previously (33), and was used as a wild-type strain in this study. Unless otherwise specified, bacteria were grown in Luria broth (LB), and ampicillin (100 µg/ml), chloramphenicol (25 µg/ml), and kanamycin (50 µg/ml) antibiotics were added as required. LB and LB-agar plates were prepared as described previously (32). Motility agar plates were prepared by adding 0.25% agar (Shoei) to 1% Bacto-Trypton (Difco) broth containing 0.5% NaCl.

A one-step inactivation method was used to construct SKI-5200 as described previously (12, 32, 33). The PCR primers used to construct SKI-5200 were 5'-AAACCCAATACGTAATCAACGACTTGCAATATAGGATAACGAATCGTGTAGGCTGGAGTGCTC-3' and 5'-ACGGGGTGCGGGTAAACCCGCA TAAAAATAAAGTTGGTTATTCTGGGTGTAGGCTGGAGCTGCTC-3'.

Construction of plasmids. pGEMGA was described previously (33). A 0.52-kbp DNA fragment of pGEMGA excised by BamHI-StuI digestion was inserted into the BamHI-NruI site of pACYC184 to yield pACGA. Plasmids pGEMF HDC and pGEMGR were constructed by cloning PCR-amplified *flhDC* and *grrR* genes, respectively (primers for pGEMFHDC, 5'-CGGGATCCCGAGAAAC GACGCAATCCCAA-3' and 5'-GAAGGCCTTCAATGTTGCGCCACACCG TAT-3'; for pGEMGR, 5'-AACAAATTGAAAGGAGTGAG-3' and 5'-ATCG ACATAAAAAACATACA-3'), into pGEM-T-Easy vector (Promega). Plasmids pACFHDC and pAC177FHDC were constructed by cloning a BamHI-StuI fragment of pGEMFHDC into the BamHI-NruI site of pACYC184 (9) and the BamHI-ScaI site of pACYC177 (9), respectively. Plasmids pRLFHD, pRLFGA, and pRLFIC contain PCR-amplified DNA fragments encoding transcriptional-regulatory sequences of *flhD*, *flgA*, and *flhC*, respectively, as deduced from *E. coli* and *Salmonella* sequences (10, 25, 29, 64, 76). These three plasmids were cut with KpnI and EcoRI to excise the promoter fragments, which were ligated into the corresponding sites of a promoter-probe vector, pRL124 (51). For pRLFHD, a 0.74-kbp DNA fragment including -717 to +23 relative to the *flhD* initiation codon (the region corresponding to -527 to +212 with respect to the transcriptional start site determined in the *E. coli* K-12 laboratory strain) (64) was similarly generated by PCR (5'-GGGGTACCCGAGAAACGACGCAATCC CAA-3'/5'-CGGAATCCGTCGGAGGTATGCATTATCC-3'). For pRLFGA, a 0.25-kbp DNA fragment including -182 to +24 relative to the *flgA* initiation codon was amplified (5'-CGGAATCCGACGCTACGTTTTATTGCCA-3'/5'-GGGGTACCCCTAAGGCGGCTCGAGCTTAT-3'). For pRLFIC, a 0.31-kbp DNA fragment including -281 to +35 relative to the *flhC* initiation codon was isolated (5'-GGGGTACCCATGAAATACTTGCCATGCGA-3'/5'-CGGAAT CCGCAGCGAGAGGCTGTTGGTAT-3'). All of these promoter fragments were similarly cloned into pRL124. pRLLER was described previously (33).

Analysis of proteins in culture supernatant and whole-cell lysates. Bacteria were grown in LB medium with or without antibiotics at 37°C with shaking until they reached an optical density at 600 nm of 0.8. Proteins in culture supernatants and whole-cell lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described previously

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant phenotype	Source or reference
Strains		
SKI-5142	Wild-type EHEC O157:H7 Sakai Δ(<i>lacZYA</i>)	33
SKI-5143	SKI-5142 Δ(<i>ler</i>): <i>kan</i>	33
SKI-5149	SKI-5142 Δ(<i>clpXP</i>): <i>kan</i>	33
SKI-5152	SKI-5142 Δ(<i>grrR</i>): <i>cat</i>	33
SKI-5153	SKI-5142 Δ(<i>grrA</i>): <i>cat</i>	33
SKI-5154	SKI-5142 Δ(<i>grrR-grrA</i>): <i>cat</i>	33
SKI-5156	SKI-5149 Δ(<i>grrR</i>): <i>cat</i>	33
SKI-5200	SKI-5142 Δ(<i>flhC</i>): <i>kan</i>	This study
Plasmids		
pGEM-T-Easy	TA cloning vector, Ap ^r	Promega
pGEM-self	Self-ligated pGEM-T-Easy	33
pGEMGA	pGEM-T-Easy- <i>grrA</i>	33
pGEMGR	pGEM-T-Easy- <i>grrR</i>	This study
pGEMFHDC	pGEM-T-Easy- <i>flhDC</i>	This study
pACYC184	Cloning vector; Cm ^r Tc ^r	9
pACGA	pACYC184- <i>grrA</i>	This study
pACYC177	Cloning vector; Ap ^r Km ^r	9
pAC177FHDC	pACYC177- <i>flhDC</i>	This study
pRL124	Promoter probe vector; Ap ^r	51
pRLFHD	pRL124- <i>flhD</i> promoter region	This study
pRLFGA	pRL124- <i>flgA</i> promoter region	This study
pRLFIC	pRL124- <i>flhC</i> promoter region	This study
pRLLER	pRL124- <i>ler</i> promoter region	33

(30, 32, 33). Western blotting was performed with polyclonal anti-H7 (FlhC) or anti-EspB antibodies (32) to detect flagellin or EspB, respectively, in whole-cell lysates. Binding of secondary anti-rabbit (or anti-mouse) immunoglobulin G antibody conjugated to horseradish peroxidase was detected using enhanced chemiluminescence Western blotting detection reagents (Amersham). All assays were performed in duplicate and were repeated at least three times.

Assay of β-galactosidase activity. β-Galactosidase activity was assayed as described previously (30, 31, 44). Bacteria grown in LB medium with or without antibiotics at 37°C with shaking were harvested at an optical density at 600 nm of 0.8. All assays were performed in triplicate and were repeated at least three times.

Motility assay and measurement of flagellar number. The motility of bacterial cells was measured by the spread of colonies on motility agar plates incubated for 5 to 6 h at 37°C. To count the flagella, bacterial cells grown to late logarithmic phase were fixed in formaldehyde, stained with a flagellum-staining reagent containing 0.2% Victoria blue B (Wako) as described previously (43), and examined in an Olympus BX50 microscope. For each stained sample, the number of flagella on more than 50 bacterial cells was determined.

Adhesion and fluorescent actin-staining tests. Fluorescent actin-staining tests were performed as described previously (38) with slight modifications. Briefly, HeLa cells maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum (Invitrogen) were plated onto coverslips (Fisher Scientific) in a 24-well plastic plate (Corning) at 1 × 10⁵ cells/ml and incubated for 24 h in the presence of 5% CO₂. After the HeLa cells were washed three times with DMEM without fetal bovine serum, 10⁷ EHEC cells cultured overnight at 37°C with shaking in DMEM containing 0.5% (wt/vol) glycerol with or without antibiotics were inoculated into each well, and the plastic plate was centrifuged at 1,000 × g for 5 minutes and then incubated for 1 h at 37°C in the presence of 5% CO₂. The cells were then washed three times with phosphate-buffered saline (PBS) and incubated for an additional 3 hours, after which the monolayers were washed six times with PBS, fixed in 3% paraformaldehyde for 15 min, permeabilized in 0.2% Triton X-100, and then incubated in blocking solution (Yukijirushi) for at least 1 hour. The cells were then washed twice in PBS and incubated with PBS containing 1 mg/ml bis-benzimide H33342 (Calbiochem) and rhodamine-phalloidin (Molecular Probes) for 30 min. After three washes with PBS and one with water, the coverslips were air dried and observed using an Olympus BX51. Images were captured with a digital charge-coupled-device camera (Hamamatsu). The adherence of each strain was evaluated quantitatively by plating adherent bacteria on LB agar plates

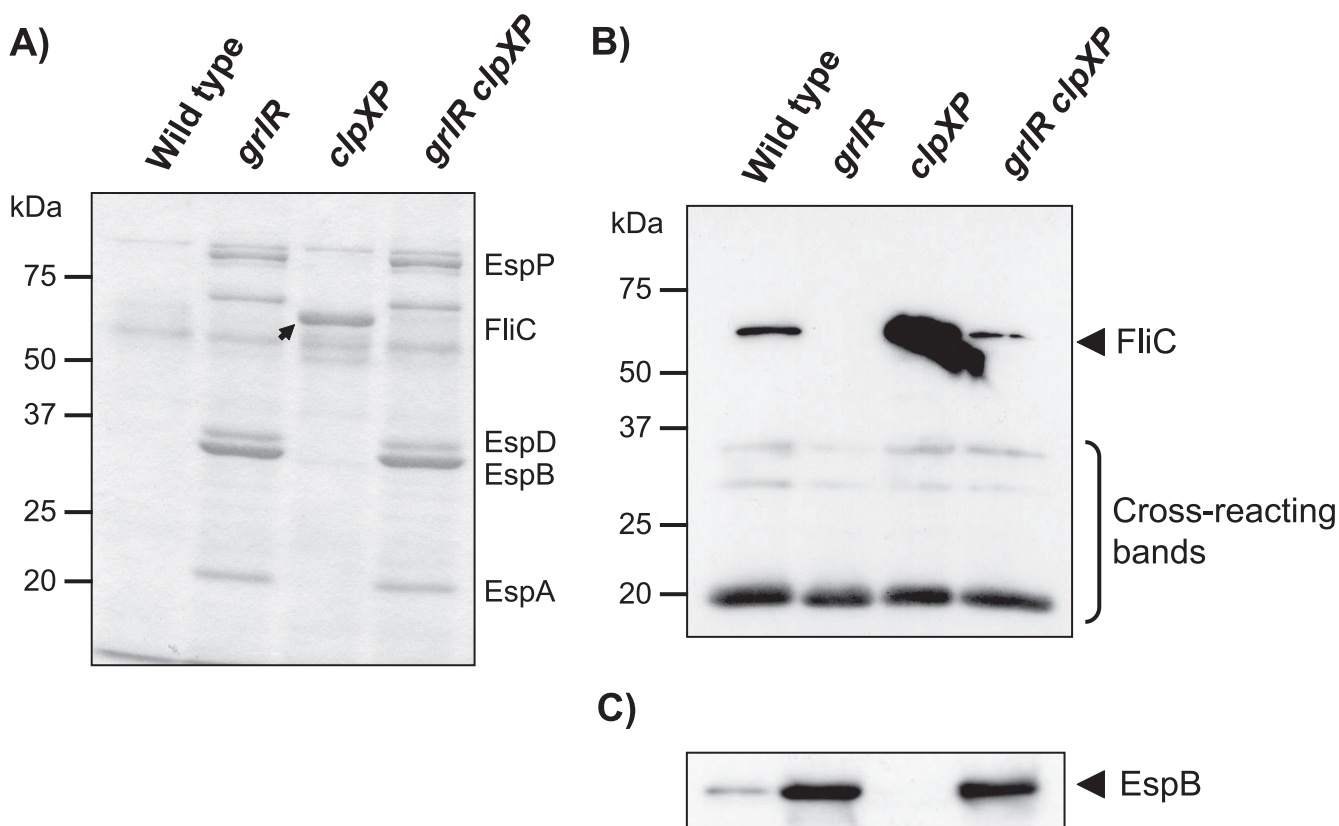


FIG. 1. Effects of *clpXP* and/or *grlR* deletions on the expression of FliC. (A) Coomassie brilliant blue-stained SDS-PAGE profiles of secreted proteins from cells cultured in LB. An arrowhead indicates the FliC band. Strains used: wild type, SKI-5142; *grlR*, SKI-5152; *clpXP*, SKI-5149; *grlR clpXP*, SKI-5156. (B and C) Western blotting analyses of (B) FliC and (C) EspB in equivalent amounts of whole-cell lysates prepared from the same strains used in panel A. Cross-reacting bands recognized by the polyclonal anti-FliC (H7) serum served as loading controls.

with ampicillin or kanamycin after incubation with 0.2% (final concentration) Triton X-100 in PBS for 20 min at room temperature. All assays were performed in duplicate and were repeated at least four times. The statistical significance was expressed as the *P* value as determined by a Student *t* test analysis.

RESULTS

The GrlR-GrIA system controls FliC expression in EHEC.

We previously reported that the ATP-dependent protease complex ClpXP positively controls LEE expression by regulating GrlR protein levels in EHEC (33). During further studies characterizing the *clpXP* mutant, we noticed that cells of the *clpXP* deletion strain secreted large amounts of protein into LB culture supernatants (Fig. 1A). The approximate molecular mass of the protein band (60 kDa), estimated by SDS-PAGE analysis was similar to that of flagellin (FliC), the component protein of the flagellar filament. Western blotting analysis using polyclonal anti-H7 (FliC) antibodies (Fig. 1B) confirmed that ClpXP in EHEC also negatively regulates FliC expression, as previously reported for *Salmonella* (71).

We further observed that the amount of FliC in culture supernatants decreased significantly in strains with a deletion of *grlR* (Fig. 1A), which encodes a negative regulator of LEE expression (13, 47). This reduction was also observed in whole-cell samples, whereas the amounts of cross-reacting protein bands were unchanged (Fig. 1B). Since steady-state levels of FliC in the wild-type strain were abolished by a *grlR* deletion

(Fig. 1B), we hypothesized that the expression of flagellar proteins is positively regulated by GrlR. In contrast, expression of LEE-encoded type III secreted translocator proteins (such as EspA, -B, and -D) in the *grlR* strain was derepressed in the same LB cultures (Fig. 1A and C), as shown in previous studies (33, 47). We also examined FliC protein levels in the same strains cultured in DMEM, conditions under which LEE expression is induced. It was found that the expression level of FliC was very low in the wild-type strain, as described previously (22, 79), and was not significantly increased even in the strain with *clpXP* deleted (data not shown).

Because induction of LEE expression in the *grlR* mutant depends on the function of GrIA (33), a positive regulator of LEE, we compared the expression level of FliC in wild-type, *grlR*, *grlA*, and *grlR grlA* strains. The FliC band was absent in the *grlR* strain but present in the *grlA* and *grlR grlA* strains (Fig. 2A). Given that *grlR* deletion upregulates the expression of GrlA, as well as other LEE-encoded proteins (13, 33, 47; our unpublished data), and that GrlR is hypothesized to interact with GrIA (11), FliC expression in the *grlR* mutant may be inhibited by GrIA. To test this hypothesis, we examined FliC expression in a strain carrying multicopy plasmids with the *grlA* gene (pGEMGA and pACGA). As predicted, FliC expression in the wild type and/or a *grlA* mutant was significantly reduced in the presence of pGEMGA (Fig. 2B) or pACGA (Fig. 2C), indicating that GrIA participates in the

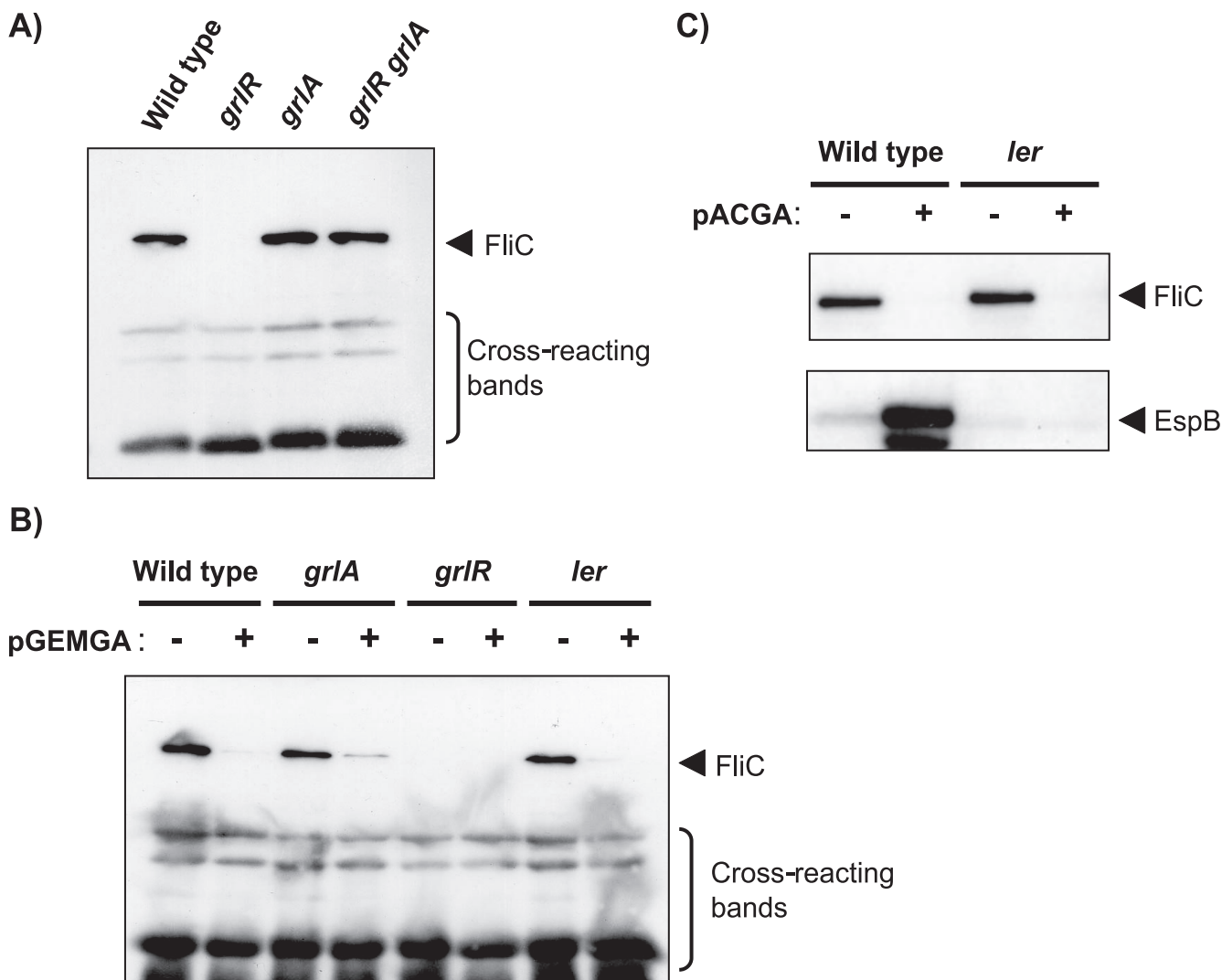


FIG. 2. (A and B) Repression of FliC expression by overproduced GrlA. Shown are Western blotting analyses of FliC prepared from whole-cell lysates containing an equivalent number of cells from the following strains: wild type, SKI-5142; *grlR*, SKI-5152; *grlA*, SKI-5153; *grlR grlA*, SKI-5154; *ler*, SKI-5143; and pGEM-self or pGEMGA (indicated by - or +, respectively, in panel B). (C) Western blotting analyses of FliC and EspB prepared from whole-cell lysates containing an equivalent number of cells from the same strains with plasmid: wild type, SKI-5142; *ler*, SKI-5143; pACYC184 or pACGA (indicated by - or +, respectively).

repression of flagellar synthesis. A similar reduction was observed in a *ler* mutant strain harboring pGEMGA (Fig. 2B) or pACGA (Fig. 2C), suggesting that unlike the requirement for Ler in the GrlA-dependent activation of LEE (13) (Fig. 2C), repression of FliC by GrlA does not require Ler function.

The GrlR-GrlA system controls the flagellar formation and function of EHEC. To examine whether the GrlR-GrlA regulatory system affects flagellar formation and function, we compared the motility phenotypes of wild-type, *grlR*, *grlA*, and *grlR grlA* EHEC strains on motility agar plates. A significant reduction in EHEC cell motility in the *grlR* strain, but not in the *grlA* or *grlR grlA* strain (Fig. 3A), indicated that GrlA negatively regulates EHEC motility in the absence of GrlR. Furthermore, because multicopy plasmids carrying *grlA*, but not *grlR*, strongly repressed motility (Fig. 3B), we concluded that GrlA-dependent FliC repression affects the flagellar function of EHEC. In

agreement with these observations, fewer flagella were stained with Victoria blue in the *grlR* mutant, with the mean number of flagella per cell being 3.1 in the wild type and 0.99 in the *grlR* mutant strain (Fig. 3C).

Transcription of the flagellar master operon is under the control of the GrlR-GrlA system. More than 50 genes are involved in the formation and function of flagella in *E. coli* and *Salmonella* (50). They are organized within more than a dozen operons and can be classified into three hierarchical classes (classes 1, 2, and 3), which together form the flagellar regulon (39, 42, 43). Class 1 is a single operon composed of the *flhD* and *flhC* genes (4, 39, 41), which are essential for the expression of all other flagellar genes (29, 48). Class 2 operons contain genes essential for the formation of the hook-basal body and the FliA-sigma factor, all of which are responsible for the transcription of class 3 genes (42, 56). The components necessary for the formation of the flagellar filament and functions

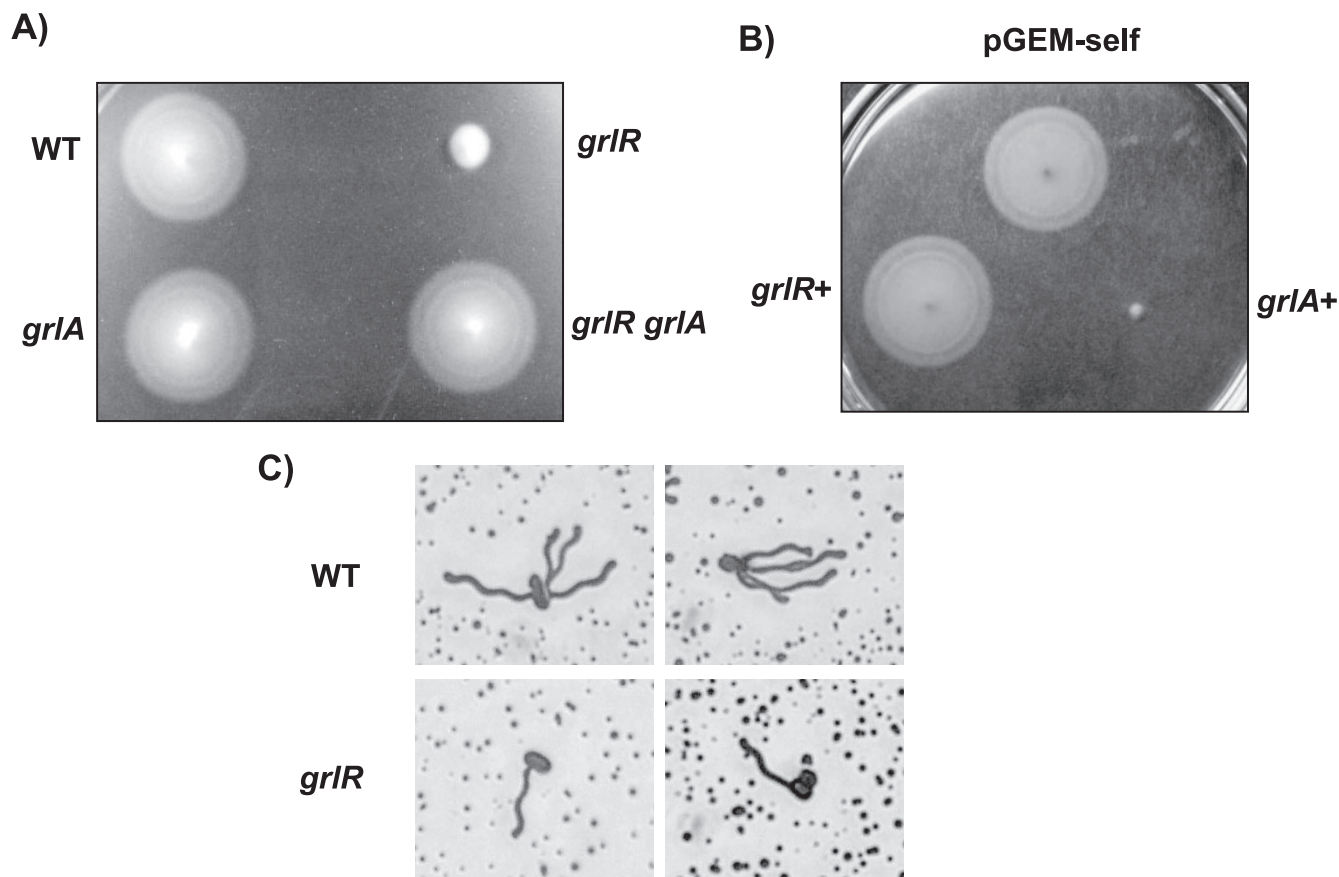


FIG. 3. Motility phenotypes of wild-type (WT), *grlR*, *grlA*, and *grlR grlA* strains of EHEC (A) and the wild-type strain with pGEM-self (control vector), pGEMGR (carrying *grlR*), or pGEMGA (carrying *grlA*) plasmid (B). (C) Cells grown in 1% Bacto-Trypton broth containing 0.5% NaCl were stained with Victoria blue as described in Materials and Methods. The following strains were used: WT, SKI-5142; *grlR*, SKI-5152; *grlA*, SKI-5153; and *grlR grlA*, SKI-5154 (A and/or C) and pGEM-self, SKI-5142 with pGEM-self; *grlR*⁺, SKI-5142 with pGEMGR; and *grlA*⁺, SKI-5142 with pGEMGA (B).

(motility and chemotaxis) are encoded by the genes belonging to class 3 operons (42). To locate a possible GrlA target site on the flagellar regulon, we measured the transcriptional activities of several flagellar promoters by fusing the transcriptional-regulatory regions of *flhD* (for measuring flagellar class 1 transcription), *flgA* (for class 2), and *fliC* (for class 3) to *lacZ* in pRL124 as described in Materials and Methods. β -Galactosidase activity mediated by each of these three promoters (on plasmids pRLFHD, pRLFGA, and pRLFIC), as well as the *ler* promoter (on pRLLER) (33), was higher than that of the promoterless vector (pRL124) in the wild-type strain (Table 2), indicating that all plasmids contained functional promoters. The activities of all the flagellar promoters were reduced in the

grlR strain and slightly increased in the *grlA* and *grlR grlA* strains relative to those in the wild type, while the activity of the *ler* promoter was inversely derepressed in the *grlR* strain but reduced in the *grlA* strain (Table 2). These results indicate that these promoters are under the control of the GrIR-GrIA system. The transcriptional activity of *flhD* in the *grlR* mutant strain was decreased only twofold compared to that in the wild type. Hence, we further examined the effects of a multicopy of *grlA* on the transcriptional activities of flagellar promoters. Although the *flgA* and *fliC* promoters were significantly reduced in the presence of multicopy *grlA* (pACGA), the *flhD* promoter in the presence of pACGA still exhibited approximately twofold-lower activity than that in the strain with

TABLE 2. GrIR-GrIA system controls promoter activity of *flhD*, *flgA*, *fliC*, and *ler* genes

Strain (no.)	β -Galactosidase activity (Miller units) (SD)				
	pRL124	pRLFHD	pRLFGA	pRLFIC	pRLLER
Wild type (SKI-5142)	15.8 (5.07)	2,670 (284)	717 (76.5)	1,720 (98.7)	7,600 (410)
<i>grlR</i> (SKI-5152)	18.8 (7.04)	1,240 (199)	87.9 (3.18)	147 (4.76)	23,800 (1,600)
<i>grlA</i> (SKI-5153)	19.3 (3.27)	3,400 (250)	869 (210)	2,130 (118)	4,390 (515)
<i>grlR grlA</i> (SKI-5154)	23.1 (5.11)	3,130 (138)	848 (87.2)	2,000 (313)	ND ^a

^a ND, not done.

TABLE 3. Promoter activities of *flhD*, *flgA*, and *fliC* genes with or without multicopy *grlA*

Strain/plasmid (no.)	β -Galactosidase activity (Miller units) (SD)			
	pRL124	pRLFHD	pRLFGA	pRLFIC
SKI-5142/pACYC184	14.6 (5.87)	5,460 (296)	608 (10.5)	1,330 (567)
SKI-5142/pACGA	13.8 (4.24)	2,620 (182)	126 (4.29)	10.7 (2.05)

pACYC184 (Table 3). Consistent with these observations, several other mutations that affect flagellar expression reduced the transcription of the *flhD* operon by 2- to 3-fold but decreased FliC expression more than 10-fold in *E. coli* K-12 strains or an EHEC O157:H7 strain (46, 61, 64, 67). Together, this evidence suggests that the reduction of the *flhD* operon observed in the *grlR* strain is due to the effect of GrlA, and the twofold reduction alone may be enough to reduce the entire activity of the flagellar regulon.

Constitutive expression of FlhD/FlhC inhibits efficient adhesion of EHEC to HeLa cells. The results presented above led us to hypothesize that repression of *flhD* transcription by GrlA may be necessary for EHEC adherence to host cells, because reduction of the flagellar regulon was observed in the *grlR* strain, a condition under which LEE expression is significantly induced (33, 47). To test this hypothesis, *flhD* and *flhC* genes cloned from the wild-type EHEC into pGEM-T-Easy or pACYC177 (designated pGEMFHDC and pAC177FHDC, respectively) were introduced into wild-type and *grlR* strains, and the effect of constitutive FlhDC expression on EHEC adhesion to HeLa cells was examined. The strains transformed with pGEMFHDC or pAC177FHDC were highly motile on motility agar and produced high levels of flagellin even when they were grown in DMEM (data not shown).

Figure 4A depicts wild-type Sakai microcolonies on cultured HeLa cells, with intense accumulations of stained actin beneath bacterial attachment sites. The *grlR* mutant cells transformed with the control vector (pGEM-self) adhered to HeLa cells more efficiently than wild-type cells with pGEM-self. Neither strain with pGEMFHDC adhered to HeLa cells or showed fluorescent actin staining, suggesting that constitutively expressed FlhD/FlhC inhibit efficient adhesion of EHEC to HeLa cells. The in vitro adherence levels of these strains were then compared by quantitative assay (Fig. 4B). The introduction of plasmid pGEMFHDC produced significantly reduced adherence in wild-type (1.9%; $P < 0.001$) and *grlR* mutant (0.93%; $P < 0.001$) strains relative to those strains with vector control (pGEM-self). We obtained the same results when the wild-type strain was transformed with pACYC177 or pAC177FHDC and used for adhesion assays (data not shown); the strain harboring pAC177FHDC did not efficiently adhere to HeLa cells. Therefore, we hypothesize that GrlA-dependent repression of the flagellar regulon is important for efficient adhesion of EHEC to host cells.

These results also suggest that constitutively expressed flagella may have a deleterious effect on efficient adhesion of EHEC to HeLa cells. To test this hypothesis, we constructed a *fliC* mutant of EHEC (designated SKI-5200) transformed with pGEM-self or pGEMFHDC and performed the same adhesion assay. The *fliC* mutant with pGEM-self showed an adhesion phenotype comparable to that of the wild-type strain car-

rying pGEM-self: constitutive expression of FlhDC reduced adhesion of EHEC to HeLa cells even in the *fliC* mutant (data not shown). These results suggest that overproduced flagellar filament is not the sole reason for the reduction of cell adhesion. Other genes under the control of FlhDC may have deleterious effects on the adhesion of EHEC to host cells.

DISCUSSION

In the present study, we demonstrate that the GrlR-GrlA regulatory system coordinately controls the expression of not only LEE genes but also the flagellar regulon in EHEC. When we compared the expression levels of FliC in several strains of EHEC, reduction of FliC expression was observed in the *grlR* mutant but not in the *grlR grlA* mutant. Additionally, overproduced GrlA from a multicopy plasmid strongly repressed FliC expression and the motility of EHEC, indicating that GrlA is responsible for reducing the expression of the flagellar regulon. Conversely, LEE expression was strongly derepressed in the *grlR* mutant and induced by the introduction of a plasmid carrying *grlA*, as reported previously (3, 13, 33, 47). Intracellular GrlR levels in wild-type EHEC have been shown to decrease gradually as culture times increase and to achieve minimum levels in stationary-phase cultures (33). Therefore, this reduction of GrlR levels, which leads to the derepression of GrlA (our unpublished data), is a key event in the inverse regulation of flagellar and LEE gene expression, as summarized in Fig. 5. Consistent with this model, maximum expression of a LEE gene transcription was observed as bacterial cells entered the stationary phase (60). As described above, flagellin expression is highly induced in *clpXP*-mutant EHEC. This may be due to the increased stability of FlhD/FlhC, as hypothesized for *Salmonella* (73). Since the protein levels of intracellular GrlR have also been regulated by ClpXP in EHEC (33), ClpXP directly and indirectly controls flagellar expression in EHEC by regulating the FlhD/FlhC and GrlR levels, respectively (Fig. 5). Other than these regulatory systems, LEE expression has been shown to be under the control of various regulatory elements: bacterial histone-like proteins, such as H-NS (8, 74), Hha (60), IHF (21), and Fis (23); the quorum-sensing system (65, 66, 68); and other factors, such as GadX (62), YhiE (GadE)/YhiF (69), BipA (24), GrvA (70), and EtrA/EivF (78). Therefore, these regulators should also be depicted properly in the regulation model in Fig. 5. Further studies integrating these regulatory networks must be done. Our results indicate that GrlA-dependent repression of flagellar synthesis does not require Ler, although this protein is a central regulator and is indispensable for the GrlA-dependent activation of LEE expression (13). To our knowledge, this is the first report that GrlA controls the expression of non-LEE genes without Ler function.

We also show that GrlA represses transcription of *flhD*, the master operon of the flagellar regulon. Although GrlA has a putative helix-turn-helix DNA-binding motif (13) and activates *ler* transcription even in an *E. coli* K-12 background (3), attempts to verify the DNA-binding activity of GrlA have been not successful (3). Therefore, the molecular mechanisms by which GrlA activates and represses *ler* and *flhD* transcription remain unknown. Since the transcription of the *flhD* operon has been shown to be under the control of multiple regulatory

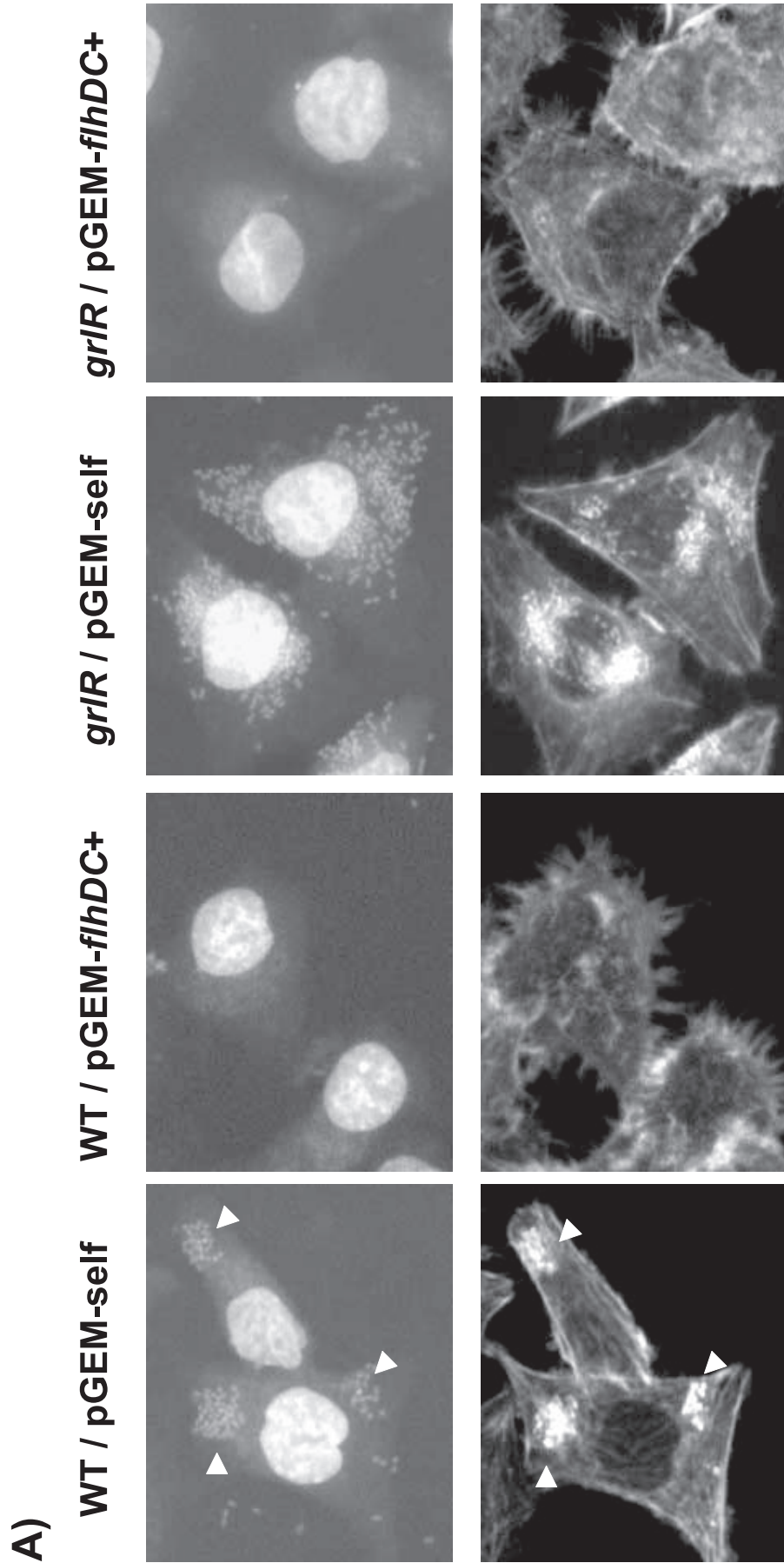


FIG. 4. Effect of constitutively expressed FlhD/FlhC on adhesion of EHEC to HeLa cells. (A) The top row was stained with bisbenzimidide to see the formation of microcolonies. The bottom row was stained with rhodamine-phalloidin to see accumulated actin under individual attached bacteria. Microcolonies or localized actin accumulations are indicated by arrowheads. The following strains were used: WT/pGEM-self; SKI-5142 with pGEM-self; WT/pGEM-FlhDC+, SKI-5142 with pGEM-self; grIR/pGEM-self; grIR/pGEM-FlhDC+, SKI-5152 with pGEM-self; grIR/pGEM-FlhDC+, SKI-5152 with pGEM-self. (B) Quantitative analysis of adhesion phenotypes of the strains used in panel A. The bars indicate the numbers of mean bacterial CFU per well. The error bars indicate standard deviations.

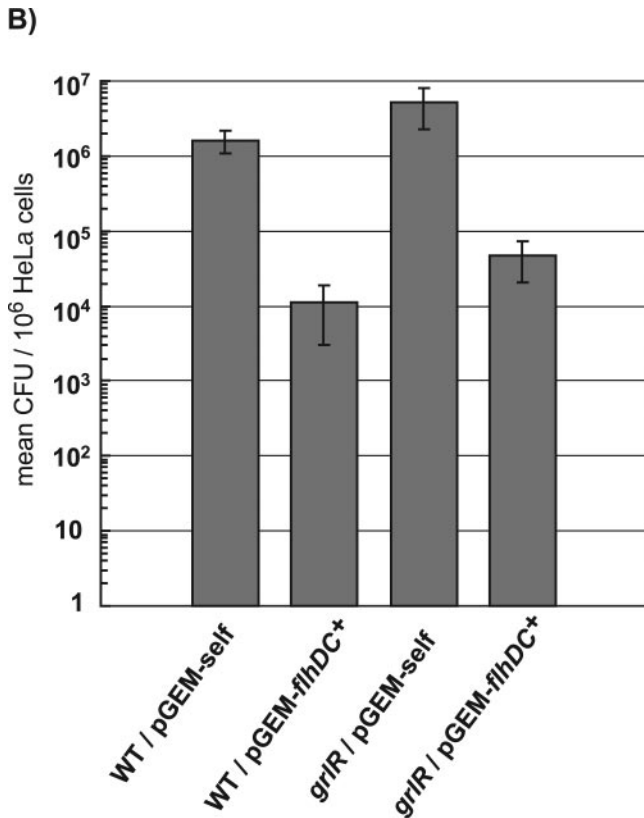


FIG. 4—Continued.

elements, such as cyclic AMP-CRP (42, 63, 64), OmpR (61), H-NS (6, 64, 76), CsrA (75), IHF (77), RcsB (19), RtsB (15), LrhA (46), and QseB (10, 67), in several bacteria, GrlA may alternatively affect the expression or activity of these regulators and thereby control the expression of the flagellar regulon in EHEC.

In a mouse model, GrlA is essential for full virulence of *C. rodentium* (13), indicating that the activation of LEE expression by GrlA is necessary for virulence. This finding raises the possibility that repression of the flagellar regulon, as well as LEE activation by GrlA, is also important for the establishment of EHEC infection. To test the effect of constitutive expression of the flagellar regulon on the LEE-mediated adhesion phenotype, we constructed EHEC strains transformed with a multicopy plasmid carrying *flhDC* and examined their adhesion phenotypes. The fact that the strain constitutively expressing FlhDC did not efficiently adhere to HeLa cells suggests that appropriate reduction of flagellar-gene expression is necessary for the adhesion phenotype of EHEC. Consistent with these observations, a mutant of *E. coli* strain K-12, MG1655, with *flhDC* deleted can colonize the mouse intestine more efficiently than the parent strain (45). In *Bordetella bronchiseptica*, a causative agent of human respiratory diseases, flagellar synthesis and virulence gene expression are coordinately regulated by a two-component regulatory system, BvgA/BvgS, in response to environmental signals. Activated BvgA, which positively regulates the transcription of several virulence genes, including adhesin and toxin, inversely represses transcription of *fliAB*, an analogue of *flhDC* (2). Constitutive ex-

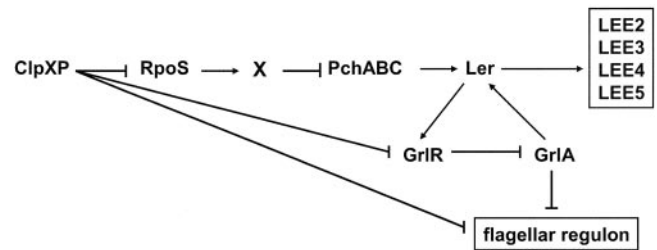


FIG. 5. Model depicting the coordinate regulation of LEE and flagellar gene expressions by the GrlR-GrlA system in EHEC. GrlA activates LEE expression via activation of *ler* transcription, whereas it inhibits transcription of the *flhD* operon in response to GrlR levels in the cell. ClpXP positively regulates LEE expression by controlling changes in GrlR and RpoS levels and directly or indirectly represses the flagellar regulon by altering the stabilities of FlhD/FlhC and GrlR. As described in the text, additional regulatory elements, such as Fis, H-NS, IHF, Hha, GadX, YhiE, YhiF, BipA, EtrA, EivF, GrvA, and quorum sensing, have also been implicated in the regulation of LEE.

pression of FrlAB in BygAS-activated cells resulted in defective tracheal colonization by *B. bronchiseptica* (1), demonstrating that *B. bronchiseptica* down-regulates FrlAB expression to circumvent the inappropriate expression of the gene under the control of FrlAB.

In the EPEC O127:H6 strain E2348/69, most of the bacterial cells attached to the actin pedestals were flagellated at an early stage of infection of HeLa cells, while many cells associated with actin pedestals at a later stage of infection did not form flagella (77). Therefore, EPEC may repress flagellar synthesis at later stages of attaching and effacing lesion formation if strain E2348/69 has the same GrlA-dependent regulatory system for reducing flagellar expression. Because purified H6 (but not H7) flagella can bind to HeLa cells (22) and nonflagellated *fliC* mutant cells of E2348/69 were less adherent and were not able to produce typical large microcolonies on HeLa cells compared with the wild-type strain (22), H6 but not H7 flagella seem to be important for initial attachment of the E2348/69 strain to HeLa cells. However, both H7 and H6 flagellins from EHEC O157:H7 strains 86-24 and E2348/69, respectively, have been shown to be the main triggers inducing the expression of a neutrophil chemoattractant, interleukin-8, in human intestinal epithelial cells (5, 79). Therefore, not only the EHEC but also the EPEC strain may need to down-regulate flagellar synthesis to circumvent the immune responses of host cells at a specific stage of their infection process. Consistent with this, inversely regulated expression of flagellar and LEE-encoded T3S systems in EPEC has been reported: BipA, a positive regulator for LEE gene expression, negatively regulates bacterial-cell motility in EPEC (24).

Given that flagellar filaments achieve lengths of more than 5 μm and that each reversibly rotating flagellum consists of approximately 20,000 subunits of flagellin (50), strict control of flagellar synthesis may be necessary to minimize the energy expense of survival in changing environments. Alternatively, repressing flagellar formation may be important to avoid steric hindrance for polymerizing the EspA filament, which is essential for translocating effector proteins into host cells (38).

When a plasmid overexpressing FlhDC was introduced into the *fliC* mutant of EHEC, the resulting transformants still did

not adhere to HeLa cells, suggesting that overproduced flagellin was not the sole reason for the reduction of EHEC cell adhesion to HeLa cells when FlhDC was constitutively expressed. This result further suggests that the function of another gene(s) under the control of FlhDC may have a deleterious effect on the adhesion of EHEC to HeLa cells. Because the flagellar regulator FlhDC and/or FliA controls the expression of several genes other than those required for flagellar formation and function in *E. coli* K-12 strains (27, 59), we can hypothesize that these nonflagellar genes under the control of FlhDC and/or FliA may inhibit EHEC cell adhesion when FlhDC is overproduced. Alternatively, these flagellar regulators may affect the expression of LEE genes. Supporting this idea, deletion of the *flhD* operon or *fliA* up-regulates the expression of several T3SS-related virulence genes, such as *yop* or *ysc* in *Yersinia enterocolitica* (7, 26).

Superstructures of the flagellar hook-basal body and needle complexes of T3SS are similar, and the genes encoding their components are phylogenetically related (40). Regulatory interactions between flagellar and other T3S systems have been reported in several bacteria (57). For example, a positive regulatory gene in the flagellar regulon, *fliZ*, also activates the expression of *Salmonella* pathogenicity island 1 genes and the virulence phenotype of *Salmonella* (31, 49). In contrast, as shown here, the positive regulator GrlA that is encoded within a LEE pathogenicity island negatively regulates flagellar expression. Additionally, putative regulators, EtrA and EivF, encoded in *E. coli* T3SS-2 (ETT2) negatively regulate LEE expression, even though ETT2 seems to be defective in the EHEC O157 Sakai strain (78). Links between T3S regulatory systems may be important for survival under various environmental conditions, including those within host cells. Future studies of coupled or mutually exclusive expression of T3S systems in various environments will further extend our understanding of the roles of these control systems in infection by each pathogenic bacterium.

ACKNOWLEDGMENTS

We thank Kazuhiro Kutsukake and Shouji Yamamoto (Okayama University) for helpful discussions and suggestions.

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Science and Technology of Japan; the Ministry of Welfare and Labor of Japan (H17-Sinkou-ippan-019); and the Japan Health Science Foundation. Yan Lu was supported by a fellowship from the Japan Health Science Foundation.

REFERENCES

- Akerley, B. J., P. A. Cotter, and J. F. Miller. 1995. Ectopic expression of the flagellar regulon alters development of the *Bordetella*-host interaction. *Cell* **80**:611–620.
- Akerley, B. J., D. M. Monack, S. Falkow, and J. F. Miller. 1992. The *bygAS* locus negatively controls motility and synthesis of flagella in *Bordetella bronchiseptica*. *J. Bacteriol.* **174**:980–990.
- Barba, J., V. H. Bustamante, M. A. Flores-Valdez, W. Deng, B. B. Finlay, and J. L. Puente. 2005. A positive regulatory loop controls expression of the locus of enterocyte effacement-encoded regulators Ler and GrlA. *J. Bacteriol.* **187**:7918–7930.
- Bartlett, D. H., B. B. Frantz, and P. Matsumura. 1988. Flagellar transcriptional activators FlhB and FlhA: gene sequences and 5' consensus sequences of operons under FlhB and FlhA control. *J. Bacteriol.* **170**:1575–1581.
- Berlin, M. C., A. Darfeuille-Michaud, L. J. Egan, Y. Miyamoto, and M. F. Kagnoff. 2002. Role of EHEC O157: H7 virulence factors in the activation of intestinal epithelial cell NF- κ B and MAP kinase pathways and the up-regulated expression of interleukin-8. *Cell. Microbiol.* **4**:635–647.
- Bertin, P., E. Terao, E. H. Lee, P. Lejeune, C. Colson, A. Danchin, and E. Collatz. 1994. The H-NS protein is involved in the biogenesis of flagella in *Escherichia coli*. *J. Bacteriol.* **43**:5537–5540.
- Bleves, S., M. N. Marenne, G. Detry, and G. R. Cornelis. 2002. Up-regulation of the *Yersinia enterocolitica* *Yop* regulon by deletion of the flagellum master operon. *flhDC*. *J. Bacteriol.* **184**:3214–3223.
- Bustamante, V. H., F. J. Santana, E. Calva, and J. L. Puente. 2001. Transcriptional regulation of type III secretion genes in enteropathogenic *Escherichia coli*: Ler antagonizes H-NS-dependent repression. *Mol. Microbiol.* **39**:664–678.
- Chang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* **134**:1141–1156.
- Clarke, M. B., and V. Sperandio. 2005. Transcriptional regulation of *flhDC* by QseBC and σ^{28} (FliA) in enterohaemorrhagic *Escherichia coli*. *Mol. Microbiol.* **57**:1734–1749.
- Creasey, E. A., R. M. Delahay, S. J. Daniell, and G. Frankel. 2003. Yeast two-hybrid system survey of interactions between LEE-encoded proteins of enteropathogenic *Escherichia coli*. *Microbiology* **149**:2093–2106.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640–6645.
- Deng, W., J. L. Puente, S. Gruenheid, Y. Li, B. A. Vallance, A. Vazquez, J. Barba, J. A. Ibarra, P. O'Donnell, P. Metalnikov, K. Ashman, S. Lee, D. Goode, T. Pawson, and B. B. Finlay. 2004. Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proc. Natl. Acad. Sci. USA* **101**:3597–3602.
- Eichelberg, K., and J. E. Galan. 2000. The flagellar sigma factor FliA (σ^{28}) regulates the expression of *Salmonella* genes associated with the centisome 63 type III secretion system. *Infect. Immun.* **68**:2735–2743.
- Ellermeier, C. D., and J. M. Slauch. 2003. RtsA and RtsB coordinately regulate expression of the invasion and flagellar genes in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **185**:5096–5108.
- Elliott, S. J., L. A. Wainwright, T. K. McDaniel, K. G. Jarvis, Y. K. Deng, L. C. Lai, B. P. McNamara, M. S. Donnenberg, and J. B. Kaper. 1998. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Mol. Microbiol.* **28**:1–4.
- Elliott, S. J., V. Sperandio, J. A. Giron, S. Shin, J. L. Mellies, L. Wainwright, S. W. Hutcheson, T. K. McDaniel, and J. B. Kaper. 2000. The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect. Immun.* **68**:6115–6126.
- Flynn, J. M., S. B. Neher, Y. I. Kim, R. T. Sauer, and T. A. Baker. 2003. Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals. *Mol. Cell* **11**:671–683.
- Francez-Charlot, A., B. Laugel, A. Van Gemert, N. Dubarry, F. Wiorowski, M. P. Castanie-Cornet, C. Gutierrez, and K. Cam. 2003. RcsCDB His-Asp phosphorelay system negatively regulates the *flhDC* operon in *Escherichia coli*. *Mol. Microbiol.* **49**:823–832.
- Frankel, G., A. D. Phillips, I. Rosenshine, G. Dougan, J. B. Kaper, and S. Knutton. 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Mol. Microbiol.* **30**:911–921.
- Friedberg, D., T. Umanski, Y. Fang, and I. Rosenshine. 1999. Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic *Escherichia coli*. *Mol. Microbiol.* **34**:941–952.
- Giron, J. A., A. G. Torres, E. Freer, and J. B. Kaper. 2002. The flagella of enteropathogenic *Escherichia coli* mediate adherence to epithelial cells. *Mol. Microbiol.* **44**:361–379.
- Goldberg, M. D., M. Johnson, J. C. Hinton, and P. H. Williams. 2001. Role of the nucleoid-associated protein Fis in the regulation of virulence properties of enteropathogenic *Escherichia coli*. *Mol. Microbiol.* **41**:549–559.
- Grant, A. J., M. Farris, P. Alefounder, P. H. Williams, M. J. Woodward, and C. D. O'Connor. 2003. Co-ordination of pathogenicity island expression by the BipA GTPase in enteropathogenic *Escherichia coli* (EPEC). *Mol. Microbiol.* **48**:507–521.
- Hanafusa, T., A. Sakai, A. Tominaga, and M. Enomoto. 1989. Isolation and characterization of *Escherichia coli* *hag* operator mutants whose *hag48* expression has become repressible by a *Salmonella* H1 repressor. *Mol. Gen. Genet.* **216**:44–50.
- Horne, S. M., and B. M. Pr ub. 2006. Global gene regulation in *Yersinia enterocolitica*: effect of FliA on the expression levels of flagellar and plasmid-encoded virulence genes. *Arch. Microbiol.* **185**:115–126.
- Ide, N., and K. Kutsukake. 1997. Identification of a novel *Escherichia coli* gene whose expression is dependent on the flagellum-specific sigma factor, FliA, but dispensable for motility development. *Gene* **199**:19–23.
- Ikebe, T., S. Iyoda, and K. Kutsukake. 1999. Structure and expression of the *fliA* operon of *Salmonella typhimurium*. *Microbiology* **145**:1389–1396.
- Ikebe, T., S. Iyoda, and K. Kutsukake. 1999. Promoter analysis of the class 2 flagellar operons of *Salmonella*. *Genes Genet. Syst.* **74**:179–183.
- Iyoda, S., and K. Kutsukake. 1995. Molecular dissection of the flagellum-

- specific anti-sigma factor, FlgM, of *Salmonella typhimurium*. Mol. Gen. Genet. **249**:417–424.
31. **Iyoda, S., T. Kamidoi, K. Hirose, K. Kutsukake, and H. Watanabe.** 2001. A flagellar gene *fljZ* regulates the expression of invasion genes and virulence phenotype in *Salmonella enterica* serovar Typhimurium. Microb. Pathog. **30**:81–90.
 32. **Iyoda, S., and H. Watanabe.** 2004. Positive effects of multiple *pch* genes on expression of the locus of enterocyte effacement genes and adherence of enterohaemorrhagic *Escherichia coli* O157:H7 to Hep-2 cells. Microbiology **150**:2357–2371.
 33. **Iyoda, S., and H. Watanabe.** 2005. ClpXP protease controls expression of the type III protein secretion system through regulation of RpoS and GrIR levels in enterohaemorrhagic *Escherichia coli*. J. Bacteriol. **187**:4086–4094.
 34. **Jarvis, K. G., J. A. Girón, A. E. Jerse, T. K. McDaniel, M. S. Donnenberg, and J. B. Kaper.** 1995. Enteropathogenic *Escherichia coli* contains a specialized secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. Proc. Natl. Acad. Sci. USA **92**:7996–8000.
 35. **Jerse, A. E., J. Yu., B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87**:7839–7843.
 36. **Jerse, A. E., and J. B. Kaper.** 1991. The *eae* gene of enteropathogenic *Escherichia coli* encodes a 94-kilodalton membrane protein, the expression of which is influenced by the EAF plasmid. Infect. Immun. **59**:4302–4309.
 37. **Kenny, B., R. DeVinney, M. Stein, D. J. Reinscheid, E. A. Frey, and B. B. Finlay.** 1997. Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. Cell **91**:511–520.
 38. **Knutton, S., T. Baldwin, P. H. Williams, and A. S. McNeish.** 1989. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohaemorrhagic *Escherichia coli*. Infect. Immun. **28**:1290–1298.
 39. **Komeda, Y.** 1986. Transcriptional control of flagellar genes in *Escherichia coli* K-12. J. Bacteriol. **168**:1315–1318.
 40. **Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galan, and S. I. Aizawa.** 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. Science **280**:602–605.
 41. **Kutsukake, K., T. Iino, Y. Komeda, and S. Yamaguchi.** 1980. Functional homology of *fla* genes between *Salmonella typhimurium* and *Escherichia coli*. Mol. Gen. Genet. **178**:59–67.
 42. **Kutsukake, K., Y. Ohya, and T. Iino.** 1990. Transcriptional analysis of the flagellar regulon of *Salmonella typhimurium*. J. Bacteriol. **172**:741–747.
 43. **Kutsukake, K., and T. Iino.** 1994. Role of FlhA-FlgM regulatory system on the transcriptional control of the flagellar regulon and flagella formation in *Salmonella typhimurium*. J. Bacteriol. **176**:3598–3605.
 44. **Kutsukake, K., S. Iyoda, K. Ohnishi, and T. Iino.** 1994. Genetic and molecular analyses of the interaction between the flagellum-specific sigma and anti-sigma factors in *Salmonella typhimurium*. EMBO J. **13**:4568–4576.
 45. **Leatham, M. P., S. J. Stevenson, E. J. Gauger, K. A. Krogfelt, J. J. Lins, T. L. Haddock, S. M. Autieri, T. Conway, and P. S. Cohen.** 2005. Mouse intestine selects nonmotile *flhDC* mutants of *Escherichia coli* MG1655 with increased colonizing ability and better utilization of carbon sources. Infect. Immun. **73**:8039–8049.
 46. **Lehnen, D., C. Blumer, T. Polen, B. Wackwitz, V. F. Wendisch, and G. Umden.** 2002. LrhA as a new transcriptional key regulator of flagella, motility and chemotaxis genes in *Escherichia coli*. Mol. Microbiol. **45**:521–532.
 47. **Lio, C.-W. J., and W.-J. Syu.** 2004. Identification of a negative regulator for the pathogenicity island of enterohaemorrhagic *Escherichia coli* O157:H7. J. Biomed. Sci. **11**:855–863.
 48. **Liu, X., and P. Matsumura.** 1994. The FlhD/FlhC complex, a transcriptional activator of the *Escherichia coli* flagellar class II operons. J. Bacteriol. **176**:7345–7351.
 49. **Lucas, R. L., C. P. Lostro, C. C. DiRusso, M. P. Spector, B. L. Wanner, and C. A. Lee.** 2000. Multiple factors independently regulate *hilA* and invasion gene expression in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **182**:1872–1882.
 50. **Macnab, R. M.** 1996. Flagella and motility, p. 123–146. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
 51. **Malo, M. S., and R. E. Loughlin.** 1988. Promoter-detection vectors for *Escherichia coli* with multiple useful features. Gene **64**:207–215.
 52. **McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper.** 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc. Natl. Acad. Sci. USA **92**:1664–1668.
 53. **McDaniel, T. K., and J. B. Kaper.** 1997. A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. Mol. Microbiol. **23**:399–407.
 54. **Mellies, J. L., S. J. Elliott, V. Sperandio, M. S. Donnenberg, and J. B. Kaper.** 1999. The Per regulon of enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). Mol. Microbiol. **33**:296–306.
 55. **Nataro, J. P., and J. B. Kaper.** 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. **11**:142–201.
 56. **Ohnishi, K., K. Kutsukake, H. Suzuki, and T. Iino.** 1990. Gene *flhA* encodes an alternative sigma factor specific for flagellar operons in *Salmonella typhimurium*. Mol. Gen. Genet. **221**:139–147.
 57. **Ottmann, K. M., and J. F. Miller.** 1997. Roles for motility in bacterial-host interactions. Mol. Microbiol. **24**:1109–1117.
 58. **Porter, M. E., P. Mitchell, A. Free, D. G. Smith, and D. L. Gally.** 2005. The LEE1 promoters from both enteropathogenic and enterohaemorrhagic *Escherichia coli* can be activated by PerC-like proteins from either organism. J. Bacteriol. **187**:458–472.
 59. **Prüß, B. M., X. Liu, H. Hendrickson, and P. Matsumura.** 2001. FlhD/FlhC regulated promoters analyzed by gene array and *lacZ* gene fusions. FEMS Microbiol. Lett. **197**:91–97.
 60. **Sharma, V. K., and R. L. Zuerner.** 2004. Role of *hha* and *ler* in transcriptional regulation of the *esp* operon of enterohaemorrhagic *Escherichia coli* O157:H7. J. Bacteriol. **186**:7290–7301.
 61. **Shin, S., and C. Park.** 1995. Modulation of flagellar expression in *Escherichia coli* by acetyl phosphate and the osmoregulator OmpR. J. Bacteriol. **177**:4696–4702.
 62. **Shin, S., M. P. Castanic-Cornet, J. W. Foster, J. A. Crawford, C. Brinkley, and J. B. Kaper.** 2001. An activator of glutamate decarboxylase genes regulates the expression of enteropathogenic *Escherichia coli* virulence genes through control of the plasmid-encoded regulator Per. Mol. Microbiol. **41**:1133–1150.
 63. **Silverman, M., and M. Simon.** 1974. Characterization of *Escherichia coli* flagellar mutants that are insensitive to catabolite repression. J. Bacteriol. **120**:1196–1203.
 64. **Soutourina, O., A. Kolb, E. Krin, C. Laurent-Winter, S. Rimsky, A. Danchin, and P. Bertin.** 1999. Multiple control of flagellum biosynthesis in *Escherichia coli*: role of H-NS protein and the cyclic AMP-catabolite activator protein complex in transcription of the *flhDC* master operon. J. Bacteriol. **181**:7500–7508.
 65. **Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper.** 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohaemorrhagic and enteropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci. USA **96**:15196–15201.
 66. **Sperandio, V., C. C. Li, and J. B. Kaper.** 2002. Quorum-sensing *Escherichia coli* regulator A: a regulator of the LysR family involved in the regulation of the locus of enterocyte effacement pathogenicity island in enterohaemorrhagic *E. coli*. Infect. Immun. **70**:3085–3093.
 67. **Sperandio, V., A. G. Torres, and J. B. Kaper.** 2002. Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. Mol. Microbiol. **43**:809–821.
 68. **Sperandio, V., A. G. Torres, B. Jarvis, J. P. Nataro, and J. B. Kaper.** 2003. Bacteria-host communication: the language of hormones. Proc. Natl. Acad. Sci. USA **100**:8951–8956.
 69. **Tatsuno, I., K. Nagano, K. Taguchi, L. Rong, H. Mori, and C. Sasakawa.** 2003. Increased adherence to Caco-2 cells caused by disruption of the *yhiE* and *yhiF* genes in enterohaemorrhagic *Escherichia coli* O157:H7. Infect. Immun. **71**:2598–2606.
 70. **Tobe, T., H. Ando, H. Ishikawa, H. Abe, K. Tashiro, T. Hayashi, S. Kuhara, and N. Sugimoto.** 2005. Dual regulatory pathways integrating the RcsC-RcsD-RcsB signalling system control enterohaemorrhagic *Escherichia coli* pathogenicity. Mol. Microbiol. **58**:320–333.
 71. **Tomoyasu, T., T. Ohkishi, Y. Ukyo, A. Tokumitsu, A. Takaya, M. Suzuki, K. Sekiya, H. Matsui, K. Kutsukake, and T. Yamamoto.** 2002. The ClpXP ATP-dependent protease regulates flagellum synthesis in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **184**:645–653.
 72. **Tomoyasu, T., A. Takaya, Y. Handa, K. Karata, and T. Yamamoto.** 2005. ClpXP controls the expression of LEE genes in enterohaemorrhagic *Escherichia coli*. FEMS Microbiol. Lett. **253**:59–66.
 73. **Tomoyasu, T., A. Takaya, E. Isogai, and T. Yamamoto.** 2003. Turnover of FlhD and FlhC, master regulator proteins for *Salmonella* flagellum biogenesis, by the ATP-dependent ClpXP protease. Mol. Microbiol. **48**:443–452.
 74. **Umanski, T., I. Rosenshine, and D. Friedberg.** 2002. Thermoregulated expression of virulence genes in enteropathogenic *Escherichia coli*. Microbiology **148**:2735–2744.
 75. **Wei, B. L., A.-M. Brun-Zinkernagel, J. W. Simecka, B. M. Prüß, P. Babitzke, and T. Romeo.** 2001. Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. Mol. Microbiol. **40**:245–256.
 76. **Yanagihara, S., S. Iyoda, K. Ohnishi, T. Iino, and K. Kutsukake.** 1999. Structure and transcriptional control of the flagellar master operon of *Salmonella typhimurium*. Genes Genet. Syst. **74**:105–111.
 77. **Yona-Nadler, C., T. Umanski, S. Aizawa, D. Friedberg, and I. Rosen-**

- shine. 2003. Integration host factor (IHF) mediates repression of flagella in enteropathogenic and enterohaemorrhagic *Escherichia coli*. *Microbiology* **149**:877–884.
78. Zhang, L., R. R. Chaudhuri, C. Constantinidou, J. L. Hobman, M. D. Patel, A. C. Jones, D. Sarti, A. J. Roe, I. Vlisidou, R. K. Shaw, F. Falciani, M. P. Stevens, D. L. Gally, S. Knutton, G. Frankel, C. W. Penn, and M. J. Pallen. 2004. Regulators encoded in the *Escherichia coli* type III secretion system 2 gene cluster influence expression of genes within the locus for enterocyte effacement in enterohemorrhagic *E. coli* O157:H7. *Infect. Immun.* **72**:7282–7293.
79. Zhou, X., J. A. Giron, A. G. Torres, J. A. Crawford, E. Negrete, S. N. Vogel, and J. B. Kaper. 2003. Flagellin of enteropathogenic *Escherichia coli* stimulates interleukin-8 production in T84 cells. *Infect. Immun.* **71**: 2120–2129.