ClpXP Protease Controls Expression of the Type III Protein Secretion System through Regulation of RpoS and GrlR Levels in Enterohemorrhagic *Escherichia coli*

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Expression of the type III protein secretion system (TTSS), encoded in the locus of enterocyte effacement (LEE) of enterohemorrhagic *Escherichia coli* (EHEC), has been shown to be controlled by various regulators. In a search for additional regulatory genes, we identified a DNA fragment containing *clpX* and *clpP* that has a positive regulatory effect on LEE expression in EHEC O157. The expression of LEE-encoded Esp proteins was significantly reduced in a *clpXP* deletion mutant. Deletion of *grlR*, a negative regulatory gene within LEE, markedly increased LEE expression even in the *clpXP* mutant. To verify the regulatory mechanism of GrlR expression, a chromosomal epitope-tagged allele of *grlR* (*grlR-FLAG*) was constructed. GrlR-FLAG expression was increased significantly in the *clpXP* deletion mutant, suggesting that the GrlR level is under the control of ClpXP, and this regulation is critical for the ClpXP-dependent expression of LEE in EHEC. Deletion of *rpoS*, the gene encoding a stationary-phase-inducing sigma factor that is a substrate for ClpXP protease, partially restored LEE expression in the *clpXP* mutant. A multicopy plasmid carrying *rpoS* strongly repressed expression of Esp proteins, suggesting that positive regulation by ClpXP is partially mediated through a negative effect of RpoS on LEE expression. We also found that *rpoS* deletion induces transcription of *pchA*, which encodes one of the positive regulators for LEE expression in EHEC. These results suggest that ClpXP controls expression of LEE through the regulation of RpoS and GrlR levels in EHEC.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are life-threatening human pathogens and cause hemorrhagic colitis, bloody diarrhea, and hemolytic uremic syndrome (40). EHEC is a member of the attaching and effacing pathogens (40, 56), a group that includes enteropathogenic *E. coli* (EPEC) (39) and the mouse pathogen *Citrobacter rodentium* (45). The attaching/effacing lesion on intestinal epithelial cells is characterized by destruction of microvilli and formation of a pedestal-like structure, triggered by rearrangement of cytoskeletal proteins (30, 42). The genes essential for causing the attaching/effacing lesion are encoded in a pathogenicity island designated the locus of enterocyte effacement (LEE). LEE consists of more than 40 genes organized into five major operons, designated LEE1 to LEE5 (6, 8, 43). LEE1, -2 and -3 operons contain mostly genes encoding the structural and auxiliary proteins necessary for formation of a dedicated type III protein secretion system (TTSS) (22). The LEE4 operon encodes several secreted proteins (e.g., EspA, EspB, EspD, and EspF), all of which are secreted through TTSS (28, 29, 34, 37). The LEE5 operon encodes an adhesion factor, designated intimin (23, 24), and Tir, a protein that is also translocated through TTSS and acts as a receptor for intimin at the host cell membrane (27).

A transcriptional activator, Ler, is encoded by the first gene of the LEE1 operon and is essential for the expression of almost all LEE genes (9, 38). Deng et al. (5) reported that LEE-encoded Orf10 (GrlR) and Orf11 (GrlA) are positive and negative regulators, respectively. Among regulatory proteins encoded outside LEE, nucleoid-associated proteins such as Fis (10), H-NS (2, 57), integration host factor (9), and Hha (46) have been shown to be involved in positive or negative control of LEE expression. Several quorum-sensing-related genes also are important for regulation of LEE expression (25, 49, 50). Other regulatory factors such as YhiE (GadR), YhiF, YhiX (GadE), EtrA, EivF, and BipA are involved in regulation of LEE gene expression in EPEC and/or EHEC (15, 47, 52, 61).

Although most of these regulatory mechanisms are thought to be common in both EHEC and EPEC, there are some differences in LEE regulation between these two organisms. For example, the EPEC adherence factor plasmid, which is widely distributed in EPEC strains (48, 51) but is not found in EHEC, contains *perA*, *B*, and *C* (11), also designated *bfpT*, *V*, and *W* (53). The functions of *perA* and *perC* have been found to be important for full activation of LEE genes (11, 38, 41). We previously reported that *perC* homologues A, B, and C (renamed *pchA*, *B*, and *C*) were identified by screening an EHEC O157 genomic library, and that *pchA*, *B*, and *C* positively regulate LEE transcription in EHEC and are essential for EHEC adhesion to HEp-2 cells (20).

In the present study, we have identified *clpX* and *clpP* as positive regulatory genes for LEE expression in EHEC O157 and found that this positive regulation partially depends on negative regulation by RpoS, a stationary-phase sigma factor and a substrate for the ClpXP protease. We further investigated ClpXP down-regulation of expression of the LEE-encoded negative regulator GrlR in strains with or without *rpoS* deletion. These results suggest that RpoS and GrlR are involved in the regulation of ClpXP-dependent expression of LEE in EHEC.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant phenotype	Source or reference
Strains		
EDL933-1	EDL933 Δ(ler)::kan	20
EDL933-5141	EDL933 Δ(lac)	20
Sakai	Wild-type EHEC O157:H7 (same as RIMD0509952)	16
SKI-5142	Sakai Δ(lacZYA)	This study
SKI-5143	SKI-5142 Δ(ler)::kan	This study
SKI-5144	SKI-5142 Δ(pchA)::kan	This study
SKI-5147	SKI-5142 Δ(clpX)::kan	This study
SKI-5148	SKI-5142 Δ(clpP)::kan	This study
SKI-5149	SKI-5142 Δ(clpXP)::kan	This study
SKI-5151	SKI-5142 Δ(rpoS)::cat	This study
SKI-5152	SKI-5142 Δ(grlR)::cat	This study
SKI-5153	SKI-5142 Δ(grlA)::cat	This study
SKI-5154	SKI-5142 Δ(grlRA)::cat	This study
SKI-5155	SKI-5149 Δ(rpoS)::cat	This study
SKI-5156	SKI-5149 Δ(grlR)::cat	This study
SKI-5180	SKI-5142 grlR::FLAG Km^r	This study
SKI-5181	SKI-5180 Δ(ler)::cat	This study
SKI-5182	SKI-5180 Δ(clpXP)::cat	This study
SKI-5183	SKI-5180 Δ(rpoS)::cat	This study
SKI-5188	SKI-5183 Km^s	This study
SKI-5189	SKI-5188 Δ(clpXP)::kan	This study
SKI-5190	SKI-5142 grlA::FLAG Km^r	This study
SKI-5191	SKI-5190 Δ(ler)::cat	This study
SKI-5192	SKI-5190 Δ(clpXP)::cat	This study
SKI-5193	SKI-5190 Δ(rpoS)::cat	This study
Plasmids		
pKD3, pKD4, pKD13	Template plasmid for lambda Red recombination system, Ap^r	4
pKD46	Red recombinase expression plasmid, Ap^r	4
pCP20	FLP recombinase expression plasmid, Ap^r	4
pSUB11	Template plasmid for FLAG epitope tagging, Ap^r	57
pLEE19	pSSVI215-LEE, espB::Tn3-lacZYA Km^r Ap^r	20
pGEM-T-Easy	TA cloning vector, Ap^r	Promega
pGEM-self	Self-ligated pGEM-T-Easy	A. Iguchi, unpublished
pGEMLER	pGEM-T-Easy-ler	20
pGEMRS	pGEM-T-Easy-rpoS	This study
pGEMGA	pGEM-T-Easy-grlA	This study
pGEMPA	pGEM-T-Easy-pchA	This study
pACYC184	Cloning vector, Cm^r Tc^r	3
pSPH4	pACYC184 carrying a 6.29-kbp SphI fragment containing clpP and clpX from EDL933-1	This study
pACXP	pACYC184-clpXP	This study
pRL124	Promoter probe vector, Ap^r	36
pRLLER	pRL124-ler promoter region	This study
pRLSL	pRL124-sepL promoter region	This study
pRLPA	pRL124-pchA promoter region	This study

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are summarized in Table 1. The wild-type EHEC O157:H7 strain used was Sakai (16). *E. coli* K-12 strain JM109 (60) was used for DNA procedures. Unless otherwise specified, bacteria were grown in Luria broth (LB). Antibiotics were added, as required, at these final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; and 5-bromo-4-chloro-3-indolyl-β-ᴅ-galactopyranoside (X-Gal), 50 μg/ml.

DNA procedures. Standard DNA procedures, including DNA sequencing and PCR, were performed as described previously (17–19, 33).

Shotgun cloning. A genomic library of strain EDL933-1 (a *ler* derivative of wild-type EHEC O157 strain EDL933) (20) was screened to identify regulatory genes for LEE expression in EHEC O157 as described previously (20).

Construction of plasmids. The reporter plasmid pLEE19 and ler-expressing plasmid pACLER were described previously (20). Plasmids pACXP, pGEMGA, pGEMPA, and pGEMRS were constructed by cloning amplified PCR fragments into pACYC184 (3) or pGEM-T-Easy (Promega). The DNA sequences of the PCR primers used in these studies are shown in Table 2.

To construct pACXP, a 2.31-kbp DNA fragment carrying *clpX* and *clpP* (including 244 bp upstream from the *clpP* initiation codon) that had been amplified

with CLPBHI/CLPSPH primers was digested with BamHI and SphI and inserted into the corresponding sites of pACYC184. To construct pGEMGA, a 0.52-kbp DNA fragment containing the *grlA* sequence (including 49 bp upstream from the *grlA* initiation codon) that had been amplified with ORF11BHI/ORF11STU primers was cloned into pGEM-T-Easy. To construct pGEMPA, a 0.41-kbp DNA fragment containing the *pchA* sequence (including 38 bp upstream from the *pchA* initiation codon) that had been amplified with PERC4STU/PERC3BHI primers (20) was cloned into pGEM-T-Easy. To construct pGEMRS, a 1.08-kbp DNA fragment containing the *rpoS* sequence (including 62 bp upstream from the *rpoS* initiation codon) that had been amplified with RPOSSTU/RPOSBHI primers was cloned into pGEM-T-Easy. The transcriptional direction of *grlA*, *pchA*, and *rpoS* was the same as that of the *lac* promoter in pGEM-T-Easy.

Plasmids pRLLER, pRLSL, and pRLPA carry PCR-amplified DNA fragments containing the putative transcriptional regulatory regions of *ler*, *sepL*, and *pchA*, respectively, inserted into the promoter-probe vector pRL124 (36). To construct pRLLER, a 0.98-kbp DNA fragment (−904 to +80 relative to the *ler* initiation codon) that had been amplified with LERKPN (20) and LERERI2 primers was digested with KpnI and EcoRI and inserted into the corresponding sites of pRL124. To obtain pRLSL, a 0.69-kbp DNA fragment (−488 to +205

TABLE 2. Oligonucleotide primers used in this study[a]

Primer	Nucleotide sequence (5′-3′)
LACIP1	GCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAATGTGTAGGCTGGAGCTGCTTC
LACAP2	CATATCGGTAAATAGCTTGCCTGCTTTTATTCTTTCTGTCATCGACATGTCATATGAATATCCTCCTTAGT
CLPPP1	AGGTTACAATCGGTACAGCAGGTTTTTTCAATTTTATCCAGGAGACGGAAGTGTAGGCTGGAGCTGCTTC
CLPPP2	AGCGTTGTGCCGCCCTGGATAAGTATAGCGGCACAGTTGCGCCTCTGGCCATATGAATATCCTCCTTAGT
CLPXP1	CATTTGCGTCGTGTGCGGCGCACAAAGAACAAAGAAGGAGGTTTTGACCCGTGTAGGCTGGAGCTGCTTC
CLPXP2	GGAGATAAAATCCCCCCTTTTTGGTTAACTAATTGTATGGGAATGGTTAACATATGAATATCCTCCTTAGT
RPOSP52	AAGAAAAAGGCCAGCCTCGCTTGAGACTGGCCTTTCTGACAGATGCTTACCTTACGCCCCGCCCTGCCAC
RPOSP6	AGGCTTTTGCTTGAATGTTCCGTCAAGGGATCACGGGTAGGAGCCACCTTCTACCTGTGACGGAAGATCA
ORF10P5	TTTTAAATAAACTTGTGGCATTCCTGTGTTTTTAATAACTAATGGCTTACGCCCCGCCCTGCCAC
ORF10P6	ATTATGAAGGATGGCATCTATAGCATTATATTTATTAGCAATGAACTACCTGTGACGGAAGATCA
ORF11P5	ACTCTCCTTTTTCCGCCTCATGATCATTTCGTTCCAGATATTAGACTTACGCCCCGCCCTGCCAC
ORF11P6	GAATCTAAAAATAAAAATGGCGACTATGTAATTCCTGACTCAGTACTACCTGTGACGGAAGATCA
ORF10P5FF	CCATTAGTTATTAAAAACACAGGAATGCCACAAGTTTATTTAAAAGACTACAAAGACCATGACGG
ORF10FR	TCTTTTTATTTGATAAATAAATCGACATAAAAAACATACATAAAACATATGAATATCCTCCTTAG
ORF11P5FF	TCTAATATCTGGAACGAAATGATCATGAGGCGGAAAAAGGAGAGTGACTACAAAGACCATGACGG
ORF11FR	GAGAAAAAGGCTTACCCTGGAAAACAAAACCCTTAAATATAGCTTCATATGAATATCCTCCTTAG
CLPBHI	CGGGATCCCGTTTACGCAGCATAACGCGCT
CLPSPH	CATGCATGCATGATCCCCCCTTTTTGGTTAAC
ORF11BHI	CGGGATCCCGTGTATGTTTTTTATGTCGAT
ORF11STU	GAAGGCCTTCACCCTAAATATAGCTTTAT
RPOSSTU	GAAGGCCTTCACTGGCCTTTCTGACAGATG
RPOSBHI	CGGGATCCCGATCGGCGGAACCAGGCTTTT
LERKPN	GGGGTACCCCTCTTTATAGAGGGGCGCATT
LERERI2	CGGAATTCCGTGCTTCCTGCTGTAGAACTG
LEE4KPN	GGGGTACCCCGTCGACTTTTGGCAGAGAAC
LEE4ERI	CGGAATTCCGTGCGATATCCCAGGCTTAGC
PERCKPN	GGGGTACCCCGTACCCAGTGTATACCGTGA
PERCERI	CGGAATTCCGTTTCTCCAGACATTCTGCCA

[a] Priming sites on pKD3, pKD4, pKD13, and pSUB11 are underlined. Restriction sites are shown in bold type.

relative to the *sepL* initiation codon) that had been amplified with LEE4KPN/LEE4ERI primers was cloned into pRL124. To construct pRLPA, a 0.52-kbp DNA fragment (−489 to + 36, relative to the *pchA* initiation codon) that had been amplified with PERCKPN/PERCERI primers was cloned into pRL124. Though the exact promoter structures are still not known for the *pchA* gene, we believe that all pRL124-derived plasmids contain functional promoters, because cells carrying these plasmids had much higher levels of β-galactosidase than cells carrying pRL124 (Table 3, data not shown). Plasmid pGEM-self is self-ligated pGEM-T-Easy (A. Iguchi, unpublished).

Construction of mutant strains. A one-step inactivation method (4) was used to construct mutant strains. PCR products containing kanamycin or chloramphenicol resistance cassettes flanked by 45 or 50 bp of homology to the 5′ and 3′ termini of each gene, were electroporated into competent cells of parent strains carrying pKD46. To construct strain SKI-5142, PCR fragments were amplified from pKD4 using primers LACIP1 and LACAP2. The resultant kanamycin-resistant colonies were examined for the Lac-negative phenotype on LB agar plates containing X-Gal. The FRT-flanked kanamycin cassette was removed after transformation with pCP20 as described previously (4).

We confirmed that the expression levels of LEE-encoded Esp proteins in wild-type Sakai and SKI-5142 cells were comparable (data not shown). To construct strains SKI-5143 and SKI-5144, PCR products amplified from pKD13 and pKD4 using primers LERP1/LERP4 and PERCP1/PERCP2, respectively, were electroporated into strain SKI-5142 carrying pKD46, as described previously (20). To construct strains SKI-5147, SKI-5148, and SKI-5149, PCR products amplified from pKD4 with primers CLPXP1/CLPXP2, CLPPP1/CLPPP2, and

CLPPP1/CLPXP2, respectively, were electroporated into SKI-5142 carrying pKD46. To construct strains SKI-5151, SKI-5152, SKI-5153, and SKI-5154, PCR products amplified from pKD3 with primers RPOSP52/RPOSP6, ORF10P5/ORF10P6, ORF11P5/ORF11P6, and ORF11P5/ORF10P6, respectively, were electroporated into SKI-5142 carrying pKD46. To construct SKI-5155 and SKI-5156, PCR products amplified from pKD3 with primers RPOSP52/RPOSP6 and ORF10P5/ORF10P6, respectively, were electroporated into SKI-5149 carrying pKD46.

To tag the chromosomal *grlR* and *grlA* genes with the DNA sequence encoding triple FLAG epitopes, the modified lambda Red recombination system was used as described previously (58). Briefly, forward primers ORF10P5FF and ORF11P5FF containing the 3′-terminal sequence (without a stop codon) of *grlR* and *grlA*, respectively, followed by a sequence encoding triple FLAG epitopes, and reverse primers ORF10FR and ORF11FR, corresponding to a chromosomal region downstream from *grlR* and *grlA*, respectively, were used for PCR amplification. PCR products containing the kanamycin resistance cassette were amplified from pSUB11 with each primer set as described above and electroporated into SKI-5142 carrying pKD46 to construct strains SKI-5180 and SKI-5190. To construct strains SKI-5181 (or SKI-5191), SKI-5182 (or SKI-5192), and SKI-5183 (or SKI-5193), the parent strain SKI-5180 (or SKI-5190) carrying pKD46 was electroporated with PCR products amplified from pKD3 with primers LERP5/LERP6 (20), CLPXP1/CLPPP2, and RPOSP52/RPOSP6, respectively. To construct strain SKI-5188, a kanamycin-sensitive derivative of SKI-5183, the FRT-flanked kanamycin cassette was removed after transformation with pCP20 as described previously (4). To construct strain SKI-5189, PCR products amplified from pKD4 with CLPPP1/CLPXP2 primers were electroporated into SKI-5188 carrying pKD46.

All the intact and mutant loci described above were verified by PCR and/or DNA sequence analysis (data not shown).

β-Galactosidase assay. β-Galactosidase activity was assayed as described previously (17, 20). Bacteria were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen) supplemented with 0.5% glycerol and appropriate antibiotics, at 37°C with shaking until they reached an optical density at 600 nm of 0.6. To evaluate the effect of an *rpoS* deletion on *pchA* transcription, stationary-phase cultures, grown as above to an optical density at 600 nm of 1.0, were used. All assays were done in triplicate and repeated at least three times.

Analysis of proteins in culture supernatants and whole-cell lysates. Proteins in culture supernatants and whole-cell lysates from stationary-phase cultures,

TABLE 3. Effect of *clpXP* deletion on transcription of LEE regulator genes

Strain	β-Galactosidase activity, Miller units[a] (SD)		
	pRL124	pRLLER	pRLPA
SKI-5142	63.7 (4.74)	43,100 (4,510)	12,500 (1,280)
SKI-5149	56 (5.43)	11,100 (1,190)	5,950 (499)

[a] β-Galactosidase activity was measured in late-log-phase cells grown in DMEM plus 0.5% glycerol.

grown as above, were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting as described previously (20). Western blotting was performed with polyclonal anti-EspB (20) or monoclonal anti-FLAG M2 (SIGMA) antibodies to probe EspB or FLAG tag, respectively. Binding of secondary anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase was detected using ECL Western blotting detection reagents (Amersham). All assays were performed in duplicate and repeated at least three times. The quantification of Orf10-FLAG levels in log-phase and stationary-phase cultures of strains with or without *clpXP* deletion was performed as follows: both spectinomycin (200 μg/ml) and tetracycline (20 μg/ml) were added to late-log-phase (5 h after initiation of culture growth) and stationary-phase (9 h after initiation of culture growth) cultures of bacteria to inhibit further protein synthesis. Proteins in whole-cell lysates sampled at 0.5, 1, 2, and 4 h after addition of antibiotics were probed with anti-FLAG antibody as described above. All assays were performed in duplicate and repeated at least three times.

RESULTS

ClpXP protease positively regulates expression of LEE-encoded TTSS. In our previous study, the *pchB* gene was identified by shotgun cloning as one of the positive regulatory gene for LEE expression (20). Using the same strategy, another putative LEE regulator was found on a 6.29-kbp SphI fragment of pACYC184 (designated plasmid pSPH4). Introduction of pSPH4 into EDL933-5141 resulted in about a twofold increase in *espB-lacZ* expression on plasmid pLEE19 (data not shown). Sequence analysis showed that pSPH4 contains intact *clpX* and *clpP* genes (data not shown, Table 1), which encode a heteromultimeric ATP-dependent protease.

To examine the effect of ClpXP on LEE expression, we isolated *clpXP* mutants. For this purpose, Sakai was used as the wild-type EHEC O157 strain because of the availability of sequence information for its *pchA* and *pchB* genes, which are important for positive regulation of LEE expression (20). We constructed *clpX*, *clpP*, and *clpXP* deletion mutants of strain SKI-5142, as described in Materials and Methods. Proteins from equal amounts of culture supernatants from strains carrying pACYC184 or pACXP were analyzed by SDS-PAGE. All *clpX* and/or *clpP* deletions abolished the expression of LEE-encoded secreted proteins, such as EspA, -B, and -D (data not shown, Fig. 1A). Introduction of pACXP restored the amount of Esp proteins to that of the wild-type strain (Fig. 1A).

The amount of EspB was examined further by Western blotting using a polyclonal anti-EspB antibody. These results confirmed that the *clpXP* deletion impaired EspB expression but did not change the amount of the cross-reacting control band (Fig. 1B, upper and lower panels). We further confirmed these results by determining the transcriptional activity of genes in the LEE4 operon. Transcriptional activities of *sepL* and *espB* decreased more than eightfold in the *clpXP* deletion strain as well as in the *ler* deletion strain (Fig. 2).

grlR deletion suppresses the negative effect of clpXP deletion on expression of the LEE-encoded TTSS. As suggested above, ATP-dependent protease ClpXP positively regulates the expression of LEE-encoded TTSS in EHEC. One explanation for this would be if ClpXP protease controls the expression level of a negative regulator for LEE expression. Since *grlR* has been shown to encode a negative regulator for LEE expression (5, 35), we examined the effect of *grlR* deletion on LEE expression. The *grlR* deletion mutant showed constitutive expression of EspB even in repressed culture conditions (Fig. 3B), confirming a previous report (35). We also constructed a *grlR*
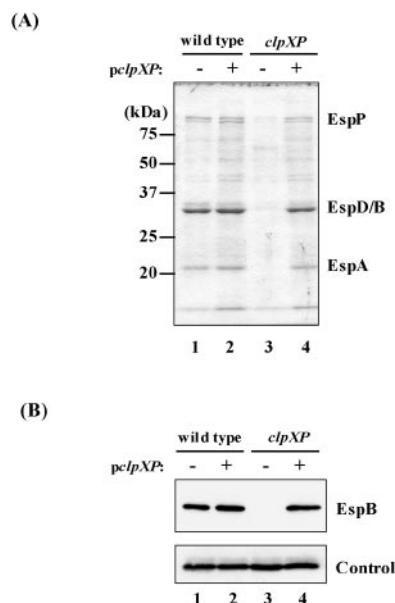

FIG. 1. Effect of *clpXP* deletion and of a multicopy plasmid carrying *clpXP* on expression of LEE-encoded Esp proteins. (A) Coomassie brilliant blue-stained SDS-PAGE profiles of Esp proteins secreted in culture supernatants. (B) Western blotting analysis of EspB in whole-cell lysates from equal amounts of wild-type (SKI-5142) and *clpXP* (SKI-5149) strains. Cultures were sampled at stationary phase. Strains used: lanes 1, SKI-5142/pACYC184; lanes 2, SKI-5142/pACXP; lanes 3, SKI-5149/pACYC184; lanes 4, SKI-5149/pACXP. The control shows a cross-reacting protein used to normalize loading.

clpXP double mutant to examine whether the negative effect of a *clpXP* deletion can be suppressed by an additional *grlR* deletion. The amount of EspB in the double mutant was comparable to that in the strain carrying the *grlR* deletion alone (Fig. 3), suggesting that ClpXP may regulate the expression level of GrlR.
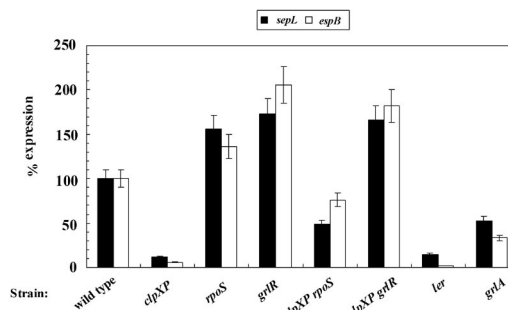

FIG. 2. Transcriptional activity of LEE4 in various mutants. β-Galactosidase activities of *espB-lacZ* in pLEE19 (open bars) and *sepL-lacZ* on pRLSL (solid bars) were calculated as percentages relative to those in wild-type SKI-5142. Cultures were sampled at late log phase. Average percentages were calculated using three or more measurements from at least five different experiments. Error bars represent standard errors of the normalized values. Typical β-galactosidase activities in Miller units (with standard deviation) of each fusion in SKI-5142 were pLEE19, 66.5 (7.11) and pRLSL, 11,900 (798). Strains used: wild type, SKI-5142; *clpXP*, SKI-5149; *rpoS*, SKI-5151; *grlR*, SKI-5152; *clpXP rpoS*, SKI-5155; *clpXP grlR*, SKI-5156; *ler*, SKI-5143; *grlA*, SKI-5153.
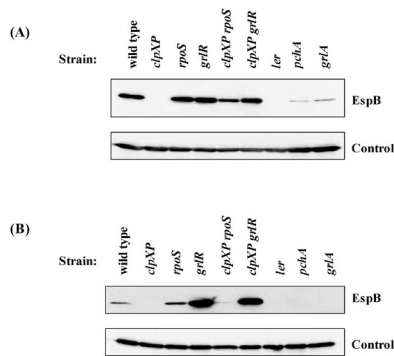
FIG. 3. Expression of EspB in whole-cell lysates from stationary-phase cultures of mutants grown in (A) DMEM and (B) LB. Strains used: wild type, SKI-5142; *clpXP*, SKI-5149; *rpoS*, SKI-5151; *grlR*, SKI-5152; *clpXP rpoS*, SKI-5155; *clpXP grlR*, SKI-5156; *ler*, SKI-5143; *pchA*, SKI-5144; *grlA*, SKI-5153. The control is as described for Fig. 1.

Amount of GrlR increases in the *clpXP* deletion strain. To examine the effect of the *clpXP* deletion on the GrlR level, we inserted FLAG epitope sequences into the chromosomal *grlR* at the 3′ terminus, as described in Materials and Methods. We also constructed a control strain, which carries a chromosomal *grlA-FLAG* fusion gene. An additional *ler*, *clpXP*, or *rpoS* deletion was introduced into these strains. The amounts of GrlA-FLAG and GrlR-FLAG were found to be decreased in the *ler* mutant (Fig. 4A and B), as also shown in a previous study (7). Although the *clpXP* deletion markedly reduced GrlA-FLAG expression, it conversely increased the amount of GrlR-FLAG (Fig. 4AB). These results suggest that ClpXP negatively controls the GrlR level.

Growth-phase-dependent expression of GrlR. We also examined the growth-phase-dependent expression of GrlR. The GrlR-FLAG level in wild-type background was minimal in stationary-phase cultures (12 h after initiation of culture growth), while the level in the *clpXP* deletion strain was constitutive (Fig. 5B, upper two panels). To investigate this fur-

FIG. 5. Growth phase-dependent expression of GrlR-FLAG. (A) Growth curves of SKI-5180 (wild type), SKI-5182 (*clpXP*), SKI-5188 (*rpoS*), and SKI-5189 (*rpoS clpXP*) in DMEM plus 0.5% glycerol. (B) Western blots of GrlR-FLAG from whole-cell lysates of each mutant sampled at 4, 5, 9, and 12 h during the growth cycle.

ther, we constructed an *rpoS clpXP* double mutant in a *grlR-FLAG*-carrying strain, as described in Materials and Methods. More GrlR-FLAG was found in the stationary-phase culture of *rpoS clpXP* mutant than in that of the *rpoS* mutant (Fig. 5B, lower two panels). These results indicate that ClpXP controls GrlR expression even in an *rpoS* deletion strain.

We also examined the GrlR-FLAG levels at different times after addition of spectinomycin and tetracycline in late-log-phase and stationary-phase cultures (Fig. 6). Although the GrlR-FLAG was quite stable in log-phase cultures of strains with or without *clpXP* deletion even 4 hours after addition of antibiotics, that in stationary-phase cultures of a strain without *clpXP* deletion was gradually decreased and minimal at 4 h after antibiotic addition. These results suggest that ClpXP may be directly involved in controlling the GrlR levels and thereby regulate LEE expression in stationary phase.

Deletion in *rpoS* partially suppresses the negative effect of the *clpXP* deletion on LEE expression. The ClpXP protease has been shown to be responsible for degradation of various regulatory proteins (12, 13). Therefore, we examined the RpoS sigma factor, which is one of these regulatory proteins and is
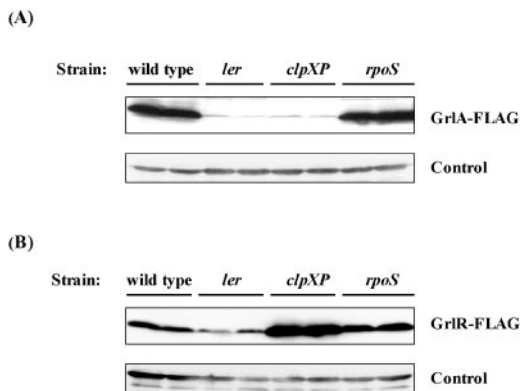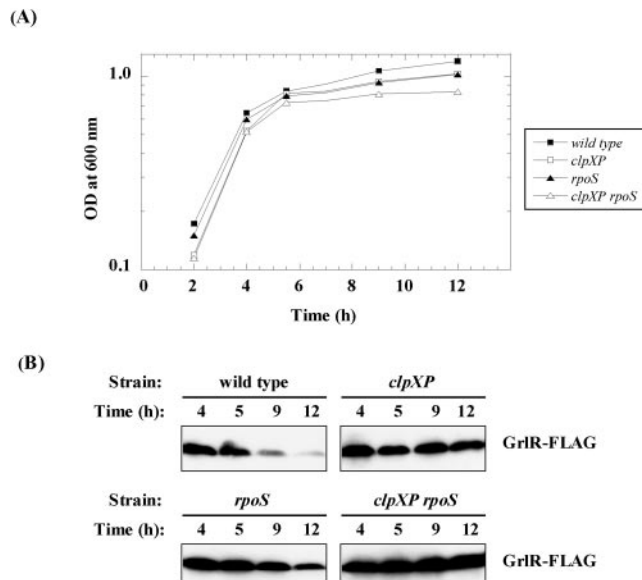

FIG. 4. Expression of GrlA-FLAG and GrlR-FLAG. The amount of (A) GrlA-FLAG and (B) GrlR-FLAG in whole-cell lysates was examined by Western blotting using monoclonal anti-FLAG antibody. Lanes received independent samples from each strain. Cultures were sampled at stationary phase. Strains used in A: wild type, SKI-5190; *ler*, SKI-5191; *clpXP*, SKI-5192; *rpoS*, SKI-5193. Strains used in B: wild type, SKI-5180; *ler*, SKI-5181; *clpXP*, SKI-5182; *rpoS*, SKI-5183. The control is as described for Fig. 1.
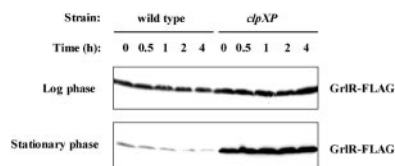

FIG. 6. Stability of GrlR-FLAG in log-phase (upper panel) and stationary-phase (lower panel) cultures of the wild type (SKI-5180) and *clpXP* mutant (SKI-5182). Western blots of GrlR-FLAG from whole-cell lysates of each strain were sampled at 0, 0.5, 1, 2, and 4 h after antibiotic addition.
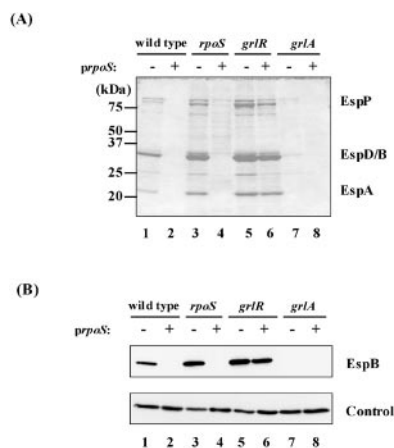
FIG. 7. Effect of *rpoS* overexpression on expression of LEE-encoded secreted proteins. (A) Coomassie brilliant blue-stained SDS-PAGE profiles of Esp proteins secreted in the culture supernatants. (B) Western blotting analysis of EspB in whole-cell lysates. Cultures were sampled at stationary phase. Strains used: wild type, SKI-5142; *rpoS*, SKI-5151; *grlR*, SKI-5152; *grlA*, SKI-5153; lane 1, SKI-5142/pGEM-self; lane 2, SKI-5142/pGEMRS; lane 3, SKI-5151/pGEM-self; lane 4, SKI-5151/pGEMRS; lane 5, SKI-5152/pGEM-self; lane 6, SKI-5152/pGEMRS; lane 7, SKI-5153/pGEM-self; lane 8, SKI-5153/pGEMRS. The control is as described for Fig. 1.
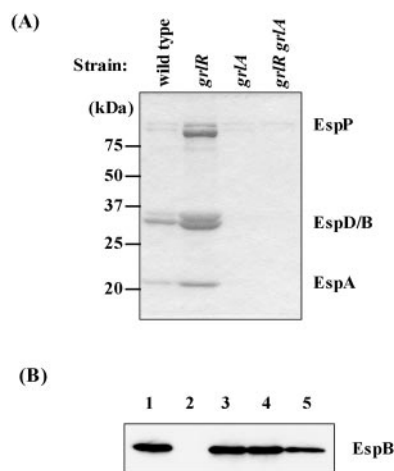

FIG. 8. Effect of *grlR* and/or *grlA* deletions on expression of secreted proteins. (A) Coomassie brilliant blue-stained SDS-PAGE profiles of Esp proteins secreted in culture supernatants. (B) Complementation of *clpXP* mutant (SKI-5149) by *ler*-, *pchA*-, or *grlA*-expressing plasmid (pGEMLER, pGEMPA, and pGEMGA, respectively) monitored by the amount of EspB in whole-cell lysates. Cultures were sampled at stationary phase. Strains used in A: wild type, SKI-5142; *grlR*, SKI-5152; *grlA*, SKI-5153; *grlR grlA*, SKI-5154; Strains used in B: lane 1, SKI-5142/pGEM-self; lanes 2, SKI-5149/pGEM-self; lane 3, SKI-5149/pGEMGA; lane 4, SKI-5149/pGEMPA; lane 5, SKI-5149/pGEMLER.

responsible for transcription of stationary-phase-induced genes. To analyze the effect of an *rpoS* deletion on LEE expression, with or without *clpXP* mutation, we constructed an *rpoS* deletion strain and an *rpoS clpXP* double deletion strain and compared the amount of EspB in whole-cell lysates from DMEM (LEE-activated) and LB (LEE-repressed) cultures with those of wild-type, Δ*clpXP*, Δ*ler*, Δ*pchA*, and Δ*grlA* strains. As shown previously (5, 7, 20), the level of LEE expression was reduced by *ler*, *pchA*, and *grlA* deletions (Fig. 3). The loss of EspB observed in the *clpXP* deletion mutant was partially suppressed by introduction of an additional deletion in *rpoS* in both DMEM and LB cultures (Fig. 3). These results suggest that RpoS negatively regulates LEE expression in EHEC. In agreement with these results, the transcriptional activities of *sepL* and *espB* were increased by an *rpoS* deletion, with or without a *clpXP* mutation (Fig. 2).

Multicopy *rpoS* significantly reduces expression of LEE-encoded TTSS. To confirm these results, we examined the effect of multicopy *rpoS* on expression of Esp proteins. As shown in Fig. 7, multicopy *rpoS* significantly reduced the amount of Esp proteins in culture supernatants and whole-cell lysates in wild-type and *rpoS* mutant strains. These results indicate that RpoS negatively regulates LEE expression.

Effect of *clpXP* deletion on LEE expression is mediated through regulation of the *pchA* gene. As described previously, LEE-encoded GrlR and GrlA have been shown to act as negative and positive regulators, respectively, for LEE expression (5, 35). Those studies also showed that the positive effect of GrlA was not observed in a *ler* mutant. Therefore, we investigated whether the negative effect of GrlR on LEE expression only occurred in the presence of GrlA.

We showed that derepression of LEE expression by the *grlR* deletion did not occur if the strain also had a *grlA* deletion (Fig. 8A). In the *clpXP* deletion mutant, a multicopy plasmid carry-

ing *ler*, *pchA*, or *grlA* restored LEE expression to the wild-type level (Fig. 8B), suggesting that *clpXP* may also regulate LEE expression by modulating the expression of *pchA* or *ler*. Therefore, we analyzed transcriptional regulation of the *pchA* and *ler* genes. The β-galactosidase activities of *pchA* and *ler* promoters decreased more than twofold in the *clpXP* deletion strain, while that of pRL124 (vector control) did not change (Table 3). These results indicate that transcription of *pchA* as well as of *ler* is under the positive control of ClpXP. We also examined the effect of *rpoS* deletion on *pchA* transcription. As shown in Table 4, transcriptional activity of *pchA* in cells from stationary-phase cultures was increased about twofold due to the *rpoS* deletion. These results suggest that ClpXP regulates LEE expression via controlling RpoS and GrlR levels (Fig. 9).

DISCUSSION

Regulation of LEE expression is induced in cells under conditions similar to those in the gastrointestinal tract, but repressed in cells grown in rich culture media such as LB (1, 26). In the studies reported here, we confirmed previous findings (35) that deletion of *grlR* overcomes the repression of LEE-

TABLE 4. Effect of *rpoS* deletion on *pchA* transcription[a]

Strain	β-Galactosidase activity, Miller units (SD)		
	pRL124	pRLPA	Ratio (pRLPA/pRL124)
SKI-5142	60.4 (8.63)	9,940 (913)	165
SKI-5151	49.5 (4.00)	15,600 (1,340)	315

[a] β-Galactosidase activity was measured in late-log-phase cells grown in DMEM plus 0.5% glycerol
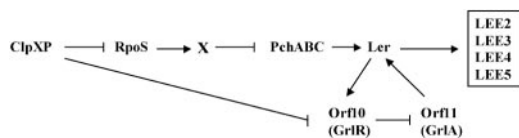
FIG. 9. Model for ClpXP-mediated regulation of LEE expression in EHEC. RpoS-dependent regulation by ClpXP is hypothesized to occur through an unidentified factor, X, which negatively regulates *pch* transcription. RpoS-independent regulation by ClpXP could be mediated through direct or indirect effects of the ClpXP protease on the GrlR level. Ler positively regulates transcription of *grlR*, whose gene product may antagonize GrlA, thereby facilitating Ler-dependent expression of other LEE operons. As described in the text, additional regulatory elements such as Fis, H-NS, integration host factor Hha, GadR, YhiF, GadE, EtrA, EivF, BipA, and quorum sensing have also been implicated in regulation of the LEE.

encoded TTSS expression in LB (Fig. 3B). Deng et al. (5) showed that mutation of *grlA* caused a significant reduction in the expression of LEE-encoded secreted proteins, and introduction of a plasmid carrying *grlA* could complement this phenotype only in the presence of Ler.

In this report, we found that the negative effect of the *grlR* deletion on LEE expression was observed only in the presence of *grlA* (Fig. 8). Since *grlA* is immediately downstream of *grlR* and these two genes probably form an operon, GrlR may directly repress GrlA by protein-protein interactions that down-regulate Ler expression. Since transcriptional expression of *grlR* is under the control of Ler (7), interactions among these regulators are summarized in the model shown in Fig. 9. Ler is central for the regulatory cascade, negative regulation by GrlR of LEE expression depends on the presence of GrlA, and GrlA activates Ler, which can induce expression of other LEE genes.

The level of GrlR-FLAG in the wild-type strain was markedly decreased in stationary-phase cultures, while the level in the *clpXP* mutant was constitutive (Fig. 4B and 5B). Therefore, ClpXP is involved in regulation of the GrlR level. One explanation for this would be if the ClpXP level is induced in stationary-phase cultures. However, this is unlikely because the ClpXP level is not significantly different in exponential and stationary-phase *E. coli* K-12 cultures (44). Another possibility may be that GrlR is a substrate for ClpXP. To test this possibility, the level of GrlR-FLAG was compared at different times after addition of spectinomycin and tetracycline in strains with and without a *clpXP* mutation (Fig. 6). The GrlR-FLAG level of a strain without *clpXP* deletion was unstable in stationary-phase cultures for several hours after antibiotic addition, suggesting that ClpXP may directly be involved in controlling the stability of GrlR. However, further experiments should be carried out to confirm this conclusion. Since we measured the level of the FLAG-tagged allele of GrlR in this study, the level of native GrlR needs to be determined to rule out the possibility that the FLAG tag may influence the stability of GrlR. Therefore, it is premature to conclude that GrlR is a direct substrate of ClpXP.

The ClpXP protease plays diverse physiological roles in degrading damaged and incomplete proteins (14, 59) and regulatory proteins in response to various stresses (12, 13). Recently, Jackson et al. (21) demonstrated that ClpXP and another ATP-dependent protease, Lon, together control TTSS expression by regulating the stability of a small histone-like

protein, YmoA, in *Yersinia pestis*. YmoA is a homologue of *E. coli* Hha protein, which has been reported to regulate LEE expression through Ler in EHEC (46). Therefore, it is possible that ClpXP controls Hha stability and thereby regulates LEE expression in EHEC. It will be important to learn whether Hha is a substrate for ClpXP in EHEC and whether Hha controls other LEE regulators such as GrlR, GrlA, and PchA/B/C.

We also found that abolishment of LEE expression in the *clpXP* deletion mutant was partially suppressed by an *rpoS* deletion. A previous report showed that expression of the LEE3 operon is positively regulated by RpoS in *E. coli* K-12 (50). We have not examined LEE3 transcription in the EHEC *rpoS* mutant used in our studies. However, we have shown that RpoS negatively regulates *ler* transcription through reduction of *pchA* transcription in EHEC (Table 4). Therefore, RpoS may also negatively regulate LEE3 transcription in EHEC O157. Because *rpoS* encodes a stationary-phase alternative sigma factor, an additional regulator, tentatively designated X in Fig. 9, should exist downstream of RpoS and negatively regulate Pch expression. Further study to identify this regulator is now in progress.

ClpXP has been shown to negatively regulate flagellar synthesis in *Salmonella enterica* serovar Typhimurium by modulating turnover of FlhD and FlhC, encoded by the flagellar master operon (54, 55). We also observed that *clpXP* deletion in strain O157 Sakai causes overproduction of flagellin, the subunit of the flagellar filament (Iyoda and Watanabe, unpublished). Therefore, *clpXP*-dependent repression of flagellar synthesis is also observed in EHEC O157. The supramolecular structure of bacterial flagella resembles that of TTSS, and they are considered phylogenetically related (31). As shown in *Salmonella*, flagellar synthesis is an energy-consuming process, and overproduced flagellin causes significant growth retardation in vitro (32). Therefore, it is possible that ClpXP controls expression of both the flagellar system and other TTSS (in this case, LEE-encoded TTSS) to minimize their expression when they are not needed by the cell.

We previously have found that the *pchABC* genes encode positive transcriptional regulators that induce *ler* transcription in EHEC (20). The molecular mechanism by which other regulators, encoded outside LEE, control LEE expression is largely unknown. However, some mechanisms may directly affect Pch expression. Analysis of the regulatory mechanisms of Pch as well as GrlR will be important for a better understanding of the regulation of LEE expression in EHEC.

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