

**Identification and evaluation of regulatory factors modulating the expression of *LEE4*  
in enterohemorrhagic *Escherichia coli* O157:H7**

by

Karla Ann Mesterhazy

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Program of Study Committee:  
Vijay K. Sharma, Co-major Professor  
Nancy Cornick, Co-major Professor  
Gregory Phillips  
Amanda Fales-Williams

Iowa State University

Ames, Iowa

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Graduate College  
Iowa State University

This is to certify that the master's thesis of

Karla Ann Mesterhazy

has met the thesis requirements of Iowa State University

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“We are starving for knowledge but drowning in information”

Unknown author

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## General Introduction

There are currently three major clinical categories of pathogenic *Escherichia coli* strains that cause disease in humans: gastrointestinal pathogens, urinary tract pathogens, and those that infect the central nervous system. The gastrointestinal tract pathogens are further characterized into six pathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), and enterohemorrhagic *E. coli* (EHEC). Each of these pathotypes causes disease by a different mechanism. The remainder of this introduction will focus mainly on EHEC O157:H7 and its ability to cause intestinal disease. EHEC is responsible for a wide range of clinical diseases including, uncomplicated diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS).

### Thesis Format

The organization chosen for this thesis is written in the alternate format. It includes a literature review and one manuscript followed by general conclusions. The manuscript has been submitted to the Journal of Bacteriology.

## Literature Review

### Reservoirs

The major reservoir for EHEC O157:H7 is ruminants such as cattle, sheep, and deer. However EHEC O157:H7 has been isolated from other species including wild birds, dogs, raccoons and swine (42). Despite the large reservoir base for EHEC, the most common source of infection for humans is the inadvertent contamination of food or water by ruminant manure (7, 5, 26, 41). In the United States cattle population, EHEC O157:H7 has a relatively low prevalence when compared to other serotypes of Shiga toxin-producing *E. coli* (STEC) (7, 15, 18, 58, 12). The estimated infectious dose of *E. coli* O157:H7 from contaminated food in humans is estimated between 10-100 cells (7, 30). The most common sources of EHEC O157:H7 infections in humans include undercooked ground beef patties, unpasteurized beverages (apple cider and milk), raw unwashed vegetables and non-chlorinated water.

Newborn animals acquire normal gastrointestinal flora from their dam and surrounding environment. One of the most prevalent bacterial species to colonize the intestinal tract of young animals includes pathogenic and non-pathogenic strains of *E. coli*. In adult ruminants, the presence of EHEC O157:H7 and STEC strains in the gastrointestinal tract do not appear to cause clinical disease (7). On occasion, experimentally and naturally inoculated STEC strains will produce clinical signs of disease such as diarrhea, hemorrhagic colitis and attaching and effacing lesions in young calves (7, 19, 25, 46). These findings of STEC disease in young calves are often confounded by concurrent infections with other pathogenic agents such as *Cryptosporidium* sp., rotavirus, coronavirus, bovine viral diarrhea virus (BVDV), and coccidia. These additional pathogenic agents complicate the

interpretation and understanding of a pure STEC infection (7, 25). In general, EHEC strain O157:H7 is only rarely associated with naturally occurring disease in calves. When challenged with a high dose ( $10^{10}$  CFU) of EHEC O157:H7, colostrum deprived calves displayed clinical disease of hemorrhagic enterocolitis and microscopically attaching and effacing lesions on intestinal mucosa (7, 13). Historically, the heaviest fecal shedders of STEC bacteria include healthy adult cattle and normal calves.

### **Surveillance**

Routine surveillance and prediction of STEC shedding by cattle does not provide adequate protection against contamination of food items. In order to increase the food safety and decrease the incidence of STEC infections the implementation of, routine pasteurization of milk and apple cider, increasing sanitation at processing plants, and proper cooking of meat products has reduced the risk of transmission (42). Previous attempts to vaccinate cattle against EHEC O157:H7 have failed due to the transient shedding and the failure to prevent subsequent re-infection (7, 11). Another approach to reduce fecal shedding of EHEC O157:H7 includes critical evaluation of specific farm management practices. Some studies have indicated that EHEC shedding maybe dependent on repeated environmental exposure such as a common drinking tank for individual animals (7, 50). Other potential reservoirs on the farm such as sheep, ponies, and flies can complicate the tracing and shedding of STEC as noted on one particular farm (7, 23).

### **Human Infections**

In 1971, the first isolate of STEC was identified in humans and the first isolate of EHEC was first recognized in 1982 (42, 30). Contaminated small bowel syndrome was a term used previously for STEC infections. In North America, the United Kingdom and Japan

serotype O157:H7 is the most common cause of HC and HUS, whereas in other countries serotype O26 and O111 are more prominent (30). Current records do not provide accurate estimates of the exact number of STEC infected people since not all laboratories test for these organisms and not all patients seek medical advice, especially when symptoms are mild. A survey of human stool samples obtained from patients with diarrhea was conducted by the Centers for Disease Control and Prevention (CDC) indicated that in 28 states, almost half of the total samples contained isolates of EHEC strain O157:H7 (42). Additional surveys to determine the prevalence of O157:H7 indicated that this bacterial pathogen represents the fourth most common pathogen found in stool samples (42).

### **Virulence Attributes**

To fully appreciate the ability of EHEC O157:H7 to infect and produce disease in humans, molecular genetics have systematically discovered essential genetic factors associated with the production of disease. Pathogenicity is often associated with virulence attributes which may be encoded on plasmids, the chromosome, bacteriophages or pathogenicity islands (PI). One major virulence attribute of STEC is its ability to produce Shiga toxins, as noted by Konowalchuck et al. (37). Shiga toxin nomenclature originated from the toxin produced by *Shigella dysenteriae*. Shiga toxins belong to the cytotoxic protein family of toxins that consist of five B subunits and one A subunit. There are two major variants of Shiga toxins, Stx1, which is almost identical to Shiga toxin of *Shigella dysenteriae* and Stx2 (58% amino acid sequence homology to Stx1). Shiga toxin genes are carried by temperate lambdoid bacteriophages (45). These phages are optimally induced at 37°C by multiple host and environmental factors and this phenomenon of phage induction is considered to contribute to substantial increases in the amount of toxin. In order for Shiga



toxins to initiate disease within the host, cells must display the Gb<sub>3</sub> (globotriaosylceramide) cell surface receptor. The host cell internalizes the toxin by receptor-mediated endocytosis, followed by fusion of toxin with lysosomes and translocation to the Golgi apparatus where it is incorporated into the rough endoplasmic reticulum and eventually released into the cytoplasm (44). Shiga toxins depurinate 28S eukaryotic rRNA which ultimately results in the inhibition of protein synthesis and thus killing of the cells. In humans, the vascular endothelium of small blood vessels especially in the digestive and urinary tracts are rich with Gb<sub>3</sub> receptors. Extensive damage to the endothelial cells lining the glomerulus leads to renal failure and the hemolytic uremic syndrome (HUS).

The second major virulence attribute of EHEC O157:H7 is the pathogenicity island (PI) termed the Locus of Enterocyte Effacement (LEE). A pathogenicity island is defined as a section of closely linked chromosomally-encoded virulence-associated genes. PIs are highly indicative of horizontal gene transfer between different species or genera that often include criteria such as encoding multiple virulence factors and occupying large sections of the genome (6). PIs regularly contain a consistent G+C content which is different from the rest of the genome (22, 30). The LEE of EHEC O157:H7 is localized on a 35-kb chromosomal segment that encodes for a type III secretion system (TTSS). The TTSS allows for bacteria/host cell interaction and is one of four major secretion systems described for gram negative bacteria (47). The type III secretion system is highly conserved among attaching and effacing (A/E) groups of pathogens and its major function is to actively transport translocator and effector proteins across the inner and outer membranes to the bacterial cell surface and into the host cell cytoplasm. These translocator and effector proteins are responsible for the formation of A/E lesions, which are characterized by

effacement of the microvilli of intestinal cells, intimate adherence of bacteria and host cells followed by conformational changes of the host cell mediated through cytoskeletal rearrangements and pedestal formation (38). Previous studies have characterized five principal operons within LEE: *LEE1* (encodes ler-LEE encoded regulator), *LEE2*, and *LEE3* (encodes TTSS, CesAB chaperone and EspH effector), *LEE4* (encodes EspA, EspD, and EspB ), and *LEE5* (encodes intimin, Tir, and CesT chaperone) (2).

### **Genetic Regulation**

The regulation of genes located within LEE is complex and multifaceted, but in general, the expression of LEE of EHEC and EPEC is controlled by unique global regulators. The most recognized global regulator is located within LEE (*LEE1*) and is an H-NS paralog termed *ler*, the LEE encoded regulator. Ler is a major factor in positively regulating *LEE2*, *LEE3*, *LEE4*, *LEE5*, *espG*, and *map* (4). Positive and negative regulation of global regulators such as *ler* is very complex and often involves many factors associated within separate regulatory cascades. Individual genes are targeted to affect LEE in either a positive or negative regulatory role. The following is a list of genes associated with the regulation of *ler*: *h-ns*, *ihf*, *fis*, *perC*, *bipA*, *gria*, *griaR*, *gadX*, *hha*, along with factors included in the quorum-sensing cascade (4, 9, 14, 17, 20, 21, 29, 35, 51, 52, 53, 55) Many of these genes influencing *ler* expression are not encoded by the LEE. It has also been reported that *ler* has the ability to auto-regulate its own synthesis by binding to the *LEE1* regulatory sequence in EPEC (4).

Individual regulatory genes are currently studied along with the mechanisms by which they alter the expression of LEE. H-NS, Fis (factor for inversion stimulation) and IHF (integration host factor) are each categorized as small bacterial DNA-binding proteins which

are implicated in the regulation of virulence factors in STEC (40). H-NS has been studied the most of the three small DNA-binding proteins and is classified as a histone-like protein. Histone-like proteins are a common structural component of the chromatin in bacterial cells and are normally abundant in cells. H-NS binds non-specifically to intrinsically curved DNA and is utilized in various cellular activities including recombination, transposition and transcription (36). Most notably, H-NS has the ability to negatively influence the transcription of virulence factors in *E. coli* (1). Unfortunately, the complete mechanism of action of N-NS is not known.

Current data suggests that Ler can act as an anti-repressor and overcome the ability of H-NS to negatively effect the transcription of LEE by forming a “repressing H-NS-nucleoprotein complex”(9). Investigations into the regulatory function of negative effectors of LEE have led to the discovery of Hha and H-NS proteins as functionally equivalent structures at the N-terminal domain (43). Hha was first described as a small protein thermo-modulator (approximately 8-kDa) of virulence factors. This category also includes small protein thermo-modulators of other pathogenic bacteria such as YmoA of *Yersinia enterocolitica*. Hha is implicated in the negative regulation of  $\alpha$ -hemolysin expression in EPEC (49) and we recently identified it as a possible substrate for the ClpXP protease.

ClpXP is an ATP dependent protease that is ubiquitous in most bacteria. There are two components to ClpXP which include a peptide-degrading unit and the ATP-binding unit, ClpP and ClpX respectively. The *clpX* gene is normally transcribed in the *clpXP* operon but there is a single weak promoter for *clpX* located between *clpP* and *clpX* (32). This protease is able to degrade abnormal proteins and specifically tagged normal proteins. RpoS and  $\lambda$  O protein are two known specific targets for the ClpXP protease (32). Recent studies have

shown that ClpXP controls the expression of type III protein secretion system by regulation of RpoS and GrIR (26). The role of the ClpXP protease system in other pathogenic bacteria include the *ail* (attachment invasion locus) gene in *Yersinia enterocolitica* and the *hla* (alpha hemolysin) gene of *Staphylococcus aureus* (16). These proteins are expressed in response to stress as a part of the stress tolerance response and are actively associated with quorum-sensing loci.

RpoS is another major regulatory molecule that is classified as an alternative sigma subunit of RNA polymerase (8). RpoS regulates the general stress response in *E. coli* and it is estimated that RpoS may affect the expression of more than 50 genes (8). RpoS mediated regulation of virulence genes is complex. For example, GadX is a RpoS dependent system and as stated below GadX is associated with *pch* gene regulation (33). RpoS is also linked to other factors such as ppGpp and DksA, which are both needed for activation of RpoS in stationary-phase regulation (8, 33).

Another positive regulator of *LEE* in EPEC includes PerC, which is part of the Per operon that includes PerA and PerB. PerC is implicated in the activation of *bfp* genes encoding the formation of the pilus needed for bacteria-bacteria attachment in EPEC. There are at least five PerC-like homologues in EHEC and three of these homologues encode 104 amino acid proteins (PchA, PchB, and PchC). These 104 amino acid proteins, when expressed from a multicopy plasmid, enhance transcription of *LEE* resulting in the increased adherence of EHEC O157:H7 to HEp-2 cells (25). In addition, genes that act as indirect regulators of *ler* have also been identified. The *gadX* gene, which is transcribed during acid stress and stationary phase growth, affects *ler* expression by altering the transcription of PerC (2).

*BipA* is characterized as another regulatory gene in STEC and is a member of the ribosome-binding GTPase superfamily. BipA is ubiquitous in both bacteria and plants and is reported to regulate both cell surface and virulence associated genes in EPEC including flagella and *espC*. The modulation of *espC* in a positive manner was accomplished through transcriptional control of *ler*. It is suggested that BipA controls virulence by acting at the top of a multi-faceted cascade or cascades in EPEC (21). Unfortunately, like many of the other regulatory genes of LEE the full mechanism or cascade of BipA-mediated regulation of *ler* is not elucidated.

Quorum-sensing is a term used for describing the response of gene expression in bacterial cells during environmental and cell density changes. Quorum-sensing is also implicated in the regulation of virulence factors associated with *LEE* in STEC. Sperandio et al. discovered the regulation of *LEE* by the quorum-sensing autoinducer-2 (AI-2). AI-2 is a hormone-like compound that alters the effects of certain regulatory proteins and is encoded by the *luxS* gene. *QseA* (quorum-sensing *Escherichia coli* regulatory A) was also implicated in the regulation of *LEE* (54). The *sidA* gene is the *E. coli* homologue for the transcriptional regulator *luxR* in quorum-sensing (29).

GlrR (*L0044*) and GlrA (*L0043*), are two recently identified negative and positive transcriptional regulators, respectively, of *LEE* in EHEC 0157:H7 (10). *Salmonella enterica* serotypes contain the only known homolog of GrlA and this homolog is a presumed product of an uncharacterized gene. Barba et al. noted that GrlA and Ler form a positive regulatory loop that controls the expression of *LEE* (2) and Iyoda et al. reported that the type III secretion system is modulated by GrlR that is regulated by the protease ClpXP (26).

Additional pathways that positively or negatively affect the transcription of individual LEE operons have also been identified. *LEE4* has been an important operon to study and multiple regulatory factors affecting the expression of *LEE4* have been identified. *LEE4* encodes for the proteins, EspA, B, and D, which are secreted by the type III secretion system. EspA is required for the formation of the pilus-like structure of the TTSS (3). Beltrametti et.al. showed that the expression of the *esp* genes is influenced by environmental stimuli such as osmolarity and temperature. Additional factors required for fine-tuning of the expression of *LEE4* include  $\sigma^s$  and the H-NS proteins that, genetically map outside of the LEE pathogenicity island.

The entire LEE pathogenicity island appears to be modulated mainly through global regulators that respond to environmental factors. These environmental factors allow bacteria to either up- or down-regulate the expression of genes necessary for attachment of bacterial cell to host and evasion of the host immune response. Further investigation of *ler* and subsequently *LEE* transcriptional regulation will allow understanding of the complex pathway of LEE expression and pathogenesis of EHEC O157:H7.

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**ClpXP protease regulates expression of locus of enterocyte effacement-encoded genes  
by modulating intracellular levels of Hha in *Escherichia coli* O157:H7**

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Karla A. Mesterhazy<sup>1,2</sup> and Vijay K. Sharma<sup>1</sup>

<sup>1</sup>*Pre-Harvest Food Safety and Enteric Diseases, National Animal Disease Center, USDA,  
Agricultural Research Service, Ames, Iowa 50010*

<sup>2</sup>*Veterinary Microbiology and Preventive Medicine, Iowa State University, Ames, Iowa  
50011*

**Abstract**

The locus of enterocyte effacement (LEE) encoded operons (*LEE1* – *LEE5*) enable enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 to produce attaching and effacing lesions on intestinal cells (A/E). We have previously reported that *hha*-deleted EHEC O157:H7 up-regulated *LEE4* through increased expression of *ler*, the regulatory gene encoded by the *LEE1* operon. In the present study, we demonstrate that a transpositional insertion in *clpXP* abrogated the increased expression of the *LEE4* in the *hha*-deleted strain. The loss of *LEE4* up-regulation in an EHEC O157:H7  $\Delta hha$ ,  $\Delta clpXP$  mutant was restored by transposon insertions immediately upstream of *grlR* of the *grlR-grlA* operon, suggesting that

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*grlR* represses the expression of *LEE4* in the absence of Hha and ClpXP. RT-PCR analysis revealed that *grlR* transcription increased by about 100-fold in strains lacking *hha* but remained the same in the presence or absence of *clpXP* thus indicating that *hha* acts as a negative regulator of *grlR* transcription. RT-PCR data also showed a 10-fold reduction in *LEE4* transcription for EHEC O157:H7  $\Delta$ *clpXP* thus suggesting that the absence of *clpXP* resulted in an increased accumulation of GrlR and presumably Hha thereby causing an additional reduction in *LEE4* transcription compared to that observed for *LEE4* in EHEC O157:H7. Western blot analysis of the cell-free extracts of EHEC O157:H7  $\Delta$ *clpXP* revealed increased accumulation of Hha. Altogether, the results described in this report indicate that ClpXP is important for maintaining a critical intracellular pool of Hha, which in turn modulates transcription of *grlR-grlA* so that a basal level of transcription could continue through *LEE4* and the other *LEE* operons.

## **Introduction**

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 causes a broad spectrum of diseases including uncomplicated diarrhea, hemorrhagic colitis (HC), and hemolytic-uremic syndrome (HUS) (19). One major virulence attribute of EHEC O157:H7 is the ability to produce Shiga toxins encoded by genes *stx1* and *stx2*. Shiga toxins are responsible for producing systemic manifestations of the disease, which are characterized by acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia in very young and elderly patients (19, 24, 26). The second attribute of virulence in EHEC is the ability to attach intimately to the host intestinal tract and produce the characteristic histopathology lesion termed attaching and effacing (A/E) (30). The genes involved in the intimate colonization are located on a 35.6-kb pathogenicity island termed the locus of enterocyte effacement



(LEE) (31). Previous studies have characterized five principal operons within LEE: *LEE1* which encodes *ler*, a key positive regulator of LEE; *LEE2* and *LEE3* which encode the type-III secretion system, CesAB chaperone and EspH effector; *LEE4* which encodes EspA, EspD, and EspB; and *LEE5* which encodes intimin, Tir, and CesT chaperone (7, 34).

EHEC utilizes the type-III secretion system for the secretion of several virulence and effector proteins including, intimin (an outer membrane protein), Tir (translocated intimin receptor), and EspA, EspD, and EspB (secreted proteins). Collectively, these proteins promote intimate attachment of bacterial cells to host intestinal cells and subsequent formation of A/E lesions. A/E pathology is characterized by the loss of enterocyte microvilli and the formation of a polymerized actin pedestal forming a cup-like structure around the invading bacteria (6).

The regulation of genes located within LEE is complex and multifaceted. LEE itself encodes several regulators such as *ler* (a positive regulator of *LEE1-LEE5*), *grlA* (a global regulator of LEE activation), and *grlR* (a global regulator of LEE repression) (1, 3, 12, 25, 40, 41). Global regulatory systems such as Fis, BipA, H-NS, PerC1, IHF and Hha (2, 5, 12, 18, 28, 37, 39) are located outside of LEE and interact with *ler* to affect the expression of LEE. Additionally, a quorum-sensing pathway activates transcription of *qseA*, a positive regulator of *ler* (18, 38, 42).

ClpXP, a caseinolytic protease, affects LEE expression by modulating the intracellular levels of RpoS and GrlR (17). ClpXP is classified in a group (AAA+, ATP-dependent proteases) that is responsible for greater than 90% of cellular proteolysis, (4, 9, 14, 20, 21, 22, 27). Other proteases in this category include Lon, ClpAP, and HslVU (21).

We have recently reported that EHEC O157:H7  $\Delta hha$  showed enhanced expression of *LEE4* and other LEE-encoded operons via the up-regulation of LEE activator *ler* (37). In this paper, we describe experiments to demonstrate that *clpXP*-mediated regulation of *GrlR* is responsible for the enhanced expression of *LEE4* and *ler* in EHEC O157:H7  $\Delta hha$ . In addition, we also describe experiments to show that *hha* functions as a negative regulator of *grlR-grlA* operon and accumulation of Hha in EHEC O157:H7  $\Delta clpXP$  caused reduced expression of *LEE4*. Based on the data presented in this study, we have proposed a model to illustrate the role of *clpXP*, *hha*, *grlR*, and *grlA* in the expression of LEE-encoded genes.

## Methods

Bacterial strains, plasmids, and growth conditions. Table 1 lists bacterial strains and plasmids used in this study. Luria-Bertani (LB) agar and LB broth were used for bacterial growth. Ampicillin (50 mg l<sup>-1</sup>), kanamycin (50 mg l<sup>-1</sup>), zeocin (25 mg l<sup>-1</sup>), and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) (40 mg l<sup>-1</sup>) were added to the media as needed.

Primer design, PCR amplification, and DNA sequencing. Primers used for isolating specific nucleotide sequences and for determining relative abundance of gene-specific transcripts by RT-PCR are listed in Table 2. EHEC O157:H7 EDL933 genome was used as reference for selecting these primers (35). Primers were synthesized by Integrated DNA Technologies (Coralville, IA). PCR amplifications of DNA  $\leq$  2 kb were performed using 25  $\mu$ l reactions, containing 2.5  $\mu$ l DNA (0.1  $\mu$ g), 1  $\mu$ l each of forward and reverse primers (0.3  $\mu$ M), and 20.5  $\mu$ l of AmpliTaq Gold master mix (PE Biosystems, Foster City, CA). DNA fragments  $\geq$  2 kb were amplified using the Failsafe PCR kit (Epicenter Technologies, Madison, WI). PCR

products were resolved on agarose gels and DNA fragments of predicted sizes were purified from agarose slices using the QIAquick Gel Extraction Kit (QIAGEN, Inc, Valencia, CA). DNA sequencing analysis was performed either at the DNA Sequencing and Synthesis Facility at the Iowa State University (Ames, IA) or by the DNA sequencing facility at the National Animal Disease Center (Ames, IA).

Construction of *LEE4::lac* transcriptional fusion. *LEE4::lac* fusion was constructed and introduced into the chromosome of EHEC O157:H7 strain 86-24  $\Delta$ *stx2* $\Delta$ *lac* by the method described previously (37).

Transposon mutagenesis. EHEC O157:H7 strain 86-24  $\Delta$ *stx2* $\Delta$ *lac* $\Delta$ *hha* *LEE4::lac* was electroporated with EZ::Tn<Kan-2>Tnp transposon (Epicenter Technologies, Madison, WI). Following incubation for 1 h in SOC broth, electroporated cells were plated on LB agar plates containing kanamycin and X-gal. After an overnight incubation at 37°C, plates were visually examined for the presence of colonies that were either dark blue or white in color. The amount of  $\beta$ -galactosidase produced by selected dark blue colonies was compared to the amount produced by the parent strain. White colonies were not selected for further studies because these colonies invariably resulted from transposon insertion in the *lac* cassette.

Quantification of  $\beta$ -galactosidase activity (quantitative colorimetric assay). Standard methods were used to determine  $\beta$ -galactosidase activity (29). Overnight cultures were diluted 1:100 in LB broth, incubated at 37°C for up to six hours (growth period long enough to detect near maximal LEE activity), and then assayed for  $\beta$ -galactosidase activity as described previously (37).

Identification of transposon insertion sites. Location of transposon insertion in the bacterial chromosome and the nucleotide sequence of the DNA flanking the insertion sites were determined by a previously described method (37).

Construction of in-frame *clpXP* deletion. To generate an in-frame deletion of *clpXP*, a 1.5-kb sequence located upstream (US) and a 1.5-kb sequence located downstream (DS) of *clpXP* were amplified by PCR from EHEC O157:H7 86-24 by using primers sets VS449-*XbaI*/VS450*SalI* and VS451-*SalI*/ VS452*XbaI* (Table 2). These fragments were cloned in pCRXL TOPO TA cloning vector (Invitrogen, Carlsbad, CA) to generate plasmid pSM222 and pSM223, respectively. The 1.5-kb DS fragment was isolated from pSM222 and cloned at the 3' end of the 1.5-kb US fragment present in pSM223 to construct plasmid pSM224. The 3-kb US-DS fragment of pSM224 was isolated using *XbaI* and cloned at the *XbaI* site of the temperature-sensitive plasmid pAM450 to generate a recombinant plasmid pSM225. Plasmid pSM225 was electroporated into EHEC 86-24  $\Delta stx2 \Delta lac$  strain and the new strain 86-24  $\Delta stx2 \Delta lac$ /pSM225 was cultured under growth conditions, as described previously (37), that facilitated deletion of the chromosomal copy of *clpXP*. Deletion of *clpXP* from the chromosome of the host strain was verified by PCR using primers VS418/VS421. The *clpXP*-deleted strain produced a 200-bp band with these primers indicating the deletion of the entire 3.24-kb *clpXP* operon (data not shown).

Complementation with multi-copy plasmids. The ORFs corresponding to disrupted genes (*clpP* or *clpXP* or *grlR*) were isolated by PCR using primers listed in Table 2. The 1.2-kb *clpP* and 2.4-kb *clpXP* ORFs were cloned into pCR2.1 (In Vitrogen, Carlsbad, CA) to generate plasmids pSM201 and pSM202, respectively. The 0.42-kb *grlR* ORF was cloned into pCRXL to produce plasmid pSM270. The complementation plasmids were

electroporated into *E. coli* Top10 electrocompetent cells (Invitrogen, Carlsbad, CA).

Recombinant plasmids carrying cloned copies of specific ORF were isolated and transformed into strains containing a Tn-Kn-disrupted copy of the corresponding ORF in the chromosome. Degree of complementation, conferred by the plasmid-borne ORF, in the host strain was measured by comparing the  $\beta$ -galactosidase activity of the complemented versus un-complemented strain.

Purification of proteins. The method for cloning of the *hha* ORF in pGEX-4T-3 (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) to produce GST (glutathione S-transferase)-Hha fusion protein and isolation of Hha moiety by thrombin treatment from the fusion protein has been reported in a previous publication (37).

Reverse-transcription polymerase chain reaction (RT-PCR) analysis. Total RNA was prepared by using RNAeasy Kit (QIAGEN, Inc, Valencia, CA). Contaminating DNA was removed from RNA preparations by using a commercially available DNA removal kit (Ambion, Inc, Austin, TX). Recovered RNA was analyzed on an agarose gel to determine the presence of distinct 23S and 16S rRNA bands. RNA concentration was determined by using NanoDrop Spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE). A single-step RT-PCR kit was used for converting RNA into cDNA and amplifying cDNA into double-stranded DNA (PE Biosystems, Foster City, CA). The primer sets VS319/VS320, VS323/VS324, VS376/VS377, VS587/VS588, and VS589/VS590 facilitated detection of amplicons specific to *gapA*, *espA*, *ler*, *grrR*, and *grrA*, respectively (Table 2). Primers used for detecting *gapA*, *espA*, and *ler* have been described in a previous publication (37). The samples were analyzed on a 4% Nusieve agarose gel containing ethidium bromide (Cambrex

Corporation, East Rutherford, NJ) and the gel was visualized using Alpha Innotech Image documentation system (Alpha Innotech Corporation, San Leandro, CA.).

Immunization of rabbits. Seven New Zealand White, female rabbits (4 kg each) were sedated with an intra-muscular (IM) injection of 10 mg per ml of acepromazine (Phoenix Pharmaceutical, St. Joseph, MO) prior to bleeding and antigen (GST-Hha) injection (GST-Hha). A pre-immune serum sample (control) was collected from the marginal ear vein prior to injecting the animal with the antigen (0.5 mg of antigen mixed with Freund's incomplete adjuvant) in thigh muscles. Booster shots of the antigen alone were administered at 2 and 4 weeks post initial injection. Blood was collected on day 36 to detect the level of specific antibody in the sera. Rabbits were sedated with Ketamine (35mg/kg BW)/Xylazine (2mg/kg BW) combination prior to exsanguination by cardiac puncture on day 50. Approximately 60 ml of blood was harvested thus resulting in 40 ml of serum per rabbit. Relative levels of the anti-Hha antibody titers in rabbit antisera were determined by using a commercially available ELISA kit (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD). In brief, 100  $\mu$ l of coating buffer containing Hha at 10  $\mu$ g per ml was applied to each well of an ELISA plate. The post and pre-inoculation sera were diluted at concentrations of 1,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$  per ml before adding to the Hha-coated wells. These plates were processed according to manufacturer's instructions (KPL, Gaithersburg, MD). This procedure produced a positive signal with  $10^{-4}$  dilutions of serum samples indicating very high titers for anti-Hha antibodies in rabbit sera (data not shown).

Western immunoblotting. Strains were grown overnight from a single colony. The overnight culture was diluted 1:100 in fresh LB broth. After incubation at 37°C for 6 hours, cells were harvested at  $6,000 \times g$  for 5 min. The pellet was resuspended in 10 ml of PBS containing

1mg of lysozyme, incubated on ice (10 min), sonicated (20 second pulses for one to two minutes), and centrifuged at  $12,000 \times g$  (10 minutes) to remove unbroken cells and cell debris. The supernatant was analyzed for protein content by using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Samples containing approximately 0.03 mg of total protein were resolved on a 15% pre-cast SDS polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins from the gel were transferred overnight (30V and 90mA) to a nitrocellulose membrane using the Bio-Rad Mini Trans-Blot system (Bio-Rad, Hercules, CA). A Western immunoblotting kit (KPL, Gaithersburg, MD) was used for detecting relative amounts of Hha in different strains. The membrane was first incubated in a blocking buffer for 1 h, followed by incubation in rabbit anti-Hha (diluted 1:10 in blocking buffer) antibodies for 1 h. The membrane was washed, incubated with HRP-conjugated goat-anti-rabbit-antibody, and treated with HRP substrate according to the instructions of the kit (KPL, Inc., Gaithersburg, MD).

## Results

Isolation and characterization of transposon mutants exhibiting reduced expression of *LEE4* (*espA*)::*lac* fusion in EHEC O157:H7  $\Delta$ *hha*. We have previously shown that the deletion of *hha* in EHEC O157:H7 (strain 86-24) resulted in the enhanced expression  $\beta$ -galactosidase activity from the *LEE4*::*lac* transcriptional fusion. Other *LEE* operons also exhibited substantial increases in their transcription in this strain (37). These increases in the transcriptional activities of *LEE4* and other *LEE* operons resulted from the enhanced expression of *ler* in the absence of *hha*. In order to identify regulatory factors responsible for the elevated expression of *LEE4*::*lac* fusion, strain VKS06 (EHEC O157:H7 strain 86-24  $\Delta$ *hha* containing *LEE4*::*lac* fusion) was subjected to transposon mutagenesis. Three pale

blue colonies were visually identified amongst the majority of the dark blue colonies that grew on LB agar plates supplemented with kanamycin and X-gal. Figure 1 shows the amount of  $\beta$ -galactosidase activity produced by one of the pale blue isolates (VKS07) relative to the parent strain (VKS06) in the quantitative colorimetric assay. The strain VKS07 demonstrated  $\beta$ -galactosidase activity (22.4 units/OD<sub>600</sub>) that was 48-fold less than the parent strain (1075.6 units/OD<sub>600</sub>) indicating that a transposon insertion had disrupted a gene encoding a factor essential for maintaining elevated expression of *LEE4::lac* fusion. Fig. 1 also shows that the level of  $\beta$ -galactosidase activity produced by the TN-KN mutant VKS07 was similar to that produced by the strain VKS05 expressing *hha*. Nucleotide sequence analysis of the DNA flanking the transposon insertion site in the mutant strain VKS07 revealed that the transposon had inserted 30-bp upstream from the 3' end of the *clpP* ORF of the *clpXP* operon.

Complementation of mutants carrying disrupted *clpP* with a plasmid cloned copy of *clpP* or *clpXP*. Since ClpXP is implicated in virulence control in *Streptococcus pneumoniae* (23), *Staphylococcus aureus* (10), *Escherichia coli* O157:H7 (36,43), and *Yersinia pestis* (17, 33), we wanted to understand the mechanism by which ClpXP facilitated enhanced expression of *LEE*-encoded virulence genes in EHEC O157:H7 lacking *hha*. First, we wanted to ascertain that the reduced expression of *LEE4::lac* fusion in EHEC O157:H7  $\Delta hha$  resulted from transpositional inactivation of *clpP* and not due to the polar effect on the expression of genes downstream of *clpXP* operon. For this, plasmid pSM201 (harboring *clpP* ORF) or pSM202 (harboring both *clpP* and *clpX* ORFs) were introduced in to the mutant strain VKS07. As shown in Fig. 1, transformation of pSM201 or pSM202 restored  $\beta$ -galactosidase activity of the mutant strain VKS07 to the levels that were equivalent to 62% (667.7 units/OD<sub>600</sub>) or



74% (804.7 units/OD<sub>600</sub>), respectively, of the activity expressed by VKS06 (1075.6 units/OD<sub>600</sub>). Transformation of vector alone (pCR2.1), however, did not bring any significant change in the  $\beta$ -galactosidase activity of strain VKS07. These results indicated that *clpP* is required for maintaining high levels of *LEE4::lac* expression in the absence of *hha*. In other words, absence of ClpXP and Hha in EHEC O157:H7 could potentially allow either the enhanced accumulation and/or expression of another repressor that reduced the expression of the *LEE4::lac* fusion to very low levels.

Identification of the gene encoding a repressor of *LEE4::lac* expression in the absence of *clpXP* and *hha*. To identify the gene encoding a repressor of *LEE4::lac* expression in the absence of *clpXP* and *hha*, the *clpXP* operon was deleted from strain VKS06 to construct strain VKS08. As shown in Fig. 2, VKS08 produced very low levels of  $\beta$ -galactosidase activity (26.6 units/OD<sub>600</sub>), that is levels similar to that produced by VKS07 (Fig. 1). The strain VKS08 was then subjected to transposon (TN-KN) mutagenesis to identify insertion mutants that have their  $\beta$ -galactosidase activity restored to the same high level as was expressed in VKS06. Among 3000 kanamycin-resistant colonies, 10 colonies were found to exhibit darker shade of blue color on LB agar plates supplemented with kanamycin and X-gal. However, when these transposon mutants were tested using quantitative colorimetric assay, only two isolates (isolate #3 and #6) showed  $\beta$ -galactosidase activity levels that were significantly higher (isolate #3, 200-fold; isolate #6, 800-fold) than VKS08. Genomic DNA from isolate #3 and #6 was fragmented with *EcoRI* and cloned in pUC18 for isolation of kanamycin-resistant (i.e. transposon-bearing) colonies. Nucleotide sequence analysis of the plasmid DNA revealed that four kanamycin-resistant isolates that originated from transposon mutant #6 had transposon insertion in the promoter region of *grlR* (4 of 6 isolates) and the

two isolates that originated from the mutant #3 contained transposon insertion in the sequence that had no homology to the nucleotide sequences in the NCBI's database (2 of 6 isolates). The mutants containing transposon insertion in *grlR* were named VKS09.

Complementation of transposon-disrupted *grlR* mutant with a plasmid-cloned copy of *grlR*.

Since *grlR* (LEE-encoded first gene of *grlR-grlA* operon) has been shown to cause repression of *ler* in *Citrobacter rodentium* and EHEC O157:H7 (5, 13, 25), we wanted to demonstrate whether the loss of *grlR* function was the major reason behind restoration of hyperexpression of *LEE4::lac* fusion in strain VKS09. For this, the complete *grlR* ORF was isolated by PCR and cloned into pCRXL TOPO TA cloning vector system to construct plasmid pSM270. As shown in Figure 2, strain VKS09 containing disrupted *grlR* produced 2248.1 units of  $\beta$ -galactosidase activity that was 48.1-fold higher than that produced by VKS08 (26.6 units/OD<sub>600</sub>). Addition of plasmid pSM270 into strain VKS09, however, reduced  $\beta$ -galactosidase activity of this strain to 24.4 units/OD<sub>600</sub>. This reduction was equivalent to the activity produced in the parent strain VKS08 (26.6 units/OD<sub>600</sub>) thus indicating that *grlR* acts as a negative regulator of *LEE4* expression in the absence of *hha* and *clpXP*.

Effect of *clpXP* and *hha* on transcriptional levels of *grlR* and *grlA*. According to a recent report, *grlR* and *grlA* encode for a repressor (GrlR) and an activator (GrlA) of *ler*, respectively, and these two genes constitute an operon that is located within the LEE region (1). In an attempt to understand the mechanisms by which *clpXP* and *hha* affect *grlR-grlA* expression, we used 10-fold serially-diluted RNA from EHEC O157:H7 strain VKS01 (*hha*<sup>+</sup> *clpXP*<sup>+</sup>), VKS02 ( $\Delta$ *hha clpXP*<sup>+</sup>), VKS03 (*hha*<sup>+</sup>  $\Delta$ *clpXP*), and VKS04 ( $\Delta$ *hha*  $\Delta$ *clpXP*) in RT-PCR assays to determine transcriptional levels of *grlR* and *grlA*. As shown in Fig. 3,

transcriptional levels of *grlR* and *grlA* were increased by 100-fold in VKS02 (lanes 1–4) in comparison to the transcriptional levels produced for these two genes in VKS01 and VKS03 (lanes 1 and 2). These results indicated that Hha acts as a repressor of *grlR-grlA* expression regardless of the presence or absence of *clpXP*. Interestingly, transcriptional levels for both *grlR* and *grlA* were 10-fold lower in strain 86-24 lacking both *hha* and *clpXP* compared to those observed in *hha*-negative but *clpXP*-positive strain. This observation would suggest that the double deletion allows accumulation of another repressor that reduces transcriptional increases of *grlR-grlA* from 100-fold (observed in VKS02) to 10-fold (VKS04).

Effect of increased expression of *grlR-grlA* on the expression of *LEE4* and *ler*. Based on the RT-PCR data described in Fig. 3, we concluded that the expression of *grlR* and *grlA* is negatively regulated by *hha* because increased transcription of *grlR-grlA* occurred in strain VKS02 ( $\Delta hha$ ). However, total increases of *grlR-grlA* transcription in the *hha*-negative strain were also affected by the presence or absence of *clpXP*. For example, *grlR-grlA* transcription increased by about 100-fold in VKS02 ( $\Delta hha clpXP^+$ ) while only a 10-fold increase was noticed for *grlR-grlA* transcription in strain VKS04 ( $\Delta hha \Delta clpXP$ ) (Fig. 3). To determine whether increased transcription of *grlR-grlA* in *hha*-negative strain would affect the expression of *LEE4* and *ler*, we used RT-PCR to detect levels of *ler*- and *LEE4*- (measured by detecting *espA*, the very first gene in the *LEE4* operon) specific amplification products in strain VKS02 ( $\Delta hha$ ) carrying or lacking *clpXP*. As shown in Fig. 4, the levels of a control gene *gapA* were identical in all four strains. On the other hand, strain VKS02 ( $\Delta hha clpXP^+$ ) showed increases of  $\geq 100$ -fold (lanes 1–5) and  $\geq 10$ -fold (lanes 1–3) for *LEE4*- and *ler*-specific amplification products, respectively, relative to the transcriptional

levels observed for *LEE4* (lanes 1–3) and *ler* (lanes 1-2) in the parent strain VKS01. Thus, these results indicated that increased transcriptional levels of *grlR-grlA* in the *hha*-negative strain did not abolish increased expression of *ler* and *LEE4* presumably because the presence of ClpXP in this strain maintained GrlR at levels unable to reduce hyperexpression of *ler* and *LEE4*. However, no differences in the *ler* expression could be detected in VKS03 (*hha*<sup>+</sup>  $\Delta$ *clpXP*) or VKS04 ( $\Delta$ *hha*<sup>+</sup>  $\Delta$ *clpXP*) relative to the *ler* expression in the parent strain. Interestingly, however, transcriptional levels of *LEE4* were 10-fold lower in VKS03 (*hha*<sup>+</sup>  $\Delta$ *clpXP*) (Fig. 4) than the levels of *LEE4* in VKS01 while the transcriptional levels of *grlR-grlA* in VKS03 were identical to the levels produced in VKS01 (*clpXP*<sup>+</sup> *hha*<sup>+</sup>) (Fig. 3). These results suggest that the lack of *clpXP* presumably allows not only GrlR but also Hha to accumulate, thereby causing a more robust repression of *LEE4* in a strain deleted of *clpXP* versus a strain expressing *clpXP*.

Accumulation of Hha in *clpXP* deletion mutants. It has been shown in previous studies that the accumulation of GrlR and RpoS in *clpXP*-deleted strains of EHEC O157:H7 causes repression of *ler* and *ler*-regulated *LEE* operons (16). Having demonstrated that Hha acts as a negative regulator of *grlR-grlA* (current study) and *LEE4* (37), we further investigated if the lack of *clpXP* in strain VKS03 is responsible for increased accumulation of Hha and enhanced repression of *LEE4*. For this, cell-free extracts from strain VKS01 (*hha*<sup>+</sup> *clpXP*<sup>+</sup>), VKS02 ( $\Delta$ *hha*<sup>+</sup> *clpXP*<sup>+</sup>), VKS03 (*hha*<sup>+</sup>  $\Delta$ *clpXP*), and VKS04 ( $\Delta$ *hha*  $\Delta$ *clpXP*), were resolved on a 15% SDS-PAGE. The proteins from the gel were transferred to a nitrocellulose membrane and the membrane was probed with an anti-Hha antibody. As shown in Fig. 5, a protein band identical to Hha in size could only be detected in strain VKS03 lacking *clpXP*. In addition, the relative location of this protein band on the membrane was identical to that

occupied by the purified Hha in the control lane, indicating that the strain lacking *clpXP* did accumulate Hha. The inability to detect Hha in the parent 86-24 *hha*<sup>+</sup> *clpXP*<sup>+</sup> strain could be attributed to the presence of very low levels of Hha, which were below the detection limits of the immunoassay used in this study.

## Discussion

The most important prerequisite for EHEC O157:H7 and other related pathogens inflicting A/E lesions on intestinal epithelium is the activation of LEE that encodes factors mediating A/E lesion formation. The expression of *ler*, the key positive regulator of LEE, is controlled by a myriad of positive and negative regulatory factors as described in this manuscript. Two important negative regulators of *ler* include *hha* and *grlR*, with the former serving as a LEE-independent regulator and the latter functioning as a LEE-encoded regulator (25, 37). Both genes appeared to be required for maintaining LEE expression to a basal minimum, which is a reasonable strategy for EHEC not to expend its energy expressing LEE at higher levels until the pathogen is in the right niche of the host that provides environmental signals conducive for up-regulation of LEE. Recent studies have implicated that the AAA<sup>+</sup> protease ClpXP might be a critical component of regulatory cascade that controls the level of negative regulator, such as *grlR*, to upregulate LEE expression under appropriate conditions (17). In pathogens like *Yersinia pestis*, ClpXP and Lon protease coordinately control the expression of type-III secretion system by regulating the stability of YmoA, a small histone-like protein similar to Hha in *E. coli* (17). ClpXP has also been shown to regulate the expression of  $\alpha$ -hemolysin (*hly*) in *E. coli* and quorum-sensing locus *agr* that regulates the stress tolerance in *Staphylococcus aureus* (11, 15).

We have previously reported that Hha exhibits specific binding in *in vitro* assays to the *ler* promoter and that Hha-*ler* promoter interactions presumably are responsible for the reduced transcription of *ler* and *ler*-regulated *LEE* operons (37). The interactions between Hha and its target promoters also form the basis for Hha-mediated reduced transcription of virulence gene-regulator *hilA* in *Salmonella typhimurium* (8) and for the expression of hemolysins in *E. coli* pathotypes causing extraintestinal infections in humans (32).

In the present study, we provide experimental evidence illustrating that ClpXP is required for LEE expression because disruption of *clpXP* in Hha-deficient EHEC O157:H7 resulted in a dramatic reduction of *LEE4* expression. This repression could be restored to wild-type levels by providing *clpXP* *in trans*. These observations suggest that the absence of ClpXP and Hha allowed another repressor to accumulate and /or express to levels capable of causing strong repression of *LEE4*. By screening a library of transposon mutants generated in VKS04 ( $\Delta clpXP \Delta hha$ ), we found elevated expression of *LEE4* in mutants containing transposon insertions immediately upstream of the start codon of *grlR*. This suggested that *grlR* must be expressed at higher levels in strains deleted of both *clpXP* and *hha*. Using RT-PCR, we were able to show that the absence of *hha* resulted in the detection of at least 100-fold higher levels of *grlR*- and *grlA*-specific amplicons compared to the levels of the same amplicons in the *hha*<sup>+</sup> strain. However, the presence or absence of *clpXP* had no effect on the transcriptional levels of *grlR* and *grlA*. These results concur with the findings of a study describing that the GrIR protein is a potential direct or indirect substrate in the pathway of ClpXP-mediated regulation of LEE in EHEC O157:H7 (16).

Additional evidence supporting the negative role of *hha* in the transcriptional regulation of *grlR* and *grlA* was obtained from experiments in which RT-PCR was used to

compare the levels of *ler*- and *LEE4* (*espA*)- specific amplicons in strains carrying or lacking *hha* or *clpXP* or both. According to the RT-PCR results, the strains lacking *hha* showed about 100-fold and 10-fold increases in the transcriptional levels of *LEE4* and *ler*, respectively. On the other hand, transcriptional levels of *LEE4* were 10-fold lower in the *hha*-positive but *clpXP*-deleted strain relative to the expression of *LEE4* in the parent strain possessing both *hha* and *clpXP*. These observations provided the basis for the following two conclusions. First, in absence of *hha*, *grlR* is presumably transcribed at higher levels but the presence of ClpXP protease does not allow GrlR to accumulate to levels high enough to have a significant negative affect on the hyperexpression of *LEE4* and *ler*. Second, the absence of *clpXP*, however, results in accumulation of Hha (as described in the present study) and GrlR (16) despite the fact the Hha reduces transcription of *grlR*. The increased intracellular levels of Hha (and presumably GrlR) might explain why the transcriptional levels of *LEE4* were lower in the *hha*<sup>+</sup> strain lacking *clpXP* compared to the levels of *LEE4* detected in the strain wild-type for both *clpXP* and *hha*. Although a recent report has described that ClpXP promotes *LEE4* expression by controlling levels of GrlR and RpoS (16) in EHEC O157:H7 Sakai strain, our study provides novel evidence that ClpXP-mediated regulation of intracellular levels of Hha might be a the part of a cascade controlling expression of the *grlR-grlA* operon. The data obtained by the Western blot analysis of the cell-free extracts confirmed that Hha accumulated in EHEC O157:H7 lacking *clpXP*. Based on the data provided in this report and previously published studies (1, 16, 37, 43), we propose a two-part model for the mechanism by which Hha, ClpXP, GrlR, and GrlA modulate expression of *ler* and *ler*-regulated LEE of EHEC O157:H7 (Fig. 6). The first part of the model depicts that the steady state expression or the basal level expression of *ler* and LEE by ClpXP is

accomplished by maintaining certain minimal (basal) intracellular amounts of Hha and GrlR. Since the intracellular levels of GrlA (which induces the expression of *ler*) are not affected by ClpXP (1), low level transcription from the *ler* promoter would ensue resulting in the steady state expression of *ler* and LEE genes. However, in strains lacking *hha*, ClpXP activity would presumably prohibit accumulation of GrlR despite a 100-fold increase in the transcription of *grlR*. A 100-fold increase in the transcriptional level of *grlA* in the *hha*-negative strain, on the other hand, will presumably enhance intracellular levels of GrlA that would facilitate hyperexpression of *ler* and *ler*-regulated genes.

In summary, the results reported in this study indicate that ClpXP is one of the key positive regulators that modulate expression of LEE-encoded genes by controlling intracellular levels of Hha in EHEC O157:H7. There appears to be hierarchy among Hha and GrlR and, based on the data provided in this and a previously published report (37), Hha represents the major negative regulator of LEE expression as it not only controls *ler* expression through its direct interactions with the *ler* promoter but also down-regulates expression of *grlR-grlA* operon. What remains to be investigated is whether Hha interacts with the *grlR-grlA* promoter in order to repress *grlR-grlA* transcription, whether Hha or GrlR or both serve as direct substrates for ClpXP, and if the levels of either or both of these repressors are affected by RpoS, which is also regulated by ClpXP.

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**Table 1.** Bacterial strains and plasmids<sup>a</sup>

<b>Strain</b>	<b>Relevant genotype and description</b>	<b>Source or Reference</b>
EHEC 86-24	<i>stx2</i> <sup>+</sup> EHEC O157:H7, strain 86-24	34
VKS01	86-24 deleted of <i>stx2</i> and <i>lac</i>	34
VKS02	86-24 $\Delta$ <i>stx2</i> $\Delta$ <i>lac</i> deleted of <i>hha</i>	34
VKS03	86-24 $\Delta$ <i>stx2</i> $\Delta$ <i>lac</i> deleted of <i>clpXP</i>	This study
VKS04	86-24 $\Delta$ <i>stx2</i> $\Delta$ <i>lac</i> $\Delta$ <i>hha</i> deleted of <i>clpXP</i>	This study
VKS05	86-24 $\Delta$ <i>stx2</i> $\Delta$ <i>lac</i> containing <i>LEE4::lac</i> transcriptional fusion	34
VKS06	86-24 $\Delta$ <i>stx2</i> $\Delta$ <i>lac</i> $\Delta$ <i>hha</i> containing <i>LEE4::lac</i> transcriptional fusion	34
VKS07	86-24 $\Delta$ <i>stx2</i> $\Delta$ <i>lac</i> $\Delta$ <i>hha</i> <i>LEE4::lac</i> containing transposon insertion in <i>clpP</i>	This study
VKS08	86-24 $\Delta$ <i>stx2</i> $\Delta$ <i>lac</i> $\Delta$ <i>hha</i> <i>LEE4::lac</i> deleted of <i>clpXP</i>	This study
VKS09	86-24 $\Delta$ <i>stx2</i> $\Delta$ <i>lac</i> $\Delta$ <i>hha</i> <i>LEE4::lac</i> containing transposon insertion in <i>grlR</i> promoter	This study
<i>E. coli</i> TOP 10	<i>endA1 recA1 hsd R17(r<sub>k</sub><sup>-</sup>m<sub>k</sub><sup>-</sup>) supE44</i> $\phi$ 80 <i>dlacZ</i> $\Delta$ M15 $\Delta$ ( <i>lacZYA-argF</i> )	Invitrogen
<b>Plasmids</b>		
pSM201	pCR2.1 containing <i>clpP</i>	This study

pSM202	pCR2.1 containing <i>clpXP</i>	This study
pSM222	pCRXL containing 1.5 kb sequence located 5' of <i>clpXP</i> (US sequence)	This study
pSM223	pCRXL containing 1.5 kb sequence located 3' of <i>clpXP</i> (DS sequence)	This study
pSM224	pCRXL containing 3 kb US-DS fragment (constructed by ligating 1.5 kb DS sequence of pSM223 at the 3' end of 1.5 kb US sequence present in pSM222)	This study
pSM225	pAM450 containing 3 kb fragment from pSM224	This study
pSM270	pCR2.1 containing 0.42 kb <i>grlR</i> ORF	This study
pSM272	0.84 kb fragment (0.42 kb <i>grlR</i> ORF and 0.42 kb sequence upstream of <i>grlR-grlA</i> ) cloned in pCR2.1	This study

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<sup>a</sup>Detailed descriptions of bacterial strains and plasmids are discussed in Materials and Methods.



**Table 2.** Primers used for PCR and RT-PCR

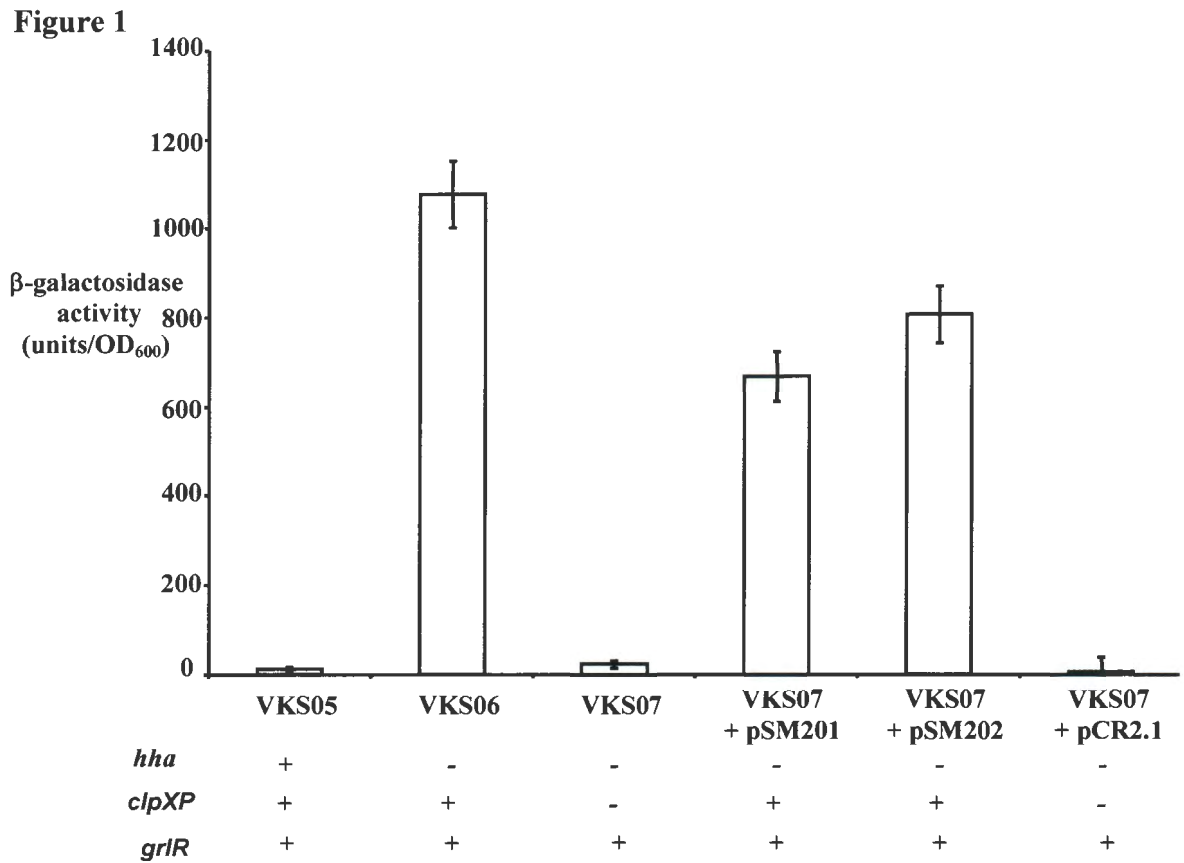
<b>Primer</b>	<b>Nucleotide sequence (5' – 3')</b>	<b>Gene</b>
VS319	CCGTTGAAGTGAAAGACGGTC	<i>gapA</i>
VS320	AACCACTTTCTTCGCACCAGC	<i>gapA</i>
VS323	TGTGGATGCCAAAATTGCTG	<i>espA</i>
VS324	CGCCTTCACTGTTTGCAGATC	<i>espA</i>
VS376	AGTCCATCATCAGGCACATTAG	<i>ler</i> ORF
VS377	CATACAACAAGTCCCATACATTC	<i>ler</i> ORF
VS418	GGTGGATCCTGGCGAAAGCGAAAGTGACTG	<i>clpP</i>
VS419	GGTGGATCCCATCTTTGCGTTTATCTGTCATG	<i>clpP</i>
VS420	GGTGGATCCAACGAGCTGATGAACCAGCAG	<i>clpXP</i>
VS421	GGTGGATCCCATTC AACGCCGAGAATAGAG	<i>clpXP</i>
VS449	CATCTAGACTATTTGAATGCTTTCGGG	<i>clpXP</i>
VS450	CAGTCGACTCCATGCAAAATTCATG	<i>clpXP</i>
VS451	CAGTCGACCATAACAATTAGTTAAC	<i>clpXP</i>
VS452	CATCTAGACAAGCAGTGCGGAAGC	<i>clpXP</i>
VS534	GATAGAACAAATTGAAAGGAGTG	<i>grlR</i>
VS535	CCGTCGACTTATTTTAAATAAACTTGTGGCATTCC	<i>grlR</i>
VS537	ACAGGCATCATTCAATAAATCATC	<i>grlR</i> - L0045 intergenic region
VS587	AATAAACTTGTGGCATTCTGTGT	<i>grlR</i>
VS588	CCCTCGGTGCTAAACATTGAAC	<i>grlR</i>
VS589	CGGTTGTTCCAGGACTTTCTATC	<i>grlA</i>

VS590      CTGAGGAGAGCATCATTATTATAAC

*grlA*

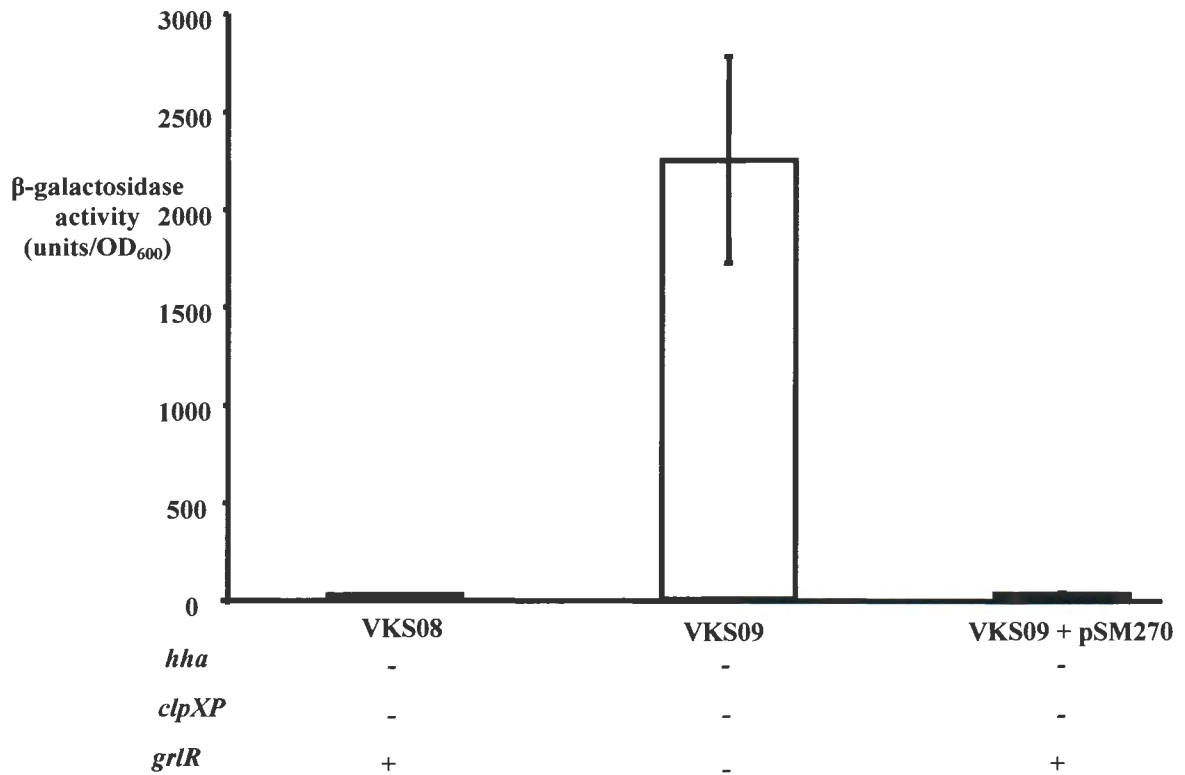
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<sup>a</sup> Position of the primer sequence represents the location in the published sequence deposited under the indicated accession numbers at NCBI. Underlined sequences GGATCC, GTCGAC, and TCTAGA represent restriction sites *Bam*HI, *Sal*I, and *Xba*I, respectively.



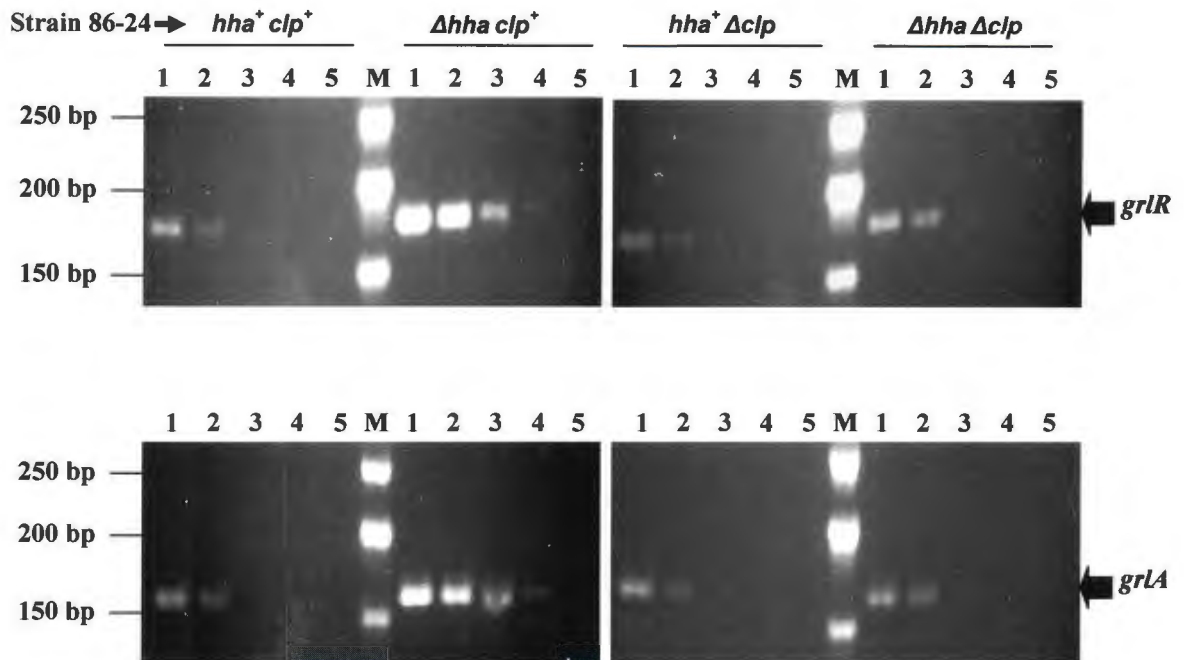
**Fig. 1.** Comparison of  $\beta$ -galactosidase activity produced by the *LEE4::lac* transcriptional fusion in various genetic backgrounds of EHEC O157:H7 strain 86-24  $\Delta stx2 \Delta lac$ . Amounts of  $\beta$ -galactosidase produced, as determined by  $OD_{600}$  values and a liquid colorimetric assay, by each strain are shown as solid bar. Error bars represent standard deviations based on three measurements.

Figure 2



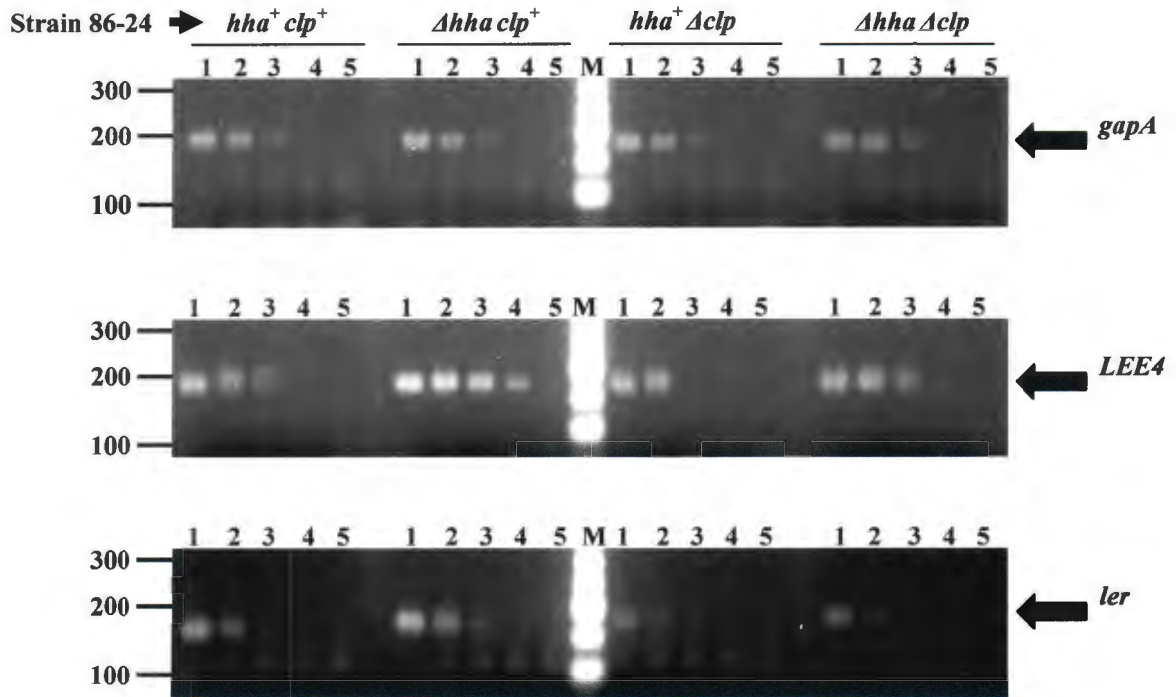
**Fig. 2.** Determination of the  $\beta$ -galactosidase activity of a EHEC O157:H7 strain 86-24  $\Delta hha$   $\Delta clpXP$  and its derivative containing transposon-disrupted *grlR* with or without a plasmid providing *grlR* in trans. Amounts of  $\beta$ -galactosidase produced by each strain are shown as solid bars. Error bars represent standard deviations based on three measurements.

Figure 3



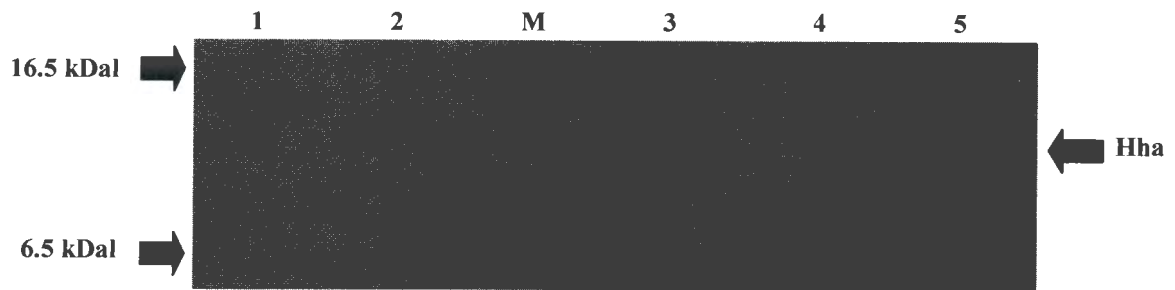
**Fig. 3.** RT-PCR-based determination of transcriptional levels of *grlR-grlA* in strains deleted of *hha* or *clpXP* or both. Amplification products generated by RT-PCR were resolved on 4% agarose gels containing ethidium bromide, and DNA bands were visualized using Alpha Innotech Image documentation system. Lanes 1-5, RT-PCR conducted in the presence of  $8 \times 10^1$ ,  $8 \times 10^0$ ,  $8 \times 10^{-1}$ ,  $8 \times 10^{-2}$ , and  $8 \times 10^{-3}$   $\mu\text{g}$  of total RNA; lanes: M, DNA size markers (size of marker bands is listed in on the left side). The arrow on the right shows the position of amplified products specific for *grlR*, and *grlA*.

Figure 4



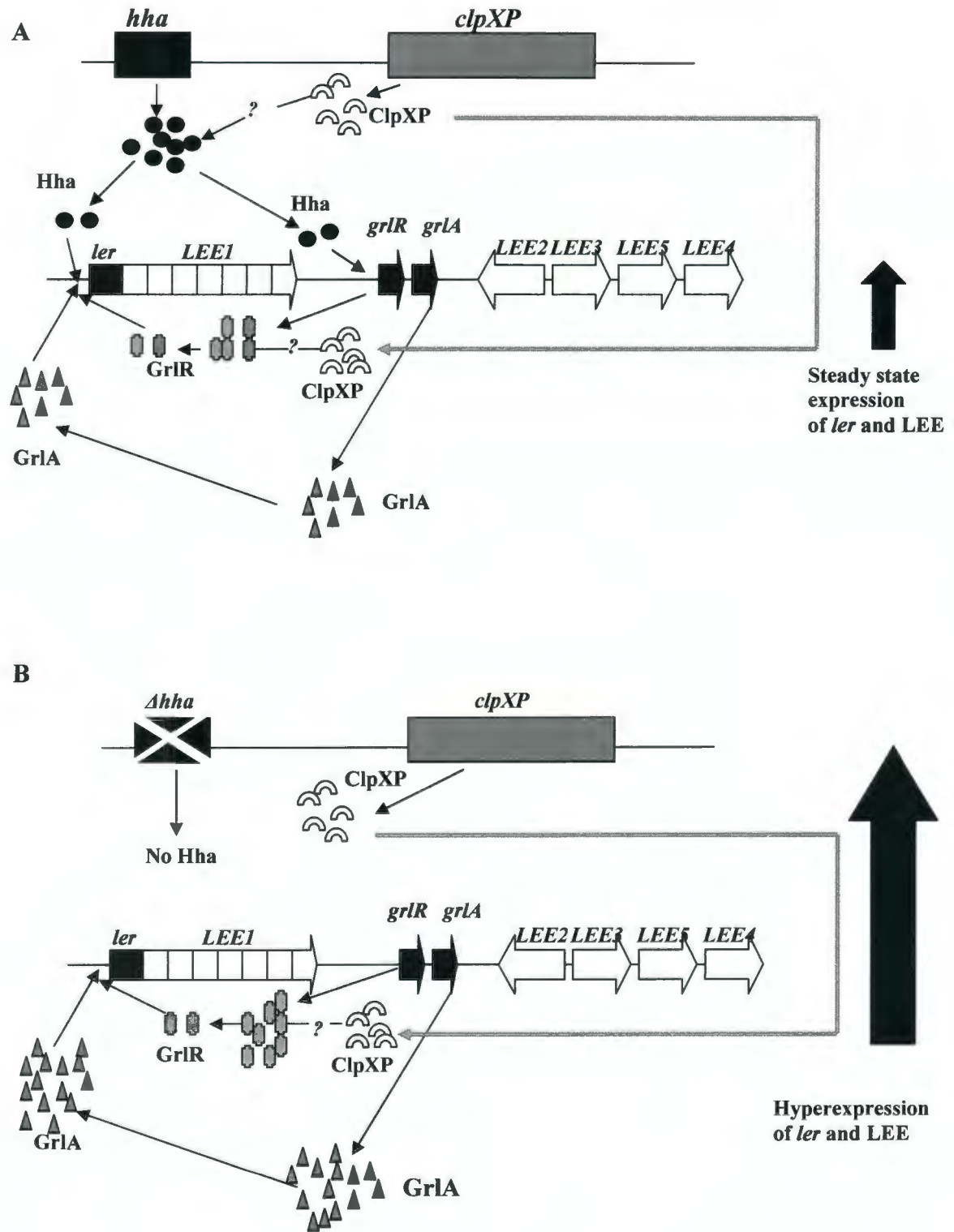
**Fig. 4.** Determination of transcriptional levels of *LEE4* and *ler* using RT-PCR. Total RNA that was determined to be free of DNA contamination was diluted in a 10-fold serial dilution and used in RT-PCR assay to amplify and detect *gapA*-, *LEE4*- and *ler*-specific amplicons. The *gapA* was used as a control because the transcriptional level of this gene remain unaltered in mutant strains examined in this study. Equal volumes of amplified samples were resolved on 4% agarose gels containing ethidium bromide, and DNA bands were visualized using Alpha Innotech Image documentation system. Lanes 1-5, RT-PCR conducted in the presence of  $8 \times 10^1$ ,  $8 \times 10^0$ ,  $8 \times 10^{-1}$ ,  $8 \times 10^{-2}$ , and  $8 \times 10^{-3}$   $\mu\text{g}$  of total RNA; lanes: M, DNA size markers (size of marker bands is listed in on the left side). The arrow on the right shows the position of amplified products specific for *gapA*, *LEE4*, and *ler*.

Figure 5



**Fig. 5.** Effect of *clpXP* deletion on intracellular levels of Hha. Cell-free extracts containing identical amounts of total proteins prepared were resolved on a 4-20% SDS-polyacrylamide gel. The resolved proteins were electroblotted to a nitrocellulose membrane, and the membrane, after treatment with a blocking solution, was incubated with rabbit anti-Hha polyclonal serum followed by incubation with a goat-anti-rabbit polyclonal antibodies conjugated to horseradish peroxidase. The specific binding of anti-Hha antibody was determined by visualization of purple bands at a position corresponding to the predicted size for Hha. Total proteins from strain VKS01, VKS03, VKS02, and VKS04 were analyzed in lane 1, 2, 3, and 4, respectively. Lane M contains molecular size (indicated as bp on the left side) markers. Arrow shows the position of Hha-specific signal.

Figure 6





**Fig. 6.** Diagrammatic representation of the ClpXP- and Hha-mediated regulation of *ler* and LEE expression in EHEC O157:H7. A) This part of the model shows that by some unknown mechanism (?), ClpXP keeps Hha at concentrations that allow very low levels of transcription to proceed from promoters transcribing *ler* and *grlR-grlA*. Since GrlA (*grlA* encoded protein) is not affected by ClpXP protease unlike GrlR (mechanism not known), increased amounts of GrlA would have an overall positive effect on *ler* transcription resulting in a steady state or low level of LEE expression. B) This part of the model shows that if Hha is not present in the cell (due to *hha* deletion in this study) or its concentration become too low to repress *ler* and *grlR-grlA*, *ler* and LEE would be up-regulated because of increased amounts of GrlA. Transcriptional levels of *grlR* would also be elevated, but ClpXP would keep GrlR at levels much lower than that of GrlA.

## General Conclusions

Enterohemorrhagic *Escherichia coli* O157:H7 is a major food borne pathogen which can lead to severe human disease. This strain in particular is a burden in the U. S. due to the difficulty in identifying and controlling the fecal shedding in our dairy and beef cattle herds. Multiple attempts at control have failed and with the expanding knowledge of molecular genetics we are opening doors to new methods of control and prevention.

This study was designed to identify important and novel regulatory agents of *LEE4*. The experiment utilized transcriptional fusions to monitor the effects of gene knockouts and identified the ClpXP protease as a positive regulator of *LEE4*. Additional experiments yielded the identification of GrlR and Rep as potential substrates for the ClpXP protease. Unfortunately, before we could publish our work these results were reproduced and published by another group. The identification of potential substrates for ClpXP included proteins, Hha and GlrR which are negative regulators of LEE. Our laboratory previously identified Hha as a transcriptional regulator of LEE. Additional experiments and the correlation with YmoA of *Yersinia pestis* as a Hha homolog made Hha a probable target. We identified that in the absence of the ClpXP protease and the presence of other proteases such as Lon, the protein Hha accumulates. This finding is indicative of Hha as a potential substrate for the ClpXP protease system and further experiments are warranted.

Upon completion of this paper, additional experiments were considered such as the use of crude protein extracts and digestion assays to identify Hha as a potential direct target for the ClpXP protease, 2-dimensional gel systems to compare protein distributions in parent and mutant strains (86-24 $\Delta$ *stx2*  $\Delta$ *lac*,  $\Delta$ *clpXP*,  $\Delta$ *hha*, and  $\Delta$ *clpXP*  $\Delta$ *hha*) and microarray

systems to identify an overall view of genetic activity in parent and mutant strains. I see the use of these techniques in the future to better characterize the regulatory and modulatory effect of ClpXP in LEE of EHEC O157:H7. The information gained in these experiments will help contribute to the knowledge of LEE regulation in EHEC O157:H7. The important discovery of *ler* in the modulation of virulence has lead to the potential production of vaccines (58). The more we learn about these regulatory cascades, the more efficient our attempts will be to reduce human exposure to EHEC O157:H7.

### **Acknowledgements**

I would like to extend thanks to everyone who made it possible for me to concurrently achieve a master's degree and my doctorate of veterinary medicine. It has been a long and challenging journey but very rewarding.

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