Regulation of *nleA* in Shiga Toxin-Producing *Escherichia coli* O84:H4 Strain 4795/97[†]

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Many Shiga toxin-producing *Escherichia coli* (STEC) strains express a type III secretion system (TTSS) encoded by the locus of enterocyte effacement (LEE). Using the TTSS, STEC is able to inject effector proteins directly into eukaryotic host cells, where they cause characteristic attaching and effacing (A/E) lesions. In addition to the LEE-encoded effectors, a number of non-LEE-encoded effectors, located on phage-associated elements, have been described. One of them, the non-LEE-encoded effector *A* (NleA), is widely distributed among pathogenic *E. coli*. In this study, we investigated the influence of environmental conditions on the expression of the phage-encoded effector *nleA* gene (designated *nleA*₄₇₉₅) present in STEC O84:H4 strain 4795/97. We demonstrated that a particular NaCl concentration and starvation stress increase the activity of the *nleA*₄₇₉₅ promoter. Moreover, several regulators that control *nleA*₄₇₉₅ expression were identified. The involvement of the LEE regulators Ler, GrlA, and GrlR show that *nleA*₄₇₉₅ is integrated in the LEE regulation circuit. Furthermore, the binding of Ler to sequences upstream of *nleA*₄₇₉₅ underlined these findings.

Enterohemorrhagic *Escherichia coli* (EHEC) and Shiga toxin-producing *E. coli* (STEC) are important human pathogens which are able to cause serious intestinal diseases, ranging from uncomplicated watery diarrhea to bloody diarrhea and hemorrhagic colitis. In up to 20% of the cases, EHEC infection is followed by extraintestinal complications such as the hemolytic uremic syndrome (33). Most EHEC strains colonize the intestinal mucosa by the formation of characteristic “attaching and effacing” (A/E) lesions (32, 48). These A/E lesions are characterized by the local destruction of microvilli, the intimate binding of the bacteria to the enterocytes, and the building of actin-rich pedestal-like structures on the host cells (48). Essential for the formation of A/E lesions is a chromosomal pathogenicity island termed the locus of enterocyte effacement (LEE) (14, 28). The LEE is organized in five polycistronic operons and encodes the components for a type III secretion system (TTSS) and several effector proteins, which are translocated by the TTSS machinery into host cells (18). Several studies have shown that the “LEE-encoded regulator” (Ler), encoded by the first gene of the *LEE1* operon, is a crucial positive regulator for the expression of LEE genes (13, 15). Two more regulators are encoded between the *LEE1* and the *LEE2* operons, the “global regulator of LEE activator” (GrlA) and the “global regulator of LEE repressor” (GrlR) (12). It was previously shown that Ler and GrlA can influence each other's expression in a positive regulatory loop (2).

In addition to the known LEE regulators, a number of regulators encoded outside the LEE were shown to be involved in

the LEE regulation circuit, an example of which is *pch*. The *pch* genes comprise a group of homologous prophage-associated genes which are similar in sequence to the plasmid-encoded PerC regulator of enteropathogenic *E. coli* (EPEC) (25, 53). Based on sequence similarities, the *pch* genes are classified in three different groups. One group is formed by the *pchA*, *pchB*, and *pchC* genes, which are nearly identical in sequence and differ in only one or two nucleotides. The shorter *pchD* and *pchE* genes constitute the other two groups (25, 53). Recently, two additional *pchABC*-like genes were defined as *pchX* and *pchY* (53), but only the *pchA*, *pchB*, and *pchC* genes were described as encoding positive regulators of the *LEE1* operon (25). Another positive regulator of LEE is QseA (quorum-sensing *E. coli* regulator A), which activates the transcription of Ler in response to quorum sensing (41). So far, three different types of signal molecules, termed “autoinducers” (AI), have been described in quorum sensing; these are designated AI-1 and AI-2 (4) and AI-3 (43). AI-1 and AI-2 are widely distributed among different species of Gram-negative bacteria and are involved in both intraspecific and interspecific communication (3, 45, 51). The AI-3-dependent quorum-sensing system responds to the hormones epinephrine and norepinephrine and is involved in interkingdom communication (43). Two examples of negative regulators encoded outside the LEE are EtrA and EivF, which are encoded by the *E. coli* type III secretion system 2 (ETT2) gene cluster. The ETT2 gene cluster is distributed functionally as a whole or truncated among the majority of *E. coli* strains, independent of their pathogenicity (38). EtrA and EivF were shown to exert strong negative effects on the expression and secretion of LEE genes and proteins at the transcriptional level (54), whereas EtrA was suggested to produce a stronger influence than EivF.

Besides the seven LEE-encoded effector proteins, a large number of type III effectors which are encoded outside the LEE have been identified (12, 47). These non LEE-encoded effectors are encoded mostly on cryptic or inducible prophages

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TABLE 1. *E. coli* strains and plasmids used in this study

<i>E. coli</i> strain or plasmid	Description ^a	Reference
Strains		
4795/97	O84:H4 <i>stx</i> ₁ ⁺ <i>nleA</i> ⁺ <i>eae</i> -ζ ⁺	55
EDL933	O157:H7 <i>stx</i> ₁ <i>stx</i> ₂ <i>eae</i> -γ ⁺	34
DH5α	<i>supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Invitrogen
BL21(DE3)	F ⁻ <i>ompT hsdSB</i> (r _B ⁻ m _B ⁻) gal dcm λ(DE3)	44
Plasmids		
pKD4	Template plasmid containing the <i>aph</i> cassette; Ap ^r Km ^r	11
pKD46	Red recombinase system under the control of <i>araB</i> promoter; Ap ^r	11
p3121	Luciferase (<i>luc</i>) template vector; Ap ^r Km ^r	21
pCP20	Helper plasmid containing FLP recombinase; Ap ^r	11
pWSK29	Low-copy-number vector, derived from pBluescript II SK(+); Ap ^r	52
pCM1	pWSK29 derivative carrying <i>ler</i> under control of its own promoter	This study
pCM2	pWSK29 derivative carrying <i>grlA</i> under control of its own promoter	This study
pCM3	pWSK29 derivative carrying <i>pchA</i> under control of its own promoter	This study
pET-22b(+)	High-copy-number vector containing His tag sequence, T7 promoter	Novagen
pET- <i>ler</i> -his	pET-22b(+) carrying <i>Ler</i> -His ₆ under control of the T7 promoter	This study
pK18	Multicopy cloning vector; Km ^r	37
pK18- <i>rrsB</i>	pK18 carrying <i>rrsB</i>	T. Slanec, unpublished data
pK18- <i>gapA</i>	pK18 carrying <i>gapA</i>	T. Slanec, unpublished data
pK18- <i>nleA</i>	pK18 carrying <i>nleA</i>	This study

^a Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

and include Cif (27), EspI/NleA (22, 30), EspJ (10), Tccp/EspFu (7, 19), and others (18). The non-LEE-encoded effector A (synonym, EspI), is widespread among pathogenic *E. coli* strains (9, 29). In the mouse pathogen *Citrobacter rodentium*, NleA was associated with a more severe course of disease (22). Furthermore, it was shown that NleA localizes with the Golgi apparatus after translocation (8, 22) into host cells and that it compromises the protein traffic and secretion from the endoplasmic reticulum through binding a subunit of the COPII coat (26). In a recent study, NleA was even found to play an important role in the destruction of tight junctions in EPEC (46).

Due to the widespread distribution of *nleA* in pathogenic *E. coli*, an aim of this study was to investigate the regulation of *nleA* in a non-O157 *E. coli* serotype (9). Therefore, we selected STEC O84:H4 strain 4795/97, which contains the *nleA*₄₇₉₅ variant, located on a *Stx*-converting prophage (8, 55). The expression of *nleA*₄₇₉₅ in response to different environmental conditions and crucial regulators was investigated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All strains and plasmids used in this study are described in Tables 1 and 2. Bacteria were grown in Luria-Bertani (LB) broth at 37°C or 30°C and 180 rpm. Modified LB broth containing NaCl at final concentrations of 0.4% and 0.1%, simulated colonic environment medium (SCM) (6), and minimal medium M9, supplemented with 44 mM NaHCO₃ (40), were used to test their influence on *nleA*₄₇₉₅ expression. SOC medium, supplemented with 5 mM MgCl₂ and 5 mM MgSO₄ (40), was used to recover cells after electroporation. When required, LB broth was supplemented with 10 mM arabinose, 1% glucose, epinephrine or AI-1 [*N*-(3-oxooctanoyl)-L-homoserine lactone] (Sigma), or antibiotics to final concentrations of 100 μg ml⁻¹ for ampicillin and 50 μg ml⁻¹ for kanamycin.

PC medium. Overnight cultures of EHEC O157:H7 strain EDL933 or strain MS-36 were diluted 1:100 in LB broth. Cultures were incubated at 37°C and 180 rpm and grown to an optical density at 600 nm (OD₆₀₀) of 1.4. Bacterial cells were separated by centrifugation at 8,000 × *g* at 4°C for 10 min. The supernatants were adjusted to a pH value of 7.5 and sterilized by filtration (pore size of 0.22 μm). Preconditioned (PC) medium was aliquoted and stored at -20°C (45).

Construction of deletion mutants. Gene deletions were constructed by site-directed mutagenesis (11, 21). The replacement of *nleA*₄₇₉₅ was performed using

plasmid p3121 (Table 1) carrying the *luc* reporter gene, followed by an *aph* cassette (21). All further deletion mutants were constructed using template plasmid pKD4 (Table 1), which carries the *aph* cassette without a reporter gene. PCRs were performed using the primers listed in Table S1 in the supplemental material. As the complete genome of the EHEC O84:H4 strain has not been sequenced so far, the primers used for site-directed mutagenesis were designed according to the respective gene sequences present in the genome of EHEC O157:H7 strain EDL933 (35). Deletions were confirmed by PCR (primers are listed in Table S2 in the supplemental material) and nucleotide sequencing using a CEQ 8000 genetic analysis system (Beckmann, Coulter) according to standard protocols. These procedures resulted in *E. coli* strains MS-10, MS-11, MS-12, MS-13, MS-14, and MS-15 (Table 2). Where necessary, the deletions were complemented by transformation with low-copy-number plasmid pCM1, pCM2, or pCM3 (Table 1). Moreover, two mutants with deletions in a second regulatory gene were generated, resulting in strains MS-1112 and MS-1313 (Table 2). In the

TABLE 2. Recombinant strains and transformants of STEC O84:H4 strain 4795/97 and EHEC O157:H7 strain EDL933^a

Strain	Description
MS-10.....	Replacement of <i>nleA</i> ₄₇₉₅ by <i>luc</i> in strain 4795/97
MS-11.....	MS-10 with in-frame deletion of <i>ler</i>
MS-12.....	MS-10 with in-frame deletion of <i>pchA</i>
MS-13.....	MS-10 with in-frame deletion of <i>grlA</i>
MS-14.....	MS-10 with in-frame deletion of <i>grlR</i>
MS-15.....	MS-10 with in-frame deletion of <i>etrA</i>
MS-16.....	MS-10 with in-frame deletion of <i>luxS</i>
MS-1112.....	MS-10 with in-frame deletions of <i>ler</i> and <i>grlA</i>
MS-1313.....	MS-10 with in-frame deletion of two <i>pch</i> genes
MS-11/pCM1.....	MS-11 complemented with pCM1
MS-12/pCM2.....	MS-12 complemented with pCM2
MS-13/pCM3.....	MS-13 complemented with pCM3
MS-21.....	4795/97 with in-frame deletion of <i>ler</i>
MS-22.....	4795/97 with in-frame deletion of <i>grlA</i>
MS-23.....	4795/97 with in-frame deletion of <i>pchA</i>
MS-2323.....	4795/97 with in-frame deletion of two <i>pch</i> genes
MS-21/pCM1.....	MS-21 containing pCM1
MS-22/pCM2.....	MS-22 containing pCM2
MS-23/pCM3.....	MS-23 containing pCM3
MS-36.....	EDL933 with in-frame deletion of <i>luxS</i>

^a All strains were constructed in this study.

latter strain, in addition to *pchA*, a deletion within a second *pch* gene was generated. For transcriptional analysis of *nleA*₄₇₉₅ by real-time PCR, deletion mutants without reporter genes were constructed in the wild-type (WT) strain 4795/97, resulting in strains MS-21, MS-22, MS-23, and MS-2323 (Table 2).

Cloning of regulatory genes for complementation studies. Vectors for expression of *ler*, *pchA*, or *grlA* under the control of its own promoter were obtained using low-copy-number plasmid pWSK29 (Table 1). The respective wild-type genes, including their 500-bp upstream regions, were amplified from *E. coli* O84:H4 strain 4795/97 by PCR using the primers listed in Table S3 in the supplemental material. Purified PCR products and vector DNA were digested with the respective restriction enzymes, ligated in a ratio of 4:1, and transformed into *E. coli* strain DH5 α by electroporation. Transformants containing inserts were selected by their white color on agar plates containing 100 μ g ml⁻¹ ampicillin, 6 μ g ml⁻¹ isopropyl- β -D-thiogalactopyranoside (IPTG), and 150 μ g ml⁻¹ 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). Since *grlA* is located as the second gene in an operon, the *grlA* open reading frame and its promoter region were ligated and transformed in a two-step cloning procedure. First, *grlA* PCR products were cloned in vector pWSK29 using restriction sites for EcoRI and BamHI. In a second step, the *grlA*-containing vector was digested with SalI and EcoRI, and promoter-containing PCR fragments restricted with the same enzymes were ligated. After confirmation of the transformants by PCR and sequencing, the constructed plasmids were transformed into the respective deletion mutants. The resulting complementation mutants, MS-11/pCM1, MS-13/pCM3, and MS-12/pCM2, were selected by ampicillin resistance and confirmed by PCR and sequencing.

Measurement of *nleA*₄₇₉₅ promoter activity. Reporter gene activity was determined using a luciferase assay system according to the manufacturer's recommendation (Promega). Briefly, overnight cultures of the reporter strains were diluted in 30 ml of the respective medium (LB broth unless otherwise noted) to an OD₆₀₀ of 0.05. Cultures were grown at 37°C and 180 rpm, OD₆₀₀ values were determined at each time point, and 1 ml of the bacterial cultures was harvested by centrifugation for 8 min at 13,000 rpm. Bacterial pellets were resuspended in 450 μ l cell culture lysis reagent, shock frozen at -80°C, and incubated for 20 min at 25°C and 250 rpm in a heating block. Fifty microliters of the cell lysates was transferred to a white microtiter plate (Lumitrac 600; Greiner), and after addition of 50 μ l luciferase assay substrate, the total reporter gene expression was immediately determined by measuring the luminescence in relative light units (RLU) in a microplate reader (Infinite 200; Tecan). To adjust the resulting values to the number of cells, we defined a "relative reporter gene activity" as the ratio of RLU and the respective OD₆₀₀ values. All experiments were carried out three times.

Isolation of total RNA and synthesis of cDNA. Overnight cultures of wild-type strain 4795/97 and deletion mutants were diluted in 30 ml medium (LB broth unless otherwise noted) to an OD₆₀₀ of 0.05. Cultures were grown at 37°C and 180 rpm, either to the mid-logarithmic growth phase (2 h) for examination of different environmental conditions or to the late exponential phase (4 h) for investigation of different deletion mutants. A culture volume corresponding to 6 $\times$ 10⁸ cells was harvested by centrifugation at 13,000 rpm and 4°C for 5 min and stabilized with 2 volumes of RNeasy lysis reagent (Qiagen) and 1 volume of Tris-EDTA buffer (Sigma) for 5 min. After centrifugation at 13,000 rpm and 4°C for 10 min, cell pellets were lysed in 200 μ l Tris-EDTA buffer with 1 mg lysozyme ml⁻¹. The total RNA was isolated using a RNeasy minikit (Qiagen) according to the manufacturer's instructions and treated with the RNase-free DNase set (Qiagen). RNA was quantified photometrically and analyzed by formaldehyde-agarose gel electrophoresis. Total RNA from each experiment was isolated on three independent days.

Subsequently, cDNA synthesis was performed using the SuperScript II reverse transcriptase system (Invitrogen). One milligram of the obtained RNA was mixed with 3 μ g random primers (Invitrogen) and denatured for 10 min at 65°C. After annealing for 10 min at room temperature and incubation on ice for 2 min, the samples were mixed with 4 μ l 5 $\times$ first-strand buffer (Invitrogen), 2 μ l deoxynucleoside triphosphate (dNTP) mix (10 mM each dNTP), 200 U SuperScript II reverse transcriptase, and DNase- and RNase-free distilled water (Gibco) in a total volume of 20 μ l. For each sample a control without reverse transcriptase was included. RNA was reverse transcribed during incubation at 42°C for 90 min and subsequently heated for 10 min at 65°C with 5 μ l 1 M NaOH to inactivate the enzyme. The obtained cDNA was then neutralized with 5 μ l 1 M HCl and 200 μ l Tris-EDTA buffer and purified using the QIAquick PCR purification kit.

Gene expression analysis. The relative expression of *nleA*₄₇₉₅ was monitored with the iQ5 real-time PCR detection system (Bio-Rad) using *gapA* (glyceraldehyde-3-phosphate dehydrogenase) and *rpsB* (16S rRNA) as reference genes. A 2.8-ng portion of cDNA was mixed with 12.5 μ l iQ SYBR green Supermix

(Bio-Rad), 0.75 μ l of each primer (10 pmol μ l⁻¹) (primers are listed in Table S4 in the supplemental material), and DNase- and RNase-free distilled water in a total volume of 25 μ l. Each sample was analyzed in triplicate. Standard curves were generated using dilutions of the respective plasmids (Table 1) from 10⁻³ to 10⁻⁹. Controls without reverse transcriptase, as well as a positive (strain 4795/97) and a negative (H₂O) control, were included in each assay. The PCR conditions for the expression of *nleA*₄₇₉₅ were 1 cycle of 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 52°C for 30 s, and 72°C for 20 s. Specificities of the primers were tested by a melting curve analysis from 52°C to 95°C. The PCR conditions for the two reference genes were as follows: for *gapA*, 1 cycle of 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 63°C for 1 min, and 72°C for 20 s, and for *rpsB*, 1 cycle of 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 20 s. Fluorescence signals were collected after each cycle, and the threshold cycle (*C*_T) value for each sample was determined using the iQ5 optical system software (Bio-Rad). The relative expression of *nleA*₄₇₉₅ was then determined in relation to the reference genes *gapA* and *rpsB* as described by Pfaffl (36). Student's *t* test (one tailed) was used for statistical analysis, and a *P* value of <0.05 was considered significant.

Labeling of Ler. His tagging of proteins was performed using vector pET-22b(+) carrying the T7 promoter (Table 1). DNA fragments containing the respective gene flanked by NdeI and XhoI restriction sites were amplified by PCR with primers listed in Table S3 in the supplemental material. Vector and DNA fragments were digested, ligated, and transformed into *E. coli* strain DH5 α by electroporation. Transformants were selected by ampicillin resistance and confirmed by PCR and sequencing. Recombinant vectors containing the His-tagged regulator genes under the control of the T7 promoter were transformed in strain BL21(DE3) (Table 1) carrying the T7 polymerase downstream of the *lac* promoter. The resulting transformant, BL21(DE3)/pET-*ler*-his, was selected by ampicillin resistance and confirmed by PCR and sequencing.

Expression, purification, and refolding of His-tagged Ler protein. An overnight culture of *E. coli* BL21(DE3)/pET-*ler*-his (Table 1) was diluted 1:60 in 50 ml LB broth supplemented with 100 μ g ml⁻¹ ampicillin and 1% glucose. The culture was grown at 37°C and 180 rpm to the mid-logarithmic phase and induced with 1 mM IPTG. After treatment, the culture was incubated at 30°C and 210 rpm for 4 h. Bacterial cells were harvested by centrifugation at 4,000 $\times$ *g* and 4°C for 15 min and frozen immediately for at least 2 h. Purification of His-tagged Ler protein was carried out under denaturing conditions with Ni-nitrilotriacetic acid (NTA) spin columns (Qiagen) according to the manufacturer's recommendation. The protein eluate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Subsequent matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry was performed by the Service Unit of the Life Science Center, University of Hohenheim. For refolding, the denatured protein eluate was applied to Microcon centrifugal filter devices (Millipore; molecular mass cutoff, 3 kDa). The protein was separated from the elution buffer by four centrifugation steps at 12,000 rpm and 4°C for 2.5 h. After each step, the concentrated protein eluate was resuspended in dialysis buffer containing 30 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 20% (vol/vol) glycerol, 240 mM NaCl, 0.1% (vol/vol) Triton X-100, and 3 mM EDTA with decreasing amounts of urea (4 M, 1 M, 0.2 M, and, after the final step, without urea) as described by Barba et al. (2) with slight modifications. The protein concentration was determined using the 2-D Quant kit (GE Healthcare), and aliquots of the purified protein were stored at -20°C.

EMSA. To investigate the binding of regulator proteins to the *nleA* promoter region, electrophoretic mobility shift assays (EMSAs) were performed as follows. DNA fragments of different lengths and locations with respect to the *nleA* promoter region were synthesized and purified (primers are listed in Table S5 in the supplemental material). Between 100 and 1,200 ng (0.3 μ M to 3.6 μ M) of the purified and renatured regulator protein Ler-His₆ was mixed with 100 ng of the corresponding DNA fragments in final volume of 10 μ l containing 1% glycerol, 2 mM NaCl, 2 mM Tris-HCl (pH 7.5), 0.2 mM EDTA (pH 7.5), 2 mM dithiothreitol (DTT), and 0.2 mM MgCl₂ (50). If required, poly(dI-dC) (Roche Applied Science) was added as a nonspecific competitor at a final amount of 0.1 μ g. The protein-DNA mixture was incubated for 50 min at 30°C and then separated by agarose gel electrophoresis. Gels were cast with 0.8% low-electroendosmosis (EEO) agarose (LE agarose; Biozym) in 1 $\times$ Tris-acetate-EDTA (TAE) buffer and run at 70 V and 4°C for 4.5 h in 1 $\times$ TAE buffer. Agarose gels were stained with ethidium bromide, and DNA bands were visualized with UV light. The *K*_d (dissociation constant) was estimated from three independent experiments with various concentrations of Ler and the DNA fragment from position -500 to +100 (-500/+100 DNA fragment) using the evaluation software of an Alpha Imager (Biozym).

FIG. 1. Reporter gene expression of *E. coli* strain MS-10 in different culture media. Cultures were grown at 37°C and 180 rpm over a time period of 4 h. Bars show the mean relative luminescence for three samples, and error bars indicate the standard errors of the means. The OD₆₀₀ values of the cultures are indicated by symbols and lines.

RESULTS

Expression of *nleA*₄₇₉₅ under different environmental conditions. In order to investigate the influence of various environmental conditions on the expression of *nleA*₄₇₉₅, we first determined its expression during growth in LB broth containing either 0.1% (17 mM), 0.4% (68 mM), or 1% NaCl (171 mM), in SCEM, and in M9 minimal medium. *E. coli* MS-10 was incubated in these media, and reporter gene activity was measured. Values obtained in these experiments were related to those of standard LB broth (1% NaCl), which was taken as reference. The most remarkable difference in reporter gene expression was obtained in the mid-exponential phase in LB broth containing 0.4% NaCl, where it showed a 4.8-fold increase (Fig. 1). In contrast, incubation in LB broth containing 0.1% NaCl yielded only a 2.5-fold increase in reporter gene activity (Fig. 2). Reporter gene activity in LB broth supplemented with 68 mM KCl was increased 2-fold, whereas the identical amount of MgSO₄ had no effect (data not shown). In order to test whether the effect is caused by monovalent ions or by osmolarity in general, we repeated the experiments using sucrose in molarities identical to those for NaCl (see above). The results of the experiments showed that the same molar concentrations of NaCl and sucrose did not induce *nleA*₄₇₉₅ to

FIG. 2. Effect of quorum sensing on the expression of *nleA*₄₇₉₅. Reporter gene expression was measured over an incubation period of 4 h. Bars show the mean relative luminescence for three samples, and error bars indicate the standard errors of the means. The OD₆₀₀ values of the cultures are indicated by symbols and lines. (A) Comparison of *nleA*₄₇₉₅ gene expression in PC medium and LB broth, using *E. coli* strain MS-10. (B) Influence of AI-1 on *nleA*₄₇₉₅ expression, using strain MS-10. (C) Dependence of *nleA*₄₇₉₅ expression on AI-2 in different PC media, using *E. coli* strains MS-10 and MS-16. Black bars depict *nleA*₄₇₉₅ gene expression of strain MS-10 in LB broth, light gray bars depict *nleA*₄₇₉₅ gene expression of strain MS-16 in LB broth, dark gray bars depict *nleA*₄₇₉₅ gene expression of strain MS-10 in PC medium produced from strain EDL933, and gray bars depict *nleA*₄₇₉₅ gene expression of strain MS-10 in PC medium produced from strain MS-36. (D) Influence of epinephrine on *nleA*₄₇₉₅ expression, using strain MS-10. Bars depict *nleA*₄₇₉₅ gene expression in LB broth alone or with the indicated concentrations of epinephrine.

comparable levels. Whereas 68 mM NaCl induced *nleA*₄₇₉₅ 4.8-fold (see above), the same molar saccharose concentration did not induce *nleA*₄₇₉₅ above the control level (data not shown).

In SCEM and M9 medium, the reporter strain grew more slowly than in LB medium. The luminescence obtained in SCEM was similar to that in LB broth, whereas incubation of MS-10 in M9 medium did not show any significant reporter gene activity over the complete incubation period. These results indicate that an NaCl concentration of 0.4% in LB broth increases *nleA*₄₇₉₅ expression. However, expression was not increased during growth in SCEM and M9 minimal medium.

Expression of *nleA*₄₇₉₅ is increased in preconditioned medium. *E. coli* MS-10 was incubated in preconditioned medium produced with strain EDL933 (PC EDL933) and processed as described above. As shown in Fig. 2A, reporter gene expression increased 4.4-fold (after 2 h) in comparison with that after incubation in LB broth. This result indicates that *nleA*₄₇₉₅ gene expression was induced by conditions provided by the PC medium.

In order to examine whether the strong increase of *nleA*₄₇₉₅ gene expression is caused by autoinducer 1 (AI-1), *E. coli* strain MS-10 was incubated in LB broth supplemented with 1 mM AI-1. As shown in Fig. 2B, only a slight, 1.3-fold increase in reporter gene expression was obtained after 3 h and 4 h of incubation by the addition of AI-1. To prove the functionality of AI-1, it was added to a *Vibrio fischeri* culture under similar conditions, resulting in an 8-fold increase in luminescence (data not shown). This suggested that the increase obtained by incubation in PC medium is not in essence caused by AI-1. To examine whether AI-2 could be the inducing component, the same strain was incubated in PC medium produced with strains EDL933 (PC EDL933) and MS-36 (PC MS-36). Additionally, strain MS-16 was generated and compared to the parent strain MS-10. As shown in Fig. 2C, incubation in both PC media led to an approximately 4-fold increase of reporter gene expression in the mid-exponential phase after 2 h. Similarly, *E. coli* strain MS-16 did not show any difference from the parent *E. coli* strain MS-10 in reporter gene activity. This suggested that the induction obtained in the PC media was not caused by AI-2. In order to investigate the role of AI-3 in *nleA*₄₇₉₅ expression, strain MS-10 was tested in LB broth supplemented with 50 μ M and 100 μ M epinephrine (Fig. 2D). The addition of either concentration of epinephrine did not yield any change of reporter gene expression over the complete incubation period, indicating that the induction of *nleA*₄₇₉₅ gene expression in PC medium is also not caused by AI-3.

In order to test the hypothesis that a reduced content of nutrients in the PC medium may be responsible for the increased expression of *nleA*₄₇₉₅, *E. coli* strain MS-10 was tested with two different dilutions of LB broth under the conditions described above. The incubation of strain MS-10 in LB broth diluted 1:2 in sterile water caused only a slight increase of reporter gene expression. Incubation in LB broth diluted 1:5 resulted in a 2.8-fold-increased reporter gene activity in the mid-exponential phase (data not shown). These results suggested that *nleA*₄₇₉₅ gene expression is induced by starvation stress in media with a small amount of nutrients.

The expression of *nleA*₄₇₉₅ is dependent on the global positive LEE regulator Ler. In order to test our hypothesis that

FIG. 3. Influence of the regulator Ler on *nleA*₄₇₉₅ gene expression. Reporter gene expression by *E. coli* strains MS-10, MS-11, and MS-11/pCM1 in LB broth was determined over an incubation period of 4 h at 37°C and 180 rpm. Bars show the mean relative luminescence for three samples, and error bars indicate the standard errors of the means. The OD₆₀₀ values of the cultures are indicated by symbols and lines.

*nleA*₄₇₉₅ could be regulated by LEE or LEE-associated regulators, the *E. coli* reporter strains MS-10 and MS-11 were used. The latter was complemented with plasmid pCM1. Reporter gene activity was measured and compared to that in the parent strain MS-10. Deletion of *ler* in strain MS-11 resulted in a 3.9-fold decrease in relative luminescence in the late exponential phase compared to that in strain MS-10 (Fig. 3). In contrast, complementation of the *ler* deletion in strain MS-11/pCM1 showed a 1.5-fold increase in reporter gene activity in the late exponential phase and a 6.5-fold increase in the mid-exponential phase. These results indicate that the expression of *nleA*₄₇₉₅ is positively regulated by Ler.

***nleA*₄₇₉₅ upregulation by environmental conditions is *nleA*₄₇₉₅ specific and independent from Ler.** Because of the strong regulatory influence of Ler on *nleA*₄₇₉₅ gene expression, the next question to answer was whether the *nleA*₄₇₉₅ upregulation by different environmental conditions is also mediated by Ler. Therefore, the previous experiments with low NaCl concentrations, PC medium, and diluted growth media were repeated with *E. coli* strain MS-11. Although at a lower level (compared to the results shown in Fig. 1 and 2A), reporter strain MS-11 showed an increased luciferase activity under these conditions compared to the reporter gene activity in MS-11 in LB broth (data not shown). This indicates that *nleA*₄₇₉₅ gene expression can be induced independently of Ler by different environmental conditions.

In order to rule out the possibility that the induction of *nleA*₄₇₉₅ gene expression resulted from a global response to the change in environmental conditions, the results were confirmed by real-time PCR. Therefore, wild-type strain 4795/97 was incubated under the conditions described above, and *nleA*₄₇₉₅ gene expression was determined at the transcriptional level in relation to the housekeeping genes *gapA* and *rpsB*. The relative expression of *nleA*₄₇₉₅ under different conditions was normalized to expression in LB broth. Relative expression of *nleA*₄₇₉₅ was increased 2.3-fold in LB broth with 0.4% NaCl, 3.6-fold in diluted LB broth (1:5), and 3.4-fold in PC medium (data not shown). Although the levels of upregulation differed

FIG. 4. Influence of various regulators on *nleA*₄₇₉₅ expression. Reporter gene expression by different $\Delta nleA$ reporter constructs was determined in LB broth over an incubation period of 4 h at 37°C and 180 rpm. Bars show the mean relative luminescence for three samples, and error bars indicate the standard errors of the means. The OD₆₀₀ values of the cultures are indicated by symbols and lines.

slightly from those measured with the luciferase reporter strain, these results confirm those of the reporter gene assays.

The LEE-encoded regulator GrlA shows a positive regulatory effect on *nleA*₄₇₉₅ gene expression. Based on the preceding results, the influence of the second positive LEE regulator, GrlA, was examined. For this purpose, *E. coli* strains MS-12 and MS-12/pCM2 were incubated as described above, and reporter gene expression was measured and compared with that in *E. coli* MS-10. The greatest differences in relative luminescence between the strains were obtained in the late exponential phase, where deletion of *grlA* resulted in a 2.1-fold reduction in reporter gene activity (Fig. 4A). A complementation experiment with strain MS-12/pCM2 showed an increase of luminescence after 2 h and 4 h up to the wild-type level, but no further increase above this level could be observed. These results show that the expression of *nleA*₄₇₉₅ is also influenced by the positive LEE regulator GrlA.

In order to characterize the observed effects of Ler and GrlA in more detail, the double mutant MS-1112 was incubated and processed together with strains MS-10, MS-11, and MS-12. As shown in Fig. 4B, deletion of *grlA* resulted in a 2.2-fold reduction of reporter strain expression compared to that in strain MS-10, whereas *E. coli* strain MS-11 showed a more than 3.9-fold decrease in the late exponential phase. Strain MS-1112 showed a 3.9-fold reduction of luminescence, similar to that in strain MS-11. The identical reduction of reporter gene activity

in these strains demonstrates that there is obviously no synergistic effect of the two regulators on *nleA*₄₇₉₅ gene expression. Moreover, Ler shows a stronger regulatory effect on the expression of *nleA*₄₇₉₅ than GrlA.

Influence of Pch regulators on the expression of *nleA*₄₇₉₅. In order to analyze the influence of the Pch regulators on *nleA*₄₇₉₅ expression, two *pch* deletion mutants were constructed. Initially, a single deletion in *pchA* was made, resulting in strain MS-13. Subsequently, a second *pch* gene was deleted in the same strain, resulting in strain MS-1313. In reporter strain MS-13, no significant change in reporter gene expression was obtained compared to that in the parental strain (Fig. 4C). In contrast, strain MS-1313 showed a 1.7-fold reduction of luciferase activity after 2 h of incubation and a 2.8-fold reduction in the late exponential phase. Interestingly, complementation of strain MS-13 with wild-type *pchA* (pCM3) resulted in a 1.7-fold increase of luminescence after 4 h of incubation. The results of these experiments demonstrate that expression of *nleA*₄₇₉₅ is positively regulated by the *pch* genes.

Effect of other regulators on *nleA*₄₇₉₅ gene expression. For a more comprehensive characterization of the *nleA*₄₇₉₅ regulation, additional genes with putative regulatory function for the expression of *nleA*₄₇₉₅ were deleted, including *fnr*, *etrA*, and *grlR*. Deletion of *fnr*, a main regulator for the anaerobic metabolism, revealed no relevant changes in *nleA*₄₇₉₅ gene expression (data not shown). The two regulators GrlR and EtrA were

FIG. 5. Expression of *nleA*₄₇₉₅ in different recombinant strains in comparison with WT strain 4795/97. The effect of different regulator genes was examined using real-time PCR in relation to the reference genes *gapA* and *rnsB*. Samples were taken after 4 h of incubation in LB broth at 37°C and 180 rpm. Bars show the normalized fold expression of *nleA*₄₇₉₅ for three samples. Error bars indicate the standard errors of the means.

previously reported to have negative regulatory effects on the expression of LEE genes (12, 54). The respective mutant strains MS-14 and MS-15 were compared with *E. coli* strain MS-10. Reporter gene expression was increased 1.5-fold in *E. coli* strain MS-14 after an incubation time of 2 h and was 1.8-fold enhanced in the late exponential phase, as shown in Fig. 4D. Deletion of *etrA* in strain MS-15 did not show any effect on luciferase activity after 2 h of incubation, and there was only a slight increase at incubation times of 3 h and 4 h. These results show that the LEE-encoded regulator GrIR has a negative regulatory effect on the expression of *nleA*₄₇₉₅, whereas EtrA obviously has no influence.

Transcriptional analysis of *nleA*₄₇₉₅ gene expression. By the analysis of various luciferase reporter strains, we were able to show that *nleA*₄₇₉₅ gene expression is regulated by LEE- and non-LEE-encoded regulators. For confirmation of these results, we constructed single deletion mutants, which do not contain reporter genes, of the wild-type *E. coli* strain 4795/97. These procedures resulted in recombinant strains MS-21, MS-22, MS-23, and MS-2323. Complementation of these mutants yielded strains MS-21/pCM1, MS-22/pCM2, and MS-23/pCM3. The expression of *nleA*₄₇₉₅ was determined at the transcriptional level in relation to that of the housekeeping genes *gapA* and *rnsB*. The relative fold expression of *nleA*₄₇₉₅ in the different deletion and complementation mutants was normalized to the expression in wild-type (WT) strain 4795/97. Expression of *nleA*₄₇₉₅ was reduced up to 20-fold in the recombinant strain with a deletion in the *ler* gene, whereas a deletion in *grlA* resulted in a 3.3-fold decrease (Fig. 5). Identical to the results reported above, a deletion in *pchA* had no detectable effect on *nleA*₄₇₉₅ expression, whereas deletion of a further *pch* gene resulted in a 9-fold reduction. The complemented strain MS-21/pCM1 showed 2.7-fold-increased expression of *nleA*₄₇₉₅, and in strain MS-22/pCM2 the expression was restored to the wild-type level. After complementation of strain MS-23 with pCM3, *nleA*₄₇₉₅ expression increased 3.3-fold, the same effect which had been observed in the preceding experiments using a reporter gene system. These results show a strong influence of the regulator proteins Ler, GrIA, and Pch on *nleA*₄₇₉₅ gene expression and therefore confirm the results obtained with the reporter gene constructs.

Ler binds to the *nleA*₄₇₉₅ promoter region. In order to investigate whether Ler influences *nleA*₄₇₉₅ gene expression by direct binding to the regulatory region of *nleA*₄₇₉₅, electrophoretic mobility shift assays (EMSAs) were performed. PCR fragments of different lengths and locations with respect to the *nleA*₄₇₉₅ start codon were used to investigate Ler binding (Fig. 6A).

Purified Ler was incubated with the respective PCR fragments under appropriate conditions. First, the concentration for optimal binding of Ler was determined using different amounts of protein with the −500/+100 DNA fragment. The results after electrophoretic separation are shown in Fig. 6B, lanes 1 to 7. A concentration of 2.4 μM Ler demonstrated the greatest binding (Fig. 6B, lane 5), and a *K_d* of 1.6 μM was estimated for the interaction of Ler with the −500/−100 DNA fragment. The specificity of the binding was confirmed by the addition of a nonspecific competitor control to the mixture of 2.4 μM Ler and the −500/+100 DNA fragment (Fig. 6B, lane 8). Figure 6C shows the EMSA results using the optimal protein concentration with different DNA fragments. Band shifts were obtained with nearly all examined fragments, except for the negative control (Fig. 6C, lanes 11 and 12). The shifted bands differed in clearness and intensity. The −500/+100 and −500/0 PCR fragments showed sharp and clear band shifts, and a signal for a second and smaller shifted band was even obtained (Fig. 6C, lanes 1 and 3). The −250/+100 and −250/0 fragments showed the strongest band shifts (Fig. 6C, lanes 8 and 9), whereas a faint shift was obtained with the −500/−250 DNA fragment (Fig. 6C, lane 5). Together, these results suggest that Ler binds to the regulatory region of *nleA*₄₇₉₅ by recognizing a DNA region between 500 bp upstream and 100 bp downstream of the *nleA*₄₇₉₅ start codon.

DISCUSSION

The analysis of bacterial virulence factor regulation is an important tool to better understand the mechanisms of virulence and to develop substances to specifically block pathogenesis. For EHEC, the regulatory circuitry controlling virulence has been intensely studied. Besides *stx* gene expression, the regulation of LEE- and non-LEE-encoded type III effectors is a current research topic (16, 39, 49).

It is known that environmental stress can influence the expression of LEE and other virulence-associated genes in EHEC and EPEC. For example, the expression of the two regulators Ler and Pch was shown to be increased by a downshift of nutrients or by entry of the bacterial culture into the stationary phase (31). It was also demonstrated that transcription of LEE genes is stimulated by the presence of bacterial autoinducers (42) or by addition of bicarbonate ions (1). In EHEC, the expression of type III effectors *espJ* and *tccP* was reported to be influenced by changes in temperature, pH, osmolarity, and O₂ pressure (17).

To investigate the influence of environmental conditions on *nleA*₄₇₉₅ expression, several culture conditions were tested. We found the highest increase in *nleA*₄₇₉₅ expression in LB broth containing 0.4% NaCl. The upregulation of *nleA*₄₇₉₅ by this NaCl concentration might be the result of changes in the osmolarity. It has been demonstrated that an

FIG. 6. Electrophoretic mobility shift assay of Ler binding. (A) Schematic demonstration of various DNA fragments within the *nleA*₄₇₉₅ promoter region used for the EMSAs. Arrows in light gray indicate sequences upstream of the *nleA*₄₇₉₅ start codon, and arrows in dark gray indicate sequences downstream of the start codon. DNA fragments were named according to their length in relation to the *nleA*₄₇₉₅ start codon. Base pairs upstream from the start codon are designated by minus and those downstream by plus. (B) EMSA with the -500/+100 DNA fragment (100 ng) and increasing concentrations of Ler, including a nonspecific competitor control. Protein and PCR fragments were incubated under the appropriate conditions as described in Materials and Methods, separated on a 0.8% agarose gel, and stained with ethidium bromide. Lane M, 1-kb DNA ladder; lane 1, 0.3 μ M protein; lane 2, 0.6 μ M protein; lane 3, 1.2 μ M protein; lane 4, 1.8 μ M protein; lane 5, 2.4 μ M protein; lane 6, 3.0 μ M protein; lane 7, 3.2 μ M protein; lane 8, 2.4 μ M protein plus nonspecific competitor [poly(dI-dC)]. (C) EMSA with purified Ler (2.4 μ M) and PCR fragments of different sizes and locations with respect to the *nleA*₄₇₉₅ start codon. Lane M, 1-kb DNA ladder; lane 1, -500/+100 fragment plus Ler; lane 2, -500/+100 fragment without protein; lane 3, -500/0 fragment plus Ler; lane 4, -500/0 fragment without protein; lane 5, -500/-250 fragment plus Ler; lane 6, -500/-250 fragment without protein; lane 7, -250/+100 fragment plus Ler; lane 8, -250/+100 fragment without protein; lane 9, -250/0 fragment plus Ler; lane 10, -250/0 fragment without protein; lane 11, NC fragment (negative control) plus Ler; lane 12, NC fragment without protein.

osmotic shift alters gene expression in *E. coli* by changes in DNA supercoiling (23, 24). The expression of the *espA*, *espD*, and *espB* genes of EHEC O157:H7 strain EDL933 was shown to be increased by high osmolarity (430 mM NaCl) (5). Interestingly, we obtained the strongest increase in *nleA*₄₇₉₅ expression in LB broth containing 0.4% (68 mM) NaCl, a concentration which corresponds to a low osmolarity. The same effect could be shown by incubating the cultures in LB broth supplemented with 68 mM KCl, but not in that with equal amounts of MgSO₄ (data not shown), indicating that this phenomenon is specific for monovalent salts. This assumption is further confirmed by the results obtained with different amounts of the osmolyte sucrose, which showed no comparable effects on the expression of *nleA*₄₇₉₅. We could also demonstrate that *nleA*₄₇₉₅ expression was increased in a *ler* deletion mutant during incubation in LB broth containing 0.4% NaCl. Thus, the upregulation of *nleA*₄₇₉₅ by a low NaCl concentration is obviously independent from Ler and is possibly controlled by other global mechanisms (20, 23). Under the conditions tested, incuba-

tion in minimal medium M9 showed a very low expression of *nleA*₄₇₉₅. An explanation might be the slow growth of bacteria in this medium, resulting in a general reduced rate of gene expression in comparison to that in LB broth.

We could also demonstrate that high levels of *nleA*₄₇₉₅ were obtained after incubation in PC medium. The fact that none of the autoinducers tested could restore this effect led us to assume that the increase in *nleA*₄₇₉₅ expression might be induced mainly by the low nutrient level in the PC medium. Incubation of the luciferase reporter strain in diluted LB broth induced the expression of *nleA*₄₇₉₅ but at a lower level than was obtained with PC medium. However, identical levels of induction for diluted LB broth and PC medium were shown by real-time PCR, perhaps resulting from the higher sensitivity of the system. Similarly to the upregulation by low NaCl concentrations, the *nleA*₄₇₉₅ expression during starvation stress was also increased in a *ler* deletion mutant. This Ler-independent upregulation indicates that *nleA*₄₇₉₅ might also be a target of the stringent response system of EHEC (31).

As the expression of LEE-encoded type III effectors is affected by global LEE regulators (12, 13), we investigated whether these regulators can also influence the expression of *nleA*₄₇₉₅. For example, Ler and GrlA were shown to affect the expression of the non-LEE-encoded effector NleH of *C. rodentium*, whereas both regulators had no significant influence at the transcriptional level (16). In EHEC O157:H7, the expression and secretion of NleA were demonstrated to be regulated by Ler in a direct or indirect way (39).

Our results clearly demonstrate that Ler acts as a positive regulator of *nleA*₄₇₉₅ gene expression through direct binding to its promoter region. Ler was shown to bind to a region between 250 bp upstream and 100 bp downstream of the *nleA*₄₇₉₅ start codon. Moreover, a second band shift was obtained in EMSAs with DNA fragments of 500 bp and longer, suggesting that there might be a second binding site within the 500 bp upstream of the start codon. For example, Ler was shown to bind to higher- and lower-affinity binding sites within the promoter region of the *grlRA* operon (2). Furthermore, a regulatory influence on *nleA*₄₇₉₅ expression was also demonstrated for regulators GrlA and GrlR, whereas GrlA acted in a positive way and GrlR in a negative way. However, the positive regulatory influence of GrlA was not as strong as was demonstrated for Ler. Interestingly, the deletion of both regulators in a double deletion mutant showed no synergistic effect on *nleA*₄₇₉₅ expression, despite the fact that Ler and GrlA regulate the expression of each other in a positive regulatory loop (2). This led us to assume that *nleA*₄₇₉₅ is regulated primarily by the direct binding of Ler and secondarily by direct or indirect regulation of GrlA.

As reported previously (25, 54), the expression of LEE genes is also under the control of several regulator proteins encoded outside the LEE. Therefore, we investigated the regulatory function of PchABC, which are homologues to the plasmid-encoded PerC of EPEC. We showed that a deletion of *pchA* had no effect on *nleA*₄₇₉₅ expression, whereas an additional deletion in one of the other *pch* genes resulted in a considerable decrease. Interestingly, complementation with vector pCM3 was sufficient to increase the expression of *nleA*₄₇₉₅. The fact that deletion of only *pchA* had no effect on *nleA*₄₇₉₅ expression, whereas its overexpression increased the level, indicates that not all of the *pchABC* variants are necessary for *nleA*₄₇₉₅ expression. Obviously, the presence of PchA is sufficient to increase the expression of *nleA*₄₇₉₅. As it has previously been reported that Ler is upregulated by PchABC (25), this positive regulatory effect on *nleA*₄₇₉₅ expression could be in either a direct or indirect way. In contrast, the regulator EtrA showed no significant effect on *nleA*₄₇₉₅ expression, although this regulator was described to influence the expression of LEE genes in a negative way (54).

The results of this study show that the expression of *nleA*₄₇₉₅ is regulated in different ways (Fig. 7). On the one hand, *nleA*₄₇₉₅ is integrated in the Ler-mediated regulation circuit of type III effector genes. In addition, the positive influence of Pch regulators, encoded outside the LEE, was shown. On the other hand, environmental factors, such as starvation stress or low osmolarity, can upregulate the expression level of *nleA*₄₇₉₅ in a way independent of the Ler regulatory cascade. Further studies will be necessary to better understand these correla-

FIG. 7. Model for the regulation circuit of *nleA*₄₇₉₅. The directions of the arrows indicate the effects of regulators and environmental conditions: ↑, upregulation, ↓, downregulation, →, no effect on *nleA*₄₇₉₅ expression. The arrow without a question mark indicates direct binding of Ler to the promoter region of *nleA*₄₇₉₅. The assumed promoter region and the upstream region of the *nleA*₄₇₉₅ open reading frame are shown at the bottom.

tions in *nleA*₄₇₉₅ expression and to identify additional regulators.

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REFERENCES

1. Abe, H., I. Tatsuno, T. Tobe, A. Okutani, and C. Sasakawa. 2002. Bicarbonate ion stimulates the expression of locus of enterocyte effacement-encoded genes in enterohemorrhagic *Escherichia coli* O157:H7. *Infect. Immun.* **70**: 3500–3509.
2. Barba, J., et al. 2005. A positive regulatory loop controls expression of the locus of enterocyte effacement-encoded regulators Ler and GrlA. *J. Bacteriol.* **187**:7918–7930.
3. Bassler, B. L., M. Wright, R. E. Showalter, and M. R. Silverman. 1993. Intercellular signalling in *Vibrio harvey*: sequence and function of genes regulating expression of luminescence. *Mol. Microbiol.* **9**:773–786.
4. Bassler, B. L., M. Wright, and M. R. Silverman. 1994. Multiple signalling systems controlling expression of luminescence in *Vibrio harvey*: sequence and function of genes encoding a second sensory pathway. *Mol. Microbiol.* **13**:273–286.
5. Beltrametti, F., A. U. Kresse, and C. A. Guzman. 1999. Transcriptional regulation of the *esp* genes of enterohemorrhagic *Escherichia coli*. *J. Bacteriol.* **181**:3409–3418.
6. Beumer, R. R., J. de Vries, and F. M. Rombouts. 1992. *Campylobacter jejuni* non-culturable coccoid cells. *Int. J. Food Microbiol.* **15**:153–163.
7. Campellone, K. G., D. Robbins, and J. M. Leong. 2004. EspFU is a translocated EHEC effector that interacts with Tir and N-WASP and promotes Nck-independent actin assembly. *Dev. Cell* **7**:217–228.
8. Creuzburg, K., et al. 2005. The Shiga toxin 1-converting bacteriophage BP-4795 encodes an NleA-like type III effector protein. *J. Bacteriol.* **187**:8494–8498.
9. Creuzburg, K., and H. Schmidt. 2007. Molecular characterization and distribution of genes encoding members of the type III effector *nleA* family among pathogenic *Escherichia coli* strains. *J. Clin. Microbiol.* **45**:2498–2507.
10. Dahan, S., et al. 2005. EspJ is a prophage-carried type III effector protein of attaching and effacing pathogens that modulates infection dynamics. *Infect. Immun.* **73**:679–686.
11. Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **97**:6640–6645.

12. Deng, W., et al. 2004. Dissecting virulence: systematic and functional analyses of a pathogenicity island. *Proc. Natl. Acad. Sci. U. S. A.* **101**:3597–3602.
13. Elliott, S. J., et al. 2000. The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect. Immun.* **68**:6115–6126.
14. Elliott, S. J., et al. 1998. The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Mol. Microbiol.* **28**:1–4.
15. Friedberg, D., T. Umanski, Y. Fang, and I. Rosenshine. 1999. Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic *Escherichia coli*. *Mol. Microbiol.* **34**:941–952.
16. Garcia-Angulo, V. A., W. Deng, N. A. Thomas, B. B. Finlay, and J. L. Puente. 2008. Regulation of expression and secretion of NleH, a new non-locus of enterocyte effacement-encoded effector in *Citrobacter rodentium*. *J. Bacteriol.* **190**:2388–2399.
17. Garmendia, J., and G. Frankel. 2005. Operon structure and gene expression of the *espI-tccP* locus of enterohemorrhagic *Escherichia coli* O157:H7. *FEMS Microbiol. Lett.* **247**:137–145.
18. Garmendia, J., G. Frankel, and V. F. Crepin. 2005. Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: translocation, translocation, translocation. *Infect. Immun.* **73**:2573–2585.
19. Garmendia, J., et al. 2004. TccP is an enterohemorrhagic *Escherichia coli* O157:H7 type III effector protein that couples Tir to the actin-cytoskeleton. *Cell. Microbiol.* **6**:1167–1183.
20. Gawande, P. V., and M. W. Griffiths. 2005. Effects of environmental stresses on the activities of the *uspA*, *grpE* and *rpoS* promoters of *Escherichia coli* O157:H7. *Int. J. Food Microbiol.* **99**:91–98.
21. Gerlach, R. G., S. U. Hölzer, D. Jackel, and M. Hensel. 2007. Rapid engineering of bacterial reporter gene fusions by using Red recombination. *Appl. Environ. Microbiol.* **73**:4234–4242.
22. Gruenheid, S., et al. 2004. Identification and characterization of NleA, a non-LEE-encoded type III translocated virulence factor of enterohemorrhagic *Escherichia coli* O157:H7. *Mol. Microbiol.* **51**:1233–1249.
23. Hatfield, G. W., and C. J. Benham. 2002. DNA topology-mediated control of global gene expression in *Escherichia coli*. *Annu. Rev. Genet.* **36**:175–203.
24. Higgins, C. F., et al. 1988. A physiological role for DNA supercoiling in the osmotic regulation of gene expression in *S. typhimurium* and *E. coli*. *Cell* **52**:569–584.
25. Iyoda, S., and H. Watanabe. 2004. Positive effects of multiple *pch* genes on expression of the locus of enterocyte effacement genes and adherence of enterohemorrhagic *Escherichia coli* O157:H7 to HEp-2 cells. *Microbiology* **150**:2357–2571.
26. Kim, J., et al. 2007. The bacterial virulence factor NleA inhibits cellular protein secretion by disrupting mammalian COPII function. *Cell Host Microbe* **2**:160–171.
27. Marches, O., et al. 2003. Enteropathogenic and enterohemorrhagic *Escherichia coli* deliver a novel effector called Cif, which blocks cell cycle G2/M transition. *Mol. Microbiol.* **50**:1553–1567.
28. McDaniel, T. K., K. G. Jarvis, M. S. Donnenberg, and J. B. Kaper. 1995. A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc. Natl. Acad. Sci. U. S. A.* **92**:1664–1668.
29. Mundy, R., C. Jenkins, J. Yu, H. Smith, and G. Frankel. 2004. Distribution of *espI* among clinical enterohemorrhagic and enteropathogenic *Escherichia coli* isolates. *J. Med. Microbiol.* **53**:1145–1149.
30. Mundy, R., et al. 2004. Identification of a novel *Citrobacter rodentium* type III secreted protein, EspI, and roles of this and other secreted proteins in infection. *Infect. Immun.* **72**:2288–2302.
31. Nakanishi, N., et al. 2006. ppGpp with DksA controls gene expression in the locus of enterocyte effacement (LEE) pathogenicity island of enterohemorrhagic *Escherichia coli* through activation of two virulence regulatory genes. *Mol. Microbiol.* **61**:194–205.
32. Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* **11**:142–201.
33. Noris, M., and G. Remuzzi. 2005. Hemolytic uremic syndrome. *J. Am. Soc. Nephrol.* **16**:1035–1050.
34. O'Brien, A. D., et al. 1984. Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis or infantile diarrhea. *Science* **226**:694–696.
35. Perna, N. T., et al. 2001. Genome sequence of enterohemorrhagic *Escherichia coli* O157:H7. *Nature* **409**:529–533.
36. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**:e45.
37. Pridmore, R. D. 1987. New and versatile cloning vectors with kanamycin-resistance marker. *Gene* **56**:309–312.
38. Ren, C. P., et al. 2004. The ETT2 gene cluster, encoding a second type III secretion system from *Escherichia coli*, is present in the majority of strains but has undergone widespread mutational attrition. *J. Bacteriol.* **186**:3547–3560.
39. Roe, A. J., et al. 2007. Analysis of the expression, regulation and export of NleA-E in *Escherichia coli* O157:H7. *Microbiology* **153**:1350–1360.
40. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
41. Sperandio, V., C. C. Li, and J. B. Kaper. 2002. Quorum-sensing *Escherichia coli* regulator A: a regulator of the LysR family involved in the regulation of the locus of enterocyte effacement pathogenicity island in enterohemorrhagic *E. coli*. *Infect. Immun.* **70**:3085–3093.
42. Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper. 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **96**:15196–15201.
43. Sperandio, V., A. G. Torres, B. Jarvis, J. P. Nataro, and J. B. Kaper. 2003. Bacteria-host communication: the language of hormones. *Proc. Natl. Acad. Sci. U. S. A.* **100**:8951–8956.
44. Studier, F. W., and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**:113–130.
45. Surette, M. G., and B. L. Bassler. 1998. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. U. S. A.* **95**:7046–7050.
46. Thanabalasuriar, A., et al. 2010. The bacterial virulence factor NleA is required for the disruption of intestinal tight junctions by enteropathogenic *Escherichia coli*. *Cell. Microbiol.* **12**:31–41.
47. Tobe, T., et al. 2006. An extensive repertoire of type III secretion effectors in *Escherichia coli* O157 and the role of lambdaoid phages in their dissemination. *Proc. Natl. Acad. Sci. U. S. A.* **103**:14941–14946.
48. Torres, A. G., X. Zhou, and J. B. Kaper. 2005. Adherence of diarrheagenic *Escherichia coli* strains to epithelial cells. *Infect. Immun.* **73**:18–29.
49. Tree, J. J., E. B. Wolfson, D. Wang, A. J. Roe, and D. L. Gally. 2009. Controlling injection: regulation of type III secretion in enterohemorrhagic *Escherichia coli*. *Trends Microbiol.* **17**:361–370.
50. Tschowri, N., S. Busse, and R. Hengge. 2009. The BLUF-EAL protein YcgF acts as a direct anti-repressor in a blue-light response of *Escherichia coli*. *Genes Dev.* **23**:522–534.
51. Walters, M., and V. Sperandio. 2006. Quorum sensing in *Escherichia coli* and *Salmonella*. *Int. J. Med. Microbiol.* **296**:125–131.
52. Wang, R. F., and S. R. Kushner. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**:195–199.
53. Yang, Z., et al. 2009. Genomic instability in regions adjacent to a highly conserved *pch* prophage in *Escherichia coli* O157:H7 generates diversity in expression patterns of the LEE pathogenicity island. *J. Bacteriol.* **191**:3553–3568.
54. Zhang, L., et al. 2004. Regulators encoded in the *Escherichia coli* type III secretion system 2 gene cluster influence expression of genes within the locus for enterocyte effacement in enterohemorrhagic *E. coli* O157:H7. *Infect. Immun.* **72**:7282–7293.
55. Zhang, W. L., et al. 2002. Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. *J. Clin. Microbiol.* **40**:4486–4492.