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Autoinducer 3 and Epinephrine Signaling in the Kinetics of Locus of Enterocyte Effacement Gene Expression in Enterohemorrhagic *Escherichia coli*

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Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is responsible for causing outbreaks of bloody diarrhea and hemolytic-uremic syndrome throughout the world. The locus of enterocyte effacement (LEE) consists of five major operons and is required for the formation of attaching and effacing lesions that disrupt intestinal epithelial microvilli. We have previously reported that expression of EHEC LEE genes is regulated by the *luxS* quorum-sensing system. The *luxS* gene in EHEC affects the production of autoinducer 3 (AI-3), which activates the LEE. Epinephrine and norepinephrine also activate the LEE in a manner similar to that of AI-3. Previous studies of quorum-sensing regulation of LEE transcription have thus far been restricted to using reporter systems in an *E. coli* K-12 background. Here, we examined the kinetics of LEE gene transcription, protein expression, and function of the LEE type III secretion apparatus in wild-type (WT) EHEC and an isogenic *luxS* mutant. The results revealed that the *luxS* mutant had diminished transcription from the LEE promoters during the mid-exponential growth phase; decreased protein levels of EscJ, Tir, and EspA; and reduced secretion of EspA and EspB. The *luxS* mutation also caused a delay in the formation of attaching and effacing lesions on cultured epithelial cells compared to the wild type. Epinephrine enhanced LEE expression in both the WT and the *luxS* mutant, but the WT still exhibited greater LEE activation. The results suggest a possible synergistic relationship between AI-3 and epinephrine. The combined effects of these two signaling molecules may lead to greater LEE expression and a more efficient infection.

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a human pathogen responsible for outbreaks of bloody diarrhea and hemolytic-uremic syndrome throughout the world. EHEC colonizes the large intestine, where it forms attaching and effacing (AE) lesions and produces Shiga toxins, which are responsible for the development of hemolytic-uremic syndrome (23, 31). The genes required for the formation of the AE lesions are located on a chromosomal pathogenicity island, termed the locus of enterocyte effacement (LEE), that is encoded in the human pathogens EHEC and enteropathogenic *E. coli* (EPEC) but is not in either *E. coli* K-12 or commensal *E. coli* (20).

The LEE is composed of 41 genes, the majority of which are organized into five polycistronic operons (*LEE1* to *LEE5*) (10, 11, 29). The first gene of *LEE1* encodes a transcriptional activator, Ler, that is required for expression of the LEE genes (6, 10, 13, 17, 29, 35, 41). The majority of the remaining genes in *LEE1*, as well as the *LEE2* and *LEE3* operons, encode structural and secondary proteins required for the formation of the type III secretion system (TTSS) (20). *LEE5* contains genes encoding an adhesin (intimin) and its cognate receptor that is translocated through the TTSS into the host cell (Tir) (21, 24). *LEE4* encodes several *E. coli* secreted proteins (Esp proteins) that make up the translocon portion of the TTSS (11, 27). The TTSS in EHEC facilitates the translocation of the LEE-encoded effector proteins Tir, EspH, EspG, EspF, SepZ, and Map (22, 24, 25, 28, 46) as well as several non-LEE-encoded (Nle) effectors such as NleA, NleB, NleC, NleD, NleE, NleF, NleG, and $EspF_U$ (7, 8, 16, 30) into eukaryotic target cells.

Regulation of the LEE involves the *luxS* quorum-sensing system (42, 44). This system is involved in the synthesis of autoinducer 2 (AI-2) and is present in both gram-negative and gram-positive bacteria (45). LuxS is an enzyme that metabolizes *S*-adenosylmethionine to yield the AI-2 precursor 4,5-dihydroxy-2,3-pentanedione (36). A *luxS* mutation also affects the production of another autoinducer, termed AI-3, which activates transcription of the LEE and motility genes in EHEC (44). AI-2 is a furanosyl-borate diester with a molecular mass of 192.9 Da (36); it is a very polar compound that does not bind to C_{18} columns. AI-3 binds to C_{18} columns and can be eluted with methanol only (44). Electrospray mass spectrometry analysis of the AI-3 fraction showed a major peak with a mass of 213.1 Da and minor peaks at 109.1, 164.9, 176.1, 196.1, 211.1, 214.1, and 222.9 Da (44). All of these are different from AI-2 (192.9 Da) (44), suggesting that AI-3 is a novel compound. Preconditioned (PC) supernatants from a *luxS*-deficient strain do not induce LEE transcription in an *E. coli* K-12 reporter system, and the *luxS* mutation leads to decreased protein secretion in EHEC (44). The addition of AI-3 to culture supernatants, but not AI-2, restores these phenotypes in the *luxS* mutant (44). Two eukaryotic hormones (epinephrine and norepinephrine) cross talk with the EHEC AI-3 quorum-sensing system and restore virulence phenotypes to the *luxS* mutant (44).

Regulation of the LEE involves factors present in both *E. coli* K-12 and EHEC as well as several EHEC-specific regula-

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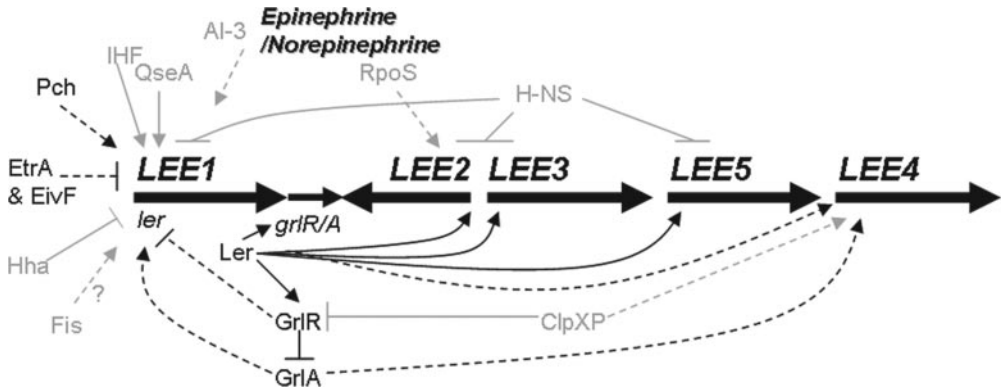

FIG. 1. Model of LEE regulation. Factors shown in gray are present in both *E. coli* K-12 and EHEC, while regulators shown in black are specific to EHEC. Solid lines represent regulators whose direct interactions with the target promoter have been biochemically defined, and dashed lines represent interactions that occur indirectly or that have not been shown to bind biochemically to the target. H-NS is a global regulator that binds to the promoters of the *LEE1*, *LEE2*, *LEE3*, and *LEE5* operons and represses transcription. Ler activates *grlR/A*, *LEE2*, *LEE3*, and *LEE5* by binding to their promoters, displacing H-NS and allowing for the transcription of these operons. Integration host factor (IHF) also activates the transcription of *LEE1*. Hha represses *LEE1* by either oligomerizing with H-NS or binding directly to the promoter sequence. The ClpXP protease regulates LEE expression through interactions with RpoS and GrlR. Fis has been shown to activate the LEE in EPEC, but its role in EHEC has not been examined. AI-3 and epinephrine/norepinephrine signal through unknown receptors to activate the transcription of the *LEE1* operon and *ler*. GrlR and GrlA, two LEE-encoded regulators, repress and promote, respectively, the transcription of the *LEE1* operon. EtrA and EivF are two regulators encoded on a second nonfunctional TTSS in EHEC that negatively influence the expression of *LEE1*. The *pch* genes are another set of EHEC-specific regulators that activate the transcription of *LEE1* and consequently the entire LEE.

tors. An overview of LEE regulation is shown in Fig. 1. H-NS is a global regulator involved in the thermoregulation of the LEE, repressing the transcription of *LEE1* at 27°C but not at 37°C (47). It also represses the transcription of the *LEE2*, *LEE3*, and *LEE5* operons by binding to the target promoter and preventing promoter recognition by the transcription machinery (6, 17, 29, 35, 41). Integration host factor, another global regulatory factor, binds to the *LEE1* promoter and activates the transcription of *ler* (13). The nucleoid-associated protein Fis has been shown to modulate LEE expression in EPEC (14), but its role in EHEC LEE expression remains to be examined. Hha and its homologues are environment-dependent regulators of gene expression that act as a negative regulator by either binding to a specific DNA sequence in the target promoter (12) or oligomerizing with H-NS and then binding the target DNA (32). Hha is a negative regulator of *ler* and, consequently, the entire LEE (38). QseA is a member of the LysR family of transcription factors and activates the transcription of *ler*, thereby promoting the expression of the other LEE genes (40). The ClpXP protease degrades damaged and incomplete proteins and also affects LEE expression (18). ClpXP is thought to regulate the LEE through interactions with RpoS and an EHEC-specific regulatory factor, GrlR (18). RpoS is a stationary-phase sigma factor and has been shown to positively regulate transcription of the *LEE3* operon in an *E. coli* K-12 background (42).

A number of regulatory pathways and factors are limited to EHEC, such as the *pch* genes, *etrA*, *eivF*, *ler*, and *grlR/A*. The *pch* genes, which are not present in *E. coli* K-12, positively regulate expression of the LEE and are necessary for full virulence of EHEC (19). The five *pch* genes are encoded outside of the LEE and are homologous to *perC* in EPEC (19). EtrA and EivF are encoded within a pathogenicity island that contains a second, nonfunctional type III secretion system in the EHEC genome (49). Both of these proteins have been shown to be negative regulators of the LEE (49). Ler, encoded by *LEE1*, is able to overcome H-NS-mediated repression and activate the transcription of the *LEE2*, *LEE3*, and *LEE5* operons (17, 35, 41). GrlR and GrlA, also encoded by the LEE, repress and activate the transcription of *ler*, respectively (8). Ler can also activate the transcription of the *grlR/A* operon, creating a positive regulatory loop (4, 10). The specific role of this regulatory loop has not been defined, but it has been suggested that it is necessary to maintain the balance of regulatory factors that help achieve optimal expression of the LEE in the host environment (4).

The numerous factors that control gene expression suggest that LEE regulation is highly complex. In the current study, we sought to examine a possible role for AI-3 and epinephrine/norepinephrine in the activation of the LEE in wild-type (WT) EHEC and an isogenic EHEC *luxS* mutant (defective in AI-3 synthesis). Previous work has examined transcription of the LEE genes only in an *E. coli* K-12 background using PC media from EHEC cultures. Herein, we directly examined the effects of AI-3/epinephrine on LEE transcription in WT EHEC and a *luxS* mutant. We found that the kinetics of LEE expression were different from those previously observed with the *E. coli* K-12 reporter strain, highlighting the importance of examining LEE regulation in a native EHEC background. Moreover, the results from these studies indicate a synergistic effect between AI-3 and epinephrine. This relationship may allow EHEC to mount a more efficient infection than responding to one signal alone.

MATERIALS AND METHODS

Strains, plasmids, and recombinant DNA techniques. WT EHEC (strain 86-24) (15), an isogenic *luxS* mutant (strain VS94) (43), and a complemented *luxS* strain (MW90) (this study) were used in this study. Strain MW90 was created by introducing plasmid pVS212 (44), containing *luxS* in the multiple cloning site of plasmid pQE30, into *luxS* mutant strain VS94. Expression of *luxS* from the

TABLE 1. Oligonucleotides used for real-time RT-PCR

Gene	Primer (5′-3′)	
	Forward	Reverse
ler	CGACCAGGTCTGCCC TTCT	GCGCGGAACTCATC GAAA
escC	GCGTAAACTGGTCCGG TACGT	TGCGGTAGAGCTATTA AAGGCAAT
escV	TCGCCCCGTCCATTGA	CGCTCCCGAGTGC AAAA
espA	TCAGAATCGCAGCCTG AAAA	CGAAGGATGAGGTGG TTAAGCT
eae	GCTGGCCTTGGTTTG ATCA	GCGGAGATGACTTCAG CACTT
rpoA	GCGCTCATCTTCTTCC GAAT	CGCGGTCGTGGTTA TGTG

pQE30 vector in MW90 was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at time zero. Dulbecco's modified Eagle's medium (DMEM) (Invitrogen), which activates the LEE, was used as the growth medium for virulence assays. All cultures grown overnight were grown aerobically at 37°C in Luria-Bertani (LB) medium and shaken at 250 rpm. These conditions have been demonstrated to not activate the LEE (2, 5). Antibiotics for selection were used at the following concentrations: ampicillin, 100 μg/ml; streptomycin, 50 μg/ml; and tetracycline, 25 μg/ml. Plasmid purification, PCR, ligation, restriction, transformation, and DNA gel electrophoresis were performed using standard methods (34).

RNA extraction and real-time RT-PCR studies. Cultures of strains 86-24, VS94, and MW90 grown aerobically in LB medium at 37°C overnight, conditions known not to induce LEE expression (2, 5), were diluted 1:100 in DMEM and grown aerobically at 37°C. For the epinephrine studies, a stock epinephrine solution of 50 mM in water was made and diluted 10^{-3} in cultures grown overnight that were diluted 1:100 in DMEM for a final concentration of 50 μM. RNA from three biological replicate cultures of each strain was extracted at the early exponential growth phase (optical density at 600 nm [OD_{600}] of 0.2), mid-exponential growth phase (OD_{600} of 0.5), and late exponential growth phase (OD_{600} of 1.0) using the RiboPure Bacteria RNA isolation kit (Ambion) according to the manufacturer's guidelines. The primers used in the real-time assays were designed using Primer Express v1.5 (Applied Biosystems) (Table 1). Real-time reverse transcription-PCR (RT-PCR) was performed in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems).

For each 20-μl reaction mixture, 10 μl 2× SYBR master mix, 0.1 μl MultiScribe reverse transcriptase (Applied Biosystems), and 0.1 μl RNase inhibitor (Applied Biosystems) were added. Amplification efficiency of each of the primer pairs was verified using standard curves of known RNA concentrations. Melting-curve analysis was used to ensure template specificity by heating products to 95°C for 15 s, followed by cooling to 60°C and heating to 95°C while monitoring fluorescence. Once the amplification efficiency and template specificity were determined for each primer pair, relative quantification analysis was used to analyze the unknown samples using the following conditions for cDNA generation and amplification: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. The *rpoA* (RNA polymerase subunit A) gene was used as the endogenous control.

Detection, quantification, and statistical analysis. Data collection was performed using ABI Sequence Detection 1.3 software (Applied Biosystems). Data were normalized to levels of *rpoA* and analyzed using the comparative critical threshold (C_T) method described previously (3). The expression level of the target genes at the different growth phases was compared using the relative quantification method (3). Real-time data are presented as the change (*n*-fold) in expression levels compared to WT levels at the early exponential growth phase. Error bars represent the standard deviations of the $\Delta\Delta C_T$ value (3). Statistical significance was determined by Student's *t* test. A *P* value of <0.05 was considered significant.

SDS-PAGE and immunoblotting. For blots using whole-cell lysates (WCLs), total proteins were extracted from strains 86-24, VS94, and MW90 grown in DMEM to OD_{600} of 0.2, 0.5, and 1.0. Briefly, 3 ml of culture was pelleted (13,000 rpm for 5 min at 4°C) and resuspended in 300 μl lysis buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5% glycerol, 1 mM dithiothreitol, and 30 mM phenylmethylsulfonyl fluoride), lysozyme was added to a final concentration of 300 μg/ml, the culture was incubated at 4°C for 4 h and DNase I treated for 45 min at 4°C, cell debris was pelleted (13,000 rpm for 10 min at 4°C), and the supernatant containing whole-cell protein was removed. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting procedures were performed as previously described (34), and blots were probed with polyclonal antisera against either EscJ (kindly provided by Gad Frankel, Imperial College London), EspA, EspB, or Tir (kindly provided by James Kaper). Proteins were detected using enhanced chemiluminescence (ECL; Bio-Rad). Equal amounts of whole-cell lysate protein were determined using the Lowry assay (34) and verified by probing blots with a monoclonal antibody against RpoA (Neoclone).

Preparation of secreted proteins. Secreted proteins from strains 86-24, VS94, and MW90 were harvested as previously described by Jarvis et al. (20). Briefly, bacteria were grown aerobically in DMEM at 37°C and collected at early exponential (OD_{600} of 0.2), mid-exponential (OD_{600} of 0.5), and late exponential (OD_{600} of 1.0) phases of growth. Total secreted protein from culture supernatants was separated by removing bacteria using centrifugation and filtration and then precipitating the secreted proteins present in the supernatant with trichloroacetic acid. The samples were then subjected to immunoblotting with rabbit polyclonal antisera to EspA and EspB (kindly provided by James Kaper) and visualized with enhanced chemiluminescence.

FAS test. Fluorescence actin staining (FAS) assays were performed as previously described by Knutton et al. (26). In brief, bacterial cultures grown aerobically overnight in LB medium at 37°C were diluted 1:100 and used to infect confluent monolayers of HeLa cells grown on glass coverslips at 37°C in 5% CO_2. Cells were grown for 6 h at 37°C in 5% CO_2, with samples being removed each hour. At the specified time points, the coverslips were washed, permeabilized with 0.2% Triton X-100, and treated with fluorescein isothiocyanate (FITC)-phalloidin to visualize actin accumulation, and propidium iodide was added to stain bacteria. Samples were visualized by immunofluorescence using a Zeiss Axiovert microscope. The entire field of two coverslips from each time point per strain was examined, and images of AE lesions were taken.

RESULTS

EHEC LEE gene transcription is reduced in a *luxS* mutant during mid-exponential growth. Expression of the LEE in EHEC is induced by both a bacterial signal, AI-3, and two eukaryotic hormones, epinephrine and norepinephrine (44). The LuxS enzyme, which is involved in the metabolism of *S*-adenosylmethionine to produce AI-2, is also required for the efficient production of AI-3 (44). Previous studies assessing AI-3/epinephrine/norepinephrine activation of LEE gene transcription were performed using a LEE::*lacZ* transcription reporter system in an *E. coli* K-12 background with PC media from the WT and a *luxS* mutant (42, 44).

Given the array of regulatory factors specific to EHEC (Fig. 1), we sought to examine LEE transcription in the WT and a *luxS* mutant in native EHEC backgrounds. For this purpose, we used real-time RT-PCR. Real-time RT-PCR avoids many of the drawbacks of plasmid-based reporter systems, such as copy number issues and coiling effects, and quantifies the amount of target transcripts. Real-time RT-PCR is also more sensitive than plasmid-based reporter systems, allowing for subtle changes in gene transcription to be detected.

The amount of *ler* (*LEE1*), *escC* (*LEE2*), *escV* (*LEE3*), *eae* (*LEE5*), and *espA* (*LEE4*) transcription was measured at early exponential (OD_{600} of 0.2), mid-exponential (OD_{600} of 0.5), and late exponential (OD_{600} of 1.0) growth points for the WT, an isogenic *luxS* mutant, and a *luxS*-complemented strain grown aerobically in DMEM, conditions known to activate LEE expression. All values are represented as expression levels (*n*-fold) with respect to strain 86-24 (WT) at the early exponential growth phase. Transcription of *ler* in the *luxS* mutant was not significantly different than that of the WT at the early exponential growth (Fig. 2A). Expression of the LEE at

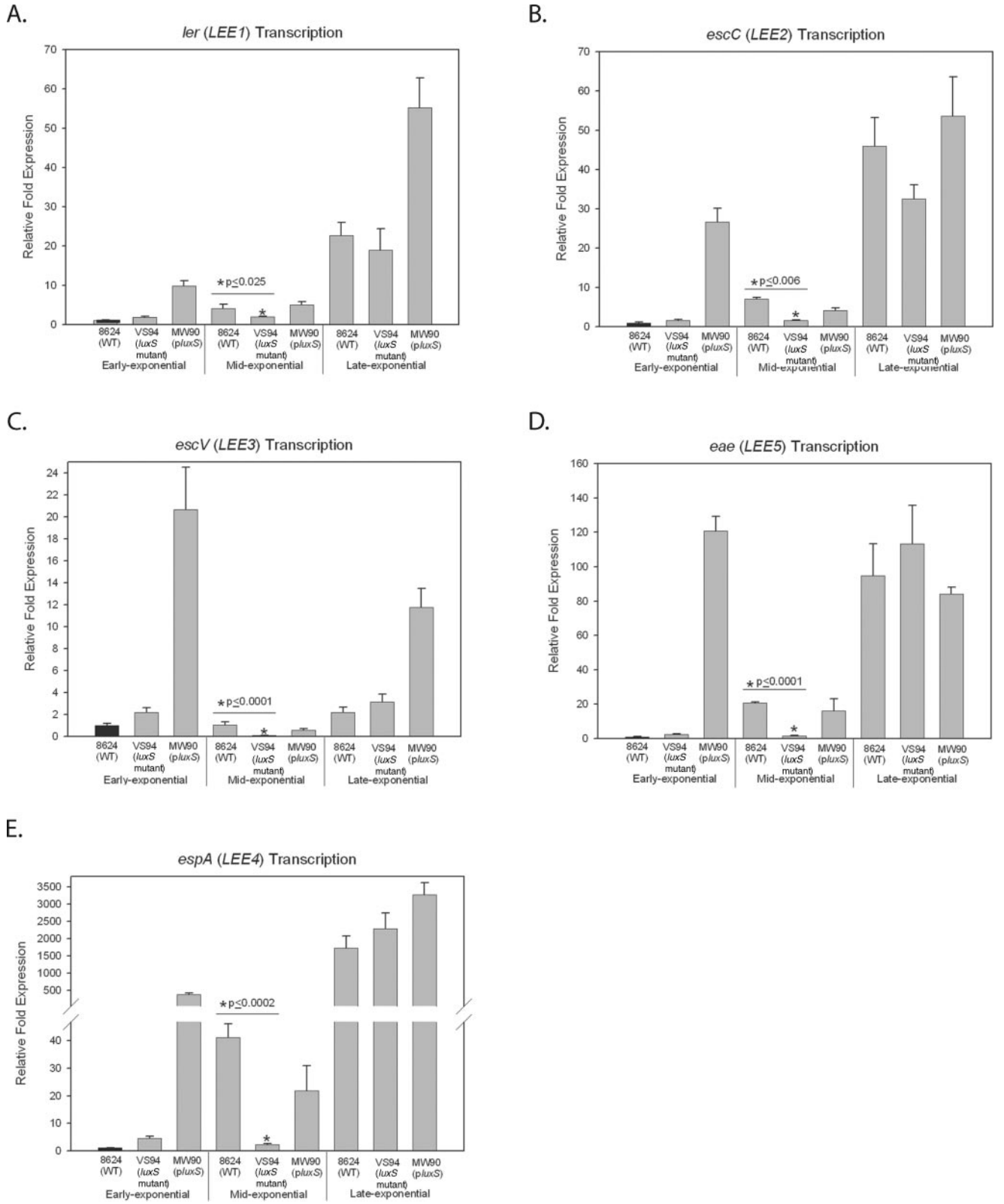

FIG. 2. Transcriptional profiles of LEE expression during early, mid-, and late exponential growth for WT EHEC, an isogenic *luxS* mutant, and a *luxS*-complemented strain (p*luxS*). Shown are transcriptional profiles of *ler* from the *LEE1* operon (A), *escC* from the *LEE2* operon (B), *escV* from the *LEE3* operon (C), *eae* from the *LEE5* operon (D), and *espA* from the *LEE4* operon (E) as measured by real-time RT-PCR. Relative fold expression represents the change (*n*-fold) in transcription compared to the 86-24 (WT) early exponential sample for each gene (black bar, value of 1.0). Results are means and standard deviations from triplicate experiments. The levels of *rpoA* transcript were used to normalize the C_T values to account for variations in bacterial numbers.

the early exponential growth phase is likely to be low because autoinducer levels are not sufficient to activate the LEE. In the *luxS*-complemented strain, transcription of *ler* was increased during early exponential growth almost 10-fold over that of the WT, implying that IPTG-induced expression of LuxS from a plasmid during early exponential growth leads to higher AI-3 levels. During mid-exponential growth, transcription of *ler* in the WT increased fourfold compared to that during early exponential growth. Transcription of *ler* at the mid-exponential growth phase in the *luxS* mutant was reduced 2.2-fold compared to the WT at the same growth phase ($P < 0.025$). The mutant's inability to synthesize sufficient amounts of AI-3 most likely led to the reduced amounts of *ler* transcript. Transcription of *ler* was restored in the *luxS*-complemented strain during mid-exponential growth. At the late exponential growth phase, *ler* transcription was the same in both the WT and the *luxS* mutant. These results suggest that AI-3-dependent regulation of *ler* occurs during mid-exponential growth. AI-3-dependent regulation does not appear to play as important a role in *ler* transcription during early and late exponential growth. There was not a significant difference in the transcription of genes within the LEE between the WT and the *luxS* mutant at these growth phases, suggesting that other factors are controlling LEE expression. When LuxS is expressed from a plasmid, transcription of *ler* is increased. The greater amounts of LuxS seem to enhance the production of AI-3 through an unknown pathway, resulting in the earlier activation of the LEE.

The other LEE operons displayed transcription patterns similar to that of *ler* (Fig. 2B to E). There was not a significant difference in the transcription of *escC* between the WT and the *luxS* mutant at the early exponential growth phase (Fig. 2B). Transcription of *escC* in the *luxS* mutant was down-regulated almost fivefold compared to the WT at the mid-exponential growth phase ($P < 0.006$), and there was no significant difference observed during late exponential growth. Transcription of *escC* in the complemented strain was induced approximately 26-fold over the WT during early exponential growth (Fig. 2B). Similar to *ler* and *escC* transcription, the *luxS* mutant had significantly decreased transcription of *escV* (13-fold; $P < 0.0001$), *eae* (13-fold; $P < 0.0001$), and *espA* (18-fold; $P < 0.0002$) at the mid-exponential growth phase compared to the WT at the same growth phase (Fig. 2C to E). Transcription of *escV*, *eae*, and *espA* was not significantly different between the *luxS* mutant and the WT during late exponential growth, analogous to the results observed for *ler* and *escC*. The complemented strain again had higher levels of *escV* (20-fold), *eae* (120-fold), and *espA* (374-fold) transcription than the WT during early exponential growth (Fig. 2C to E).

IPTG-induced expression of LuxS led to an earlier activation of genes within each of the five LEE operons at the early exponential growth phase. Normalization with the constitutively transcribed *rpoA* revealed that transcription of all genes tested increased with growth of the WT strain (Fig. 2A to E). This trend was not observed in the *luxS* mutant. We consistently observed significantly lower levels of transcription by the *luxS* mutant during mid-exponential growth, suggesting that AI-3-dependent regulation plays a major role in LEE transcription during mid-exponential growth (when bacteria are rapidly dividing). Growth curves did not reveal any difference in growth among the three strains (data not shown), indicating that these results are not due to differences in growth kinetics.

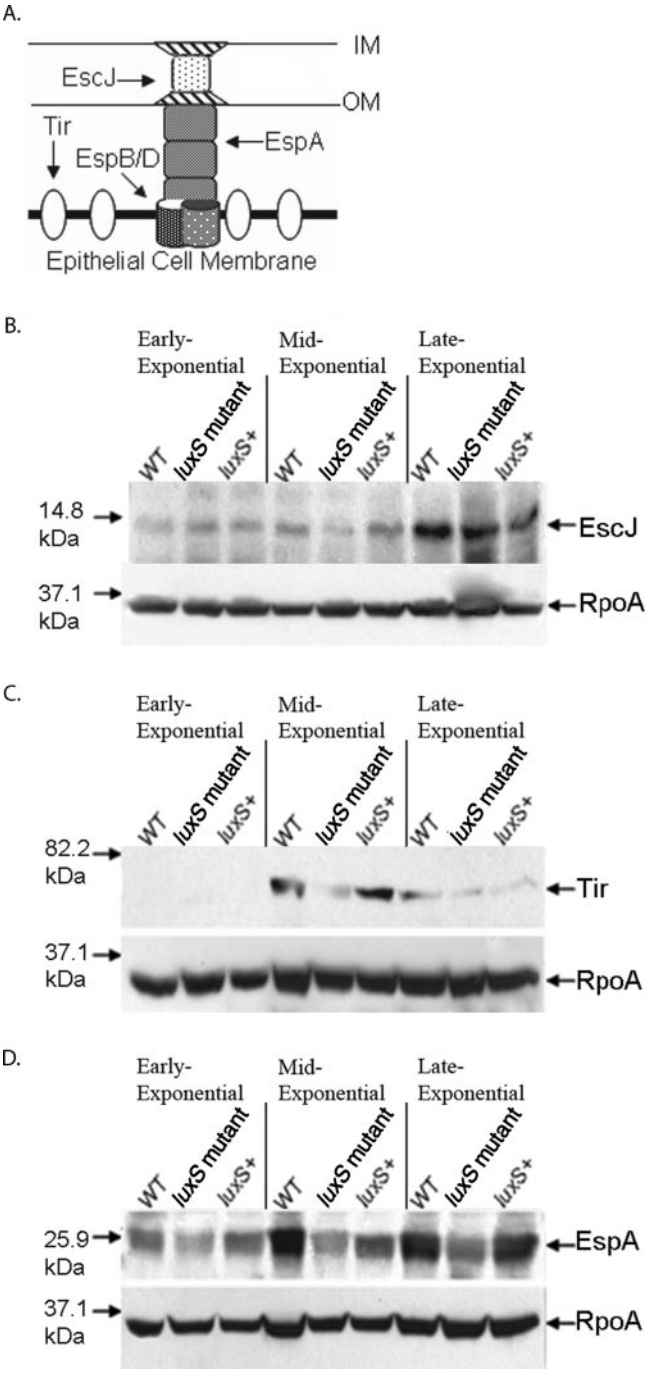

FIG. 3. Immunoblot analysis of WCLs of the wild type, an isogenic *luxS* mutant, and *luxS* complement (*luxS*+). (A) Schematic of the proteins examined and their role in the formation of the TTSS. (B to D) Protein expression in WCL during early, mid-, and late exponential growth using antibodies against EscJ encoded by the *LEE2* operon (B), Tir encoded by the *LEE5* operon (C), and EspA encoded by the *LEE4* operon (D). Each blot was stripped after probing with the EscJ, Tir, and EspA antibodies and reprobed with an antibody against RpoA to verify that equal amounts of protein were loaded.

TTSS protein expression is decreased in a *luxS* mutant. To establish a relationship between our transcript results and LEE

protein expression, we isolated bacterial whole-cell lysates of WT, *luxS* mutant, and *luxS*-complemented strains from early, mid-, and late exponential growth stages. We examined the major components of the TTSS by performing immunoblot analysis using rabbit polyclonal antisera directed against a structural component of the TTSS (EscJ), a translocated effector protein (Tir), and the outer filament of the TTSS (EspA). Hence, we were able to examine the expression of proteins that compose three distinct portions of the TTSS machinery (Fig. 3A). A mouse monoclonal antibody to the constitutively expressed *E. coli* RNA polymerase alpha subunit (RpoA) was used to verify that equal amounts of proteins were loaded.

Figure 3B shows that the expression of EscJ was decreased in the *luxS* mutant during mid-exponential growth, in agreement with the transcription data for *escC* (Fig. 2B). An antibody to EscJ was used to examine protein expression of the *LEE2* operon because the generation of an antibody against EscC was unsuccessful. Since both *escJ* and *escC* are encoded in the same operon, it is expected that they would be transcribed together and that they would share similar expression patterns. There appears to be no significant difference in the expression of EscJ at the late exponential growth phase, further supporting the transcriptional data for *escC*. Surprisingly, although transcription of *LEE2* was highly up-regulated in the complemented strain during early exponential growth, we did not observe an increase in EscJ expression at this growth phase in this strain. The reason for this disparity between the transcription of *LEE2* and protein levels of EscJ is unknown, and future experiments will further examine this phenomenon.

Similar results were observed when the expression of Tir (*LEE5*) in bacterial whole-cell lysates was examined. Our transcription data showed a significant decrease in the amount of *LEE5* transcription (Fig. 2D) during mid-exponential growth in the *luxS* mutant compared to either the WT or the complemented strain. Indeed, the levels of Tir in the WCL were decreased during mid-exponential growth (Fig. 3C). Although the levels of *tir* transcription were high during late exponential growth, we observed a decrease in the amount of Tir present in whole-cell lysates during the same growth phase. The difference between transcript and protein levels may have resulted from the secretion of Tir into the culture medium by the TTSS. Hence, lower amounts of Tir would be present within the bacterial whole-cell lysates used for immunoblot analysis. In accordance with this finding, we have previously reported significant secretion of Tir during late exponential growth (44).

EspA protein expression was decreased in the *luxS* mutant during both mid- and late exponential growth (Fig. 3D). Interestingly, despite there being no significant difference in the transcription of *espA* between the WT and the *luxS* mutant at the late exponential growth phase (Fig. 2E), there was less EspA protein produced by the *luxS* mutant during this phase of growth (Fig. 3D). This may be a result of differences in the posttranscriptional regulation of *espA* in the WT and the *luxS* mutant. Roe et al. previously demonstrated that EspA secretion is phase variable and controlled at the posttranscriptional level through an uncharacterized mechanism (33). A constraint seems to be placed on the *espADB* transcript so that it is translated only when the appropriate signals are present. The *luxS* mutant may not be capable of producing these signals to allow for the *espADB* transcript to be translated, causing the observed decrease in the levels of EspA protein present in the whole-cell lysate. Transcription of *espA* was much higher at the late exponential growth phase than during the mid-exponential growth phase (Fig. 2E), but a comparable increase in EspA expression was not observed in whole-cell lysates (Fig. 3D). Cellular levels of EspA were also influenced by its secretion, similar to Tir. Indeed, we found that the greatest amount of EspA and EspB secretion occurred during late exponential growth in WT, *luxS* mutant, and complemented strains (see Fig. 5A). Transcription of *LEE5* (*tir*) and *LEE4* (*espA*) were also up-regulated in the complemented strain during early exponential growth. However, the levels of Tir and EspA in WCLs in this strain were comparable to those of the WT at this growth phase (Fig. 3B and C). Since the complemented strain is already secreting these proteins through the TTSS during early exponential growth (see Fig. 5A), our inability to observe an increase in the levels of Tir and EspA in the complemented strain's WCL was again due to the fact that they have been secreted into the media.

Epinephrine increases transcription of the LEE. To examine the effects of epinephrine in an EHEC background, we performed real-time RT-PCR analysis of genes within the *LEE1*, *LEE2*, and *LEE3* operons in the WT and an isogenic *luxS* mutant in the presence and absence of epinephrine. The addition of epinephrine to a final concentration of 50 μM at time zero (previously shown to induce the maximal signal in *E. coli* K-12) (44) increased the transcription of *ler* (*LEE1*) in both the WT and the *luxS* mutant nearly 100-fold more than medium alone during early exponential growth (Fig. 4A). At the early exponential phase, it is likely that there is not sufficient AI-3 to endogenously activate *LEE1* transcription (Fig. 2A). Hence, epinephrine increases transcription of *LEE1* in both the WT and the *luxS* mutant to the same extent. By the mid-exponential growth phase, transcription of *ler* in the *luxS* mutant with epinephrine is significantly less than that in the WT with epinephrine ($P < 0.05$) (Fig. 4A). Since the *luxS* mutant cannot readily produce AI-3, epinephrine is the main signal present to activate expression of the LEE. In the WT, both AI-3 and epinephrine are present to activate *LEE1* transcription. The combination of these two signals results in the increased expression of *ler* and the rest of the LEE. These results suggest that there may be an agonistic relationship between AI-3 and epinephrine to activate *LEE1* transcription, inasmuch as significant additive effects are observed in the WT with epinephrine during mid-exponential growth. During late exponential growth, *ler* transcription was activated over 1,000-fold in the WT and the *luxS* mutant with epinephrine compared to the respective strains without epinephrine at the same growth phase. There was not a significant difference in the levels of *ler* transcription between the WT and the *luxS* mutant during late exponential growth.

We also examined the effect of epinephrine on the transcription of the other downstream LEE operons. Specifically, we measured the transcription of *escC* (*LEE2*) and *escV* (*LEE3*) in response to epinephrine (Fig. 4B and C). Epinephrine increased the transcription of *escC* in both the WT and the *luxS* mutant only during late exponential growth (Fig. 4B). When epinephrine was present, transcription of *escC* during late exponential growth was significantly higher in the WT than in the

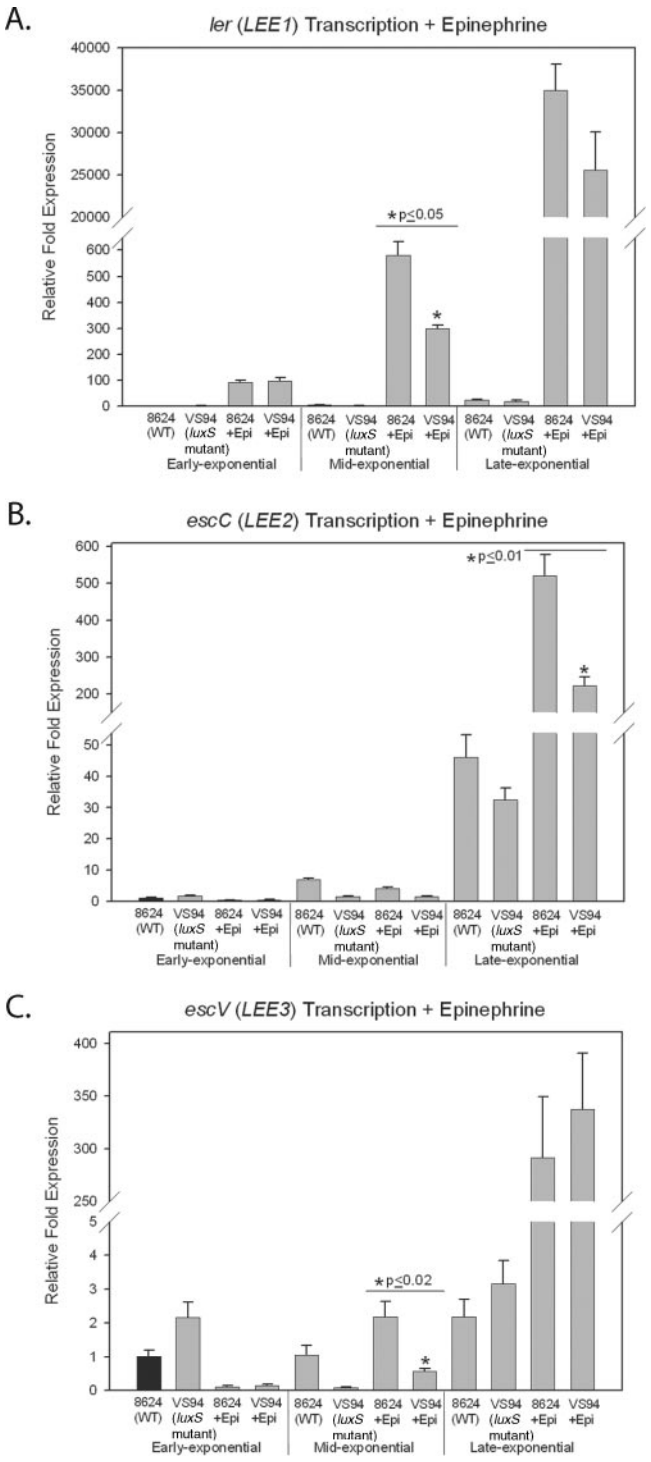

FIG. 4. Addition of epinephrine to the growth media increased transcription of the *LEE1*, *LEE2*, and *LEE3* operons. Shown are transcriptional profiles of *ler* from the *LEE1* operon ± 50 μM epinephrine (A), *escC* from the *LEE2* operon ± 50 μM epinephrine (B), and *escV* from the *LEE3* operon ± 50 μM epinephrine (C) for WT EHEC and an isogenic *luxS* mutant during early, mid-, and late exponential growth as measured by real-time RT-PCR. Relative fold expression represents the change (*n*-fold) in transcription compared to the 86-24 (WT) sample for each gene during the early exponential phase (black bar, value of 1.0). Results are means and standard deviations from triplicate experiments. The levels of *rpoA* transcript were used to normalize the C_T values to account for variations in bacterial numbers.

luxS mutant ($P < 0.01$). The delay in increased *escC* transcription suggests that the effect of epinephrine on *LEE2* transcription may be indirect and a result of the increased amounts of Ler over time. During early exponential growth, epinephrine decreased the transcription of *escC*. By the mid-exponential growth phase, epinephrine did not significantly affect the transcription of *escC* in the WT or *luxS* mutant compared to cultures without epinephrine.

The *escV* gene (*LEE3*) revealed a transcriptional pattern similar to that of the *escC* gene of *LEE2* (Fig. 2C). During early exponential growth, the addition of epinephrine resulted in a decrease in the amount of *escV* being transcribed in both the WT and the *luxS* mutant. By the mid-exponential phase of growth, the addition of epinephrine resulted in an increase of two- and sevenfold for the WT and the *luxS* mutant, respectively (compared to the WT and the *luxS* mutant with no epinephrine at the same growth phase). The WT displayed significantly higher transcription than the *luxS* mutant in response to epinephrine during mid-exponential growth ($P < 0.02$). This result is similar to those for transcription without epinephrine and further suggests that AI-3 is responsible for the increased transcription observed in the WT during mid-exponential growth. The addition of epinephrine resulted in over a 100-fold increase of *escV* transcription during late exponential growth for both the WT and the *luxS* mutant compared to the WT and the *luxS* mutant with no epinephrine at the same growth phase. There was no significant difference in the transcription of *escV* between the WT with epinephrine and the *luxS* mutant with epinephrine during late exponential growth.

The *luxS* mutation reduces TTSS-dependent phenotypes. To examine the function of the LEE-encoded TTSS as a whole in the WT and the *luxS* mutant, we assessed the amounts of EspA and EspB actively secreted from cultures grown in the presence and absence of epinephrine. EspA composes the filament of the TTSS (27), while EspB helps to form a pore in the eukaryotic membrane that is necessary to translocate effector proteins into the eukaryotic cell (37, 39, 48) (Fig. 3A). Both of these proteins are required for virulence and the formation of AE lesions on the intestinal epithelium (1). Previous studies examining EspA and EspB secretion in the WT and the *luxS* mutant used a primary antiserum against total secreted proteins (44). Protein secretion in the *luxS* mutant could not be detected with these antisera against total secreted proteins. The studies presented here employ a specific anti-EspA antibody and a specific anti-EspB antibody. The specific antisera allow for more sensitive detection of secreted EspA and EspB in culture supernatants, resulting in the detection of secreted proteins from the *luxS* mutant.

Secretion of EspA and EspB occurred in the early growth phase in the *luxS*-complemented strain when expression of *luxS* was induced with 1 mM IPTG (Fig. 5A), linking the early increase in transcription of the five LEE operons observed by real-time RT-PCR (Fig. 2A to E) with earlier TTSS activity. At the mid-exponential growth phase, the WT strain secreted more EspA and EspB protein than the *luxS* mutant. A more pronounced difference was observed at the late exponential growth phase. Despite transcription of the LEE being significantly lower in the *luxS* mutant only during mid-exponential growth, a defect in TTSS activity was most prominently observed during late exponential growth.

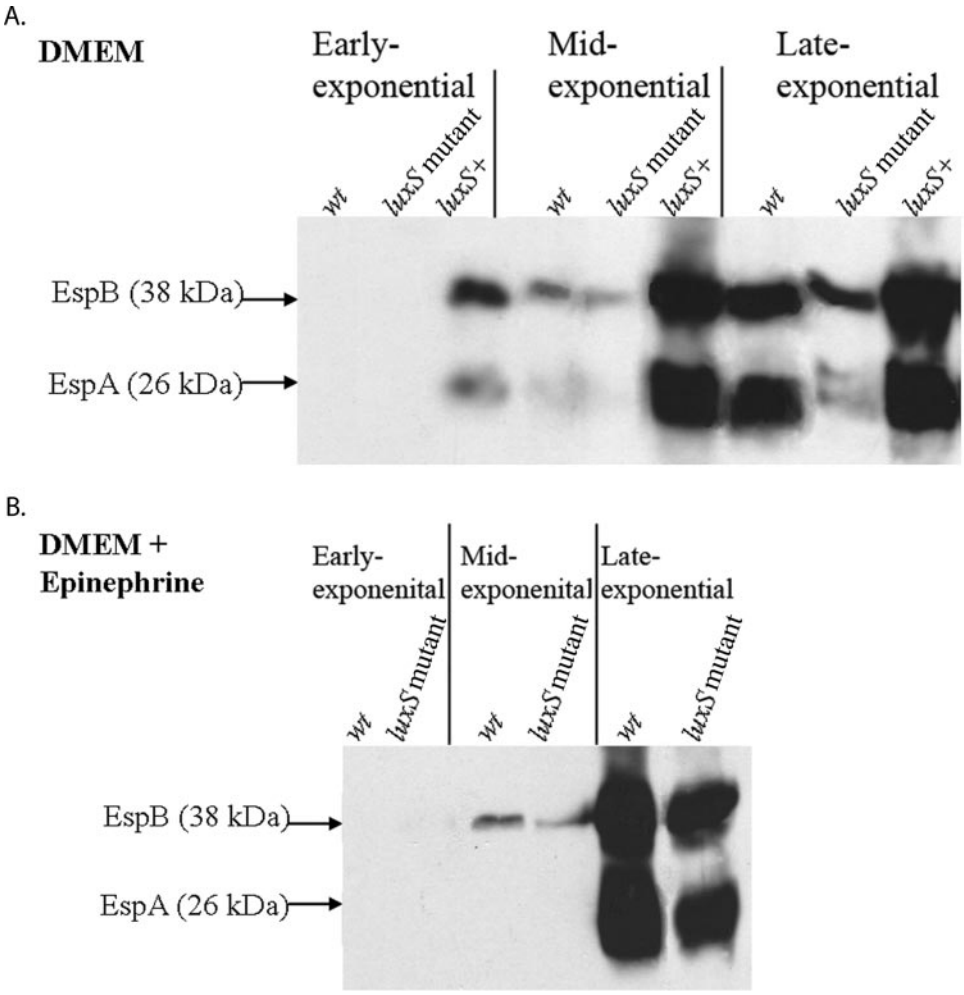

FIG. 5. Total protein secreted in equal culture volumes was trichloroacetic acid precipitated and examined by SDS-PAGE and immunoblot. Shown are immunoblots of secreted EspB and EspA proteins from WT EHEC and *luxS* mutant culture supernatants without epinephrine (A) and with the addition of 50 μM epinephrine (B).

The addition of epinephrine increased the amount of EspA and EspB secreted by the WT and the *luxS* mutant (Fig. 5B), in agreement with previous findings (44). The WT secreted more EspA and EspB than the *luxS* mutant did in response to epinephrine. The greater amount of protein secreted by the WT again suggests that there may be a synergistic relationship between epinephrine and AI-3 since the *luxS* mutant is deficient in AI-3 production. Epinephrine did not appear to result in increased EspA and EspB secretion during mid-exponential growth, consistent with the transcription data from the *LEE2* and *LEE3* operons in response to epinephrine.

EHEC is able to produce AE lesions on eukaryotic epithelial cells. The LEE encodes the factors necessary to induce the formation of these AE lesions. To assess the entire production and expression of the LEE, the abilities of the WT, an isogenic *luxS* mutant, and the complemented *luxS* strain to form AE lesions were observed using FAS assays (Fig. 6). EHEC cells (red) were stained with propidium iodide, while actin (green) was visualized with FITC-phalloidin. WT and *luxS*-complemented bacteria formed AE lesions at 3 h postinfection, between the early and mid-exponential growth phases. The *luxS* mutant was delayed 2 h in AE lesion formation in this tissue culture model and did not display the AE phenotype until 5 h postinfection, corresponding to a time between the mid- and late exponential growth phases used in the transcriptional studies. In previous studies using these strains, only the late exponential (6 h) growth phase was examined for the presence of AE lesions, and no difference between the WT and the *luxS* mutant was detected (44). This work provides the first phenotypic difference in AE lesion formation between the WT and a *luxS* mutant.

DISCUSSION

The *luxS* gene is necessary for the efficient production of the AI-3 quorum-sensing signal (44). However, the *luxS* mutation does not affect the ability of EHEC to respond to AI-3 and epinephrine/norepinephrine signals (44). In our in vitro studies, the only AI-3 present is produced by the bacteria. The *luxS* mutant allows us to study the relationship of LEE activation and AI-3 production in vitro. We have shown here that the *luxS* mutation leads to decreased transcription of the LEE promoters during mid-exponential growth. This is in contrast to the

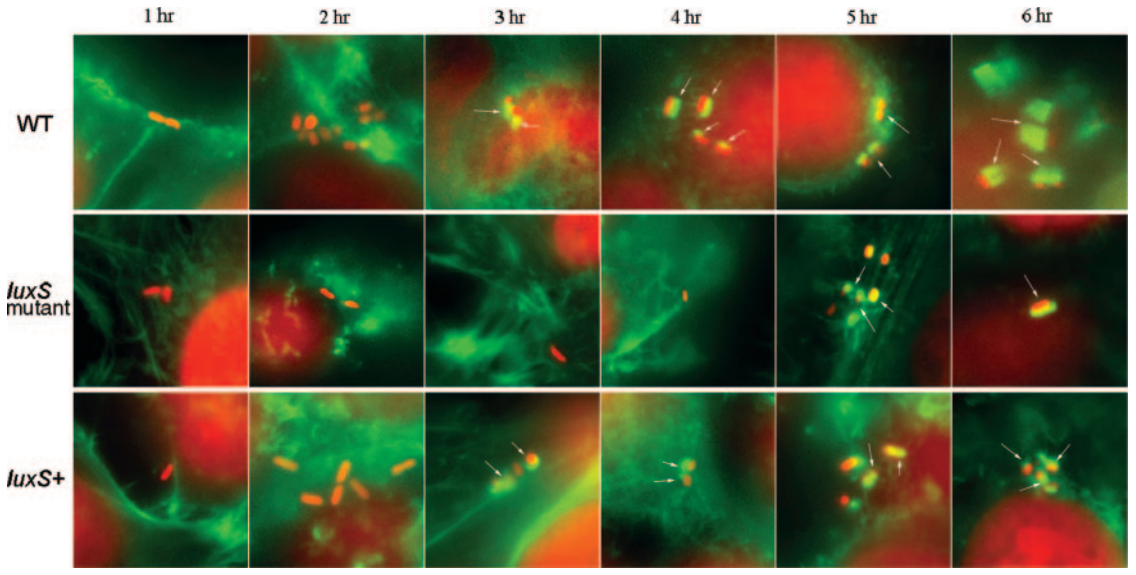

FIG. 6. FAS to measure AE lesion formation of the WT, an isogenic *luxS* mutant, and the *luxS* complement in a HeLa cell infection model. Two hours postinfection corresponds with early exponential growth, 4 h corresponds with mid-exponential growth, and 6 h corresponds with late exponential growth. EHEC is stained red with propidium iodide, and the actin cytoskeleton is stained green with FITC-phalloidin. AE lesions are indicated by arrows.

activation of the LEE promoters by this signaling system previously observed during the late exponential phase in an *E. coli* K-12 background (42). The difference in the kinetics of activation between the two backgrounds can most likely be attributed to the additional regulators of the LEE present in EHEC but not in *E. coli* K-12 (Fig. 1). Pch, EtrA, EivF, GrlR/A, and Ler are several of the known LEE regulators present in EHEC and not *E. coli* K-12. Furthermore, there may be additional uncharacterized regulatory factors specific to EHEC that influence LEE expression. The reduced LEE transcription led to a reduction in protein expression in the *LEE2*, *LEE5*, and *LEE4* operons. EspA and EspB protein secretion by the LEE TTSS was also reduced in the *luxS* mutant. The *luxS* mutant exhibited reduced amounts of EspA and EspB secretion compared to the WT at the late exponential growth phase (Fig. 5A), although no difference in transcription was observed (Fig. 2E). This may be a result of the *luxS* mutant's decreased ability to properly assemble functional TTSS machinery due to the decreased transcription of the LEE genes during mid-exponential growth, or this may be a result of the posttranscriptional regulation of the *espADB* operon, which has been described previously (33). Complementation of the *luxS* mutation restored the transcriptional activity of the LEE promoters as well as cognate protein production and secretion.

When the WT and the *luxS* mutant were grown in the presence of epinephrine, transcription of *LEE1*, *LEE2*, and *LEE3* increased. The *ler* gene is the only gene examined that shows a direct increase in transcription at the early exponential growth phase in response to the addition of epinephrine. WT bacteria exhibited a greater increase in the transcription of *ler* (*LEE1*) at the mid-exponential growth phase, presumably because of the ability of the WT to produce the AI-3 signal. Epinephrine and AI-3 seemed to signal in a synergistic fashion to activate the transcription of *ler*. We did not observe AI-3-dependent regulation during early and late exponential growth (Fig. 2A), and the effect of epinephrine appeared to be comparable for both the WT and the *luxS* mutant during these growth phases (Fig. 4A).

Both *escC* and *escV* transcription levels increased during late exponential growth in response to epinephrine (Fig. 4B and C). Interestingly, the addition of epinephrine to the WT only resulted in a larger increase of *escC* transcription (compared to the *luxS* mutant plus epinephrine) during late exponential growth. There was not a significant difference in the transcription of *escV* between these two strains at this growth phase. AI-3 may influence the expression of another repressor that diminishes the transcription of the *LEE3* operon but not the *LEE2* operon at this growth phase. The addition of epinephrine also increased EspA and EspB protein secretion in the WT and the *luxS* mutant, in agreement with previous data (44). Epinephrine and AI-3 increased the secretion of these proteins to a larger extent in the WT than was observed for the *luxS* mutant, again suggesting a synergistic relationship between these signals. The result of the decreased transcription and expression of the LEE was a delay in the formation of AE lesions by the *luxS* mutant on cultured epithelial cells.

It is important that the *luxS* mutation does not abolish LEE expression, and the mutant is still able to respond to exogenous activating signals such as epinephrine. AI-3 and epinephrine/norepinephrine appear to play a large role in the proper expression and possibly the coordinated production of the LEE to yield a functional TTSS. The work presented here reveals for the first time the effects of the *luxS* mutation on the transcription of the LEE in a native EHEC background (containing all regulators of LEE expression). The disruption of *luxS* leads to a defect in the production of AI-3 (44) and to lower levels of transcription of the LEE operons in EHEC.

EHEC infects the colon and has a very low infectious dose, estimated to be as few as 10 to 100 organisms. Because so few organisms are able to cause an infection, it is unlikely that

EHEC relies on the small amount of self-produced AI-3 early during infection to activate the expression of the LEE. The more likely scenario is that EHEC uses both the AI-3 produced by the normal flora of the colon (44) and epinephrine/norepinephrine naturally present in the intestine (9) to recognize that it is within the host. The precise epinephrine/norepinephrine concentrations in the gastrointestinal tract are not known, although substantial amounts of both epinephrine and norepinephrine have been shown to be present in the intestine (9). Epinephrine from the bloodstream may spill out from enterocytes or may reach the lumen after the first round of infection and the resultant disruption of the intestinal epithelium and blood entering the colon. Norepinephrine is produced in the gastrointestinal tract by adrenergic neurons in enteric nervous systems. The concentration of norepinephrine in the lumen may also increase after destruction of the intestinal epithelium.

The data from this study suggest that there is a synergistic effect between AI-3 and epinephrine. Such combined signals would then likely activate LEE expression in the same manner. This relationship would allow for a more efficient infection than responding to one signal alone. During the initial infection, the first wave of EHEC would sense the AI-3 produced by the normal flora as well as any epinephrine/norepinephrine that may be present in the intestinal lumen, resulting in the activation of the LEE. As the intestinal epithelium becomes more disrupted, more epinephrine/norepinephrine would be released into the gastrointestinal tract. This increased amount of epinephrine/norepinephrine, as well as the AI-3 synthesized by the escalating EHEC population, would be detected by EHEC, leading to increased activation of the LEE and another wave of infection.

In summary, this study further characterizes the effects of the *luxS* mutation by examining LEE transcription in an EHEC background and also for the first time provides evidence of a synergistic relationship between AI-3 and epinephrine/norepinephrine. A better understanding of the signals that activate EHEC pathogenesis will help to direct new therapeutic approaches.

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