Global Effects of the Cell-to-Cell Signaling Molecules Autoinducer-2, Autoinducer-3, and Epinephrine in a luxS Mutant of Enterohemorrhagic Escherichia Coli

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ABSTRACT

Intrakingdom and interkingdom cell-to-cell communication play essential roles in the virulence of enterohemorrhagic E. coli (EHEC). Four signals, autoinducer 2 (AI-2), autoinducer 3 (AI-3) and the human hormones epinephrine and/or norepinephrine, are important in this communication. The effect of these signaling compounds on the transcriptome of EHEC is examined in this study. We demonstrate that the \textit{luxS} mutation affects primarily central metabolic genes in both pathogenic and nonpathogenic strains of \textit{E. coli} and that addition of exogenous AI-2 does not fully restore the expression profile in a \textit{luxS} deficient strain lacking the ability to synthesize AI-2. Addition of AI-3 or epinephrine increased expression of the locus of enterocyte effacement (LEE) regulon, known to play a pivotal role in EHEC virulence. Moreover, when epinephrine was added to the culture medium, the greatest number of gene alterations was observed. These alterations included a greater proportion of EHEC genes when compared to MG1655, suggesting that epinephrine may be a global virulence signal. Detailed examination with real-time RT-PCR confirmed the increases in virulence gene expression with the addition of AI-3 and epinephrine. Additional studies with real time RT-PCR examining the EHEC secreted effectors and putative fimbrial gene expression showed a variable expression profile indicating that there is differential regulation of these secreted molecules. This study begins to examine the global signaling networks in EHEC and reveals expression profiles that are signal- and pathogen-specific.
INTRODUCTION

The human pathogen enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) colonizes the human colon, resulting in the development of hemorrhagic colitis and hemolytic uremic syndrome that may be fatal (35). Upon colonization of the colon, EHEC forms attaching and effacing (AE) lesions on the epithelial cells and produces Shiga toxin. Most of the genes involved in the formation of the AE lesions are encoded in a chromosomal pathogenicity island named the locus of enterocyte effacement (LEE) (40). The LEE encodes a type III secretion system (TTSS) and effector proteins that are translocated into epithelial cells and cause extensive cytoskeletal rearrangements resulting in the formation of AE lesions (32, 33, 36). In addition to the LEE, EHEC’s arsenal of virulence factors includes non-LEE encoded effector proteins that are secreted through the LEE-encoded TTSS (8, 14, 21, 24, 51, 63) and may also include fimbriae that increase adherence or mediate colonization of epithelial cells (64, 65).

Regulation of the LEE genes is extremely complex and includes the global regulators H-NS (7, 25, 42, 49, 56, 66), integration host factor (19) and the environment-dependent regulator Hha (52) that act to repress LEE transcription. Other regulators include the LysR transcriptional regulator QseA that positively regulates LEE by binding to ler (53, 55), the ClpXP protease (30) that increases transcription of LEE by inhibiting GrlR repression, and also through interacting with the stationary-phase sigma factor RpoS. RpoS also positively regulates transcription of LEE3 (30, 57), and the signaling molecule ppGpp can also increase transcription of LEE (45). Many of the aforementioned regulators are common to both pathogenic and nonpathogenic strains of *E. coli*; however, a number of regulators are unique to EHEC. Encoded in the LEE, Ler
(LEE-encoded regulator) is able to overcome H-NS-mediated repression and activate expression of the LEE2, LEE3, and LEE5 operons (25, 49, 56), and GrlR and GrlA, respectively, repress and activate transcription of ler (3, 14). The pch genes that are homologous to perC in EPEC increase expression of the LEE genes (31). Finally, the transcriptional regulators eivF and etrA that are encoded within a second, non-functional TTSS (ETT2) are negative regulators of the LEE (76).

EHEC also utilizes quorum sensing (QS) to regulate expression of its virulence and flagellar and motility genes (57-60). Initial investigations suggested that the autoinducer (AI)-2 was the QS signal responsible for regulating expression of virulence genes in EHEC (57, 58); however, subsequent research using purified and in vitro synthesized AI-2 demonstrated that the signaling molecule affecting TTSS and motility was not AI-2, but was a distinct compound named AI-3 (59). Differences in these molecules have been revealed by biochemical assays: the polar furanone, AI-2, does not bind to C-18 columns, whereas AI-3 binds to C-18 columns and can be eluted only with methanol; electrospray mass spectrometry revealed structural differences between AI-2 and AI-3 (9, 59). Moreover, the transcriptional assay for AI-2 is based on the production of bioluminescence in Vibrio harveyi, and AI-3 does not show any activity for this assay. Conversely, AI-3 activates transcription of the EHEC virulence genes, whereas AI-2 has no effect on this assay (59, 68).

AI-2 production is dependent upon the LuxS enzyme (4, 5, 61). LuxS plays a role in the metabolism of S-adenosyl-methionine by converting S-ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (dpd). Dpd is unstable and spontaneously cyclizes to form several different furanones, one of which is believed to be
AI-2 (50). AI-3 is not dependent upon LuxS for synthesis; however, a luxS mutation leaves the cell with only one pathway to produce homocysteine which may lead to diminished production of AI-3 (68). Additional regulation occurs through crosstalk between EHEC and its host (59). EHEC senses the host hormones epinephrine and norepinephrine through the membrane protein QseC (11). QseC senses both AI-3 and epinephrine, and thus functions in interkingdom cross-signaling (11). QseC is part of a two-component system, QseB/C in which QseC is the sensor kinase and QseB is the response regulator. QseB/C activates transcription of the flagella regulon responsible for swimming motility in EHEC (10). Furthermore, QseC plays an important role in EHEC pathogenesis, as the virulence of a qseC mutant was attenuated in a rabbit infection model (11).

Previous gene array analysis was performed in order to elucidate the role that QS plays in the regulation of EHEC virulence and physiology by comparing a luxS mutant strain of EHEC to wild type (WT) EHEC (58). That analysis demonstrated that luxS regulation was pleiotropic and regulated numerous basic physiological functions, including cell division, motility, and genes involved in metabolism as well as virulence (58). The fact that EHEC produces two AI molecules was not recognized at that time, nor was it known that EHEC responded to human hormones, thus the specific role that each signaling molecule played in gene regulation was not fully elucidated. The specific aim of this study was to determine more precisely how cell signaling by AI-2, AI-3, and epinephrine affect global gene expression in EHEC. Transcriptome analyses were performed to compare global gene expression in WT EHEC to gene expression in a luxS
mutant grown without QS molecules added to the culture medium or grown with the signaling molecules AI-2, AI-3, or epinephrine added to the medium.

MATERIALS AND METHODS

Strains and culture/growth conditions. The WT EHEC strain 86-24 was used in this study. Strain 86-24 was isolated in 1986 from a patient in Seattle experiencing hemorrhagic colitis (23) and has been used extensively to study EHEC infection in animal models (13, 16, 34, 41, 54). The isogenic luxS mutant (strain VS94) (58) was also used in this study. Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) was used as the growth medium in all assays.

RNA extraction. Cultures of strains 86-24 and VS94 were grown aerobically in LB medium at 37°C overnight, and then were diluted 1:100 in DMEM and grown in a shaking incubator at 37°C. The tested compounds were added to the media at the following concentrations: 100 µM of dpd (AI-2), 30 µM of AI-3, and 50 µM of epinephrine. RNA from three biological replicate cultures of each strain/condition was extracted at the late exponential growth phase (OD_{600} of 1.0) using the RiboPure Bacteria RNA isolation kit (Ambion).

Microarrays. The GeneChip E. coli Genome 2.0 array system by Affymetrix system was used to compare gene expression in strain 86-24 to strain VS94 (luxS mutant) (with and without addition of signaling molecules to culture media). The GeneChip E. coli Genome 2.0 Array includes approximately 10,000 probe sets for all 20,366 genes present in four strains of E. coli: K-12 (lab strain, MG1655), CFT073 (uropathogenic strain), O157:H7-EDL933 (enterohemorrhagic strain), and O157:H7-Sakai (enterohemorrhagic
The RNA processing, labeling, hybridization and slide scanning procedures were preformed as described in the Affymetrix Gene Expression Technical Manual (http://www.affymetrix.com/support/technical/manual/espression_manual.affx).

**Microarray data analysis.** The output from the scanning of the single replicate of the Affymetrix GeneChip® E. coli 2.0 for each of the biological conditions was obtained using GCOS v 1.4 according to manufacturers instructions. Data were normalized using the Robust Multiarray analysis (RMA) (6, 29) via the RMAExpress website (http://rmaexpress.bmbolstad.com/). The resulting data were compared to obtain features that were increased or decreased in response to either the QS stimuli or the inactivation of the luxS gene. Custom analysis scripts were written in Perl to complete multiple array analyses. These array analyses were further confirmed using real-time RT-PCR described below. The authors note that the isolate used in these studies has not been sequenced, and thus, is not fully contained on the array and that differences in genome content are evident. Expression data can be accessed using accession number GSE7439 at the NCBI GEO database.

**Real-time reverse transcriptase-PCR (real-time RT-PCR).** The primers used in the real-time assays were designed using Primer Express v1.5 (Applied Biosystems) (Table 1). Amplification efficiency and template specificity of each of the primer pairs were validated as described previously (69). Real-time reverse transcription-PCR was performed in a one-step reaction using an ABI 7500 sequence detection system (Applied Biosystems), and reaction mixtures were prepared as previously described (69).
Real-time RT-PCR detection, quantification, and statistical analysis. Data was collected using the ABI Sequence Detection 1.3 software (Applied Biosystems). All data were normalized to levels of *rpoA* and analyzed using the comparative critical threshold (*C*₇) method (1). The expression level of the target genes under the various culture conditions was compared using the relative quantification method (1). Real-time data are presented as the change (*n*-fold) in expression levels compared to WT levels. Error bars represent the standard deviations of the ∆∆*C*₇ value (1). Statistical significance was determined by Student’s *t* test, and a *P* value of ≤ 0.05 was considered significant.

RESULTS

Previous analyses of gene expression in WT EHEC and the luxS mutant used spotted amplicon based arrays and hybridized EHEC cDNA with the *E. coli* K-12 array (58). We used the Affymetrix GeneChip technology as a starting point to examine the expression profiles of the entire *E. coli* genome with and without the addition of QS signaling molecules.

Transcriptome comparison of the WT 86-24 and luxS mutant VS94. The inactivation of the luxS gene in *E. coli* 86-24 interrupts homocysteine synthesis from SRH, preventing the production of AI-2 and diminishing the AI-3 QS signaling pathway in EHEC (58, 68). In the current study, a total of 710 genes were differentially expressed in the luxS mutant as compared to the WT strain (Table 2), and a greater proportion of these genes showed decreased expression than increased expression (480 decreased vs. 230 increased).
The majority of the genes with an altered profile were derived from the *E. coli* MG1655 strain (~39%). These features represent a common *E. coli* backbone conserved among all *E. coli* pathovars, and many of the features are associated with central metabolism and core biological processes. Not surprisingly, the proportion of altered features from the EHEC isolates, *E. coli* EDL933 (10.4%) and *E. coli* Sakai (8.3%), were higher than that of *E. coli* CFT073 (3.7%), suggesting that the *E. coli* 86-24 strain is more similar to the other EHEC strains than to the uropathogenic isolate (39, 46, 72). The ratios of increased and decreased features are similar in all of the pathovar subgroupings (Table 3), with the exception of the intergenic regions which had a significantly greater proportion of features decreased (7.7% decreased vs 1% increased). The reason for this altered profile is unclear and was not observed with any of the other stimuli. Perhaps the bias was a result of the probe selection process, since the intergenic regions are selected and the array represents an incomplete set. As a whole, these data suggest that the *luxS* mutation causes a metabolic deficiency that would affect the central metabolism of most *E. coli* strains.

Previously, Sperandio et al. (58) identified ~400 MG1655 genes that were altered in the *luxS* mutant when compared to WT, representing ~10% of the genes on that array using a conservative 5-fold threshold for altered expression. If the analysis in this study is limited to the *E. coli* K-12 genes, a total of 280 genes have an altered profile. This is significantly less than the number of genes, 736, with an altered expression profile in the previous study when a 2-fold threshold was utilized. Although the numbers of genes with an altered profile in these two studies are different, the array designs (amplicon versus 25
mer oligonucleotides) and analysis thresholds (absolute fold change versus normalization and algorithmic analysis) are also different.

Transcriptome modification with dpd. The changes on gene expression caused by the luxS mutation in E. coli VS94 that are due to AI-2 signaling should be functionally complemented by the addition of dpd to the growth medium (68). Indeed, the fewest differences in gene expression between 86-24 and VS94 occurred when dpd was added to the culture medium (Table 2). These data indicate that of the signaling molecules AI-2 best complements the luxS mutation under the conditions examined. However, differences in gene expression between WT and VS94 grown with dpd were evident, thus the addition of dpd to the growth medium does not completely compensate for the luxS mutation.

Comparisons between VS94 + dpd and VS94 revealed the fewest differences in the transcriptional profile, with 403 altered genes (Table 2). Interestingly, when we compared the genes with the altered expression profile for the addition of dpd (i.e. 86-24 vs VS94 + dpd and VS94 vs VS94 + dpd), we observed 951 genes that were differentially regulated between these conditions and only 18 genes that were similarly regulated by the addition of dpd. The genes that were regulated similarly in these conditions represent the minimal dpd responsive set of genes. Further examination of the distribution of the pathovar-expressed genes for the addition of dpd to VS94 culture did not reveal any significant alterations in gene expression profiles.
Transcriptome modification with epinephrine. The greatest amount of transcriptome alteration was observed when epinephrine was added to the growth medium (5204 genes were altered when VS94 was compared to VS94 + epinephrine, Table 2). Activated genes included the LEE, stx2, flagella regulon (including flhDC), iron uptake systems, the gene encoding the Hfq protein (chaperone involved in small regulatory RNAs post-transcriptional regulation), and genes encoding several nucleoid proteins (H-NS, HU, FIS, and Hha, all reported to be involved in the regulation of the LEE). Although initially overwhelming, the fact that epinephrine is inducing activation of several nucleoid proteins, mostly involved in global repression of gene transcription, is consistent with this observation. The observed alterations in the great number of genes suggests that to assemble energetic expensive structures such as the LEE encoded TTSS and flagella (up-regulated by epinephrine), there is down-regulation of homeostatic genes.

Interestingly, a greater proportion of the EHEC-specific genes appeared to have an expression profile in a pathovar-specific manner (Table 3). Nearly 50% and 56% of the EHEC-specific genes from *E. coli* EDL933 and *E. coli* Sakai, respectively, were altered in expression when epinephrine was added to the medium. Additionally, the CFT073 genes exhibited an altered expression profile with epinephrine treatment. These data contrast with the increased expression in ~39% of the *E. coli* MG1655 genes and suggest that epinephrine preferentially activates virulence genes. This is consistent with previous studies that showed that *E. coli* senses and responds to this important hormone signal (11, 59, 68).

Additionally, the intergenic regions exhibited a significant proportion of increased transcription; approximately 56% of the intergenic regions demonstrated increased
expression. Most likely, there is increased regulation of upstream regions of the activated
genes as well as other features on the array such as sRNAs
(http://www.affymetrix.com/products/arrays/specific/ecoli2.affx). While there were a
significant number of increased genes and features with the epinephrine treatment, the
proportion of decreased genes among the *E. coli* MG1655 dataset was also the greatest
proportion observed in any culture condition (Table 3).

**Transcriptome modification with AI-3.** Although the VS94 strain may still produce
AI-3, the concentration of synthesized AI-3 has been shown to be diminished compared
to WT (68). When the expression profile is examined for the pathovar-specific
distribution, a significant bias in terms of altered gene expression profiles was not
apparent.

**Effects on expression of LEE and Shiga toxin genes.** Real-time RT-PCR analyses
revealed interesting insights as to how QS molecules contribute to regulation and
expression of LEE-encoded genes (Fig 1A-F). When VS94 was compared to WT EHEC,
no significant differences in expression of LEE1 or LEE2 were apparent (Fig 1A).
However, expression of LEE3 was significantly increased whereas expression of LEE4
and LEE5 were significantly decreased (Fig 1A). Addition of dpd had variable effects on
expression of LEE genes. When dpd was added to the medium, expression of LEE1-3
genes was similar to the VS94 grown without any signaling molecules (Fig 1B-D).
Expression of LEE4 and LEE5 was significantly higher in the cultures grown with dpd;
however, dpd did not enhance expression to the extent that was seen when either AI-3 or epinephrine was added to the growth medium.

Addition of either exogenous AI-3 or epinephrine increased expression of all of the LEE genes (Fig 1B-F). AI-3 significantly increased expression of the LEE2-5 genes at late-exponential phase of growth, and also increased expression of LEE1 compared to VS94; however, the increased expression of LEE1 was not considered to be significant. A previous study showed that the most significant regulation of the LEE genes in WT vs a luxS mutant occurred at mid-exponential growth (69). This is most likely because a luxS mutant can still produce AI-3, albeit at a lower level than the WT, but by the time the strain reaches late-logarithmic, enough AI-3 has been produced to stimulate expression of the LEE (69). The current study shows that exogenous AI-3 still contributes to regulation of the LEE, even at late-exponential growth phase and further underscores the importance of AI-3 in EHEC pathogenesis.

Addition of epinephrine had the greatest effect on LEE gene expression. Expression of all five of the LEE operons was significantly increased when epinephrine was added to the culture medium. These data are congruous with the array data and reinforce epinephrine’s role in EHEC pathogenesis.

The luxS mutation had no effect on expression of the stx2A gene (Fig 1A); however, addition of any signaling molecule greatly decreased expression of Stx2A in the luxS mutant cultures (Fig 1G). Although expression was decreased, a similar trend was observed when expression of this gene was analyzed: expression of Stx2A was most similar between VS94 and VS94 + dpd and the greatest differences in expression occurred between VS94 and VS94 + epinephrine.
Non-LEE-encoded effectors. EHEC encodes many non-LEE effector proteins that are thought to be secreted (63) and which result in an enhancement of virulence in the host. Several of these genes were selected for detailed analysis by real-time RT-PCR. NleA is encoded outside of LEE, but it is secreted through the LEE encoded TTSS. Once NleA enters the host, it localizes to the golgi apparatus (24). Although its precise function is not understood, NleA appears to play a role in virulence in mouse model experiments as virulence of an nleA mutant strain is attenuated (24, 44). Both the microarray and real-time RT-PCR data indicated that expression of nleA was not affected in the luxS mutant compared to the WT strain. However, addition of AI-2, AI-3, or epinephrine to luxS mutant cultures significantly decreased nleA expression (Fig 2A & B). Again, the most significant differences were observed in cultures to which AI-3 or epinephrine was added to the medium than was observed when dpd was added.

Recently, several novel effectors proteins were identified in EHEC (63), and we performed real-time RT-PCR on the effectors, EspX3’ (Z5212 and Z5213) and EspY5’ (Z5214). Similar to nleA, the luxS mutation did not alter expression of these genes (Fig 2B), but the addition of any of the signaling molecules AI-2, AI-3, or epinephrine reduced expression (Fig 2B). Expression of Z5212 and Z5214 was significantly decreased in the cultures where exogenous signaling molecules were added, and there were no significant differences dependent on the type of QS molecule added. Expression of Z5213 was significantly decreased when either dpd or AI-3 was added to the medium. Expression of this gene was decreased when epinephrine was added when compared to
VS94 grown in DMEM, but this was not considered significant. The addition of external signaling molecules had a decreased effect on the secreted effector genes.

**ETT2-encoded regulators.** In addition to the LEE-encoded TTSS, EHEC contains a non-functional type III secretion system (ETT2) (26, 46). When expression of these genes was compared between WT and VS94, expression was significantly decreased in the luxS mutant (Fig 2A). Then, when we compared VS94 to VS94 grown with signaling molecules, addition of the signaling molecules further decreased expression of these genes (Fig 2B). The transcriptional regulators, *eivF* and *etrA*, encoded in the ETT2 have been shown to be negative regulators of the LEE (76). These data suggest that AI-3 and epinephrine may function not only to increase expression of the LEE directly but also by inhibiting factors such as *eivF* and *etrA* that work to repress LEE (i.e. repressing the repressors).

**Fimbrial genes.** Attachment is the first step in colonization, and thus we wanted to examine the fimbrial genes contained on the array to determine if they were being alternatively regulated in response to the quorum signals. Overall, epinephrine seemed to have the greatest effect of expression of known (*fimZ*, *fimH*, *fimG*, *fimF*, *fimD*, and *fimC*) and putative fimbrial genes. We performed real-time RT-PCR on three putative fimbrial genes. The putative major fimbrial subunit (Z4971) and a putative fimbrial chaperone (Z5223) were significantly down-regulated in the VS94 culture grown without signaling molecules when compared to the WT (Fig 3A). Addition of signaling molecules to cultures of VS94 caused further repression of these fimbrial genes. In contrast, no
significant differences existed between the WT and the *luxS* mutant in the expression of
the putative fimbrial-like protein Z3279 (Fig 3A). Addition of dpd or AI-3 caused further
repression of this gene; however, expression was rescued to near WT levels when
epinephrine was added to the medium (Fig 3B).

**DISCUSSION**

The data presented in this study provide a more complete picture of the
transcriptional modifications that occur in *E. coli* due to bacterial signaling via AI-2 and
AI-3 and interkingdom signaling with the host hormone epinephrine. A previous
transcriptome analysis of the *E. coli* 86-24 and the *luxS* mutant (VS94) revealed that there
was an alteration of expression of ~ 400 genes, by more than 5-fold (235 up-regulated
and 169 down-regulated) and 736 genes using a less stringent 2-fold threshold (58). We
observed 280 MG1655 genes with an altered profile in the *luxS* mutant in our arrays. It
must be noted that the previous array utilized single *E. coli* K-12 (MG1655) amplicons
for each feature, whereas the current Affymetrix *E. coli* 2.0 array contains the complete
non-redundant gene complement of the laboratory adapted isolate *E. coli* MG1655, both
EHEC isolates, *E. coli* EDL933 and *E. coli* Sakai, and the uropathogenic *E. coli* isolate
CFT073. Additionally, the Affymetrix *E. coli* 2.0 array contains 1297 intergenic
features, which the previous amplicon-based array did not. It was interesting to note that
while the strain utilized in this study, *E. coli* 86-24, has not been sequenced and thus
could not be completely contained on the array we do see a significant number of *E. coli*
CFT073 genes with an altered transcriptional profile. This would suggest that *E. coli* 86-
24 contains some regions that are also shared with *E. coli* CFT073 and thus not with
other sequenced EHEC strains. This is a further example of the mosaic nature of the *E. coli* genomes (72).

The function of AI-2 in bacterial signaling is an issue of debate. Some studies have implicated AI-2 to be involved in biofilm formation and motility (15, 22, 27); however, it has also been suggested that AI-2 signaling is involved primarily in the regulation of metabolic processes (67, 71, 73, 74). In *Salmonella*, AI-2 regulation only involves genes that encode an ABC transporter termed Lsr (LuxS-regulated) (62). This transporter has also been found in *E. coli* (75). In both *Salmonella* and *E. coli*, *lsr* shares a high level of sequence homology, and functionally they resemble sugar transporters. Similarly to other sugar transporters, import of AI-2 is strictly controlled (70, 75). AI-2 is synthesized and secreted during exponential growth and is imported in stationary phase when glucose becomes limiting (70, 75). In the presence of glucose, AI-2 is not imported because the *lsr* operon is not transcribed due to camp-CAP mediated repression (70, 75). Indeed, gene expression profiles comparing *E. coli* MG1655 grown in glucose-containing or glucose-free medium found that the *lsr* operon was induced only in the absence of glucose, and that the *luxS* mutation in *E. coli* MG1655 mainly affected genes related to AI-2 production and transport (71). Moreover, a study using phenotype microarrays observed that the *luxS* mutation resulted in numerous metabolic changes, especially in those processes that involve nitrogen and carbon metabolism (68). Our data are congruous with studies that suggest a metabolic role for AI-2.

The AI-3 signaling molecule has been shown to activate the LEE and flagellar and motility genes in EHEC (11, 12, 59, 69). Addition of exogenous AI-3 significantly increased expression of the LEE in a *luxS* mutant; however, significant changes in global
gene expression were not apparent under these conditions. This work demonstrates that even though the wild type and *luxS* mutant may be making AI-3 there is not a saturation of the receptor or the response mechanism as the addition of exogenous AI-3 resulted in the exacerbation of the virulent phenotype. Future work is required to determine the level of AI-3 required for saturation of the EHEC system.

The greatest changes in gene expression occurred when epinephrine was added to the medium. The stress hormones epinephrine and norepinephrine are present in and modulate smooth muscle contraction, submucosal blood flow, and chloride and potassium secretion in the gastrointestinal tract (28). Norepinephrine is produced within adrenergic neurons present in the enteric nervous system (20), whereas epinephrine is synthesized in both the central nervous system as well as in the adrenal medulla and is involved in systemic responses (47). The levels of norepinephrine and epinephrine in the intestine are in the micromolar range (17) similar to what was used as a signal in the present study. Moreover, during an EHEC infection, the integrity of the epithelial cell layer is compromised, causing bloody diarrhea and stressing the host; therefore, concentrations of epinephrine and norepinephrine may be even higher.

Previous work has shown that AI-3 and epinephrine act synergistically (59, 69). Upon entry into the colonic lumen EHEC responds to bacterial (commensal as well as pathogen)-produced AIs and activates motility. Then upon close proximity to the host epithelium, the host hormones sustain and further alter the expression profile, allowing attachment though expression of the *LEE*, resulting in clinical disease presentation. This fine-tuning may allow the bacteria to respond favorably to various environmental situations.
The effect of the luxS mutation with or without added signals on the LEE operons was examined in detail. Comparisons of the luxS mutant and WT revealed significant differences only in the expression of LEE4 and LEE5 suggesting that additional mechanisms and/or signals are involved in the regulation of these operons. The addition of dpd had no effect on LEE1-LEE3, but increases expression of LEE5 and LEE4, suggesting that these latter operons are controlled through AI-2 signaling in addition to the AI3/epinephrine system. Although, a luxS mutant still synthesizes a low level of AI-3, addition of exogenous AI-3 to the culture medium significantly increases expression of all the LEE genes at late-exponential growth phase (Fig 1). Of the stimuli examined, epinephrine affected expression of the LEE genes to the greatest degree. Additionally, the identification of multiple stimuli that can activate the LEE regions suggests a complex regulatory network exists for the virulence genes of EHEC.

Additional genes involved in EHEC virulence were also affected by addition of signaling molecules. Consistent with our observation that regulation of the stx2A/B genes occurs through several qse genes (unpublished data), signaling molecules directly affected expression of the shiga toxin-producing genes. Other known regulatory proteins are produced in response to QS stimuli. For example, QseA was increased when epinephrine was added to the medium (59). QseA increases expression of the LEE (55) as well as QseE, a response regulator that controls transcription of the EspFu/TccP effector (8, 48, 51). Taken together these data suggest that AI-3 and epinephrine are important regulators of TTSS and virulence in EHEC.

The repertoire of EHEC’s virulence factors extends beyond the LEE-encoded effectors, and similar to the LEE, these virulence factors are probably subjected to
multiple complex levels of regulation. Interestingly, the addition of the signaling
molecules repressed expression of many non-LEE encoded effectors. The luxS mutation
did not have significant effects on gene expression of non-LEE effectors; however,
addition of the signaling molecules significantly decreased expression.

While fimbriae are important virulence factors for EPEC (7, 23, 42, 55) and for
UPEC (2, 43), their role in EHEC adherence is not fully understood. In EHEC, fimbriae
putatively function to increase adherence to epithelial cells (65) or aiding in the formation
of stable microcolonies (64). Other studies propose that fimbriae may be more important
in effectively colonizing the bovine GI tract (37, 38). Mutation of ler in EHEC was
associated with enhanced fimbrial expression (18). Our data show that regulation of
known and putative fimbrial genes are regulated differently under varying conditions.

The results reported in this study demonstrate that the addition of exogenous AI-2
(in the form of dpd) cannot fully restore the metabolic defect caused by the luxS
mutation. Epinephrine has an EHEC-specific alteration of many genes including those
related to the pathogenesis of EHEC in humans (LEE, toxins, and fimbriae). Additional
virulence traits seem to be activated by exogenous AI-3 even in the presence of
endogenous AI-3 indicating that the system is not fully saturated by the production of
endogenous AI-3. These gene sets altered by epinephrine and AI-3 are not entirely
overlapping suggesting that regulation of the virulence traits in EHEC infection may
follow a program based on the signal and level of signal sensed by the bacterium and may
contain a temporal component.
This study accurately maps the transcriptome of EHEC in the presence of major QS signals and provides novel insight into the QS control of virulence in the presence of these stimuli.

ACKNOWLEDGMENTS

We would like to thank J. R. Falck, from the University of Texas Southwestern Medical Center at Dallas, for very generously providing the AI-3 used in this work. This work was supported by the Burroughs Wellcome Fund, the Ellison Medical Foundation, and the NIH grant AI053067.
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Table 1. Oligonucleotides used for real-time RT-PCR.

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<tr>
<th>Gene</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
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<td>eae</td>
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<td>AACCACGCAGTTCCCACAA</td>
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<td>espY5'</td>
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<td>rpoA</td>
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Table 2: Number of genes altered as measured by Affymetrix GeneChip

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<th>Decrease</th>
<th>No Change</th>
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<tbody>
<tr>
<td>8624_VS94</td>
<td>230</td>
<td>480</td>
<td>9497</td>
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<tr>
<td>8624_VS94+dpd</td>
<td>143</td>
<td>260</td>
<td>9804</td>
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<tr>
<td>8624_VS94+Epi</td>
<td>2394</td>
<td>2722</td>
<td>5091</td>
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<tr>
<td>8624_VS94+AI3</td>
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<td>369</td>
<td>9556</td>
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<tr>
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<td>9804</td>
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Table 3. Pathovar specific distribution of genes

<table>
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<th></th>
<th>MG1655 (n=4070)</th>
<th>EDL933 (n=1787)</th>
<th>Sakai (n=373)</th>
<th>CFT073 (n=2486)</th>
<th>Intergenic (n=1297)</th>
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</thead>
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<td>50</td>
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<td>6</td>
<td>16</td>
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<tr>
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<td>2299</td>
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<td>4070</td>
<td>1787</td>
<td>373</td>
<td>2486</td>
<td>1297</td>
</tr>
<tr>
<td><strong>VS94 v VS94+dpd</strong></td>
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<td></td>
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<td></td>
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<tr>
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<td>373</td>
<td>2486</td>
<td>1297</td>
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<td><strong>VS94 v VS94+epi</strong></td>
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<td>373</td>
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<td><strong>VS94 v VS94+AI3</strong></td>
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<tr>
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<td>69</td>
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<td>1787</td>
<td>373</td>
<td>2486</td>
<td>1297</td>
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</tbody>
</table>

The gene number assigned to specific genomes listed in this table total 10013. There are an additional 96 features that are used as controls and 99 features that are associated with phage and plasmids and thus not directly linked to a genome project.

Total number of features on the array is 10208.
FIGURE LEGEND

Figure 1. Transcriptional profiles of LEE and stx2A expression for WT EHEC, an isogenic luxS mutant (A), and the luxS mutant grown with AI-2 (DPD), AI-3, or epinephrine (B) as measured by real-time RT-PCR measured in fold difference normalized to WT 86-24 (A) and the luxS mutant (B). Significance values are as follows:

*p ≤ 0.05; **p ≤ 0.005; ***p ≤ 0.0005.

Figure 2. Transcriptional profiles of nleA, etrA, eivF, and the secreted effectors EspX3’ (Z5212 and Z5213) and EspY5’ for WT EHEC and an isogenic luxS mutant (A), and the luxS mutant grown with AI-2 (dpd), AI-3, or epinephrine (B) as measured by real-time RT-PCR measured in fold difference normalized to WT 86-24 (A) or the luxS mutant (B). Significance values are as follows: *p ≤ 0.05; **p ≤ 0.005; ***p ≤ 0.0005.

Figure 3. Transcriptional profiles of fimbrial gene expression for WT EHEC and an isogenic luxS mutant (A), and the luxS mutant grown with AI-2 (DPD), AI-3, or epinephrine (B) as measured by real-time RT-PCR measured in fold difference normalized to WT 86-24 (A) or the luxS mutant (B). Significance values are as follows:

*p ≤ 0.05; **p ≤ 0.005; ***p ≤ 0.0005.
Fig. 2
Fig. 3
Author’s Correction

Global Effects of the Cell-to-Cell Signaling Molecules Autoinducer-2, Autoinducer-3, and Epinephrine in a luxS Mutant of Enterohemorrhagic Escherichia coli

Melissa M. Kendall, David A. Rasko, and Vanessa Sperandio

University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390-9048

Volume 75, no. 10, p. 4875–4884, 2007. Page 4882, column 1, lines 12 to 14 from the bottom: “The levels of norepinephrine and epinephrine in the intestine are in the micromolar range (17), similar to the levels that were used as signals in the present study” should read “The level of norepinephrine in the intestine is in the micromolar range (17), while the level of epinephrine is largely unknown; thus, at basal levels, these hormones could induce EHEC virulence, as demonstrated in this study.”