

# Temporal regulation of enterohemorrhagic *Escherichia coli* virulence mediated by autoinducer-2

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**Abstract** The autoinducer-2 (AI-2) molecule is produced by many bacterial species, including various human gastrointestinal (GI) tract commensal bacteria, and has been proposed to be involved in interspecies communication. Because pathogens are likely to encounter AI-2 in the GI tract, we studied the effects of AI-2 on various phenotypes associated with enterohemorrhagic *Escherichia coli* (EHEC) infections. AI-2 attracted EHEC in agarose plug chemotaxis assays and also increased swimming motility, as well as increased EHEC attachment to HeLa cells. The molecular basis underlying the stimulation of EHEC chemotaxis, motility, and colonization by AI-2 was investigated at the transcriptome level using DNA microarrays. We found that exposure to AI-2 altered the expression of 23 locus of enterocyte effacement (LEE) genes directly

involved in the production of virulence determinants, as well as other genes associated with virulence (e.g., 46 flagellar/fimbrial genes, 24 iron-related genes), in a temporally defined manner. To our knowledge, this is the first study to report AI-2-mediated regulation of EHEC chemotaxis and colonization, as well as temporal regulation of EHEC transcriptome by AI-2. Our results suggest that AI-2 is an important signal in EHEC infections of the human GI tract.

**Keywords** AI-2 · Virulence · Enterohemorrhagic *E. coli*

## Introduction

The gastrointestinal (GI) tract is colonized by approximately  $\sim 10^{14}$  bacteria belonging to hundreds of species that exist in a symbiotic relationship with the host without leading to host inflammatory response (Collier-Hyams and Neish 2005). However, the introduction of pathogens, such as enterohemorrhagic *Escherichia coli* (EHEC), into the GI tract disturbs this delicate balance between prokaryotes and eukaryotes (Collier-Hyams and Neish 2005) and causes bloody diarrhea and hemolytic uremic syndrome (HUS) (Karch et al. 2005). Currently, EHEC infections are not treated with antibiotics as this has been shown to increase development of HUS in infected children (Wong et al. 2000), as well as lead to the increased release of shiga-like toxins (Boyce et al. 1995). In the USA, there are approximately 70,000 infections annually and 2,000 hospitalizations, with an overall cost of \$405 million (Frenzen et al. 2005), which makes EHEC infections a serious problem.

EHEC infections have been reported to proceed in three stages: migration of the pathogen toward the epithelial cell surface, colonization of epithelial cells, and infection of

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cells by releasing shiga-like toxins (Torres and Kaper 2003). It is becoming increasingly evident that prokaryotic and eukaryotic cell–cell communication signals present in the GI tract impact the different steps involved in EHEC infections. Bacterial signals present in the GI tract include molecules produced by the commensal bacterial flora (e.g., indole, a stationary phase signal secreted by commensal *E. coli*) (Lee et al. 2007) and those involved in quorum sensing [e.g., autoinducers-2 and -3 (AI-2 and AI-3)] (Kendall et al. 2007). Recent results from our laboratory have shown that indole attenuates EHEC chemotaxis, motility, biofilm formation, colonization of epithelial cells, and the expression of genes related to virulence (Bansal et al. 2007), suggesting that indole signaling could be important in EHEC infections. Similarly, AI-3 has also been shown to regulate EHEC virulence and flagellar gene expression (Sperandio et al. 2003) and function similar to the human hormone epinephrine (Walters and Sperandio 2006). However, the effect of AI-2, which has been proposed as an interspecies signaling molecule and is produced by many species of commensal bacteria (Clarke and Sperandio 2005), on EHEC virulence and infection has not been fully understood.

AI-2 is synthesized in bacteria through the conversion of *S*-adenosylhomocysteine to *S*-ribosylhomocysteine, and then *S*-ribosylhomocysteine to (*S*)-4,5-dihydroxypentane-2,3-dione (DPD) by the enzymes Pfs and LuxS, respectively (Chen et al. 2002). DPD spontaneously cyclizes to form a number of molecules, one of which is AI-2. While purified AI-2 (DPD) has been shown by us to be directly involved in nonpathogenic *E. coli* biofilm formation (González Barrios et al. 2006) and three groups have shown that the inability to form AI-2 stimulates motility (DeLisa et al. 2001; Ren et al. 2004; Sperandio et al. 2001), its effect on pathogenic *E. coli* has been inconsistent. For example, a recent study (Kendall et al. 2007) reported that the addition of AI-2 to an EHEC *luxS* mutant did not significantly alter the expression of virulence genes other than locus of enterocyte effacement (LEE) 4 and LEE5 and concluded that AI-2 does not impact EHEC virulence. However, this study did not account for temporal regulation of AI-2 uptake and signaling.

The goal of this study was to investigate the effect of AI-2 on phenotypes related to EHEC virulence and infection. Because AI-2 concentration is likely to be high in the human GI tract, we hypothesized that AI-2 will significantly impact EHEC colonization and infection. We studied the relative changes in EHEC chemotaxis, motility, and attachment to HeLa cells – important components of EHEC infections – in the presence of AI-2 using a low-nutrient medium that mimics the GI tract environment (Lyte et al. 1996). The temporal response of EHEC gene expression in response to AI-2 exposure was also investigated using a

series of DNA microarrays. Our results suggest an important role for AI-2 in EHEC infections. To our knowledge, this is the first study to report AI-2-mediated regulation of EHEC chemotaxis and colonization and the temporal response of EHEC to AI-2.

## Materials and methods

### Bacterial strains, materials, and growth

Strain VS94 (EHEC  $\Delta luxS$ ) (Sperandio et al. 2003) was kindly provided by Dr. V. Sperandio. Plasmids pCM18 (Hansen et al. 2001) and pDS-RedExpress (Clontech, Mountain View, CA, USA) were used to constitutively express the green fluorescent protein and the red fluorescent protein. Luria–Bertani (LB) medium was used to grow overnight cultures of bacteria. Ten micrograms per milliliter of tetracycline, 100  $\mu\text{g mL}^{-1}$  kanamycin, and 150  $\mu\text{g mL}^{-1}$  erythromycin were used for plasmid selection. AI-2 as (*S*)-4,5-dihydroxy-2,3-pentanedione was purchased from Omm Scientific (Dallas, TX, USA).

### Agarose plug chemotaxis assay

Chemotaxis assay in agarose plugs was performed as described previously (Bansal et al. 2007). Briefly, an overnight culture of VS94 expressing pCM18 was used to inoculate 10 mL growth medium with erythromycin, and the culture was grown to midexponential phase (turbidity of 0.7 at 600 nm). The cells were harvested, washed, and resuspended in chemotaxis buffer with kanamycin-killed *E. coli* TG1/pDS-RedExpress cells in a 2:1 ratio and were exposed to a plug containing AI-2. Migration of bacteria was imaged on a Zeiss Axiovert 200 fluorescence microscope (Thornwood, NY, USA) over a period of 20 min. Images were analyzed using Zeiss Axiovert software, version 4.3.

### Motility assay

Swimming motility was assayed as previously described (Bansal et al. 2007). Briefly, overnight culture of VS94 was subcultured to a turbidity of 0.05 at 600 nm in LB medium and grown to a turbidity of  $\sim 1.0$  at 600 nm at 37 °C. AI-2 (1  $\mu\text{M}$  to 100  $\mu\text{M}$ ) was added to the motility agar plates (1% tryptone, 0.25% NaCl, and 0.3% agar), and the sizes of the halos were measured after 8 h. Five motility plates were used for each AI-2 concentration.

### *In vitro* adhesion assays

Adhesion of EHEC to HeLa S3 cells was performed using a previously described protocol (Bansal et al. 2007). HeLa S3

cells (ATCC, Manassas, VA, USA) were cultured and propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated bovine serum according to standard protocols (ATCC). Low-passage-number HeLa cells were cultured in standard 24-well tissue culture plates and grown at 37 °C in 5% CO<sub>2</sub> until ~80% confluence. HeLa cell monolayers were washed two times with sterile PBS to remove unattached cells, and the growth medium was replaced with antibiotic-free DMEM with 10% bovine serum. Approximately 10<sup>6</sup> cells of a freshly grown VS94 culture (turbidity of ~0.8 at 600 nm) were added to each well and incubated for 3 h at 37 °C and 5% CO<sub>2</sub>. Loosely attached cells were removed by washing the wells two times with sterile PBS, and the HeLa cells were lysed in the wells using 0.1% Triton X-100 in PBS. The cell suspension in each well was vigorously vortexed, and serial dilutions of the bacteria were plated on LB plates. Colonies were counted after 24 h incubation at 37 °C.

#### AI-2 uptake assay

Overnight cultures of VS94 were diluted in Standard American Petroleum Institute (SAPI) medium (Lyte et al. 1996) to a turbidity of 0.1 at 600 nm. SAPI medium contains limited nutrients (2.77 mM dextrose, 6.25 mM ammonium nitrate, 1.84 mM monobasic potassium phosphate, 3.35 mM potassium chloride, 1.01 mM magnesium sulfate, and 10 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid); hence, it was used here to mimic the in vivo milieu of the human GI tract (Lyte et al. 1996). The medium pH was adjusted to 7.5 and filter-sterilized. Thirty percent (v/v) bovine serum was then added. The cells were grown to a turbidity of approximately 0.5 at 600 nm at 37 °C before adding 100 μM AI-2. Turbidity was measured and supernatants were collected every hour for 7 h after addition of AI-2. Three independent cultures were used, and the *Vibrio harveyi* autoinducer assay was performed as described previously (Surette and Bassler 1998). Briefly, an overnight culture of *V. harveyi* was diluted 1:5,000 in fresh AB medium, mixed with supernatants in a ratio 9:1, and grown for 4 h at 30 °C with shaking. Luminescence was then measured using TD-20e luminometer (Turner Biosystems, Sunnyvale, CA, USA) to determine the amount of extracellular AI-2 remaining at each time point.

#### RNA isolation and DNA microarrays

Overnight cultures of VS94 were diluted in SAPI medium to a turbidity of 0.1 at 600 nm. The cells were allowed to grow to a turbidity of 0.5 at 600 nm at 37 °C and 100 μM AI-2 was added to the culture. In the control flasks, no AI-2 was added. The cultures were then allowed to grow for 3.5,

4, 4.5, or 5.5 h before cell pellets were collected by centrifugation and stored at –80 °C.

Total RNA was isolated from the cell pellets and RNA quality was assessed using gel electrophoresis. *Escherichia coli* Genome 2.0 arrays (Affymetrix, Santa Clara, CA, USA) containing 10,208 probe sets for all 20,366 genes present in four strains of *E. coli*, including EHEC, were used to profile changes in gene expression using RNA samples for each treatment. Hybridization was performed for 16 h and the total cell intensity was scaled automatically in the software to an average value of 500. The data were inspected for quality and analyzed according to the procedures described by the manufacturer (Affymetrix Data Analysis Fundamentals), which include using premixed polyadenylated transcripts of the *Bacillus subtilis* genes (*lys*, *phe*, *thr*, and *dap*) at different concentrations. Genes were identified as differentially expressed if the expression ratio (between AI-2 and the control cells at different time-points) was greater than 1.5 (based on the standard deviation between values measuring relative changes in expression) (Ren et al. 2004) and if the change in the *p* value was less than 0.05. The differentially expressed genes were annotated using gene ontology definitions available in the Affymetrix NetAffx Analysis Center (<http://www.affymetrix.com/analysis/index.affx>). The expression data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE9388.

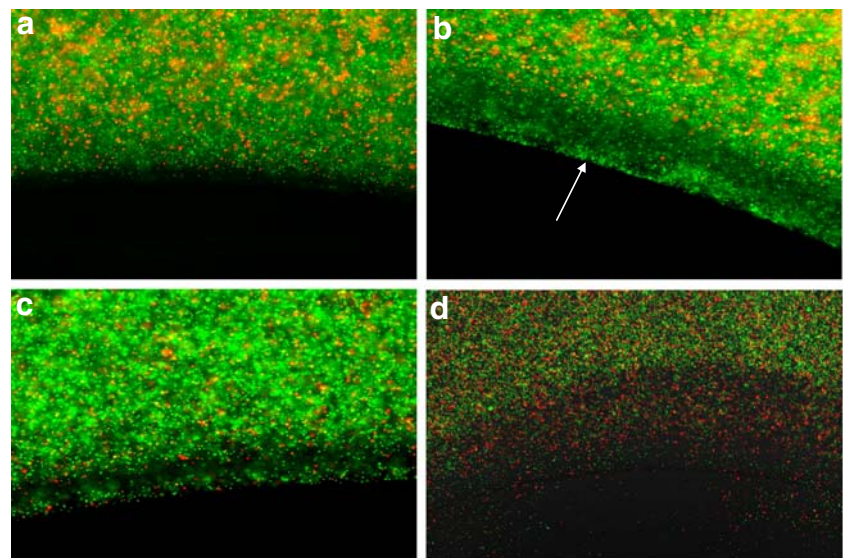
A total of eight microarrays were used in this study at the four time points for samples without and with externally added 100 μM AI-2. The genes were sorted into various functional categories (Supplemental Table 1), and the role of AI-2 in regulation of these genes was analyzed.

## Results

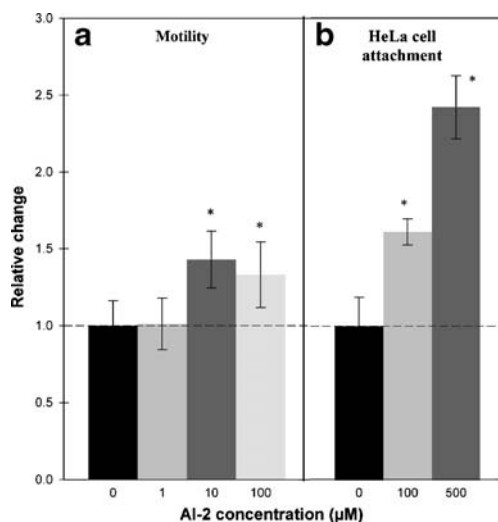
### Effect of AI-2 on VS94 chemotaxis, motility, and colonization

A two-fluorophore agarose plug assay was used to determine the effect of AI-2 on EHEC chemotaxis. AI-2 attracted VS94 in a concentration-dependent manner, where migration towards AI-2 was increasingly pronounced as the concentration of AI-2 in the plug was increased to 500 μM (Fig. 1b); i.e., more live (green) cells move to the plug containing AI-2 than dead cells (red). When casamino acids (positive control) were used in the plug, VS94 was similarly attracted (Fig. 1c), whereas exposure to nickel sulfate (negative control) caused VS94 to move away from the plug (Fig. 1d). Exposure to no chemical (experimental control) elicited no response from VS94 (Fig. 1a). Motility

**Fig. 1** Agarose plug chemotaxis. Response of VS94 to various chemicals was observed using a modified agarose plug assay in which dead cells are red (red fluorescent protein) and live cells are green (green fluorescent protein). Fluorescent images are shown from: experimental control (no AI-2) (a), AI-2 (500  $\mu$ M) (b), casamino acids (positive control) (c), and nickel sulfate (negative control) (d). Data are a representative image from three independent experiments. Arrow indicates chemoattractant ring (b)



assays showed that low concentrations of AI-2 (1  $\mu$ M) did not evoke a response from the pathogen, while AI-2 at 100  $\mu$ M increased the swimming motility by 1.3-fold (Fig. 2a). Because AI-2 impacted EHEC motility and chemotaxis, we also tested the effect of presence of AI-2 on EHEC adherence to HeLa cells. Consistent with the changes in chemotaxis and motility, AI-2 at concentrations of 100 and 500  $\mu$ M caused a statistically significant increase in attachment of VS94 to HeLa cells by 1.6- and 2.4-fold, respectively (Fig. 2b). Hence, AI-2 increases EHEC chemotaxis, motility, and attachment to HeLa cells. These are the first results to show regulation of EHEC chemotaxis and colonization by AI-2.



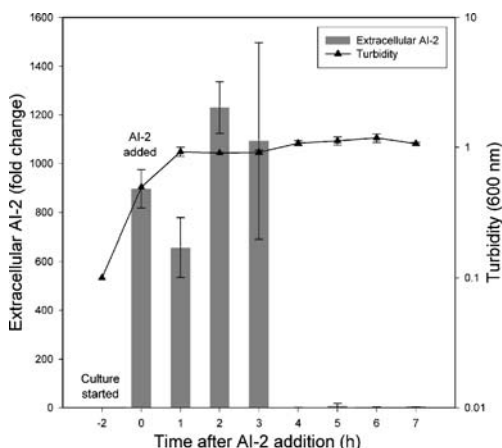
**Fig. 2** AI-2 effects on VS94 motility and attachment to HeLa cells. Relative change in VS94 motility in the presence of AI-2 (a). Motility data are mean  $\pm$  one standard deviation for five plates for each concentration. Relative change in VS94 attachment to HeLa cells in the presence of AI-2 (b). Data are from six HeLa cell culture wells for each concentration. Asterisk indicates statistical significance determined using student *t* test at  $p < 0.01$

#### Uptake of AI-2 in VS94

As a first step towards determining the genes that are altered in expression by AI-2, we investigated the time at which externally added AI-2 is imported by the EHEC *luxS* mutant. Extracellular AI-2 levels were determined at different time points corresponding to various growth phases of VS94 using the AI-2 bioassay (Surette and Bassler 1998). The concentration of AI-2 in the GI tract is not known; therefore, experiments were performed with 100  $\mu$ M AI-2, as this concentration was sufficient to increase chemotaxis, motility, and attachment of VS94, and has also been used in a recent study (Kendall et al. 2007). Our data show that ~99% of the externally added AI-2 was still present in the medium after 3 h. However, after 4 h, the extracellular AI-2 concentration dropped precipitously to less than 5% of the initial value (Fig. 3); this indicates that AI-2 is imported by VS94 between 3 and 4 h after addition. Based on these results, transcriptome analyses were performed at different time points, including when extracellular AI-2 was actively being absorbed by the cells (3.5 h), was reduced to zero (4 h), and was likely processed and cleared from the cells (4.5 and 5.5 h) (Xavier and Bassler 2005).

#### Transcriptome profiling of the effect of AI-2

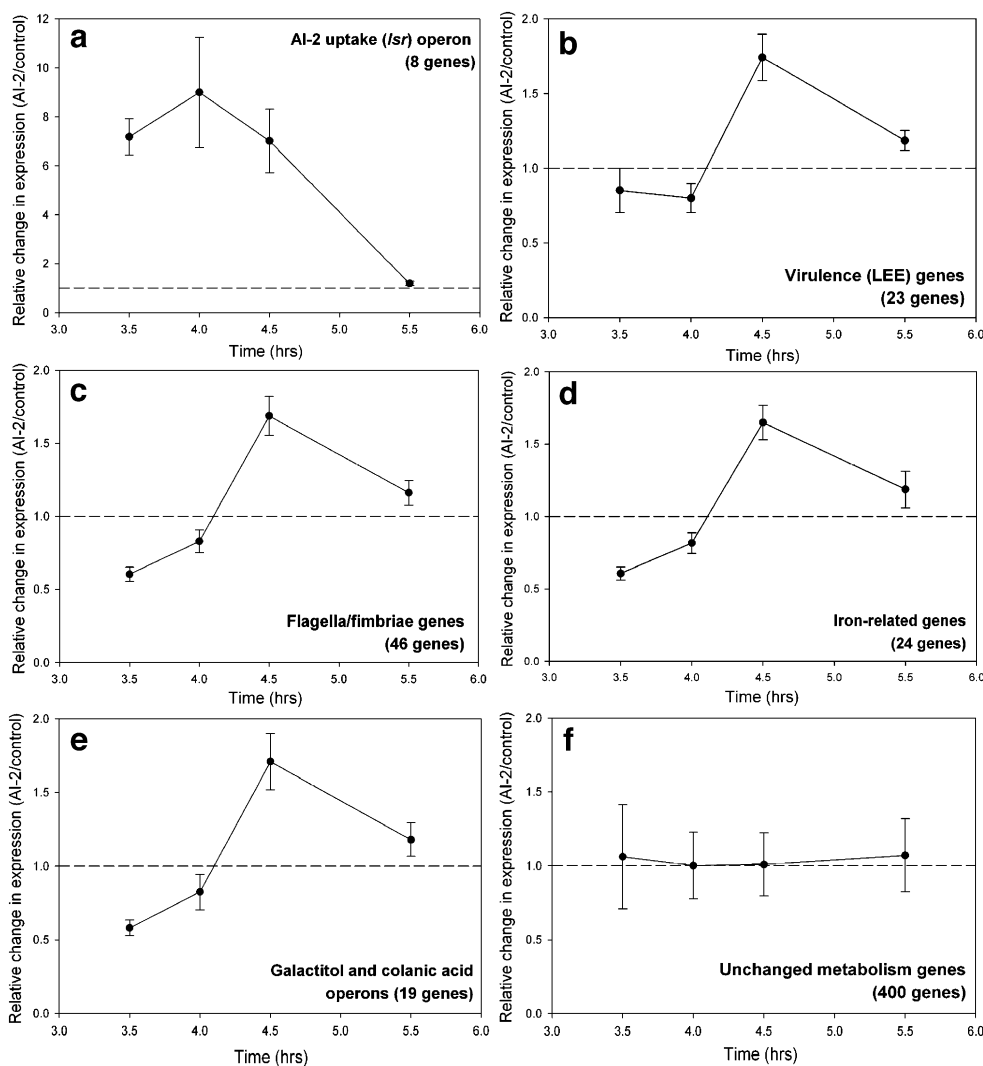
We investigated the molecular basis underlying the effect of AI-2 on VS94 chemotaxis, motility, and attachment to epithelial cells using DNA microarrays. VS94 was grown both in the absence (negative control) and presence of 100  $\mu$ M AI-2, and cells were isolated at various times based on the AI-2 uptake assay (Fig. 3). Genes whose expression was altered by 1.5-fold [based on the standard deviation of our data (Ren et al. 2004), which was less than



**Fig. 3** AI-2 uptake assay. VS94 was grown in SAPI medium to a turbidity of 0.5 at 600 nm and 100  $\mu$ M AI-2 was added. Supernatants were collected, and the *V. harveyi* autoinducer assay was performed to determine the amount of extracellular AI-2 remaining. Three independent cultures were used to generate the AI-2 uptake profile

1.5] in the presence of AI-2 were selected and sorted into operons or various functional groups (e.g., virulence genes, flagellar genes, iron uptake genes etc.; Supplemental Table 1), and the temporal expression trends were analyzed (Fig. 4).

The data show that, after 3.5 h of exposure to AI-2, 1,441 genes were down-regulated and 127 genes were up-regulated as compared to the negative control with no AI-2. The genes that were down-regulated included flagellar and fimbrial genes (average fold-change of 0.60-fold for 46 genes), iron uptake genes (average fold-change of 0.61-fold for 24 genes), and operons related to biofilm formation, galactitol (average fold-change of 0.59-fold for 11 genes), and colanic acid operon (average fold-change of 0.56-fold for eight genes). The *lsr* operon, which is involved in AI-2 uptake (Wang et al. 2005b, Xavier and Bassler 2005), was the most induced at this time point (average fold-change of six- to ninefold).



**Fig. 4** Changes in VS94 gene expression with AI-2 as a function of time. Effect of AI-2 (100  $\mu$ M) on genes related to the *lsr* operon (a), virulence (b), flagella/fimbriae (c), iron (d), biofilm (e), and metabolism (f)

At 4 h, the gene expression data showed that fewer genes were significantly altered in expression in VS94 exposed to AI-2 as compared to the negative control with no AI-2. The expression of 48 genes was significantly induced, while 145 genes were down-regulated after 4 h. As expected, the *lsr* operon was again induced by 6- to 13-fold, but all the other operons that were down-regulated at 3.5 h (e.g., LEE genes, flagella genes, etc.) were unchanged from the negative control.

In contrast to the gene expression data at 3.5 h, a significant number of genes (1,619 genes) were up-regulated at 4.5 h in the presence of AI-2, while only 71 genes were repressed compared to the negative control with no AI-2. The *lsr* operon was induced (five- to ninefold) along genes related to the flagella, iron uptake, and biofilm formation. Most importantly, 23 LEE genes that are involved in EHEC virulence (Elliott et al. 1998) were also significantly induced (average fold-change of 1.74-fold) at this time point. At 5.5 h, after all the extracellular AI-2 had been taken up by VS94 cells (Fig. 3), only 207 genes were found to be repressed and 64 genes were induced by AI-2 as compared to the negative control. The *lsr* operon was no longer differentially expressed as no more extracellular AI-2 was available for uptake.

## Discussion

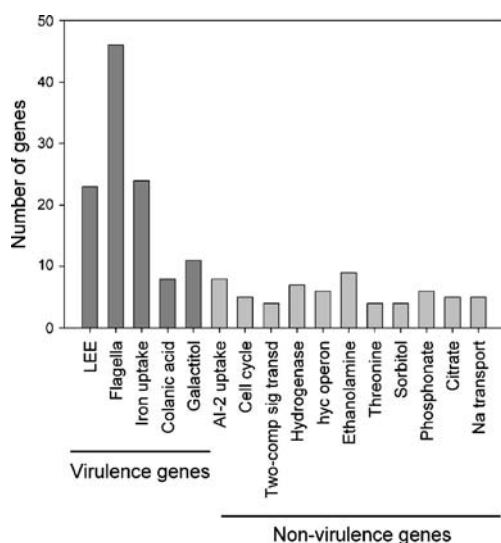
It is becoming increasingly evident that the signaling environment in the GI tract is an important determinant of pathogen colonization and infection. This is not surprising, as the GI tract is colonized by hundreds of bacterial species (Collier-Hyams and Neish 2005) that produce a diverse range of signals. We (Bansal et al. 2007) have recently demonstrated for the first time that the bacterial signal indole is important in EHEC infections by demonstrating that indole down-regulated EHEC chemotaxis, motility, biofilm formation, and adherence to epithelial cells in a manner directly opposite to that observed with the eukaryotic hormones epinephrine and norepinephrine. Here, we demonstrate that the bacterial quorum sensing signal AI-2 is also important in EHEC infections.

It is highly likely that EHEC encounters AI-2 in the GI tract, as this interspecies communication signal is produced by several commensal bacterial species that reside in the GI tract (Clarke and Sperandio 2005). Although the concentration of AI-2 in the GI tract is not known, unpublished data from our laboratory show that the concentration used in this study (100  $\mu$ M) is comparable to that produced by  $\sim 10^8$  cells/mL of nonpathogenic *E. coli*, which is the reported density of commensal *E. coli* in the GI tract (Hartl and Dykhuizen 1984; Leclerc et al. 2001; Wang et al. 2006). However, this only provides an approximate

estimate of in vivo AI-2 levels, as commensal *E. coli* constitutes only 1% of the GI tract flora (Hartl and Dykhuizen 1984; Leclerc et al. 2001) and does not account for secondary interactions (i.e., AI-2 production and consumption) by other bacteria present in the GI tract.

The chemotactic recognition of AI-2 and the increase in motility by EHEC in the presence of AI-2 suggest that this signal is involved in the initial migration of EHEC to epithelial cells. Consistent with this increase in chemotaxis and motility, the presence of AI-2 also causes an increase in adherence to HeLa cells. Given the direct correlation between colonization to epithelial cells and increased virulence (Lyte et al. 1996; Lyte et al. 1997), our data strongly suggest that the presence of AI-2 leads to increased EHEC colonization of host cells and virulence. This is also corroborated by the gene expression data showing that genes involved in bacterial colonization and biofilm formation (colonic acid and galactitol transport genes) are up-regulated by AI-2 (Fig. 4e). Colonic acid is a capsular exopolysaccharide that is involved in colonization (Prigent-Combaret et al. 1999). Similarly, genes associated with galactitol transport are important during early developmental stages of bacterial colonization of abiotic surfaces (Domka et al. 2007). The up-regulation of these genes is consistent with increased attachment of EHEC to HeLa cells in our experiments.

The regulation of virulence genes (LEE genes) and several other operons important in virulence strongly suggests the importance of AI-2 in EHEC infections. Several studies (DeLisa et al. 2001; González Barrios et al. 2006; Kendall et al. 2007; Ren et al. 2004; Widmer et al. 2007) have used whole transcriptome analysis to understand the effect of AI-2 on gene expression in both nonpathogenic and pathogenic bacteria. While the role of AI-2 in nonpathogenic *E. coli* biofilm formation has been established (DeLisa et al. 2001; González Barrios et al. 2006; Ren et al. 2004), its effect on EHEC phenotypes is less well understood. One study (Widmer et al. 2007) recently observed that AI-2 down-regulated several virulence genes including *rpoS* (positive regulator of LEE3 operon) and *prgH* (cell invasion protein) in *Salmonella enterica* serotype Typhimurium *delta luxS*. However, another recent study (Kendall et al. 2007) observed that several metabolism genes, but only two virulence genes, *espA* and *eae*, belonging to LEE4 and LEE5, respectively, were altered in expression when VS94 was exposed to AI-2 and concluded that AI-2 is not involved in EHEC virulence. On the other hand, our results clearly show that LEE genes and other operons involved in virulence are up-regulated in VS94 upon exposure to AI-2 (Fig. 5). Irrespective of the effect exerted by AI-2 (i.e., up-regulation or down-regulation of virulence) or the extent to which virulence genes were altered in expression, our data and these studies



**Fig. 5** Overall changes in VS94 gene expression in presence of AI-2. Genes significantly regulated by AI-2 were divided into two groups—virulence-related and non-virulence-related. The genes were further classified based on operons and functions

clearly indicate that AI-2 is an important signal in GI-tract infections.

The addition of AI-2 induced the expression of 23 virulence genes (belonging to all five LEE operons—LEE1 through LEE5) by an average of twofold (Fig. 4b) after 4.5 h. Induction of flagellar and fimbrial genes has been associated previously with virulence (Lane et al. 2005), and in our studies, we also observed that 46 fimbrial genes were regulated by AI-2 over time (Fig. 4c). The same was true for 24 iron-related genes (Fig. 4d). Iron has been reported to increase virulence of certain strains of *E. coli* along with other species such as *Vibrio*, *Neisseria*, and *Hemophilus*, through the production of cytotoxins and leukotoxins (Telang et al. 2001). Together, the transcriptome profiling data strongly support our hypothesis that AI-2 is involved in the regulation of EHEC virulence. The temporal aspect of AI-2 signaling in virulence gene expression is not a nonspecific effect that is seen with all genes, as approximately 400 genes involved in phenotypes other than virulence were not altered in expression at any of the four time points at which transcriptome analysis was performed (Fig. 4f).

The regulation of EHEC virulence by AI-2 was also temporally regulated, as LEE genes and other genes related with virulence were up-regulated upon 4.5 h AI-2 exposure but down-regulated at earlier time points (3.5 and 4 h). This is intriguing, as the AI-2 uptake data clearly show that almost all of the externally added AI-2 is still present in the culture supernatant at this time point (Fig. 3). One possible explanation for this observation could be that additional mechanism(s) for AI-2 signal recognition exist. For

example, AI-2 could be sensed by a membrane-bound receptor that transduces, but does not transport into the cell, the AI-2 signal. The idea of a *lsr*-independent AI-2 recognition mechanism has also been proposed (Taga et al. 2003; Wang et al. 2005a). These observations could also reflect the need for a different signal for AI-2 to up-regulate virulence, in the absence of which AI-2 down-regulates certain phenotypes. Taken together, our data suggest that AI-2 signaling in EHEC is tightly regulated, which is reasonable, considering that AI-2 is likely to be abundant in the GI tract and the additional layers of regulation likely prevent early up-regulation of virulence genes (i.e., prior to colonization).

A notable difference between this study and prior work (Kendall et al. 2007) is that we specifically account for temporal aspects of AI-2 signaling. This is important, as several groups have demonstrated that AI-2 is taken up by bacterial cells only during the early stationary phase of growth (Wang et al. 2005a, Xavier and Bassler 2005) and is rapidly phosphorylated and cleared from the system (Xavier and Bassler 2005). Therefore, it is possible that the effects of AI-2 on virulence gene expression were not evident at the single time points at which transcriptome analysis was performed in these studies (Kendall et al. 2007). The choice of growth medium can also explain the contradiction between our results and the work of Kendall et al. (2007) on the effects of AI-2 on EHEC virulence. In our study, we used serum supplemented-SAPI medium, which has limited nutrients, in an effort to mimic the *in vivo* milieu of the human GI tract (Lyte et al. 1996). More importantly, SAPI medium also contains ~10-fold less glucose than standard glucose supplemented DMEM medium, which was used in Kendall et al. (2007). The presence of glucose in culture is important, as it is well established that AI-2 uptake is regulated by catabolite repression in the presence of glucose through down-regulation of the *lsr* operon (Wang et al. 2005a; Xavier and Bassler 2005); hence, glucose can mask the impact of AI-2 on gene expression.

The validity of our data is clearly evident from the temporal expression pattern of the *lsr* operon, which consists of eight genes that are involved in uptake of extracellular AI-2 during the stationary phase. These genes were all up-regulated between 6- and 12-fold at the first three time-points (i.e., only when extracellular AI-2 was available) but not at 5.5 h because no extracellular AI-2 was remaining at that time point (Fig. 4a). The transcriptome analysis data revealed that, in addition to altering the expression of genes involved in virulence, AI-2 also controlled the expression of genes involved in other phenotypes and functions (e.g., metabolism). In this regard, our data are consistent with that of several prior reports that have reported on the role of AI-2 in metabolism (Kendall et al. 2007; Wang et al. 2005b).

Although the relative changes in expression of genes other than the *lsr* operon are not very high, they are statistically significant. Furthermore, it should also be noted that entire operons associated with these phenotypes were differentially expressed. For example, 23 virulence (LEE) genes were differentially expressed at 4.5 h after AI-2 addition, as were 46 flagellar and fimbrial genes, 24 iron related genes, and 19 genes associated with colonization. A possible reason for the relatively small changes in expression of the virulence genes could be the choice of the experimental system. Although we used a *luxS* mutant that does not produce AI-2 to study the effect of external addition of AI-2, AI-3 synthesis is not affected in these cells, as it has been reported to take place independent of *luxS* (Walters and Sperandio 2006). Because AI-3 has been reported to be involved in EHEC virulence (Kendall et al. 2007), the reduced fold-change in AI-2-treated cells relative to control could be a result of high levels of AI-3-mediated virulence gene expression in the control cells.

In summary, we show here that AI-2 is an important determinant of EHEC virulence and infection. Phenotypic assays for chemotaxis, motility, and colonization clearly demonstrate the importance of AI-2 in EHEC pathogenesis and are strongly supported by the gene expression data. To our knowledge, this is the first temporal study of EHEC gene expression in presence of AI-2.

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