Proteomic Analysis to Identify the Role of LuxS/AI-2 Mediated Protein Expression in *Escherichia coli* O157:H7

Kamlesh Soni, Palmy Jesudhasan, Martha Cepeda, Brad Williams, Michael Hume, William K. Russell, Arul Jayaraman, and Suresh D. Pillai

**Abstract**

Microorganisms employ autoinducer molecules to modulate various bacterial processes including virulence expression, biofilm development, and bioluminescence. The universal autoinducer molecule AI-2 is hypothesized to mediate cell signaling in *Escherichia coli* O157:H7. We investigated the role of AI-2 on the *E. coli* O157:H7 cellular proteins using a two-dimensional (2D) gel electrophoresis-based proteomic approach. The protein expression patterns between two experimental comparisons were studied namely, 1) a wild type *E. coli* O157:H7 and its isogenic *luxS* mutant, and 2) the *luxS* mutant and the *luxS* mutant supplemented with AI-2 molecules. Eleven proteins were differentially expressed between the wild type and the *luxS* mutant strain, whereas 18 proteins were differentially expressed in the *luxS* mutant strain when supplemented with AI-2. The tryptophan repressor binding protein (WrbA), phosphoglycerate mutase (GpmA), and a putative protein YbbN were found to be differentially expressed under both experimental comparisons. The FliC protein which is involved in flagellar synthesis and motility was up-regulated in the wild type strain but was not influenced by the addition of synthetic AI-2 molecules to the *luxS* mutant suggesting the involvement of signaling molecules other than AI-2 on flagellar synthesis and motility.

**Introduction**

Bacterial cells utilize autoinducer molecules for cell--cell communication in a process termed quorum sensing (Lu *et al.*, 2004; Camilli and Bassler, 2006; Pillai and Jesudhasan, 2007). Autoinducer molecules play a key role in bacterial metabolism, virulence, and sporulation (Sperandio *et al.*, 2003; Zhao *et al.*, 2006). Autoinducer AI-2 has been considered the universal signaling molecule since it is recognized by several bacterial species (Camilli and Bassler, 2006). The *luxS* gene, which is involved in the production of AI-2, is widely conserved among different bacterial species including *Escherichia coli* (Xavier and Bassler, 2003). The LuxS synthase is involved in the production of AI-2 molecules as part of the *S*-adenosyl methionine (SAM) degradation pathway.

*E. coli* O157:H7 infection is a major cause of foodborne illness around the world (Moxley, 2004). Studies are focused on delineating the role of autoinducer molecules such as AI-2 molecules in *E. coli* O157:H7 regulation (Sperandio *et al.*, 2001; DeLisa and Bentley, 2002; Sperandio *et al.*, 2003; Ren *et al.*, 2004; Wang *et al.*, 2005).
There is also a growing interest in understanding how autoinducer molecules, pathogens and foods interacts (Cloak et al., 2002; Lu et al., 2004; Medina-Martinez et al., 2007). Sperandio and coworkers suggest that E. coli O157:H7 senses two classes of signals to activate its virulence and motility genes, namely AI-3, and a host-derived catecholamine class of hormones (Sperandio et al., 2003). DNA microarray analysis using cell-free supernatants has been used to identify the role of autoinducer molecules in various cellular processes of E. coli (DeLisa et al., 2001; Sperandio et al., 2001; Ren et al., 2004). Recently, Kim et al. (2007) also used cell-free supernatants and a proteomic approach to understand the role of AI-2-like activity in E. coli virulence regulation. The objective of this study was to use in vitro synthesized AI-2 molecules and proteomic analysis to identify the specific role(s) of LuxS/AI-2 mediated signaling in E. coli O157:H7.

Materials and Methods

Preparation and quantification of AI-2 molecules

In vitro synthesized AI-2 was prepared and quantified as described previously (Sperandio et al., 2003). Briefly, His-tagged LuxS and Pfs (proteins required for the formation of AI-2) were isolated and purified using a nickel resin column (Qiagen Inc., Valencia, CA). The purified enzymes were incubated with 1 mM S-adenosyl homocysteine (Sigma-Aldrich, St. Louis, MO) for 1 hour at 37°C, and AI-2 was further separated from the enzymes using a centrifuge column (Biomax-5, Millipore, Billerica, MA) and its concentration determined.

Bacterial strain and growth conditions

E. coli O157:H7 wild type strain (86-24) and its isogenic luxS mutant (VS-94) strains (provided by V. Sperandio, Southwestern Medical Center, Dallas, TX) were used in this study. The luxS mutant strain lacks the capacity to synthesize LuxS protein and thereby lacks the ability to synthesize AI-2 molecules. The strains were grown at 37°C at 100 rpm in LB medium supplemented with 0.5% glucose until an OD600 of 1.2 was attained (which occurred within 3 hours). In vitro synthesized AI-2 (25 μM) was added to LB broth from the beginning of growth period to expose the luxS mutant E. coli O157:H7 cells to an exogenous source of AI-2.

AI-2 activity measurement

AI-2 activity was measured as relative light units (RLU) using the Vibrio harveyi BB 170-based AI-2 activity bioassay (Lu et al., 2004). The RLU was calculated based on the ratio of the bioluminescence of the test sample to the negative control. The wild type cells produced a significant amount (139 ± 3.7 RLU) of AI-2 activity at the late-log phase (OD600 ~ 1.2) compared to the luxS mutant cells which failed to produce the luminescence in the reporter strain. The LB medium supplemented with 25 μM of AI-2 produced an activity of 120 ± 7.5 RLU (data not included).

Soluble protein extraction

Bacterial strains were grown OD600 (~ 1.2) in triplicate for each experimental treatment (the wild type, the luxS mutant, and the luxS mutant grown with 25 μM AI-2), and the soluble protein fractions were extracted from each sample independently using B-Per® bacterial protein extraction reagent (Pierce, Rockford, IL). The Ready Prep® 2-D cleanup-kit (Bio-Rad, Hercules, CA) was used to reduce the ionic contaminants in the protein preparation. The purified proteins were dissolved in 100 μL of rehydration buffer (9.5 M urea, 2% w/ v CHAPS, 18 mM 1,4-dithio-DL-threitol (DTT), 0.5% ampholytes and one tablet of protease inhibitor [Roche Diagnostics, Mannheim, Germany]), and insoluble proteins and cell debris were removed by centrifugation.

Two-dimensional gel electrophoresis

Protein concentrations were measured using the Bradford protein assay kit (Pierce). Two hundred microliters of Bradford reagents were mixed with 1.7 μL of the protein samples and absorbance was measured at 580 nm. Preliminary studies using immobilized pH gradient (IPG) strips in the range of pH 3–10 indicated that the majority of the soluble proteins were detectable in the pH 4–7 range. Hence for sub-
sequent analysis, IPG strips in the pH 4–7 range were employed. Protein loads of 35 μg in 125 μL of rehydration buffer and 800 μg in 250 μL of rehydration buffer were used for 7-cm and 13-cm IPG strips (pH 4–7) respectively. The IPG strips were rehydrated overnight in a rehydration tray (Bio-Rad).

For the first dimensional electrophoresis, the isoelectric focusing of 7-cm IPG strips were conducted at a linear voltage gradient with ~15,000 final V-h (250 V in 15 minutes, 4000 V in 2 hours, 10,000 V in 2.5 hours, and holding at constant 500 V/h) using Protean IEF cell (Bio-Rad). For 13-cm IPG strips, the isoelectric focusing was conducted in linear mode to achieve ~65,000 final V-h (Amersham Bioscience, Piscataway, NJ). After the required V-h was applied, the IPG strips were incubated for 15 minutes in equilibration buffer I (6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS) [w/v], 50 mM Tris-HCl [pH 8.8], 1% dithiothreitol, and approximately 5 mg of bromophenol blue) followed by 15 minutes in equilibration buffer II (6 M urea, 30% glycerol, 2% SDS [w/v], 50 mM Tris-HCl [pH 8.8], 2.5% iodoacetamide, and bromophenol blue as color indicator). Second dimension electrophoresis was performed at 150 constant volts using 10% SDS–polyacrylamide gel electrophoresis (PAGE) gel. Protein spots were visualized using Sypro Ruby fluorescence stain (Molecular Probe, Eugene, OR) for the 7-cm IPG strip gel. GelCode® blue stain reagent (Pierce, Rockford, IL) was used to stain the 13-cm IPG strip gel.

**Data analysis**

The extracted and purified proteins from three experimental replicates of each treatment (wild type, luxS mutant, luxS mutant grown with 25 μM/mL AI-2) were run in duplicate using 7-cm IPG strips, resulting in six independent gels for each treatment. Additional two-dimensional (2D) gels were run from the 13-cm IPG strips to assist in spot excision. The gels were scanned using Gel Doc (Bio-Rad) and the raw images were analyzed using advanced PDQuest 2-D gel analysis software version-8 (Bio-Rad). The protein expression patterns of 1) the wild type E. coli O157:H7 against the luxS mutant (an AI-2 deficient strain), and 2) the luxS mutant against the luxS mutant grown with AI-2 molecules were compared. Only those spots with spot intensities exhibiting ±1.5-fold change difference were short-listed for identification (Arevalo-Ferro et al., 2005).

**In-gel proteolytic digestion and MALDI-TOF**

The protein spots of interest were manually excised (approximately 1 mm in size) and placed in a 96-well microtiter plate for in-gel digestion. Proteolytic digestion was performed overnight using trypsin (20 μg/mL) at 37°C. The digested samples were spotted onto matrix-assisted laser desorption/ionization (MALDI) targets using a ProMS™ (Genomic Solutions, Ann Arbor, MI) robot capable of sample clean-up prior to MS analysis. All MALDI-MS experiments were performed using a model 4700 Proteomics Analyzer MALDI–time of flight (TOF)/TOF (Applied Biosystems, Foster City, CA) instrument. The mass spectrometry (MS) data for each gel spot was acquired using the reflectron detector and 20 tandem MS spectra per spot were acquired. All MS and MS/MS data were queried against the Swiss-Prot protein sequence database using the GPS Explorer (Applied Biosystems) software. The parameters for database searching were as follows: taxonomy, Escherichia coli; database, Swiss Prot; enzyme, trypsin; maximum missed cleavages, 1; variable modifications, oxidation (Met); peptide tolerance, 85 ppm; and MS/MS fragment tolerance, 0.3 Da. To verify the reproducibility of MALDI-MS data, 10 spots were reanalyzed.

**Motility assay**

Motility assays were performed using motility agar media (Acumedia, Baltimore, MD) to validate the expression of specific proteins observed in the proteomic data. In vitro synthesized AI-2 molecules (25 μM/mL) were added to motility agar at around 37°C prior to pouring the plates. Late-log phase culture (~10^9 cfu/mL) of wild type and luxS mutant E. coli O157:H7 cells were inoculated in the center of motility agar plates using sterilized toothpicks. The plates were incubated for 16 hours at 37°C, and ability of the cells to swim in semisolid motility agar medium was recorded by measuring the diameter of the formed halos.
Results

A total of 312 protein spots were detectable across the different experimental treatment groups (Figs. 1 and 2). The reproducibility of the individual 2D gels was evaluated by correlation coefficient analysis. Average correlation coefficient among individual gel comparisons of different treatment groups were > 0.7, suggesting high similarity in spotting pattern (Bland et al., 2006). Since each treatment had six gels, protein spots appearing in at least four out of six gels

![Comparative 2DGE of soluble proteins fraction of the luxS mutant (VS 94) and wild type (86-24) strain of E. coli O157:H7.](image1)

**FIG. 1.** Comparative 2-dimensional gel electrophoresis (2DGE) of soluble proteins fraction of the luxS mutant (VS 94) and wild type (86-24) strain of E. coli O157:H7.

![Comparative 2DGE of soluble proteins fraction of luxS mutant (VS 94) and luxS mutant supplemented with AI-2 molecules.](image2)

**FIG. 2.** Comparative 2DGE of soluble proteins fraction of luxS mutant (VS 94) and luxS mutant supplemented with AI-2 molecules.
were short-listed for further analysis. When spots exhibiting significant changes on intensity (±1.5-fold) were identified using MALDI-MS, they resulted in protein score confidence interval between 95% and 100% (Choe et al., 2005).

Influence of luxS mutation on protein expression in Escherichia coli O157:H7 strain

The differentially expressed proteins between the wild type and luxS mutant strain are shown in Fig. 1 and Table 1. Eleven proteins were identified as differentially expressed. Among these, five proteins were up-regulated and six proteins were down-regulated. Proteins associated with carbohydrate metabolism (GpmA, Ta1B, FumB, and Mak) were affected by the luxS mutation. The gpmA and talB gene products which catalyze reactions in the glycolysis pathway were up-regulated and down-regulated respectively in the wild type as compared to the luxS mutant strain. The fumB gene product (malate dehydratase) involved in the interconversion of malate to fumarate during the TCA cycle was up-regulated in the wild type strain. The results also indicate that proteins related to amino acid metabolism (PepD), cellular processes and signaling (YbbN, FliC, and YbhF), protein biosynthesis (TufA), nucleotide transport and processing (DeoA), and yet to be fully characterized protein (WrbA) were also affected by the luxS mutation.

Influence of AI-2 on protein expression in Escherichia coli O157:H7 strain

The differentially expressed proteins between the luxS mutant and luxS mutant supplemented with AI-2 are shown in Fig. 2 and Table 2. Eighteen proteins (four up-regulated and 14 down-regulated) were differentially expressed. The proteins related to carbohydrate metabolism (PfkA, Eda, Eno, FruB, and GpmA), and

Table 1. Differentially Expressed Proteins in the Wild Type Strain Compared to luxS Mutant Strain of E. coli O157:H7

<table>
<thead>
<tr>
<th>ID</th>
<th>pI</th>
<th>MW</th>
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<th>Gene</th>
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<tbody>
<tr>
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<td>2</td>
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<td>deoA</td>
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<td>18</td>
<td>Trp repressor binding protein</td>
<td>wrbA</td>
<td>-11</td>
<td>Repressor binding protein</td>
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</table>

aID refers to spot shown in Fig. 1.
bIsoelectric point (pI) and molecular weight (MW) obtained in the experiment (practical).
cFold difference in the protein expression of wild type strain compared to luxS mutant strain.
Table 2. Differentially Expressed Proteins in *E. coli* O157:H7 *luxS* Mutant in the Presence and Absence of AI-2

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<td>4.5</td>
<td>28</td>
<td>Protein ybbN</td>
<td>ybbN</td>
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<td>Posttranslational modification, protein turnover, chaperone</td>
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<td>Cell wall/envelop biogenesis/membrane</td>
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<td>66</td>
<td>Glucosamine-fructose-6-phosphate aminotransferase</td>
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<td>3</td>
<td>Cell wall/envelop biogenesis/membrane</td>
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**Metabolism**

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**Poorly characterized**

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<td>wrbA</td>
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<td>Repressor binding protein</td>
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*a ID refers to spot shown in Fig. 2.

*p*Isoelectric point (pI) and molecular weight (MW) obtained in the experiment (practical).

`Fold difference in the protein expression of luxS mutant supplemented with AI-2 compared to the luxS mutant strain.*

amino acid metabolism (SpeE, NikA, and IscC), were affected by the presence of AI-2 molecules. Proteins related to cellular processes and signaling (YbbN, UspE, Tsx, and GlmS), and some yet-to-be fully characterized proteins (SspB, YnjE, and WrB) were also influenced by the presence of AI-2 molecules.

**Motility assays**

The wild type cells produced larger halos (25.1 ± 1.9 mm) in the motility agar compared to that of luxS mutant (19.8 ± 0.8 mm) or when the luxS mutant was supplemented with AI-2 (18.7 ± 0.9 mm) (Fig. 3).

**Discussion**

The aminoacyl-histidine dipeptidase (PepD) protein is involved in the synthesis of amino acids such as alanine, aspartate, arginine, proline, and histidine. Brombacher et al. (2003) reported that repression of pepD gene is necessary for better growth of biofilm. Gonzalez et al. (2006) has reported that biofilm formation is linked to quorum sensing. In this study, we
observed a twofold down-regulation of PepD in the wild type (as compared to the luxS mutant). The down-regulation of pepD gene was observed in microarray analysis (Sperandio et al., 2001). The results reported in this study in combination with these previous studies confirm the linkage between quorum sensing and biofilm formation via the involvement of the PepD protein. We observed the flavoprotein WrbA being down-regulated 11-fold in the wild type compared to the luxS mutant (Table 1). This means that the WrbA protein is over-expressed in the luxS mutant. The tryptophan repressor TrpR is involved in regulating tryptophan biosynthesis whereas WrbA acts as a tryptophan repressor binding protein (Gorman and Shapiro, 2005). Though the exact biological role of WrbA has not yet been clearly identified, it has been reported that binding of WrbA with TrpR improves the stability between the TrpR and operator region of the Trp operon thereby repressing biosynthesis of tryptophan (Han and Lee, 2006). This suggests that in the luxS mutant the tryptophan biosynthesis is repressed due to the over-expression of WrbA. This repression could explain the altered metabolic pathways in luxS mutants that have been reported by previous investigators (Winzer et al., 2002; Walters et al., 2006).

We noticed that polyamine biosynthesis protein (SpeE) was repressed (-2.2-fold) in the presence of AI-2 (Table 2). Krin et al. (2006) also noticed the repression of speE gene by AI-2 molecules in Photobacterium luminescens which also harbors the LuxS/AI-2 system. We observed repression of universal stress protein UspE (-2.2-fold), and stringent starvation protein SspB (-3.2-fold) in the presence of AI-2 molecules (Table 2). Kim et al. (2007) noticed down-regulation (-3.0-fold) of another universal stress protein UspG in E. coli O157:H7 cells in the presence of cell-free supernatant containing AI-2-like molecules. Microarray analysis has shown the repression of universal stress proteins in Porphyromonas gingivalis by cell signaling molecules (Yuan et al., 2005). These results imply that if AI-2 is present, the bacterial cell does not perceive the presence of stress factor(s) and consequently the stress response proteins are down-regulated. It is only in the absence of AI-2 either due to low cell numbers or when nutrients are depleted (when AI-2 is rapidly metabolized) that the stress response is activated.

The two-component system QseBC is known to regulate genes for flagella and motility using quorum sensing molecules. Protein FliC is involved in flagellar synthesis and is regulated by a QseBC system in enterohemorrhagic E. coli (EHEC) (Sperandio et al., 2003). The FliC protein was up-regulated in the wild type (Table 1) and addition of AI-2 molecules to the luxS mutant did not show change in FliC expression (Table 2). The motility assay results were in agreement with the protein expression data in that the addition of AI-2 molecules to the luxS mutant did not influence the size of motility halo formed by luxS mutant strain. Sperandio et al. (2003) claim that flagellar and motility genes are up-regulated not by AI-2, but rather another autoinducer molecule, AI-3. The three proteins

FIG. 3. Motility assay showing formed halos for wild type, luxS Mutant and luxS mutant amended with AI-2.
(WrbA, YbbN, GpmA) were present across the two experimental comparisons (LuxS mutation and presence of extraneous AI-2) (Table 1 and 2) indicating that AI-2 molecules produced by luxS plays a specific role in the expression of these proteins. The gpmA gene product belongs to phosphoglycero mutase family and is involved in carbohydrate transport and metabolism (Turlin et al., 2006). We observed 2.3-fold increase in GpmA protein expression in the wild type compared to the luxS mutant (Table 1). Furthermore, a 1.8-fold increase in GpmA levels was observed when the luxS mutant was supplemented with synthetic AI-2 (Table 2). DeLisa et al. (2001) reported the higher expression (3.2-fold) gpmB which codes for a protein belonging to the phosphoglycero mutase family when luxS mutant strain was supplemented with cell-free supernatant (CFS) containing AI-2 like molecules. Compared to the 11-fold down-regulation of WrbA protein in the wild type (Table 1), we observed only 1.8-fold down-regulation of luxS mutant when supplemented with AI-2 molecules (Table 2). This difference in expression levels of WrbA among these two experimental comparisons highlights two possibilities namely, 1) WrbA expression is more actively controlled by luxS gene itself than AI-2 molecules, and 2) there may be autoinducer molecules other than AI-2 (in the wild type strain) which are also involved in modulating expression of WrbA. The observation that not many proteins are regulated by AI-2 in E. coli O157:H7 raises the possibility that E.coli O157:H7 may be under the control of other autoinducer molecules. This is not surprising. Sperandio and coworkers (2003) have reported that another autoinducer molecule, AI-3 is presumably controlling some of the traits in E. coli O157:H7.

Conclusion

The key finding of this study was in E. coli O157:H7 only three proteins appear to be under the direct influence of the LuxS/AI-2 system. Nevertheless, the identified proteins are involved in carbohydrate, amino acid metabolism, and stress response. It would be interesting to identify whether the protein expression in E. coli O157:H7 would vary depending on the food matrix. There is a need for proteomic studies such as this to evaluate the interaction of food matrices and autoinducer molecules in controlling pathogen survival and virulence on foods.

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