Autoinducer AI-2 Is Involved in Regulating a Variety of Cellular Processes in *Salmonella* Typhimurium

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

Abstract

*Salmonella* Typhimurium is known to exhibit LuxS/AI-2-mediated cell signaling. We investigated the role of LuxS/AI-2 system on *Salmonella* Typhimurium protein expression using a proteomics approach based on two-dimensional gel electrophoresis (2DGE)—MALDI-MS. The global protein expression profiles of the wild-type, a luxS mutant, and a luxS mutant strain supplemented with AI-2 were compared. Seven proteins were differentially expressed when comparing the wild-type and luxS mutant strains, whereas 13 proteins were differentially expressed when comparisons were made between luxS mutant strains with and without AI-2 supplementation. The seven proteins that were differentially expressed between the wild-type and the luxS mutant strain were also differentially expressed in the luxS mutant strain supplemented with AI-2. The level of PhoP, a virulence determinant, was higher in the presence of AI-2. Proteins associated with the carbohydrate metabolism (pfkA, gpmI, and taaB) and ATP synthesis (Pla gene product) were up-regulated by the presence of AI-2 molecules. These results provide experimental evidence that AI-2 molecules regulate a variety of cellular processes in *Salmonella* Typhimurium.

Introduction

Autoinducer (AI) molecules such as AI-1, AI-2, and AI-3 are thought to influence a variety of bacterial processes including pathogenicity, biofilms, motility, and bioluminescence (Surette and Bassler, 1998; Arevalo-Ferro et al., 2003; Henke and Bassler, 2004; Gonzalez et al., 2006; Pillai and Jesudhasan, 2007). Optimal levels of these cell signaling molecules and interaction of these molecules with regulatory proteins are thought to be key steps involved in the coordination of gene expression. Among these different autoinducer molecules, AI-2 is synthesized by LuxS synthase. The luxS gene has been identified in more than 55 species of gram-negative and gram-positive bacteria, including *Salmonella* Typhimurium (Xavier and Bassler, 2003). The luxS gene is involved in the synthesis of AI-2 molecules by catalyzing the reaction from S-ribosyl homocysteine to homocystein and 4,5-dihydroxy-2,3-pentanedione (DPD). Reaction of these unstable DPD molecules with water leads to cyclation and consequent formation of furanones known as AI-2 molecules (Schauder et al., 2001). The AI-2 molecules are thought to exist in two different forms depending on the presence or absence of boron molecules (Chen et al., 2002; Miller et al., 2004).
Salmonella Typhimurium is a key foodborne pathogen (Gomez et al., 1997). While the role of LuxS/AI-2 system in different bacterial species has been associated with genes responsible for virulence, motility, and biofilm formation, the only known role of AI-2 in Salmonella Typhimurium is the regulation of the lsr operon (Taga et al., 2003). The objective of this work was to identify the proteins that are influenced by LuxS/AI-2 in Salmonella Typhimurium using a two-dimensional gel electrophoresis (2DGE)-based proteomic approach. Transcriptome analysis with a DNA microarray can be used to identify genes influenced by the autoinducer molecules. However, a microarray approach provides information at the mRNA level only and not at the functional protein level (Kim et al., 2007). To the best of our knowledge this is the first report correlating the presence of AI-2 with protein expression patterns and their relationship to cellular processes in Salmonella.

**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 the Biomax-5 (Millipore, Billerica, MA) column and the AI-2 concentration was determined. The concentration of *in vitro* synthesized AI-2 was 125 µM, and 20% (25 µM) of this AI-2 preparation was used in experiments that involved the addition of AI-2 molecules.

**Bacterial strains and growth condition**

Salmonella Typhimurium (Accession no. 87-26254, National Veterinary Service Laboratory, Ames, IA) and its isogenic luxS mutant (PJ 002) were used in this study. The luxS mutant strain was created in our laboratory as described previously (Widmer et al., 2007). Salmonella Typhimurium cells (wild-type and luxS mutant) were cultured into Luria-Bertani (LB) medium supplemented with 0.5% glucose at 37°C with moderate agitation (100 rpm) until the late-log phase of growth was reached (optical density [OD] ~1.2). *In vitro* synthesized AI-2 (25 µM) was added to the LB broth from the beginning of growth period. Vibrio harveyi strain BB170 (luxN::Tn5 sensor 1-, sensor 2+), a reporter strain used to determine AI-2 activity, was grown at 30°C with aeration in autoinducer bioassay (AB) medium (Lu et al., 2004).

**AI-2 bioassay to verify the inability of the luxS mutant to produce AI-2**

Overnight cultures of Salmonella Typhimurium (wild-type and luxS mutant) were inoculated (1:100) in fresh LB broth (0.5% glucose) and cultured at 37°C with shaking (100 rpm). Optical densities (OD600nm) of these cultures were noted and the cell-free supernatants (CFS) were collected by centrifugation (10,000 g for 2 minutes) at different time points. The supernatants were then passed through 0.22-µm syringe filters (Corning® Inc., Corning, NY) and stored at −20°C until used in the AI-2 bioassay. The V. harveyi reporter strain BB170, which produces luminescence in the presence of AI-2 molecules, was used as a bioassay reporter strain to verify the lack of AI-2 production by the luxS mutant strain. The AI-2 bioassay was performed as described earlier (Lu et al., 2004). Briefly, 90 µL of the freshly diluted (1:5000) culture of the reporter strain in AB medium was mixed with 10 µL of test samples (CFS) or 10 µL of AB media (negative control) in a 96-well plate. The plates were incubated at 30°C with moderate shaking (100 rpm) and the luminescence response of the reporter strains was monitored using a Wallac 1420 plate reader (PerkinElmer, Shelton, CT). The *in vitro* synthesized AI-2 molecules were also verified using the similar bioassay to confirm their ability to induce the luminescence response.

**Sample preparation**

Late log-phase cultures of the wild-type (no. 87-26254), luxS mutant, and the luxS mutant grown in the presence of AI-2 were obtained. The cells were centrifuged (2 minutes at
Protein concentration (Pierce). Preliminary studies using immobilized pH gradient (IPG) strips in the range of pH 3–10 indicated that the majority of the soluble proteins were in the pH 4–7 range. Hence for subsequent analysis, IPG strips in the pH 4–7 range were employed. The protein samples were adjusted to 35 μg in 125 μL of rehydration buffer for the rehydration of 7-cm IPG strips (Bio-Rad). The isoelectric focusing of the 7-cm IPG strips was performed at a linear voltage gradient with approximately 15,000 final V-hours in a Protean IEF Cell (Bio-Rad). For spot excision, 13-cm IPG strips (pH 4–7) with a total protein load of 800 μg in a final volume of 250 μL (rehydration buffer) were used. Equilibration of the IPG strips was performed as previously mentioned (Arevalo-Ferro et al., 2003). The second dimension gel electrophoresis was performed at a linear voltage gradient with approximately 15,000 final V-hours in a Protean IEF Cell (Bio-Rad). For spot excision, 13-cm IPG strips (pH 4–7) with a total protein load of 800 μg in a final volume of 250 μL (rehydration buffer) were used. Equilibration of the IPG strips was performed as previously mentioned (Arevalo-Ferro et al., 2003). The second dimension gel electrophoresis was performed using 10% (w/v) SDS-PAGE gel at 150V constant voltage. The protein spots were visualized by staining the gels with Sypro Ruby fluorescence stain (Molecular Probe, Eugene, OR).

Data analysis

Three independent 2DGE analyses (i.e., three biological replications) were performed for each of the three experimental treatments; namely, the wild-type strain, the luxS mutant strain, and the luxS mutant strain grown in the presence of AI-2. The 2D gels were scanned using Gel Doc Image System (Bio-Rad) and the raw images were analyzed using the PDQuest™ 2-D gel analysis software version-8 (Bio-Rad). The data was analyzed to achieve two different comparisons, namely 1) protein profile of the wild-type compared to protein profile of the luxS mutant, and 2) protein profile of the luxS mutant compared to the protein profile of the luxS mutant grown in the presence of AI-2. Only those protein spots that showed significant differences in intensities (i.e., ±1.5-fold change) were identified using MALDI-MS (Arevalo-Ferro et al., 2005).

Protein identification using MALDI-MS

The differentially expressed protein spots were manually excised (approximately 1 mm in size) and placed in a 96-well microtiter plate for proteolytic digestion. The proteolytic digestions were performed overnight at 37°C using trypsin (20 μg/mL in 25 mM ammonium bicarbonate) (Promega, Madison, WI). The digests were spotted onto MALDI targets using a ProMS™ robot capable of sample cleanup prior to MALDI-MS analysis (Genomic Solutions, Ann Arbor, MI). The MALDI-MS experiments were performed in a 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystem, Foster City, CA). Twenty tandem MS spectra per spot were acquired. To confirm the generated MALDI-MS data, five spots were reanalyzed.

Results

The AI-2 bioassay verified that CFS from wild-type Salmonella Typhimurium cells were able to induce luminescence response in V. harveyi reporter strain. Luminescence decreased as the culture transitioned from log-phase to the stationary phase (Fig. 1). Luminescence by the CFS from the luxS mutant remained at a negligible level (<100) during the growth period, confirming that the luxS mutant strain was unable to produce AI-2 molecules. The growth pattern of both wild-type and luxS mutant cells were similar, confirming that the mutant strain was not metabolically defective as compared to the wild-type. The in vitro synthesized AI-2 molecules were able to induce luminescence (3.0 × 10⁶) light units.

Approximately 198 protein spots were detectable in the experimental gels (Figs. 2 and 3). The similarity in spotting patterns between individual gels resulted in correlation coefficients
in the range of 0.66 to 0.99, implying strong correlation of the protein spotting patterns among the different gels (Bland et al., 2006). Seven proteins were found to be differentially expressed (six up-regulated and one down-regulated) between the wild-type and luxS mutant strain (Table 1; Fig. 2) and 13 proteins were found to be differentially expressed (10 up-regulated and three down-regulated) when luxS mutant was supplemented with AI-2 (Table 1; Fig. 3). The protein spots were identified using MALDI-MS and resulted in protein hits with confidence intervals of 95% and 100%. We were unable to identify one of the spots from the protein database (ID-1, Table 1).

Seven proteins that were differentially expressed between the wild-type and mutant strain were also differentially expressed between the mutant strain and the mutant strain supplemented with AI-2 (Table 1). The pattern of

FIG. 1. Comparison of AI-2 activity and growth patterns between Salmonella Typhimurium wild-type (accession no. 87-26254) and its isogenic mutant. The histograms represent the luminescence while the line graphs indicate the growth pattern.

FIG. 2. Comparative two-dimensional gel electrophoresis patterns of the soluble proteins fraction of the luxS mutant (PJ 002) and the wild-type strain (87-26254) of Salmonella Typhimurium.
up-regulation of six of these proteins (TiG, YaeT, PhoP, GpmI, TrxB, and one unidentified spot) were similar; however, the protein identified as 6-phosphofructokinase isozyme I (PfkA) was down-regulated in the wild-type and up-regulated in the mutant strain supplemented with AI-2 (Table 1). There was no difference in the expression of six proteins (GroL, TalB, OmpF, RpoA, Pta, and YiiM) between the wild-type and luxS mutant strain. However, in the presence of AI-2, three of these proteins (TalB, Pta, and YiiM) were up-regulated and three (GroL, OmpF, and RpoA) were down-regulated.

TABLE 1. IDENTIFIED QUORUM SENSING REGULATED PROTEINS USING MALDI-MS.

<table>
<thead>
<tr>
<th>ID</th>
<th>Protein name</th>
<th>Gene name</th>
<th>Wild-type</th>
<th>AI-2</th>
<th>Clusters of orthologous (COG) categories</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Unidentified protein spots</td>
<td></td>
<td>+1.53</td>
<td>+2.15</td>
<td>Carbohydrate transport and metabolism</td>
</tr>
<tr>
<td>2</td>
<td>6-phosphofructokinase isozyme I</td>
<td>pfkA</td>
<td>-4.34</td>
<td>+1.81</td>
<td>Cellular processes and signaling</td>
</tr>
<tr>
<td>3</td>
<td>Trigger factor (TF)</td>
<td>tig</td>
<td>+2.69</td>
<td>+4.69</td>
<td>Cellular processes and signaling</td>
</tr>
<tr>
<td>4</td>
<td>60 kDa chaperonin</td>
<td>groL</td>
<td></td>
<td>-2.17</td>
<td>Cellular processes and signaling</td>
</tr>
<tr>
<td>5</td>
<td>Outer membrane protein precursor</td>
<td>yaeT</td>
<td>+2.86</td>
<td>+3.02</td>
<td>Cellular processes and signaling</td>
</tr>
<tr>
<td>6</td>
<td>Transcriptional regulatory protein</td>
<td>phoP</td>
<td>+3.49</td>
<td>+5.72</td>
<td>Cellular processes and signaling</td>
</tr>
<tr>
<td>7</td>
<td>Transadolase B</td>
<td>talB</td>
<td></td>
<td>+2.26</td>
<td>Carbohydrate transport and metabolism</td>
</tr>
<tr>
<td>8</td>
<td>Outer membrane protein F precursor</td>
<td>ompF</td>
<td></td>
<td>-2.7</td>
<td>Cellular processes and signaling</td>
</tr>
<tr>
<td>9</td>
<td>2,3-biphosphoglycerate mutase</td>
<td>gpmI</td>
<td>+8.01</td>
<td>+5.85</td>
<td>Carbohydrate transport and metabolism</td>
</tr>
<tr>
<td>10</td>
<td>DNA directed RNA polymerase alpha chain</td>
<td>rpoA</td>
<td></td>
<td>-3.03</td>
<td>Information storage and processing</td>
</tr>
<tr>
<td>11</td>
<td>Thioredoxin reductase</td>
<td>trxB</td>
<td>+4.04</td>
<td>+2.84</td>
<td>Cellular processes and signaling</td>
</tr>
<tr>
<td>12</td>
<td>Phosphate acetyl transferase</td>
<td>pta</td>
<td></td>
<td>+2.24</td>
<td>Metabolism</td>
</tr>
<tr>
<td>13</td>
<td>Hypothetical protein yiiM</td>
<td>yiiM</td>
<td></td>
<td>+2.53</td>
<td>Putative hypothetical protein</td>
</tr>
</tbody>
</table>

*ID refers to spot shown in Figs. 1 and 2.*
Discussion

The *Salmonella* Typhimurium wild-type cells produced maximal amount of AI-2 luminescence between mid- and early log phases of growth (Fig. 1). The AI-2 level decreased as the culture entered into stationary phase. This decreased level of AI-2 in stationary phase could be due to degradation or uptake of AI-2 molecules by bacterial cells (Xavier and Bassler, 2005). The two component regulatory system PhoP/PhoQ is a key transcriptional regulatory system that controls *Salmonella* pathogenicity (Aguirre et al., 2006; Nishino et al., 2006; Tu et al., 2006). We observed that PhoP was up-regulated in the presence of AI-2 molecules compared to its absence, suggesting that AI-2 is involved in modulating PhoP expression in *Salmonella* Typhimurium (Table 1). The two-component regulatory system OmpR/EnvZ regulates the porin genes *ompF* and *ompC* in response to osmolarity changes in *Salmonella* (Mills et al., 1998). In this study, the OmpF protein was down-regulated in the luxS mutant supplemented with AI-2. Ren et al. (2004), however, reported that in *E. coli*, *ompF* was down-regulated as a function of signaling molecules other than AI-2 implying that the *Salmonella* Typhimurium and *E. coli* may use an alternate signaling system to modulate OmpF expression.

Proteins associated with the carbohydrate transport and metabolism also appear to be influenced by the presence of AI-2 molecules. The gene products of *pkA*, *gpmI*, and *talB*, which catalyze reactions in glycolysis, were up-regulated in the mutant strain supplemented with AI-2 molecules. Compared to the −4.34 fold down-regulation of PfkA protein in the wild-type (Table 1), we observed a 1.81-fold up-regulation of PfkA protein in the luxS mutant supplemented with AI-2 molecules (Table 1). This difference in PfkA expression levels raises the possibility that there may be autoinducer molecules other than AI-2 (in the wild-type strain) which are involved in modulating expression of PfkA. Sperandio et al. (2003) have reported that the LuxS gene controls another autoinducer molecule (AI-3) in addition to AI-2 in *E. coli* O157:H7. In this study, there was a 2.24-fold up-regulation of the Pta protein in the presence of AI-2 molecules. Pta gene product is involved in the phosphotransacetylase-ATP-acetate phosphotransferase (Pta-AckA) pathway to generate ATP molecules (Hardie et al., 2003). The observed up-regulation of the Pta protein by AI-2 implies that AI-2 may have some role in *Salmonella* Typhimurium metabolism. Furthermore, consistent with our observation, recent studies have also suggested that the LuxS/AI-2 system in bacterial cells may have an important metabolic role (Winzer et al., 2002a, 2002b; Walters and Sperandio, 2006).

The *lsr* operon, consisting of seven genes (*lsr*ACDBFGE) has been suggested to be involved in the cellular uptake of AI-2 molecules in *Salmonella* Typhimurium (Taga et al., 2003). However, Xavier and Bassler (2005) report that if glucose is present in the medium, the *lsr* operon is not influenced by the AI-2 molecules. They hypothesized that catabolite repression occurring in the presence of glucose, prevents regulation of the *lsr* operon by AI-2 molecules. This could explain the results we observed when we supplemented LB media with 0.5% glucose and did not observe the differential expression of the *lsr* operon–related proteins (Table 1).

Our study focused only on the soluble protein fraction and we detected approximately 198 protein spots. The possibility that the LuxS/AI-2-mediated system in *Salmonella* Typhimurium may control expression of some other (non-soluble) proteins cannot be ruled out. These results confirm the importance of AI-2 molecules as a key metabolic intermediate controlling a variety of proteins related to metabolism and virulence. Thus it can be expected that any alteration in metabolic pathways involving AI-2 will be manifested in the pathogen’s cellular processes.

Acknowledgments

This work was supported by Hatch grant H0858. We would like to thank Dr. Larry Dan-gott of the Protein Chemistry Laboratory, Texas A&M University for his valuable suggestions. The advice by Dr. Sperandio regarding AI-2 synthesis is greatly appreciated.

References

Aguirre A, Cabeza ML, Spinelli SV, McClelland M, Garcia Vescovi E, and Soncini FC. PhoP-induced genes within...
**PROTEOMIC ANALYSIS IN S. TYPHIMURIUM**


