Comparative Proteomic Analysis of *Salmonella enterica* Serovars Enteritidis, Typhimurium and Gallinarum

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**ABSTRACT.** *Salmonella enterica* includes several related serovars which have different host ranges and cause diseases of different severities. However, their pathogenic potential is unknown, and it is not clear what mechanisms are activated or inhibited during adaptation to a specific host environment. Some proteins are involved in the mechanism of pathogenicity at a molecular level and provide the functional aspects that create the diverse phenotypes. To compare proteomic analyses of the total proteins of *Salmonella Enteriditis* (SE), Typhimurium (ST), and Gallinarum (SG), two-dimensional gel electrophoresis (2-DGE) was performed using a pH 4–10 immobilized pH gradient (IPG) strip, and some proteins were identified by mass spectrometry (MS). After staining the gels, the proteins that were expressed at 10-fold or higher levels compared to other spots on the gel were characterized. Some of the identified proteins were related to virulence, such as β-lactamase, RfbH protein, and shikimate kinase. Additionally, there was a high level of variation between serovars despite the similarities in the expression patterns. Furthermore, this study shows that 2-DGE combined with MS is a useful tool for identifying proteins differentially expressed between serovars with different host ranges and pathogenic potential.

**KEYWORDS:** 2-dimensional gel electrophoresis (2-DGE), Proteome, *Salmonella Enteritidis*, *Salmonella Gallinarum*, *Salmonella Typhimurium.*

The Gram-negative bacterial pathogen *Salmonella enterica* is a leading cause of several illnesses, including typhoid fever, food poisoning, gastroenteritis and septicemia. The genus *Salmonella enterica* subspecies enterica is routinely divided into more than 2300 serovars based on the expression of three surface antigens, the somatic O antigen, the flagellar H1 and H2 antigens and the capsular Vi antigen, according to the Kauffmann-White scheme \[17\]. Most *S. enterica* serotypes infect a broad range of hosts, such as cattle, pigs, poultry and humans, while other serotypes have adapted to specific hosts. In contrast, *S. Gallinarum* (SG) is a non-motile host-adapted serovar that causes fowl typhoid, a severe systemic disease responsible for heavy economic losses in poultry production because of its high morbidity and mortality and the resulting reduction in egg production \[16\]. The pathogenicity of most of the host-adapted serotypes remains unclear, and little is known about the host-specific adaptation of different variants to specific environments.

Recently, proteomics has emerged as a powerful and widely used method to investigate protein expression. Knowing the protein expression patterns under different environmental conditions may increase our understanding about the virulence of this organism at the molecular level and provide insights into many other aspects of *Salmonella* biology. Edward et al. reported that each *Salmonella* serovar genome has numerous insertions and deletions, varying in size up to 50 kb, despite their similar genomes \[3\]. Furthermore, identical genes may be regulated differently, further diversifying the phenotypes of *Salmonella* strains within a specific serovar. Therefore, analyzing the protein expression patterns that result from the observed genomic differences is of great importance. The protein expression patterns of ST and SE have been extensively studied, and annotated reference maps of the 2 serovars have been published \[3\]. Additionally, the differences in protein expression between SE and SG have been studied \[13\]. However, the protein expression patterns of SE, ST, and SG, which belong to different serogroups, have yet to be compared. To our knowledge, SE isolates from different hosts have not been compared by proteomics. In a previous study, we isolated SE strains from chickens and humans and characterized several phenotypes and genotypes \[8\]. From these isolates, we selected and analyzed two representative SE isolates, one human isolate and one chicken isolate. Comparing the proteomes of SE isolates from humans and chickens, ST, and SG may help reveal factors that enable *Salmonella* to overcome species barriers and adapt to new hosts, and should ultimately lend insight into the process of host adaptation and the emergence of new pathogens. Therefore, the purpose of our investigation was to examine the level of variability in the protein patterns of SE, ST, and SG. Thus, in this study, we used a standard proteomic approach involving 2-dimensional gel electrophoresis (2-DGE). Furthermore, we used Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) analysis.
to identify the proteins with different expression profiles from serovars with different host specificity and pathogenic potential.

MATERIALS AND METHODS

**Bacterial strains and growth conditions**: Three serovars were used in this study (SE, ST, and SG). The SG vaccine strain 9R was selected to represent SG, and two SE isolates, one isolated from a human and the other from a chicken, were used. Human ST and SE isolates were isolated from fecal samples from patients showing diarrhea. The chicken SE isolate was isolated from cecal contents from chickens showing diarrhea. The bacteria were grown on Luria-Bertani (LB) agar plates for 18 hr at 37°C, a single colony was transferred to 10 ml LB broth, and the culture was incubated for 18 hr with shaking at 37°C. Cultured bacteria were harvested by centrifugation at 5,000 × g for 20 min at 4°C. The pellets were washed with phosphate buffered saline (PBS, pH 7.2) three times, and the resulting cell pellets were used for the protein extraction.

**Protein extraction**: The cell pellets were washed twice with ice-cold PBS, resuspended in PBS, and sonicated for 10 sec with a Sonoplus sonicator (Bandelin electronic, Germany). The cells were collected by centrifugation at 5,000 × g for 20 min. The resulting cell pellet was resuspended in sample lysis solution, which was composed of 7 M urea, 2 M thiourea containing 4% (w/v) 3-[3-cholamidopropyl]dimethyammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT) 2% (v/v) pharmalyte, and 1 mM benzamidine. Proteins were extracted for 1 hr at room temperature with vortexing. After centrifugation at 15,000 × g for 1 hr at 15°C, the insoluble material was discarded, and the soluble fraction was harvested and used for 2-DGE.

**Two-dimensional gel electrophoresis (2-DGE)**: Immobilized pH gradient (IPG) dry strips were equilibrated for 12-16 hr with 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, and 1% pharmalyte, and 200 μg of sample was loaded onto the strip. Isoelectric focusing (IEF) was performed at 20°C, and the voltage was linearly increased from 150 to 3,500 V over 3 hr to allow sample entry, followed by a constant voltage of 3,500 V, with focusing being complete after 96 kVh. Prior to running the second dimension, the strips were incubated for 10 min in equilibration buffer (50 mM Tris-Cl pH 6.8, 6 M urea, 2% SDS, and 30% glycerol). Equilibrated strips were inserted into 10% SDS-PAGE gels. Electrophoresis was carried out at a constant voltage of 200 V for 1 hr. The 2D gels were run at 20°C for 1,700 Vh and silver stained as described by Oakley et al. [11].

**Analysis of the 2D gel profiles**: Selected spots were drown out and destained prior to swelling the gel for 30 min at 4°C in digestion buffer (100 mM NH₄HCO₃, 12.5 ng/mg trypsin). The spectra were evaluated using Peak Explorer 3.0 (Applied Biosystems) software. The resulting data were analyzed using GPS Explorer TM 3.5 software and the values obtained from this software were analyzed by Excel (Microsoft). The proteins were identified by searching the Salmonella database from the National Center for Biotechnology Information (NCBI).

RESULTS

**Profiles of 2-DGE from Salmonella serovars**: The protein expression profiles of SE, ST, and SG9R were visualized by 2-DGE. For the chicken and human SE isolates, approximately 270 spots could be detected by silver staining (Fig. 1a & 1b). Approximately 342 spots were detected with SG9R, and approximately 284 spots were detected with ST (Fig. 1c & 1d). All of the spots were distributed in the pH 4–10 range and had relative molecular weights (MWs) between 10–200 kDa.

**Identified proteins**: Expression levels were assessed by silver staining each gel, and all of the spots were evaluated. For each gel, the spot with the highest intensity was given a value of 100%, and the other spots on that gel were given values based on their relative intensities. The 10 spots with the greatest differences in protein expression levels between the four tested Salmonella strains were selected. These 10 spots were excised, digested with trypsin, and subjected to MALDI-TOF MS protein identification analysis. The peptide mass peaks were compared with those in the NCBI database, and the protein identification data, including the genebank ID, molecular weight, pI value, and sequence coverage ratio (%) were determined. Because two spots could not be identified, only eight proteins were ultimately identified (Fig. 2). Table 1 shows the identified proteins from the four Salmonella strains. The identified spots were located in the range of 30–50 kDa and pI 5–6 and represented various functional activities including metabolism and energy production. When comparing between serovars, isocitrate hydrogenase is unique to the chicken and human SE isolates. The expression of β-lactamase, on the other hand, was 7–8 fold higher in the human SE isolate than in the chicken SE isolate, even though both isolates are from the same serovar.

DISCUSSION

The complete genome sequence of Salmonella is already known. However, most of the predicted open reading frames remain uncharacterized. A previous study reported differences between the proteomes of SG and SE [13]. Another study compared the cytosolic proteins of ST and Salmonella serovar Pullorum (SP) [5]. These studies focused on defining the mechanism behind host adaptation by comparing host-adapted serovars (SP and SG) with serovars with broad host specificity (SE and ST). In this study, we analyzed four strains from three serovars, SE, ST and SG, simultaneously. For the SE serovar, we compared SE strains isolated from human and chicken sources. Comparisons of the proteomic analyses of the SE isolates from different hosts may provide insight into the mechanism of host adaptation. Studies of the mechanisms behind host adaptation usually examine both DNA sequences and protein
expression levels, but proteomics can reveal modifications and expression level differences that are not detectable from the DNA data [3].

The 2-DGE analysis of the three serovars, SE, ST, and SG, resulted in 270, 284, and 324 valid spots, respectively. In a previous report, Kamelia et al. found approximately 719 protein spots by 2-DGE analysis of SG and 577 protein spots with SE, which is greater than the number of spots found in our study [13]. In another report by Park et al. analysis of SE resulted in approximately 400 protein spots, which is similar to the number of spots found in our study [15]. Some studies suggested that the differences in the numbers of spots is not only related to the Salmonella serovars, host specificity, and evolution, but may also be related to the origin of the isolates and the effects of various external factors such as media composition, temperature, and storage [4].

Despite the variations in protein expression among the characterized serovars, several serovar-specific factors were also observed. Isocitrate dehydrogenase, a participant (Fig. 3, spot no. 1) in the citric acid cycle, was detected as a prominent protein in the profiles of chicken and human SE isolates but was not expressed by ST or SG9R. Furthermore, the human SE isolate showed a significantly higher expression level than the chicken SE isolate for this spot.

Another difference in the expression patterns was the absence of transaldolase in the ST expression map (Fig. 4, spot no. 7). Transaldolase has been annotated as part of the pentose phosphate cycle and is encoded by TALDO1 in humans [19]. Additionally, in Gram-negative bacteria, this enzyme is necessary for the synthesis of heptose moieties in the lipopolysaccharide layer of the outer membrane [1].

The over-expression of β-lactamase (Fig. 4, spot no. 6) in the human SE isolate results in greater pathogenicity because this enzyme is responsible for resistance to β-lactam antibiotics such as penicillins, cephemcins, and carbapenems. This enzyme reflects the important role antibiotic resistance plays in Salmonella virulence. Based on the antibiotic resistance profile determined in our previous study, this human SE isolate is highly resistant to β-lactam antibiotics [8]. Also, pathogenic Salmonella are facultative intracellular pathogens that survive and replicate in the mononuclear phagocytes of infected hosts, and it is thought that β-lactamase contributes to bacterial survival within macrophages by stabilizing bacterial macromolecular complexes after exposure to the toxic and degradative products found within macrophages [13].

The over-expression of shikimate kinase (Fig. 4, spot no. 8) in ST suggests that this protein may play a role in pathogenicity. However, further detailed characterization should
be performed to confirm this supposition [10].

The protein RfbH was strongly expressed in SG and the human SE isolate (Fig. 3, spot no. 2). The rfb gene cluster encodes glycosyl synthase and transferase enzymes that are used in the biosynthesis of oligosaccharide-repeat units. In recent years, the entire rfb loci of Salmonella serovars Typhimurium, Paratyphi A, Typhi, Muenchen, and Anatum, representing serogroups B, A, D1, C2, and E1, respectively, have been cloned and sequenced [13]. Additionally, the rfb genes are required for the biosynthesis of the bacterial
poly saccharide O-antigen [7].

Enzymes related to phosphate metabolism were overexpressed in ST (Fig. 3, spot no. 3 and Fig. 4, spot no. 5). Several enzymes in the phosphate pathway were identified, including phosphofructokinase and phosphoglucomutase, which suggests that this pathway is also active under our growth conditions.

In a similar study, a response regulator protein, the T-cell inhibitor protein, and *Salmonella* pathogenicity island 1 (SPI-1) were over-expressed in SG as compared to SE [14]. These proteins have an important role in pathogenicity, as effector proteins are directly inserted into the host cell cytoplasm where they control host cell signaling. According to our study, however, the three abovementioned proteins were not over-expressed in SG, SE, or ST.

Comparing the protein maps from the three serovars provides a basis for more detailed analysis of the serovar-specific differences and the potential roles of the identified proteins in the host adaptation and pathogenicity of *Salmonella*. This study indicates that protein expression profiles differ based on the host, even for strains within the SE serovar. In a previous report, the expression patterns of laboratory ST strains and clinical ST isolates differed, and such differences may be due to the clinical isolates gradually adapting to laboratory conditions while undergoing continuous subculturing [5]. Further studies are required to identify
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