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Proteomic Comparison between *Salmonella* Typhimurium and *Salmonella* Typhi[§]

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The genus *Salmonella* contains more than 2500 serovars. While most cause the self-limiting gastroenteritis, a few serovars can elicit typhoid fever, a severe systemic infection. *S. enterica* subsp. *enterica* serovar Typhimurium and *S.* Typhi are the representatives of the gastroenteritis and typhoid fever types of *Salmonella*. In this study, we adopted Stable Isotope Labeling with Amino acids in Cell culture (SILAC) technology to quantitatively compare the proteomes of the two serovars. We found several proteins with serovar-specific expression, which could be developed as new biomarkers for clinical serotype diagnosis. We found that flagella and chemotaxis genes were down-regulated in *S.* Typhi in comparison with *S.* Typhimurium. We attributed this observation to the fact that the smooth cellular structure of *S.* Typhi may better fit its systemic lifestyle. Instead of known virulence factors that were located within *Salmonella* Pathogenecity Islands, a number of core genes, which were involved in metabolism and transport of carbohydrates and amino acids, showed differential expression between the two serovars. Further studies on the roles of these differentially-expressed genes in the pathogenesis should be undertaken.

***Keywords*:** SILAC, proteome, *Salmonella*, typhoid, biomarker

Introduction

Salmonella species are important pathogens in both humans and animals. As a genus, *Salmonella* includes more than 2,500 serovars. Different *Salmonella* serovars can cause a variety of diseases in human, all of which are collectively called salmonellosis. For example, *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) usually causes self-limited gastroenteritis, whereas *S.* Typhi causes life-threatening typhoid fever. Genomic comparison of the two serovars revealed that, despite the high degree of similarity of the core genome, each serovar possesses a unique accessory gene inventory (McClelland *et al*., 2001; Parkhill *et al*., 2001). These unique genes are likely to be associated with virulence, but the differences observed at the DNA level have not yet been correlated with protein expression. In fact, the serovar-specific genes, many of which are horizontally acquired, are not always translated into proteins, presumably because these exogenous elements are incompatible with the translational machinery of the cell (Taoka *et al*., 2004). In addition, the differential regulation of identical gene content may also play a role in the diverse phenotypes of the different serovars. Therefore, it would be of great interest to compare their global expression patterns at the proteomic level.

Selection of an appropriate model for proteomic study is very important for understanding the pathogenesis of *Salmonella* infection. *Salmonella* infection involves two steps: the bacterium first enters the intestinal epithelial cells, then it can be taken up by and survive within phagocytic cells of the host immune system. The latter step characterizes *Salmonella* as an intracellular pathogen and is considered to be more important than the former step. However, when macrophages were used as a cellular model for proteomic research, only 300 proteins were from bacterial cells, with most identified proteins belonging to the host cell b (Shi *et al*., 2006, 2009b). Moreover, the majority of the identified bacterial proteins were housekeeping proteins, as shown by their relatively high abundance, and most well-documented virulence factors were not detected. Likewise, in animal models where protein samples were extracted from mouse spleen or cecum, there was also a very low coverage of the bacterial proteome (Becker *et al*., 2006). Consequently, most studies have used an *in vitro* medium to mimic the *in vivo* situation, hoping to provide an important foundation for the much more complex dual-organism investigations.

In this study, we chose the RPMI medium to culture bacteria and compared the proteomic expression of different *Salmonella* serovars. RPMI has less amino acid contamination than the commonly used Luria-Bertani (LB) broth and therefore is better for proteomic research. Using Stable Isotope Labeling with Amino acids in Cell culture (SILAC) technology we identified proteins that were differentially expressed between *S.* Typhimurium and *S.* Typhi. These proteins, and their corresponding genes, are likely to explain the distinct syndromes caused by different serovars and could

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serve as biomarkers for clinical diagnosis.

Materials and Methods

Bacterial strains and SILAC culture conditions

S. Typhimurium LT2 (abbr. STM) and *S.* Typhi Ty2 (abbr. STY) were used in this study. A mono-clone of the bacteria was cultured in LB broth at 37°C overnight. 25 μl of each overnight culture was transferred to 50 ml SILAC medium and grew to late log phase. The composition of SILAC medium was as follows: the L-Lys- and L-Arg-depleted RPMI media (Invitrogen, USA) with the addition of 20 mM HEPES buffer plus either $12C_6$-L-Lys/$12C_6$-L-Arg (light medium, Isotec, USA) or $13C_6$-L-Lys/$13C_6$-L-Arg (heavy medium, Isotec). STM and STY were cultured by heavy medium and light medium, respectively. Equal amount of STM and STY bacteria were blended for protein extraction, and the extraction was fulfilled with 0.1% SDS solution by sonication. The BCA kit (Thermo Fisher, USA) was used to determine the protein concentration. 50 μg protein extracts were alkylated with dithiothreitol and iodoacetamide, and then were digested with 1 μg modified trypsin (Promega, USA) at 37°C overnight.

The LC-MS/MS experiment

Bacterial proteins were separated by 2D-SCX/RPLC system (Dionex, Germany). Briefly, the protein samples were dissolved in 50% acetonitrile containing 0.1% formic acid and then were loaded onto SCX column (Phenomenex, USA). An ammonium chloride concentration gradient with 0.1% formic acid and 30% acetonitrile was used to elute the peptides. The salt gradient was sectionalized in 15 steps, each of which took 90 min, and went on to the second dimensional reverse phase separations. Prior to the arrival at the trapping column, the isocratic loading pump was utilized for diluting the effluent of SCX column. Meanwhile, the other RP-trapping column, which was installed on the same valve, was linked with the RP-separating column. Before finishing the salt gradient step, the binary pump for the SCX separation was turned off and the six-port valve was switched to permit the loading pump to wash the residual salt solution away in the RP-trapping column. After the trapping column was switched to the RP analytical column, the bound peptides were eluted with an acetonitrile gradient in the presence of 0.05% formic acid.

The effluent obtained from the above was analyzed by a LTQ-Orbitrap hybrid mass spectrometer (Thermo Electron, Germany). The IDA mode was adopted when operating the mass spectrometer. Full scan MS spectra (the m/z value ranged from 300 to 2,000) were acquired in the Orbitrap at a resolution of 60,000. The ten most intense ions in each MS spectrum were selected for fragmentation in the linear ion trap (MS/MS). Each precursor ion would be analyzed twice and excluded in the next minute. The MS/MS isolation width and the maximum precursor accumulation time for MS/MS were set to be 2 Da and 150 ms, respectively.

Protein identification and quantification

The entire experiment, from the culturing of the bacteria to the LC/MS, was repeated twice. The raw MS files from the LTQ-Orbitrap were analyzed by Mascot v2.3 and MaxQuant v1.2 (Matrix Science, USA). The protein sequences of STM and STY, which were downloaded from NCBI database, were put together to constitute a database, against which Mascot search engine searched the MS/MS spectra. The accession number of STM and STY genome were NC_003197 and NC_004631, respectively. The parameters setting for the Mascot search were as follows: cysteine carbamidomethylation was chosen as a fixed modification, while N-terminal acetylation and methionine oxidation were selected as variable modifications; two missed cleavages were allowed at most. The mass deviation of 5 p.p.m. and 0.5 Da were set for parent mass and fragment ions, respectively. The Mascot result was further analyzed by MaxQuant to acquire the quantitative ratio between STM and STY. The following parameter settings were adopted: peptides should contain at least 6 amino acids; false discovery rate must be lower than 0.01; and a posterior error probability for each MS/MS spectrum must be 0.1 or lower.

qPCR validation

The bacteria were cultured in SILAC medium as described in SILAC labeling procedure. Then the bacteria was washed with PBS and stored in TRIzol reagent (Invitrogen). The RNA was isolated according to the phenol-base method. In order to avoid the contamination from genomic DNA, the RNA was treated with DNAse I (Epicentre, USA) and purified again with the Nucleospin RNA clean-up kit (Macherey-nagel, Germany). Approximately 1 μg of total RNA were reverse transcribed to first-strand cDNA with iScript cDNA synthesis kit (Bio-Rad, USA). The qPCR was performed according to the manufacturer's instructions on CFX96 instrument with SybrGreen Supermix (Bio-Rad). The final concentration of primers was 0.25 μM in a total volume of 25 μl. The thermal cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of 30 sec at 95°C and 30 sec at annealing temperature. The expression of each gene was normalized to *putP*, the expression of which was not affected in different growth conditions.

Results and Discussion

Salmonella proteins identified by SILAC

STM and STY were cultured in RPMI medium, and late-log cultures were collected for proteome analysis. SILAC was used to compare the proteomes of the two serovars. The genomes of STM and STY contain 4425 and 4318 protein-encoding genes, respectively, with 3749 genes shared between the two serovars that can be regarded as the "core genome" of *S. enterica* subspecies I. In this study, 1591 and 1542 proteins were identified for STM and STY, respectively (Supplementary data Table S1). A total of 1506 proteins were common between the two serovars. Most of the proteins were involved in basic biological functions; only seven were encoded by genes present on *Salmonella* Pathogenicity

Island (SPI), i.e., SitA and SitB by SPI-1, STM1388 and STM1389 by SPI-2, STM3755 by SPI-3, and STM0307 and STM0308 by SPI-6. While SitA and SitB can enhance bacterial survival ability in macrophages in the Nramp1^{G169} murine typhoid model (Zaharik *et al*., 2004), the other five proteins have not been associated with *Salmonella* virulence.

We found 85 and 36 proteins that were expressed uniquely in STM and STY, respectively. A large proportion of these serovar-specific genes were accessory genes rather than housekeeping genes. Apart from those encoded by *Salmonella* Pathogenicity Island (SPI), many were also originated from laterally transferred elements, such as Fels phage and Gifsy phage (Figueroa-Bossi *et al*., 2001). However, very few of the serovar-specific proteins have previously been shown to be involved in pathogenesis. Meanwhile, many of the known virulence factors were not detected in our analysis. For example, *S.* Typhi characteristically produces Vi capsule, which is encoded by the *viaB* operon carried within SPI-7; but we did not identify Vi proteins in our study. We attributed to the failure to detect known virulence factors to the following reasons. Firstly, the expression level of these proteins may be so low that the sensitivity of our protocol was not sufficient to detect them. Second, certain virulence factors are part of the cytoskeleton and are therefore insoluble. Our detection method may be better able to detect soluble proteins and therefore not identify insoluble proteins. Finally, RPMI medium does not mimic *in vivo* conditions; therefore virulence factors may not be induced under these conditions.

Table 1. **Proteins specifically expressed in STM**

Locus_tag	Gene	Product
PSLT031	*rsdB*	Resolvase
PSLT043	-	Type II secretion system protein
PSLT046	-	Putative carbonic anhydrase
PSLT048	*tlpA*	Alpha-helical coiled-coil protein
PSLT051	-	Putative cytoplasmic protein
PSLT052	*parA*	Plasmid partition protein A
PSLT053	*parB*	Plasmid partition protein B
PSLT103	*traT*	Surface exclusion protein
STM0327	-	Putative cytoplasmic protein
STM0920	-	Ail/OmpX-like protein
STM0924	-	Putative CuZn superoxide dismutase
STM0928	*nanH*	Neuraminidase
STM1012	-	Probable regulatory protein
STM1044	*sodC*	Superoxide dismutase precursor
STM1549	-	Putative translation initiation inhibitor
STM1572	*nmpC*	Putative outer membrane porin precursor
STM1627	-	Alcohol dehydrogenase class III
STM1959	*fliC*	Flagellar biosynthesis protein
STM2232	*oafA*	O-antigen acetylase
STM2238	-	Hypothetical protein
STM2610	-	Hypothetical protein
STM2724	-	Hypothetical protein
STM2726	-	Hypothetical protein
STM2746	-	Putative ATPase
STM2771	*fljB*	Flagellar biosynthesis protein
STM3084.S	-	Putative regulatory protein
STM3533	-	Putative transcriptional regulator
STM3753	*sugR*	ATP binding protein
STM4417	-	Putative transcriptional regulator
STM4421	-	Putative NAD-dependent aldehyde dehydrogenase
STM4491	-	Putative ATP-dependent Lon protease
STM4492	-	Putative cytoplasmic protein
STM4495	-	Putative type II restriction enzyme
STM4496	-	Putative DNA repair ATPase
STM4498	-	Putative inner membrane protein
STM4524	*hsdS*	Type I restriction enzyme specificity protein

Potential biomarkers distinguishing *Salmonella* serovars

Not all genes that are expressed in a serovar-specific manner can be directly used as biomarkers. Certain genes may exist in the genome but require specific culture conditions to activate their expression. For example, if one protein was detected only from STY under the current experimental conditions but the corresponding gene was also present in the genome of STM, it would not be considered to be a suitable biomarker for identification of STY. We therefore further filtered the specifically expressed proteins by aligning their sequences against the genomic sequences of the two serovars. As a result, only 36 and 15 proteins were truly specific to STM and STY, respectively, at both the genomic and proteomic level, and can be regarded as candidate biomarkers for clinical diagnosis (Tables 1 and 2). FliC and RfbE, which are proteins associated with flagella and somatic antigen, were identified as being serovar-specific and are currently used in the Kauffman-White *Salmonella* serotyping scheme. Serotyping is one of the most commonly used methods for typing *Salmonella* lineages. Nevertheless, this method often generates false-positives or false-negatives because the expression of flagella and somatic antigen is not always stable. Therefore, it is perhaps necessary to develop new biomarkers to complement the current serotyping scheme.

Although we didn't detect the expression of Vi capsule in STY, we did identify the peptides from the gene t4290 and t4325, both of which are located in SPI-7 but not in Vi biosynthetic operon. SPI-7 has only been found in three *Salmonella* serovars, i.e., *S.* Typhi, *S.* Paratyphi C and *S.* Dublin (Pickard *et al*., 2003). In *S.* Typhi, SPI-7 comprises four parts:

Table 2. **Proteins specifically expressed in STY**

Locus_tag	Gene	Product
t0466	-	Putative lipopolysaccharide modification acyltransferase
t0784	*rfbE*	CDP-tyvelose-2-epimerase
t0786	*rfbV*	Putative glycosyl transferase
t0918	*fliC*	Flagellar biosynthesis protein
t1874	-	Putative bacteriophage protein
t1896	-	Putative bacteriophage protein
t2842	-	Hypothetical protein
t3402	*cI*	Repressor protein
t3413	-	Possible lipoprotein
t3414	-	Hypothetical protein
t3415	-	Hypothetical protein
t3588	*yiiF*	Putative CopG-family DNA-binding protein
t4290	-	Hypothetical protein
t4325	-	Hypothetical protein
t4521	-	Hypothetical protein

Table 3. **Expression of SPI-1 and SPI-2 in STM and STY**

Locus_tag	Location	Gene	Product	Ratio (STY/STM)
STM2873	SPI-1	prgI	Needle complex major subunit	0.97678
STM2882	SPI-1	sipA	Secreted effector protein	1.51928
STM2898	SPI-1	invG	Outer membrane secretin precursor	1.17821
STM1392	SPI-2	ssrA	Sensor kinase	0.77128
STM1405	SPI-2	sseG	Secreted effector protein	0.96788
STM1416	SPI-2	ssaO	Type III secretion system apparatus protein	1.06587

the type IVB pilus operon, the SopE prophage, the Vi biosynthetic operon, and a 15 kb phage. In contrast, in *S.* Paratyphi C and *S.* Dublin, only the Vi biosynthetic operon is intact (Pickard *et al.*, 2003; Tam *et al.*, 2004). We next searched the genomes of *S.* Paratyphi C str. RKS4594 (accession no. NC_012125) and *S.* Dublin str. CT_02021853 (accession no. NC_011205) for the t4290 and t4325 sequences, and determined that t4290 is present in *S.* Paratyphi C but absent from *S.* Dublin, whereas t4325 is absent from both *S.* Paratyphi C and *S.* Dublin. Based on these observations, we propose that t4325 is a good candidate to be developed as a biomarker for diagnosing *S.* Typhi.

Although the serovar-specific genes can be used as biomarkers, it is unlikely that the differences in clinical symptoms caused by *S.* Typhimurium and *S.* Typhi are caused by such a small number of specific proteins, especially when most of these genes were annotated as hypothetical proteins. It is much more likely that the differential expression of common genes contributes to the differences in phenotype between the two serovars.

Expression of SPI-1 and SPI-2

SPI-1 and SPI-2 are SPIs that are conserved in the entire *S.* enterica sp. I lineage. Although they both harbor a type three secretion system (TTSS), SPI-1 and SPI-2 function at different stages of the infection: the former is required for invading non-phagocytic cells, while the latter protects the *Salmonella*-containing vacuole (SCV) from the effects of the phagocytic defense enzymes (Shea *et al.*, 1996; Galan, 2001). Previous studies have shown that *S.* Typhimurium grown to late logarithmic phase in rich medium expresses SPI-1 genes, while in contrast, the acidic, iron-, magnesium-, and phosphate-depleted minimal medium (MgM) would induces the up-regulation of SPI-2 genes (Adkins *et al.*, 2006; Shi *et al.*, 2009a).

The RPMI medium used in the current study is a kind of rich medium similar to LB broth, and therefore mimics the in vivo environment of the epithelial cells, in which *Salmonella* would be expected to express SPI-1 genes. However, neither SPI-1 nor SPI-2 proteins could be detected in both STM and STY, possibly because the levels of these virulence factors were too low to be detected by our protocol. We then examined the mRNA expression of SPI-1 and SPI-2 by quantitative PCR (qPCR). The SPI-1, 2 genes selected for qPCR encode a TTSS needle complex and secreted effector, and therefore represent the function of the entire SPI.

The qPCR analysis showed that the SPI-1 and SPI-2 genes were expressed at similar levels in STM and STY (Table 3). There are at least two possible explanations for this phenomenon. First, the different clinical outcomes of STY and STM are not caused by differential expression of SPI-1 and SPI-2 genes but by that of other genes, though the role of SPI-1 and SPI-2 in pathogenesis is generally important in both two serovars. Second, the progression of typhoid fever can be divided into several environmentally diverse stages for the bacteria, e.g. the initial invasion of intestinal epithelial cells, the internalization within macrophages, and the dissemination through the circulatory system. The RPMI medium, like any other type of medium, only mimics one stage of the infection. The detail stage that distinguishes STY from STM remains unknown, therefore the proteome of the bacteria grown in the RPMI medium does not necessarily reflect the most typical expression pattern of STY. The above two explanations do not contradict each other, and may each account for a part of the difference.

Quantitative proteomic expression

The bacterial regulatory system is so complex that even tiny genomic difference can lead to differential expression of identical genes, and thereby cause entirely different phenotypes. Here we used SILAC technology to quantify protein expression patterns with the aim of identifying the proteins that are commonly present but differentially expressed between serovars. Because SILAC technology assesses the abundance ratio by measuring the peak intensities of the same peptide within the compared samples, the final number of proteins carrying a quantitative value was 1459, which was slightly less than the number of identified proteins shared by both STM and STY. The log ratio values (Log_2) followed a normal distribution pattern, with the peak at 0 (Fig. 1). This pattern fulfilled the general assumption of genome-scale comparative expression experiments (including SAGE and microarray) that only a small number of proteins were differentially expressed and were responsible for the phenotypic difference.

When we set two-fold change as the threshold, 310 proteins were differentially expressed. When the threshold was

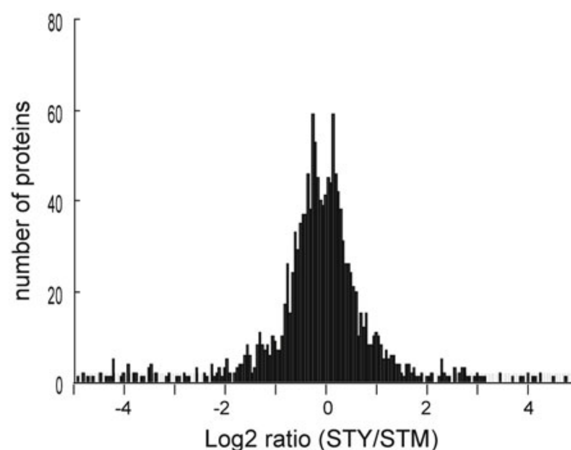

Fig. 1. **Distribution of expression ratio between STY and STM.**

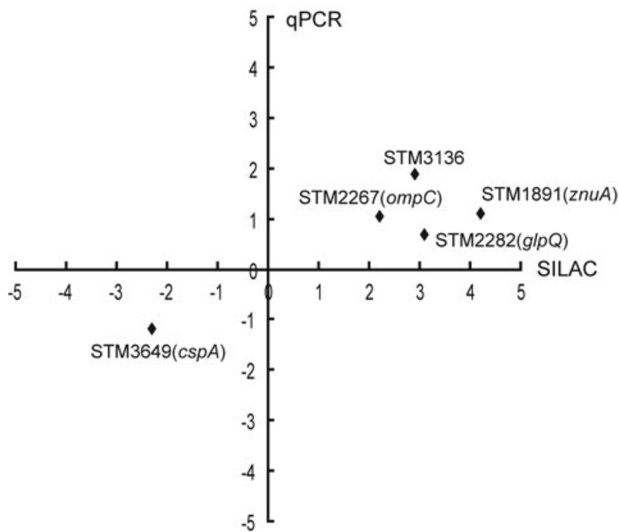

Fig. 2. Comparison of expression ratio by SILAC and qPCR. Both were represented in the form of Log2 ratio (STY/STM).

increased to four-fold, 122 proteins were differentially expressed, nearly one tenth of the total proteins that had quantitative value. Of these, 72 proteins were down-regulated in STY in comparison to in STM, while the remaining 51 proteins were up-regulated in STY (Supplementary data Table S2). Several up-regulated proteins, such as OmpC and CadA, are involved in enhancing bacterial survival *in vivo* (Park *et al*., 1996; Negm and Pistole, 1999). However, no SPI-encoded proteins appeared in the list of differentially expressed proteins. We therefore suspected that the core genes, which comprise the ubiquitous backbone of *Salmonella* genomes, are responsible for the serovar-specific phenotypes through their differential expression.

We noticed that many differentially expressed proteins were adjacent to each other in terms of their positions in the genome. For example, flagella proteins (FlgN, FlgE, FliG, FliL, FliM) and chemotaxis proteins (CheZ, CheY, CheB, CheM, CheW, CheA) were down-regulated in STY compared to in STM. This phenomenon is perhaps related to the different habitats of STM and STY: the former is restricted to the gut, while the latter can disseminate systemically. Decreased flagella and chemotactic activity can lead to a smoother cell surface, assisting with cellular invasion and escape from the host's pro-inflammatory responses (Jones *et al*., 1992). A number of differentially expressed genes were concentrated within some operons that are involved in the metabolism and transport of carbohydrates and amino acids. For example, *ilvI* and *ilvH* participate in acetolactate synthesis, *trpB* and *trpA* are involved in tryptophan synthesis, *glnQ* and *glnH* are responsible for transporting glutamine, and *oppA*, *oppB*, *oppD*, and *oppF* transport oligopeptides. For part of them, STY had a higher expression level than STM; but for the others, STM had a higher expression level. These results indicate that the two serovars may utilize different carbohydrate sources and amino acids, and that this difference in metabolic capability is likely to be the source of the different clinical manifestations of the two serovars.

Several differentially expressed genes were selected for qPCR analysis to verify if gene expression was consistent between the mRNA and protein levels. All genes showed a consistent trend between proteomic data and qPCR results (Fig. 2), indicating the reliability of the SILAC technique. However, the fold change value shown by qPCR was always a little lower than that shown by SILAC, which we could not explain satisfactorily.

In conclusion, SILAC is an efficient and reproducible quantitative proteomic technology, and is being increasingly applied in the broad field of cell biology. In this study, we used SILAC technology to compare the proteomes of representative *Salmonella* serovars. To the best of our knowledge, this is the first direct quantitative comparison of protein expression between *Salmonella* serovars. We identified several proteins that were likely responsible for the characteristic pathogenesis of each serovar, or which could be used as potential biomarkers for clinical diagnosis. However, further experimental confirmation is required. Interestingly, the differentially expressed genes were found to be involved in basic cellular metabolism rather than virulence. While this finding also requires further study to assess whether it is also the case *in vivo*, it at least reminds us to examine the role of these core genes in pathogenesis.

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