Int. J. Mol. Sci. **2007**, *8*, 116-135

International Journal of
Molecular Sciences
ISSN 1422-0067

www.mdpi.org/ijms/

Genome-Wide Scan of the Gene Expression Kinetics of *Salmonella enterica* Serovar Typhi during Hyperosmotic Stress

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Received: 10 December 2006 / Accepted: 12 February 2007 / Published: 16 February 2007

Abstract: *Salmonella enterica* serovar Typhi is a human enteroinvasive pathogen that can overcome the stress caused by the high osmolarity of the human small intestine and cause systemic infection. To investigate the global transcriptional regulations of *S. enterica* serovar Typhi exposed to a hyperosmotic environment, a genomic oligo-DNA microarray containing 4474 *Salmonella* genes was prepared. A wild strain of *S. enterica* serovar Typhi GIFU10007 was grown in LB medium containing 50 mM NaCl to simulate a low osmotic environment. The hyperosmotic stress was simulated by an osmotic up-shift, which increased the concentration of NaCl in the LB from 50 mM to 300 mM. Genome-wide gene expressions of *S. enterica* serovar Typhi at 15 min, 30 min, 60 min, and 120 min after the osmotic up-shift were investigated by the microarray analysis. Gene expression profiles in somewhat later stage (60 ~120 min) of the stress were quite different from those in the early stage (0 ~ 30 min) of the stress. At 120 min after the osmotic stress, the expression levels of 889 genes were obviously changed. However, expression levels of only 382 genes were significantly changed at 15 min after the osmotic stress. The expression levels of most SPI-1 genes associated with invasion of the pathogen were increased at 120 min after the osmotic up-shift, but were not obviously changed at 15 min or 30 min after the osmotic stress. Expressions of a central regulatory gene, *phoP*, and sigma factor genes *rpoE*, *rpoD*, and *rpoS* were also changed with different profiles during the osmotic stress. These results indicated that the invasive ability of the pathogen is significantly increased after 2 h of

hyperosmotic stress, and regulator PhoP and sigma factors RpoE, RpoD appear to participate in the network regulatory mechanisms that benefit the pathogen to adapt hyperosmotic environmental conditions. The later increased invasive ability of *S. enterica* serovar Typhi after hyperosmotic stress may be one reason why the pathogen performs invading in the distal ileum of human and not in areas of the upper small intestine.

Keywords: *Salmonella enterica* serovar Typhi; hyperosmotic stress; microarray; gene expression.

1. Introduction

Salmonella enterica serovar Typhi is a gram-negative, enteroinvasive pathogen that may cause typhoid fever. The infection is initiated when *Salmonella* enter the host from contaminated water or food in the gastrointestinal tract. The bacteria cells reach the distal ileum and enter the specialized intestinal epithelial M cells of Peyer's patches. Following intestinal invasion, the pathogen migrates into the mesenteric lymph nodes and reaches the liver, spleen, and bone marrow though the blood and lymph systems, where they replicate themselves and cause systemic infection [1-3]. Intestinal invasion by *Salmonella* is associated with many pathogen-specific factors, including Type III secretion systems, secretion of invasion related proteins, flagella and motility, and reducing of Vi polysaccharide [3-7].

Most invasion factors of *Salmonella* are affected by the osmolarity of the surrounding environment [7-9]. When *S. enterica* enters the intestine lumen of host, the bacterium is opposed to an extreme environment, including an increase in osmolarity. Therefore, the regulation of *Salmonella* in hyperosmotic environment is very important during the invasion through the small intestine.

The cross-regulation of different regulators demonstrates that the mechanisms of response to stress are quite complex [10, 11]. At present, the mechanisms by which these bacteria adapt to changes in environment remain unclear. Microarray analysis is an effective strategy for genome-wide screens of gene expression in bacteria. Although some microarray studies described bacterial gene expression changes in response to hyperosmotic stress [12, 13], however, kinetics of gene expressions of enteroinvasive pathogens after onset of the osmotic stress is rarely presented.

In the present study, to investigate systemic gene expression of *S. enterica*, we prepared a *Salmonella* genomic oligo microarray system that includes 4474 specific 40-mer oligonucleotides as the available genomic sequence information of *S. enterica* serovar Typhi Ty2 and serovar Typhimurium LT2. We used this system to investigate the kinetics of gene expression of *S. enterica* serovar Typhi during hyperosmotic stress. Many interesting gene expression profiles associated with invasion, motility and some regulators of the pathogen after hyperosmotic stress were found in this study.

2. Materials and Methods

2.1 Bacterial cultures

A wild strain of *S. enterica* serovar Typhi GIFU10007 isolated in Japan [14] was utilized in this study. Bacteria were grown with shaking at 37°C in Luria-Bertani (LB) broth (pH 7.0) containing 50 mM or 300 mM NaCl to simulate a low or high osmolarity environment, respectively. Cultures were incubated overnight, and then grown in fresh LB of same osmolarity to log phase (0.5 OD at 600 nm). Total RNA was extracted for investigation of gene expression under sustained high and low osmotic conditions. To simulate a stress of osmotic up-shift, NaCl was added to a final concentration of 300 mM to the low osmotic bacterial cultures at log phase, and the bacteria were then incubated with shaking at 37°C for 120 min. Total RNAs of bacteria were extracted at 15 min, 30 min, 60 min, and 120 min to investigate the kinetics of genome-wide changes in gene expression in response to hyperosmotic stress.

2.2 Preparation of Salmonella oligo microarray and treatment of slides

Salmonella genomic oligo microarray was constructed, which contains 4370 genes of *S. enterica* serovar Typhi Ty2, 102 genes of plasmid of *S. enterica* serovar Typhimurium LT2, and 2 *fljBA*-like genes identified in z66 antigen-positive strains of *S. enterica* serovar Typhi [15]. Plus-chain-specific oligonucleotides were designed according to genomic information for *Salmonella* (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html.) All 40-mer oligonucleotides were synthesized by TaKaRa company (TaKaRa, Tokyo, Japan), and decorated with an amino group at each 5'-terminus to fix the oligo on chip slides. To make the chips, 50 μM oligonucleotides stock solution was mixed with an equal volume of N6 spotting solution (Toyo Kohan, Tokyo, Japan), and stamped on GENE DIA slides (75 × 25 mm, Toyo Kohan, Tokyo, Japan) with a robotic slide printer MicroGrid II (BioRobotics, UK). According to the manufacture's instructions, the spotted oligonucleotides were fixed on the chips at 80°C for 2 h. Slides were blocked just before hybridization by incubation in blocking buffer (5× SSC, 0.2% SDS) at 95°C for 5 min, rinsed with distilled water and dried by centrifugation (1000 rpm for 10 min) at room temperature.

2.3 RNA extraction and cDNA probe labeling

Bacterial cells were cooled on ice for 10 min, harvested by centrifugation (4000 rpm for 10 min at 4°C), and lysed in 100 μl of lysozyme-TE buffer (0.6 mg/ml lysozyme, pH 8.0) within 5 min at 25°C. Total RNA was extracted with an RNeasy mini-column (QIAGEN), according to the manufacturer's instructions. The quantity and quality of the extracted RNA were checked with an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). The extracted total RNA was treated with 1 U of RNase-free DNase I (TaKaRa) at 37°C for 10 min to diminish the trace mixed DNA, and then incubated at 85°C for 15 min to inactivate the DNase. cDNA probes were synthesized with a CyScribe First-Strand cDNA Labeling Kit (Amersham Pharmacia). The reverse transcription and labeling was performed in a 20-μl reaction containing 20 μg total RNA, 50 ng random nonamers, 1 μmol of cy3- or cy5-conjugated dCTP, and others as the manufacturer's instructions. After a 90-min reaction at 42°C, RNA was degraded by addition of 2 μl 2.5 N NaOH and a 15-min incubation at 37°C

followed by neutralization with 10 µl 2 M Hepes free-acid. The labeled cDNAs were purified over AutoSeq G-50 columns (Amersham Pharmacia).

2.4 Hybridization and scanning

The cy3-and cy5-labeled cDNAs derived from the wild strain of *S. enterica* serovar Typhi grown under different osmotic conditions were pooled and dried with a speed vacuum, and then dissolved in 40 µl of hybridization buffer (5× SSC, 0.2% SDS, 1 mg dextran sulfate, 80 µg salmon sperm DNA, 40 µg bovine serum albumin, 40 µg ficoll). Each slide was covered with a cover glass (22 × 40 mm, Matusnami, Osaka, Japan), and then hybridized in a small humidified chamber (78 × 28 × 5 mm) at 50°C for 15 h. The cover glass was removed in 0.1× SSC, and the slides were washed for 15 min in pre-warmed (45°C) washing buffer (2× SSC, 0.1% SDS), rinsed with distilled water, and then dried by centrifugation (1000 rpm for 10 min). Slides were scanned with a microarray analysis system ScanArray 4000 (GSI Lumonics, USA) with two channels, the cy3 and cy5 appropriate lasers. Images were exported as TIFF files for digital analysis. Each experiment was performed on duplicate slides and at least three times with different RNA samples.

2.5 Data analysis

The TIFF files of data for two channels for each slide were converted to the digital density data with DNasis-Array software (Hitachi, Tokyo, Japan). After a visual check, the intensity of the signal from each spot was normalized with the total intensity in each channel. The digital data were exported and transferred to a Microsoft Excel file. The quality control and subsequent analysis was performed essentially as described previously [16, 17]. In brief, 96 negative control spots that only spotted buffer were stamped, were used to correct local backgrounds. After local background subtraction, only signals that showed a two-fold higher than the average of negative controls in each channel were used to calculate the ratio of two channels to view expression differences. The average of ration for different slides was calculated, and a 2-fold difference was necessary for the change in expression to be considered significant. The results were then expressed as $\log_2$ (ratio) on profile plots, and heat maps with the Avadis Explor software (Strand Genomics, Bangalore, India).

2.6 RT-PCR

RNA extraction and treatment were performed as described above. Reverse transcription was performed with random hexamers and specific reverse primers by using SuperScript II (Invitrogen), according to the manufacturer's instructions. Specific primers used in this RT-PCR are described in Table 1. Each 20 µl reaction contained 2 µg of RNA, 10 ng random hexamers and 10 nmol specific reverse primers. One microliter of product was subjected to the quantitative PCR assay, which was performed in Mx3000P QPCR Systems (Stratagene) with SYBR green master mix (Applied Biosystems), according to the manufacturer's instructions. Fluorescence was measured in an additional step (80°C for 10 sec) after synthesis each cycle. Serious diluted genomic DNAs were used to make a standard curve at same times to calculate reference mRNA copies in samples. Each experiment was performed with four different samples.

Table 1. Specific primers using in the RT-PCR.

Genes	Forward primers	Reverse Primers
iagA	5'-TTCATGGCTGGTCAGTTG	5'-GGGATCCTGTTTCCATCTT
invF	5'-TGTGAAGGCGATGAGTAA	5'-CGTTGTCGCACCAGTATC
invH	5'-TTTTACTGATCGGCTGTG	5'-TAAGGCTTGCAGTCTTTCAT
spaM	5'-CTCCTCTGACTCGGCCTCT	5'-ATTCGCTGACCAGAATTA
phoP	5'-GAAGGCTGGCAGGATAAAGT	5'-CCGTGAGTTTGATGACCTCT
mig-14	5'-AACCTTCCCGCTATCTTC	5'-AGTGGCCTGCATTCATTT

3. Results

3.1 Profiles of genome-wide expression kinetics under osmotic up-shift conditions

Of 4474 genes on the microarray, approximately 3300 usable digital data was gotten in profiles of genomic transcriptional expression of the wild strain of *S. enterica* serovar Typhi incubated at low and high osmolarity to log phase. Other genes were not detected in most of experiments of the study, due to the intensities of one or two channel less than 2-flod of the relative negative control after correction for the background.

We used our microarray system to investigate systemic gene expressions of *S. enterica* serovar Typhi at various time points during 120 min after an osmotic up-shift and the expression in sustained high osmotic condition. Profiles of genome-wide expression are shown in Figure 1 and Figure 2A. Expression of 382 genes was changed at 15 min. Expression levels of 170 genes and 212 genes were decreased and increased, respectively (Figure 2B). Expression levels of approximately 40% of these genes had been returned to the pre-stress levels by 120 min after the osmotic up-shift stress. At 120 min after the osmotic up-shift, differential expression of 889 genes was observed. More than 700 of these genes had not obvious change in expression at 15 min (Figure 2C). Most of them were also no obvious change under the sustained high osmolarity conditions. A few of them, however, were reversely expressed under the sustained hyperosmotic conditions (Figure 2D). When the bacteria were incubated in LB medium containing 300 mM NaCl overnight, only 85 genes increased expression and 112 genes decreased expression.

Genes with altered expression at 15 min and 120 min after the up-shift and under sustained hyperosmotic conditions are listed in table 2. These expressional profiles revealed that the majority of changes in gene expression in *S. enterica* serovar Typhi appear somewhat later after the osmotic up-shift stress. We will describe the differential expression of some particularly interesting genes in following sections.

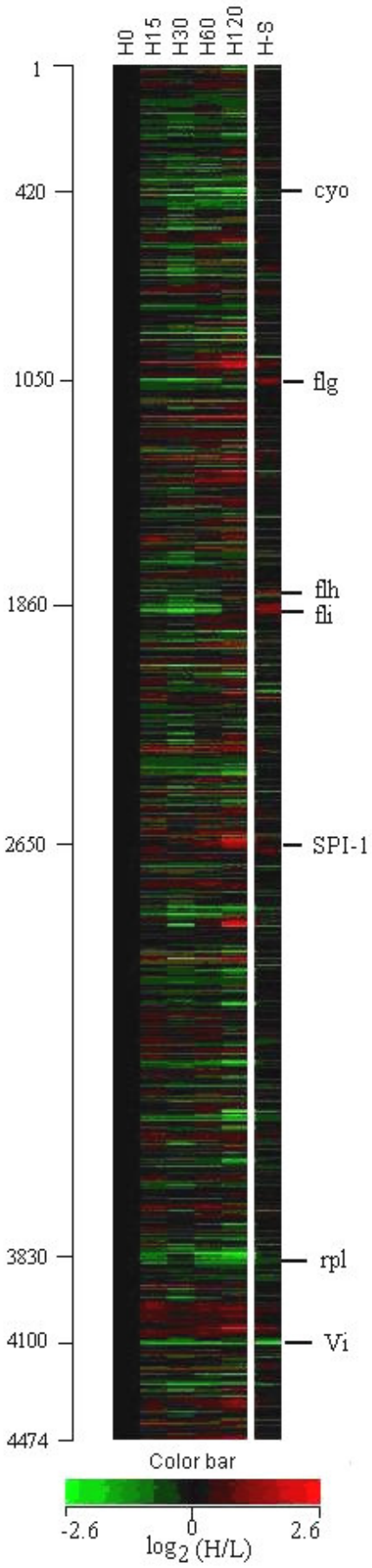

Figure 1. Heat map of genomic expressions of *S. enterica* serovar Typhi under up-shift and sustained hyperosmotic conditions. A wild strain of *S. enterica* serovar Typhi GIFU 10007 was cultured to log phase in LB broth (pH 7.0) containing 50 mM NaCl as a low osmotic environment, and then grown under an osmotic up-shift conditions when NaCl was added to a final concentration of 300 mM. Total RNAs were then extracted at 15, 30, 60, and 120 min after addition of NaCl to investigate the kinetics changes in gene expression in response to hyperosmotic stress. The expression difference between high and low osmolarity is indicated by different colors; colors from green to red indicate changes of -2.6 to 2.6 of the $\log_2$ ratio. In other words, the change in expression from 5-fold repression to 5-fold stimulation is indicated by a color scale. H0, H15, H30, H120, and H-S indicate 0 min, 15 min, 30 min, 60 min, and 120 min after the up-shift and the sustained hyperosmotic conditions, respectively. Expression change of each gene at H0 was set as zero (unchanged). The gene order number as determined by the genomic location is indicated on the side of the figure. Some gene clusters of interest are indicated on the right.

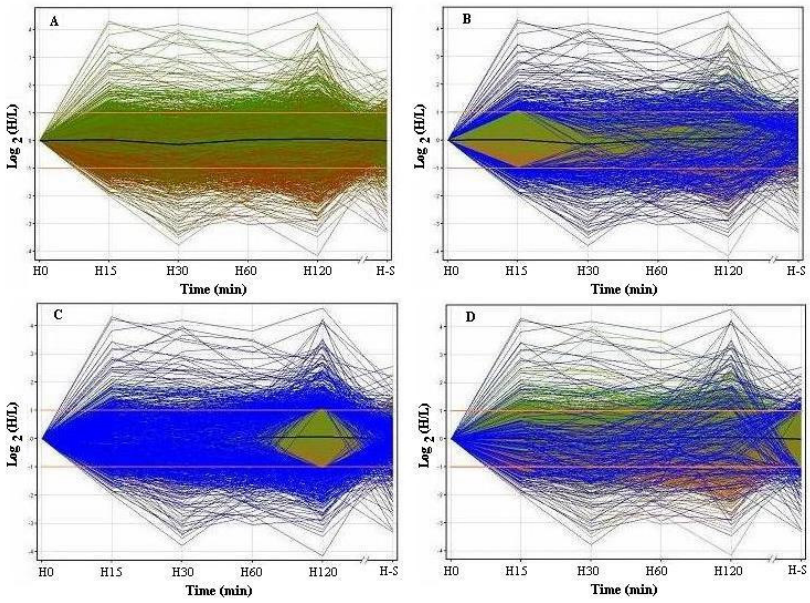

Figure 2. Profile plots of genome-wide expression kinetics during an osmotic up-shift stress. The ratio of the level of expression of each gene between high and low osmotic conditions was calculated, and the change in expression under hyperosmotic conditions were shown as the $\log_2$ ratio on profile plots. H0, H15, H30, H120, and H-S of X-axis of plots indicate 0 min, 15 min, 30 min, 60 min, and 120 min after the up-shift and the sustained high osmotic conditions, respectively. (A) Plot of expression kinetics of total 3323 genes or ORFs (effective data). (B) Plot of kinetics of expression for genes with obviously altered expression at 15 min after the osmotic up-shift. One hundred seventy repressed and 212 induced genes or ORFs are indicated as blue lines. By 120 min of the up-shift, approximately 40% of them returned to the pre-stress levels. (C) Plot of expression kinetics of genes or ORFs with obviously altered expression at 120 min after osmotic up-shift. Expression of 889 genes or ORFs including 400 repressed and 489 induced was altered. More than 700 of them did not have obviously altered expression at 15 min after the osmotic up-shift. (D) Plot of expression kinetics of genes or ORFs with obviously altered expression during culture under sustained high osmotic conditions. Expression of 197 genes was changed, including 112 repressed and 85 promoted genes or ORFs.

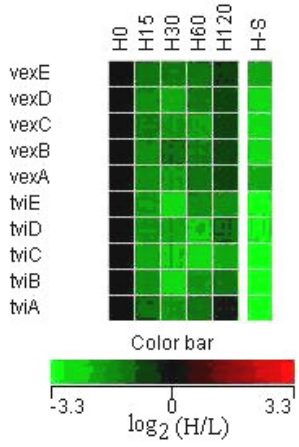

Figure 3. Expression of Vi capsular antigen genes. The difference between expression under high and low osmolarities is indicated by different colors; color bar from green to red indicate changes of -3.3 to 3.3 of the $\log_2$ ratio. In other words, the change in expression from 10-fold repression to 10-fold induction is indicated by a color scale. H0, H15, H30, H120, and H-S indicate 0 min, 15 min, 30 min, 60 min, and 120 min after the up-shift and the sustained high osmotic condition, respectively. Expression change of each gene at H0 was regarded zero. Expressions of *vex* and *tiv* operon genes were repressed soon after the increase in osmolarity, and this repression was high under sustained high osmotic conditions. Repression of expression of *vex* operon appears to be weakened at later time points after the increase in osmolarity.

Table 2. Genes of *S. enterica* serovar Typhi with altered expression after cultured under sustained hyperosmotic and osmotic up-shift conditions.

Functional kinds	Up-shift 15 min		Up-shift 120 min		Steady Hyperosmotics	
	decreased	induced	decreased	induced	decreased	induced
Total number	170	212	400	489	112	85
Regulators and putative regulators	4	12	17	22	5	6
Virulence and putative virulence proteins	4	1	1	15		8
Flagella and chemotaxis cluster proteins	15			1		26
Secreted and putative secreted proteins	1	5	2	20	1	1
Membrane and putative membrane proteins	6	24	18	45	5	3
Vi antigen relative proteins	9		3		10	
Fimbrial relative proteins		4		1		
Ribosomal proteins	11	1	46	1		
DNA replication related proteins		1	7			1
drug resistance proteins	2		6	1	1	
Metabolic enzymes and putative enzymes	57	51	136	147	38	15
Heat shock and cold shock proteins		5	1	1		1
Other functional proteins	31	30	83	58	18	10
Unknown function proteins	30	78	80	177	34	14

3.2 Expression of Vi capsular antigen genes

Vi capsular polysaccharide of *S. enterica* serovar Typhi is an important factor that allows the bacterium to survive in human macrophages [18, 19]. Vi capsular antigen was expressed at relatively high levels under conditions of low osmolarity. This expression was dependent on OmpR, a central regulatory protein that is a part of a two-component regulatory system with the osmosensor EnvZ [20]. After the osmotic stress, expression levels of all of 10 Vi-cluster genes, *tviA*, *tviB*, *tviC*, *tviD*, *tviE*, *vexA*, *vexB*, *vexC*, *vexD*, and *vexE* were decreased obviously (shown in Figure 3). The expression levels of those genes in the low osmolarity were 3- to 10-fold higher than those at high osmolarity. These results suggest that all Vi cluster genes were immediately and continually down-regulated after the osmotic up-shift.

3.3 Expression of flagella and chemotaxis genes

Flagella are necessary for motility of *Salmonella* and are an important pathogenic factor of *Salmonella*. Approximately 50 genes are associated with flagellar structure and function, have been divided into four regions on the basis of genomic location, and divided into three classes as expression regulation [21-23]. Most *S. enterica* serovars have two flagellin genes, *fliC* and *fljB*. However, *S. enterica* serovar Typhi is thought to have only *fliC*. The wild strain of *S. enterica* serovar Typhi GIFU10007 used in the present study is a z66 antigen-positive strain. The gene encoding z66 antigen was recently identified as an *fljB* gene, following with a downstream *fljA*-like gene [15]. The transcriptional regulation of *fljB*:z66 and *fljA*-like gene remains unclear.

With the present microarray analysis, most flagella-related and chemotaxis-associated genes were detected. Results are shown in Figure 4. The location of *fljBA*, which is separated from others in *S. enterica* serovar Typhimurium, is not yet known in *S. enterica* serovar Typhi. Expression levels of flagellar and chemotaxis genes of *S. enterica* serovar Typhi under the osmotic up-shift conditions were quite different from levels under the sustained high osmolarity conditions. Profiles of flagellar and chemotaxis gene expression are described in Figure 4. Expression levels of most region I and region III genes were decreased immediately after the increase in osmolarity. Expression of *flhC*, a global flagellar transcriptional activator gene, was mildly reduced after the shift, and a class-2 gene *fliA*, which encodes an RNA polymerase sigma factor for expression of the flagellar operon, was significantly decreased. In contrast, expression of *flgM*, *flgN*, *cheA*, and *cheW* was not changed, and expression of *fliC* and *fliE* was increased slightly. Under the sustained hyperosmotic conditions, expression levels of most flagella and chemotaxis genes were significantly higher than those in low osmolarity conditions. However, expression of some regulator genes, such as *fliA* and *flgM*, was shown little change.

3.4 Expression of invasion relative genes

High osmolarity could induce the expression of some SPI-1 genes [9]. In the present study, we found that expression of most SPI-1 genes was quite low under conditions of low osmolarity. Expression patterns of 21 SPI-1 genes (from *prgK* to *invH*) and several other invasion-related genes are shown in Figure 5. Expression kinetics during the osmotic up-shift showed that expression of most SPI-1 genes, including regulator genes *iagA* and *invF*, was greatly increased at 120 min after the onset of hyperosmotic stress, but not increased at 15 min or 30 min of the stress. Expression levels of *iagA*, *invF*, *invH*, and *spaM* at 15 min and 120 min of the stress were also investigated by RT-PCR. The RT-PCR results were similar to those of microarray analysis (Figure 7). Under sustained hyperosmotic conditions, expression levels of only a few SPI-1 genes, including *invH*, *spaM* and *prgH*, were mildly increased.

Expression of other invasion-related genes was examined (bottom of Figure 5). Expression level of *sirA*, which regulates SPI-1 genes by inducing *iagA* expression, was slightly decreased at early stages after the osmotic up-shift, but no change was observed in later stage of the osmotic stress. Expression levels of two oxygen inducing invasion genes, *orgAa* and *orgAb*, were not changed under any hyperosmotic conditions. *sopE* is locating outside of SPI-1 loci and encoding an toxin protein SopE, which is another important invasion factor and secreted into host cells by SPI-1 relative type III secretion system [24, 25]. The expression of *sopE* was identical to the expression of most SPI-1 genes, greatly promoted at 120min but not in early stage of the stress. Expression of other two invasion relative genes *sigE* and *sigD*, which were activated by InvF [26], was also induced at same time.

These results suggested that *S. enterica* serovar Typhi may increase the invasive ability by increasing expression of invasion-related genes at 120 min after entering a high osmotic surrounding.

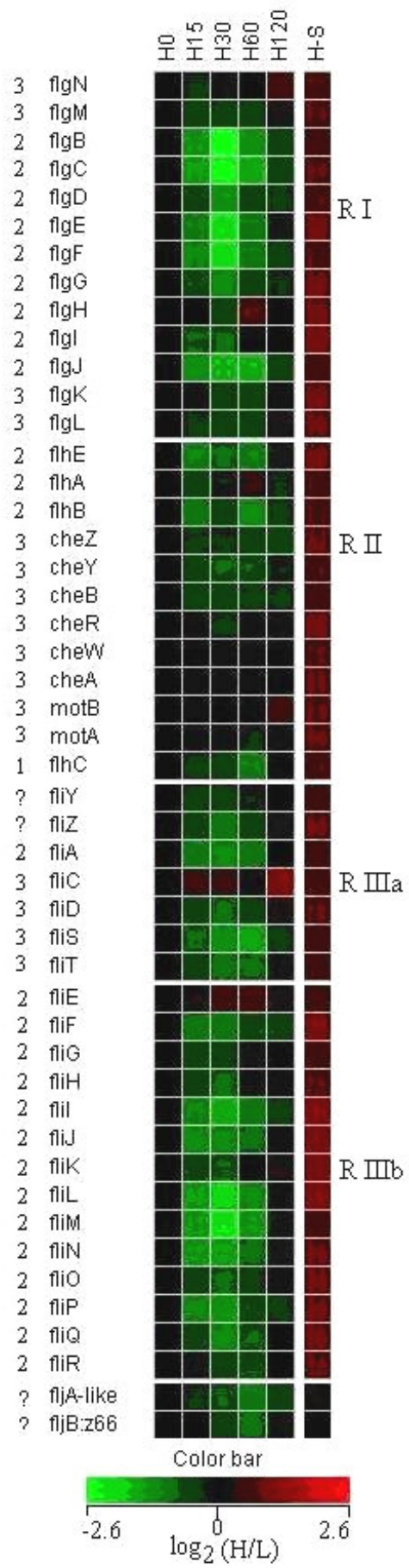

Figure 4. Expression of flagella and chemotaxis genes. The expression difference between high and low osmolarity is indicated by different colors; colors from green to red indicate changes of -2.6 to 2.6 of the $\log_2$ ratio. In other words, the change in expression from 5-fold repression to 5-fold induction is indicated by a color scale. H0, H15, H30, H120, and H-S indicate 0 min, 15 min, 30 min, 60 min, and 120 min after the up-shift and the sustained high osmotic condition, respectively. Expression change of each gene at H0 was set as zero. Flagella and chemotaxis genes are separated by genomic region, region I, II, IIIa, and IIIb and *fljBA* (unknown), respectively. The gene name and regulation class are listed on the left. Early after the up-shift, expression of most class 2 genes was repressed, especially at 30 min. By 120 min, expression of most genes had return to the pre-stress levels. Under sustained hyperosmotic conditions, expression of most genes was mildly increased.

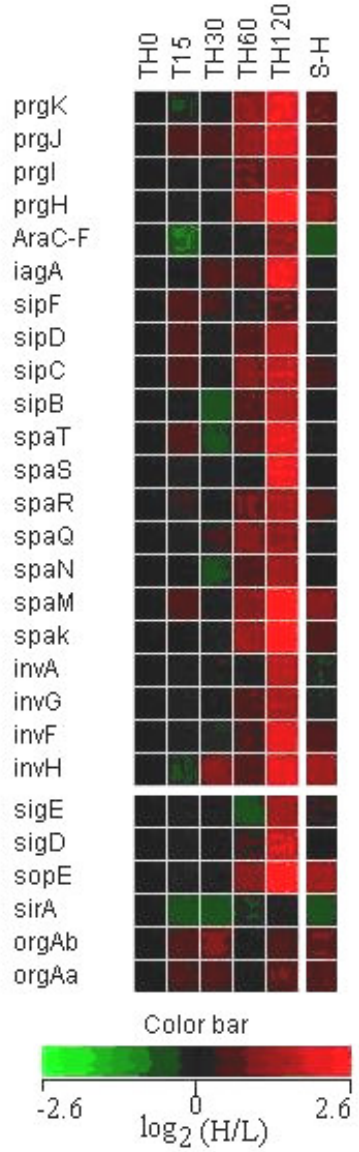

Figure 5. Expression of SPI-1 genes. The expression difference between high and low osmolarity is indicated by different colors; colors from green to red indicate changes of -2.6 to 2.6 of the $\log_2$ ratio. In other words, the change in expression from 5-fold repression to 5-fold induction is indicated by a color scale. H0, H15, H30, H120, and H-S indicate 0 min, 15 min, 30 min, 60 min, and 120 min after the up-shift and the sustained high osmotic condition, respectively. Expression change of each gene at H0 was set as zero. Gene names are listed on the left, and classified into two groups, SPI-1 genes and others. Under sustained hyperosmotic conditions, expression of only *invH*, *spaM*, and *prgH* was mildly induced. During the up-shift osmotic conditions, expression of most SPI-1 genes, including regulatory gene *iagA* and *invF* was greatly enhanced later but not immediately after the up-shift. Expression of the regulatory gene *sirA* was repressed slightly during the early stage after the up-shift.

3.5 Expression of regulatory genes

All regulatory and putative regulatory genes expressed under hyperosmotic conditions were identified from the effective data acquired in the present study. In addition to the regulatory genes described above, there were some sigma-factor genes, two-component regulatory system genes, and some other putative regulatory genes that appeared expression change during the osmotic up-shift conditions. Expression profiles of forty regulatory or putative transcription regulatory genes are shown in Figure 6. Most expression changes occurred transiently during the osmotic up-shift.

Expression of *rpoS*, which encodes sigma factor σ^S, under sustained hyperosmotic conditions was 2.1-fold higher than that under the low osmolarity, whereas it was not changed during the osmotic up-shift. Expression of *rpoE*, which encodes sigma factor σ^{24}, was decreased at 15 min and 30 min after the osmotic up-shift. Similar results were obtained for *rseA*, which is a negative regulator gene of sigma-24. Expression of *rpoD* was decreased later after exposure to osmotic stress but was not changed at the early stage of the osmotic stress or under the sustained hyperosmotic conditions.

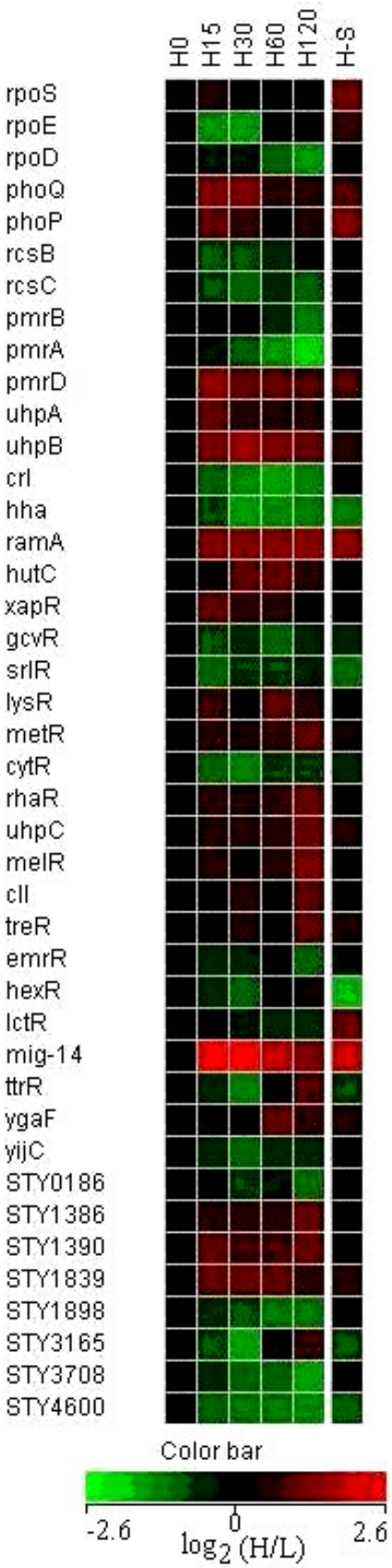

Figure 6. Expression of regulator genes. Differences between high and low osmolarity is indicated by different colors; colors from green to red indicate changes of -2.6 to 2.6 of the $\log_2$ ratio. In other words, the change in expression from 5-fold repression to 5-fold induction is indicated with a color scale. H0, H15, H30, H120, and H-S indicate 0 min, 15 min, 30 min, 60 min, and 120 min after the osmotic shift and sustained high osmotic condition, respectively. Expression change of each gene at H0 was set as zero. Gene names were listed on the left, and the STY numbers are synonymous to ORFs and that proposed functions are based on information contained in the NCBI database.

Virulence-related PhoP-PhoQ is a pleiotropic two-component regulatory system, and *phoP* is considered as a central regulatory gene [27]. Expression of *phoP* and *phoQ* was slightly increased immediately after the onset of osmotic stress and under the sustained hyperosmotic conditions. Expression levels of two-component regulatory system genes *pmrA* and *pmrB* were reduced in later stages of the osmotic stress, and this pattern was opposite to that of *phoP* and *phoQ*. Expression of *prmD* was increased during the osmotic up-shift, peaking at 15 min with a level 2.9-fold higher than that under low osmotic conditions. Expression of *mig-14* was greatly increased at early stage of the osmotic stress. Increased expression levels of *phoP* and *mig-14* at 15 min of the osmotic stress were verified by RT-PCR (Figure 7). Expression of *rcsB* and *rcsC*, which encode two-component regulatory proteins related to osmo-regulation expression of Vi antigen cluster genes [28], was mildly decreased during early osmotic stress, but not changed significantly under the sustained hyperosmotic conditions. Expression of another set of two-component regulatory system genes, *uhpA* and *uhpB*, was mildly induced immediately after the shift.

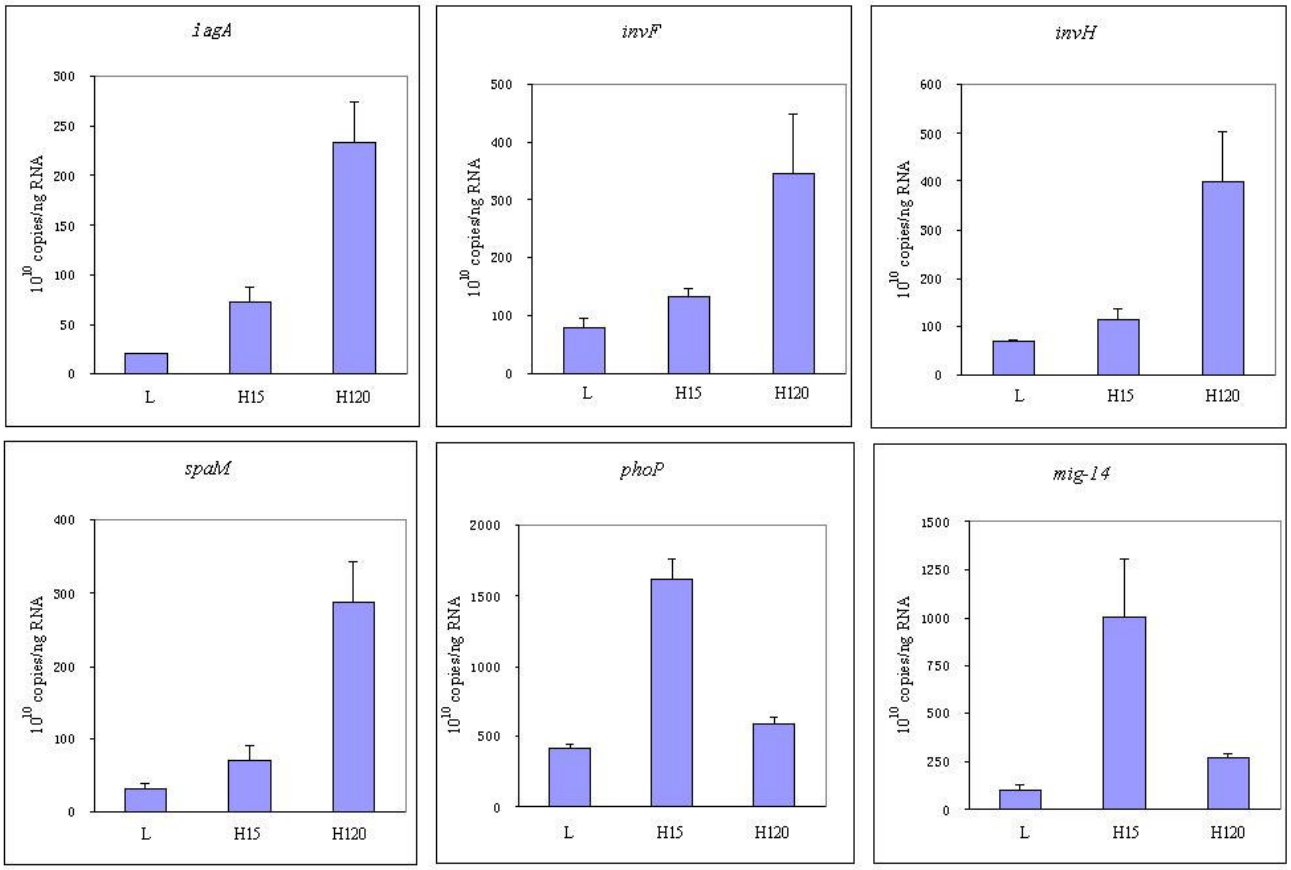

Figure 7. RT-PCR results. Expression of 6 invasion relative genes and *phoP* and *mig-14* was investigated by RT-PCR. RNAs were isolated from *S. enterica* serovar Typhi wild strain incubated in low osmolarity and at 15 min and 120 min after the osmotic shift, respectively. Error bars represent the standard error of the mean.

4. Discussion

Salmonella is one of the most extensively studied bacteria in terms of its genetics, cell structure and physiology, pathogenesis and host interactions, and development. *S. enterica* serovar Typhi is a human enteroinvasive pathogen. After invasion of the intestinal epithelium, *S. enterica* serovar Typhi can survive host defenses and cause severe systemic infection [1, 3]. During movement from the natural surroundings to the host cell, *S. enterica* serovar Typhi is subjected to severe environmental stresses, including acidic conditions in the gut, hyperosmotic conditions in the small intestine, and the oxidative attack by host defense cells. The osmolarity surrounding of *Salmonella* pathogen in food and in the lumen of the small intestine is approximately 50 and 300 mM NaCl, respectively [20]. On the basis of published genomic information, we prepared a *Salmonella* microarray to investigate genome-wide gene expression under environments of the low osmolarity, the osmotic up-shift, and sustained high osmolarity. The present results indicate that expression of a large number of genes changes in response to the osmotic up-shift, and the expression profile at 120 min after the osmotic up-shift are significantly different from those soon after the shift (15 min to 30 min).

Vi polysaccharide is an important factor in *S. enterica* serovar Typhi against host defense systems and environmental stresses. Vi gene cluster of *S. enterica* serovar Typhi includes 10 genes, polysaccharide-biosynthesis related genes *tviA*, *tviB*, *tviC*, *tviD*, and *tviE* and polysaccharide-export related genes *vexA*, *vexB*, *vexC*, *vexD*, and *vexE* [29]. Previous research found that expression of Vi capsular antigen is OmpR dependent and affected by the osmolarity of environment [20]. In the present study, we found that expression of Vi-cluster genes was rapidly inhibited by the shift to hyperosmotic conditions, and suppressed under sustained hyperosmotic conditions also. Invasion ability of *S. enterica* serovar Typhi is negatively affected by Vi polyssacrylide [7], and reduced expression of Vi antigen at high osmolarity may promote invasion of *S. enterica* serovar Typhi in the small intestinal lumen.

Some regulators and factors including SirA, BarA, and RcsC/B influence expression of flagellar genes by regulating FlhDC, which is the global regulator of flagellar and motility-related chemotaxis genes in *E. coli* and *Salmonella* [30-32]. Sigma factor FliA and anti-sigma factor FlgM form the FliA-FlgM regulatory system in response to FlhDC and can regulate expression of most class-3 flagellar genes [21, 33, 34]. The expression of flagella-related genes is also affected by environmental factors, such as osmotic or acid stress [35, 36]. Our present microarray analysis revealed that expression of *fliA* and most other class-2 flagellar and chemotaxis genes in regions I and III is repressed immediately after the onset of osmotic stress. At 120 minutes after the up-shift, expression levels of most flagellar genes, including *flhC* and *fliA*, have return to those at low osmolarity before the stress. However, expression of most flagellar and chemotaxis genesis is elevated slightly under sustained hyperosmotic conditions. It appears that *S. enterica* serovar Typhi gradually adapts to hyperosmotic conditions and recovers the motility at 120 min after the high osmotic stress.

FljA is a post-transcriptional repressor of FliC through binding 5'-termini of the mRNA of FliC in *S. enterica* serovar Typhimurium [37]. The expression of *fliC* is obviously different from the expression of *fljB*:z66, *fljA*-like gene and most of other flagellar and chemotaxis genes. Expression of the *fljA*-like gene is reduced at the later stage after the osmotic up-shift, whereas the expression of *fliC* is just

increased at the same time. More detail experiments are necessary to clarify the principle of the expression of the *fljA*-like gene and the expression of *fliC* in *S. enterica* serovar Typhi.

Previous studies revealed that expression of SPI-1 genes was optimal under conditions of high osmolarity during late-log phase growth, and promoted by HilA and SirA in *S. enterica* serovar Typhimurium [38, 39]. SirA is a response regulator of the BarA-SirA regulatory system that directly induces expression of the central regulator gene *hilA* and indirectly reduces FlhD/C through CsrB/A [31, 40]. InvF, another important regulator encoded by a SPI-1 gene *invF*, regulates the expression of most SPI-1 genes [41, 42]. Expression of *iagA* (named as *hilA* in *S. enterica* serovar Typhimurium), *invF*, and most of the detected SPI-1 genes is increased at the later stage after the osmotic up-shift. However, expression of *sirA* is unchanged at that time. We suspect that change in expression of most SPI-1 genes is not caused directly by the SirA in that case, and some other regulatory factors are likely involved in activation of expression of *iagA* or *invF*. It is unclear why expression of SPI-1genes is increased at 120 min after the onset of osmotic stress and what regulatory systems or regulators are involved in this expression.

After checking all expression data from the present study, we found that the expression levels of near 40 regulatory genes, including some sigma factors, two-component regulatory systems, and putative transcriptional regulators were changed with different patterns after the hyperosmotic stress. RpoS is the master regulator of the general stress response, which provides cells with the ability to survive stresses including starvation, acid, high osmolarity and oxidative stress [43-45]. In the present study, the expression of *rpoS* is promoted under sustained hyperosmotic conditions, but is not changed during the osmotic up-shift. We suspect that RpoS is not a major regulator playing direct roles in the early response to hyperosmotic stress. RpoE, an RNA polymerase sigma factor 24 encoded by *rpoE*, is produced under some conditions of stress, e.g. heat shock, starvation, and oxidative stress, in *E. coli* and *S. enterica* serovar Typhimurium [46-49]. RpoE can be regulated by RseA, RseB, and RseC in *E. coli* [50]. RpoD, an RNA polymerase sigma factor 70, could affect expression of *mer* and *pan* operon that is required for synthesis of pantothenate [51, 52]. However the genome-wide function is not understood. Expression of *rpoE* and *rse* operon is temporarily reduced immediately after the up-shift, whereas expression of *rpoD* is repressed at a later stage of the shift. It appears that RpoE and RpoD temporarily affect gene expression in response to hyperosmotic stress in *S. enterica* serovar Typhi.

Two-component regulatory systems in bacteria mostly transduce signals from the external environment via membrane sensors [53]. PhoP-PhoQ, a two-component regulatory system, regulates numerous cellular functions in several Gram-negative species and is important for virulence of *Salmonella* [27, 54-56]. PhoP–PhoQ is connected with another two-component regulatory system, PmrA–PmrB through PmrD, which is promoted by PhoP and can post-translationally regulate the *prmAB* operon [11]. Interestingly, expression of *phoP*, *phoQ*, and *prmD* is elevated transiently after the increase in osmolarity; however, expression of *pmrA* and *pmrB* is reduced at a later stage after onset of osmotic stress when *phoP* and *prmD* are not induced. We suspect that some factors negatively regulate expression of *pmrA* and *pmrB* against the activation of PrmD during the osmotic stress. Another regulatory system, RcsC-RcsB that is connected to the PhoP-PhoQ system has been found in *E. coli* [57]. Expression of *rcsC* and *rcsB* is decreased immediately after the shift to hyperosmotic conditions, whereas expression of *phoP* and *phoQ* is elevated. The relation between PhoP-PhoQ and RcsC-RcsB

in *S. enterica* requires further research to identify. Expression of *mig-14*, which is activated by PhoP [58], is increased in the early stage of the osmotic stress. This result also supports the importance of the PhoP in response to hyperosmotic conditions. The fact that many transcriptional regulators appeared expression changes in response to hyperosmotic stress represents the complexity of the osmo-regulatory network of *S. enterica* serovar Typhi.

The OmpR-EnvZ two-component regulatory system is a well-understood osmo-regulatory system, and OmpR is considered as a central regulator [59-62]. EnvZ and other regulators PhoB, FadD, FliZ, and SirA independently regulate expression of *hilA* and invasion in *S. enterica* serovar Typhimurium [63]. Though we did not observe any obvious change in *ompR* expression in response to hyperosmotic stress, the expression of *ompF*, a phosphated OmpR negative controlled gene [64], is reduced immediately after onset of the osmotic up-shift stress, and the effect continually exists in all stages of the stress. The result suggests that increasing phosphorylation of OmpR appears in the early stage of the osmotic stress and may be an important initial event in the osmotic regulatory network. A genome-wide examination of OmpR-EnvZ function during osmotic stress will be useful to reveal the relation among regulatory proteins and systems.

In conclusion, when *S. enterica* serovar Typhi encounters an osmotic up-shift environment, regulatory cascade is activated, and appears rapid and later responses. *S. enterica* serovar Typhi immediately reduce the expression of Vi-cluster genes and some flagellar and chemotaxis genes in early stage, and increasing expression of invasion-related genes and most flagellar and chemotaxis genes gradually in somewhat later stage. Many regulators, e.g. PhoP, RpoE, and RpoD, are perhaps involved in these responses. *S. enterica* serovar Typhi will increase the invasive ability in somewhat later after enter the hyperosmotic environment of small intestine of host. This may explain why invasion by the pathogen occurs mainly in distal portion of the ileum of the small intestinal. It is also suggests that bacterial infection of the intestine is dependent on expression of pathogenic genes altered in response to hyperosmotic surroundings.

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