J. Microbiol. Biotechnol. (2005), **15**(1), 175–182

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Analysis of *Salmonella* Pathogenicity Island 1 Expression in Response to the Changes of Osmolarity

LIM, SANGYONG[1], KYEONGHWA YONG, AND SANGRYEOL RYU*

Department of Food Science and Biotechnology, School of Agricultural Biotechnology, and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, Korea
[1]Radiation Food Science and Biotechnology Team, Korea Atomic Energy Research Institute, Daejeon 305-600, Korea

Received: September 1, 2004
Accepted: November 19, 2004

Abstract *Salmonella* pathogenicity island 1 (SPI1) gene expression is regulated by many environmental signals such as oxygen, osmolarity, and pH. Here, we examined changes in the expression level of various regulatory proteins encoded within SPI1 in response to three different concentrations of NaCl, using primer extension analysis. Transcription of all the regulatory genes tested was activated most when *Salmonella* were grown in Luria Broth (LB) containing 0.17 M NaCl. The expression of *hilA*, *invF*, and *hilD* was decreased in the presence of 0.47 M NaCl or in the absence of NaCl, while *hilC* expression was almost constant regardless of the NaCl concentration when *Salmonella* were grown to exponential phase under low-oxygen condition. The reduced expression of *hilA*, *invF*, and *hilD* resulted in lower invasion of *hilC* mutant to the cultured animal cells when the mutant was grown in the presence of 0.47 M NaCl or in the absence of NaCl prior to infection. Among the proteins secreted via the SPI1-type III secretion system (TTSS), the level of *sopE2* expression was not influenced by medium osmolarity. Various effects of osmolarity on virulence gene regulation observed in this study is one example of multiple regulatory pathways used by *Salmonella* to cause infection.

Key words: SPI1, *hilC*, *sopE2*, osmolarity

During infection of its hosts, *Salmonella enterica* serovar Typhimurium enters the epithelial cells of the small intestine. This process requires a type III secretion system (TTSS) encoded on *Salmonella* pathogenicity island 1 (SPI1), a 40 kb stretch of DNA located near 63 centisome of chromosome [27, 29]. The genes of SPI1 are under the complex control of many transcriptional regulators encoded within SPI1, including HilA, HilC, HilD, and InvF. HilA plays a crucial role in the expression of genes encoding SPI1-TTSS apparatus, *prg* and *invlspa* operon, by binding upstream of *prgH* and *invF* [24]. Expression of *invF* controlled by HilA leads to induction of several effector genes encoded both within (*sic/sip* operon) and outside SPI1 (*sigD/sopB* and *sopE*), and InvF is known to require SicA as a cofactor [13]. The effector proteins translocated into the epithelial cells by SPI1-TTSS can induce rearrangements of actin cytoskeleton and internalization of *Salmonella* into host cells [10, 19, 20, 38]. HilD, a member of the AraC/XylS family of transcription regulators, has been postulated to act as a derepressor of *hilA* expression by counteracting the action of negative regulatory elements at the *hilA* promoter [31, 32]. Recent studies, however, showed that HilD provided an essential activating function for *hilA* in the absence of negative regulators [7]. Although another AraC/XylS family of transcription regulators, HilC (also called SprA or SirC), has been known to be required to derepress *hilA* expression like HilD, a *hilC* null mutation has little effects on invasion gene expression or the invasive phenotype, whereas the *hilD* mutant strain does not express *hilA* even in the presence of HilC [16, 30, 29, 32]. Expression of SPI1 invasion and effector genes respond to multiple environmental signals and is decreased under conditions of high oxygen, low osmolarity, low pH, and stationary phase growth [4, 17, 22]. Although the sensors and transcription factors responsible for the environmental regulation of SPI1 have not yet been clearly identified, these signals have been shown to affect the transcription of *hilA*, perhaps through modulation of expression of *hilD* and *hilC* or activity of their gene products [25]. Thus, we checked the effect of medium osmolarity on the expression of SPI1 transcriptional regulators in order to gain more information about how the environmental signals are transferred to regulate virulence gene expression in the pathogenesis of *Salmonella*.

*Corresponding author
Phone: 82-2-880-4856; Fax: 82-2-873-5095;
E-mail: sangryu@snu.ac.kr

Table 1. The bacterial strains and plasmid used in this study.

Strain and plasmid	Relevant genotype	Reference and source
S. typhimurium		
SL1344	Wild-type *rpsL hisG*	4
EE635	SL1344 *hilC*::Tn*5lacZY-9* (Tet^r)	32
EE639	SL1344 *invF12-5*::Tn*5lacZY* (Tet^r)	Dr. C. A. Lee
EE658	SL1344 *hilA080*::Tn*5lacZY* (Tet^r)	25
YK3039	UK1 *ompR132*::MudJ (Kan^r)	5
SR1013	SL1344 *ompR132*::MudJ (Kan^r)	This study
SR1025	SL1344 containing pCJ20	This study
SR1026	EE658 containing pCJ20	This study
Plasmid		
pCJ20	Containing 4.4 kb region of *hilC* region for overexpression of HilC (Amp^r)	30

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains and plasmid used in this study are listed in Table 1. Isogenic *ompR* mutant strain, SR1013, was made by P22HT-mediated transduction of *ompR* mutation allele from YK3092 to wild-type, SL1344 [8]. *S. typhimurium* were routinely cultivated at 37°C in Luria Broth (LB) containing 1% Bacto-tryptone, 0.5% yeast extract, and 1% NaCl. To study the effect of HilC overexpression on SPI1 expression, a plasmid, pCJ20, carrying the *hilC* gene was used (Table 1). Invasion assay was performed with bacterial cultures grown statically; that is, defined as low-oxygen condition with various medium osmolarity [1, 4, 22]. Antibiotics at the following concentrations were used when necessary: ampicillin, 50 μg/ml; tetracycline, 15 μg/ml; kanamycin, 50 μg/ml.

RNA Extraction

RNA was isolated from cultures of serovar Typhimurium SL1344 grown statically in LB containing 0, 0.17, or 0.47 M NaCl. Briefly, cultures were begun from stationary-phase cells grown overnight with shaking. Low-oxygen cultures were obtained by inoculating the fresh medium at 1:100 dilution, and cells were harvested at 2-h intervals between 2 and 8 h after inoculation. Cells from 50 ml of culture were collected by centrifugation, and RNAs were isolated using the Trizol® reagent (Life Technologies, Inc.) according to the manufacturer's instruction. For comparison of *hilC* expression between low- and high-oxygen conditions, RNA was isolated from high-oxygen cultures prepared by inoculating fresh LB broth at 1:100 dilution and then growing with aeration to late log phase (~4 h; $OD_{600} \cong$ 3.0).

Primer Extension Analysis

Primers used for primer extension analysis in this study are listed in Table 2. Purified ^{32}P end-labeled primers were co-precipitated with 30 μg of total cell RNA. Primer extension analysis was carried out as described previously [23]. The pellet was resuspended in 20 μl of 250 mM KCl, 2 mM Tris (pH 7.9), and 0.2 mM EDTA. The mixture was heated to 60°C and then allowed to cool to room temperature over a period of 1 h. After annealing, 50 μl of reaction solution containing 5 μg of actinomycin D, 700 μM dNTPs, 10 mM $MgCl_2$, 5 mM dithiothreitol, 20 mM Tris (pH 8.7), and 150 units of Superscript reverse transcriptase (Life Technologies, Inc.) were added. The mixture was incubated at 42°C for 70 min and treated with ribonuclease T1 (Invitrogen, Carlsbad, CA, U.S.A.) at 37°C for 15 min. The DNA was

Table 2. Oligonucleotide primers used for primer extension analysis.

Gene	Nucleotide sequences (5'→3')	Complementary region[a]
hilA	TAATAATATTGTTATAACTAACTGTGATTA	-216~-235
invF	CATTGTGTCGGCTTTCAGAAAATGACATAT	-1~+28
hilC	GGAAATTTGTTCGGCTGTTGAAGGTGATTA	+45~+74
hilD	TTTAATTTGCTGCCGGGTATTTGTCAAAAG	+73~+102
prgH	CTGTCAGCAATGGAAACTCACAGCCGTTCA	+71~+100
sicA	TTCACTAACGGCATCCCAAATCATTTCCGC	+37~+66
sigD	AGGTTTTTTGTAGGCTTTTAAAAGCCTCCT	+44~+73
sopE	ACGGTAATGATCCTTTTATATGTACATAAT	-27~+2
sopE2	TCTGTAGTGCTGGGTGGATAGTGTTATGTT	+7~+36

[a]Numbers of nucleotides were determined relative to the translational start codon of each gene.

precipitated, washed with 70% ethanol, and resolved on an 8% polyacrylamide and 8 M urea gel. sopE2 primer (Table 2) was also used for sequencing the *sopE2* promoter region, using a double-stranded DNA cycle sequencing system (Life Technologies, Inc.).

Cell Culture

HEp-2 (ATCC CCL-23) cells, a line established from a human epidermoid carcinoma, were maintained in RPMI1640, containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml), at 37°C under 5% CO_2. Cell cultures were replaced after passage #20. Invasion and intracellular replication assays were set up by seeding 2×10^5 cells into each well of a 24-well plate and incubated overnight.

Invasion Assay (Gentamicin Protection Assay)

Bacteria (5×10^6) washed with PBS and suspended in prewarmed RPMI media were then added onto cell monolayers at a multiplicity of infection of about 1 to 10. Following a 1 h incubation at 37°C, cell monolayers were washed three times with prewarmed PBS to remove extracellular bacteria and incubated for 2 h with prewarmed RPMI supplemented with 100 μg of gentamicin (Gm) per ml to kill extracellular adherent bacteria. Then, the monolayer was washed three times with PBS, lysed in 1% Triton X-100 for 10 min, and diluted with PBS. Dilutions of the suspension were plated on LB agar medium to enumerate cfu.

RESULTS

hilC Expression was Insensitive to the Medium Osmolarity

Using primer extension analysis, we tested the expression of four transcriptional regulators in the presence of three different NaCl concentrations under low-oxygen conditions. LB broth was prepared as usual, except that the NaCl concentration was altered to 0, 0.17, or 0.47 M. The transcriptional start sites of *hilA*, *invF*, *hilC*, and *hilD* have previously been reported [24, 28, 32]. The expression of *hilA* peaked at exponential phase (4 h after inoculation) and decreased when cells entered the stationary phase, whereas *invF* expression was increased slightly once when it was activated 4 h after inoculation in the presence of 0.17 M NaCl (Fig. 1). However, the transcription of *hilA* and *invF* was very low when the concentration of NaCl was 0 or 0.47 M, suggesting that their expression is highly sensitive to the NaCl concentration. But, the NaCl effects were not observed in *hilC* and *hilD* expression. The level of *hilC* expression was maximum 4 h after inoculation, but the level was almost unchanged regardless of the medium osmolarity (Fig. 1). *hilC* expression was decreased at the stationary phase in the presence of 0 or 0.47 M NaCl, compared with 0.17 M NaCl (Fig. 1). *hilD* expression also peaked at 4 h after inoculation and decreased gradually as

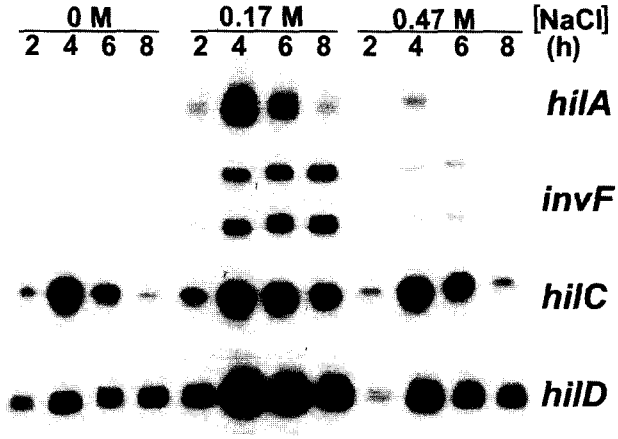

Fig. 1. *In vivo* transcription analysis of the transcriptional regulators of SPI1 responding to medium osmolarity.
Cultures were grown statically in LB containing 0, 0.17, or 0.47 M NaCl. Total RNA was isolated from *S. typhimurium* SL1344, grown in LB medium supplemented with different NaCl concentrations, by 2 h intervals between 2 and 8 h after inoculation. Aliquots (30 μg) of RNA were subjected to primer extension analysis.

the cells entered the stationary phase, but maximal activation was observed in the presence of 0.17 M NaCl. These results demonstrate the complex regulation of SPI1 gene expression in response to environmental signals such as osmolarity and growth phase.

Influence of Osmolarity and Oxygen Tension on *hilC* Expression and Pathogenesis of *Salmonella*

Under high-oxygen condition, SPI1 gene expression and corresponding invasive phenotype is activated only in a narrow growth phase; that is, in the transition between logarithmic and stationary growth phases [26, 35]. We compared the expression of *hilC* under low- and high-oxygen conditions in order to know whether oxygen tension affects the *hilC* expression in the presence of different

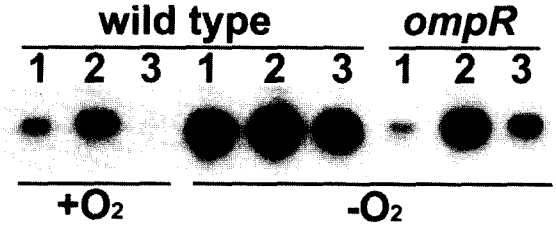

Fig. 2. *In vivo* transcription analysis of the expression of *hilC* responding to oxygen tension.
High-oxygen cultures ($+O_2$) were aerobically grown to late-log phase (~4 h; $OD_{600}\cong3.0$) in LB broth supplemented with different NaCl concentrations. Low-oxygen cultures ($-O_2$) were obtained by growing statically to log phase (~4 h; $OD_{600}\cong0.6$). After total RNA was isolated from *S. typhimurium*, 30 μg of RNA were subjected to primer extension analysis. Lane 1, LB without NaCl; lane 2, LB containing 0.17 M NaCl; and lane 3, LB containing 0.47 M NaCl.

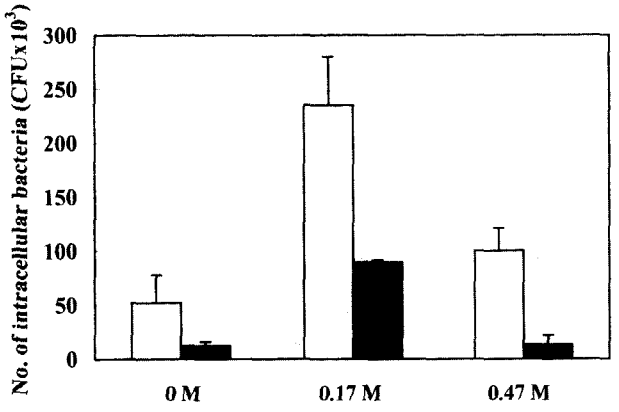

Fig. 3. Effects of *hilC* mutation on *S. typhimurium* invasion of HEp-2 cells.
HEp-2 epithelial cells (2×10^5) suspended in RPMI1640 medium with 10% fetal bovine serum (FBS) were seeded into each well. At a multiplicity of infection of about 10:1, cell monolayers were infected with wild-type (SL1344; white bars) and *hilC* mutant strain (EE635; black bars) grown statically to log phase in LB supplemented with 0 M, 0.17 M, or 0.47 M NaCl, respectively, as indicated. The error bars represent mean standard deviation from one representative experiment performed with triplicate samples of three independent experiments.

concentrations of NaCl (Fig. 2). Interestingly, the expression of *hilC* decreased only in the presence of 0 and 0.47 M NaCl, if *Salmonella* were grown under high-oxygen condition, implying that HilC may play a role in pathogenesis when *Salmonella* meets with limited condition. It is well known that OmpR-EnvZ responds to changes in osmolarity [3]. Thus, we checked the role of OmpR in *hilC* expression in response to changes in osmolarity. When cells were grown in the presence of 0 M or 0.47 M NaCl, *hilC* expression decreased in the absence of OmpR, compared to 0.17 M NaCl (Fig. 2). These results imply that OmpR-EnvZ is required to maintain the *hilC* expression in the presence of different concentrations of NaCl.

In order to test the influence of osmolarity to the invasive phenotype of *Salmonella*, we next examined the ability of *hilC* mutant grown in the presence of different NaCl concentrations to invade cultured HEp-2 epithelial cells (Fig. 3). It has earlier been reported that a mutation in *hilC* results in only a slight decrease (about 3-fold) of invasive activity when cells were grown in LB supplemented with 0.17 or 0.3 M NaCl prior to infection [16, 30, 32]. Our results also showed that the *hilC* mutant was about 2.7-fold less invasive than SL1344 grown in the presence of 0.17 M NaCl prior to infection (Fig. 3). When cells were grown in the presence of 0 or 0.47 M NaCl, however, *hilC* mutation lowered *Salmonella* invasion by 6.6- 7.5-fold. These results suggest that HilC may play an important role, if the expression of other SPI1 transcriptional regulators such as HilA were not permitted by growing *Salmonella* under low or high osmolarity (Fig. 1).

hilC Overexpression Resulted in the Activation of *hilA* and *invF* Regardless of Osmolarity

It has been suggested that HilC and HilD are functionally redundant, because overexpression of *hilC* can suppress a *hilD* mutation even though HilC is known to play a minor role in regulation of SPI1 gene expression [25, 32]. We studied the effect of *hilC* overexpression on *hilA* and *invF* regulation in the presence of different NaCl concentrations (Fig. 4). Overexpression of HilC was achieved by expression of the *hilC* gene from its own promoter on a low-copy-number plasmid, pCJ20 [30]. As expected, overexpression of *hilC* strikingly activated *hilA* transcription in the presence of 0.17 M NaCl, but the activation was also seen in the presence of 0 or 0.47 M NaCl, even though the effect was smaller (Fig. 4A). The pattern of *invF* expression was similar to that of *hilA*. Overexpression of *hilC* activated *invF*, regardless of the osmolarity, and the effect was the largest in the presence of 0.17 M NaCl (Fig. 4B). Moreover, *invF* expression was not active in EE658, a *hilA* mutant strain, even in the presence of pCJ20 (Fig. 4B). Collectively, it is evident that *hilC* overexpression can induce *hilA* transcription and then *invF* transcription via elevated HilA, irrespective of the medium osmolarity.

Regulation of *sopE2* Expression is Different from other Effector Genes

We studied the effects of osmolarity on the expression of effector genes such as *sigD*, *sopE*, and *sicA/sipBCDA* operon and the apparatus gene, *prgH* (Fig. 5). The transcriptional start sites of *prgH*, *sicA*, *sigD*, and *sopE* have previously been reported [13, 24]. As shown in Fig. 5, expression of these genes in the absence of NaCl was almost negligible (Fig. 5). The expression of *prgH*, *sicA*, and *sigD* was activated 4 h after inoculation, similar to the case of SPI1

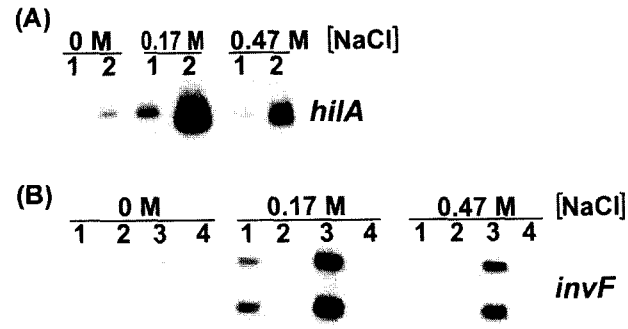

Fig. 4. *In vivo* transcription analysis of the effect of overexpression of *hilC* on *hilA* and *invF* expression responding to medium osmolarity.
S. typhimurium were cultivated statically to log phase (~4 h; $OD_{600}\cong0.6$) in LB broth supplemented with different NaCl concentrations. Total RNA was isolated from *S. typhimurium*, and 30 μg of RNA were used for primer extension analysis. (A) Lane 1, SL1344; lane 2, SR1025 (SL1344 containing pCJ20). (B) Lane 1, SL1344; lane 2, EE658 (*hilA* mutant); lane 3, SR1025; and lane 4, SR1026 (EE658 containing pCJ20).

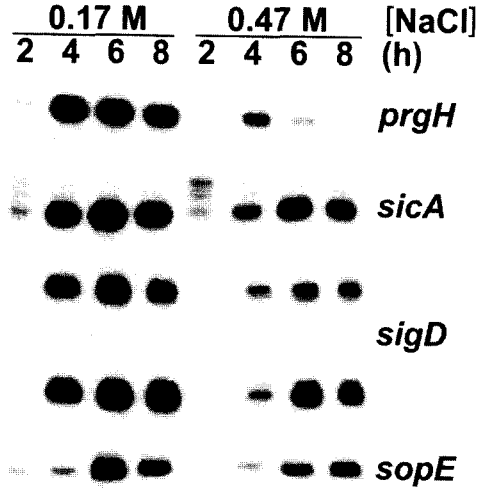

Fig. 5. *In vivo* transcription analysis of the expression of effector genes responding to medium osmolarity.
Cultures were grown statically in LB containing 0, 0.17, and 0.47 M NaCl. Total RNA was isolated from *S. typhimurium* SL1344, grown in LB medium supplemented with different NaCl concentrations, by 2-h intervals between 2 and 8 h after inoculation. Aliquots (30 μg) of RNA were subjected to primer extension analysis.

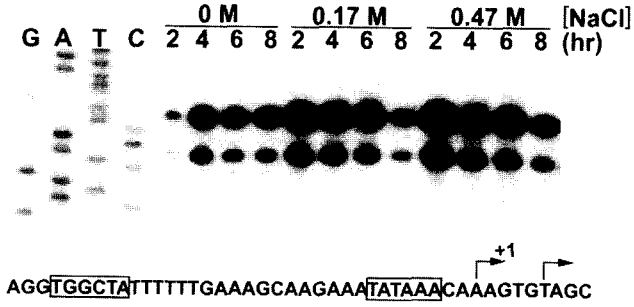

Fig. 6. *In vivo* transcription analysis of *sopE2* expression responding to medium osmolarity.
Cultures were grown statically in LB containing 0, 0.17, and 0.47 M NaCl. Total RNA was isolated from *S. typhimurium* SL1344, grown in LB medium supplemented with different NaCl concentrations, by 2-h intervals between 2 and 8 h after inoculation. Aliquots (30 μg) of RNA were subjected to primer extension analysis. Sequence ladder was generated using the same end-labeled primer used for primer extension analysis. The nucleotide sequences of the *sopE2* promoter region are shown. The transcription start sites (+1) are indicated by an arrow and the possible - 10 and - 35 regions are boxed.

regulator genes, whereas maximal expression of *sopE* occurred 6 h after inoculation (compare Figs. 1 and 5). Interestingly, the pattern of *prgH* transcription, known to be directly regulated by HilA, was different from that of *hilA*; that is, *prgH* expression was not decreased, when the cells entered the stationary phase in the presence of 0.17 M NaCl. Effector genes such as *sicA/sipBCDA* operon, *sigD*, and *sopE* showed higher level of expression during the course of growth, compared to the apparatus gene, *prgH*, in the presence of 0.47 M NaCl. We detected two primer extension products from *sigD* mRNA, and found that the longer one corresponded to the primer extended to the previously identified +1 site [13], and that the shorter product newly identified in this study corresponded to the primer extended to the site 13 bp downstream of the +1 site. These results suggest the possibility that there are two promoters in the *sigD* gene, because the relative band intensity of these two primer extension products was different in the presence of 0.17 and 0.47 M NaCl.

SopE2, highly homologous to SopE, is known to be an important invasion-associated effector and conserved in most pathogenic strains of *Salmonella* [5]. We characterized the *sopE2* promoter and examined the effect of osmolarity on *sopE2* expression (Fig. 6). Two primer extension products were seen whose transcription start sites were mapped to the positions 39 and 34 nucleotides upstream of the *sopE2* ORF. Since the distance between the two transcription start sites is only 4 nucleotides and the band intensity of the shorter products was proportional to that of the longer products, we speculate that the shorter product might have been generated by degradation of longer mRNA. We could identify a putative - 10 element of the promoter from which the longer transcript was made, however could not find a conspicuous - 35 element located 16- 18 bp upstream of the - 10 region, indicating that the transcription regulators may possibly be involved in the *sopE2* expression. Thus, we monitored the expression of *sopE2* in *hilA* (EE658), *invF* (EE639), and *hilC* (EE635) mutant strains, however, there were no changes in *sopE2* expression, compared with wild-type strain (data not shown). As shown in Fig. 6, the *sopE2* expression showed some unique features unlike other SPI1 genes. (i) The level of *sopE2* expression was high regardless of the medium osmolarity; (ii) *sopE2* expression was maximally activated 2 h after inoculation in the presence of 0.17 and 0.47 M NaCl; (iii) the level of *sopE2* expression was not reduced, whereas the expression level of effector genes was significantly reduced in the presence of 0.47 M NaCl (Figs. 5 and 6).

DISCUSSION

Virulence genes are thought to be regulated in the host, and they are expressed only at those sites where their products are needed. During infection of its hosts, *Salmonella* encounters diverse environments in the intestinal lumen, within epithelial cells, and inside the phagosome of macrophages. It is believed that the lumen of the distal ileum is a low-oxygen and high-osmolarity environment, and approximate osmolality (OsM=moles per kilogram) of human colonic contents is known to be 0.3 to 0.6 OsM, whereas LB broth has about 0.4 OsM [33, 34]. When grown under a low-oxygen and high-osmolarity environment, *Salmonella* enters cultured

animal cells much more efficiently than *Salmonella* grown aerobically or in low-osmolarity media [11, 36]. In addition, conditions that maximally induce *hilA* expression include low oxygen tension and LB medium containing 1% (≈0.17 M) NaCl [4]. In this study, we investigated the expression pattern of SPI1 regulatory genes under suboptimal condition for their activation; that is, various salt concentrations. The expression of *hilA*, *invF*, and *hilD* at the exponential phase was decreased in high (0.47 M NaCl) or low (0 M NaCl) osmotic conditions, when cells were grown statically, but *hilC* expression was not decreased under the same condition (Fig. 1). This type of regulation may be useful to maintain the invasive phenotype even under suboptimal conditions, because the HilC-regulatory pathway is active under different osmotic conditions. However, when β-gal fusion was used to monitor the *hilC* expression, the expression of *hilC* was reported to decrease under 0 M NaCl, similar to other SPI regulators [16, 25]. When *hilC* expression was monitored by measuring the levels of β-galactosidase using chromosomal *hilC*::Tn5*lacZY* fusion strain, EE635, we also found in the present study that *hilC* expression was reduced by low osmolarity condition (data not shown). The discrepancy in the primer extension assay and the β-galactosidase fusion results may arise from the inactivation of HilC in the *hilC*::Tn5*lacZY* fusion strain. We suggest that HilC may be involved in the autoregulation of *hilC* expression, because *hilC* promoter contains *HilC* binding sites, and that HilC and HilD proteins can change the choice of promoter at *hilC in vitro* [28]. However, *hilC* mutation had no effect on the expression of *hilC* itself as well as SPI1-transcriptional regulators, including HilA, InvF, and HilD [unpublished data; 16, 30, 32]. Since 10 genes, from *prgH* to *avrA* containing *hilC* promoter, are transcribed as a single polycistronic message driven by the *prgH* promoter [21] and this *prgH* promoter responds to changes in osmolarity like *hilA* (Fig. 5), we speculate that the level of *hilC* expression, which was measured by chromosomal *hilC*::Tn5*lacZY* fusion, may be the sum of transcription from *prgH* and *hilC* promoters.

OmpR was required to maintain the almost constant *hilC* expression level regardless of the changes in osmolarity (Fig. 2). Lucas and Lee reported that EnvZ/OmpR regulates *hilA* by altering the expression and/or activity of *hilC* [25], and *ompR* mutation can attenuate *Salmonella* virulence [9, 14], implying that one of the major functions of OmpR in virulence gene regulation of *Salmonella* is to modulate *hilC* expression.

HilA activates the transcription of components of the SPI1 type III secretion apparatus, whereas the expression of effector proteins is regulated differentially by both InvF and HilA [12, 15]. Recently, it has been found that *invF* expression can be upregulated by overexpression of HilC even in the absence of *hilA* [16, 26, 30], because HilC and HilD activate the transcription of *invF* from an alternative promoter, that is far upstream of its HilA-dependent promoter, through direct binding [2]. Therefore, HilC overexpression can induce *invF* expression through both HilA-dependent *invF* promoter tested in this study (Fig. 4) and HilA-independent *invF* promoter. It is likely that the ability of overexpressed HilC to activate *hilA* and *invF* expression regardless of osmolarity may help maintain the expression of SPI1 genes in nonpermissive condition and rapidly activate SPI1 expression after transition to a permissive environment.

While SopE is encoded by a gene within a temperate bacteriophage present in relatively few strains of *Salmonella*, SopE2 which is highly homologous to SopE is present and conserved in most pathogenic strains of *Salmonella*, showing that SopE2 is an important invasion-associated effector [5]. In responding to osmolarity changes, *sopE2* can be highly expressed regardless of medium osmolarity and growth stage, compared to other effector genes such as *sigD* and *sopE* (Figs. 5 and 6). SigD, SopE, and SopE2 exhibit overlapping function to mediate rearrangements of actin cytoskeleton and subsequent bacterial entry. *Salmonella* strains deficient in any one of these proteins can normally enter into host cells, whereas a strain lacking all three effectors is completely inert for entry [37]. This bacterial internalization is due to modulation of the GTP-binding proteins activity, in particular Cdc42 and Rac. SopE is highly efficient in activating both Cdc42 and Rac1, whereas SopE2 can efficiently activate Cdc42, but not Rac1 [18]. Altogether, these observations imply that the differential expression of effector proteins in response to an environmental signal such as osmolarity could make *Salmonella* arm itself with a good weapon to cause disease at different sites in the host and in different growth stages.

Acknowledgments

The authors are grateful to Drs. Y. K. Park, B. D. Jones, and C. A. Lee for their gifts of bacterial strains and plasmids. K. Yong is a recipient of graduate fellowships from the Ministry of Education through the Brain Korea 21 Project. This study was supported by a grant (01-PJ1-PG1-01CH14-0001) from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea.

References

1. Ahn, J. B., H.-J. Hwang, and J. H. Park. 2001. Physiological responses of oxygen-tolerant anaerobic *Bifidobacterium longum* under oxygen. *J. Microbiol. Biotechnol.* **11:** 443-451.
2. Akabar, S., L. M. Schechter, C. P. Lostroh, and C. A. Lee. 2003. AraC/XylS family members, HilD and HilC, directly activate virulence gene expression independently of HilA in *Salmonella typhimurium*. *Mol. Microbiol.* **47:** 715-728.

3. Alphen, W. V. and B. Lugtenberg. 1977. Influence of osmolarity of the growth medium on the outer membrane protein pattern of *Escherichia coli*. *J. Bacteriol.* **131:** 623-630.
4. Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee. 1996. Co-ordinate regulation of *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. *Mol. Microbiol.* **22:** 703-714.
5. Bakshi, C. S., V. P. Singh, M. W. Wood, P. W. Jones, T. S. Wallis, and E. E. Galyov. 2000. Identification of SopE2, a *Salmonella* secreted protein which is highly homologous to SopE and involved in bacterial invasion of epithelial cells. *J. Bacteriol.* **182:** 2341-2344.
6. Bang, I. S., B. H. Kim, J. W. Foster, and Y. K. Park. 2000. OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **182:** 2245-2252.
7. Boddicker, J. D., B. M. Knosp, and B. D. Jones. 2003. Transcription of the *Salmonella* invasion gene activator, *hilA*, requires HilD activation in the absence of negative regulators. *J. Bacteriol.* **185:** 525-533.
8. Chan, R. K., D. Botstein, T. Watanabe, and Y. Ogata. 1972. Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium*. II. Properties of a high frequency transducing lysate. *Virology* **50:** 883-898.
9. Chatfield, S. N., C. J. Dorman, C. Hayward, and G. Dougan. 1991. Role of *ompR*-dependent genes in *Salmonella typhimurium* virulence: Mutants deficient in both OmpC and OmpF are attenuated *in vivo*. *Infect. Immun.* **59:** 449-452.
10. Chung, T. W., U. H. Jin, and C. H. Kim. 2003. *Salmonella typhimurium* LPS confers its resistance to antibacterial agents of baicalin of *Scutellaria baicalensis* George and novobiocin: Complementation of the *rfaE* gene required for ADP-L-*glycero*-D-*manno*-heptose biosynthesis of lipopolysaccharide. *J. Microbiol. Biotechnol.* **13:** 564-570.
11. Collazo, C. M. and J. E. Galán. 1997. The invasion-associated type-III protein secretion system in *Salmonella*; a review. *Gene* **192:** 51-59.
12. Darwin, K. H. and V. L. Miller. 1999. InvF is required for expression of genes encoding proteins secreted by the SPI1 type III secretion apparatus in *Salmonella typhimurium*. *J. Bacteriol.* **181:** 4949-4954.
13. Darwin, K. H. and V. L. Miller. 2001. Type III secretion chaperone-dependent regulation: Activation of virulence genes by SicA and InvF in *Salmonella typhimurium*. *EMBO J.* **20:** 1850-1862.
14. Dorman, C. J., S. Chatfield, C. F. Higgens, C. Hayward, and G. Dougan. 1989. Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated *in vivo*. *Infect. Immun.* **57:** 2136-2140.
15. Eichelberg, K. and J. E. Galán. 1999. Differential regulation of components and substrates of the cetiosome 63 type III secretion system of *Salmonella typhimurium* by the transcription activators InvF and HilA. *Infect. Immun.* **67:** 4099-4105.
16. Eichelberg, K., W. D. Hardt, and J. E. Galán. 1999. Characterization of SprA, an AraC-like transcriptional regulator encoded within the *Salmonella typhimurium* pathogenicity island 1. *Mol. Microbiol.* **33:** 139-152.
17. Ernst, R. K., D. M. Domboski, and J. M. Merrick. 1990. Anaerobiosis, type I fimbriae, and growth phase are factors that affect invasion of Hep-2 cells by *Salmonella typhimurium*. *Infect. Immun.* **58:** 2104-2106.
18. Friebel, A., H. Ilchmann, M. Aepfelbacher, K. Ehrbar, W. Machleidt, and W.-D. Hardt. 2001. SopE and SopE2 from *Salmonella typhimurium* activate different sets of RhoGTPases of the host cell. *J. Biol. Chem.* **276:** 34035-34040.
19. Hardt, W.-D., L.-M. Chen, K. E. Schuebel, X. R. Bustelo, and J. E. Galán. 1998. *Salmonella typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell* **93:** 815-826.
20. Hong, K. H. and V. L. Miller. 1998. Identification of a novel *Salmonella* invasion locus homologous to *Shigella ipgDE*. *J. Bacteriol.* **180:** 1793-1802.
21. Klein, J. R., T. F. Fahlen, and B. D. Jones. 2000. Transcriptional organization and function of invasion genes within *Salmonella enterica* serovar Typhimurium pathogenicity island 1, including the *prgH*, *prgI*, *prgJ*, *prgK*, *orgA*, *orgB*, and *orgC* genes. *Infect. Immun.* **68:** 3368-3376.
22. Lee, C. A. and S. Falkow. 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proc. Natl. Acad. Sci. USA* **98:** 1847-1851.
23. Lim, S., H. Seo, H. Yoon, S. Choi, S. Heu, and S. Ryu. 2003. Molecular analysis of *Salmonella* enterotoxin gene expression. *J. Microbiol. Biotechnol.* **13:** 598-606.
24. Lostroh, C. P., V. Bajaj, and C. A. Lee. 2000. The *cis* requirements for transcriptional activation by HilA, a virulence determinant encoded on SPI-1. *Mol. Microbiol.* **37:** 300-315.
25. Lucas, R. L. and C. A. Lee. 2001. Roles of *hilC* and *hilD* in regulation of *hilA* expression in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **183:** 2733-2745.
26. Lundberg, U., U. Vinatzer, D. Berdnik, A. von Gabain, and M. Baccarini. 1999. Growth phase-regulated induction of *Salmonella*-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. *J. Bacteriol.* **181:** 3433-3437.
27. Mills, D. B., V. Bajaj, and C. A. Lee. 1995. A 40 kilobase chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 genome. *Mol. Microbiol.* **15:** 749-759.
28. Oleknovich, I. N. and R. J. Kadner. 2002. DAN-binding activities of the HilC and HilD virulence regulatory proteins of *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **184:** 4148-4160.
29. Pancetti, A. and J. E. Galán. 2001. Characterization of the *mutS*-proximal region of the *Salmonella typhimurium* SPI-1 identifies a group of pathogenicity island-associated genes. *FEMS Microbiol. Lett.* **197:** 203-208.
30. Rakeman, J. L., H. R. Bonifield, and S. I. Miller. 1999. A HilA-independent pathway to *Salmonella typhimurium* invasion gene transcription. *J. Bacteriol.* **181:** 3096-3104.

31. Schechter, L. M. and C. A. Lee. 2001. AraC/XylS family members, HilC and HilD, directly bind and derepress the *Salmonella typhimurium hilA* promoter. *Mol. Microbiol.* **40:** 1289-1299.
32. Schechter, L. M., S. M. Damrauer, and C. A. Lee. 1999. Two AraC/XylS family members can independently counteract the effect of repressing sequences upstream of the *hilA* promoter. *Mol. Microbiol.* **32:** 629-642.
33. Shin, J. W., J. K. Kang, K. I. Jang, and K. Y. Kim. 2002. Intestinal colonization characteristics of *Lactobacillus* spp. isolated from chicken cecum and competitive inhibition against *Salmonella typhimurium*. *J. Microbiol. Biotechnol.* **12:** 569-576.
34. Stanton, T. 2000. Ecological aspects of host colonization: Coaggregation, osmoadaptation, and acid tolerance or resistance. pp 61-75. *In* Brogden K. A. *et al.* 3rd eds. *Virulence Mechanisms of Bacterial Pathogens*. American Society for Microbiology Press, Washington, DC, U.S.A.
35. Steele-Mortimer, O., S. Méresse, J.-P. Gorvel, B.-H. Toh, and B. B. Finaly. 1999. Biogenesis of *Salmonella typhimurium*-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. *Cell Microbiol.* **1:** 33-49.
36. Tartera, C. and E. S. Metcalf. 1993. Osmolarity and growth phase overlap in regulation of *Salmonella typhi* adherence to and invasion of human intestinal cells. *Infect. Immun.* **61:** 3084-3089.
37. Zhou, D., L.-M. Chen, L. Hernandez, S. B. Shears, and J. E. Galán. 2001. A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. *Mol. Microbiol.* **39:** 248-259.
38. Zhou, D., M. Mooseker, and J. E. Galán. 1999. Role of the *S. typhimurium* actin-binding protein SipA in bacterial internalization. *Science* **283:** 2091-2095.