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Molecular Analysis of *Salmonella* Enterotoxin Gene Expression

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Abstract *Salmonella* encodes an enterotoxin (Stn) which possesses biological activity similar to the cholera toxin. Stn contributes significantly to the overall virulence of *S. typhimurium* in a murine model. The production of Stn is enhanced in a high-osmolarity medium and by contact with epithelial cells. In the present study, the *in vitro* and *in vivo* transcriptional regulations of the *stn* promoter revealed two promoters, P1 and P2. The P1 promoter identified by a primer extension analysis of *stn* mRNA exhibited a switching mechanism *in vivo*. Depending on the growth stage, transcription was initiated from different start sites termed $P1_S$ and $P1_E$. $P1_S$, recognized by RNA polymerase containing σ^S ($E\sigma^S$), and $P1_E$, recognized by $E\sigma^{70}$, were activated during the stationary and exponential phases, respectively, while $P1_S$ and $P1_E$ were both negatively regulated by CRP·cAMP and H-NS. Results revealed that $P1_S$ was the responsible promoter activated under a high osmolarity and low pH. The P2 promoter was identified 45 nucleotides downstream from $P1_E$ and negatively controlled by CRP·cAMP *in vitro*. No P2 activity was detected *in vivo*. The regulation of *stn* expression monitored using a P*stn*::*egfp* fusion indicated that $E\sigma^S$ was required for the induction of *stn* and various factors were involved in *stn* regulation inside animal cells.

Key words: Gene regulation, *Salmonella* enterotoxin (Stn), *rpoS*

Salmonella enterica serovar Typhimurium is a major cause of enteritis in humans. Although studied by many researchers, however, the pathogenic mechanism of *Salmonella*-associated enteritis has not yet been fully elucidated [49]. One of the potential virulence factors responsible for causing diarrhea is *Salmonella* enterotoxin (Stn). Previous studies have shown that *S. typhimurium* encodes an enterotoxin, which possesses biological activity similar to that of the cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin (LT-I) [14]. Stn causes the elongation of Chinese hamster ovary cells, evokes a fluid secretory response in ligated rabbit ileal loops, and induces the production of 3',5'-adenosine monophosphate (cAMP) and prostaglandin (e.g. PGE_2) in the mucosa [39, 40]. Chopra *et al.* [4, 6] reported on the cloning, sequence analysis, and expression of a *Salmonella* enterotoxin gene (*stn*) encoding a 29-kDa protein from *S. typhimurium* strain Q1, and demonstrated that Stn contributes significantly to the overall virulence of *S. typhimurium* using a murine model. The role of Stn in the pathogenesis of *Salmonella*, including enteritis, is controversial, because the level of fluid secretion in calf ileal loops and cytotoxicity on cultured macrophages were not found to decrease in an *stn* mutant of *Salmonella* [28, 48, 50]. However, considering the functional redundancy of a bacterial virulence factor, the possibility that Stn only contributes to diarrhea under certain conditions still remains. Although the production of Stn is enhanced in a high-osmolarity medium and through contact with epithelial cells [5, 9, 42], the mechanism of *stn* regulation is not yet known. Since the expression of bacterial virulence factors depends on environmental factors [23, 33], some transcriptional factors formed upon suitable environmental signals are likely necessary for inducing Stn expression.

Many bacteria can survive different growth conditions using alternative sigma factors that mediate the expression of specific sets of genes. During transition to the stationary growth phase, RpoS is produced as an alternative sigma factor, also known as σ^S, σ^{38}, or *katF* [41]. Rapid growing, stress-free cells contain very little σ^S, yet the σ^S level dramatically increases as exponentially growing cells encounter various environmental signals, including the deprivation of nutrients and stress conditions, such as a high osmolarity,

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heat shock, oxidative shock, and low pH [18, 22, 27, 35]. The mechanism controlling the level of σ^S under different growth conditions is very complex. Various environmental stress signals affect *rpoS* translation and σ^S turnover differentially as well as the transcription of *rpoS* [34, 35]. Furthermore, since σ^S regulates a variety of genes in response to a number of environmental stress conditions, σ^S is well known as the key regulator of general stress responses rather than as an alternative sigma factor [19, 30].

In the current work, the expression of *stn* was studied *in vivo* as well as *in vitro* to understand the regulation mechanism of the *stn* transcription. It was found that multiple promoters were involved and $E\sigma^S$ played an important role in the regulation of *stn* expression, which was also studied using green fluorescent protein (GFP) as a reporter upon the invasion of *Salmonella* into animal cells.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains and plasmids used in the current study are listed in Table 1. *E. coli* and *S. typhimurium* were routinely cultivated at 37°C in a Luria broth (LB) overnight and used as seed cultures at a 1:100 dilution. The effects of hyperosmolarity were tested in the LB supplemented with 0.5 M NaCl [22]. The cells were grown to an exponential phase (A_{600}=0.3) in a minimal E medium containing 0.4% glucose at pH 7.7, which was then adjusted to 4.3 for acid shock [26]. For a carbon starvation analysis, a morpholinopropane sulfonic acid (MOPS)-buffered salt minimal medium containing 0.01% (w/v) glucose was used [37]. For heat shock, the culture was grown at 30°C and shifted to 42°C at A_{600}=0.3 and incubated for 15 min [44]. Antibiotics were used at the following concentrations: ampicillin 100 µg/ml, tetracycline 15 µg/ml, chloramphenicol 30 µg/ml, and kanamycin 100 µg/ml.

Cell Culture

RAW264.7 (ATCC TIB-71), a murine macrophage-like cell line, and IEC-6 (ATCC CRL-1592), a murine epithelial cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C under 5% CO_2.

Plasmid Construction

A supercoiled plasmid pStn containing the *stn* promoter region was constructed by inserting a DNA fragment from positions - 345 to +36 relative to the translation start codon between the *Eco*RI and *Pst*I sites in front of the *rpoC* terminator in plasmid pSA600 [43]. The linear DNA was prepared by cutting pStn with the restriction enzyme *Apa*I and verified by agarose gel electrophoresis. To generate the pSEC plasmid for the expression of the enhanced green fluorescent protein gene (*egfp*) under the control of the *stn* promoter, a PCR fragment of about 400 bp of the *stn* promoter region was amplified from the plasmid pStn using the forward primer (5'-GCTCGTCGACCCGGG-TACCGAGCA-3') and the reverse primer (5'-AACAGG-

Table 1. Strains and plasmids used in the current study.

Strain and plasmid	Relevant genotype	Reference or source
S. typhimurium		
SF530 (χ3761)	Wild-type UK1	Y. K. Park
JF2690	UK1 *rpoS*::Amp^r	Y. K. Park
JF2997	UK1 *cya*::Tn10	Y. K. Park
YK3064	UK1 *hns105*::Tn10	Y. K. Park
SR1002	UK1 *rpoS*::Amp^r *cya*::Tn10	This study
SR1003	UK1 *rpoS*::Amp^r *hns105*::Tn10	This study
SR1201	UK1 P*stn*::*egfp* Cm^r	This study
SR1202	UK1 P*stn*::*egfp* Cm^r, *cya*::Tn10	This study
SR1203	UK1 P*stn*::*egfp* Cm^r, *hns105*::Tn10	This study
SR1204	UK1 P*stn*::*egfp* Cm^r, *rpoS*::Amp^r	This study
E. coli		
DH5α	See reference	Gibco-BRL
SA1751	F$^-$ *relA rpsL lac trp* (λ *int*$^+$ *xis439 cI857* [*cro-chlA*] ΔH1)	16
Plasmids		
pEGFP	pUC ori, *lac* promoter, *lacZ*-EGFP, Amp^r	CLONTECH Lab. Inc.
pSA600	ColE1 ori, *rpoC* terminator, Amp^r	43
pStn	*stn* promoter in pSA600	This study
pSE1	*stn* promoter::EGFP in pSA600	This study
pSEC	Cm^r, *stn* promoter::*egfp* in pSA600 *for* chromosome insertion of *stn*::*egfp*	This study
pKO3	pSC101-ts ori *sacB* Cm^r	29
pRpoS30	RpoS in pQE30 (Qiagen), Amp^r	R. Hengge-Aronis

ATTACCATGGGTGATA*CCTCCTTA*AATCGATAT-3'), which contained *Sma*I and *Nco*I sites, underlined. The 'TTG' start codon of *stn* was replaced with an 'ATG' start codon (bold letters), and the SD sequence with a consensus sequence 'TAAGGAGG' (in italic letters) for higher translation efficiency [31]. After digestion with *Sma*I and *Nco*I, the fragments were ligated to pEGFP vectors. The resulting construct was digested by *Eco*RI to generate the *stn* promoter and *egfp* fusion (P*stn*::*egfp*) fragment and cloned into pSA600 to yield pSE1. A 1.3-kb fragment containing the chloramphenicol acetyltransferase (*cat*) gene was generated from pKO3, treated with *Ava*I, and blunt-ended by treatment with the Klenow fragment of DNA polymerase I. The fragment was cloned near P*stn*::*egfp* in pSE1 treated with *Sma*I. The pSEC plasmid was then used for the generation of *egfp* reporter gene fusion.

Primer Extension Analysis

Total RNA was isolated from *Salmonella* grown to the exponential and stationary phases using the Trizol reagent (Life Technologies, Inc). To study *stn* transcription, 50,000 cpm of a ^{32}P-labeled STN5 primer (5'-ATTGAGGGTAA-AGGCGCCGTCTTTACTATC-3', complementary to +44 to +73 relative to the translation start site) was coprecipitated with 30 µg of total RNA. The primer extension reactions were performed as described by Shin *et al.* [44].

***In Vitro* Transcription Assay**

The reactions were performed as described by Ryu and Garges [43] in a 25-µl total volume containing 20 mM Tris acetate (pH 8.0), 3 mM magnesium acetate, 200 mM potassium glutamate, 1 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 0.2 mM CTP, 0.02 mM UTP, 10 µCi of [α-^{32}P] UTP (800 Ci/mmol), 2 nM supercoiled DNA template, 20 nM RNA polymerase saturated with σ^{70} or σ^{S}, 100 µg/ml bovine serum albumin, 40 nM CRP, and 5% glycerol. One-hundred µM cAMP was added to the reactions as needed. All components, except the nucleotides, were incubated at 37°C for 10 min. Transcription was started by the addition of the nucleotides and terminated after 10 min by the addition of 25 µl of a formamide loading buffer (80% formamide, 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The RNA was resolved by electrophoresis on an 8 M urea, 6% polyacrylamide gel.

Construction of Chromosomal *stn* Promoter-*egfp* Fusion

The P*stn*::*egfp* fusion was inserted into the *Salmonella* chromosome through homologous recombination using minicircle DNA with no origin of replication [16]. The minicircle DNA was generated *in vivo* by exploiting the mechanism of the site-specific integrative recombination of bacteriophage λ. The plasmid pSEC, containing the *stn* promoter joined with the *egfp* and *cat* genes between the phage λ attachment site *attP'OP* and the corresponding bacterial site *attB'OB*, was introduced by transformation at 32°C into *E. coli* strain SA1751. To prepare the DNA minicircle, SA1751 containing pSEC was grown to the late logarithmic phase (A_{600}=1.0) at 32°C, which was then shifted to 42°C for 10 min to induce the synthesis of the λ Int protein from the cryptic λ prophage by inactivating the temperature-sensitive *cI857* repressor. The culture was cooled down to 32°C for maximal recombination between the *attB'OB* and *attP'OP* elements catalyzed by the λ integrase. After 30 min of incubation at 32°C. the cells were harvested and total plasmid DNA isolated using a Concert kit (Life Technologies, Inc.). The recombinant product including the origin of replication was linearized with *Apa*I and the supercoiled DNA containing the P*stn*::*egfp* fusion and *cat* gene, whereas that without the origin of replication was introduced into *S. typhimurium*. The homologous recombination resulted in the insertion of the minicircle DNA without the origin of replication into the *stn* locus, creating the P*stn*::*egfp* transcriptional fusion.

Fluorescence-activated Cell Sorter (FACS) Analysis

The flow cytometry experiments were performed using an FACS Vantage SE (Becton Dickinson) machine equipped with an argon laser emitting at 488 nm. The bacteria were detected as described previously [47]. *Salmonella* containing the P*stn*::*egfp* fusion was grown to the exponential phase and used to infect animal cells. RAW264.7 or IEC-6 cells (2×10^{5}) were seeded into each well of a 24-well plate and incubated for 24 h at 37°C under 5% CO_2. The bacteria (2×10^{6}) were washed with PBS and suspended in prewarmed DMEM, then added to the cell monolayer at a multiplicity of infection of about 1 to 10. Following 1 h of incubation at 37°C, the cell monolayer was washed three times with prewarmed PBS to remove any extracellular bacteria and incubated for 2 h in prewarmed DMEM supplemented with 100 µg of gentamicin per ml to kill any extracellular adherent bacteria. The monolayer was washed three times with PBS 5 h after infection, lysed in 1% Triton X-100 for 10 min, then diluted with PBS. To determine the levels of induction inside the mammalian cells, the fluorescence level of detergent-released bacteria was compared to that of bacteria exposed to the tissue culture media alone. After 10,000 bacteria-sized particles were collected, the fluorescence intensities of the bacteria were monitored and analyzed using CellQuest software (Becton Dickinson).

RESULTS

RpoS (σ^{S}), CRP·cAMP Complex, and H-NS are Involved in Transcriptional Control of *stn* Gene

To study the effects of various transcription factors on the expression of *stn*, the transcription of *stn* was analyzed

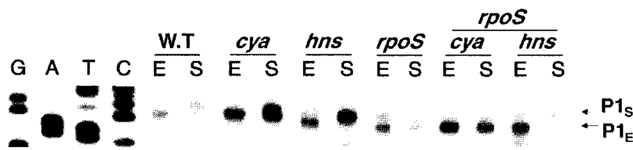

Fig. 1. *In vivo* transcription analysis of *stn* promoter in wild-type and various *S. typhimurium* mutants.

Total RNA was extracted from SF530 (wild-type), JF2997 (*cya*⁻), YK3064 (*hns*⁻), JF2690 (*rpoS*⁻), SR1002 (*rpoS*⁻/*cya*⁻), and SR1003 (*rpoS*⁻/*hns*⁻) grown in an LB medium to different stages of the growth cycle. E, early exponential phase (A_{600}=0.3); S, stationary phase (A_{600}=2.5). Fifty µg of the RNA was subjected to a primer extension analysis as described under Materials and Methods. The start site of each promoter was marked with the name of that promoter. A sequence ladder was generated using the same end-labeled primer used for the primer extension analysis.

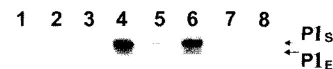

Fig. 2. Transcription of *stn* P1 promoter under different environmental conditions.

The primer extension analysis was performed using total RNA prepared from *S. typhimurium* strain SF530 grown to the exponential phase (A_{600}=0.3) under different stress conditions. Lane 1, SF530 grown at 30°C in an LB medium; Lane 2, SF530 grown in an LB medium at 30°C, then at 42°C for heat shock; Lane 3, SF530 grown in an LB medium with a normal NaCl concentration (10 g/l); Lane 4, SF530 grown in an LB medium with 0.5 M NaCl; Lane 5, SF530 grown in an E medium (pH 7.7) containing 0.4% glucose (EG); Lane 6, SF530 grown in an EG medium (pH 4.3) under acidic conditions; Lane 7, SF530 grown in a MOPS medium containing 0.2% (w/v) glucose; Lane 8, SF530 grown in a MOPS medium containing 0.01% (w/v) glucose for glucose starvation. No $P1_E$ band was detected, because the gel was underexposed to X-ray film, compared to the gel in Fig. 1, due to the strong activity of $P1_S$ in lanes 4 and 6.

through a primer extension assay. Total RNA was purified from *S. typhimurium* strain UK1 and its isogenic mutants in the exponential and stationary phases. Two transcription start points separated by three nucleotides were discovered depending on the growth stage of the bacteria (Fig. 1). The transcription initiation sites that appeared in the exponential and stationary phases were designated as $P1_E$ and $P1_S$, respectively. The activities of both $P1_E$ and $P1_S$ increased in the *cya* mutant strain, JF2997, which cannot produce cAMP required for the activation of CRP (Fig. 1). Interestingly, the transcriptions from both $P1_E$ and $P1_S$ were activated simultaneously in the stationary phase of JF2997. The activity of $P1_E$ increased, whereas that of $P1_S$ disappeared in the stationary phase of the *rpoS*⁻ *cya*⁻ double mutant. $P1_E$ and $P1_S$ also increased in the *hns* mutant, yet the transcription from the $P1_S$ promoter was only increased in the stationary phase in the presence of RpoS. As such, these results indicate that $P1_E$ and $P1_S$ are transcribed by RNA polymerase containing σ^{70} ($E\sigma^{70}$) and $E\sigma^S$, respectively.

$P1_S$ is Promoter Induced Under High Osmolarity and Acidic pH

The expression of *stn* under various culture conditions was studied through a primer extension analysis to identify the environmental factors affecting *stn* transcription. The effects of high salt, a low pH, carbon starvation, and heat shock on *stn* expression were examined. Significant induction of *stn* was observed under osmotic upshifts and acidic conditions, and $P1_S$ was the main promoter induced (Fig. 2). Environmental cues, such as the osmolarity, pH, oxygen, temperature, and iron concentration, induce changes during the course of transition from the external environment to the host, among different locations in the host, and among different hosts. *Salmonella* responds to a complex environment by integrating the signals from several of these cues to sense their locations and express the appropriate genes [33]. The cellular level of σ^S increases both upon entry into the stationary phase and also in response to various stress conditions [34, 35]. Thus, the induction of $P1_S$ responding to environmental conditions suggests its importance in the expression of *stn* within the host.

In Vitro Transcription Analysis of *stn*

To investigate the effect of CRP·cAMP on *stn* transcription, *in vitro* transcription assays were performed using a supercoiled template, purified RNA polymerase, and CRP. Although transcripts from P1 were observed, the exact transcription start point could not be distinguished, because the activity was weak and the band was not sharp enough

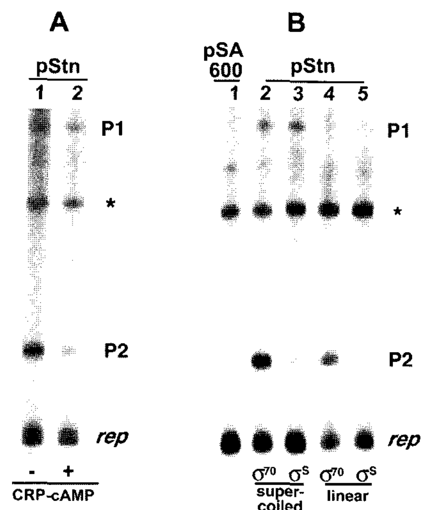

Fig. 3. *In vitro* transcription analysis of *stn* promoter.

(A) A supercoiled DNA template, pStn, was used for the *in vitro* transcription assay using purified $E\sigma^{70}$ in the absence of both CRP and cAMP (lane 1), and in the presence of a CRP·cAMP complex (lane 2). The transcripts from the plasmid origin of replication (106/107 nucleotides) are marked as *rep*. A 165-nucleotide transcript from P1 and 120-nucleotide transcript from P2 are indicated. (B) Supercoiled DNA (lanes 2 and 3) and linear DNA (lanes 4 and 5) templates transcribed by $E\sigma^{70}$ (lanes 2 and 4) and $E\sigma^S$ (lanes 3 and 5) were used for the *in vitro* transcription analysis. The band labeled with * is not *stn*-related, because it also appeared when pSA600 that did not contain the *stn* promoter was used as the template.

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                                          -35                    -10              P1E
  1  ATCTCCTTCAGCGTGGCGATACCGTCCATCTCCGCCATACGCACATCGCACAGTACCAGA
 61  TCAAAGACTTTCTCACGCACCTGAGCCAACGCATCATGCCCACTGTAGGCCAGAGCGACG
        -35                  -10            P2                 P1S
121  TTATAGCCCCAGCCCCGAAGCAGCGCCTGTAAAATCGTGCAGTGGCTTACATCATCATCC
                       CRP consensus  AAATGTGA         TCACATTT
181  ACAACCAGAATATCGATTTTTCCGCGTATCACCCTTGTTAATCCTGTTGTCTCGCTATCA
                                      translation
                                      start codon
```

Fig. 4. Nucleotide sequences of promoter region of *stn*. The transcription start points for $P1_E$, $P1_S$, and P2 are marked with arrows.

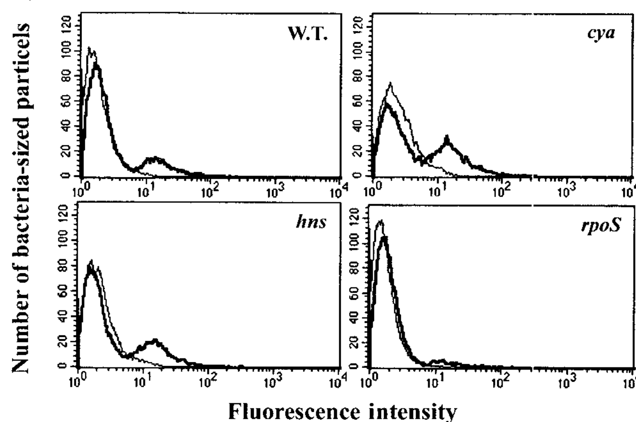
The - 10 and - 35 regions of the $P1_E$ and P2 promoters are overlined, while the 10 and 35 regions of the $P1_S$ promoter are underlined. Sequences corresponding to known consensus CRP sites are indicated with a dot. The translation start codon of *stn*, TTG, is boxed.

(Fig. 3). The exact transcription start site of P1 was studied through a primer extension analysis of the *in vitro* transcription product (data not shown). The results revealed that the initiation of the transcription occurred at both $P1_E$ and $P1_S$, regardless of the RNA polymerase used ($E\sigma^{70}$ or $E\sigma^S$). A new transcription start site was also observed 45 nucleotides downstream from $P1_E$. The downstream promoter detected only through the *in vitro* transcription assay was named P2. The CRP·cAMP complex exhibited a negative effect on the P2 transcription, yet did not affect the P1 transcription *in vitro* (Fig. 3A). The two promoters identified by the *in vivo* and *in vitro* analyses of the *stn* gene are shown in Fig. 4. Nucleotide sequences showing a partial homology with a known consensus CRP binding site were found near the +1 site of the P2 promoter, indicating that CRP·cAMP repressed the P2 promoter by simply inhibiting the RNA polymerase binding to the promoter.

The *in vitro* transcription assay was also used to examine the σ^S-dependent transcription of the *stn* P1 promoter using purified RNA polymerase containing σ^S. Although transcripts were made from both the P1 and P2 promoters when $E\sigma^{70}$ was used (Fig. 3B, lane 2), only $E\sigma^S$ could initiate transcription from P1. In general, a σ^S-dependent promoter is recognized by $E\sigma^{70}$ due to the similarity in the - 10 regions of σ^S- and σ^{70}-dependent promoters and the homology between the domains responsible for promoter recognition in the two sigma factors [11, 46]. The CRP·cAMP complex did not affect the $E\sigma^S$ transcription of P1 (data not shown). These results together with those of the primer extension analysis of *stn* expression in the *cya*⁻ strain (Fig. 1) suggest that CRP·cAMP may indirectly repress the *stn* P1 transcription *in vivo*. The activity of the *stn* promoter was also dependent on the superhelical density of the DNA template. Transcription experiments performed using linear DNA templates revealed that the transcriptions of P1 and P2 decreased compared to the supercoiled plasmid. No transcription from P1 by either $E\sigma^S$ or $E\sigma^{70}$ was detected and the activity of P2 decreased when linear DNA was used as the template (Fig. 3B, lanes 4 and 5).

stn Expression in Animal Cells

Even though it was revealed that $E\sigma^S$ played a significant role in *stn* expression and various factors were involved in its regulation, this regulation can differ inside animal host cells. Previous studies have established that *stn* transcription is induced inside animal cells [9, 42]. However, a primer extension analysis could not be used to study the mechanism of *stn* induction inside animal cells, because the separation of bacterial mRNA from the RNA of animal cells is difficult. Therefore, *stn* gene expression in animal cells was studied using the *egfp* reporter gene and flow cytometry. A P*stn*::*egfp* transcriptional fusion, in which *egfp* expression is controlled by the *stn* promoter, was generated through the homologous integration of the plasmid-borne *stn*::*egfp* fusion into the *S. typhimurium* chromosome. The effects of *cya*, *hns*, and *rpoS* mutations on *stn* expression inside animal cells were studied using IEC-6



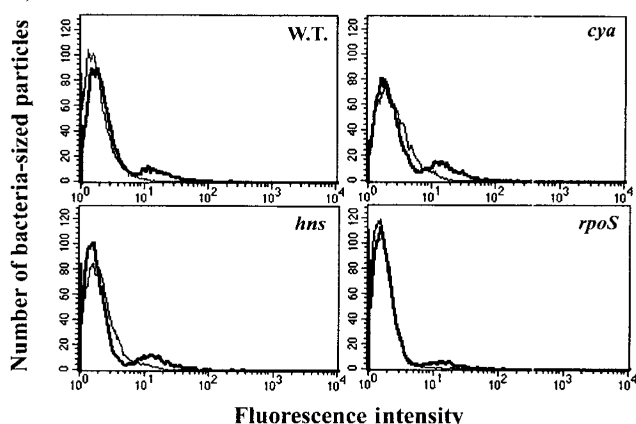


Fig. 5. Analysis of *stn* gene expression in IEC-6 cells (A) and RAW 264.7 cells (B) using flow cytometry.

The animal cells were infected for 7 h with strains bearing the reporter gene, *egfp*, in the chromosome. The strains used were SR120 (wild-type), SR1202 (*cya*⁻), SR1203 (*hns*⁻), and SR1204 (*rpoS*⁻). The histograms show EGFP expression from extracellular bacteria (thin lines) and intracellular bacteria (thick lines).

epithelial cells and murine macrophage-like RAW 264.7 cells. SR1201 (UK1, *Pstn::egfp*), SR1202 (SR1201, *cya*::Tn10), SR1203 (SR1201, *hns105*::Tn10), and SR1204 (SR1201, *rpoS*::Amp^r) strains grown to the exponential phase were used to infect IEC-6. The bacteria were released from the cells 7 h post-infection by treatment with a mild detergent, then the expressions of *egfp* from the extracellular and intracellular bacteria were compared. The intracellular wild-type bacteria could easily be distinguished from the extracellular ones, and *stn* expression only increased in the bacteria inside the animal cells (Fig. 5A). The induction of *stn* expression by *cya*$^-$ and *hns*$^-$ strains inside the animal cells was slightly higher than that by the wild-type. As expected, *stn* expression did not increase in the *rpoS* mutant strain. Therefore, these results imply that *rpoS*-dependent $P1_S$ was the predominant promoter responsible for Stn production inside IEC-6 epithelial cells. A similar pattern of *stn* expression was also observed in RAW 264.7 cells, but the number of bacteria expressing *stn* was lower than that in IEC-6 (Fig. 5B).

DISCUSSION

Role of RpoS in Virulence of *Salmonella*

It is already known that RpoS plays a key role in *Salmonella* virulence in mice [8, 24, 36]. RpoS (σ^S) regulates various gene expressions in response to the conditions encountered during the course of infection [3]. The *rpoS* mutation in *Salmonella* causes a reduction in the ability to colonize spleens [8, 24] and Peyer's patches in infected mice [8, 36], resulting in an attenuated virulence in mice [13]. RpoS-dependent virulence determinants are also found in other pathogenic bacterial species. The expressions of *yst*, which encodes a secreted heat-stable enterotoxin of *Yersinia enterocolitica* [20], and *flaA*, which contributes to the motility of *Legionella pneumophila*, are dependent on σ^S [2]. The *rpoS* mutation leads to a lower production or secretion of hemagglutinin/protease (HA/protease) of *Vibrio cholerae* [52] and *vvpE*, which encodes elastase in *V. vulnificus* [21]. In the current study, it was found that the $P1_S$ promoter of *stn* was recognized by σ^S both *in vivo* and *in vitro* (Figs. 1 and 3) and *stn* expression only increased inside animal cells in the presence of RpoS (Fig. 5).

The transcription of the $P1_S$ of *stn* increased under both a low pH and high osmolarity (Fig. 2). Once inside the intestine, pathogens encounter a low oxygen level, intestinal bile salts, weak acids (owing to fermentation), and increased osmolarity. At least 16 *rpoS*-dependent proteins are induced by increasing the osmolarity in *E. coli* [17]. However, the σ^S-mediated stress response is often accompanied by σ^S-independent mechanisms responding to the stress conditions. It has been shown that the sustained acid tolerance response (ATR) of *S. typhimurium* requires σ^S, whereas the transient development of acid tolerance is not dependent on σ^S [26]. Conter *et al.* [7] have reported that σ^S-dependent *osmE*p stationary phase induction is abolished in the absence of σ^S, whereas the osmotic effect is still observed in *E. coli* due to changes in the DNA superhelical density involved in the osmotic induction of *osmE*p in stressed cells. Environmentally induced changes in the DNA superhelical density are important to the coordinated control of virulence gene expression [10, 15]. The sensitivity of the *stn* promoters to the DNA topology, revealed through an *in vitro* transcription assay (Fig. 3), showed that alterations in the DNA topology in response to environmental signals appeared to play an important role in regulating *stn* expression. RpoS-dependent *stn* expression may be a partial explanation as to why only epidemic strains are able to express Stn, although all strains of *Salmonella enterica* carry the *stn* gene [38]. The *rpoS* gene in the strain *S. typhimurium* LT2 has been shown to contain a rare UUG strart codon [26], contributing to the avirulence of LT2 [45, 51].

Negative Regulation of *stn* by H-NS and CRP·cAMP Complex

Both the P1 and P2 promoters of *stn* exhibited complex regulation by CRP·cAMP. The activity of the $P1_S$ promoter increased in *cya*$^-$ strains, however, no CRP binding site was found near $P1_S$, and CRP·cAMP had no effect on $P1_S$ activity *in vitro* (Figs. 1 and 3). As such, these results suggest that the effect of CRP·cAMP on $P1_S$ transcription would appear to be indirect, probably through the modulation of the intracellular concentration of RpoS. The amount of RpoS significantly increases in *cya*$^-$ strains [25]. The transcription from $P1_E$ significantly increased in both *cya* and *rpoS cya* double-mutant strains regardless of the growth phase (Fig. 1), suggesting that CRP·cAMP plays an important role in the selective repression of $P1_E$ in the stationary phase. Interestingly, P2 activity was only observed *in vitro* (Fig. 1 and lane 4 of Fig. 3). In addition, the activity of the P2 promoter was stronger than that of P1 and repressed by CRP·cAMP. Thus, the P2 activity was likely repressed under the assay conditions used for the primer extension analysis, yet activated when the cells encountered specific signals, such as contact with animal cells.

H-NS repressed transcription from both the $P1_E$ and $P1_S$ promoters. The expression of *stn* increased in the *hns* mutant, although not significantly when the mutant invaded animal cells (Fig. 5). Thus, the level of *stn* expression from the *hns*$^-$ mutant was likely similar to that of the wild-type in the infected host, because the infection of macrophages by *S. typhimurium* induced the expression of the *hns* promoter, which then decreased within 2 h of infection [32]. Atlung and Ingmer [1] suggested that one of the purposes of H-NS could be to repress target gene expression

under conditions characteristic of a non-intestinal environment, yet allow the expression of specific genes in response to certain stimuli in the intestinal environment, thereby leading to the induction of positive transcription factors as well as the derepression of H-NS. In *Shigella* and enteroinvasive *E. coli*, H-NS represses both *virB*, which encodes the AraC-like protein that activates the expression of a number of virulence genes, and *virF*, which encodes the positive transcriptional regulator of *virB* under conditions typical of a non-intestinal environment [12].

stn Regulation Inside Animal Cells is Complex

Stn transcription is induced inside animal cells [9, 42]. An stn mutant strain displayed a decreased virulence in a mice model and caused less fluid secretion in mouse intestinal loops [6]. In the present study, *Stn* expression, upon bacterial invasion of animal host cells, was studied using P*stn::egfp* fusion, because growth in LB cannot mimic the growth conditions inside animal cells. *Stn* was not induced in the *rpoS* mutant inside the animal cells, which suggests that RpoS plays an important role in *stn* expression, as revealed by a primer extension analysis. However, both the *cya* and *hns* mutant strains exhibited *stn* induction patterns similar to that of the wild-type inside animal cells, even though the P1 expression was higher when grown in LB. Furthermore, $P1_E$ and $P1_S$ were not distinguished by $E\sigma^{70}$ and $E\sigma^S$ in an *in vitro* transcription assay, suggesting that unknown factors were involved in the growth phase-dependent regulation of $P1_E$ and $P1_S$ transcription. Accordingly, the current results imply that additional factors to RpoS, CRP, and H-NS are involved in the regulation of *stn* expression inside animal cells. Thus, further study is necessary to identify these factors in order to understand the mechanism of virulence gene induction inside animal cells.

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