

## Combinational Role of Oxidant Response Genes *rpoS*, *ssrAB* and *hmp* in the Virulence of Highly Pathogenic *Salmonella typhimurium* Isolated in Korea

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**Abstract:** This study was designed to elucidate the role of oxidant response genes in the virulence of attenuated *Salmonella typhimurium* (ST). After ST mutants with single or multiple deletions were constructed: the susceptibility of ST to hydrogen peroxide and S-nitrosoglutathione, expression of Salmonella pathogenicity island-2 genes and the virulence of attenuated ST were assessed in this study. Unexpectedly, no difference was observed between the growth of *rpoE* mutant ST98 and Wild Type (WT) however, the *rpoS* mutant was hypersusceptible to hydrogen peroxide. The expression and Nitrogen Oxide (NO)-detoxifying activity of the flavohemoglobin *hmp* were associated with the mechanism of resistance of ST98 to NO. A triple mutant of ST98 in which *rpoS*, *ssrAB* and *hmp* genes were deleted showed a permanent phenotype with no modification of other characteristics. In a mouse infection test, the survival of ST98 triple mutant was lower than that of WT. The virulence of ST was shown to be associated with the oxidant response genes. This study provides useful information for understanding the combinational role of oxidant response genes in the virulence of Salmonella.

**Key words:** Live attenuated vaccine, oxidant response genes, *Salmonella typhimurium*, virulence, *rpoS* mutant

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### INTRODUCTION

Prevention of infectious diseases in animals is a crucial issue for the livestock industry. In particular, the emergence of antibiotic-resistant bacteria hampers the control of infectious diseases of animals and above all, threatens public health by circulation of the resistant bacteria to human hosts (Gyles, 2008; Weese, 2008). This highlights the need for vaccines which can provide a far safer method than antibiotic treatment for preventing infectious disease in animals. The food-borne enteric pathogen *Salmonella enterica* Serovar Typhimurium (ST) causes a typhoid-like disease in mice and also causes a wide spectrum of zoonotic diseases in livestock animals ranging from local gastroenteritis to systemic septicemia (Stevens *et al.*, 2009). Extensive studies using mouse infection models have provided a successful model to study bacteria-host interactions and have generated considerable understanding of the pathogenesis of ST. Animals generally acquire ST from contaminated feed/water or by contact with carriers. ST can invade the intestinal barrier, replicate in phagocytes and even persist in host tissues (Gopinath *et al.*, 2012). ST which survives

stomach acidity because of its acid tolerance response, reaches the intestinal epithelial cells where it induces Type III Secretion Systems (TTSS) and secretes effectors encoded by Salmonella Pathogenicity Island (SPI)-1 to invade host epithelial cells. After penetration of the intestinal epithelial barrier, ST can be engulfed by phagocytic cells such as macrophages in which bacteria survive and replicate inside the Salmonella-Containing Vacuole (SCV) by inducing SPI-2-directed evasion of phagocyte antimicrobial molecules. ST inside phagocytes can then travel throughout the body and disseminate to host systemic sites such as liver and spleen.

Despite many studies of the pathogenesis of ST, the development of an effective vaccine strain has not been successful in either clinical or industrial applications. One reason seems to be the genetic variation between natural isolates and laboratory strains which leads to differences in their adaptation to and virulence in the host (Beltran *et al.*, 1991; Rabsch *et al.*, 2002; Swearingen *et al.*, 2012). Based on the findings from experimental studies on ST pathogenesis, this study aimed to examine the contribution of this genetic variation to the virulence of endemic ST isolated in Korea. This is

a major part of attempts to develop a fully attenuated strain useful as a live vaccine to control ST infection. Researchers selected genes known to be required for survival in murine macrophages and for virulence in mice because survival in macrophages is essential for both acute and persistent infection of ST in animal hosts. However to maintain the immunogenicity of ST, researchers excluded genes also required for invasion of epithelial cells. Of the antimicrobial molecules produced in macrophages, free radicals such as Reactive Oxygen Species (ROS) and NO-mediated Reactive Nitrogen Species (RNS) can damage ST *in vitro* and abolishing generation of these radicals in macrophages or evasion of them by SPI-2 effectors promotes ST replication in macrophages (Fang, 2004). Therefore, mutation of the ST genes encoding molecules that antagonize ROS/RNS and those responsible for SPI-2 expression decreases both survival in macrophages and virulence in mice (Bang *et al.*, 2005, 2006; Kim *et al.*, 2010; Testerman *et al.*, 2002; Vazquez-Torres *et al.*, 2000). For this study, researchers chose the alternative sigma factors rpoE and rpoS which are essential for governing ROS resistance, the principal NO metabolizing enzyme flavohemoglobin hmp and ssrA-ssrB, two component response regulators that activate SPI-2 effectors and examined their role in the virulence of highly pathogenic ST isolated in Korea.

**MATERIALS AND METHODS**

**Bacterial strains and media:** The ST strains and plasmids used in this study are listed in Table 1. The ST98 strain which was isolated from ileocecal lymph nodes of pigs in Korea was used as the Wild Type (WT) parental strain. Bacteria were grown in Luria-Bertani (LB) broth (Difco, Detroit, MI) or minimal E medium containing 0.2% glucose (EG medium) (Vogel and Bonner, 1956). Salmonella-Shigella (SS) agar (BD Biosciences, Sparks, MD) was used to enumerate Colony-Forming Units (CFU) of ST isolated from mouse tissues. Antibiotics were added to the culture media if the bacteria contained antibiotic resistance cassettes, at concentrations of 30 µg mL<sup>-1</sup> for ampicillin, 50 µg mL<sup>-1</sup> for kanamycin and 30 µg mL<sup>-1</sup> for chloramphenicol. Chemicals including antibiotics were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. An NO congener, S-nitrosoglutathione (GSNO) was synthesized by the reaction of glutathione and acidified sodium nitrite according to the method reported previously (Bang *et al.*, 2005; Hart, 1985).

**Construction of ST mutants:** To construct gene mutations in ST98, researchers used a Polymerase Chain Reaction (PCR)-mediated one-step mutation method

Table 1: Bacterial strains and plasmids

Groups	Genotypes	Sources/References
<b>Strains</b>		
FB280	ST98 wild type	Isolates from pigs (33)
FB289	ST98 rpoE::cm	This study
FB324	ST98 ssrAB::km	This study
FB327	ST98 ssrAB	This study
FB328	ST98 ssrAB rpoS::cm	This study
FB329	ST98 ssrAB rpoS	This study
FB330	ST98 ssrAB rpoS hmp::cm	This study
FB331	ST98 ssrAB rpoS hmp	This study
<b>Plasmids</b>		
pKD3	cat cassette	(Datsenko and Wanner, 2000)
pKD4	kan cassette	(Datsenko and Wanner, 2000)
pKD46	λ Red recombinase	(Datsenko and Wanner, 2000)
pCP20	FLP recombinase	(Datsenko and Wanner, 2000)

Table 2: Oligonucleotide primers used in this study

Primers	Sequence (5'-3')
rpoS-P1	tftcgcgacagcggcgcaggccttaacctgaatctgacgggtgaggctggagctccttc
rpoS-P2	tgatttaaatgaagacgcgggaattgatgagaacggagctacatagaatcctccttag
rpoS-Fw	cagtgctgac attgtctgta
rpoS-Rev	cagctctacaagcttcatt
ssrA-P2	atatgtactaagaacatcaacggttgaagaagctgaacgcatatgaatcctccttag
ssrB-P1	acctcattctcggcagcaagtaagtaactctgctactttgtgaggctggagctgcttc
ssrA-Fw	caggcgattctatcattcgg
ssrB-Rev	ggattttgctgacgatgagca
hmp-P1	gcttgacgcacaacatcgctacagtaaaagccaccattgtgaggctggagctccttc
hmp-P2	tggggctgactgctgctgcccggcagggtgactctttatgcatatgaatcctccttag
hmp-Fw	cataacgtaaacgagagaag
hmp-Rev	gctcgttagggcatgctttat
rpoD-RT-Fw	gtgaatgggcactgtgaaactg
rpoD-RT-Rev	ttccagcagataggaatggcttc
sseJ-RT-Fw	ctttaccaccacacatgcag
sseJ-RT-Rev	tggcctgggatgattga
ssaB-RT-Fw	ggatcagggcgaagagta
ssaB-RT-Rev	aaatgcaagttaaacgacggtg
sseA-RT-Fw	gaggggaatgatgataaagaaa
sseA-RT-Rev	ggggcttgagcattaagt
katE-RT-FW	ctgagccagcgtgacatcaa
katE-RT-Rev	gcgttcagcttgaactggg
hmp-RT-FW	tggacgaaatgtcaacccg
hmp-RT-Rev	aatctcgcttcccgatgaa

mediated by the λ Red recombinase (Datsenko and Wanner, 2000). All primers used to generate mutations in ST are listed in Table 2. To construct the rpoS mutation in ST98, linear DNA fragments were obtained by PCR reaction with primers rpoS-P1 and rpoS-P2 using pKD4 plasmid as a template and then transformed to ST98 harboring pKD46. The mutation was confirmed by PCR with primers rpoS-Fw and rpoS-Rev. To construct mutations in the ssrA/ssrB operon and hmp, the primer pairs ssrA-P2/ssrB-P1 and hmp-P1/hmp-P2 were used, respectively. Primer pairs ssrA-Fw/ssrB-Rev and hmp-Fw/hmp-Rev were used to confirm the mutation by PCR. All mutations constructed were transduced to fresh WT ST98 strain bacteria with bacteriophage P22HT105/int and nonlysogenic colonies sensitive to a lytic P22 variant H5 were selected for further studies. To construct the rpoS ssrAB hmp triple mutant, the kanamycin resistance cassette in the ssrAB::Km mutant

was first removed using FLP recombinase encoded from the pCP20 plasmid, resulting in an *ssrAB* mutant without kanamycin resistance. The *rpoS ssrAB hmp* triple mutants were then constructed by sequential transduction of *rpoS::Cm* and *hmp::Cm* into this *ssrAB* mutant with removal of the antibiotic resistance cassette by FLP recombinase after each transduction.

**Measurement of ST susceptibility to hydrogen peroxide and S-nitrosoglutathione:** ST grown overnight in LB broth were diluted in Phosphate Buffered Saline (PBS) to adjust the optical density at 600 nm (O.D.<sub>600</sub>) to a value of 1.0 then bacterial cells (O.D.<sub>600</sub> = 0.2) were inoculated into wells of a microtiter plate containing LB or EG medium. Hydrogen peroxide was added to LB medium as an oxidative agent and a nitric oxide congener GSNO was added to EG medium. Bacterial growth under both stress conditions was monitored by measuring the optical density of bacterial cultures at 37°C with shaking in a Bioscreen C Microbiology Microplate reader (Labsystems, Helsinki, Finland).

**Quantification of gene transcription by Real-Time Reverse Transcription (RT)-PCR:** *SPI-2* gene transcription was induced by shifting the bacteria from culture media containing high Mg concentration to that containing low Mg as described (Deiwick *et al.*, 1999). Briefly, ST cells cultured overnight in LB broth were washed with high Mg<sup>2+</sup> N salts medium [5 mM KCl, 7.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 mM K<sub>2</sub>SO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub>, 38 mM glycerol, 0.1% casamino acids, 10 mM MgCl<sub>2</sub> and 100 mM Tris-HCl, pH 7.6]. The bacteria were subcultured into high Mg<sup>2+</sup> N salts medium and grown at 37°C to early log phase (O.D.<sub>600</sub> = 0.5). ST cells were then harvested and resuspended in a low Mg<sup>2+</sup> (8 µM MgCl<sub>2</sub>) N salts medium (pH 6.9) and incubated for another 3 h. For maximal *katE* transcription, bacterial cells were cultured overnight in LB broth and *hmp* transcription was induced by treating log-phase bacterial cells (O.D.<sub>600</sub> = 0.5) with or without GSNO (1 mM) for 1 h. Bacterial transcription was stopped by adding 1/5×volumes of phenol/ethanol (5:95) solution before harvesting cells. Total RNA was purified with an RNAiso Plus (Takara) according to the manufacturer's protocol. To measure the transcription levels, quantitative real-time RT-PCR was employed. Real-time RT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) as described earlier (Bang *et al.*, 2005). DNA sequences of primer pairs used in this study are as listed in Table 2.

**Virulence assay in mice:** Two groups of 20, 7 weeks old C57BL/6 female mice (control group, 3 mice) were used

according to protocols approved by the Kangwon University Institute Animal Care and Use Committee (Permit number: KW-130924-2). To determine the virulence of *rpoS ssrAB hmp* triple mutant ST98, mice were infected either intraperitoneally or orally with 1×10<sup>3</sup> CFU FB331 or WT. At 3, 6, 9 and 12 days post infection (dpi), 5 mice per group were euthanized by cervical dislocation and the liver and spleen collected. The tissue samples were homogenized 3 times for 3 min at 30 Hz with peptone buffered saline in a tissue lyser (Qiagen, Hilden, Germany). Diluted samples were inoculated on SS agar and incubated at 37°C for 24 h and CFU of cultured bacteria were counted.

## RESULTS AND DISCUSSION

**RpoS is required for hydrogen peroxide resistance of ST98:** To test whether alternative sigma factors play roles in the resistance of ST98 to oxidative stress, researchers constructed ST98 mutants lacking *rpoE* and *rpoS*, respectively and compared the susceptibility of these ST mutants to ROS by monitoring their growth in hydrogen peroxide-containing cultures. The growth of the *rpoE* mutant ST98 was comparable to that of WT whereas the *rpoS* mutant strain was hypersusceptible to hydrogen peroxide (Fig. 1a).

Furthermore, quantitative real-time RT-PCR analysis demonstrated that transcription of *katE* which encodes a catalase was increased up to 10 fold in the WT ST98 following hydrogen peroxide exposure and that this induction was absolutely dependent on a functional *rpoS* (Fig. 1b). These results imply that *rpoE* plays a minor role but *rpoS* regulates the major antioxidant defense as has been reported previously for laboratory strains of ST (Fang *et al.*, 1992).

**Flavo-hemoglobin hmp is required for nitrosative stress resistance of ST98:** To protect against NO-mediated nitrosative stress, many bacteria contain genes encoding flavo-hemoglobin *hmp* to detoxify NO to NO<sub>3</sub><sup>-</sup>. The WT laboratory strain ST 14028S expresses high levels of *hmp* in response to NO and mutant strains lacking this enzyme show decreased virulence in an NO-dependent manner (Bang *et al.*, 2006; Forrester and Foster, 2012). To test the role of flavo-hemoglobin *hmp* in ST98, researchers measured the susceptibility to NO of *hmp* mutant ST98. As shown in Fig. 2a, in NO-producing culture media the growth of *hmp* mutants was completely abolished while WT cells replicated only slightly less than in normal media. In addition, real-time RT-PCR showed that the canonical induction of *hmp* transcription by NO treatment was reproduced in WT ST98

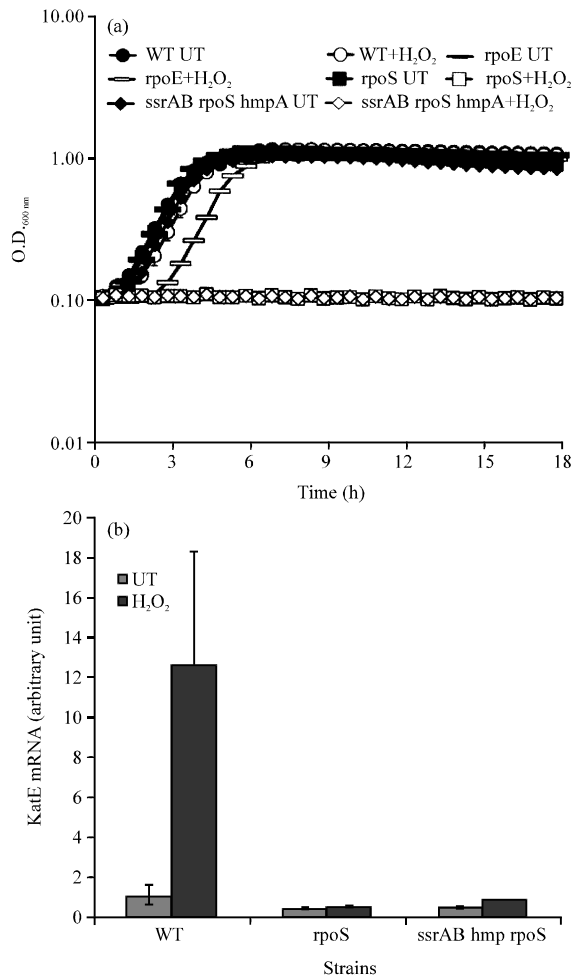


Fig. 1: *rpoS*-dependent hydrogen peroxide resistance of *ST98*; a) *ST* strains were grown in microplates containing LB broth in the presence and absence of H<sub>2</sub>O<sub>2</sub>. Optical density was monitored in the Bioscreen C microplate reader. Data were collected at 30 min intervals and are shown as the mean±SD of three independent experiments; b) log-phase cultures grown in LB media were or were not treated with H<sub>2</sub>O<sub>2</sub> (1 mM) for 30 min and then both cultures were mixed with phenol/ethanol solution to stop bacterial gene transcription. The mRNA levels of *katE* were measured by quantitative real-time RT-PCR. The housekeeping gene *rpoD* was used as a normalization control. Data are the mean±SD of three independent experiments. UT: H<sub>2</sub>O<sub>2</sub>-untreated

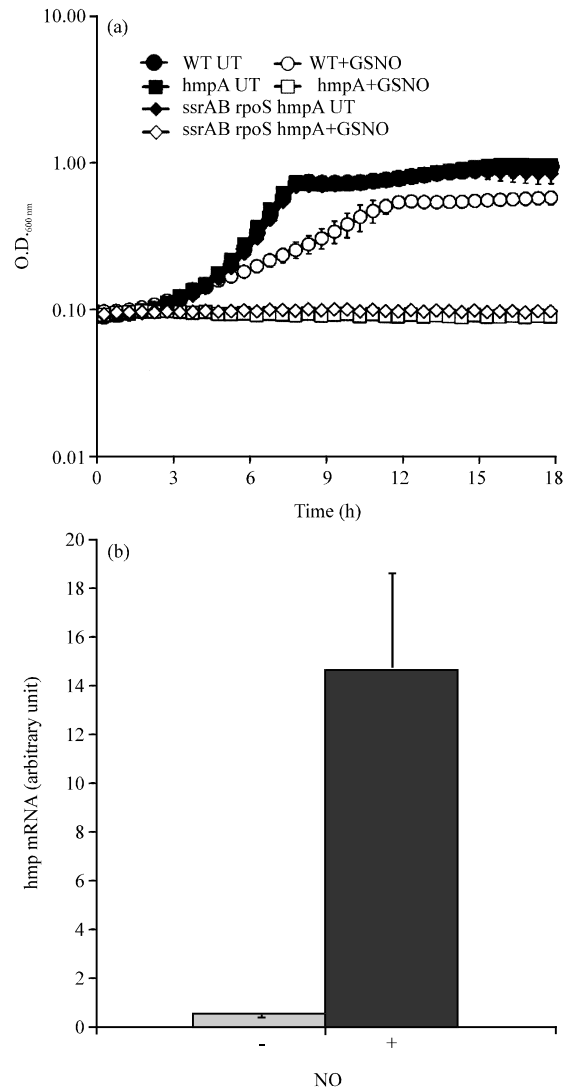


Fig. 2: *hmp-d* dependent nitrosative stress resistance of *ST98*; a) the growth of strains was monitored during culture in E minimal media in the presence and absence of GSNO as described for Fig. 1. Data shown are the mean±SD of three independent experiments; b) log-phase cultures grown in E media were treated with or without GSNO for 1 h. Preparation of RNA samples and quantitative real-time RT-PCR were performed as described in Fig. 1 and in the methods. Data are the mean±SD of three independent experiments. UT: GSNO-untreated

(Fig. 2b). These results clearly show that the expression and NO-detoxifying activity of flavohemoglobin *hmp* is required for NO resistance of *ST98*.

**ssrAB is required for SPI-2 gene expression in *ST98*:**

To measure SPI-2 expression in *ST98*, researchers selected representative genes of SPI-2 for study. Genes *sseJ* and *ssaB* encode effector proteins and *sseA* encodes

chaperones for the SPI-2 encoded TTSS (Srikanth *et al.*, 2011). Transcription of these genes and other *SPI-2* genes increases during phagocytosis and is dependent on the *ssrA/ssrB* two component response regulator system encoded by the *ssrAB* operon (Fass and Groisman, 2009). Some *in vitro* conditions including acidic pH and low concentrations of the divalent cations  $Ca^{2+}/Mg^{2+}$  and of phosphate which mimic the microenvironment of the SCV in phagocytes, induce *SPI-2* gene transcription (Deiwick *et al.*, 1999). Researchers tested SPI-2 induction in ST98 under a low  $Mg^{2+}$  condition. The 3 h after the shift of cultured cells from high to low  $Mg^{2+}$  media, there were 80, 60 and 7 fold increases in transcription of *sseJ*, *ssaB* and *sseA*, respectively. This induction was completely abolished in *ssrAB* mutants, demonstrating that the ST98 strain conserves the *ssrA/ssrB*-dependent regulatory system for SPI-2 induction (Fig. 3).

**Stable phenotype of *rpoS ssrAB hmp* triple mutant ST98:**

An important prerequisite for applications of a strain containing multiple gene mutations is stable maintenance of each phenotype caused by the respective gene mutation because any one mutation, especially in global regulators can cause spontaneous suppressor mutations or compromise the phenotypic effects of different gene mutations by affecting the regulatory circuits for gene expression. To examine this possibility in the *rpoS ssrAB hmp* triple mutant ST98, researchers repeated the susceptibility and gene expression tests for hydrogen

peroxide and GSNO exposure and examined the *ssrA/ssrB* dependence of SPI-2 induction. As shown in Fig. 1-3, the ST mutant lacking all three genes shows phenotypes comparable to those observed after mutation of each single gene, demonstrating that no genetic alteration affecting phenotypes has occurred.

**Attenuated virulence of *rpoS ssrAB hmp* triple mutant ST98:**

Groups of mice were infected with the *rpoS ssrAB hmp* triple mutant ST98 to evaluate virulence of this ST mutant (Fig. 4). Compared with mice infected with WT ST, the number of CFU recovered from the liver of mice infected with *rpoS ssrAB hmp* triple mutant ST98 via the intraperitoneal route were markedly decreased by 1.99, 1.59, 1.63 and 1.11 log at 3, 6, 9 and 12 dpi, respectively, (Fig. 4a). CFU counts from the spleen showed a similar trend compared with WT ST with reductions in the range of 0.48-0.72 log at 12 dpi (Fig. 4b). However, the differences in CFU recovered from liver and spleen of mice infected with WT and the *rpoS ssrAB hmp* triple mutant ST98 were not statistically significant. The number of CFU recovered from the liver of mice infected with the *rpoS ssrAB hmp* triple mutant ST98 via the oral route was reduced compared with that from mice infected with the WT strain by 2.38, 0.28 and 0.57 log at 3, 9 and 12 dpi, respectively (Fig. 4c). Numbers of CFU recovered from the spleen also showed a similar trend but with greater reductions in the range of 1.78-2.98 log at 12 dpi (Fig. 4d). The number of CFU recovered from the liver and spleen of mice infected orally with *rpoS ssrAB hmp* triple mutant ST98 showed no reduction at 6 dpi compared with those infected with WT (Fig. 4c and d).

Bacterial virulence depends largely on the expression of virulence factors that resist antimicrobial factors produced by host immunity and thereby help bacteria to establish within target host tissues. Studies on gene mutations altering the expression and activity of virulence factors for *Salmonella* pathogenesis have demonstrated a close relationship between bacterial gene regulation identified *in vitro* and their roles in infection *in vivo*. All genes selected and examined in this study are essential for virulence in mouse infection models of laboratory WT ST strains such as 14028S and LT2. The extra cytoplasmic function of sigma factor *rpoE* activated by envelope stresses such as ethanol and heat is also increased by oxidative stress in aerobic stationary phase cultures and promotes transcription of genes required for the antioxidant defenses of ST and for virulence in ROS-producing mice (Bang *et al.*, 2005; Rowley *et al.*, 2006; Testerman *et al.*, 2002). The stationary-phase sigma factor *rpoS* is activated in response to several environmental changes and governs gene transcription

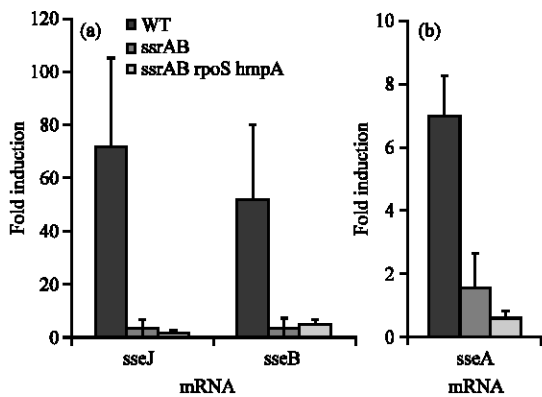


Fig. 3: *SPI-2* gene transcription in ST98. The mRNA levels of *sseJ*, *ssaB* and *sseA* were measured from total RNA isolated from ST strains incubated under SPI-2 inducing conditions. Fold induction was calculated as the levels of each mRNA expressed after induction divided by the levels before induction. The housekeeping gene *rpoD* was used as a normalization control. Data are the mean±SD of three independent experiments

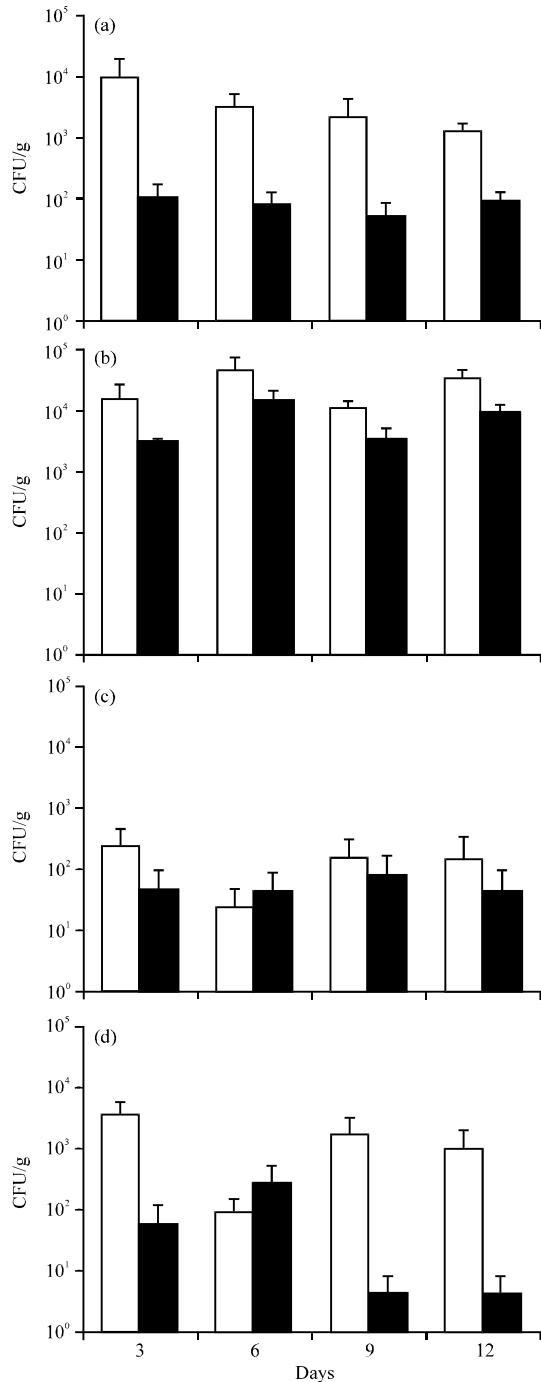


Fig. 4: Enumeration of viable ST WT (□) and mutant (■) recovered from the liver and spleen in mice. Female 7 weeks old C57BL/6 mice were intraperitoneally (panels a and b) or orally (panels c and d) immunized with rpoS srrAB hmp triple mutant ST98 and WT-infected control. Results are shown as mean CFU per gram of liver (panel a and c) spleen (panel b and d)

required for resistance of many pathogens to various antimicrobial factors (Dong and Schellhorn, 2010; Hengge-Aronis, 2002). rpoS expression in ST is also induced by a range of environmental stresses and is essential for virulence in several mouse strains. As global regulators of bacterial gene transcription, both rpoE and rpoS are involved in expression of many sets of genes that depends on environmental cues including oxidative stress. Although, gene mutations causing a lack of function of either sigma factor cause reductions in target gene transcription and attenuate virulence in the mouse, there is also indirect regulation of genes containing no cognate consensus sequences for binding of the respective sigma factors. One reason that researchers chose these sigma factors for this study is that mutations in genes for either sigma factor indirectly increase *SPI-1* gene transcription by unknown mechanisms (Bang *et al.*, 2005; Karlinsey *et al.*, 2012). This is important for the development of an attenuated vaccine strain, to maintain or promote its immunogenicity following epithelial invasion.

Unexpectedly, this study showed less dependence of resistance to oxidative stress on rpoE than reported previously (Bang *et al.*, 2005; Testerman *et al.*, 2002). Considering the fact that rpoE is essential for survival of *Escherichia coli* at all temperatures but mutant ST lacking rpoE is viable (De Las Penas *et al.*, 1997; Humphreys *et al.*, 1999), it seems possible that the role of rpoE might also differ among ST strains.

Flavo-hemoglobin hmp plays a central role in protecting many bacteria, fungi and protozoa (Forrester and Foster, 2012) against another potent radical species, NO and NO-mediated RNS. This study confirmed the role of hmp in NO resistance of the ST98 strain suggesting that evolutionary conservation of this enzyme is required for bacterial pathogenesis in NO-producing host animals.

After SPI-1-mediated invasion, ST can be phagocytosed by macrophages where it encounters dramatic changes in the microenvironment that trigger expression and secretion of SPI-2 effector proteins (Srikanth *et al.*, 2011). Translocation of SPI-2 effectors across the SCV inhibits recruitment of phagocyte NADPH oxidase to the SCV membrane resulting in evasion by ST of major ROS stressors in phagocytes (Vazquez-Torres and Fang, 2001). Phosphorylated response regulator srrB, activated by cognate histidine kinase srrA, binds to the promoters of all SPI-2 genes and activates their expression (Worley *et al.*, 2000). In addition, a recent study showed that nitrosylation of srrB by NO modulates SPI-2 gene expression both *in vitro* and during infection (Husain *et al.*, 2010), potentiating the

function of *ssrB* in the strategy of ST for evading radical species. Researchers showed that deletion of both *ssrA* and *ssrB* genes abolished SPI-2 induction in ST98 suggesting that in addition to the deleterious effect of mutations of *rpoS* and *hmp*, *ssrAB* mutations would cause additional damage to ST98 that would prevent its survival within macrophages.

In the virulence test in mice, the number of CFU of the *rpoS ssrAB hmp* triple mutant ST98 recovered from the liver and spleen in mice infected via the intraperitoneal route were reduced while the WT strain retained full virulence for 12 days (Fig. 4a and b). Even though the numbers of CFU of *rpoS ssrAB hmp* triple mutant ST98 recovered from the liver and spleen in mice infected via the oral route were not stable over 12 days, *rpoS ssrAB hmp* triple mutant ST98 showed a trend towards attenuated virulence compared with the WT strain except at day 6. These observations are in good agreement with a previous study where mice were inoculated with *hmp* mutant *Salmonella* and WT strain and the *hmp* mutant showed a 100 fold decrease in virulence compared with WT even though the virulence at day 5 was not significantly different (Bang *et al.*, 2006).

These results demonstrated that the flavohemoglobin *hmp* promotes ST virulence over 12 days. This observation is also in good agreement with a previous report that an ST *rpoS* single mutant was highly attenuated in mice compared with virulent ST, although the mutant retained significant ability to protect mice against salmonellosis (Coynault *et al.*, 1996; Coynault and Norel, 1999).

## CONCLUSION

This study highlighted the roles of oxidant response genes in virulence of an endemic ST strain isolated in Korea. Except for the *rpoE* mutant, all mutants examined in this study showed phenotypes in the ST98 strain that were comparable with earlier findings in other strains. This demonstrates that the functions of these virulence factors identified from studies with laboratory strains are conserved in a naturally occurring ST isolate from pigs and can play significant roles in antioxidant defenses.

In the virulence test in mice, the *rpoS ssrAB hmp* triple mutant ST98 was highly attenuated compared with fully virulent WT but was still able to invade the organs of the host. This study sheds new light on the understanding of the phenotypic and genotypic characteristics of virulent ST causing endemic disease in Korea and provides the basis for development of a live ST vaccine.

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