σS-Mediated Stress Response Induced by Outer Membrane Perturbation Dampens Virulence in Salmonella enterica serovar Typhimurium

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Salmonella alters cellular processes as a strategy to improve its intracellular fitness during host infection. Alternative σ factors are known to rewire cellular transcriptional regulation in response to environmental stressors. σS factor encoded by the rpoS gene is a key regulator required for eliciting the general stress response in many proteobacteria. In this study, Salmonella Typhimurium deprived of an outer membrane protein YcfR was attenuated in intracellular survival and exhibited downregulation in Salmonella pathogenicity island-2 (SPI-2) genes. This decreased SPI-2 expression caused by the outer membrane perturbation was abolished in the absence of rpoS. Interestingly, regardless of the defects in the outer membrane integrity, RpoS overproduction decreased transcription from the common promoter of ssrA and ssrB, which encode a two-component regulatory system for SPI-2. RpoS was found to compete with RpoD for binding to the PssrA region, and its binding activity with RNA polymerase (RNAP) to form EσS holoenzyme was stimulated by the small regulatory protein Crl. This study demonstrates that Salmonella undergoing RpoS-associated stress responses due to impaired envelope integrity may reciprocally downregulate the expression of SPI-2 genes to reduce its virulence.

Keywords: Salmonella Typhimurium, RpoS (σS), ssrA, Salmonella pathogenicity island-2, virulence
σ\(^{37}\) (σ\(^{28}\), FliA), heat-shock response-specific σ\(^{47}\) (σ\(^{12}\), RpoH), stationary-phase nutrient-starvation-specific σ\(^{35}\) (σ\(^{38}\), RpoS), and nitrogen-starvation-specific σ\(^{N}\) (σ\(^{34}\), RpoN). The abundance of alternative σ factors available for Eσ complex formation is regulated not only by environmental signals (Shimada et al., 2017) but also by the interaction with two types of inhibitory proteins, anti-σ factors, and adaptor proteins (Trevino-Quintanilla et al., 2013). Different σ factors operate discrete regulatory circuits containing cognate genes and operons in response to specific environmental cues, but some transcriptional regulations are coordinated by multiple σ factors that function in a regulatory cascade or by competitive interactions. In response to the diverse stimuli encountered by bacterial pathogens upon host infection, multiple alternative σ factors interact with each other to promote bacterial adaptation in hostile conditions. σ\(^{i}\) can activate one of the rpoH promoters (Hiratsu et al., 1995; Vanaporn et al., 2008) and σ\(^{ii}\), in turn, stimulates the transcription of Hfq (Muffler et al., 1996), which is required for efficient translation of rpoS mRNA (Bang et al., 2005), indicating sequential activation of multiple regulons by a regulatory cascade of σ\(^{i}\), σ\(^{ii}\), and σ\(^{iii}\) under certain circumstances. Besides, there is a trade-off between self-preservation and nutritional competence and genes required for membrane integrity maintenance and genes associated with metabolism are reciprocally controlled by competitive action between σ factors (Ferenci, 2005; Levi-Meyrueis et al., 2015). In the context of competitive action between σ factors, σ\(^{20}\) and σ\(^{3}\) recognize almost identical –35 and –10 promoter elements, especially the –10 region (Hengge-Aronis, 2002b). Therefore, competitive binding of Eσ\(^{3}\) to the overlapping promoter regions may occlude transcription initiation by Eσ\(^{20}\), inducing the transcription of a repertoire of genes by σ\(^{3}\) under stressful environments (Levi-Meyrueis et al., 2015).

Many genes whose promoters bind to both σ\(^{20}\) and σ\(^{3}\) show stronger transcription activities with σ\(^{20}\) binding than with σ\(^{3}\) binding, implying a negative role of σ\(^{3}\) in gene expression (Levi-Meyrueis et al., 2015; Grove et al., 2017; Yin et al., 2018). Interestingly, nullifying the negative effects of σ\(^{3}\) is beneficial to bacterial growth in the absence of environmental stressors (Zambrano et al., 1993; Notley-McRobb et al., 2002). The attenuated expression associated with Eσ\(^{3}\) may confer fitness advantages to bacteria during unfavorable conditions. σ\(^{3}\) is induced under nutrient-depleted stationary phase or in response to various stressors, and its activity in Salmonella is known to alter transcription or protein production of more than 20% of its genome (Levi-Meyrueis et al., 2014; Lago et al., 2017). σ\(^{3}\) upregulates or downregulates the expression of a myriad of genes involved in carbohydrate and amino acid metabolism, stress resistance, and membrane integrity directly or indirectly. In contrast to the essential roles of σ\(^{2}\) in bacterial stress response, the requirement of σ\(^{3}\) for bacterial virulence varies between bacterial species (Dong and Schellhorn, 2010). Salmonella Typhimurium lacking rpoS gene showed reduced virulence, and σ\(^{3}\) factor was found to activate the transcription of sprR and sprABC sectors virulence plasmid genes (Fang et al., 1992; Kowarz et al., 1994).

In this study, we induced outer membrane perturbation on S. Typhimurium by deleting ycfR to stimulate σ\(^{2}\)-mediated adaptation responses. YcfR is a putative outer membrane protein that is expressed under stressful conditions in enteric pathogens and is known as a multiple stress resistance protein (Zhang et al., 2007; Salazar et al., 2013). Our previous study demonstrated that the deletion of ycfR caused structural alterations in lipopolysaccharide and destabilized Salmonella envelope integrity (Kim and Yoon, 2019). Salmonella devoid of YcfR tremendously increased rpoS transcription and showed an increase in curli fibers, cellulose, and c-di-GMP production and a decrease in motility, implicating comprehensive transcriptional alterations by σ\(^{3}\) in response to stress on the cellular envelope (Kim and Yoon, 2019). Besides the known repertoires of σ\(^{3}\) regulatory circuits, such as biofilm formation, this study revealed that virulence genes of Salmonella pathogenicity island-2 (SPI-2) were downregulated by σ\(^{3}\). SPI-2 is a locus responsible for the type III secretion system (T3SS) injectosome-mediated delivery of virulence factors from Salmonella to host cells and is critical for bacterial survival and replication inside host cells (Jennings et al., 2017). The negative role of σ\(^{3}\) in SPI-2 regulation was influenced by a small regulatory protein Crl. Crl is known to be required for σ\(^{3}\)-dependent transcriptional initiation at the promoters of adrA and csgD genes, whose products activate curli and cellulose production (Robbe-Saule et al., 2006). The role of σ\(^{3}\) in Salmonella virulence regulation was elucidated by examining the interaction between σ\(^{3}\) and the ssrAB promoter, which encodes the two-component regulatory system SsrAB for SPI-2.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions**

*Salmonella enterica* serovar Typhimurium ATCC 14028 was used as the parent strain. *Salmonella* mutants of ΔycfR and ΔrpoS were constructed using the phage lambda (λ) Red recombination system as described in previous studies (Yoon et al., 2009; Kim and Yoon, 2019) and a mutant lacking both ycfR and rpoS was constructed using P22 HT105/1 int-201-mediated transduction (Kwok and Kemper, 1978). The phage λ Red recombination system was also used for the construction of Salmonella strains producing HA-tagged SPI-2 proteins (SseC and SsaN) as described in the previous study (Kim et al., 2018). In brief, the kanamycin resistance (kan) cassette of pKD13-2HA was amplified by PCR using primers designed to provide 40-nucleotide sequences homologous to target genes at both termini of the resultant PCR products. The PCR products were introduced into Salmonella cells harboring pKD46 to insert the HA-coding sequences with a kan cassette prior to the stop codon sequences. The kan marker was subsequently removed using pCP20 providing a flip recombinase. Primers used for the construction of HA-tagged SPI-2 genes are listed in Supplementary Table 1. *Escherichia coli* DH5α strain was used for plasmid cloning and protein purification.

To express rpoS in *trans*, the rpoS gene was cloned into pACYC184 (Chang and Cohen, 1978) and pBbA2sk-RFP vectors (Lee et al., 2011). For the construction of pRpoS, the rpoS
CDS and its promoter region were amplified by PCR using primers pPspS-CF and pPspS-CR and inserted into pACYC184 through BamHI and SalI restriction enzyme sites. In cloning pPspS2, the rpoS gene was amplified using PCR with pPspS-CF2 and pPspS-CR2 primers, digested with EcoRI and BglII, and ligated with EcoRI/BglII digested pBBA2k-RFP plasmid. Primer sequences are listed in Supplementary Table 1.

To construct transcriptional lacZ fusion to the P_ssa and P_ssa regions, the promoter regions of ssrA (from −253 to +290) and ssrB (from −90 to +303) were amplified by PCR using primers (Supplementary Table 1) of psrA-lacZ-CF and psrA-lacZ-CR for ssrA and primers (Supplementary Table 1) psrB-lacZ-CF and psrrB-lacZ-CR for ssrB, as described by Feng et al. (2003). The amplified promoter regions were cloned into the pRS415 plasmid (Simons et al., 1987) using EcoRI and SalI restriction enzyme sites.

RpoS, RpoD, and Crl proteins were tagged with His, at their N-termini by cloning three genes into the pUHE21-lacI plasmid (Soncini et al., 1995) via EcoRI and HindIII and inducing their expression using IPTG. The primers used for the construction of His-tagged proteins are listed in Supplementary Table 1. All restriction enzymes and ligases were purchased from Takara Bio, Inc. (CA, United States).

Salmonella cells were cultured in Luria-Bertani (LB) medium broth or acidic minimal medium (AMM) broth at 220rpm at 37°C, as described in previous studies (Yoon et al., 2009, 2011). For AMM cultivation, bacterial cells at the stationary growth phase in LB medium broth were washed twice with PBS, diluted in pH 7.0 minimal medium broth at a 1:100 ratio, and cultivated overnight. Pre-cultured Salmonella cells in minimal medium broth (pH 7.0) were diluted in minimal medium broth (pH 5.0) at a 1:20 ratio and cultivated for 3 h to mimic intracellular conditions (Yoon et al., 2009). Antibiotics were purchased from Sigma-Aldrich (MO, United States) and used when required: ampicillin (Amp, 50 μg/ml), chloramphenicol (Cm, 35 μg/ml), kanamycin (Kan, 50 μg/ml), and anhydrotetracycline (aTc, 0.2 or 0.5 ng/ml).

Mammalian Cell Infection

To assess bacterial invasiveness, HeLa human epithelial cell line (ATCC CCL-2) was infected as described in the previous study (Kim et al., 2018). HeLa cells were seeded in 24-well plate at 2 × 10^5 cells/well and incubated in Dulbecco’s modified Eagle’s medium (DMEM; Corning cellgro, Thermo Scientific Inc., IL, United States) supplemented with 4.5 g/L glucose (Thermo Scientific Inc.) and 10% fetal bovine serum (FBS; Gibco, Thermo Scientific Inc.) at 37°C with 5% CO2. After overnight incubation, HeLa cells were treated with 1.5 h. The infected macrophages were washed with PBS three times and incubated in fresh DMEM containing 20 μg/ml gentamicin for additional 8 h. To enumerate intracellular bacteria, RAW264.7 cells were lysed, and the lysates were spread on LB agar as described above.

qRT-PCR Analysis

Bacterial total RNA was isolated from Salmonella cultivated in LB medium and AMM broth or RAW264.7 cells infected with Salmonella. Bacterial cells cultivated in vitro were treated with Rnaprotect Bacteria Reagent (Qiagen, Hilden, Germany) and subjected to total RNA extraction using RNeasy mini kit (Qiagen). For RNA extraction from intracellular bacteria, infected macrophage cells were treated with RnAlater™ Stabilization Solution (Invitrogen, Thermo Scientific Inc.) and processed with RNeasy mini kit according to the manufacturer’s recommendations. Isolated total RNA was treated with RNase-free DNase (Ambion, TX, United States) at 37°C for 30 min and used to synthesize cDNA using RNA to cDNA EcoDry™TM Premix (Takara Bio United States, Inc.). cDNA corresponding to 10 ng of input RNA was used as a template in each qRT-PCR, and the primer sequences are listed in Supplementary Table 2. qRT-PCR was conducted using the StepOnePlus Real-time PCR system (Applied Biosystems, MA, United States) with Power SYBR Green PCR Master Mix (Applied Biosystems), and the levels of amplified PCR products were normalized to those of gyrB (Yoon et al., 2009).

β-Galactosidase Assay

The β-galactosidase assay was conducted using the Miller method (Smale, 2010). Bacterial cells were cultivated in LB medium broth, and β-galactosidase activity normalized to the number of input bacteria was represented by Miller units. Miller units were computed as follows: Miller unit = [1,000 × (OD_{590}−1.75 × OD_{600})]/ (t × V × OD_{600}), where t is time (min) and V is volume (ml).

Immunoblot Assay

Bacterial cells were pelleted and resuspended in 1× Laemmli sample buffer (Bio-Rad Laboratories, Inc., CA, United States). The aliquots were loaded on 10% SDS-PAGE gels, and the separated proteins were transferred to PVDF membranes (Bio-Rad Laboratories, Inc.). The membrane was blocked with 5% skim milk solution and treated with anti-RpoS antibody (anti-E. coli RNA Sigma S antibody, BioLegend, CA, United States) at a 1:200 dilution ratio or anti-DnaK antibody (Enzo Life Science, NY, United States) at a 1:10,000 dilution ratio in combination with horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad Laboratories, Inc.) at
a 1:3,000 dilution ratio. SPI-2 proteins tagged with HA were identified using anti-HA antibody (1:10,000 dilution; Sigma, United States) as a primary antibody. Immunoblotting was conducted using ECL™ Western Blotting Detection Reagents kit (GE Healthcare, Thermo Scientific Inc.), and the blot images were visualized using the ChemiDoc™ MP System (Bio-Rad Laboratories, Inc.). The intensity of the blot images was analyzed using ImageJ software.1

**Chromatin Immunoprecipitation Assay**
The Chromatin immunoprecipitation (ChIP) assay was performed as previously described (Gu et al., 2016; Yin et al., 2018) with minor modifications. Briefly, *Salmonella* cells cultivated in the stationary growth phase in LB medium broth were fixed with 1% formaldehyde solution for 10 min and subsequently treated with 100 mM glycine for 5 min. Cells were washed with cold PBS and resuspended in SDS lysis buffer (50 mM Tris–HCl, 10 mM EDTA, 1% SDS, and pH 8.0) containing 1× protease inhibitor. After 10 min of incubation, the cell extract was sonicated to fragment genomic DNA into 200 bp to 1 kb and centrifuged at 12,000 × g for 10 min. The supernatant solution was used as input DNA, and the aliquots were further processed for pre-clearing and immunoprecipitation (IP) samples. The lysate solution containing DNA-protein complexes was pre-incubated with Protein A/G Plus-Agarose (Santa Cruz Biotechnology, Inc. TX, United States) at 4°C for 2 h to remove DNA or proteins non-specifically bound to Protein A/G Plus-Agarose and centrifuged at 800 × g for 3 min. The resultant pellet fraction was used as a pre-clearing sample, and the supernatant solution was further incubated with the anti-RpoS antibody at 4°C overnight, followed by Protein A/G Plus-Agarose at 4°C for 2 h, and centrifuged at 800 × g for 3 min to immunoprecipitate DNA-RpoS complexes bound to the agarose. The pellet fraction was used as an IP sample. The pre-clearing and IP samples were washed with LiCl wash buffer (100 mM Tris–HCl, pH 8.0, 2% Triton X-100, and 250 mM LiCl), twice with high-salt buffer (100 mM Tris–HCl, pH 8.0, 600 mM NaCl, and 2% Triton X-100), twice with low-salt buffer (100 mM Tris–HCl, pH 8.0, 300 mM NaCl, and 2% Triton X-100), and with TE wash buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA). The precipitated DNA-protein complexes were eluted with ChIP elution buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, and 1% SDS) and incubated with 0.2 M NaCl at 65°C overnight to resolve DNA-protein cross-links. All samples were treated with RNase A (10 mg/ml) at 37°C for 30 min and further incubated with a protease solution (1 μg/ml Tris–HCl, pH 8.0, 500 mM EDTA, proteinase K, and 5 M NaCl) at 65°C for 4 h. DNA from pre-clearing and IP samples was extracted using phenol: chloroform: isomyl alcohol (25: 24: 1) solution, precipitated with EtOH and NaOAc (pH 5.2), and resuspended in distilled water.

**ChIP-Quantitative PCR Assay**
DNA cross-linked to RpoS was analyzed using quantitative PCR (qPCR), as previously described (Hermans et al., 2016). Relative enrichment (RE) of the promoter of interest was computed using differences in Ct values (ΔCt) with gyrB gene as an endogenous control as follows: RE = 2^((ΔCtIP−ΔCtPre-clearing)/μ)

1https://imagej.nih.gov/ij/

**Purification of His<sub>6</sub>-Tagged Protein**
*Escherichia coli* strains producing His<sub>6</sub>-tagged RpoS, RpoD, and Crl proteins were cultivated in LB medium broth, and the proteins were induced by adding 0.05 mM (RpoS and RpoD) or 1 mM (Crl) isopropyl β-D-1-thiogalactopyranoside for 7 or 3 h at 30°C. Bacterial cells were centrifuged at 10,000 × g for 10 min and resuspended in lysis buffer (50 mM NaHPO<sub>4</sub>, 300 mM NaCl, 20 mM imidazole, and pH 8.0) containing 1 mg/ml lysozyme. After 30 min incubation on ice, the cells were sonicated and centrifuged at 10,000 × g and 4°C for 20 min. The resultant soluble lysate fraction was treated with Ni<sup>2+</sup>-nitrilotriacetic acid (Ni<sup>2+</sup>-NTA) agarose beads (Qiagen) at 4°C for 1 h with rotation and loaded onto a Ni<sup>2+</sup>-NTA agarose affinity column (Qiagen). The column was washed with washing buffer (50 mM NaHPO<sub>4</sub>, 300 mM NaCl, 40 mM imidazole, and pH 8.0) three times, and the proteins were eluted with elution buffer (50 mM NaHPO<sub>4</sub>, 300 mM NaCl, 300 mM imidazole, and pH 8.0). The eluted protein fraction was packed into SnakeSkin™ Dialysis tubing with 10 K MWCO (Thermo Scientific Inc.) and subjected to dialysis at 4°C in dialysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1 mM EDTA, 5 mM DTT, and 20% glycerol). Purified proteins were quantified using Bradford assay.

**Electrophoretic Mobility Shift Assay**
The binding between σ factors and the P<sub>σ</sub> region was investigated using His<sub>6</sub>-RpoS or His<sub>6</sub>-RpoD in combination with His<sub>6</sub>-Crl. The P<sub>σ</sub> region was PCR-amplified using primers srrA-electrophoretic mobility shift assay (EMSA)-F and srrA-EMSA-R. The csgBA promoter region amplified using primers csgBA-EMSA-F and csgBA-EMSA-R was employed as a positive control, whereas the STM14_1978 (putative ABC transporter permease component) CDS region amplified using primers STM14_1978 EMSA-F and STM14_1978 EMSA-R was used as a negative control. The primer sequences used in the EMSA are listed in Supplementary Table 3. EMSA was performed as described previously (Bougdour et al., 2004; Storvik and Foster, 2010) with the following modifications. To reconstitute the RNAP holoenzyme, 20 nM RNAP core enzyme (*E. coli* RNAP Core Enzyme; NEB, MA, United States) was incubated with 300 nM His<sub>6</sub>-RpoS or His<sub>6</sub>-RpoD in a binding buffer (200 mM Tris–HCl, pH 8.0, 30 mM KCl, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and BSA 20 μg/ml) at 30°C for 45 min.
DNA of 20 ng was incubated with the reconstituted RNAP holoenzyme in a binding buffer (50 mM Tris–HCl, pH 8.0, 200 mM KCl, 3 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, BSA μg/ml, and Poly (di-dc) 12 ng/μl) at 25°C for 30 min. The reactant was analyzed by electrophoresis using 5% native polyacrylamide gel, and DNA fragments were stained with EtBr solution and detected using the ChemiDoc MP System.

In the competitive binding assay between His₆-RpoS and His₆-RpoD, one σ factor was used at a constant concentration of 75 nM and the other competitor σ factor was used at incremental concentrations from 12.5 to 150 nM. After RNAP holoenzyme reconstitution with different concentrations of σ factors, DNA corresponding to the P₆₅ region was added to the binding reaction and analyzed as described above. To localize His₆-RpoS after electrophoresis on a native polyacrylamide gel, proteins on the gel were transferred to a PVDF membrane and processed as described in the immunoblot assay above. Anti-E. coli RNA sigma S antibody was used as a primary antibody at a 1:2,000 dilution ratio, and HRP-conjugated goat anti-mouse IgG was used as a secondary antibody at a 1:3,000 dilution ratio.

In the competition assay using His₆-RpoS in combination with His₆-Crl, His₆-RpoS (25, 50, and 280 nM) was pre-incubated with 280 nM His₆-Crl at 25°C for 15 min and then used to compete with 50 nM His₆-RpoD in the RNAP holoenzyme reconstitution reaction. After the addition of P₆₅ DNA, the locations of P₆₅ DNA and His₆-RpoS were identified using EtBr staining and immunoblotting methods, respectively, as described above.

**Statistical Analysis**

All assays were repeated at least three times, and the average values were presented with their SDs. To determine the significant differences, Student's t-test was applied, and the value of p was calculated.

**RESULTS**

**Outer Membrane Perturbation in ΔycfR Decreased SPI-2 Expression**

YcfR, which is expressed in response to multiple stress conditions, is a putative outer membrane protein important for stress resistance in enteric pathogens such as *Salmonella* spp. and *E. coli* (Zhang et al., 2007; Salazar et al., 2013). In this study, we observed that *Salmonella* lacking YcfR was significantly attenuated in virulence during host cell infection. The lack of YcfR did not influence bacterial growth in vitro, but significantly reduced the ability of bacteria to invade host epithelial cells and survive inside phagocytic cells (Figure 1A). The transcription of SPI-1 genes, which produce a distinct T3SS (T3SS1) and promote *Salmonella* invasion into host cells (Raffatellu et al., 2005), decreased remarkably in the ΔycfR strain (Figure 2A). Besides the attenuated SPI-1 expression, the physiological changes caused by the lack of YcfR, including cellular aggregation and reduced motility (Kim and Yoon, 2019), might impair bacterial invasion ability. Interestingly, the lack of YcfR also decreased the transcription of SPI-2 genes not only inside macrophage cells (Supplementary Figure 1A) but also in LB and AMM cells in vitro (Figure 2B; Supplementary Figure 1B, respectively), which partially reproduce the intestinal lumen and intracellular milieu, respectively (Beuzon et al., 1999; Yoon et al., 2011).

**Downregulation of SPI-2 in ΔycfR Was Attributable to RpoS**

In order to figure out a transcriptional regulator that coordinates bacterial virulence in response to outer membrane perturbation, we assessed the expression of 21 regulators associated with SPI-1 or SPI-2 regulation in the ΔycfR strain (Supplementary Figure 2) and found that rpoS showed a dramatic increase in its transcription. The levels of RpoS were compared between wild-type and ΔycfR strains in LB and AMM conditions. RpoS increased in the ΔycfR strain grown in both media (1.5-fold in LB; 2.7-fold in AMM; Figure 3). To examine whether an increase in RpoS could downregulate virulence genes associated with SPI-2 T3SS (T3SS2), the transcription of ssrAB encoding the two-component regulatory system for T3SS2 and its cognate effectors was compared. *Salmonella* deprived of RpoS slightly increased the expression of ssrAB, but the introduction of pRpoS expressing rpoS under its own promoter significantly decreased the transcription of ssrAB, implicating overall downregulation of their cognate T3SS2-associated genes by RpoS (Figure 4A). In addition, the decreased transcription of ssrAB in the absence of YcfR was nullified by the additional rpoS deletion, suggesting the possibility of σ²-mediated SPI-2 downregulation in the ΔycfR strain (Figure 4A). The ssrA and ssrB genes, located adjacent to each other, encode a sensor kinase and its response regulator, respectively, and are regarded to be transcribed in a polycistronic...
FIGURE 2 | Expression of SPI-1 and Salmonella pathogenicity island-2 (SPI-2) genes in ΔycfR mutant. (A) Transcription levels of SPI-1 genes were examined using RNA isolated from Salmonella strains grown in LB medium broth for 2h. The Ct values of qRT-PCR were normalized using those of gyrB and the fold-change between wild-type and ΔycfR mutant strains was plotted. (B) To analyze the expression of SPI-2 genes, Salmonella strains were cultivated in LB medium broth for 10h and subjected to RNA extraction. Ct values of each gene were subtracted from those of gyrB for normalization, and the fold-change (ΔycfR/wild-type) was calculated. An asterisk indicates a difference of a value of p<0.05.

FIGURE 3 | Expression of RpoS in ΔycfR mutant. Salmonella wild-type and ΔycfR mutant strains were cultivated in LB medium broth for 10h or acidic minimal medium (AMM) broth for 3h, and the expression of RpoS was compared using immunoblot assay with anti-RpoS antibody. The cytosolic protein DnaK was used as a control to standardize the protein amounts between the lanes. The abundance of RpoS was normalized to that of DnaK using ImageJ, and the ratios from three independent assays are depicted below the representative blot images.

mRNA under the same promoter (Bustamante et al., 2008; Fass and Groisman, 2009). However, the identification of a distinct promoter upstream of ssrB revealed the possibility that the expression of ssrA and ssrB could be uncoupled depending on the growth conditions (Feng et al., 2003, 2004). Therefore, the negative role of RpoS was reexamined using lacZ transcriptional fusion constructs, where the promoters of ssrA and ssrB were separately analyzed (Figure 4B). The promoter strength of ssrA was much stronger than that of ssrB in wild-type Salmonella harboring intact rpoS and ycfR genes, and deletion of rpoS alone did not alter ssrA or ssrB transcription. However, ycfR deletion, which led to an increase in RpoS, abolished ssrA transcription but not ssrB, and the additional rpoS deletion in ΔycfR mutant derepressed ssrA only, indicating differential regulation of ssrA and ssrB by σ8 (Figure 4B).

Again, overexpression of RpoS by the introduction of pRpoS2 decreased Pmar::lacZ expression in proportion to αTc concentration. These results suggest that σ8 at high concentrations dampen transcription activity at the promoter region upstream of ssrAB.

σ8 Binds Directly to the ssrA Promoter Region

To examine whether σ8 directly controls the transcription of ssrA, a ChIP assay was performed on Salmonella cells in the stationary growth phase using σ8 as a bait. DNA fragments bound to σ8 were co-precipitated using anti-RpoS antibody and used as templates in PCR using primers targeting the promoter regions of ssrA and ssrB (Figure 5A; Supplementary Figure 2). DNA fragments containing the PssrA region were bound to σ8 and amplified by PCR, but the PssrB region did not co-precipitate with σ8 (Figure 5B). When five different primer sets from R1-F/R to R5-F/R were used to dissect the ssrA promoter region, only two primer sets, R3-F/R and R4-F/R, resulted in significant PCR amplification (Figure 5C), inferring that σ8 binds to DNA sequences covering −61 to +136bp at least from the transcription start site of ssrA (Feng et al., 2003). It is believed that the ssrA promoter requires RNAP holoenzyme harnessing σ70, and the consensus −10 and −35 regions for σ70 binding were also predicted (Ramachandran et al., 2012; Banda et al., 2019). Our results raised the possibility that the ssrA promoter could recruit σ8 as well as σ70. The possibility of σ8 binding to the PssrA region was also proposed in silico in a previous study (Ramachandran et al., 2012). We further investigated transcription initiation at Pmar, which is controlled by mechanical interaction with σ factors.
σS Competes With σ70 for Binding to the ssrA Promoter Region

The promoter recognition sequences for σS and σ70 are nearly identical, and a strong functional similarity between σS and σ70 has been suggested. Many σS-regulated genes, such as the csgBA operon, can be transcribed by either σS or σ70 in vitro (Arnqvist et al., 1994; Typas et al., 2007b). The possibility of biphasic ssrA transcription initiation by σS and σ70 was examined in vitro. A DNA fragment of 172 bp encompassing the PssrA region targeted by ssrA regulators was incubated with each σ factor (His6-RpoS or His6-RpoD) in the presence or absence of RNAP core enzyme E. The PcsgBA region recognized by either σS or σ70 was used as a positive control, while a DNA fragment of the STM14_1978 gene devoid of the canonical sequences recognized by σS and σ70 was used as a negative control. The core enzyme alone could form a complex with DNA fragments of the PssrA or PcsgBA regions in a non-specific manner, as predicted elsewhere, and the addition of either σ factor (σS or σ70) retarded the mobility of the DNA-protein complex. This indicated that σ factor was engaged in the complex formation between RNAP holoenzyme (Eσ 2ββ′σω) and the DNA fragments (Figure 6). Interestingly, the addition of σS produced two bands, presumably a lower one between the core enzyme E and the PssrA and an upper one between the EσS and the PssrA, whereas σ70 incorporation produced a single shifted band, representing robust complex formation between...
Eσ^70 and P_{ssrA} (compare Figure 6A, lane 5 and Figure 6B, lane 5). These results stimulated us to compare the binding affinities between Eσ^5 and Eσ^70 at the P_{ssrA} region.

The P_{ssrA} DNA fragment was incubated with the core enzyme E and different concentrations of σ factors (His_{6}-RpoS and His_{6}-RpoD), and the levels of Erσ^5 associated with the P_{ssrA}
region were determined using an anti-RpoS antibody. When His$_s$-RpoS was used at a constant concentration of 75 nM, but His$_s$-RpoD was increased from 0 to 50 nM, the band representing the complex between Er$^s$ and P$_{ssrA}$ gradually diminished and disappeared at 50 nM His$_s$-RpoD (Figure 7A). On the other hand, when His$_s$-RpoD was maintained at 75 nM but His$_s$-RpoS was increased from 0 to 150 nM, Er$^s$ failed to bind to the P$_{ssrA}$ region even at a 2-fold higher concentration of His$_s$-RpoS than His$_s$-RpoD (Figure 7B). This result suggests that the P$_{ssrA}$ region preferentially recruits Er$^70$ when Er$^s$ and Er$^{70}$ are present at equivalent concentrations in vitro.

**Crl Promotes $\sigma^s$ Competitiveness for Binding to the ssrA Promoter Region**

For investigating the possibility that $\sigma^s$ replaces $\sigma^{70}$ and lowers the ssrA transcription, we searched for a co-regulator that could promote $\sigma^s$ activity under stressful conditions and Crl was chosen as a candidate co-regulator of $\sigma^s$-mediated ssrA transcriptional regulation. Crl is a small protein known to interact directly with $\sigma^s$ in vitro (Bougdour et al., 2004). The P$_{ssrA}$ fragment was incubated with His$_s$-tagged $\sigma$ factors ($\sigma^s$ and $\sigma^{70}$) and Crl individually or in combination, and the $\sigma^s$ bound to P$_{ssrA}$ was localized using an anti-RpoS antibody. In the absence of competition with $\sigma^{70}$, Crl addition enabled $\sigma^s$ (50 nM) to form a complex between Er$^s$ and the P$_{ssrA}$ region, whereas $\sigma^s$ alone at 50 nM were insufficient to form the Er$^s$-P$_{ssrA}$ complex (Figure 8: compare lanes 4 and 5). In the absence of Crl, His$_s$-RpoS even at 280 nM was defeated in the competition with 50 nM His$_s$-RpoD and failed to form the protein-DNA complex (Figure 8, lane 8). However, pre-incubation of His$_s$-RpoS with Crl rendered His$_s$-RpoS competitive in forming the complex between Er and P$_{ssrA}$, showing a shifted band (Figure 8, lane 10). Crl binding to $\sigma^s$ might facilitate the formation of the RNAP holoenzyme incorporating $\sigma^s$ instead of $\sigma^{70}$, as suggested previously (Gaal et al., 2006; Typas et al., 2007a).

**$\sigma^s$ Abundance Led to a Comprehensive Transcriptional Alteration of SPI-2 in Host Cells**

Our results comparing the ability of $\sigma^s$ and $\sigma^{70}$ to form the Er$^s$-P$_{ssrA}$ complex in vitro demonstrated that Er$^{70}$ bound to the P$_{ssrA}$ region preferentially than Er$^s$. Given the limited cellular resources of the RNAP core enzyme E, ssrA transcription may be dampened when $\sigma^s$ stimulated by drastic stressors diverts the RNAP core enzyme E to its cognate regulatory circuit, which is critical for surviving the challenging stressors. SPI-2 genes controlled by SsrAB regulators are known to be activated under hostile conditions such as a nutrition-deprived environment and intracellular milieu (Beuzon et al., 1999; Deiwick et al., 1999), which are prone to stimulate $\sigma^s$-mediated adaptation responses. We investigated the transcriptional response of SPI-2 genes when $\sigma^s$ levels surged in response to stress and Er$^{70}$-mediated transcriptional initiation overwhelmed the transcriptional activity of other Er complexes. Salmonella wild-type and ΔrpoS strains were added to macrophage cells, and the transcription levels of rpoS, rpoD, and SPI-2 genes were compared at 2, 4, and 10 h after phagocytosis. $\sigma^s$ was overexpressed by introducing pRpoS into the ΔrpoS mutant. The absence of rpoS increased rpoD transcriptional levels at 4 h post-infection (Figure 9). Comparing mRNA levels of SPI-2 genes between wild-type and ΔrpoS strains showed that most SPI-2 genes increased their transcription in the absence of $\sigma^s$ and addition
of pRpoS nullified these alterations (Figure 9), indicating a negative role of σS in SPI-2 transcription. In accordance with the transcriptional regulation by σS, the levels of T3SS2-associated proteins were decreased by the overexpression of σS (Supplementary Figure 6). However, the transcriptional response to σS abundance was different among the SPI-2 genes. Many genes, including sscB, sseFG, ssaG, sseI, and sspH2, showed negative transcriptional regulation by σS abundance throughout the assay, whereas sseCD genes encoding the translocon components of T3SS2 (Chakravortty et al., 2005) showed minimal transcriptional alteration by σS abundance (Figure 9). Differential requirements among T3SS effectors depending on time and site during infection have been proposed in previous studies (Brawn et al., 2007; Nunez-Hernandez et al., 2014). The differential influence of σS between T3SS2-associated genes suggests that SsrA-mediated regulation is not the only mechanism by which σS participates in controlling SPI-2 T3SS-associated genes.

**DISCUSSION**

During host infection, *Salmonella* undergoes various stress conditions, such as gastric acidity, bile salts, oxidative stress, and nutrient starvation. Alternative σ factors are prominent
regulatory proteins that enable bacteria to cope with diverse stresses by redirecting RNAP core enzymes to the transcription of genes required for survival and adaptation in these conditions. RpoS or σS, a σ factor comprising the RNAP holoenzyme, is known to activate the transcription of genes associated with general stress resistance (Hengge-Aronis, 2002a). However, the regulatory roles of σS are not only restricted to stress-resistance genes. In Salmonella, σS was found to directly or indirectly control the expression of genes that make up more than 20% of the genome (Levi-Meyrueis et al., 2014; Lago et al., 2017), implying its multifaceted roles ranging from physiological remodeling against cellular damage to metabolic regulation of sugars, amino acids, and fatty acids (Ibanez-Ruiz et al., 2000; Lago et al., 2017). In addition, σS is involved in Salmonella virulence regulation. Rice et al. (2015) observed genes comprising SPI-1 and SPI-2, which are essential for Salmonella invasion into host cells and intracellular survival, were upregulated in the absence of σS, indicating a negative role of σS in SPI-1 and SPI-2 expression. We found that σS could bind to the P_vra region directly (Figure 5), and a surplus of σS repressed its transcription. This led to an overall downregulation of SPI-2 T3SS-associated genes (Figure 9). Direct negative regulation by σS was recently reported in the transcription of esrB in Edwardsiella piscicida (Yin et al., 2018). Edwardsiella piscicida, which is phylogenetically close to Salmonella enterica, exploits T3SS and T6SS to translocate virulence factors into host cells, and the expression of these virulence machineries is activated by the two-component regulatory system EsrAB, homologs for SsrAB in S. enterica (Wang et al., 2009; Yin et al., 2018). Edwardsiella piscicida σS was proposed to mediate a trade-off between stress adaptation and virulence by inhibiting esrB expression through a direct interaction between σS and the P_vra region (Yin et al., 2018). Binding of the Es complex to gene promoter regions is typically presumed to activate transcription initiation. Therefore, the negative regulation by σS may be attributed to the competition between σ factors for binding to the RNAP core enzyme (Farewell et al., 1998; Hsu, 2002). A surge in σS caused by bacterial adaptation to general stresses can exclusively occupy the core enzyme E and impede transcriptional events mediated by other σ factors. However, alternatively to this passive regulation via competitive binding between σ factors, adhesion of the Es complex to promoter regions may sterically hinder binding of the EsS70 complex and directly attenuate transcription, as demonstrated in E. piscicida esrB gene (Yin et al., 2018) and S. enterica serovar Typhimurium sdh gene (Levi-Meyrueis et al., 2015).

The promoter region of ssrA is occupied by multiple regulators, including HiiD, SlyA, OmpR, and H-NS, and its transcription is controlled by the competitive binding of these regulators to the overlapping DNA (Banda et al., 2019). The consensus promoter sequences recognized by σS70 are also accessible to H-NS, whose binding blocks the access of EsS70 and transcriptional activators such as OmpR. Anti-repressors such as HiiD and SlyA relieve H-NS-mediated silencing by competitive binding to the P_vra region (Banda et al., 2019). Considering the similar recognition motifs at −10 and −35 elements between σS and σS70 and the functional inter-compatibility between two σ factors in some genes, the downregulation of ssrAB by binding of σS to the P_vra region can be achievable through several mechanisms. Firstly, binding of EsS70 to the P_vra region may not exert transcriptional initiation, but instead sterically hinder EsS70-mediated transcription as demonstrated in the E. piscicida esrB gene (Yin et al., 2018). A σ factor associated with RNAP core enzyme directs transcription initiation at a specific promoter region but is assumed to dissociate upon transition from transcription initiation to transcription elongation because of a steric clash between the growing RNA product and the σ factor (Hsu, 2002). σS that is not released on time may impede promoter escape of the core enzyme E and hinder transcription elongation. Alternatively, the binding of EsS70 to P_vra region may produce incorrect transcripts, as shown in the transcriptional regulation of crl gene (Zafar et al., 2014). The crl gene with overlapping promoters sensed by two different σ factors of σS70 and σS may shut down its expression by association with EsS70. σS70 increases in response to nitrogen limitation, forming a DNA-EsS70 complex at the crl promoter region, but its binding results in a long noncoding RNA transcript lacking a ribosome binding site, thereby preventing EsS70 from binding to the overlapped promoter and producing translatable crl mRNA (Zafar et al., 2014). We observed that DNA fragments bound to EsS in vivo covered a long region from the known +1 site of ssrA transcript to the start codon for SsrA (Figure 5). To differentiate between these two possibilities, it is important to examine whether EsS bound to the P_vra region can lead to ssrA transcription and whether the resultant transcript can successfully be translated.

Another possibility is the competitive EsS binding among promoters with different binding affinities due to recognition motif preference and topological characteristics. Considering that the cellular σS concentration is low even in the stationary phase of growth (Fishege et al., 1996) and its affinity for RNAP core enzyme is the lowest among σ factors in vitro (Maeda et al., 2000), the P_vra region occluded by multiple regulators may be less competent in recruiting EsS and other promoter sites, which are preferentially responsive to σS, may outcompete the ssrA promoter.

In order to cope with limited resources, bacteria allocate cellular resources between reproduction and maintenance in response to environmental cues. In the absence of nutrient depletion and hostile stressors, bacteria proliferate and deploy resources for reproduction. Under these favorable conditions, σS70 is exclusively used for the transcription initiation of housekeeping genes. On the other hand, bacteria challenged by stressful stimuli divert cellular resources to maintenance and resistance, replacing σS70 with alternative σ factors for comprehensive transcription alteration. σS orchestrates the expression of a large number of genes under conditions of starvation and general stress caused by pH, temperature, and osmolarity. SPI-2 T3SS and its cognate effectors critical for Salmonella intracellular survival and replication are thought to be induced by unfavorable stimuli encountered inside host cells (Lober et al., 2006; Fass and Groisman, 2009), which would also likely promote σS-mediated stress adaptation processes. However, we observed that excessive σS production rather decreased the transcription of SPI-2 and its associated genes. Virulence effectors translocated via SPI-2 T3SS help intracellular Salmonella to compromise the host defense systems and facilitate
intracellular proliferation and cell-to-cell spread (Grant et al., 2012; Jennings et al., 2017). However, overgrowth of Salmonella, which is less competent to manage hostile stresses, poses a disadvantage to long-term persistence inside hosts because the intense immune responses provoked by the proliferation may eliminate defective bacteria rapidly after all (Nunez-Hernandez et al., 2014). Salmonella executing σS-mediated stress adaptation may attenuate aggressive virulence ascribed to SPI-2 to achieve a trade-off between stress adaptation and virulence. Notably, the growth rates of intracellular Salmonella vary depending on the infected cell types; Salmonella proliferated exclusively in CD18-expressing phagocytes in vivo (Richter-Dahlfors et al., 1997), while restraining its growth in non-professional phagocytes such as subepithelial fibroblasts (Cano et al., 2001). Therefore, the importance of SPI-2 T3SS for Salmonella survival varies depending on infection foci or cell types. For example, SifA, an effector translocated via SPI-2 T3SS, is essential for bacterial growth inside macrophages but is dispensable for survival inside fibroblast cells (Nunez-Hernandez et al., 2014). Interestingly, Grant et al. showed that Salmonella lacking SPI-2 T3SS remained inside phagocytes at a high replication rate but failed to leave the infected cells, suggesting a new role for SPI-2 T3SS in bacterial dissemination to other sites (Grant et al., 2012). Premature escape from infected host cells may impose unaffordable expenses on Salmonella to resist severe host defense systems and constrain its successful host colonization. In this context, it is an energy-effective strategy for Salmonella to employ σS as a dual-purpose regulator that aids in adaptation and resistance against unfavorable conditions and lowers unnecessary virulence attributable to SPI-2 at the same time.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SK designed and conducted the experiment and interpreted the data. EK performed and analyzed the experiment. HY conceived and coordinated the study. SK and HY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.750940/full#supplementary-material

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