The Iron-Sensing Fur Regulator Controls Expression Timing and Levels of *Salmonella* Pathogenicity Island 2 Genes in the Course of Environmental Acidification

Eunna Choi,* Hyunkeun Kim,* Hwiseop Lee,* Daesil Nam,* Jeongjoon Choi,*+ Dongwoo Shin*

Division of Microbiology, Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, South Korea; Department of Agricultural Biotechnology, Seoul National University, Seoul, South Korea

In order to survive inside macrophages, *Salmonella* produces a series of proteins encoded by genes within *Salmonella* pathogenicity island 2 (SPI-2). In the present study, we report that Fur, a central regulator of iron utilization, negatively controls the expression of SPI-2 genes. Time course analysis of SPI-2 expression after the entry of *Salmonella* into macrophages revealed that SPI-2 genes are induced earlier and at higher levels in the absence of the Fur regulator. It was hypothesized that Fur repressed the SPI-2 expression that was activated during acidification of the phagosome. Indeed, as pH was lowered from pH 7.0 to pH 5.5, the lack of Fur enabled SPI-2 gene expression to be induced at higher pH and to be expressed at higher levels. Fur controlled SPI-2 genes via repression of the *ssrB* response regulator, a primary activator of SPI-2 expression. Fur repressed *ssrB* expression both inside macrophages and under acidic conditions, which we ascribe to the direct binding of Fur to the *ssrB* promoter. Our study suggests that *Salmonella* could employ iron inside the phagosome to precisely control the timing and levels of SPI-2 expression inside macrophages.

To accomplish systemic infection of mammalian hosts, the pathogenic bacterium *Salmonella enterica* serovar Typhimurium (here referred to as *Salmonella*) can survive within host immune cells such as macrophages (1). As a means for survival, *Salmonella* employs a series of proteins produced from *Salmonella* pathogenicity island 2 (SPI-2). SPI-2 consists of multiple operons that encode a type III secretion (TTSS) apparatus and its substrate effector proteins (2, 3). Once produced within the phagosome, the effector proteins are delivered into the macrophage cytoplasm via the TTSS apparatus, where these proteins interfere with host bacterium-killing processes (2, 3).

*Salmonella* elaborately controls the expression of SPI-2 genes using a number of regulatory proteins (4). Among these, the SsrA/SsrB two-component system encoded within SPI-2 is known to play a pivotal role in SPI-2 expression. Although the mechanism of how the SsrA/SsrB two-component system is activated within the phagosome is currently unclear, the SsrB response regulator promotes expression of SPI-2 genes when *Salmonella* is grown in minimal medium at acidic pH (5, 6). SsrB regulation of SPI-2 expression is direct, as evidenced by the finding that the C-terminal DNA-binding domain of SsrB binds to SPI-2 promoters that control genes encoding the TTSS apparatus and effector proteins (5). In addition, the SsrB regulator also directly binds and autoregulates the *ssrA* and *ssrB* promoters to activate their expression (7).

Regulatory proteins that negatively control SPI-2 expression have also been reported. H-NS and YdgT are DNA-binding proteins with low molecular weights. These proteins bind to AT-rich regions of horizontally acquired genes, including those within SPI-2, and repress their transcription (8, 9). Negative regulation of SPI-2 expression also occurs at the posttranscriptional level. EIIA^NM^, a component of the nitrogen-metabolic phosphotransferase system, directly interacts with SsrB to prevent it from activating SPI-2 gene transcription (10). The lack of YdgT- or EIIA^NM^-mediated SPI-2 regulation impairs the ability of *Salmonella* to replicate in both murine macrophages and mouse organs (8, 10).

The Fur protein is a primary transcription factor that controls expression of genes for iron acquisition (11). The Fur regulator requires iron binding for its activity and binds to promoters of iron-responsive genes to repress their transcription under iron-replete conditions (12, 13). Genes within *Salmonella* pathogenicity island 1 (SPI-1) encode a TTSS that mediates *Salmonella* invasion into epithelial cells (14). It was reported that the Fur regulator positively controls expression of the SPI-1 genes (15, 16). Fur directly binds to the *hilD* promoter and enhances its own expression (15), and *hilD* functions as an activator of SPI-1 expression (17). Fur also contributes to SPI-1 TTSS production by reducing the levels of H-NS that represses SPI-1 expression (16).

SPI-2 expression is also under the control of an iron-sensing regulator. Iron depletion increases expression of the SPI-2 genes, and this control is reversed by iron supplementation (18). The PmrA/PmrB two-component system consists of the PmrA response regulator in the cytoplasm and the PmrB sensor protein in...
the inner membrane. Of two forms of iron, ferrous iron [Fe(II)] and ferric iron [Fe(III)], only Fe(III) plays as a signal for activation of the PmrA/PmrB system (19). When PmrB is activated by Fe(III) binding (19), PmrA is activated via PmrB-dependent phosphorylation (20). The PmrA protein controls expression of the lipopolysaccharide modification genes (19). Recently, the PmrA response regulator has been reported to repress SPI-2 expression in the presence of iron (21), suggesting that PmrA could be an iron mediator to repress expression of the SPI-2 genes. Here, we report that the iron-sensing Fur regulator also controls expression of the SPI-2 genes. We found that Fur represses SPI-2 expression that is activated inside macrophages and under acidic conditions. We reveal that Fur controls SPI-2 genes via repression of SsrB activity and provide evidence that Fur represses SsrB production by binding to the ssrB promoter.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in the present study are listed in Table 1. The S. enterica serovar Typhimurium strains used were derived from strain 14028s. Phase P22-mediated transductions were performed as described previously (22). Bacteria were grown at 37°C in Luria-Bertani (LB) or in N-minimal medium [50 mM Tris, 50 mM Bis-Tris, 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 1.0 mM KH₂PO₄] (23) supplemented with 0.1% Casamino Acids, 38 mM glycerol, and 1.0 mM MgCl₂. When necessary, ampicillin and kanamycin were used at 50 μg/ml. To induce genes from plasmids, IPTG (isopropyl-β-D-thiogalactopyranoside) was used.

Construction of bacterial strains. Salmonella strains carrying a gene deletion were constructed using the one-step gene inactivation method (24). For deletion of the ssrB gene, the kanamycin resistance (Km) cassette from plasmid pKD4 (24) was amplified by using the DEL-ssrB-F/DEL-ssrB-R primer pair. The resulting PCR products were integrated into the chromosome of strain 14028s as described previously (24). Deletion of the corresponding genes was verified by colony PCR. The HK427 strain encodes the SsrB protein with a hemagglutinin (HA) tag at the C terminus in the normal ssrB chromosomal location. For its construction, the Km cassette from plasmid pKD4 was amplified by using the ssrB- HA-F/ssrB-HA-R primer pair, and the resulting PCR products were integrated into the chromosome of strain 14028s. The Km cassette was removed using plasmid pCP20 (24), and the presence of codons for the HA tag at the C terminus of SsrB was confirmed by nucleotide sequencing. The sequences of primers used for strain construction are listed in Table 2.

Construction of plasmids. Plasmid pFur expressing the fur gene from the lac promoter was constructed. The fur open reading frame carrying extra four and two nucleotides at the 5’ and 3’ ends, respectively, was amplified using the EX-fur-F/EX-fur-R primer pair and chromosomal DNA from the 14028s strain. The PCR products were purified and introduced between the BamH I and Pst I restriction sites of pUHE21-2-lac(25). Plasmid pFur(H90R) is a derivative of pFur and produces a variant of Fur with a H90R substitution. This plasmid was constructed using a QuickChange II site-directed mutagenesis kit (Stratagene) with the SM-fur(H90R)-F/SM-fur(H90R)-R primer pair and pFur as the DNA template. Recombinant plasmid gene sequences were confirmed by nucleotide sequencing. The sequences of primers used for plasmid construction are listed in Table 2.

Immunoblot analysis. Bacteria were grown in N-minimal medium to an optical density at 600 nm (OD₆₀₀) of 0.5. Equivalent amounts of bacterial cell lysates were normalized by OD₆₀₀ values were washed, followed by phosphate-buffered saline (PBS), suspended in 0.5 ml of PBS, and opened by sonication. Cell lysates were resolved on 12% sodium dodecyl sulfate-polyacrylamide gels, transferred to nitrocellulose membranes, and analyzed by immunoblotting using anti-HA (Sigma) or anti-DnaK (Stressgen) antibody. Blots were developed using anti-rabbit IgG horseradish peroxidase-linked antibodies (GE Healthcare) and an ECL detection system (GE Healthcare). The results of immunoblots were quantified by the ImageJ program.

RNA isolation and quantitative real-time reverse transcription-PCR (qRT-PCR) analysis. Bacteria were grown in N-minimal medium to an OD₆₀₀ of 0.5. The culture (0.5 ml) was removed and mixed with 1.0 ml of RNase-free DNase (Ambion). cDNA was synthesized using the PrimeScript RT reagent kit (TaKaRa), random primers (Invitrogen), and 0.1 μg of template RNA. Amounts of cDNA were quantified by real-time PCR.
using SYBR green real-time PCR master mix (Toyobo) with an ABI7300 sequence detection system (Applied Biosystems). The primer pairs used for the detection of cDNA corresponding to saaB, ssaG, ssaM, ssaA, ssaR, and enTC mRNA and 16S rRNA were Q-ssaB-F/Q-ssaB-R, Q-ssaG-F/Q-ssaG-R, Q-ssaM-F/Q-ssaM-R, Q-ssaA-F/Q-ssaA-R, Q-ssaR-F/Q-ssaR-R, Q-enTC-F/Q-enTC-R, and Q-ssaH-F/Q-ssaH-R, respectively. Transcription levels of each gene were calculated from a standard curve obtained by PCR with the same primers and serially diluted genomic DNA. mRNA levels of target genes were normalized to 16S rRNA levels. The sequences of primers used are listed in Table 2.

Analysis of bacterial gene expression inside macrophages. J774A.1 macrophage cells were grown in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% Antibiotic-Antimycotic (Gibco). A monolayer of 10^6 J774A.1 cells was prepared in a six-well tissue culture plate and incubated in DMEM with FBS and antibiotics for 24 h. Prior to bacterial infection, J774A.1 cells were washed with PBS and incubated in DMEM with FBS for 1 h. Bacterial cells grown overnight in LB broth with aeration were washed with PBS, suspended in prewarmed DMEM, and added to the cell monolayer at a multiplicity of infection of 1. After 30 min of incubation, the wells were washed three times with prewarmed PBS to remove extracellular bacteria and then incubated for 1 h in the prewarmed medium supplemented with 150 μg of gentamicin/ml to kill extracellular bacteria. Afterward, the wells were washed with PBS and incubated in the prewarmed medium with 15 μg of gentamicin/ml. At the desired time points, the wells were washed with PBS and treated with RNase protect bacterial reagent (Qiagen) containing 1% Triton X-100 for 30 min. The resulting cell lysis mixture was centrifuged at 13,000 rpm for 5 min, and bacterial RNA was isolated from the pellet using the RNeasy minikit. The reverse transcription reaction and cDNA quantification using real-time PCR were conducted as described above.

EMSA. Electrophoretic mobility shift assays (EMSAs) were conducted as described previously (10). DNA fragments corresponding to the srbB promoter region were amplified by PCR using the EMISA-srbB-F/EMISA-srbB-R primer pair and 14028s chromosomal DNA as a template. The srbB promoter DNA was purified from agarose gel using a gel extraction kit (Qiagen) and labeled with [γ-32P]ATP (Perkin-Elmer). Unincorporated radiolabeled probe was removed using ProbeQuant G-50 Micro-Columns (GE Healthcare). The radiolabeled DNA probe (10 fmol) was incubated with the purified His6-Fur protein at room temperature for 30 min in 20 μl of binding buffer (20 mM Tris [pH 7.5], 50 mM KCl, 0.1 mM MnCl₂, 100 μg of bovine serum albumin/ml, 5% glycerol) containing 25 μg of poly(dI-dC)/ml. The reaction mixtures were resolved on a 6% polyacrylamide gel, and the radiolabeled DNA fragments were visualized by autoradiography.

Purification of the His6-Fur protein. The His6-Fur protein used for EMSA was purified as described previously (26). Escherichia coli BL21(DE3) cells harboring pHis-parallel1-Fur plasmid (26) were grown in LB broth at 37°C. When the culture O_{D_{600}} value reached 0.5, IPTG (0.5 g of ml) was added to the culture for induction of His6-Fur protein production, followed by another 16 h of incubation at 21°C. The His6-Fur protein was purified by Ni-NTA affinity chromatography. The cell pellet was suspended in cold buffer A (50 mM Tris [pH 8.0], 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride) and disrupted by sonication. The cell extract was loaded onto a Ni-NTA column equilibrated with buffer A. After discarding the flowthrough fraction, the column was washed with 500 ml of washing buffer (buffer A containing 20 mM imidazole), and the adsorbed His6-Fur protein was eluted with elution buffer (buffer A containing 200 mM imidazole). The eluted proteins were dialyzed with buffer A containing 10% glycerol.

Statistical analysis. Statistical analyses were using an analysis of variance (ANOVA) test. The results were analyzed by the unpaired t test. The data are presented as means ± the standard deviations. A P value of <0.05 was considered statistically significant.
affected ssaA expression inside macrophages in a fashion similar to ssaG expression (Fig. 1B). Taken together, these results suggest that the Fur regulator negatively controls the timing and levels of SPI-2 expression after Salmonella entry into macrophages.

**Fur represses iron-responsive genes inside macrophages.** Iron binding is necessary for the function of Fur as a transcription factor (12, 13). Fur represses the expression of iron-responsive genes in a form associated with iron when bacteria are grown under iron-replete conditions (11). Therefore, Fur repression of SPI-2 genes inside macrophages raises the possibility that iron is present inside the Salmonella-containing phagosome. We further reasoned that if this were the case, Fur would repress iron-responsive target genes inside macrophages. To investigate this issue, we determined the mRNA levels of the entC gene, a representative iron-responsive Fur target gene encoding a siderophore synthesis protein (27) after Salmonella entry into macrophages. In contrast to the expression of SPI-2 genes that was induced after phagocytosis (Fig. 1A and B), expression of the entC gene in the wild-type strain was maintained at constant levels inside macrophages (Fig. 1C). However, entC mRNA levels in the fur deletion strain were constitutively higher than those observed in the wild type (Fig. 1C), indicating that Fur functions to repress entC expression inside macrophages.

The Fur regulator represses expression of SPI-2 genes during environmental acidification. After formation of the Salmonella-containing phagosome, the phagosome luminal pH decreases as a function of time (28, 29). The acidification of the phagosome is critical for SPI-2 induction (30), and SPI-2 expression is promoted when Salmonella is grown in acidicified medium (5, 6). Therefore, we reasoned that the Fur regulator might control SPI-2 expression in a fashion associated with environmental acidification.

To test this idea, we determined levels of ssaG mRNA in Salmonella strains that were grown in N-minimal medium adjusted to pH values between 7.0 and 5.5. An initial rise in ssaG mRNA levels in the fur deletion strain was observed under pH 6.5 conditions, whereas it occurred in the wild-type strain under more acidic (pH 6.0) conditions (Fig. 2A, inset). The ssaG mRNA levels were similar regardless of fur deletion under neutral conditions, whereas ssaG levels were higher in the absence of fur than in its presence under acidic conditions (Fig. 2A). Deletion of the fur gene also affected expression of three other SPI-2 genes, ssaA, ssaB, and ssaM, in a fashion similar to ssaG (see Fig. S1 in the supplemental material), suggesting that the fur effect is general to all SPI-2 genes. The entC mRNA levels that were maintained at constant levels in the wild-type strain greatly increased at similar levels in the fur deletion strain between pH 7.0 and pH 5.5 (see Fig. S1 in the supplemental material), suggesting that Fur constitutively represses iron-responsive genes during environmental acidification. Derepression of SPI-2 expression displayed by the fur mutant was due to the function of Fur, as evidenced by the fact that heterologous expression of the fur gene from the plasmid-linked lac promoter complemented ssaG mRNA expression levels in the fur mutant strain similar to wild-type levels under pH 6.0 conditions (Fig. 2B).

We also tested whether Fur regulation of SPI-2 expression is dependent on iron. The levels of ssaG mRNA in the wild-type strain increased by 5-fold when the growth medium was supplemented with the iron chelator dipipryridyl, but this increase disappeared when iron was added to the medium (Fig. 2C). In contrast, iron repression of the ssaG gene did not occur in the fur deletion mutant (Fig. 2C), suggesting that Fur represses SPI-2 expression in an iron-dependent fashion. Fur is necessary for the acid tolerance response of Salmonella (31, 32). A variant of Fur with an H90R substitution is blind to iron but is still functional to mediate the acid tolerance response (32). Fur(H90R) production from the plasmid-linked lac promoter could no longer complement ssaG expression in the fur deletion strain under pH 6.0 conditions (Fig. 2B), further indicating that Fur repression of SPI-2 expression under acidic conditions is dependent on iron. Taken together, these results illustrate that the iron-sensing Fur regulator negatively controls expression of SPI-2 genes during acidification and a lack of this regulation enables the SPI-2 genes to be induced, even under intermediate acidic conditions that would not normally allow for SPI-2 induction.

**Fur represses expression of the ssrB gene encoding the SsrB response regulator under acidic conditions.** SsrB, the response regulator of the SsrA/SsrB two-component system, directly binds to the promoters of SPI-2 genes (5) and promotes their expression
under acidic conditions (5, 6). Notably, the fur deletion strain produced ssrB mRNA at higher levels than the wild-type strain 6 h after macrophage entry (Fig. 3A), suggesting that Fur represses SsrB production under acidic conditions.

To explore this possibility, we determined levels of ssrB mRNA in Salmonella grown under neutral or acidic conditions. The wild-type and fur deletion strains produced ssrB mRNA at similar levels under pH 7.0 conditions (Fig. 3B). In contrast, ssrB mRNA levels in the fur deletion strain were 12-fold higher than the wild-type strain under pH 6.0 conditions (Fig. 3B). The DNAK protein served as an internal loading control. (D) Levels of the SsrB protein determined in panel C were quantified. Means and standard deviations from three independent experiments are shown.

The Fur regulator directly binds to the ssrB promoter. Recently, a computational analysis predicted that the consensus DNA sequence recognized by Fur (i.e., Fur box) (11, 12) exists on the ssrB promoter (Fig. 4A) (33). Based on this finding, we explored whether the Fur regulator could directly bind to the ssrB promoter. We conducted EMSA using purified Fur protein and radiolabeled ssrB promoter DNA. As the amount of Fur protein gradually increased, electrophoretic mobility shifts of the DNA probe were observed, indicating the formation of a Fur-DNA complex (Fig. 4B). The interaction between the Fur protein and the ssrB promoter DNA was specific, as evidenced by the finding that unlabeled ssrB promoter DNA competed with the labeled probe from the shifted bands (Fig. 4B). The putative 19-bp Fur box is located close to the known SsrB-binding site (7) on the ssrB promoter (Fig. 4A), and Fur actually binds to DNA regions larger than the Fur box (~31 bp) (11). Taken together, these lines of evidence suggest that the Fur repressor could interfere with binding of the SsrB activator to the ssrB promoter, leading to repression of ssrB transcription.

Fur negatively controls SPI-2 expression via repression of ssrB transcription. Given that SsrB functions as an activator for expression of SPI-2 genes (4) and that Fur represses ssrB expression (Fig. 3), it is possible that the Fur regulator controls SPI-2 genes in a fashion dependent on the SsrB regulator. SsrB was active and promoted ssaG transcription under pH 6.0 conditions, whereas the levels of ssaG mRNA were 16-fold lower in an ssrB deletion strain than in the wild-type strain (Fig. 5A). However, the ssaG mRNA levels displayed by the ssrB mutant were unaffected by fur deletion under the same growth condition, which was in contrast to the Fur repression of ssaG expression occurring in the presence of ssrB (Fig. 5A). Therefore, this result indicates that Fur repression of SPI-2 expression is dependent on SsrB.

We further reasoned that if Fur controls SPI-2 genes via the repression of ssrB transcription, Fur repression of SPI-2 expression would be impaired when the ssrB gene is ectopically expressed from a heterologous promoter. To test this idea, we constructed an ssrB chromosomal deletion strain and expressed ssrB from a
Fur repression of ssrB expression leads to repression of SPI-2 expression. *Salmonella* strains were grown in N-minimal medium adjusted to pH 6.0. (A) qRT-PCR was used to determine *ssaG* mRNA levels in the wild-type (WT, 14028s), fur deletion (Δfur, JH352), *ssaB* (ΔssaB, EN504) deletion, and *ssaB* fur deletion (ΔssaBΔfur, DN235) strains. Means and standard deviations from three independent experiments are shown. ***, P < 0.001, relative to the wild type. (B) *ssaG* mRNA levels in the wild-type (WT, 14028s), fur deletion (Δfur, JH352), *ssaB* deletion (ΔssaB, EN504), and *ssaB* fur deletion (ΔssaBΔfur, DN235) strains and *ssaB* deletion (ΔssaB, EN504) deletion and *ssaB* fur deletion (ΔssaBΔfur, DN235) strains carrying the empty plasmid vector pUHE21-3lacI (pUHE) or SsrB expression plasmid (pSsrB). IPTG was used at 10 μM for induction of SsrB expression from the pSsrB plasmid. Means and standard deviations from three independent experiments are shown (**, P < 0.01; ***, P < 0.001).

**FIG 5**

Fur repression of ssrB expression leads to repression of SPI-2 expression. (A) qRT-PCR was used to determine *ssaG* mRNA levels in the wild-type (WT, 14028s), fur deletion (Δfur, JH352), *ssaB* (ΔssaB, EN504) deletion, and *ssaB* fur deletion (ΔssaBΔfur, DN235) strains. Means and standard deviations from three independent experiments are shown. ***, P < 0.001, relative to the wild type. (B) *ssaG* mRNA levels in the wild-type (WT, 14028s), fur deletion (Δfur, JH352), *ssaB* deletion (ΔssaB, EN504), and *ssaB* fur deletion (ΔssaBΔfur, DN235) strains and *ssaB* deletion (ΔssaB, EN504) deletion and *ssaB* fur deletion (ΔssaBΔfur, DN235) strains carrying the empty plasmid vector pUHE21-3lacI (pUHE) or SsrB expression plasmid (pSsrB). IPTG was used at 10 μM for induction of SsrB expression from the pSsrB plasmid. Means and standard deviations from three independent experiments are shown (**, P < 0.01; ***, P < 0.001).

**FIG 6**

Model illustrating how the Fur regulator controls SPI-2 expression during the course of environmental acidification. When activated upon acidic pH, the SsrB response regulator binds to the promoters of multiple operons within SPI-2 and promotes their expression (5). Because SsrB expression is positively autoregulated (7), the levels of SsrB gradually increase as the external pH is lowered from pH 7.0 to pH 5.5. (A) To overcome Fur repression on ssaB, the degree of SsrB production is insufficient under intermediate acidic conditions (i.e., pH 6.5 and pH 6.0) but becomes sufficient under full acidic conditions (i.e., pH 5.5). Consequently, Fur hampers expression of SPI-2 operons until the environmental pH is lowered enough to fully activate the SsrA/SsrB system. (B) In contrast, in the absence of Fur, levels of SsrB gradually increase during environmental acidification, which then leads to the gradual induction of SPI-2 expression.

**DISCUSSION**

*Salmonella* employs two distinct TTSSs during systemic infection of the mammalian host: the TTSS produced from SPI-1 mediates *Salmonella* invasion into epithelial cells (14), while the TTSS from SPI-2 is necessary for *Salmonella* to survive inside macrophages (2, 3). Fur, a central regulator of iron utilization (11), has been reported to enhance SPI-1 expression (15, 16). In addition, we demonstrate here that Fur suppresses SPI-2 expression.

After entry into macrophages, *Salmonella* begins to promote expression of SPI-2 genes (30). We observed that a lack of fur enables *Salmonella* to express SPI-2 genes earlier and at higher levels inside macrophages (Fig. 1). The lumen of the phagosome is acidified during the course of phagosome maturation (34). Likewise, the luminal pH of the *Salmonella*-containing phagosome is lowered to pH 5.0–6.0 h after phagocytosis (29). Phagosome acidification is critical for SPI-2 induction, as evidenced by the finding that inhibition of acidification greatly impairs SPI-2 expression inside the phagosome (30). Moreover, growth of *Salmonella* in acidified medium promotes expression of SPI-2 genes (5, 6), further supporting the role of acidic pH in the activation of SPI-2 expression. As the growth medium was gradually acidified, the lack of fur enabled SPI-2 genes to be induced at higher levels under less acidic conditions (Fig. 2 and see Fig. S1 in the supplemental material). Taken together, these results suggest that the Fur regulator limits expression of SPI-2 genes in the course of acidic activation.

SsrB, the response regulator of the SsrA/SsrB two-component system, directly activates expression of SPI-2 genes under acidic conditions (5). We determined that Fur represses *ssrB* transcription (Fig. 3) by directly binding to the *ssrB* promoter (Fig. 4B). By this action on SsrB production, Fur was able to negatively control SPI-2 expression (Fig. 5). We propose the following model for how Fur controls the timing and levels of SPI-2 expression during the course of environmental acidification (Fig. 6). Because *ssrB* expression is positively autoregulated (7), the levels of SsrB grada...
ually increase as the pH is lowered from pH 7.0 to pH 5.5. Because the putative 19-bp Fur box is located only 5 bp upstream of the SsrB binding site (7, 33) on the srrB promoter (Fig. 4A), and because Fur binds to regions larger than the Fur box (~31 bp) (11), it is possible that the Fur repressor competes with the SsrB activator for binding to the srrB promoter. The degree of SsrB production would be insufficient to overcome Fur repression on the srrB promoter under intermediate acidic conditions (i.e., pH 6.5 to 6.0) but would become sufficient under more acidic conditions (i.e., pH 5.5). Therefore, expression of SPI-2 genes is not induced until the pH is lowered to 5.5, where levels of SsrB would markedly increase (Fig. 6A). In contrast, in the absence of Fur repression on the srrB promoter, levels of SsrB protein would gradually increase over the course of environmental acidification (Fig. 6B). Therefore, SPI-2 expression is induced even under intermediate acidic conditions and reaches higher levels under the fully acidic condition (Fig. 6B).

Consistent with the notion that Fur requires iron-binding to act as a transcriptional regulator (12, 13), Fur was able to control SPI-2 expression when Salmonella was grown in medium containing iron (Fig. 2C). Fur represses siderophore synthesis genes such as entABCE and iroDEN under iron-replete conditions (27). Expression of these genes is downregulated in Salmonella growing inside macrophages (35), to which Fur could contribute, as evidenced by the finding that Fur represses expression of the entC gene inside macrophages (Fig. 1C). Therefore, these lines of evidence suggest that iron inside the phagosome could serve as a signal for controlling SPI-2 expression.

PmrA is a response regulator of the PmrA/PmrB two-component system. When this system is activated by iron, the PmrA protein controls expression of the lipo polysaccharide modification genes (19). Interestingly, the PmrA protein repressed SPI-2 expression in a manner similar to the Fur regulator; PmrA was shown to bind to a region overlapping with the SsrB-binding site on the srrB promoter (Fig. 4A) and repress srrB transcription (21). Therefore, the reason Salmonella uses two iron-sensing regulatory systems to control SPI-2 expression remains a question. Iron is present in either a reduced Fe(II) or an oxidized Fe(III) form. The PmrA response regulator is active in the presence of Fe(III) but not in the presence of Fe(II) because the membrane sensor protein PmrB can only recognize Fe(III) as a signal (19). Because the Fur regulator senses iron in the cytoplasm (56), both Fe(III) and Fe(II) imported by their corresponding transport systems could be a source for Fur binding. Therefore, these two iron-sensing regulatory systems could enable Salmonella to accomplish iron regulation of SPI-2 genes during growth inside macrophages regardless of the iron oxidation state inside the phagosome.

Vireulence attenuation of the fur mutant in an intraperitoneal infection (16, 37) reflects the fact that Fur could play an important role in infection processes taking place after Salmonelle penetrates epithelial barriers. For instance, Salmonella is exposed to reactive oxygen species (ROS) produced within the macrophage phagosome (38), and the lack of Fur renders Salmonella highly susceptible to ROS-mediated killing (39). Therefore, the regulatory functions of Fur could protect Salmonella from oxidative stress inside the phagosome (40). In addition, Fur might contribute to Salmonella virulence by controlling expression timing and the expression level of SPI-2 genes. This idea could be supported by the finding that ectopic expression of the SPI-2 genes caused by the lack of YdgT- or EIExIII-mediated regulation leads to Salmonella virulence attenuation in mice infected intraperitoneally (8, 10). Finally, because both Fur and PmrA regulators control SPI-2 expression in a similar fashion, Fur might delay SPI-2-mediated macrophage killing as PmrA does for macrophages (21). However, even if this were the case, Fur ultimately contributes to Salmonella virulence, in contrast to the finding that PmrA-controlled macrophage killing limits Salmonella virulence (21). This could be explained by the possibility that the defensive roles of Fur against the hostile actions of a host mask the potential role of Fur regulation of SPI-2 in macrophage killing.

ACKNOWLEDGMENT

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science, and Technology (2012R1A2A2A01013521).

REFERENCES


