



The Ferric Uptake Regulator Represses Type VI Secretion System Function by Binding Directly to the *clpV* Promoter in *Salmonella enterica* Serovar Typhimurium

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ABSTRACT Type VI secretion systems (T6SSs) are highly conserved and complex protein secretion systems that deliver effector proteins into eukaryotic hosts or other bacteria. T6SSs are regulated precisely by a variety of regulatory systems, which enables bacteria to adapt to varied environments. A T6SS within *Salmonella* pathogenicity island 6 (SPI-6) is activated during infection, and it contributes to the pathogenesis, as well as interbacterial competition, of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*). However, the regulation of the SPI-6 T6SS in *S. Typhimurium* is not well understood. In this study, we found that the SPI-6 T6SS core gene *clpV* was significantly upregulated in response to the iron-depleted condition and during infection. The global ferric uptake regulator (Fur) was shown to repress the *clpV* expression in the iron-replete medium. Moreover, electrophoretic mobility shift and DNase I footprinting assays revealed that Fur binds directly to the *clpV* promoter region at multiple sites spanning the transcriptional start site. We also observed that the relieving of Fur-mediated repression on *clpV* contributed to the interbacterial competition activity and pathogenicity of *S. Typhimurium*. These findings provide insights into the direct regulation of Fur in the expression and functional activity of SPI-6 T6SS in *S. Typhimurium* and thus help to elucidate the mechanisms of bacterial adaptability and virulence.

KEYWORDS Fur, *S. Typhimurium*, type VI secretion system, *clpV*, regulation

Pathogenic organisms use several common strategies to compete with other species and accomplish systemic infections. The conserved type VI secretion system (T6SS) is present in more than one-fourth of Gram-negative pathogens (1, 2). The structure of the T6SS apparatus is composed of 13 core components, and additional components may be involved in effector translocation. The transenvelope complex of the T6SS consists of stacked Hcp protein hexamers, which are capped by the VgrG protein. Moreover, Hcp and VgrG act not only as secreted effector proteins but also as machine components. The ClpV ATPase is essential for T6SS secretion and the contraction of the surrounding sheath (3–8). Pathogens such as *Vibrio cholerae* (*V. cholerae*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and pathogenic *Escherichia coli* employ T6SSs to deliver effector proteins into eukaryotic or prokaryotic cells (9), and these effectors play a role in a broad variety of functions, including interbacterial interaction, stress sensing, biofilm formation, and virulence (10–20). *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is an important human and animal pathogen that causes human gastroenteritis and a typhoid-like disease in mice. Moreover, it is a leading etiological agent associated with foodborne illness outbreaks worldwide, which commonly result in heavy economic losses globally (21–23). Five different T6SSs encoded within *Salmonella*

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pathogenicity islands (SPIs) were identified among *Salmonella* lineages, whereas *S. Typhimurium* only harbors a single SPI-6-encoded T6SS (24–28). Recent studies demonstrated that the SPI-6 T6SS is encoded by two divergent operons with opposite orientations (24, 26, 29). It has been reported that SPI-6 T6SS expression is activated during infection, which is associated with the intracellular replication, systemic dissemination, and virulence of *S. Typhimurium*. Moreover, the bactericidal activity mediated by the SPI-6 T6SS contributes to the survival and interbacterial competition of *S. Typhimurium* *in vitro* and *in vivo* (19, 25, 29–31).

To adapt to various environments or stresses, bacteria can precisely control gene expression through intricate regulatory networks. In addition, the physiological control of virulence gene expression is essential for bacterial pathogenesis. Previous studies have indicated that T6SSs are tightly regulated (4, 32). Various regulatory mechanisms for T6SSs at transcriptional, translational, and posttranslational levels have been identified and characterized (4, 33). *P. aeruginosa* regulates T6SS transcripts posttranscriptionally via the Gac/Rsm pathway, whereas the threonine phosphorylation pathway activates the T6SS (34, 35). A broad variety of regulators, such as the quorum-sensing system, alternative sigma factors, H-NS, and ferric uptake regulator (Fur), were found to directly or indirectly modify the transcription of T6SSs (29, 36–42). Quorum-sensing regulators were shown to positively or negatively regulate the expression of T6SSs based on cell population density. It has been shown that T6SS gene clusters are positively controlled by the alternative sigma factor RpoN, sigma factor 54, and cognate bacterial enhancer binding proteins (36–38, 43). In *Yersinia pseudotuberculosis*, the two-component system regulator OmpR positively regulates T6SS-4 to maintain intracellular pH homeostasis (39). The PhoB/PhoR two-component system and Fur act as an activator and a repressor, respectively, for T6SS expression in *Edwardsiella tarda* (*E. tarda*) (40). H-NS is a global regulator that controls the expression of multiple secretion systems and virulence genes. *S. Typhimurium* SPI-6 T6SS gene cluster is repressed by the H-HS through preventing the binding of RNA polymerase (29, 41).

Iron is essential for bacterial survival and pathogenesis. Bacteria modulate gene expression by various regulators that are sensitive to environmental cues, such as iron concentration. Fur is a global regulator that controls genes involved in iron homeostasis and virulence in many bacteria. Fur regulates gene expression through Fe²⁺-dependent binding to a specific sequence, namely, the Fur box, in the promoter regions of the regulated genes. The Fur box, which contains a 19-bp consensus sequence, is well characterized. The Fur regulon contains genes involved in iron acquisition, flagellar chemotaxis, the tricarboxylic acid cycle, type III secretion systems (T3SSs) and T6SSs, and it is essential for bacterial multiplication and pathogenicity (44–48). In *S. Typhimurium*, Fur was shown to directly activate SPI-1 expression and repress SPI-2 expression. Moreover, Fur indirectly regulates SPI-1 expression by repressing H-NS (49–51). Previous evidence showed that T6SS expression is repressed directly by Fur in *E. tarda*, *P. aeruginosa*, enteroaggregative *E. coli* (EAEC) and avian pathogenic *E. coli* (APEC) (20, 38, 40, 52). This prompted us to determine the regulation of the SPI-6 T6SS mediated by Fur in *S. Typhimurium*.

In the present study, we demonstrated that Fur bound directly to the *clpV* promoter region and repressed its expression. Furthermore, we showed that the Fur-mediated regulation of SPI-6 T6SS expression was important for the interbacterial competition and virulence of *S. Typhimurium*. Taken together, these observations reveal that Fur plays a negative regulatory role in *clpV* expression and T6SS function in *S. Typhimurium*.

RESULTS

T6SS core gene *clpV* expression is upregulated during infection and under iron-depleted conditions. It has been indicated that the expression of SPI-6 T6SS was increased during infection, suggesting that T6SS contributes to the pathogenesis of *S. Typhimurium* (19, 25, 30, 53). In an initial experiment, we analyzed the transcript levels of T6SS core genes during infection in RAW 264.7 cells by quantitative real-time PCR (qRT-PCR). Similarly, the expressions of SPI-6 T6SS core genes *clpV* and *vgrG* were

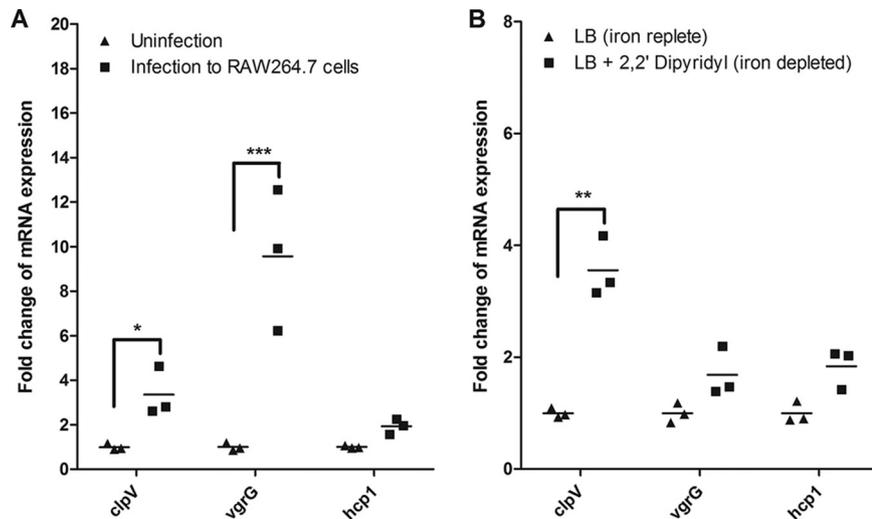


FIG 1 Infection and iron-depleted conditions significantly upregulate SPI-6 T6SS core gene *clpV* expression in *S. Typhimurium*. (A) The transcript levels of T6SS core genes during infection in RAW 264.7 cells were analyzed by qRT-PCR. The expressions of SPI-6 T6SS core genes *clpV* and *vgrG* were significantly enhanced during infection of macrophages. (B) Effect of culture in iron-depleted medium on T6SS core gene expression. The supplementation of iron chelator 2,2'-dipyridyl lead to significantly increased *clpV* expression. Statistical significance was assessed by using one-way ANOVA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

significantly enhanced during infection ($P < 0.05$ or $P < 0.001$) (Fig. 1A). During infection, the low-iron environment could induce the expression of T6SS required for survival and virulence. Thus, we next sought to determine whether T6SS gene expression was upregulated in the iron-depleted medium. The expression levels of *clpV*, *vgrG*, and *hcp1* genes were compared in Luria-Bertani (LB) medium with or without iron chelator 2,2'-dipyridyl. The depletion of free iron leads to significantly increased *clpV* expression ($P < 0.01$) (Fig. 1B). These results suggested that T6SS *clpV* gene expression was increased during infection and in iron-depleted medium.

Fur represses the expression of *clpV* in *S. Typhimurium* under iron-replete conditions. The regulator Fur has been known as the major regulator of iron metabolism. Fur complexes with iron and binds to the Fur box, and it subsequently participates in the regulation of various genes involved in environmental tolerance and virulence (44, 46–48). Hence, we intend to determine whether SPI-6 T6SS genes are potentially regulated by Fur. We constructed the *fur* gene deletion and complemented strains. Then, the expressions of the T6SS core genes were monitored by qRT-PCR. Inactivation of *fur* led to significantly higher transcription of *clpV* ($P < 0.01$), which is essential and provides energy for the secretion process of the T6SS. Moreover, complementation of the *fur* gene restored *clpV* transcription ($P < 0.01$) (Fig. 2A). However, the transcript levels of other T6SS core genes, namely, *dotU*, *vgrG*, *icmF*, *hcp1*, *hcp2*, *tae4*, and *stm0271*, were slightly upregulated (<2-fold) in the Δfur mutant strain (Fig. 2B), possibly because these core genes are transcribed by different potential promoters (29). In addition, we performed Western blotting to further validate the production levels of ClpV in these strains. Consistent with the qRT-PCR results, the ClpV production was enhanced in the Δfur mutant strain compared to the wild-type and complemented strains. These results indicate that Fur is a negative regulator of *clpV* expression. Previous studies have indicated that Fur acts as a regulator for the increased T6SS-1 transcription in response to iron limitation in EAEC (20). Thus, we wanted to test whether the transcription of *clpV* was enhanced by relieving Fur-mediated repression under iron-depleted conditions. Our results showed that the transcript level of *clpV* was increased in iron-depleted medium (Fig. 2A). Moreover, Fur was not able to repress *clpV* transcription and expression under the iron limitation condition (Fig. 2A and C).

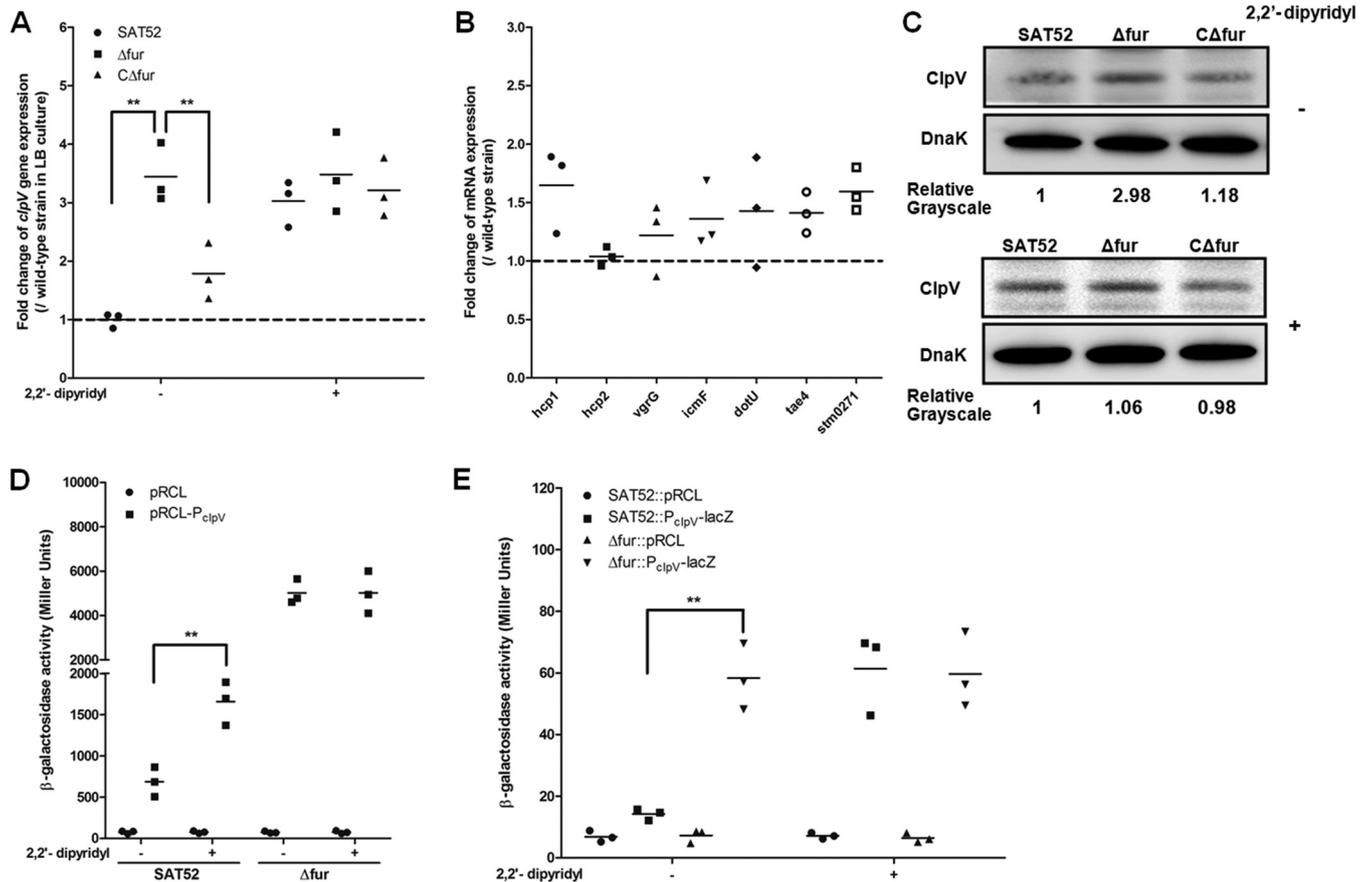


FIG 2 Fur represses the expression of the T6SS *clpV* gene in *S. Typhimurium*. (A) The transcript levels of *clpV* under the indicated conditions were analyzed by qRT-PCR. The results are shown as relative expression ratios compared to that of the wild-type strain SAT52 in LB medium. (B) qRT-PCR analysis for the transcriptional levels of the SPI-6 T6SS core genes *hop1*, *hop2*, *vgrG*, *icmF*, *dotU*, *tae4*, and *stm0271* in wild-type and *fur* mutant strains. (C) The production levels of ClpV in *Salmonella* strains were determined by Western blotting. An anti-DnaK antibody was used as a control. The expression levels of the ClpV protein were determined by quantifying the integrated optical density using ImageJ software. (D) β -Galactosidase activities of bacteria containing plasmid-encoded promoterless pRCL-*lacZ* fusions and P_{clpV} -*lacZ* fusions with or without treatment by 2,2'-dipyridyl. (E) The β -galactosidase activities of the chromosomal P_{clpV} -*lacZ* reporter fusions were measured in wild-type and *fur* mutant strains in iron-replete and iron-depleted media. Statistical significance was assessed by using one-way ANOVA (**, $P < 0.01$).

Furthermore, we constructed a *lacZ* transcriptional reporter fusion plasmid in which *lacZ* is under the control of the *clpV* promoter, and it was transformed into wild-type and *fur* mutant strains. Similarly, deletion of *fur* significantly activated the transcription of *clpV* ($P < 0.01$). Moreover, the activity of the P_{clpV} -*lacZ* reporter fusion increased in the presence of 2,2'-dipyridyl (Fig. 2D). To avoid artifactual effects of the plasmid on *lacZ* fusion activity, we constructed a chromosomal P_{clpV} -*lacZ* transcriptional reporter fusion in the wild-type and *fur* mutant strains. As expected, the activity of the P_{clpV} -*lacZ* fusion was significantly higher in the Δfur mutant strain than in the wild-type strain SAT52 in the LB rich medium but not in iron-depleted medium ($P < 0.01$) (Fig. 2E). Taken together, these findings suggest that Fur represses the expression of *clpV* in *S. Typhimurium* under iron-replete conditions.

The Fur protein binds directly to the *clpV* promoter region. To investigate whether Fur regulation of *clpV* expression is direct or indirect, we first analyzed the putative Fur binding boxes in the promoter sequence of the SPI-6 T6SS clusters in *S. Typhimurium* strain SAT52. Our inspection revealed that four putative Fur binding boxes were located upstream of the *clpV* open reading frame. Moreover, a double Fur binding box was present at positions +25 to +59 downstream of the *clpV* transcriptional start site (Fig. 3A). However, no Fur binding box was found in other promoters of the SPI-6 T6SS cluster. These putative Fur binding boxes showed 63 to 84%

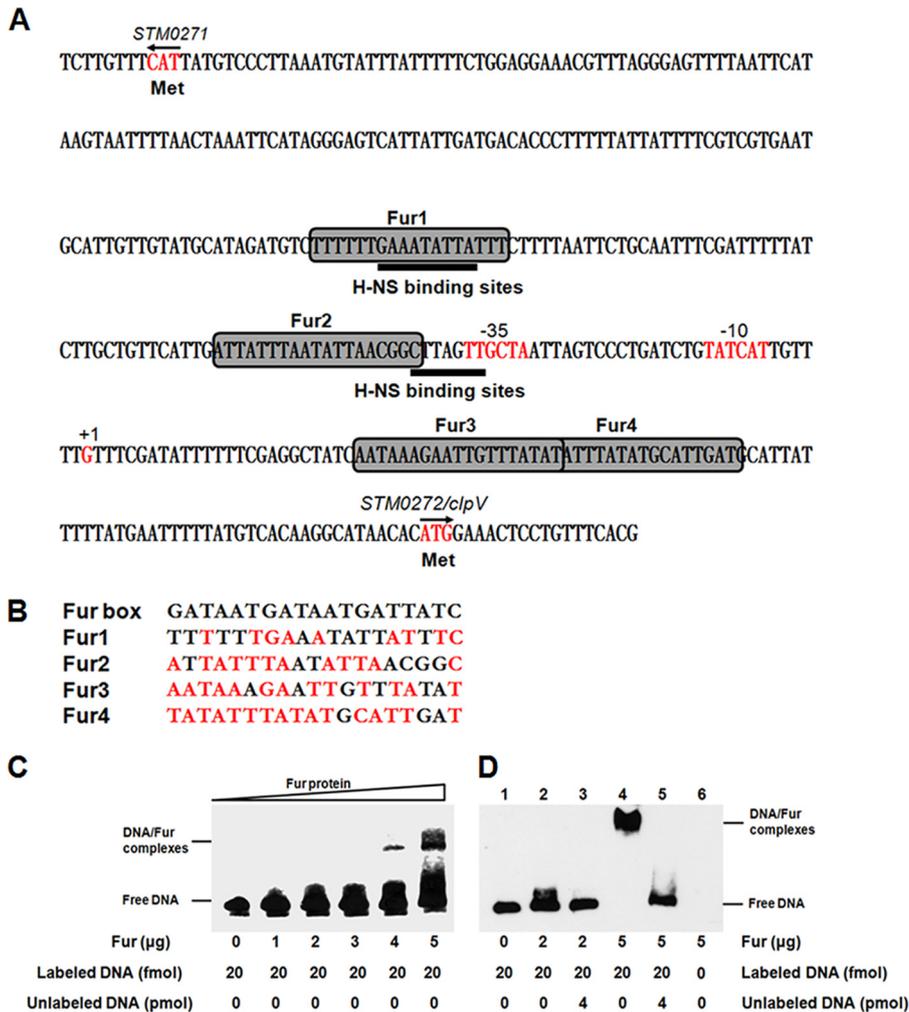


FIG 3 Fur binds directly to the *clpV* promoter region. (A) *In silico* analysis for Fur binding box prediction in the *clpV* promoter region. The putative Fur binding boxes are indicated as gray boxes. H-NS binding sites are indicated by solid lines. The transcriptional initiation site, the corresponding -10 and -35 boxes, and the translational start site are indicated in red letters. (B) Sequence alignment of these putative Fur binding boxes with the Fur box consensus sequence. Bases identical to the consensus are shown in red. (C) EMSA. The *clpV* promoter DNA fragment (270 bp) was amplified, biotin labeled, and used as a probe, and the biotin-labeled probe was mixed with increasing amounts of purified Fur. (D) Competitive EMSA. The Fur protein was incubated either with the biotin-labeled probe or with both the biotin-labeled and the unlabeled DNA probe. The biotin-labeled DNA was detected using a chemiluminescent substrate. The concentrations of Fur and the probes are shown below the figure.

homology to the consensus Fur binding site (Fig. 3B). Thus, the sequence analysis suggests that Fur may bind directly to the *clpV* promoter and regulate its expression.

To verify the direct binding of Fur to the *clpV* promoter region, a PCR fragment, including the four putative Fur binding boxes, was amplified, biotin labeled, and subjected to an electrophoretic mobility shift assay (EMSA) using purified Fur. As shown in Fig. 3C, the *clpV* promoter probe was retarded in the presence of increasing amounts of Fur protein. In addition, the binding specificity was confirmed via a competitive EMSA using a specific competitor consisting of unlabeled *clpV* promoter fragments. The binding of Fur to biotin-labeled probes was abolished by an excess of the specific competitor (Fig. 3D). Collectively, these results demonstrate that Fur could directly bind to the *clpV* promoter region in *S. Typhimurium*.

Determination of Fur binding sites required for regulating *clpV* expression. To identify the precise binding sites of Fur in the *clpV* promoter region, DNase I footprinting assays were performed. The results revealed that Fur bound to a 20-bp region (the

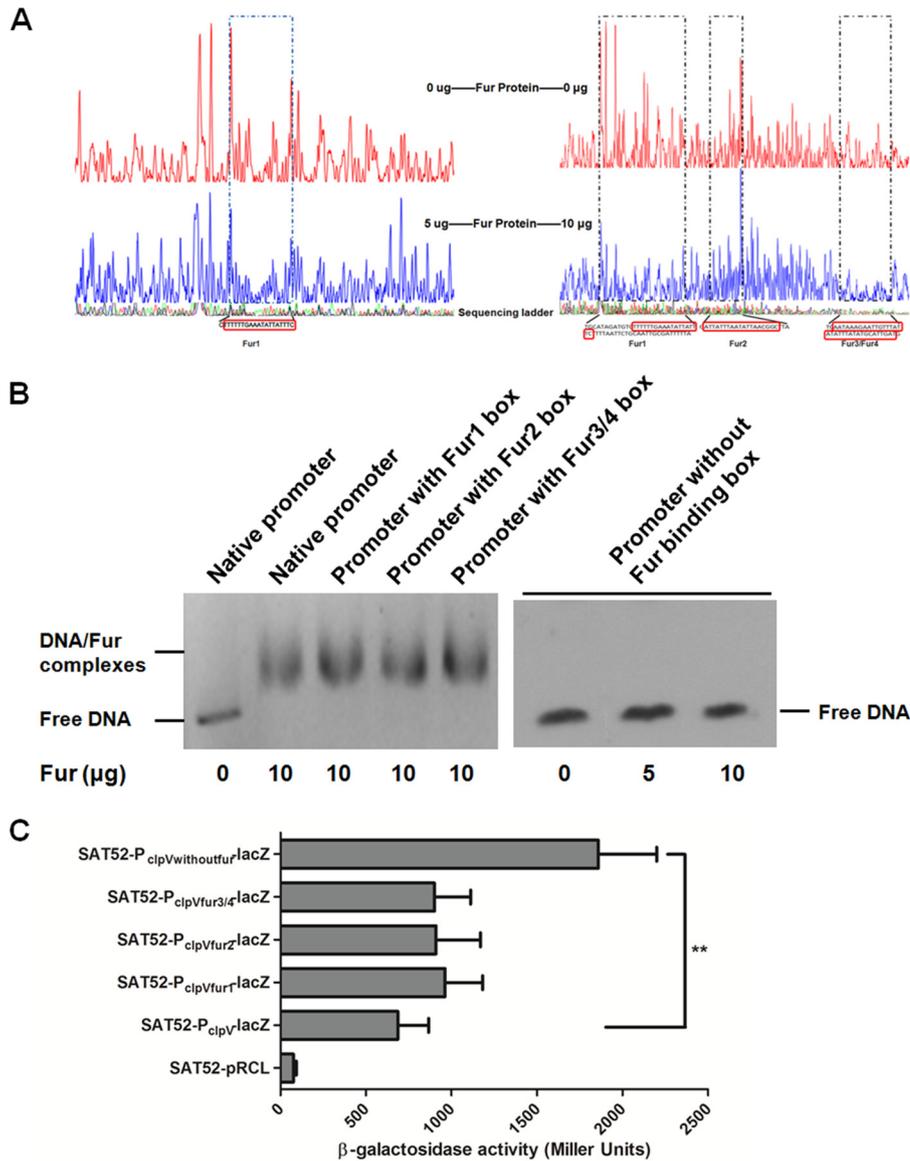


FIG 4 Determination of Fur binding sites in the *clpV* promoter region required for binding and regulation. (A) Mapping Fur binding sites in the *clpV* promoter region by DNase I footprinting assays. The 5-carboxyfluorescein (FAM)-labeled DNA fragment corresponding to the *clpV* promoter was incubated with (blue) or without (red) purified Fur. The base sequences of the protected regions are indicated, and the putative Fur binding boxes are shown in the red panes. (B) EMSA demonstrated that the Fur boxes are required for the Fur binding activity. The fragments containing mutated *clpV* promoter were synthesized and used for the EMSA analysis. Probe with deletion of these Fur binding sites could not be shifted by Fur protein. (C) Effect of Fur binding box mutation on P_{clpV}-lacZ fusion activity. The *lacZ* transcriptional reporter fusion plasmids containing these mutated *clpV* promoters were introduced into *S. Typhimurium* SAT52, and the β -galactosidase activity was analyzed. Statistical significance was assessed by using one-way ANOVA (**, $P < 0.01$).

Fur1 binding box) of the *clpV* promoter at a low concentration (5 μ g). In contrast, three separate protected regions (positions -129 to -73, positions -58 to -36, and positions +24 to +61, relative to the *clpV* transcriptional start site), containing the four predicted Fur binding boxes, were identified in the *clpV* promoter region at a higher concentration (10 μ g) of Fur (Fig. 4A). The results indicate that these Fur binding boxes are recognized by Fur; however, the affinity of Fur for the Fur1 binding box was higher than that of the other Fur binding boxes. Interestingly, the upstream and downstream regions of the Fur1 binding box were protected at higher Fur concentrations, suggesting that Fur may bind cooperatively to an initial, high-affinity site and then spread to

adjacent sites. Moreover, Fur could bind upstream and downstream of the *clpV* transcriptional start site (Fur2 and Fur3/4 binding boxes) (Fig. 3A) and regulate the *clpV* gene transcription. These regulation characteristics of Fur have already been studied in previous works (48).

To further demonstrate whether these Fur binding boxes are required for binding activity and regulation, we synthesized the *clpV* promoter fragments and constructed P_{*clpV*}-*lacZ* fusion plasmids containing mutations in these Fur binding boxes. The binding of Fur to the mutant *clpV* promoter probes was determined by EMSA, which demonstrated that Fur protein could bind directly to these putative Fur binding boxes. However, the *clpV* promoter fragment without the Fur binding box was no longer shifted by the Fur protein (Fig. 4B). Then, the activities of these mutant P_{*clpV*}-*lacZ* fusions were compared, which indicated that deletion of Fur binding boxes led to a significantly increased *lacZ* activity in LB medium (Fig. 4C). Overall, these results demonstrate that Fur could repress the *clpV* expression by directly binding to these Fur binding sites in the *clpV* promoter region of *S. Typhimurium*.

Relieving the Fur-mediated repression of *clpV* contributes to the interbacterial competition activity of *S. Typhimurium*. Recently, it was reported that T6SSs provide a fitness advantage to bacteria, including *P. aeruginosa*, *V. cholerae*, EAEC, and *S. Typhimurium*, through their antibacterial activities (11, 12, 15, 16, 18). Indeed, *S. Typhimurium* utilizes the SPI-6 T6SS as an antibacterial weapon to compete with other microorganisms *in vitro* and in colonization of the mouse gut (29, 53). Our finding that the expression of the SPI-6 T6SS core gene *clpV* was regulated by Fur in *S. Typhimurium* prompted us to investigate whether antibacterial activity was affected in the Δfur mutant strain. We first determined the interbacterial competition activity of *S. Typhimurium* using *E. coli* DH5 α strain as prey in LB medium. When cocultured with the wild-type strain SAT52, the prey strain *E. coli* DH5 α did not show significantly decreased survival. This might be due to the repression and weak expression of the SPI-6 T6SS in *S. Typhimurium*, which is consistent with previous studies (29, 53). In contrast, DH5 α survival was significantly reduced when the Δfur mutant was used as an attacker strain compared to the wild-type and complemented strains ($P < 0.001$). As expected, the mutant $\Delta clpV$ and $\Delta fur \Delta clpV$ strains had no impact on DH5 α growth, suggesting that the intoxication of prey strain DH5 α by *S. Typhimurium* Δfur mutant strain is due to the SPI-6 T6SS (Fig. 5A). The interbacterial competition activity of *S. Typhimurium* was further tested in iron-depleted medium. No significant difference in the interbacterial competition activity was observed among the *fur* mutant, wild-type, and complemented strains; this might be due to the derepression of T6SS mediated by Fur under iron-depleted conditions. Similarly, the $\Delta clpV$ and $\Delta fur \Delta clpV$ strains had no impact on the growth and survival of the prey strain DH5 α (see Fig. S1 in the supplemental material).

The T6SS component proteins form a complex membrane tubular structure, which delivers the effectors targeting eukaryotic hosts or bacteria. The effector Tae4 has been demonstrated previously to function via antibacterial amidase activity in *S. Typhimurium* (53). Moreover, our results demonstrate that Fur directly represses the expression of T6SS *clpV* in *S. Typhimurium*. Hence, we sought to determine whether the decreased interbacterial activity of the Δfur mutant strain was due to changes in Tae4 expression and secretion. The *tae4* gene was introduced into the plasmid pBAD/Myc-His (Invitrogen) and transformed into wild-type and *fur* mutant strains. The effect of Fur on the expression and secretion of the Tae4-His fusion protein was then assessed. The expression of Tae4 increased to 1.36-fold in the *fur* mutant strain, consistent with the qRT-PCR results (Fig. 2B). Moreover, increased Tae4-His production was detected in culture supernatants of the Δfur mutant strain (Fig. 5B). These data demonstrate that relieving the Fur-mediated repression of *clpV* facilitates the assembly, secretion, and antibacterial activity of SPI-6 T6SS in *S. Typhimurium*.

Fur and ClpV are required for the infection and pathogenicity of *S. Typhimurium*. Our results and previous data indicated that the SPI-6 T6SS expression was enhanced and involved in the pathogenesis of *S. Typhimurium* (19, 25, 30, 53). Thus, we

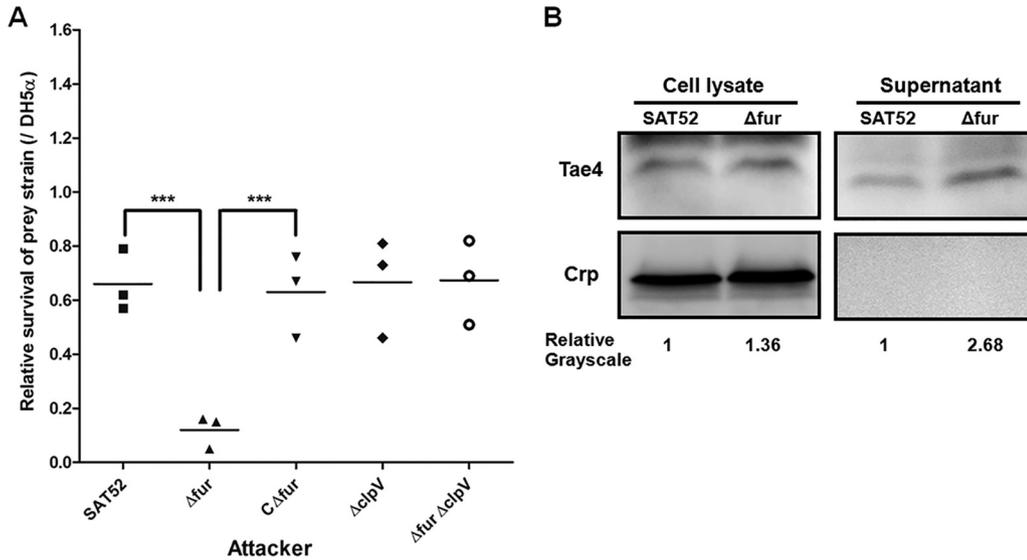


FIG 5 Derepression of *clpV* mediated by Fur contributes to the interbacterial competition activity of *S. Typhimurium*. (A) Interbacterial competition assay. The prey strain *E. coli* DH5α was mixed with the indicated attacker cells at a ratio of 1:4. After incubation for 4 h at 37°C, the surviving *E. coli* DH5α cells were recovered and enumerated. The ratio compared to the prey strain *E. coli* DH5α was calculated as the relative survival. Statistical significance was assessed by using one-way ANOVA (***, $P < 0.001$). (B) Expression and secretion of SPI-6 T6S antibacterial effector Tae4 was increased in the mutant Δfur strain. Crp (cyclic AMP receptor protein) is used as the cytoplasmic protein marker.

investigated the influence of *clpV* and *fur* on bacterial survival in RAW 264.7 macrophages. Consistent with a previous study (19), the *clpV* and *fur* mutant strains were defective in intramacrophage survival compared to *S. Typhimurium* wild-type and complemented strains (Fig. 6A). We also tested whether addition of free iron ($FeCl_2$) affects the intramacrophage survival of *S. Typhimurium*, which showed similar results (Fig. S2). The intracellular iron concentration is relatively stable; thus, the addition of

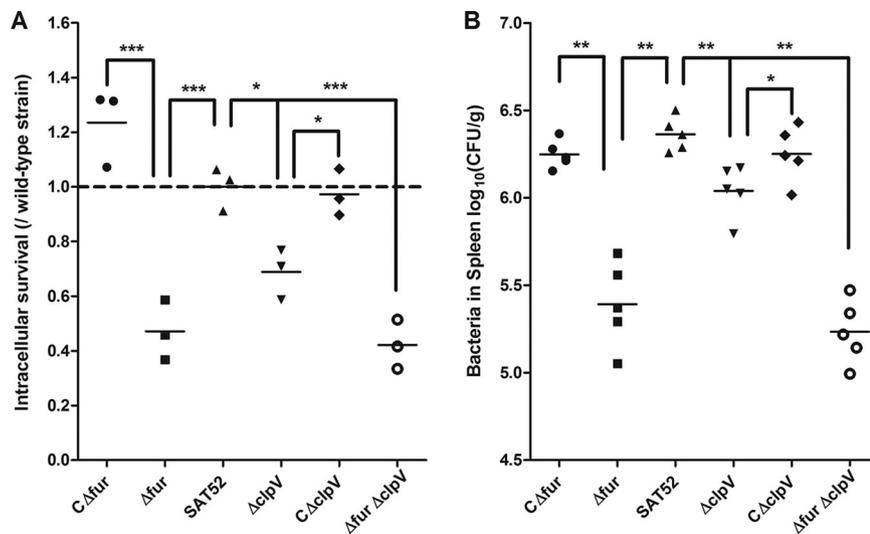


FIG 6 Bacterial intramacrophage survival and systemic dissemination in mice. (A) Assessment of intramacrophage survival. Intracellular survival of mutant and complemented strains in RAW 264.7 cells was expressed as a fraction of the wild-type strain at 10 h postinvasion. Decreased intracellular survival for the mutant strain was observed, whereas the complemented strain restored the capacity of intracellular survival. Statistical significance was assessed by using one-way ANOVA. (B) Bacterial systemic dissemination in mice. Mice were infected intraperitoneally with *S. Typhimurium* variants. At 2 days postinfection, the bacterial loads in the spleens were determined. Horizontal bars indicate the means for each sample group. Statistical analyses were performed by using a Mann-Whitney test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

TABLE 1 LD₅₀ calculations for each *S. Typhimurium* strain

Strain ^a	No. dead/no. tested at challenge dose					LD ₅₀
	10 ⁸ CFU	10 ⁷ CFU	10 ⁶ CFU	10 ⁵ CFU	10 ⁴ CFU	
SAT52	8/8	8/8	8/8	5/8	3/8	3.16 × 10 ⁴
<i>Δfur</i>	8/8	7/8	6/8	0/8	0/8	7.50 × 10 ⁵
<i>CΔfur</i>	8/8	8/8	8/8	6/8	0/8	5.62 × 10 ⁴
<i>ΔclpV</i>	8/8	8/8	7/8	3/8	0/8	1.33 × 10 ⁵
<i>CΔclpV</i>	8/8	8/8	7/8	6/8	0/8	7.50 × 10 ⁴
<i>Δfur ΔclpV</i>	8/8	7/8	4/8	0/8	0/8	1.33 × 10 ⁶

^aC, complemented strain.

free iron might not be able to significantly affect intramacrophage survival of *S. Typhimurium*.

It has been shown that the antibacterial activity of SPI-6 T6SS contributes to early colonization within the host gut for *S. Typhimurium* (53). Here, we determined the roles of T6SS ClpV in the virulence and colonization capacity of *S. Typhimurium* during systemic infection. As shown in Table 1, the median lethal dose (LD₅₀) values of the wild-type strain SAT52 and the *Δfur* and *ΔclpV* mutant strains were 3.16 × 10⁴ CFU/mouse, 7.50 × 10⁵ CFU/mouse, and 1.33 × 10⁵ CFU/mouse, respectively. Moreover, the mortality rates of the mutant strains were restored by transcomplementation with the *fur* or *clpV* genes. These observations further demonstrate that Fur and ClpV are involved in the virulence of *S. Typhimurium*. Notably, the deletion of both *fur* and *clpV* genes led to attenuated virulence compared to single-gene mutant strains (Table 1). Although inactivation of Fur derepressed ClpV function, which subsequently decreased the pathogenicity of the *Δfur ΔclpV* dual gene mutant strain, the roles of other virulence factors, such as that of the SPI-1 T3SS, might also be affected. Furthermore, the single or dual deletions of the *fur* and *clpV* genes decreased bacterial systemic dissemination *in vivo*. The bacterial loads of the mutant strains in the spleen were significantly lower than those of wild-type and complemented strains ($P < 0.05$ or $P < 0.01$) (Fig. 6B).

DISCUSSION

Bacteria precisely modulate gene expression to facilitate their survival in various environments or host cells. T6SSs are present in more than one-fourth of Gram-negative bacteria, and they are involved in a variety of processes, including signal transduction, motility, colonization, secretion of virulence factors, and interbacterial competition (10–20). Previous studies have highlighted that T6SSs are tightly regulated (4, 32). Indeed, it has been shown that multiple regulatory networks, including quorum-sensing systems, two-component systems, alternative sigma factors, H-NS, and Fur, regulate T6SS expression (4, 29, 33, 36–43). Among these regulators, Fur is an extensively investigated global regulator that controls gene expression by binding to the Fur binding box in the promoters of target genes under iron-rich conditions (44, 47). Recently, Fur binding boxes were identified in the promoter regions of the T6SSs in EAEC, APEC, and *E. tarda*, and their expression was under the control of Fur (20, 38, 40, 52). It has been shown that the SPI-6 T6SS is essential for intramacrophage survival, virulence, and interbacterial competition of *S. Typhimurium* (19, 25, 30, 53). However, the roles of Fur in the regulation of the SPI-6 T6SS in *S. Typhimurium* remained unknown. Here, we provided evidence that Fur binds directly to the *clpV* promoter region and represses the expression and function of the SPI-6 T6SS in *S. Typhimurium*.

Our and previous studies indicated that T6SS expression was upregulated during infection (19, 25, 30, 53), in which the environments are characterized by low iron. Moreover, we found that the expression of T6SS core gene *clpV* was enhanced in the iron-depleted medium. Our current knowledge of Fur is as a well-known global regulator facilitating bacterial iron availability and virulence (44–48). Thus, we intended to determine whether Fur regulates the expression of T6SS and *clpV*. The expression of *clpV* was significantly higher in the *fur* gene mutant strain than in the wild-type strain, as determined by qRT-PCR and Western blot analyses. To verify our results, the

β -galactosidase activities of different *lacZ* fusion reporter strains were measured. As expected, the deletion of *fur* activated P_{clpV} -*lacZ* expression. In addition, the *clpV* transcription was significantly enhanced in iron limitation medium; this might be due to the relieving of Fur-mediated repression. These observations demonstrate that Fur plays a negative regulatory role in *clpV* expression in *S. Typhimurium*. Similarly, the repression of the T6SS by regulator Fur was also identified in EAEC, APEC, *E. tarda*, and *P. aeruginosa* (20, 38, 40, 52).

Furthermore, this work demonstrates by using EMSA and DNase I footprinting analyses that Fur directly binds the *clpV* promoter. Extensive evidence showed that Fur works as a transcriptional regulator by binding to cognate sites located at the promoter region (44, 54). This study identified four Fur binding boxes in the *clpV* promoter region of the SPI-6 T6SS in *S. Typhimurium*. These Fur binding sites were distributed in the *clpV* promoter region, suggesting that Fur acts cooperatively to repress gene transcription. Among these binding sites, Fur2 and Fur3/4 bind to and spread on the transcriptional start site in the *clpV* promoter region; this observation is consistent with the mechanism of Fur-mediated repression (44, 54). In contrast, the Fur1 box is located upstream of the promoter but displayed high affinity for the Fur protein. Thus, it might interact initially with Fur and then stimulate further Fur binding at the adjacent downstream binding sites. Previous studies also showed similar observations, indicating that Fur could bind upstream of the promoter and repress gene expression by unknown mechanisms (20, 50). The presence of multiple Fur binding sites in the *clpV* gene promoter region suggests that several Fur dimers bind to this region. Indeed, several Fur dimers can bind to an extended promoter region, which might efficiently and rapidly alter gene expression to enable bacteria to adapt to the environment (44). Notably, some of these Fur binding boxes overlapped with the H-NS binding sites (29). Moreover, the SPI-6 T6SS gene cluster was shown to be silenced by H-NS via its binding to the *clpV* promoter region (29). These observations suggest that Fur and H-NS might synergistically control *clpV* expression in *S. Typhimurium*. Furthermore, experimental evidence has validated the interplay between Fur and H-NS in controlling SPI-1 gene expression and virulence in *S. Typhimurium* (49, 50, 55). In addition, Fur could counteract the repression of H-NS by removing H-NS from the repressive site in *E. coli*. However, H-NS cannot displace Fur from the *ftnA* promoter, even at high concentrations, indicating that Fur acts as an H-NS antagonist (56). Fur binds to the promoter region of the *hns* gene, and Fur might be able to indirectly control gene expression by repressing *hns* gene expression (50). Indeed, expression of the *hns* gene was upregulated 2.4-fold in the *fur* mutant strain compared to wild-type strain SAT52. Overall, these data suggest that the expression of SPI-6 T6SS genes might be tightly regulated by a complex regulatory network to optimize gene expression in different environments.

The use of T6SSs is a common strategy for *V. cholerae*, *P. aeruginosa*, EAEC, and *Salmonella* to mediate successful infections in eukaryotic hosts. Moreover, pathogens use this antibacterial weapon to compete efficiently with indigenous microbiota for limited resources (10–20, 53). We therefore sought to determine whether Fur-mediated T6SS regulation provides competition and pathogenesis fitness to *S. Typhimurium*. Consistent with previous studies (29), the wild-type strain SAT52 did not display sufficient antibacterial activity, which might be repressed by Fur and H-NS. On the other hand, the derepression of the *clpV* gene in the *fur* mutant strain caused the lysis of prey *E. coli* cells. The ClpV ATPase recycles the contracted sheath and was necessary for T6SS assembly and injection. Our results showed that ClpV was required for the interbacterial competition function of SPI-6 T6SS in *S. Typhimurium*. The relieving of Fur-mediated repression on *clpV* gene expression might produce and assemble a functionally T6SS structure that is used to deliver the effectors targeting bacteria. Indeed, the Δfur mutant strain showed increased secretion of effector Tae4, which is responsible for its enhanced antibacterial competition activity. In addition, Fur regulates the genes involved in iron acquisition, fimbria, flagella, the SPI secretion system, and metabolism (44–48), which might provide the competition fitness for bacterial growth of the Δfur mutant strain. Our and previous data demonstrated that the low-iron environment inside the

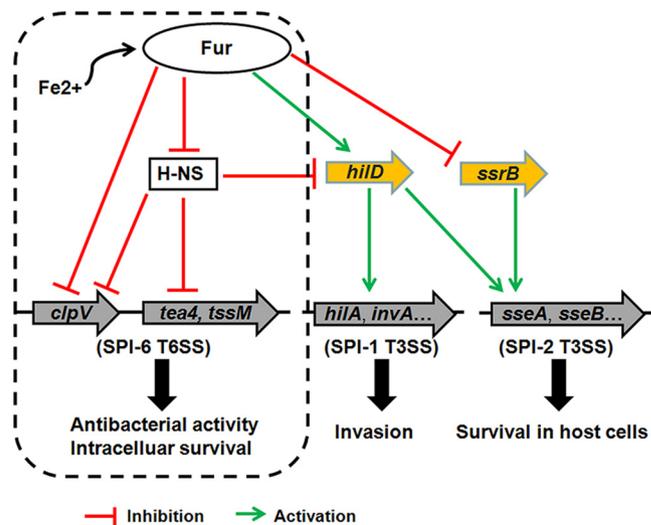


FIG 7 Schematic diagram of the Fur-mediated regulation of SPIs in *S. Typhimurium*. The regulatory mechanisms by which Fur controls the expression of T3SSs (SPI-1 and SPI-2) and H-NS represents the SPI-6 T6SS have been elucidated previously (29, 49–51). Based on our data, Fur could repress directly the expression of the SPI-6 T6SS *clpV* gene cluster under iron-rich conditions to keep the energy and source for *S. Typhimurium*. However, iron-limited environments during infection may relieve the Fur-mediated transcriptional repression of *clpV* and activate the SPI-6 T6SS as an antibacterial weapon, subsequently facilitating the survival and virulence of *S. Typhimurium*.

host could relieve Fur-mediated transcriptional repression of *clpV*, strongly suggesting that SPI-6 T6SS contributes to the survival and infection of *S. Typhimurium* (19, 25). Indeed, increased expression of the SPI-6 T6SS is required for intramacrophage survival and the successful establishment of *S. Typhimurium* in the gut during infection (25, 31, 53). Similarly, our data showed that the disruption of the SPI-6 T6SS core component ClpV resulted in defective intramacrophage survival, attenuated virulence, and diminished systemic dissemination in mice. Despite the derepression of the *clpV* gene in the *fur* mutant strain, the deletion of global regulator Fur extensively dysregulated the expression profile of virulence and metabolic genes (44–48), which might be responsible for the attenuated virulence of the Δfur mutant strain.

Based on our data and previous studies (29, 49–51), we propose a model to illustrate the regulation of the SPI-6 T6SS by Fur in *S. Typhimurium* (Fig. 7). Under iron-rich conditions, Fur binds to the SPI-6 T6SS *clpV* promoter region and represses its expression stably. In contrast, iron is scarce inside host cells. Accordingly, Fur is displaced from the promoter region, and the expression of the SPI-6 T6SS increases to promote the interbacterial competition, survival, and infection of *S. Typhimurium*. In addition, Fur could directly or indirectly control the gene expression of SPIs and other virulence genes, which is also essential for the peak fitness of bacteria in various environments. Taken together, these findings demonstrate that the expression of *clpV* in the SPI-6 T6SS is strictly controlled by Fur. In response to environmental signals, bacteria could optimize their gene expression through the combinatorial and synergistic actions of multiple regulators. Thus, elucidating the mechanisms of multiple regulatory networks on gene expression will be crucial for understanding bacterial adaptability and virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 2. *S. Typhimurium* isolate SAT52 was used for the functional analysis (57). *E. coli* DH5 α was used for cloning procedures and competition assays, and *E. coli* BL21(DE3) was used for the expression procedures. *E. coli* and *Salmonella* strains were grown routinely in LB medium at 37°C with aeration. When necessary, ampicillin (100 $\mu\text{g ml}^{-1}$) or chloramphenicol (30 $\mu\text{g ml}^{-1}$) was added to the medium. Iron-depleted medium was prepared by supplementing the iron chelator 2,2'-dipyridyl (200 $\mu\text{mol liter}^{-1}$) in LB medium.

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
SAT52	Wild-type strain	57
Δfur	<i>fur</i> deletion mutant of SAT52	This study
$\Delta C\Delta fur$	Δfur mutant with plasmid pSTV28- <i>fur</i>	This study
$\Delta clpV$	<i>clpV</i> deletion mutant of SAT52	This study
$\Delta C\Delta clpV$	$\Delta clpV$ mutant with plasmid pSTV28- <i>clpV</i>	This study
$\Delta fur \Delta clpV$	<i>fur</i> and <i>clpV</i> deletion mutant of SAT52	This study
SAT52-pRCL	SAT52 with plasmid pRCL	This study
SAT52-pRCL-P _{<i>clpV</i>}	SAT52 with plasmid pRCL-P _{<i>clpV</i>}	This study
Δfur -pRCL	Δfur mutant with plasmid pRCL	This study
Δfur -pRCL-P _{<i>clpV</i>}	Δfur mutant with plasmid pRCL-P _{<i>clpV</i>}	This study
SAT52::pRCL- <i>lacZ</i>	pRCL- <i>lacZ</i> transcriptional reporter fusion in SAT52	This study
SAT52::P _{<i>clpV</i>} - <i>lacZ</i>	pRCL-P _{<i>clpV</i>} - <i>lacZ</i> transcriptional reporter fusion in SAT52	This study
Δfur ::pRCL- <i>lacZ</i>	pRCL- <i>lacZ</i> transcriptional reporter fusion in Δfur mutant	This study
Δfur ::P _{<i>clpV</i>} - <i>lacZ</i>	pRCL-P _{<i>clpV</i>} - <i>lacZ</i> transcriptional reporter fusion in Δfur mutant	This study
SAT52-pBAD- <i>tae4</i>	SAT52 with plasmid pBAD- <i>tae4</i>	This study
Δfur -pBAD- <i>tae4</i>	Δfur mutant with plasmid pBAD- <i>tae4</i>	This study
DH5 α	F ⁻ $\Delta(lacZYA-argF)U169 recA1 endA1 hsdR17(r_K^- m_K^+) phoA supE44 \lambda^-$	Tiangen
BL21(DE3)	F ⁻ <i>ompT hsdS(r_B^- m_B^-) gal dcm</i> (DE3)	Tiangen
Plasmids		
pET28a(+)	Kan, F1 origin, His tag	Novagen
pET28a- <i>fur</i>	pET28a(+) carrying <i>fur</i> gene	This study
pET28a- <i>clpV</i>	pET28a(+) carrying <i>clpV</i> gene	This study
pSTV28	Cm; <i>lacZ</i>	Takara
pSTV28- <i>fur</i>	pSTV28 derivative harboring <i>fur</i> gene	This study
pSTV28- <i>clpV</i>	pSTV28 derivative harboring <i>clpV</i> gene	This study
pRCL	Promoterless <i>lacZ</i> ; Cm gene	65
pRCL-P _{<i>clpV</i>}	pRCL harboring <i>clpV</i> promoter	This study
pBAD/Myc-HisA	Amp; ColE1 derivative cloning vector, pBAD (<i>ara</i>) promoter	Invitrogen
pBAD- <i>tae4</i>	pBAD/Myc-HisA-expressing <i>Tae4</i> -His from the pBAD (<i>ara</i>) promoter	This study
pKD46	Amp; expresses λ red recombinase	58
pKD3	Cm gene; template plasmid	58
pCP20	Cm Amp; yeast F1p recombinase gene, FLP	58

DNA and protein sequence analyses. DNA sequences were analyzed using the online Basic Local Alignment Search Tool (BLAST) programs BLASTN and BLASTX from the National Center for Biotechnology Information website (<http://blast.ncbi.nlm.nih.gov/>). Promoter prediction analyses were conducted with the prediction program tools available at http://www.fruitfly.org/seq_tools/promoter.html. The 500-bp upstream sequence of the *clpV* gene (from positions -500 to 0 relative to the translation initiation site) was used to search the Fur binding box.

Expression and purification of recombinant proteins. The open reading frames of the *fur* and *clpV* genes of *S. Typhimurium* SAT52 were amplified and respectively cloned into the pET28a(+) vector. The recombinant proteins were expressed in *E. coli* BL21(DE3) cells after IPTG (isopropyl- β -D-thiogalactopyranoside) induction at a final concentration of 1 mM. Protein purification was performed using a HisTrap high-performance column (GE Healthcare) according to the manufacturer's guidelines. The concentrations of the purified proteins were assessed using Pierce BCA protein assay reagent (Thermo Fisher Scientific).

Construction of mutant and complemented strains and plasmids. The gene deletion mutants were constructed by the Lambda Red recombinase method as described previously (58), with some modification and appropriate primers (Table 3). To generate the chromosomal transcriptional reporter strains, the *clpV* gene was replaced with a promoterless *lacZ* gene in wild-type and *fur* mutant strains using the same procedure. For complementation, the gene coding sequences and their putative promoter regions were amplified and cloned into plasmid pSTV28. The resulting plasmids were transformed into the corresponding mutants to generate complemented strains. The obtained mutant and complemented strains were confirmed using PCR and sequence analysis. To determine the expression and secretion of effector *Tae4*, the *tae4* gene was cloned into plasmid pBAD/Myc-His (Invitrogen, Carlsbad, CA) and transformed into wild-type and *fur* mutant strains. The growth kinetics of all strains were determined as described previously (17).

To construct the transcriptional *lacZ* fusion plasmid, the promoter of the *clpV* gene (from bp -150 to bp +125 relative to the transcriptional start site) was amplified and cloned into the promoterless *lacZ* plasmid pRCL. The recombinant plasmid, in which the *lacZ* gene is under the control of *clpV* promoter, was transformed into wild-type and *fur* mutant strains. The promoterless plasmid pRCL was used as a control.

β -Galactosidase assays. β -Galactosidase activity was measured as described previously (59). Briefly, the bacteria were collected and resuspended in Z buffer. A Miller assay for β -galactosidase activity was

TABLE 3 Primers used in this study

Analysis and primer	Sequence (5' to 3') ^a	Target gene(s)	Product size (bp)
Gene expression, deletion, and complementation			
clpVEx-F	TCCGAATTCGAAACTCCTGTTTCACGCAG	<i>clpV</i>	2,634
clpVEx-R	GTGCTCGAGGCAAACGATCTCAAAAACAA	<i>clpV</i>	
furEx-F	TCCGAATTCATGACTGACAACAATACCGC	<i>fur</i>	453
furEx-R	GTGGTCGACTTATTTAGTCGCGTCATCGTGCG	<i>fur</i>	
furMu-UF	GCTGTTCCGCATATCTGCC	Upstream region of <i>fur</i>	912
furMu-UR	GAAGCAGCTCCAGCCTACACCATGCGGAATCTGCTGTT	<i>fur</i>	
furMu-CF	AACAGGACAGATTCGCATGGTGTAGGCTGGAGCTGCTTC	pKD3	1,014
furMu-CR	CTTCGAAAAGATTTACACTTACATATGAATATCCTCCTTAG	pKD3	
furMu-DF	CTAAGGAGGATATTCATATGTAAGTGTAAATCTTTCGAAG	<i>fur</i>	870
furMu-DR	CGCGTCGTTATCCAGATGAG	Downstream region of <i>fur</i>	
furup-F	AACTCAACGCACAAGCGGTTG	Upstream region of <i>fur</i>	2,704
furdown-R	CCTATCACCAGTACATCAACC	Downstream region of <i>fur</i>	
fur-F	TTTGACGATGCCGGTATCGT	<i>fur</i>	219
fur-R	AGCGCAGTGGCCGTAAGATA	<i>fur</i>	
furCo-F	TACGAATTCGCGCATCAATAGACAAGACC	Upstream region of <i>eivC</i>	726
furCo-R	GCCAAAGCTTGGCTCTTCGAAAAGATTTAC	Downstream region of <i>eivC</i>	
clpVMu-UF	TTCTGCGGATTGAGCTGTT	Upstream region of <i>clpV</i>	792
clpVMu-UR	GAAGCAGCTCCAGCCTACACCATGTGTTATGCCTTGACATA	<i>clpV</i>	
clpVMu-CF	TGTCACAAGGCATAACACATGGTGTAGGCTGGAGCTGCTTC	pKD3	912
clpVMu-CR	ACACTTCGAACGGCCGGTTTCACATATGAATATCCTCCTTAG	pKD3	
clpVMu-DF	CTAAGGAGGATATTCATATGTGAAACCGCCGTTTCGAAGTGT	<i>clpV</i>	1,014
clpVMu-DR	CGGTTGCCTGCATATTACTG	Downstream region of <i>clpV</i>	
clpVup-F	CATATCCTGTAAACGTGTCGGT	Upstream region of <i>clpV</i>	969
clpVdown-R	TGGGTAGTCTGGGTCCGAA	Downstream region of <i>clpV</i>	
clpVCo-F	TACGAATTCCTGCGGATTGAGCTGTTGTT	Upstream region of <i>clpV</i>	3,810
clpVCo-R	TACGTCGACAACCTGACCTTCACCCGTCAG	Downstream region of <i>clpV</i>	
tae4-F	GAGCTCGAGAATGAACAGACCTTCATTCAA	<i>tae4</i>	496
tae4-R	GTCGAAGCTTCGGCAGTATCCACAGTGTC	<i>tae4</i>	
qRT-PCR			
rpoD RT-F	GTGAAATGGGCACTGTTGAACTG	<i>rpoD</i>	131
rpoD RT-R	TTCCAGCAGATAGGTAATGGCTTC	<i>rpoD</i>	
clpV RT-F	CCAGCGCCATTAGTGATTTTTC	<i>clpV</i>	219
clpV RT-R	CGATCAACGAGGGCAGTATTTTC	<i>clpV</i>	
hcp1 RT-F	TAAGGTCTCCGTCACCAACC	<i>hcp1</i>	240
hcp1 RT-R	AAGGAGAGCTCCACCGTTTC	<i>hcp1</i>	
hcp2 RT-F	TCTTCCGGTAAGCACATTCC	<i>hcp2</i>	195
hcp2 RT-R	CACCACGTATTCCTGCTTC	<i>hcp2</i>	
dotU RT-F	ACCAGCACAGTAGGGTGGTC	<i>dotU</i>	198
dotU RT-R	CCGACAGTACCAGGTTGGAT	<i>dotU</i>	
icmF RT-F	GCTGGCGTAAAATCTTCGAG	<i>icmF</i>	200
icmF RT-R	GGTAAACCACAGTCGCAGT	<i>icmF</i>	
vgrG RT-F	TGGCGGTAAACGACATATC	<i>vgrG</i>	183
vgrG RT-R	TATTCGCCCAGAACCTCATC	<i>vgrG</i>	
tae4 RT-F	CTCTCCGTATGCAAAGGTTAGT	<i>tae4</i>	105
tae4 RT-R	ATAAGATCAGGCTTGCCCATAG	<i>tae4</i>	
STM0271 RT-F	GCGGCTGAATGATGGAGATA	<i>STM0271</i>	104
STM0271 RT-R	GAACCAACTGAGGAAACGAAAC	<i>STM0271</i>	
EMSA and DNase I footprinting			
furEMSA-F	CGTCGTGAATGCATTGTTGTA	Upstream region of <i>clpV</i>	253
furEMSA-R	CATGTGTTATGCCTTGTGACA	<i>clpV</i>	
<i>lacZ</i> fusion			
clpVlacZ-UF	CCAGTCCAGATCCAGGTACTIONCAGC	Upstream region of <i>clpV</i>	759
clpVlacZ-UR	GAATCCGTAATCATGGTATGTGTTATGCCTTGTGACATA	<i>clpV</i>	
lacZ-F	TATGTCACAAGGCATAACACATGACCATGATTACGGATTTC	<i>lacZ</i>	3,075
lacZ-R	GAAGCAGCTCCAGCCTACACGTCGACATAAAAAAGGGGACCT	<i>lacZ</i>	
clpVlacZ-CF	AGGTCCTTTTTATGTGCGAGCTGTAGGCTGGAGCTGCTTC	pKD3	1,014
clpVlacZ-CR	GACACTTCGAACGGCCGGTT CATATGAATATCCTCCTTAGTT	pKD3	
clpVlacZ-DF	AACTAAGGAGGATATTCATATG AACCGGCCGTTTCGAAGTGTG	<i>clpV</i>	812
clpVlacZ-DR	CAGCGTCTGCACCGCGCTTCCACG	Downstream region of <i>fur</i>	
PclpV-F	TTAAAGCTTCGTGTAATGCATTGTTGTA	Upstream region of <i>clpV</i>	275
PclpV-R	CAGGGATCCCACTGCGTGAACAGGAG	<i>clpV</i>	

^aRestriction sites are underlined.

conducted using *ortho*-nitrophenyl- β -galactoside as the substrate. This assay was performed three times in triplicate.

EMSA. To determine the binding of Fur to the *clpV* promoter, an EMSA was conducted using an EMSA kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, the sequence of the *clpV* promoter region was amplified and labeled with biotin, purified, and quantified. An EMSA was performed in a 20- μ l reaction volume that contained biotin-labeled DNA probe (20 fmol) and various amounts of purified Fur in binding buffer [10 mM Tris, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM MnCl₂, 2.5% glycerol, and 50 ng/ μ l poly(dI-dC)]. After incubation for 30 min at room temperature, the reaction system was loaded onto a 2% agarose gel, electrophoresed, and transferred to a nylon membrane. The biotin-labeled DNA was detected using a chemiluminescent substrate (Amersham Pharmacia Biotech). A competitive EMSA was performed by simultaneously incubating the biotin-labeled *clpV* promoter region and the unlabeled *clpV* promoter region with the Fur protein.

DNase I footprinting assay. The *clpV* promoter fragment was amplified and cloned into the pMD18T vector to generate plasmid pMD18T-P-*clpV*. To prepare fluorescent FAM-labeled probes, the promoter region of the *clpV* gene was PCR amplified with Dpx DNA polymerase from the plasmid pMD18T-P-*clpV* using the primers M13F-47 (FAM labeled) and M13R-48. Then, the FAM-labeled probes were purified and quantified using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific). DNase I footprinting assays were performed as described previously (60). The FAM-labeled probe (200 ng) was incubated with different concentrations of the Fur protein for 30 min at 25°C in binding buffer. DNase I digestion was performed by adding 10 μ l of DNase I (0.015 U; Promega) and 100 nmol of CaCl₂. After incubation for 1 min at 25°C, the reaction was stopped by adding 140 μ l of DNase I stop solution (200 mM sodium acetate, 30 mM EDTA, and 0.15% sodium dodecyl sulfate). DNA extraction and electrophoresis were performed as described previously (60).

qRT-PCR. The expression levels of T6SS genes were investigated using qRT-PCR as described previously (17, 61). Briefly, total RNA was extracted using TRIzol reagent (Invitrogen). RNase-free DNase I (TaKaRa) was added to the RNA samples to remove the contaminating DNA, followed by cDNA synthesis using the PrimeScript RT reagent kit (TaKaRa) according to the manufacturer's protocol. qRT-PCR was conducted using gene-specific primers (Table 3) as described previously. Data analysis was conducted according to the $\Delta\Delta C_T$ method (62) via normalization to the expression of the reference gene *rpoD*. The relative expression level is shown as the ratio compared to that of the wild-type strain SAT52.

Preparation of secretory proteins. Secretory proteins were prepared as described previously with modifications (17, 63). Fresh bacteria were diluted 1:100 in LB medium at 37°C with shaking. Bacterial cultures were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatants were then collected and filtered through a 0.22- μ m-pore-size membrane to remove bacterial cells. Secretory proteins were precipitated using 10% trichloroacetic acid and washed with acetone. The quality of secretory proteins was verified for the absence of the cytosolic marker cyclic AMP receptor protein (Crp).

Antibody production and Western blotting. Polyclonal anti-ClpV antiserum was raised in BALB/c mice by subcutaneous immunization with purified ClpV protein mixed with an equal volume of Montanide ISA 206 adjuvant (SEPPIC). The mice were immunized twice, and serum samples were collected 10 days later. For Western blotting, bacterial cultures were concentrated (100 times) by centrifugation at 12,000 rpm and boiled for 10 min. The protein samples were subjected to SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech) as described previously (17, 64). Anti-ClpV, anti-DnaK (Enzo Life Sciences), anti-His (Cell Signaling Technology), and anti-Crp (Santa Cruz Biotechnology) were used as primary antibodies. An IRDye 680RD-conjugated donkey anti-mouse polyclonal antibody (LI-COR Biosciences) was used as the secondary antibody. The antigen-antibody complex was visualized with the Odyssey two-color infrared imaging system (LI-COR Biosciences).

Bacterial competition assay. A bacterial competition assay was performed as described previously (52, 65) with some modifications. In brief, the bacterial strains were grown and adjusted to an optical density at 600 nm of 0.5, and then the fresh attacker (*S. Typhimurium*) and prey (*E. coli* DH5 α) were mixed at a 4:1 ratio, followed by incubation in LB low-salt plates with nitrocellulose membranes at 37°C for 4 h. Bacteria were collected, diluted, and spotted onto LB plates with or without antibiotics that allowed the selection of attacker or prey bacteria. The survival of prey strain was then calculated. Assays were performed three times in triplicate.

Cell infections. The intracellular survival assays were carried out as described previously (19) with some modifications. Briefly, murine macrophage RAW 264.7 cells were infected at a multiplicity of infection of 10 for 1 h at 37°C and 5% CO₂. Extracellular bacteria were killed with Dulbecco modified Eagle medium (DMEM) containing gentamicin (100 μ g/ml) for 1 h, following culture with DMEM containing 10 μ g/ml gentamicin for 10 h. The infected cells were then washed and lysed with 0.5% Triton X-100, and surviving bacteria were enumerated by plating on LB agar plates.

Ethical statement. All animal experiments were conducted in strict accordance with the Guidelines on the Humane Treatment of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China, policy 2006 398) and were approved by the Institutional Animal Care and Use Committee at the Shanghai Veterinary Research Institute (permit Shvri-Mo-0980). Mice were cared for under specific-pathogen-free conditions at the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. To minimize suffering, dying mice were euthanized humanely with an intravenous injection of sodium pentobarbital.

Animal infection experiments. The LD₅₀ was measured to determine the differences in virulence among the *S. Typhimurium* strains. Exponential-phase cultures were collected, washed, and adjusted to the appropriate doses in phosphate-buffered saline. Eight-week-old BALB/c mice ($n = 8$ per group) were infected intraperitoneally with bacterial suspensions containing different numbers of CFU. The numbers

of CFU in the injected inoculum were confirmed by plating on LB agar. Negative controls were injected with phosphate-buffered saline. Mortality was monitored daily until 14 days postinfection.

To determine the systemic dissemination of *S. Typhimurium*, bacterial loads in the spleens of infected mice were measured as described previously, with some modifications (17, 57, 61, 64). Briefly, mice infected with *S. Typhimurium* were euthanized and dissected at 2 days postinfection. The spleens were collected, weighed, and homogenized in phosphate-buffered saline containing 0.5% Triton X-100. Serial 10-fold dilutions of the homogenates were diluted and plated onto LB agar to count the bacteria.

Statistical analyses. Statistical analyses were conducted using the GraphPad Software package. One-way analysis of variance (ANOVA) and two-way ANOVA were used to analyze the qRT-PCR, Western blotting, β -galactosidase activity, and bacterial competition assays. Analysis of the animal infection study was performed using a nonparametric Mann-Whitney U test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00562-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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No potential conflicts of interest were disclosed.

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