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Fur Negatively Regulates *hns* and Is Required for the Expression of HilA and Virulence in *Salmonella enterica* Serovar Typhimurium[∇]†

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Iron is an essential element for the survival of living cells. However, excess iron is toxic, and its uptake is exquisitely regulated by the ferric uptake regulator, Fur. In *Salmonella*, the *Salmonella* pathogenicity island 1 (SPI-1) encodes a type three secretion system, which is required for invasion of host epithelial cells in the small intestine. A major activator of SPI-1 is HilA, which is encoded within SPI-1. One known regulator of *hilA* is Fur. The mechanism of *hilA* regulation by Fur is unknown. We report here that Fur is required for virulence in *Salmonella enterica* serovar Typhimurium and that Fur is required for the activation of *hilA*, as well as of other HilA-dependent genes, *invF* and *sipC*. The Fur-dependent regulation of *hilA* was independent of PhoP, a known repressor of *hilA*. Instead, the expression of the gene coding for the histone-like protein, *hns*, was significantly derepressed in the *fur* mutant. Indeed, the activation of *hilA* by Fur was dependent on 28 nucleotides located upstream of *hns*. Moreover, we used chromatin immunoprecipitation to show that Fur bound, *in vivo*, to the upstream region of *hns* in a metal-dependent fashion. Finally, deletion of *fur* in an *hns* mutant resulted in Fur-independent activation of *hilA*. In conclusion, Fur activates *hilA* by repressing the expression of *hns*.

Salmonella enterica serovar Typhimurium is a Gram-negative facultative anaerobe capable of causing food-borne gastroenteritis. Two serovars, *S*. Typhimurium and *S*. Enteritidis, are reported as the primary human isolates of nontyphoid *Salmonella* infections (8). *S*. Typhimurium, like many pathogens, contains genetic material acquired through horizontal transfer, usually phage mediated, which enhance virulence within the host (reviewed in reference 6). These horizontal gene transfer events culminated in the acquisition of *Salmonella* pathogenicity islands (SPIs). SPI-1 is a DNA segment of ~40 kb and encodes regulators of SPI-1, a type three secretion system (T3SS), chaperone proteins, and effector proteins that *Salmonella* secretes into host cells and are required for initiating host cytoskeletal rearrangement and the eventual uptake of the pathogen (19, 32, 61).

Regulation of SPI-1 is complex. Several environmental signals regulate the expression of SPI-1 (i.e., low O_2, high osmolarity, and acidic pH) (3, 36). Presumably, these environmental signals are similar to the conditions in the ileum of the small intestine, the preferred site for invasion by *S*. Typhimurium (32, 51). Oxygen tension is a critical signal that promotes invasion of epithelial cells (36), and in a previous study we demonstrated that a mutation in the oxygen-sensitive transcriptional regulator fumarate nitrate reduction (Fnr) is fully attenuated in mice and is unable to survive in macrophages (24). In addition, microarray data revealed reduced expression of several genes located within SPI-1 in the *fnr* mutant (24). Whether the two observations (i.e., increased invasion in low O_2 and avirulence of the *fnr* mutant) are connected requires further work.

Three AraC/XylS-type regulators, HilC, HilD, and RtsA (also known as STM4315) are important for the activation of SPI-1 in *S*. Typhimurium. These three regulators are capable of activating each other independently, though HilD appears to be critical following per oral inoculation (20). In addition, these three regulators (i.e., HilC, HilD, and RtsA) activate a regulator within SPI-1, HilA, which shows homology with the OmpR/ToxR family of DNA-binding proteins and induces transcriptional activation of SPI-1 genes in response to a variety of stimuli (1, 59). Specifically, HilA activates *prgH* and *invF*, driving expression of the *prg/org* and *inv/spa* genes (34, 37). Like HilC and HilD proteins, InvF belongs to the AraC/XylS family of transcriptional regulators, and its gene lies within SPI-1 (28, 33). When bound with SicA, InvF is capable of activating expression of downstream *sic/sip* genes, the effector gene, *sigD*, and the chaperone gene, *sigE*, which lies outside of SPI-1 (16–18). In addition to indirectly activating *sic/sip* expression through InvF, HilA is capable of directly activating *sic/sip* expression via readthrough transcription of *invF* (16, 19). This indicates that regulation of HilA is important for determining the activation of SPI-1.

HilA expression was shown to be repressed by addition of an intracellular iron chelator, deletion of the ferric uptake regulator (Fur), or by phosphorylated PhoP (3, 22). Deletion of *phoP* also renders the cells susceptible to iron-mediated oxi-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Source or reference
S. Typhimurium strains		
14028s	Wild type	ATCC[a]
RM5938	*hilA-lacZY*	3
KLM001	*fur*::*bla*	65
NC1091	*fur*::*bla hilA-lacZY*	This study
NC1137	*hilA-lacZY* pACYC184	This study
NC1153	*hilA-lacZY* p*fur-ha*	This study
NC1141	*fur*::*bla hilA-lacZY* pACYC184	This study
NC1154	*fur*::*bla hilA-lacZY* p*fur-ha*	This study
NC1038	*fur*::*bla* p*fur-ha*	This study
AV0474	*phoP*::*FRT*	5
NC1092	*phoP*::*FRT hilA-lacZY*	This study
NC1119	*fur*::*bla phoP:FRT hilA-lacZY*	This study
NC1148	*hilA-lacZY* p*hns*$_{-385}$	This study
NC1156	*hilA-lacZY* p*hns*$_{-357}$	This study
NC1149	*hilA-lacZY* p*hns*$_{-190}$	This study
NC1147	*fur*::*bla hilA-lacZY* p*hns*$_{-385}$	This study
NC1150	*fur*::*bla hilA-lacZY* p*hns*$_{-357}$	This study
NC1140	*fur*::*bla hilA-lacZY* p*hns*$_{-190}$	This study
WN153	*rpoS*^{low}	46
WN341	*rpoS*^{low} *hns*::*kan*	46
NC1133	*rpoS*^{low} *hilA-lacZY*	This study
NC1132	*rpoS*^{low} *hns*::*kan hilA-lacZY*	This study
NC1131	*rpoS*^{low} *fur*::*bla hilA-lacZY*	This study
NC1130	*rpoS*^{low} *hns*::*kan fur*::*bla hilA-lacZY*	This study
Plasmids		Source
pACYC184	*cat* ori p15A *tet*	12
p*fur-ha*	*fur* with in-frame expression of hemagglutinin epitope in AvaI HindIII sites of pACYC184 (*cat*)	This study
p*hns*$_{-385}$	−385 bp from ATG of *hns* to 20 bp after TAA cloned into AvaI HindIII sites of pACYC184 (*cat*)	This study
p*hns*$_{-357}$	−357 bp from ATG of *hns* to 20 bp after TAA cloned into AvaI HindIII sites of pACYC184 (*cat*)	This study
p*hns*$_{-190}$	−190 bp from ATG of *hns* to 20 bp after TAA cloned into AvaI HindIII sites of pACYC184 (*cat*)	This study

[a] ATCC, American Type Culture Collection.

dative stress and profound attenuation *in vivo* (10, 41). Because Fur controls iron uptake in many bacteria and there is fierce competition between the pathogen and the host for free iron, an understanding of how iron homeostasis regulates *hilA* is crucial to our knowledge of the role of Fur in the activation of SPI-1 and virulence in *Salmonella*.

In a recent transcriptome analysis of *S.* Typhimurium growing in anaerobiosis, we found that the expression of *hns* is significantly upregulated in a *fur* mutant (64). A putative Fur binding site was identified upstream of *hns*, but no evidence was presented for Fur binding to this region (64). In the present study, we tested the hypothesis that derepression of *hns* in the *fur* mutant is responsible for the reduced expression of *hilA* previously noted under conditions of iron chelation or Fur deletion (22, 62). We also tested whether Fur was required for systemic infection in mice. Our data indicated that Fur was required for full virulence in mice regardless of the presence or absence of the natural resistance-associated macrophage protein 1, Nramp1. In addition, we determined that under anaerobic conditions the expression of *hilA* was dependent on Fur, and we demonstrated that PhoP was not involved in this regulation. We also identified 28 nucleotides upstream of *hns* that contained the Fur binding motif responsible for the Fur-dependent activation of *hilA*. Moreover, using a modified chromatin immunoprecipitation (ChIP) assay, we determined that binding of Fur upstream of *hns* is dependent on metal. Finally, we demonstrated that in the absence of H-NS the expression of *hilA* was independent of Fur.

MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strains and plasmids used in the present study are listed in Table 1. P22-mediated transduction transferred mutations into the appropriate genetic backgrounds and transductants were purified on Evans blue-uranine agar plates. *S. enterica* serovar Typhimurium 14028s and its isogenic strains were used throughout the present study. Transductants containing the *hilA-lacZ* transcriptional fusion were selected for tetracycline resistance and a *lac*$^+$ on 0.4% lactose MacConkey agar plates. *hilA-lacZ* was also transduced into strains AV0474 (*phoP*::*FRT*), WN153, which contained an in-frame deletion of amino acids 61 to 65 of RpoS, conferring reduced function of RpoS (*rpoS*^{low}) (46), and WN341 (*rpoS*^{low} *hns*::*kan*) (46). Constructed strains were confirmed by PCR, lac$^+$ phenotype, and antibiotic resistance of linked markers. Strains with mutation in Fur were checked for siderophore secretion on Tris-buffered 0.3% xylose chrome azurol (CAS) plates (54).

Truncations of the *hns* promoter were constructed using the primers listed in Table 2. Forward primers contained a 5′ AvaI site (underlined), and the reverse

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TABLE 2. Primers

Primer	Sequence (5′–3′)	Relevance
AvaI*hns*$_{-385}$ fwd	AATAAATCCCGAGTTCATCAACAATGCTTATCA	p*hns*$_{-385}$
AvaI*hns*$_{-357}$ fwd	AATAAATCCCGAGTTACAATATGAAAACCTTGTAC	p*hns*$_{-357}$
AvaI*hns*$_{-190}$ fwd	AATAAATCCCGAGGAGTATCCCCCCTGCCAA	p*hns*$_{-190}$
HindIII*hns* rev	ATATAAAGCTTTTAAGCATCCAGGAAGTAAA	All p*hns* constructs
hns fwd	TTGTTACCGTTACTCATACCC	ChIP
hns rev	TACGCTTTATTAAGCACACG	ChIP
hns fwd	TAAAATTCTGAACAACATCC	qRT-PCR
hns rev	GATACTGTTGCAGTTTACG	qRT-PCR
rpoD fwd	TGCTGCTGGCTGAAAATACC	ChIP
rpoD rev	TTCAGGGTCAATGCTGTTGT	ChIP
rpoD fwd	CGATGTCTCTGAAGAAGTGC	qRT-PCR
rpoD rev	TTCAACCATCTCTTTCTTCG	qRT-PCR
dinP fwd	GCTTGTTCCTTGATAAAACG	ChIP
dinP rev	GACATATTCGAAGGTATGACG	ChIP

primer contained a 5′ HindIII site (underlined). Genomic DNA of 14028s was used as a template for PCR amplification of fragments that contained the *hns* promoter and the open reading frame. PCR products were extracted from agarose gels and digested with AvaI and HindIII. Purified inserts were ligated to AvaI/HindIII-digested pACYC184 and cloned into 14028s with selection on LB agar plates containing chloramphenicol. Plasmids were confirmed by restriction enzyme digest and tetracycline-sensitive (Tet^s) phenotype. Plasmid DNA and genomic DNA was purified with miniprep and DNA extraction kits (Qiagen, Valencia, CA), respectively. The p*fur-ha* plasmid contained the *fur* open reading frame expressed in-frame with the hemagglutinin (HA) epitope. This plasmid restored Fur^+ wild-type (WT) siderophore production to the *fur* mutant; as phenotypically confirmed on CAS plates (54) (see Fig. S1 in the supplemental material).

Anaerobic cultures were maintained in a Coy Anaerobic chamber filled with anaerobic gas mixture (10% H_2, 5% CO_2, 85% N_2). Growth was monitored by measuring changes in optical density at 600 nm (OD_{600}) over time. The medium used throughout was buffered LB medium containing 100 mM morpholinepropanesulfonic acid and 20 mM xylose at pH 7.4 (LB-MOPS-X) and equilibrated in the anaerobic chamber for at least 48 h prior to experiments (24). Tetracycline, chloramphenicol, ampicillin, and kanamycin were used at 20, 20, 120, and 55 μg/ml, respectively.

Pathogenicity assays. Immunocompetent 6- to 8-week-old C3H/HeN ($Nramp^{+/+}$) or C57BL/6 ($Nramp^{-/-}$) mice purchased from Charles River were tested at the animal facility of the University of Colorado Denver according to Institutional Animal Care and Use Committee guidelines. Stationary-phase serovar Typhimurium (WT and *fur* mutant) cultures grown aerobically in LB-MOPS-X broth were used. The bacteria were diluted in phosphate-buffered saline (PBS). Intraperitoneal (i.p.) challenge consisted of groups of five C3H/HeN mice inoculated with approximately 3,000 CFU in 500 μl of PBS/mouse or groups of five C57BL/6 mice inoculated with 250 CFU in 500 μl of PBS/mouse, and mortalities were scored over 21- and 30-day periods, respectively.

β-Galactosidase assays. Reporter assays of the transcriptional *hilA-lacZ* fusion were determined as described previously (40). Initial experiments were conducted by a 200-fold dilution of cultures into medium under anaerobic conditions containing the appropriate antibiotics. After ~14 h of anaerobic growth, reporter activity was determined from at least four independent cultures. Empty vector controls were included when plasmid constructs were used in experiments. In order to determine the growth phase-dependent regulation (i.e., kinetics) and the rate of expression of gene fusions, we used differential plots as previously described (14, 15, 31, 44). This was accomplished by monitoring the growth of the culture (from an initial OD_{600} of 0.02 to an OD_{600} of ~1) and the reporter activity (U ml^{-1}) in the growing cultures. The data were plotted in the form of differential plots, where reporter activity (U ml^{-1}) was plotted versus growth (OD_{600}) of the culture, and the slopes of the linear portion of these plots were used to determine the rate of expression of the reporter gene. This is beneficial for the identification of the precise OD_{600} at which point the induction of the *hilA-lacZ* reporter gene occurred. This also allowed for determining enzyme induction of the reporter as defined by the differential rate of synthesis in proportion of the increase in total protein measured as OD_{600} (42, 47). A paired Student *t* test was used to determine significance and graphs were compiled by using GraphPad Prism 4.0.

qRT-PCR and ChIP assays. For quantitative reverse transcription-PCR (qRT-PCR) experiments, three independent cultures were grown to an OD_{600} of ca. 0.35 to 0.4 and treated with RNAlater. Total RNA was extracted from each culture with TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with RNase free DNase (Promega, Madison, WI) according to the manufacturer's specifications. DNase-treated RNA was purified by using an RNeasy miniprep kit (Qiagen), the RNA quality was determined by agarose gel electrophoresis, and the RNA was quantified by determining the absorbance at 260 nm. qRT-PCR analyses were performed in triplicate using a Quantitect SYBR green RT-PCR kit (Qiagen) that contained 50 ng of total RNA in an iCycler (Bio-Rad, Hercules, CA). Reaction conditions were 49°C for 50 min for cDNA synthesis, followed by 95°C for 15 min with 45 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 30 s. Melting-curve analysis was performed beginning at 72°C and ending at 95°C and confirmed a single PCR product. Primers were used at 0.5 μM. The data were analyzed by using Bio-Rad Optical System Software, version 3.1, according to the manufacturer's specifications. The absence of genomic DNA was confirmed by PCR using primers targeting within the coding gene of *hns*. A standard curve of *rpoD* was used to determine transcript copy number. The primers are listed in Table 2.

Antibodies for ChIP included rabbit polyclonal antibody (pAb) to HA tag (ab9110-100; Abcam, Cambridge, MA) and an IgG isotype-matched control (ab6721-1; Abcam). For ChIP analyses, nucleoprotein complexes from anaerobic samples in LB-MOPS-X at an OD_{600} of ~0.25 in the presence or absence of 2,2′ dipyridyl (dip; 200 μM) were reversibly cross-linked in 1% formaldehyde for 10 min at room temperature. Cross-linked reactions were neutralized in glycine (125 mM) for 5 min at room temperature. The cells were pelleted and washed in 1× PBS and sonicated extensively on ice to produce DNA fragments 300 to 500 bp in size.

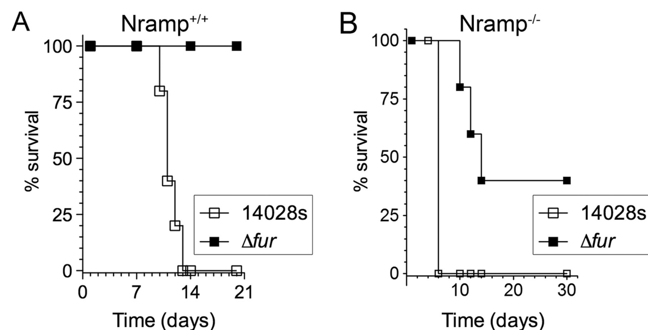

FIG. 1. Fur is required for virulence in mice. (A) C3H/HeN ($Nramp^{+/+}$) mice were inoculated i.p. with 3,000 CFU of WT 14028s or *fur* mutant. Mice were observed for 21 days after infection. (B) C57BL/6 ($Nramp^{-/-}$) mice were inoculated i.p. with 250 CFU of WT 14028s or *fur* mutant. Mice were observed for 30 days after infection.

ChIP was performed as previously described (55). Prior to ChIP, each antibody (1 μg) was incubated (1 h at 4°C) with protein A- and protein G-coupled Dynabeads (Invitrogen) at 5 μl/IP in 50 μl of radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.1% sodium deoxycholate) supplemented with 0.5 mg of sheared salmon sperm DNA/ml, 1× protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and 1 mM phenylmethylsulfonyl fluoride. Conjugated antibodies were washed and combined with equivalent chromatin amounts in 100 μl of supplemented RIPA buffer. Protein-DNA complexes were immunoprecipitated 2 h at 4°C and then washed three times in RIPA buffer and twice in Tris-EDTA (pH 8.0). Protein-DNA complexes were eluted for 15 min (100 mM $NaHCO_3$, 1% SDS). and cross-links were reversed for 15 min at 95°C in the presence of 200 mM NaCl. Protein was digested with 10 μg of proteinase K/ml 1 h at 37°C, and DNA was purified using QIAquick nucleotide removal columns (Qiagen) according to the manufacturer's instructions.

For real-time PCR, 3-μl IP and input control samples were amplified by using 1× SensiMix Plus (Quantace, London, United Kingdom) and primers specific for *rpoD*, *dinP*, and *hns* (Table 2). The cycling parameters for 20-μl reactions were as follows: 95°C for 10 min, followed by 40 to 50 cycles of 95°C for 20 s, 53°C for 30 s, and 72°C for 30 s. Fold enrichment in the bound fractions relative to input was calculated as previously described (13). Binding signals using anti-HA pAbs were normalized to ChIP signals using control IgG and are expressed as the fold enrichment over those obtained from cells cultured in the presence of 200 μM dip.

RESULTS

Fur is required for systemic infection of mice. The importance of Fur in virulence was determined in a systemic model of infection. Two strains of mice: $Nramp^{+/+}$ (C3H/HeN) and $Nramp^{-/-}$ (C57BL/6) were inoculated via i.p. with 14028s or the *fur* mutant. This approach allowed us to determine the contribution of a functional Nramp1 (for natural resistance-associated macrophage protein 1) to *S.* Typhimurium with or without the Fur protein. Mutations in Nramp1 increase host susceptibility to intracellular pathogens (57). Nramp1 is a transporter of Mn^{2+}, Fe^{2+}, and Co^{2+} that also enhances acidification of the phagosome (25, 29). Figure 1 A demonstrates that Fur is required for virulence in $Nramp^{+/+}$ (C3H/HeN) mice. The mice succumbed to infection with 14028s in less than

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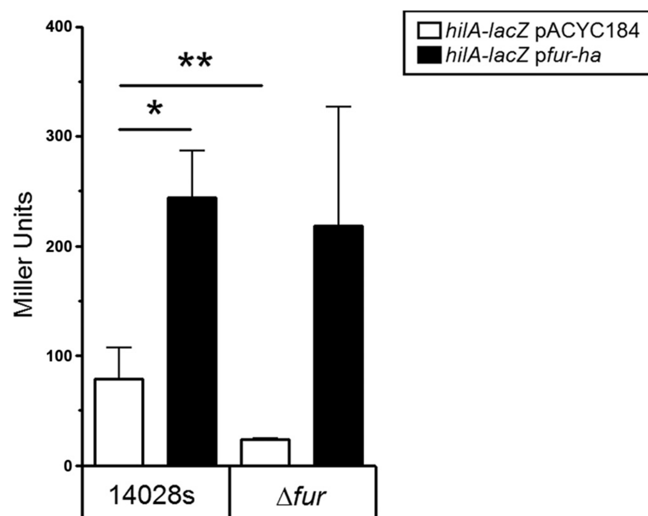

FIG. 2. Fur is required for the expression of *hilA-lacZ*. WT and the *fur* mutant strains carrying a single-chromosomal copy of *hilA-lacZ* were used. Expression of *hilA-lacZ* was determined in cells harboring empty vector (pACYC184) and compared to cells harboring p*fur-ha*. Cultures were grown anaerobically for ~14 h before β-galactosidase was determined. A paired Student *t* test was used to determine significance (*, $P \leq 0.05$; **, $P \leq 0.01$; $n = 5$). Mean values ± the standard deviations (SD) are shown.

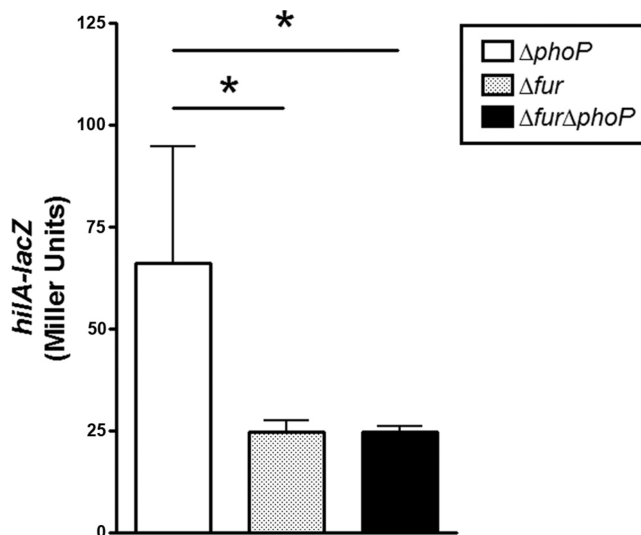

FIG. 3. Fur regulates *hilA-lacZ* independent of the response regulator PhoP. β-Galactosidase activity was determined in the Δ*phoP*, Δ*fur*, or Δ*phoP* Δ*fur* mutant strains with single-copy *hilA-lacZ*. Activity was determined as in Fig. 2. A paired Student *t* test was used to determine significance (*, $P \leq 0.05$; **, $P \leq 0.01$; $n = 4$). Means ± the SD are shown.

2 weeks, but mice infected with the *fur* mutant survived the entire study. On the other hand, the *fur* mutant was partially attenuated in Nramp$^{-/-}$ (C57BL/6) mice (Fig. 1B). Thus, all mice infected with 14028s succumbed in <1 week, but 40% of mice infected with the *fur* mutant survived the 30-day experiment. Our findings indicated that Fur-dependent regulation was important to the virulence of *S.* Typhimurium and are in agreement with a previous report in the C3H/HeN mice (65).

Fur is required for expression of *hilA*. Previous studies have suggested that activation of *hilA* is Fur dependent (22, 62). Inspection of our Fur microarray data revealed that the expression of genes within SPI-1 (*prgK*, *prgH*, *sipADCB*, *sicA*, and *invJICBA*) was significantly (1.4- to 2-fold) reduced in the *fur* mutant (64). However, in that study a 2.5-fold change was arbitrarily set to identify genes regulated by Fur. Thus, under this 2.5-fold constraint, the microarray data did not identify *hilA* as differentially regulated by Fur. To confirm the regulatory role of Fur in the regulation of *hilA* in anaerobically cultured cells, we assessed the impact of a deletion in *fur* on the expression of a *hilA-lacZ* reporter. The data in Fig. 2, show that the expression of *hilA-lacZ* in the *fur* mutant was >3-fold lower than that in the parent strain. Moreover, introduction of a low-copy-number plasmid that contained a functional *fur* gene increased the activity of *hilA-lacZ* by ~3-fold in the parent strain and by ~10-fold in the *fur* mutant (Fig. 2). This confirmed previous findings and demonstrated that the copy number of *fur* has an impact on the expression of *hilA*.

Effect of Fur on *hilA* is independent of PhoP. Several pathways regulate *hilA* expression (reviewed in references 2 and 21) and a major regulator of SPI-1 is PhoP. PhoP is a possible candidate for the observed Fur-dependent regulation of *hilA* because a deletion of *phoP* renders the cell sensitive to iron-mediated oxidative stress (10), suggesting a potential role for Fur in mediating this sensitivity. Therefore, we examined how PhoP affects Fur regulation of *hilA-lacZ.* The data in Fig. 3 show that deletion of *phoP* in the *fur* mutant background did not restore expression of *hilA-lacZ*. Therefore, PhoP-dependent regulation was not involved in the observed activation of *hilA-lacZ* by Fur. This is not surprising because activation of PhoP by its cognate histidine kinase, PhoQ, is dependent on a low [Mg^{2+}], and it is likely that Mg^{2+} is plentiful in the LB media used here (9, 58).

Previous reports indicated that induction of *hilA* is dependent on intracellular divalent cations, especially ferrous iron (22). Indeed, we found that growth in the presence of the ferrous iron chelator 2,2′-dipyridyl resulted in reduced expression of *hilA-lacZ* (data not shown). To rule out the possibility that 2,2′-dipyridyl may scavenge Mg^{2+} from the cell, we assessed the effect of the chelator on the expression of a PhoP activated gene (*pagP*) using a *pagP-lacZ* fusion, where PhoP binds *pagP* and is essential for induction of this gene (4, 52). Unlike with the *hilA* fusion, the expression of *pagP-lacZ* was not differentially affected by the addition of 200 μM 2, 2′dipyridyl to the growth media (see Fig. S2 in the supplemental material). This clearly demonstrated that PhoP is not involved in the iron- and Fur-dependent activation of *hilA*.

Fur regulation of *hns*. The microarray data (64) identified *hns*, a gene encoding the histone-like protein H-NS, as part of the Fur regulon in anaerobically grown *S.* Typhimurium. The expression of *hns* was significantly upregulated in the *fur* mutant, and a putative Fur binding site was identified upstream of the gene (64). To confirm the role of Fur in regulating *hns*, we used qRT-PCR to quantify the expression of *hns* in the presence or absence of Fur. The data showed that the expression of *hns* was >14-fold higher in the *fur* mutant than in the parent strain (Fig. 4A). We next assessed the binding of Fur to site(s) upstream of *hns* using a modified ChIP assay. Cross-linked

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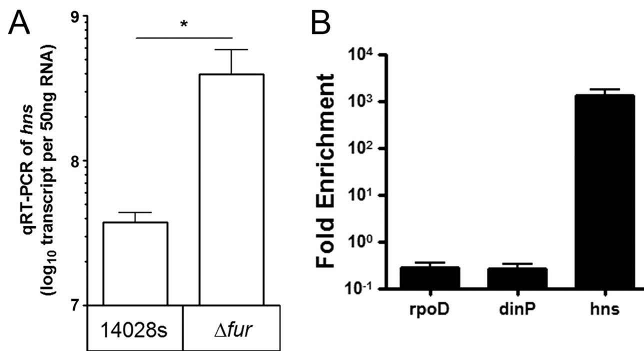

FIG. 4. Fur binds upstream of *hns* in a metal-dependent manner. (A) Expression of *hns* was significantly elevated in the *fur* mutant determined by qRT-PCR. A paired Student *t* test was used to determine significance (*, $P \leq 0.05$; $n = 3$). (B) Fur enrichment upstream of *hns* in *S.* Typhimurium. ChIP was performed on chromatin from the *fur* mutant that contained the p*fur-ha* plasmid. Resultant DNAs were analyzed by qPCR for antibody-mediated enrichment of Fur-HA at *rpoD*, *dinP*, and *hns*. Amplicon enrichment with anti-HA pAb was normalized to matched signal in nonspecific IgG control immunoprecipitations and expressed as the fold enrichment over that obtained from cells treated with dip (200 μM). Bars indicate means ± the SD of triplicate qPCRs and are representative of four independent ChIP experiments.

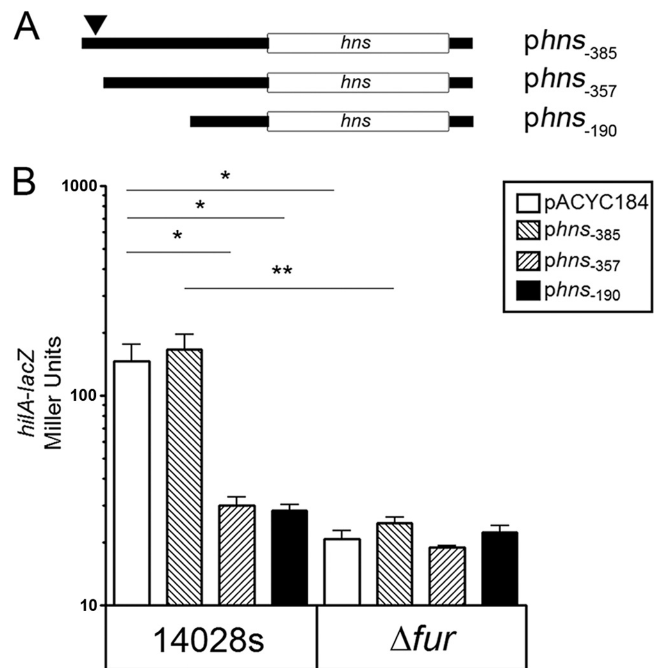

FIG. 5. Fur regulates *hilA-lacZ* through nucleotides upstream of *hns*. (A) The truncated constructs used to determine the role of the upstream region of the *hns* promoter in the Fur-dependent regulation of *hilA-lacZ*. A filled triangle depicted a predicted Fur binding site. (B) The β-galactosidase activity was determined in the parent strain and in the *fur* mutant that contained single-copy *hilA-lacZ* and harbored the empty vector (pACYC184) or in the different truncated constructs listed in panel A. β-Galactosidase activity was determined as in Fig. 2. A paired Student *t* test was used to determine significance (*, $P \leq 0.05$; **, $P \leq 0.01$; $n = 6$). Means ± the SD are shown.

nucleoprotein complexes were isolated from independent, anaerobic cultures of the *fur* mutant complemented with a plasmid expressing an HA-tagged Fur protein. This plasmid expressed an ~18-kDa protein that cross-reacted with the HA antibodies and is capable of restoring the WT siderophore secretion phenotype on CAS plates (see Fig. S1 in the supplemental material). The data in Fig. 4B show that the HA-tagged Fur was not detected at the control genes, *rpoD* or *dinP*, but was enriched $>10^3$-fold at *hns*. Importantly, Fur enrichment to the upstream region of *hns* was metal dependent, noting that the data in Fig. 4B were normalized to 2,2′-dipyridyl-treated controls.

Activation of *hilA-lacZ* by Fur is dependent on the putative Fur-binding site upstream of *hns*. H-NS has been shown to repress the transcription of *hilA*, a number of its activators, and other horizontally acquired SPIs (38, 46, 48, 49, 53). We hypothesized that the upregulation of *hns* in the *fur*-deficient cells may explain the observed Fur-dependent expression of *hilA* (Fig. 2) (22, 62). To test this hypothesis, we examined the physiological role of the putative Fur binding site in the *hns* promoter on the expression of *hilA-lacZ*. For the present study, we constructed a set of low-copy-number plasmids that contained the full-length *hns* gene downstream of progressive 5′ promoter deletions (Fig. 5A). The predicted Fur binding site is located within 385 and 357 nucleotides upstream of *hns* (signified by a filled triangle in Fig. 5A). The different *hns* plasmids were transformed into the WT and *fur* mutant strains carrying *hilA-lacZ* reporters. The data in Fig. 5B, show that the expression of *hilA-lacZ* in the WT background was the same in cells carrying the empty vector control and in cells with a construct that contained 385 nucleotides upstream of *hns* (relative to the ATG codon). The expression of *hilA-lacZ* in the WT background decreased in cells carrying a construct that contained the upstream 357- or 190-nucleotide segments (Fig. 5B). In the Δ*fur* background, the expression of *hilA-lacZ* in cells carrying the empty vector or the construct with the 385 nucleotides upstream of *hns* was much lower than in the WT background, and shorter constructs had no further effect on the expression of *hilA-lacZ* in the mutant (Fig. 5B). This indicated that the DNA segment within 385 and 357 nucleotides upstream of *hns* was important for Fur-dependent activation of *hilA-lacZ*.

H-NS reduces *hilA-lacZ* expression in the *fur* mutant. Next, we wanted to examine the effect of Fur on the expression of *hilA-lacZ* in a strain that lacked H-NS. It has been reported that, in *S.* Typhimurium, a deletion of *hns* is lethal, and certain mutations in the alternative sigma factor (*rpoS*^{low}) can compensate for this lethality (38, 46, 53). Therefore, we used P22 transduction to move *hilA-lacZ* into strains WN153 (*rpoS*^{low}) and WN341 (*rpoS*^{low}Δ*hns*) that were derived from 14028s (38, 46). We then introduced the Δ*fur* mutation into each strain to eliminate Fur expression. We monitored the expression of *hilA-lacZ* throughout anaerobic growth (see Fig. S3 in the supplemental material). These data are presented in the form of a differential plot (47), where the slopes were used to determine the rate of expression of the reporter (Fig. 6). Using this approach, we determined that *hilA-lacZ* induction occurs across a cell density that corresponds to the mid-log to the onset of stationary phase (i.e., an optical density at 600 nm [OD_{600}] of ~0.25 to ~1 [under our conditions]; see Fig. S3 in the supplemental material).

The requirement of Fur for the activation of *hilA* was ap-

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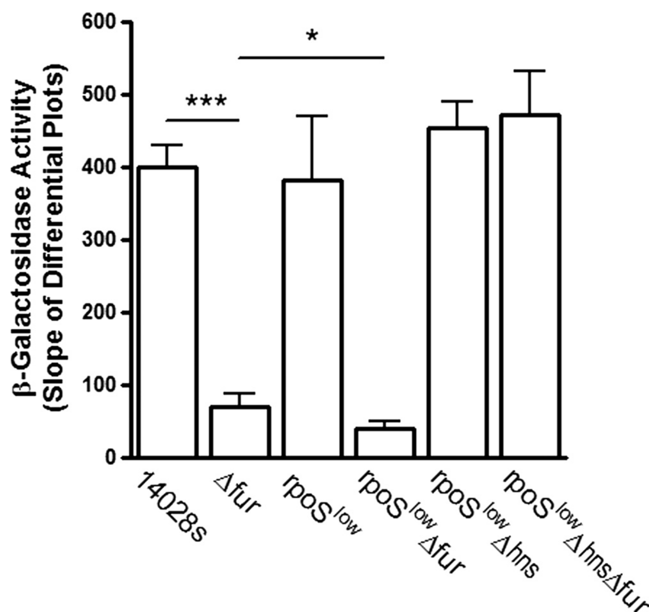

FIG. 6. Deletion of *hns* ablates Fur-dependent regulation of *hilA-lacZ*. β-Galactosidase activity was determined under anaerobic conditions. The data are presented as slopes from the differential plots (see Fig. S3 in the supplemental material). Means ± the SD are shown (*, $P \leq 0.05$; ***, $P \leq 0.001$; $n = 6$). The expression of *hilA-lacZ* in *rpoS*^{low} was biphasic (see the text and Fig. S3 for details).

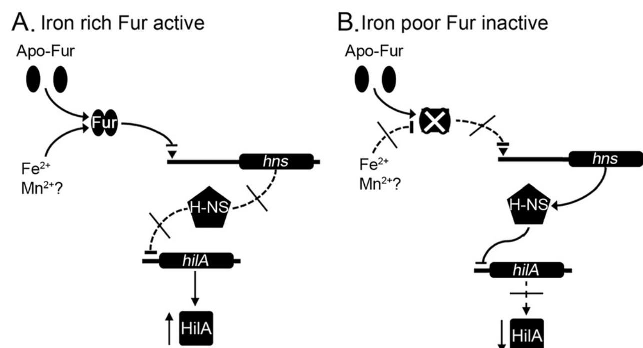

FIG. 7. Model depicting the role of Fur in the regulation of *hilA*. (A) Conditions that activate Fur (i.e., the presence of iron) repress the expression of *hns* (solid arrow), and allow the expression of *hilA* (blocked dotted arrows) and increased HilA. (B) Mutation in Fur or lack of the cofactor (i.e., Fe^{2+}) results in the expression of *hns* and the H-NS protein (blocked dotted arrows) and the reduced expression of *hilA* (solid arrows) and HilA. Implicit in this model is the role of ferrous iron transport and iron homeostasis in the activation of *hilA*/HilA and virulence.

parent as the rate of synthesis of *hilA-lacZ* was significantly reduced in Δ*fur* compared to 14028s ($P \leq 0.001$, Fig. 6), and lack of Fur resulted in decreased expression of HilA-activated genes (i.e., *invF* and *sipC*) (see Fig. S4 in the supplemental material). Interestingly, reporter activity exhibited biphasic expression in *rpoS*^{low} genetic background (see Fig. S3 in the supplemental material). Thus, during the early phase of growth the expression of *hilA-lacZ* in the *rpoS*^{low} background was similar to that of the wild type (Fig. 6) but was significantly reduced during the later phase compared to 14028s (i.e., 146.2 ± 54.7 U $OD_{600}{}^{-1}$ versus 399.8 ± 29.6 U $OD_{600}{}^{-1}$, $P \leq 0.01$; see Fig. S3 in the supplemental material). In addition, reporter activity was significantly reduced in *rpoS*^{low}Δ*fur* relative to Δ*fur* ($P \leq 0.05$; Fig. 6). Together, these findings strongly indicate a role for RpoS in *hilA-lacZ* induction during stationary phase, under anaerobic conditions. As expected, deletion of *hns* in the *rpoS*^{low} background restored reporter activity to wild-type expression (Fig. 6). Reporter activity was similar to wild type when *fur* was deleted in the *rpoS*^{low}Δ*hns* background (Fig. 6). Collectively, these data demonstrate that Fur-dependent activation of *hilA-lacZ* during anaerobic growth is due to the repression of *hns* by Fur.

DISCUSSION

In *Salmonella*, virulence genes are clustered in the SPIs that are acquired via horizontal gene transfer. H-NS has been shown to serve as a silencer of horizontally transferred genes (46, 49). SPI-1 is an important facet of *Salmonella* virulence because it facilitates the invasion and penetration of the gastrointestinal epithelium. A major regulator of SPI-1 is the DNA-binding protein HilA, which activates all components necessary for a T3SS. Indeed, *in vivo* competition experiments indicate deletion of either *hilA* or SPI-1 result in the same phenotype (20). A previous study showed that the *fur* mutant of *S*. Typhimurium was avirulent in C3H/HeN ($Nramp^{+/+}$) mice infected i.p. (65), and other work showed that Fur is required for the activation of *hilA* (22, 62). However, the identity of the Fur-regulated gene(s) responsible for the positive effect of Fur on the regulation of *hilA* and virulence in *S*. Typhimurium remained unknown. In the present study, we report that Fur regulates the expression of H-NS and this in-turn regulates the expression of *hilA* and virulence in mice.

Fur and HilA. Normally, Fur acts as a repressor of genes required for iron homeostasis (30). In *E. coli*, Fur has been shown to positively affect the expression of few genes (e.g., *sodB*) via its negative regulation of a small RNA, RyhB (39). The possibility that the *Salmonella* sRNAs (RfrA and RfrB) or the RNA-binding protein (Hfq) were involved in the activation of HilA by Fur was ruled out by the excellent work of Ellermeier and Slauch (22). We confirmed that Hfq has no role in the activation of *hilA-lacZ* under anaerobic conditions (see Fig. S4 in the supplemental material). The nature of the factor required for the Fur activation of HilA remained elusive until the present study. Thus, we showed that Fur negatively regulates the expression of *hns* and that the positive effect of Fur on the expression of *hilA* is dependent on an upstream sequence that contained the putative Fur-binding site in *hns*. In addition, a deletion in *hns* abrogated the effect of Fur on the expression of *hilA*. These results are summarized in the diagram depicted in Fig. 7, where the presence of active Fur (i.e., Fur-Fe^{2+}) results in decrease in [H-NS] and increased the expression of *hilA* (Fig. 7A). On the other hand, under conditions where Fur is inactive (i.e., via deletion of *fur* or iron deprivation) [H-NS] increases and results in repression of *hilA* (Fig. 7B). In addition, the growth-phase-dependent regulation of *hilA* (Fig. S3) may explain the small change (i.e., <2.5-fold) observed in our Δ*fur* microarray analysis (64).

Earlier work determined that Fur activation of *hilA* operated

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through HilD and that overexpression of HilD resulted in Fur-independent induction of *hilA* (22). This may be due to HilD countersilencing H-NS repression. H-NS displacement is needed to allow access of RNA polymerase to *hilA*. This distinguishes H-NS from other regulators, for instance, those that require an appropriate metal for DNA binding, e.g., Fur, which can be displaced from DNA-binding sites by removal of the metal. However, the identity of the factor(s) required for H-NS displacement is currently unknown. It has recently been shown that HilD does countersilence H-NS repression of another SPI (SPI-2) during *in vitro* growth (7). This suggests the removal of H-NS by HilD may also be responsible for activation of *hilA* in a mutated *fur* background. Thus, countersilencing of H-NS repression appears to be a common aspect of its regulation.

Fur and virulence. We found the *fur* mutant to be avirulent in the C3H/HeN ($Nramp^{+/+}$)-infected mice, in agreement with (65), but it was attenuated in C57BL/6 ($Nramp^{-/-}$) mice (Fig. 1). This result could be attributed to the metal restriction imposed on intracellular pathogens in the $Nramp^{+/+}$ background. However, as expected, we detected upregulation of Mn^{2+}, Fe^{2+}, and Fe^{3+} acquisition genes (64) and increased siderophore production in the *fur* mutant (see Fig. S1B in the supplemental material). This indicates that metal restriction in the $Nramp^{+/+}$ may not be the cause for the avirulent phenotype of the *fur* mutant. Instead, the difference in the severity of the infection in the two types of mice may be due to the Nramp1-dependent acidification of the phagosome in the $Nramp^{+/+}$ mice instead of the contribution to metal restriction from the pathogen (29). Indeed, Fur is essential during acid stress in *Salmonella* (26, 27). However, it could be suggested that reduced virulence of the *fur* mutant is related to the increased concentration of free iron in the cells and oxidative stress (50, 63, 65). In the present study, we demonstrated the role of Fur in activating a regulator of SPI-1, HilA, which may contribute to the reduced virulence of the Fur mutant. However, one may argue that in the present and the previous (65) studies mice were infected via i.p. routes that bypass the need for invasion genes found in SPI-1. Although the classical role of SPI-1 is traversing the host epithelial cell layer, several lines of evidence show that genes within SPI-1 are necessary for survival in phagocytic cells (11, 24, 35, 56). In addition, since H-NS is a major silencer of horizontally acquired genes (46), we postulate that overexpression of H-NS in the *fur* mutant results in the reduced expression of genes within SPI-1 (i.e., HilA-dependent genes), as well as genes within other SPIs, and collectively contribute to the reduced virulence of the Fur mutant in mice.

Regulation of H-NS by Fur. The nucleoid-associated protein, H-NS, is important for the selective silencing and regulation of horizontally acquired genes in enteric bacteria (23, 46, 60). Indeed, expression of horizontally acquired genes at the right time and in response to the appropriate environmental signals is essential for peak fitness of the organism (23, 46, 60). There are many reports on the antisilencing mechanisms deployed by enteric bacteria (for a review, see reference 60). We reported here, for the first time, on the regulation of H-NS by Fur. Thus, suggesting that the regulation of horizontally acquired genes in *Salmonella* is indeed integrated into the preexisting regulatory network for iron homeostasis. However, evidence presented here raises two major questions regarding H-NS regulation.

First, to what extent does H-NS interact with iron metabolism? The metal-dependent regulation of *hns* by Fur suggests that iron concentrations (possibly other metals such as manganese) within the cell may be key signals in H-NS regulation. Indeed, evidence in *Escherichia coli* suggests that H-NS represses a major iron-storing ferritin (*ftnA*) and that Fur counteracts this repression by physically removing H-NS from the repressive site (45). Also, in *E. coli*, the effect of H-NS on iron acquisition is divergent; it acts as a repressor of salmochelin (IroN) and activator of the hemin receptor (ChuA), while having no effect on the expression of enterobactin receptor (FebA) (43). In addition, in *S.* Typhimurium, activation of *ftnA* depends on iron in a Fur-dependent fashion (65). It is also noteworthy that Fur is required for induction of the least studied ferritin-like protein in *S.* Typhimurium, FtnB (64). H-NS binds the region of *ftnB* and represses transcription (46), which indicates a possible mechanism for Fur activation of *ftnB*. H-NS also binds to the promoter of the ferrous iron transport genes, *feoAB*, as well as the activator of *feoAB* transcription, RstA (46). Given that RstA (STM1475) is a member of the OmpR family of response regulators, it may not be surprising that H-NS regulation is iron dependent and indicates that H-NS may reduce ferrous iron acquisition and transport, thereby reducing Fur activity. We speculate that a primary role of RstA activation of Fur in acidic conditions is to reduce ferrous iron transport, repress *hns*, and promote Fur-mediated hydroperoxidase activity (64, 66). Collectively, our data and the reports cited above suggest an interaction between H-NS and Fur in iron homeostasis.

Second, how does Fur regulate expression of *hns* at such a distant regulatory site? The predicted -35 and -10 sites of *hns* are located 70 and 50 nucleotides upstream of ATG, respectively, 309 and 292 nucleotides downstream from our putative Fur binding site. This indicates that Fur may repress *hns* expression by a novel mechanism, perhaps in combination with other, yet-to-be discovered regulators. The recent evidence that Fur displaces H-NS from regulatory sites of *ftnA* and not vice versa (45) indicates that once Fur is activated the cell is committed to countersilencing and repression of H-NS until iron concentrations are reduced enough to inhibit Fur binding. Subsequent work should determine the exact mechanism of Fur regulation of *hns*. For instance, does Fur binding to *hns* require additional regulators? Can Fur bind to multiple regulatory sites of *hns*? The benefit of our ChIP assays was the determination of Fur binding to the regulatory region of *hns*, *in vivo*. However, *in vitro* work is needed to determine the extent of Fur affinity to our predicted Fur binding site and perhaps other sites located upstream of *hns.*

In conclusion, we provided, for the first time, evidence that Fur represses *hns* expression. In addition, Fur-controlled activation of *hilA* through 28 bp located at a distant site upstream of *hns*. Our findings advance the current model of SPI-1 regulation (2, 21) and identified the previously unexplained relationship between iron and Fur in the regulation of *hilA* (signified as "X" in reference 22). Also, our data provide a plausible explanation of the reduced virulence of the *fur* mutant. Further studies are needed to define the exact mechanism of H-NS regulation via iron homeostasis.

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