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PUBLIC AND ENVIRONMENTAL HEALTH MICROBIOLOGY

Regulation of Iron Uptake by Fine-Tuning the Iron Responsiveness of the Iron Sensor Fur

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ABSTRACT Iron is one of most abundant environmental metal ions but is highly limited in organisms. It is an important metal ion as it facilitates various biological processes, including catalysis of metabolic enzymes and DNA biogenesis. In bacteria, the ferric uptake regulator (Fur) protein controls iron uptake by regulating genes coding for iron transporters in response to iron concentration. This iron response is ascribed to Fur's intrinsic affinity for iron because its binding to iron dictates its regulatory function. However, we now report that the pathogen *Salmonella* achieves a proper response of Fur to changes in environmental iron concentrations via EIIA^{Ntr} (a nitrogen metabolic phosphotransferase system component). We establish that EIIA^{Ntr} increases expression of iron transporter-coding genes under low-iron conditions (i.e., nanomolar ranges) in a Fur-dependent manner, which promotes *Salmonella* growth under such conditions. EIIA^{Ntr} directly hampers Fur binding to DNA, thereby inducing expression of those genes. This regulation allows *Salmonella* to express Fur-regulated genes under low-iron conditions. Our findings reveal a potentially widespread control mechanism of bacterial iron uptake systems operating in response to iron availability.

IMPORTANCE Iron is a fundamental metal ion for living organisms as it facilitates various biological processes. The ferric uptake regulator (Fur) protein controls iron homeostasis in various bacterial species. It is believed that Fur's iron-dependent regulatory action is sufficient for it to function as an iron sensor. However, we now establish that the bacterial pathogen *Salmonella* enables Fur to properly reflect changes in surrounding iron availability by fine-tuning its responsiveness to iron. This process requires a protein that hampers Fur DNA binding at low iron concentrations. In this way, *Salmonella* broadens the range of iron concentrations that Fur responds to. Our findings reveal a potentially widespread control mechanism of bacterial iron homeostasis.

KEYWORDS EIIA^{Ntr}, *Salmonella enterica*, nitrogen metabolic PTS

Iron is an abundant metal on Earth but is very limited in organisms due to its poor solubility (1). Because this metal is an essential cofactor for many biological processes, including reactions catalyzed by metabolic enzymes and DNA biogenesis (2), iron homeostasis is important for living organisms (1, 2). Not surprisingly, mammalian hosts and bacterial pathogens compete for this limiting metal ion during infection (3, 4). Therefore, it is important for bacteria to properly modulate their iron acquisition system in response to changes in iron availability, especially when the available iron concentration is low. Here, we report how a microorganism tunes the responsiveness of an iron sensor to properly control the iron uptake system.

The transcription factor ferric uptake regulator (Fur) functions as an iron sensor and plays a leading role in maintaining iron homeostasis in various bacterial species by

Citation Choi J, Ryu S. 2019. Regulation of iron uptake by fine-tuning the iron responsiveness of the iron sensor Fur. Appl Environ Microbiol 85:e03026-18. https://doi.org/10.1128/AEM.03026-18.

Editor Haruyuki Atomi, Kyoto University

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Received 17 December 2018
Accepted 23 February 2019
Accepted manuscript posted online 1 March 2019
Published 18 April 2019

controlling iron transporter-coding genes (5, 6). Fur represses expression of iron transporter genes under high-iron conditions where iron-bound Fur binds to its target promoters, thereby repressing gene transcription (5–7). It is reported that external iron concentrations below 5 to 10 μM cause dissociation of Fur from its targets, allowing expression of iron transporter-coding genes (5). Fur influences the expression of a variety of genes, including those that participate in iron acquisition and virulence (5, 8).

The *ptsN* gene encodes EIIA^{Ntr}, a component of the nitrogen-metabolic phosphotransferase system (PTS) (9). This system lacks the membrane-bound complex that would normally control the activities of sugar PTSs in response to particular sugar availabilities (9). EIIA^{Ntr} plays regulatory functions in various bacterial species by interacting with proteins involved in a variety of cellular processes. EIIA^{Ntr} controls potassium uptake via TrkA and KdpD (10, 11), phosphate uptake via PhoR (12), virulence via SsrB (13), the stringent response via SpoT (14, 15), and amino sugar homeostasis via GlmS (16). The phosphorylation status of EIIA^{Ntr} contributes some of those EIIA^{Ntr}-mediated regulatory functions (10, 11, 15, 16). The phosphorylation of EIIA^{Ntr} is known to be controlled by the extracellular abundance of nitrogen sources or by the cellular concentration of glutamine (15, 17).

The intracellular pathogen *Salmonella enterica* serovar Typhimurium is the etiologic agent of human gastroenteritis and murine typhoid fever. *Salmonella* resides inside macrophage phagosomes (18) where iron availability is limited due to the iron transporters being recruited by the mammalian host pumping out iron from those vesicles (19). However, the phagosome does not represent an iron-depleted condition given that iron-responding systems are activated in that compartment (20–22). Lack of the iron sensor Fur attenuates *Salmonella* virulence (23), suggesting that *Salmonella* must manage the low iron availability inside the phagosome and that the iron-sensing ability of Fur is critical for its virulence. Fur's iron-sensing function is ascribed to its intrinsic affinity to iron (6, 24) given that iron binding dictates its DNA binding ability (6). The affinity of Fur for iron is in the low micromolar range (6, 25), which is believed sufficient for Fur to operate as an iron sensor.

Here, we now report that Fur's response to iron requires EIIA^{Ntr} in addition to its own ability to sense iron. EIIA^{Ntr} tunes the responsiveness of Fur to iron by hampering Fur binding to DNA. This allows expression of the iron uptake system when surrounding iron concentrations drop to the nanomolar range (hereafter, low-iron conditions), which enables the intracellular pathogen *Salmonella enterica* to properly control its iron uptake system in response to iron availability.

RESULTS

Expression of iron uptake genes requires EIIA^{Ntr} under low-iron conditions. A recent proteomic study reported that the *Escherichia coli ptsN* mutant reduces the abundance of proteins involved in iron uptake system (26), which leads us to question whether EIIA^{Ntr} has a role in iron response. To test this idea, we first examined expression of genes involved in iron importation (including *fhuA*, *fepA*, and *iroB*) in the wild-type strain and the *ptsN* mutant grown in acidified defined media to mimic the experience of *Salmonella* in acidic phagosomes, where iron uptake is important (18, 19). The wild-type strain displayed higher mRNA abundance of those genes than the *ptsN* mutant (Fig. 1A), indicating that EIIA^{Ntr} is involved in controlling iron response genes. We further investigated the expression of the *fhuA* gene by measuring β-galactosidase activity produced by *Salmonella* strains with a p_{fhuA}-*lacZ* fusion from its normal chromosomal location; the wild-type strain had ~9-fold-higher activities than the *ptsN* mutant (Fig. 1B). Moreover, the defective *fhuA* expression of the *ptsN* mutant was due to a lack of EIIA^{Ntr} protein because a plasmid expressing EIIA^{Ntr} from a heterologous promoter restored *fhuA* expression to wild-type levels, but the empty vector did not (Fig. 1B).

We next investigated *fhuA* expression under conditions of various iron concentrations. Wild-type *Salmonella* induced an ~18-fold increase in *fhuA* expression when iron concentrations dropped from 100 μM to 8 nM (Fig. 1C). Surprisingly, however, the *ptsN*

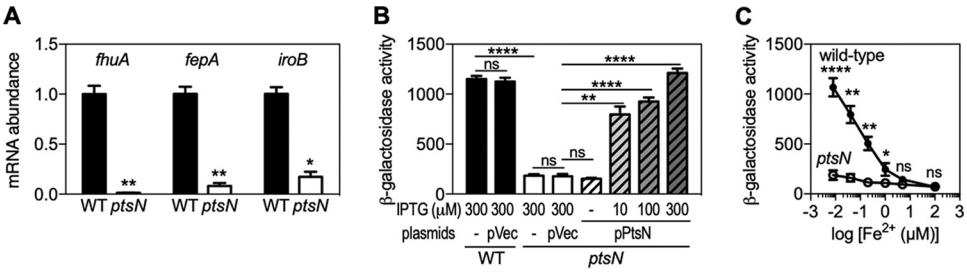

FIG 1 EIIA^{Ntr} G expression of iron uptake genes under low-iron conditions. (A) mRNA abundance of *fhuA*, *fepA*, and *iroB* genes was determined in the wild-type (WT) and the *ptsN* mutant strains grown in acidified M9 medium (pH 5.8) to mid-log phase. (B and C) β-Galactosidase activities of *Salmonella* with a p_{fhuA}-*lacZ* fusion in the normal chromosomal location and isogenic *ptsN* mutants with denoted plasmids (empty vector [pVec] or plasmid expressing EIIA^{Ntr} from a heterologous promoter [pPtsN]) were determined. Bacteria were grown to mid-log phase in acidified M9 medium (pH 5.8) with 8 nM $FeSO_4$ and IPTG at the denoted concentrations (B) or in acidified M9 medium (pH 5.8) with $FeSO_4$ at the denoted concentrations (C). The means and standard deviations (SD) of results from at least three independent experiments are shown as follows: symbols or bars, mean values; error bars, SD. Two-tailed *t* tests were performed for comparisons between the wild-type strain and the *ptsN* mutant or between indicated strains, and statistical significance is indicated as follows: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

mutant failed to do so under conditions of the same changes in iron concentrations even though it has the iron sensor Fur (Fig. 1C); the *ptsN* mutant displayed only an approximately 2-fold increase in *fhuA* expression (Fig. 1C). This EIIA^{Ntr}-dependent regulation of *fhuA* expression was evident when iron concentrations were lower than 1 μM (Fig. 1C). These results suggest that *Salmonella* EIIA^{Ntr} controls the iron uptake genes under low-iron conditions.

EIIA^{Ntr} controls iron uptake gene expression in a Fur-dependent manner. Given that expression of those iron uptake genes (including *fhuA*) is repressed by Fur (24, 27), we hypothesized that EIIA^{Ntr} controls the iron uptake gene expression via Fur. Consistent with the previous notion that Fur represses its target genes under iron-replete conditions (5–7), a deletion of the *fur* gene did not alter *fhuA* expression under low-iron conditions. Lack of Fur abrogated the regulatory effects of EIIA^{Ntr} on *fhuA* expression (Fig. 2A), indicating that Fur is necessary for EIIA^{Ntr}-mediated regulation of *fhuA* expression. If EIIA^{Ntr} regulates *fhuA* expression by altering Fur expression (i.e., if changes in Fur expression could in turn control expression of *fhuA* gene), heterologous EIIA^{Ntr}-independent expression of Fur should abolish regulatory effects of EIIA^{Ntr} on *fhuA* expression. However, lack of EIIA^{Ntr} reduced *fhuA* transcription even when Fur was expressed from a heterologous promoter (Fig. 2A), indicating that EIIA^{Ntr} likely controls *fhuA* expression independently of Fur expression and that Fur represses *fhuA* expression when EIIA^{Ntr} is absent under low-iron conditions. Moreover, *fur* transcription

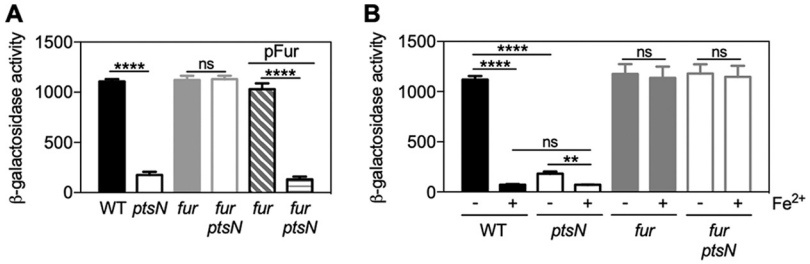

FIG 2 EIIA^{Ntr}-mediated regulation of *fhuA* requires Fur. β-Galactosidase activities of *Salmonella* with a p_{fhuA}-*lacZ* fusion in the normal chromosomal location and isogenic strains deleted for *ptsN*, *fur*, and *fur ptsN* genes with or without a plasmid expressing Fur from a heterologous promoter (pFur) were determined. Bacteria were grown to mid-log phase in acidified M9 medium (pH 5.8) (A) or the same medium with (+) or without (−) 100 μM $FeSO_4$ (B). The means and standard deviations (SD) of results from at least three independent experiments are shown as follows: bars, mean values; error bars, SD. Two-tailed *t* tests were performed for comparisons between indicated strains, and statistical significance is indicated as follows: ns, not significant; **, $P < 0.01$; ****, $P < 0.0001$.

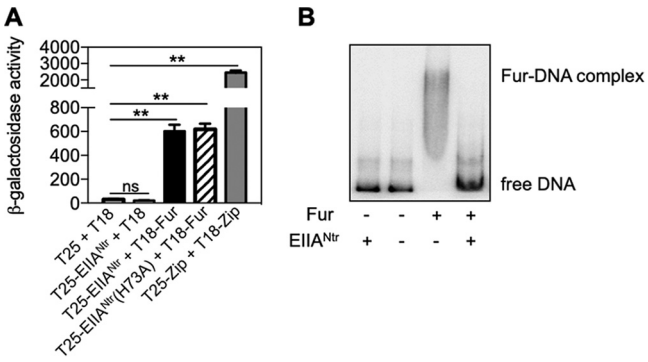

FIG 3 EIIA^{Ntr} inhibits Fur binding to DNA. (A) β-Galactosidase activities were determined from *cya E. coli* mutant strains harboring the denoted plasmid combinations grown in acidified M9 medium containing 0.5 mM IPTG. The means and standard deviations (SD) of results from at least three independent experiments are shown as follows: bars, mean values; error bars, SD. Two-tailed *t* tests were performed for comparisons between indicated strains, and statistical significance is indicated as follows: ns, not significant; **, $P < 0.01$. (B) *In vitro* binding of Fur to the *fhuA* promoter with or without EIIA^{Ntr}. The *fhuA* promoter DNA (80 fmol) was incubated with Fur (2 μM) and EIIA^{Ntr} (10 μM) proteins. Data are representative of results from at least three independent experiments.

occurred independently of EIIA^{Ntr} (see Fig. S1A in the supplemental material), and Fur protein amounts were also comparable in the wild-type and the *ptsN* mutant strains (Fig. S1B). These findings suggest that the regulatory effect of EIIA^{Ntr} occurs at post-transcriptional levels.

Given that iron renders Fur's regulatory function (6) and that Fur repressed *fhuA* expression in the *ptsN* mutant under low-iron conditions (Fig. 2A), we next investigated the effects of exogenous iron on EIIA^{Ntr}- and Fur-mediated regulation of *fhuA*. The addition of iron (100 μM of $FeSO_4$) greatly reduced *fhuA* expression levels in the wild-type strain (Fig. 2B). Under this condition, the *ptsN* mutant showed *fhuA* expression comparable to that exhibited by the wild-type strain (Fig. 2B). Moreover, lack of Fur resulted in high levels of *fhuA* expression independently of both EIIA^{Ntr} and iron levels (Fig. 2B). Taken together, these findings indicate that EIIA^{Ntr} promotes expression of iron uptake genes (including *fhuA*) via Fur under low-iron conditions.

EIIA^{Ntr} interacts with Fur. Since EIIA^{Ntr} controls its targets via protein-protein interaction (10, 11, 13–16), we wondered whether it interacts with Fur. To examine interactions of EIIA^{Ntr} and Fur, a bacterial two-hybrid assay was used in which β-galactosidase levels are dependent on the proximity of fused proteins to fragments (i.e., T25 and T18) of the *Bordetella pertussis* adenylate cyclase in an *E. coli* strain lacking its own adenylate cyclase (28). Coexpression of T25-EIIA^{Ntr} and T-18-Fur produced ~33-fold-higher levels of β-galactosidase activity than the strain expressing T25-EIIA^{Ntr} and the T-18 fragment or empty vectors (Fig. 3A). However, β-galactosidase activities from the strain expressing T25-EIIA^{Ntr} and T-18-Fur were lower than seen with expression of positive-control plasmids. Given that some EIIA^{Ntr}-mediated regulation of biological functions is dependent on its phosphorylation status (10, 11, 15, 16), we wondered if phosphorylation is necessary for the interaction of EIIA^{Ntr} with Fur. The unphosphorylatable EIIA^{Ntr} variant (H73A) gave expression levels of β-galactosidase activities similar to those seen with wild-type EIIA^{Ntr} (Fig. 3A), indicating that the phosphorylation status of EIIA^{Ntr} is not critical for the interaction with Fur. This is similar to results revealing other roles of EIIA^{Ntr} occurring independently of its phosphorylation status (12–14, 16, 29). Taken together, these results indicate that EIIA^{Ntr} interacts with Fur.

EIIA^{Ntr} controls iron uptake gene expression by hampering Fur binding to DNA. Given that EIIA^{Ntr} interacts with Fur (Fig. 3A), it is possible that EIIA^{Ntr} controls *fhuA* expression by interfering with Fur binding to DNA. To test this, a gel shift assay was conducted using purified Fur and EIIA^{Ntr} proteins with the *fhuA* promoter DNA. Purified Fur bound to the *fhuA* promoter DNA, which formed a complex *in vitro* (Fig. 3B). Addition of EIIA^{Ntr} to this reaction hampered Fur binding to the *fhuA* promoter

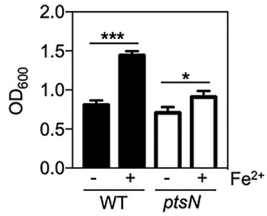

FIG 4 EIIA^{Ntr} promotes *Salmonella* growth under low-iron conditions. Bacterial growth was determined for the *Salmonella* wild-type strain and the *ptsN* mutant in acidified M9 medium with (+) or without (−) 0.25 μM $FeSO_4$. The optical density at 600 nm (OD_{600}) of bacteria was measured. The means and standard deviations (SD) from three independent experiments are shown as follows: bars, mean values; error bars, SD. Two-tailed *t* tests were performed for comparisons between indicated strains, and statistical significance is indicated as follows: *, $P < 0.05$; ***, $P < 0.001$.

(Fig. 3B). However, EIIA^{Ntr} alone did not bind to the *fhuA* promoter (Fig. 3B), indicating that prevention of Fur binding to DNA by EIIA^{Ntr} was not due to competition between EIIA^{Ntr} and Fur for binding the promoter. These results suggest that EIIA^{Ntr} promotes iron uptake gene expression by relieving Fur-mediated repression via inhibition of Fur binding to its target promoter regions under low-iron conditions.

Control of iron uptake gene expression by EIIA^{Ntr} under low-iron conditions ensures *Salmonella* growth. Given that EIIA^{Ntr} is required for expression of iron uptake genes under low-iron conditions (Fig. 1C; see also Fig. 2B) and that EIIA^{Ntr} hampers Fur binding to DNA by interacting with Fur (Fig. 3), we wondered if excess amounts of iron might alter interaction between EIIA^{Ntr} and Fur. To test this idea, the bacterial two-hybrid assay was done with a supply of 100 μM iron. Although coexpression of T25-EIIA^{Ntr} and T-18-Fur produced ~33-fold-higher levels of β-galactosidase activity than were seen with the control strains (Fig. 3A), the same strain showed basal levels of β-galactosidase activity in the presence of excess iron (Fig. S2A). Furthermore, the addition of iron to the mixture of purified Fur with or without EIIA^{Ntr} and the target promoter DNA abolished EIIA^{Ntr} effects on Fur binding to target DNA (Fig. S2B). This is consistent with the finding that regulatory effects of EIIA^{Ntr} were not observed under conditions of high iron concentrations (Fig. 1C; see also Fig. 2B). These results further support the notion that EIIA^{Ntr} plays a critical role in controlling iron uptake gene expression under low-iron conditions, such as inside the host phagosome.

We then wondered whether EIIA^{Ntr}-mediated regulation of iron uptake genes could provide any advantage with respect to *Salmonella* physiology. Given that iron supports bacterial growth (30, 31), we examined iron-dependent growth phenotypes of the *Salmonella* wild-type strain and the *ptsN* mutant using iron concentrations differentially activating iron uptake genes in those two strains (Fig. 1C). As expected, supply of low concentrations of iron (250 nM $FeSO_4$) increased growth of wild-type *Salmonella* by >80% (Fig. 4). However, it resulted in an increase of only <30% for the *Salmonella ptsN* mutant (Fig. 4). These results suggest that the EIIA^{Ntr}-mediated activation of iron uptake genes promotes *Salmonella* growth under low-iron conditions.

DISCUSSION

Signal-sensing regulatory systems respond to environmental changes. The output of such systems is dependent on the sensitivity of the system to environmental signals. How does an organism tune the sensitivity of certain sensory systems? Here, we have established that EIIA^{Ntr} allows the iron sensor Fur to appropriately respond to environmental iron concentrations. Our findings suggest that *Salmonella* fine-tunes the responsiveness of Fur to iron via EIIA^{Ntr} (Fig. 5), thereby properly controlling the iron uptake system and potentially other biological functions (5, 8, 27). This provides a new insight into the mechanism of Fur's iron sensing. Given that EIIA^{Ntr} and Fur proteins coexist in >600 different bacterial organisms (see Table S1 in the supplemental material), the EIIA^{Ntr}-mediated tuning of Fur's iron response is likely a widespread mechanism in other bacteria.

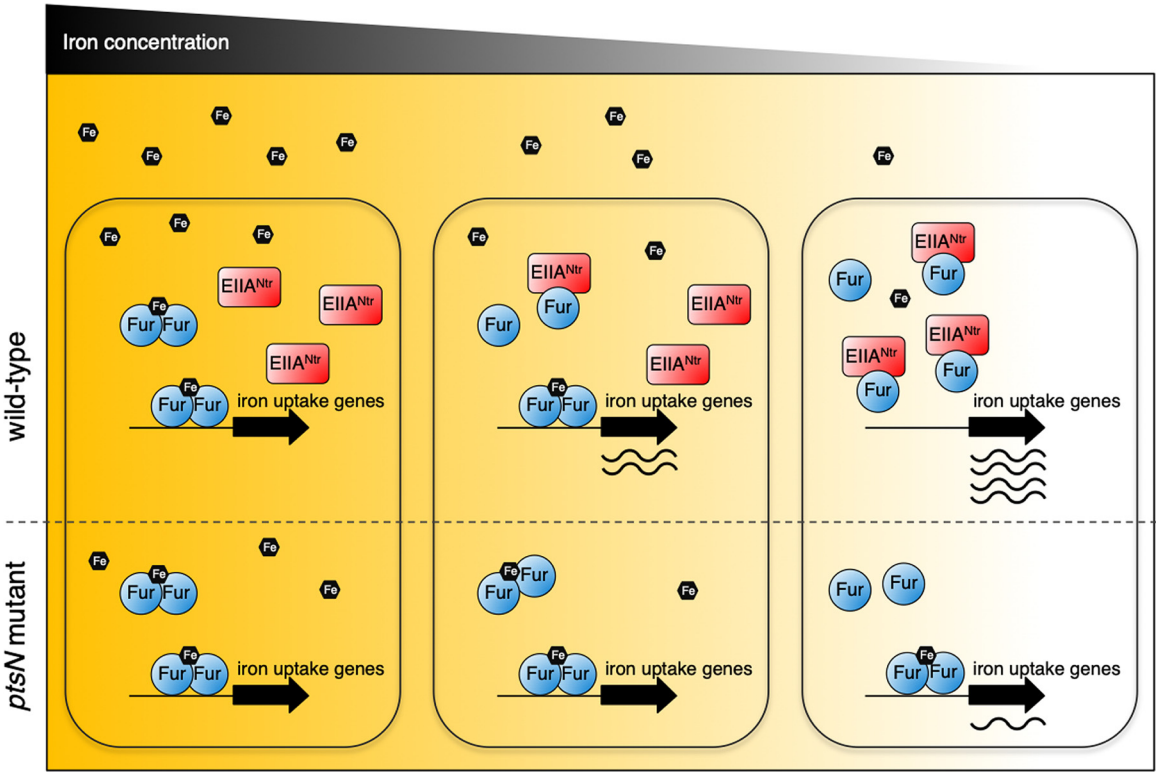

FIG 5 EIIA^{Ntr} enables *Salmonella* to properly control iron uptake gene expression in response to iron availability by modulating Fur. A model depicting iron-dependent activation of iron uptake gene expression by Fur and EIIA^{Ntr} is depicted. Transcription of iron uptake genes (including *fhuA*) is repressed by Fur at high iron concentrations. When iron concentrations decrease, iron-unbound Fur levels increase, but only a low level of expression of those genes is seen in the *ptsN* mutant. In the wild-type strain, EIIA^{Ntr} facilitates expression of iron uptake genes by hampering binding of Fur to its target promoters. This enables *Salmonella* to properly control the iron uptake system under low-iron conditions.

As described above, it has been believed that Fur is sufficient to operate as an iron sensor by itself because Fur binding to iron dictates its DNA binding ability (5, 6). We have now established that Fur actually requires an additional protein factor, EIIA^{Ntr}, to properly respond to changes of iron concentrations (Fig. 1; see also Fig. 5) in addition to its own ability to sense iron. In the absence of EIIA^{Ntr}, the high affinity of Fur for iron (6, 25) favors formation of the iron-bound form and repression of iron uptake genes even under conditions of low iron concentrations (Fig. 1A). When iron concentrations drop to below certain levels, iron-unbound Fur levels increase due to less availability of iron. Under this condition, EIIA^{Ntr} promotes iron uptake gene expression (Fig. 1A), possibly because Fur has a chance of interacting with EIIA^{Ntr} than iron. Alternatively or in addition, EIIA^{Ntr} binding to Fur may block Fur's binding to iron.

Bacteria have perhaps chosen to evolve by tuning the activity of Fur through the use of auxiliary protein instead of altering the affinity of Fur for iron, which might be feasible with respect to adjusting Fur-mediated iron metabolism in various bacterial species, depending on their habitats. As a result of tuning of Fur's activity via EIIA^{Ntr}, Fur not only attains better resolution in reflecting iron availability but also could potentially assimilate signals sensed and/or processed via EIIA^{Ntr}. The human pathogen *Acinetobacter baumannii* controls iron metabolism by altering the activity of Fur via BlsA (32), which is a photoreceptor that responds to light and temperature signals (33). Thus, BlsA allows *A. baumannii* to integrate light and temperature signals into iron uptake systems. Our findings suggest that EIIA^{Ntr} may enable *Salmonella* to control iron metabolism in response to environmental changes potentially impacting EIIA^{Ntr}, such as changes in nitrogen or amino sugar sources (16, 17) or other potential signals changing cellular amounts of EIIA^{Ntr}.

In addition to Fur, there is a membrane-bound iron sensor, PmrB, that is a constituent of the PmrA/PmrB two-component regulatory system (34). Interestingly, the Fur and PmrA iron-activated regulators negatively control *Salmonella* pathogenicity island 2 (SPI-2) gene expression (20, 21). As PmrB and Fur sense extracytoplasmic iron and cytoplasmic iron, respectively (5, 35), *Salmonella* controls SPI-2 gene expression in response to both extracytoplasmic and cytoplasmic iron levels via those iron sensors. As $EIIA^{Ntr}$ hampers Fur binding to DNA (Fig. 3B), it probably favors induction of SPI-2 gene expression by relieving Fur-mediated negative regulation. Paradoxically, $EIIA^{Ntr}$ reduces the transcription of SPI-2 genes by interfering with the binding of the major regulator of SPI-2, SsrB, to SPI-2 gene promoters (13). By impeding both Fur and SsrB, *Salmonella* probably achieves appropriate expression of virulence genes under low-iron conditions inside the host (19, 36).

We have shown that $EIIA^{Ntr}$ plays an important role in controlling iron uptake systems under low-iron conditions. This ability to cope with low iron availability is probably critical for *Salmonella* during infection given that mammalian hosts utilize a strategy to withhold iron (37), because an increase of iron availability results in enhanced growth and/or virulence of many bacterial pathogens *in vitro* and *in vivo* (30, 31) and iron depletion reduces intracellular bacterial growth (38). As NRAMP1 protein removes iron from macrophage phagosomes (19), an *Nramp1*$^{-/-}$ mouse lacking this iron transporter is highly sensitive to *Salmonella* infection (39). Moreover, lack of Fur highly attenuates *Salmonella* virulence in *Nramp1*$^{+/+}$ mice whereas this defect in virulence is reduced in *Nramp1*$^{-/-}$ mice (23). We previously reported that $EIIA^{Ntr}$ promotes virulence by preventing hyperactivation of *Salmonella* pathogenicity island 2 (SPI-2) gene expression (13). Our findings reported here suggest that the attenuated virulence of the *ptsN* mutant *Salmonella* in mice (13) might be due to both ectopic expression of SPI-2 genes and failure to import sufficient iron inside the host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *S. enterica* serovar Typhimurium strains used in this study were derived from strain SL1344. The strains and plasmids used in this study are listed in Table 1. Phage P22-mediated transduction was performed as described previously (40). All *Salmonella* strains were grown aerobically at 30 or 37°C in LB or M9 minimal medium at pH 5.8 supplemented with 0.5% Casamino Acids. Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; kanamycin (Km), 50 μg/ml; and streptomycin, 50 μg/ml. Primers used for the construction of bacterial strains and plasmids, reverse transcription-quantitative PCR (qRT-PCR), and electrophoretic mobility shift assay (EMSA) are listed in Table 2.

Construction of bacterial strains. The method of Datsenko and Wanner (41) was used for chromosomal gene deletion and epitope tagging.

For construction of the *fur* deletion strain, the kanamycin resistance (Km^r) cassette from plasmid pKD13 was amplified using primers fur-RED-F and fur-RED-R. The resulting PCR products were introduced into the SL1344 strain containing plasmid pKD46, followed by selection for Δ*fur*::kan transformants. The Km^r cassette was removed using plasmid pCP20 (41).

A *Salmonella* strain expressing the Fur protein with a FLAG tag at the C terminus in the normal *fur* chromosomal location was constructed. The Km^r cassette from plasmid pKD13 was amplified using primers Fur-FLAG-F and Fur-FLAG-R, and the PCR products were introduced into the SL1344 strain harboring plasmid pKD46. The Km^r cassette was removed using plasmid pCP20.

A strain carrying a *lacZ* fusion to the *fhuA* gene was constructed as described previously (42). The Km^r cassette from plasmid pKD13 was amplified using primers fhuA-RED-F and fhuA-RED-R. The resulting PCR products were introduced into the SL1344 strain harboring plasmid pKD46, and the Km^r cassette was removed using plasmid pCP20. Finally, the *lacZY* genes were introduced into the flippase recognition target (FRT) site using plasmid pCE70 (43).

A strain carrying a *lacZ* fusion to the *fur* gene was constructed as described previously (42). The Km^r cassette from plasmid pKD13 was amplified using primers fur-*lacZ*-F/fur-*lacZ*-R. The resulting PCR products were introduced into the SL1344 strain harboring plasmid pKD46, and the Km^r cassette was removed using plasmid pCP20. Finally, the *lacZY* genes were introduced into the FRT site using plasmid pCE70 (43).

Plasmid construction. A plasmid expressing Fur protein with a His_6 tag at N terminus from the *lac* promoter was constructed as follows: the *fur* gene was amplified from wild-type *Salmonella* (SL1344) using primers pHis6x-Fur-F/pHis6x-Fur-R, and the PCR fragments were introduced between the EcoRI and BamHI sites of the pUHE21-2*lacI*^{q} plasmid vector.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Strains		
Salmonella enterica SL1344	Wild type, Str^r	46
Salmonella enterica SR3203	Δ*ptsN*	13
Salmonella enterica SR4101	p_{fur}-*lacZ* (Km^r)	This work
Salmonella enterica SR4102	p_{fur}-*lacZ* (Km^r) Δ*ptsN*	This work
Salmonella enterica SR4103	*fur-FLAG*	This work
Salmonella enterica SR4104	*fur-FLAG* Δ*ptsN*	This work
Salmonella enterica SR4125	p_{fhuA}-*lacZ* (Km^r)	This work
Salmonella enterica SR4125	p_{fhuA}-*lacZ* (Km^r) Δ*ptsN*	This work
Salmonella enterica SR4131	p_{fhuA}-*lacZ* (Km^r) Δ*fur*	This work
Salmonella enterica SR4132	p_{fhuA}-*lacZ* (Km^r) Δ*fur* Δ*ptsN*	This work
Escherichia coli DH5α	F^- *supE44* Δ*lacU169* (φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	47
Escherichia coli BTH101	F^- *cya-99 araD139 galE15 galK16 rpsL1* (Str^r) *hsdR2 mcrA1 mcrB1*	28
Plasmids		
pCP20	rep_{pSC101}ts Ap^r Cm^r FLP^+ $cI857^+$	41
pCE70	rep_{R6K} Km^r Cm^r FRT *tnpR lacZY*	43
pKD3	rep_{R6K} Ap^r FRT Cm^r FRT	41
pKD13	rep_{R6K} Ap^r FRT Km^r FRT	41
pKD46	rep_{pSC101}ts Ap^r p_{araBAD} γ β exo	41
pUHE21-2lacI^q	rep_{pMB1} Ap^r $lacI^q$	48
pFur	rep_{pMB1} Ap^r $lacI^q$ *fur*	21
pJJ37	rep_{pMB1} Ap^r $lacI^q$ *ptsN-His6*	13
pJJ48	rep_{pMB1} Ap^r $lacI^q$ *fur-His6*	This work
pKT25	Km^r rep_{p15A}	44
pUT18	Ap^r rep_{pMB1}	44
pT25-ptsN	Km^r rep_{p15A} *ptsN*	13
pT25-ptsN(H73A)	Km^r rep_{p15A} *ptsN*(H73A)	12
pT25-zip	Km^r rep_{p15A} *zip*	44
pT18-fur	Ap^r rep_{pMB1} *fur*	This work
pT18-zip	Ap^r rep_{pMB1} *zip*	44

[a]Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Str^r, streptomycin resistance.

A plasmid expressing T18-Fur fusion protein was constructed as follows: the *fur* gene was amplified from wild-type *Salmonella* (SL1344) using primers pUT18C-Fur-F/pHis6x-Fur-R and then introduced between the BamHI and EcoRI sites of pUT18C (44).

β-Galactosidase assay. β-Galactosidase assays were carried out with at least three biological replicates with technical duplicates, and the activity was determined as described previously (45).

RNA isolation and reverse transcriptase-quantitative PCR (qRT-PCR). *Salmonella* strains were grown as described above, and total RNA was isolated using an RNeasy minikit (Qiagen). After DNase treatment of the isolated RNA, cDNA was synthesized using Omni Transcript reverse transcription reagents (Qiagen) and random hexamers (Invitrogen). Quantification of the cDNA was carried out using 2× iQ SYBR green Supermix (Bio-Rad), and real-time amplification of the PCR products was performed using an iCycler iQ real-time detection system (Bio-Rad). The primers used for detection of the gene transcripts are listed in Table 2. Data were normalized to 16S rRNA expression levels.

Western blotting. *Salmonella* strains encoding the Fur-FLAG protein from the normal chromosomal location were grown under the indicated conditions. Bacteria were collected by centrifugation, and cell lysates were prepared using B-PER solution (Pierce). Proteins from cell lysates were resolved by 12% SDS-PAGE, and the Fur and DnaK proteins were detected using anti-FLAG (Sigma; 1:2,000) and anti-DnaK (Abcam; 1:5,000) antibodies. The blots were developed using anti-mouse IgG horseradish peroxidase-linked antibody (GE Healthcare; 1:5,000) with an ECL detection system (Amersham Biosciences).

Bacterial two-hybrid assay. *E. coli* BTH101 (which lacks the *cya* gene) was used for this assay. Derivatives of plasmids pUT18 and pKT25 were introduced into BTH101 cells as indicated. These cells were grown overnight in M9 minimal media containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml). They were transferred to 1 ml of the same fresh medium containing 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at a dilution of 1:100 and grown for 8 h with shaking at 30°C. β-Galactosidase activities were determined as described above.

Protein purification. His6-tagged Fur protein was expressed in *E. coli* strain BL21(DE3) as follows: bacterial cells were grown in LB at 37°C until the optical density at 600 nm (OD_{600}) reached 0.5, and expression of those proteins was induced by addition of IPTG (0.5 M) followed by growth at 30°C for 5 h.

TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)
Strain construction	
fur-Red-F	CGC TTC CTC GTT TAA AAA TTC TGG AAG TTC TTC AGG AAC CTG TAG GCT GGA GCT GCT TCG
fur-Red-F	TCG TGA TGA TGC TGT TGC GTC AGT TCA AAA ACG GAT TTA CAT TCC GGG GAT CCG TCG ACC
fur-lacZ-F	GCG ACT GCC GCG AAG ACG AGC ACG CGC ACG ATG ACG CGA CTA AAT AAG TGC CCG TCG TTT TAC AAC GTC G
fur-lacZ-R	CAA CAT CAA GCG GCA GGA AAG AGG AGG ATA TAA AAA AGC CAA CCG GGC GGC GTG TAG GCT GGA GCT GCT TC
fhuA-Red-F	ATC GTT TAC GTT ATC ATT CAC TTT CAT CAG AGA TAT ACC ATG TAG GCT GGA GCT GCT TCG
fhuA-Red-R	CCT GCG CTA ATG GGT TGG TTG GAT CGG CGG TCA GGT TAT TAT TCC GGG GAT CCG TCG ACC
Plasmid construction	
pHis6x-Fur-F	CAT GTT CTG AAT TCA AAT TAT GCA TCA CCA TCA CCA TCA CGC AAT GAC TGA CAA CAA TAC CGC ATT AAA
pHis6x-Fur-R	ACC GGG CGG TTG GAT CCT CGA AAG ATT T
pUT18C-Fur-F	TTA GCA ACA GGA GGA TCC CCG CAT GAC T
qRT-PCR	
fhuA-qRT-F	GTT CAA CCG AAA GAA GAA ACC ATT A
fhuA-qRT-R	GTT TTT TCG ATA GGT GTA TCA GTT TTG
fepA-qRT-F	AGA AGA TTC ATT CCC TGA CCT TAC TG
fepA-qRT-R	TAT CGG TTT TGT CTT CCG CCA TCA
iroB-qRT-F	ATG CGT ATT CTG TTT GTC GGT CCA
iroB-qRT-R	CAG TAC TTC ATG GCC ATT AAC ACG A
rrs-qRT-F	CCA CAA AAC TTA TGG ATT TAT GCG T
rrs-qRT-R	TTT ACG CCC AGT AAT TCC GAT T
EMSA	
Fur-EMSA-F1	CTC GAC GAC ATC CTC AAC GCC TAA TCT
Fur-EMSA-R1	AAA CGA GGA AGC GTT ACT TTC AGG CCA G

Cells were harvested, washed, and suspended in buffer A (20 mM Tris [pH 8.0], 150 mM NaCl, 20 mM imidazole). The cells were then disrupted by sonication, and cell debris was removed by centrifugation at 20,000 × *g* at 4°C for 30 min. The supernatant was applied to a 1.5-ml nickel-nitrilotriacetic acid (Ni-NTA) agarose column equilibrated in buffer A, washed with 25 column volumes of the same buffer, and eluted using a gradient of buffer A and buffer B (20 mM Tris [pH 8.0], 150 mM NaCl, 250 mM imidazole). The fractions were then collected and analyzed by SDS/PAGE, and selected fractions were dialyzed against buffer C (20 mM Tris [pH 8.0], 150 mM NaCl, 10% glycerol).

Electrophoretic mobility shift assay (EMSA). EMSAs were performed to determine binding of Fur to DNA *in vitro*. DNA fragments corresponding to the *fhuA* promoter was amplified by PCR using ^{32}P-labeled primers EMSA-fhuA-F1/EMSA-fhuA-R1 with wild-type *Salmonella* chromosomal DNA as a template. The promoter DNA was purified from agarose gels using a gel extraction kit (Qiagen). The labeled DNA probe (16 fmol) was incubated with the His_6-Fur protein in the presence or absence of $EIIA^{Ntr}$-His_6 at room temperature for 20 min in 20 μl of binding buffer (10 mM Tris [pH 7.5], 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], 2.5% glycerol) containing 50 ng/μl poly(dI-dC). The reaction mixtures were resolved by 6% PAGE, and the radiolabeled DNA fragments were visualized using a BAS2500 system (Fuji Film).

Protein co-occurrence. Co-occurrence of $EIIA^{Ntr}$ and Fur proteins across sequenced organisms was analyzed using STRING software version 11.0.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03026-18.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

ACKNOWLEDGMENTS

We thank Dongwoo Shin for providing a plasmid, pFur.

This research was supported by a grant (14162MFDS972) from the Ministry of Food and Drug Safety of Korea in 2018. This work was also supported by the BK21 Plus Program of the Department of Agricultural Biotechnology, Seoul National University, Seoul, South Korea.

We declare that we have no conflict of interest.

J.C. and S.R. designed the research; J.C. performed the experiments; J.C. and S.R. analyzed the data; and J.C. and S.R. wrote the paper.

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