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RstA-Promoted Expression of the Ferrous Iron Transporter FeoB under Iron-Replete Conditions Enhances Fur Activity in *Salmonella enterica*▽†

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The Fur protein is a primary regulator that monitors and controls cytoplasmic iron levels. We now report the identification of a regulatory pathway mediated by the *Salmonella* response regulator RstA that promotes Fur activity. Genome-wide expression experiments revealed that under iron-replete conditions, expression of the RstA protein from a plasmid lowered transcription levels of various genes involved in iron acquisition. The RstA protein controlled iron-responsive genes through the Fur-Fe(II) protein because deletion of the *fur* gene or iron depletion abrogated RstA-mediated repression of these genes. The RstA protein maintained wild-type levels of the Fur protein but exceptionally activated transcription of the *feoAB* operon encoding the ferrous iron transporter FeoB by binding directly to the *feoA* promoter. This FeoB induction resulted in increased ferrous iron uptake, which associates with the Fur protein because lack of RstA-dependent transcriptional activation of the *feoA* promoter and *feoB*-deletion abolished repression of the Fur target genes by the RstA protein. Under iron-replete conditions, RstA expression retarded *Salmonella* growth but enabled the Fur protein to repress the target genes beyond the levels which were simply accomplished by iron.

Bacterial cells often use two-component signal transduction systems to express sets of genes that ensure their rapid adaptation to particular environmental changes. The RstA/RstB system appears to be a typical two-component regulatory system consisting of the membrane sensor RstB and its cognate response regulator RstA. Signal transduction between RstA and RstB has been shown in vitro: the purified *Escherichia coli* RstA protein could be phosphorylated by the cytoplasmic domain of RstB in a phosphotransfer experiment (31). It has been reported that the PhoP protein, a response regulator of the PhoP/PhoQ two-component system, directly activates transcription of the *rstA* gene encoding the response regulator RstA in *E. coli* (17). The *rstA* gene also seems to be a member of the PhoP regulon in *Salmonella* because a computational approach discovered the *rstA* promoter features shared with a group of PhoP-regulated promoters (35, 36). Several genes whose transcription is controlled by the RstA protein have been uncovered in recent studies. In *E. coli*, analysis of DNA fragments captured by the purified RstA protein identified the *asr* and *csgD* genes as RstA targets harboring the "RstA box" (i.e., the consensus sequences recognized by RstA) on their promoter regions (19). Transcription of the *asr* gene was activated when *E. coli* cells were grown in acidified Luria-Bertani (LB) or minimal medium, which was dependent on chromosomal expression of RstA (19). However, low pH does not seem to produce sufficient levels of active RstA (i.e., phosphorylated RstA) in *E. coli* because repression of *csgD* transcription occurred only upon overexpression of the RstA protein from a plasmid (19). In *Salmonella enterica*, overexpression of the RstA protein lowered the levels of the alternative sigma factor RpoS by an unknown mechanism, which consequently downregulated transcription of three RpoS-controlled genes, *narZ*, *spvA*, and *bapA*, in stationary phase (4). Consistent with the role of the BapA protein in biofilm formation (14, 29), RstA expression impaired *Salmonella*'s ability to develop a biofilm (4).

Iron is present in either an oxidized ferric [Fe(III)] or a reduced ferrous [Fe(II)] form. Although it is an essential metal for bacterial viability, iron excess is toxic due to formation of hydroxyl radicals by reaction of free iron with reduced forms of oxygen (1). To balance these dual aspects, bacterial cells must tightly regulate cytoplasmic iron concentrations, which are sensed by the key regulatory protein Fur (1, 9). The Fe(II)-associated Fur protein resulting from sufficient levels of intracellular iron binds to its specific DNA sequences on the target promoters to repress transcription of genes encoding proteins that are involved in iron acquisition (1, 9, 11). In this study, we report that the *Salmonella* RstA protein directly binds to the *feoA* promoter and activates expression of the *feoAB* operon encoding an Fe(II) transporter, whereby more iron can be imported into the bacterial cell, thus increasing the Fur-Fe(II) levels. Consequently, RstA activation results in hyperrepression of the Fur-regulated genes.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
S. enterica serovar Typhimurium strains		
14028s	Wild type	10
JH101	Δ*rstA*::Km^r	This study
JH352	Δ*fur*	This study
JH353	Δ*rstA*::Km^r Δ*fur*	This study
HK702	*fur*-FLAG	This study
HK703	Δ*rstA*::Km^r *fur*-FLAG	This study
JH358	P*feoA*$_{mt}$	This study
JH359	Δ*rstA*::Km^r P*feoA*$_{mt}$	This study
JH362	Δ*feoB*	This study
JH367	Δ*rstA*::Km^r Δ*feoB*	This study
Plasmids		
pUHE21-2*lacI*^{q}	rep$_{pMBI}$ Ap^r *lacI*^{q}	23
pT7-7	rep$_{pMBI}$ Ap^r P$_{T7}$	24
pKD3	repR$_{6K\gamma}$ Ap^r-FRT Cm^r-FRT[a]	6
pKD4	repR$_{6K\gamma}$ Ap^r-FRT Km^r-FRT	6
pKD46	rep$_{pSC101}^{ts}$ Ap^r P$_{araBAD}$ γ β *exo*	6
pCP20	rep$_{pSC101}^{ts}$ Ap^r Cm^r *c*I857 λ P$_R$*flp*	6
pJH4	rep$_{pMBI}$ Ap^r *lacI*^{q} *rstA*	This study
pT7-7-*rstA*-His$_6$	rep$_{pMBI}$ Ap^r P$_{T7}$ *rstA*-His$_6$	This study

[a] FRT, Flp recombinase target.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *S. enterica* serovar Typhimurium strains were derived from strain 14028s. Phage P22-mediated transductions were performed as described previously (7). Bacteria were grown at 37°C in LB medium or M9 minimal medium supplemented with 0.1% Casamino Acids and 0.5% glycerol. Ampicillin, chloramphenicol, kanamycin, and isopropyl-β-D-thiogalactopyranoside (IPTG) were used at 50 μg/ml, 25 μg/ml, 50 μg/ml, and 0.5 mM, respectively.

Construction of bacterial strains. The *Salmonella* strain JH101 with a deletion of the *rstA* gene was constructed using the one-step gene inactivation method (6). The Km^r cassette from plasmid pKD4 (6) was amplified using primers DE-*rstA*-F and DE-*rstA*-R. Primer DE-*rstA*-F (5′-AGGCGGTGTATGTTG GCGTTTTCTATTCTCCATTTATAATTGTAGGCTGGAGCTGCTTCG-3′) carries the sequence immediately upstream of the start codon of the *rstA* gene following the priming site 1 sequence of pKD4 (6). Primer DE-*rstA*-R (5′-ACTATGACTTGATAAAGGCAGGTAAAATCTGTCGGCTAACCAT ATGAAATATCCTCCTTAG-3′) harbors the sequence immediately downstream of the stop codon of the *rstA* gene linked to the priming site 2 sequence of pKD4 (6). The resulting PCR product was integrated into the *rstA* region in strain 14028s (6). The JH352 strain with *fur* deleted was constructed by introduction of PCR products, which were obtained from the reaction of primers DE-*fur*-F (5′-TCTAATGAAGTGAATCGTTTAGCAA CAGGACAGATTCCGCTGTAGGCTGGAGCTGCTTCG-3′) and DE-*fur*-R (5′-AAAAGCCAACCGGGCGGTTGGCTCTTCGAAAGATTTACA CCATATGAAATATCCTCCTTAG-3′) with pKD4 as a template, into strain 14028s. The Km^r cassette was removed from the resulting strain using plasmid pCP20 as described previously (6). For construction of the JH362 strain with *feoB* deleted, PCR was performed with primers DE-*feoB*-F (5′-AGTGGAA GCGGTTTCCTGTTAATACAGTGAGTCTATAAAATGTAGGCTGGAG CTGCTTCG-3′) and DE-*feoB*-R (5′-GATCGCGAACCTGTATCAATGAA GCCATTTTTTACATCCCATATGAAATATCCTCCTTAG-3′) and pKD3 as a template. The resulting PCR products were integrated into the *feoB* region of strain 14028s from which the Cm^r cassette was removed using plasmid pCP20. Deletion of the corresponding genes was verified by colony PCR.

The HK702 strain encoding the Fur protein with a FLAG tag at the C terminus of the normal *fur* chromosomal location was constructed as described previously (21). The Km^r cassette was amplified by PCR using primers *fur*-FLAG-F (5′-CTGCCGCGAAGACGAGCACGCGCACGATGACGCG ACTAAAGACTACAAGGACGACGATGACAAGTGATGTAGGCTGG AGCTGCTTCG-3′) and *fur*-FLAG-R (5′-AAAAGCCAACCGGGCGGTT GGCTCTTCGAAAGATTTACACCATATGAATATCCTCCTTAG-3′) and pKD4 as a template; the PCR product was integrated into the *fur* region of the wild-type strain. After pCP20-mediated removal of the Km^r cassette, the presence of a FLAG tag at the C terminus of Fur was confirmed by nucleotide sequencing.

By using a strategy similar to one described previously (20), the JH358 strain was constructed by mutating the nucleotide sequences corresponding to the RstA-binding site of the *feoA* promoter in the chromosome. We first integrated the Km^r cassette obtained by PCR, using the primers RSM-*feoA*-1 (5′-GATGGACGC GCTGGCAGCCGCAATGGGAAAAAAACGCTAATGTAGGCTGGA GCTGCTTCG-3′) and RSM-*feoA*-2 (5′-CAGGCCGGATAAGAAGTAACCCG GCCTGCGTATTACCCGACATATGAATATCCTCCTTAG-3′) and pKD4 a as template, immediately downstream of the stop codon of *yhgF*, which is a gene located upstream of *feoA*. By employing chromosomal DNA from the resulting strain as a template, PCR was performed using primers RSM-*feoA*-1 and RSM-*feoA*-4 (5′-GGCGACGGAAGAGCCAAATAAGCGATAATTTGCGCCGATG TTGATATGGCTC-3′). Secondly, another PCR was carried out using the primers RSM-*feoA*-3 (5′-CAAATTATCGCTTATTTGGCTCTTCCGTCGCCTTTTAAT CGTTGAAGATAGAAACCATTCTC-3′) and RSM-*feoA*-6 (5′-CTGATTTCAC GCGCAAAGCCGGTGATTTTC-3′) and chromosomal DNA from wild-type 14028s strain as the template. In the third PCR, the two PCR products obtained above were mixed and amplified using primers RSM-*feoA*-1 and RSM-*feoA*-6. The resulting DNA fragments were integrated into the wild-type strain 14028s harboring plasmid pKD46 (6). The intactness of the *feoA* promoter region except for mutation of the nucleotide sequences in the putative RstA binding site was verified by nucleotide sequencing. Finally, the Km^r cassette was removed from the resulting strain using pCP20 as described previously (6).

Plasmid construction. To construct plasmid pJH4 in which the RstA protein is expressed from the *plac* promoter, the *rstA* gene was amplified by PCR using the primers CD-*rstA*-F (5′-GCGGATCCAATATGAACCGCATTGTATTTGT TGAAG-3′) and CD-*rstA*-R (5′-CGCTGCAGTTATCCCGTCGTTTCGTCCC AGGCATG-3′) and chromosomal DNA from strain 14028s as a template, and the product was introduced between the BamHI and PstI restriction sites of pUHE21-2*lacI*^{q} (23). Plasmid pT7-7-*rstA*-His$_6$ encoding the RstA protein with a six-His tag at the C terminus was constructed. The *rstA* coding region was amplified by PCR using primers *rstA*-His$_6$-F (5′-CATTTATCATATGAACCG CATTGTATTTGTTG-3′) and *rstA*-His$_6$-R (5′-CGGGATCCTCAGTGGTGG TGGTGGTGGTGTCCCGTCGTTTCGTCCCAGGCATGAGG-3′) and chromosomal DNA from strain 14028s as a template, and the product was introduced between the NdeI and BamHI restriction sites of the pT7-7 vector (24). Sequences of the *rstA* coding region on the recombinant plasmids were confirmed by nucleotide sequencing.

DNA microarray analysis. The *Salmonella* strains that carried deletions of the *rstA* gene and harbored the plasmid vector pUHE21-2*lacI*^{q} (23) or the RstA expression plasmid pJH4 were grown in LB medium supplemented with 0.5 mM IPTG. When the cells' optical density at 600 nm (OD_{600}) reached 0.5 to 0.6, 0.5 ml of the culture was removed and mixed with 1 ml of RNAprotect Bacteria Reagent (Qiagen), and total RNA was extracted using an RNeasy Mini Kit (Qiagen). The RNA sample was treated further with RNase-free DNase (Ambion) to remove residual DNA. cDNA synthesis, modification, hybridization, and labeling with Cy5 dye on a DNA chip was performed using a 3DNA Array 900MPX kit (Implen) as described in the manufacturer's instructions. We used a DNA chip (CombiMatrix) that harbored 4,781 oligonucleotides specific to the open reading frames of the *S. enterica* serovar Typhimurium strain LT2 genome in duplicates and probes for negative and quality controls. Six microarray experiments were conducted on three independent cultures of two bacterial strains. Data were analyzed by global normalization using genes displaying a median intensity value greater than zero in at least two samples for each group. A *t* test and the relative change in expression were used to determine differentially expressed genes between two groups of bacterial strains.

Quantitative real-time PCR analysis. mRNA levels were determined using quantitative real-time PCR as described previously (22). Isolation and DNase treatment of RNA was conducted as described above. cDNA was synthesized using Omnitranscript Reverse Transcription reagents (Qiagen) and random primers (Invitrogen) and quantified using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI7300 Sequence Detection System (Applied Biosystems). The cDNA concentrations were determined using a standard curve obtained from PCR on serially diluted genomic DNA as templates. Expressed mRNA levels of target genes were normalized to the *gyrB* transcript levels. The sequences of the primers used are shown in Table S1 of the supplemental material.

Western blot analysis. For detection of the FLAG-tagged Fur protein, *Salmonella* strains harboring the *fur*-FLAG gene were grown in LB medium to an

OD_{600} of 0.5 to 0.6. For preparation of cell extracts, aliquots of cells (i.e., 0.6 OD_{600} units) were suspended in 0.1 ml of B-PER solution (Pierce). Total protein concentrations were determined by the bicinchoninic acid method, and the cell extracts containing 15 μg of total proteins were resolved on a 12% sodium dodecyl sulfate-polyacrylamide gel; the Fur protein was detected using anti-FLAG antibody (Sigma) as described previously (21).

Purification of the RstA protein. *E. coli* BL21(DE3) cells harboring plasmid pT7-7-*rstA*-His_6 was grown to an OD_{600} of 0.6; then 1 mM IPTG was added to the culture for RstA-His_6 protein induction, and another 3 h of incubation followed. The C-terminal His-tagged RstA protein was purified by Ni^{2+} affinity chromatography. The cell pellet was suspended in lysis buffer containing 10 mM Tris (pH 8.0) and 0.3 M NaCl and disrupted by sonication. After removal of cell debris by centrifugation, the cell extract was applied onto a column with 2 ml of Ni-nitrilotriacetic acid resin. The column was washed two times, once with lysis buffer containing 20 mM imidazole and a second time with lysis buffer containing 30 mM imidazole, and the adsorbed His-tagged protein was eluted with elution buffer (i.e., lysis buffer containing 200 mM imidazole). Finally, the eluted proteins were dialyzed with lysis buffer containing 25% glycerol and stored at −70°C.

Gel shift analysis. The DNA fragments corresponding to the *feoA* promoter region were generated by PCR amplification using primers GS-*feoA*-F (5′-AAA CGGTGAATATTTGCACATTAG-3′) and GS-*feoA*-R (5′-TTACTAACTGGA TGTATACCTCAT-3′) and wild-type *Salmonella* chromosomal DNA as a template. To obtain the mutant *feoA* promoter DNA lacking the putative RstA-binding site ($PfeoA_{mt}$), PCR was conducted using the same primers and chromosomal DNA from the JH358 strain as a template. The *feoA* promoter DNA was purified from agarose gel using a gel extraction kit (Qiagen) and labeled with [γ-^{32}P]ATP (GE Healthcare), and unincorporated radio isotope was removed using a MicroSpinG-25 column (GE Healthcare). The ^{32}P-labeled DNA probe (0.2 pmol) was incubated with the purified RstA-His_6 protein (0, 4, 8, and 16 pmol) at 37°C for 15 min in 20 μl of gel shift assay buffer (20 mM Tris acetate, pH 8.0, 3 mM magnesium acetate, 100 mM potassium glutamate, 1 mM dithiothreitol, 100 μg/ml of bovine serum albumin, and 1% sucrose) containing 50 μg/ml of poly(dI-dC). For a competition assay, a fourfold molar excess of the unlabeled *feoA* promoter DNA was added to the reaction mixture containing 0.2 pmol of the same labeled probe and 16 pmol of the RstA protein. For phosphorylation of the RstA protein, 10 mM acetyl phosphate was added into the reaction mixture. The reaction mixtures were resolved on a 6% polyacrylamide gel, and the radiolabeled DNA fragments were visualized using BAS2500 system (Fuji film).

Iron uptake assay. Ferrous iron uptake levels in *Salmonella* strains were determined as described previously (32) with appropriate modifications. Bacterial cells grown in LB medium to an OD_{600} of 0.5 were washed with M9 medium, suspended in the same medium, and kept on ice. One milliliter of the cell suspension at an OD_{600} of 1.0 was placed at 37°C for 10 min, and an Fe(II) transport assay was started by the addition of 0.5 μM ^{55}Fe(II). The ^{55}Fe(II) stock solution was prepared in M9 medium and contained 50 μM $^{55}FeCl_3$ (Perkin Elmer) and 100 mM sodium ascorbate to reduce iron. After 5 min of incubation, bacterial cells were harvested, washed twice with M9 medium, suspended in 100 μl of 1% Triton X-100, and mixed with 1 ml of scintillation fluid. Activity as counts per minute was determined using a Wallac 1400 liquid scintillation counter (Turku) and converted into picomoles of ^{55}Fe(II) using a standard curve.

RESULTS

Expression of the RstA protein affects transcription of genes that are involved in iron acquisition. We used DNA microarray analysis to search for genes that are under the control of the RstA protein, a response regulator of the *Salmonella* RstA/RstB two-component system. As the environmental signal(s) activating the RstA/RstB regulatory system is not known, we reasoned that overexpression of a response regulator, RstA, could significantly alter expression levels of its regulated genes, mimicking a situation where the RstB sensor promotes RstA phosphorylation in response to an environmental signal. Indeed, this has been demonstrated for other response regulators (13, 15). Thus, we constructed two isogenic *Salmonella* strains with deletions of the chromosomal copy of the *rstA* gene and harboring either plasmid pJH4 expressing the RstA protein from the *lac* promoter or the plasmid vector pUHE21-2*lacI*^{q}. We then performed DNA microarray experiments using RNA isolated from bacterial cells grown to mid-exponential phase (i.e., OD_{600} of 0.5 to 0.6) in LB medium containing IPTG.

We found that in the RstA-expressing strain, the transcription levels of several iron-responsive genes that are implicated in iron uptake, metabolism, and storage were two- to eightfold lower than those in the strain harboring the plasmid vector (see Table S2 in the supplemental material). By contrast, the mRNA levels corresponding to the *feoAB* operon encoding an Fe(II) transporter (5, 12) were increased upon expression of the RstA protein (see Table S2 in the supplemental material). By conducting quantitative real-time PCR analysis, we investigated further the transcription levels of *fhuA* and *fhuF* in bacterial strains grown under the conditions used for DNA microarray experiments. As shown in Fig. 1A, expression of the RstA protein in the *rstA* deletion strain lowered the mRNA levels of the *fhuA* and *fhuF* genes six- and sevenfold, respectively. However, transcription of these genes was hardly affected by deletion of the *rstA* gene. Thus, our experiments suggest that activation of the *Salmonella* RstA/RstB two-component system resulting from overexpression of the RstA protein globally affects expression of genes that are involved in iron metabolism.

The regulatory effect of RstA on iron-responsive genes is dependent on the Fur-Fe(II) protein. The Fur protein is known to be the primary regulator of iron metabolism (1, 9). In *Salmonella* cells grown under iron-rich conditions, Fe(II) associates with Fur, and then the Fur-Fe(II) protein binds to its target promoters to repress transcription of various iron-responsive genes (1, 9, 11). Therefore, we explored whether downregulation of iron-responsive genes resulting from RstA expression is dependent on the Fur-Fe(II) protein.

Real-time PCR analysis showed that the transcription levels of *fhuA* and *fhuF* were significantly increased in the *fur* deletion strain grown in LB medium (Fig. 1B), which was consistent with the previous reports that the Fur protein functions as a transcriptional repressor of these genes (2, 16). We next examined transcription of the two Fur-repressed genes in the strain that carried deletions of the *rstA* and *fur* genes and harbored either the RstA expression plasmid or the plasmid vector; analysis determined that the mRNA levels of *fhuA* and *fhuF* were similar to those in the *fur* deletion strain regardless of RstA expression (Fig. 1B).

Iron depletion also eliminated the regulatory effect of RstA on the Fur-repressed genes: in bacterial cells grown in LB medium containing the iron-specific chelator dipyridyl, the transcription levels of the *fhuA* and *fhuF* genes were increased to the levels observed in the *fur* deletion strain even when the RstA protein was overexpressed (Fig. 1B).

To verify these results further, the *fhuA* mRNA levels were determined in cells that were exponentially growing in minimal medium with or without iron. In the wild-type strain, the *fhuA* transcripts were approximately threefold higher than those in LB medium (Fig. 1C), indicating that a low iron concentration reduced Fur activity in the minimal medium. Under the same growth conditions, the RstA protein was unable to repress transcription of *fhuA* (Fig. 1C), which resembled the result in LB medium treated with the iron chelator (Fig. 1B). Supplying iron, however, reproduced the regulatory effect of RstA on the Fur protein: the *fhuA* transcripts of wild-type cells were ap-

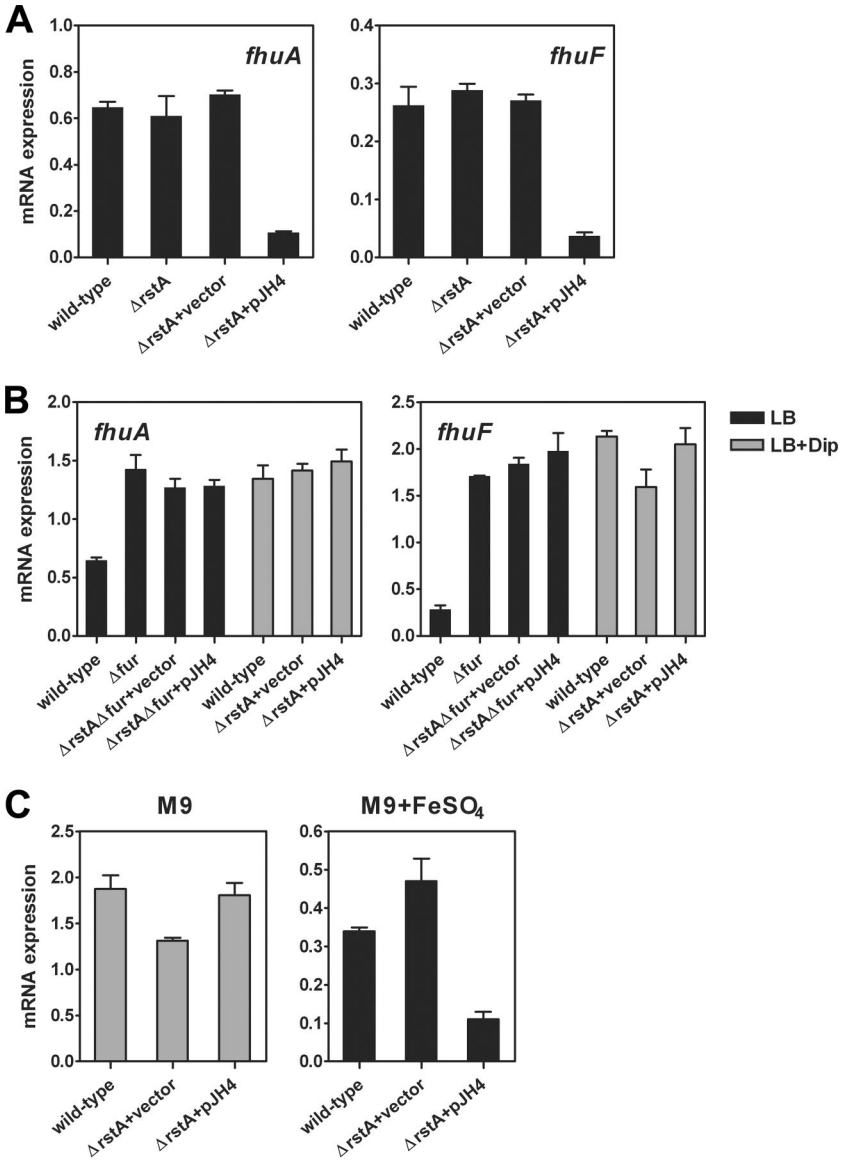

FIG. 1. Expression of the RstA protein represses transcription of iron-responsive genes via the Fur-Fe(II) protein. mRNA levels expressed from the *fhuA* and *fhuF* genes were determined in the *Salmonella* strains 14028s (wild-type), JH101 (Δ*rstA*::Km^r), JH352 (Δ*fur*), JH353 (Δ*rstA*::Km^r Δ*fur*), and JH101, JH352, and JH353 strains harboring the plasmid vector pUHE21-2*lacI*^{q} or the RstA expression plasmid (pJH4) by using real-time PCR analysis. (A) Strains were grown in LB medium. (B) Strains were grown in LB medium or LB medium containing 0.2 mM dipyridyl (+Dip). (C) The *fhuA* mRNA levels were determined in strains grown in M9 minimal medium with or without 20 μM $FeSO_4$. All strains were grown to an OD_{600} of 0.5 to 0.6, and 0.5 mM IPTG was added to the strains harboring the plasmid. Shown are the mean values and standard deviations of three independent experiments.

proximately sixfold lower in the presence of 20 μM ferrous sulfate ($FeSO_4$) than in its absence, and expression of the RstA protein reduced these mRNA levels even further (Fig. 1C). Thus, our experiments demonstrated that the RstA protein downregulates expression of iron-repressed genes via the Fur-Fe(II) protein.

Fur levels are not affected upon overexpression of the RstA protein. We hypothesized that the RstA-promoted Fur-dependent repression of iron-responsive genes might be due to an increase in the Fur protein levels. To test this idea, we constructed a strain that expressed the Fur protein tagged with a FLAG epitope at its C terminus from its normal chromosomal location. Real-time PCR analysis revealed that upon RstA expression, transcriptional repression of the *fhuF* gene by the FLAG-tagged Fur protein was as efficient as in the strain with the wild-type Fur protein (see Fig. S1 in the supplemental material), suggesting that introduction of an epitope tag does not affect the Fur protein functions.

Transcription of the *fur* gene per se was little affected by RstA expression (see Fig. S1 in the supplemental material). We next carried out Western blot analysis using the cell extracts prepared from strains grown under the same conditions used for transcription experiments (i.e., growth in LB medium to mid-exponential phase). Consistent with the transcription

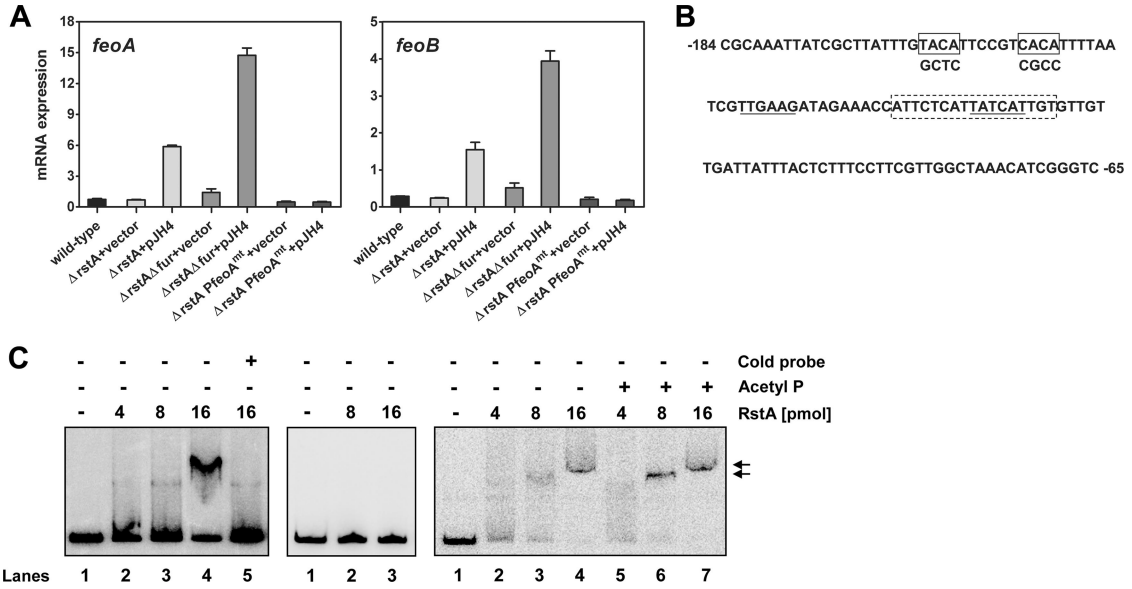

FIG. 2. Transcription of the *feoAB* operon is directly activated by the RstA protein overcoming the Fur-mediated repression. (A) The transcription levels of the *feoA* and *feoB* genes were determined by real-time PCR analysis using RNA isolated from cells grown to an OD_{600} of 0.5 to 0.6. The wild-type strain was grown in LB medium while the JH101 (Δ*rstA*::Km^r), JH353 (Δ*rstA*::Km^r Δ*fur*), and JH359 (Δ*rstA*::Km^r P*feoA*$_{mt}$) strains harboring pUHE21-2*lacI*^{q} (vector) or RstA-expression plasmid (pJH4) were grown in LB medium containing IPTG. Shown are the mean values and standard deviations of three independent experiments. (B) Indicated are nucleotide sequences of the *feoA* promoter region. The DNA sequences corresponding to the putative RstA-binding site (19) are boxed with solid lines, and their substitutions in the *feoA* promoter mutant strain (JH359) are shown below. The predicted −10 and −35 elements are underlined, and the sequences within the dotted box indicate the putative site for Fur-binding as proposed by Escolar et al. (8). Numbering here is based on the start codon of the *feoA* gene. (C) A gel shift assay was performed to examine the interaction between the RstA protein and the *feoA* promoter. Concentrations of the purified RstA protein are indicated on top of the figure, and 0.2 pmol of the 5′ end-labeled *feoA* promoter DNA was used as a probe. The labeled wild-type *feoA* promoter was incubated with increasing concentrations of the RstA protein (left panel, lanes 1 to 4). For competition analysis, 0.8 pmol of the same but unlabeled *feoA* promoter DNA was added into the reaction shown in lane 5. The labeled mutant *feoA* promoter DNA lacking the putative RstA-binding site was used as a probe (middle panel). In the experiment shown in the right panel, the indicated concentration of RstA was incubated with the labeled wild-type *feoA* probe in the absence (lanes 1 to 4) or the presence (lanes 5 to 7) of acetyl phosphate. Arrows indicate the RstA-DNA complexes.

data, the Fur protein levels were similar in the strains harboring the RstA expression plasmid and the plasmid vector (see Fig. S1 in the supplemental material). Therefore, we concluded that the RstA protein represses transcription of iron-responsive genes using a mechanism that affects the activity (as opposed to the levels) of the Fur protein.

RstA expression activates transcription of the *feoAB* operon, overcoming Fur-mediated repression. The *feoAB* operon encodes an Fe(II)-transporter, FeoB, and the FeoA protein of unknown function (5, 12). Transcription of the *feoAB* operon is negatively regulated by the Fur protein (12). Contrary to the regulatory behaviors of other Fur-repressed genes, transcription of the *feoAB* operon was increased upon RstA expression in our DNA microarray experiment (see Table S2 in the supplemental material). By conducting real-time PCR analysis on RNA isolated from *Salmonella* cells that were exponentially growing in LB medium, we verified that the RstA protein does activate transcription of the *feoAB* operon: the mRNA levels of the *feoA* and *feoB* genes were eight- and sixfold higher in the RstA-expressing strain than levels in the wild-type strain and the *rstA* deletion strain harboring a plasmid vector (Fig. 2A). We also determined that the Fur protein was acting as a transcriptional repressor of the *feoAB* operon because deletion of the *fur* gene resulted in a twofold increase of the wild-type *feoAB* transcripts (Fig. 2A). Consistent with this, transcription of the *feoA* and *feoB* genes reached the maximum levels in the strain carrying a *fur* deletion and the RstA-expressing plasmid (Fig. 2A). Thus, our data showed that the RstA protein activates *feoAB* transcription independently of the repression mediated by the Fur protein.

The RstA protein directly activates transcription of the *feoAB* operon. Recently, it has been proposed that the *E. coli* RstA protein recognizes the consensus DNA sequence called the RstA box (i.e., TACA-N_6-TACA) for its binding (19). Examination of the promoter region of the *Salmonella feoA* gene revealed DNA sequences resembling the *E. coli* RstA box (Fig. 2B), which prompted us to explore whether the RstA protein could directly interact with the *feoA* promoter. Incubation of the purified RstA protein and the 5′ end-labeled *feoA* promoter DNA resulted in electrophoretic mobility shifts of the DNA molecules (Fig. 2C, left panel). The RstA-*feoA* promoter interaction was specific because addition of the unlabeled *feoA* promoter DNA competed with the labeled probe from the shifted band (Fig. 2C, left panel). In addition, the RstA protein could not gel shift the *feoA* promoter DNA that lacks the putative RstA-binding sequences (Fig. 2B and C, middle panel). As it has been known that phosphorylation of many response regulators increases their DNA-binding affinity in vitro, we also tested the effect of phosphorylation on the RstA and DNA interaction. When acetyl phosphate was used

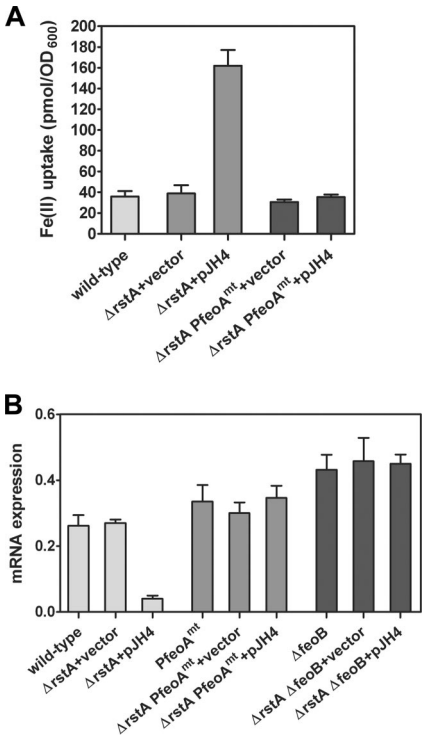

FIG. 3. Induction of the *feoB* gene is necessary for downregulation of the Fur-repressed genes upon RstA activation. (A) ^{55}Fe(II) uptake levels were determined in the wild-type strain and JH101 (Δ*rstA*::Km^r) and JH359 (Δ*rstA*::Km^r P*feoA*$_{mt}$) strains harboring pUHE21-2*lacI*^{q} (vector) or the RstA expression plasmid (pJH4) that were grown to an OD_{600} of 0.5 in LB medium. IPTG was added to the strains carrying plasmids. Shown are the mean values and standard deviations of three independent experiments. (B) mRNA levels corresponding to the *fhuF* gene were determined in cells grown to an OD_{600} of 0.5 to 0.6 by using real-time PCR. The JH358 (P*feoA*$_{mt}$) and JH362 (Δ*feoB*) strains were grown in LB medium, and JH359 (Δ*rstA*::Km^r P*feoA*$_{mt}$) and JH367 (Δ*rstA*::Km^r Δ*feoB*) strains carrying pUHE21-2*lacI*^{q} (vector) or the RstA expression plasmid (pJH4) were grown in LB medium supplemented with IPTG. Shown are the mean values and standard deviations of three independent experiments.

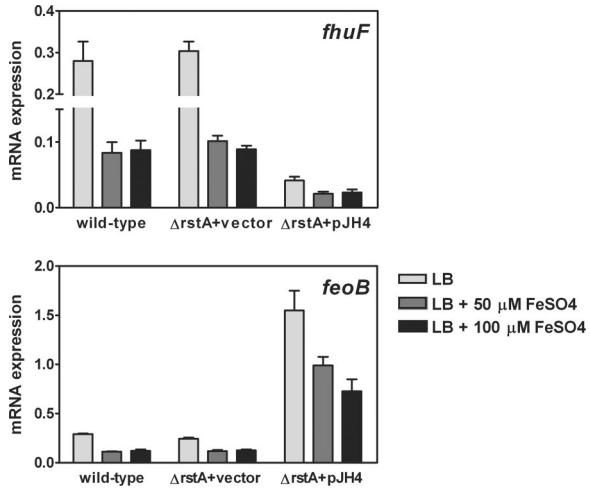

FIG. 4. RstA-activated FeoB expression increases Fur-promoted repression. mRNA levels corresponding to the *fhuF* and *feoB* genes were determined using real-time PCR analysis. The RNA samples were prepared from the wild-type strain and the JH101 (Δ*rstA*::Km^r) strain harboring pUHE21-2*lacI*^{q} (vector) or the RstA expression plasmid (pJH4) grown to an OD_{600} of 0.5 to 0.6 in LB medium supplemented with $FeSO_4$. IPTG was added to the strains harboring plasmids. Shown are the mean values and standard deviations of three independent experiments.

as a phospho-donor in the gel shift assay, the same concentration of RstA increased the amount of RstA-DNA complex (Fig. 2C, compare lanes 3 and 6 in the right panel), suggesting that phosphorylation of the RstA protein by acetyl phosphate could enhance its binding to the target DNA.

To assess the relevance of RstA binding in *feoAB* transcription in vivo, we constructed a mutant strain in which the nucleotide sequences corresponding to the putative RstA-binding site of the *feoA* promoter were replaced on chromosome (Fig. 2B). Real-time PCR analysis revealed that in the *feoA* promoter mutant strain, the RstA protein was unable to promote *feoAB* transcription (Fig. 2A). In sum, our experiments demonstrated that the RstA protein activates transcription of the *feoAB* operon via its direct binding to the *feoA* promoter.

Activation of *feoB* transcription by the RstA protein is necessary for repression of iron-responsive genes. We hypothesized that FeoB induction by the RstA protein might enable bacterial cells to import more external Fe(II), elevating levels of the Fur-Fe(II) complex, which consequently enhances Fur-dependent repression of the target genes. Consistent with this idea, Fe(II) uptake levels were approximately fourfold higher in the RstA-expressing strain than in the wild-type strain or the strain harboring the plasmid vector (Fig. 3A). This result was dependent on RstA-activated *feoAB* transcription because Fe(II) uptake in the *feoA* promoter mutant strain reached wild-type levels upon RstA expression (Fig. 3A).

We next compared the mRNA levels of *fhuF* between the wild-type and *feoA* promoter mutant strains grown in LB medium and found that the lack of RstA-activated *feoAB* transcription abrogates repression of the Fur target gene even in the strain expressing the RstA protein (Fig. 3B). To investigate further whether the Fe(II) transporter FeoB is necessary for repression of iron-responsive genes by the RstA protein, we constructed a strain with a deletion of the *feoB* gene. Real-time PCR analysis determined that this mutation was nonpolar because transcription of the *yhgG* gene, which is transcribed in the same direction and thus seems to constitute an operon with *feoA* and *feoB*, occurred at the wild-type levels (data not shown). The *feoB* deletion prevented the RstA-promoted repression of transcription levels of *fhuF* (Fig. 3B). In sum, our data suggest that induction of FeoB expression is responsible for repression of the iron-responsive genes taking place upon RstA activation.

RstA activation results in hyperrepression of the Fur-regulated genes in response to iron. We hypothesized that growth of wild-type *Salmonella* under high-iron conditions might increase Fur activity to the levels displayed by the RstA-expressing strain. As expected, addition of 50 μM $FeSO_4$ reduced transcription levels of the *fhuF* gene approximately threefold in the wild-type strain grown in LB medium (Fig. 4). However, it seemed that the Fur-Fe(II) levels were already close to the maximum at this iron concentration because the mRNA levels of this Fur-repressed gene were hardly altered in bacterial cells

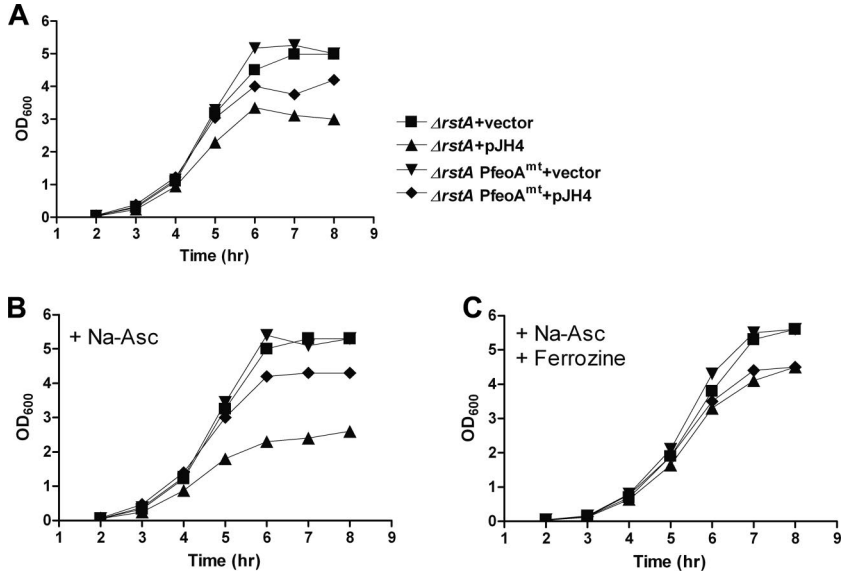

FIG. 5. RstA expression under iron-replete conditions results in a *Salmonella* growth defect. OD_{600}s of the JH101 (Δ*rstA*::Km^r) and JH359 (Δ*rstA*::Km^r P*feoA*$_{mt}$) strains harboring pUHE21-2*lacI*^{q} (vector) or an RstA expression plasmid (pJH4) were determined at different times. Strains were grown in LB medium (A), LB medium containing 2 mM sodium ascorbate (Na-Asc) (B), and LB medium supplemented with sodium ascorbate and 0.25 mM ferrozine (C). IPTG was also added to all bacterial cultures. Shown is the result of one of the three independent experiments that gave similar results. Growth of the wild-type strain 14028s was similar to that of the JH101 and JH359 strains harboring a plasmid vector (data not shown).

grown with 100 μM $FeSO_4$ (Fig. 4). Interestingly, it turned out that the *fhuF* messages in the RstA-expressing strain grown in LB medium were kept lower than those in wild-type cells cultured in the medium supplemented with iron, which were reduced further by iron addition (Fig. 4). This result was correlated with regulation of the *feoB* gene in response to iron and RstA activation: in the wild-type strain, iron decreased *feoB* transcription whereas RstA expression highly increased it, overcoming the Fur-Fe(II) protein-mediated repression (Fig. 4). Together with the finding that induction of the *feoB* gene was necessary for repression of the iron response by the RstA protein (Fig. 3B), our results suggest that iron signaling via the RstA-induced FeoB promotes Fur activity beyond the levels which are simply accomplished by iron.

The *Salmonella* strain expressing the RstA protein displays a growth defect under iron-replete conditions. During the course of experiments, we found a growth defect of the *rstA* deletion strain expressing the RstA protein in LB medium: it showed slower growth and finally stopped growing at a lower optical density than the *rstA* mutant harboring the plasmid vector, which grew like the wild-type strain (Fig. 5A). Based on the finding that the RstA protein promotes expression of the *feoAB* operon (Fig. 2A), we reasoned that increased FeoB expression might import Fe(II) to levels that were toxic to *Salmonella*. Indeed, lack of RstA-dependent transcriptional activation of the *feoA* promoter partially restored growth of the strain expressing the RstA protein (Fig. 5A). We next added the reducing agent sodium ascorbate to increase the Fe(II) levels in LB medium; this step retarded further growth of the *rstA* deletion strain harboring an RstA-expressing plasmid but not carrying a plasmid vector (Fig. 5B). By contrast, the Fe(II) chelator significantly compromised RstA-mediated growth inhibition. In the ferrozine-containing medium, growth of the *rstA* deletion strain was recovered similarly to that of the *feoA* promoter mutant strain upon RstA activation (Fig. 5C). Thus, our experiments suggest that under iron-rich conditions, RstA-mediated FeoB induction provides *Salmonella* with additional Fe(II) that not only associates with the Fur protein but is toxic to the bacterial cell.

DISCUSSION

Iron is essential for bacterial viability. However, the reduced (i.e., ferrous) form of this metal reacts with hydrogen peroxide to generate highly deleterious hydroxyl radicals (1). Thus, cells must regulate the internal concentration of iron under aerobic conditions in which a reduction of oxygen inside inevitably occurs. The regulatory protein Fur has been known to sense cytoplasmic iron levels and to regulate vast numbers of genes involved in iron metabolism (1, 9). We have now identified a novel regulatory pathway that controls Fur activity in *S. enterica*.

We have established that expression of the RstA protein, which is the response regulator of the RstA/RstB two-component system, results in repression of various genes encoding proteins that are implicated in iron uptake, storage, and metabolism (see Table S2 in the supplemental material). Iron-responsive genes are negatively regulated by the Fur protein when complexed with Fe(II) (2, 16). The regulatory effect of the RstA protein occurred through the Fur protein because the RstA protein failed to repress transcription of the iron-regulated genes (i.e., *fhuA* and *fhuF*) in a *fur* deletion strain (Fig. 1B). The RstA protein appears to affect Fur activity because the Fur protein levels were not affected upon RstA expression (see Fig. S1 in the supplemental material).

We have now determined that RstA overexpression pro-

motes transcription of the *feoAB* operon (Fig. 2A), which encodes the FeoB protein, a high-affinity Fe(II)-transporter in several bacterial species including *Salmonella* (3, 5, 12). A recent study showed that the *E. coli* RstA protein specifically binds to promoters harboring the DNA sequences of the RstA box, which consists of a tandem repeat of TACA sequences with six nucleotides of a spacer (i.e., TACA-N_6-TACA) (19). Based on the predicted −10 and −35 sequences, we found that the RstA box-like sequences (underlined residues in TACAT TCCGTCACA) are located approximately between −45 and −60 upstream of the transcription initiation site of the *Salmonella feoAB* operon (Fig. 2B). Indeed, the RstA protein bound to the *feoA* promoter, and mutation of this putative site prevented RstA binding (Fig. 2C), which in turn abolished the RstA-mediated activation of *feoAB* transcription (Fig. 2A). Considering the binding position, the *Salmonella* RstA protein is likely to act as a class I transcription factor for *feoAB* transcription as proposed for the *E. coli asr* gene, where the RstA protein bound at a site between −55 and −68 upstream of the *asr* promoter and activated its transcription (19).

We propose that the RstA-induced *feoB* expression allows *Salmonella* cells to take up more Fe(II), thereby promoting Fur activity [as evidenced by Fur-Fe(II) levels] based on the following. First, when iron was depleted by the Fe(II)-specific chelator (i.e., dipyridyl), the RstA protein failed to repress iron-responsive genes (Fig. 1B). Second, in cells grown in minimal medium, the RstA protein was able to repress the Fur-regulated genes, depending on the iron supply (Fig. 1C). Third, the RstA-dependent activation of *feoAB* transcription increased Fe(II) uptake, whereas mutation of the RstA-binding sequences on the *feoA* promoter or deletion of the *feoB* gene abolished repression of Fur-regulated genes in cells expressing the RstA protein (Fig. 3). It should be noted that the *feoA* promoter mutant strains grown in LB medium still displayed the wild-type levels of Fur repression (Fig. 3B). This emphasizes that, in the presence of iron, the *feoB* gene should be induced by the RstA protein to repress iron-responsive genes and differs from the finding that iron chelator inactivated the Fur protein to abolish the regulatory effect of RstA on its regulated genes (Fig. 1B).

The fact that Fe(II) is rapidly oxidized into Fe(III) under aerobic conditions at neutral pH raises a question of how the Fe(II) pool could be formed in *Salmonella* that was aerobically growing in LB medium. It has been demonstrated that even siderophore-producing bacteria such as *Salmonella*, *E. coli*, and *Pseudomonas aeruginosa* harbor extracellular Fe(III) reductase activities to solubilize iron (28). Therefore, this enzyme activity would be another determinant for the RstA-controlled iron response in *Salmonella* and also a reason that expression of the *E. coli feoB* gene from plasmid results in equally enhanced Fe(II) uptake in cells grown aerobically or anaerobically (12).

Salmonella uses several different iron uptake systems. The siderophore-captured Fe(III) is bound to the outer membrane receptors and transported into cells in a TonB-ExbB-ExbD complex-dependent manner (1), whereas Fe(II) uptake is mediated by the FeoB and SitABCD transporters (3, 34). As proposed in previous studies (25, 26), our data suggest that Fe(II) imported by the FeoB protein functions as an activating signal for the Fur repressor. When *Salmonella* is grown under iron-rich conditions, the Fur-Fe(II) levels seem to attain the steady state while Fe(II) transport through the FeoB protein is repressed. These steady-state levels of Fur-Fe(II) protein are unlikely to be altered in cells grown in the presence of even higher concentrations of iron where iron import might continuously occur through other, marginally expressed iron uptake systems (Fig. 4). Under these circumstances, FeoB induction by RstA activation could provide the Fur protein with additional Fe(II) to repress the iron response beyond the levels normally mediated by the Fur protein (Fig. 4 and Fig. 6).

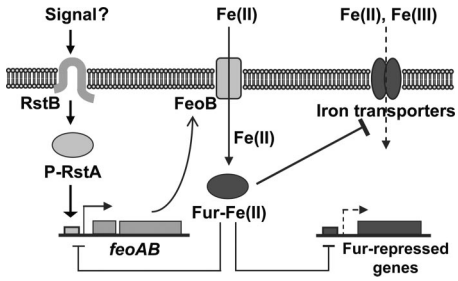

FIG. 6. Model illustrating the RstA-mediated regulatory pathway that controls Fur activity in *Salmonella enterica*. When activated under iron-replete conditions by unknown signal(s), the RstA protein binds to the *feoA* promoter to activate transcription of the *feoAB* operon encoding the Fe(II) transporter FeoB. The RstA-promoted FeoB expression imports additional Fe(II), elevating the active Fur [i.e., Fur-Fe(II)] levels, which in turn represses further transcription of Fur-repressed genes encoding other Fe(II)/Fe(III) transporters and proteins involved in iron assimilation.

In addition to providing a means to control Fur activity, the increase in Fe(II) uptake via the FeoB protein appeared to be a source of a *Salmonella* growth defect under iron-replete conditions. This phenotypic defect became more prominent when cells expressing the RstA protein were grown with the iron-reducing agent, whereas lack of the RstA-dependent activation of the *feoA* promoter or the Fe(II) chelator significantly eliminated the RstA-mediated growth inhibition (Fig. 5).

What else could be the consequences of RstA activation for *Salmonella* growing aerobically with iron? In response to oxidative stress, expression of the Fur protein is upregulated by the OxyR and SoxRS regulators (33). Hydrogen peroxide is likely to oxidize Fe(II) that is complexed with the Fur protein, which inactivates Fur regulation (27). Under iron-rich environments, this would cause an increase in the levels of free Fe(II) that reacts with the reduced oxygen. However, an *E. coli* mutant accumulating endogenous hydrogen peroxide could grow aerobically in LB medium because the OxyR protein promoted Fur expression to maintain the normal Fur-Fe(II) levels whereby the TonB-ExbB-ExbD complex-mediated iron uptake was repressed (27). In this context, if the RstA-induced FeoB protein imported Fe(II) to levels that allowed it to serve as a cofactor for the OxyR-promoted Fur protein, the resulting hyperrepression of other iron-uptake systems could minimize the toxic iron levels.

The PmrA/PmrB two-component system is a major determinant for *Salmonella*'s survival under high Fe(III) environments (30). It has been demonstrated that the PmrB sensor protein recognizes extracellular Fe(III) as a specific signal and

promotes phosphorylation of the PmrA response regulator (30). The phospho-PmrA protein activates expression of sets of proteins that are implicated in lipopolysaccharide modification, which reduces the association of Fe(III) with the outer membrane (18). Our present work demonstrates that *Salmonella* controls cytoplasmic iron metabolism using the RstA/RstB two-component regulatory system.

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