RstA-promoted expression of the ferrous iron transporter FeoB under iron-replete conditions enhances fur activity in Salmonella enterica

Jihye Jeon
Sungkyunkwan University School of Medicine

Hyunkeun Kim
Sungkyunkwan University School of Medicine

Jiae Yun
Seoul National University

Sangryeol Ryu
Seoul National University

Eduardo Groisman
Washington University School of Medicine in St. Louis

See next page for additional authors

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Authors
Jihye Jeon, Hyunkeun Kim, Jiae Yun, Sangryeol Ryu, Eduardo Groisman, and Dongwoo Shin
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RstA-Promoted Expression of the Ferrous Iron Transporter FeoB under Iron-Replete Conditions Enhances Fur Activity in Salmonella enterica

Jihye Jeon, Hyunkeun Kim, Jiae Yun, Sangryeol Ryu, Eduardo A. Groisman, and Dongwoo Shin

Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon 440-746, South Korea; Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, South Korea; and Department of Molecular Microbiology, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri 63110

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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 Rstd/RstB system appears to be a typical two-component regulatory system consisting of the membrane sensor Rstd and its cognate response regulator RstA. Signal transduction between RstA and RstdB has been shown in vitro: the purified Escherichia coli Rstd protein could be phosphorylated by the cytoplasmic domain of RstdB 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 RstdA protein identified the asr and csgD genes as RstdA targets harboring the “RstdA box” (i.e., the consensus sequences recognized by RstdA) 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 RstdA (19). However, low pH does not seem to produce sufficient levels of active RstdA (i.e., phosphorylated RstdA) in E. coli because repression of csgD transcription occurred only upon overexpression of the RstdA protein from a plasmid (19). In Salmonella enterica, overexpression of the RstdA 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), RstdA 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 RstdA protein directly binds to the feoB operon 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, RstdA activation results in hyperrepression of the Fur-regulated genes.
TABLE 1. Bacterial strains and plasmids used in this study

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<tr>
<th>Strain or plasmid</th>
<th>Description</th>
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<tr>
<td>S. enterica serovar Typhimurium strains</td>
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<tr>
<td>14028s</td>
<td>Wild type</td>
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<tr>
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<td>ΔrstA::Km^r</td>
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<td>JH367</td>
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**Plasmids**

- pUHE21-2lac^f^T^F^ (P_{lac}^f^T^F^) 23
- T7-7 (P_{T7}^f^T^F^) 24
- pKD3 (P_{FRT}^f^T^F^) 6
- pKD4 (P_{FRT}^f^T^F^) 6
- pCP20 (P_{Cm}^f^T^F^) 6
- pJH4 (P_{I857}^f^T^F^) 6
- pT7-7-7-4-His^m (P_{I857}^f^T^F^rstA^m5-His^m) 6

**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′-AGGCGGGTGATGTGTCGCGTTATCCATCGGATATGCACCCGATGACTGACTGACGCGCTGCGTATTACCCGACATATGAATATCCTCCTTAG-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′-CTGATTTCACATTTATCATATGAACCGGCCTGACGGTAACTGGTTTCGTTTC-3′) was amplified by PCR using primers rstA^f^T^F^ and rstA^f^T^F^rstA^m5. The resulting PCR fragments were integrated into the wild-type strain 14028s as a template. The resulting strain was confirmed 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 pH4 in which the RstA protein is expressed from the lac promoter, the rstA gene was amplified by PCR using the primers CD-rstA-1 and RSM-rstA-6 (5′-CGCCATGGATGATATGTTG-3′ and 5′-CTGATTTCACATTTATCATATGAACCGGCCTGACGGTAACTGGTTTCGTTTC-3′) and chromosomal DNA from strain 14028s as a template, and the product was introduced between the BamHI and PstI restriction sites of pT7-7 vector (24). Sequences of the P_{I857} 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 pUHE21-2lac^f^T^F^ (23) or the RstA protein expression plasmid pH4 were grown in LB medium supplemented with 0.5 mM IPTG. When the cells' optical density at 600 nm (OD600) 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 Omniscript 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 relative fold changes of target genes were calculated relative to the untreated control level. 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 pTT7-rtst-His<sub>t</sub> were grown to an OD_{600} of 0.6; then 1 mM IPTG was added to the culture for RstA-His<sub>t</sub> protein induction, and another 3 h of incubation followed. The C-terminal His-tagged RstA protein was purified by Ni<sup>2+</sup> 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-NTA agarose resin. The column was washed two times, once 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 fetoA promoter region were generated by PCR amplification using primers GS-fetoA-F (5′-AAA CGGTTAATTTGCACTTG-3′) and GS-fetoA-R (5′-TTATTAATCTGGA TGTATACCTCAT-3′) and wild-type Salmonella chromosomal DNA as a template. To obtain the mutant fetoA promoter DNA lacking the putative RstA-binding site (P<sub>fetoA</sub>), PCR was conducted using the same primers and chromosomal DNA from the JH358 strain as a template. The fetoA promoter DNA was purified from agarose gel using a gel extraction kit (Qiagen) and incubated with 50 pmol of poly(dI-dC). For a competition assay, a fourfold molar excess of the unlabeled fetoA 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, determined as described previously (32) with appropriate modifications. Bacterial cells were grown to mid-exponential phase (OD_{600} of 0.5 to 0.6) in LB medium containing IPTG.

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 fetoAB 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 results 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 dipipryridyl, 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-
proximately sixfold lower in the presence of 20 μM ferrous sulfate (FeSO₄) 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. 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 (ΔrstA::Km'), JH352 (Δfur), JH353 (ΔrstA::Km' Δfur), and JH101, JH352, and JH353 strains harboring the plasmid vector pUHE21-2lacIq 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 FeSO₄. All strains were grown to an OD₆₀₀ 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.
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-N6-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 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.
FeoAB demonstrated that the RstA protein activates transcription of the FeoAB promoter mutant strain, the RstA protein was unable to promote dependent repression of the target genes. Consistent with this idea, FeoB induction by the RstA protein might enable necessary for repression of iron-responsive genes.

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 wild-type strain and the JH101 (Δrsta::Km) strain harboring pUHE21-2lacPq (vector) or the RstA expression plasmid (pJH4) grown to an OD600 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.

We next compared the mRNA levels of fluF 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). 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, FeoB induction by the RstA protein was approximately fourfold higher in the RstA-expressing strain than in the wild-type strain or the strain harboring the plasmid vector (Fig. 3A).

We next compared the mRNA levels of fluF 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, 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 fluF (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 FeSO4 decreased transcription levels of fluF 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
grown with 100 μM FeSO₄ (Fig. 4). Interestingly, it turned out that the fluF 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 now determined that RstA overexpression pro-
motes transcription of the \textit{feoAB} operon (Fig. 2A), which encodes the FeoB protein, a high-affinity Fe(II)-transporter in several bacterial species including \textit{Salmonella} (3, 5, 12). A recent study showed that the \textit{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\text{s}-TACA) (19). Based on the predicted −10 and −35 sequences, we found that the RstA box-like sequences (underlined residues in TACAT TCGTTCACA) are located approximately between −45 and −60 upstream of the transcription initiation site of the \textit{Salmonella} \textit{feoAB} operon (Fig. 2B). Indeed, the RstA protein bound to the \textit{feoA} promoter, and mutation of this putative site prevented RstA binding (Fig. 2C), which in turn abolished the RstA-mediated activation of \textit{feoAB} transcription (Fig. 2A).

Considering the binding position, the \textit{Salmonella} RstA protein is likely to act as a class I transcription factor for \textit{feoAB} transcription as proposed for the \textit{E. coli} \textit{asr} gene, where the RstA protein bound at a site between −55 and −68 upstream of the \textit{asr} promoter and activated its transcription (19).

We propose that the RstA-induced \textit{feoB} expression allows \textit{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 \textit{feoB} transcription increased Fe(II) uptake, whereas mutation of the RstA-binding sequences on the \textit{feoA} promoter or deletion of the \textit{feoB} gene abolished repression of Fur-regulated genes in cells expressing the RstA protein (Fig. 3). It should be noted that the \textit{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 \textit{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 \textit{Salmonella} that was aerobically growing in LB medium. It has been demonstrated that even siderophore-producing bacteria such as \textit{Salmonella}, \textit{E. coli}, and \textit{Pseudomonas aeruginosa} harbor extracellular Fe(III) reductase activities to solubilize iron (28). Therefore, the enzyme activity would be another determinant for the RstA-controlled iron response in \textit{Salmonella} and also a reason that expression of the \textit{E. coli} \textit{feoB} gene from plasmid results in equally enhanced Fe(II) uptake in cells grown aerobically or anaerobically (12).

\textit{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 SitABC 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 \textit{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).

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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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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