

The FeoC Protein Leads to High Cellular Levels of the Fe(II) Transporter FeoB by Preventing FtsH Protease Regulation of FeoB in *Salmonella enterica*

Hyunkeun Kim, Hwiseop Lee, Dongwoo Shin

Division of Microbiology, Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, South Korea

In the gammaproteobacteria, the FeoA, FeoB, and FeoC proteins constitute the Feo system, which mediates ferrous iron [Fe(II)] import. Of these Feo proteins, FeoB is an inner membrane Fe(II) transporter that is aided by the small protein FeoA. However, the role of another small protein, FeoC, has remained unknown. Here we report that the FeoC protein is necessary for FeoB protein-mediated Fe(II) uptake in *Salmonella* experiencing low levels of oxygen and iron. The FeoC protein was found to directly bind to the FeoB transporter, leading to high cellular levels of FeoB. Depletion of the FtsH protease enabled high levels of FeoB in the absence of FeoC, suggesting that the FeoC protein protects the FeoB transporter from FtsH-mediated proteolysis. Our present study provides a singular example of bacteria that can control expression of iron uptake systems posttranslationally by employing a small iron transporter-binding protein.

Iron, an essential metal for all forms of life, is present in an oxidized (ferric) or a reduced (ferrous) form depending on the oxidation state. Thus, iron mostly exists in an insoluble ferric or as a soluble ferrous form under aerobic or anaerobic conditions, respectively. To utilize ferric iron [Fe(III)], bacteria synthesize and secrete Fe(III)-specific chelators called siderophores (1). Gram-negative bacteria then uptake Fe(III)-siderophore complexes via outer membrane receptors in a process that requires the TonB-ExbB-ExbD protein complex (1). In contrast, bacteria directly import ferrous iron [Fe(II)] via membrane transporters (1).

In the gammaproteobacteria, the *feoA*, *feoB*, and *feoC* genes are likely to constitute an operon (2). The *Escherichia coli* *feo* operon encodes the small (8.4-kDa) protein FeoA, the large (84.5-kDa) protein FeoB, and another small (8.7-kDa) protein, FeoC (2). The FeoB protein is an authentic Fe(II) transporter (3) consisting of an N-terminal cytoplasmic domain and a C-terminal transmembrane domain (2). The FeoB N terminus possesses a eukaryotic G-protein function and hydrolyzes GTP using GTPase activity, which is essential for FeoB to import Fe(II) (4). We recently reported that the FeoA protein interacts with the FeoB transporter and is necessary for FeoB-mediated Fe(II) uptake in *Salmonella enterica* (5). FeoB-mediated Fe(II) uptake appears to play an important role in *Salmonella* pathogenesis, as evidenced by the fact that a *Salmonella feoB* mutant was attenuated for virulence in mice (6).

The deduced amino acid sequence of the FeoC protein possesses a motif resembling DNA-binding domains of transcriptional regulators and a putative binding site for an iron-sulfur (Fe-S) cluster (2). On the basis of these features, the FeoC protein was initially proposed as a Fe-S cluster-dependent transcription factor controlling the *feo* operon (2); however, a *feoC* mutation was shown to have no effect on transcription of the *feo* promoter in *Yersinia pestis* (7). Nonetheless, evidence suggests that the FeoC protein plays a role as the FeoB transporter, because the FeoC protein and the N-terminal domain of FeoB from *Klebsiella pneumoniae* were shown to interact with each other (8).

Expression levels of the *feoB* gene change in response to iron

and oxygen, which results from the action of the iron-sensing transcriptional regulator Fur (9) and the oxygen-sensing transcriptional regulator Fnr (10). The Fur protein was shown to repress *feoB* transcription under iron-replete conditions (3, 11), while the activator Fnr protein promotes *feoB* transcription under anaerobic conditions (3). In the present study, we report that the FeoC protein is another factor that determines cellular levels of the FeoB Fe(II) transporter in *Salmonella enterica*. We reveal that the FeoC protein contributes to an increase in FeoB protein levels at the posttranslational level. We demonstrate direct interaction between the FeoC and FeoB proteins and provide evidence that the FeoC protein leads to high cellular levels of FeoB by protecting it from FtsH protease-mediated proteolysis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Salmonella enterica* serovar Typhimurium strains were derived from strain 14028s. Phage P22-mediated transductions were performed as described previously (12). *S. Typhimurium* strains were grown at 37°C in Luria-Bertani (LB) medium. Bacteria were grown in Erlenmeyer flasks with vigorous shaking for the preparation of aerobic cultures or grown in screw-cap tubes fully filled with medium without agitation for the preparation of anaerobic cultures. The iron chelator deferoxamine was used at 0.2 mM to reduce the iron levels in LB medium. When necessary, ampicillin (Ap), chloramphenicol (Cm), and kanamycin (Km) were used at 50 µg/ml, 25 µg/ml, and 50 µg/ml, respectively. For induction of genes from plasmids, arabinose and

Received 22 March 2013 Accepted 16 May 2013

Published ahead of print 24 May 2013

Address correspondence to Dongwoo Shin, shind@skku.edu.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.00343-13>.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JB.00343-13

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics ^a	Reference or source
<i>S. enterica</i> serovar Typhimurium		
14028s	Wild type	31
JH352	14028s Δfur	11
JH362	14028s $\Delta feoB$	11
JH363	14028s $\Delta feoC$	This study
JH323	14028s $\Delta feoC::Km^r$	This study
HK715	14028s $\Delta feoBC$	This study
HK708	14028s Δfur	This study
HK333	14028s $\Delta ftsH::Km^r/plac-FtsH$	This study
HK334	14028s $\Delta ftsH::Km^r \Delta feoC/plac-FtsH$	This study
DN281	14028s $\Delta clpA::Km^r$	This study
DN297	14028s $\Delta clpX$	This study
DN296	14028s $\Delta clpP$	This study
DN283	14028s $\Delta hslUV::Km^r$	This study
DN282	14028s $\Delta lon::Km^r$	This study
DN299	14028s $\Delta clpA::Km^r \Delta feoC$	This study
DN311	14028s $\Delta clpX \Delta feoC$	This study
DN310	14028s $\Delta clpP \Delta feoC$	This study
DN301	14028s $\Delta hslUV::Km^r \Delta feoC$	This study
DN300	14028s $\Delta lon::Km^r \Delta feoC$	This study
<i>E. coli</i> BTH101	F ⁻ <i>cya99 araD139 galE15 galK16 rpsL1 (Str^r) hsdR2 mcrA1 mcrB1</i>	19
Plasmids		
pUHE21-2 <i>lacI</i> ^q	P _{lac} rep _{pMB1} Ap ^r <i>lacI</i> ^q	14
plac-FeoC	pUHE21-2 <i>lacI</i> ^q <i>feoC</i>	This study
plac-FtsH	pUHE21-2 <i>lacI</i> ^q <i>ftsH</i>	This study
pBAD33	P _{BAD} pACYC184 <i>ori</i> Cm ^r	15
pBAD-FeoB	pBAD33 <i>feoB</i>	This study
pHis-parallel1	P _{T7} rep _{pMB1} Ap ^r	16
pHis-parallel1-FeoC	pHis-parallel1 <i>feoC</i>	This study
pHis-parallel1-Fur	pHis-parallel1 <i>fur</i>	This study
pKD3	rep _{R6Kγ} Cm ^r FRT Km ^r FRT	13
pKD4	rep _{R6Kγ} Ap ^r FRT Km ^r FRT	13
pKD46	rep _{pSC101} (Ts) Ap ^r P _{araBAD} γ β <i>exo</i>	13
pKD46-CmR	rep _{pSC101} (Ts) Cm ^r P _{araBAD} γ β <i>exo</i>	This study
pCP20	rep _{pSC101} (Ts) Ap ^r Cm ^r <i>cl857</i> λ P _R <i>flp</i>	13
pKT25	P _{lac} p15A <i>ori</i> Km ^r	17
pUT18	P _{lac} ColEI <i>ori</i> Ap ^r	17
pT18-FeoC	pUT18 <i>feoC</i>	This study
pT25-FeoB	pKT25 <i>feoB</i>	5
pT25-MgtA	pKT25 <i>mgtA</i>	This study
pT18-Zip	pUT18 <i>zip</i>	19
pT25-Zip	pKT25 <i>zip</i>	19

^a FRT, Flp recombination target; rep, replicon.

isopropyl 1-thio- β -D-galactoside (IPTG) were used at various concentrations.

Construction of bacterial strains. *S. Typhimurium* strains carrying a gene deletion were constructed using the one-step gene inactivation method (13). For deletion of the *feoC* and *feoBC* genes, the Km^r cassette from plasmid pKD4 (13) was amplified using primer pairs DEL-*feoC*-F/DEL-*feoC*-R and DEL-*feoB*-F/DEL-*feoC*-R, respectively. For deletion of the *fnr* gene, the Km^r cassette from plasmid pKD4 was amplified using the primer pair DEL-*fnr*-F/DEL-*fnr*-R. For deletion of the *clpA*, *clpX*, *clpP*, *hslUV*, and *lon* genes, the Km^r cassette from plasmid pKD4 was amplified using the primer pairs DEL-*clpA*-F/DEL-*clpA*-R, DEL-*clpX*-F/DEL-*clpX*-R, DEL-*clpP*-F/DEL-*clpP*-R, DEL-*hslUV*-F/DEL-*hslUV*-R, and DEL-*lon*-F/DEL-*lon*-R, respectively. The resulting PCR products were

integrated into the chromosome of strain 14028s as described previously (13). When necessary, the Km^r cassette was removed using plasmid pCP20 (13). The *ftsH* gene encoding the FtsH protease was essential in *S. Typhimurium*. Thus, a chromosomal deletion of the *ftsH* gene was generated in strains producing the FtsH protease from the plasmid-linked *lac* promoter as follows. The Km^r cassette from plasmid pKD4 was amplified using the primer pair DEL-*ftsH*-F/DEL-*ftsH*-R. The resulting PCR products were integrated into the chromosome of strains expressing the λ Red recombinase and the FtsH protease from pKD46-CmR and *lac*-FtsH plasmids, respectively. Deletion of the corresponding genes was verified by colony PCR. The sequences of all primers used for strain construction are listed in Table S1 in the supplemental material.

Construction of plasmids. Plasmid *plac-FeoC*, expressing the *feoC* gene from the *lac* promoter, was constructed. The *feoC* gene was amplified using the primer pair EX1-*feoC*-F/EX1-*feoC*-R and chromosomal DNA from the 14028s strain. The PCR products were purified and introduced between the BamHI and PstI restriction sites of pUHE21-2*lacI*^q (14). To construct plasmid pBAD-FeoB, in which the *feoB* open reading frame (ORF) is expressed from the P_{BAD} promoter, the *feoB* ORF was amplified using the primer pair EX2-*feoB*-F/EX2-*feoB*-R and chromosomal DNA of strain 14028s. The PCR products were then introduced between the XbaI and PstI restriction sites of pBAD33 (15). Plasmid *plac-FtsH* expresses the *ftsH* gene from the *lac* promoter. For its construction, the *ftsH* gene was amplified using the primer pair EX-*ftsH*-F/EX-*ftsH*-R and chromosomal DNA from the 14028s strain, and the PCR products were introduced between the BamHI and PstI restriction sites of pUHE21-2*lacI*^q. Plasmids pHis-parallel1-FeoC and pHis-parallel1-Fur express the *feoC* and *fur* genes, respectively, with nucleotides encoding a His₆ tag at their N termini. For construction of these plasmids, the *feoC* and *fur* genes were amplified using the primer pairs EX2-*feoC*-F/EX2-*feoC*-R and EX-*fur*-F/EX-*fur*-R, respectively, with 14028s chromosomal DNA as the template. The resulting PCR products were introduced between the NcoI and NotI restriction sites of the pHis-parallel1 vector (16). For the bacterial two-hybrid assay, plasmids pT18-FeoC and pT25-MgtA were constructed. The *feoC* and *mgtA* genes were amplified using the primer pairs TH-*feoC*-F/TH-*feoC*-R and TH-*mgtA*-F/TH-*mgtA*-R, respectively. The resulting PCR products were introduced between the PstI and BamHI sites of the plasmid vector pUT18 (17) or between the XbaI and BamHI sites of pKT25 (17). Plasmid pKD46-CmR, a derivative of plasmid pKD46 (13), expresses the λ Red recombinase gene from the P_{BAD} promoter. For its construction, the *bla* gene on pKD46 plasmid was inactivated by restriction enzyme digestion with XmnI as described previously (18). Then, the Cm^r cassette from plasmid pKD3 (13) was amplified using the primer pair EX-CmR-F/EX-CmR-R, which was introduced into the XmnI-digested pKD46 via blunt-end DNA ligation. Recombinant plasmid gene sequences were confirmed by nucleotide sequencing. The sequences of all primers used for plasmid construction are listed in Table S1 in the supplemental material.

Immunoblot analysis. *S. Typhimurium* strains were grown in LB medium to optical density at 600 nm (OD₆₀₀) values of approximately 0.5. Equivalent amounts of bacterial cells normalized by OD₆₀₀ values were removed, washed with phosphate-buffered saline (PBS), suspended in 0.5 ml PBS, and opened by sonication. Whole-cell lysates were resolved on 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and analyzed by immunoblotting using anti-FeoB antibodies (5). Blots were developed using anti-rabbit IgG horseradish peroxidase-linked antibodies (GE Healthcare) and an enhanced chemiluminescence (ECL) detection system (GE Healthcare).

RNA isolation and quantitative real-time RT-PCR (qRT-PCR) analysis. Bacteria were grown in LB medium to an OD₆₀₀ of 0.5 to 0.6. The culture (0.5 ml) was removed and mixed with 1 ml RNAprotect bacterial reagent (Qiagen), and RNA was isolated using the RNeasy minikit (Qiagen). The RNA sample was treated further with RNase-free DNase (Ambion). cDNA was synthesized using the PrimeScript RT reagent kit (TaKaRa), random primers (Invitrogen), and 0.5 μ g of template RNA.

Amounts of cDNA were quantified by real-time PCR using SYBR green real-time PCR master mix (Toyobo) with an ABI7300 sequence detection system (Applied Biosystems). Primers used for the detection of cDNA corresponding to the *feoB* mRNA, *feoC* mRNA, and *gyrB* mRNA were Q-*feoB*-F/Q-*feoB*-R, Q-*feoC*-F/Q-*feoC*-R, and Q-*gyrB*-F/Q-*gyrB*-R, respectively. Transcription levels of each gene were calculated from a standard curve obtained by PCR with the same primers and serially diluted genomic DNA. mRNA levels of the *feoB* and *feoC* genes were normalized to those of the *gyrB* gene. The sequences of primers used are listed in Table S1 in the supplemental material.

Bacterial two-hybrid (BACTH) assay. To assess protein-protein interactions *in vivo*, a BACTH assay was conducted as described previously (19). The *E. coli* BTH101 (*cydA*) strain was cotransformed with derivatives of the pUT18 and pKT25 plasmids. The strains were grown overnight at 30°C in LB supplemented with Ap (50 µg/ml), Km (50 µg/ml), and IPTG (1 mM). β-Galactosidase activity was determined in the cultures as described previously (20).

Purification of the His6-FeoC and His6-Fur proteins. *E. coli* BL21(DE3) cells harboring pHis-parallel1-FeoC plasmid were grown in LB medium at 37°C. When the culture OD₆₀₀ value reached approximately 0.5, IPTG (0.5 mM) was added to the culture for induction of His6-FeoC protein production, followed by another 16 h of incubation at 21°C. The His6-FeoC protein was purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. The cell pellet was suspended in cold buffer A [50 mM Tris (pH 8.0), 300 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and disrupted by sonication. The cell extract was loaded onto a Ni-NTA column equilibrated with buffer A. After the flowthrough fraction had been discarded, the column was washed with 500 ml washing buffer (buffer A containing 20 mM imidazole), and the adsorbed His6-FeoC protein was eluted with elution buffer (buffer A containing 200 mM imidazole). The His6-Fur protein was purified from *E. coli* BL21(DE3) cells harboring pHis-parallel1-Fur plasmid by the same method. Finally, the eluted proteins were dialyzed with buffer A containing 10% glycerol and stored at -80°C.

His tag protein pulldown assay. To assess protein-protein interaction *in vitro*, a pulldown assay was conducted. Two proteins of interest, one with a His₆ tag and another without a His₆ tag, were mixed at a 1:1 molar ratio (25 µM each). The protein mixture was incubated at 4°C for 3 h in buffer A [50 mM Tris (pH 8.0), 300 mM NaCl, and 1 mM PMSF] and then loaded onto a column packed with Ni-NTA agarose resin (Qiagen). After the flowthrough fraction had been discarded, the column was washed twice with washing buffer (buffer A containing 20 mM imidazole), and the bound proteins were eluted with elution buffer (buffer A containing 200 mM imidazole). The flowthrough, wash, and elution fractions were boiled in SDS sample loading buffer for 10 min and resolved on 15% SDS-polyacrylamide gels. Protein bands were visualized by silver staining.

Iron uptake assay. Fe(II) uptake levels in *S. Typhimurium* strains were determined as follows. Bacterial cells grown in LB to an OD₆₀₀ of 0.5 were washed twice with N-buffer [N-minimal medium (21), pH 7.0, supplemented with 0.1% Casamino Acids, 38 mM glycerol, 1 mM MgCl₂, and 0.1 M sodium ascorbate], suspended in the same buffer, and kept on ice. One ml of the cell suspension at an OD₆₀₀ of 1.0 was placed at 37°C for 15 min, and the Fe(II) transport assay was started by addition of 0.5 µM ⁵⁵Fe(II). The ⁵⁵Fe(II) stock solution was prepared in N-buffer and contained 50 µM ⁵⁵FeCl₃ (PerkinElmer). After 15 min of incubation, bacterial cells were loaded onto a 0.45-µm membrane filter (Whatman) placed on a 1225 sampling manifold (Millipore) and washed twice with 0.1 mM LiCl. The filter with bacterial cells was transferred into a vial containing 2 ml scintillation cocktail fluid. Activity as counts per minute was determined using an LS6500 scintillation counter (Beckman) and converted into picomoles of ⁵⁵Fe(II) using a standard curve.

RESULTS

The FeoB protein is highly produced when levels of both oxygen and iron are low. We wanted to determine the amount of *Salmo-*

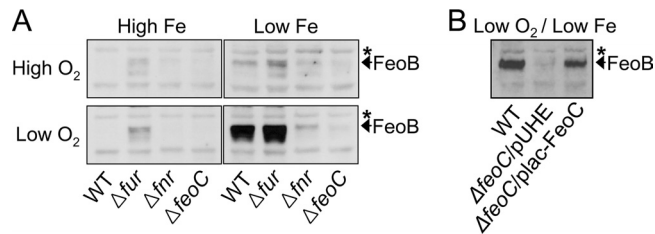


FIG 1 The FeoC protein is necessary for high cellular levels of the FeoB Fe(II) transporter under low-oxygen and -iron conditions. *Salmonella* strains were grown aerobically (high O₂) or anaerobically (low O₂) in LB medium (high Fe) or in LB medium supplemented with 0.2 mM deferoxamine (low Fe). By conducting immunoblot analysis, levels of the FeoB protein were determined in wild-type (WT, 14028s), *fur* deletion (Δfur , JH352), *fnr* deletion (Δfnr , HK708), and *feoC* deletion ($\Delta feoC$, JH363) strains (A) and in the wild-type strain (14028s) and the *feoC* deletion strain ($\Delta feoC$, JH363) carrying the empty plasmid vector pUHE21-2*lacI*⁹ (pUHE) (14) or FeoC-expression plasmid (plac-FeoC) (B). The band indicated with an asterisk corresponds to a protein displaying cross-reactivity against anti-FeoB antibody and serving as an internal loading control.

nella FeoB Fe(II) transporter produced at different levels of oxygen and iron. For this purpose, we grew *Salmonella* strains aerobically and anaerobically in LB (i.e., high-iron medium) and in LB supplemented with the iron chelator deferoxamine (i.e., low-iron medium). In the wild-type strain, FeoB protein production was negligible under both aerobic and anaerobic conditions with high iron and weak under aerobic conditions with low iron (Fig. 1A). However, FeoB was highly produced under anaerobic conditions with low iron (Fig. 1A).

The iron-sensing Fur regulator represses *feoB* transcription under iron-replete conditions (3, 11) while the oxygen-sensing Fnr regulator promotes *feoB* transcription under anaerobic conditions (3). Consistent with these findings, the lack of Fur under anaerobic conditions enhanced FeoB production under high-iron conditions, whereas the lack of Fnr impaired FeoB production under low-iron conditions (Fig. 1A). Taken together, these results suggest that two events that occur upon *feoB* transcription, the relief of Fur repression and the occurrence of Fnr activation, lead to high-level production of the FeoB transporter when levels of both iron and oxygen are low.

FeoC is necessary for high cellular levels of the FeoB transporter. Together with the FeoB Fe(II) transporter, the FeoA and FeoC proteins constitute the Feo system in gammaproteobacteria, including *Salmonella* (2). We explored the possibility that the FeoC protein plays a role in regulating FeoB protein levels. Interestingly, a *feoC* deletion strain produced very low levels of the FeoB protein under anaerobic conditions with low iron, in contrast to the wild-type strain, which contained high levels of the FeoB protein (Fig. 1A). The defective FeoB production displayed by the *feoC* deletion mutant was due to the function of FeoC, as evidenced by the fact that ectopic production of the FeoC protein from a plasmid plac-FeoC restored FeoB levels in the *feoC* deletion mutant (Fig. 1B). Therefore, these results indicate that high cellular levels of the FeoB transporter are dependent on the FeoC protein.

FeoC contributes to FeoB production at the posttranslational level. We initially explored the possibility that the FeoC protein regulates transcription of the *feoB* gene. Consistent with FeoB protein levels, the wild-type strain produced the *feoB* mRNA at high levels under anaerobic conditions with low iron

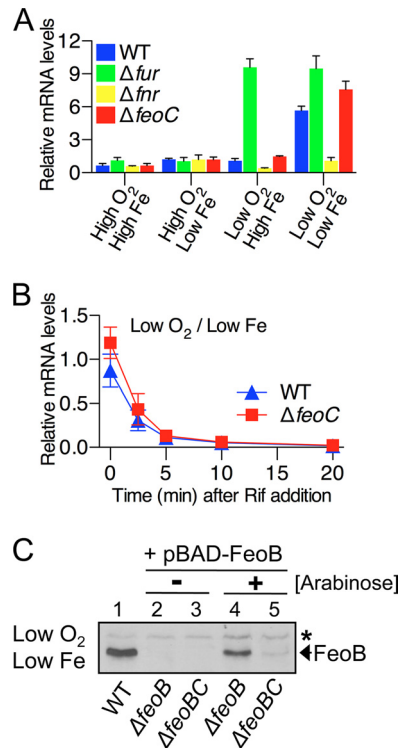


FIG 2 The FeoC protein contributes to cellular FeoB transporter levels at the posttranslational level. *Salmonella* strains were grown aerobically (high O₂) or anaerobically (low O₂) in LB (high Fe) or in LB containing 0.2 mM deferoxamine (low Fe). (A) qRT-PCR analysis determined mRNA levels of the *feoB* gene in wild-type (WT, 14028s), *fur* deletion (Δfur , JH352), *fnr* deletion (Δfnr , HK708), and *feoC* deletion ($\Delta feoC$, JH363) strains. (B) The stability of the *feoB* mRNA was examined in the wild-type (WT, 14028s) and *feoC* deletion ($\Delta feoC$, JH363) strains. RNA synthesis was inhibited with 200 μ g/ml rifampin (Rif). qRT-PCR determined *feoB* mRNA levels in total RNA isolated at the indicated time points. Values (A and B) are the means and standard deviations (SD) of three independent experiments. (C) Immunoblot analysis determined FeoB protein levels in the wild-type (WT, 14028s), *feoB* deletion ($\Delta feoB$, JH362), and *feoBC* deletion ($\Delta feoBC$, HK715) strains. The JH362 and HK715 strains harbored the FeoB expression plasmid (pBAD-FeoB) and were grown with (+) or without (–) the inducer arabinose (60 μ M). The band indicated with an asterisk corresponds to a protein displaying cross-reactivity against anti-FeoB antibody and serving as an internal loading control.

(Fig. 2A). Under the same conditions, the *feoC* deletion strain was able to express the *feoB* mRNA slightly more than the wild-type (Fig. 2A), despite the fact that this mutant hardly produced the FeoB protein (Fig. 1A). This result was also in contrast to the finding that an *fnr* mutant was defective for the expression of *feoB* mRNA and the production of FeoB protein under the same growth conditions (Fig. 1A and 2A). Thus, unlike the transcriptional regulator Fnr, the FeoC protein did not act on *feoB* transcription. The FeoC protein also had no effect on the stability of the *feoB* mRNA. Once RNA synthesis was inhibited with rifampin, the *feoB* mRNA produced under anaerobic conditions with low iron was degraded at similar rates in both the wild-type and *feoC* deletion strains (Fig. 2B).

We then reasoned that if the FeoC protein contributes to FeoB protein levels at the posttranslational level, FeoC-dependent FeoB production would still occur even when the only *feoB* ORF was expressed from a heterologous promoter. To test this idea, we determined FeoB protein levels in strains deleted for the chromo-

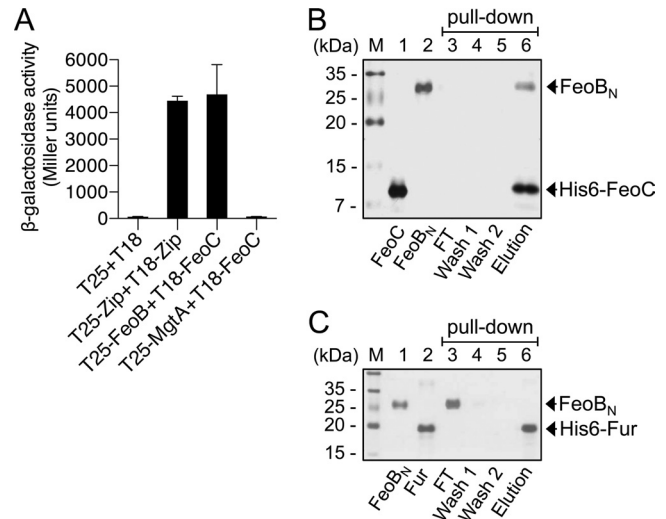


FIG 3 The FeoC protein directly interacts with the FeoB transporter. (A) FeoB-FeoC interaction was assessed using a BACTH system (19). β -Galactosidase activity was determined in *cya E. coli* (BTH101) strains carrying BACTH plasmids coexpressing T25 and T18, T25-Zip and T18-Zip, T25-FeoB and T18-FeoC, and T25-MgtA and T18-FeoC. β -Galactosidase activity resulting from the coproduction of T25 and T18 or T25-Zip and T18-Zip (19) served as negative and positive controls, respectively. Values are the means and SD of three independent experiments. (B) His tag protein pulldown experiments determined interaction between FeoB_N and FeoC. The same amounts of purified FeoB_N (without a His tag) and His₆-tagged FeoC protein (each at 25 μ M) were incubated and subjected to Ni-NTA affinity chromatography. (C) Interaction between purified FeoB_N and His₆-Fur proteins was assessed as described for panel B. Proteins in the flowthrough (FT), wash (wash 1 and wash 2), and elution fractions were resolved by SDS-PAGE and visualized by silver staining.

somal copy of the *feoB* gene and expressing the *feoB* ORF from the plasmid-linked P_{BAD} promoter. Under anaerobic conditions with low iron, the FeoB protein was produced at levels similar to the wild-type upon the addition of the inducer arabinose in the *feoC*⁺ strain but was hardly produced in the isogenic *feoC* mutant (Fig. 2C). Taken together, these results indicate that the FeoC protein contributes to FeoB production at the posttranslational level.

The FeoC protein directly interacts with the FeoB transporter. We explored whether the FeoC protein directly acts on the FeoB protein via protein-protein interaction. To assess protein-protein interaction *in vivo*, a bacterial two-hybrid (BACTH) assay was conducted. We constructed a series of plasmids where proteins are expressed as forms fused to the T18 or T25 domain of *Bordetella pertussis* adenylate cyclase (19). The *cya E. coli* strain BTH101 (19) coexpressing the T25-FeoB and T18-FeoC fusion proteins expressed approximately 70-fold higher levels of β -galactosidase than the control strain coproducing the T18 and T25 domains (Fig. 3A). This result indicates that the FeoC and FeoB proteins interact with each other to complement adenylate cyclase activity, which results in cyclic AMP (cAMP)-dependent *lacZ*⁺ expression. In contrast, the FeoC protein did not bind to the Mg²⁺ transporter MgtA protein (22); therefore, coproduction of the T25-MgtA and T18-FeoC fusion proteins failed to generate β -galactosidase activity relative to the control (Fig. 3A).

To further investigate whether the FeoB-FeoC interaction is direct, we conducted His tag pulldown experiments. The purified N-terminal domain of the FeoB protein (FeoB_N) and His-tagged

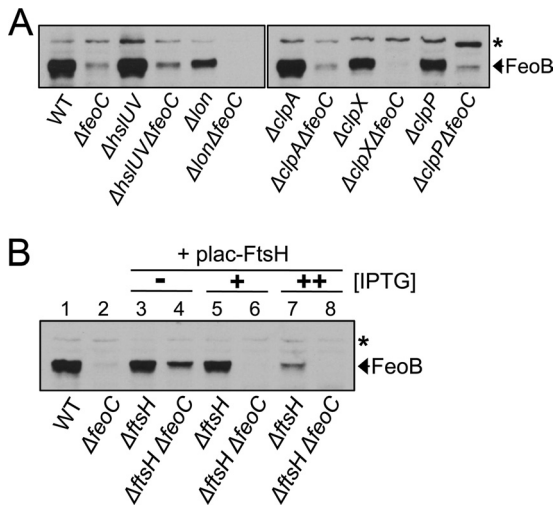


FIG 4 Depletion of the FtsH protease enables high levels of FeoB in the absence of FeoC. *Salmonella* strains were grown anaerobically in LB medium with low iron (i.e., LB supplemented with 0.2 mM deferrioxamine). Immunoblot analysis determined FeoB protein levels in the wild-type (WT, 14028s), *feoC* deletion (Δ *feoC*, JH363), *hslUV* deletion (Δ *hslUV*, DN283), *hslUV feoC* deletion (Δ *hslUV* Δ *feoC*, DN301), *lon* deletion (Δ *lon*, DN282), *lon feoC* deletion (Δ *lon* Δ *feoC*, DN300), *clpA* deletion (Δ *clpA*, DN281), *clpA feoC* deletion (Δ *clpA* Δ *feoC*, DN299), *clpX* deletion (Δ *clpX*, DN297), *clpX feoC* deletion (Δ *clpX* Δ *feoC*, DN311), *clpP* deletion (Δ *clpP*, DN296), and *clpP feoC* (Δ *clpP* Δ *feoC*, DN310) strains (A) and wild-type (WT, 14028s) and *feoC* deletion (Δ *feoC*, JH363) strains and *ftsH* deletion mutants carrying (Δ *ftsH*, HK333) or lacking (Δ *ftsH* Δ *feoC*, HK334) the *feoC* gene (B). Note that *ftsH* mutants harbored the FtsH expression plasmid (plac-FtsH). *ftsH* mutants were grown to saturation in the presence of the inducer IPTG (30 μ M), and cultures diluted to OD₆₀₀ values of approximately 0.01 were grown to OD₆₀₀ values of approximately 0.5 with 30 μ M (+) or 60 μ M (++) IPTG or without (–) IPTG. The band indicated with an asterisk corresponds to a protein displaying cross-reactivity against anti-FeoB antibody and serving as an internal loading control.

FeoC protein (His6-FeoC) were mixed and subjected to Ni-NTA affinity chromatography. The FeoB_N protein was detected together with the His6-FeoC protein in the elution fraction (Fig. 3B), indicating that FeoB_N bound to His6-FeoC. In contrast, when the experiment was performed using the His-tagged Fur regulatory protein, the FeoB_N protein was mostly detected in the flow-through fraction but not in the elution fraction, indicating no interaction between these two proteins (Fig. 3C). Taken together, our results demonstrate that the FeoC protein directly and specifically binds to the FeoB transporter.

The FeoC protein prevents FtsH protease-mediated degradation of the FeoB protein. Based on the findings that the FeoC protein contributes to FeoB protein levels at the posttranslational level and directly interacts with the FeoB transporter, we reasoned that FeoC might function to protect FeoB from proteolytic control mediated by a protease. We further predicted that a lack of such protease activity would recover FeoB protein levels in the *feoC* mutant.

Five proteases—ClpAP, ClpXP, HslUV, Lon, and FtsH—are known to play roles in regulated proteolysis in Gram-negative bacteria (23). We initially examined FeoB levels in a series of *feoC* deletion mutants carrying an additional deletion of the *clpA*, *clpP*, *clpX*, *hslUV*, or *lon* gene. However, all of these mutants produced the FeoB protein at low levels, similar to the *feoC* deletion mutant (Fig. 4A), indicating that a lack of the ClpAP, ClpXP, HslUV, or Lon protease does not affect FeoB levels in the *feoC* mutant.

FtsH is a membrane-bound protease that controls membrane protein substrates (24). Thus, we next investigated the role of the FtsH protease in FeoC-dependent FeoB protein levels. Because the *ftsH* gene is essential in *E. coli* (25), we anticipated that this might be the case in *Salmonella* as well. Therefore, we generated an *ftsH* deletion in a strain expressing the FtsH protease from the plasmid-linked *lac* promoter. Indeed, the resulting *ftsH* deletion mutants were able to form colonies on agar plates only when FtsH was induced from the plac-FtsH plasmid by adding IPTG (see Fig. S1 in the supplemental material).

We grew the *ftsH* mutant to saturation in the presence of IPTG and then diluted the cultures in fresh medium containing 0 to 60 μ M IPTG. Interestingly, when grown without IPTG, the *ftsH* mutant lacking the *feoC* gene produced the FeoB protein at markedly increased levels compared to the *feoC* deletion strain (Fig. 4B, lane 4), indicating that FtsH depletion enables FeoB stabilization even in the absence of FeoC. In contrast, FtsH induction by IPTG complemented FtsH depletion, so that the *ftsH* mutant contained hardly any FeoB protein in the absence of FeoC (Fig. 4B, lanes 6 and 8). Collectively, these results suggest that FeoB transporter levels are negatively controlled by the FtsH protease, where the FeoC protein protects FeoB from FtsH. Regardless of whether FtsH is induced by 30 μ M IPTG, the *ftsH* mutant carrying the *feoC* gene maintained FeoB protein levels similar to those in the wild-type (Fig. 4B, lanes 3 and 5). In contrast, further FtsH induction by 60 μ M IPTG greatly lowered FeoB content in this strain (Fig. 4B, lane 7), suggesting that FtsH overproduction surpasses FeoC protection of FeoB.

***Salmonella* lacking the *feoC* gene is impaired for FeoB-mediated Fe(II) uptake.** Because the FeoC protein was necessary for high levels of the FeoB transporter under anaerobic conditions with low iron, we reasoned that the *feoC* mutant might be defective for FeoB-mediated Fe(II) uptake after growth under such conditions. The wild-type strain was able to import Fe(II) at much higher (approximately 11-fold) levels after growth under anaerobic conditions with low iron than under aerobic conditions with high iron (Fig. 5A). This phenotype required both the FeoB and FeoC proteins, as evidenced by the fact that the *feoB* and *feoC* deletion strains imported Fe(II) approximately 6.5- and approximately 4.5-fold less than the wild-type strain, respectively (Fig. 5A). The FeoC protein contributed to Fe(II) uptake in *Salmonella* via the FeoB transporter as evidenced by the fact that FeoC production from the plac-FeoC plasmid restored Fe(II) uptake in the *feoC* deletion strain but failed to do so in the *feoBC* double deletion strain (Fig. 5B). Cumulatively, these results demonstrate that FeoC-dependent stabilization of the FeoB transporter is important for *Salmonella* to take up Fe(II) under conditions of both oxygen and iron depletion.

DISCUSSION

Because iron is present mostly in the ferrous form under anaerobic conditions and because production of metal transporters is often induced as levels of their cognate metals are lowered, production of the FeoB Fe(II) transporter should be induced when levels of both oxygen and iron are low. Indeed, the FeoB protein was highly produced in *Salmonella* only when these two environmental factors (i.e., low oxygen and low iron) were satisfied (Fig. 1A). For such FeoB production, Fur repression should be relieved and Fnr activation should occur on *feoB* transcription (Fig. 1A and 2A), as previously reported (3, 11). In addition to this regulation,

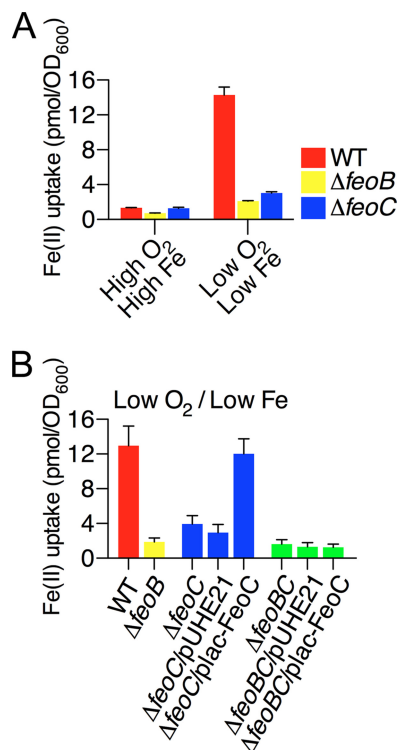


FIG 5 FeoC-dependent FeoB production enables *Salmonella* to take up Fe(II) under anaerobic conditions with low iron. ⁵⁵Fe(II) uptake levels were determined in *Salmonella* strains that were grown aerobically (high O₂) or anaerobically (low O₂) in LB (high Fe) or LB containing 0.2 mM deferoxamine (low Fe). Bacterial strains used in the experiments were wild-type (WT, 14028s), *feoB* deletion ($\Delta feoB$, JH362), and *feoC* deletion ($\Delta feoC$, JH363) strains (A) and wild-type (WT, 14028s) and *feoB* deletion ($\Delta feoB$, JH362) strains and *feoC* deletion ($\Delta feoC$, JH363) and *feoBC* deletion ($\Delta feoBC$, HK715) strains carrying the empty plasmid vector pUHE21-2lacI^q (pUHE) or FeoC-expression plasmid (plac-FeoC) (B). Values are the means and SD of three independent experiments.

we have now revealed that even under conditions where the *feoB* mRNA is highly expressed (Fig. 2A), the FeoB transporter cannot be maintained at high levels in the absence of the FeoC protein (Fig. 1).

Based on the notion that FeoC possesses a putative DNA-binding motif and a putative binding site for a Fe-S cluster, this protein was initially proposed as a Fe-S cluster-dependent transcription factor controlling the *feo* operon (2). However, the FeoC protein does not affect *feoB* transcription (Fig. 2A) (7). Instead, the FeoC protein regulates FeoB protein levels at the posttranslational level (Fig. 2). The FeoC protein was shown to interact with the FeoB transporter *in vivo* (Fig. 3A). Moreover, this protein-protein interaction was direct, as evidenced by the fact that the FeoC protein bound to the N-terminal domain of the FeoB protein *in vitro*, whether these proteins were from *Salmonella* (Fig. 3B) or from *Klebsiella* (8).

FtsH is a membrane-bound protease that degrades proteins in the membrane as well as those in the cytoplasm (24). FtsH depletion restored FeoB cellular levels even in the absence of FeoC, while its overproduction impaired FeoB levels even in the presence of FeoC (Fig. 4B), indicating that the FtsH protease negatively controls FeoB levels, and this control is hindered by the FeoC protein. It is known that the FtsH protease initiates proteol-

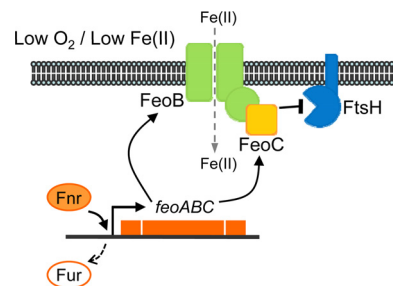


FIG 6 Model illustrating the role of FeoC in FeoB cellular levels. In *Salmonella* experiencing environmental conditions with low levels of oxygen and iron, Fur repression is relieved on the *feo* operon while Fnr promotes expression of the *feoC* mRNA (see Fig. S2 in the supplemental material) together with the *feoB* mRNA. The FeoB transporter is under proteolytic control by the membrane-bound FtsH protease. The FeoC protein binds to the cytoplasmic N-terminal domain of FeoB, which protects the FeoB transporter from FtsH-mediated proteolysis. Consequently, the FeoC protein leads to high cellular levels of the FeoB transporter, which enables *Salmonella* to take up Fe(II).

ysis at a cytoplasmic region of the substrate when degrading a membrane protein (24, 26). Therefore, we propose that FeoC binding to the cytoplasmic N-terminal domain of FeoB protects the FeoB transporter from FtsH-mediated proteolysis initiated at the FeoB N terminus (Fig. 6). Consequently, the action of FeoC on FeoB may stabilize the FeoB transporter under certain circumstances, which enables *Salmonella* to take up Fe(II) under anaerobic conditions with low iron (Fig. 5 and 6).

It has been a long-standing notion that bacteria control expression of iron uptake systems at the transcriptional level (27). Likewise, *feoB* expression is under dual transcriptional control by the iron-sensing Fur and oxygen-sensing Fnr regulators whereby FeoB-mediated Fe(II) uptake is maintained at appropriate levels in response to different levels of iron and oxygen. What then is the physiological relevance of FeoC regulation of FeoB? We note that FeoC could be a Fe-S cluster-binding regulator (2). Fe-S clusters in some regulatory proteins are known to serve as redox sensors or as iron sensors. The Fe-S cluster of Fnr is an example of redox sensors (10). A shift from anaerobic to aerobic conditions oxidizes the Fe-S cluster to cause its disassembly in the Fnr protein, whereby Fnr loses the activity of a transcription factor (10). Moreover, the Fnr regulator that lacks the Fe-S cluster becomes vulnerable to ClpXP protease-mediated proteolysis (28). Although aconitase B (AcnB) is a tricarboxylic acid cycle enzyme carrying a Fe-S cluster, the apo form of AcnB plays a regulatory role by binding to the *acnB* mRNA (29). AcnB was reported to be demetallated upon iron depletion even under anaerobic conditions, suggesting that the Fe-S cluster of AcnB could serve as an iron sensor (30). Therefore, if the Fe-S cluster of FeoC acts as a redox sensor and/or as an iron sensor, exposure to oxygen or iron might reduce the activity (i.e., FeoB-binding ability) or the levels of FeoC, resulting in rapid elimination of FeoB by the FtsH protease. Given that *Salmonella* can be exposed to various concentrations of oxygen and iron, this scenario might be particularly important for *Salmonella* to prevent sudden Fe(II) influx when it experiences a radical environmental change, that is, a growth shift from anaerobic conditions with low iron to aerobic conditions with high iron.

ACKNOWLEDGMENTS

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by

the Ministry of Education, Science and Technology (2011-0021585 and 2012R1A2A2A01013521).

REFERENCES

- Wandersman C, Delepelaire P. 2004. Bacterial iron sources: from siderophores to hemophores. *Annu. Rev. Microbiol.* 58:611–647.
- Cartron ML, Maddocks S, Gillingham P, Craven CJ, Andrews SC. 2006. Feo—transport of ferrous iron into bacteria. *Biometals* 19:143–157.
- Kammler M, Schon C, Hantke K. 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. *J. Bacteriol.* 175:6212–6219.
- Marlovits TC, Haase W, Herrmann C, Aller SG, Unger VM. 2002. The membrane protein FeoB contains an intramolecular G protein essential for Fe(II) uptake in bacteria. *Proc. Natl. Acad. Sci. U. S. A.* 99:16243–16248.
- Kim H, Lee H, Shin D. 2012. The FeoA protein is necessary for the FeoB transporter to import ferrous iron. *Biochem. Biophys. Res. Commun.* 423:733–738.
- Boyer E, Bergevin I, Malo D, Gros P, Cellier MF. 2002. Acquisition of Mn(II) in addition to Fe(II) is required for full virulence of *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* 70:6032–6042.
- Fetherston JD, Mier I, Jr, Truszczyńska H, Perry RD. 2012. The Yfe and Feo transporters are involved in microaerobic growth and virulence of *Yersinia pestis* in bubonic plague. *Infect. Immun.* 80:3880–3891.
- Hung KW, Tsai JY, Juan TH, Hsu YL, Hsiao CD, Huang TH. 2012. Crystal structure of the *Klebsiella pneumoniae* NFeoB/FeoC complex and roles of FeoC in regulation of Fe²⁺ transport by the bacterial Feo system. *J. Bacteriol.* 194:6518–6526.
- Escobar L, Perez-Martin J, de Lorenzo V. 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. *J. Bacteriol.* 181:6223–6229.
- Bauer CE, Elsen S, Bird TH. 1999. Mechanisms for redox control of gene expression. *Annu. Rev. Microbiol.* 53:495–523.
- Jeon J, Kim H, Yun J, Ryu S, Groisman EA, Shin D. 2008. RstA-promoted expression of the ferrous iron transporter FeoB under iron-replete conditions enhances Fur activity in *Salmonella enterica*. *J. Bacteriol.* 190:7326–7334.
- Davis RW, Bolstein D, Roth JR. 1980. *Advanced Bacterial Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* 97:6640–6645.
- Soncini FC, Vescovi EG, Groisman EA. 1995. Transcriptional autoregulation of the *Salmonella typhimurium* *phoPQ* operon. *J. Bacteriol.* 177:4364–4371.
- Guzman LM, Belin D, Carson MJ, Beckwith J. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol.* 177:4121–4130.
- Sheffield P, Garrard S, Derewenda Z. 1999. Overcoming expression and purification problems of RhoGDI using a family of “parallel” expression vectors. *Protein Expr. Purif.* 15:34–39.
- Karimova G, Ullmann A, Ladant D. 2001. Protein-protein interaction between *Bacillus stearothermophilus* tyrosyl-tRNA synthetase subdomains revealed by a bacterial two-hybrid system. *J. Mol. Microbiol. Biotechnol.* 3:73–82.
- Doublet B, Douard G, Targant H, Meunier JY, Cloeckert A. 2008. Antibiotic marker modifications of lambda Red and FLP helper plasmids, pKD46 and pCP20, for inactivation of chromosomal genes using PCR products in multidrug-resistant strains. *J. Microbiol. Methods* 75:359–361.
- Karimova G, Pidoux J, Ullmann A, Ladant D. 1998. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc. Natl. Acad. Sci. U. S. A.* 95:5752–5756.
- Miller JH. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Snively MD, Gravina SA, Cheung TT, Miller CG, Maguire ME. 1991. Magnesium transport in *Salmonella typhimurium*. Regulation of *mgtA* and *mgtB* expression. *J. Biol. Chem.* 266:824–829.
- Hmiel SP, Snively MD, Florer JB, Maguire ME, Miller CG. 1989. Magnesium transport in *Salmonella typhimurium*: genetic characterization and cloning of three magnesium transport loci. *J. Bacteriol.* 171:4742–4751.
- Gur E, Biran D, Ron EZ. 2011. Regulated proteolysis in Gram-negative bacteria—how and when? *Nat. Rev. Microbiol.* 9:839–848.
- Ito K, Akiyama Y. 2005. Cellular functions, mechanism of action, and regulation of FtsH protease. *Annu. Rev. Microbiol.* 59:211–231.
- Tomoyasu T, Yuki T, Morimura S, Mori H, Yamanaka K, Niki H, Hiraga S, Ogura T. 1993. The *Escherichia coli* FtsH protein is a prokaryotic member of a protein family of putative ATPases involved in membrane functions, cell cycle control, and gene expression. *J. Bacteriol.* 175:1344–1351.
- Chiba S, Akiyama Y, Ito K. 2002. Membrane protein degradation by FtsH can be initiated from either end. *J. Bacteriol.* 184:4775–4782.
- Andrews SC, Robinson AK, Rodriguez-Quinones F. 2003. Bacterial iron homeostasis. *FEMS Microbiol. Rev.* 27:215–237.
- Mettert EL, Kiley PJ. 2005. ClpXP-dependent proteolysis of FNR upon loss of its O₂-sensing [4Fe-4S] cluster. *J. Mol. Biol.* 354:220–232.
- Tang Y, Guest JR. 1999. Direct evidence for mRNA binding and post-transcriptional regulation by *Escherichia coli* aconitases. *Microbiology* 145:3069–3079.
- Varghese S, Tang Y, Imlay JA. 2003. Contrasting sensitivities of *Escherichia coli* aconitases A and B to oxidation and iron depletion. *J. Bacteriol.* 185:221–230.
- Fields PI, Swanson RV, Haidaris CG, Heffron F. 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc. Natl. Acad. Sci. U. S. A.* 83:5189–5193.