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The FeoA protein is necessary for the FeoB transporter to import ferrous iron

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ABSTRACT

In many bacterial *feo* loci, the *feoA* gene is associated with the *feoB* gene. While the *feoB*-encoded FeoB protein has been demonstrated as a ferrous iron [Fe(II)] transporter, the function of the *feoA* gene product, FeoA, is unknown. In the present study, we report that the FeoA protein interacts with the FeoB Fe(II) transporter, which is required for FeoB-mediated Fe(II) uptake in *Salmonella enterica*. Iron uptake assay revealed that in the absence of FeoA, FeoB import of Fe(II) is impaired. Bacterial two-hybrid assay determined that the FeoA protein directly and specifically binds to the FeoB transporter *in vivo*. This FeoA-FeoB interaction appeared necessary for FeoB-mediated Fe(II) uptake because *Salmonella* expressing the mutant FeoA that cannot interact with FeoB failed to uptake Fe(II) via the FeoB transporter. Finally, we showed that the FeoA protein does not affect expression of the FeoB transporter *per se*.

1. Introduction

Iron is an essential metal for all forms of life. Bacteria transport iron into the cytoplasm via multiple routes, depending on the oxidation state of iron. Under oxic environments, iron mostly exists in an oxidized ferric form [Fe(III)]. As Fe(III) is insoluble at neutral pH, for its import, bacteria synthesize and secrete Fe(III)-specific chelators called siderophores [1]. In Gram-negative bacteria, such as *Escherichia coli*, siderophore-bound Fe(III) is recognized by the outer membrane receptors and is then transported into cells in a process that requires the TonB–ExbB–ExbD protein complex [1]. On the other hand, ferrous iron [Fe(II)], which is in a soluble and reduced form, is directly imported via Fe(II) transporters [2].

Feo (Ferrous iron transport) is the first Fe(II) transport system that has been characterized in bacteria [3]. Although Fe(II) is a major form of iron under anaerobic environments, the Feo system can also function under aerobic conditions [3]. In *E. coli*, FeoB is an inner membrane Fe(II) transporter with an N-terminal cytoplasmic domain and a C-terminal transmembrane domain [4]. The N-terminal domain of FeoB (FeoB-N) has been proposed to possess regulatory function for Fe(II) uptake. FeoB-N shares amino acid sequence similarity with eukaryotic G-proteins that harbor GTPase activity [4]. Consistent with this notion, the purified FeoB-N protein hydrolyzes GTP, and this enzymatic activity is necessary for the FeoB protein to import Fe(II) [4].

An interesting feature of the *E. coli feo* locus is that the *feoA* and *feoC* (or *yhgG*) genes, whose products are two small proteins FeoA and FeoC with unknown functions, are located up- and downstream the *feoB* gene, respectively [5]. The *feoA*, *feoB* and *feoC* genes are likely to constitute an operon. Although the presence of the *feo* locus is found in large numbers of bacterial genomes, its organization varies among bacteria [5]. In particular, the *feoC* gene seems to exist in a certain bacterial species, such as γ-proteobacteria, whereas the *feoA* gene is linked to the *feoB* gene in 80% of bacteria that possess the *feo* locus [5]. Although this finding suggests that the function of FeoA might be closely related to that of FeoB, this possibility has not been tested experimentally.

In the present study, we investigate how the FeoA protein affects FeoB function in *Salmonella enterica*. We find that the FeoA protein is necessary for the FeoB transporter to import Fe(II). Moreover, we reveal that the FeoA protein directly binds to the FeoB protein *in vivo* and that the interaction with FeoA is crucial for FeoB uptake of Fe(II).

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *S. enterica* serovar Typhimurium (*S.* Typhimurium) strains were derived from strain 14028s. Phage P22-mediated transductions were performed as described [6]. Bacteria were grown at 37 °C in Luria–Bertani (LB) medium or M9 minimal medium supplemented with 0.1% casamino acids and 10 mM glucose. Ampicillin (Ap), chloramphenicol (Cm) and kanamycin (km) were used at 50, 25 and 50 μg/ml, respectively. For induction of 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		
14028s	Wild-type	[22]
HK733	*ΔfeoA*	This study
JH362	*ΔfeoB*	[12]
HK735	*ΔfeoAB*	This study
JH380	*ΔmntH ΔsitABCD*	[17]
HK741	JH380 *ΔfeoA*	This study
HK743	JH380 *ΔfeoB*	This study
E. coli		
BTH101	F-cya-99 araD139 galE15 galK16 rpsL1 (Strr) hsdR2 mcrA1 mcrB1	[13]
Plasmids		
pUHE21	P_{lac} rep_{pMBI} Ap^R *lacI*^{q}	[8]
pFeoA	pUHE21 *feoA*	This study
pFeoA(L26Q)	pUHE21 *feoA*(L26Q)	This study
pBAD33	P_{BAD} pACYC184 *ori* Cm^R	[9]
pFeoB	pBAD33 *feoB*	This study
pHis-parallel1	P_{T7} rep_{pMBI} Ap^R	[10]
pHis-parallel1-*feoB*(N)-His6	pHis-parallel1 *feoB*(N)-His6	This study
pKD4	$repR_{6K\gamma}$ Ap^R FRT Km^R FRT	[7]
pKD46	$rep_{pSC101}{}^{ts}$ Ap^R P_{araBAD} γ β exo	[7]
pCP20	$rep_{pSC101}{}^{ts}$ Ap^R Cm^R *cI857* λP_R*flp*	[7]
pKT25	P_{lac} p15A *ori* Km^R	[11]
pUT18	P_{lac} ColEI *ori* Ap^R	[11]
pT18-feoA	pUT18 *feoA*	This study
pT18-feoA(L26Q)	pUT18 *feoA*(L26Q)	This study
pT25-feoA	pKT25 *feoA*	[23]
pT25-feoB	pKT25 *feoB*	This study
pT18-mgtA	pUT18 *mgtA*	[23]
pT18-fur	pUT18 *fur*	This study
pT18-zip	pUT18 *zip*	[11]
pT25-zip	pKT25 *zip*	[11]

from plasmids, arabinose and isopropyl 1-thio-β-D-galatoside (IPTG) were used at 10 and 0.1 mM, respectively.

2.2. Construction of bacterial strains

S. Typhimurium strains carrying a gene deletion were constructed by using the one-step gene inactivation method [7]. For deletion of the *feoA* and *feoAB* genes, the Km^R cassette from plasmid pKD4 [7] was amplified using primer pairs DEL-feoA-F/DEL-feoA-R and DEL-feoA-F/DEL-feoB-R, respectively. The resulting PCR products were integrated into the chromosome of strain 14028s as described [7]. The Km^R cassette was removed using plasmid pCP20 [7]. Deletion of the corresponding genes was verified by colony PCR. The sequences of primers used are indicated in Table S1.

2.3. Plasmid construction

Plasmid pFeoA expressing the FeoA protein from the *lac* promoter was constructed. The *feoA* gene was amplified using primer pairs EX-feoA-F/EX-feoA-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} [8].

To construct plasmid pFeoB in which the FeoB protein is expressed from the P_{BAD} promoter, the *feoB* gene was amplified using primer pairs EX-feoB-F/EX-feoB-R and chromosomal DNA of the 14028s strain. The PCR products were then introduced into the XbaI and PstI restriction sites of pBAD33 [9].

Plasmid pHis-parallel1-*feoB*(N)-His6 encodes the N-terminal domain of FeoB (FeoB-N) with a His6-tag at the N-terminus. For its construction, the *feoB* coding region corresponding to the FeoB-N was amplified by using primer pairs EX-feoB(N)-F/EX-feoB(N)-R and the 14028s chromosomal DNA as a template, which was introduced between the NcoI and NotI restriction sites of the pHis-parallel1 vector [10].

For the bacterial two-hybrid assay, plasmids pT18-feoA, pT25-feoA, pT25-feoB and pT18-fur were constructed. The *feoA*, *feoB* and *fur* genes were amplified by using primer pairs TH-feoA-18-F/TH-feoA-18-R, TH-feoA-25-F/TH-feoA-25-R TH-feoB-F/TH-feoB-R and TH-fur-F/TH-fur-R, respectively. After purification, the PCR products were introduced between the PstI and BamHI sites of the plasmid vector pKT25 [11] or pUT18 [11].

Plasmids pFeoA(L26Q) and pT18-feoA(L26Q) are derivatives of pFeoA and pT18-feoA, respectively. The former expresses a variant of FeoA with a L26Q substitution [FeoA(L26Q)], and the letter expresses the T18-FeoA(L26Q) fusion protein. These two plasmids were constructed using a QuikChange II site-directed mutagenesis kit (Stratagene) with primer pairs SM-feoA(L26Q)-F/SM-feoA(L26Q)-R and pFeoA and pT18-feoA as DNA templates, respectively.

Sequences of particular genes on the recombinant plasmids were confirmed by nucleotide sequencing. The sequences of primers used are indicated in Table S1.

2.4. Iron uptake assay

Ferrous iron uptake levels in *Salmonella* strains were determined as described [12] with appropriate modifications. Bacterial cells grown in LB to OD_{600} = 0.5 were washed with M9 medium, suspended in the same medium, and kept on ice. One ml of the cell suspension with OD_{600} = 1.0 was placed at 37 °C for 15 min, and Fe(II) transport assay was started by 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 10 min of incubation, bacterial cells were loaded to a 0.45 μm membrane filter (Whatman) placed on the 1225 Sampling Manifold (Millipore) and washed twice with 0.1 mM LiCl buffer. The membrane with bacterial cells was transferred into a vial containing 2 ml of scintillation cocktail fluid. Activity as counts per minute was determined using LS6500 Scintillation Counter (Beackman) and converted into picomoles of ^{55}Fe(II) using a standard curve.

2.5. Bacterial two-hybrid (BACTH) assay

To assess protein–protein interactions *in vivo*, a BACTH assay was conducted as described [13]. The *E. coli* BTH101 (*cya*$^-$) strain was co-transformed 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 [14].

2.6. Purification of the FeoB-N protein and preparation of anti-FeoB-N antibody

E. coli BL21(DE3) cells harboring pHis-parallel1-*feoB*(N)-His6 plasmid were grown in LB medium at 37 °C. When the culture's OD_{600} value reached ~0.5, IPTG (0.5 mM) was added to the culture for induction of the FeoB-N-His6 protein, and another 16 h of incubation was followed at 21 °C. The FeoB-N-His6 protein was purified by Ni^{2+} affinity chromatography. The cell pellet was suspended in cold buffer A [50 mM Tris (pH 8.0), 300 mM NaCl and 1 mM PMSF] and disrupted by sonication. The cell extract was bound onto a Ni–NTA column equilibrated with buffer A. After discarding the flow through fraction, the column was washed with 500 ml of washing buffer (buffer A containing 20 mM imidazole), and the adsorbed His-tagged protein was eluted with elution buffer (buffer A containing 200 mM imidazole). Finally, the eluted proteins were dialyzed with buffer A containing 10% glycerol and

stored at −80 °C. By using the purified FeoB-N protein, preparation of polyclonal rabbit anti-FeoB-N antibody was serviced by Abfrontier (Korea).

2.7. Western blot analysis

Salmonella strains were grown in 30 ml of M9 minimal medium. When OD_{600} values of the cultures reached ~0.5, bacterial cells were washed with phosphate-buffered saline (PBS), suspended in 0.5 ml of PBS and opened by sonication. Total protein concentrations were determined by the BCA method. Whole-cell lysates containing 25 μg of total protein were resolved on 12% SDS polyacrylamide gels, transferred to nitrocellulose membranes and analyzed by Western blot using anti-FeoB-N or anti-DnaK antibodies (Stressgen). Blots were developed by anti-rabbit (or anti-mouse) IgG horseradish peroxidase-linked antibody (GE Healthcare) and the ECL detection system (GE Healthcare).

3. Results

3.1. The FeoA protein is necessary for FeoB-mediated Fe(II) uptake

In numbers of the bacterial *feo* loci, the *feoA* gene is linked to the *feoB* gene [5]. While the inner membrane FeoB protein has been demonstrated as a ferrous iron [Fe(II)] transporter [3], the function of FeoA, which is predicted to be a small (i.e., ~8.5 kDa) cytoplasmic protein, is unknown.

We explored whether the FeoA protein affects FeoB-mediated Fe(II) import by direct measurement of Fe(II) uptake in *Salmonella*. Other than FeoB, *Salmonella* can also import Fe(II) via the SitABCD and MntH transporters [15,16]. Therefore, to assess Fe(II) import via the FeoB transporter precisely, we initially employed the strain JH380, where both the *sitABCD* and *mntH* genes were deleted [17]. Introduction of *feoB* deletion into the JH380 strain reduced its Fe(II) uptake levels by ~3-fold (Fig. 1A). Lack of the *feoA* gene conferred the *feoB*$^-$-like phenotype to *Salmonella*: in the JH380 strain carrying *feoA* deletion, the Fe(II) uptake levels were as low as those in the strain with *feoB* deletion (Fig. 1A). This defective phenotype on Fe(II) uptake was due to the FeoA function because heterologous expression of FeoA from the *lac* promoter increased Fe(II) uptake levels similar to those displayed by the JH380 strain (Fig. 1A).

We next wanted to test whether the effect of FeoA on Fe(II) uptake is dependent on FeoB. For this, we constructed the two compatible plasmids, pFeoA and pFeoB, where the FeoA and FeoB proteins are expressed from the *lac* and P_{BAD} promoters, respectively. The Fe(II) uptake levels were ~3-fold lower in the *feoAB* deletion strain expressing FeoB alone than in the wild-type strain (Fig. 1B). In contrast, co-expression of FeoA and FeoB increased Fe(II) uptake of the *feoAB* deletion mutant to a little higher than in the wild-type (Fig. 1B). These results indicated that both the FeoA protein and FeoB transporter were necessary for normal Fe(II) uptake in *Salmonella*. The FeoA function in Fe(II) import required the FeoB transporter because expression of the FeoA protein alone did not affect Fe(II) uptake of the *feoAB* deletion strain (Fig. 1B). Cumulatively, these results demonstrate that the FeoA protein is required for the FeoB transporter to import Fe(II).

3.2. The FeoA protein interacts with the FeoB transporter in vivo

We hypothesized that the FeoA protein might contribute to FeoB-mediated Fe(II) uptake via direct protein–protein interaction. Therefore, we explored whether the FeoA protein could bind to the FeoB transporter by employing a bacterial two-hybrid (BACTH) system. In the BACTH experiment, two proteins of interest are expressed as forms fused to the T18 and T25 catalytic domains of *Bordetella pertussis* adenlyate cyclase, respectively, and interaction between the two proteins can be assessed by complementation of adenylate cyclase activity in the *cya*$^-$ *E. coli* strain BTH101 [13].

Co-expression of the T18-FeoA and T25-FeoB fusion proteins conferred the Lac$^+$ phenotype to the BTH101 strain that had displayed the Lac$^-$ phenotype upon co-expression of the T18 and T25 domains only (Fig. S1). Consistent with this, the BTH101 strain co-expressing T18-FeoA and T25-FeoB produced ~23-fold higher levels of β-galactosidase than the control co-expressing T18 and T25 (Fig. 2). These results indicated that in the BTH101 strain, the FeoA and FeoB proteins interacted and recovered their adenylate cyclase activity, resulting in cAMP-dependent expression of the *lacZ* gene. To further validate the FeoA-FeoB interaction, we exam-

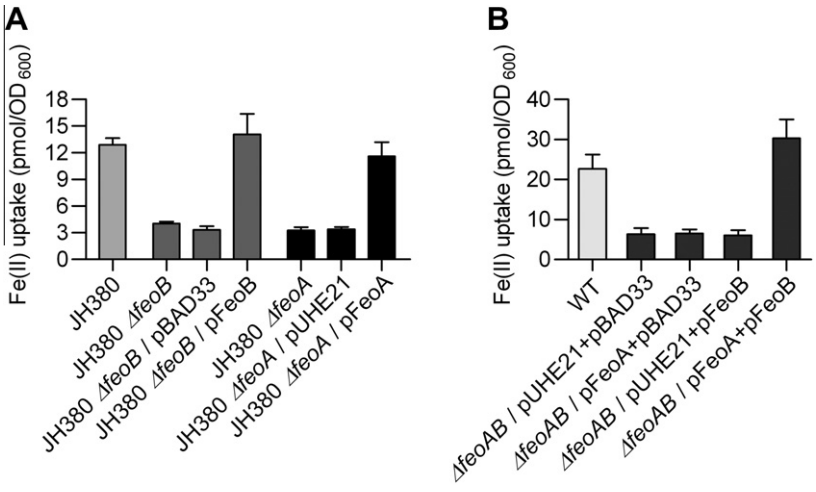

Fig. 1. The FeoA protein is necessary for the FeoB transporter to import Fe(II). ^{55}Fe(II) uptake levels were determined in *Salmonella* strains that were grown in M9 medium. Bacterial strains used in the experiments were (A) JH380 which is deleted for the *mntH* and *sitABCD* genes, JH380 with *feoB* deletion (JH380Δ*feoB*, HK743), HK743 carrying pBAD33 (JH380Δ*feoB*/pBAD33) or pFeoB (JH380Δ*feoB*/pFeoB), JH380 with *feoA* deletion (JH380Δ*feoA*, HK741), HK741 carrying pUHE21 (JH380Δ*feoA*/pUHE21) or pFeoA (JH380Δ*feoA*/pFeoA); (B) wild-type strain (WT, 14028s) and *feoAB* deletion strains that carried sets of two compatible plasmids, pUHE21 + pBAD33, pFeoA + pBAD33, pUHE21 + pFeoB and pFeoA + pFeoB, respectively. IPTG (0.1 mM) and arabinose (10 mM) were added to the bacterial cultures for induction of FeoA and FeoB from pFeoA and pFeoB plasmids. The mean values and standard deviations of three independent experiments are shown.

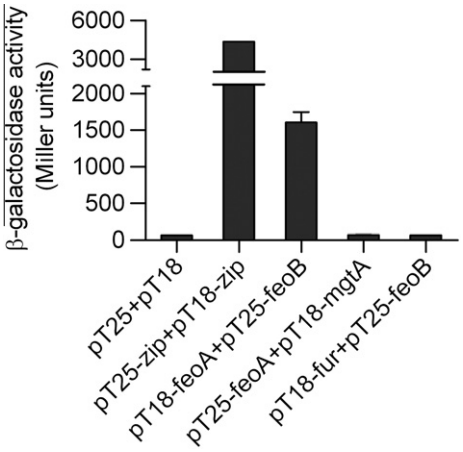

Fig. 2. The FeoA protein interacts with the FeoB transporter *in vivo*. A bacterial two-hybrid (BACTH) assay [13] was conducted to assess protein–protein interactions. β-galactosidase activity (Miller units) was determined in *E. coli* BTH101 strains harboring sets of BACTH plasmids pT25 + pT18, pT25-zip + pT18-zip, pT18-feoA + pT25-feoB, pT25-feoA + pT18-mgtA and pT18-fur + pT25-feoB. The mean values and standard deviations of three independent experiments are shown.

ined the interaction between the FeoA protein and the MgtA Mg^{2+} transporter and also between the Fur regulator and the FeoB transporter. However, in these two sets of experiments, the BTH101 strains expressed β-galactosidase to levels similar to the control (Fig. 2) and displayed the Lac⁻ phenotype (Fig. S1). This finding suggested that the FeoA protein exclusively binds to the FeoB transporter. The results of the BACTH experiment were further supported by the reproduction of the known protein–protein interaction between the Zip proteins (Fig. 2 and Fig. S1) [11]. Cumulatively, our results suggest that the FeoA protein directly and specifically binds to the FeoB transporter *in vivo*.

3.3. Identification of mutations on the FeoA protein that prevent the FeoA–FeoB interaction

We wanted to investigate whether the FeoA–FeoB interaction correlates to FeoB-mediated Fe(II) uptake. To this end, we searched for amino acid substitutions on the FeoA protein that prevent the interaction between FeoA and FeoB by employing the BACTH screening method. We first created random mutations on the portion of the *feoA* gene on the BACTH plasmid pT18-feoA by using error-prone PCR. Mutant pT18-feoA plasmids were then introduced into the BTH101 strain harboring pT25-feoB, and Lac⁻ colonies were selected. In two mutant pT18-feoA plasmids that conferred the Lac⁻ phenotype to the BTH101 strain with pT25-feoB, one possessed P20A and L26Q substitutions and another possessed W9G and L26Q substitutions on the *feoA* gene (Fig. S2). Consistent with the phenotype, in the BTH101 strain that harbored pT25-feoB and any of the two mutant pT18-feoA plasmids, the β-galactosidase activity levels were ~28-fold lower than those in the strain that carried the pT25-feoB and wild-type pT18-feoA plasmids (Fig. 3A).

As the L26Q mutation commonly existed in the two mutant plasmids, we reasoned that the L26 amino acid residue might be crucial for FeoA interacting with FeoB. To test this, by conducting site-directed mutagenesis, we constructed a derivative of pT18-feoA expressing the T18-FeoA fusion protein with a L26Q substitution on the portion of FeoA [i.e., T18-FeoA(L26Q)]. The BTH101 strain co-expressing the T18-FeoA(L26Q) and T25-FeoB proteins displayed the Lac⁻ phenotype (Fig. S2) and produced β-galactosidase in amounts similar to the control (Fig. 3A). This result

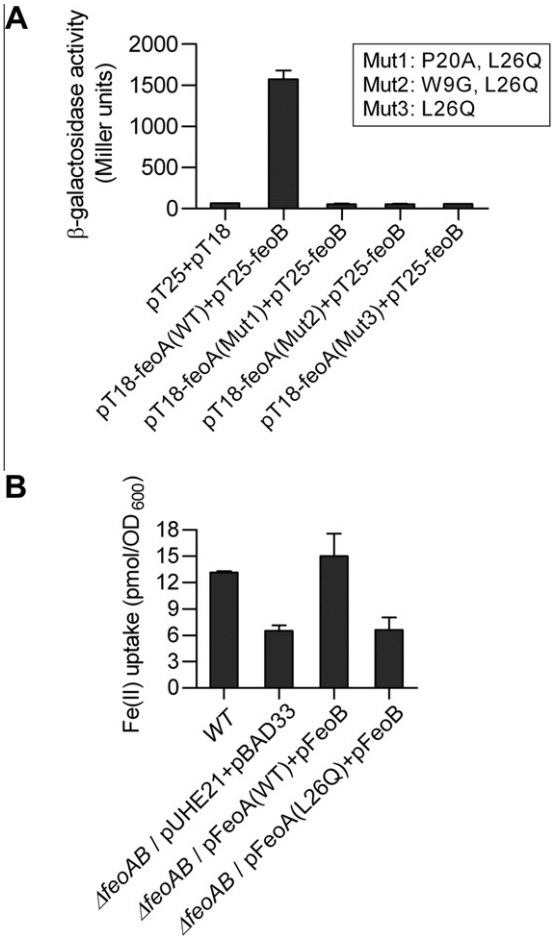

Fig. 3. Interaction between FeoA and FeoB is necessary for FeoB-mediated Fe(II) uptake. (A) *In vivo* interactions between the derivatives of FeoA and FeoB protein were examined using the BACTH assay. β-Galactosidase activity (Miller units) was determined in *E. coli* BTH101 strains harboring sets of plasmids pT25 + pT18, pT18-feoA(WT) + pT25-feoB, pT18-feoA(Mut1) + pT25-feoB, pT18-feoA(Mut2) + pT25-feoB and pT18-feoA(Mut3)+pT25-feoB. In T18 fusion plasmids, feoA(WT), feoA(-Mut1), feoA(Mut2) and feoA(Mut3) encode wild-type FeoA, mutants FeoA with P20A and L26Q substitutions, W9G and L26Q substitutions and an L26Q substitution, respectively. (B) $^{55}Fe(II)$ uptake levels were determined in wild-type (WT, 14028s) strain and *feoAB* deletion strains carrying sets of two plasmids, pUHE21 + pBAD33, pFeoA(WT) + pFeoB and pFeoA(L26Q) + pFeoB, respectively. pFeoA(WT) and pFeoA(L26Q) express wild-type FeoA and mutant FeoA with an L26Q substitution, respectively. Strains were grown in M9 medium, and IPTG (0.1 mM) and arabinose (10 mM) were added to the cultures of strains carrying plasmids. The mean values and standard deviations of three independent experiments are shown.

suggests that a single L26Q amino acid substitution on FeoA is sufficient to abolish the FeoA–FeoB interaction.

3.4. Salmonella expressing the mutant FeoA protein that cannot interact with FeoB fails to uptake Fe(II) via the FeoB transporter

We next explored how the mutation of the FeoA protein that prevents its interaction with FeoB affects the FeoA function in FeoB-mediated Fe(II) uptake. We constructed a derivative of pFeoA expressing a variant of FeoA with an L26Q amino acid substitution [i.e., FeoA(L26Q)]. In the *feoAB* deletion strain co-expressing the FeoA(L26Q) protein and the FeoB transporter, the Fe(II) uptake levels were ~2.5-fold lower than the wild-type levels (Fig. 3B). This result was in contrast to the observation that the *feoAB* deletion mutant co-expressing the wild-type FeoA and FeoB proteins imported Fe(II) as efficiently as the wild-type strain (Fig. 3B).

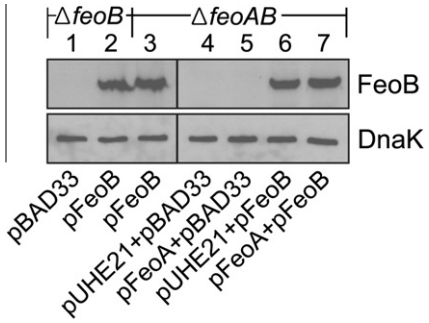

Fig. 4. Expression of the FeoB transporter is unaffected by the FeoA protein. By conducting Western blot analysis, FeoB protein levels were determined in *feoB* deletion (Δ*feoB*, JH362) strains carrying pBAD33 and pFeoB plasmids respectively (lanes 1 and 2), *feoAB* deletion (Δ*feoAB*, HK735) strain carrying pFeoB plasmid (lane 3) and the HK735 strains carrying sets of two plasmids, pUHE21 + pBAD33, pFeoA + pBAD33, pUHE21 + pFeoB and pFeoA + pFeoB, respectively (lanes 4–7). Bacteria were grown in M9 medium. Expression of the FeoA and FeoB proteins was induced by IPTG (0.1 mM) and arabinose (10 mM), respectively. The DnaK protein served as a control.

Cumulatively, these results suggest that the FeoB transporter interacts with the FeoA protein to import Fe(II).

3.5. The FeoA protein does not affect expression levels of the FeoB transporter

We explored the possibility that the failure of Fe(II) uptake observed in the *Salmonella* strains lacking the *feoA* gene (Fig. 1) might result from reduced amounts of the FeoB transporter in the absence of the FeoA protein. Western blot analysis determined that levels of the FeoB protein, which was expressed from the pFeoB plasmid, were similar in both the *feoB* and *feoAB* deletion strains (Fig. 4, compare lanes 2 and 3). Moreover, in the *feoAB* deletion strain, the FeoA protein expressed from the pFeoA plasmid did not change levels of the FeoB protein from the pFeoB plasmid (Fig. 4, compare lanes 6 and 7). Therefore, these results indicate that the FeoA protein does not affect the levels of the FeoB transporter and thus suggest that the FeoA function could contribute to the FeoB activity of importing Fe(II).

4. Discussion

The *feoA*, *feoB* and *feoC* genes in *E. coli* appear to constitute the *feoABC* operon [5]. It has been reported that an *E. coli feoA*::Tn*5* mutant displays reduced Fe(II) uptake ability compared to its parental *feoA*$^+$ strain [3]. Although this observation implies that the FeoA protein might be involved in Fe(II) uptake, it is still possible that the phenotype is due to the polar effect of the *feoA*::Tn*5* mutation on *feoB* expression. In the present study, our results provide direct evidence that the FeoA protein is required for FeoB-mediated Fe(II) transport in *S. enterica*. First, the strain lacking the *feoA* gene was defective for Fe(II) uptake, but recovered this function with FeoA expression from a plasmid (Fig. 1A). And second, in the mutant strain lacking both the *feoA* and *feoB* genes, the wild-type levels of Fe(II) uptake were accomplished by co-expression of FeoA and FeoB but not by sole expression of FeoA or FeoB (Fig. 1B).

How could the FeoA protein contribute to Fe(II) uptake via the FeoB transporter? The FeoB protein is composed of the N-terminal cytoplasmic domain and the C-terminal transmembrane domain. The FeoB N-terminus has been demonstrated as a G-protein that possesses GTPase activity [4]. GTP binding seems to be essential for FeoB function because a mutation on the predicted GTP binding motif abolished the FeoB-mediated Fe(II) uptake [4]. Similar to eukaryotic G-proteins, the FeoB G-protein possesses weak GTPase activity and displays very slow GTP hydrolysis [4]. On the basis of this finding, it has been proposed that if the FeoB protein is energized by GTP hydrolysis to transport Fe(II), a factor that can promote FeoB's GTPase activity might exist [5,18]. In eukaryotic systems, GTPase activating proteins (GAPs) directly interact with G-proteins and stimulate their GTPase activity [19]. Although the presence of such GAPs is unknown in bacteria, our results raise the possibility that the FeoA protein might target the GTPase activity of FeoB. The FeoA protein directly bound to the FeoB transporter *in vivo* (Fig. 2), and this protein–protein interaction was necessary for FeoB-mediated Fe(II) uptake (Fig. 3). Moreover, given that FeoA was not necessary for FeoB expression (Fig. 4), the FeoA protein is likely to control the activity of FeoB.

In 80% of bacteria that are predicted to possess the *feo* genes, the FeoA protein seems to constitute the Feo system together with the FeoB transporter [5]. Interestingly, the FeoA protein shares amino acid sequence similarity with the Src-Homology 3 (SH3) domain of DtxR [5]. Although the amino acid homology between FeoA and DtxR is weak [5], the presence of the SH3 domain-like fold in FeoA is further evidenced by the tertiary structure of FeoA from *Stenotrophomonas maltophilia* [20]. In eukaryotes, SH3 domains are present in many signaling proteins including GAPs and known to mediate protein–protein interactions [19]. An L26 residue of FeoA was found to be crucial for FeoA interacting with FeoB and mediating FeoB-dependent Fe(II) uptake (Fig. 3). Notably, in comparison of the amino acid sequences between the FeoA proteins and DtxR SH3 domains, both from various bacterial origins, the FeoA L26 residue was highly conserved [5]. Therefore, all of these lines of evidence suggest that the FeoA protein resembles eukaryotic GAPs, thus supporting the hypothesis that the FeoA protein acts as a modulator for the FeoB's G-protein function.

What is the biological significance of FeoA regulation of FeoB-mediated Fe(II) uptake? Fe(II) overload in the cytoplasm is highly toxic to cells living with oxygen. To avoid such circumstances, the Fur regulator represses expression of iron uptake systems including Feo under iron-replete conditions [12,21]. Together with this gene expression control, as discussed by Cartron et al. [5], if the FeoA protein modulates the FeoB activity in response to intracellular Fe(II) levels, this mechanism can prevent an excess of Fe(II) influx and thus protect cells from Fe(II)-mediated toxicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.027.

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