

The *Bradyrhizobium japonicum* *frcB* Gene Encodes a Diheme Ferric Reductase^{∇†}

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Iron utilization by bacteria in aerobic environments involves uptake as a ferric chelate from the environment, followed by reduction to the ferrous form. Ferric iron reduction is poorly understood in most bacterial species. Here, we identified *Bradyrhizobium japonicum frcB* (bl13557) as a gene adjacent to, and coregulated with, the *pyoR* gene (blr3555) encoding the outer membrane receptor for transport of a ferric pyoverdine. FrcB is a membrane-bound, diheme protein, characteristic of eukaryotic ferric reductases. Heme was essential for FrcB stability, as were conserved histidine residues in the protein that likely coordinate the heme moieties. Expression of the *frcB* gene in *Escherichia coli* conferred ferric reductase activity on those cells. Furthermore, reduced heme in purified FrcB was oxidized by ferric iron *in vitro*. *B. japonicum* cells showed inducible ferric reductase activity in iron-limited cells that was diminished in an *frcB* mutant. Steady-state levels of *frcB* mRNA were strongly induced under iron-limiting conditions, but transcript levels were low and unresponsive to iron in an *irr* mutant lacking the global iron response transcriptional regulator Irr. Thus, Irr positively controls the *frcB* gene. FrcB belongs to a family of previously uncharacterized proteins found in many proteobacteria and some cyanobacteria. This suggests that membrane-bound, heme-containing ferric reductase proteins are not confined to eukaryotes but may be common in bacteria.

Iron is essential for the growth and survival of most organisms. The metal is required for many cellular processes or incorporated into heme and iron sulfur clusters that function as cofactors of proteins. Iron exists in the insoluble ferric form in neutral or alkaline pH environments (6), and therefore, organisms have evolved highly specific systems for iron acquisition from their natural environments. However, too much iron can lead to the production of reactive oxygen species, which damage proteins and DNA (6). Thus, iron acquisition, storage, and usage are tightly regulated (1).

Siderophores are high-affinity compounds produced and secreted by microorganisms to chelate ferric iron from the environment (7). Many microorganisms produce siderophores, and the siderophores from *Escherichia coli*, *Pseudomonas putida*, *Pseudomonas aeruginosa* (7), and the smut fungus *Ustilago maydis* (39) have been particularly well characterized. In Gram-negative bacteria, Fe³⁺-siderophores are bound by TonB-dependent receptors on the outer membrane (17). TonB, ExbB, and ExbD transfer energy from a proton motive force of the inner membrane to the receptor on the outer membrane. The siderophore-specific receptor transports the ferric iron-bound siderophore into the periplasm. The siderophore is then bound by a periplasmic binding protein and delivered to an ABC transporter on the cytoplasmic membrane (5) for transport into the cytoplasm (10). Incorporation of the bound ferric iron into cellular components requires its release from the siderophore and reduction to the ferrous (Fe²⁺)

form. These latter steps are less well understood than the systems that transport iron into the cell.

Ferric reductase activity has been demonstrated in whole cells or cell fractions in many bacterial species (31), but the proteins responsible for this activity have been identified only in a few cases (21, 32, 40). Most bacterial assimilatory ferric reductases are soluble and require flavin for activity. An exception is FhuF, an iron-sulfur cytoplasmic protein that reduces ferric ferrioxamine B (21).

Unlike bacterial ferric reductases, the eukaryotic enzymes are membrane-bound diheme proteins, some of which also contain flavin (9, 22, 28). Dancis et al. (9) first linked ferric reductase activity to ferric uptake in the yeast *Saccharomyces cerevisiae*. This activity is catalyzed by the heme- and flavin-containing proteins Fre1 and Fre2. The mammalian heme protein Dcytb is expressed under iron-limiting conditions in duodenal cells (22). Homologs of Dcytb are found in other mammalian cell types as well as other eukaryotic species (3, 4, 13, 36, 42). Although Fre1 and Dcytb have been well described and are necessary for ferric iron reduction in whole cells, ferric reductase activity has not been demonstrated in the purified proteins.

Bradyrhizobium japonicum exists as a free-living organism in the soil or as a symbiont of soybean. It is a member of the *Alphaproteobacteria*, a taxonomic group that includes symbionts, pathogens, and photosynthetic organisms. Regulation of iron metabolism differs substantially in *B. japonicum* and in the proteobacteria in general compared with other well-studied model organisms as described below. Moreover, *B. japonicum* can utilize ferric siderophores produced by other organisms as an iron source, but it does not synthesize them. This may make *B. japonicum* more competitive in the rhizosphere. Ferrichrome and rhodotorulic acid are siderophores secreted by fungi in the soil and are utilized by *B. japonicum* (27, 34). Five

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putative siderophore receptor genes have been identified in *B. japonicum* based on homology and on induction of their transcript levels under iron-limiting conditions (34, 41). The cognate siderophore has been identified for one receptor, ferrichrome, but the others are unknown (19, 34). The other components involved in ferric iron transport into cells and its subsequent reduction to the ferrous form have not been described in *B. japonicum*.

Induction of *B. japonicum* ferric siderophore receptors is dependent on the iron response regulator (Irr) protein, which is the major regulator of iron homeostasis in *B. japonicum* and in numerous other alphaproteobacteria (16, 33). This is markedly different from Fur-dependent iron regulation in *E. coli* and many other well-studied bacteria. The Fur protein is functional as a transcriptional repressor when bound to iron. Irr accumulates and functions under iron-limiting conditions and degrades in the presence of iron in response to heme. Whole-genome microarray analysis shows that Irr functions as a global regulator of iron homeostasis by upregulating genes involved in iron acquisition and downregulating genes encoding iron-containing proteins (29, 41). Irr binds to the iron control element (ICE), a *cis*-acting element in the promoters of target genes (25, 29, 30, 34).

In the present study, we identify a gene necessary for ferric iron reduction in *B. japonicum* that encodes a diHEME membrane protein, features characteristic of eukaryotic ferric reductases. In addition, homologs of this protein are widely distributed among the proteobacteria.

MATERIALS AND METHODS

Strains and media. *Bradyrhizobium japonicum* strain LO is a spontaneous nalidixic acid-resistant derivative of strain USDA122. LO was the parent strain used in the present work. All mutants used in this study are derivatives of the parent strain LO (14, 34). A mutant disrupted in the *blj3557* (*frcB*) gene was made by replacing the open reading frame (ORF) with the Ω cassette, which confers resistance to streptomycin and spectinomycin, as described previously (34). *B. japonicum* strains were routinely grown at 29°C in GSY medium (11). Strain LODTM5 was grown in medium supplemented with 50 μ g/ml kanamycin and 50 μ g/ml streptomycin. The mutants constructed in this study were grown in medium supplemented with 50 μ g/ml streptomycin and 100 μ g/ml spectinomycin. For the iron experiments, modified GSY medium was used, which contains 0.5 g/liter yeast extract instead of 1 g/liter, and either no exogenous iron was added for low-iron medium or 12 μ M FeCl₃ · 6H₂O was added for high-iron medium. The actual iron concentration of the unsupplemented medium was 0.3 μ M, as determined with a Perkin-Elmer model 1100B atomic absorption spectrometer.

E. coli strains were grown in standard LB medium (10 g/liter tryptone, 5 g/liter yeast extract, and 5 g/liter NaCl) or YT medium (8 g/liter tryptone, 5 g/liter yeast extract, and 5 g/liter NaCl). Strain S905 is a *hemA* deficient derivative of strain DK905 (18) and requires media supplemented with 40 μ g/ml δ -aminolevulinic acid (ALA). Strains harboring pBluescript SK+ or pET14b plasmid derivatives were grown in media supplemented with 100 μ g/ml ampicillin.

Construction of plasmids. All primers used are listed in Table S1 in the supplemental material. The open reading frame of *blj3557* (*frcB*) was obtained by PCR using genomic DNA as the template. The forward primer included the NdeI restriction site, and the reverse primer included the XhoI site. The product was ligated into the NdeI and XhoI sites of the pET14b expression vector, yielding pET14b-3557 (pET14b with the open reading frame of *blj3557*). The plasmid was transformed into *E. coli* strain C43(DE3) for overexpression. *E. coli* C43(DE3) is more tolerant to expression of toxic or membrane proteins and was purchased from Lucigen Corporation (Middleton, WI).

pET14b-3557 was digested with XbaI and XhoI to release the ORF plus the plasmid-borne His tag and ribosome binding site. The fragment was cloned into the XbaI and XhoI sites of pBluescript SK+ for expression of the gene under the control of the plasmid-borne *lac* promoter in *E. coli* strain DH5 α . The plasmid and empty vector were transformed into *E. coli* DH5 α .

Alignment of annotated cytochromes b561 from diverse organisms. Homologs from the following species were compared (NCBI accession numbers are shown in parentheses): *Bradyrhizobium japonicum* (NP_770197.1) *Burkholderia glumae* (YP_002907809), *Shewanella oneidensis* (NP_718925), *E. coli* (AP_002041.1), *Brucella abortus* (YP_222970.1), *Neisseria gonorrhoeae* (YP_002002877.1), *Synechocystis* sp. strain PCC 6803 (NP_440882.1), *Helicobacter pylori* (NP_207427.1), and *Desulfuromonas acetoxidans* (ZP_01313790.1).

Disk assay. Utilization of various siderophores as iron sources by wild-type and mutant *B. japonicum* bacteria was assessed as growth on solid, modified GSY medium containing 25 μ M EDDHA (ethylenediamine-di-*o*-hydroxyphenylacetic acid). Six-millimeter Whatman paper disks that had been presoaked with siderophores, 10 mM FeCl₃, or double-distilled water were placed in the center of the plates. Cells (1×10^5) cells of strains LO, LODTM5, and mutants defective in the ferric siderophore receptor-encoding genes were spotted 3.3 cm away from the disk and visually assessed for growth. The siderophores were a generous gift from J.-M. Meyer (Université Louis-Pasteur, Strasbourg, France) and are listed in Table S2 in the supplemental material.

Overexpression and purification of FrcB. Five milliliters of a culture of *E. coli* C43(DE3) harboring pET14b-3557 grown overnight was used to inoculate 1 liter of YT medium. One-liter cultures were incubated with shaking (225 rpm) at 37°C until mid-log phase, at which time 0.5 mM IPTG (isopropyl-1-thio- β -D-galactopyranoside) was added to induce protein expression. Cultures were shaken at 37°C for 4 additional hours. Cells were harvested by centrifugation at $10,000 \times g$, washed with 100 mM Tris (pH 7.5), and resuspended in 10 ml Tris, 1 mM phenylmethylsulfonyl fluoride, and 25.5 μ g aprotinin per 5 g of cells. Cells were disrupted by passage twice through a French pressure cell at 1,200 lb/in². The lysate was clarified by centrifugation at $4,000 \times g$, solubilized by gentle rocking at 4°C for 1 h in 5% glycerol, 150 mM NaCl, and 1.2% dodecyl maltoside (DM) (Thermo Fisher Scientific), and further clarified by centrifugation for 90 min at $45,000 \times g$. The cleared lysate was added to 1 ml of a 50% nickel-nitrilotriacetic acid (Ni-NTA) slurry (Qiagen Inc., Valencia, CA) in a sealed column, and rocked for 75 min at 4°C. The Ni-nitrilotriacetic acid slurry-protein mixture was washed four times with 5 ml of phosphate wash buffer (50 mM NaH₂PO₄ [pH 8.0], 300 mM NaCl, 20 mM imidazole, 0.2% DM) and once with 5 ml of phosphate wash buffer containing 10% glycerol. Purified His-tagged proteins were eluted with phosphate elution buffer (50 mM NaH₂PO₄ [pH 8.0], 300 mM NaCl, 250 mM imidazole, 10% glycerol, 0.2% DM). Eluted protein fractions were dialyzed twice, for 10 h each time against 400 ml buffer containing 100 mM Tris (pH 7.5), 10% glycerol, and 0.2% DM. The purified proteins were stored at -80°C.

Absorption spectroscopy and heme quantification. Ferrous heme bound to protein was determined by measuring the absorption of 8 μ M purified, recombinant protein in 100 mM Tris (pH 7.5) after the addition of several crystals of sodium dithionite, which reduced ferric heme to ferrous heme. All spectra were recorded between 360 and 600 nm on a SpectraMax MS spectrophotometer (Molecular Devices).

Quantification of heme was performed using the pyridine hemochromagen method (35). A 500- μ l sample contained 8 μ M purified, recombinant protein, 100 mM NaOH, and 8.5 μ l pyridine. Dithionite crystals were added to reduce the heme. A cuvette containing no protein was used as a reference. The heme-to-protein ratio was calculated using a published millimolar molecular extinction coefficient of 192 at 419 nm.

Mutagenesis of FrcB. Mutations were made by single-round PCR in pSKBluescript-3557 (pBluescript SK+ with the open reading frame of *blj3557*) using complementary primers containing the nucleotide changes. The H20, H55, H148, and H169 codons were changed from CAC to the alanine (Ala) codon GCC. The H162 codon was changed from CAC to GCG. PCR products were digested with DpnI to remove the parent plasmids and transformed into *E. coli* DH5 α . Nucleotide mutations were confirmed by sequencing.

Measurement of the accumulation of FrcB and mutant derivatives in E. coli. Two hundred fifty microliters of stationary-phase, overnight culture of *E. coli* DH5 α harboring pSKBluescript-3557 (wild-type pSKBluescript-3557 or mutant plasmids with histidine mutations) was used to inoculate 50 ml of YT medium. Cultures were grown to mid-log phase with shaking at 37°C, followed by the addition of 0.5 mM IPTG and continued incubation with shaking for 4 h. To investigate heme-dependent protein stability, pSKBluescript-3557 was transformed into the heme-deficient *E. coli* strain S905. *E. coli* S905 harboring pBluescript-3557 was grown overnight in 5 ml LB medium plus different concentrations of 5-aminolevulinic acid as stated in the text. We determined empirically that supplementation with 5 μ g/ml of ALA was the minimum concentration that allowed growth of the heme auxotroph S905. Growth of the mutant with 5 μ g/ml ALA lagged by about two doubling times compared to cells grown with 25 μ g/ml ALA. In both sets of experiments, cells were harvested by centrifugation. Sam-

ples were mixed with 2× sodium dodecyl sulfate (SDS) sample buffer (20 mM sodium phosphate, 20% glycerol, 4% SDS, 0.2 M dithiothreitol, and 0.001% bromophenol blue) and boiled for 10 min. Aliquots were loaded onto a 15% SDS-polyacrylamide gel, the gel was run for 1 h at 160 V, and the contents of the gel were transferred to a polyvinylidene difluoride (PVDF) membrane and probed with a horseradish peroxidase (HRP)-conjugated, nickel-charged probe which has high affinity to the histidine (His) tag, followed by chemiluminescence and visualized by exposure to X-ray film. Probing with antibodies against GroEL (StressGen, Vancouver, Canada) was performed by standard immunoblot procedures (12).

Whole-cell ferric reductase assay. *E. coli* strain DH5 α cells harboring pBluescript-3557 or empty vector were grown in 500 ml LB medium overnight to stationary phase. Two liters of *B. japonicum* strain LO and mutants were grown to mid-log phase in either iron-limiting or 12 μ M FeCl₃ · 6H₂O conditions in modified GSY medium as indicated in the text. Cells were harvested by centrifugation at either 8,000 \times g (*E. coli*) or 9,500 \times g (*B. japonicum*), washed twice with double-distilled H₂O, and resuspended in 100 mM Tris (pH 7.5) to a concentration of 1 \times 10¹⁰ cells/ml. Ferric reductase activity was measured in cells using a modified version of the protocol of Dailey and Lascelles (8). Ferrozine (125 mM), FeCl₃ (100 mM), succinate (100 mM), and iron-nitritotriacetic acid (Fe-NTA) (100 mM) were made fresh in 100 mM Tris buffer (pH 7.5). Fe-NTA stock was made by first dissolving 1.64 g NaHCO₃ in 80 ml double-distilled H₂O, followed by the addition of 1.91 g NTA and 2.7 g FeCl₃ · 6H₂O. Double-distilled H₂O was added to bring the final volume to 100 ml. One-milliliter *E. coli* reaction mixtures contained 7.5 \times 10⁹ cells, 200 μ M FeCl₃, 100 μ M succinate, and 1 mM ferrozine. One milliliter of the *B. japonicum* reaction mixture contained 7.5 \times 10⁹ cells, 200 μ M Fe-nitritotriacetic acid, and 1 mM ferrozine. Reactions were started by the addition of whole cells, blanked against cells harboring empty vector (*E. coli*) or parent strain LO (*B. japonicum*) at time zero. The reaction mixtures were then incubated in a 37°C water bath, and the absorbance at 562 nm was measured over time. Ferrous ferrozine produced was calculated using the ferrozine molar extinction coefficient 27.9 mM⁻¹ cm⁻¹ at 562 nm.

Iron-dependent oxidation of FrcB. Oxidation of FrcB was discerned spectrophotometrically by comparing the reduced sample to that oxidized by FeCl₃. Dithionite (final concentration of 200 μ M) was added to 20 μ M protein. Fifty microliters of the reduced protein was then aliquoted into the reaction mixture containing 500 μ M FeCl₃ or air-saturated buffer in a final volume of 500 μ l and a final FrcB concentration of 2 μ M. The reduced spectrum between 390 and 600 nm was recorded immediately. The oxidizing reaction mixture was incubated at 29°C for 30 min before the spectrum was recorded and then subtracted from the reduced spectrum. A background spectrum of dithionite plus FeCl₃ minus FeCl₃ in the absence of protein was subtracted from the reduced minus FeCl₃-oxidized spectrum. This was done to eliminate spectral features of oxidized FeCl₃ below 500 nm.

Determination of steady-state mRNA levels. Parent strain LO, *irr*, or blr3557 mutant cells were grown under iron-limiting and iron-replete conditions. RNA extraction, cDNA synthesis, and PCR were carried out as described previously (34). Primers were constructed to amplify a region within the open reading frame. *gapA* is a housekeeping gene which is not iron or Irr responsive. The data are expressed as the relative starting quantities (SQ) of the mRNA normalized to *gapA* and presented as the averages plus standard deviations for three samples.

5' RACE. The transcription start site for *frcB* was determined by rapid amplification of 5' cDNA ends (5' RACE) using a kit from Invitrogen (Carlsbad, CA) according to the manufacturer's instructions. RNA was prepared from cells grown in iron-limited media, where the genes of interest were expressed at high levels.

RESULTS

Identification of outer membrane receptors necessary for utilization of rhodotorulic acid and pyoverdine PL-8 as iron sources. Previous work identified five putative outer membrane ferric siderophore receptor genes based on homology and on their induction under iron-limiting conditions (34, 41). However, these data cannot accurately predict which siderophores the receptors bind and transport into the periplasm. Moreover, *B. japonicum* does not contain genes that encode recognizable siderophore synthesis proteins, and thus, the genome does not yield clues to the identity of the receptors.

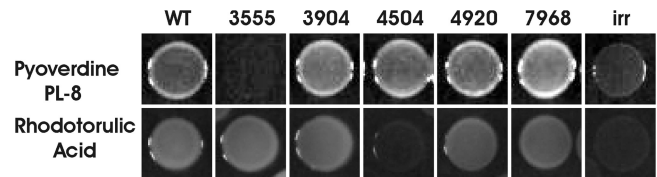


FIG. 1. Growth of wild-type (WT) and putative outer membrane receptor mutants on pyoverdine PL-8 or rhodotorulic acid as the sole iron source. The parent *B. japonicum* strain LO, *irr* mutant strain, and the ferric siderophore receptor mutants, defective in the blr3555 (3555), blr3904, blr4504, blr4920, or blr7968 genes were spotted on plates where the sole iron source was pyoverdine PL-8 or rhodotorulic acid, and the ability of the strains to grow was assessed visually.

Among the five putative proteins, only Blr4920 was identified as a ferrichrome receptor (19, 34). Rhodotorulic acid was shown previously to be an iron source for *B. japonicum* (27). Thus, we tested the ability of mutants defective in each of the receptor genes to grow on rhodotorulic acid as an iron source (Fig. 1). To do this, a paper filter disk containing rhodotorulic acid was placed at the center of an agar medium plate, and cells of the wild type or mutant strain were spotted onto the plate 3.3 cm from the disk and allowed to grow at 29°C. Whereas the parent strain grew with rhodotorulic acid, the blr4504 mutant strain did not. The wild type and all mutants grew with FeCl₃ as an iron source (data not shown). Thus, Blr4504 is very likely the rhodotorulic acid receptor. We designated the blr4504 gene *fhuE* in keeping with the *E. coli* name for the gene encoding the rhodotorulic acid receptor and because Blr4504 is the *B. japonicum* protein with the highest homology with the *E. coli* FhuE protein.

We tested 28 additional siderophores from several *Pseudomonas* species (gift of J.-M. Meyer) (see Table S2 in the supplemental material) obtained on paper disks in the same manner as described above for rhodotorulic acid. Only one of them, pyoverdine PL-8, supported growth of the parent strain. Pyoverdine PL-8 is a siderophore produced by *Pseudomonas fluorescens* strain PL8; its structure has been determined, but the cognate outer membrane receptor has not been identified in that organism (23). Unlike the *B. japonicum* parent strain, the blr3555 mutant did not grow on pyoverdine PL-8 (Fig. 1), indicating that Blr3555 is the outer membrane receptor for that siderophore. The annotated genome shows blr3556 immediately downstream of blr3555 (<http://genome.kazusa.or.jp/rhizobase/Bradyrhizobium>). However, resequencing that region reveals that blr3555 and blr3556 comprise a single open reading frame (GenBank accession no. FJ430786.1), with a likely frameshift error in the annotated genome. This gene was designated *pyoR*.

The *pyoR* and *fhuE* genes are regulated by the Irr protein and thus are not activated by iron limitation in an *irr* mutant (34). Consistent with those observations, the *irr* strain grew very poorly on rhodotorulic acid or pyoverdine PL-8 as iron sources (Fig. 1).

The blr3557 gene found within the *pyoR* gene cluster encodes a heme protein. Microarray analysis shows that blr3555 is clustered with eight other genes from blr3553 to blr3562 that are all upregulated 3- to 23-fold at the RNA level under iron-limiting conditions (41) (Fig. 2A) (see Table S3 in the supplemental material). blr3558 and blr3559 are predicted to encode

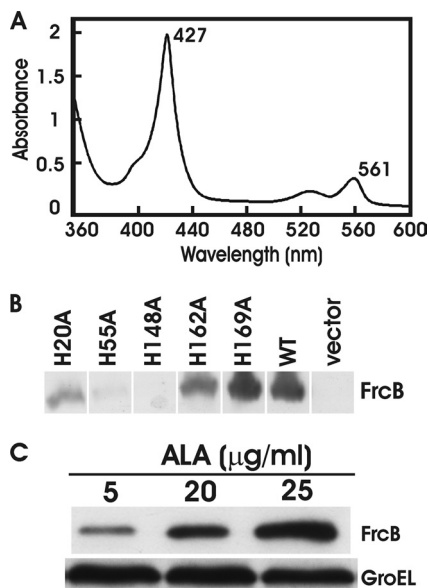


FIG. 3. *frcB* encodes a diheme protein, and heme-bound FrcB was analyzed. (A) The absorption spectrum of purified, recombinant FrcB reduced with sodium dithionite by light between wavelengths of 360 and 600 nm. Peaks at 427 and 561 nm are noted. (B) Analysis of FrcB and histidine-to-alanine (His-to-Ala) mutants in *E. coli* cells by immunoblot analysis. An HRP-conjugated anti-His probe was used to detect His-tagged proteins. (C) Analysis of FrcB accumulation in *E. coli* *hemA* strain S905 whole cells, grown in media supplemented with 5, 20, or 25 $\mu\text{g/ml}$ δ -aminolevulinic acid (ALA), by immunoblot analysis. An HRP-conjugated anti-His probe was used to detect His-tagged proteins. GroEL was used as a control for a protein not controlled by heme.

FrcB(H169A) contained 1.9 heme molecules per monomer, as was found in the wild-type protein (data not shown).

To assess whether the inability to obtain pure protein for the four His-to-Ala variants was due to accumulation levels in *E. coli*, we examined the gene variants borne on pBluescript SK for expression in *E. coli* strain DH5 α by Western blot analysis using antibodies directed against the His tag. Protein accumulation was much lower for proteins carrying variants H20A, H55A, H148A, and H162A compared to the wild type (Fig. 3B), but variant His169A protein was expressed at approximately wild-type levels (Fig. 3B).

The low level of expression of the four His-to-Ala variants suggested that those amino acid residues may be necessary for protein stability. Moreover, if they are heme-binding ligands, then heme may be required for protein stability. To test this idea, we expressed wild-type *frcB* in the *E. coli* heme-deficient mutant strain S905. This mutant is defective in the *gtrA* (*hemA*) gene encoding the heme synthesis enzyme glutamyl tRNA reductase, which synthesizes the heme precursor δ -aminolevulinic acid. Thus, the *gtrA* mutant requires exogenous ALA for growth. We expressed the *frcB* gene in the *gtrA* strain and grew cells in media with various concentrations of ALA (Fig. 3C). FrcB protein levels were low in cells grown with 5 $\mu\text{g/ml}$ ALA but were substantially higher in cells grown with 25 $\mu\text{g/ml}$ ALA. These findings are consistent with the hypothesis that heme is required for FrcB stability and that conserved histidine

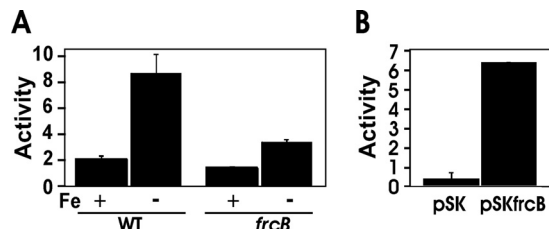


FIG. 4. Ferric reductase activity in *E. coli* and *B. japonicum* cells. The production of ferrous ferrozine produced by whole cells was measured and is presented as nanomoles of ferrous iron produced per hour. Ferric reductase activity is expressed as nanomoles of Fe^{2+} produced/hour/ 10^9 cells, and the values are presented as the averages plus standard deviations (error bars) of three replicate samples. (A) *B. japonicum* wild-type (WT) or *frcB* mutant strains were grown in low-iron media (Fe $-$) or supplemented with 12 μM FeCl_3 (Fe $+$). (B) *E. coli* DH5 α harboring empty vector (pSK) or vector plus bl3557 (*frcB*) (pSK*frcB*), grown in LB medium.

residues 20, 55, 148, and 162 are likely required for heme coordination.

The *frcB* gene is necessary for maximum ferric reductase activity in iron-limited cells. The arrangement and spacing of the nine genes in the blr3555 gene cluster indicate that they do not comprise an operon. Nevertheless, the arrangement and spacing of the genes suggest that the clustered and coregulated genes may be involved in a common process. Moreover, ferric reductase activity is required for iron assimilation into cells, and known eukaryotic ferric reductases contain heme as a prosthetic group (9, 22, 28). Although FrcB does not share sequence homology with the eukaryotic reductases, it is expressed under conditions where that activity would be needed. To address this further, we measured ferric reductase activity in whole cells of the parent strain and in a mutant strain containing a deletion in most of the *frcB* open reading frame and replaced the sequence with an antibiotic-encoding cassette (see Materials and Methods). Ferric reductase was measured using the ferrozine assay as described previously (8). Ferric reductase activity was detected in wild-type cells grown in iron-replete media and was induced by about 4-fold in cells from iron-limited cultures (Fig. 4A). However, this activity was diminished in the *frcB* mutant, suggesting that FrcB is involved in inducible ferric reductase activity. Deletion of *frcB* did not abolish activity, which may explain the lack of a growth phenotype of the mutant under iron-limiting conditions (data not shown).

Expression of *frcB* in *E. coli* from a high-copy-number plasmid confers ferric reductase activity on cells. The *frcB* gene was introduced into *E. coli* strain DH5 α on pBluescript SK and under the control of the plasmid-borne *lacZ* promoter. Strain DH5 α harboring the empty vector showed low ferric reductase activity (Fig. 4B), but cells that expressed *frcB* from the plasmid had much higher activity. Thus, *frcB* confers ferric reductase activity on a heterologous host.

Oxidation of purified recombinant FrcB by iron *in vitro*. Ferric reductases mediate electron transfer from a reductant to ferric iron. In our hands, reductants that reduced the heme groups of purified FrcB, such as dithionite or ascorbate, were also able to reduce ferric iron nonenzymatically in the absence of protein. To circumvent this problem, we measured the ox-

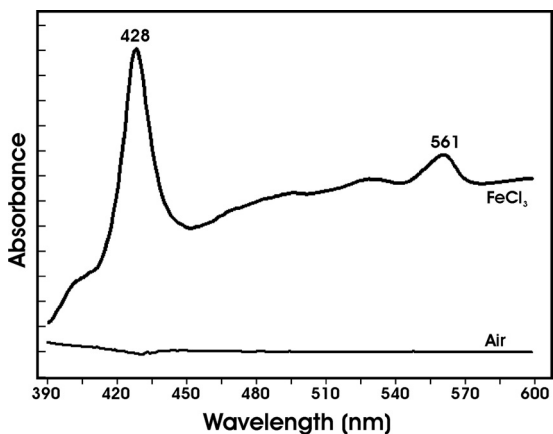


FIG. 5. Dithionite-reduced FrcB is oxidized in the presence of FeCl₃. FrcB (2 μM) reduced with dithionite as described in the text was treated with 500 μM FeCl₃ or air-saturated buffer, and the absorption spectrum of each was subtracted from the reduced spectrum. Peaks at 428 and 561 nm are noted. The tick marks on the y axis represent a change in absorbance of 0.05.

idation of reduced FrcB by ferric iron rather than ferric iron reduction. To do this, FrcB was reduced with dithionite, and the spectrum was compared to the spectrum of dithionite-treated protein in the presence of 100 μM FeCl₃ (Fig. 5). The reduced minus FeCl₃-treated spectra gave absorption peaks at 561 and 424 nm, showing that iron oxidized the heme groups of the protein. The control with air only yielded no spectral features, thus FrcB was not auto-oxidized. These findings, along with the *in vivo* data, strongly support the conclusion that FrcB is a ferric reductase.

The *frcB* gene is regulated by Irr. The nine-gene cluster from blr3553 to blr3562 is regulated by iron on the basis of the results of microarray analysis (41), and this was confirmed for *frcB* by quantitative real-time PCR (qPCR) (Fig. 6). Transcript levels were very low in cells grown in iron-replete media but were induced under iron-limiting conditions, consistent with the pattern of ferric reductase activity. Control of the *pyoR* (blr3555) gene by iron is mediated by Irr (34), and therefore, we wanted to determine whether *frcB* was regulated similarly. Thus, we measured steady-state levels of *frcB* in cells of the parent strain and an *irr* mutant, using *pyoR* as a control for an Irr-regulated gene (Fig. 6). The induction of *frcB* transcripts under iron-limiting conditions was not observed in the *irr* strain. Thus, Irr is normally a positive effector of that gene, and *frcB* is coregulated with *pyoR*.

DISCUSSION

Prokaryotic ferric reductases are mostly soluble, flavin-containing proteins based on the characterization of enzyme activity in cell fractions (31) and on the few proteins that have been purified (32, 40). In contrast, FrcB is bound to the membrane and contains two heme groups per monomer, as is observed for eukaryotic ferric reductases (9, 22, 28). Association of FrcB with the membrane raises the possibility that iron reduction occurs concomitantly with transport into the cytoplasm, and additional work is needed to clarify this. Ferric reductase activity has not been demonstrated for any of the

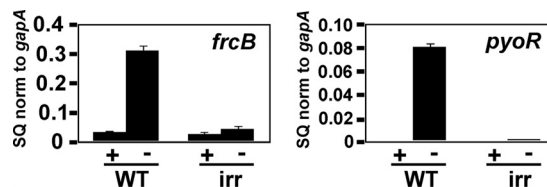


FIG. 6. Effects of an *irr* mutation on iron-dependent expression of *frcB* mRNA. mRNAs from cells of the parent strain or the *irr* mutant grown in medium supplemented with no added iron (–) or with 12 μM FeCl₃ (+) were analyzed by quantitative real-time PCR (qPCR). The data are expressed as the relative starting quantity (SQ) of the mRNA normalized (norm) to the housekeeping gene *gapA*, and the values are presented as the averages plus standard deviations (error bars) of three replicate samples. *pyoR* was used for comparison.

purified eukaryotic heme proteins. It is plausible that ferric reductase activity requires the integrity of the membrane or cell or requires additional proteins. In the current study, we demonstrate that iron was able to oxidize reduced FrcB; therefore, it has reductase activity. Further work will be needed to determine the physiological reductant substrate and whether additional protein components are needed to reduce FrcB.

FrcB is part of the cytochrome *b561* (cytb561) family of proteins, and homologs are found throughout the proteobacteria and in some cyanobacterial species. Thus, the current work suggests that ferric reductase heme proteins are not confined to eukaryotes but may be common in prokaryotes. Mammalian Dcytb is a member of a protein family also designated cytb561. However, the common designation of the prokaryotic and eukaryotic proteins is fortuitous and based on the absorption peak of the heme moieties, not on amino acid homology. Cytochromes with a protoheme prosthetic group will typically absorb in the 555- to 565-nm region of the spectrum and are functionally diverse proteins.

Accumulation of FrcB in *E. coli* cells was strictly dependent on heme, as determined by its dependence on exogenous δ-aminolevulinic acid (ALA) in a heme-dependent strain. This suggests that heme is required for FrcB stability. Moreover, mutants containing conserved histidine substitutions accumulated to only very low levels in cells. These amino acids are likely to be coordinating residues. Heme stabilizes cytochrome *c*-type cytochromes, which bind the protein covalently through cysteine ligation (12), and stabilizes some *b*-type cytochromes as well (24).

Iron metabolism is globally controlled by the Irr protein in *B. japonicum* (41), and genes involved in iron acquisition are positively regulated in an Irr-dependent manner under iron-limiting conditions (34). The current study shows that *frcB* is clustered with a ferric siderophore receptor gene and is coregulated with it. Collectively, these findings support the hypothesis that the ferric reductase activity of FrcB is involved in iron acquisition and metabolism in iron-limited cells. The *frcB* mutant showed diminished but measurable activity (Fig. 4A), suggesting the presence of an additional reductase in *B. japonicum*. This likely explains the lack of a growth phenotype of the mutant under the conditions tested.

Pyoverdines are a group of structurally related siderophores produced by fluorescent *Pseudomonas* species (38), and rhodotorulic acid is fungal in origin. We found that *B. japonicum*

has a specific outer membrane receptor for each of these compounds. Unlike *Sinorhizobium meliloti* and *Rhizobium leguminosarum*, *B. japonicum* does not have a known siderophore biosynthesis pathway (26). Because pyoverdines and rhodotorulic acid are specific to limited microbial genera, it is extremely unlikely that the ancestor of *B. japonicum* had the capacity to synthesize them, followed by loss in the extant species. Rather, *B. japonicum* probably acquired the ability to transport pyoverdine and rhodotorulic acid as an adaptation to its availability in the rhizosphere.

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