A bacterial iron exporter for maintenance of iron homeostasis

Siva Sankari and Mark R. O’Brien*

From the Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York 14214, USA

*Running title: Bacterial iron export

*To whom correspondence should be addressed: Mark R. O’Brien, Department of Biochemistry, 140 Farber Hall, State University of New York at Buffalo, Buffalo, NY 14214 USA Tel: 716.829.3200; Fax: 716.829.2725; Email: mrobrian@buffalo.edu.

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Background: Iron export has not been previously demonstrated in a bacterium.

Results: A bacterial iron exporter was identified and shown to be important for iron-dependent processes. The N-terminal cytoplasmic domain is essential for function.

Conclusions: Iron export activity participates in the maintenance of bacterial homeostasis

Significance: Iron export is likely to be a common homeostatic mechanism in prokaryotes.

ABSTRACT

Nutritional iron acquisition by bacteria is well-described, but almost nothing is known about bacterial iron export even though it is likely to be an important homeostatic mechanism. Here, we show that Bradyrhizobium japonicum Mbfa (Blr7895) is an inner membrane protein expressed in cells specifically under high iron conditions. Mbfa contains an N-terminal ferritin-like domain (FLD) and a C-terminal domain homologous to the eukaryotic vacuolar membrane Fe²⁺/Mn²⁺ transporter CCC1. An mbfa deletion mutant is severely defective in iron export activity, contains over 2-fold more intracellular iron than the parent strain, and displays an aberrant iron-dependent gene expression phenotype. B. japonicum is highly resistant to iron and H₂O₂ stresses, and Mbfa contributes substantially to this as determined by phenotypes of the mbfa mutant strain. The N-terminal FLD was localized to the cytoplasmic side of the inner membrane. Substitution mutations in the putative iron-binding amino acid residues E20A and E107A within the N-terminal FLD abrogate iron export activity and stress response function. Purified soluble FLD oxidizes ferrous iron (Fe²⁺) to incorporate ferric iron (Fe³⁺) in a 2:1 iron to protein ratio, which does not occur in the E20A/E107A mutant. The FLD fragment is a dimer in solution, implying that the Mbfa exporter functions as a dimer. Mbfa belongs to a protein family found in numerous prokaryotic genera. The findings strongly suggest that iron export plays an important role in bacterial iron homeostasis.
The ability of bacteria to sense nutrient availability and adapt accordingly contribute to their success in diverse environments. Iron is an essential nutrient required for many cellular processes. Bioavailability of iron is low in aerobic environments because it is mostly oxidized, and therefore insoluble. High affinity iron acquisition systems are expressed under iron limitation to scavenge the metal. Iron can also be toxic, as it catalyzes the generation of reactive oxygen species. Thus, metal homeostasis must be maintained.

Because of the low bioavailability of iron, studies on the maintenance of iron homeostasis have focused almost exclusively on acquisition of the metal. However, arguments can be made *prima facie* that control of the cellular iron content by uptake alone would limit the ability of the cell to adapt to changes in the environment. For example, exposure to $\text{H}_2\text{O}_2$ would likely lower the optimal iron content because it can be toxic under that condition, but attenuating uptake alone cannot lower the iron content. The cellular iron concentration can be lowered by dilution through cell division, but factors that mitigate division, such as nutrient limitation, may result in iron toxicity even when iron availability is low. Sequestration by the iron storage proteins ferritin or bacterioferritin can manage iron stress, but they will saturate in the absence of dilution.

Little is known about iron export in prokaryotes, and to our knowledge iron efflux activity by bacterial cells has not been demonstrated. An *E. coli* mutant defective in the *yiiP* gene has iron-related phenotypes (1), but the *YiiP* protein and its eukaryotic homologs are $\text{Zn}^{2+}$ exporters (1,2). *E. coli* mutants defective in *fetA* and *fetB* are sensitive to $\text{H}_2\text{O}_2$ stress, and overexpression of those genes decreases the free intracellular iron concentration (3), but no ion transport studies have been described for that system.

*B. japonicum* lives as a free-living soil organism or as the endosymbiont of soybean, where it fixes atmospheric nitrogen to ammonia to fulfill the nitrogen requirements of the host. Soils are highly variable ecosystems, and symbiosis represents a niche with specific nutritional requirements. Thus, *B. japonicum* and other rhizobia must be able to accommodate changes in metal availability. *B. japonicum* belongs to the alpha-Proteobacteria, a large taxonomic group that occupies diverse niches, including within eukaryotic cells in a symbiotic or pathogenic context. *B. japonicum* serves as a model system to understand metal metabolism and homeostasis in many alpha-Proteobacterial species (4).

The iron response regulator (Irr) is the major transcriptional regulator of iron-responsive gene expression in *B. japonicum*, and is found in many Alphaproteobacterial species. In those bacteria, Irr has replaced Fur as the global iron responsive regulator. *B. japonicum* Irr accumulates in iron-limited, manganese replete cells, and serves as both a positive and negative regulator of gene expression (5,6). When the iron level is sufficient, heme binds directly to Irr leading to its degradation in *B. japonicum* and *Brucella abortus* (7,8), whereas the *Rhizobium leguminosarum* Irr level is not substantially altered by the iron status, but its binding activity is affected by heme, at least in vitro (9).

Identification of the Irr regulon has provided a means to address fundamental questions in iron metabolism in the alpha-Proteobacteria, and in prokaryotes more generally. It has led to the discovery of a ferric iron reductase in bacteria (10), heme degradation in many Gram-negative organisms (11), an understanding of how the metabolism of iron and manganese are integrated (12,13), and the identification of novel regulatory elements and proteins that control the iron stimulon (14,15).

In the current study, we address the hypothesis that iron export is an essential
bacterial iron homeostatic mechanism, and we identify an iron exporter in B. japonicum.

EXPERIMENTAL PROCEDURES

Strains and media—B. japonicum USDAI110 is the parent strain used in this study. B. japonicum strains were routinely grown at 29°C in GSY medium as described previously (16). The actual iron concentration of the unsupplemented medium was 0.3 µM, as determined with a Perkin-Elmer model 1100B atomic absorption spectrometer.

Construction of strains and plasmids—For creating the mbfA mutant, the open reading frame plus 600 bp of flanking DNA was isolated by PCR, using genomic DNA as a template and ligated into pBluescript SK2. A deletion removing the open reading frame was constructed by inverse PCR as described previously (10), and the deleted fragment was replaced with a DNA cassette carrying genes for spectinomycin and streptomycin resistance. The construct was introduced into pLO1, mobilized into the parent strain and selected for double recombinants as previously described (10). The mutant was confirmed by PCR and antibiotic resistance and was grown in media supplemented with 50 µg/ml streptomycin and 50 µg/ml spectinomycin.

For creating pRK290X mbfA, the open reading frame of mbfA along with 100 bp flanks on either side of the gene (encompassing the promoter of mbfA), was amplified from genomic DNA and the PCR product was ligated into pSK, to make pSK mbfA. The construct was then ligated into the low copy number plasmid pRK290X to make pRK290X mbfA. For the construction of pRK290X mbfA-HA, the reverse primer was engineered to have HA tag sequence in frame after the fifth amino acid of mbfA ORF. The final product was amplified using a reverse primer having a HA tag sequence in frame with the mbfA ORF. The product was ligated to pSK and then subsequently ligated to pRK290X to make pRK290X His-mbfA-HA. pSK mbfA E20A/E107A was made by carrying out quick change mutagenesis (Stratagene) on pSK mbfA. The modified DNA was then ligated into pRK290X to make pRK290X mbfA E20A/E107A. To make pSK mbfA E20A/E107A-HA, pSK mbfA E20A/E107A was used as template and a HA tag was engineered to the reverse primer. The PCR product was cloned into pSK and ligated into pRK2909X to make pRK290X mbfA E20A/E107A HA. Strains with pRK290X were regularly grown in media supplemented with 50 µg/ml of tetracycline.

mRNA determination—Cells were grown to mid log phase and RNA was isolated by hot phenol method as previously described (6). 1 µg of RNA from each strain was used to make cDNA using Bio Rad cDNA synthesis kit. qPCR were performed as previously described (18). Data were normalized to gapA and are expressed as average of triplicates, with standard deviation represented by the error bars.

Western blot analysis—Cells were harvested by centrifugation at 9000 rpm for 5 minutes, washed twice in phosphate buffered saline (10 mM Na2HPO4, 2 mM KH2PO4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) and resuspended in the same buffer. The protein concentrations were measured by BCA protein assay (Pierce, Rockford, IL). 15 µg of protein from each sample were boiled in SDS loading buffer and loaded on 12% polyacrylamide gel and immune blotting was carried out. Anti-HA antibody (Sigma) was used at a dilution of 1:2500. Anti-GroEL (Enzo life sciences) was
used at a dilution of 1:8000. HRP conjugated goat anti-rabbit IgG (SouthernBiotech, Birmingham, AL) was used as secondary antibody and the blot was detected using Immobion chemiluminescence system (Millipore).

**Cellular localization of MbfA** - Cultures were grown to mid log phase and inner membrane, outer membrane and cytoplasmic fractions were separated previously described (19). 15 μg of protein from each fraction were resolved by 12% PAGE and immune blotting was carried out. Anti-Myc (Sigma) was used at a titer of 1:2500.

**Iron export assay** - Cultures were grown to mid log phase. 30ml of culture was aliquoted into 250 ml flasks and maintained at 29°C with shaking. At time zero, radioactive 55Fe was added to a final concentration of 1 μM. 1 ml of cells were removed at different time points and quenched in ice cold quench buffer (0.1 M Tris, 0.1 M succinate, and 10 mM EDTA). The quenched cells were immediately collected on 0.45-mm filters presoaked in quench buffer containing 40 mM Fe-EDTA, and then counted on a LKB γ-counter. EDTA was added to a final concentration of 3 mM when necessary.

**Metal content determination** - Forty milliliters of wild type or the mbfA mutant cells were grown in GSY with either no added iron or 20 μM FeCl3 added. The cells were harvested at mid log phase by centrifugation at 9000 rpm for 5 minutes. The pellet was washed twice with ice cold quench buffer (0.1 M Tris, 0.1 M succinate and 10 mM EDTA). To remove excess salt, the pellets were washed with double distilled H2O. In order to lyse the cells completely, the pellets were treated with 100 μl of 70% nitric acid and incubated at 98°C for 3 hrs. 1 ml of double distilled water was added to this preparation and centrifuged at 13000 rpm for 5 minutes to remove cell debris. The supernatant was sent for ICP-MS analysis (Penn State Institutes of Energy and the Environment)

**Iron and H2O2 sensitivity assays** - Cells were grown up to mid log phase in regular GSY. They were then treated with either 5 mM FeSO4, 10 mM FeSO4, 5 mM H2O2, 5 mM CoSO4, 5 mM ZnSO4 or 5 mM MnSO4 for 2 hrs. Cells were then harvested by centrifugation, washed with PBS twice and serial diluted in PBS. The serial dilutions were then spotted on regular GSY plates.

**Spheroplast preparation and Proteinase K treatment** - 50 ml of cells were grown to mid log phase. 10 ml of culture was spun down, washed twice and resuspended in 3 ml of PBS. This preparation is maintained as whole cells. 20 ml of the remaining culture was spun down and gently resuspended in 20 ml of 0.1 M Tris, 20% sucrose solution. The cells were maintained at 30°C with gentle shaking. After 1 hour, lysozyme was added drop wise to a final concentration of 2 mg/ml for 20 mins. Then, pre-warmed EDTA was added drop wise over a 2 hour period, to a final concentration of 3 mM. The formation of spheroplasts was examined and verified by phase contrast microscopy, where cells were oblong and spheroplasts were circular in shape. This spheroplast culture was then divided into two halves and spun down. In one half of the culture, the integrity of spheroplasts was maintained by very gently resuspending in 3 ml of Tris sucrose buffer pH 7.8. The other half was resuspended in 3 ml water and vortexed for a minute. Cells lysis in this suspension was verified by a drop in O.D of cells and also visually by light microscopy. This preparation is called the broken cells. Proteinase K was added to a final concentration of 0.5 mg/ml to the remaining of all three preparation and incubated in ice for 2 hours. All three preparations were then TCA precipitated and heated at 95°C for 10 minutes along with SDS loading buffer. Equal amount of samples were loaded on 12% SDS gels and Western blots were carried out with respective antibodies. Anti-Sco1 antibody was used at a titer of of 1:10000. Anti-His (Invitrogen) antibody was
Bacterial iron export

Overexpression and purification of the FLD and FLD E20A/E107A- For making pET14b FLD, the ORF of mbfA encoding the first 180 amino acids was amplified from genomic DNA and cloned in frame into pET14b containing an N terminal 6xHis tag. The FLD E20A/E107A mutant was made from pET14b FLD by Quick change mutagenesis (Stratagene). The vector with insert was transformed into chemical competent BL21 (DE3) E. coli cells. Cells were inoculated from an overnight culture grown in Luria-Bertani media containing 200 μg/ml ampicillin and 25 μg/ml chloramphenicol into 1 liter of fresh 2×YT medium (Sambrook et al., 1989) containing the same antibiotics and grown at 37°C. Overexpression was induced in cells at the midlog phase by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 37 °C for 4 hours with shaking. The cells were washed in 20 mM HEPES (pH 7) and 100 mM NaCl and resuspended in the same buffer. The cells were broken in a French pressure cell and the soluble fraction was collected by centrifugation at 9000 rpm for 15 mins. The supernatant was then mixed with Ni-NTA slurry and rotated at 4°C for 1hour. The slurry –protein mixture was then poured into a column and washed with wash buffer (20 mM HEPES, 100 mM NaCl, 10% glycerol and 20 mM imidazole), 10 times the column volume. A step wise elution was performed with 1 column volume of wash buffer with 40 mM, 80 mM, 120 mM, 160 mM and 200 mM imidazole in the order of increasing imidazole concentrations. Wild type and the FLD E20A/E107A mutant were eluted with 120 mM imidazole. Proteins were then dialyzed using Snake skin pleated dialysis tubing 3500MWCO (Thermo Scientific) in dialysis buffer (20 mM HEPES, 100 mM NaCl, 10% glycerol). The His-tags were removed with thrombin. Equal amount of both proteins were run on SDS PAGE gels to confirm purity of the samples. Apo form of the proteins were made by dialyzing against 0.3% Sodium dithionite and 1 mM 2,2’-Dipyridyl for 24 hours as previously described (20,21).

Iron oxidation- Iron oxidation by FLD and the E20A/E107A mutant carried out as previously described (20). All reactions were carried out in aerated 20 mM MOPS buffer at pH 7. Freshly prepared ferrous ammonium sulfate was used within minutes of preparation. Iron oxidation was measured at 310 nm in kinetic mode of Thermo Scientific Genesys 10 bio UV/Visible spectrophotometer. Protein added to the buffer was calibrated as blank. Iron oxidation stoichiometry was calculated as previously described (22).

Molecular weight determination by size exclusion chromatography- Size exclusion chromatography was performed with a Superose 12(10/300) column using Akta Prime FPLC (GE). The column was equilibrated with 20 mM MOPS pH 7, 100 mM NaCl, 10% glycerol. Standards (Bio-Rad) and subsequently 200 µg of purified FLD and FLD E20A/E107A were eluted with the same buffer at a flow rate of 1 ml/min. Elutions were followed spectrophotometrically by tracing UV absorbance at 280 nm. The equation of the standard curve was y = -0.5353x + 2.503 with an R² value of 0.9933.

RESULTS

MbfA (Blr7895) protein is expressed in media supplemented with high iron- The blr7895 gene was originally identified as a member of the Irr regulon that contains a very high affinity Irr binding site in its promoter necessary for repression in iron-limited media (23,24). blr7895 mRNA is expressed under high iron conditions where Irr levels are low. The function of blr7895 is not known, but its homolog in Agrobacterium tumefaciens contributes to resistance to hydrogen peroxide stress by an unknown mechanism (25). Blr7895 is predicted to contain an N-terminal
domain homologous to the uncharacterized ferritin-like AB protein family. The X-ray crystal structure of a family member from *Magnetospirillum magnetotacticum* has been solved as a dimer (PDB Id. no. 2OH3). The C-terminal portion of Blr7895 shares 46% similarity to CCC1, a vacuolar membrane protein that transports Fe$^{2+}$ and Mn$^{2+}$ from the cytoplasm into the vacuole in *Saccharomyces cerevisiae* and other eukaryotes (26,27). Blr7895 is not related to *E. coli* FetA, FetB or YiiP, but homologs of it are found in many eubacterial and archaeal genera. Although CCC1 is a eukaryotic transporter, no eukaryotic proteins containing both domains of Blr7895 were found in homology searches.

In assigning names to Irr regulon genes based solely on homology, Rodionov et al named blr7895 and its homologs mbfA for membrane-bound ferritin (28), and this designation was used subsequently for the *A. tumefaciens* homolog (25). As described below, blr7895 is not a bona fide ferritin, but we adopted the gene designation mbfA herein nevertheless based on the established precedent.

We found previously that mbfA mRNA is maximally expressed when grown in high iron media (24). Here, we grew cells supplemented with 100 µM of either Fe, Co, Zn or Mn, and examined mbfA transcripts by qualitative real time PCR (qPCR) (Fig 1A). Only iron was able to derepress the mbfA gene. To examine protein levels, we expressed a mbfA-HA gene in trans in *B. japonicum* cells grown in media supplemented with 20 µM or 100 µM Fe, Mn Co, and Zn (Fig. 1B). MbfA protein was detectable on Western blots in cells grown in high iron media, but not in media supplemented with the other metals, and thus mRNA and protein levels correlated with each other and demonstrate that mbfA expression is specifically iron-dependent.

* MbfA is an inner membrane protein- *B. japonicum* cells harboring mbfA-HA grown in high iron media were fractionated into cytoplasmic, inner and outer membrane fractions. The integrity of each fraction was confirmed by Western blot analysis using MntH, MnoP and Mur as markers for the inner membrane, outer membrane and cytosol, respectively (Fig. 2). MbfA-HA localized exclusively to the inner membrane. As described below, the mbfA-HA gene complemented an mbfA mutant, thus its use here is physiologically meaningful.

**MbfA is required for iron export**- Bacterial iron transporters that have been characterized previously are expressed under iron limitation, and are required for high affinity uptake. Thus, the expression of mbfA under high iron conditions made it a good candidate for an iron exporter. Moreover, transport from the cytoplasm to the vacuole in eukaryotes is analogous to transport from inside the cytoplasm to outside. To test this idea, we added 1 µM $^{55}$FeCl$_3$ to cultures of *B. japonicum* cells at mid log phase, harvested them at various time points, and measured radiolabel in them (Fig. 3A). In the parent strain, $^{55}$Fe was taken up by cells for about 20 minutes after addition, followed by efflux. The mbfA mutant showed similar initial $^{55}$Fe uptake, but little subsequent efflux was observed.

Because the observed transport of $^{55}$Fe in cells at a given time is the net result of its import and export, we wanted to examine export alone. To do this, we added EDTA to chelate extracellular iron in the media 20 minutes after addition to prevent further uptake (Fig. 3A). We found that the rate of export with EDTA by the parent strain was only modestly greater than without it, suggesting little uptake after 20 minutes even without EDTA addition. In the mbfA mutant, little efflux was observed after the addition of EDTA, similar to what was observed without the chelator. The data show that MbfA is required for iron export from cells.

To confirm that deletion of the mbfA gene was responsible for the Fe export phenotype, we complemented the mutant with the mbfA
gene in trans (Fig. 3C). In addition, we found that the mbfA-HA gene also complemented the mutant (Fig. 3C), showing that the tagged protein is active as an exporter, and thereby justifying its use in the localization analysis (Fig. 2).

**MbfA is required to maintain iron homeostasis and gene expression in cells grown under high iron conditions.** We measured iron levels in cells grown in media supplemented with no iron or with 20 μM FeCl₃ in the parent strain and the mbfA mutant (Fig. 4). The iron levels between the wild type and mutant were similar in cells grown in media with no added iron. However, the mutant contained about 2.5-fold more iron than the parent strain in cells grown in media containing 20 μM FeCl₃. This phenotype is consistent with a defect in iron export activity. We also measured the content of Mn, Ni, Zn and Co in those cells (Fig. 4). The parent strain and mbfA mutant were similar to each other with respect to those metals in cells grown in 20 μM FeCl₃. Under iron limited growth, a small but statistically significant difference in the Zn content was observed, for which we offer no explanation because mbfA expression is very low in the parent strain under that growth condition (Fig. 2). The findings show that MbfA is required for maintaining cellular iron levels when availability is high.

If the higher total iron content in the mutant strain also results in an increase in the available or regulatory iron, then aberrations in iron-responsive gene expression should be observed in the mbfA strain. We examined transcript levels of 5 known Irr-regulated genes that are derepressed by iron in the parent and mbfA strains grown in media supplemented with no, 20 μM or 100 μM FeCl₃ (Fig. 5). For cells grown in media with no added iron (0.3 μM Fe as determined by atomic absorption spectroscopy), transcript levels were repressed similarly in both strains. In 20 μM iron, blr0466 and blr4339 transcripts were increased about 2- and 2.5-fold greater, respectively in the mutant than in the parent strain. The remaining 4 genes were slightly elevated in the mutant. For cells grown in 100 μM iron, bfr, blr0512 and leuC transcripts were increased about 2-fold in the mutant and bli0466 was 3-fold higher. blr4339 mRNA did not increase further in the mutant upon an iron increase from 20 μM to 100 μM, presumably because it was already fully derepressed at the lower concentration. We found previously that differences in affinity of Irr for target promoters results in differential responses of Irr-repressible genes to iron (12), which likely explains why some genes were more fully derepressed in the mbfA strain at 20 μM iron than others.

Although differences in iron-responsive gene expression between the parent and mbfA strains were modest, the cumulative effects throughout the iron stimulon genes may be substantial.

**The mbfA mutant is sensitive to iron and H₂O₂ stresses.** Iron is toxic at high concentrations because the ferrous (Fe²⁺) form can react with H₂O₂ to generate the very reactive hydroxyl radical (OH·). Because the mbfA mutant is defective in iron export activity and maintains a higher cellular iron content, we asked whether it was sensitive to exposure to high concentrations of the metal or of H₂O₂ in terms of cell viability. To address the former, cells were grown to mid log phase and then treated with FeSO₄ for 2 h, washed and then serial dilutions were spotted onto nonselective plates to estimate relative cell viability (Fig. 6A). We carried out pilot experiments to determine the range of iron in the media that was lethal to the parent strain upon a 2 h exposure, and found that *B. japonicum* is resistant, showing little loss of viability at 5 mM or 10 mM FeSO₄ (Fig 6A). This concentration is likely to be higher than what *B. japonicum* encounters in the environment. However, the mbfA strain was about 2- and 4-orders of magnitude more sensitive to 5 mM and 10 mM iron, respectively, relative to the parent strain.
Thus, MbfA confers substantial resistance to cells at high iron concentrations.

We repeated these experiments with the sulfate salts of Co, Zn and Mn (Fig. 6B). Both Co and Zn were more toxic than Fe, but Zn and Mn showed no differences between the parent strain and the mutant, and the mutant was only very slightly more sensitive to Co. Thus, the role of MbfA in managing iron stress is specific to that metal.

To address the role of MbfA in H$_2$O$_2$ stress response, we carried out the experiments described above, adding 5 mM H$_2$O$_2$ rather than metal to cultures (Fig. 6C). The mbfA mutant was much more sensitive to H$_2$O$_2$ exposure than was the wild type, showing that MbfA has a role in the management of this oxidative stress. The mbfA mutant of A. tumefaciens was also found to be more sensitive to H$_2$O$_2$ stress (25).

The N-terminal ferritin-like domain (FLD) of MbfA is required for iron export activity and stress responses- The ferritin-like AB protein family is widely dispersed in prokaryotes, but its function is unknown. This family is similar to bona fide ferritins with respect to having a four alpha-helix bundle with a conserved glutamate residue within each helix involved in di-iron binding. The crystal structure of the ferritin-like AB protein from Magnetospirillum magnetotacticum was resolved bound to zinc, confirming that the conserved histidines bind metal (PDB Id. no. 2OH3).

Glutamate at positions 20 and 107 of MbfA within the N-terminal FLD correspond to two of the conserved glutamates in ferritins. A mutant mbfA gene was constructed that encodes Glu to Ala substitutions at positions 20 and 107, and the plasmid borne gene was introduced into the mbfA deletion strain. Whereas the wild type mbfA gene was able to complement the mutant strain in trans with respect to iron export activity, the mbfAE20A/E107A mutant gene was unable to do so (Fig. 3C), showing that the FLD of MbfA is necessary for export activity. We note that an HA-tagged derivative of the MbfA E20A/E107A variant targeted to the inner membrane (Fig. 2), showing that loss of function was not due to aberrant localization.

We also examined whether the E20A/E107A variant was able to complement the mbfA mutant strain with respect to sensitivity to iron stress (Fig. 7). Cell viability in response to iron treatment in the mutant harboring the E20A/E107A variant was similar to that bearing the empty vector were similar to each other, showing that the FLD is necessary for iron stress response.

The ferritin-like domain of MbfA is located on the cytoplasmic side of the inner membrane-To determine the location of the N-terminal FLD, we constructed a mbfA gene variant that encodes a C-terminal HA tag and a 6-His tag starting at position 6 of the N terminus, and expressed it in trans in the mbfA strain. This gene variant complemented an mbfA mutant with respect to iron export activity (Fig. 3C), confirming that it encodes a functional protein.

Whole cells, spheroplasts in which the outer membrane was removed, or broken cells were prepared, treated with proteinase K and then probed with antibodies raised against the HA- or His-tag (Fig. 8). Sco1 is an inner membrane-anchored protein that is located mostly in the periplasm (29), and Mur is a cytosolic protein (18); they were used as controls. Both the N- and C-terminal tags, as well as Sco1 and Mur were detected on Western blots in whole cells untreated or proteinase K-treated, and in untreated spheroplasts or broken cells. The Sco1 signal was lost in spheroplasts upon protease treatment, expected of a protein found predominantly in the periplasm. Only about 27 amino acids of the 196 amino acid protein are predicted to be in the inner membrane, and we did not resolve the proteins on SDS-PAGE in a manner that would detect the small peptide. The N-terminal His-tag remained detectable in protease-treated spheroplasts at the same size of the untreated samples. Consistent with this,
the C-terminal HA tagged protein did not decrease in size as would have been expected if the N-terminal domain were digested. Only in broken cells were signals lost for the MbfA tags, as well as for the Mur control. We conclude that the N-terminal FLD is located on the cytoplasmic side of the inner membrane.

The purified FLD domain oxidizes Fe\(^{2+}\) to Fe\(^{3+}\) in an iron to peptide ratio of 2-

Incorporation of iron into ferritins involves oxidation of the ferrous iron (Fe\(^{2+}\)) to the ferric (Fe\(^{3+}\)) form, concomitant with coordination of the metal with protein ligands in a 2 Fe\(^{3+}\) per monomer ratio (22). We overexpressed in E. coli the portion of the \(mbfA\) gene encoding the FLD, and purified the peptide (Fig. 9A). Ferrous iron (Fe(NH\(_4\))\(_2\)(SO\(_4\))\(_2\)) was titrated into a reaction containing FLD, and ferric iron production was monitored as described in the Experimental Procedures (Fig. 9B). The Fe\(^{3+}\) produced increased with Fe\(^{2+}\) added until a 2 iron per monomer ratio was reached, and then no further Fe\(^{2+}\) was oxidized. Thus 2 Fe\(^{3+}\) molecules were produced per FLD monomer.

We also measured Fe\(^{2+}\) oxidation under increasing concentrations of FLD in the presence of excess FeSO\(_4\) (Fig. 9C). Fe\(^{3+}\) was produced in a 2:1 ratio with FLD. The experiments were repeated with a purified FLD E20A/E107A derivative (Fig 9), and we found very little Fe\(^{2+}\) oxidation in the mutant protein. Thus, the putative iron-binding residues are required for incorporation of iron into the protein.

Bona fide ferritins have an enzymatic ferrous iron oxidase (ferroxidase) activity, but the stoichiometric ratio of ferric iron to protein shows no catalytic turnover of the FLD domain, and thus it does not behave as an enzyme.

Purified FLD is a dimer in solution- The ferritin-like protein from Magnetspsirillum magnetotacticum was crystalized as a dimer. We determined the oligomerization state of the purified FLD of MbfA by size exclusion chromatography (Fig. 10) and found it to be exclusively a dimer. The FLD E20A/E107A mutant was also a dimer (data not shown), suggesting that the putative iron binding sites do not control the oligomerization state of the protein. It also implies that the mutations do not drastically alter the tertiary structure of that domain. These findings suggest that MbfA functions as a dimer.

DISCUSSION

In the present study, we identified an iron exporter that functions under high iron conditions, and is needed to maintain the internal iron concentration, and protect cells against oxidative and iron stress. Mbf homologs are distributed throughout the eubacterial and archaeal kingdoms. The findings strongly suggest that the cellular iron status cannot be regulated solely by acquisition of the metal, at least under certain conditions, and that iron export is required to maintain bacterial iron homeostasis.

Iron export becomes more important as the extracellular iron concentration increases, but it also functions under oxidative stress, where iron can react with \(H_2O_2\) to form more reactive oxygen species. Thus, the iron content at which MbfA is beneficial is not likely to be an absolute value, but depends on the metabolic state of the cell more generally. In the current study, we used severe iron and \(H_2O_2\) conditions to demonstrate the degree to which MbfA preserves viability. Iron conditions that are not immediately lethal nevertheless affect metabolism in terms of the cellular iron content and alteration of iron-dependent gene expression. It was shown previously that \(H_2O_2\) generated from respiration can lower the cellular level of Irr, the transcriptional repressor of \(mbfA\) expression (30), suggesting that cells may be able to respond to this stress at the level of iron export. Although not experimentally addressed here, it is plausible that cells subjected to nutrient limitation may be more sensitive to iron toxicity at even a low or moderate extracellular level because iron...
dilution through cell division would be mitigated.

We found that the N-terminal ferritin-like domain of MbfA is located in the cytoplasm, is necessary for function, and the purified domain is a dimer in solution. Thus, it is plausible that MbfA functions as a dimer based on interactions with the N-terminal FLD (Fig. 11). This is an appealing model because the protein is predicted to contain either 4 or 5 transmembrane alpha-helical domains, depending on the algorithm used, which is not sufficient to form a functional channel. However, 8 or 10 helices would be sufficient. The bacterial K⁺ channel contains 8 alpha helices resulting from tetramerization of identical subunits with two helices each (31). The bacterial SemiSWEET sugar transport protein family contains 3 helical domains, and oligomerizes to form a channel (32).

The FLD oxidizes 2 iron molecules per monomer upon incorporation into the protein, as do ferritins, but it does not behave as a ferroxidase enzyme. The FLD E20A/E107A mutant cannot oxidize iron, and the intact MbfA E20A/E107A variant is not functional as an exporter, nor can it protect the cell from iron stress. The function of iron oxidation and binding is unknown, but it may have a structural or sensing function. The absence of ferroxidase activity makes it unlikely that the ferrous iron is oxidized upon export from the cytoplasm, although we acknowledge that the intact protein may behave differently than the purified N-terminal domain.

Bacteria are known to remove ions from the cytoplasm via inner membrane RND efflux pumps, P-type ATPases, or cation diffusion facilitator proteins (33). The CCC1-like channel of MbfA and other prokaryotes are not homologous to those proteins. We found that MbfA protects B. japonicum from exposure to iron in the millimolar range, and therefore iron export likely requires energy to transport against a concentration gradient. The energy requirements of eukaryotic CCC1-dependent ion transport from the cytoplasm to the vacuole are not known.

B. japonicum is a gram-negative bacterium, and therefore MbfA transports iron to the periplasm. A future challenge will be to learn how iron is transported to the outside the cell. Many Gram-negative bacteria use a tripartite efflux pump complex to export heavy metals and antibiotics from the cytoplasm to the medium (eg. (34). In addition to an RND family inner membrane pump, the complex contains a periplasmic adaptor protein and an outer membrane beta-barrel outer membrane channel. The genes encoding this system are often organized in an operon, but mbfA is not part of an operon, nor are there adjacent genes in the genome that suggest these auxiliary functions.

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FIGURE LEGENDS

FIGURE 1. Expression of the \textit{mbfA} gene. A. Steady-state transcript levels of the \textit{mbfA} gene in the wild type strain, grown in GSY media supplemented with either 100 \(\mu\)M of FeCl\(_3\) (Fe), CoCl\(_2\) (Co), ZnCl\(_2\) (Zn), MnCl\(_2\) (Mn) or no added metal were analyzed by quantitative real time PCR (qPCR). The data are expressed as relative starting quantities (SQ) of respective mRNAs normalized to the housekeeping gene \textit{gapA}, and presented as average of three replicates \pm the standard deviation. B. Western blot analysis of MbfA-HA from cells grown in media supplemented with no, 20 \(\mu\)M or 100 \(\mu\)M metal as described in panel A. The protein was detected with anti-HA antibodies. GroEL was used as a control for an unregulated protein, and was detected using anti-GroEL antibodies. Fifteen micrograms of protein was loaded per lane.

FIGURE 2. Localization of MbfA to the inner membrane. Cells were fractionated into the inner membrane, outer membrane and cytosol, and western blots analysis was performed on the three fractions. Fifteen micrograms of protein was loaded per lane. Anti-HA antibodies was used to detect C-terminal HA tagged MbfA (MbfA), C-Terminal HA tagged MbfA E20A/E107A mutant (MbfA mutant) and C-Terminal HA tagged MntH. Anti-Myc antibody was used to detect C-terminal Myc tagged MnoP. Anti-Mur antibody was used to detect Mur.

FIGURE 3. Iron export by \textit{B. japonicum} strains. A. Cells of the wild type strain (squares) or the \textit{ΔmbfA} strain (circles) were grown in GSY media. At time zero, 1 \(\mu\)M \(^{55}\)Fe was added to the assay medium, and cells were harvested at various time points and the radioactivity was counted. Closed squares and closed circles represent data points of experiments in which 3 mM EDTA was added at 20 minutes (marked by an arrow) and open circles and open squares represent data points of experiments in which EDTA was not added. Diamonds represent data points of experiments in which 3 mM EDTA was added to the wild type strain, immediately after the addition of \(^{55}\)Fe (t=0). Each time point is the average of triplicate samples \pm the standard deviation. B. Complementation of \textit{ΔmbfA} strain in trans. Wild type strain with an empty vector pRK290X (open squares), \textit{ΔmbfA} strain with an empty vector pRK290X (open circles) and \textit{ΔmbfA} strain complemented with pRK290X \textit{mbfA} (closed triangles) were grown in GSY media and iron export assay was performed as described in panel A. The arrow represents the time of addition of 3 mM EDTA. C. Complementation of \textit{ΔmbfA} strain in trans with tagged \textit{mbfA} constructs. Cells of the \textit{ΔmbfA} strain complemented with either pRK290X \textit{mbfA} E20A/E107A (open circles), pRK290X \textit{mbfA}-HA (closed squares), pRK290X \textit{mbfA} E20A/E107A-HA (closed circles), pRK290X His-\textit{mbfA}-HA (closed inverted triangles) were grown in GSY media and iron export assay was performed as in panel A. The time of addition of 3 mM EDTA is indicated by an arrow. Each time point is the average of triplicate samples \pm the standard deviation.

FIGURE 4. Measurement of total intracellular metal contents of the wild type and the \textit{ΔmbfA} strain. Cells of the wild type strain (closed bars) and \textit{ΔmbfA} strain (open bars) were grown in GSY media supplemented with either no added iron or 20 \(\mu\)M FeCl\(_3\). Total amount Fe, Co, Mn, Ni, and Zn were determined by ICP-MS analysis. The values represented are an average of three runs \pm the standard deviation. The bracket with an asterisk denotes significant differences between the parent strain and mutant based on the Students t test using a confidence level of p<0.01.
FIGURE 5. Expression of iron responsive genes in the wild type and the ΔmbfA strain. Cells of the wild type strain (closed bars) and ΔmbfA strain (open bars) were grown in GSY media supplemented with either no added iron, 20 µM or 100 µM FeCl₃. The steady state transcript levels of *leuC*, *bfr*, *blr4339*, *bll0466* and *blr0512* were analyzed by qPCR. The data are expressed as relative starting quantities (SQ) of respective mRNAs normalized to the housekeeping gene *gapA*, and presented as average of three replicates ± the standard deviation.

FIGURE 6. Increased sensitivity of ΔmbfA strain to iron and peroxide stress. Cells of the wild type strain and ΔmbfA strain were grown in GSY media. A. Cells treated with either 5 mM FeSO₄ or 10 mM FeSO₄ for 2 hours, were serial diluted and spotted on GSY plates. B. Cells were treated with 5mM CoSO₄, 5 mM MnSO₄ or 5 mM ZnSO₄ and spotted as in Panel A. C. Cells were treated with 5 mM H₂O₂ for 2 hours and spotted as in panel A. Untreated cells were serial diluted and spotted along with treated cells for reference.

FIGURE 7. Requirement of FLD in resistance to iron stress. Cells of the wild type strain with empty vector pRK290X, ΔmbfA strain with empty vector pRK290X, ΔmbfA strain complemented with pRK290X mbfA and ΔmbfA strain complemented with pRK290X mbfA E20A/E107A were grown in regular GSY, treated with 5 mM FeSO₄ for 2 hours and serial diluted and spotted on GSY plates. Untreated cells of each strain were serial diluted and spotted along with treated cells for reference.

FIGURE 8. Localization of FLD to the cytoplasmic side of the inner membrane. Western blot analysis of whole cells (W), spheroplasts (S) and broken cells (B) of cells expressing MbfA with a N-terminal His tag and a C-terminal HA tag, either treated or not treated with Proteinase K. Anti-HA antibody was used to identify the C-terminal end and Anti-His antibody was used to detect N-terminal end of MbfA. Sco1 (periplasm) and Mur (cytoplasm) were used as controls. Anti-Sco1 was used to detect Sco1 and Anti-Mur was used to detect Mur.

FIGURE 9. Iron oxidation by FLD. A. SDS PAGE gel containing the protein ladder (lane 1), purified FLD (lane 2) and purified FLD E20A/E107A (lane 3). B. Iron oxidation was spectroscopically measured at 310 nm. Increasing concentrations of iron in the form of ferrous ammonium sulphate was added to 20 mM MOPS buffer (pH 7) containing 30 µM of FLD (open circles) or FLD E20A/E107A (closed squares). Absorbance at 310 nm was noted after 2 minutes of Fe²⁺ addition for each iron concentration added. Number of iron atoms formed is calculated using an ε value of 2.5 mM⁻¹ cm⁻¹, which is used for other ferritins, and plotted against number of iron atoms added per monomer. The tangent lines intersect at a Fe/monomer ratio of 2. C. 5 µM iron in the form of ferrous ammonium sulfate was added to different FLD and FLD E20A/E107A concentrations (ranging from 0.5 to 5 µM). Absorbance at 310 nm was measured spectrophotometrically. Number of iron atoms formed is calculated as in panel B and plotted against the respective protein concentrations used.

FIGURE 10. Dimerization of FLD in solution. Determination of molecular weight of FLD and FLD E20A/E107A were determined by size exclusion. Molecular weight standards are represented as tick marks on the upper X axis. B. The standard curve was generated from the elution volumes of the standards divided by the elution volume of the thyroglobulin (Ve/Vo) plotted against the log of the molecular weights of the standards (squares). The elution of FLD (open circles) and FLD E20A/E107A (closed circles) are shown, but the overlap makes them visually indistinguishable.
Ve/Vo of FLD is 1.623 and the calculated molecular weight is 43.1 kDa which is 2.05 times the predicted molecular weight of the monomer. Ve/Vo of FLD E20A/E107A is 1.630 and the calculated molecular weight is 42.7 kDa which is 2.03 times the predicted molecular weight of the monomer.

**FIGURE 11.** Proposed model of MbfA dimerization. The model shows dimerization through the N-terminal ferritin-like domain (FLD) to form a functional channel. TMPRED and Sosui predict 5 and 4 alpha transmembrane helices, respectively, and 4 are shown in the diagram.
Figure 1
Figure 2

|   | I   | O   | C   |
|---|-----|-----|-----|
|   | MbfA| MbfA mutant | MntH |
|   | MnoP | Mur |     |
Figure 3
Figure 4
Figure 5
Fig 6
Figure 7
Figure 8
Figure 9
Figure 10
Figure 11
A bacterial iron exporter for maintenance of iron homeostasis
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