Roles of *Agrobacterium tumefaciens* membrane-bound ferritin (MbfA) in iron transport and resistance to iron under acidic conditions

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*Agrobacterium tumefaciens* membrane-bound ferritin (MbfA) is a member of the erythrin (Er)–vacuolar iron transport family. The MbfA protein has an Er or ferritin-like domain at its N terminus and has been predicted to have five transmembrane segments in its C-terminal region. Analysis of protein localization using PhoA and LacZ reporter proteins supported the view that the N-terminal di-iron site is located in the cytoplasm whilst the C-terminal end faces the periplasm. An *A. tumefaciens* mbfA mutant strain had 1.5-fold higher total iron content than the WT strain. Furthermore, multi-copy expression of *mbfA* reduced total iron content two- and threefold in WT and *mbfA* mutant backgrounds, respectively. These results suggest that MbfA may function as an iron exporter rather than an iron storage protein. The *mbfA* mutant showed 10-fold increased sensitivity to the iron-activated antibiotic streptonigrin, implying that the mutant had increased accumulation of intracellular free iron. Growth of the *mbfA* mutant was reduced in the presence of high iron under acidic conditions. The expression of *mbfA* was induced highly in cells grown in iron-replete medium at pH 5.5, further supporting the view that *mbfA* is involved in the response to iron under acidic conditions. *A. tumefaciens* MbfA may play a protective role against increased free iron in the cytoplasm through iron binding and export, thus preventing iron-induced toxicity via the Fenton reaction.

**INTRODUCTION**

*Agrobacterium tumefaciens* is a soil bacterium that induces the formation of crown gall tumours on dicotyledonous plants by transferring T-DNA from the bacterium’s tumour-inducing (Ti) plasmid into host plant cells (Zhu et al., 2000). The virulence (*vir*) genes on the Ti plasmid are activated by plant-derived phenolic compounds, such as acetosyringone, and by an acidic environment at plant wound sites. Plants compete with invading bacteria for essential metals, including iron (Fones & Preston, 2013). Plant pathogens are exposed to reactive oxygen species as part of host defence responses (Wojtaszek, 1997). Therefore, the abilities of pathogenic bacteria to cope with fluctuations of iron availability and oxidative stress are important for bacterial survival and successful infection.

Although iron is an essential element for bacterial growth, an excess of intracellular free iron causes cell damage due to its ability to catalyse the production of reactive hydroxyl radicals through the Fenton reaction (Inlay et al., 1988). Bacteria can store free iron in a non-reactive state to prevent iron participation in the Fenton reaction, thus inhibiting the formation of hydroxyl radicals. Ferritins are iron storage proteins that play an important role in bacterial survival under oxidative stress conditions and iron reserves in ferritins can be released when cells need them during iron-limiting conditions (Boughammoura et al., 2008; Chen & Morse, 1999; Velayudhan et al., 2007).
Importantly, reduced virulence associated with iron storage protein deficiencies has been reported in many bacteria (Boughammoura et al., 2008; Velayudhan et al., 2007; Waidner et al., 2002).

The erythrin–vacuolar iron transport (Er-VIT1) family is a member of the ferritin-like superfamily (Andrews, 2010). The Er-VIT1 family has two major domains: the N-terminal Er or ferritin-like domain and the C-terminal membrane-embedded VIT1 domain. The Er domain contains conserved motifs (E-6-Y and ExxH), which are ligands for the di-iron site. The VIT1 domain is homologous to Arabidopsis VIT1, which is involved in transporting iron into vacuoles (Kim et al., 2006). The domain is also similar to yeast Ca22+-sensitive cross-complementer 1 (CCCI) protein, which is localized in vacuolar membranes, and is an iron/manganese transporter that transfers iron from the cytoplasm and into vacuoles (Li et al., 2001). Unlike the ferritins, very little is known about the Er-VIT1 family. The functional mechanism of the Er-VIT1 family remains unknown.

The membrane-bound ferritin (MbfA) protein belongs to the Er-VIT1 family. Previous studies in Bradyrhizobium japonicum, Rhizobium leguminosarum, A. tumefaciens and Rhodobacter sphaeroides demonstrated that transcription of the mbfA gene is regulated by iron levels, and is negatively controlled by the iron response regulator (irr) under iron-limiting conditions (Hibbing & Fuqua, 2011; Peuser et al., 2012; Rudolph et al., 2006; Todd et al., 2006). It has been shown that the expression of A. tumefaciens mbfA is also inducible upon exposure to H2O2 and MbfA plays a role in H2O2 resistance, possibly by removing intracellular free iron and thus preventing the Fenton reaction (Ruangkiattikul et al., 2012). Here, the topology and function of A. tumefaciens MbfA were further investigated. The N-terminal domain containing the iron-binding site was likely responsible, at least in part, for controlling the cytoplasmic levels of free iron. The MbfA was shown to confer protection against acid-enhanced iron toxicity.

### METHODS

#### Bacterial growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. Luria–Bertani (LB) medium was used for the routine culture of bacteria. Escherichia coli and A. tumefaciens were grown aerobically at 37 and 28 °C, respectively, with shaking at 150 r.p.m. Bacteria grown overnight in LB medium were subcultured into fresh LB medium to OD600 0.1. After incubation for 4 h to OD600 0.4, the cells were used as exponential-phase cells, as indicated. LA refers to LB medium containing 1.5% agar. When the pH of the LB medium was adjusted to pH 5.5, the medium was designated LB 5.5. Induction broth pH 5.5 (IB 5.5) is a medium that mimics the vir-inducing condition of a plant wound (Cangelosi et al., 1991). When required, medium supplemented with 100 μg carbenicillin ml−1, 20 μg chloramphenicol ml−1, 60 μg gentamicin ml−1 or 30 μg kanamycin ml−1 was used for A. tumefaciens. Medium supplemented with 100 μg ampicillin ml−1 or 20 μg chloramphenicol ml−1 was used for E. coli.

#### Molecular techniques

General molecular techniques were performed using standard protocols (Sambrook et al., 1989). The sequence of cloned DNA was confirmed by DNA sequencing (Macrogen). Plasmid DNA was electroporated into A. tumefaciens strains (Cangelosi et al., 1991).

#### Construction of mbfA–phoA and mbfA–lacZ fusions

The E. coli 5′-truncated phoA gene lacking its signal peptide sequence was amplified using primers BT3586 (5′-GGGCCGTGTTCTGGAAAAACCG-3′)/BT3587 (5′-GGGCCACGGTTTTTATTTATGCC-3′) and cloned into the Smal site of pBBR1MCS-4 (Kovach et al., 1995) to generate the plasmid p’PhoA. Primers BT1707 (5′-CGCGTTGCCGATGATAATGCC-3′)/BT3880 (5′-GGTCAGAAGGAACTGCGCGT-3′) were used to amplify the mbfA promoter region and sequences encoding the amino acid residues of M1–T180 (the DNA fragment was named M1–T180). Primers BT1707/ BT3776 (5′-CCGGTGGCGATGATAATGCC-3′) were used to amplify

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
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<td><em>Agrobacterium tumefaciens</em></td>
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<tr>
<td>NTL4</td>
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<tr>
<td>WK074</td>
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<tr>
<td>DH5α</td>
<td>Host for general DNA cloning</td>
<td>Grant et al. (1990)</td>
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<td>pNR114C</td>
<td>Full-length mbfA coding region cloned into pBBR1MCS-4, Ap′</td>
<td>Ruangkiattikul et al. (2012)</td>
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<td>Plasmids for topology analysis</td>
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<td>pPR9TT</td>
<td>Reporter vector, LacZ as a reporter, Ap′</td>
<td>Santos et al. (2001)</td>
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<td>This study</td>
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<td>This study</td>
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<td>p’PhoA</td>
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<td>This study</td>
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<td>MbfA aa 1–327 fused to PhoA of p’PhoA, Ap′</td>
<td>This study</td>
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the mbfA promoter region and sequences encoding the amino acid residues of M1–A327 (the DNA fragment was named M1–A327). Next, mbfA–phoA and mbfA–lacZ in-frame fusions were generated. The DNA fragments of mbfA (M1–T180 and M1–A327) were cloned into the SmaI site of pPhoA to generate plasmids pM1-T180PhoA and pM1-A327PhoA, respectively. The DNA fragments M1-T180 and M1-A327 were cloned into the SmaI site of a reporter vector pPR9TT (Santos et al., 2001) to generate plasmids pM1-T180LacZ and pM1-A327LacZ, respectively.

Alkaline phosphatase activity assay. The cell culture grown overnight in LB medium was adjusted to OD_{600} 1. The cell suspension (1 ml) was harvested by centrifugation at 12 000 g for 1 min. The cell pellet was resuspended in 400 μl 1 M Tris/ HCl, pH 8.0. Cells were permeabilized by the addition of 50 μl 0.1% SDS and 50 μl chloroform, and incubated at 28 °C for 5 min. Reactions were started by adding 400 μl 0.8 mg p-nitrophenyl phosphate ml⁻¹ solution in 1 M Tris/HCl, pH 8.0, and they were incubated at 28 °C for 60 min. Reactions were stopped with 100 μl 1 M K_{2}HPO_{4} and centrifuged at 12 000 g for 1 min to remove cells. The supernatant was collected and the OD_{420} was measured. Units of alkaline phosphatase activity were defined as the change in OD_{420} min⁻¹ per OD_{600} of culture (Brickman & Beckwith, 1975). The data were reported as the mean ± SD of biological triplicates.

β-Galactosidase activity assay. The assay was based on the previously reported protocol (Miller, 1972). Overnight cell culture grown in LB medium was permeabilized by SDS and chloroform, as described above. Aliquots of 50 μl of the treated cells were incubated with 950 μl ONPG solution (3.3 mM ONPG, 1 mM MgCl₂, 50 mM 2-mercaptoethanol in 50 mM phosphate buffer, pH 7.0) at 28 °C. The OD_{420} was measured. Units of β-galactosidase activity were defined as the change in OD_{420} min⁻¹ per OD_{600} of culture. The data were reported as the mean ± SD of biological triplicates.

Subcellular fractionation and Western blot analysis of MbFA–PhoA and MbFA–LacZ fusion proteins. Overnight cell cultures grown in LB medium were harvested and subcellular fractionation was performed using the method described previously (Nilavongse et al., 2006). Protein concentrations were determined using the Bradford Bio-Rad protein assay and BSA protein standard. The total proteins (50 μg) from subcellular fractions were separated by 7.5 % SDS-PAGE and transferred onto Hybond-P PVDF membranes (Amersham Pharmacia Biotech) using a Bio-Rad semi-dry blotting apparatus. The PhoA fusion protein was detected using mouse anti-bacterial alkaline phosphatase antibody (Abcam) and sheep anti-mouse IgG–horseradish peroxidase (Bio-Rad) using a Bio-Rad semi-dry blotting apparatus. The PhoA fusion protein was detected using mouse anti-bacterial alkaline phosphatase antibody (Abcam) and sheep anti-mouse IgG–HRP conjugate (Qiagen). Proteins were visualized using TMB Stabilized Substrate for HRP (Promega). The experiment was repeated twice to ensure the reproducibility of the results.

Measurement of total cellular metal content. Cells were grown in LB or in LB supplemented with, individually, 50 μM CoCl₂, FeCl₃, MnCl₂ or ZnCl₂ for 4 (exponential-phase cells) and 24 h (stationary-phase cells). Cells were washed with 50 mM potassium phosphate buffer, pH 7.0 (KPB) and 10 mM EDTA a total of three times. Cells were then washed with 50 mM KPB twice and finally resuspended in KPB to achieve OD_{600} 1. Aliquots of 2 μl of the cell suspension were harvested and digested with 300 μl 65 % ultrapure nitric acid at 55 °C for 18 h. The samples were brought up to a total volume of 2.5 ml with metal-free MilliQ water. Standard solutions of CdCl₂, CoCl₂, CuSO₄, FeCl₃, MnCl₂, NiCl₂ or ZnCl₂ were prepared in a solution with a final nitric acid concentration similar to that of the digested samples and were used to prepare calibration curves. Metal levels were measured in parts per billion (ppb) by using inductively coupled plasma (ICP)-MS (Agilent Technologies 7500c equipped with a G3160A integrated autosampler and a Babington nebuliser). The excitation power of the plasma was 1500 W; the gas flow rates for plasma gas, carrier gas and make-up gas were 15.0, 0.9 and 0.3 l min⁻¹, respectively. Hydrogen and helium gases were used as reaction gases at flow rates of 4.5 and 5.0 ml min⁻¹, respectively. The data were reported as the mean ± SD of biological triplicates.

Sensitivity to streptonigrin (SNG). The SNG sensitivity test was performed as described previously (Ngok-Ngam et al., 2009). Cells were treated with SNG at a final concentration of 150 μg ml⁻¹ in the presence of either 50 μM FeCl₃ or 100 μM 2,2'-dipyridyl (Dy) at 28 °C for 48 h. SNG was prepared as a 10 mg ml⁻¹ stock solution in DMSO. Cells treated with DMSO were used as a control. The experiment was repeated twice to ensure the reproducibility of the results. It should be noted that treatment with either 50 μM FeCl₃ or 100 μM Dy only did not inhibit bacterial growth.

Measurement of growth in the presence of high iron under acidic conditions. Overnight cell cultures were washed and adjusted to OD_{600} 0.1 in fresh LB and LB 5.5 in the absence or presence of 1.25 mM of FeCl₃. Cell cultures were incubated at 28 °C with shaking for 24 h and the OD_{600} was measured. Each strain was tested in biological triplicates and the experiment was repeated twice to ensure the reproducibility of the results.

Quantitative real-time (qRT)-PCR analysis. Exponential-phase NTL4 cells grown in LB medium (pH 7.0), LB 5.5 and IB 5.5 were untreated or treated with 50 μM FeCl₃ at 28 °C for 15 min. Total RNA was extracted and reverse transcribed to cDNA as described previously (Ngok-Ngam et al., 2009). qRT-PCR was performed as described previously (Bhubhanil et al., 2014). Gene-specific primers for mbfA [BT1666 (5'–AACGTCTCGGTAGTCTGCG–3')/BT1667 (5'–CGCTTCAACGGTGATCCAG–3')] and the 16S rRNA gene [BT1421 (5'–GAATCTACCATCTCTGCGG–3')/BT1422 (5'–AAGGCCTTACATCAGCCG–3')] were used. The data were reported as the mean ± SD of biological triplicates.

RESULTS AND DISCUSSION

Topology analysis of A. tumefaciens MbFA

The A. tumefaciens MbFA protein is 327 aa in length (Fig. S1a, available in the online Supplementary Material); it is a member of the Er-VIT1 family that has an N-terminal ferritin-like domain that is likely involved in binding to iron and is proposed to have five transmembrane segments in the C-terminal domain (Andrews, 2010). However, the membrane topology of MbFA has not been verified experimentally. The membrane topology of A. tumefaciens MbFA protein was predicted using four different programs: HMMTOP (Tusnady & Simon, 2001), TMpred (Hofmann & Stoffel, 1993) and MEMSAT (Jones, 2007). All four programs predicted that there would be five membrane segments in the C-terminal half of MbfA (Fig. S1b), and the presence of five transmembrane segments in the C-terminal half of MbFA (Fig. S1b), and the presence of five transmembrane segments in the C-terminal half of MbFA (Fig. S1b). Furthermore, the PhoBius program also predicted that the N-terminal ferritin-like domain of MbFA (aa 1–184) is found in the cytoplasmic side.

Alkaline phosphatase (PhoA) and β-galactosidase (LacZ) have been used widely as reporter proteins to study protein...
localization (Cunneen & Reeves, 2008; Daniels et al., 1998; Haardt & Bremer, 1996; Sarsero & Pittard, 1995). PhoA is enzymically active when it is translocated to the periplasm but inactive when it is retained in the cytoplasm (Derman & Beckwith, 1991). If PhoA is fused to a protein target site normally facing the periplasm, the PhoA is likely to be exported to the periplasm and to exhibit enzymic activity (Manoil & Beckwith, 1986). Alternatively, fusion of PhoA to a cytoplasmic site of a target protein results in the PhoA remaining in the cytoplasm and being inactive. In contrast to PhoA, LacZ is active in the cytoplasm, whereas the periplasmic domain–LacZ fusion causes the hybrid enzyme to remain embedded within the membrane and renders it inactive (Froshauer et al., 1988; Lee et al., 1989; Manoil, 1990). Therefore, the alkaline phosphatase and β-galactosidase activities of the hybrid proteins could be used as indicators for the periplasmic and cytoplasmic locations, respectively, of the target protein fusion sites.

To confirm the topology prediction of MbfA, the polypeptide arrangement was determined. The N-terminal iron-binding domain of MbfA (residues M1–T180) and full-length MbfA (residues M1–A327) were used for in-frame fusions between MbfA and reporter proteins PhoA and LacZ, to generate MbfA–PhoA and MbfA–LacZ hybrid proteins. Enzymic activity was assayed using WT NTL4 carrying plasmids that express the hybrid proteins MbfA–PhoA (pM1-T180PhoA and pM1-A327PhoA) and MbfA–LacZ (pM1-T180LacZ and pM1-A327LacZ). The alkaline phosphatase and β-galactosidase activities corresponding to the fusion sites are shown in Fig. 1(b, grey and white boxes, respectively). The M1–T180PhoA fusion protein (~0.4 U) had very low levels of alkaline phosphatase

Fig. 1. (a) The predicted topological model of MbfA. Amino acid residues T180 and A327 of MbfA selected for the fusion site are indicated. (b) The proposed topological model of A. tumefaciens MbfA tested by MbfA–PhoA and MbfA–LacZ fusions. The putative iron-binding motifs (E-6-Y and ExxH) in the N-terminal domain are indicated by circles. The fusion sites at aa T180 and A327 of MbfA along with enzyme activities of alkaline phosphatase (MbfA–PhoA fusions, grey boxes, units) and β-galactosidase (MbfA–LacZ fusions, white boxes, units) are indicated. The values of enzyme activities are the mean ± SD of biological triplicates. The five transmembrane segments (TM1, TM2, TM3, TM4 and TM5) are shown as black boxes. (c) Western blot analysis. Overnight cell cultures of WT-carrying plasmid expressing MbfA–PhoA (M1–T180PhoA and M1–A327PhoA) or MbfA–LacZ (M1–T180LacZ and M1–A327LacZ) fusion proteins were harvested and fractionated into periplasm (P), cytoplasm (Cy) and membrane (M) fractions. Cellular fractions (50 μg proteins) were separated by 7.5 % SDS-PAGE. The fusion proteins were detected using antibodies against alkaline phosphatase (Anti-PhoA) and β-galactosidase (Anti-LacZ). The estimated sizes of the detected bands are indicated.
activity compared with the M1-A327PhoA fusion protein (~198 U), whilst the M1-T180LacZ fusion protein (~44 U) exhibited higher levels of β-galactosidase activity compared with the M1-A327LacZ fusion protein (~6 U). These results suggested that the T180 and A327 locations of MbfA are in the cytoplasm and the periplasm, respectively (Fig. 1b). WT NTL4 containing either the p’PhoA or pPR9TT plasmid was used as a negative control that showed no detectable activity of alkaline phosphatase or β-galactosidase.

Western blot analyses confirmed that all the MbfA–PhoA and MbfA–LacZ fusion proteins were produced successfully (Fig. 1c). Protein bands of the estimated sizes for the M1-T180PhoA, M1-T180LacZ and M1-A327LacZ fusions (68, 136 and 151 kDa, respectively) were detected. The M1-A327PhoA protein band was observed at a higher position than its estimated size (83 kDa), possibly due to polar residues in transmembrane segments of the hybrid protein, which in another protein has been shown to cause abnormal mobility (Walkenhorst et al., 2009).

Cells expressing MbfA–PhoA and MbfA–LacZ fusion proteins were fractionated into the periplasm, cytoplasm and membrane. None of the MbfA–PhoA or MbfA–LacZ fusion proteins were detected in the periplasmic fraction. The M1-T180PhoA and M1-T180LacZ fusion proteins were detected only in the cytoplasmic fraction (Fig. 1c), supporting the computational predictions that there is no transmembrane segment in the N-terminal iron-binding domain of MbfA (Fig. 1a) and also supporting the Phobius prediction that the N-terminal domain of MbfA is in the cytoplasm. However, the M1-A327PhoA and M1-A327LacZ fusion proteins were detected in the membrane fraction (Fig. 1c). Taking the results together, it was proposed that the N-terminal ferritin-like domain of A. tumefaciens MbfA is located in the cytoplasm, whilst the C-terminal end faces the periplasm (Fig. 1b).

**mbfA mutant has higher levels of total iron content than WT**

To assess the influence of mbfA on the total cellular iron content, WT NTL4 and the mbfA mutant (NR114) (Ruangkiattikul et al., 2012) were analysed for total iron content using ICP-MS. The results showed that exponential-phase cells of WT and mutant strains grown in LB medium or LB supplemented with 50 μM FeCl₃ had no obvious difference in total iron content (data not shown). Using stationary-phase cells grown in LB, the mutant carrying plasmid vector pBBR1MCS-4 (NR114/pBBR, ~94 p.p.b.) had a slightly higher total iron content than that of the WT carrying a plasmid vector (NTL4/pBBR, ~84 p.p.b.) (Fig. 2a). The difference in total iron content was more obvious when stationary-phase cells were grown in LB supplemented with 50 μM FeCl₃, in that the mutant NR114/pBBR had an iron content (~200 p.p.b.) that was much higher than that of the WT NTL4/pBBR (~134 p.p.b.) (Fig. 2a).

Next, the metal specificity response of mbfA was tested using WT and mutant stationary-phase cells grown in LB supplemented with 50 μM MnCl₂, ZnCl₂ and CoCl₂. The total contents of manganese, zinc and cobalt in the mutant NR114 were not significantly different from those of WT NTL4 (P>0.05 via unpaired Student’s t-test, Fig. 2b), demonstrating that A. tumefaciens mbfA responds specifically to iron.

The increased total iron content of the mutant NR114 could be a result of increased iron uptake. To test this idea, the expression levels of iron-uptake genes (fatB and irp6A) and siderophore biosynthesis genes (Atu3673 and Atu3675) were determined using qRT-PCR. It was found that the expression levels of the iron-uptake genes in the mutant NR114 and WT grown in LB supplemented with 50 μM FeCl₃ were similar (data not shown), suggesting that the increased total iron content of the mutant NR114 was not due to the increased expression of iron-uptake genes. This is because expression of iron-uptake genes in the mbfA mutant would be expected to be repressed by RirA (rhizobial iron regulator) under the high-iron conditions tested.

Typically, bacterial strains lacking iron-storage genes have a reduction in total iron content (Boughammoura et al., 2008; Chen et al., 2010; Velayudhan et al., 2007; Waidner et al., 2002). In contrast, the A. tumefaciens mbfA mutant has increased total iron content, indicating that MbfA does...
not act in iron storage despite its N-terminal, ferritin-like domain. Therefore, MbfA may function as an iron efflux transporter. Supporting this idea, WT cells expressing mbfA multi-copies from the plasmid pNR114C, NTL4/pNR114C, contained less total iron than NTL4/pBBR (Fig. 2a). In the complemented strain, NR114/pNR114C also showed lower total iron content than the mutant NR114/pBBR (Fig. 2a).

mbfA mutant has increased accumulation of intracellular free iron

It was proposed that the increased hypersensitivity of NR114 to H₂O₂ resulted from enhanced levels of the iron-catalysed reaction (Ruangkiattikul et al., 2012). To test if the mutant NR114 exhibited increased accumulation of iron in the reactive form, which can drive the Fenton reaction, the sensitivity to SNG was tested. SNG is an antibiotic that requires iron for its bactericidal action (Yeowell & White, 1982). Higher levels of intracellular free iron are correlated with increased sensitivity to SNG (Bates et al., 2005; Ngok-Ngam et al., 2009; Yeowell & White, 1982). The mutant NR114/pBBR was 10-fold more sensitive to SNG + Fe than WT NTL4/pBBR (Fig. 3), suggesting that the mbfA mutant had intracellular free iron levels that were higher than that of WT. Conceivably, cells exhibiting hypersensitivity to SNG could have reduced superoxide dismutase (SOD) activity (Gregory & Fridovich, 1973); however, this is not the case for the mutant NR114. Determination of SOD activity using the SOD activity gel assay (Ngok-Ngam et al., 2009) revealed that the WT and mutant NR114 had similar levels of SOD activity (data not shown).

Cells expressing multi-copies of the mbfA gene, NTL4/pNR114C and NR114/pNR114C, were 10²- and 10⁴-fold more resistant to SNG + Fe than NTL4/pBBR and NTL4/pBBR, respectively (Fig. 3). In the presence of Dy, a cell-membrane-permeable iron chelator that chelates intracellular iron, the mutant NR114/pBBR showed a similar level of SNG resistance to that of WT NTL4/pBBR (Fig. 3, SNG + Dy). These results support the view that the mbfA gene plays an important role in controlling intracellular iron levels. The loss of mbfA led to increased total iron content (Fig. 2a) and increased sensitivity to SNG + Fe treatment (Fig. 3), suggesting that the extra iron in the mbfA mutant was likely in the free form. Unexpectedly, the presence of Dy increased the sensitivity of cells to SNG. One possible explanation for this is that Dy might reduce the amount of iron available for iron-containing enzymes or proteins that are involved in SNG resistance.

Growth of mbfA mutant was reduced in the presence of high iron under acidic conditions

It has been shown previously that mutation of the mbfA gene does not affect the growth of A. tumefaciens in LB medium (pH 7.0) under either high-iron (100 μM FeCl₃) or low-iron (300 μM Dy) conditions (Ruangkiattikul et al., 2012). Acidic conditions help to increase iron toxicity by stabilizing the more soluble and reactive form of iron (Fe²⁺). A. tumefaciens typically encounters low pH environments (~pH 5.5) at plant wound sites during infection (Zhu et al., 2000). The question of whether mbfA plays a role in iron resistance under acidic conditions has been raised. To answer this question, the iron resistance of cells grown in LB 5.5 was determined.

The results showed that all strains grew similarly in LB 5.5, suggesting that the mbfA mutation does not affect acid tolerance (Fig. 4a). The growth of the mbfA mutant (NR114/pBBR) was reduced in LB 5.5 supplemented with 1.25 mM FeCl₃ compared with WT (NTL4/pBBR). At iron concentrations <1.25 mM, the growth of the mbfA mutant was similar to that of WT (data not shown). Importantly, the reduced growth of the mutant at 1.25 mM FeCl₃ was reversed in the complemented strain (NR114/pNR114C) (Fig. 4a), demonstrating that MbfA plays a role in iron resistance in A. tumefaciens under acidic conditions.

Expression of mbfA is highly inducible when cells are grown in an iron-replete acidic medium

The expression of mbfA is inducible by an iron response regulator (Irr) in response to high iron levels (Hibbing & Fuqua, 2011; Ruangkiattikul et al., 2012). Consistent with the previous finding, qRT-PCR analysis showed that the expression of mbfA in WT NTL4 was inducible under high iron concentrations.

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<td>NR114/pBBR</td>
<td>-3</td>
<td>-3</td>
</tr>
<tr>
<td>NR114/pPNR114C</td>
<td>-5</td>
<td>-4</td>
</tr>
</tbody>
</table>

Fig. 3. Sensitivity to SNG. Cells were treated with streptonigrin (150 μg ml⁻¹) in the presence of 50 μM FeCl₃ (SNG + Fe) or 100 μM Dy (SNG + Dy) at 28 °C for 48 h. SNG was prepared as a stock solution in DMSO. Cells treated with DMSO were used as control. Cells were then diluted and spotted on LA plates. Plates were incubated at 28 °C for 2 days. Tenfold serial dilutions are indicated above each column.
iron conditions (LB grown in LB (pH 7.0), LB 5.5 and IB 5.5 were untreated (−) or treated with 50 μM FeCl₃ (Fe) at 28 °C for 15 min. Fold changes in mbfA expression are relative to untreated cells grown in LB (regarded as one). The results are the mean ± SD of biological triplicates. *Significantly different from untreated cells grown in LB (P<0.05 via unpaired Student’s t-test).

Fig. 4. (a) Growth in the presence of high iron under acidic conditions. Cells were grown in LB 5.5 in the absence and presence of 1.25 mM FeCl₃ (LB 5.5 + 1.25 mM Fe). The bacterial growth at 24 h was determined by measuring OD₆₀₀. The data are the mean ± SD of biological triplicates. *Significantly different when comparing NR114/pBBR with NTL4/pBBR grown in LB 5.5 + 1.25 mM Fe (P<0.05 via unpaired Student’s t-test). (b) qRT-PCR analysis of mbfA expression. Exponential-phase cells of WT NTL4 grown in LB medium (pH 7.0), LB 5.5 and IB 5.5 were untreated (−) or treated with 50 μM FeCl₃ (Fe) at 28 °C for 15 min. Fold changes in mbfA expression are relative to untreated cells grown in LB (regarded as one). The results are the mean ± SD of biological triplicates. *Significantly different from untreated cells grown in LB (P<0.05 via unpaired Student’s t-test).

iron conditions (LB + 50 μM FeCl₃) (Fig. 4b). As mbfA was shown to be required for iron resistance under acidic conditions (Fig. 4a), qRT-PCR analysis was performed to determine if acidic conditions had any further effect on the expression levels of mbfA. The results in Fig. 4(b) show that the expression of mbfA from cells grown in LB 5.5 and IB 5.5 was not significantly different from cells grown in LB (P>0.05 via unpaired Student’s t-test), indicating that acidic conditions do not affect the expression of mbfA. In LB medium, mbfA expression increased ~1.5-fold in response to iron. This iron-induced expression of mbfA was further enhanced under acidic conditions ~2-fold in LB 5.5 + Fe and ~2.5-fold in IB 5.5 + Fe (Fig. 4b). The high induction of mbfA expression observed during exposure to iron under acidic conditions further supports the notion that A. tumefaciens mbfA is involved in protection against acid-enhanced iron toxicity. An irr mutant strain, WK074, (Ruangkiattikul et al., 2012) exhibited increased expression of mbfA 75-fold in LB 5.5 and 100-fold in LB 5.5 + Fe compared with WT in LB 5.5, which is consistent with the repressor role of Irr. The fact that iron-mediated mbfA induction under acidic conditions was still observed in an irr mutant indicates that the induction was Irr independent.

In conclusion, topology analysis of A. tumefaciens Mbfa provides additional information that leads to a better understanding of its previously reported role in protecting cells from iron and H₂O₂ stress (Ruangkiattikul et al., 2012). Our data suggest that Mbfa may be an iron efflux transporter that controls cytoplasmic iron concentrations and thus prevents iron-induced toxicity via the Fenton reaction. The N-terminal iron-binding domain of Mbfa located in the cytoplasm may be involved in sequestering cytoplasmic free iron, which can then be transported out of the cytoplasm through the C-terminal-membrane-embedded VIT1 domain. The direction of iron transport must be confirmed by additional experiments, such as monitoring iron radioisotope uptake into everted membrane vesicles (Grass et al., 2005), which is an interesting subject for future study. Here, Mbfa has been shown to play a role in the resistance to iron under acidic conditions. The response of A. tumefaciens to iron under low pH is of interest because the induction of its virulence genes occurs only under acidic conditions, which are typical for the environmental pH of A. tumefaciens wound infection sites on host plants (Zhu et al., 2000). The role of A. tumefaciens Mbfa in maintaining levels of essential iron at suitable ranges for bacterial growth but not at levels toxic to cells may be important during infection. Therefore, a virulence assay of the mbfA mutant should also be performed in a future study. Compared with the model gammaproteobacteria, alphaproteobacteria, to which A. tumefaciens belongs, display atypical regulation of iron homeostasis genes that is mediated by Irr and RirA under low- and high-iron conditions, respectively (Rodionov et al., 2006). Expression of A. tumefaciens mbfA is negatively regulated by Irr but not RirA (Ruangkiattikul et al., 2012). Furthermore, Mbfa is mainly present in alphaproteobacteria, with only a very few other gammaproteobacteria (such as Kushneria aurantia and Serratia sp. ATCC 39006) have been found to contain closely related sequences and these have yet to be characterized. It therefore seems that Mbfa is very much a signature polypeptide of alphaproteobacteria.

ACKNOWLEDGEMENTS

S. B. received a Royal Golden Jubilee Scholarship (PHD52K0207) from the Thailand Research Fund. This work is supported by Chulabhorn Research Institute and the Thailand Research Fund (grant no. RSA5380004) to R.S.

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Edited by: J. Cavet