

***Bradyrhizobium japonicum* senses iron through the status of haem to regulate iron homeostasis and metabolism**

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Summary

The *Irr* protein from the bacterium *Bradyrhizobium japonicum* is expressed under iron limitation to mediate iron control of haem biosynthesis. The regulatory input to *Irr* is the status of haem and its precursors iron and protoporphyrin at the site of haem synthesis. Here, we show that *Irr* controls the expression of iron transport genes and many other iron-regulated genes not directly involved in haem synthesis. *Irr* is both a positive and negative effector of gene expression, and in at least some cases the control is direct. Loss of normal iron responsiveness of those genes in an *irr* mutant, as well as a lower total cellular iron content, suggests that *Irr* is required for the correct perception of the cellular iron status. Degradation of *Irr* in iron replete cells requires haem. Accordingly, control of *Irr*-regulated genes by iron was aberrant in a haem-defective strain, and iron replete mutant cells behave as if they are iron-limited. In addition, the haem mutant had an abnormally high cellular iron content. The findings indicate that *B. japonicum* senses iron via the status of haem biosynthesis in an *Irr*-dependent manner to regulate iron homeostasis and metabolism.

Introduction

Iron is an essential element for most living organisms. It is required for many cellular processes in catalysis, elec-

tron transfer, oxygen metabolism, regulation and signal transduction. Iron availability can be limiting because it is predominantly in the insoluble ferric form in aerobic environments. On the other hand, excessive intracellular iron can be deleterious because it generates reactive oxygen species that damage cellular components (Braun and Killmann, 1999; Touati, 2000). Iron homeostasis is strictly regulated so that iron acquisition, storage and consumption are geared to iron availability, and that intracellular levels of free iron do not reach toxic levels (reviewed in Andrews *et al.*, 2003).

In bacteria, the Fur (ferric-uptake regulator) protein is regarded as a global regulator of iron metabolism and homeostasis. It has been particularly well-studied in the γ -proteobacteria *Escherichia coli* (Crosa, 1997; Escolar *et al.*, 1999; Hantke, 2001) and *Pseudomonas aeruginosa* (Ochsner *et al.*, 1995; Ochsner and Vasil, 1996), and the prevailing model of Fur function is based largely on work from those organisms. In the presence of iron, the metal binds Fur directly, which enhances its DNA-binding activity to represses genes under its control.

Rhizobia belong to the α -Proteobacteria, a taxonomic group comprising numerous members that form close or intracellular associations with higher eukaryotes in a symbiotic or pathogenic context. Rhizobia live as free-living soil organisms or in symbiosis with leguminous plants, where they convert atmospheric nitrogen to ammonia to fulfil the nutritional nitrogen requirement of the plant host (Nap and Bisseling, 1990). Recent work with rhizobial species shows that control of iron metabolism differs substantially from the *E. coli* model. Some rhizobia have iron regulators in addition to Fur, or have structural Fur homologues that appear not to be iron regulators (Hamza *et al.*, 1998; 1999; Todd *et al.*, 2002; Chao *et al.*, 2004; 2005; Diaz-Mireles *et al.*, 2004; Platero *et al.*, 2004; Viguier *et al.*, 2005).

The *Irr* protein has been characterized in *Bradyrhizobium japonicum* and *Brucella abortus* (Hamza *et al.*, 1998; Martinez *et al.*, 2005), and homologues are present in other α -Proteobacterial genomes. Haem is an iron protoporphyrin and is the end product of a multistep biosynthetic pathway. *Irr* mediates iron control of the haem

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biosynthetic pathway to prevent the accumulation of toxic porphyrin precursors from exceeding iron availability (Hamza *et al.*, 1998). Irr accumulates under iron limitation, where it negatively regulates the pathway. Irr is a conditionally stable protein that degrades rapidly when cells are exposed to iron, allowing derepression of haem synthesis (Hamza *et al.*, 1998; Qi *et al.*, 1999). This iron-dependent turnover is mediated by haem (Qi *et al.*, 1999), which binds directly to Irr to promote degradation. Accordingly, Irr persists in haem synthesis mutant strains in the presence of iron, and mutations in Irr that affect the haem binding site stabilize the protein in the presence of the metal (Qi *et al.*, 1999; Yang *et al.*, 2005). Thus, haem is an effector molecule in Irr degradation that reflects the availability of iron for haem synthesis. Irr interacts directly with the haem biosynthesis enzyme ferrochelatase (Qi and O'Brian, 2002). In the presence of iron, ferrochelatase inactivates Irr followed by haem-dependent degradation. Under iron limitation, protoporphyrin relieves the inhibition of Irr by ferrochelatase, probably by promoting protein dissociation, allowing genetic repression. Thus, Irr responds to iron via the status of protoporphyrin and haem locally at the site of haem synthesis.

In the present study, we show that Irr controls many iron-regulated genes, and is not restricted to haem biosynthesis. Furthermore, the findings suggest that the status of haem biosynthesis may be a regulatory input signal to control iron metabolism.

Results

The Irr protein controls ferric iron transport genes

The Irr protein negatively controls haem biosynthesis when iron is limiting. Irr accumulates in cells grown under iron deprivation where it is needed, but degrades when cells are exposed to iron. We found previously that an *irr* mutant is deficient in an inducible ferric iron transport activity (Hamza *et al.*, 1998), suggesting that Irr may have a broader role in regulating iron-dependent processes. Iron deficiency induces high-affinity transport systems in bacteria to scavenge available iron (Andrews *et al.*, 2003). The *B. japonicum* genome encodes five ferric iron chelate receptors. We used quantitative real time polymerase chain reaction (qRT-PCR) to measure mRNA levels of the five ferric iron chelate receptor genes in the parent and *irr* strains grown in high or low-iron media (Fig. 1). These

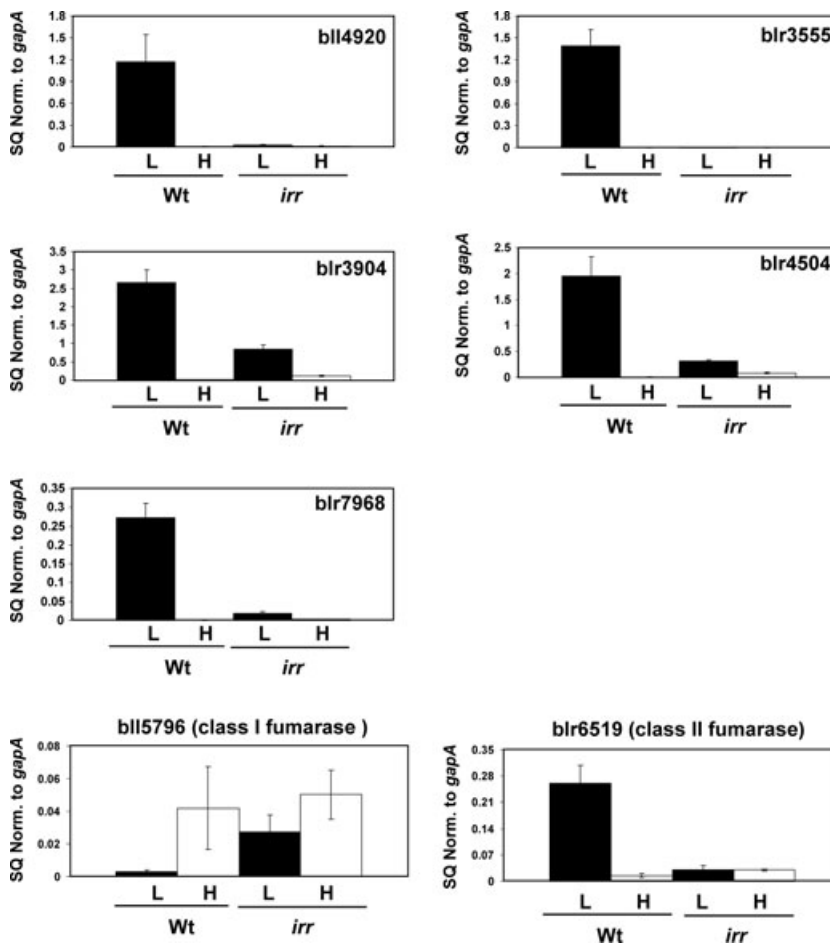


Fig. 1. Iron-dependent expression of the ferric receptor genes and fumarase genes in the wild-type strain LO (Wt) or the *irr* mutant LODTM5 (*irr*). mRNAs were analysed by quantitative real-time PCR from cells grown in media supplemented with no iron (L) or with 12 μ M FeCl₃ (H). The data are expressed as the relative starting quantity (SQ) of the respective mRNAs normalized to the housekeeping gene *gapA*. The data are expressed as the average of three replicates \pm the standard deviation.

data were normalized to the *gapA* gene, which was not iron-regulated or affected in the *irr* strain (This was corroborated in the microarray experiments described below). The data showed strong induction of these genes in response to iron limitation in the parent strain. However, induction of the iron transport genes was abolished or diminished in the *irr* strain (Fig. 1), showing that Irr normally has a positive effect on their expression. These findings agree with the previous observation that an *irr* strain is defective in iron transport (Hamza *et al.*, 1998). Induction of *blr3555* and *blI4920* appeared to be strictly dependent on Irr because there was little transcript in the mutant in low-iron cells. Levels of *blr3904* and *blr4504* mRNA were diminished but measurable in the *irr* strain (Fig. 1). This remaining activity was iron-responsive in the mutant as judged by the approximately threefold decrease in mRNA levels in the presence of iron. This suggests some Irr-independent iron regulation of those genes. A similar result was observed previously using reporter fusions of the *hmuR* haem receptor gene (Nienaber *et al.*,

2001). In that study, promoter activity was diminished in an *irr* strain, but the remaining activity was iron responsive.

The Irr protein affects many genes within the iron regulon

Iron transport is central to iron homeostasis. The observation that Irr controls the expression of iron transport genes led us to ask whether Irr affects iron metabolism more generally. To do this, we first characterized the iron regulon in parent strain LO by using whole genome microarray analysis to identify changes in gene expression in cells in response to iron limitation. (Table S1) Then, we compared strain LO to the *irr* mutant LODTM5 grown in iron-deficient media using microarrays (Table S2), and focused on those genes that were also iron-regulated in the wild type (Table 1).

Comparison of the parent strain grown under high- or low-iron conditions detected 343 genes upregulated or downregulated in response to iron limitation (Table S1).

Table 1. Genes within the iron regulon that are aberrantly regulated in *irr* strain LODTM5 grown in iron-limited media compared with parent strain LO grown in iron-limited media.^a

Genes that are downregulated in the <i>irr</i> mutant strain compared with the parent strain			
Gene ^b	<i>irr/irr</i> ⁺	SD	Gene product
blr0056	-2.41	0.10	Unknown protein
blI0233	-2.11	0.32	Hypothetical protein
blI0620	-2.02	0.39	Two-component response regulator
blr0697	-5.68	1.63	Hypothetical protein
blr0698	-8.86	3.44	Putative hydroxymethylglutaryl-CoA lyase
bsr0858	-3.10	1.64	Hypothetical protein
bsr0859	-3.20	1.82	Hypothetical protein
blI1076	-3.50	0.43	Hypothetical protein
blr1180	-2.66	0.37	Two-component response regulator
blI1555	-2.99	0.51	Unknown protein
blI2216	-2.77	0.11	Transcriptional regulatory protein TetR family
blr3445	-3.78	0.72	Putative enoyl-CoA hydratase
blr3553	-11.51	3.77	Unknown protein
blr3554	-6.36	1.71	Unknown protein
blr3555	-42.60	12.93	Ferric-siderophore receptor precursor
bsr3556	-101.67	39.67	Hypothetical protein
blI3557	-9.66	1.00	Putative cytochrome b561
blI3558	-3.90	0.96	Two-component hybrid sensor and regulator
blI3559	-2.71	1.12	Two-component response regulator
blr3561	-18.14	14.14	Hypothetical protein
blr3562	-22.00	17.63	Hypothetical protein
blr3904	-2.32	0.44	Ferric-siderophore receptor precursor
blr3905	-2.17	0.41	Hypothetical protein
blr3906	-2.01	0.28	Biopolymer transport protein (ExbB)
blr4146	-5.94	1.59	Hypothetical protein
blI4177	-6.76	7.15	Hypothetical protein
blr4504	-4.14	0.73	Ferric-siderophore receptor precursor
blr4505	-4.31	0.53	Hypothetical protein
blI4708	-5.61	1.22	Probable ATP-binding protein
blI4920	-31.91	11.85	Ferric-siderophore receptor precursor (FegA)
blr5540	-8.19	0.33	Hypothetical protein
blr5541	-2.65	0.31	Hypothetical protein
blI5595	-2.78	1.34	Hypothetical protein
blr6519	-3.27	0.45	Fumarase, class II (FumC)
blr6801	-5.53	2.16	Unknown protein
blr6802	-2.92	0.49	Hypothetical protein
blI6994	-3.33	0.13	Putative phosphatidylethanolamine N-methyltransferase

Table 1. cont.

Genes that are downregulated in the <i>irr</i> mutant strain compared with the parent strain			
Gene ^b	<i>irr/irr</i> ⁺	SD	Gene product
blI6995	-2.60	0.40	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase
blI7074	-2.30	0.16	Hypothetical protein
blI7076	-2.06	0.34	Haemin receptor precursor (HmuR)
blr7077	-2.73	0.48	Haemin ABC transporter haemin-binding protein (HmuT)
blr7078	-2.11	0.31	Haemin ABC transporter permease protein (HmuU)
blr7079	-2.19	0.09	Haemin ABC transporter ATP-binding protein (HmuV)
blr7296	-20.34	6.07	Hypothetical protein
blr7297	-26.70	2.87	Unknown protein
blI7310	-4.01	3.46	Probable ArcA2 arginine deiminase
blI7311	-3.33	2.75	Probable ArcD2 arginine-ornithine antiporter
blr7314	-4.81	4.40	Unknown protein
blr7315	-3.78	2.79	Unknown protein
blr7321	-2.33	0.81	Hypothetical protein
blI7322	-2.67	1.69	Hypothetical protein
bsr7328	-2.80	1.60	Unknown protein
blr7329	-2.45	1.12	Putative multidrug resistance protein
blI7414	-2.66	0.37	Probable Elongation factor G
blr7418	-4.99	1.52	Hypothetical glutathione S-transferase like protein
blr7471	-2.52	0.10	Phosphatidylglycerophosphate synthase
bsr7472	-2.16	0.30	Molybdopterin converting factor small subunit
blr7730	-5.20	0.79	Hypothetical protein
blr7746	-2.77	0.11	Capsule expression protein
blI7967	-4.65	2.02	Uncharacterized iron-regulated protein
blI7968	-12.75	1.33	Ferric-siderophore receptor precursor

Genes that are upregulated in the <i>irr</i> mutant strain compared with the parent strain			
Gene ^b	<i>irr/irr</i> ⁺	SD	Gene product
blr0095	4.75	0.84	Hypothetical protein
blr0488	8.90	1.81	3-isopropylmalate dehydratase large subunit
blr1080	2.79	0.48	Hypothetical protein
blI1200	2.84	0.35	5-aminolevulinic acid synthase
blr2581	3.12	2.24	Putative D-fructose-1,6-bisphosphatase protein
blI2737	2.30	0.16	Oxidoreductase with iron-sulphur subunit
blI3190	2.53	0.27	ABC transporter ATP-binding protein
blI3192	2.54	0.35	ABC transporter substrate-binding protein
blI3193	2.41	0.25	Unknown protein
blr4582	14.38	7.22	Acetylornithine aminotransferase
blr5037	5.07	0.71	Delta-aminolevulinic acid dehydratase
blI5796	3.35	0.47	Fumarase, class I
blI5913	4.35	0.90	Cytochrome c
blr5962	2.17	0.41	ABC transporter ATP-binding protein
blr6742	4.02	0.50	Putative glutamate synthase small subunit
blr6743	3.52	0.67	Putative ferredoxin oxidoreductase alpha subunit
blr6744	3.43	0.51	Putative ferredoxin oxidoreductase beta subunit

a. The list is a subset of genes regulated in the *irr* strain compared with the parent strain under iron limitation. Only genes that were also upregulated in the parent strain in iron limited cells compared with the parent strain grown in iron replete media were included.

b. Genes in bold were analysed further in the article.

Thus, *B. japonicum* has a large iron regulon, which is similar to what has been observed in other bacteria (Baichoo and Helmann, 2002; Ochsner *et al.*, 2002; Gri-fantini *et al.*, 2003; McHugh *et al.*, 2003). Many of the regulated genes are organized in clusters in the genome (indicated by shading in Table S1), which may give some insight into the roles of hypothetical or unknown genes that are proximal to genes of known or putative function. Here, we refer to a cluster as simply a group of adjacent genes regardless of orientation. Moreover, 64 of the 176

genes induced by iron limitation in the wild type were found in clusters that include transporter genes.

About one-third (61 of 176) of the genes that increased in response to iron limitation in the wild type were down-regulated in the *irr* mutant (Table 1), suggesting that *Irr* is normally involved in the positive regulation of those genes. Furthermore, 30 of the 45 genes that show at least a 10-fold increase in expression in the wild type in response to iron limitation are regulated by *Irr*. These findings further indicate that regulation by *Irr* is not restricted to genes

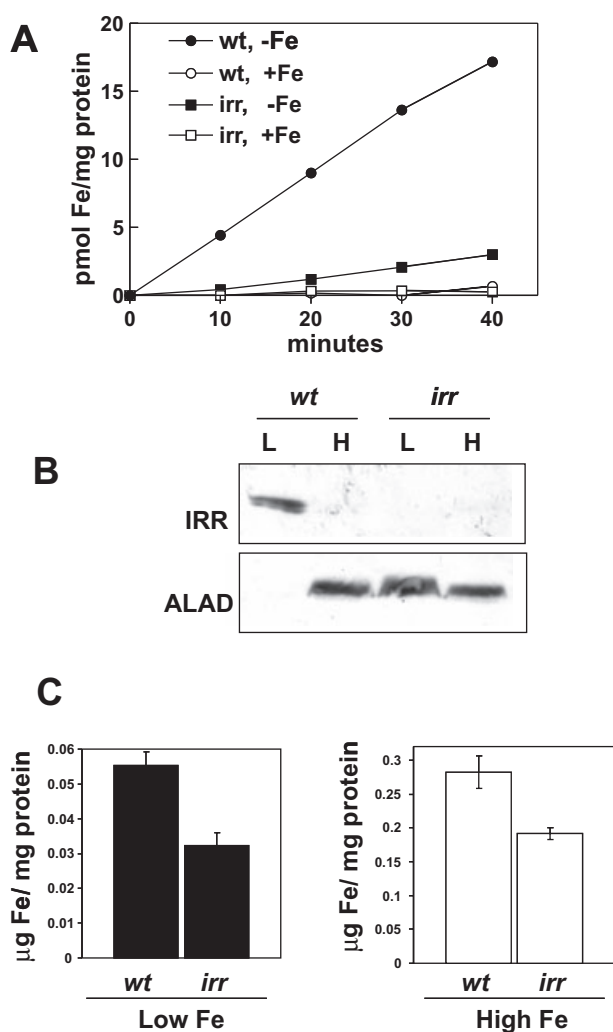


Fig. 2. Phenotypes of the *irr* mutant.

A. Ferric iron transport. Parent strain LO (circles) or *irr* mutant LODTM5 (squares) were grown in media containing no added iron (filled symbols) or 12 μM iron (open symbols). At time zero, 0.05 μM ^{59}Fe was added to the assay medium, and aliquots were subsequently taken at various time points and counted. Each point is the average of triplicate samples, and the standard deviations were less than 10%.

B. Immunoblot analysis of the *hemB* product ALA dehydratase (ALAD) and Irr in cells of parent strain LO and *irr* mutant LODTM5 grown in low- or high-iron media as described above. Fifty micrograms of protein were loaded per lane.

C. Measurement of the iron content of the parent strain LO and *irr* strain LODTM5 by atomic absorption spectrometry. Cells grown in media supplemented with no iron (L) or with 12 μM FeCl_3 (H). The data were based on five to six replicates with error bars within 95% confidence window. The data are shown in two panels to expand the y-axis for the low-iron cells.

involved in haem biosynthesis. In agreement with the quantitative RT-PCR analysis (Fig. 1), the microarray study detected the five ferric iron chelate receptor genes. We measured ^{59}Fe transport in cells of the parent and mutant strains prepared in the same manner as were cells for the microarray analysis (Fig. 2A). High-affinity iron

transport activity was induced in cells of the parent strain grown in low-iron media. However, this activity was greatly diminished in the *irr* strain. Thus, the transport activity is in good agreement with the quantitative RT-PCR and microarray studies.

Among the 167 genes that were downregulated in response to iron limitation in the wild type (Table S1), 17 of them were identified that involve Irr (Table 1). In these cases, mRNA levels were higher in the *irr* mutant than in the wild type, suggesting that Irr normally represses those genes. The class I fumarase, 3-isopropylmalate dehydratase and glutamate synthase are iron sulphur proteins (Flint *et al.*, 1992; Prodromou *et al.*, 1992; Vanoni and Curti, 1999), and genes encoding three additional iron-sulphur proteins were identified in this analysis as well (*blt2737*, *blr6743*, *blr6744*). Haem is an iron-containing cofactor; genes encoding the haem biosynthetic enzymes δ -aminolevulinic acid (ALA) synthase and ALA dehydratase were affected in the *irr* strain, as was a cytochrome *c*. We measured ALA dehydratase protein by immunoblotting in the parent strain and the *irr* mutant grown under the same conditions employed for the microarray analysis (Fig. 2B). ALA dehydratase accumulated to high levels in iron replete cells of the wild type, but not under iron limitation. However, in the *irr* strain, ALA dehydratase was expressed highly independently of the iron status. These observations agree with the microarray analysis (Table 1), and are similar to previous findings (Hamza *et al.*, 1998). It makes physiological sense for iron-containing proteins to be downregulated when iron is scarce, and Irr mediates this control for the genes described above.

The *B. japonicum* genome encodes both a class I and class II fumarase, a tricarboxylic acid cycle enzyme (Acuna *et al.*, 1991), and both genes are iron-regulated in an Irr-dependent manner (Table 1, Fig. 1). Class I fumarases (*blt5796*) are iron-sulphur proteins (Flint *et al.*, 1992) whereas class II fumarases (*blr6519*) do not contain iron (Ueda *et al.*, 1991). Quantitative RT-PCR showed that both of these genes are regulated by iron, but in an opposite manner (Fig. 1). The class II fumarase mRNA was highly induced under iron limitation in the wild type, but not in the *irr* mutant, indicating that Irr is necessary for activation in the parent strain. The iron-containing class I fumarase gene was also controlled by iron, but was downregulated in iron-limited cells, showing 15-fold less mRNA compared with iron-replete cells. This level was elevated 10-fold in the *irr* strain in low iron compared with the parent strain, indicating that Irr normally downregulates this gene. Some control by iron was observed in the *irr* mutant, suggesting some Irr-independent regulation. It is plausible that the iron-independent fumarase substitutes for the iron-sulphur protein when iron is limiting. We suggest that Irr is involved in control of energy metabolism at the level of the tricarboxylic acid cycle.

Evidence that the irr mutant cannot correctly perceive the cellular iron status

The data presented suggest that an *irr* mutant does not correctly perceive the iron status based on the loss of normal responses to iron limitation with respect to many genes (Table 1, Fig. 1). Specifically, the mutant behaves as if there is sufficient available iron when grown in iron-deficient media. To address this directly, we measured the iron content of parent strain LO and *irr* strain LODTM5 in cells grown under iron limitation by atomic absorption spectroscopy (Fig. 2C). The *irr* strain is even more iron deficient than the parent strain when grown in low-iron media, containing about 40% less iron. This decrease is likely to be significant because the parent strain is already iron-limited. The lower iron levels in the *irr* mutant are probably due to the lack of induction of iron transport (Table 1, Figs 1 and 2A). Furthermore, the microarray data suggest that downregulation of chemotaxis genes is indicative of iron deficiency (Table S1). We found that many of these chemotaxis genes were downregulated in the *irr* strain compared with the wild type when the cells were grown under iron limitation (Table S2), supporting the conclusion that the mutant is more iron deficient than the parent strain. We suggest that Irr is necessary for perception of the iron status to regulate iron metabolism and homeostasis.

Evidence for both positive and negative regulatory activities for Irr

Inactivation of the *irr* gene results in both increases and decreases in genes compared with wild-type cells, and thus Irr can have both a positive and negative role in gene expression. Therefore, it is plausible that Irr has two regulatory activities. Previously, an iron control element (ICE) was identified upstream of the divergent haem transport genes *hmuR* and *hmuT* (Nienaber *et al.*, 2001), and it is a target for Irr (Rudolph *et al.*, 2006). An ICE motif is an AT-rich imperfect palindrome (Fig. 3A). Here, we identified putative ICE motifs upstream of several Irr-regulated genes. We chose to assess the putative elements upstream of *blI5796* (class I fumarase), which is downregulated by iron limitation in the wild type, and *blr3904* (iron transport protein), which is normally upregulated in those cells. Double stranded DNA probes containing the respective elements were radiolabelled and analysed by electrophoretic mobility shift assays (EMSA) using purified recombinant Irr (Fig. 3B). Irr bound both elements, but not a non-specific DNA control, which agrees with mRNA analyses showing that those genes are controlled by Irr. Collectively, these findings suggest that Irr can be a direct positive and negative regulator of gene expression. Numerous genes affected in the *irr* strain do not contain an

A



B

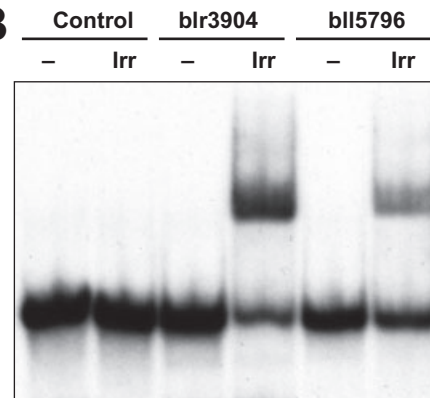


Fig. 3. Irr binds to ICE-like motifs upstream of Irr-regulated genes. A. ICE-like motif upstream of *blr3904* and *blI5796*. B. Electrophoretic mobility shift assays (EMSA) of Irr with ICE-like motifs. The model ICE motif is the nearly perfect inverted repeat found upstream of *blr7895*. EMSA analysis was carried out using 500 nM purified recombinant Irr and 100 pM ³²P-labelled probes. Complexes were resolved on 5% non-denaturing gels and visualized by autoradiography. A non-specific DNA sequence from the multiple cloning site of plasmid pSK was used as a negative control. '-' denotes free probe.

obvious ICE motif, suggesting either that Irr can recognize disparate sequences, or that the control by Irr is indirect.

Evidence that Irr-regulated genes depend on the status of haem

Our findings indicate that Irr is involved in the control of many iron-regulated genes, and not only those directly involved in haem biosynthesis. We showed previously that Irr responds to iron via the status of haem and protoporphyrin by interacting with the enzyme ferrochelatase at the site of haem synthesis (Qi and O'Brian, 2002). Therefore, the current work suggests that the control of iron-regulated genes not directly related to haem synthesis may also be dependent on the status of haem.

To test this idea, we measured iron-dependent expression of several Irr-regulated genes in the haem-defective mutant Δ hemAH, and in its parent strain I110 by quantitative RT-PCR (Fig. 4). Irr accumulates to high levels independently of iron in haem synthesis mutants because haem is required for its degradation, and the protein is active under both iron conditions (Qi *et al.*, 1999; Qi and

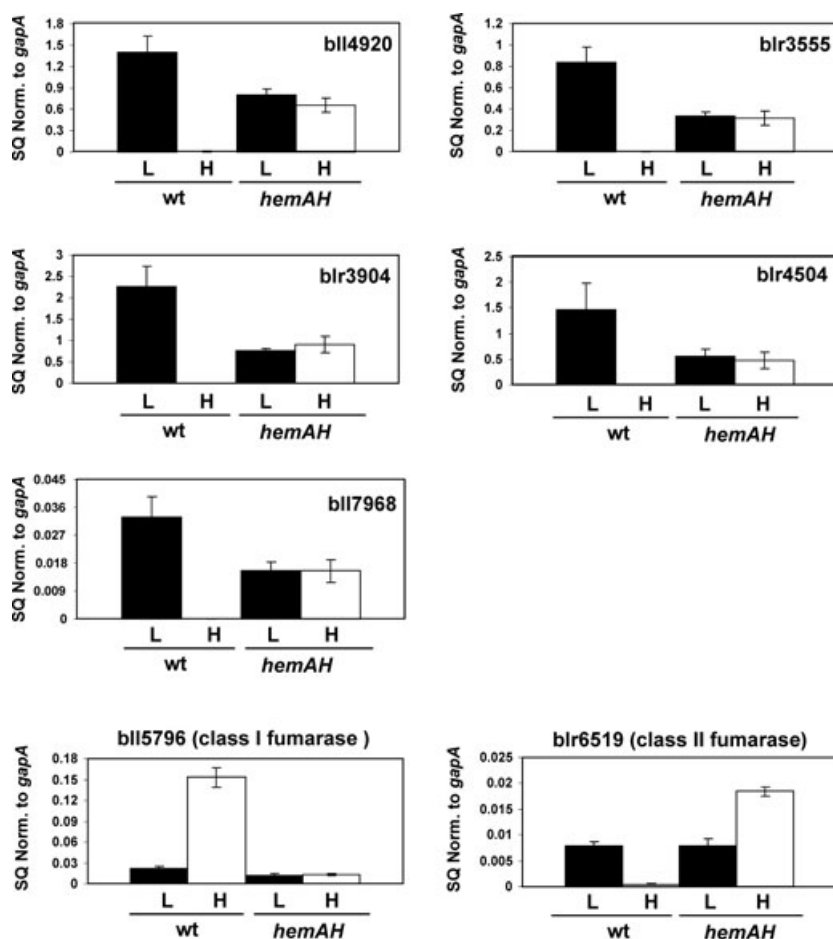


Fig. 4. Iron-dependent expression of the five ferric receptor genes and fumarase genes in the wild-type strain I110 (wt) or the *hemA hemH* mutant strain Δ hemAH. mRNAs were analysed by quantitative real-time PCR from cells grown in media supplemented with no iron (L) or with 12 μ M FeCl₃ (H). The data are expressed as the relative starting quantity (SQ) of the respective mRNAs normalized to the housekeeping gene *gapA*. The data are expressed as the average of three replicates \pm the standard deviation.

O'Brian, 2002). Here, we found that the five ferric iron transporter genes strongly upregulated by Irr in the wild type were aberrantly expressed in the haem mutant (Fig. 4). In all cases, the respective mRNAs were partially activated in the haem mutant regardless of the iron status. Similarly, the class II fumarase gene (*blr6519*) that was induced under iron limitation in the parent strain was highly expressed in the haem mutant in the presence or absence of iron (Fig. 4). We do not know why *blr6519* mRNA is greater in high-iron cells than low-iron cells in Δ hemAH.

Whereas the iron transport genes and the class II fumarase are normally activated under iron limitation, expression of the class I fumarase gene (*bll5796*) is normally downregulated in iron deficient cells. In the haem mutant, *bll5796* mRNA was low irrespective of the iron status (Fig. 4). Thus, mutant strain Δ hemAH has a low-iron phenotype with respect to genes that are normally upregulated or downregulated in the wild type, which is consistent with a constitutively active Irr. The findings suggest that these iron-regulated genes are affected by the status of haem even though they are not directly involved in haem biosynthesis.

A haem biosynthesis mutant is defective in maintaining iron homeostasis

The low-iron phenotype of mutant strain Δ hemAH led us to ask whether those cells are in fact iron-deficient compared with the wild type. Therefore, we measured cellular iron levels in those cells (Fig. 5). We found that the haem mutant was not iron-deficient, but rather contained almost four times more iron than the parent strain when grown in low-iron media. The reason for the higher iron was not determined, but the constitutively expressed iron transporters may result in greater iron accumulation in the mutant compared with the wild type. Regardless of the reason, the findings show that the haem-defective strain cannot maintain normal iron homeostasis or respond correctly to the cellular iron status. Collectively, the data suggest that *B. japonicum* senses iron through the status of haem biosynthesis to regulate iron metabolism.

Discussion

Previous work demonstrated that Irr interacts directly with the haem biosynthetic enzyme ferrochelatase, and

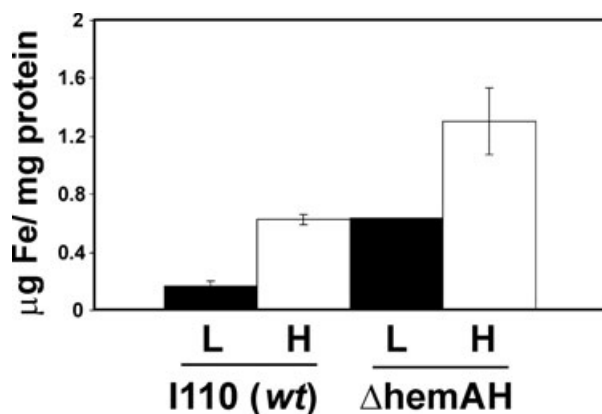


Fig. 5. Measurement of the iron content of the parent strain I110 and the haem synthesis mutant strain Δ hemAH by atomic absorption spectrometry. Cells grown in media supplemented with no iron (L) or with 12 μ M FeCl_3 (H). The data were based on five replicates with error bars within 95% confidence window.

responds to iron via the status of haem and its precursors at the site of synthesis (Qi *et al.*, 1999; Qi and O'Brian, 2002). Thus, the finding that Irr affects many genes within the iron regulon strongly suggests that *B. japonicum* senses iron via the status of haem biosynthesis to control iron transport, the tricarboxylic acid cycle, expression of iron-containing proteins, and other processes not directly related to haem metabolism. Mutants defective either in Irr or in haem synthesis resulted in loss of normal iron-dependent control, and in aberrant total cellular iron content. The *irr* mutant has a high-iron phenotype with respect to genes normally regulated by iron, but those cells are iron-deficient. On the other hand, a haem synthesis mutant, which expresses Irr constitutively, has a low-iron phenotype with respect to Irr-regulated genes, and contains more cellular iron than the wild type. These findings indicate that iron metabolism and homeostasis are linked to the status of haem in an Irr-dependent manner. This mechanism differs substantially from iron-dependent control of gene expression described in other well-studied bacteria. In those cases, iron homeostasis is mediated to a large extent by Fur (Baichoo *et al.*, 2002; Ochsner *et al.*, 2002; Grifantini *et al.*, 2003; McHugh *et al.*, 2003), which binds iron directly to regulate its activity. We propose that *B. japonicum* senses iron by the status an iron-dependent process. We are not aware of a similar control mechanism in other bacteria, but it is interesting to note that a haem biosynthesis mutant of the yeast *Saccharomyces cerevisiae* cannot induce transcription of iron transport genes (Crisp *et al.*, 2003). Although this phenotype is essentially the opposite of what we observed for *B. japonicum* (Fig. 4), it nevertheless suggests a link between iron transport and haem. It would be interesting to learn the basis of that control in yeast, and whether other iron-regulated processes are affected in the haem synthesis mutant.

The *irr* strain was shown to contain less iron than the parent strain when grown under iron limitation (Fig. 2C), which is likely due to the low expression levels of iron transport in the mutant (Figs 1 and 2A). Ironically, a *fur* mutant of *E. coli* expresses iron transporter genes constitutively, but is also iron deficient (Abdul-Tehrani *et al.*, 1999). This was shown to be the result of the repression of many genes encoding iron-containing proteins (McHugh *et al.*, 2003). By contrast, numerous genes involved in the expression of iron-containing proteins are upregulated in the *irr* mutant (Table 1). Thus, in *B. japonicum* the cellular iron deficiency of the *irr* strain appears to be caused by defective iron acquisition, whereas in the *E. coli fur* mutant, it is the result of low incorporation of the metal into proteins. The findings suggest diverse strategies for maintaining iron homeostasis in bacteria.

The iron regulon of *B. japonicum* is large, and Irr appears to control only a subset of those genes. *B. japonicum* contains a functional *fur* gene as well, but preliminary data suggest that Fur cannot account for all of the Irr-independent regulation (J. Yang and M.R. O'Brian, unpubl. obs.). Furthermore, the genome contains a gene encoding a protein that is homologous in its C-terminus to Irr (*blr1216*). However, we have been unable to detect *blr1216* mRNA in wild-type cells grown under the conditions used in the present study (data not shown). Thus, we speculate the presence of a yet unidentified iron regulator in *B. japonicum*. Interestingly, *blr1216* was upregulated in the *irr* strain in microarray analysis (Table S2). It is possible that conditions created by the *irr* deletion, such as a low-iron content, can induce that gene.

Experimental procedures

Strains and media

Bradyrhizobium japonicum I110 and LO were parent strains used in the present work. Strain LODTM5 is a mutant derivative of LO that contains a transposon Tn5 within the *irr* gene (Hamza *et al.*, 1998). Strain Δ hemAH is a double mutant of I110 that is defective in *hemA* and *hemH* genes, encoding δ -aminolevulinic acid synthase and ferrochelatase respectively (Qi *et al.*, 1999). *B. japonicum* strains were routinely grown at 29°C, 150 r.p.m. in GSY medium as described previously (Frustaci *et al.*, 1991). Strain LODTM5 was grown with 50 μ g ml⁻¹ kanamycin and 50 μ g ml⁻¹ streptomycin. Strain Δ hemAH was grown with 50 μ g ml⁻¹ kanamycin, 50 μ g ml⁻¹ streptomycin, 100 μ g ml⁻¹ spectinomycin and 15 μ M haemin to fulfil its haem auxotrophy. For low-iron conditions, modified GSY medium was used, which contains 0.5 g l⁻¹ yeast extract instead of 1 g l⁻¹ and no exogenous iron source. The actual iron concentration of the medium was 0.3 μ M, as determined with a Perkin-Elmer model 1100B atomic absorption spectrometer. The parent strain LO and *irr* strain LODTM5 have doubling times of about 8 and 9.5 h under low-iron conditions. Strain Δ hemAH was grown with 0.05 μ M haemin for low-iron

conditions. The mutant grows similarly to the wild type. The medium was supplemented with 12 μM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ for high-iron conditions.

Microarray analysis. Sample preparation, RNA isolation, cDNA synthesis, fragmentation, terminal labelling and hybridization

Three biological replicates of LO and LODTM5 grown under either high- (12 μM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) or low-iron conditions were used for microarray procedures. Forty millilitres of cultures grown to mid-exponential phase (OD_{540} 0.4–0.6) were quickly transferred to chilled centrifuge tubes containing 8 ml of RNA protect (Qiagen), mixed by inversion and centrifuged at 7000 r.p.m., 4°C for 10 min to pellet cells. Supernatants were discarded and pellets were quickly frozen in liquid nitrogen and stored at –80°C. To isolate RNA, frozen cell pellets were resuspended in 1.5 ml of cold buffer A [0.02 M NaOAc (pH 5.3), 1 mM EDTA (pH 8.0)] and additional 200 μl of RNA protect (50 μl per 10 ml of culture) was added. The cell suspension was transferred to the acid phenol solution (160 μl of 10% SDS, 2 ml of buffer A and 3.5 ml of acid phenol) that was preheated at 65°C for 5 min, vortexed for 30 s, incubated at 65°C for 2 min, vortexed for 1 min, and incubated at 65°C for additional 5 min. The mixture was centrifuged at 7000 r.p.m., 4°C for 5 min and the upper aqueous phase was extracted with 3 ml of phenol : chloroform : isoamyl alcohol (25:24:1) and 3 ml of chloroform successively. RNA was precipitated from the aqueous phase by addition of 1/10 vol. of 3M sodium acetate and 2 vols of 100% ethanol at –80°C overnight; followed by centrifugation at 13 000 r.p.m., 4°C for 30 min. The pellet was washed once with 70% ethanol, and then it was dissolved in 100 μl of RNase-free H_2O . RNA samples were treated with RQ1 Rnase-free DNase I (Promega) and purified using RNeasy bacterial RNA purification kits (Qiagen). The genomic DNA contamination was examined by PCR using 500 ng of total RNA as templates.

cDNA synthesis, fragmentation, terminal labelling and hybridization were carried out using GeneChip *P. aeruginosa* Genome Array Expression Analysis protocol (Affymetrix) with slight modifications indicated below. One thousand five hundred units of M-MLV reverse transcriptase (Promega) were used for 10 μg of total RNA in a 60 μl cDNA synthesis reaction. A mixture of *in vitro* transcripts was spiked into RNA samples as internal controls. Subsequently, 3 μg of synthesized cDNA was fragmented by DNase I (0.2 U μg^{-1} cDNA) (Amersham Biosciences) at 37°C for 2.5–3 min and the reaction was immediately inactivated at 98°C for 10 min. 2.8 μg of cDNA fragments were then labelled with biotinylated GeneChip® DNA labelling reagent (Affymetrix) using the terminal deoxynucleotidyl transferase (Promega) at 37°C for 75 min and the reaction was stopped by the addition of 2 μl of 0.5 M EDTA. The target is ready to be hybridized onto probe arrays.

Labelled cDNA fragments (2.5 μg) and an internal standard (spike-in) hybridization control were used as targets in the microarray analysis. *B. japonicum* probe arrays (BJAPETHa520090, Affymetrix) were incubated in the 140 μl of hybridization cocktail in a hybridization oven at 60 r.p.m., 48°C for 16 h. Arrays were washed and stained with streptavidin, biotinylated antistreptavidin antibody and streptavidin phycoerythrin successively in a fluidics station 450 (Affyme-

trix) and scanned in a GeneChip® scanner 3000 (Affymetrix) according to Affymetrix protocols.

GeneChip data analysis

Signals from scanned images were processed using GeneChip Operating Software (GCOS) version 1.1 (Affymetrix). The Affymetrix GeneChip used represents the 8317 annotated open reading frames as well as 51 RNA genes and intergenic regions. For single array analysis, global scaling was applied by scaling signals to a target signal intensity of 500 using all probe sets. To compare expression under different conditions, we used comparison array analysis where an experimental array was compared with a baseline array. The comparison array data were exported from GCOS and analysed in Microsoft Excel. A decrease was deemed only when signal log ratio was ≤ -1 and the comparison was statistically evaluated as significantly changed ($P \geq 0.98$) for all three replicates, or an increase was deemed only when signal log ratio was ≥ 1 and the comparison was statistically evaluated as significantly changed ($P \leq 0.02$) for all three replicates. The fold change was calculated from the signal log ration ($\text{FC} = 2^{\text{SLR}}$) and the average fold change of three biological replicates were shown in the results table. The intergenic regions were filtered thus not included in the results. Microsoft Access was further used to compare data from different comparison array analysis. Gene names and annotations are from the published genome database (Kaneko *et al.*, 2002, <http://www.kazusa.or.jp/rhizobase/index.html>).

Quantitative real-time PCR

We determined expressions of selected genes by real-time qPCR with iQ™ SYBR Green supermix (Bio-Rad) using iCycler thermal cycler (Bio-Rad). RNA was isolated and treated with DNase I as above for microarray analysis. Total RNA of 2.5–5 μg was used for cDNA synthesis using Superscript first-strand synthesis system (Invitrogen). cDNA was purified using MinElute PCR purification kit (Qiagen) as above for microarray analysis and various dilutions of cDNA were used as PCR templates. Each PCR reaction contained 10 μl of 2 \times SYBR Green supermix, 0.2 μM primers (IDT DNA Technology) and appropriate templates in a 20 μl reaction. PCR reactions were heated to 95°C for 3 min and then for 40 cycles with steps of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s. The generation of specific PCR products was confirmed by melting curve analysis and gel electrophoresis. Samples in which the reverse transcriptase was omitted in reverse transcriptase reactions were used as negative controls. The standard curve method was employed for relative quantification and *gapA* was a housekeeping gene control. Genomic DNA from parent strains LO or I110 was used as PCR templates to generate a standard curve for each gene. Relative starting quantities (SQ) of mRNAs for interested genes and *gapA* were calculated from corresponding standard curves. Quantity of interested genes was then normalized to quantity of *gapA* for each condition. The results were based on the average of triplicates and standard deviation was shown as the error bar in the results.

Iron uptake assays

Cells were grown to mid-log phase in low- or high-iron media, centrifuged, washed twice and resuspended in uptake buffer to a OD₅₄₀ of 0.4. Uptake buffer contained 0.2 M MOPS, 20 mM citrate and 2% (w/v) glucose, pH 6.8. Thirty millilitres of cells were placed into a 125 ml flask and preincubated at 30°C with shaking. At time zero, ⁵⁹FeCl₃ was added to a final concentration of 0.05 µM (1.8 µCi). One millilitre aliquots (c. 125 µg protein) were removed at various times and added to 3 ml quench buffer precooled on ice. The quench buffer contained 0.1 M Tris, 0.1 M succinate and 10 mM EDTA, pH 6.0. The quenched cells were collected on 0.45 µm filters presoaked in quench buffer containing 40 µM Fe-EDTA, and then counted on an LKB gamma-counter.

Iron content measurement using atomic absorption spectroscopy

Forty millilitres of cultures grown to mid-log phase (OD₅₄₀ 0.4–0.6) were harvested by centrifugation at 7000 r.p.m., 4°C for 10 min. The pellet was washed twice with 20 ml of ice-cold quench buffer (0.1 M Tris, 0.1 M succinate and 10 mM EDTA) and once with metal-free double distilled water to remove salts. To lyse cells, the pellet was resuspended in 100 µl of 70% HNO₃ (J.T. Baker, AAS grade), lightly vortexed and heated at 75°C for 3–5 min until cells were completely lysed. One millilitre of metal-free double distilled water was added to the lysed cells and mixed by vortex. Samples were centrifuged at 13 000 r.p.m. for 5 min and the supernatant was analysed for Fe content. Atomic absorption was performed in the furnace mode on a Perkin Elmer Atomic Absorption Spectrometer model 1100B equipped with a model HGA 700 graphite furnace. All samples were diluted with metal-free double distilled water to contain 1% HNO₃. To set up a Fe standard curve, the stock solution Fe 2 ng/10 µl in 2% HNO₃ (Perkin Elmer) was diluted to 0.25 ng/10 µl, 0.5 ng/10 µl, 0.75 ng/10 µl, 1 ng/10 µl in 1% HNO₃. Ten microlitres of samples were used for each measurement and 4–6 replicates were performed for each sample. The program for Fe element included five steps: 90°C for 45 s, 1400°C for 30 s, 30°C for 20 s, 2400°C for 10 s and 2650°C for 5 s. Protein concentrations were determined from another 40 ml cells by BCA assay (Pierce) using bovine serum albumin standards. The Fe content was normalized to the protein concentration.

Electrophoretic mobility shift analysis of ICE motifs

The ICE motifs were identified using the FUZZNUC program (<http://emboss.sourceforge.net/apps/fuzznuc.html>) using the imperfect inverted repeat sequence in the *hmuR* promoter as the input data (Nienaber *et al.*, 2001) to find motifs within the *B. japonicum* genome (<http://www.kazusa.or.jp/rhizobase/index.html>). EMSAs were carried out to determine Irr binding to putative ICE motifs upstream of the *blr3904* and *blt5796* genes as previously described (Friedman and O'Brian, 2003). The purified recombinant Irr protein (500 nM) that was overexpressed and purified as previously described (Qi *et al.*, 1999) and 100 pM ³²P-labelled probes were used. A non-specific DNA fragment from plasmid pSK was used as a negative control. Protein–DNA complexes were resolved on 5% non-denaturing gels and visualized by autoradiography.

DNA oligonucleotides used as probes are: *blr3904* (5'-CAGG AATTCGATACTTTAGAACCGTTTGAACCTGACCTC-3', 5'-GTCGAG-GTCAGTTTCAAACGGTTCTAAAGTATCGAATTC-3'); *blt5796* (5'-CAGGAATTCGATA-GCTTAGAACGCTTCTA TGCCGACCTC-3', 5'-GTCGAGGTCGGCATAGAAGCGTTC TA-AGCTATCGAATTC-3'); control (5'-CAGGAATTCGATATC AAGCTTATCGATACCGTTCG-ACCTC-3', 5'-GTCGAGGTCG ACGGTATCGATAAGCTTGATATCGAATTC-3').

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Note added in proof

The microarray data have been deposited in NCBI Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE4156.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Comparison of parent strain LO grown in iron-limited or iron replete media.

Table S2. Comparison of Irr mutant strain LODTM5 to parent strain LO grown in iron-limited media.

This material is available as part of the online article from <http://www.blackwell-synergy.com>